MX2, a Morpholin Anthracycline, as a New Antitumor Agent against Drug-sensitive and Multidrug-resistant Human and Murine Tumor Cells

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ABSTRACT

MX2, a new morpholin anthracycline, showed similar or superior chemotherapeutic effects to Adriamycin (ADM) against several experimental murine tumors. i.v. administration of MX2 against L1210-bearing mice induced a prolongation of life-span by twice or more compared to ADM. MX2 was equally or slightly more effective against Lewis lung carcinoma and colon adenoscarcinomas 26 and 38 than ADM when either drug was given i.v. The antitumor activity of MX2 against human tumor xenografts was similar to that of ADM, and the compound was effective against three out of four gastric adenoscarcinomas, one out of two non-small-cell lung carcinomas, and two out of two mammary adenocarcinomas. In particular, this compound exhibited a marked effect against MX-1, a mammary adenocarcinoma. MX2, in contrast to ADM, was effective against sublines of P388 leukemia resistant to ADM or aclacinomycin A in vivo as well as in vitro. A maximum percentage increase in life-span of about 90% was obtained in mice bearing these resistant tumors. MX2 is a unique anthracycline antibiotic effective on drug-sensitive as well as multidrug-resistant murine and human cells.

INTRODUCTION

MX2, 3'-deaminomorpholio-13-deoxo-10-hydroxycarmycininycin, is a new morpholin anthracycline, and its parent compound is obtained from the culture broth of strain RNM134-13, a blocked mutant of Actinomadura roseoviolacea 1029-AV1. It is modiﬁed at the amino sugar of its 3'-position by morpholin derivation (1). The compound shows a similar antitumor activity to ADM2 against i.p.-inoculated P388 leukemia by i.v. administration, and exhibits nearly equal activity by p.o. administration to that obtained by i.v. administration (1, 2). MX2 induces the same level of myelosuppression as ADM at the same doses during 28-day repeated injection using a rat model. The subacute cardiotoxicity, however, was found to be much weaker than that of ADM using a rabbit model. The major dose limiting factor of MX2 seems to be a myelosuppression, not a cardiotoxicity. MX2 may be used for longer time than ADM.

In this study, we examined the antitumor activities of MX2 against various experimental murine tumors and human tumor xenografts. MX2 showed a superior activity to ADM against L1210 leukemia, Lewis lung carcinoma, and colon adenoscarcinomas 26 and 38. This drug also showed a comparable activity to ADM against human tumor xenografts. We have also examined the antitumor activity of MX2 against drug-resistant P388 leukemias in vitro and in vivo. MX2 was effective against P388/ADM, P388/ACR, and P388/MMC in vitro and in vivo, whereas ADM was not effective against P388/ADM and P388/ACR, and was inferior to MX2 against P388/MMC in vivo. MX2 seems to warrant consideration for further development as a new chemotherapeutic agent.

MATERIALS AND METHODS

Drugs. MX2 was prepared as described previously (1) and its hydrochloride was used in this study. The structure of MX2-HCl is shown in Fig. 1. ADM and MMC formulated for clinical use were obtained from Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan. ADM was from Sanraku Co., Ltd., Tokyo, Japan. Drugs were dissolved in 0.9% NaCl solution.

Animals. Male DBA/2Cr and C57BL/6J, and female BALB/c, C57BL/6J × DBA/2Cr F1 (hereafter called BD2Fl) and BALB/c × DBA/2Cr F1 (hereafter called CD2Fl) mice were obtained from Charles River Japan, Inc., Tokyo, Japan. Female BALB/c-nu/nu athymic nude mice were obtained from Nihon Clea Inc., Tokyo, Japan.

Murine Tumors. P388 leukemia, L1210 leukemia, Lewis lung carcinoma, and colon adenoscarcinomas 26 and 38 were kindly provided by the National Cancer Institute, NIH, Bethesda, MD. These tumors were maintained according to the protocol of the National Cancer Institute. L1210 leukemia was maintained in male DBA/2Cr mice;colon adenoscarcinoma 26 was maintained in female BALB/c mice; and Lewis lung carcinoma and colon adenoscarcinoma 38 were maintained in male C57BL/6J mice.

