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Antitumor Activity and Pharmacokinetics of a Morpholino-anthracycline Derivative (KRN8602) against Human Breast Carcinoma Xenografts Serially Transplanted into Nude Mice

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The antitumor activity and pharmacokinetics of (7*R*, 8*S*, 10*S*)-10-((3-deamino-3-(4-morpholino)-2,3,6-trideoxy-α-L-*lyxo*-hexopyranosyl)oxy)-8-ethyl-7,8,9,10-tetrahydro-1,6,7,8,11-pentahydroxy-5,12-naphthacenedione hydrochloride (KRN8602) were evaluated using five human breast carcinoma xenografts in nude mice. The maximum non-toxic dose of KRN8602 was 2 mg/kg by q4d×3 intraperitoneal and peroral administration. KRN8602 showed significant antitumor activity against MX-1, which is less sensitive to adriamycin, with the chemotherapeutic indices of 13.0 for po administration and 9.5 for ip injection. Although KRN8602 also inhibited the growth of T-61 significantly, the antitumor activity of this agent against the other three breast carcinoma xenografts was limited. To elucidate this discrepancy, pharmacokinetic analysis and MTT assay were conducted using the KRN8602-sensitive MX-1 and KRN8602-insensitive R-27. While no differences were observed in the area under the curve and the peak concentration of KRN8602 for each tumor, a difference in the sensitivity of the tumor strains was obvious in MTT assay. The efficacy of this agent seemed to depend on the sensitivity of each type of tumor cell rather than the concentration of agent in tumor tissues. If it were possible to select patients with sensitive tumor cells to this agent by the MTT assay, the phase II trial might be completed within a short period by reducing the number of studied patients.

Key words: KRN8602 — Anthracycline — Nude mouse — Breast carcinoma

KRN8602 is a newly developed anthracycline with a morpholino moiety, which was originally synthesized by Umezawa *et al.*[1] Following a phase I study of this agent, a phase II study on leukemic disease and breast carcinomas was initiated. The present study dealt with the antitumor activity and pharmacokinetics of KRN8602 against human breast carcinoma xenografts serially passaged in nude mice.

MATERIALS AND METHODS

Mice Female nude mice with a BALB/cA genetic background were purchased from CLEA Japan Inc., Tokyo. The mice were maintained under specific pathogen-free conditions using an Isorack, and fed on sterile food and water *ad libitum* in our experimental animal center. Six- to eight-week-old mice weighing 20–22 g were used for the experiments.

Tumor MCF-7 was established as a cultured cell line in 1970 by Soule *et al.*,[2] and was successfully transplanted into nude mice treated with estrogen and progesterone in 1983 by the authors.[3] R-27 was also transplanted into nude mice from a tamoxifen-resistant variant of MCF-7[4] by the same procedure as that for MCF-7. Br-10 was established from floating cancer cells in the pleural effusion of a 43-year-old female with a common ductal carcinoma in 1974 by Hirohashi *et al.*[5] and was kindly supplied to our institute. T-61 was derived from cancerous tissue of a 54-year-old female patient with breast cancer in Frankfurt am Main and was kindly supplied by Dr. N. Brünner,[6] Copenhagen University. MX-1 was established by Giovanella *et al.*[7] from cancerous tissue of a 29-year-old female patient, and was kindly supplied to our institute by Dr. K. Inoue, Cancer Chemotherapy Center, Tokyo, in 1979.

Agent (7*R*, 8*S*, 10*S*)-10-((3-Deamino-3-(4-morpholino)-2,3,6-trideoxy-α-L-*lyxo*-hexopyranosyl)oxy)-8-ethyl-7,8,9,10-tetrahydro-1,6,7,8,11-pentahydroxy-5,12-naphthacenedione hydrochloride (KRN8602) was kindly supplied by Kirin Brewery Co., Ltd., Tokyo. The molecular structure of the compound is shown in Fig. 1 in comparison with that of adriamycin.

