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, (+)-(7S,9S)-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 amrubcin 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 amrubcin- or doxorubicin-treated mice were analyzed by Matsushita’s method with modifications.11) Plasma was diluted 1:9 in 0.1 M NH₄·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 amrubcin 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 N₂ gas flow. The dried samples were dissolved in 200 µl of MeOH and 200 µl of 50 mM NaH₂PO₄ (pH 3.0) containing 2% (CH₃)₂NCl:CH₃CN (73:27). One hundred microliter aliquots 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 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):CH₃CN (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.

**Pharmacokinetic analysis**

A pharmacokinetic analysis of the disappearance of amrubcin 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 amrubcin and its metabolites**

Methods for determining the tissue levels of amrubcin and its metabolites were studied in order to clarify the pharmacokinetics of amrubcin. 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 amrubcin, 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 amrubcin. We also measured the levels of amrubcin and its metabolites in other tissues, and the results are described below.

**Analysis of blood levels of drugs in non-tumor-bearing mice given amrubcin intravenously**

Amrubcin 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 amrubcin and metabolites after the intravenous injection of amrubcin are shown in Fig. 3. Amrubcinol was detected in the plasma and blood cells until 8 h after administration, showing that amrubcin was metabolized to its 13-hydroxy alcohol and aglycones, as found for other anthra-
cyclines. 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. 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.
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. 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 daunorubicin exceeded that of daunorubicin from 6 h after administration in the rat, and that the area under the concentration curve (AUC) of idarubicinol in plasma of patients was greater than that of idarubicin. 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. 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. 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-
Tissue Distribution of Amrubcinol

Amrubcinol was found to be superior to that of doxorubicin in a system of human tumor xenografts transplanted into nude mice. We therefore conclude that the selective distribution of amrubcinol in tumors is an important factor in the higher efficacy of amrubcin 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.

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