Table 3 Objective response rate and time to progression

	N	CR	PR	NC	PD	Response (%)	95% CI	TTP (day)	Range
Phase II portion	19	0	14	3	2	73.7	48.8–90.9%	179	24–384
Total	25	0	19	4	2	76.0	54.9–90.6%	162	24–384

CR = complete response; PR = partial response; NC = no change; PD = progressive disease; CI = confidence interval; TTP = time to progression (median).

Pharmacokinetics

Plasma PK analysis was performed on samples obtained from 12 patients during the first course of the phase I portion for S-1 components and total platinum (Table 4). There were no significant differences between the two PK parameters of S-1 components on days 6 and 8.

Table 4 Pharmacokinetic parameters of S-1 component after oral administration of S-1 alone, or with CDDP

	CDDP: 60 mg m^{-2}		CDDP:70 mg m^{-2}	
	$AUC_{(0-8)}$ (ng h ml^{-1})	C_{max} (ng ml^{-1})	$AUC_{(0-8)}$ (ng h ml^{-1})	C_{max} (ng ml^{-1})
Day 6 (S-1 alone)				
FT	22724±10693	3517±1392	23860±12059	3378±1574
5-FU	670.9±155.8	136.8±40.3	860.6±466.4	166.3±78.2
CDHP	1193.6±258.1	308.9±95.1	1031.9±125.2	219.7±17.2
Oxo	373.0±196.1	89.9±62.4	291.1±112.9	61.3±26.2
Day 8 (with CDDP)				
FT	20930±8631	3236±1119	21192±11401	3104±1572
5-FU	573.8±148.7	111.9±33.6	782.0±326.6	144.9±42.6
CDHP	1054.9±144.8	241.6±62.6	1127.0±191.4	258.2±66.5
Oxo	282.8±99.1	63.2±25.7	335.1±177.2	68.1±35.4

Values are mean+s.d. ($n = 6$). CDDP = cisplatin; FT = tegafur; 5-FU = 5-fluorouracil; CDHP = 5-chloro-2, 4-dihydroxypyridine; oxo = potassium oxonate.

DISCUSSION

Two phase II studies of S-1 for AGC patients who had not previously received chemotherapy as a single agent were conducted in Japan (Sakata *et al*, 1998; Koizumi *et al*, 2000). Examining those pooled results, the RR rate was 44.6% (45/101, 95% CI: 35.2–54.3), MST was 244 days (95% CI: 172–319), and 1- and 2-year survival rates were 36.6 and 16.5%, respectively. Subgroup analysis of the objective RR in the two S-1 phase II studies revealed that the well-differentiated cell type (intestinal type) was less sensitive to S-1 (RR = 35%, 16/46) than the poorly differentiated type (diffuse type, RR = 53%, 29/55). The results of phase II studies suggested that S-1 is one of the most active antitumour single agents for AGC patients and is even comparable to recent combination therapies. Based on these data, to achieve more survival benefit, we therefore planned combination therapy of S-1 with another antitumour agent with a different mechanism of action, because this might obtain better efficacy including longer survival, as a clinical benefit.

We selected CDDP as the combination agent to be used with S-1, because CDDP has been widely used in combination therapy for AGC patients (Rougier *et al*, 1994), and synergistic activity with 5-FU and its derivatives has been reported in animal models (Scanlon *et al*, 1986; Yamada *et al*, 1990; Shirasaka *et al*, 1993).

As most toxicities of S-1 in phase II studies appeared at 4 weeks of consecutive S-1 administration, a new combination therapy of S-1 with CDDP was planned in which S-1 was to be administered daily for 3 consecutive weeks, that is, 1 week less than the period at which toxicities such as leucopenia appeared. In addition, CDDP showed the best activity when given 8 days after the start of daily UFT administration (Ichinose *et al*, 1995). Therefore, CDDP was administered on day 8 of 21-day consecutive S-1 administration. In this combination phase I/II study, S-1 was administrated at the RD (80 mg m^{-2} in a day) and the CDDP dose was escalated from 60 mg m^{-2} as level 1 to 70 mg m^{-2} at level 2. According to the results of the phase I portion, the RD of CDDP combined with S-1 was designated as 60 mg m^{-2} with a DLT of myelosuppression, and in the phase II trial S-1 was orally administered daily for 21 consecutive days followed by a 2-week rest, and CDDP (level 1) was intravenously administered on day 8 of every 5-week period. The severity of neutropenia increased with the dose of CDDP in this study, and grade 4 neutropenia was seen in one of six patients (16.7%) when 70 mg m^{-2} of CDDP was administered with S-1. Furthermore, thrombocytopenia became slightly more marked, although it had been infrequent in S-1 single therapy. These results may lead to the conclusion that CDDP dose-dependently increases the myelosuppressive toxicity of S-1.

For the nonhaematological toxicity, GI toxicity, that is, diarrhoea was observed in this combination therapy. The incidence of diarrhoea was 9.9% (total) and 2.0% (grades 3 and 4) by S-1 single therapy (Sakata *et al*, 1998; Koizumi *et al*, 2000). It suggests that in combination with CDDP, the total incidence of diarrhoea was increased a little; however, severe diarhoea was nearly the same and uncommon, and in any study diarrhoea was manageable, similar to those reported for UFT (Ichinose *et al*, 1995; Borner *et al*, 2002).

The PK results of drugs were similar to the previous results obtained from single-agent therapy (Hirata *et al*, 1999). As there was no PK difference for any S-1 component on day 6, before CDDP administration, and on day 8 after CDDP administration, no PK interaction of CDDP in S-1 metabolism was suggested.

In this study, the overall RR of all eligible patients was 76%. The RR in this combination therapy was high, not only in the diffuse type subgroup, 76%, but also in the intestinal-type subgroup, 75%. The MST (383 days) of our study was longer than in the S-1 single-agent phase II study, or other combination chemotherapy phase II results for AGC patients (Sakata *et al*, 1998; Koizumi *et al*, 2000; Kulke, 2000). Based on these data, combination chemotherapy using S-1 and CDDP was suggested to be worthwhile.

It is reported that the vascular endothelial growth factor (VEGF) is more frequently expressed in well-differentiated tumours, and that it promotes angiogenesis in human gastric carcinoma (Tanigawa *et al*, 1997) and also that the prognosis in the group with high VEGF-C expression was significantly poorer than that in the group with lower VEGF-C expression (Takahashi *et al*, 2002). The combination of CDDP with S-1 therapy is reported to show higher response than S-1 alone, in VEGF-positive gastric cancer patients, which may also support the high objective RR and long survival in this phase I/II combination study (Hironaka *et al*, 2002). Due to its potent angiogenic activity, VEGF is supposed to contribute to metastasis including peritoneal metastasis, a representative life-threatening condition in gastric cancer. S-1 showed superior therapeutic efficacy against peritoneal metastasis in nude mice (Mori *et al*, 2003) and clinically, alone (Iwazawa *et al*, 2002) or in combination with CDDP (Nakamura *et al*, 2002). The results suggest good therapeutic effect of S-1 in terms of survival

benefit and increased QOL, as a result of improving several symptoms related to peritoneal metastasis.

The effect of S-1 in combination with CDDP in the present study was shown to be as good as in previous studies based on 5-FU (Kulke, 2000), with no increasing toxicity. Even though the present study is only a limited experience, a further phase III study to confirm the efficacy of combination therapy of S-1 with CDDP is warranted.

Oral chemotherapy has the advantage of greater patient convenience and acceptance with potential cost saving. It is reported that if equivalent response is achieved, patients prefer oral to intravenous medication (Liu *et al*, 1997). For fluoropyrimidine, most patients selected oral UFT, which is a kind of DIF rather than intravenous 5-FU (Borner *et al*, 2002), and UFT has been proposed to raplace i.v. 5-FU as a first-line therapy for metastatic colorectal cancer (Stabuc, 2003). The same appears to be true for not only oral S-1 alone but also in combination with CDDP therapy, which needs limited hospitalisation.

In Japan, several phase II studies using 5-FU combined with CDDP have been tested for AGC, employing various dosage and treatment schedules. However, based on the results of the JCOG9205 phase III study, 5-FU single therapy is still recognised as the standard first-line chemotherapy (Ohtsu *et al*, 2003). In addition, currently, a randomised phase III study (5-FU *vs* S-1 *vs* CPT-11 with CDDP) for AGC patients as the first-line chemotherapy is underway in Japan (JCOG9912). We also initiated a randomised phase III study comparing S-1 alone, and with CDDP for AGC. From these two phase III studies, we may be able to evaluate the clinical benefit of the regimen using S-1 in terms of survival benefits and improving the QOL for AGC patients.

ACKNOWLEDGEMENTS

We are grateful to Drs Tetsuo Taguchi, Minoru Kurihara, Masahiko Shibuya, and Keisuke Aiba for kind advices, and Drs Yuh Sakata and Junji Tanaka for extramural reviewing, and Miwa Nonaka, Toshiko Kaneko, and Noriko Kanno for good support as CRC. We also thank T Tahara, K Hoashi, K Ono, K Urata, A Fukushima, and H Anbe for assistance in data management and analysis, and K Ikeda for pharmacokinetic analysis. We are indebted to Professor J Patrick Barron of the International Medical Communications Center of Tokyo Medical University for ireviewing this manuscript.

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Antitumor effect of MCC-465, pegylated liposomal doxorubicin tagged with newly developed monoclonal antibody GAH, in colorectal cancer xenografts

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(Received April 14, 2004/Revised April 30, 2004/Accepted May 1, 2004)

MCC-465 is an immunoliposome-encapsulated doxorubicin. The liposome is tagged with polyethylene glycol and the F(ab')$_2$ of a monoclonal antibody named GAH, a human antibody obtained by the hybridoma technique. The epitope recognized by GAH is not well characterized, but human gastric, colorectal, and mammary cancer cells were GAH-positive, while the normal counterparts were GAH-negative. Pegylated liposome doxorubicin (PLD) and MCC-465 did not show significant antitumor activity against GAH-negative Caco-2 xenografts. On the other hand, MCC-465 exhibited significantly superior antitumor effects against GAH-positive WiDr-Tc and SW837 xenografts, compared with PLD. Immunohistochemistry with GAH revealed that 94% (100 of 106) of surgical specimens of colorectal cancer were GAH-positive. These results warrant a phase I clinical trial of MCC-465 for patients with metastatic colorectal cancer. (Cancer Sci 2004; 95: 608–613)

Colorectal cancer is increasing, and is the third leading cause of cancer death in men and the second in women in Japan. Approximately 37,200 people died of it in Japan in 2001.[1)] The primary curative therapy of colorectal cancer is surgical resection. Although 40–50% of patients may be cured with surgery, many will develop metastatic disease. The limitation of systemic therapy for such recurrent or metastatic colorectal cancer is well recognized. Few chemotherapeutic options are available and the median survival time is only 12–20 months. Although new agents such as irinotecan and oxaliplatin have been introduced into clinical practice for colorectal cancer recently, 5-fluorouracil (5-FU) still remains the mainstay of treatment for patients with advanced colorectal cancer. Therefore, there is still much room for improvement of the current therapy.