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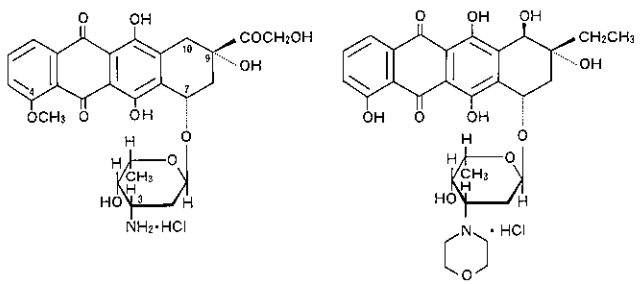

Fig. 1. The molecular structures of adriamycin and KRN-8602.

A 2 mg/kg dose of KRN8602 was administered intraperitoneally (ip), intravenously (iv) or perorally (po) dissolved in 0.2 ml of physiological saline against MX-1. Because 2 mg/kg q4d×3 po could induce the complete disappearance of MX-1 tumors without toxicity, the other four breast carcinoma strains were tested using this dose and the effect of the compound was compared with that of adriamycin, which was given iv at a dose of 8 mg/kg. This route and dose of adriamycin were reported to be the most appropriate for antitumor activity in the human tumor xenograft-nude mouse system.[8]

Tumor inoculation, measurement of tumor size and evaluation of agent activity One (MX-1) or two tissue fragments, each approximately 3×3×3 mm in size, were inoculated into the subcutaneous tissue of the lateral dorsum of ether-anesthetized nude mice, using a trocar needle. The tumors were measured (length and width) with sliding calipers three times weekly by the same observer.

According to the method of Geran *et al.*,[9] the tumor weight was calculated from the linear measurements obtained using the formula: tumor weight (mg)=length (mm)×(width (mm))^2/2. When the tumors reached 100–300 mg, the tumor-bearing mice were randomly allocated to test groups each consisting of 4 to 6 mice. The relative mean tumor weight (RW) was calculated as $RW=W_i/W_0$, where W_i is the mean tumor weight at any given time and W_0 is the mean tumor weight at the time of initial treatment. The antitumor effects of the agents were evaluated in terms of the lowest T/C value (%) during the experiment, where T is the relative mean tumor weight of the treated group and C the relative mean tumor weight of the control group at any given time. The antitumor activity was evaluated as positive when the lowest T/C was equal to or less than 42%, which was calculated from $(0.75)^3$, corresponding to a 25% reduction of each diameter.[8]

In MX-1, the regression equation was derived as ln(the lowest T/C in percent)$=a-b\times$(total dose in mg) and the minimum effective dose (MED) was calculated from the formula; $MED=(a-\ln 42)/b$.

At the end of the experiments, all the mice were killed, the tumors and spleens were weighed and the differences were analyzed statistically using Student's *t* test. The toxicity of the agent was assessed in terms of the mortality of mice, the lowest body weight loss during the experiments and the weight loss of the spleen in the tumor-bearing mice.

MTT assay The MTT assay was performed according to the method of Mosmann with some modifications as reported elsewhere.[10] In brief, the xenografts (R-27 and MX-1) were resected aseptically from nude mice and single-cell suspensions were prepared enzymatically using 0.5 mg/ml pronase, 0.2 mg/ml collagenase type I and 0.2 mg/ml DNase. The primarily cultured human tumor cells were suspended in RPMI1640 supplemented with 20% FCS and plated into 96-well microplates at a volume of 50 μl/well. KRN8602 dissolved in RPMI1640 was also added at a volume of 50 μl/well at concentrations of 0.03, 0.3, 3 and 30 μg/ml. After incubation of the plates for 48 h, 10 μl of MTT dissolved in 5 mg/ml of phosphate-buffered saline was applied to each well, and then the plates were further incubated for 4 h. The formazan products were solubilized in 0.04 *N* HCl in isopropanol by sonification for a few seconds. The plates were read on a Bio-Rad (Richmond, CA) model 2550 EIA reader at 600 nm. All drug concentrations were tested in six replicate wells. The inhibition rate at each concentration was calculated as follows: inhibition rate (%)=(1−absorbance of the treated well/mean absorbance of the control wells)×100. The 50% inhibition concentration (IC_{50}) was calculated from the linear regression equation between concentration and inhibition rate.

