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Reduction of the Side Effects of an Antitumor Agent, KRN5500, by Incorporation of the Drug into Polymeric Micelles

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For intravenous (i.v.) injection of a water-insoluble antitumor drug, KRN5500, we have successfully incorporated KRN5500 into polymeric micelles. In the present study, *in vitro* and *in vivo* antitumor activity against several human tumor cell lines and toxicity in mice of polymeric micelles incorporating KRN5500 (KRN/m) were evaluated in comparison with those of the prototype KRN5500. KRN/m was found to express similar antitumor activity to KRN5500 in the *in vitro* and *in vivo* systems. However, the vascular damage and liver focal necrosis observed with KRN5500 i.v. injection were not seen when KRN/m was administered i.v. Therefore, we expect that KRN/m will be superior to KRN5500 for clinical use and that the methodology of polymeric micelle drug carrier systems can be applied to other water-insoluble drugs.

Key words: DDS — Micelles — KRN5500

Although numerous anticancer agents have been developed, solid tumors in general still respond poorly to treatment. There is a need to find ways by which anticancer agents can be selectively targeted to tumors. Solid tumors generally possess the following pathophysiological characteristics: (a) hypervasculature; (b) incomplete vascular architecture; (c) secretion of 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.[1–5] These characteristics of solid tumor are believed to be the basis of the so-called EPR effect (enhanced permeability and retention effect).

It is well known that small molecules easily leak from normal vessels in the body and are rapidly filtered in kidney glomeruli. Thus, if one injects hydrophilic low-molecular-weight drugs intravenously (i.v.), the drugs almost completely disappear without being subjected to the above-mentioned EPR effect before they reach the target organs (solid tumors). On the other hand, macromolecules and strongly hydrophobic low-molecular-weight drugs which bind plasma proteins tightly have long plasma half-lives because they are too large to pass through the normal vessel walls, unless they are trapped by the reticuloendothelial system in various organs. Such macromolecular drugs can diffuse out of tumor blood vessels, reach the solid tumor tissue effectively and be retained for a long time due to the EPR effect. In that regard, hydrophobic anticancer drugs are superior to hydrophilic low-molecular-weight ones. However, there is at least one disadvantage to the i.v. injection of hydrophobic anticancer agents, that is, the drugs can not be dissolved in saline solution because of their hydrophobicity. Thus, a mixture of organic solvents and chemicals must be used to dissolve the water-insoluble drugs for i.v. injection.

KRN5500, 6-[4-deoxy-4-(2*E*,4*E*)-tetradecadienoylglycyl]-amino-L-glycero-*b*-L-mannoheptopyranosyl]amino-9*H*-purine, is a water-insoluble anticancer drug of which the main mechanism of anticancer activity is an inhibitory effect on protein synthesis.[6] KRN5500 itself has little effect on protein synthesis in rabbit reticulocyte lysates.[6] However, 4-*N*-glycylspicamycin aminonucleoside (SAN-Gly), which has no fatty acid chain and is thought to be generated from KRN5500 by a cytosomal enzyme, exhibited a marked inhibitory effect on protein synthesis in the cell-free system.[6] Nevertheless, SAN-Gly showed 1000-fold weaker cytotoxicity than KRN5500 *in vitro* because of the poor intracellular incorporation of SAN-Gly.[6] Therefore, to obtain an antitumor effect *in vivo* KRN5500 should be administered i.v. Since KRN5500 is highly water-insoluble, a mixture of organic solvents and chemicals (the composition of which has not been divulged by the pharmaceutical company) must be used to dissolve the drug for injection.

Phase I clinical trials of KRN5500 are now under way at the National Cancer Center Hospital in Japan and the National Cancer Institute in the USA. The injection of the

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drug is being limited to the central vein in the clinical trials in order to minimize angiitis, which is probably caused by the organic solvents and chemicals. To overcome this problem, we recently succeeded in incorporating KRN5500 into polymeric micelles.[7] In the present study, we examined the antitumor activity and the toxic effect of micelles incorporating KRN5500 (KRN/m) *in vitro* and *in vivo* in comparison with those of KRN5500.

