Development of a novel anti-tumor drug 'amrubicin', a completely synthetic anthracycline

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Amrubicin is a completely synthetic anthracycline derivative. In contrast, however, the anthracyclines used clinically thus far have been produced by fermentation or semisynthesis. Amrubicin is structurally distinguishable from other anthracyclines by the amino group at the 9-position and its unique sugar moiety. In April 2002, Amrubicin was approved in Japan for the treatment of non-small cell lung cancer and small cell lung cancer.

Introduction

Anthracycline derivatives such as doxorubicin (DXR), daunorubicin (DNR) and epirubicin have been widely used for a variety of carcinomas in the clinical context. In order to discover safer, more effective anti-tumor derivatives, numerous carbon side chain replacement and amino sugar conversions have been conducted since DNR and DXR were discovered from actinomyces.[1] However, despite such attempts many derivatives have not shown better profiles than DXR. Because these anthracycline derivatives are either natural products or semi-synthetic products, they are structurally limited. In order to have more effective derivatives, we have discovered hydrochloric acid amrubicin (AMR, **Fig. 1**) by screening the derivatives created through total synthesis. It is the world's first anthracycline anti-cancer agent produced through total chemical synthesis. AMR possesses the amino group instead of the hydroxyl group at the 9-position. The structure of AMR is such that it possesses a simpler carbohydrate part instead of amino sugar.[2] AMR demonstrates a higher anti-tumor effect than that of DXR against the human tumor xenografts that have been implanted subcutaneously into nude mice.[3] It has been confirmed that its active metabolite amrubicinol (AMR-OH, Fig. 1) plays an important role in its anti-tumor effect.[4] It is a distinctive characteristic of AMR, given that it cannot be seen in any other anthracycline derivative. The major action mechanism of AMR is the
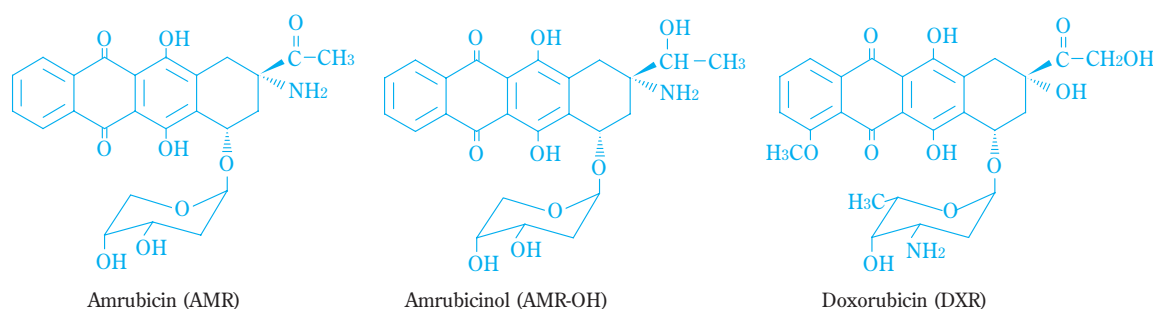
Chemical structures of amrubicin (left), amrubicinol (center) and doxorubicn (right)

Fig. 1 Chemical structures

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stabilization of cleavable complex via DNA topoisomerase II (Topo II).[5]

The manufacture of AMR for the application to non-small cell lung cancer and small cell lung cancer was approved in April 2002. The efficacy and pharmacological action of AMR and the results of clinical testing conducted on AMR are described below:

Efficacy and Pharmacological Action of Amrubicin Hydrochloride

1. *In Vitro* Cell Proliferation Inhibitory Effect

As with other anthracycline agents, it has been found that AMR also generates amurubicinol (AMR-OH) in which the 13-position ketone group has been replaced with the alcohol group, as well as aglycone metabolite in which the carbohydrate part has been detached.[6] Comparing the cell-proliferation inhibitory effects of these metabolites and that of their parent compound AMR against four human tumor-cell lines, it has been observed that AMR-OH was more active than AMR and that the activity levels of all three aglycones and a deaminoderivative were low (**Table 1**). Although the reduced form of the 13-position of idarubicin hydrochloride (IDR) retains the same activity level as that of the parent compound,[7] it has been reported that in other anthracycline derivatives the activity levels of metabolites in which the 13-position ketone had been reduced are lower than that of the parent compound.[8] Daunorubicine (DNR) and IDR having structures similar to DXR are launched as a drug for leukemia. While the hydroxyl group is bound at the 14-position of the side chain in the DXR structure, such hydroxyl group is not seen in DAU, IDR or AMR. It appears that as compared to DXR the ketone group at

the 13-position ketone is more easily reduced in the above three agents. It also appears that the hydroxyl group at the 14-position participates in the reduction. Moreover, the common characteristic seen in IDR and AMR is that neither of the agents possesses the methoxy group at the 4-position. This characteristic seems to influence activity in a reduced form at the 13-position. It is speculated that the fact the metabolism at the 13-position enhances activity is one of the major characteristics unique to AMR.

