ABSTRACT

We previously reported that MX2, a new morpholino anthracycline, showed marked effects on pleotropic drug-resistant sublines of murine P388 leukemia in vivo as well as in vitro. In this study we examine the in vitro cytotoxicity against pleotropic drug-resistant sublines of human tumor cell lines. MX2 was effective against multidrug-resistant sublines of four human tumor cell lines; these cells, having a 4.8- to 200-fold cross-resistance to Adriamycin (ADM) showed only a 0.7- to 2.3-fold resistance to MX2 compared with the sensitive cells.

To elucidate the mechanism by which MX2 overcomes multidrug resistance, the intracellular pharmacology of MX2 in human myelogenous leukemia K562 and its ADM-resistant subline (K562/ADM) was examined. Both K562 and K562/ADM cells accumulated MX2 more easily than ADM, and the intracellular accumulation of MX2 attained a steady state in both cell lines within 30 min of incubation at 37°C. The amount of MX2 that accumulated in K562/ADM at a steady state was only 1.3 times lower than that in K562. However, ADM was accumulated slowly in both cell lines compared with MX2, and the intercellular concentration reached a steady state in K562/ADM after 90 min of incubation and in K562 after more than 120 min. K562/ADM cells accumulated a 3.3-fold lower concentration of ADM than K562 after 120 min of exposure. The steady-state concentration of ADM in K562/ADM was 8.3 times lower than that of MX2. In addition, >70% of MX2 was accumulated a 3.3-fold lower concentration of ADM than K562 after 120 min of exposure.

Pleiotropic drug-resistant cells have not been exposed and are called "pleiotropic drug-resistant cells" (1-3). In general, reduced intracellular accumulation and retention of antitumor drugs through an active outward transport are observed in these cells, which result in a decrease in cytotoxicity and an escape from cell death (4, 5). Pleiotropic drug-resistant cells selected by natural products are usually accompanied by overexpression of a M, 7,000-18,000 surface glycoprotein termed P-glycoprotein (6-9). Growing lines of evidence indicate that this protein is directly involved in drug transport mechanisms in these cells (10-14).

Enormous efforts to search for effective agents against pleiotropic drug-resistant cells have been made in many laboratories. Recently, we reported that MX2, 3'-deamino-3'-morpholino-13-deoxo-10-hydroxycarminomycin, may be a candidate anti-tumor agent effective against multidrug-resistant tumor cells (15-17). MX2 showed prominent effects in vitro and in vivo compared with ADM on the multidrug-resistant sublines of murine P388 leukemia selected by ADM, aclacinomycin A, or mitomycin C (17). In the present study we investigated the effect of MX2 on human pleiotropic drug-resistant cell lines and the intracellular pharmacology of MX2 in both sensitive and resistant cell lines of human myelogenous leukemia K562 to clarify the mechanism by which MX2 circumvents multidrug resistance.

INTRODUCTION

The frequent emergence of resistant tumor cells during treatments with naturally occurring antitumor drugs such as anthracyclines and Vinca alkaloids is one of the major problems resulting in failure of cancer chemotherapy. These cells usually show cross-resistance to other antitumor drugs to which the cells have not been exposed and are called "pleiotropic drug-resistant cells" or "multidrug-resistant cells" (1-3). In general, reduced intracellular accumulation and retention of antitumor drugs through an active outward transport are observed in these cells, which result in a decrease in cytotoxicity and an escape from cell death (4, 5).

P-leukemia (6-9). Growing lines of evidence indicate that this protein is directly involved in drug transport mechanisms in these cells (10-14). Enormous efforts to search for effective agents against pleiotropic drug-resistant cells have been made in many laboratories. Recently, we reported that MX2, 3'-deamino-3'-morpholino-13-deoxo-10-hydroxycarminomycin, may be a candidate anti-tumor agent effective against multidrug-resistant tumor cells (15-17). MX2 showed prominent effects in vitro and in vivo compared with ADM on the multidrug-resistant sublines of murine P388 leukemia selected by ADM, aclacinomycin A, or mitomycin C (17). In the present study we investigated the effect of MX2 on human pleiotropic drug-resistant cell lines and the intracellular pharmacology of MX2 in both sensitive and resistant cell lines of human myelogenous leukemia K562 to clarify the mechanism by which MX2 circumvents multidrug resistance.
MX2 AND HUMAN MULTIDRUG-RESISTANT CELLS

