

***In vivo* Efficacy and Tumor-selective Metabolism of Amrubicin to Its Active Metabolite**

Toshihiro Noguchi,¹ Shinji Ichii,¹ Shinya Morisada,¹ Takashi Yamaoka¹ and Yoshikazu Yanagi²

¹Research Center, Sumitomo Pharmaceuticals Co., Ltd., 1-98 Kasugadenaka 3-chome, Konohana-ku, Osaka 554-0022 and ²Clinical R & D Division, Sumitomo Pharmaceuticals Co., Ltd., 2-8, Doshomachi 2-chome, Chuo-ku, Osaka 541-8510

The tissue distribution of a novel antitumor anthracycline antibiotic, amrubicin, was studied using seven human tumor xenografts implanted into nude mice, in order to identify the principal factors determining its therapeutic efficacy. We found a good correlation between the level of the metabolite amrubicinol in the tumor and the *in vivo* efficacy. High metabolic activity of amrubicin to amrubicinol was detected in tumor tissue homogenates, especially in cell lines highly sensitive to amrubicin *in vivo*. In contrast to amrubicin, the administration of amrubicinol showed less tumor-selective toxicity in these human tumor xenograft models. These data indicate that the tumor-selective metabolism of amrubicin to amrubicinol resulted in a tumor-selective disposition of amrubicinol, leading to good efficacy in *in vivo* experimental therapeutic models.

Key words: Anthracycline — Amrubicin — SM-5887 — Active metabolite — Pharmacokinetics

Amrubicin hydrochloride (SM-5887) is a completely synthetic 9-aminoanthracycline derivative.¹⁾ Its antitumor activity was found to be superior to that of doxorubicin in experimental therapeutic models using human tumor xenografts.²⁾ All nine human tumors tested showed significant responses to amrubicin. Compared with doxorubicin, amrubicin was significantly more effective in five tumors, and less potent in two tumors. Clinical studies currently being conducted against 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 was shown that amrubicin had more than 5 times lower *in vitro* activity than doxorubicin against human tumor cell lines in a cell growth inhibition assay,⁵⁾ but a putative metabolite, amrubicinol, had about 10 to 100 times higher activity than its parent compound, exhibiting cytotoxic activity similar to that of doxorubicin.⁶⁾ In a previous study, we detected amrubicinol in plasma, normal tissues and also tumor tissues of mice treated with amrubicin. Amrubicinol was distributed more in tumor tissues than in normal tissues compared to doxorubicin.⁷⁾ We thus suspected that amrubicinol is an important contributor to the antitumor activity of amrubicin *in vivo*.

To characterize further the biological properties of amrubicinol, we measured the levels of amrubicinol in seven human tumor xenografts, and examined the relationship between the tumor level of amrubicinol and the efficacy of amrubicin *in vivo*. We also examined the metabolism of amrubicin to amrubicinol *in vitro* using tumor and normal tissue homogenates. In order to eluci-

date the pharmacokinetics and metabolism of amrubicinol after the administration of amrubicin, we compared the tumor-selective toxicity and tissue distribution after the administration of amrubicinol with those of amrubicin. We also discuss the role of amrubicinol in the antitumor activity of amrubicin.

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 Fig. 1.

Animals and tumors BALB/c \times DBA/2 F1 (CDF1) mice were obtained from Charles River Japan, Inc. (Kanagawa). Male and Female BALB/c nude mice (4 weeks old) were supplied by CLEA Japan Inc. (Tokyo). The animals were maintained under standard laboratory conditions.

A mouse leukemia cell line, P388, a human breast cancer cell line, MX-1 and two lung cancer cell lines, LX-1 and QG-56, were supplied by the Cancer Chemotherapy Center, Japanese Foundation for Cancer Research (Tokyo). The five human gastric cancer cell lines, SC-6,

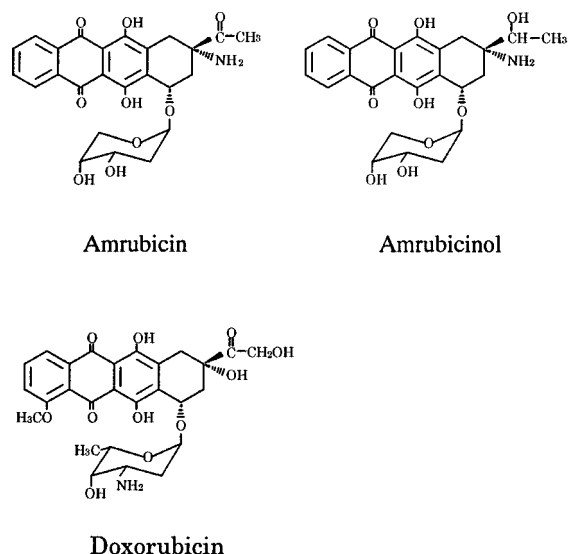


Fig. 1. Chemical structures of amrubicin, amrubicinol, and doxorubicin.