Pharmacokinetic analysis KRN8602 (2 mg/kg) was administered po to tumor (R-27 or MX-1)-bearing nude mice. The mice were killed at intervals, and the plasma and tumors were collected for pharmacokinetic assay by high-performance liquid chromatography. The specimens were obtained at 15, 30, 60, 120, 240, 360, 480, 1440 and 2880 min after administration. At each point, the concentration in the tumor was determined from two mice each with two tumors. The concentration in plasma was the average of two mice, because the amount of plasma from one mouse was insufficient for assessment.

RESULTS

The antitumor activity of KRN8602 against MX-1 is shown in Table I. When 2 mg of KRN8602 per kg was administered on a schedule of q4d×3 ip, po or iv, all

Table I. Antitumor Activity of KRN8602 against MX-1

Total dose (mg/kg)	Schedule	Route	TW (%)	T/C (%)	RW	Effect	Spleen wt. (%)	Death	Body wt. loss (%)
6	0.3×20	ip	0	0	0	++	80.1	0/5	23.9
	0.3×20	po	0	0	0	++	70.6	0/5	8.0
	2×3	ip	0	0	0	++	106.3	0/4	6.3
	2×3	po	0	0	0	++	88.7	0/4	9.5
	2×3	iv	0	0	0	++	108.7	3/4[a]	22.4
3	1×3	ip	3.2	0.30	0.05	++	67.6	0/5	0.8
	1×3	po	0.3	0.12	0.02	++	87.1	2/5[b]	17.8
	1×3	iv	0.4	0.02	0.03	++	60.9	0/5	3.0
1.5	0.5×3	ip	12.8	8.5	0.53	++	72.2	0/5	2.6
0.45	0.15×3	ip	57.7	52.9	1.61	−	71.2	0/4	0
	0.15×3	po	37.2	26.4	1.42	+	100	0/5	0
0.3	0.1×3	po	85.8	95.5	1.87	−	97.7	0/5	3.0

Abbreviations: Schedule, dose in mg/kg×days; 3, q4d×3; 20, qd×20; TW, T/C of actual tumor weight in percent; RW, relative mean tumor weight; T/C, T/C ratio of relative mean tumor weight in percent; spleen wt., T/C of actual spleen weight at the end of experiments in percent; body wt. loss, maximum body weight loss during the experiments in percent.
a) Toxic death.
b) Accidental death.

Table II. Antitumor Activity of KRN8602 against Breast Carcinoma Xenografts

Tumor	Group[a]	Actual TW[b]	TW (%)	T/C[c] (%)	on Day	RW[d]	on Day	Effect
MCF-7	control	848.0±343.0	100					
	KRN8602	772.0±232.0	91.1	76.6	7	1.64	7	(−)
R-27	control	279.2±163.8	100					
	KRN8602	302.2±139.3	108.2	90.2	14	1.42	6	(−)
Br-10	control	219.0±25.1	100					
	KRN8602	172.5±125.1	78.8	95.5	9	1.08	2	(−)
T-61	control	499.5±551.3	100					
	KRN8602	29.8±24.6	6.0	10.5	21	0.21	21	(++)
MX-1	control	2992.0±435.9	100					
	KRN8602	0	0	0	16	0	16	(++)

a) Two mg of KRN8602 per kg was administered po in a schedule of q4d×3.
b) Actual tumor weight in mg at the end of experiments (M±SD).
c) Lowest T/C ratio of relative mean tumor weight during the experiments in percent.
d) Lowest relative mean tumor weight during the experiments.
Underline: positive antitumor activity.

treated tumors disappeared completely, while 3 of 4 mice died after iv administration, indicating drug toxicity. Complete regression of the tumors was also observed in the group treated with 0.3 mg/kg KRN8602 administered on a schedule of qd×20 ip and po. There was no regrowth of the regressed tumors within the experimental period of three weeks. Since the maximum body weight loss was within 20% except for the 0.3 mg/kg qd×20 ip and 2 mg/kg q4d×3 iv groups, the total dose of 6 mg/kg in the other treated groups could be regarded as the maximum tolerated dose, which resulted in complete disappearance of all tumors. The minimum effective doses of KRN8602 on MX-1 were calculated to be 0.46 and 0.62 mg/kg in total dose for po and ip administration, respectively.

