Cancer Chemother Pharmacol (1993) 33: 10–16

Cancer Chemotherapy and Pharmacology



# Effects of the methoxymorpholino derivative of doxorubicin and its bioactivated form versus doxorubicin on human leukemia and lymphoma cell lines and normal bone marrow

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Received 4 February 1993/Accepted 8 June 1993

**Abstract.** The methoxymorpholino derivative of doxorubicin (MMDX; FCE 23672) has recently entered clinical trials because of its broad spectrum of preclinical antitumor activity and non-cross-resistance in multidrug-resistant (MDR) tumor models. MMDX is activated in the liver to a >10 times more potent metabolite that cross-links DNA. To assess the potential of this drug in hematologic malignancies, we studied the myelotoxicity in vitro and antitumor effect of MMDX as well as its bioactivated form (MMDX+) in a panel of 14 different human leukemia and lymphoma cell lines. The tumor specificity of MMDX in CEM and K562 cells was similar to that of doxorubicin (DOX), and that of MMDX+ was slightly superior. All of the 14 cell lines were found to be more sensitive to MMDX and MMDX+ than were granulocyte-macrophage progenitors. On a molar basis, MMDX was approximately 3–100 times more active than DOX, and MMDX+ was 10–1,000 times more potent than DOX. The cytotoxic effect of MMDX and MMDX+ in two P-glycoprotein-positive MDR sublines was greatly improved in comparison with that of DOX. Whereas the response to DOX in the different leukemia and lymphoma cell lines was highly heterogeneous, the response to MMDX and MMDX+ was rather homogeneous. The novel anthracycline MMDX and its bioactivated form MMDX+ are highly active against this panel of human leukemia and lymphoma cell lines and demonstrate potentially greater selectivity for tumor cells in vitro as compared with normal bone marrow precursors.

This work was supported in part by American Cancer Society grant CH-411 and a grant from Farmitalia Carlo Erba. One of the authors (J.S.K.) was supported by grant Ku 664/1-1 from the Deutsche Forschungsgemeinschaft

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*Abbreviations:* DOX, doxorubicin; CFU-GM, granulocyte-macrophage colony-forming unit; L-CFU, leukemia colony-forming unit; CSA, cyclosporine; $IC_{50}/IC_{90}$, drug concentrations that inhibit growth or plating efficiency to 50%/90% as compared with control levels; MDR, multidrug resistance; MMDX, 3′-deamino-3′-[2-(*S*)-methoxy-4-morpholino]doxorubicin (FCE 23762); MMDX+, MMDX bioactivated by human liver microsomes in the presence of reduced nicotinamide adenine dinucleotide phosphate; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoline bromide; P-gp, P-glycoprotein; PCR, polymerase chain reaction

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## Introduction

Anthracyclines such as doxorubicin (DOX) are important therapeutic agents in hematologic malignancies. However, their value is limited by myelosuppression, cardiotoxicity, and development of drug resistance, including overexpression of the *mdr1* gene. Consequently, attempts have been made to synthesize derivatives lacking these disadvantages [1]. The 3′-deamino-morpholino derivatives of doxorubicin are potent cytotoxins that are active in animal models and not cardiotoxic at therapeutic doses [1, 19]. Their lack of cross-resistance in multidrug-resistant (MDR) cell lines [9, 18, 20] is at least in part due to their higher lipophilicity [1] and increased intracellular accumulation [21]. Among the MDR active compounds of this class, methoxymorpholino doxorubicin (MMDX; FCE 23672) has displayed the most promising preclinical antitumor activity in vitro and in vivo [10]. The >10-fold greater potency of MMDX in the P388 leukemia model in vivo suggests a bioactivation of this compound to more active metabolites [10]. One isoenzyme of the cytochrome P-450 multigene family, CYB3A1 according to the new nomenclature [16], was recently identified as being capable of potentiating MMDX [8, 14]. This bioactivation probably results in O-demethylation of MMDX with subsequent opening of the morpholino ring [8]. The process can be simulated in vitro by incubating MMDX with human liver microsomes in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH). The active metabolite forms DNA

interstrand cross-links, in contrast to MMDX, which primarily induces protein-associated DNA single-strand breaks [14].

Preliminary in vitro experiments in our laboratory using classic MDR [P-glycoprotein(P-gp)-mediated] as well as atypical MDR (topoisomerase II-mediated) variants of P388 cells had indicated (1) a relatively high tumor cell selectivity for MMDX and (2) non-cross-resistance with DOX in these sublines. The aim of the present study was to investigate the antitumor selectivity of MMDX as well as its activated form in a panel of different human leukemia and lymphoma cell lines and normal human bone marrow and to compare it with that of DOX.

## Materials and methods

*Drugs and drug preparation.* DOX and MMDX (FCE 23672; both obtained from Farmitalia Carlo Erba, Milan, Italy) were dissolved at 250 μ*M* in ethanol and stored at –20° C. For studies with the activated metabolite, MMDX was incubated with human liver microsomes in the presence of NADPH as described elsewhere [13]. The reaction mixture was filtered through a 0.2-μm Acrodisc filter (Gelman Sciences Inc., Ann Arbor, Mich.) and stored at –20° C. Controls included the reaction mixture without NADPH. Cyclosporine (CSA) was obtained from Sandoz (Basel, Switzerland). Appropriate dilutions were prepared prior to each experiment in complete medium. If not indicated otherwise, chemicals were purchased from Sigma (St. Louis, Mo.), and tissue-culture reagents from Gibco (Grand Island, N. Y.).