SC-7, St-4, St-15 and 4-1ST were provided by the Central Institute for Experimental Animals (Kanagawa).

Evaluation of antitumor activities The evaluation of antitumor activities was performed as described in a previous report.^{8,9)} Briefly, P388 leukemia cells were implanted intraperitoneally (ip) in CDF1 mice at 10^6 cells/mouse, and drugs were administered intravenously (iv) 24 h after tumor implantation. Antitumor activity against P388 leukemia was determined by comparing the median survival time of the treated group ($n=6$) with that of a control group ($n=6$) and expressed as the percentage of increased life span (ILS%).

Human tumors were transplanted subcutaneously (sc) into the right flank of nude mice. The estimated tumor volume (V) was expressed as $V = 1/2 \times \text{length (mm)} \times [\text{width (mm)}]^2$. When the tumor volume reached 100–300 mm^3 , the mice were separated into groups consisting of 6 mice after randomization, and drugs were administered iv. The growth of tumors was measured twice a week. The treated/control (T/C) % values were calculated by comparing the average tumor volume of the treated group with that of the control group at each time that the tumors were measured.

Assay of drugs and metabolites in plasma and tissues

The drugs in plasma and tissues were analyzed by Matsushita's method with modifications.¹⁰⁾ Plasma was diluted 1:9 in 0.1 M $\text{NH}_3\text{-HCl}$ (pH 9.0), 9% NaCl, and 5% bovine serum albumin (BSA), and tissues were homogenized in the same buffer (1:19) with a Polytron homogenizer (Kinematica, Luzern). One hundred microliters of serially diluted standard solution containing amrubicin

and metabolites was added to 1 ml 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 mM NaH_2PO_4 (pH 3.0) containing 2% $(\text{CH}_3)_4\text{NCl}:\text{CH}_3\text{CN}$ (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 mM sodium 1-heptanesulfonate, 2.3 mM acetic acid:tetrahydrofuran:dioxane (15:2:6) pumped at a flow rate of 1 ml/min. The eluate was monitored with a fluorescence detector set 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.

Reductase assay of the tissue homogenates The 10% tumor tissue homogenates were prepared with 50 mM phosphate buffer (pH 7.4) containing 0.25 M sucrose and 5 mM mercaptoethanol in a Polytron homogenizer. After centrifugation (10,000g, 20 min, 4°C), the protein contents in the cleared supernatants were assayed by the biuret method using BSA as a standard, and adjusted to 2.5 mg/ml protein solution. The reaction mixtures contained 0.3 ml of these adjusted extracts, 0.1 ml of 10 $\mu\text{g}/\text{ml}$ of amrubicin, and 0.1 ml of 5 mM NADPH in 50 mM phosphate buffer (pH 7.4). After incubation at 37°C for 30, 60 or 120 min, the reactions were stopped by mixing each solution with 2.5 ml of MeOH and 0.5 ml of 0.1 M $\text{NH}_3\text{-HCl}$ (pH 9.0), 9% NaCl, and 5% BSA. The metabolites and the mother compound were extracted into 4.7 ml of chloroform with shaking for 30 min. The drugs in the organic phase were assayed by HPLC as described above. The amount of amrubicinol produced from amrubicin by homogenate containing 1 mg of protein in 1 h was considered to represent the reductase activity.