Additionally, using 17 human tumor cell lines we examined the *in vitro* cell proliferation inhibitory effects of AMR, AMR-OH and DXR.[9] Two drug treatment methods were utilized: three-day continuous drug exposure and one-hour drug exposure. The concentration (IC_{50} value) at which cell proliferation is inhibited by 50% was then obtained for each cell line (**Fig. 2**). Subsequently, activity levels were compared using the IC_{50} value as an index. The activity of AMR-OH was 5 – 20 times higher than that of AMR, which was a level of activity similar to DXR. From the results of experimentation using these cell lines, no significant difference was observed between the DXR spectrum and the AMR/AMR-OH spectrums.

Next, cellular pharmacokinetics were examined in order to elucidate the mechanism behind the phenomenon in which the activity level of AMR-OH is greater than that of AMR.[9] Using four human tumor-cell lines, intracellular drug concentrations were measured after one hour of drug exposure to AMR and AMR-OH at various concentrations. **Fig. 3** shows the intracellular concentrations compared to the drug concentrations within the culture medium. From the result of this experiment it has been found that the AMR-OH concentration within the culture medium is one-tenth that

Table 1　Growth Inhibition of Human Tumor Cells Following 3-Day Continuous Exposure to AMR, Its Metabolites, and DXR [a]

| Drug	IC_{50} (μM) [b]			
	CCRF-CEM	U-937	PC-8	A-549
Amrubicin (AMR)	0.58　± 0.03	0.48　± 0.06	0.26　± 0.16	0.062　± 0.008
Amrubicinol (AMR-OH)	0.017 ± 0.008	0.0071 ± 0.0011	0.021　± 0.015	0.0079 ± 0.0022
7-Deoxyamrubicin aglycone	1.1　± 0.1	13　± 0	1.3　± 0.4	0.80　± 0.21
Amrubicinol aglycone	0.79　± 0.04	0.76　± 0.08	0.76　± 0.27	0.45　± 0.25
7-Deoxyamrubicinol aglycone	0.73　± 0.02	0.93　± 0.00	0.92　± 0.25	0.77　± 0.16
9-Deaminoamrubicin	1.2　± 0.3	2.3　± 0.2	9.2　± 5.4	0.70　± 0.08
Doxorubicin (DXR)	0.034 ± 0.001	0.010　± 0.001	0.010　± 0.004	0.0057 ± 0.0000

a) Cells were grown in medium containing various concentrations of the drugs for 3 days.

b) The data are the mean IC_{50} vallue (μM) ± standard deviation of two experiments. CCRF-CEM and U-937 are hematopoietec cell lines; A549 and PC-8 are lung cancer cell lines.

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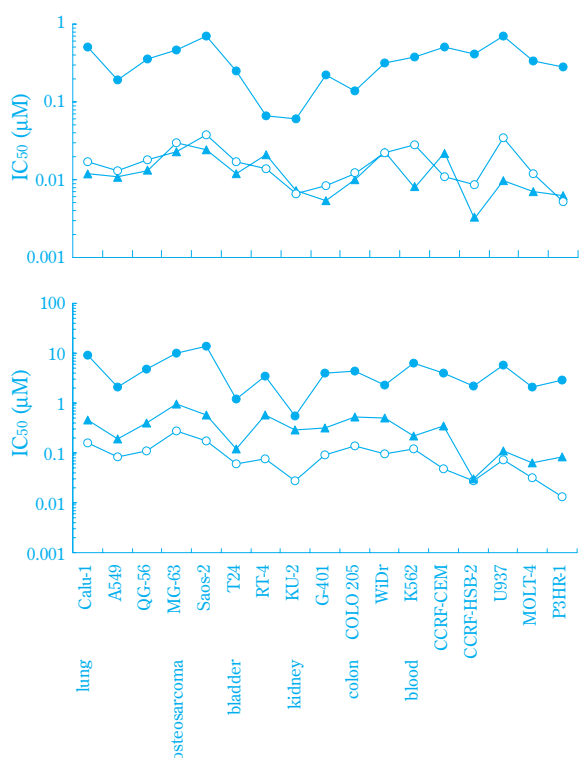
Growth-inhibitory activities of AMR (●), AMR-OH (○) and DXR (▲) on human tumor cells with a 1-h (lower) or 3-day (upper) drug exposure. In the 1-h drug exposure test, cells were incubated for 1 h with drugs, and grown in drug-free medium for 3 days. In the 3-day continuous drug exposure test, cells were grown in the medium containing drugs for 3 days. Results are expressed as mean IC_{50} value of two or three experiments.

Fig. 2   Growth-inhibitory activities
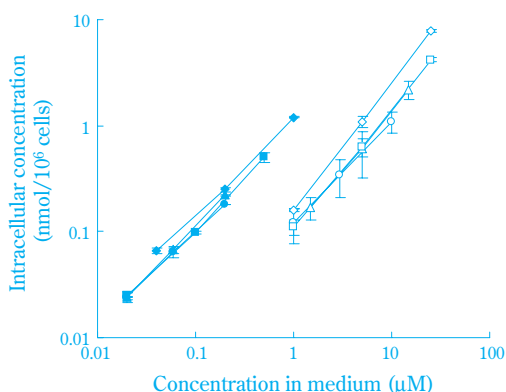
CCRF-CEM (●,○), U-937 (▲,△), QG-56 (◆,◇) or G-401 cells (■,□) were incubated in medium containing various concentrations of AMR (open symbols) or AMR-OH (closed symbols) in triplicate for 1 h, and the intracellular concentrations were measured by HPLC. Each point represents the mean value ± standard deviation of triplicate wells.

