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Preclinical report

Antitumor activity of KF22678, a novel thioester derivative of leinamycin

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KF22678, a novel thioester derivative of leinamycin with the 1-oxo-1,2-dithiolane-3-one moiety, was examined for antitumor activity, toxicity in mice and activation mechanism. KF22678 showed a broad antitumor spectrum against human carcinoma xenografts (lung, colon, ovary and prostate). The efficacy of KF22678 was significantly higher than that of cisplatin. KF22678 exhibited low cross-resistance against various drug-resistant cell lines of *MDR1* or *MRP* overexpressing human tumors, and, in addition, exhibited more potent antitumor activity *in vivo* than ADM against A2780/ADM and KB/MRP xenograft. DL-Buthionine sulfoximine (BSO) pretreatment significantly reduced intracellular glutathione (GSH) level in human lung carcinoma A549 cells, leading to decrease in the cytotoxicity of KF22678, whereas the cytotoxicity of melphalan was augmented by BSO pretreatment. DNA single-strand breaks (SSB) were observed in A549 cells treated with KF22678 and bleomycin. DNA SSB induced by KF22678 was greatly reduced in the presence of BSO in the cells, whereas DNA SSB induced by bleomycin was not. In addition, the antitumor activity of KF22678 against BSO-pretreated human lung carcinoma PC-9 tumor was significantly decreased. These results suggest that the activation of KF22678 by intracellular GSH might be important for DNA SSB and antitumor activity *in vitro* and *in vivo*. [© 1999 Lippincott Williams & Wilkins.]

Key words: Antitumor, cisplatin, KF22678, mouse.

Introduction

Leinamycin (Figure 1), a novel antitumor antibiotic with an unusual 1-oxo-1,2-dithiolan-3-one moiety, was isolated from a culture broth of *Streptomyces* sp and exhibited antitumor activity against murine experimental tumors.[1] The DNA cleavage and DNA alkylation activity by leinamycin is caused in the presence of thiol cofactors such as glutathione (GSH).[2] Aiming at finding clinically useful leinamycin analogs, we have synthesized a variety of derivatives of leinamycin with thiol-mediated DNA cleavage activity and antitumor activity.[3] Among them, we have selected KF22678 (Figure 1)[3] as a candidate which possesses excellent antitumor activity *in vitro* and *in vivo*, and very low bone marrow toxicity in animal studies. In preliminary experiments, KF22678 showed potent antitumor activity against Sarcoma 180 and marked antiprolieferative activity against human uterine carcinoma HeLa S3 cells.[3]

In the present study, we examined the antitumor activity of KF22678 against various human tumor models in detail, including some anti-cancer drug-resistant human tumor cells *in vitro* and *in vivo*, and the comparison with that of cisplatin and melphalan. The role of thiol in intracellular activation of KF22678 was also examined using DL-buthionine sulfoximine (BSO) (a specific inhibitor of the rate-limiting enzyme of GSH synthesis, γ-glutamylcysteine synthetase) *in vitro* and *in vivo*.

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Materials and methods

Drugs and reagents

KF22678, leinamycin, adriamycin (ADM) and mitomycin C (MMC) were prepared by Kyowa Hakko Kogyo Co (Tokyo, Japan). Vincristine (VCR) was purchased from Shionogi Pharmaceutical (Tokyo, Japan). Cisplatin, melphalan and BSO were purchased from Sigma (St Louis, MO). Etoposide (VP-16) was purchased from Nihon Kayaku Co (Tokyo, Japan). These drugs were dissolved and diluted in sterile 0.9% NaCl solution (Otsuka Pharmaceutical, Tokyo, Japan) just prior to their use.

Animals and tumors

Male BALB/c-*nu/nu* mice (nude mice, 5–7 weeks old), weighing 20–25 g, were obtained from Clea Japan (Tokyo, Japan). They were housed in an exclusive experimental room, and given sterilized food and water *ad libitum*. All animal experiments were conducted using five mice in a group except for the lethal toxicity studies.

