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3′-Deamino-3′-morpholino-13-deoxo-10-hydroxycarminomycin Conquers Multidrug Resistance by Rapid Influx following Higher Frequency of Formation of DNA Single- and Double-Strand Breaks[1]

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ABSTRACT

The mechanism of action of 3′-deamino-3′-morpholino-13-deoxo-10-hydroxycarminomycin (MX2) was examined in a human leukemia cell line (K562) and its Adriamycin (ADM)-resistant subline (K562/ADM). ADM and MX2 showed an equivalent antitumor effect against K562. K562/ADM was highly resistant to ADM. In cellular pharmacokinetic studies, MX2 showed faster and greater influx than did ADM in both K562 and K562/ADM. The efflux of ADM was rapid in K562/ADM but not in K562. On the other hand, the efflux of MX2 was rapid in both cell lines. The formation of DNA single-strand breaks and double-strand breaks by ADM was significantly lower in K562/ADM than K562. On the other hand, formation of those breaks by MX2 was not decreased. Although some of the DNA breaks induced by MX2 were resealed, there was no difference in the degree of resealing in K562 and K562/ADM cells. On the other hand, most of the small number of DNA breaks in K562/ADM induced by ADM were resealed. The topoisomerase II activity in K562 and K562/ADM was not significantly different. It is concluded that MX2 conquers multidrug resistance by rapid influx following a higher frequency of formation of DNA single- and double-strand breaks in K562/ADM cells.

INTRODUCTION

The anthracycline antibiotics, ADM[3] and DAU, are widely used antitumor agents that are effective for acute leukemia, lymphomas, and a variety of solid tumors (1, 2). However, the use of these agents is restricted by dose-limiting cardiotoxicity (3) and myelosuppression (4), although these problems could partially be reduced by manipulating administration of the drugs. Since the initial observations on P-glycoprotein by Ling and coworkers (5–7), there have been many reports on the presence of P-glycoprotein in drug-resistant cells, and the amino acid sequences of P-glycoprotein have been determined (8–10). The expression of P-glycoprotein coded by the *mdr*-1 gene has been reported to be connected with pleiotropic drug resistance in cell lines, including increased resistance to anthracyclines and *Vinca* alkaloids (10–12). A recent study showed that a high frequency of the multidrug resistance phenotype is associated with prior chemotherapy, clinical drug resistance, and poor prognosis and that a 5′ cDNA probe for *mdr*-1 and monoclonal antibody for P-glycoprotein detected the glycoprotein in 46% (12 of 26) of multiple myeloma, malignant lymphoma, and metastatic breast cancer cases (13). We believe it is very important to find compounds which can conquer this multidrug resistance. MX2 (Fig. 1), developed by Umezawa *et al.*, shows antitumor effects similar or superior to those of ADM against several murine and human tumor cell lines and has been found to be effective even against multidrug-resistance tumor cells (14–17).

In this study we evaluated the antitumor activities and cellular pharmacokinetics of MX2 and ADM, as well as the DNA damages caused by these agents and its repair, in a human leukemia cell line and its ADM-resistant variant (18) in order to determine the mechanism by which MX2 overcomes multidrug resistance. The effects of these drugs on topoisomerase II activity of both cell lines were also evaluated, because both drugs are considered to be topoisomerase II inhibitors.

MATERIALS AND METHODS

Materials. MX2 was obtained from Kirin Brewery Co., Ltd., Tokyo, Japan. ADM formulated for clinical use was from Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan. The drugs were dissolved in distilled water and stored in the dark at −20°C. RPMI 1640 medium was obtained from Nissui Pharmaceutical Co., Ltd., Tokyo, Japan. Fetal bovine serum was obtained from Immunobiological Laboratories, Gunma, Japan. [^{14}C]Thymidine (specific activity, 50 to 60 mCi/mmol) was obtained from Amersham Japan, Tokyo, Japan. Proteinase K was from E. Merck AG, Darmstadt, West Germany, and tetrapropylammonium hydroxide was from Eastman Kodak Co., Rochester, NY. Polycarbonate filters (2.0 μm) were obtained from Nucleopore Corp., Pleasanton, CA. Aquasol-2 was obtained from New England Nuclear Research Products, Boston, MA. Coomassie protein assay reagent was obtained from Pierce Chemical Co., Rockford, IL. kDNA was kindly provided by Professor N. Kuwano (Oita University).