Fig. 3   Cellular incorporation of AMR and AMR-OH

of the AMR concentration in order to reach the same intracellular concentration, thus indicating that AMR-OH is more easily taken into the cells. Also, in a similar

test the drug concentrations within a culture medium (IC_{50} value) that possesses a cell proliferatory effect of 50% and the intracellular drug concentrations at this level of effect were compared as the ratio between AMR and AMR-OH. While the ratio of medium concentration became 27 to 67 times greater, that of intracellular concentration became 3 to 7 times greater. This result indicates that AMR-OH is absorbed into the cells approximately 10 times more easily than AMR. The result also suggests that the activity level of AMR-OH is several times higher than that of AMR without the difference of cellular uptake ratio.

2. *In Vivo* Anti-Tumor Effect and Myelosuppression

The *in vivo* anti-tumor effects of anti-cancer agents are usually compared at the maximum tolerated dosage of each agent. Therefore, a dosage at which no death but weight loss of a maximum 3g (15%) occurs within two weeks after the administration was determined as the maximum tolerated dosage of AMR for a single intravenous administration. From the results of experiments conducted on four types of mice (BALB/c, ICR, CDF1 and BDF1), the maximum tolerated dosages of AMR and DXR were determined as 25 mg/kg and 12.5 mg/kg, respectively.[10] The results of animal experiments conducted using these maximum tolerated dosages are shown below.

In anthracycline agents, myelosuppression and local injury in the area of injection are considered toxic effects. Therefore, we conducted the following experiment: The maximum tolerated dose or one-half the maximum tolerated dose of AMR or DXR was administered to a BALB/c mouse intravenously only once, and a femur was periodically excised in order to measure the number of bone marrow cells.[10] As shown in **Fig. 4**, a strong bone-marrow inhibitory effect manifested after administration of the maximum tolerated dose of AMR. Although the degree of such effect decreases when one-half the maximum tolerated dose was administered, the suppressive effect of AMR was nearly the same as that of DXR when the maximum tolerated dose was administered. On the other hand, when comparing the recovery periods from the myelosuppression of AMR and DXR, while it requires only eight days to recover when AMR is administered, it requires only 11 days to recover when DXR is administered. This means that although the myelosuppression of AMR is stronger than that of DXR, recovery from the effect of

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AMR is more rapid than recovery from the effect of DXR. From the results of examining the myelosuppression of AMR by counting the number of stem cells (CFU-GM) of an ICR mouse, while it started to decrease one day after administration, recovery was noticeable three days after administration.[11] In contrast, with DXR, the recovery did not occur until 10 days after administration. Although further research is still required, it can be assumed that while the effect of AMR is stronger on stem cells that are in the differentiation stage after CFU-GM, the effect of DXR is stronger on stem cells that are in the differentiation stage before CFU-GM.
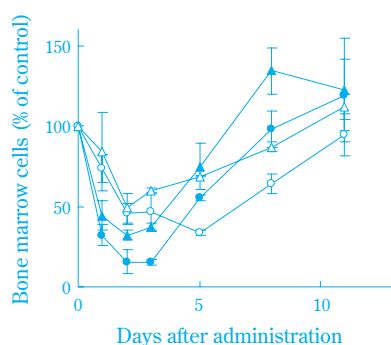

Time-course of the number of bone marrow cells after single i.v. injection of AMR and DXR. Male BALB/c mice were treated with AMR (25mg/kg, ●, or 12.5mg/kg, ▲) or DXR (12.5mg/kg, ○, 6.25mg/kg, △). The femurs were removed rapidly at definite intervals, and then bone marrow cells were flushed with 0.85% NaCl. The number of them was counted with a Coulter counter. Results are expressed as mean ± SD of three or four mice.

Fig. 4 Myelosuppression of AMR

Next, the local tissue toxicity of AMR was compared to that of DXR using the method that Siegel and other researchers have employed in examining the toxic effects of DXR when administered to the local area.[10] Toxicity was evaluated by observing the amount of inflammation after injecting subcutaneously with 10µl of either AMR or DXR solution in the plantar region of hind paw of CDF1 mice. The results indicate that the toxicity of AMR is lower than that of DXR (**Fig. 5**). Given that necrosis occurred accidentally with DXR due to leakage from the vein at the time of administration, it can be concluded that AMR is a relatively safe agent in terms of local toxicity.

Using a mouse experimental tumor, the *in vivo* AMR anti-tumor effect was compared to that of DXR.[3] In an experiment to measure the increased life span of each agent administered intravenously after implanting the
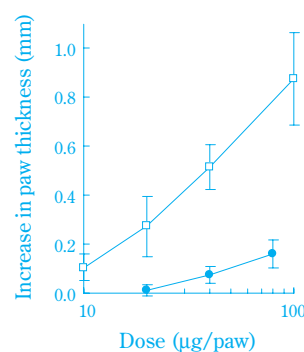

Dose-response relationship for CDF1 mouse hind paw inflammation induced by s.c. injection of AMR and DXR. CDF1 mice were injected s.c. with the indicated doses of AMR (●) or DXR(□). Each point represents the mean ± SD of 9 or 10 mice.

