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Tumor-selective Distribution of an Active Metabolite of the 9-Aminoanthracycline Amrubicin

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It has been reported that the 9-aminoanthracycline amrubicin shows good efficacy in human tumor xenograft models. We studied the disposition and metabolism of amrubicin in mice, in comparison with those of doxorubicin. Amrubicinol, a 13-hydroxy metabolite of amrubicin, which is 10 to 100 times more cytotoxic than amrubicin, was detected as a major metabolite in blood and tissues, and aglycones of amrubicin were also detected. A pharmacokinetic study revealed that amrubicin had a smaller distribution volume and a shorter half-life than doxorubicin. In several normal tissues, the levels of amrubicin and amrubicinol were lower than those of doxorubicin. In contrast, the tumor levels of amrubicinol in the mice treated with amrubicin were higher than those of doxorubicin in the mice treated with that drug, in tumors that are sensitive to amrubicin. These results suggest that the potent therapeutic activity of amrubicin is caused by the selective distribution of its highly active metabolite, amrubicinol, in tumors.

Key words: Anthracycline — Amrubicin — SM-5887 — Active metabolite — Tissue distribution

Anthracycline anticancer agents such as doxorubicin are among the most important drugs in the treatment of solid tumors. A novel 9-aminoanthracycline, amrubicin, a completely synthetic anthracycline drug,[1)] exhibited antitumor activity superior to that of doxorubicin in human tumor xenograft models[2)]; all nine human tumors tested showed significant responses to amrubicin. Relative to doxorubicin, amrubicin was significantly more effective in five tumor lines, less potent in two, and equipotent in the others. Clinical studies currently being conducted on malignant lymphoma, non-small cell lung carcinoma, small cell lung carcinoma, and superficial bladder carcinoma show the activity of amrubicin to be very promising.[3, 4)]

It is reported that the major metabolites of anthracycline are C-13-hydroxy alcohol and aglycones.[5)] We found that the 13-hydroxy alcohol, amrubicinol, showed about 10 to 100 times higher activity in an *in vitro* cell growth inhibition assay using 17 human tumor cell lines.[6)] This characteristic of amrubicin distinguishes it from doxorubicin, daunorubicin, epirubicin and also idarubicin. Kuffel *et al*. showed that doxorubicinol, daunorubicinol and epirubicinol were 7–52 times less active than the respective parent drugs, and that idarubicinol was essentially equipotent to idarubicin against three human tumor cell lines in growth inhibition assays.[7)] Generally, aglycones and the 13-hydroxy metabolites of anthracyclines show low cytotoxic activity,[8, 9)] but in the case of idarubicin, the pathway of metabolism to idarubicinol was not inactivating.[10)] In this paper we present methods for determining the tissue levels of amrubicin, amrubicinol and its aglycones. We examined the plasma and tissue levels of amrubicin and amrubicinol in mice treated with amrubicin and compared them with those of doxorubicin in doxorubicin-treated mice. The results suggest that the toxicity and efficacy of amrubicin are greatly influenced by the distribution of amrubicinol.

MATERIALS AND METHODS

Chemicals Amrubicin hydrochloride, (+)-(7*S*,9*S*)-acetyl-9-amino-7-[(2-deoxy-β-D-*erythro*-pentopyranosyl)oxy]-7,8,9,10-tetrahydro-6,11-dihydroxy-5,12-naphthacenedione hydrochloride (SM-5887), and its derivatives, amrubicinol hydrochloride (diastereoisomeric mixture), amrubicin aglycone, amrubicinol aglycone, 7-deoxyamrubicin aglycone, and 7-deoxyamrubicinol aglycone were prepared by Sumitomo Pharmaceuticals Co. (Osaka).[1)] Doxorubicin hydrochloride was purchased from Kyowa Hakko Co. (Tokyo). The chemical structures of amrubicin, amrubicinol, and doxorubicin are shown in Ref. 19.

Animals and tumors Male BALB/c mice, 9 week old, were purchased from Charles River Japan Inc. (Kanagawa). Female 4-week-old BALB/c nude mice were supplied by CLEA Japan Inc. (Tokyo). The animals were maintained under standard laboratory conditions.