RESULTS

Relationship between the *in vivo* efficacy of amrubicin and the tumor level of the active metabolite

Amrubicin and doxorubicin were administered at 25 and 12.5 mg/kg, iv, respectively. The tumor levels of amrubicin, its metabolites and doxorubicin at 2, 5 and 24 h after drug administration were measured using 7 human tumor xenograft cell lines, 4-1ST, SC-6, St-4, St-15, SC-7, LX-1 and QG-

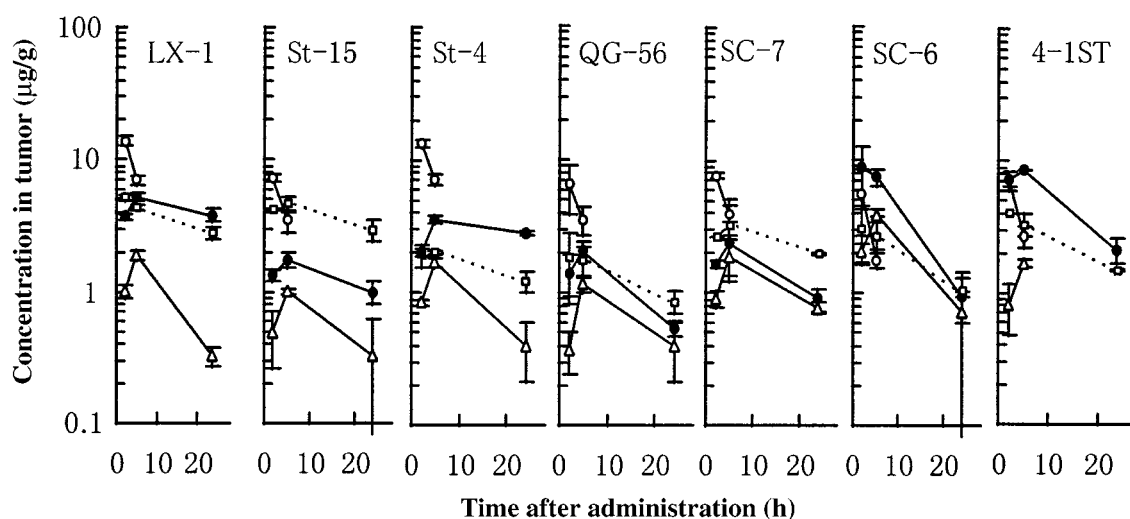


Fig. 2. Tissue levels of amrubicin, its metabolites and doxorubicin in tumor-bearing mice. The plots show the mean concentrations of amrubicin (○), amrubicinol (●), and aglycones of amrubicin (△) after the administration of amrubicin hydrochloride (25 mg/kg), and that of doxorubicin (□) after the administration of doxorubicin (12.5 mg/kg). Bars represent the standard deviation around the mean values. Each human tumor cell line was implanted sc in nude mice. The nude mice were separated into groups after the tumor volume reached about 100–1000 mm³. Two or three mice were killed at each time point after injection of 25 mg/kg of amrubicin or 12.5 mg/kg of doxorubicin. Amrubicin, its metabolites and doxorubicin were extracted from tissues and analyzed by HPLC as described in “Materials and Methods.”

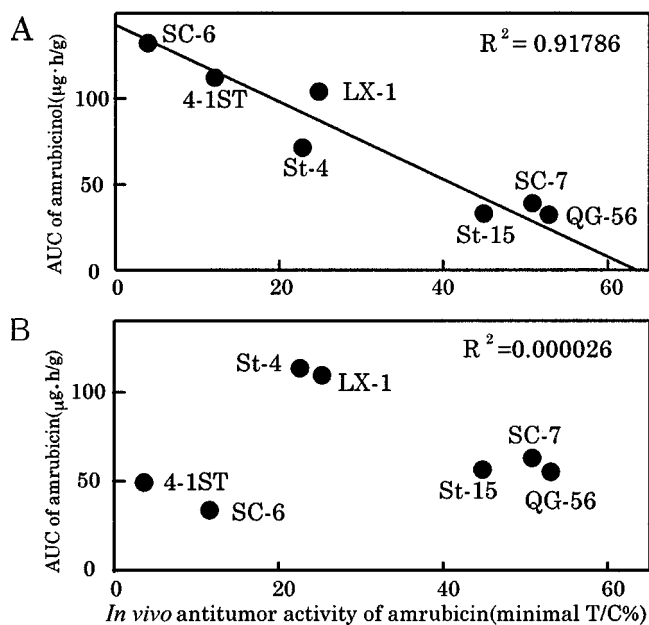


Fig. 3. Correlation between the *in vivo* activities and tumor levels of amrubicinol (A) and amrubicin (B). The AUC values of amrubicinol and amrubicin were calculated by summing trapezoids. The *in vivo* antitumor activity is expressed as the value of minimal T/C%, from the data of Morisada *et al.*²⁾ ● indicates the value for each indicated cell line. *R* is the correlation coefficient.