Human lung large cell carcinomas Lu-65, Lu-99 and PC-14 were kindly provided by Dr S Hirohashi (National Cancer Center, Tokyo, Japan), human lung carcinoma A549, human prostate carcinoma PC-3 and human colon adenocarcinoma HCT 116 were obtained from ATCC through Dainippon Pharmaceutical (Osaka, Japan) human ovary carcinoma A2780 and ADM-induced resistant lines A2780/ADM[4] were kindly provided by Dr T Tsuruo (Tokyo University, Tokyo, Japan), and ADM-induced resistant human nasopharynx carcinoma KB lines KB-A1[5] and KB/MRP[6] were kindly provided by Dr K Ueda (Kyoto University, Kyoto, Japan).

These tumor cell lines were established by inoculating the cells from each cell line cultured s.c. into nude mice.

Evaluation of antitumor activity

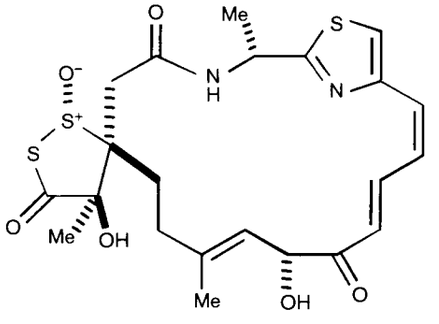
Human solid tumors were s.c. inoculated into the flank of BALB/c-*nu/nu* mice. To evaluate the antitumor activity against s.c. inoculated tumors, the tumor volume was calculated by the following formula according to the method of the National Cancer Institute[7] after measuring the lengths and widths of the tumors: tumor volume (mm^3)={length (mm) × [width (mm)]^2}/2.

Drug efficacy against human tumors inoculated into nude mice was expressed as the mean V/V_0 value, where V is the tumor volume on the day of evaluation and V_0 is that on the day of treatment. The T/C value was calculated by the mean V/V_0 value of the treated group versus that of the untreated group.

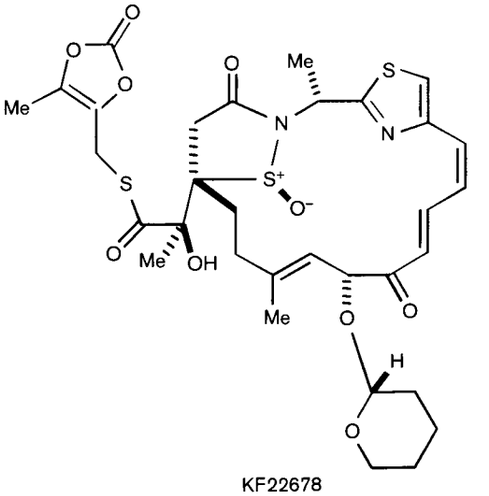
Antiproliferative activity

Human lung normal cell lines WI-38 and CCD-18Lu, human colon normal cell line CCD-18Co and human uterine carcinoma HeLa S3 (ATCC through Dainippon Pharmaceutical), human nasopharynx carcinoma cell line KB and teniposide (VM-26)-induced resistant cell line KB/VM-4[8] were kindly provided by Dr M Kuwano (Kyushu University, Fukuoka, Japan), human breast carcinoma MCF-7 and its ADM-induced resistant cell line MCF-7/ADM[9] were kindly provided by Dr KH Cowan (National Cancer Institute, Bethesda, MD) and human lung carcinoma cell line PC-9 was kindly provided by Dr K Kasahara (Kanazawa University, Ishikawa, Japan).

The cells were precultured for 24 h in 96-well microplates (Nunc, Roskilde, Denmark) containing 0.1 ml of culture medium at 37°C in a humidified atmosphere containing 5% CO_2 in air. Then cells were treated with each compound for 72 h. The antiproliferative activity was evaluated by the XTT method.[10] The IC_{50} (compound concentration causing 50%



Leinamycin



KF22678

Figure 1. Structure of leinamycin and KF22678.

decrease of cell growth) was calculated from the regression line of the dose-response. The degree of resistance (RD) for test compounds was calculated from a ratio of IC_{50} between the parent and its resistant line.