The subline of P388 leukemia resistant to ADM (P388/ADM) was obtained from the National Cancer Institute, NIH, Bethesda, MD. The sublines of P388 leukemia resistant to ACR (P388/ACR) and MMC (P388/MMC) were kindly provided by Dr. M. Inaba in this center. These three tumors were maintained in female CD2Fl mice.

Evaluation of Antitumor Activity against Murine Tumors. A sample, 0.1 ml, of cell suspension in PBS containing 107 L1210 leukemia cells was inoculated i.p. in i.v. into female CD2Fl mice on Day 0. MX2 and ADM were given i.v. on Days 1, 5, and 9. Antitumor activity was determined by comparing the mean survival time of the test group with that of the control group (C) and was expressed as an increase in life-span [(T/C-1) × 100%]. Tumor-free survivors, which had no tumor macroscopically or histopathologically in abdominal cavity, in blood, or at the inoculation site on Day 60, were excluded from calculation.

Tumor fragments (2 x 2 x 2 mm) of Lewis lung carcinoma and colon adenoscarcinoma 26 were implanted s.c. in the flank of female BD2Fl mice and CD2Fl mice on Day 0, respectively. A portion, 0.2 ml, of tumor brei of colon adenoscarcinoma 38, prepared by passing the tumor pieces through an 18-gauge needle and diluted in HBSS (33% w/v), was inoculated s.c. into the flank of female BD2Fl mice on Day 0. MX2 and ADM were given i.v. on Days 1, 5, and 9 for Lewis lung carcinoma, on Day 13 for colon adenoscarcinoma 26, or on Day 21 for colon adenoscarcinoma 38. The tumor volume (v) was calculated as follows: v = πab2/6, where a and b are long and short diameter of the tumor mass in mm. Antitumor activity was determined by: (a) the percentage mean tumor volume compared to controls taking controls as 100%; and (b) ILS calculated by the median survival time in comparison to controls.

Cell suspension, 0.2 ml, in HBSS containing 10⁶ cells of the sublines of resistant P388 leukemia cells was inoculated into female CD2Fl mice.

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1 Supported by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan.
2 To whom requests for reprints should be addressed.
3 The abbreviations used are: ADM, Adriamycin; ACR, aclacinomycin A; MMC, mitomycin C; P388/ADM, P388 leukemia resistant to Adriamycin; P388/ACR, P388 leukemia resistant to aclacinomycin A; P388/MMC; P388 leukemia resistant to mitomycin C; PBS, Dulbecco's phosphate-buffered saline; HBSS, Hank's balanced salt solution; ILS, percentage increase in life-span; IC50, concentration of drug necessary to reduce growth by 50%.
4 Unpublished data.


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mice on Day 0. Drugs were given i.v. on Days 1, 5, and 9 in doses decreasing by 67 or 50% each time. Antitumor activity was determined by comparing the median survival times of the treated groups with that of the control groups and expressed as an ILS.

Human Tumor Xenograft Model and Experimental Design. Human tumor xenografts used in this study were as follows: four gastric adenocarcinomas (Sc-2, Sc-6, Sc-9, and Sc-4); one small-cell lung carcinoma (LX-1); two non-small-cell lung carcinomas (Lu-24 and Lu-99); two mammary adenocarcinomas (MX-1 and H-31). These tumors were maintained in female BALB/c-nu/nu athymic nude mice.

Chemotherapeutic experiments were performed as described previously (3). Fragments of xenografts were implanted s.c. into the right subaxillary region of athymic nude mice. When the tumors had grown to a palpable size (100–300 mm³) the mice were randomly allocated to several experimental groups consisting of six animals each and MX2 at a dose of 2 mg/kg and ADM at a dose of six mg/kg were given i.v. every 4 days up to a total of three injections. Each tumor volume was calculated using the above formula twice a week and expressed as an ILS.

Evaluation as “effective” was based on a T/C (%) of 50% or less with graded concentrations of MX2 or ADM, reincubated for 72 h in a humidified atmosphere containing 5% CO₂. Then the cells were treated with graded concentrations of MX2 or ADM, reincubated for 72 h in the presence of drugs and counted with a Model ZBI Coulter Counter (4). Three tubes were used for each drug concentration. MX2 was dissolved in ethanol to a concentration of 1 mg/ml and diluted with PBS. IC₅₀ was determined by plotting the logarithm of the drug concentration versus the growth rate of the treated cells (4).