Fig. 5 Dose-response relationship for inflammation

P388 leukemia cell line intraperitoneally, AMR demonstrated strong life prolongation, as did DXR. In experiments to compare the tumor-growth inhibitory effects of AMR and DXR, Ehrlich carcinoma, Sarcoma 180, Lewis lung carcinoma, B16 melanoma and Colon adenocarcinoma 38 were implanted either intramuscularly or subcutaneously into mice. The results are as follows: AMR showed a stronger effect than DXR with two cell lines (Ehrlich carcinoma, Sarcoma 180); the effects of AMR and DXR were nearly the same with two cell lines (Lewis lung carcinoma, Colon adenocarcinoma 38); AMR also showed a weaker effect than DXR with one cell line (B16 melanoma).

The anti-tumor effect of AMR and that of DXR were compared using human tumor xenografts implanted subcutaneously in nude mice.[3] In the experiment with a single intravenously administration of the maximum tolerated dose of either AMR or DXR (25mg/kg and 12.5mg/kg, respectively), AMR demonstrated a stronger anti-tumor effect than DXR against the breast cancer (MX-1), small-cell lung cancer (LX-1) and gastric cancer (SC-6, SC-9 and 4-1ST). **Fig. 6** shows the anti-tumor effects of AMR and DXR against the human gastric cancer known as 4-1ST. From the result of our experiments of myelosuppression, one course was determined as 10 days, which was the time period required for a mouse to recover from the myelosuppression. When AMR was administered at maximum tolerated dose for three courses into nude mice implanted subcutaneously with human gastric cancer 4-1ST[3], no aggravation was observed in the toxicity with weight loss used as an index. Furthermore, a strong anti-tumor effect was observed including com-

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plete tumor regression in five out of six cases. With respect to DXR, after giving the maximum tolerated dose with a single administration at a 10-day interval, several mice died, indicating that toxicity had become more intense.
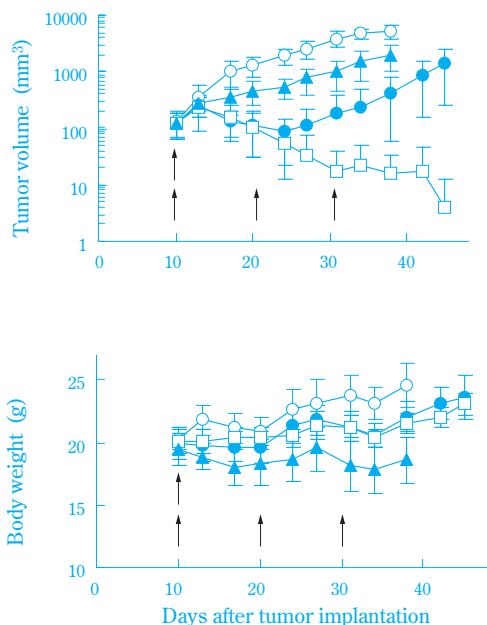
The growth of human tumor xenograft 4-1ST and body weight change in tumor-bearing nude mice after i.v. injection of AMR and DXR. Groups of 6 nude mice implanted with tumor were untreated (○), given DXR i.v. at a dose of 12.5mg/kg with a single administration(▲), given AMR i.v. at a dose of 25 mg/kg with a single administration (●), or 3 administrations every 10 days (□).

**Fig. 6** In vivo anti-tumor activity of AMR

The enhancement effect of the administration schedule toward the drug efficacy of AMR was examined through a similar drug efficacy evaluation method using nude mice.[12] In total dosages, the maximum tolerated dose of five days consecutive administration became 1.5 times greater than a single-dose administration, thus indicating that the anti-tumor effect had been enhanced. However, the myelosuppression lasted longer and was enhanced as a result of the extended administration period. Taking these factors into account, a three-day consecutive administration method is currently clinically applied to lung cancer.

3. Importance of Active Metabolite

As described in the previous section, it has been discovered that AMR demonstrates a stronger *in vivo* anti-tumor effect than DXR. As a result of examining the metabolic mechanism of AMR, we have obtained data

suggesting that AMR-OH plays an important role in anti-tumor activity of AMR. This data is introduced below:

Drug distributions of AMR and DXR to the tumor tissues and normal tissues were compared using nude mice bearing the human gastric cancer 4-1ST.[4] The result showed that DXR distribution was greater in the normal tissues of the lung, liver and kidney than that in the tumor tissues (**Fig. 7**). In contrast, after administration of AMR, the concentration of AMR-OH was higher in the tumor tissue than in the normal tissue. Moreover, when AMR-OH was directly administered intravenously, unlike the AMR administration method, the greater distribution of AMR-OH was observed in
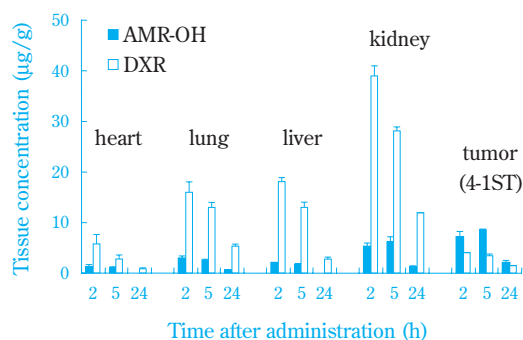
Tissue levels are expressed as the mean concentration of AMR-OH (■) after administration of AMR (25mg/kg) and DXR (□) after administration of DXR (12.5mg/kg) from two or three mice. Bars represent SD. At 2, 5 or 24 h after injection of drugs, AMR-OH and DXR were extracted from tissues and analyzed by HPLC.