Intracellular Accumulation and Retention of Drugs. K562 and K562/ADM cells were suspended in HBSS containing 20 mM HEPES buffer (pH 7.4) at 3 x 10^6 cells/ml and incubated with 150 nM [14C]MX2 or [14C]ADM at 37°C. In retention experiments, both cells were incubated with [14C]MX2 for 30 min at 37°C, washed with phosphate-buffered saline, and suspended in HBSS containing 20 mM HEPES (pH 7.4). The cells (3 x 10^6 cells/ml) were then incubated at 25°C. Five hundred μl of cell suspension were withdrawn at appropriate time points, loaded on 500 μl of an oil layer consisting of Toray silicon SH550 (Toray Silicon Co., Ltd., Tokyo, Japan) and liquid paraffin (Wako, Ltd., Tokyo, Japan) at a 4:1 volume ratio, and centrifuged at 1500 x g for 30 s. The cell pellet was dissolved in 0.5 ml of 0.5 N KOH and neutralized with 2 M acetic acid. The radioactivity was counted with a Beckman Model LS-355 liquid scintillation counter after the addition of 10 ml of ACSII (Amersham). The amounts of intracellular drug were determined as described previously (5).

Binding Assay. Preparation of plasma membrane from K562/ADM cells and a binding experiment of [3H]VCR to the plasma membrane preparation were performed as previously described (13).

RESULTS

Growth-inhibitory Effect of MX2 on Sensitive and Multidrug-resistant Human Tumor Cell Lines. The cytotoxicity of MX2 against pleiotropic drug-resistant human tumor lines is of interest for the development of this drug for clinical use. The IC50 values of MX2 on four human tumor cell lines and their drug-resistant sublines are summarized in Table 1. Two pleiotropic drug-resistant human leukemias, K562/ADM and K562/VCR, showed a 200- and 25-fold resistance to ADM, respectively, when the IC50 values were compared. In contrast, both sublines showed only a 1.3-fold resistance to MX2. Similar results were obtained with 2780AD, an ADM-resistant subline of human ovarian carcinoma A2780. This line showed an 83-fold resistance to ADM, whereas those lines showed a 4.8- and 8.8-fold resistance to MX2, indicating almost an equal sensitivity to ADM but only a 2.3-fold resistance to MX2. CEM-VLB100, vinblastine-resistant subline of human leukemia CCRF-CEM, and KB4-4, colchicine-resistant subline of human epidermoid carcinoma KB3-1, showed only a 0.7- and 0.9-fold resistance to MX2, indicating almost an equal sensitivity to sensitive lines, whereas those lines showed a 4.8- and 8.8-fold resistance to ADM, respectively. These data indicate that MX2 is an effective antitumor drug against human pleiotropic drug-resistant tumor cells.

Table 1 In vitro cytotoxicity of MX2 and ADM against drug-sensitive and pleiotropic drug-resistant human tumor cell lines

<table>
<thead>
<tr>
<th>Tumor cells</th>
<th>IC50 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K562</td>
<td>32 ± 3.5*</td>
</tr>
<tr>
<td>K562/ADM</td>
<td>43 ± 7.2 (1.3)*</td>
</tr>
<tr>
<td>K562/VCR</td>
<td>40 ± 4.2 (1.3)</td>
</tr>
<tr>
<td>CCRF-CEM</td>
<td>43 ± 3.7</td>
</tr>
<tr>
<td>CEM-VLB100</td>
<td>30 ± 2.6 (0.7)</td>
</tr>
<tr>
<td>A2780</td>
<td>22 ± 0.6</td>
</tr>
<tr>
<td>2780AD</td>
<td>50 ± 2.9 (2.3)</td>
</tr>
<tr>
<td>KB3-1</td>
<td>30 ± 2.6</td>
</tr>
<tr>
<td>KBC-4</td>
<td>27 ± 0.3 (0.9)</td>
</tr>
</tbody>
</table>

* Mean ± SD of 3 independent determinations.
# Numbers in parentheses, degree (X-fold) of resistance compared with parent cells.