MATERIALS AND METHODS

Chemicals KRN5500 (its chemical structure is shown in Fig. 1A) powder was kindly supplied by the Kirin Brewery Co., Ltd., Japan. Also, KRN5500 dissolved in several organic solutions and chemicals, currently being used for the phase I clinical study, was a gift from the company. Other chemicals were of reagent grade and were used as purchased.

Incorporation of KRN5500 into micelles As shown in Fig. 1B, KRN5500 was incorporated into polymeric micelles formed from poly(ethylene glycol)-poly(*b*-benzyl L-aspartate-to-*b*-cetyl L-aspartate) block copolymer (PEG-P(BLA, C16)) by physical entrapment utilizing hydrophobic interactions between the drug and the poly(amino acid) chain of the block copolymers, as described previously.[7] Briefly, the block copolymers were dissolved in dimethylsulfoxide (DMSO) and mixed with a KRN5500 solution in DMSO. The mixture was stirred at room temperature for 10 min, and then dialyzed against distilled water for at least 5 h using a cellulose dialysis membrane (molecular weight cut-off=12,000–14,000). Then sonication was carried out with a probe type sonicator model VC 100 (Sonics & Materials Inc., Danbury, CT) with a cycle of sonication for 1 s and standby for 1 s at 4°C. Finally, polymeric micelles containing KRN5500 with a relatively narrow size distribution were obtained. The weight-fractioned average diameter of the particles was 71±32 nm (mean±SD), which was considered appropriate for passive targeting to solid tumors. KRN5500 content was determined spectroscopically by absorption in distilled water at 263.5 nm using the molar extinction coefficient of 4.93×10^4 ($cm^{-1}M^{-1}$), which was obtained with KRN5500 dissolved in *N*,*N*-dimethylacetamide.

Cell culture Five colonic cancer cell lines (COLO201, COLO320, DLD-1, HT-29, LOVO), 5 gastric cancer cell lines (MKN-28, MKN-45, MKN-72, TMK-1, KATO III) and 4 breast cancer cell lines (MCF-7, MDA-MB-435, T-47-D, SST) were used in this study. All the cell lines were maintained in monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum in a humidified atmosphere containing 5% CO_2 at 37°C. For cytotoxicity analysis, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used. Ten thousand cells of each cell line in 198 μl of culture medium were plated in 96-well plates 24 h prior to drug treatment. Then 2 μl of various doses of free KRN5500 or KRN/m was added. Cells were treated in triplicate for 24 h, 48 h or 72 h.