BSO-induced antiproliferative activity

Tumor cells were precultured with or without 0.2 mmol/l BSO for 24 h. The cells were treated with test drugs for 1 h after exchanging the medium to HBSS (SH- and FBS-free medium) and further incubated for 71 h in the culture medium. On day 4, the antiproliferative activity was determined by a cell count and the neutral red dye-uptake method. The IC_{50} was calculated from the regression line of the dose-response.

Measurement of intracellular thiol

Thiol levels in HeLa S3 cells were measured according to a previous method.[11] Thiols were measured with 1×10^6 cells of logarithmically growing HeLa S3. Cells were washed, resuspended, and extracted with 5% trichloroacetic acid (TCA). Then 200 μl aliquots of the extracts were used for the reactions of fluorescence detection of thiols. The final reaction mixture (50 μl) was subjected to a Hitachi F1000 HPLC system using an inertsil ODS-2 column equipped with a fluorescence detector. Levels of thiols were calculated from standard curves and data were expressed as nmol/10^6 cells.

Alkaline elution

Human lung carcinoma A549 cells (8×10^4/dish) were precultured with or without 0.2 mmol/l BSO for 24 h. The cells were labeled with [^{14}C]thymidine (60 mCi/mmol; NEN, Boston, MA) for 24 h, washed with PBS(−) and further incubated in culture medium. Then the cells were treated with each compound at 37°C for 1 h, washed with ice-cold PBS(−), detached with 0.02% EDTA on ice and collected on a polycarbonate filter. These cells were treated with proteinase K in the presence of 2% sodium lauryl sulfate and 25 mmol/l EDTA (pH 9.6) for 1 h at room temperature. The filter was washed by 20 mmol/l EDTA (pH 11.9) at a rate of 0.05 ml/min. Fractions were collected directly into scintilation vials. The radioactivity of each fraction was measured and expressed as proportion of total radioactivity.

Toxicity

Lethal toxicity was observed for 30 days after KF22678 i.v. administration and LD_{10} (10% lethal dose) or LD_{50} (50% lethal dose) values were calculated by the probit method. The body weight of mice was measured by an electronic balance FX-300 (A&D, Tokyo, Japan).

Statistical analysis

Analysis with a SAS statistical program was conducted using the Mann-Whitney's rank-sum test.

Results

Antiproliferative activity of KF22678 against human normal and carcinoma cells

Leinamycin and KF22678 exerted antiproliferative activity through thiol activation in the cells. We

Table 1. Relationship between antiproliferative activity of KF22678 and GSH content in human carcinoma cell lines

Origin	Histology	Cell line	*n*	GSH content[a]	IC_{50} (nmol/l)[b]	
					KF22678	Cisplatin
Lung	carcinoma		6	46±27	6.0±5.7	7245±893
	normal	WI-38	1	4.0	72	22500
		CCD-18Lu	1	4.0	45	18350
Colon	carcinoma		3	35±2.2	25±32	25803±348
	normal	CDD-18Co	1	34	34	30500

Tumor cells were cultured on day 0 and treated with each compound for 1 h on day 1. On day 4, the antiproliferative activity was determined by the XTT method.
[a]GSH content (nmol/mg protein), mean±SD.
[b]50% inhibitory concentration: values were obtained from dose-response curves evaluating different drug concentrations, mean±SD.

have already reported that GSH is one of the thiol activation molecules of leinamycin.[2,12] Thus, we examined the relationship between the intracellular GSH content and the antiproliferative activity of KF22678 against human normal and carcinoma cells. Human lung carcinoma and human colon carcinoma cells, which have a higher GSH content than human normal cells, were more sensitive to KF22678 (Table 1), but not to cisplatin.

Effect of BSO on the antitumor activity of KF22678 *in vitro* and *in vivo*

The depletion of total intracellular GSH content by treatment with BSO *in vitro* was significantly different between the untreated group and the treated group (data not shown). The treatment with 0.2 mmol/l BSO for 24 h caused 10% depletion of intracellular GSH. KF22678 showed significant antiproliferative activity against human lung carcinoma A549 and human uterine carcinoma HeLa S3. Contrary to KF22678, the cytotoxicity of melphalan was enhanced by BSO treatment (Figure 2A and B).