Fig. 7 Tissue levels of AMR-OH and DXR in tumor-bearing mice.
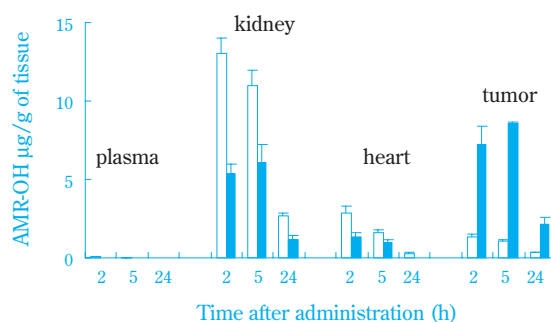
AMR and AMR-OH were i.v. administered at 25 or 12.5 mg/kg, respectively, to mice bearing 4-1ST human tumor xenografts. After 2, 5 or 24 h, the plasma, kidney, heart and tumor tissue were excised and the levels of AMR-OH were determined. The closed and open columns indicate AMR-OH level after AMR and AMR-OH administration, respectively. Each point represents the mean and standard deviation of data for 3 mice per group.

Fig. 8 Tissue levels of AMR-OH after administration of AMR-OH

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the normal tissues than in the tumor tissues (**Fig. 8**).[13] It can therefore be concluded that the tumor selectivity decreased with AMR-OH, indicating that its pharmacokinetic profile is somewhat similar to that of DXR. Thus we have examined the conversion activity of AMR into AMR-OH using the homogenate of normal tissues and tumor tissue. As a result, the conversion activity was observed not only in the liver and kidney but also in tumor tissues at a high rate (**Table 2**). Considering the above fact, as well as the result of pharmacokinetic profole in a mouse administered intravenously with AMR, it can be assumed that not only the normal tissues such as the liver but also the tumor tissues greatly contribute to the conversion activity of AMR into AMR-OH.

The AUC values of AMR-OH and AMR were calculated by summing trapezoids.
The in vivo antitumor activity is expressed as the value of minimal T/C%, from the data of ref.3. R is the correlation coefficient.

Fig. 9　Correlation between the in vivo activities and tumor levels of AMR-OH

**Table 2**　*In vitro* Metabolizing Activity of AMR to AMR-OH by Tumor Tissues

	Origin	Enzymatic activity[a]
Human tumor		
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 AMR to AMR-OH /h/mg of protein.

Tissue homogenates were incubated with AMR and NADPH. The concentration of metabolized AMR-OH was determined by HPLC. The converting rate (%) from AMR to AMR-OH was calculated.

Furthermore, the following correlation has been found between *in vivo* anti-tumor activity of AMR against the 7 human tumor xenografts and AMR-OH concentration within the tumor: the higher the AMR-OH concentration within the tumor, the higher becomes the anti-tumor activity.(**Fig. 9**)[13]

These factors suggest that AMR administered intravenously demonstrates the anti-tumor effect after converted into AMR-OH within the tumor, and that its conversion level affects the degree of anti-tumor activity. Additionally, it can be assumed that the level of conversion from AMR to AMR-OH occurring in the normal tissues or within the blood is lower than that occurring in tumor tissues, showing an anti-tumor activity with higher selectivity. In other words, it appears that the active metabolite AMR-OH greatly contributes to the development of the *in vivo* AMR anti-tumor activity.

4. Action Mechanism

The following actions have been reported as the action mechanisms of anthracycline anti-cancer agents: intercalation activity, topo II inhibitory effect and radical production action. Based on this discovery, we conducted the following experiment:

As a result of the experiment to examine the DNA binding capacities of AMR, AMR-OH and DXR by spectrometric titration, we found that the binding constants of AMR and AMR-OH toward DNA were similar (1.4×10^5 and 1.8×10^5 M^{-1}, respectively). However, the binding constant of DXR was 10.2×10^5 M^{-1}, showing a DNA affinity five times greater than that of AMR or AMR-OH.[5]

Because anthracycline agents emit fluorescent light, the distributions of AMR, AMR-OH and DXR within cells can be directly observed through a fluorescent microscope. The distributions of these agents within the cells were measured using the mouse leukemia-cell line P388.[14] As shown in **Fig. 10**, after a short period of drug exposure it was observed that while AMR and AMR-OH were distributed more to cytoplasmic granules than to nuclei, DXR was mainly distributed to nuclei. Furthermore, using the same P388 cells, the distributions of these agents to cytoplasm and cell nuclei were compared through the quantifications of the agents taken into the whole cell, as well as the separated nuclei, following drug exposure (**Table 3**). As a result, approximately 20% of AMR or AMR-OH, and approximately 80% of DXR were distributed to nuclei. This is somewhat consistent with the results of observation using a fluorescent microscope. This fact reflects the aforementioned significant affinities of the agents with DNA. It can therefore be concluded that on

