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# Uptake and Intracellular Distribution of Amrubicin, a Novel 9-Aminoanthracycline, and Its Active Metabolite Amrubicinol in P388 Murine Leukemia Cells

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**Amrubicin, a 9-aminoanthracycline anti-cancer drug, and its C-13 hydroxyl metabolite amrubicinol, were examined for growth-inhibitory activity as well as cellular uptake and distribution in P388 murine leukemia cells and doxorubicin-resistant P388 cells. Also discussed are the differences in the mechanisms of action among amrubicin, amrubicinol and doxorubicin in terms of their cellular pharmacokinetic character. In P388 cells, amrubicinol was about 80 times as potent as amrubicin, and about 2 times more potent than doxorubicin in a 1-h drug exposure growth-inhibition test. A clear cross-resistance was observed to both amrubicin and amrubicinol in doxorubicin-resistant P388 cells, though the resistance index was lower for amrubicin. The intracellular concentration of amrubicinol was about 6 times and 2 times higher than those of amrubicin and doxorubicin, respectively. Compared to doxorubicin, amrubicin and amrubicinol were released rapidly after removal of the drugs from the medium. A clear correlation was found between the growth-inhibiting activity and the cellular level of amrubicin and amrubicinol in P388 cells. About 10 to 20% of amrubicin or amrubicinol taken up by the cells was detected in the cell nuclear fraction, whereas 70 to 80% of doxorubicin was localized in this fraction. These results suggest that amrubicin and amrubicinol exert cytotoxic activity via a different mechanism from that of doxorubicin.**

Key words: Anthracycline — Amrubicin — SM-5887 — Metabolite

Amrubicin hydrochloride (SM-5887) is a completely synthetic 9-aminoanthracycline anti-cancer drug.[1)] It has potent antitumor activities, being more potent than doxorubicin against various mouse experimental tumors and human tumor xenografts.[2)] Phase II clinical trials of amrubicin for the treatment of malignant lymphoma, superficial bladder cancer, small cell lung cancer and non-small cell lung cancer are in progress.[3, 4)] The response rates were 25% and 79% against non-small cell lung cancer and small cell lung cancer, respectively.

In our previous studies, amrubicinol, the C-13 hydroxy metabolite of amrubicin, was much more potent than the parent compound, amrubicin, and as potent as doxorubicin in inhibiting the growth of human tumor cells.[5)] In nude mouse-human tumor models, amrubicinol was detected in plasma, normal tissues and tumor tissues of mice treated with amrubicin. Amrubicinol was distributed more in tumor tissues than in normal tissues compared to doxorubicin, and the level of amrubicinol in the tumor correlated to the *in vivo* efficacy of amrubicin.[6, 7)] We thus suppose that amrubicinol plays an important role in the *in vivo* antitumor effect of amrubicin as an active metabolite.

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A major metabolic pathway of anthracyclines is known to be the reduction of the C-13 carbonyl group to a hydroxyl group by cytoplasmic carbonyl reductase.[8–10)] Generally, the C-13 hydroxy metabolite of an anthracycline shows lower growth-inhibitory activity than the respective parent compound. Doxorubicinol, epirubicinol and daunorubicinol are less potent than doxorubicin, epirubicin and daunorubicin, respectively.[11–15)] In contrast, idarubicinol is essentially equipotent to idarubicin.[11–13, 16)] It is thought that the growth-inhibitory activity of the C-13 hydroxy metabolite of an anthracycline is associated with the degree of cellular uptake.[12, 14)] The intracellular concentrations of doxorubicinol, epirubicinol and daunorubicinol were found to be much lower than those of the respective parent compound, while the intracellular concentration of idarubicinol was in the same range as that of idarubicin. Amrubicin is unique among the anthracyclines in that the growth-inhibitory activity of its C-13 hydroxy metabolite is much greater than that of the parent compound. It was also found that the intracellular concentration of amrubicinol was higher than that of amrubicin in human tumor cells.[5)] In the present study, the growth-inhibitory activities, cellular uptake and release, and intracellular distribution of amrubicin and amrubicinol were further examined using P388 murine leukemia cells and doxorubicin-resis-

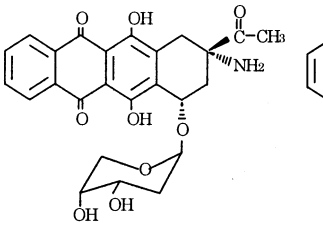
tant P388 cells, and were compared to those of doxorubicin.