Liposomes are closed vesicular structures that are capable of encapsulating water-soluble molecules, and were initially described in the 1960s.[2)] They may serve as a vehicle for delivering cytotoxic agents more specifically to tumors and for limiting exposure of normal tissues to the drug. Extensive studies have yielded promising results with pegylated liposomal doxorubicin (PLD), and recently one PLD formulation, "Doxil," was approved in the United States and Europe for the treatment of Kaposi's sarcoma and ovarian cancer. Relative to conventional doxorubicin (DXR), PLD has a very limited volume of distribution because of its confinement to the vascular space, slower clearance from the circulation, prolonged plasma half-life, and a remarkably greater area under the curve compared to free DXR.[3–6)] Some clinical studies have shown better tumor localization and penetration in solid tumors such as head and neck cancer, lung cancer, Kaposi's sarcoma, malignant effusions, and metastatic bone lesions from breast cancer.[7,8)]

This selective tumor targeting of liposomes containing cytotoxic agents is believed to be achieved through the so-called EPR (enhanced permeability and retention) effect. The EPR effect was named on the basis of the following pathophysiological characteristics of solid tumors: (a) hypervasculature; (b) incomplete vascular architecture; (c) several vascular permeability factors stimulating extravasation within the cancer; (d) little drainage of macromolecules and particulates, which results in their long-term retention in the cancer tissue.[9–11)] Several comparative studies have indicated that PLD produces less nausea, vomiting, alopecia, stomatitis, and cardiotoxicity than conventional DXR, while providing comparable or better objective responses and clinical survival benefits.

MCC-465 is immunoliposome-encapsulated DXR. The liposome is tagged with polyethelene glycol (PEG) and a newly developed monoclonal antibody, GAH. GAH is a human antibody and was obtained using the hybridoma technique.[12)] The hybridomas were originally established by fusion of lymphocytes from regional lymph nodes of colon cancer with mouse myeloma cells. At the present time, the antigen recognized by GAH has not been identified, but it is immunohistochemically well characterized. The antibody binds to a novel cancerous cell surface antigen expressed in human gastric, colorectal, and mammary cancer tissues. Most gastric cancer tissues are positively stained with GAH, while normal tissue or cells, such as GI tract, liver, lung, uterine, thyroid gland, or blood cells do not show any significant reactivity with GAH.[12)] MCC-465 is supposed to accumulate around cancerous tissue by utilizing the EPR effect and binds to the cancer cell surface, then it is internalized to the cytoplasm by GAH-antigen interaction.[12)] Therefore we anticipated that MCC-465 would possess superior antitumor activity to conventional DXR or PLD against GAH-positive human cancers.

In the present study, in order to evaluate whether MCC-465 is worthy of clinical trial against colorectal cancer, which is generally resistant to DXR, we examined GAH-positivity immunohistochemically on formalin-fixed, paraffin-embedded archival colorectal adenocarcinoma, and we studied the *in vivo* and *in vitro* antitumor activity of MCC-465 in comparison with free DXR and PLD against colorectal cancer.

Materials and Methods

Antibody. A human monoclonal antibody (GAH) was prepared from a human mouse hybridoma as described previously.[12)] The antibody was biotin-labeled with an ECL Protein

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Biotinylation Module (Amersham Life Sciences, Buckinghamshire, England) for immunohistochemistry. The $F(ab')_2$ fragment of GAH was obtained by pepsin digestion for MCC-465 preparation.

Chemicals. MCC-465 was obtained from Mitsubishi Pharma Corporation. Briefly, the GAH fragment and 2-chain-type PEG were incorporated in DPPC gel phase liposomes containing DXR.[12] The incorporated amounts of the GAH fragment and the PEG were 0.2 mg and 0.8 mg per 1 mg of DXR, respectively. The average size of the immuno-liposomes (MCC-465) was determined by dynamic light scattering, and it was 143 nm. PLD was prepared in the same way as MCC-465 except for incorporation of the GAH fragment. Doxorubicin hydrochloride was purchased from Mercian Corporation (Tokyo). Other chemicals were of reagent grade.

Cell lines. Colorectal cancer cell lines, SW837 (obtained from Immuno-Biological Laboratories Co., Ltd., Gunma, Japan), WiDr-Tc (obtained from Tohoku University, Institute of Development, Aging and Cancer, Miyagi, Japan), and Caco-2 (obtained from American Type Culture Collection) were grown in D-MEM/F12 medium (GIBCO BRL Life Technologies, Inc., Rockville, MD) supplemented with 10% fetal calf serum.

Tumor xenografts. Four- to five-week-old male BALB/c *nu/nu* mice were purchased from Nihon Clea Co. (Tokyo) and were kept in standard housing. All animal studies were carried out according to the guidelines for the care and use of experimental animals drawn up by the Committee for Animal Experimentation of the National Cancer Center, which meet the ethical standards required by law and by current guidelines dealing with the treatment of experimental animals in Japan. WiDr-Tc, SW837, and Caco-2 were injected subcutaneously onto the backs of the mice. When the tumors had grown, they were resected and a block of each tumor a few millimeters in diameter was implanted directly into the exposed left subcapsular kidney. The subcapsular renal xenograft (SRC) model was used to evaluate the anti-tumor activity of liposomes. Since Aamdal *et al.* reported that many human tumors grow well and retain the morphology and characteristics of the parent tumors in the SRC model,[13] we adopted the SRC model for studying the antitumor effect depending on the antibody and the antigen interaction. SRC tumors were also obtained to prepare paraffin-embedded sections for immunohistochemistry with GAH.

Antitumour activity against subcapsular kidney xenografts. The mice bearing tumors at the subcapsular kidney were randomly allocated to drug treatment groups of 6 animals each. DXR at a dose level of 3 mg/kg, PLD or MCC-465 at an equivalent dose of DXR was injected intravenously on days 1, 8, and 15 after tumor implantation. Control mice were injected with saline. Twenty-two days after tumor implantation, all mice were sacrificed under anesthesia, and each tumor in the subcapsular kidney was excised and weighed. Antitumor activity was evaluated in terms of tumor weight.

Patient population. Archival paraffin-embedded tissue blocks of colorectal cancer from patients treated in 1991 were retrieved from the Department of Pathology of the National Cancer Center Hospital, Tokyo. Stage and histological classification, and survival times were available for all primary colorectal cancers. Patients who were lost follow-up or who underwent surgery after endoscopical mucosal resection were excluded from the study.

Immunohistochemistry. Immunostaining was performed with the use of biotinylated GAH and streptavidin-PerCP. Sections were placed on slides, deparaffinized, rehydrated, and microwaved. Slides were cooled at room temperature for 15 min. Sections were blocked with 5% (w/v) bovine serum albumin-supplemented PBS, then incubated with biotinylated GAH solution at 37°C for 2 h, and reacted with streptavidin-PerCP solution (Becton Dickinson Immunocytometry System, CA) in an ice-cold light-resistant container for 30 min. Red fluorescence of PerCP from clinical sections was observed using a biological fluorescence microscope BX-50 (Olympus Optical Co., Tokyo) equipped with filters for FITC and that from experimental sections was observed using an Axiovert 35 (Carl Zeiss, Inc., Germany). Human IgG was used in place of GAH to prepare the negative staining control. The quantity and intensity of red fluorescence were graded 3+, 2+, 1+, and 0, by the use of imaging software (Adobe Photoshop ver 5.5). When the red fluorescence from the section was at the level of the detection limit or below, and was equal to that in an adjacent normal gland, the section was graded 0 and regarded as negative. Any section which was graded 1+ or over, based on the intensity of the red fluorescence, was regarded as positive.

Statistical methods. Differences of *in vivo* antitumour activity were analyzed by means of Dunnett's test to compare each group with the MCC-465 treated group. Overall survival (OS) and colorectal cancer-corrected survival were estimated by the Kaplan-Meier method. The log-rank method was used to estimate the equivalence of the survival curves of different subgroups. Distributions of prognostic factors in different groups were compared with the Kruskal-Wallis test. Differences were assessed with a two-sided test, with an α level of 0.05. All statistical analysis was done using SAS version 6.12 (SAS Institute, Inc., NC) running on "Windows" 98.

Results

Immunohistochemical staining and correlation between GAH-positivity and clinicopathological indices. The staining in carcinoma cells occurred mostly on the cell membrane or, less frequently, as a diffuse cytoplasmic staining. GAH-positive staining was observed in 94% (100 of 106). Among positive specimens, 36 (34%) were found to be weakly positive (1+), and 64 (60%) were strongly positive (2+, 3+) (Table 1 and Fig. 1). There was no statistically significant difference between carcinomas graded by GAH reactivity in terms of clinical staging (Dukes' classification), tumor histopathological grading (Table 1) or OS (Fig. 2).

Table 1. GAH reactivity of 106 patients with primary colorectal cancer

	GAH reactivity					GAH reactivity			
	0	1+	2+	3+		0	1+	2+	3+
Dukes					Histologic classification				
A	2	14	11	1	Wel	3	27	29	3
B	2	8	15	5	Mod	1	9	22	5
C	2	13	25	6	Por	1	0	1	2
D	0	1	1	0	Muc	1	0	0	1
Total	6	36	52	12	Undiff	0	0	0	1

There was no statistically significant difference between carcinomas graded by GAH reactivity in terms of clinical staging or tumor histopathological grading.