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A

B

C
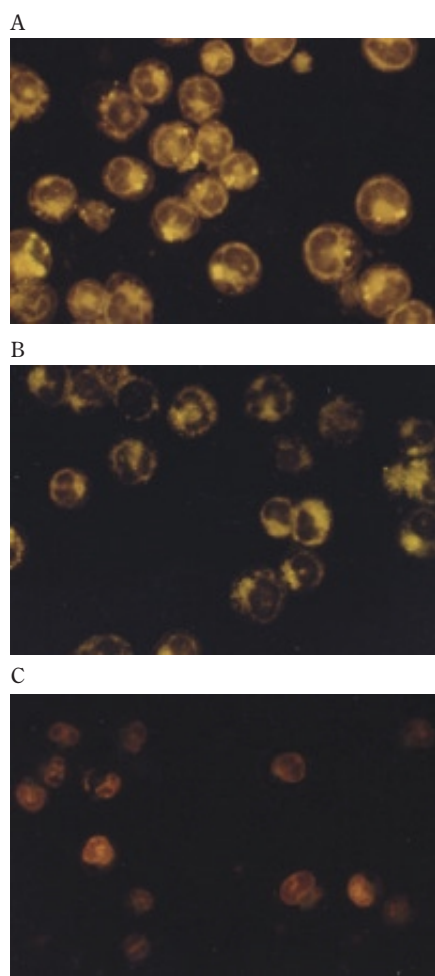
Intracellular localization of AMR (A), AMR-OH (B) and DXR (C) in P388 cells. Cells were treated with 10 µg/ml of the drugs for 10 min, and were observed by fluorescence microscopy.

Fig. 10   Intracellular localization

Table 3   Distribution of AMR, AMR-OH and DXR in P388 cells.[a]

drug	concentration (µmol/10^6 cells)[b]		nuclei/ whole cell
	whole cell	nuclei	
AMR	0.32 ± 0.02	0.05 ± 0.00	0.16
AMR-OH	2.31 ± 0.03	0.37 ± 0.04	0.16
DXR	1.65 ± 0.02	1.30 ± 0.10	0.79

a) Cells were incubated with medium containing 10 µg/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 triplicate drug treatments.
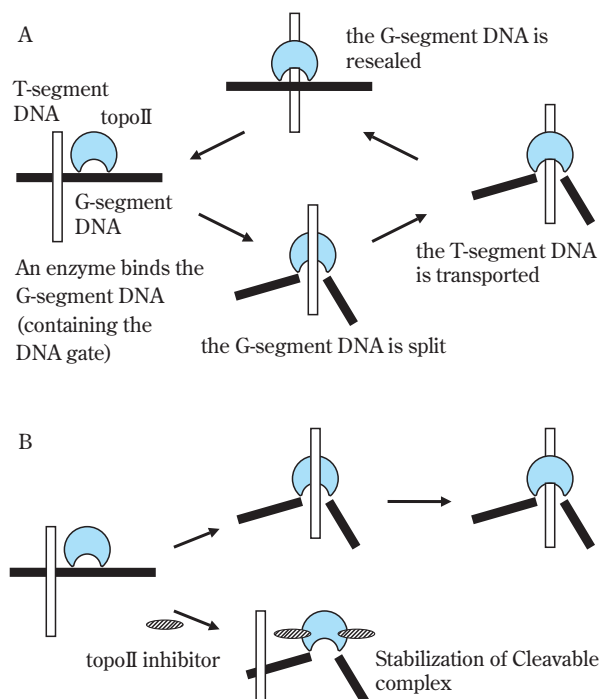
the cell level the characteristic differences among agents are indicated in the differences in cellular localization.

It has been reported that anthracycline agents interact with DNA by intercalation. Specifically, the plane part (which is the nucleus of a molecule) has slipped into a crack between the bases in the planar structure. As a result of measurement using the NMR it has been confirmed that, as was the case with DXR, AMR was bound to DNA through intercalation.[15] Furthermore, the intercalation activity of AMR toward DNA was examined using the DNA unwinding effect as an index. Although AMR and AMR-OH demonstrated intercalation activity at the concentrations of 40 µM and 35 µM or lower, respectively, the activity levels were approximately seven times lower than that of DXR, which demonstrated intercalation activity at the concentration of 5µM or lower.[5] Therefore, both AMR and AMR-OH possess binding activity to DNA through intercalation, although this activity is weaker than that of DXR.