*Cell lines and culture conditions.* A panel of 14 established human leukemia and lymphoma cell lines [7 T-cell lines (Molt3/4, HSB, Lac-1, Jurkat, CCRF-CEM and HPB-ALL); 3 B-cell lines (DHL4, Daudi, and 697); 1 non-T-, non-B-cell line (Reh); and 3 myeloid cell lines (K562, HL60, and U937)] were used. Moreover, MDR sublines of K562 and CCRF-CEM (CEM) cells, R7 [6] and CEM/VLB100 [2], respectively, were included in the panel. Cell lines were kindly provided by Drs. M. Cleary, A. Krensky, R. Levy (all Stanford University), and W. Beck (St. Jude's Childrens' Hospital, Memphis, Tenn.).

Cells were maintained in the logarithmic growth phase as standard suspension cultures in a humidified atmosphere containing 5% $CO_2$ at 37° C. Complete medium consisted of RPMI 1640 (Whittaker, Walkersville, Md.) supplemented with 10% heat-inactivated fetal calf serum (FCS; Hyclone laboratories, Logan, UT), 2 m*M* L-glutamine, 100 IU penicillin/streptomycin/ml and 50 μ*M* β-mercaptoethanol.

*Clonogenic assays.* To assess the myelotoxicity of the drugs used, tumor colony-forming units (L-CFU) were compared with granulocyte-macrophage colony-forming units (CFU-GM) as described elsewhere [12]. Bone marrow cells were taken from healthy volunteers at the time of bone marrow donation after informed consent and approval from the Human Subjects Committee had been obtained. Mononuclear cells were recovered after separation on a Ficoll-Hypaque density gradient. These cells or harvested leukemic cells were washed twice in phosphate-buffered saline, resuspended in Iscove's modified Dulbecco's medium (IMDM), and incubated with various drug concentrations for 2 h at 37° C under frequent shaking. The final cell concentration was adjusted to $4 \times 10^6$ cells/ml. After incubation, cells were washed twice in cold phosphate-buffered saline and resuspended in complete IMDM. K562 and CEM cells as well as their respective MDR variants were used for L-CFU assays. Leukemic cells were cultured in 1% methylcellulose and complete medium supplemented with 20% FCS. The cell suspension (0.25 ml/well) was plated in triplicate in 24-well plates (Costar, Cambridge, Mass.) with a final number of $0.2-10 \times 10^4$ tumor cells/well and incubated in a humidified atmosphere containing 5% $CO_2$ at 37° C. For CFU-GM assays, bone marrow cells were cultured at a final concentration ranging from $0.25 \times 10^5$ to $2.0 \times 10^5$ cells/well with placenta-conditioned medium containing 0.01% endotoxin as a stimulant. Colonies consisting of >50 cell/aggregate were scored on day 7 (L-CFU) or day 10 (CFU-GM) under an inverted microscope. Under the conditions used, the plating efficiency for normal CFU-GMs was $252 \pm 39$ colonies/$10^5$ cells; for leukemic cells the plating efficiency varied between 5 and $8 \times 104$ colonies/$10^5$ cells. Survival was calculated as the percentage of the number of colonies formed after drug incubation in comparison with untreated control values.

*MTT assays.* Drug effects among all cell lines were compared by employing the MTT assay [15]. Depending on the cell line, from 1 to $2 \times 10^4$ cells/well were plated in a 96-well plate (Costar) and allowed to enter logarithmic growth overnight before the addition of the appropriate drug concentration. After a continuous drug exposure for 48 h, MTT at a final concentration of 0.1 mg/ml was dispensed into each well. The reaction was stopped by adding acidified isopropanol (0.05 *N* HCl). After vigorous mixing, plates were read with a Thermomax reader (Molecular Devices, Menlo Park, Calif.) at 570 nm, with 630 nm being the reference wavelength. Within each experiment, determinations were done in quadruplicate, and experiments were repeated at least twice. Cytotoxicity was calculated from the optical density (OD) as follows:

$$\text{Cytotoxicity (\%)} = \frac{(OD_{tested} - OD_{blank})}{(OD_{untreated\ control} - OD_{blank})} \times 100,$$

with blank wells containing only medium. Drug sensitivity was compared according to the $IC_{50}$ values, the drug concentration that reduces specific absorbance to 50% of control levels.