Next, the antitumor activities of KF22678 and cisplatin with or without BSO against human lung carcinoma PC-9 were examined (Figure 3). The antitumor activities [the lowest T/C ratio (%)] of BSO alone (500 mg/kg i.p., on days 0, 1 and 3), KF2267 alone (LD_{10}; 9.4 mg/kg i.v., day 0) and cisplatin alone (LD_{10}; 11 mg/kg i.v., day 0) and KF22678 with BSO or cisplatin with BSO against PC-9 were 81, 41, 57, 64 and 50%, respectively. Although the antitumor activity of BSO alone was negligible, the antitumor activity of KF22678 was significantly decreased by combined treatment with BSO (Figure 3A). On the other hand, cisplatin with BSO showed more potent activity than that of cisplatin alone (Figure 3B). No death and no body weight loss in excess of 20% were encountered during these experiments.

Activation of KF22678 by intracellular GSH in human lung carcinoma

The activity of DNA cleavage and DNA alkylation by leinamycin is caused by thiols including GSH in cell-free systems.[2] To elucidate the activation mechanism of KF22678, DNA in the cells treated with KF22678, bleomycin or X-rays was analyzed by the alkaline elution method, which can detect SSB of DNA. X-ray (3000 cGy irradiation) and bleomycin (IC_{50} value at 1 h exposure) were used as positive control (Figure 4). DNA in the cells treated with 20 nmol/l of KF22678 (IC_{50} value at 1 h exposure) was eluted more rapidly than that treated with X-ray. On the contrary, the elution pattern of DNA in the cells treated with 20 μmol/l bleomycin (IC_{50} value at 1 h exposure) was quite similar to that in untreated cells, indicating that bleomycin did not cause a SSB under the condition of 1 h exposure. GSH depletion by BSO sufficiently decreased the SSB by KF22678 in the cells (Figure 4). Contrary to KF22678, the SSB by bleomycin were enhanced by BSO.

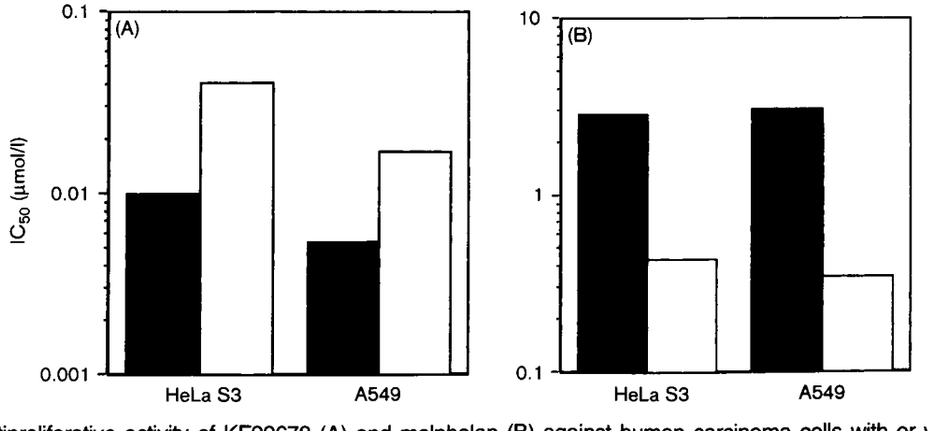


Figure 2. Antiproliferative activity of KF22678 (A) and melphalan (B) against human carcinoma cells with or without BSO. HeLa S3 cells and A549 cells were precultured with (open bar) or without (solid bar) 0.2 mmol/l BSO for 24 h, treated with each compound for 1 h and further incubated for 71 h in the culture medium.