## MATERIALS AND METHODS

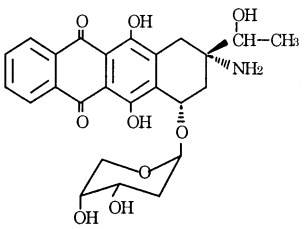
**Chemicals** Amrubicin hydrochloride and amrubicinol hydrochloride (a diastereoisomeric mixture of 13-hydroxyamrubicin hydrochloride) were prepared by Sumitomo Pharmaceuticals Co. (Osaka). Doxorubicin hydrochloride was purchased from Kyowa Hakko Co. (Tokyo). The chemical structures of amrubicin, amrubicinol, and doxorubicin are shown in Fig. 1.

**Cells** P388 murine leukemia cell line and doxorubicin-resistant P388 cell line (P388/ADR) were provided by the Cancer Chemotherapy Center, Japanese Foundation for Cancer Research (Tokyo). Cells were maintained as ascite tumor cells by serial transplantation. Cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 5 $\mu M$ 2-hydroxyethyl disulfide, at 37°C under humidified 5% $CO_2$ in air.

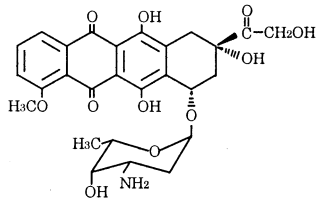
**Cell growth inhibition test** Cell growth inhibition was examined by means of a 1-h drug exposure test. Cells were seeded at $3\times10^5$ cells/ml in growth medium containing different concentrations of drugs and incubated at 37°C for 1 h. After that, the cells were collected by centrifugation at 1,000 rpm for 5 min and washed twice with ice-cold phosphate-buffered saline (PBS(−)). The resulting cells were suspended in fresh growth medium and were grown for 2 days. Cell growth inhibition was assessed by counting the cell number with a Coulter Counter (Coulter Electronics, Luton Beds, England). Assays were carried out in triplicate and the $T/C$(%) value was calculated by use of the following formula. $T/C$(%) = [(mean cell number of drug-treated cells at day 2)−(mean cell number of control cells at day 0)]/[(mean cell number of control cells at day 2)−(mean cell number of control cells at day 0)]×100.



Amrubicin



Amrubicinol



Doxorubicin

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

**Determination of intracellular drug concentration** Cellular uptake was analyzed using spectrofluorometry as described by Egorin *et al*. and Kunimoto *et al*. with slight modifications.[17, 18] Cells were seeded at $10^6$ cells/ml in growth medium containing the drugs at 10 $\mu$g/ml, and incubated at 37°C. At the indicated time, duplicate aliquots of 1 ml of cell suspension were centrifuged, and the cells were washed twice with ice-cold PBS(−). In some experiments, the cells were incubated for 5 min in ice-cold Hanks' balanced salt solution containing 0.1% Nonidet P-40, and cell nuclei were obtained by centrifugation at 12,000 rpm for 30 s. The drugs were extracted from the pelleted cells or cell nuclei using 0.3 *N* HCl containing 50% ethanol. The drugs were detected by fluorescence using a Model RF-540 spectrofluorometer (Shimadzu, Kyoto) with an excitation wavelength of 470 nm and emission wavelengths of 550 nm for doxorubicin and 540 nm for amrubicin and amrubicinol. Drug concentrations were calculated against a standard curve and related to $10^6$ cells.

**Fluorescence microscopy** Cells were seeded at $10^6$ cells/ml in growth medium containing the drugs at 10 $\mu$g/ml, and incubated for 10 min at 37°C. The cells were centrifuged at 1,200 rpm for 1 min, then resuspended in 0.1 ml of the drug-containing medium. The cells were observed under an VANOX-S-equipped AH2-RFL-T microscope (Olympus, Tokyo).

## RESULTS

**Cell growth inhibition** Amrubicin, amrubicinol and doxorubicin were examined for growth inhibitory activity towards P388 and doxorubicin-resistant P388 (P388/ADR) cells. Table I shows the $IC_{50}$ values in a 1-h drug exposure test. Amrubicinol was about 80 times more potent than amrubicin, and about 2 times more potent than doxorubicin in P388 cells. In P388/ADR cells, amrubicinol was about 4 times more potent than amrubicin, and as potent as doxorubicin. The degree of resistance, represented as the ratio of the $IC_{50}$ values obtained in P388/ADR and P388 cells, was 65 for doxorubicin, 5 for amrubicin and 121 for amrubicinol.