Intracellular Accumulation and Retention of MX2 on K562 and K562/ADM. The intracellular accumulation of MX2 and ADM in K562 and K562/ADM at 37°C was compared using 150 nM of the [14C]labeled drugs. The time-dependent accumulation of MX2 was similar in both cell lines. A rapid accumulation of the drug occurred, and a steady state was attained within 30 min (Fig. 1A). The amount of MX2 in K562/ADM cells at a plateau was 24 pmol/1 x 10^6 cells, which was only 1.3-fold lower than that in K562 cells. On the other hand, these cell lines accumulated ADM more slowly (Fig. 1B). The accumulation of ADM in K562/ADM cells reached a steady state after 90 min of incubation at 37°C, and the amount of ADM at a steady state was 2.9 pmol/1 x 10^6 cells, which was 8.3 times lower than that of MX2. The drug accumulation in K562 cells increased gradually and did not attain a steady state during the duration of the experiment (120 min). K562/ADM cells accumulated a 3.3-fold lower concentration of ADM than did K562 cells after 120 min of incubation.

Partial elimination of intracellular MX2 rapidly occurred during 30 min of incubation in the absence of MX2; however, 77% and 71% of MX2 in K562 and K562/ADM, respectively, were retained during the subsequent incubation for 120 min (Fig. 2). ADM was transported very rapidly from K562/ADM, and >90% of the drug was lost from the cells after 60 min of incubation under the same conditions, whereas 80% of ADM was retained in the sensitive K562 cells during 120 min of incubation (data not shown). These results suggest that MX2 has a strong affinity for sensitive and multidrug-resistant tumor cells, possibly due to its high lipophilicity.

Effect of Verapamil on the Cytotoxicity and the Intracellular Accumulation of MX2. Verapamil is well known to augment the cytotoxicity of naturally occurring antitumor drugs against multidrug-resistant cells through an enhancement of the intra-
The enhancement of the cytotoxicities of both drugs decrease in the IC50 value of ADM on this cell line. In contrast, ent manner, and verapamil at 10 mM resulted in a 36-fold verapamil are summarized in Table 2. The cytotoxicity of ADM cellular accumulation of MX2 was examined. The IC50 values of MX2 against K562/ADM and the intracellular concentration of the drugs. The effect of verapamil on the cytotoxicity of MX2 against K562/ADM and the intracellular accumulation of MX2 was examined. The IC50 values of MX2 and ADM on K562 and K562/ADM in the presence of verapamil are summarized in Table 2. The cytotoxicity of ADM on K562/ADM was potentiated by verapamil in a dose-dependent manner, and verapamil at 10 μM resulted in a 36-fold decrease in the IC50 value of ADM on this cell line. In contrast, the IC50 value of MX2 on K562/ADM was hardly affected by verapamil and was only 1.3-fold lower in the presence of 10 μM verapamil. The enhancement of the cytotoxicities of both drugs against K562 cells by verapamil was scarcely observed.

The time-dependent intracellular accumulation of MX2 in K562 and K562/ADM was also barely influenced in the presence of 10 μM verapamil (Fig. 3). The amount of MX2 that accumulated in K562/ADM cells at a steady state was 1.3 to 1.6 times lower than that in K562 at 30, 100, and 300 nM of [3H]MX2. Verapamil at 10 μM did not change the intracellular concentration of MX2 in both cells and, in contrast, a steady state concentration of ADM in K562/ADM was effectively increased by this drug (Fig. 4).

Effect of MX2 on [3H]VCR Binding to K562/ADM Membrane. We previously reported that [3H]VCR bound to a high-affinity site on the plasma membrane of K562/ADM, depending on ATP and Mg2+, and that certain drugs to which K562/ADM showed cross-resistance inhibited binding (13, 21). MX2 inhibited ATP/Mg2+-dependent [3H]VCR binding to the K562/ADM membrane preparations. The apparent Kd of MX2 was 4 ± 3 nM, which was 6 times lower than that of ADM (Table 3).