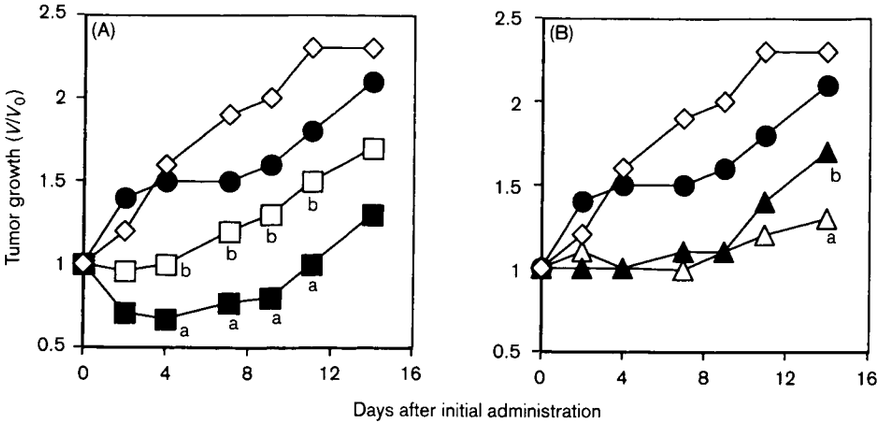


Figure 3. Tumor growth patterns of KF22678 (A) and cisplatin (B) against human non-small cell lung carcinoma PC-9 cells inoculated into nude mice without (closed symbols) and with (open symbols) treated with BSO. KF22678 was administered i.v. as follows: 9.4 mg/kg, once (squares); cisplatin 11 mg/kg, once (triangles); BSO 500 mg/kg, once daily i.p. for 3 days (circles); untreated control (diamonds). T/C (%) ≤ 50. $p < 0.05$ by Mann–Whitney's rank-sum test as compared with the untreated group (a). $p < 0.05$ by Mann–Whitney's rank-sum test as compared with the KF22678-treated group and cisplatin-treated group (b), respectively.

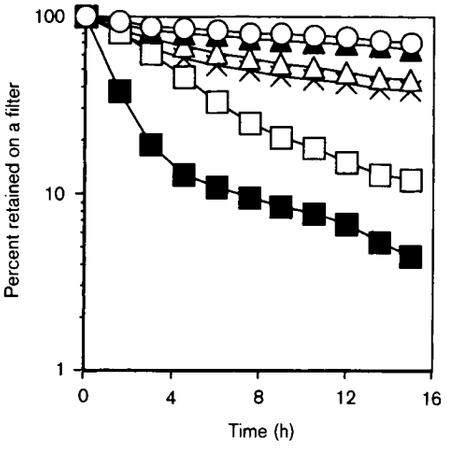


Figure 4. Alkaline elution method detecting DNA SSB caused by KF22678, bleomycin or X-rays. A549 cells were pre-cultured with (open symbols) or without (closed symbols) 0.2 mmol/l BSO for 24 h. The cells were treated for 1 h with 20 nmol/l KF22678 (squares), 20 μmol/l bleomycin (triangles), 3000 cGy X-ray (crosses) and untreated (circles), and subjected to alkaline elution as described in Materials and methods.

Antitumor activity of KF22678 against human tumors

The antitumor activity of KF22678 was further evaluated against human tumors inoculated into nude mice (Table 2).

The efficacy of KF22678 and that of cisplatin were compared by single i.v. administration at their LD_{10} value (KF22678, 9.4 mg/kg; cisplatin, 11 mg/kg), because the body weight loss in host mice by KF22678 or cisplatin treatment was almost the same at this dose.

KF22678 showed a broad antitumor activity against all eight human tumors tested. The efficacy (response) rate of KF22678 was significantly higher than that of cisplatin. Human non-small cell lung carcinoma was more sensitive to KF22678. Its activity was remarkable against large cell lung carcinoma Lu-65 and Lu-99 with T/C values less than 20%, and the suppression of tumor growth was observed at approximately 10–14 days after drug administration (data not shown). In addition, the myelotoxicity of KF22678, estimated by counts of peripheral blood cells and bone marrow cells, was less than that of MMC by single i.v. administration at the LD_{10} (data not shown).