Topo II is a nuclear protein that regulates DNA topology through strand breakage, strand passage and religation of double strand DNA. Thus, topo II is extensively involved in DNA metabolisms including replication, transcription, recombination and sister chromatide segregation.[16] It is also well known that mammalian topo II is the primary cellular target of a number of potent anti-tumor agents such as anthracycline, acridine, ellipticine and epipodophyllotoxin (etoposide).[17] For example, during the replication process of DNA having a helical structure, the duplicated DNA chain and the original DNA chain usually become spirally intertwined. Using topo II, however, these chains are untwined (**Fig. 11**). When these double strand are passing, it is necessary to temporarily cleave one of the DNA double strand. During this process the reaction intermediate is produced through the covalent binding between the phosphate group (at the end of the cleaved DNA) and the topo II protein. This reaction intermediate is referred to as a cleavable complex. Topo II inhibitors are generally classified into two types, depending on the presence of the stabilization effect of this cleavable complex. Etoposide is one of the two types. It is generally considered that since etoposide is a topo II inhibitor (topo II poison) possessing the cleavable complex stabilization action, stabilization of the cleavable complex is more important for cytotoxicity of etoposide than inactivation of the topo II function.[18] This theory is also confirmed by the fact that in etoposide-resistant cells, for example, the decrease in topo II expression is often observed. In other words, cells become resistant not through the enhancement of enzyme reaction (increase in topo II expression) but through decreased production of the cleavable com-
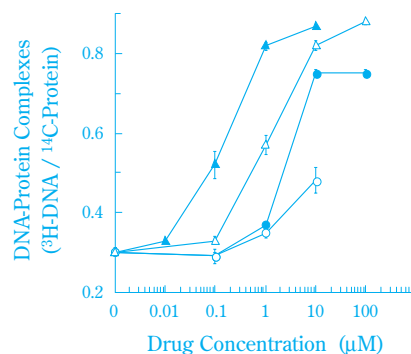
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plex. The other type is represented by topo II inhibitors (topo II catalytic inhibitors), which inhibit enzyme reaction without stabilizing cleavable complex. These inhibitors include merbarone, aclarubicin and suramin.[19]–[21]

A

T-segment DNA

topoII

G-segment DNA

An enzyme binds the G-segment DNA (containing the DNA gate)

the G-segment DNA is resealed

the T-segment DNA is transported

the G-segment DNA is split

B

topoII inhibitor

Stabilization of Cleavable complex

Fig. 11 A molecular model for the catalytic reaction of topoisomerase II

Since decatenation reaction using kinetoplast DNA requires the cleavage, passing and religation of double strand DNA, it can be concluded that it is a reaction unique to topo II , which cannot be seen in topo I. AMR, AMR-OH and DXR have all inhibited this decatenation reaction at 25μM or greater, and etoposide has inhibited the reaction at 125μM or greater.[5] Moreover, in the cell free experiment to evaluate the cleavable complex stabilization effect caused by the topo II inhibition using fragmented DNA as an index, the acceleration of DNA fragmentation through topo II was recognized in the AMR or AMR-OH treatment at 5μM or greater. The DNA fragmentation used as an index was carried out through the following procedure: Linear plasmid DNA was reacted with the topo II derived from a human, then the stabilized cleavable complex was treated with proteinase K. Under the same conditions DXR demonstrated almost no DNA fragmentation. From these results it has been suggested that both AMR and AMR-OH are topo II inhibitors that possess the cleavable complex stabilization effect.

Regarding the cleavable complex stabilization effect on cell system via topo II inhibition, the quantity of the DNA-protein complexes that have been stabilized by generating cleavable complexes can be measured through the K-SDS precipitation method, which recovers DNA that is covalently bound with protein. Additionally, the effect can be found as a scission of chromosome DNA using the pulsed-field electrophoresis that separates DNA chains at the unit of several thousand kb (kilo base pairs). It is known that topo II inhibitors including teniposide and m-AMSA stabilize the DNA-protein complexes at the cell level.[22], [23] When treating human leukemia cell lines CCRF-CEM with AMR or AMR-OH, as with etoposide, the formation of DNA-protein complexes increased, accompanied by the increase in drug concentration (**Fig. 12**).[5] The IC$_{50}$ values of cell-proliferation inhibitory effects of AMR, AMR-OH, DXR and etoposide against CCRF-CEM cell lines were 3.3μM, 0.060μM, 0.40μM (Fig. 2) and 2.3μM, respectively. Accordingly, under conditions where cell growth was inhibited by AMR, AMR-OH and etoposide, considerable amount of DNA-protein complexes were formed. On the other hands, DXR failed to form DNA-protein complexes at the concentration that induced cell growth inhibition. Furthermore, through pulsed-field electrophoresis it has been observed that chromosome DNA can be cleaved by AMR or AMR-OH at the concentrations that induce DNA-protein complexes formation.[5] It has been reported by Rowe, Long and other researchers that the cytotoxic effects of acridine derivative and epipodophillotoxin derivative are related to the quantity of DNA-pro-

Acute lymphoblastic leukemia CCRF-CEM cells were treated with drugs 1 h. The ability of drugs to stabilize DNA-protein complexes was measured by means of the K-SDS precipitation assay. ● AMR, ▲ AMR-OH, ○ DXR, △ etoposide. Results are expressed as mean ± SD of triplicates.

**Fig. 12** DNA-protein complex formation in human tumor cells

tein complex formation and the quantity of cleavage of chromosome DNAs.[22),24)] These discoveries suggest that AMR and AMR-OH induce cytotoxicity due to the cleavable complex stabilization effect via topo II. On the other hand, both the DNA-protein complex stabilization effect and the chromosome DNA scission of DXR were weaker compared to AMR and AMR-OH.