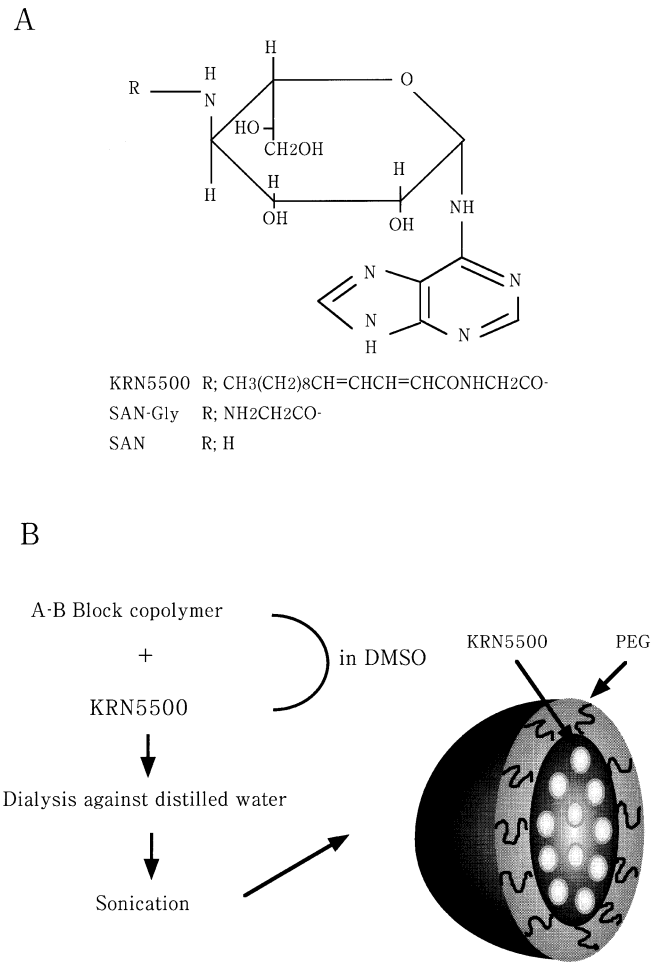

Fig. 1. Chemical structure of KRN5500 and the method of incorporating KRN5500 into polymeric micelles. A) The fatty acid chain of KRN5500 is pivotal for the drug internalization into cancer cells. 4-*N*-Glycyl spicamycin amino nucleoside (SAN-Gly), which has no fatty acid and is obtained after metabolization of KRN5500 by a cytosomal enzyme, exhibited a marked inhibitory effect on protein synthesis in the cell-free system (see text). B) Block copolymer, PEG-P(BLA, C-16) was dissolved in dimethyl sulfoxide (DMSO) and mixed with KRN5500 in DMSO. The mixture was stirred at room temperature for 10 min, and then dialyzed against distilled water for at least 5 h using a cellulose membrane. Sonication was then carried out to obtain uniformly sized micelle particles (approximate size, 70 nm).

Animals BALB/c *nu*/*nu* female mice were obtained from CRJ Co., Ltd. (Kanagawa). One million cells of the human colonic cancer cell line HT-29 were injected into a

Table I. IC_{50} Value (μM) of KRN5500 and KRN/m in Various Cell Lines

	Exposure time					
	24 h		48 h		72 h	
	KRN5500	KRN/m	KRN5500	KRN/m	KRN5500	KRN/m
Colonic cancer						
COLO201	>3.0	>3.0	0.060	0.13	0.036	0.021
COLO320	>3.0	>3.0	0.058	0.105	0.044	0.053
DLD-1	>3.0	>3.0	>3.0	>3.0	>3.0	>3.0
HT-29	>3.0	>3.0	0.046	0.050	0.038	0.051
LoVo	>3.0	>3.0	>3.0	2.400	0.820	0.823
Stomach cancer						
MKN-28	>3.0	>3.0	0.122	0.250	0.045	0.041
MKN-45	>3.0	>3.0	0.037	0.042	0.019	0.021
MKN-72	>3.0	>3.0	0.085	0.156	0.065	0.062
TMK-1	>3.0	>3.0	0.035	0.059	<0.01	0.010
KATOIII	>3.0	>3.0	<0.01	0.017	<0.01	0.010
Breast cancer						
MCF-7	>3.0	>3.0	>3.0	3.0	0.730	0.33
MDA-MB-435	>3.0	>3.0	>3.0	2.45	>3.0	0.40
T-47-D	>3.0	>3.0	>3.0	>3.0	0.160	0.17
SST	>3.0	>3.0	0.066	0.05	0.010	0.01

Each cell line was treated in triplicate for 24 h, 48 h and 72 h.
MTT assay was used for obtaining IC_{50} value.

subcutaneous (s.c.) site on the abdominal skin of 5-week-old mice. Two days later, when the tumor size had reached approximately 3 mm diameter, the tumor-bearing mice were randomly allocated to drug treatment groups of 5 animals each.

In experiment 1, free KRN5500 at the dosage level of 5.6 mg/kg or KRN/m at the equivalent dose was injected i.v. on day 2. In experiment 2, 2 days later, when the tumor size had reached approximately 3 mm in diameter, KRN/m was injected i.v. daily for 4 days at the dosage level of 5.6 mg/kg/day. The anticancer effect was evaluated by measuring tumor size ($a \times b$. a: long diameter; b: short diameter) at various times. Statistical significance of differences was determined by using Student's t test.

Toxicity of KRN5500 and KRN/m Free KRN5500 at 5.6 mg/kg or KRN/m at the equivalent dose was injected i.v. once into female ddY mice. At 24 h or 2 weeks after administration of the drugs, the mice were killed. The tail, heart, lung, liver, spleen, kidney, stomach, colon, skin and femoral bone were obtained and the organs were immersed in 10% formalin solution. Paraffin-embedded sections were stained with hematoxylin-eosin and examined microscopically. Toxicity was evaluated by measurement of body weight changes after the drug treatment.