Antitumor activity of KF22678 against the various anticancer drug-resistant human tumor cell line

We compared the chemosensitivity of KF22678 with that of cisplatin against the various anticancer drug-resistant tumor cells. KF22678 and cisplatin showed a very low degree of cross-resistance to various *MDR* and *MRP* overexpressed human carcinoma cell lines (Table 3). To elucidate whether the low cross-resistance property of KF22678 *in vitro* is therapeutically beneficial, an *in vivo* experiment on KF22678 was conducted using A2780/ADM and KB/MRP cells

Table 2. Spectrum of antitumor activity of KF22678 against human carcinomas inoculated into nude mice

Origin	Histology	Tumor	Antitumor activity[a]	
			KF22678	Cisplatin
Lung	large cell carcinoma	Lu-65	++++[b]	+[b]
	large cell carcinoma	Lu-99	++++[b]	+++[b]
	large cell carcinoma	PC-14	++[b]	++++[b]
	carcinoma	A549	++[b]	+[b]
	adenocarcinoma	PC-9	+[b]	−
Colon	carcinoma	HCT 116	+[b]	−
Ovary	carcinoma	A2780	+[b]	−
Prostate	adenocarcinoma	PC-3	+[b]	−
Response rate			8/8 (100%)[c]	4/8 (50%)

Tumor cells were inoculated s.c. into BALB/c-*nu/nu* mice. When tumors had grown to a size between 50 and 300 mm^3 (day 0), the LD_{10} of drugs was administered i.v.
[a]Scoring criteria: −, T/C $(\%)_{min} > 51$; +, $TC(\%)_{min}$ 40−50; ++, $TC(\%)_{min}$ 30−40; +++, $TC(\%)_{min}$ 20−30; ++++, $T/C(\%)_{min} < 19$.
[b]$TC(\%) \leqslant 50$ and $P < 0.05$ by Mann−Whitney's rank-sum test as compared with the untreated group.
[c]Number of positive responses in total xenografts tested.

Table 3. Antiproliferative activity of KF22678 against various multidrug-resistant human carcinoma cell lines

Cell line	Inducing agent	Gene expression	Degree of resistance[a]		
			KF22678	Cisplatin	PD[b]
A2780/ADM	ADM	*MDR1*	15	6.0	151
MCF-7/ADM	ADM	*MDR1*	13	0.4	47
KB-A1	ADM	*MDR1*	17	0.7	59
KB/MRP		*MRP*	2.2	0.4	28
KB/VM-4	VM-26	*MRP*	23	4.4	156

Tumor cells were cultured on day 0 and treated with each compound on day 1 for 72 h. On day 4, the antiproliferative activity was determined by the cell count and XXT method, respectively.
[a]Degree of resistance was calculated as IC_{50} for resistant cells/IC_{50} for sensitive cells.
[b]Parent drug which induced the resistance.

inoculated in nude mice (Figure 5A and B). KF22678 exhibited statistically significant antitumor activity against all tumors tested in this experiment.

Discussion

Leinamycin, a novel antitumor antibiotic,[1] causes single-strand scission of plasmid DNA in the presence of thiol cofactors including GSH.[2] In addition, thiol-mediated DNA alkylation and DNA cleavage by leinamycin have been reported.[2] We synthesized novel thioester derivatives of leinamycin aiming at discovering clinically useful leinamycin analogs. In the course of the study, we have selected KF22678 showing enhanced stability and potent antitumor activity. GSH depletion by BSO treatment sufficiently caused the decrease of the cytotoxicity of KF22678 in cells. On the other hand, potentiation of melphalan cytotoxicity in human ovarian cancer cells has been reported by thiol depletion.[13] Thus we compared the antiproliferative activity of KF22678 with that of melphalan against human carcinooma cells with or without BSO. As a result the cytotoxicity of melphalan was enhanced by BSO treatment. In the analysis of DNA in the KF22678-treated cells using the alkaline elution method, some DNA SSB were detected without adding thiol molecules (Figure 4). The physiological concentration of thiol molecules in the cells is supposed to be sufficient to activate KF22678.