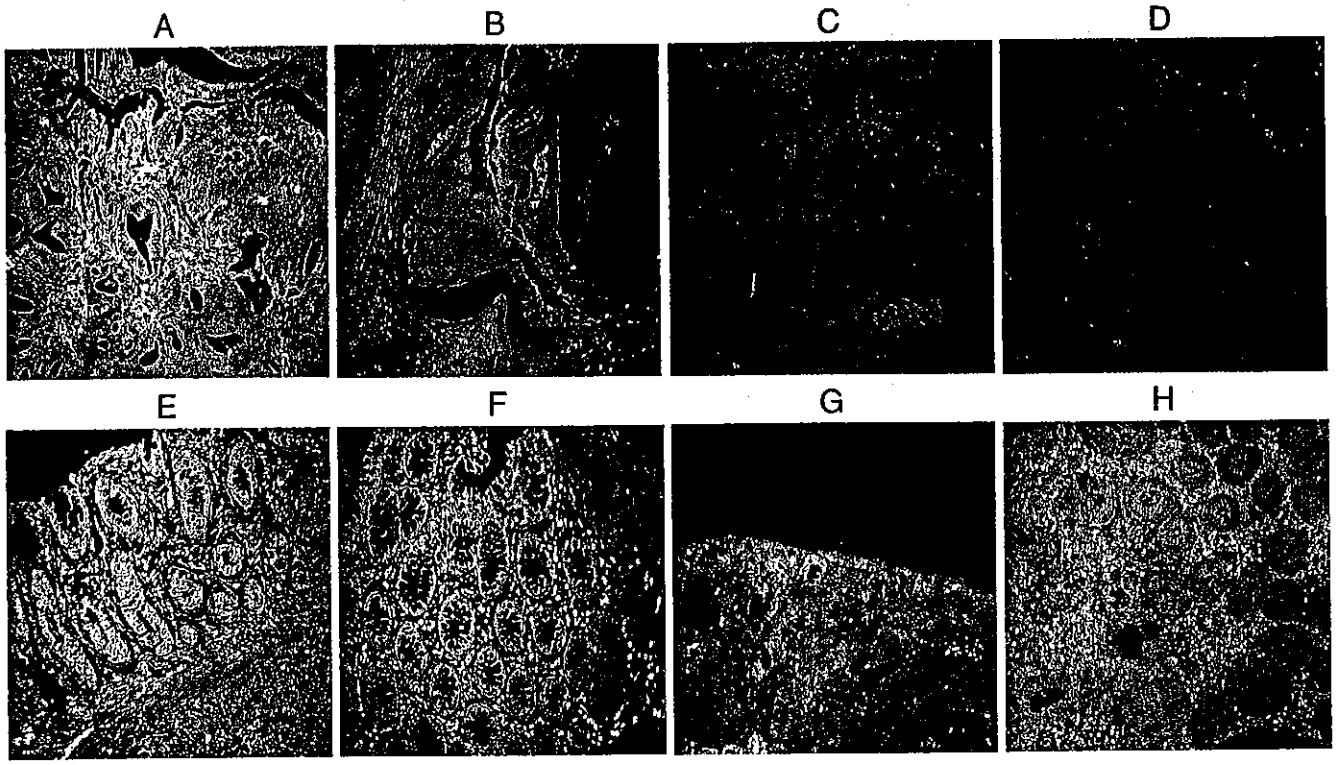

Fig. 1. Immunohistochemistry with GAH antibody. Four pairs of colonic cancer tissues (A–D) and their normal counterparts (E–H) are presented. A, B, C, and D are representatives of negative, weak positive (1+), positive (2+), and strong positive (3+), respectively.

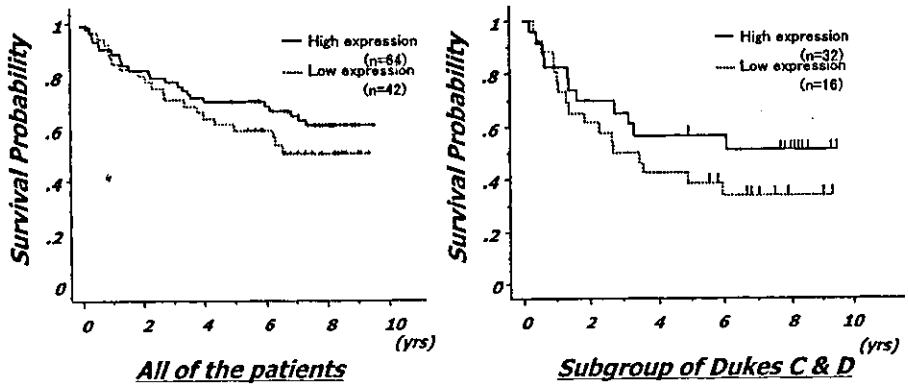

Fig. 2. Overall survival (OS) was compared in patients with no or low expression (0 or 1+) and with high expression (2+ or 3+). *P* value was determined with the use of the log-rank test (left). OS in patients with Dukes' C and D was compared (right).

GAH reactivity against tumor xenograft. Strong red fluorescence of GAH revealed that the antigen recognized by the antibody existed at the cell membrane, as well as in the cytoplasm of WiDr-Tc and SW837 cells (Fig. 3, inset A and B). In contrast, weak fluorescence was observed at both sites of Caco-2 cells, regarded as GAH-negative (Fig. 3, inset C).

***In vivo* antitumor activity.** We evaluated the difference of antitumor activity among the three drugs after injection of each drug three times at weekly intervals. MCC-465 did not show any significant antitumor activity towards GAH-negative Caco-2 xenografts in comparison with DXR, PLD, and the saline control group. In the case of GAH-positive WiDr-Tc and SW837 xenografts, the equivalent dose of MCC-465 showed significantly superior antitumor activity in terms of tumor growth inhibition, while there was no significant tumor growth inhibition with DXR or PLD treatment in comparison with the control. As shown in Fig. 3, treatment of tumor-bearing mice with MCC-465 resulted in a 70–80% reduction in the tumor weight compared with the control group.

Discussion

For any cytotoxic agent to be effective, it should accumulate in target cells at the optimal concentration for the necessary duration of time in order to exert its cell killing effect. Unfortunately, physicochemical and physiological barriers for cytotoxic agents can lead to heterogeneous accumulation in solid tumors, and failure to kill a small fraction of cells can result in tumor regrowth. Jain postulated that there are at least four physiological barriers to delivery of a blood-borne agent; (a) heterogeneous angiogenesis and blood flow in tumors, (b) heterogeneous permeability of tumor vessels, (c) interstitial compartment in tumors, and (d) the cell membrane and the cytoplasm.[14]

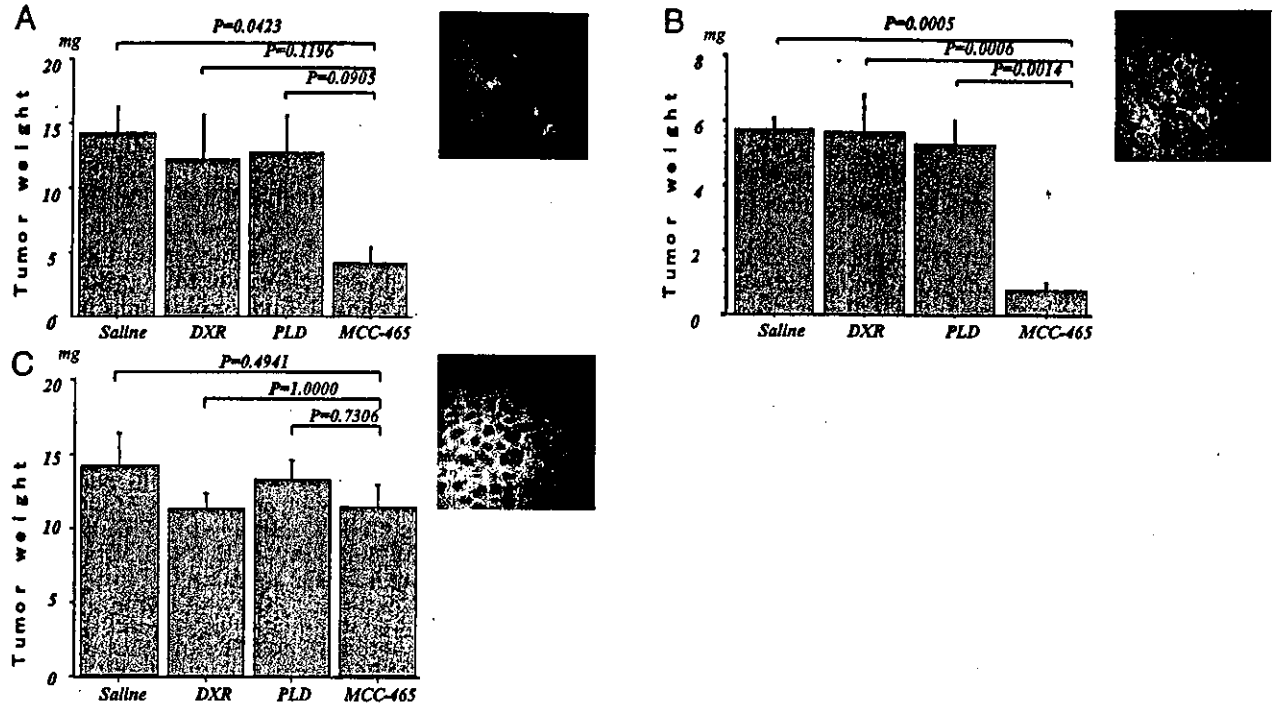

Fig. 3. The *in vivo* antitumor activities of DXR, PLD, and MCC-465 in athymic mice bearing WiDr-Tc (A), SW837 (B), or Caco-2 (C) xenograft. Treatment began 1 day after implantation and was repeated on days 8 and 15. On day 22, mice were sacrificed and tumors were excised and weighed. Bars: means±SE. n=6 except for MCC-465-treated group (n=5) of WiDr-Tc (A). Immunohistochemistry of each tumor xenograft with GAH is shown in the corresponding inset. Red fluorescence denotes GAH-positive.

DXR has been used clinically for the treatment of various kinds of cancer, including breast and ovarian cancer, and hematologic malignancies such as malignant lymphoma. The major side effects of DXR are myelosuppression and cumulative cardiotoxicity. Because DXR is cleared from the blood rapidly after conventional intravenous injection, only a very small amount of the injected DXR reaches the tumor tissues, while the vast majority is distributed to normal tissues and consequently damages normal organs. Further, at the cellular level, P-glycoprotein and multidrug resistant protein can mediate drug resistance. Drug pumping by these proteins across the plasma membrane results in lower intracellular DXR concentrations. To improve these issues, the only alternative to conventional injections has been prolonged infusional treatment until recently. An infusional DXR regimen, etoposide, vincristine, and DXR over 96 h with bolus cyclophospamide and oral prednisone (EPOCH), was highly effective in patients who had previously received most or all of the same drugs, and produced durable remission of relapsed and resistant lymphoma.[15]

Several different formulations of DXR have been investigated to improve its delivery to tumors and to decrease the toxicity, especially cardiotoxicity. One of the most investigated formulations is PLD and recently a commercial formulation, "Doxil" (Alza Corporation), has been approved by the US Food and Drug Administration (FDA) for the treatment of Kaposi's sarcoma and ovarian cancer. Pharmacological studies with PLD demonstrated that it resulted in the prolonged presence of DXR in the blood, like continuous infusion, and it appeared to have less cardiotoxicity due to a difference in tissue distribution from that of conventional DXR.[3-6,16] The density and permeability of tumor vasculature are generally increased and the development of the lymph system as a drainage system of extravasated molecules is poor in comparison with that of normal tissue.[9-11] Therefore, PLD can extravasate through the leaky microvessels and is retained in the tumor tissue over a prolonged period. Several phase II and III clinical trials of "Doxil" have demonstrated comparable activity and reduced toxicity in comparison with DXR, in particular in AIDS-related Kaposi's sarcoma,[17,18] soft tissue sarcoma,[19,20] ovarian cancer,[21-23] and breast cancer.[24]

PLD accumulated in cancer xenografts to a greater extent than DXR, but it still was not able to show significant antitumor activity in comparison with a saline control in our study using xenografted colorectal carcinoma. The *in vitro* cytotoxicity of DXR against human colorectal cancer cell lines is ten to hundreds of times lower than against human gastric or breast cancer cell lines. "Doxil" is considered to be too stable a liposomal formulation for efficient release of free DXR into tumor tissue. As a result, accumulation and retention of DXR in the tumor tissues following "Doxil" injection are not sufficient to suppress tumor growth. Indeed, in a clinical trial of "Doxil" for colorectal cancer, no evidence of efficacy was seen by objective measurement of tumor size by imaging.[25]

Use of monoclonal antibodies (mAbs) with specificity for tumor-associated markers is a relatively new and exciting modality in cancer therapy. Such mAbs may have an adequate antitumor activity when used alone, and they can also be used to deliver conjugated cytotoxic agents, such as chemotherapeutic drugs, toxins, and radionucleotides, to the tumors. Several mAbs have already been entered into clinical trials. Herceptin[26,27] and Rituxan[28-30] have been approved by the FDA.