RESULTS

Chemotherapeutic Effect of MX2 against L1210 Leukemia

In the previous study we demonstrated that the antitumor activity of MX2 against P388 leukemia was dependent on the treatment schedule and the best efficacy was obtained when MX2 was given on Days 1, 5, and 9 (2). ADM also having showed the best efficacy under this regimen, we administered MX2 and ADM at intervals in this study.

MX2 administered i.v. on Days 1, 5, and 9 showed a higher activity than ADM against i.p.-inoculated L1210 leukemia (Table 1). The impressive activity of MX2 was obtained at doses of 2.22 and 3.33 mg/kg, and these doses of drug produced ILSs of the deceased mice of 206 and 270%, respectively. One and three out of six mice were cured (examined on Day 60) at MX2 doses of 2.22 and 3.33 mg/kg, respectively. A dose of 5 mg/kg was toxic in this experiment. The maximum activity of ADM was observed at 13.3 mg/kg and an ILS of 109% was obtained. Tumor-free survivors could not be obtained with ADM.

The chemotherapeutic effect of MX2 against i.v.-inoculated L1210 leukemia was also superior to that of ADM (Table 2). The most effective dose of MX2 was 3 mg/kg, where six of seven mice became tumor free and an ILS of 102% of one deceased mouse was obtained. At 1.5 mg/kg, a high ILS (240%) was observed but no cured mice was obtained. The maximum activity of ADM was observed at 10 mg/kg, at which an ILS of 78% with no survivors was observed.

Chemotherapeutic Effect of MX2 against Solid Tumors

Lewis Lung Carcinoma. MX2 and ADM were given i.v. on Days 1, 5, and 9 after s.c. inoculation of a tumor fragment, which was suited for the inhibition of tumor growth for both drugs. On Day 14, the tumor volumes of the drug-treated and the control mice were determined (Table 3). The best T/C

![Fig. 1. Structure of MX2-HCl.](image-url)
Table 3: Antitumor activity of MX2 and ADM against s.c.-implanted Lewis lung carcinoma

<table>
<thead>
<tr>
<th>Drug</th>
<th>Tumor volume on Day 14 (mm³)</th>
<th>T/C (%)</th>
<th>Cured mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1470 ± 80*</td>
<td>100</td>
<td>0/23</td>
</tr>
<tr>
<td>MX2</td>
<td>0.375</td>
<td>1570 ± 588</td>
<td>107/100</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>1260 ± 176</td>
<td>86/100</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>372 ± 111</td>
<td>25/100</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>57 ± 28</td>
<td>4/100</td>
</tr>
<tr>
<td>ADM</td>
<td>1.25</td>
<td>1710 ± 123</td>
<td>116/0/10</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>884 ± 143</td>
<td>60/0/10</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1060 ± 103</td>
<td>72/0/10</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>295 ± 174</td>
<td>20/1/10</td>
</tr>
</tbody>
</table>

* Mean ± SD.

Table 6: Antitumor activity of MX2 and ADM against human xenografts

<table>
<thead>
<tr>
<th>Tumor xenograft</th>
<th>Relative tumor volume (T/C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastric adenocarcinoma</td>
<td>Sc-2</td>
</tr>
<tr>
<td></td>
<td>Sc-6</td>
</tr>
<tr>
<td></td>
<td>Sc-9</td>
</tr>
<tr>
<td></td>
<td>St-4</td>
</tr>
<tr>
<td>Lung carcinoma, small non-small</td>
<td>LX-1</td>
</tr>
<tr>
<td></td>
<td>Lu-24</td>
</tr>
<tr>
<td></td>
<td>Lu-99</td>
</tr>
<tr>
<td>Mammary adenocarcinoma</td>
<td>MX-1</td>
</tr>
<tr>
<td></td>
<td>H-31</td>
</tr>
</tbody>
</table>

* Tumor volume was determined on Day 14.
* Significant at P < 0.01 (one sided) by Mann-Whitney's U-test.