Since 2 mg of KRN8602 per kg q4d×3 po and ip was estimated to be the most effective dose without toxicity, and po administration seemed to be more effective than ip

injection, the other four breast carcinoma xenografts were treated po with KRN8602 at a dose of 2 mg/kg on a schedule of q4d×3. The effects of KRN8602 against the five human breast carcinoma xenografts are shown in Table II. KRN8602 significantly suppressed the growth of T-61; the T/C of the actual tumor weight was 6.0% and the lowest T/C of the relative mean tumor weight was 10.5% with tumor regression. However, the remarkable antitumor activity of this agent against MX-1 and T-61 was not observed against MCF-7, R-27 and Br-10, the growth of which in the treated group was essentially identical to that in the control group.

Table III. Antitumor Activity of KRN8602 and Adriamycin against Human Breast Carcinoma Strains in Nude Mice

Tumor	T/C by KRN8602[a]	T/C by adriamycin[b]
MCF-7	76.6[c]	60.5
R-27	90.2	70.5
Br-10	95.5	90.0
T-61	10.5[d]	52.0
MX-1	0[d]	39.8[d]

a) T/C of relative mean tumor weight in percent by 2 mg of KRN8602 per kg administered po in a schedule of q4d×3.
b) T/C of relative mean tumor weight in percent by 8 mg of adriamycin per kg administered iv once.
c) Data are shown as the lowest T/C of relative mean tumor weight during the experiments.
d) Positive antitumor effect where T/C is equal to or less than 42%.

The antitumor activity of KRN8602 against breast carcinoma xenografts was compared with the effect of adriamycin at a dose of 8 mg/kg iv (Table III). Both drugs were ineffective against MCF-7, R-27 and Br-10, but effective against MX-1, and the coefficient of correlation between T/C by KRN8602 and T/C by adriamycin was 0.882 with statistical significance ($t=3.238$, $P<0.05$), suggesting similar antitumor spectra of these two agents. However, T-61, which was insensitive to adriamycin, was significantly suppressed by KRN8602, and MX-1, with only marginal sensitivity to adriamycin, was remarkably suppressed by KRN8602.

The results of pharmacokinetic analysis of KRN8602 in plasma of nude mice bearing R-27 and MX-1, and in R-27 and MX-1 tumors are shown in Table IV. The pharmacokinetic parameters of KRN8602 in plasma were essentially identical in mice bearing R-27 and MX-1. The shift of KRN8602 from plasma to tumor tissue was remarkable for both tumors, and the concentration/time profiles of KRN8602 in R-27 and MX-1 were also very similar. While statistically significantly higher concentrations were observed in MX-1 tumor than T-61 tumor at 30, 60, 240, 360 and 480 min after the drug administrations, the areas under the curves (AUCs) and the peak concentrations of KRN8602 in tumor tissue

Table IV. Pharmacokinetic Parameters of KRN8602

Time[a] (min)	Plasma		Tumor	
	MX-1[b]	R-27[b]	MX-1	R-27
15	7.39[c]	13.59	0.014±0.006[d]	0.017±0.003
30	17.94	21.15	0.070±0.013	0.050±0.009*
60	25.31	16.75	0.237±0.028	0.135±0.015***
120	11.46	7.68	0.208±0.025	0.229±0.068
240	6.19	4.41	0.270±0.049	0.119±0.039**
360	5.33	2.17	0.251±0.045	0.125±0.016**
480	5.36	1.04	0.239±0.010	0.124±0.009***
1440	UD	UD	0.095±0.002	0.089±0.006
2880	UD	UD	0.054±0.008	0.049±0.008
Tmax[e]	60	30	240	120
Cmax[f]	0.025	0.021	0.270	0.229
AUC[g]	0.073	0.050	6.26	5.22

a) Minutes after the administration of KRN8602.
b) Data for R-27- or MX-1-bearing mice.
c) Data were shown in ng/ml. UD: undetectable.
d) Data were shown as M±SD in μg/g.
e) Time until the peak concentration in min.
f) Peak concentration in μg/ml or g.
g) Area under the curve from 0 to 48 h in μg·h/ml or g.
* $P<0.05$ to MX-1 tumor. ** $P<0.01$ to MX-1 tumor. *** $P<0.001$ to MX-1 tumor.