Cell Lines. K562, a human myelogenous leukemia cell line, and the subline of K562 resistant to ADM (K562/ADM) were kindly donated by Dr. T. Tsuruo, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research. These cell lines were grown in suspension culture in RPMI 1640 medium supplemented with 10% fetal bovine serum as reported previously. K562/ADM showed a typical multidrug resistance phenotype (18).

Cell Viability. Tumor cells were treated for various lengths of time with 0.001 to ~300 μmol of MX2 or ADM and incubated for 72 h in the absence of drugs, and the tumor cells were counted with a Model ZBI Coulter Counter. The IC_{50} was determined by plotting the logarithm of the drug concentration *versus* the percentage of survival of the treated cells.

Cellular Uptake and Efflux of ADR and MX2. K562 and K562/ADM, 2×10^6 each, were incubated with 5 μmol of ADM and MX2 for 15 to 120 min. To study efflux, the tumor cells were further incubated in growth medium without drug for 15 to 120 min and then washed with cold saline buffer. The incorporated anthracyclines were extracted with 200 μl of dimethyl sulfoxide, and then cellular proteins

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[3] The abbreviations used are: ADM, Adriamycin; DAU, daunorubicin; SSB, DNA single-strand breaks; DSB, DNA double-strand breaks; kDNA, kinetoplast DNA; IC_{50}; 50% inhibitory concentration; NB, nuclear buffer; cDNA, complementary DNA; MX2, 3′-deamino-3′-morpholino-13-deoxo-10-hydroxycarminomycin; SDS, sodium dodecyl sulfate.

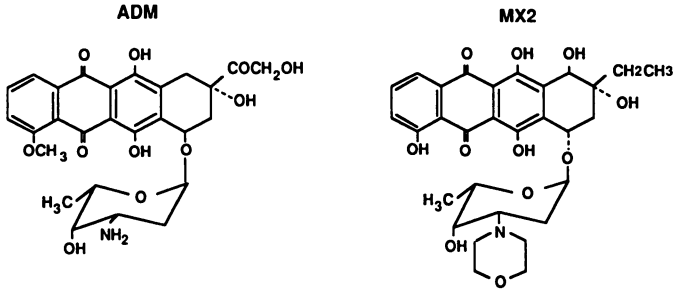

Fig. 1. Structure of ADM and MX2.

were precipitated by the addition of 1.8 ml of absolute methanol. The fluorescence intensity of the extracts was determined with a fluorescence spectrophotometer (Spectrofluorometer FP-770F; Japan Spectroscopic Co., Ltd., Tokyo, Japan) at excitation and emission wavelengths of 470 and 550 nm, respectively. High-pressure liquid chromatography analysis of the cellular extracts did not show the presence of metabolites of any anthracycline after 2 h of exposure.

DNA Strand Breaks. DNA strand breaks were measured by filter elution assay. Filter elution procedures were essentially the same as reported by Kohn *et al.* (19, 20) and described elsewhere (21). Briefly, 1×10^5 cells/ml were labeled with 0.8 μCi/ml of [^{14}C]thymidine for 48 h, followed by a 4-h chase period. For γ-ray irradiation, cells were irradiated on ice with ^{60}Co γ-irradiation at a dose rate of 0.36 Gy/min or 1.7 Gy/s. Drug treatments of cells were carried out at the concentration of 5 μmol. To study DNA resealing, the cells were further incubated in growth medium without drug for 120 min. Six $\times 10^5$ cells were layered on polycarbonate membranes, washed with cold phosphate-buffered saline, and lysed with a solution of 2% SDS, 50 mM glycine, 25 mM disodium EDTA (pH 10.0), 50 mM Tris, and 0.5 mg/ml of proteinase K (5 ml for the SSB study, 2.5 ml for the DSB study). After cell lysis, to remove the lysis solution for the study of SSB, DNA was washed with 0.02 M disodium EDTA (pH 10.0). For the study of DSB, the lysis solutions were discarded through the filters, and the DNA was not washed. DNA on the filter was eluted at 0.04 ml/min for 15 h with 20 mM EDTA (acid form) and tetrapropylammonium hydroxide, pH 12.1 or 9.6, for SSB and DSB, respectively. Fractions were collected at 90-min intervals and mixed with 17 ml of Aquasol-2 containing 0.5% acetic acid for scintillation counting. SSB and DSB frequencies were calculated as previously reported and expressed in rad-equivalents (19, 20).