We here examined the effect of cancer-specific-antibody conjugation onto PLD on the antitumor efficacy against human colorectal cancers using the SRC model, in which many human tumors grow well and retain the morphology and characteristics of the parent tumors.[13] In agreement with our previous results using a stomach cancer cell line in SRC and SC (subcutaneous) models,[12] MCC-456 showed strong antitumor activity against GAH-reactive colorectal cancers compared to free DXR or PLD.

The monoclonal antibody, GAH, recognizes a cell surface

antigen, thought to be linked to a cytoskeletal component. The positive reaction rate of GAH to human colorectal cancer tissues was approximately 90%, when evaluated in comparison with the staining intensity of the epithelial cells of adjacent normal glands. The biological significance of the antigen recognized by GAH is so far unknown, since there was no statistically significant difference between carcinomas graded by GAH reactivity in terms of clinical staging, tumor histological grading, or OS (Table 1).

No positive staining was observed in various normal tissues, the GI tract, liver, lung, uterine cells, thyroid gland, and blood cells, that were examined previously. GAH does not possess antitumor activity itself, but might induce internalization of liposomal DXR into the cytoplasm,[12] where it can exhibit antiproliferative activity. Though there were no difference in accumulation into tumor tissue between MCC-465 and PLD, only MCC-465 stood a better chance of binding to GAH-positive cancer cells. In fact, *in vivo*, MCC-465 had better selectivity to target GAH-positive xenografts than did PLD. In contrast, in GAH-negative xenografts, MCC-465 and PLD did not show any antitumor activity compared to free DXR (Fig. 3). There may be other advantages of immunoliposomes, as the internalization into cancer cells may also bypass drug efflux mechanisms on the plasma membrane of DXR-resistant cancer cells. For example, Suzuki *et al.* reported that anti-transferrin receptor antibody immunoliposomes could modulate DXR resistance in human leukemia cells.[31] Such immunoliposomal formulations may be useful for endocytotic internalization of other chemotherapeutic agents or genes.

We concluded that MCC-465 exhibited an enhanced antitumor activity towards GAH-positive xenografts due to its ability of specific binding to and internalization into cancer cells. Although "Doxil" or DXR itself exhibited no activity against colorectal cancer in clinical study, our results suggest that MCC-465 improves the antitumor activity against colorectal cancer. Thus, we intend to start a clinical trial using MCC-465 against colorectal cancer.

This work was supported partially by Grants-in-Aid from the Ministry of Health, Labour and Welfare of Japan and by Mitsubishi Pharma Corporation. We thank Miss M. Araake and Mrs. H. Koike for their technical assistance. We also thank Mrs. K. Shiina, Miss H. Orita, and Miss M. Okano for their secretarial assistance.

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Cancer Chemother Pharmacol (2004) 54 (Suppl 1): S78–S82
DOI 10.1007/s00280-004-0891-1

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New approaches for pancreatic cancer in Japan

Published online: 14 August 2004

Abstract Pancreatic cancer is the fifth leading cause of cancer-related mortality in Japan, with an estimated annual incidence rate of approximately 20,000 cases. Even in patients with resectable disease, the long-term outcome remains unsatisfactory due to early recurrence after resection. However, surgical resection has offered the only curative strategy for pancreatic cancer. Currently available chemotherapeutic agents have little impact on survival, although the development of gemcitabine has renewed interest in clinical research for pancreatic cancer. To further improve the prognosis of patients with pancreatic cancer, the development of more effective nonsurgical treatment is essential. Studies to identify more effective treatments, such as chemotherapy, interventional therapy and gene therapy, are ongoing in Japan. The expanding understanding of molecular and genetic biology should facilitate research to develop novel molecular-targeted agents and to establish individualized therapy regimens for this disease.

Keywords Pancreatic cancer · Chemotherapy · Gemcitabine · Gene therapy

This work was presented at the 19th Bristol-Myers Squibb Nagoya International Cancer Treatment Symposium, "State of the Arts for Digestive Organs", 14–15 November 2003, Nagoya, Japan.

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Introduction

Pancreatic cancer is the fifth leading cause of cancer-related mortality in Japan. The estimated annual incidence is approximately 20,000 cases, which is similar to its mortality [26]. Of all the treatment modalities for pancreatic cancer, only resection offers the opportunity for cure. However, because of local extension and/or metastatic disease, only a small minority of pancreatic cancer patients are candidates for resection with curative intent. Moreover, even for these selected patients, the prognosis remains unsatisfactory because of postoperative recurrence, indicating that surgery alone has limited value in the treatment of pancreatic cancer. Accordingly, to improve the overall survival of patients with pancreatic cancer, there is an urgent need to develop effective nonsurgical treatment for this disease. Various studies have been conducted to identify more effective nonsurgical treatments for pancreatic cancer in Japan. This review focuses on new approaches for chemotherapy in patients with advanced pancreatic cancer, and introduces other approaches including nonmyeloablative allogeneic stem cell transplantation and gene therapy.

Fluoropyrimidine-based chemotherapy in Japan

Of all chemotherapeutic drugs, the thymidylate synthase inhibitor fluorouracil (5-FU) has been the most extensively evaluated and most widely used agent for pancreatic cancer in Japan. Since the results with this agent remain poor, with reported response rates reaching 20% [17], there have been various attempts at biochemical modulation to enhance the antitumor activity of 5-FU through different agents. In Japan, sequential administration with methotrexate and 5-FU has been examined, but the antitumor activity of this regimen appears to be only marginal [9]. UFT is an orally administered drug developed in Japan that is a combination of tegafur, a prodrug of 5-FU, and uracil,

a competitive inhibitor of dihydropyrimidine dehydrogenase. Unfortunately, clinical trials of this agent have demonstrated little superiority in therapeutic effect to 5-FU alone against advanced pancreatic cancer [22, 31].

S-1 is an oral anticancer drug, which consists of tegafur (FT), 5-chloro-2,4-dihydroxypyridine (CDHP), and potassium oxonate (Oxo). The drug was developed in Japan to improve the tumor-selective toxicity of 5-FU by two biochemical modulators, CDHP and Oxo. CDHP is a competitive inhibitor of dihydropyrimidine dehydrogenase involved in degradation of 5-FU, and maintains efficacious 5-FU concentrations in plasma and tumor tissues. Oxo, a competitive inhibitor of orotate phosphoribosyltransferase, inhibits phosphorylation of 5-FU in the gastrointestinal tract and reduces the serious gastrointestinal toxicity of 5-FU. S-1 has already demonstrated a potent antitumor effect in various solid tumors in clinical studies [7, 11, 12, 16, 25, 27]. We conducted an early phase II study of S-1 in patients with metastatic pancreatic cancer [19]. This study showed promising results with a 21% response rate in 19 evaluable patients and a manageable toxicity profile of this agent. We are conducting a multi-institutional late phase II study of S-1 for metastatic pancreatic cancer to confirm these results.

There has been hope that improved therapeutic results might be obtained with 5-FU-based multiagent chemotherapy, since several agents having at least some activity have been identified. Cisplatin has been the most extensively used agent as a potential modulator of 5-FU, and has itself demonstrated some antitumor activity against pancreatic cancer. The combination of continuous infusion of 5-FU and bolus administration of cisplatin has been found to have limited antitumor activity, with only an 8% response rate in 37 Japanese patients [15]. With this treatment, 4 (21%) of 21 patients obtained remarkable symptom relief [20]. Based on laboratory data suggesting a profound schedule dependency for the cytotoxicity of this combination, Tsuji and colleagues conducted a phase II trial of continuous-infusion 5-FU and low-dose consecutive cisplatin in 39 patients with advanced pancreatic cancer [30]. 5-FU (160 mg/m^2 per day) was continuously infused over 24 h for seven consecutive days and cisplatin (3 mg/m^2 per day) was administered over 30 min for 5 days followed by a 2-day rest period, every 4 weeks. The objective response rate was 28.2%, with a clinical benefit response rate of 48.7% and a median survival time of 6.5 months.

Most studies of 5-FU-based multiagent chemotherapy have documented little reproducible impact on patient survival, while all of these regimens exhibit great toxicity. Takada and coworkers failed to demonstrate a survival benefit for combination chemotherapy consisting of 5-FU, doxorubicin and mitomycin for Japanese patients with unresectable pancreatic and biliary tract cancer compared to palliative surgery alone [29]. Based on the results to date, 5-FU-based multiagent chemotherapy cannot be recommended outside clinical trials.

Chemotherapy using gemcitabine

Gemcitabine is a deoxycytidine analog that is capable of inhibiting DNA replication and repair. Gemcitabine has the potential for great activity against various solid tumors including pancreatic cancer. This is because of gemcitabine's prolonged inhibition of both cell synthetic function and progression through the cell cycle. In a randomized trial comparing gemcitabine with 5-FU, gemcitabine showed significantly better results in terms of clinical benefit and survival [3]. Accordingly, gemcitabine has been accepted as first-line chemotherapy for advanced pancreatic cancer. In the phase I trial conducted in Japan before this randomized trial, the recommended dose schedule of gemcitabine was 800 mg/m^2 weekly ×3 followed by 1 week of rest, with leukocytopenia as the dose-limiting toxicity [28]. However, in most trials of gemcitabine for pancreatic cancer including the previous randomized study, a dose of 1000 mg/m^2 has been employed and approved in Western countries. Therefore, we conducted a phase I trial to confirm the tolerability of a weekly schedule of gemcitabine at a dose of 1000 mg/m^2 in Japanese patients with advanced pancreatic cancer [18]. This study showed a low incidence of dose-limiting toxicity, suggesting that gemcitabine at 1000 mg/m^2 weekly ×7 followed by 1 week rest and weekly ×3 every 4 weeks may be tolerated in Japanese patients with advanced pancreatic cancer. In this trial, a partial response was obtained in 2 (18%) of the 11 enrolled patients with metastatic pancreatic cancer and a clinical benefit response was achieved in 2 (29%) of the 7 evaluable patients. Based on the consistency in response and toxicity of this study with those of previous Western trials, gemcitabine was approved in Japan for the treatment of pancreatic cancer in 2001.