For blood analysis, blood samples were taken from the inferior vena cava of ddY mice under anesthesia 3 days after the i.v. administration of 5.6 mg/kg of KRN5500 or KRN/m at the equivalent dose. In each blood sample, levels of total protein, albumin, glutamic-oxaloacetic transaminase (GOT), glutamic-pyrubic transaminase (GPT), total bilirubin, blood urea nitrogen (BUN) and creatinine were measured with a Hitachi 7170. White blood cells (WBCs), red blood cells (RBCs) and platelets were also counted with a Hitachi 7170 automatic counter.

RESULTS

***In vitro* antitumor activity** IC_{50} values for KRN5500 and KRN/m in 5 human colonic cancer cell lines, 5 human stomach cancer cell lines and 4 human breast cancer cell lines are shown in Table I. The cell lines were treated with both drugs for 24 h, 48 h or 72 h. The level of antitumor activity was time-dependent for each drug. There was no remarkable difference between the IC_{50} values of KRN5500 and KRN/m at any exposure time.

***In vivo* antitumor activity** In experiment 1, the activity of KRN5500 or KRN/m was evaluated with human colonic cancer line, HT-29. 5.6 mg/kg KRN5500 could be administered i.v. only once because this dose of KRN5500 always induced irreversible inflammatory change in the tail of mice. Therefore, we evaluated the difference of antitumor activity between KRN5500 and KRN/m after a single injection of the two drugs. One bolus i.v. injection of 5.6 mg/kg KRN5500 did not show