The nude mice received subcutaneous transplants of human tumor xenografts (stomach cancer: 4-1ST, provided by the Central Institute for Experimental Animals, Kanagawa) into the right flank and were used for tissue

distribution studies when the tumors were palpable (about 5–15 mm in diameter). Aqueous solutions of amrubicin hydrochloride or doxorubicin hydrochloride were injected intravenously at the maximum tolerated dose (MTD) of 25 or 12.5 mg/kg, respectively.

Three BALB/c mice for each time point were anesthetized with ether, and blood samples were obtained by heart puncture at 0.5, 1, 3, 10, 30 min and 1, 2, 4, 8 and 24 h after treatment; in another experiment, the tumor and tissues (heart, liver, kidney, lung, small intestine and bone marrow) were removed from 2 or 3 mice at 2, 5 and 24 h after treatment. The plasma was separated from blood cells by centrifugation at 3,000 *g* for 20 min. Bone marrow cells were removed from the femur. All samples were stored at −80°C until drug extraction and quantitative analysis.

Assay of drugs and metabolites in plasma and tissues Plasma and tissues from the amrubicin- or doxorubicin-treated mice were analyzed by Matsushita's method with modifications.[11] Plasma was diluted 1:9 in 0.1 *M* NH_3-HCl (pH 9.0), 9% NaCl and 5% bovine serum albumin (BSA), and blood cells and tissues were homogenized in the same buffer as described above (1:19) with a Polytron homogenizer (Kinematica, Luzern). One hundred microliter aliquots of serially diluted standard solutions containing amrubicin and metabolite were added to 1 ml aliquots of diluted plasma or tissue homogenate to obtain a calibration curve.

The samples were then extracted with 7 ml of chloroform:MeOH (2:1) with shaking for 30 min at room temperature. After centrifugation, the organic layer was evaporated at 35°C under an N_2 gas flow. The dried samples were dissolved in 200 μl of MeOH and 200 μl of 50 m*M* NaH_2PO_4 (pH 3.0) containing 2% $(CH_3)_4NCl$:CH_3CN (73:27). One hundred microliters of the solution was injected into a high-performance liquid chromatography (HPLC) system (Shimadzu, Kyoto) with a Sumipax ODS A-212 column (Sumika Chemical Analysis Service, Osaka). The mobile phase consisted of 4 m*M* sodium 1-heptanesulfonate, 2.3 m*M* acetic acid:tetrahydrofuran:dioxane (15:2:6) pumped at a flow rate of 1 ml/min. The eluate was monitored with a fluorescence detector at an excitation wavelength of 465 nm and a detection wavelength of 560 nm. Doxorubicin was analyzed with a μ-Bondapack phenyl column (Sumika Chemical Analysis Service). The mobile phase consisted of 0.035 *M* HCOOH (pH 3.0):CH_3CN (65:35), and the flow rate was 1.3 ml/min. Fluorescence was detected at 550 nm with an excitation wavelength of 470 nm. The quantitative analysis was performed using external standard methods.

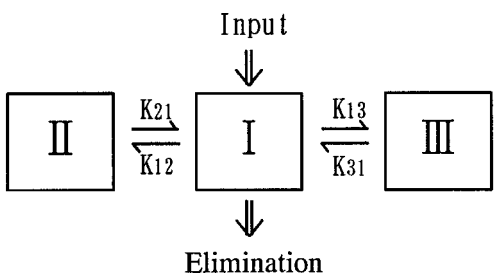

Fig. 1. Three-compartment open model. I, central compartment; II, rapidly equilibrated peripheral compartment; III, slowly equilibrated peripheral compartment. The data were fitted to the following equation: $C=Ae^{-pt}+Be^{-qt}+De^{-rt}$, where C is the plasma concentration of drug.

Pharmacokinetic analysis A pharmacokinetic analysis of the disappearance of amrubicin from plasma was performed according to a three-compartment open model (Fig. 1).[12] The experimental results, represented by the average of the concentrations of three animals per time point, were analyzed by means of the MULTI program.[13]

RESULTS

Determination of the tissue level of amrubicin and its metabolites Methods for determining the tissue levels of amrubicin and its metabolites were studied in order to clarify the pharmacokinetics of amrubicin. Generally, anthracycline compounds are metabolized to aglycones and compounds reduced at the 13-C ketone group. We therefore synthesized aglycones and 13-hydroxy derivatives related to amrubicin, and set up procedures for quantitative analysis by HPLC. We found the optimum HPLC conditions for measuring simultaneously all of the metabolites mentioned above. These 6 compounds were clearly separated using this system. Fig. 2a shows the chart of standard samples. To check the specificity of signals, we analyzed control tissues using this system. We did not find any interfering peaks in the chromatographic chart. Fig. 2b shows the chart of extracts from normal mouse heart without administration of drugs. Fig. 2c shows a typical chart of an extract of heart from an animal given amrubicin. We also measured the levels of amrubicin and its metabolites in other tissues, and the results are described below.