Topoisomerase II Activity. Nuclear extracts were prepared by the method reported before (22, 23). In brief, exponentially growing cells were collected by centrifugation and washed in ice-cold NB, 2 mM K_2HPO_4, 5 mM $MgCl_2$, 150 mM NaCl, 1 mM ethyleneglycol-bis(β-aminoethylether)-*N,N'*-tetraacetic acid, and 0.1 mM dithiothreitol, pH 6.5. The washed cells were resuspended in NB, and 9 ml of NB supplemented with 0.35% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride were added slowly down the side of the tube. The cell suspension was mixed by rotation for 5 min at 4°C with ice-cold NB containing 0.35 M NaCl. DNA and nuclear debris were pelleted by centrifugation at 17,000 $\times$ *g* for 10 min, and the supernatant was collected. Protein concentration in the extract was determined with the Coomassie protein assay reagent. Topoisomerase II catalytic activity was assayed by decatenation of kDNA into free minicircles. Decatenation was carried out by incubating 5 μl of nuclear extract (containing 1.5 μg of total protein) with 1 μg of kDNA, various dilutions of drug, 50 mM Tris-Cl (pH 7.5), 85 mM KCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM EDTA, 30 μg/ml of bovine serum albumin, and 1 mM ATP in a final volume of 20 μl of reaction mixture, at 30°C for 30 min. Samples were treated with 5 μl of a dye solution consisting of 2% SDS, 50% glycerol, and 0.05% bromophenol blue. The samples were then electrophoresed through 1% agarose in 40 mM Tris-acetate-1 mM EDTA (pH 7.2) at 90 V for 5 h. After being stained with ethidium bromide (10 μg/ml) the gels were photographed under UV light.

RESULTS

Cytotoxicity of ADM and MX2 for K562 and K562/ADM. *In vitro* cytotoxicity of MX2 and ADM for K562 and K562/ADM was compared by exposing these cells for 15, 30, 60, and 120 min to each anticancer drug (Table 1). K562/ADM showed 44.7- to 150.0-fold resistance to ADM, depending on the exposure time. On the other hand, IC_{50} values of MX2 for K562/ADM were similar to those for K562.

Cellular Pharmacokinetics. The uptake of ADM and MX2 by K562 and K562/ADM was examined in the presence of 5 μmol of drug over a period of 2 h (Fig. 2, *A* and *B*). The uptake of ADM by K562 cells was shown to be time dependent during 2 h, but that by K562/ADM reached a plateau within 15 min, and this concentration was lower than that in K562. The difference in uptake between them was about 3-fold at 120 min. The uptake of MX2 by K562 and K562/ADM was also shown to reach a plateau within 15 min, but the maximum concentrations in both cell lines were about 4.5-fold higher than the concentration of ADM in K562 after 120 min of exposure. The efflux of both drugs after 120 min of drug exposure was determined by incubating both tumor cells in RPMI-fetal calf serum without drug for 120 min (Fig. 2, *C* and *D*). The amount of ADM in K562 decreased slowly, and more than 50% of the drug was present after 60 min, but the amount of the ADM in K562/ADM decreased very quickly, and less than 50% of ADM remained after 15 min. On the other hand, there was no difference in the efflux of MX2 between K562 and K562/ADM.