Table V. Antitumor Activity of KRN8602 against MX-1 and R-27 *in vitro* Assessd by MTT Assay

Tumor[a]	0.03[b]	0.3	3	30	IC_{50}[c]
MX-1	0[d]	26.6±4.1	62.9±3.2*	100	1.1
R-27	0	0	4.5±0.01*	36.3±0.1	>30

a) Twenty thousand (MX-1) or fifty thousand (R-27) primarily cultured cells were incubated with various concentrations of KRN8602 for 48 h and the effect of KRN8602 was assessed by MTT assay.
b) Concentration of KRN8602 in μg/ml.
c) The 50% inhibitory concentration in μg/ml.
d) Data are shown as inhibitory rate (%) in M±SD.
* Statistically significant at $P<0.001$.

were essentially identical to each other in R-27 and MX-1. Although the active metabolites of KRN8602, M1 and M2 in tumor tissues were also assessed by high-performance liquid chromatography, the AUCs of these metabolites were less than 1/20 that of the unchanged compound in both tumors, and the concentrations of these metabolites were also very similar to each other in R-27 and MX-1 (data not shown). These results indicated that the pharmacokinetic changes in KRN8602 and its active metabolites were essentially identical in insensitive R-27 and sensitive MX-1.

Since no differences were observed in the pharmacokinetics of KRN8602 and its active metabolites in insensitive and sensitive breast carcinoma strains, MTT assay was performed using primarily cultured R-27 and MX-1 tumor cells, which showed different sensitivities to KRN8602 *in vivo*. Table V shows the *in vitro* sensitivity of R-27 and MX-1 tumor cells to KRN8602 detected by MTT assay. A difference in efficacy of the drugs was obvious in this assay, and no inhibition of R-27 cells was found even using 0.3 μg of KRN8602 per ml, a level which was achieved in R-27 tumor *in vivo*. On the other hand, MX-1 cells were suppressed at the same concentration of KRN8602 with an IC_{50} of 1.1 μg/ml. Since the red color of KRN8602 interfered with the optical density determination of formazan at more than 30 μg/ml, the IC_{50} of R-27 could not be obtained in the MTT assay.

DISCUSSION

Adriamycin is one of the key drugs used against breast carcinomas and is widely applied in combination chemotherapy with cyclophosphamide and 5-fluorouracil as "CAF" therapy. Although adriamycin has an excellent antitumor activity against various kinds of solid tumor including breast, lung and gastrointestinal carcinomas, its adverse effects, including cardiotoxicity, alopecia and bone marrow suppression, prevent the drug from being used above a cumulative dose of 500 mg/m^2, since fatal cardiotoxicity, in particular, may ensue. In addition, long-term adjuvant cancer chemotherapy with anthracyclines is limited, because most of these agents only show antitumor activity when given by iv injection. If a new anthracycline could be used effectively by po administration without reduction of its antitumor activity, it would be useful for postoperative long-term adjuvant cancer chemotherapy, like masked compounds of fluoropyrimidines which are available for postoperative adjuvant chemotherapy against gastrointestinal carcinomas.

KRN8602 was developed following a survey of morpholino-anthracyclines, and was reported to be effective against adriamycin-resistant P388 leukemia *in vivo*.[11] The antitumor activity of KRN8602 given by po administration was confirmed, and its cardiotoxicity was found to be significantly lower than that of adriamycin in animal experiments. In fact, KRN8602 was highly and dose-dependently effective against MX-1, and the efficacy of the agent did not depend on the route of administration, though the toxicity in the case of iv injection seemed to be slightly higher than that with ip or po administration. It is noteworthy that KRN8602 could suppress the growth of MX-1 even at 1/13 of the maximum administered dose (0.15 mg/kg q4d×3 po), and the minimum effective total doses were calculated to be 0.46 mg/kg by po administration and 0.62 mg/kg by ip injection. If the maximum tolerated total dose of KRN8602 was assumed to be 6 mg/kg, the chemotherapeutic indices for this agent were concluded to be 13.0 for the po route and 9.7 for the ip route.