Analysis of blood levels of drugs in non-tumor-bearing mice given amrubicin intravenously Amrubicin was administered via the tail vein of non-tumor-bearing mice. The animals were killed by bleeding from the heart under ether anesthesia at different times after the drug administration. The plasma and blood cell levels of amrubicin and metabolites after the intravenous injection of amrubicin are shown in Fig. 3. Amrubicinol was detected in the plasma and blood cells until 8 h after administration, showing that amrubicin was metabolized to its 13-hydroxy alcohol and aglycones, as found for other anthra-

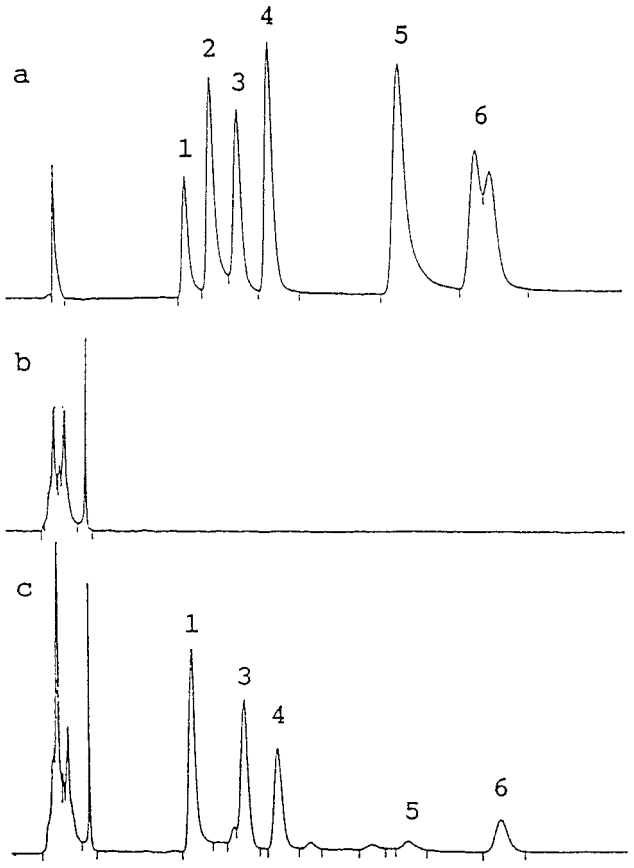

Fig. 2. HPLC chromatogram of typical sample. a: Typical HPLC elution pattern for a standard mixture of amrubicin and its metabolites. Column: Sumipax ODS A-212. Mobile phase: 4 m*M* sodium 1-heptanesulfonate, 2.3 m*M* acetic acid-dioxane-tetrahydrofuran (15:6:2). Detection: excitation 465 nm, emission 560 nm. Peak 1, amrubicin; peak 2, amrubicin aglycone; peak 3, amrubicinol; peak 4, amrubicinol aglycone; peak 5, 7-deoxyamrubicin aglycone; peak 6, 7-deoxyamrubicinol aglycone (two peaks). b: Blank chromatogram from heart extract before administration of amrubicin. c: Chromatogram of heart extract from mice after administration of amrubicin.