Table 2  Effect of verapamil on the cytotoxicities of MX2 and ADM against K562 and K562/ADM

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Verapamil (μM)</th>
<th>IC50 (nM) MX2</th>
<th>IC50 (nM) ADM</th>
</tr>
</thead>
<tbody>
<tr>
<td>K562</td>
<td>0</td>
<td>34</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>34</td>
<td>9.6</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>28</td>
<td>8.6</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>27</td>
<td>7.6</td>
</tr>
<tr>
<td>K562/ADM</td>
<td>0</td>
<td>39</td>
<td>2800</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>36</td>
<td>680</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>30</td>
<td>220</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>29</td>
<td>77</td>
</tr>
</tbody>
</table>

DISCUSSION

In our previous paper we demonstrated that MX2 was almost equally effective against murine P388 leukemia and its sublines resistant to ADM, aclacinomycin A, or mitomycin C in vitro and showed a marked therapeutic effect on these tumors in vivo compared with ADM (17). In the present study we reported the in vitro cytotoxic effect of the drug against human pleiotropic drug-resistant cell lines, which exhibited 4- to 200-fold resistance to ADM. The pleiotropic drug-resistant cell lines selected by ADM usually show a high degree of resistance to ADM, whereas the degree of resistance to ADM is lower in cell lines selected by other antitumor agents inducible pleiotropic drug-resistance such as VCR, vinblastine, or colchicine (Table 1). Drug-resistant tumor cells usually show a high degree of resistance to the drug used for selection. Pleiotropic drug-resistant tumor cells induced by VCR, vinblastine, or colchicine might have specific resistant mechanism(s) to the drug used for selec-

Table 3  Apparent Kd values of MX2 and ADM for [3H]VCR binding

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Kd (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MX2</td>
<td>4 ± 3a</td>
</tr>
<tr>
<td>ADM</td>
<td>25 ± 15</td>
</tr>
<tr>
<td>VCR</td>
<td>0.5 ± 0.6</td>
</tr>
</tbody>
</table>

* We used the total concentration of agents instead of the free concentration for the calculation of Kd values and the apparent Kd values described above therefore might be somewhat higher than the real Kd values.

* Mean ± SD of 3 determinations.
tion, in addition to the expression of P-glycoprotein. Thus, these cells show rather a lower degree of resistance to ADM. MX2 showed a potent cytotoxicity to pleiotropic drug-resistant cell lines selected by ADM as well as those selected by other drugs. For example, K562/ADM showed a 200-fold resistance to ADM but only a 1.3-fold resistance to MX2. 2780AI, which was the most resistant cell line to MX2, exhibited only a 2.3-fold resistance. Ohe et al. (22) showed the effectiveness of MX2 against cisplatin-resistant sublines of human non-small and small cell lung carcinomas and K562/ADM by using a clonogenic assay. These data indicate that MX2 is a potent antitumor drug against human pleiotropic drug-resistant tumors.

The time-dependent accumulation of MX2 in both K562 and K562/ADM was apparently different from that of ADM (Fig. 1). Both cell lines showed an enhanced accumulation of MX2, and the amount of MX2 in K562/ADM at a steady state was only 1.3 times lower than that in K562. In contrast, the intracellular concentration of ADM in K562/ADM was at least 3.3 times lower than that in K562, and the steady-state level of ADM in K562/ADM was 8.3 times lower than that of MX2. Interestingly, >70% of MX2 was retained in both cell lines after 150 min of incubation in the medium without drug (Fig. 2), indicating the high affinity of MX2 to tumor cells. Komeshima et al. (16) demonstrated that MX2 possessed a high log \( P \) value \([\log P = \log(\text{concentration of drug in the organic phase/}} \text{concentration in the aqueous phase})\]. The ability of MX2 to bind to DNA in P388 cells, however, was 10 times lower than that of ADM (23). These findings suggest that the high intracellular accumulation and retention of MX2 may be explained by its high lipophilicity.

Pleiotropic drug-resistant cells usually transport antitumor drugs to outside the cells by an active efflux pump, thereby decreasing the intracellular concentration of drugs and diminishing their damage (4, 5). Reduced intracellular accumulation of antitumor drugs such as anthracyclines and \textit{Vinca} alkaloids (18) is observed in K562/ADM and is probably one of the major mechanisms by which K562/ADM shows cross-resistance to these drugs. Morpholino derivation of ADM was demonstrated to increase its intracellular concentration in human colon carcinoma HT-29 (24) and murine P388 leukemia and P388/ADM (25) and to decrease the degree of resistance in P388/ADM (25, 26). The amount of morpholino ADM that accumulated in P388/ADM was reported to be much higher than that of ADM but only 1.5 times lower than that in P388 (25). These findings indicate a similarity in the cellular pharmacology of both MX2 and morpholino ADM in drug-sensitive and multidrug-resistant cells. Relatively high accumulation and retention of MX2 in K562/ADM may be one of the reasons why MX2 is effective against pleiotropic drug-resistant cells.