Although the remarkable antitumor activity of KRN8602 against MX-1 was also observed against T-61, which is insensitive to adriamycin, MCF-7, R-27 and Br-10 were resistant to the agent and the growths of these strains treated with KRN8602 did not differ from those of the control groups. To clarify this difference in sensitivity of the strains to KRN8602, pharmacokinetic analysis of the agent was conducted using a resistant strain, R-27, and a sensitive strain, MX-1. However, no significant differences were observed in the area under the curves and the maximum concentrations of KRN8602 in R-27 and MX-1 tumor tissues, though slightly higher concentrations of the agents in MX-1 tumor were observed. Because there were no significant differences in the plasma levels of this agent in mice bearing R-27 and MX-1, it was apparent that there was no difference in the shift of KRN8602 from plasma to tumor tissue in the resistant and sensitive tumor strains. We have reported that the maximum concentrations of ACNU in tumor tissues are almost equivalent to each other in ACNU-resistant and -sensitive tumor strains, whereas the elimination of ACNU is more rapid in resistant strains.[12] In this study, however, the changes in

the concentrations of KRN8602 with time in R-27 and MX-1 tumors were essentially the same and the different sensitivities of these strains to KRN8602 could not be explained in terms of the pharmacokinetics, while the cellular basis of drug sensitivity differences between tumors is not clear in this experiment. One of the reasons for this discrepancy might be that "the drug concentration in the tumor" strictly means "the drug concentration in the tumor block," which also contains the tumor vessels and interstitial tissues.

The sensitivity of primarily cultured resistant R-27 and sensitive MX-1 tumor cells was analyzed by MTT assay, and the difference in sensitivity between the two found *in vivo* was also clearly demonstrated *in vitro*. Whereas the IC_{50} of R-27 was more than 30 μg/ml, which is not achievable under physiological conditions, the IC_{50} of MX-1 was concluded to be 1.1 μg/ml, which was actually achieved in our pharmacological experiment. This suggested that the difference in sensitivity between R-27 and MX-1 was due to the different sensitivity of these strains as detected *in vitro* by MTT assay, and not to different pharmacokinetic patterns of the agent in these strains.

Although the antitumor spectrum of KRN8602 was similar to that of adriamycin, KRN8602 showed remarkable antitumor activity against T-61 and MX-1, which were insensitive or less sensitive to the maternal compound, adriamycin. This result is compatible with a report that KRN8602 was effective against adriamycin-resistant P388 leukemia *in vivo*.[11] While the distinct non-cross-resistance found in rodent tumors would not necessarily apply in a human tumor xenograft-nude mouse system,[13] the present results indicated that the growth of adriamycin-insensitive T-61 is significantly suppressed by KRN8602, suggesting that KRN8602 is a promising agent for further preclinical and clinical trials against breast carcinomas. Also, the finding that this agent showed antitumor activity by po administration, as well as by ip and iv injection, suggested that KRN8602 might be useful for long-term adjuvant cancer chemotherapy against breast carcinoma in outpatient clinics.

However, it should be noted that KRN8602 was completely ineffective against three of five xenografts at the maximum tolerated dose, which significantly inhibited the growth of sensitive strains. If KRN8602 were to be submitted to a clinical phase II trial against breast carcinoma, some patients with insensitive tumor cells would obtain no benefit from the agent, but would suffer only adverse effects. Although a comparative study using freshly isolated tumors should be done, if it were possible to select patients with sensitive tumor cells by an *in vitro* chemosensitivity test such as the MTT assay, the phase II trial might be completed within a short period by reducing the number of studied patients and we would be able to spare patients with insensitive tumor cells from harmful and unnecessary side effects. Such a phase II trial guided by chemosensitivity tests is thought to be a promising approach for the future.

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