Table I. *In vitro* Activity for Metabolism of Amrubicin to Amrubicinol by Tumor Tissues

Human tumor	Origin	Enzymatic activity ^{a)}
MX-1	Mammary	7.7±0.1
LX-1	Lung	3.1±0.0
QG-56		5.1±0.8
SC-6	Stomach	49±18
St-15		1.0±0.6
4-1ST		13±1

a) Conversion rate (%) from amrubicin to amrubicinol (/h/mg of protein).

Tissue homogenates were incubated with amrubicin and NADPH. The concentration of amrubicinol was determined as described in “Materials and Methods.” The conversion rate (%) from amrubicin to amrubicinol was calculated.

56 (Fig. 2). In all cell lines except SC-6, the tumor levels of amrubicin were highest at 2 h and undetectable at 24 h after administration. The concentrations of amrubicinol, aglycones and doxorubicin at 5 h were higher than those at 2 h and 24 h. The estimated area under the concentration curve (AUC) values of the tumor levels of amrubicinol or doxorubicin was calculated by summing trapezoids, and plotted against the *in vivo* efficacy (minimum T/C% values), as shown in Fig. 3. The AUC values of amrubicin-

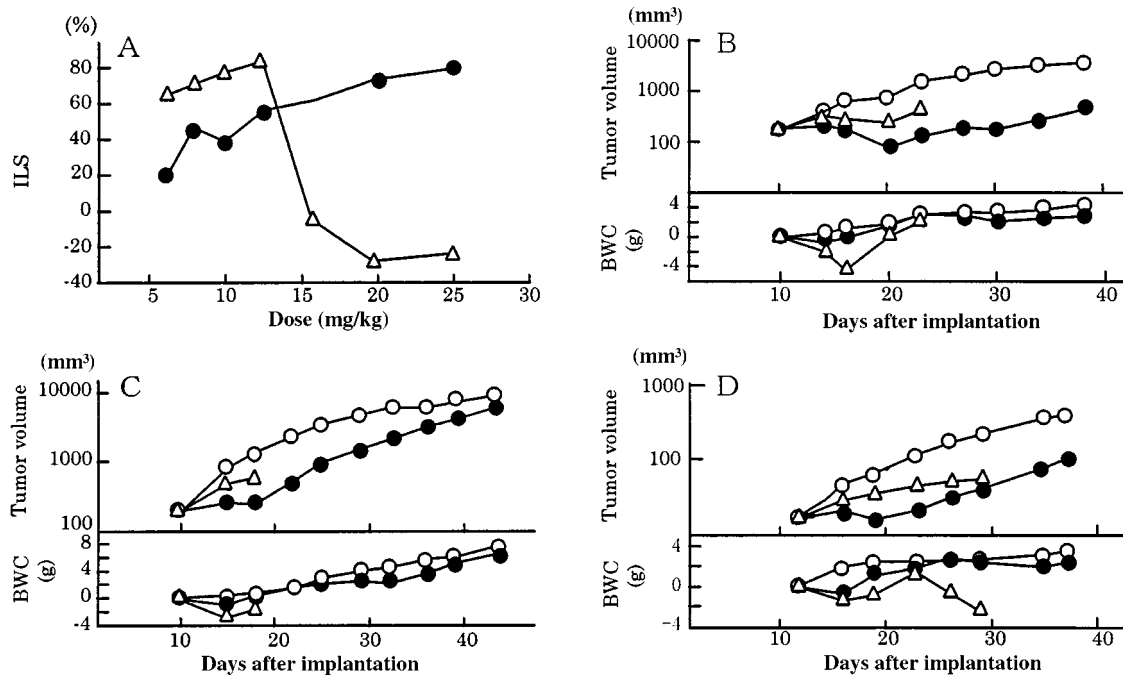


Fig. 4. *In vivo* antitumor activity following the administration of amrubicin or amrubicinol. A: The antitumor activity was expressed in terms of increased life span (ILS%). P388 mouse leukemia cells were inoculated ip, and drugs were administered one day after inoculation ($n=6$, each group). ● amrubicin, Δ amrubicinol. B: (4-1ST), C: (MX-1), D: (SC-6): Effects of amrubicin, and amrubicinol on the growth of human tumor xenografts in nude mice. Groups of 6 nude mice implanted with a tumor were not treated (○), or given amrubicin iv at a dose of 25 mg/kg (●), or amrubicinol iv at a dose of 12.5 mg/kg (B), or 10 mg/kg (C, D) (Δ). All values are mean values.