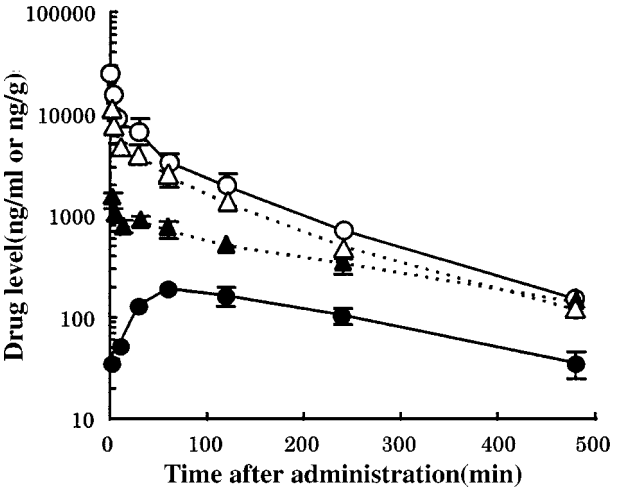

Fig. 3. Plasma and blood cell levels of amrubicin and amrubicinol in mice. Amrubicin hydrochloride was administered to mice at a dose of 25 mg/kg, i.v. Plasma and blood cell levels were assayed by HPLC methods. ○ amrubicin in plasma (ng/ml), ● amrubicinol in plasma (ng/ml), △ amrubicin in blood cells (ng/g), ▲ amrubicinol in blood cells (ng/g). Bars represent SD around the means of values from three mice.

Table I. Pharmacokinetic Parameters of Amrubicin in Mice

Parameters		Amrubicin
$t_{1/2}$[a)] (α)	(h)	0.012
$t_{1/2}$[a)] (β)	(h)	0.27
$t_{1/2}$[a)] (γ)	(h)	1.6
Cl[b)]	(liters/h·kg)	1.7
Vd[c)]	(liters/kg)	3.2
AUC[d)]	(μg·h/ml)	14

Plasma levels were simulated by a three-compartment open model as shown in the following equation: $C=Ae^{-pt}+Be^{-qt}+De^{-rt}$.
a) $t_{1/2}$, half-life of the drug.
b) Vd, apparent volume of distribution at the steady state.
c) Cl, plasma clearance.
d) AUC, area under the plasma concentration-time curve.

cyclines.[5)] Amrubicinol aglycone, 7-deoxyamrubicin aglycone and 7-deoxyamrubicinol aglycone were also detected in plasma and blood cells (data not shown). The plasma levels of amrubicin were almost equal to the blood cell levels until 8 h, but higher levels of amrubicinol in the blood cells than in the plasma were observed. The pharmacokinetic parameters are summarized in Table I. We found that the tissue distribution volume was 3.2 liters/kg, and the half-life of the γ phase was 1.6 h.

Analysis of tissue levels of drugs in tumor-bearing mice given amrubicin or doxorubicin intravenously A human tumor, 4-1ST, was transplanted into nude mice subcutaneously, and amrubicin or doxorubicin was administered via the tail vein at the MTD as mentioned in "Materials and Methods." It was reported that this tumor cell line is very susceptible to amrubicin. The minimum treated/control (T/C) % value of tumor volume was 4% for amrubicin and 26% for doxorubicin.[2)] Fig. 4 shows the levels of amrubicin, amrubicinol and total aglycones in tissue samples excised at 2, 5 or 24 h after amrubicin administration. In the tumor tissues, the levels of amrubicinol at 2 or 5 h after administration were higher than 7.2 μg/g, and the amrubicinol level at each time point was about half the total level of amrubicin and its metabolites.