Formation of DNA Strand Breaks. SSB and DSB are shown

Table 1 *In vitro cytotoxicity of MX2 and ADM for K562 and K562/ADM*

Exposure time (min)	ADM, IC_{50} (μM)		MX2, IC_{50} (μM)	
	K562	K562/ADM	K562	K562/ADM
15	1.8	>300	4.4	4.5 (1.0)[a]
30	1.5	224.0 (150.0)	2.7	3.0 (1.1)
60	0.9	67.3 (74.8)	1.9	2.4 (1.3)
120	0.7	31.3 (44.7)	1.8	1.8 (1.0)

[a] Numbers in parentheses, (K562/ADM)/(K562) ratio.

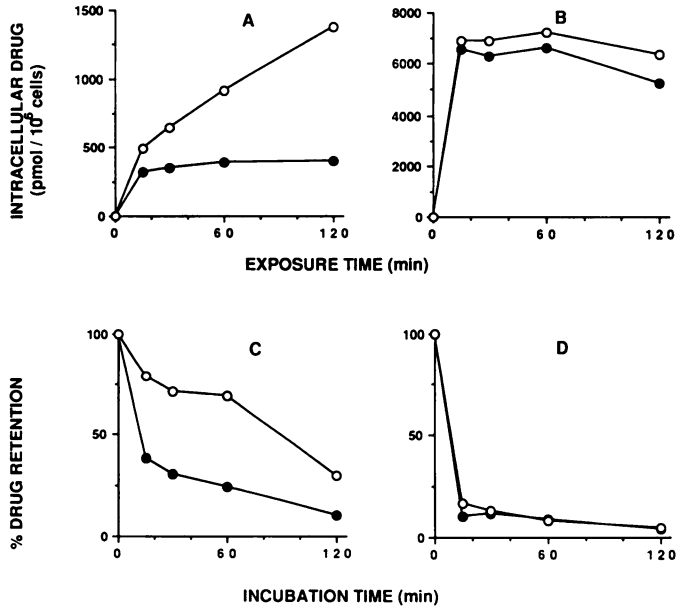

Fig. 2. Time course of cellular drug uptake and retention of MX2 and ADM. K562 (○) or K562/ADM (●) cells were treated with 5 μmol of drug. In *A* and *B*, cells were treated with ADM and MX2, respectively, for the indicated time periods and then processed as described in "Materials and Methods"; in *C* and *D*, cells were treated with ADM and MX2 for 2 h and then cultured in drug-free medium for the indicated time periods.

as rad-equivalents in Fig. 3. The formation of SSB and DSB by ADM was significantly lower in K562/ADM compared with K562 at 30- and 120-min incubation. On the other hand, that by MX2 was not decreased in K562/ADM. The formation of SSB by MX2 in K562 and K562/ADM was the same as that by ADM in K562 after 30-min exposure. However, the formation of DSB by MX2 after 30-min exposure and that of SSB and DSB by MX2 after 120-min exposure was significantly higher than that by ADM in K562.

Persistence of DNA Strand Breaks. Following 120-min exposure under conditions used in studies for SSB and DSB formation, removal of the drug and continued incubation of the cells in drug-free medium for 120 min did not result in significant DNA break resealing in the case of ADM in K562, but DNA breaks were partially resealed in the case of MX2 in K562 and K562/ADM (Fig. 3), reflecting the rapid efflux of MX2 from both cell lines. ADM produced a small number of DNA breaks in K562/ADM, and most of the DNA breaks were resealed (Fig. 3). In addition, we did the experiments of SSBs by ADM at the concentration of 0.5 and 50 μmol for K562 and K562/ADM, respectively, which were considered to be nearly equitoxic concentrations for both cells. SSBs (rad-equivalents) by ADM at 120 min were 184 ± 40.2 and 217 ± 37.1 for K562 and K562/ADM, respectively, and persistences of 120 min after washing were 321.9 ± 68.2 and 65.4 ± 15.2 for each cell, respectively.