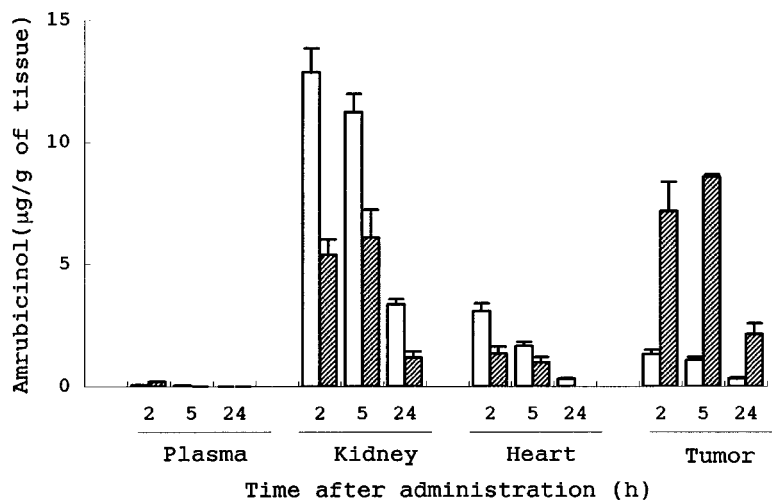


Fig. 5. Amrubicinol levels in mouse tissues after the administration of amrubicin or amrubicinol. Amrubicin and amrubicinol were iv-administered at 25 or 12.5 mg/kg, respectively, to mice bearing 4-1ST human tumor xenografts. After 2, 5 or 24 h, the plasma, kidney, heart and tumor tissue were excised and the levels of amrubicinol were determined. The hatched and open columns indicate amrubicinol level after amrubicin and amrubicinol administration, respectively. Each point represents the mean and standard deviation of data for 3 mice per group.

nol well reflected the *in vivo* efficacy of amrubicin. The R^2 value (R =correlation coefficient) was 0.91786.

Reductase activities in the tumor xenografts To estimate the degree of metabolism of amrubicin in tumor tissues, the supernatants of the homogenates were incubated with amrubicin *in vitro*, and the amrubicinol concentrations in the incubation mixtures were measured. The degree of *in vitro* metabolism was determined and the results are listed in Table I. The SC-6 and 4-1ST tumors were shown to have higher metabolizing activity than the other tumors, and the amrubicinol levels in these tumors were higher than those in the other tumors after administration of amrubicin; further, these tumors were highly sensitive to amrubicin. These data indicated that the degree of metabolism of amrubicin to amrubicinol by tumor homogenates *in vitro* is well correlated with the tumor level of amrubicinol after the administration of amrubicin *in vivo*.

***In vivo* tumor-selective toxicity of amrubicinol** To assess the significance of the conversion of amrubicin to amrubicinol in tumor tissues, the tumor-selective toxicity of *in vivo* administration of amrubicinol was compared to that of amrubicin. The tumor-selective toxicity of amrubicinol to P388 leukemia was determined in terms of ILS (%) values, as plotted in Fig. 4A. The tumor-selective toxicity of amrubicinol was not superior to that of amrubicin, and the dose of amrubicinol giving the maximum tumor-selective toxicity was found to be 12.5 mg/kg, iv; doses higher than 12.5 mg/kg caused toxic death. This result also indicated that the maximum tolerated dose of amrubicinol was 12.5 mg/kg.

Tumor-selective toxicity to a human tumor xenograft, 4-1ST, was evaluated at a dose of 25 mg/kg of amrubicin or 12.5 mg/kg of amrubicinol. The tumor growth-inhibitory activity of amrubicinol was lower than that of amrubicin, and all of the mice treated with amrubicinol died by day 27 (Fig. 4B). We examined the tumor-selective toxicity of a reduced dose of amrubicinol using other tumor xenografts, i.e., MX-1 and SC-6 (Fig. 4, C and D). At the dose of 10 mg/kg of amrubicinol, the growth-inhibitory activity was lower than that of the mother compound, and toxic deaths were again observed in the amrubicinol-treated group. These results indicated that the tumor-selective toxicity of externally administered amrubicinol was lower than that of the mother compound.