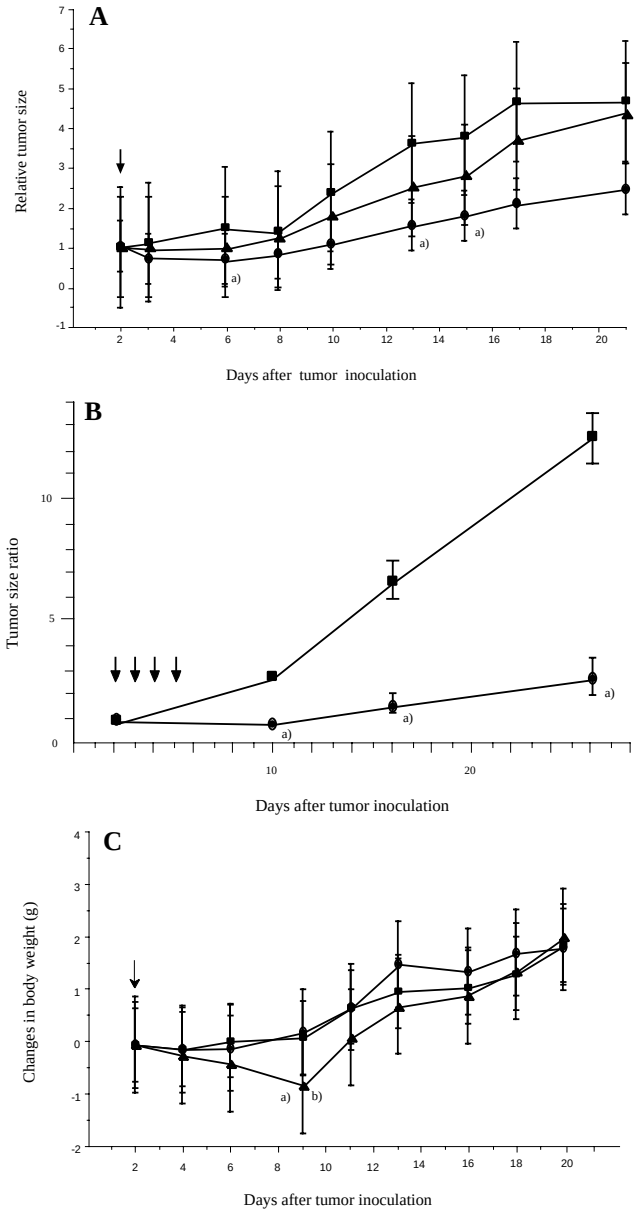

Fig. 2. A) Changes in relative tumor (HT-29) size in abdominal skin of nude mice after treatment with KRN5500 or KRN/m. KRN5500 at 5.6 mg/kg (▲), KRN/m in an equivalent amount to KRN5500 (●), or saline (■) was given i.v. on day 2 (arrow). Points, mean values; bars, ±SD. a) Significant differences between KRN/m and control (P<0.05). B) Changes in relative tumor (HT-29) size in abdominal skin of nude mice after 4 injections of KRN/m. KRN/m in an equivalent amount to 5.6 mg/kg KRN5500 (●) and saline (■) were given daily for 4 days starting on day 2 after tumor inoculation. a) Significant differences between KRN/m and control (P<0.0006). C) Body weight change of the nude mice. The data were from the same mice used in the treatment experiment. Points are the means of 5 determinations. a) Significant difference between KRN5500 and control (P<0.05). b) Significant difference between KRN5500 and KRN/m (P<0.05).

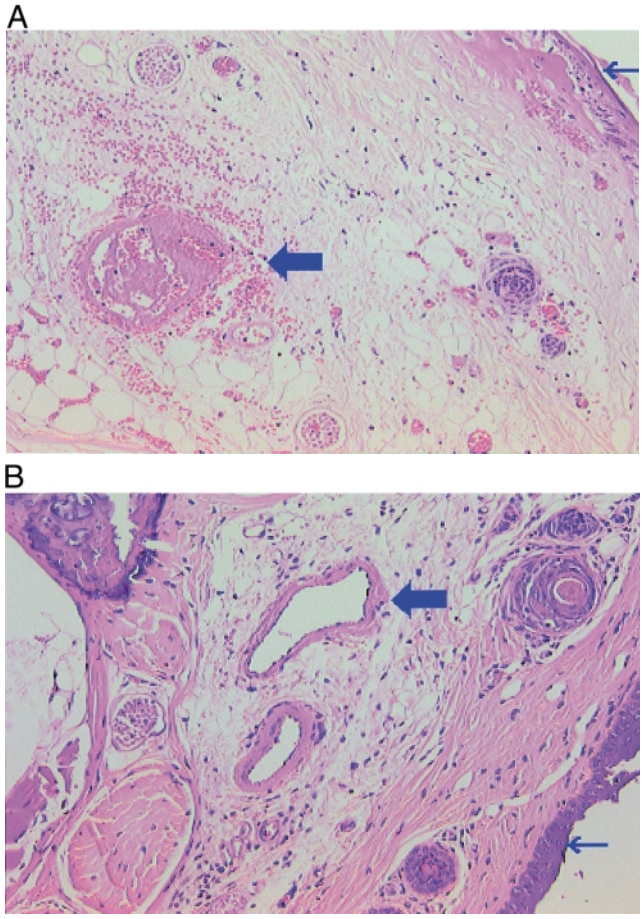

Fig. 3. Vascular damage caused by i.v. injection of KRN5500, but not KRN/m. The tail of each mouse was cut in cross-sectional slices at 1 cm proximal to the injection site after the i.v. administration of 5.6 mg/kg of KRN5500 or an equivalent amount of KRN/m. A) KRN5500: vascular necrosis with fibrin clot was observed (large arrow). Also, the skin of the tail had degenerated (small arrow). B) KRN/m: there was no pathological change in the blood vessel (large arrow) or the skin of the tail (small arrow).

any significant antitumor activity on HT-29 xenografts in comparison with the control, whereas the equivalent dose of KRN/m was significantly superior to the control. However, there was no statistically significant difference between the effects of these two drugs on HT-29 xenografts (Fig. 2A).

In experiment 2, since KRN/m could be administered i.v. without vascular damage, we injected KRN/m i.v. daily for 4 days. KRN/m showed highly significant antitumor activity in comparison with the control group (Fig. 2B).

Toxicity of KRN5500 and KRN/m The weight of the

mice injected with free KRN5500 was significantly lower than that of control or KRN/m-injected mice on day 7 after administration (Fig. 2C). As shown in Fig. 3, necrosis with a fibrin clot and skin degeneration were observed when KRN5500 was administered. Also, several focal necrotic lesions were found in the liver 2 weeks after the injection of KRN5500 (Table II). On the other hand, no pathological change was seen in any resected organ when KRN/m was administered (Fig. 3 and Table II). As regards blood analysis, a significant increase of the BUN value was found after administration of KRN5500, compared to the control or KRN/m (Table III). No other significant differences in blood analysis values were found among the control, KRN5500- or KRN/m-injected mice (TableIII).