Therefore BSO pretreatment leads to a dramatic decrease of DNA SSB in A549 cells by KF22678. These results suggests that intracellular GSH could be involved in the activation of KF22678.

KF22678 showed broad antitumor activity against all eight human carcinoma xenografts tested. In particular, human non-small cell lung carcinomas were sensitive to KF22678 (Table 2). Tumor regression was observed in mice bearing Lu-65 and Lu-99 lung carcinomas, and was significantly higher than that of cisplatin. In addition, the bone marrow toxicity

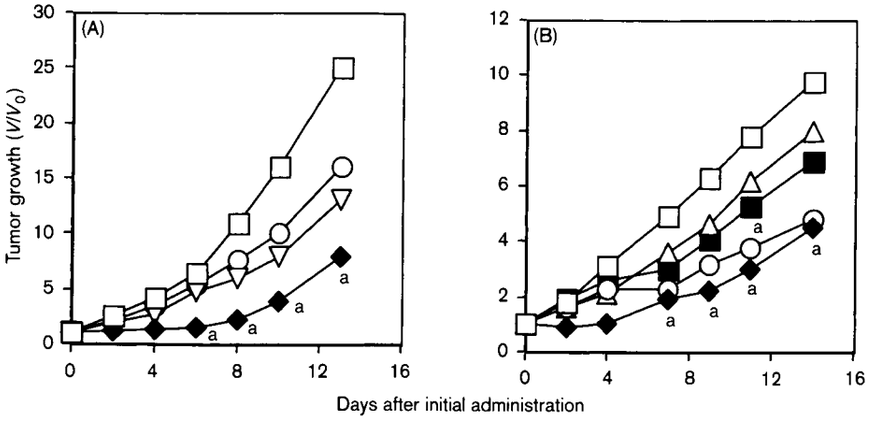


Figure 5. Tumor growth patterns of drug-resistant human ovary carcinoma A2780/ADM (A) and human nasopharynx carcinoma KB/MRP (B) cells inoculated into nude mice. KF22678 was administered i.v. as follows: 9.4 mg/kg, once (◆); cisplatin 11 mg/kg, once (▽); VP-16 13 mg/kg, once daily i.p. for 5 days (■); VCR 2.2 mg/kg, once (△); ADM 14 mg/kg, once (○); untreated control (□). T/C (%) ≤50. $p<0.05$ by Mann–Whitney's rank-sum test as compared with the untreated group (a).

induced by KF22678 was less than that of MMC (data not shown).

One of the remarkable features of antitumor activity of KF22678 is low cross-resistance against multidrug-resistant (*MDR*) lines of three human tumors (A2780/ADM, MCF-7/ADM and KB-A1) which are confirmed to express P-glycoprotein.[14]

Interestingly, KF22678 exhibited as high sensitivity to teniposide-resistant KB/VM-4[15] cells and KB/MRP[6] cells overexpressing multidrug-resistant associated protein (*MRP*) as to their parent cells, respectively. In addition, this compound exhibited effectiveness against ADM-resistant human ovary carcinoma A2780/ADM and nasopharynx carcinoma KB/MRP xenograft *in vivo*. Thus, it could be concluded that KF22678 shows potent antitumor activity against *MDR* cells with a high level of P-glycoprotein and *MRP* over-expressed cells.[6,13] The superior *in vivo* antitumor activity of KF22678 to that of ADM against A2780/ADM and KB/MRP cells could be explained by its superior *in vitro* cytotoxicity.

Drug resistance has been reported to be correlated with the increased cellular GSH content.[16] Because KF22678 was activated by thiol molecules and the higher GSH content cell line was more sensitive to KF22678, KF22678 could be an interesting compound to overcome the drug resistance caused by GSH. These results also indicate that the mechanisms of action of KF22678 are somewhat different from those of cisplatin and melphalan.

In the present experiment, KF22678 was demonstrated to be more potent in various human xenograft tumor models compared with cisplatin.

The reasons for the superiority of KF22678 to cisplatin are of interest. These features of KF22678 might contribute to the superior antitumor activity.

Acknowledgments

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