To further examine the relationship between the cytotoxicity and cleavable complex stabilization effect of AMR, several experiments were conducted as described below. It has already been found that the cytotoxicities of etoposide, m-AMSA and DNR can be canceled by aclarubicin [25), 26)], and that the cytotoxicity and cleavable complex stabilization effect of etopocise can be restrained by the dioxisopiperazine derivative ICRF-193.[27)] Both aclarubicin and ICRF-193 are topo II catalytic inhibitors, which can restrain topo II poison. Based on these findings, we have examined the effects of ICRF-193 toward the cleavable complex stabilization effects of AMR, AMR-OH and etoposide. It has been found that the cleavable complexes stabilized by AMR, AMR-OH and etoposide in the human leukemia cell strain CCRF-CEM can be restrained by adding ICRF-193, depending on the dosage (**Fig. 13**).[5)] Furthermore, under the same conditions we have examined the influence of ICRF-CEM to the *in vivo* cell-proliferation inhibitory effects of AMR and DXR. Although the cell reproduction rate of CCRF-CEM was reduced by as much as 20% in 5μM AMR, it recovered to 70% following the addition of 10μM ICRF-193 (**Fig. 14**). While the cell-proliferation inhibitory effects of AMR-OH and etoposide were also restrained by ICRF-193, the same effect of DXR was not affected by ICRF-193.
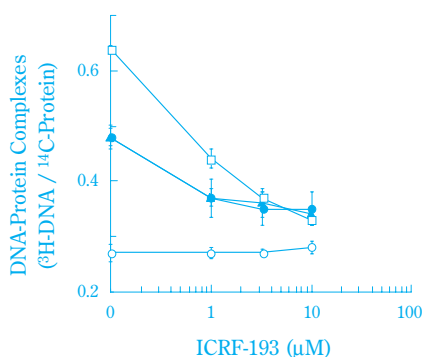
Cells were preincubated with ICRF-193 for 30 min followed by incubation with both ICRF-193 and drugs. After 1 h, the formation of DNA-protein complexes was quantitated. ● 5 μM AMR, ▲ 0.2 μM AMR-OH, ○ ICRF-193 alone, □ 5 μM etoposide.

Fig. 13 Antagonistic effect of ICRF-193 on DNA-protein complex formation
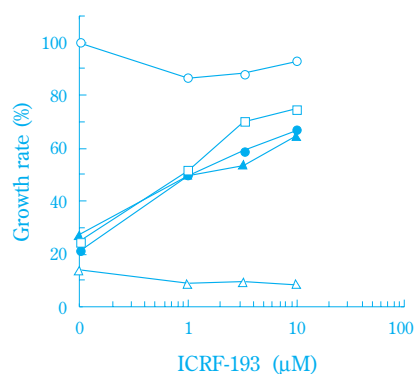
Cells were treated as described in Fig.13. After 1 h, cells were re-incubated for 3 days in the absence of drugs, and then counted in a Coulter counter. ● 5 μM AMR, ▲ 0.1 μM AMR-OH, ○ 1 μM DXR, △ 5 μM etoposide.

Fig. 14 Antagonistic effect of ICRF-193 on cell growth inhibition

It has therefore been clarified that AMR and AMR-OH restrain the proliferation of tumor cells mainly by stabilizing cleavable complexes through topo II. Since DXR has a weaker cleavable complex stabilization effect by comparison to AMR or AMR-OH, it can be assumed that the DXR's cell-proliferation inhibitory effect depends on the intercalation activity toward DNA, not on the cleavable complex stabilization effect.

Results of Clinical Study

A clinical study of monotherapy with AMR demonstrated a high response rate of 76% against untreated extensive desease small-cell lung cancer (ED-SCLC), which was a result similar to that of the existing standard combination therapy. Moreover, the response rate of AMR against untreated non-small cell lung cancer was 23%. The major side effect was myelosuppression. In particular, the incidence rate of neutropenia of grade 3 or higher was 77%. Accordingly, AMR has received indication against both small-cell lung cancer and non-small cell lung cancer. Currently, the post-manufacture/sales clinical study of AMR against small-cell lung cancer and non-small cell lung cancer is being conducted. It can be expected that the efficacy against small-cell lung cancer will continue to improve through further clinical study, including combined therapy, thereby allowing AMR to gain wide acceptance as a standard therapeutic agent against small-cell lung cancer. Moreover, the late phase II clinical study of AMR against non-Hodgkin lymphoma has already been completed. In the basic testing it has been discovered that AMR

has low cardiotoxicity, which is a problem common to anthracycline anti-tumor agents, and evidences of low cardiotoxicity of AMR has also been collected in clinical study. It can therefore be expected that AMR will eventually become a useful therapeutic agent for other cancers, taking advantage of low carciotoxicity.

Conclusion

DXR is an anthracycline anti-cancer agent that has been widely used. It is one of the indispensable drugs for current cancer treatment. However, to design an anti-tumor agent stronger than DXR through the total synthesis of anthracycline, screening was conducted on chemical compounds having configurations that have never been achieved in the past, merely by using the conventional methods of fermentation and semisynthesis. AMR is the agent created as a result of such a screening process. It has properties that are different than those of conventional anthracycline agents: First, when it is metabolized its activity becomes enhanced. Secondly, its tumor selectivity increases when it becomes metabolized and when the metabolites become distributed. Although the initial intention was not to achieve an agent having these metabolic characteristics, we believe the creation of this unique agent AMR owes significantly to our senior researchers' persistent effort, strong enthusiasm to develop an agent that can fight cancer, and the ability to identify the effective anti-cancer agents. This agent possesses indication against lung cancer. Furthermore, it is anticipated that its indication will extend to non-Hodgkin lymphoma. It is our hope that the indication of AMR will attain further cancer type, and that AMR will contribute to the well-being of greater numbers of cancer patients.

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