Table II. Pathological Findings after i.v. Administration of KRN5500 or KRN/m

Organs	Time after drug administration			
	1 day		2 weeks	
	KRN5500	KRN/m	KRN5500	KRN/m
Lung	N	N	N	N
Heart	N	N	N	N
Liver	N	N	Focal necrosis	N
Kidney	N	N	N	N
Spleen	N	N	N	N
Stomach	N	N	N	N
Colon	N	N	N	N
Bone marrow	N	N	N	N
Skin	N	N	N	N
Tail	Necrosis	N	ND	N

The change of each organ of the ddY mouse was determined microscopically one day or 2 weeks after the administration of 5.6 mg/kg KRN5500 or KRN/m in an equivalent amount to KRN5500.
N: normal. ND: not done.

DISCUSSION

Most cancer chemotherapeutic agents also have toxic effects on normal cells, especially on the bone marrow, mucous membrane and hair follicle cells. Since there is no anticancer agent which has a killing effect on cancer cells alone while preserving normal host cells, ways by which anticancer agents can be selectively targeted to solid tumors are urgently needed.

Conventional low-molecular-weight anticancer agents freely traverse cell membranes by diffusion, leading to their rapid leakage from normal blood vessels and consequently a uniform body distribution.[1)] This means that low-molecular-weight anticancer agents disappear before reaching tumor tissues and exerting their cell-killing effects. On the other hand, macromolecules and small particles have long circulation times in plasma because they do not leak from normal vessels and, if not captured by the reticuloendothelial system,[1–3)] they should have time to reach and exit from tumor capillaries, utilizing the EPR effect. To make use of the EPR effect, several techniques have been developed to modify the structure of drugs and to make so-called carriers, which result in longer half-lives of the drugs in blood. Some of these drug delivery systems (DDS) have been approved by regulatory authorities as anticancer treatments, including polyethyleneglycol (PEG)-L-asparaginase, for the treatment of acute lymphocytic leukemia,[8)] poly(styrene-maleic anhydride)-neocarzinostatin (SMANCS) in "Lipiodol" for the treatment of hepatoma,[9)] and liposomal antracycline formulations for the treatment of Kaposi's sarcoma.[10–13)]

N-(2-Hydroxypropyl)methacrylamide (HPMA) copolymer-doxorubicin conjugate has been developed and tested through preclinical trials.[14, 15)] Yokoyama *et al*. reported that polymeric micelles could be effectively used as a carrier for doxorubicin.[16)] Originally, Ringsdorf *et al.* proposed that AB block copolymer-drug conjugates might form micellar structures that could be useful for drug solubilization.[17)] The polymeric micelle system has the

Table III. Blood Chemistry in ddy Mice 3 Days after i.v. Injection of Drugs

Drugs	Control (saline)	KRN5500	KRN/m
T-pro (g/dl)	4.47±0.15	4.47±0.21	4.67±0.42
Alb (g/dl)	2.90±0.10	2.77±0.21	3.00±0.27
T-Bil (mg/dl)	0.40±0.10	0.37±0.12	0.33±0.12
GOT (IU/liter)	56.67±2.08	53.67±3.22	49.33±5.13
GPT (IU/liter)	29.33±5.77	23.00±6.25	25.33±6.11
BUN (mg/dl)	23.03±1.17	29.50±3.54[a)]	22.93±3.22
Creatinine (mg/dl)	0.17±0.06	0.17±0.06	0.13±0.06

Values are mean±SD (n=3).
a) KRN5500 vs. Control (P<0.05). KRN5500 vs. KRN/m (P<0.05).

advantage that the diameter of the polymeric micelle is approximately 10–100 nm, which is smaller than that of liposomes.[18] The smaller particles are expected to show higher vascular leakage at the target sites, while still being large enough to avoid renal excretion.[19] In addition, the micelle systems can evade nonspecific capture by the reticuloendothelial system because the outer shell of the micelle is covered with PEG, which is electrically neutral, and because the micelle is small.[20] Consequently, polymeric micelles containing anticancer drugs have long half-lives in the bloodstream, which permits large amounts of the micelles to reach the target sites.[21] Adriamycin (ADR)-containing polymeric micelles showed dramatically higher antitumor activity *in vivo* than free ADR, and highly selective delivery to a solid tumor was achieved by a passive targeting mechanism utilizing the EPR effect.[21] A preclinical study of these ADR-containing polymer micelles is under way.