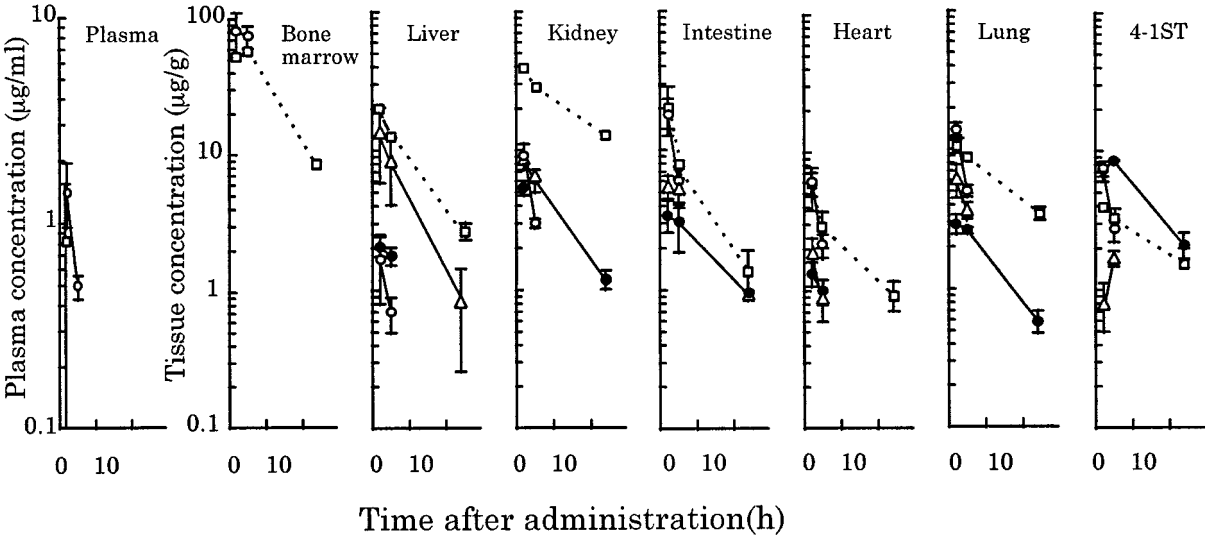

Fig. 4. Tissue levels of amrubicin, its metabolites and doxorubicin in tumor-bearing mice. The plots represent the mean concentrations of amrubicin (○), amrubicinol (●), and aglycones of amrubicin (△) after administration of amrubicin hydrochloride (25 mg/kg), and of doxorubicin (□) after administration of doxorubicin (12.5 mg/kg). The drug concentration in bone marrow cells is shown as ng in 10^9 cells. Bars represent SD around the means of values from two or three mice. Mice that received amrubicin or doxorubicin were killed at 2, 5 or 24 h after injection of drugs. Amrubicin, its metabolites, and doxorubicin were extracted from tissues and analyzed by HPLC as described in "Materials and Methods."

This indicated that amrubicinol was a major metabolite in the tumor tissue of amrubicin-treated mice. However, amrubicinol was not a major component in the normal tissues except kidney, with levels of less than 6.1 μg/g. We observed that the clearance rates of amrubicinol from tissues, especially from tumor tissues, were lower than those of amrubicin. The tumor level of doxorubicin (3.3 μg/g at 5 h) was lower than that of amrubicinol (8.6 μg/g at 5 h), but in normal tissues the concentrations of doxorubicin were considerably higher than those of amrubicinol. For example, the kidney levels of amrubicinol and doxorubicin were 6.1 and 28 μg/g at 5 h, respectively.

DISCUSSION

In this report we present the pharmacokinetics of amrubicin. The tissue distribution volume was 3.2 liters/kg, and the half-life of the γ phase was 1.6 h. It was reported that the distribution volume of doxorubicin was about 36 liters/kg and the half-life of the γ phase was about 17 h in mice treated with doxorubicin at a dose of 5 mg/kg.[12] These data indicated that amrubicin accumulated in the tissues at a lower level than doxorubicin. In fact, tissue pharmacokinetic experiments confirmed the lower tissue distribution of amrubicin compared with doxorubicin, considering that the dose of amrubicin was twice that of doxorubicin. To elucidate the reason for the better efficacy of amrubicin than of doxorubicin in a tumor-bearing mouse model, despite the lower tissue distribution of amrubicin, we examined the pharmacokinetics of its metabolites.

We detected amrubicinol and aglycones as well as the parent compound in plasma and blood cells. The ratio of amrubicinol to amrubicin levels in plasma was about 0.1 from 1 h after administration. As for other anthracyclines, it was reported that the plasma concentration of daunorubicinol exceeded that of daunorubicin from 6 h after administration in the rat,[14] and that the area under the concentration curve (AUC) of idarubicinol in plasma of patients was greater than that of idarubicin.[15, 16] These data indicate that the ratio of the 13-hydroxy metabolite to the parent compound of amrubicin in plasma is lower than that of daunorubicin or idarubicin. Together with the 13-hydroxy metabolite, aglycones of amrubicin were also detected in tissues, as in the case of daunorubicin.[5, 14] The level of amrubicinol in the blood cells was higher than that in plasma. We found that the blood cells showed some metabolic activity to convert amrubicin to amrubicinol [unpublished result]. Therefore, we suggest that a part of amrubicinol in the blood cells is produced in the cells. Thus, amrubicin is metabolized to amrubicinol and aglycones, like daunorubicin and idarubicin, but amrubicinol is less susceptible to further metabolism or is retained in tissues for a longer period than the 13-hydroxy metabolites of daunorubicin and idarubicin. It is noteworthy that a level of amrubicinol higher than that of the parent com-