The tissue distribution of amrubicinol after amrubicinol administration was compared with that after amrubicin administration (Fig. 5). In the system using 4-1ST human tumors, the *in vivo* tumor-selective toxicity of amrubicinol was less than that of amrubicin, as mentioned above. The tumor level of amrubicinol after the administration of amrubicinol at a dose of 12.5 mg/kg was lower than that after the administration of amrubicin at a dose of 25 mg/kg, whereas the kidney and heart levels of amrubicinol

were higher after the administration of amrubicinol than after the administration of amrubicin. In experiments using SC-6 or MX-1 tumors, we obtained similar results (data not shown).

DISCUSSION

We previously reported that amrubicinol was detected as a major metabolite in mice treated with amrubicin, and that it was distributed in tumors at a higher level than in normal tissues.⁷ Moreover, the cytotoxic activity of amrubicinol was 10–100 times higher than that of the mother compound *in vitro*.⁶ On the basis of these data we suggested that amrubicinol is an active metabolite of amrubicin. In the present study, we measured the levels of amrubicinol in 7 human tumor xenografts and found that they well reflected the *in vivo* efficacy of amrubicin. Considering that the activity of amrubicinol inside the cells was higher than that of amrubicin, and that the retention period of amrubicinol in the tumor was longer than that of amrubicin, metabolism to amrubicinol in the tumor is suggested to increase the antitumor activity of amrubicin *in vivo*. On the other hand, a good correlation between the tumor levels of amrubicin and the efficacy was not observed. The effect of amrubicin may be masked by the coexisting amrubicinol in the tissue, because the cytotoxic effect of amrubicinol is higher than that of amrubicin. The role of amrubicin itself in *in vivo* antitumor activity should be elucidated by further experiments. The host toxicity of amrubicinol is about 2 times stronger than that of amrubicin, although the cytotoxicity of amrubicinol is 10–100 times more potent. It is suggested that the discrepancy may be related to the difference between the level of amrubicinol produced in tissues after injection of amrubicin and that in cultured cells in the cytotoxicity assay. The level of amrubicinol was higher than the level of amrubicin in kidney 5 h after administration of amrubicin. But the level of amrubicinol in cultured cells was obviously lower than that of amrubicin 5 h after incubation with amrubicin.⁶ Thus, we assume that the contribution of amrubicinol in normal tissues to the host toxicity after administration of amrubicin is higher than that in the cultured cells treated with amrubicin.

We next evaluated the tumor-selective toxicity of amrubicinol *in vivo*. Amrubicinol did not show superior tumor-selective toxicity against mouse leukemia and human tumor xenografts compared to amrubicin. In order to elucidate the greater tumor-selective toxicity of amrubicin than amrubicinol *in vivo*, the pharmacokinetic profile of amrubicinol after amrubicinol administration was compared with that after amrubicin administration. The amrubicinol level in the tumors after the administration of amrubicinol was lower than that after the administration of amrubicin. This is consistent with the difference in

tumor-selective toxicity between amrubicin and amrubicinol. Moreover, distribution of amrubicinol was more selective for tumors after amrubicin administration than after amrubicinol administration. We suspect that a higher degree of metabolism to amrubicinol in tumor tissue accounts for the higher level of amrubicinol in the tumor after the administration of amrubicin.

We therefore measured the metabolic activity for amrubicin using tissue homogenates. We found that high activity was detected in tumors which showed a high degree of distribution of amrubicinol. In the case of daunorubicin, it was suggested that C-13 carbonyl group reduction is catalyzed by carbonyl reductase.¹¹⁾ The distribution of the enzyme has been found by immunohistochemical techniques to be ubiquitous in human tissues.¹²⁾ It is not yet known whether higher carbonyl reductase activity is present in tumor tissue, but our data suggest that the tumor tissue contained a high level of carbonyl reductase.

At this stage we speculate that the high antitumor activity was caused by the selective metabolism of amrubicin to amrubicinol in the tumor. If we can develop methods to activate further the enzymatic activity in tumor tissue alone, the efficacy of amrubicin might be considerably improved.

In conclusion, we found that the administration of the mother compound caused lower systemic toxicity, more effective accumulation of amrubicinol in the tumor tissue and higher antitumor activity than the administration of active metabolite amrubicinol itself.

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