A New Antitumor Agent Amrubicin Induces Cell Growth Inhibition by Stabilizing Topoisomerase II-DNA Complex

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Amrubicin is a novel, completely synthetic 9-aminoanthracycline derivative. Amrubicin and its C-13 alcohol metabolite, amrubicinol, inhibited purified human DNA topoisomerase II (topo II). Compared with doxorubicin (DXR), amrubicin and amrubicinol induced extensive DNA-protein complex formation and double-strand DNA breaks in CCRF-CEM cells and KU-2 cells. In this study, we found that ICRF-193, a topo II catalytic inhibitor, antagonized both DNA-protein complex formation and double-strand DNA breaks induced by amrubicin and amrubicinol. Coincidentally, cell growth inhibition induced by amrubicin and amrubicinol, but not that induced by DXR, was antagonized by ICRF-193. Taken together, these findings indicate that the cell growth-inhibitory effects of amrubicin and amrubicinol are due to DNA-protein complex formation followed by double-strand DNA breaks, which are mediated by topo II.

Key words: Amrubicin — Anthracycline — DNA-protein complex — Double-strand DNA break — DNA topoisomerase II

DNA topoisomerase II (topo II) is a nuclear enzyme that regulates DNA topology through strand breakage, strand passage and religation. Thus, topo II is extensively involved in DNA metabolism, including replication, transcription, recombination and sister chromatid segregation. Mammalian topo II is the primary cellular target of a number of potent antitumor agents such as doxorubicin (DXR), daunorubicin (DNR), etoposide and amsacrine (m-AMSA). These agents interfere with the breakage-reunion reaction of topo II by trapping a covalent enzyme-DNA complex, termed “cleavable complex,” in which DNA strands are broken and their 5′ termini are covalently linked to the protein. In general, the cytotoxicity of these topo II poisons is dependent on stabilization of the cleavable complex followed by topo II-mediated DNA damage, rather than inactivation of topo II cellular functions.

Amrubicin is a novel, completely synthetic 9-aminoanthracycline derivative, and has antitumor activities in murine experimental tumor systems and human tumor-nude mouse systems. Like other anthracycline derivatives, such as DXR and DNR, amrubicin is converted to its C-13 alcohol metabolite, amrubicinol. In contrast to doxorubicinol and daunorubicinol, amrubicinol has much higher antitumor activity than the parent drug in vitro. In addition, amrubicin showed much weaker cardiotoxicity than DXR in a rabbit chronic experimental model. Phase II clinical trials of amrubicin for the treatment of malignant lymphoma, superficial bladder, small cell lung carcinoma, and non-small cell lung carcinoma are in progress. Amrubicin showed substantial activity (response rate of 25%) against non-small cell lung cancer, and a response rate of 78.8% was obtained against small cell lung cancer.

The mechanisms underlying the antitumor activities of amrubicin and amrubicinol are not well understood. Our present study has shown that amrubicin and amrubicinol target human topo II by stabilizing the cleavable complex. Furthermore, amrubicin and amrubicinol induced DNA-protein complex formation and double-strand DNA breaks in cultured cells. We also investigated the antagonistic effect of ICRF-193, a topo II catalytic inhibitor, on the cell growth inhibition, DNA-protein complex formation and double-strand DNA breaks induced by amrubicin and amrubicinol.

MATERIALS AND METHODS

Materials Amrubicin hydrochloride (Fig. 1), (+)-(7S,9S)-9-acetyl-9-amino-7-[(2-deoxy-β-D-erythro-pentopyranosyl)-oxy]-7,8,9,10-tetrahydro-6,11-dihydroxy-5,12-naphthacenecidine hydrochloride (SM-5887), amrubicinol hydrochloride and ICRF-193 were prepared by Sumitomo Pharmaceuticals Co., Ltd. (Osaka). DXR and etoposide were obtained from Kyowa Hakko Co., Ltd. (Tokyo) and Nihon Kayaku Co., Ltd. (Tokyo), respectively. Kinetoplast DNA and plasmid pBR322 DNA were purchased from Wako Pure Chemical Industries, Ltd. (Osaka). Human type II topoisomerase was purchased from Topogen Inc. (Columbus, OH). L-[U-14C]Leucine (1.85 MBq/
ml) and [methyl-3H]thymidine (37 MBq/ml) were from Amersham International plc (Buckinghamshire, England). DNA type I from calf thymus was from Sigma Chemical Co. (St. Louis, MO).