Despite worldwide agreement on the role of gemcitabine as a first-line treatment in advanced pancreatic cancer, only a minority of patients obtain clear benefits such as symptom relief and prolongation of survival from the administration of gemcitabine. Accordingly, it is important to establish effective methods for estimating individual drug response and toxicity. We are currently conducting a pharmacogenomics study for gemcitabine to identify polymorphisms of genes encoding drug-metabolizing enzymes and membrane-transporter proteins for gemcitabine and its metabolites, and their correlation with pharmacokinetics, toxicity and tumor response in pancreatic cancer patients. In this study, evidence for functional single-nucleotide polymorphisms responsible for gemcitabine metabolism is accumulating. This gene-based information has the potential to aid in the establishment of individualized therapy regimens using gemcitabine for pancreatic cancer.

Based on preclinical and clinical data showing the favorable antitumor effects of gemcitabine in combination with other cytotoxic agents, additional trials of gemcitabine-based regimens including gemcitabine plus S-1 are in progress in Japan.

Other new agents

Several novel chemotherapeutic agents developed in Japan, such as irinotecan, exatecan, UCN-01, NK911, capecitabine and S-1, have been evaluated in clinical trials for pancreatic cancer in Japan and/or other countries. It is hoped that improved therapeutic results might be obtained using these agents either singly or in combination with gemcitabine. This section focuses on irinotecan and NK911, clinical trials of which are ongoing for pancreatic cancer patients in Japan.

Irinotecan, a semisynthetic, water-soluble derivative of the plant alkaloid camptothecin, induces antitumor activity by inhibition of topoisomerase I. The single-agent antitumor activity of irinotecan in pancreatic cancer has been demonstrated in two phase II studies [24, 33]. In the first study conducted in Japan, administration of irinotecan at 100 mg/m^2 weekly or 150 mg/m^2 every other week to previously untreated patients resulted in a response rate of 11% in the 35 assessable patients treated [24]. In the second study, conducted by the European Organization for Research and Treatment of Cancer (EORTC), an irinotecan regimen of 350 mg/m^2 every 3 weeks induced partial responses in 9% of the 32 assessable patients [33]. A confirmatory phase II study is now underway in Japan. While no significant survival improvement with the combination of irinotecan and gemcitabine over gemcitabine alone has been reported recently [23], this agent may have the potential to be used in gemcitabine-refractory patients.

A new agent, developed based on the pathobiology of pancreatic cancer, is also being studied in a clinical trial for treatment of this disease. NK911 is a doxorubicin-encapsulated polymeric micellar nanoparticle [10]. The polymeric micelle carrier of NK911 consists of a block copolymer of polyethyleneglycol and polyaspartic acid. Polyethyleneglycol is expected to be in the outer shell of the micelle. NK911 has a highly hydrophobic inner core, and therefore can entrap a sufficient amount of doxorubicin. After the NK911 is extravasated from the tumor vessels, doxorubicin is released from NK911. It is suggested that pegylated liposomal doxorubicin (known as Doxil) can deliver doxorubicin to a solid tumor, via the enhanced permeability and retention (EPR) effect, more efficiently than NK911. This is because pegylated liposomal doxorubicin is more stable in the bloodstream. However, it is expected that NK911 can distribute more doxorubicin into cancer cells distant from the tumor vessel than can pegylated liposomal doxorubicin, once NK911 is extravasated from the tumor vessel. It is, therefore, suggested that NK911 may be more effective against cancers where the tumor vessel network is rough due to an abundant collagen-rich matrix, e.g. pancreatic cancer. In a phase I trial, NK911 was well tolerated and produced only moderate nausea and vomiting at myelosuppressive dosages. A partial response was obtained in one patient with gemcitabine refractory pancreatic cancer [13]. A phase II study of NK911 is ongoing in Japan.

A novel arterial infusion chemotherapy

Homma and coworkers have reported a novel arterial infusion chemotherapy for advanced pancreatic cancer [8]. To restrict the blood flow into the pancreas, the peripancreatic blood vessels were embolized superselectively with microcoils. The catheter tip for continuous arterial infusion of 5-FU and cisplatin is placed in the splenic artery just proximal to the branching of the great pancreatic artery for treatment of the primary tumor, and in the common hepatic artery for treatment of metastatic liver lesions. In 31 patients with advanced pancreatic cancer, 2 achieved a complete response and 16 showed a partial response. The median survival period of all patients was 18.3 months. They concluded that this treatment is effective against both primary tumor and metastatic lesions in unresectable pancreatic cancer patients.

Other approaches in Japan

Allogeneic stem-cell transplantation has been proven to have potent antitumor effects not only in patients with hematologic malignancies but also in those with solid tumors [6, 32]. Successful nonmyeloablative allogeneic peripheral blood stem-cell transplantation has been reported in patients with metastatic renal cell carcinoma, and the results with this treatment are consistent with a graft-versus-tumor effect [4, 5]. Omuro and colleagues described a patient who showed continuous regression of unresectable pancreatic tumor following nonmyeloablative allogeneic peripheral blood stem-cell transplantation, which was considered to be attributed to a graft-versus-tumor effect [21]. Based on the results of the report and those for other malignancies, clinical trials of nonmyeloablative allogeneic peripheral blood stem-cell transplantation are being conducted with pancreatic cancer patients in several institutes in Japan.

Increased understanding of the biology of pancreatic cancer could provide the potential to develop entirely novel treatment options. One innovative approach for therapy is a combination of interferon α and antisense K-ras [14]. We have shown that interferon α gene transduction into pancreatic cancer cells induces growth suppression and cell death in the cells; an effect that appears to be more prominent when compared with other types of cancers and normal cells. Another strategy developing for pancreatic cancer targets its characteristic genetic aberration, K-ras point mutation. It has been reported that the expression of antisense K-ras RNA significantly suppresses the growth of pancreatic cancer cells [1, 2]. When these two gene therapy strategies are combined, the expression of antisense K-ras

RNA significantly enhances interferon α-induced cell death (1.3- to 3.5-fold), and suppresses subcutaneous growth of pancreatic cancer cells in mice. Because the 2′,5′-oligoadenylate synthetase/RNaseL pathway, which is regulated by interferon and induces apoptosis of cells, is activated by double-strand RNA, it is plausible that the double-strand RNA formed by antisense and endogenous K-ras RNA enhances the antitumor activity of interferon α. This study suggested that the combination of interferon α and antisense K-ras RNA is a promising gene therapy strategy against pancreatic cancer.

Conclusion

Pancreatic cancer is a major cause of cancer-related mortality in Japan. At present, nonsurgical therapy is of limited value in the treatment of pancreatic cancer, but various approaches are being attempted that we hope will result in improved patient survival. The evolving understanding of molecular and genetic biology should facilitate research to develop novel target-based agents and to establish individualized therapy regimens for this disease.

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British Journal of Cancer (2004) 91, 1775–1781

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Phase I clinical trial and pharmacokinetic evaluation of NK911, a micelle-encapsulated doxorubicin

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NK911 is a novel supramolecular nanocarrier designed for the enhanced delivery of doxorubicin (DXR) and is one of the successful polymer micelle systems to exhibit an efficient accumulation in solid tumours in mice. The purpose of this study was to define the maximum-tolerated dose (MTD) and dose-limiting toxicities (DLTs) of NK911 and to evaluate its pharmacokinetic profile in man. NK911 was given intravenously to patients with solid tumours every 3 weeks using an infusion pump at a rate of 10 mg DXR equivalent min^{-1}. The starting dose was 6 mg DXR equivalent m^{-2}, and the dose was escalated according to the accelerated titration method. A total of 23 patients participated in this study. Neutropenia was the predominant haematological toxicity, and grade 3 or 4 neutropenia was observed at doses of 50 and 67 mg m^{-2}. Common nonhaematological toxicities were mild alopecia, stomatitis, and anorexia. In the dose identification part of the study, DLTs were observed at a dose of 67 mg m^{-2} (grade 4 neutropenia lasting more than 5 days). Thus, this dosage level was determined to be the MTD. Infusion-related reactions were not observed in any cases. The $C_{5\,min}$ and area under the concentration curve parameters of NK911 exhibited dose-dependent characteristics. Among the 23 patients, a partial response was obtained in one patient with metastatic pancreatic cancer. NK911 was well tolerated and produced only moderate nausea and vomiting at myelosuppressive dosages. The recommended phase II dose was determined to be 50 mg m^{-2} every 3 weeks.

British Journal of Cancer (2004) **91**, 1775–1781. doi:10.1038/sj.bjc.6602204 www.bjcancer.com
Published online 12 October 2004

Keywords: DDS; pharmacokinetics; NK911; micelle; doxorubicin; EPR effect

Agents categorised as drug delivery systems (DDSs) have been developed based on the characteristic macroscopic features of solid tumours, such as hypervascularity, an irregular vascular architecture, the presence of several vascular permeability factors stimulating extravasation within the cancer, and the relatively poor drainage of macromolecules and particulates from cancer tissue. These characteristics, which are unique to solid tumours, constitute the basis of the enhanced permeability and retention (EPR) effect (Matsumura and Maeda, 1986; Maeda *et al*, 2000). Macromolecules have long plasma half-lives because they are too large to pass through normal vessel walls unless they are trapped by the reticuloendothelial system (RES) in the cells of various organs. Such macromolecular agents can diffuse out of tumour blood vessels, reach the solid tumour tissue, and be retained for a long period because of the EPR effect.

To maximise the EPR effect, several techniques have been developed to modify the structures of drugs and to construct drug carriers. Doxil is comprised of doxorubicin (DXR) encapsulated in STEALTH™ liposomes, which are composed of a phospholipid bilayer with surface-bound methoxypolyethyleneglycol. Doxil recently received the US Food and Drug Administration's (FDA) approval for use in the treatment of Kaposi sarcoma or ovarian cancer after the clinical benefits of this drug were clearly shown in recent clinical trials (Muggia *et al*, 1996; Stewart *et al*, 1998; Gordon *et al*, 2001).