**Cellular uptake and release** The cellular uptake and release were examined by measuring the intracellular drug

Table I. Growth Inhibition of P388 and P388/ADR Cells by Amrubicin, Amrubicinol and Doxorubicin[a)]

| Drug | $IC_{50}$ ($\mu M$)[b)] | |
|---|---|---|
| | P388 | P388/ADR |
| Amrubicin | 2.3±0.1 | 12±2 |
| Amrubicinol | 0.028±0.012 | 3.4±0.9 |
| Doxorubicin | 0.060±0.016 | 3.9±0.0 |

*a*) Cells were incubated with the drugs for 1 h, and grown in drug-free medium for 2 days.
*b*) The data are the mean $IC_{50}$ value±standard deviation of two or three experiments.

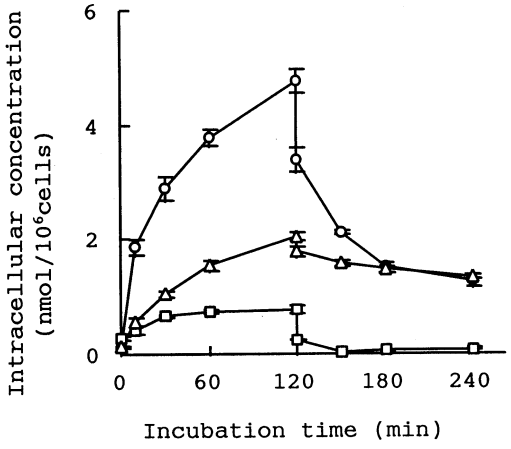



Fig. 2. Uptake of amrubicin, amrubicinol and doxorubicin by P388 cells. Cells were incubated with 10 $\mu$g/ml of amrubicin hydrochloride (□), amrubicinol hydrochloride (○), and doxorubicin hydrochloride (△) for 2 h and then the drugs were removed. The cells were then incubated in the drug-free medium for an additional 2 h. The intracellular drug concentrations were determined at the indicated time. The symbols indicate the mean values and standard deviations. Similar results were obtained in an additional experiment.

concentrations using spectrofluorometry. Fig. 2 shows intracellular drug concentrations during a 2-h incubation of P388 cells with the drugs at 10 $\mu$g/ml and a further 2-h incubation of the cells after removal of the drugs from the medium. Amrubicinol and doxorubicin in the cells increased during the 2-h drug incubation. In contrast to amrubicinol and doxorubicin, amrubicin reached a maximum level within 30 min. The intracellular concentration of amrubicinol was about 6 and 2 times higher than those of amrubicin and doxorubicin, respectively, at 1 h. The intracellular concentrations of amrubicin and amrubicinol were 7% and 40% of the respective maximum levels at 30 min after drug removal. On the other hand, a substantial amount of doxorubicin, 62% of the maximum level, remained even 2 h after drug removal. Fig. 3 shows the intracellular drug concentrations in P388 and P388/ADR cells after a 1-h incubation. The intracellular concentrations of doxorubicin and amrubicinol in P388/ADR cells

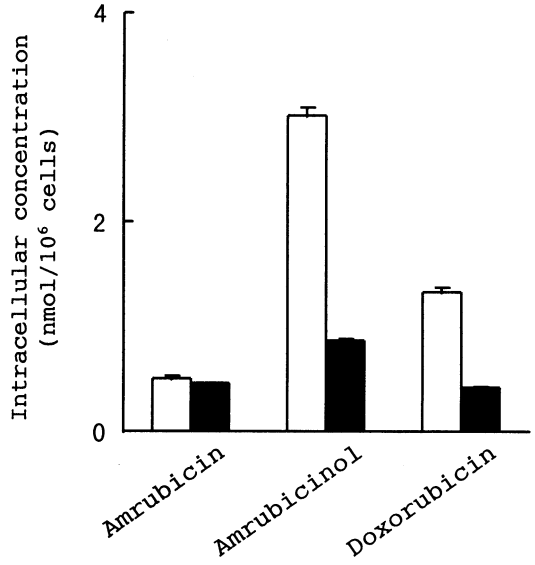



Fig. 3. Uptake of amrubicin, amrubicinol and doxorubicin by P388 and P388/ADR cells. P388 cells (open bars) or P388/ADR cells (closed bars) were incubated with 10 $\mu$g/ml of amrubicin hydrochloride, amrubicinol hydrochloride and doxorubicin hydrochloride. The intracellular drug concentrations were determined after 1 h of incubation with the drugs.