Cell cultures Acute lymphoblastic leukemia CCRF-CEM was obtained from the American Type Culture Collection (Rockville, MD), and grown in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS). Renal carcinoma KU-2, kindly provided by Dr. Hiroshi Tazaki (Keio University), was grown in Eagle’s minimum essential medium supplemented with 10% heat-inactivated FCS. Cells were cultured at 37°C in 5% CO2 /95% air.

DNA interactions The binding studies of amrubicin, amrubicinol and DXR were performed by spectrometric titration. Reaction buffers, containing 135 mM sucrose, 20 mM Tris-HCl (pH 7.4), 50 mM NaCl, 100 mM KCl, 1 mM MgCl2 and 1 mM CaCl2, were mixed with drugs, as previously described by Terasaki et al.14) Aliquots (5–20 µl) of DNA solution (0.2 mg/ml), up to 150 µl, were added to a cuvette filled with 3 ml of reaction buffer. The reaction mixture was stirred for 5 min, and the absorbance was measured on a spectrometer V-520-SR (Nihon Bunkoh, Tokyo) at 460 nm for amrubicin and amrubicinol, and 470 nm for DXR. The drug affinity for DNA was determined by quantitating the change in absorbance. The affinity constants and the number of binding sites were obtained by the least-squares method.

DNA unwinding effects caused by intercalation were assayed according to the method of Ono et al.15) Reaction mixtures (10 µl) containing 10 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 1 mM dithiothreitol (DTT), 1 mM ATP, 0.2 µg of pBR322 DNA linearized with EcoRI, 0.4 units of T4 DNA ligase and drugs were incubated at 4°C. After 24 h, DNA samples were precipitated with ethanol and analyzed on a 1% agarose gel in Tris-borate EDTA (TBE) buffer. After electrophoresis, gels were stained with ethidium bromide and photographed under UV illumination.

Kineto-plast-DNA decatenation assay Reaction buffer containing 40 mM Tris-HCl (pH 7.4), 100 mM KCl, 10 mM MgCl2, 0.5 mM EDTA, 0.5 mM DTT, 30 µg/ml of bovine serum albumin (BSA) and 0.5 mM ATP was mixed with 0.2 µg of kinetoplast DNA (k-DNA). 0.5 units of human topo II and drugs in a total volume of 10 µl and the reaction mixtures were incubated at 37°C. After 30 min, reactions were terminated by the addition of 10 µl of a solution containing 40 mM Tris-HCl (pH 7.4), 0.4 mM EDTA, 20% sucrose, 2% sodium dodecyl sulfate (SDS) and 0.01% bromophenol blue, and electrophoresis was then carried out in 1% agarose gel in TBE buffer.

Topo II-mediated DNA cleavage assay Topo II cleavage reactions were carried out in mixtures (50 µl) containing reaction buffer, 0.2 µg of pBR322 DNA linearized with EcoRI, 25 units of human topo II and drugs. One unit corresponds to the activity that decatenates 0.2 µg of k-DNA in 30 min at 37°C. Reaction mixtures were incubated at 37°C for 30 min and then treated with 2.5 µl of 10% SDS and 2.5 µl of 3 mg/ml proteinase K at 37°C for 60 min. DNA samples were precipitated with ethanol and analyzed on a 1% agarose gel in TBE buffer.

DNA-protein complex formation assay in intact cells The formation of covalent DNA-protein complexes was quantitated using the K-SDS precipitation assay as previously described by Chen and Beck.16) Briefly, cells were labeled with both [methyl-3H]thymidine (26 kBq/ml) and [U-14C]leucine (7.4 kBq/ml) overnight. After having been treated with drugs for 1 h at 37°C, the cells were lysed in 1 ml of 5 mM EDTA (pH 8.0), 1.25% SDS and 0.4 mg/ml salmon sperm DNA. Cell lysates in tubes were heated at 65°C for 15 min, and then treated with 2.5 µl of 10% SDS and 2.5 µl of 3 mg/ml proteinase K at 37°C for 60 min. DNA samples were precipitated with ethanol and analyzed on a 1% agarose gel in TBE buffer.