Inhibition of Topoisomerase II Activity by ADM and MX2. The effect of MX2 on topoisomerase II activity was examined by decatenation assay (Fig. 4). In the absence of a nuclear extract, catenated kDNA was observed as a band close to the well (*Lane C*). The decatenated DNA appeared as a fast-moving band in the presence of the nuclear extract from K562 or K562/ADM without drugs (*Lane 0*). It disappeared in the presence of ADM and MX2 at the concentrations of 3 and 10 μmol in K562/ADM and K562, respectively. At the lower concentrations of 1 and 0.3 μmol of ADM and MX2, topoisomerase II activity was not suppressed. From these data it was suggested that the topoisomerase II activity of K562 and K562/ADM could be inhibited by both drugs to the same degree.

DISCUSSION

The development of drug resistance in tumor cells is one of the biggest obstacles in cancer chemotherapy. In experimental systems, resistance to multiple drugs most frequently involves P-glycoprotein. Transfection and expression of P-glycoprotein cDNA sequences in different cell types result in the expression of the multidrug resistance phenotype, directly implicating P-glycoprotein as the causative molecule of multidrug resistance. There have been several trials to reverse P-glycoprotein-mediated multidrug resistance. The drugs used include calcium-channel blockers, calmodulin antagonists, and other cardiac agents such as quinidine, etc. The development of new anthracycline derivatives with different cellular pharmacokinetics may be another approach to reversing multidrug resistance. In earlier studies it was found that morpholino derivation of ADM and DAU makes the derivatives much more lipophilic (24) and that the intracellular concentration of morpholino Adriamycin and morpholino daunorubicin is greater than that of the corresponding parent compounds (25, 26). In Japan, Otake developed a new morpholino anthracycline derivative named MX2. Although K562/ADM, which has a typical multidrug resistance phenotype, was highly resistant to ADM, it showed similar IC_{50} values to MX2 compared with K562. The uptake of MX2 was quick compared with that of ADM, and there was no difference in the uptake of MX2 by the two cell lines. The efflux of MX2 was also very fast in both cell lines. Again there was no difference in the speed of efflux between these two cell lines. On the other hand, ADM was retained for a long time in K562 but not in K562/ADM.

The frequencies of SSB and DSB induced by MX2 were equivalent in K562 and K562/ADM. On the other hand, the frequency of induction by ADM was significantly lower in K562/ADM than in K562. These results suggest that the initial rapid uptake of MX2 in both cells is the cause of equivalent formation of SSB and DSB as well as the equivalent cytotoxicity of MX2 for both cells. On the other hand, the low uptake and fast efflux of ADM in K562/ADM resulted in the lower formation of SSB and DSB and in the resistance to ADM.