Table II. Distribution of Amrubicin, Amrubicinol and Doxorubicin in P388 and P388/ADR Cells[a)]

| Drug | Concentration (nmol/$10^6$ cells)[b)] | | Nuclei/ whole cell |
|---|---|---|---|
| | Whole cell | Nuclei | |
| P388 | | | |
| Amrubicin | 0.32±0.02 | 0.05±0.00 | 0.16 |
| Amrubicinol | 2.31±0.03 | 0.37±0.04 | 0.16 |
| Doxorubicin | 1.65±0.02 | 1.30±0.10 | 0.79 |
| P388/ADR | | | |
| Amrubicin | 0.28±0.01 | 0.04±0.00 | 0.14 |
| Amrubicinol | 0.75±0.09 | 0.07±0.02 | 0.09 |
| Doxorubicin | 1.37±0.04 | 1.04±0.14 | 0.76 |

*a*) Cells were incubated with medium containing 10 $\mu$g/ml of the drugs for 1 h, and the concentrations of whole cells and nuclei were determined.
*b*) The data are the mean value±standard deviation of quadruplicate drug treatments.

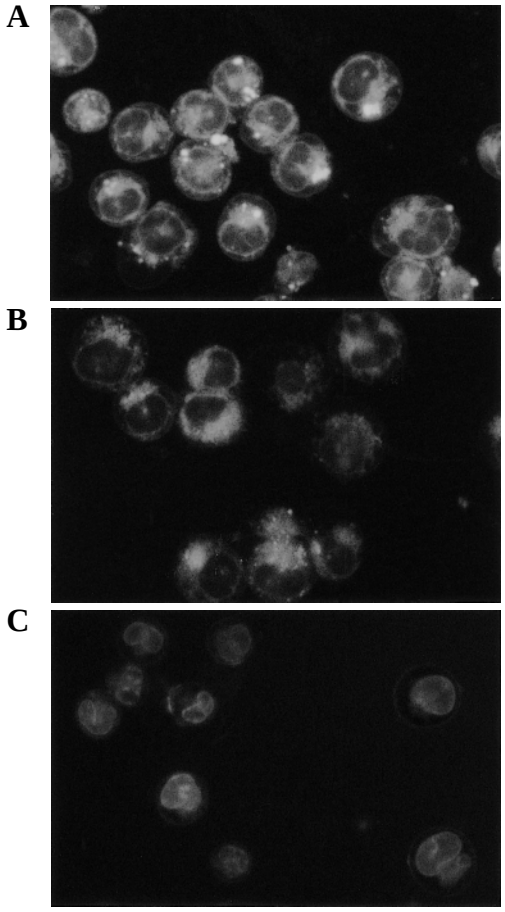



Fig. 4. Intracellular localization of amrubicin (A), amrubicinol (B) and doxorubicin (C) in P388 cells. Cells were treated with 10 $\mu$g/ml of the drugs for 10 min, and were observed by fluorescence microscopy (×330).

were about 2–3 times lower than those in P388 cells, whereas the intracellular concentrations of amrubicin in P388/ADR were in almost the same range as those in P388 cells.

**Intracellular distribution** The intracellular distribution of the drugs was examined. First, P388 and P388/ADR cells were incubated with the drugs at 10 $\mu$g/ml for 1 h, and the drug concentration in the cell nucleus and that in whole cells were determined by spectrofluorometry (Table II). Doxorubicin was shown to be accumulated at higher concentration in the nuclear fraction than were amrubicin and amrubicinol in both P388 and P388/ADR cells. The proportions of the drug detected in the nuclear fraction as a percentage of total drug taken up by the cells during 1-h drug incubation were 76–79% for doxorubicin, 14–16% for amrubicin and 9–16% for amrubicinol. Next, P388 cells incubated with the drugs for 10 min at the same concentration were observed by fluorescence microscopy. As shown in Fig. 4, orange fluorescence was seen, localized in the nucleus of doxorubicin-treated cells. In the case of amrubicin- and amrubicinol-treated cells, the fluorescence was detected mainly in the cytoplasmic compartments, and seemed to be localized in cytoplasmic organelles. The fluorescence microscopic observations were consistent with the results of quantitative analysis by spectrofluorometry described above.