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CA). Results were expressed as the ratio of $^{3}$H-DNA/$^{14}$C-protein.

**Pulsed-field gel electrophoresis** Cells (4×10$^6$) were exposed to drugs for 1 h and washed twice with Ca$^{2+}$, Mg$^{2+}$-free phosphate-buffered saline (PBS). They were then centrifuged at 200g for 5 min at 4°C and resuspended in 100 µl of 50 mM EDTA (pH 8.0). The cell suspension was mixed with 100 µl of 1.6% low melting point agarose and left at 4°C for 30 min. The agarose blocks were placed in 10 mM Tris-HCl (pH 7.5), 500 mM EDTA, 1% N-lauroylsarcosine, 1 mg/ml proteinase K and incubated at 50°C for 12 h. The incubation was followed by rinsing in three changes of 50 mM EDTA (pH 8.0). Plugs of about 5 mm×5 mm were cut from the agarose blocks and used for electrophoresis. Electrophoresis was carried out using the CHEF-DR II system (Bio-Rad Laboratories, Hercules, CA). DNA fragments were separated on a 1% agarose gel in 0.5×TBE buffer at 14°C for 20 h. Switch time was ramped from 60 to 90 s at 200 V. Gels were stained with SYBR Green I Nucleic Acid Gel Stain (Molecular Probes, Inc., Eugene, OR).

**Cell growth inhibition assay** Exponentially growing cells were preincubated with ICRF-193 for 30 min, and then treated for 1 h with both ICRF-193 and each drug. After treatment, drugs were removed and cells were washed twice with Ca$^{2+}$, Mg$^{2+}$-free PBS. The resulting cells were then reincubated for 3 days in the absence of drugs, and counted in a model ZM Coulter Counter (Coulter Electronics Inc., Hieleah, FL).

**RESULTS**

**DNA binding affinity and DNA intercalation** Binding parameters of amrubicin, amrubicinol and DXR to DNA are shown in Table I. The affinity constant of amrubicin for DNA binding was 1.4×10$^5$ M$^{-1}$, which was comparable to the value for amrubicinol. DXR, on the other hand, gave about a 7-fold higher affinity constant than amrubicin and amrubicinol. Although both amrubicin and amrubicinol bound to DNA, they displayed lower affinity for DNA than did DXR. DNA unwinding measurements were subsequently used for the determination of DNA intercalation. As determined from the appearance of supercoiled DNA, amrubicin and amrubicinol produced a DNA unwinding effect at 40 µM and 35 µM, respectively, whereas DXR had this effect at 5 µM (Fig. 2). These results indicate that both amrubicin and amrubicinol interact with DNA by intercalation, although their potencies are lower than that of DXR.

**Inhibition of topo II catalytic activity and stimulation of topo II-induced DNA cleavage** The decatenation of k-DNA into free minicircles is a highly specific assay of topo II, the enzyme that catalyzes the double-strand-passing reaction. Amrubicin and amrubicinol inhibited the

Table I. Binding Parameters of Amrubicin, Amrubicinol and DXR to DNA

| Drugs   | $K_a$ (×10$^5$ M$^{-1}$)$^a$ | $n$$^b$ |
|---------|-----------------------------|--------|
| Amrubicin | 1.4±0.3$^{(n)}$ | 0.20±0.05 |
| Amrubicinol | 1.8±0.1 | 0.20±0.05 |
| DXR     | 10.2±0.2 | 0.18±0.01 |

$^a$ $K_a$, the affinity constant; $n$, the number of binding sites.

$^b$ Mean±SD of triplicate samples.

Fig. 2. DNA unwinding effect. Linearized pBR322 DNA (0.2 µg) was incubated with 0.4 units of T4 DNA ligase in the presence of drugs for 24 h at 4°C.
strand-passing activity of topo II (Fig. 3). In this purified system, the inhibition was evident at 25 µM amrubicin, amrubicinol and DXR, and at 125 µM etoposide.