pound was detected in the 4-1ST tumor tissues (Fig. 4). Boven *et al*. found that daunorubicin was easily metabolized to daunorubicinol, and the AUC of daunorubicin and that of daunorubicinol in tumor xenografts in nude mice were almost the same,[17] while Cusack *et al*. reported that the AUC of daunorubicin was rather lower than that of daunorubicinol in rats.[14] In the case of doxorubicin, doxorubicinol was found at low levels in plasma, normal tissues and tumor tissues.[18] In contrast, the amrubicinol levels were higher in tumor tissue than in normal tissues, after the administration of amrubicin to mice bearing tumors which were sensitive to amrubicin, as shown in this report. These data suggest that the 13-hydroxy metabolite of amrubicin is more selective for tumors than is that of daunorubicin or doxorubicin, and less of it is transferred to normal tissues. We found that the amrubicinol levels were lower in tumor tissue than in kidney after administration of amrubicinol.[19] Therefore the selective distribution of amrubicinol to tumors after administration of amrubicin seemed to be due to the high production of amrubicinol in the tumor tissue, but not to the high uptake of amrubicinol formed in normal tissues.

In our previous study, the antitumor activity of amrubicin was found to be superior to that of doxorubicin in a system of human tumor xenografts transplanted into nude mice.[2] Compared with doxorubicin, amrubicin was significantly more effective in five tumors, less potent in two, and equipotent in two others. We attempted to elucidate the reason for this efficacy of amrubicin by examining its pharmacokinetic behavior in tissues. It has been reported that the activities of metabolites of doxorubicin are less than one-tenth of that of the parent compound in the growth inhibition of various human tumor cell lines.[7] It is thus likely that doxorubicin itself contributes mainly to the cytotoxicity. We found that amrubicinol, in contrast, was more than ten times more active than the parent compound and was almost as active as doxorubicin *in vitro*,[6] and the levels of amrubicinol in all tissues except bone marrow and plasma were more than 10% of the total of amrubicin and its metabolites in this study. Considering these data, we think that it is appropriate to compare the tissue level of amrubicinol with that of doxorubicin in order to evaluate the cytotoxic effects of amrubicin and doxorubicin. The tumor level of amrubicinol was higher than that of doxorubicin (Fig. 4), whereas the normal tissue levels of doxorubicin were higher than those of amrubicinol. Comparing the normal tissue levels with tumor tissue levels for each drug, we found a more selective distribution of amrubicinol than of doxorubicin in the tumors. We therefore conclude that the selective distribution of amrubicinol in tumors is an important factor in the higher efficacy of amrubicin than doxorubicin in this experimental therapeutic model using human tumor xenografts in nude mice.

Recent evidence suggests that the 13-hydroxy metabolite of anthracycline contributes to its cardiotoxicity.[14, 17, 20] Cusack *et al*. reported that cardiac dysfunction was associated with the cardiac concentration of daunorubicinol but not that daunorubicin, and also showed that a higher inhibition rate of Ca^{2+} uptake into cardiac sarcoplasmic reticulum vesicles *ex vivo* is produced by daunorubicinol than by daunorubicin.[21] The heart level of daunorubicinol after 24 h was reported to be considerably higher than that of daunomycin,[14] but here we found the heart level of amrubicinol to be lower than that of the parent compound. We suspect that the lower cardiotoxicity[22] of amrubicin was caused by the lower distribution of the 13-hydroxy metabolite, as well as the parent compound, in the heart. Of particular interest is the lower amrubicinol level in normal tissues (especially in the heart) than in tumor tissue. It is noteworthy that the tumor level of the sum of amrubicin and its metabolites was higher than that of doxorubicin. This suggests that unchanged amrubicin is selectively accumulated in the tumor, though this could not be confirmed because the 4-1ST tumor had high metabolic activity in the present experiments.

In conclusion, we found that the active metabolite of amrubicin is more selective for tumors than doxorubicin and is less distributed in normal tissues. Amrubicin thus appears to be a very promising anticancer drug, the toxicity of which can be easily controlled because of the lower distribution of the active metabolite in normal tissues, and the antitumor activity of which is high because of the higher level of active metabolite in the tumor.

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