Polymeric micelles have also been utilised as a drug carrier system. The original form of micellar DXR contained two trapped components: a DXR monomer and a DXR dimer in the inner core (Yokoyama *et al*, 1990a, b). However, the lyophilised micelle containing DXR dimers became insoluble after long periods of storage. To improve the solubility of this drug carrier system, a new type of polymeric micelle containing only the DXR monomer, known as NK911, has been developed (Nakanishi *et al*, 2001). The DXR monomers, rather than the DXR dimers, were thought to play a major role in the antitumour activity of the original micellar DXR drug preparation. The DXR dimers, on the other hand, were thought to stabilise the drug's conformation. Thus, NK911, which only contains DXR monomers, is less stable in aqueous media than the original form of micellar DXR (Nakanishi *et al*, 2001; Tsukioka *et al*, 2002).

Both polyethyleneglycol (PEG)-liposomal and micellar DXR have longer plasma half-lives, accumulate in tumours more effectively because of the EPR effect, and exhibit a stronger

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Revised 26 August 2004; accepted 2 September 2004; published online 12 October 2004

antitumour activity than free DXR when administered in mice. Both the plasma area under the concentration–time curve (AUC) and the tumour AUC of NK911 are, however, lower than those of doxil because NK911 is less stable in the bloodstream than doxil (Working *et al*, 1994; Nakanishi *et al*, 2001). At this stage, however, we have no definitive idea as to which formulation exerts a superior antitumour activity *in vivo* because an evaluation of the activity *in vivo* cannot be based solely on the enhanced tumour AUC; several additional factors, including the efficiency of the free drug inside the formulation and the distribution of the free drug throughout the tumour tissue, must also be taken into consideration. Proper selection of the type of DDS formulation is likely to depend on the tumour vessel density of the tumour tissue. With this in mind, we conducted a phase I clinical trial on the use of NK911 in patients with solid tumours. The study was performed at the National Cancer Center Hospital, Tokyo, Japan. Our objectives were to assess the safety and toxicity profile of NK911 and to determine the maximum-tolerated dose (MTD), the phase II recommended dose, and the pharmacokinetics of NK911 in man.

PATIENTS AND METHODS

The study protocol was reviewed and approved by the Institutional Review Board of the National Cancer Center, Tokyo.

Therapeutic agent

Figure 1 shows the schematic structure of NK911, a DXR-entrapped polymeric micelle formulation. The polymeric micelle consists of a PEG–poly(aspartic acid) block copolymer conjugated with DXR. Polyethyleneglycol is believed to form the outer shell of the micelle, producing a 'stealth' effect that prevents NK911 from being captured by the RES. The DXR-conjugated poly(aspartic acid) chain is hydrophobic and is believed to form the hydrophobic inner core of the micelles in aqueous media. The hydrophobic inner core enables NK911 to entrap a sufficient amount of DXR. NK911 has a diameter of about 40 nm (Nakanishi *et al*, 2001).

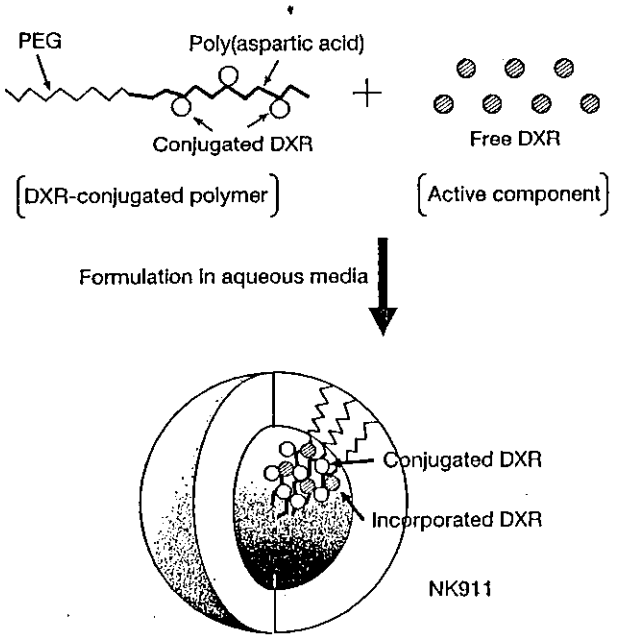

Figure 1 Schematic structure of NK911. A polymeric micelle carrier of NK911 consists of a block copolymer of PEG (molecular weight of about 5000) and poly(aspartic acid) (about 30 units). Polyethyleneglycol is believed to be the outer shell of the micelle. NK911 has a highly hydrophobic inner core, and therefore can entrap sufficient amounts of DXR.

Patients

Patients with metastatic or recurrent solid tumours refractory to conventional chemotherapy and for whom no effective therapy was available were eligible for enrolment in this study, provided that the following criteria were met: a histologically confirmed malignant tumour; a performance status of $\leqslant 2$; an age of $\geqslant 20$ and <75 years; a normal haematological profile (neutrophil count $\geqslant 2000\,\mu l^{-1}$, platelet count $\geqslant 100\,000\,\mu l^{-1}$, haemoglobin $\geqslant 8\,g\,dl^{-1}$); normal hepatic function (total bilirubin level $\leqslant 1.5\,mg\,dl^{-1}$, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) $\leqslant 2.5$ times the upper normal limit); normal renal function (serum creatinine $\leqslant 1.5\,mg\,dl^{-1}$); normal cardiac function (New York Heart Association (NYHA) classification of $\leqslant 1$); normal pulmonary function ($PaO_2 \geqslant 60$ mgHg); no chemotherapy within 4 weeks (6 weeks for nitrosourea or mitomycin C chemotherapy) of the administration of NK911; and a life expectancy of more than 2 months. Patients with any serious infection (including hepatitis B, hepatitis C, or HIV), symptomatic brain metastasis, and pre-existing cardiac disease (including congestive heart failure, myocardial infarction, or angina pectoris within 3 months) were ineligible for enrolment in the study. Patients were also excluded if they were pregnant or lactating or showed signs of gastrointestinal bleeding. Additionally, any patients whom the investigators considered ineligible were excluded. Written informed consent was obtained from all patients.

Drug administration

NK911 was dissolved in sterile phosphate-buffered saline for injection at room temperature at a concentration of 2 mg DXR equivalent ml^{-1}. NK911 solution was infused intravenously using an electric pump at a speed of 10 mg DXR equivalent min^{-1}.

Dosage and dose escalation

The starting dosage of NK911 was 6 mg DXR equivalent, which is one-tenth of the LD_{10} in rats. NK911 was administered once every 3 weeks, and the treatment was continued unless a severe adverse event or disease progression was observed. Dose escalation was performed according to the previously described accelerated titration method (Simon *et al*, 1997). Toxicity was graded from 1 to 4 using the NCI common toxicity criteria (version 12). Intrapatient dose escalation was not permitted. The dose-limiting toxicity (DLT) was confirmed in at least six out of 23 patients. The MTD was defined as the level at which three out of six patients experienced a DLT. The recommended dosage for a phase II trial was defined by the Efficacy and Safety Assessment Committee, based on the safety and efficacy results of this trial. The DLT was defined as grade 4 neutropenia lasting more than 5 days or an associated neutropenic fever of more than 38.5°C, a platelet count of less than $25\,000\,\mu l^{-1}$, or grade 3 or higher nonhaematological toxicity, with the exception of nausea, vomiting, appetite loss, constipation, and hyperglycaemia.

Pretreatment assessment and follow-up care

A complete medical history and physical examination, performance status evolution, complete blood cell count (CBC), blood chemistry, urinalysis, electrocardiogram (ECG), and a computed tomography (CT) examination or an upper gastrointestinal (GI) series were performed in each patient. Other examinations were

performed only in the presence of a specific clinical indication. Patients were physically examined everyday until the second administration of NK911; CBC and blood chemistry tests were performed on days 1, 2, and 4 and weekly thereafter. Electrocardiogram and ultrasonic cardiography studies were repeated prior to each administration of NK911. Tumour markers were also measured prior to each administration. Tumour response was evaluated according to the WHO guidelines (World Health Organization, 1979). A complete response (CR) was defined as the disappearance of the cancerous lesion(s), and a partial response (PR) was defined as a reduction of more than 50% of the sum of the bidimensional products when the results for two observations, separated by at least 4 weeks, were compared. Stable disease (SD) was defined as a reduction of less than 50% or a growth of less than 25% over a period of at least 4 weeks. Progressive disease (PD) was defined as a growth of more than 25%, the appearance of new malignant lesion(s), or the unequivocal worsening of other clinical evidence of malignancy.

High-performance liquid chromatography determination of DXR and its metabolites

Doxorubicin and its metabolites (doxorubicinol and aglycones) were extracted from human plasma and urine using Abselut NEXUS cartridges (Varian), pretreated with methanol (2 ml), 1% phosphoric acid (1 ml), and sodium phosphate buffer (2 ml of 25 mmol l^{-1} NaH_2PO_4 (pH 4) containing 0.2% sodium lauryl sulphate). Daunorubicin was added to the plasma (0.5 ml) or urine (0.1 ml) samples as an internal standard. The diluted plasma or urine was applied to the above-mentioned cartridges and then washed with distilled water (1 ml) or 40% methanol (2 ml) and eluted with methanol (2 ml). The eluate was evaporated to dryness at 40°C under a stream of nitrogen. The residue was dissolved in sodium phosphate buffer (200 μl of 25 mmol l^{-1} NaH_2PO_4 (pH 4) containing 0.2% sodium lauryl sulphate/acetonitrile = 75/25, v v^{-1}), and 50 μl of the resulting solution was injected onto the analytical column. The analyses of DXR and its metabolites in plasma and urine samples were performed using high-performance liquid chromatography (HPLC) with fluorescence detection. The DXR concentrations determined in the present Phase I study represented the total drug concentrations (both micelle-entrapped and nonencapsulated). The HPLC system (HP1100 series, Hewlett Packard) consisted of a binary pump, an automatic sample injector, a reversed-phase CAPCELL PAK C_8 (2.0 mm i.d. × 150 mm, 5 μm, SHISEIDO), and a fluorescence detector with excitation and emission wavelengths of 500 and 550 nm, respectively. A gradient elution was employed, consisting of 25 mmol l^{-1} NaH_2PO_4 (pH 4) containing 0.2% sodium lauryl sulphate/acetonitrile at ratios of 75/25 to 55/45 (v v^{-1}), at a flow rate of 0.2 ml min^{-1}.

Pharmacokinetic analysis

The pharmacokinetic parameters were calculated after fitting the data to a three-compartment model using the Win Nonlin program. The plasma DXR concentration, $C(t)$, at each time (t), was computed using the equation

$$C(t) = D/T\,[A/\alpha(e^{\alpha T}-1)\,e^{-}\alpha\,(t+T) + B/\beta\,(e^{\beta T}-1)\,e^{-\beta(t+T)} + C/\gamma\,(e^{\gamma T}-1)\,e^{-\gamma(t+T)}]$$

where D is the dose and T is the infusion time.