Resealing of SSB and DSB was not observed in K562 exposed

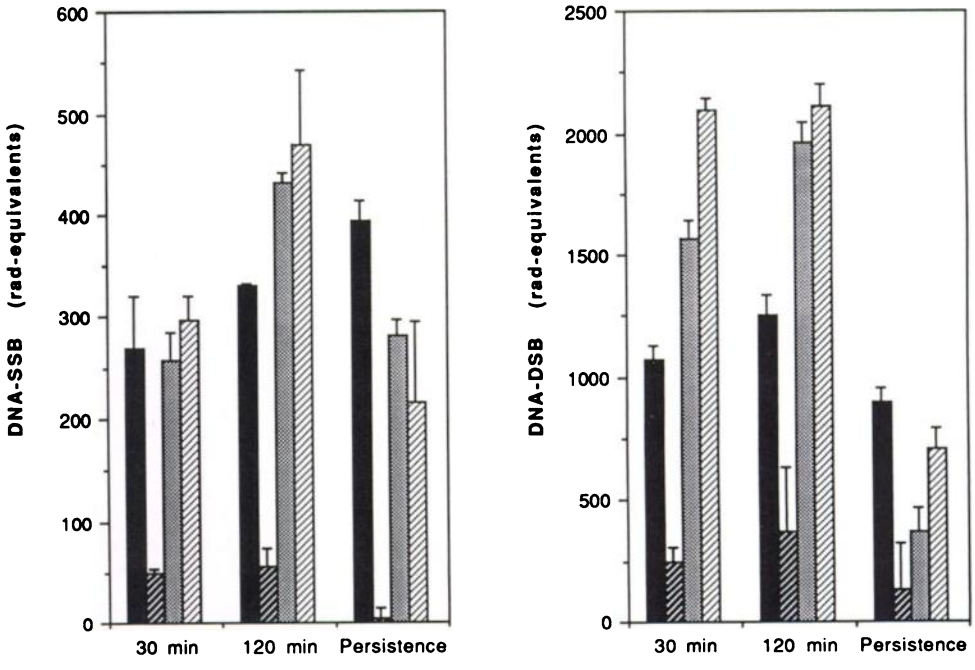

Fig. 3. SSB and DSB and their persistence. Tumor cells were treated with 5 μmol of drug for 30 and 120 min, and after drug removal to obtain the persistence they were incubated in drug-free medium for 120 min. SSBs in K562 produced by ADM (■) and MX2 (▩); SSBs in K562/ADM produced by ADM (▨) and MX2 (▨). *Columns*, mean; *bars*, SD.

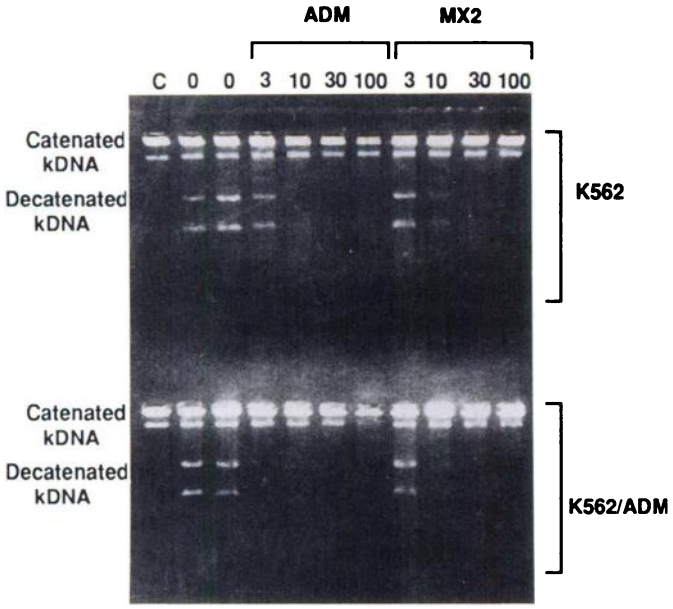

Fig. 4. The effect of ADM and MX2 on topoisomerase II-mediated decatenation activity of nuclear extracts from K562 and K562/ADM cells. Extracts were prepared from 1×10^7 cells of K562 and K562/ADM as described in "Materials and Methods." Decatenation of kDNA by the enzyme from both cells was followed by agarose gel electrophoresis. Decatenated minicircles enter the gel, whereas catenated circles remain near the well. Equal concentrations of protein were used in each *lane*. The concentrations of ADM and MX2 used are indicated.

to ADM. On the other hand, partial resealing was demonstrated in both K562 and K562/ADM exposed to MX2. Even at nearly equitoxic concentrations of ADM, the data for the SSBs and persistences were the same as shown in Fig. 3. These findings could be explained by the slow and rapid efflux of ADM and MX2 in K562 and both in K562 and K562/ADM, respectively.

Although almost no drug is left in the cells, much MX2 cleavage remains 2 h after drug removal. This might be caused by the formation of irreversible DNA damage, because MX2 cleavage remains more than 8 h after drug removal in our preliminary experiments.

Alternative forms of multidrug resistance have become apparent in recent studies, particularly resistance associated with topoisomerase II (23, 27, 28). In the study the topoisomerase II activity in K562 and K562/ADM was almost the same, and it was completely suppressed by ADM and MX2 in both cell lines.

In summary it can be concluded that MX2 conquered the multidrug resistance of K562/ADM by rapid influx following higher frequency of single and double strand breaks.

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