There is another advantage of the polymeric micelle system over the liposomal system. In the liposomal system, hydrophobic drugs are preferentially retained in the lipid bilayer of liposomes, not in the inner aqueous phase. Hydrophobic drugs incorporated in the lipid bilayer may destabilize the liposomal structure, and so incorporation must be limited to a small amount of drug per liposome. In contrast to the liposomal system, the polymeric micelle system can incorporate hydrophobic drugs by utilizing hydrophobic interactions between the drug and the inner core, which is composed of the hydrophobic chain of block copolymers. Recently we have successfully incorporated KRN5500 into polymeric micelles formed from poly(ethylene glycol)-poly(amino acid) block copolymers by physical entrapment utilizing hydrophobic interactions between the drug and the poly(amino acid) chain of the block copolymers.[7] In this study, we investigated the antitumor activity of KRN/m *in vitro* and *in vivo*, and compared the acute and chronic adverse effects of the KRN/m and free KRN5500.

In the *in vitro* study there was no remarkable difference between the IC_{50} values of KRN5500 and KRN/m at any exposure time (Table I). Initially, we expected that free KRN5500 might have greater *in vitro* antitumor activity than KRN/m at the same dose, because we had speculated that KRN/m would release KRN5500 molecules gradually into the cell culture medium, so that it might take longer for KRN/m to achieve an antitumor effect equivalent to that of free KRN5500. However, KRN/m appeared to possess similar antitumor activity to free KRN5500 in the *in vitro* assay system at all exposure times examined (Table I). There are 2 possible reasons for this result. One is that some micelles were destroyed immediately after KRN/m was added to the medium and KRN5500 was thereby released into the medium immediately. The other possibility is that KRN/m particles were internalized by endocytosis as effectively as free KRN5500, and the micelles were degraded inside the cells to release KRN5500 into the cytoplasm. The former possibility seems unlikely because studies of ADR/micelles in a very similar setting showed that the micelles were stable and only gradually degraded in the bloodstream.[21] We are now investigating the mechanism of internalization of KRN/m into the cells.

In the *in vivo* study, a single i.v. bolus injection of KRN/m showed significant antitumor activity compared to the control, whereas free KRN5500 showed only a marginal antitumor effect. However, there was no statistically significant difference between the effects of KRN/m and KRN5500. Since repeated administration of free KRN5500 was not possible because of irreversible inflammation in the tail of mice, caused by the first i.v. injection, we examined the antitumor activity after a single injection of the drugs. Consequently, neither of the drugs showed remarkable antitumor effects. However, we can at least conclude that incorporation of KRN5500 into micelles did not reduce the antitumor activity of the parent KRN5500. The most remarkable difference between KRN5500 and KRN/m concerns the vasculitis of the tail vein at about 1 cm proximal to the injection site on day 1 after the injection. Necrosis with a fibrin clot and skin degeneration were observed when KRN5500 was administered (Fig. 3). Further, several focal necrotic lesions were found in the liver of a mouse 2 weeks after the injection of KRN5500 (Table II). On the other hand, no pathological change was seen in any resected organ of animals given KRN/m (Table II). The body weight of the mice injected with free KRN5500 was significantly lower than that of control or KRN/m-injected mice on day 7 after drug administration (Fig. 2C). A significant increase of the BUN value was found after administration of KRN5500, compared to control or KRN/m group. Since the administered dose of KRN5500 in KRN/m was equivalent to that of the free KRN5500, the toxicity of free KRN5500 may have been caused by the organic solvents or chemicals used for dissolving the water-insoluble drug.

As reported previously, we succeeded in incorporating ADR into micelle cores, and we verified that the micelle system could selectively deliver ADR in micelles to mouse solid tumors and that the micelle systems incorporating ADR showed antitumor effects superior to those of free ADR. In addition, we have shown here that incorporation of the water-insoluble drug KRN5500 into micelles reduced adverse effects which may be caused at least partially by organic solvents or chemicals used for dissolving KRN5500. It may also be possible to apply the micelle system to other water-insoluble anticancer drugs, such as taxol.

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