The antitumor activity against non-small-cell lung carcinoma was similar for both drugs, and MX-2 was effective against Lu-99 but not Lu-24. In contrast, ADM was effective against Lu-24, but not Lu-99. The antitumor activity of MX2 against mammary adenocarcinoma was superior to that of ADM. Notably, MX2 showed a marked therapeutic effect against MX-1.

Growth-inhibitory Effect of MX2 on the Sublines of Drug-resistant P388 Leukemia. The cytotoxicities of MX2 and ADM against three sublines of P388 leukemia resistant to ADM, ACR, or MMC, were examined (Fig. 2). P388/ADM and P388/MMC were also examined in selected tumors. Each drug was more effective on P388/MMC cell than ADM. This tumor showed a twofold resistance to ADM and only a 1.2-fold resistance to MX2. Furthermore, MX2 was more effective on P388/MMC cell than ADM. This tumor showed a twofold resistance to ADM and only a 1.2-fold resistance to MX2. These results indicate that these resistant tumor cells do not exhibit cross-resistance to MX2 and MX2 is more effective on drug-resistant tumor cell lines than ADM.

Chemosensitizing Effect of MX2 against Human Tumor Xenografts. The antitumor activity of MX2 and ADM against s.c.-implanted human xenografts is summarized in Table 6. The antitumor effect of MX2 against four gastric adenocarcinomas was slightly inferior to that of ADM. In the evaluation criteria described in "Materials and Methods," MX2 was effective against three out of four xenografts (Sc-2, Sc-9, and St-4), while ADM was effective against four xenografts. Both drugs were ineffective against LX-1, a small-cell lung carcinoma. The antitumor activity against non-small-cell lung carcinoma was similar for both drugs, and MX-2 was effective against Lu-99 but not Lu-24. In contrast, ADM was effective against Lu-24, but not Lu-99. The antitumor activity of MX2 against mammary adenocarcinoma was superior to that of ADM. Notably, MX2 showed a marked therapeutic effect against MX-1.

Chemosensitizing Effect of MX2 against Drug-resistant Tumor-bearing Mice. The antitumor effects of MX2 and ADM against i.p.-inoculated P388 leukemia resistant to ADM, ACR, or MMC were examined. The antitumor effects of ACR and MMC were also examined in selected tumors. Each drug was more effective in vivo in animals bearing drug-resistant tumors.
ANTITUMOR ACTIVITY OF MX2

Table 7 In vitro cytotoxicity of MX2 and ADM on sensitive and resistant P388 leukemias

<table>
<thead>
<tr>
<th>Tumor cell</th>
<th>MX2 (nM)</th>
<th>ADM (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P388</td>
<td>8.5 ± 0.27†</td>
<td>10.5 ± 0.26</td>
</tr>
<tr>
<td>P388/ADM</td>
<td>12.9 ± 2.51 (1.5)‡</td>
<td>1420 ± 265 (140)</td>
</tr>
<tr>
<td>P388/ACR</td>
<td>12.7 ± 1.61 (1.5)</td>
<td>769 ± 35.8 (73)</td>
</tr>
<tr>
<td>P388/MMC</td>
<td>10.1 ± 0.42 (1.2)</td>
<td>22.4 ± 0.86 (2.1)</td>
</tr>
</tbody>
</table>

† Mean ± SD for three determinations.
‡ Numbers in parentheses, resistant P388/sensitive P388.

DISCUSSION

In the present study we evaluated the antitumor activity of MX2, a new morpholino anthracycline antibiotic, against four murine tumor systems and nine human xenograft systems comparing the results with those of ADM, the most widely used anthracycline. The antitumor activity of MX2 against murine tumor cells was superior to that of ADM in achieving the maximum ILS, the number of cured mice, and inhibition of s.c.-implanted tumor growth. Chemotherapy with MX2 against i.v.-inoculated L1210 leukemia showed an ILS of 240-102% with six out of seven tumor-free survivors whereas that of ADM produced a maximum ILS of 78% without tumor-free survivors. The chemotherapeutic efficacy of MX2 against s.c.-implanted Lewis lung carcinoma, colon adenocarcinomas 26 and 38 also exceeded that of ADM in the inhibition of tumor growth. The effectiveness of MX2 against human tumor xenografts implanted into nude mice was similar to that of ADM. MX2 was effective on three out of four gastric tumor xenografts, one out of two non-small-cell lung carcinomas, and two