To examine whether amrubicin stimulates the enzyme-linked DNA cleavage, linearized pBR322 DNA was incubated with human topo II in the presence of amrubicin. It

![Fig. 3. Inhibition of the strand-passing activity of human topo II. k-DNA (0.2 µg) was incubated with 0.5 units of human topo II in the presence of drugs for 30 min at 37°C. NW, kinetoplast DNA network; MC, minicircular monomeric DNA.](image)

![Fig. 4. Topo II-mediated DNA cleavage. Human topo II (25 units) was incubated with 0.2 µg of linearized pBR322 DNA in the presence of drugs for 30 min at 37°C. Arrowhead indicates linearized pBR322 DNA.](image)

![Fig. 5. DNA-protein complex formation in cells. Cells treated with drugs indicated for 1 h at 37°C. The ability of drugs to stabilize DNA-protein complexes was measured by means of the K-SDS precipitation assay. A, CCRF-CEM; B, KU-2. • amrubicin, ▲ amrubicinol, ○ doxorubicin, △ etoposide. Points, the mean of triplicate samples; bars, SD.](image)
was shown that both amrubicin and amrubicinol stimulated topo II-mediated DNA cleavage at concentrations of 5 \( \mu \text{M} \) or above, like etoposide (Fig. 4). At higher concentrations of amrubicin and amrubicinol, DNA cleavage was actually inhibited, presumably due to DNA intercalation. In contrast, little DNA cleavage stimulated by DXR could be detected under the same conditions.

Fig. 6. Double-strand DNA breaks in cells. Cells were incubated with drugs for 1 h at 37°C, and chromosomal DNA was analyzed by pulsed-field gel electrophoresis in 1% agarose.

Fig. 7. Antagonistic effect of ICRF-193 on DNA-protein complex formation. Cells were preincubated with ICRF-193 for 30 min, and then incubated with both ICRF-193 and drugs. After 1 h, the formation of DNA-protein complexes was quantitated. A, CCRF-CEM. ○ ICRF-193 alone, ● +5 \( \mu \text{M} \) amrubicin, ▲ +0.2 \( \mu \text{M} \) amrubicinol, □ +5 \( \mu \text{M} \) etoposide. B, KU-2. ○ ICRF-193 alone, ● +4 \( \mu \text{M} \) amrubicin, ▲ +0.8 \( \mu \text{M} \) amrubicinol, □ +20 \( \mu \text{M} \) etoposide. Points, the mean of triplicate samples; bars, SD.
DNA-protein complex formation in cultured cells. It is known that teniposide and m-AMSA stabilize DNA-protein complexes in cultured cells. We tested the ability of amrubicin to stabilize DNA-protein complexes in cultured cells by K-SDS precipitation assay. As shown in Fig. 5, amrubicin and amrubicinol, like etoposide, induced DNA-protein complex formation in cultured CCRF-CEM cells and KU-2 cells in a dose-dependent manner. By contrast, DXR formed few complexes under the same conditions. The concentration of each agent required to inhibit the growth of cells by 50% (IC50) was determined for each cell line. The IC50 values of amrubicin, amrubicinol, DXR and etoposide for CCRF-CEM cells are 3.3 µM, 0.060 µM, 0.40 µM and 2.3 µM, respectively. These four agents inhibited the growth of KU-2 cells with IC50 values of 0.63 µM, 0.046 µM, 0.42 µM and 5.9 µM, respectively. Accordingly, under conditions where cell growth was inhibited by amrubicin, amrubicinol and etoposide, considerable amounts of DNA-protein complexes were formed. On the other hand, DXR failed to form DNA-protein complexes at concentrations that induced cell growth inhibition.

Double-strand DNA breaks in cultured cells. To elucidate whether amrubicin can induce double-strand DNA breaks in cells, chromosomal DNA prepared from cells treated with amrubicin was resolved by pulsed-field gel electrophoresis. Irrespective of cell line, 1 h of incubation with amrubicin caused an accumulation of DNA fragments of about 2000 kb and lower, indicating that amrubicin induced double-strand DNA breaks in the cells (Fig. 6). A similar pattern of DNA cleavage was observed after 1 h of incubation with amrubicinol and etoposide. DXR was less effective in inducing double-strand DNA breaks than amrubicin, amrubicinol and etoposide. In addition, amrubicin and amrubicinol induced double-strand DNA breaks in other human cancer cell lines such as lung carcinoma.