The AUC, total clearance (CL_{tot}), volume of distribution at steady state (V_{ss}), area under the first moment curve (AUMC), volume of distribution of the central compartment ($V1$), half-lives ($t_{1/2\alpha}$, $t_{1/2\beta}$, $t_{1/2\gamma}$), and mean residence time (MRT) of DXR were calculated using the equations listed below.

$$\mathrm{AUC} = A/\alpha + B/\beta + C/\gamma\ (\mathrm{ng\,h\,ml^{-1}})$$
$$\mathrm{CL_{tot}} = \mathrm{dose/AUC}\ (\mathrm{ml\,min^{-1}\,kg^{-1}})$$
$$V_{ss} = \mathrm{dose} \times \mathrm{AUMC}/(\mathrm{AUC})^2\ (\mathrm{l\,kg^{-1}})$$
$$V1 = \mathrm{dose}/A + B + C\ (\mathrm{l\,kg^{-1}})$$
$$T_{1/2} = 0.693/k\ (\mathrm{h})\ (k = \alpha, \beta, \gamma)$$
$$\mathrm{MRT} = \mathrm{AUMC/AUC\ (h)}$$

RESULTS

Patient characteristics

A total of 23 eligible patients were recruited for the study. Their clinical characteristics are shown in Table 1. With the exception of one patient with a leiomyosarcoma, all the patients had received chemotherapy prior to enrolment in the study. Prior therapies ranged from 0 to 6 regimens of chemotherapy. None of the patients had received anthacycline chemotherapy. As a deviation from the ordinary phase I trial protocol, patients with metastatic pancreatic cancer were deliberately recruited, based on the previously described characteristics of NK911. All patients were included in the safety and response analyses.

Dosing

Dosage escalation started at 6.0 mg DXR equivalent m^{-2} and was increased up to 67 mg DXR equivalent m^{-2}. Infusion time ranged from 58 s to 12 min and 15 s depending on the absolute dosage of NK911. In total, 63 administrations were performed in 23 patients. A total of 14 patients received more than two administrations. The maximum number of treatments was 10 courses at level 3; the average number of administrations at all levels was 2.7 courses. Up until the third level, grade 2 toxicity was not observed during the first course of chemotherapy. According to the original protocol, the dosage of NK911 should have been doubled for each escalation. However, the safety committee recommended that the dosage should be raised by 50%, instead of 100%, at level 4 and that a modified Fibonacci escalation method should be implemented. Therefore, we recruited three patients at this dosage level and restarted the dose identification study using a modified Fibonacci method.

Haematological toxicity

As shown in Table 2, significant myelosuppression was not observed up to level 4. At level 5, two patients died because of tumour progression. The progression of the disease was confirmed

Table 1 Patient characteristics

Number of patients	23
Male/female	15/8
Age (years)	
Median	61.5
Range	48–72
ECOG PS	
Median	1
0	6
1	17
Prior treatment chemotherapy regimens	
Median	2
Range	0–6

Table 2 Haematological toxicity: cycle 1

		Leucocytopenia				Neutropenia				Thrombocytopenia			
		Grade				Grade				Grade			
Dose level ($mg\,m^{-2}$)	n	1	2	3	4	1	2	3	4	1	2	3	4
6	1	0	0	0	0	0	0	0	0	0	0	0	0
12	1	0	0	0	0	0	0	0	0	0	0	0	0
24	1	1	0	0	0	1	0	0	0	0	0	0	0
36	3	1	0	0	0	0	1	0	0	0	0	0	0
50	11	3	5	3	0	1	3	5	2	2	0	0	0
67	6	0	1	5	0	0	0	0	6	3	0	1	0
Total	23	5	6	8	0	2	4	5	8	5	0	1	0

by autopsy in both cases. Since these two patients could not be assessed for safety, an additional two patients were enrolled at level 5. Two patients developed grade 3 neutropenia and one patient developed grade 4 neutropenia. However, none of the patients developed a DLT at this dosage level. At level 6, all six patients who entered at this level developed grade 4 neutropenia; three of the six patients appeared to have acquired a DLT (grade 4 neutropenia lasting for more than 5 days). Based on these results, level 6 was considered to be the MTD, with neutropenia as the DLT. Since a dosage of $50\,mg\,m^{-2}$ was considered to be the recommended dosage for phase II studies, an additional six patients were enrolled at a dosage of $50\,mg\,m^{-2}$; one of these six patients developed a DLT in the form of febrile grade 4 neutropenia.

Nonhaematological toxicity

The NK911 injection was generally uneventful and well tolerated. The major nonhaematological toxicities were nausea, vomiting, and anorexia. All of these toxicities were controllable. Severe mucositis and skin toxicity in the form of hand-foot syndrome did not occur. Alopecia was also mild, and only three patients experienced grade 2 alopecia after repeated doses of NK911 at levels 5 and 6 (Table 3). A few patients at level 5 or 6 developed a grade 2 elevation in AST or ALT, but these changes were transient. No pain or local toxicity in the area of injection was observed in any of the patients treated with NK911, except in one patient treated at level 2. No infusion-related reactions were observed in any cases; such reactions sometimes occur during liposomal drug administration. Cardiac function was monitored at baseline and serially in all patients enrolled in the study. Clinical congestive heart failure did not occur. The left ventricular ejection fraction did not decrease significantly from the baseline level in any of the patients except for one patient treated at level 5 whose LVEF decreased to 45% after one cycle. Since this patient was transferred to another hospital, this change could not be confirmed.

Pharmacokinetics

The plasma concentrations of DXR after the intravenous infusion of NK911 were determined in all of the patients enrolled in the present phase I study; the results are shown in Figure 2. The $C_{5\,min}$ and AUC parameters increased at doses between 6 and $67\,mg\,m^{-2}$, as shown in Figure 3. The peak plasma concentrations ranged from $586.8\,ng\,ml^{-1}$ at a dose level of $6\,mg\,m^{-2}$ to $6188.2\,ng\,ml^{-1}$ at a dose level of $67\,mg\,m^{-2}$.

The pharmacokinetic parameters are summarised in Table 4. The initial distribution half-life ($t_{1/2\alpha}$) was about 5–8 min, $t_{1/2\beta}$ was 1.6–4.7 h, and $t_{1/2\gamma}$ was 29.4–241.4 h. The $V1$ was 0.116 – $0.1831\,kg^{-1}$. The CL_{tot} was $3.9–9.8\,ml\,min^{-1}\,kg^{-1}$, and the V_{ss} was $11.7–52.31\,kg^{-1}$. No significant differences in these parameters were seen among patients, with the exception of patients receiving doses of 6, 12, and $24\,mg\,m^{-2}$. These observations may be attributed to the fact that DXR was difficult to detect at 168 h after dosing at levels of below $24\,mg\,m^{-2}$, possibly resulting in an underestimation or an overestimation of the tails of the clearance curve. The half-lives ($t_{1/2\alpha}$, $t_{1/2\beta}$, and $t_{1/2\gamma}$) were longer for NK911 than for free DXR (Mross *et al*, 1988). The AUC of NK911 was two-fold larger than that of free DXR at a dose of $50\,mg\,m^{-2}$. The V_{ss} and CL_{tot} of NK911 were lower than those of free DXR. As expected, the parameters for NK911 were more than 100-fold lower than those previously described for doxil (Gabizon *et al*, 1994).

The cumulative urinary excretion rates of DXR and its metabolites (0–72 h) after the administration of NK911 were 7.1–16.6%, similar to those after the administration of free DXR.

Table 3 Nonhaematological toxicity

	Grade				
	1	2	3	4	Total
Nausea	10	2	3	0	15
Vomiting	5	2	1	0	8
Anorexia	11	3	3	0	17
Fatigue	1	1	0	0	2
Stomatitis	5	0	0	0	5
Alopecia	12	0	—	—	12

There was no DLT regarding nonhaematological toxicities within this trial.

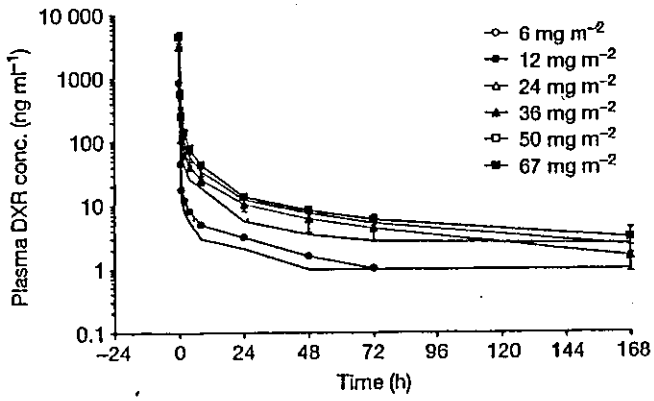

Figure 2 Mean plasma levels of doxorubicin following the intravenous administration of NK911 at dosages of 6, 12, 24, 36, 50, and $67\,mg\,m^{-2}$ in 23 patients.

Therapeutic response

At the time of the study's completion, eight patients (including one patient with colonic cancer and two patients with stomach cancer) had exhibited a stable disease for longer than 4 weeks (Table 5). A partial response was seen in one patient with metastatic pancreatic cancer who had been treated at a dosage level of 6; the size of the liver metastasis had decreased by more than 50%, compared to the baseline scan, in this patient (Figure 4A and B). The tumour marker (CA19-9 and CEA) levels in this patient had also decreased remarkably (Figure 4C). This patient had previously undergone gemicitabine chemotherapy. Initially, this patient received an NK911 dosage of 67 mg m^{-2}. The dosage was decreased to 50 mg m^{-2} for the second course, however, because the patient experienced grade 4 neutropenia. The antitumour response was maintained even after the dosage was reduced.