MX2 (nM)
100 1000
ADM (nM)
60
Fig. 2. Growth-inhibitory effects of MX2 and ADM on drug-sensitive and -resistant P388 leukemia. P388 (●), P388/ADM (○), P388/ACR (△), and P388/MMC (▲) leukemia cells were seeded at 2 x 10⁴ cells in 2 ml of growth medium and were treated with graded concentrations of MX2 (A) or ADM (B) for 72 h. Cell numbers were counted with a Coulter Counter. Points, mean of three determinations.

ADM, in a dose range of 0.78 to 20 mg/kg, showed no chemotherapeutic effect in mice bearing P388/ADM (Fig. 3). ADM was toxic above 13.3 mg/kg. MX2 resulted in an ILS of about 87% being achieved at a dose of 1.48 mg/kg. Although this survival advantage was less than that produced by the drug at similar doses in the sensitive P388 leukemia-bearing mice, it is evident that MX2 is effective against ADM-resistant tumor cells in vivo.

ADM and ACR showed no chemotherapeutic effect in mice bearing P388 leukemia resistant to ACR at dose range from 1.25 to 2.5 mg/kg with an ILS of 81 to 86% whereas that of ADM was obtained at 5 to 10 mg/kg with an ILS of 44%. MMC, in dose range of 0.25 to 4 mg/kg, showed no chemotherapeutic effect in mice bearing P388/MMC.

MX2 (O), ADM (●), or ACR (★) was administered i.v. on Days 1, 5, and 9 after tumor inoculation. ADM, in a dose range of 0.78 to 20 mg/kg, showed no chemotherapeutic effect in mice bearing P388/ADM (Fig. 3). The maximum activity of MX2 was obtained at 1.25 to 2.5 mg/kg with an ILS of 81 to 86% whereas that of ADM was obtained at 5 to 10 mg/kg with an ILS of 44%. MMC, in dose range of 0.25 to 4 mg/kg, showed no chemotherapeutic effect in mice bearing P388/MMC.

MX2, ADM, and ACR against ACR-resistant P388 leukemia. 10⁶ P388/ACR leukemia cells were inoculated i.p. on Day 0. MX2 (O), ADM (●), or ACR (★) was administered i.v. on Days 1, 5, and 9. Median survival time of the control group was 9.8 days.

Fig. 5. Antitumor activity of MX2, ADM, and MMC against MMC-resistant P388 leukemia. 10⁶ P388/MMC leukemia cells were inoculated i.p. on Day 0. MX2 (O), ADM (●), or MMC (▲) was administered i.v. on Days 1, 5, and 9. Median survival time of the control group was 11.8 days.
out of two mammary tumor xenografts. The antitumor effect of MX2 against MX-1, a mammary tumor xenograft, was particularly superior to that of ADM.

As the in vitro results suggested, MX2 was effective against multidrug-resistant P388 leukemia cells in vivo. In previous studies it was reported that morpholino derivation of ADM and daunorubicin makes the derivatives much more lipophilic than the corresponding parent compounds (5) and the intracellular concentration of morpholinodoxorubicin and morpholino-daunorubicin is greater than that of the corresponding parent compounds (6, 7). MX2 also possesses a similar log P value \[ \log P = \log(\text{concentration of drug in the organic phase/concentration of drug in the aqueous phase}) \] to the other morpholino compounds (2) and is more lipophilic than ADM. These findings suggest the possibility that the intracellular accumulation and retention of MX2 in both sensitive and resistant cells could be greater than those observed for ADM. However, morpholinodoxorubicin has been reported to be not fully effective against P388/ADM cells (8). The mechanisms of effectiveness of MX2 against pleiotropic drug-resistant tumor cells could be complicated. We are currently investigating the mechanisms taking the above possibility into account. Although the mechanisms still remained to be elucidated, MX2 seem to warrant considerable interest in further development against multidrug-resistant tumors.

ACKNOWLEDGMENTS

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