Fig. 8. Antagonistic effect of ICRF-193 on DNA cleavage. Cells were preincubated with (+) or without (−) ICRF-193 for 30 min followed by incubation with both ICRF-193 and drugs. After 1 h, they were processed for pulsed-field gel electrophoresis.
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QG-56, bladder carcinoma T24 and colon adenocarcinoma WiDr (data not shown), suggesting that double-strand DNA breaks are commonly induced by amrubicin and amrubicinol in various cancer cell lines.

**Antagonistic effect of ICRF-193 on DNA-protein complex formation and double-strand DNA breaks induced by amrubicin**
ICRF-193 is the most potent topo II inhibitor among bis(2,6-dioxopiperazine) derivatives and inhibits topo II-mediated DNA cleavage induced by etoposide.11) The antagonistic effect of ICRF-193 on amrubicin-induced DNA-protein complex formation was studied. We found that ICRF-193 suppressed DNA-protein complex formation induced by amrubicin, amrubicinol and etoposide in a dose-dependent manner (Fig. 7). Preincubation with 1 µM ICRF-193 resulted in a nearly 50% reduction of DNA-protein complex formation when compared to that in cells treated with amrubicin, amrubicinol or etoposide alone. To elucidate further the effect of ICRF-193 observed in cultured cells, we also investigated the influence of ICRF-193 on amrubicin-stimulated double-strand DNA breaks. As shown in Fig. 8, preincubation of cells with ICRF-193 inhibited DNA cleavage induced by amrubicin, amrubicinol and etoposide. These results suggest that both DNA-protein complex formation and double-strand DNA breaks induced by amrubicin and amrubicinol are associated with topo II.

**Antagonistic effect of ICRF-193 on the cell growth inhibition by amrubicin**
Amrubicin inhibited purified human topo II, and ICRF-193 antagonized amrubicin-induced DNA-protein complex formation and double-strand DNA breaks in cells. These findings led us to speculate that ICRF-193 would block the cytotoxicity of amrubicin, if amrubicin exerts its cytotoxic effect via stabilizing a cleavable complex in cells. The cytotoxic potency of amrubicin in the presence or absence of ICRF-193 was evaluated by cell growth inhibition assay. When CCRF-CEM cells or KU-2 cells were preincubated with ICRF-193, the growth-inhibitory effect of amrubicin was diminished (Fig. 9). At 5 µM amrubicin, 10 µM ICRF-193 increased the growth rate of CCRF-CEM cells from about 20 to 70%. Furthermore, ICRF-193 antagonized the growth-inhibitory effects of amrubicinol and etoposide, but not that of DXR, indicating that amrubicin and amrubicinol exert growth-inhibitory effects in quite a different manner from DXR. In cultured cells, thus, by reducing both DNA-protein complex formation and double-strand DNA breaks, ICRF-193 antagonized the growth inhibition induced by amrubicin, amrubicinol and etoposide. Accordingly, these findings indicate that amrubicin and amrubicinol, as well as etoposide, inhibit cell growth by inducing DNA-protein complex formation followed by double-strand DNA breaks.

Fig. 9. Antagonistic effect of ICRF-193 on cell growth inhibition. Cells were preincubated with ICRF-193 for 30 min followed by incubation with both ICRF-193 and drugs. After 1 h, cells were reincubated for 3 days in the absence of drugs, and then counted in a model ZM Coulter Counter. A, CCRF-CEM. ○ ICRF-193 alone, ● +5 µM amrubicin, ▲ +0.1 µM amrubicinol, Δ 1 µM doxorubicin, □ +5 µM etoposide. B, KU-2. ○ ICRF-193 alone, ● +2 µM amrubicin, ▲ +0.1 µM amrubicinol, Δ 1 µM doxorubicin, □ +20 µM etoposide.
DISCUSSION

A number of antitumor agents such as DXR, ellipticines, m-AMSA and actinomycin D have been shown to interact intimately with DNA by intercalation.\(^1\) The findings in purified systems presented here indicate that amrubicin and amrubicinol also interact with DNA by intercalation, although their potencies are lower than those of DXR. Since the DNA binding affinity constants of anthracycline derivatives such as DNR, idarubicin and epirubicin are comparable to the value for DXR,\(^1\) it seems that amrubicin and amrubicinol have lower DNA binding affinity than other anthracycline derivatives.