## DISCUSSION

It is generally accepted that the cellular pharmacokinetics of drugs affects their pharmacological activity. Therefore, amrubicin and its C-13 hydroxy metabolite amrubicinol were examined for growth-inhibitory activity, cellular uptake and distribution using P388 and P388/ADR cells, in comparison with doxorubicin.

Amrubicinol was shown to be about 80 times as potent as the parent compound, and about 2 times more potent than doxorubicin in inhibiting the growth of P388 cells, which corresponds well with the findings in human tumor cell lines.[5] It has been shown that the intracellular drug concentration reflects the degree of cell growth inhibition by an anthracycline and its C-13 hydroxy metabolite.[12, 14] In contrast with other anthracyclines, it was demonstrated that amrubicinol was accumulated in the cells at higher concentration than the parent compound. This result suggests that amrubicinol exhibits higher growth-inhibitory activity than amrubicin due, at least in part, to its increased cellular uptake.

Cross-resistance was observed for both amrubicin and amrubicinol, whereas the degree of cross-resistance was reduced for amrubicin in P388/ADR cells. The intracellular drug levels were decreased for both doxorubicin and amrubicinol in P388/ADR cells, indicating that doxorubicin resistance was mediated, at least in part, by alterations of the plasma membrane properties. It is reported that P-glycoprotein is over-expressed in various multidrug-resistant cells.[19–21] In our preliminary experiment using flow cytometry, P-glycoprotein reactive to C-219 antibody was not over-expressed on the P388/ADR cells (data not shown). Further studies are necessary to clarify the expression level of the P-glycoprotein on these resistant cells and the affinity of amrubicin and amrubicinol for the P-glycoprotein.

The intracellular distribution of amrubicin and amrubicinol was markedly different from that of doxorubicin. Less than 20% of amrubicin and amrubicinol incorporated in the cells was located in the nucleus, whereas more than 70% of doxorubicin was located in the nucleus according to previous studies.[14, 22] Cellular distribution was confirmed by observation of the drug fluorescence in the cells using a fluorescence microscope. Amrubicin and amrubi-

cinol resemble aclarubicin and idarubicin rather than doxorubicin and daunorubicin in the pattern of intracellular distribution.[23, 24] We have found the DNA-binding affinities of amrubicin and amrubicinol to be about 7 times lower than that of doxorubicin.[25] Thus, it seems that decreased proportions of amrubicin and amrubicinol in the cell nucleus result from their decreased DNA binding affinities compared to doxorubicin. These observations, along with the results on intracellular drug concentration and the cell growth-inhibitory activity described above, suggest that a similar degree of cell growth inhibition is caused through binding of a smaller amount of amrubicinol to DNA, compared to doxorubicin. Therefore, the cell growth inhibition by amrubicin and amrubicinol seems to be mediated by a different mechanism from that of doxorubicin. It is thought that doxorubicin has several mechanisms of antitumor action, including DNA intercalation, inhibition of topoisomerase II, and generation of free radicals. Our previous study showed that doxorubicin, in contrast to amrubicin and amrubicinol, formed few DNA-protein complexes and produced few DNA strand breaks in cultured human tumor cells at concentrations that cause cell growth inhibition.[25] Further studies are necessary to clarify the cellular pharmacokinetics and the mechanisms by which amrubicin and amrubicinol inhibit cell growth.

In conclusion, we found that amrubicinol, the C-13 hydroxy metabolite of amrubicin, has higher activity in growth inhibition of tumor cells and accumulates in tumor cells at higher levels than its parent compound. This is in contrast to the C-13 hydroxy metabolites of other anthracyclines, such as daunorubicin, doxorubicin and also idarubicin, which are as active or less active than their parent drugs. Hence, the metabolism of amrubicin to amrubicinol plays an important role in the antitumor effect of amrubicin. Further, the mode of action of amrubicin differs from that of doxorubicin, in contrast to the cellular pharmacokinetic character of amrubicin and amrubicinol.

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