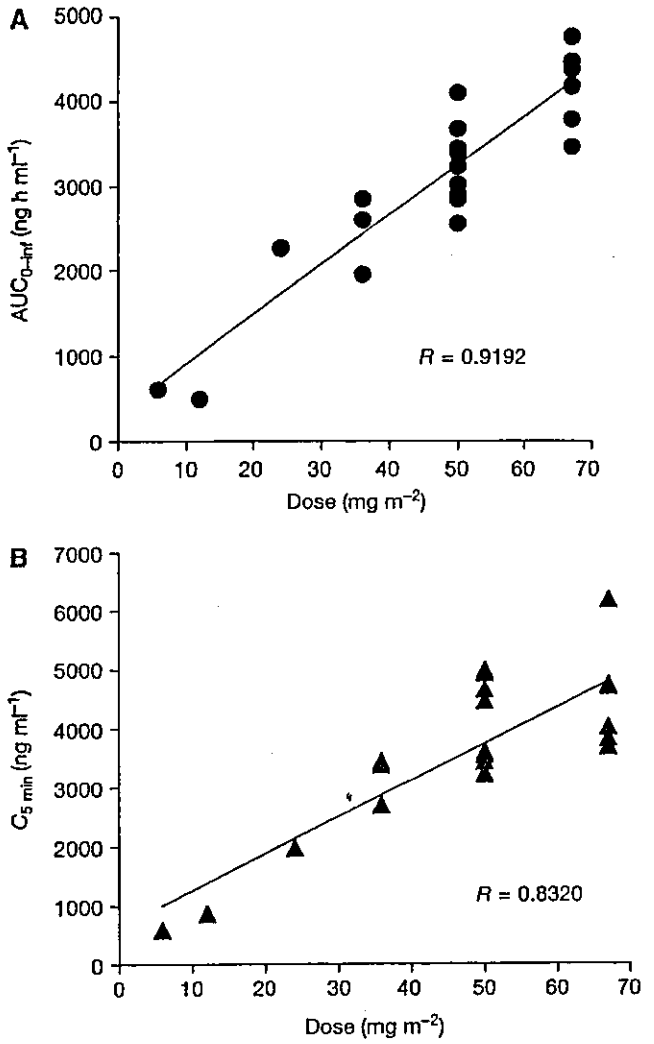

Figure 3 Correlations between dosage and AUC_{0-inf} (A) and $C_{5\,min}$ (B) after a single intravenous administration of NK911.

DISCUSSION

The utility of polymeric micelles in cancer chemotherapy was demonstrated in mice for the first time using DXR-incorporated polymeric micelles in the early 1990s (Yokoyama *et al*, 1990a, b, 1991). The original form of micellar DXR contained two entrapped components, DXR monomers and DXR dimers, in the inner core. The DXR dimers were thought to contribute to the stabilisation of the micellar DXR conformation. However, the DXR dimers in this formulation caused freeze-dried samples of micellar DXR to become water-insoluble after prolonged storage. To improve the solubility of micellar DXR, a new polymeric micellar preparation, NK911, containing only DXR monomers was created (Nakanishi *et al*, 2001).

In this phase I study, the toxicity spectrum of NK911 resembled that of free DXR: the DLT was neutropenia, and no adverse effects appeared other than those also encountered with the use of free DXR. Regarding nonhaematological toxicities, nausea and vomiting were mild. Mucositis was also rare and mild. No infusion-related reactions, which are sometimes seen in cases of liposomal drug administration (Uziely *et al*, 1995; Muggia *et al*, 1996; Stewart *et al*, 1998; Gordon *et al*, 2001), occurred in this trial. Ultimately, this phase I study showed that the recommended dosage of NK911 (50 mg m^{-2}) using a 3-week schedule was similar to the recommended dosage of free DXR (40–60 mg m^{-2}). In preclinical studies using several kinds of animals, the pharmacokinetics of NK911 differed from those of free DXR (Nakanishi *et al*, 2001).

Table 5 Antitumour activity

Primary tumour	*n*	PR	SD	PD
Pancreas	7	1	1	5
Colorectal	7	0	4	3
Oesophagus	3	0	1	2
Gall bladder	3	0	0	3
Stomach	2	0	2	0
Leiomyosarcoma	1	0	0	1

Table 4 Pharmacokinetic parameters

Dose (mg m^{-2})	*n*	$t_{1/2\alpha}$ (min)	$t_{1/2\beta}$ (h)	$T_{1/2\gamma}$ (h)	AUC_{0-inf} (ng h ml^{-1})	CL_{tot} (ml min^{-1} kg^{-1})	V_{ss} (l kg^{-1})	V_1 (l kg^{-1})	MRT_{0-inf}
NK911									
6	1	5.1	2.3	159.7	601.4	3.9	36.8	0.116	158.2
12	1	5.0	1.6	29.4	495.3	9.8	11.7	0.153	19.9
24	1	6.8	4.7	241.4	2257.7	46.1	52.3	0.163	210.9
36	3	6.6±0.5	2.8±0.0	54.8±4.8	2460.5±469.9	6.4±1.2	12.0±2.3	0.183.13±0.030	32.0±8.8
50	11	7.5±0.7	2.8±0.3	64.2±8.9	3262.7±425.2	6.7±1.1	14.9±3.6	0.167±0.035	37.0±8.2
67	6	8.1±1.1	2.9±0.5	73.6±21.4	4174.1±471.2	6.8±1.1	16.3±6.1	0.183±0.051	41.2±17.8
DXR[a]									
50	7	2.4±0.9	0.8±1.1	25.8±11.4	1620.3±1062.9	14.4±5.6	24±12	—	—
PLD[b]									
50	14	84	45.9	—	902000	0.02	0.08	—	62.7

[a]Cited from Mross *et al* (1998). [b]Cited from Gabizon *et al* (1994).

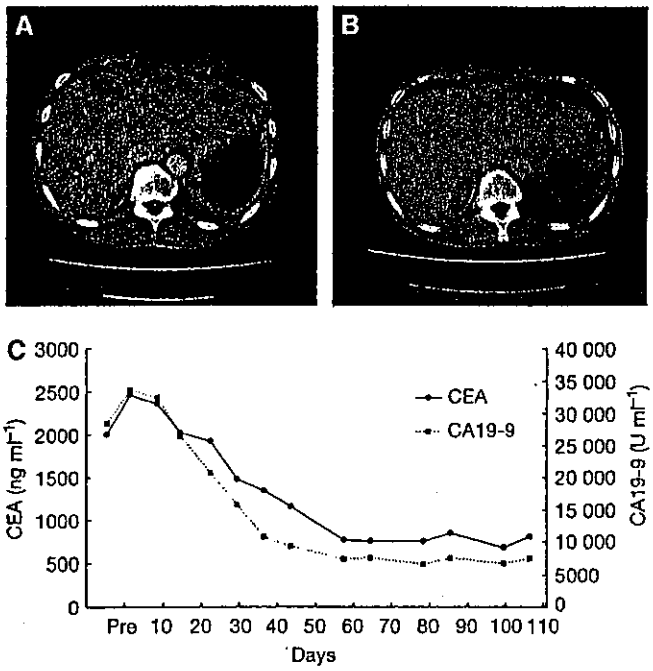

Figure 4 Serial CT scans of a 69-year-old male with pancreatic cancer who was treated with NK911 at an initial dosage level of 67 mg m^{-2} during the first course and at 50 mg m^{-2} after the second course. Changes in tumour marker levels are also shown. (**A**) Baseline scan showing a metastasis in the left lateral lobe. (**B**) Partial response, characterised by a more than 50% decrease in the size of the liver metastasis compared with the baseline scan. (**C**) Tumour markers, CA19-9 and CEA, decreased remarkably after treatment.

When compared with free DXR, NK911 exhibited longer half-lives ($t_{1/2\alpha}$, $t_{1/2\beta}$, and $t_{1/2\gamma}$), a lower CL_{tot}, and a larger AUC; these findings suggest that the circulation of NK911 in plasma was prolonged. Furthermore, the extent of the NK911 distribution in the tumour tissue was thought to differ from that of free DXR because of the smaller V_{ss} of NK911 compared with that of free DXR. The $V1$ of NK911 was smaller than the volume of extracellular fluid in humans, which may lead to a smaller distribution of NK911 in tumour tissue during the early phase of chemotherapy. Although the value of $V1$ in humans after the injection of free DXR has not been reported, the $V1$ of free DXR was two-fold larger than that of NK911 in a dog model and a different distribution in the early phase was observed (data not shown); a similar tendency in humans is expected. As mentioned above, the micelle-forming ability of NK911 seems to result in different physicochemical behaviours and a specific retention in plasma, compared with DXR, because NK911 circumvents the early distribution phase. The early pharmacokinetic phase may represent a very important stage in the overall behaviour of DXR in the body (Robert *et al*, 1987). Therefore, the inherent characteristic pharmacokinetics of NK911 in human subjects seems to be useful and significant for the enhancement of clinical responses to DXR.

Concerning the release of DXR from the conjugated block copolymer, the DXR concentrations in plasma were assessed after administering a DXR-conjugated polymer in a dog model. The released DXR concentration from the polymer was estimated to be 100-fold less than that of NK911 containing the same amount of DXR-conjugated polymer in dogs. Consequently, conjugated DXR is likely to have little effect on the plasma DXR concentration after the injection of NK911 in patients. When [^{14}C]DXR-conjugated polymer was intravenously administered to rats, the polymer was excreted via both urine and faeces (urine : faeces = 2 : 1). In the rat urine, several kinds of fragmented polymers derived from the DXR-conjugated polymer were observed, as well as a nonfragmented polymer. In rat faeces, only the fragmented polymers were observed, and [^{14}C]DXR was not seen in either urine or faeces. These results indicated that the DXR-conjugated polymer was excreted after extensive metabolism, but that the free DXR was hardly released from the polymer. Until now, the structure of the excreted high-molecular fragments could not be determined because of the difficulty in developing an analytical methodology for these molecular species. In the present clinical trial, which examined the urinary excretion of the physically trapped DXR in NK911, the excretion rate was similar to that of free DXR over a 72-h period. The present results suggest that the major route of excretion of DXR and its metabolites after NK911 injection in human subjects is likely biliary, the same as for DXR administration.

When compared with the historical data for doxil (Gabizon *et al*, 1994), the plasma AUC of NK911 was more than 100-fold lower than that of doxil, and the plasma clearance of NK911 was approximately 400-fold higher than that of doxil at a DXR equivalent dosage of 50 mg m^{-2}. These results indicate that NK911 is less stable in plasma than doxil, since the DXR dimers in the micellar DXR have not been included in NK911 for the reason described earlier. Thus, doxil appears to deliver DXR to solid tumours via the EPR effect more efficiently than NK911, since doxil is more stable in the bloodstream. However, the V_{ss} of NK911 at a dose of 50 mg m^{-2} was about 180-fold higher than that of doxil at the same dose level. This observation suggests that the distribution of DXR in tumour tissue may be wider in the case of NK911 administration compared to that of doxil, once each formulation extravasates from the tumour vessels. Therefore, to determine whether doxil is more effective for the treatment of solid tumours than NK911, several factors must be considered, including drug pharmacokinetics, pharmacodynamics, tumour vasculature, the tumour interstitium, the efficiency of drug release from the formulation, and the distribution of free drug throughout the tumour tissue.

In conclusion, the toxicity characteristics and antitumour activity of NK911 justify its continued clinical evaluation. A phase II clinical trial of NK911 for the treatment of metastatic pancreatic cancer is ongoing.

ACKNOWLEDGEMENTS

This study was supported by Nippon Kayaku Co. Ltd. We thank Drs K Kataoka and M Yokoyama for their scientific advice. We also thank Mrs K Shiina and Miss H Orita for their secretarial assistance.

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