Mammalian topo II is the target of antitumor agents, such as anthracyclines, acridines, ellipticines and epipodophyllotoxins, that interfere with the breakage-reunion reaction of the enzyme by stabilizing the cleavable complex.\(^1\) In purified systems, both amrubicin and amrubicinol inhibited the decatentation of k-DNA by topo II and stimulated topo II-mediated DNA cleavage. Therefore, amrubicin and amrubicinol are topo II poisons, which stabilize the cleavable complex. On the other hand, neither amrubicin nor amrubicinol inhibited DNA topoisomerase I (data not shown).

There have been several reports on topo II catalytic inhibitors, which lack the ability to stabilize the cleavable complex. These agents include bis(2,6-dioxopiperazine) derivatives, merbarone, aclarubicin, festrinertin and suramin.\(^2\) It has been shown that aclarubicin prevented the cytotoxicity of topo II poisons such as m-AMSA, etoposide and DNR.\(^3\)\(^-\)\(^6\) This effect of aclarubicin is suggested to be caused by inhibition of the non-covalent DNA binding reaction of topo II.\(^7\) ICRF-193, a bis(2,6-dioxopiperazine) derivative, stabilizes topo II in the closed-clamp form, preventing it from opening again, and therefore inhibits cleavable complex formation and growth inhibition induced by etoposide.\(^1\)\(^,\)\(^11\)\(^,\)\(^30\)\(^,\)\(^31\) In cultured CCRF-CEM cells and KU-2 cells, amrubicin and amrubicinol, as well as etoposide, induced both DNA-protein complex formation and double-strand DNA breaks, both of which were antagonized by the topo II catalytic inhibitor ICRF-193. These results confirm that amrubicin and amrubicinol inhibit intracellular topo II by stabilizing the cleavable complex. Although as potent as amrubicin in inhibiting purified topo II, amrubicinol was much more effective in inducing both DNA-protein complex formation and double-strand DNA breaks than amrubicin. One possible explanation for these results is the cellular pharmacokinetic difference between two drugs, with amrubicinol accumulating in the cells at higher levels than amrubicin (unpublished observation).

Rowe et al. previously demonstrated that the level of DNA-protein complexes or DNA strand breaks induced by acridine derivatives correlated with drug cytotoxicity.\(^7\) Long et al. also found a correlation between cytotoxicity and DNA breakage activity of the different congeners of epipodophyllotoxin.\(^8\) Among amrubicin, amrubicinol and etoposide, the ability to induce DNA-protein complex formation and double-strand DNA breaks in cells appears to correlate with that to inhibit cell growth, since these three agents caused DNA-protein complex formation at concentrations that cause cell growth inhibition. Furthermore, ICRF-193 antagonized cell growth inhibition induced by amrubicin, amrubicinol and etoposide. These results indicate that, like etoposide, amrubicin and amrubicinol induce cell growth inhibition by stabilizing protein-DNA complexes followed by double-strand DNA breaks, which are mediated by topo II.

Much effort has been devoted to unraveling the mechanism of antitumor action of DXR. It has been suggested that DXR has several mechanisms of action which cause cellular damage. One of these involves generation of free radicals, which appear to play a major role in the development of cardiomyopathy.\(^9\) Another mechanism is mediated by the interaction with DNA by intercalation and the inhibition of topo II.\(^1\) DXR has been previously shown to induce double-strand DNA breaks and protein-DNA complex formation in cells.\(^9\) However, our studies showed that DXR, in contrast to amrubicin, amrubicinol and etoposide, could form few DNA-protein complexes and produce few DNA strand breaks at concentrations that cause cell growth inhibition. In addition, ICRF-193 could not antagonize cell growth inhibition induced by DXR. Thus, it is suggested that the mechanism by which amrubicin and amrubicinol induce cell growth inhibition is quite different from that in the case of DXR, although the chemical structures of these agents are quite closely related.\(^9\)

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