Recently, Naito et al. (13, 21) reported that \[^{3}H\]VCR showed ATP/Mg\(^{2+}\)-dependent high-affinity binding to the plasma membrane preparations of K562/ADM cells, and the binding was effectively inhibited by ADM, VCR, and actinomycin D to which K562/ADM shows cross-resistance. This VCR binding appeared to be involved in the active drug efflux by P-glycoprotein of K562/ADM (10-14). In this study MX2 also inhibited the \[^{3}H\]VCR binding with an apparent \( K_f \) of 4 ± 3 \( \mu M \), suggesting that MX2 could be excluded from K562/ADM cells by the active efflux pump. Taking the apparent \( K_f \) into consideration, MX2 seems to be a more potent inhibitor of binding than ADM. It has been reported that ADM is not as effective as VCR in competing with verapamil photoaffinity analogues for binding to P-glycoprotein, suggesting that the drug may be binding to more than one site (27). MX2 also might be binding to P-glycoprotein at more than one site.

Calcium channel blocker verapamil is well known to reverse multidrug resistance by inhibiting active drug transport (5). This drug certainly augmented the cytotoxicity of ADM against K562/ADM in a dose-dependent manner (Table 2) and increased the intracellular accumulation of ADM (Fig. 4). Recent studies have demonstrated that verapamil directly bound to P-glycoprotein of K562/ADM (27) and inhibited \[^{3}H\]VCR binding to K562/ADM membrane preparations (21). These data suggest that verapamil restores the cytotoxicity against resistant cells by competitively inhibiting the binding of drugs to P-glycoprotein. Of great interest is that verapamil scarcely enhanced the cytotoxicity of MX2 against K562/ADM (Table 2) and the intracellular accumulation of MX2 in K562/ADM (Figs. 3 and 4). These results and the fact that MX2 and verapamil were competitive inhibitors of \[^{3}H\]VCR binding to K562/ADM membrane preparations are apparently paradoxical. A similar paradoxical finding on the effect of verapamil was reported in morpholino ADM; i.e., verapamil did not enhance the \textit{in vitro} cytotoxicity of morpholino ADM against P388/ADM (26) but increased the intracellular accumulation of the drug in P388/ADM cells to a level equivalent to that in sensitive P388 cells (25).

One explanation for the abolition of verapamil effect on the accumulation of MX2 in K562/ADM is that MX2 rapidly flows into cells according to the concentration gradient and that the majority of the drug bound to the internal and plasma membrane, possibly due to its high lipophilicity, even if free MX2 were actively transported to the outside by P-glycoprotein. The amount of intracellular free MX2 might be very low; hence, the effect of verapamil could not be observed. The second explanation is that membrane-bound MX2 greatly contributes to the cytotoxicity of MX2 and that free MX2 is not important in cytotoxicity. The effect of verapamil on the cytotoxicity of MX2 in K562/ADM may, hence, be hardly observed. Wassermann et al. (28) reported that morpholino ADM preferentially inhibited ribosomal gene transcription, which was not observed with ADM. The ability of MX2 to bind to DNA in P388 cells was demonstrated to be 10 times lower than that of ADM (23). These findings suggest that the major cytotoxic mechanism of MX2 is somewhat different from that of ADM. It has been demonstrated that ADM enhanced membrane lipid peroxidation in nuclei, mitochondria, and microsomes (29, 30). Membrane lipid peroxidation by MX2 is likely to play an important role in its cytotoxicity.

Although the precise mechanisms by which MX2 exerts its cytotoxic effects on tumor cells remain to be solved, the lack of cross-resistance to pleiotropic drug-resistant tumor cells may be explained by the fact that drug efflux via P-glycoprotein is not rate limiting for MX2 as it is for ADM, because of the very rapid accumulation of the drug. This drug warrants considerable clinical interest for further development against multidrug-resistant tumor cells.

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Cellular Pharmacology of MX2, a New Morpholino Anthracycline, in Human Pleiotropic Drug-resistant Cells

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