*PCR studies.* In some of the cell lines, semiquantitative detection of *mdr1* gene expression was performed as described by Noonan and Roninson [17], with minor modifications. Briefly, total cellular RNA was isolated [5] and cDNA, synthesized using random hexadeoxynucleotide primers. The *mdr1*-specific message was detected using the sense-strand primer CCCATCATTGCAATAGCAGG (GenBank position 3020-3037) and the antisense-strand primer AGCATACATATGTTCAAACT (3168-3187), yielding a 168-bp product. To maximize sensitivity, 40 cycles of polymerase chain reaction (PCR; Perkin-Elmer/Cetus, Emeryville, Calif.) were carried out using cDNA derived from 200 ng of RNA. Moreover, the $MgCl_2$ concentration was increased to 18 m*M*. For quantitation, 2 μCi (1 Ci = 37 GBq) of [$\alpha$-$^{32}$P]-deoxycytidine triphosphate (Amersham, Arlington Heights, Ill.) was added to each reaction mixture. Expression was standardized on the basis of the rRNA message. The rRNA-specific primer set (GenBank positions 1501–1520 and 1827–1846) resulted in a 346-bp fragment [7]. For rRNA studies, 25 cycles of PCR were performed under identical conditions using 0.8 ng of cDNA as the template. Negative controls prepared using $H_2O$ instead of the template were included in all experiments. Samples were electrophoresed in an 8% polyacrylamide gel and stained with ethidium bromide. Corresponding bands were cut out and the radioactivity was determined by Cerenkov counts in a scintillation counter (Beckman LS 8000, Palo Alto, Calif.). The ratio of message for *mdr1* versus rRNA was calculated and arbitrarily set to 100 units for R7 cells.

*Statistical analysis.* $IC_{50}$ and $IC_{90}$ values were calculated from semilogarithmic dose-response curves by linear interpolation. Mean values and standard deviations of MTT data were calculated from the common logarithm of the $IC_{50}$ values, and the 95% confidence interval was determined. Values differed significantly at $P < 0.05$ when their 95% confidence intervals did not overlap. Performance indices were calculated involving products or ratios of $IC_{50}/IC_{90}$ values.

## Results

### *Characteristics of the cell lines tested*

The origins and doubling times of the leukemia and lymphoma cell lines used in the present study are summarized

**Table 1.** Characteristics of the 14 human leukemia and lymphoma cell lines

| Cell line | Type | Doubling time (h) | *mdr1* expression (U)[b] |
|---|---|---|---|
| HSB | T-cell | 23.6 | ND |
| Lac-1 | T-cell | 24.1 | 0.1 |
| Molt3 | T-cell | 23.5 | ND |
| Molt4 | T-cell | 22.1 | ND |
| Jurkat | T-cell | 29.6 | 0.0 |
| CEM | T-cell | 21.2 | 0.4 |
| CEM/VLB100[a] | T-cell | 28.5 | 77.4 |
| HPB-ALL | T-cell | 39.6 | 0.0 |
| 697 | B-cell | 24.0 | ND |
| Daudi | B-cell | 26.7 | 0.0 |
| DHL4 | B-cell | 34.8 | 0.1 |
| Reh | Non-T, non-B-cell | 39.2 | ND |
| U937 | Myeloid | 18.7 | 0.0 |
| K562 | Myeloid | 14.1 | 0.0 |
| R7[a] | Myeloid | 20.3 | 100.0 |
| HL60 | Myeloid | 37.8 | 0.0 |

[a] MDR variant of K562
[b] *mdr1* expression standardized for rRNA expression and calculated relative to R7 (amount for R7 arbitrarily set to 100 U)
ND, Not determined

in Table 1. Cell lines in which an $IC_{50}$ value of >50 n*M* was obtained for DOX (see Table 4) were assayed for expression of the *mdr1* gene by PCR. It was confirmed that all those cell lines except for the two known MDR sublines were negative for *mdr1*.

### *Specificity of MMDX and bioactivated MMDX versus DOX*

The specific antitumor effect of MMDX and bioactivated MMDX was assessed by comparing the inhibition of L-CFU to that of CFU-GM using the lymphoid CEM and the myeloid K562 cell lines. Figure 1 illustrates the dose-response curves generated for DOX, MMDX, and MMDX+; and Table 2 summarizes the $IC_{50}$ and $IC_{90}$ values calculated from these curves.

Both CEM and K562 cells showed sensitivity to DOX with an exponential reduction in L-CFU (Fig. 1 A). However, the therapeutic ratio (ratio of the $IC_n$ value for CFU-GM to the $IC_n$ value for L-CFU) was low (approx. 2-fold; Table 3). The respective MDR sublines were highly resistant to DOX, with $IC_{50}$ values of 4.5 μ*M* (CEM/VLB100) and 25.5 μ*M* (R7) being obtained as compared with the 0.35 μ*M* value found for CFU-GM. With regard to the sensitive parental cells, MMDX displayed a tumor specificity similar to that of DOX [therapeutic ratios, 1.5 (K562) and 3.3 (CEM); Fig. 1B, Table 3]. The cytotoxic effect on the two resistant sublines was significantly improved, with potency ratios (ratio of the $IC_n$ value for DOX versus the $IC_n$ value for MMDX) of >100 being recorded. However, R7 cells remained resistant, with $IC_{50}/IC_{90}$ values being twice as high as those obtained for CFU-GM, and CEM/VLB100 cells displayed a dose response similar to that of bone marrow progenitor cells. Bioactivation of

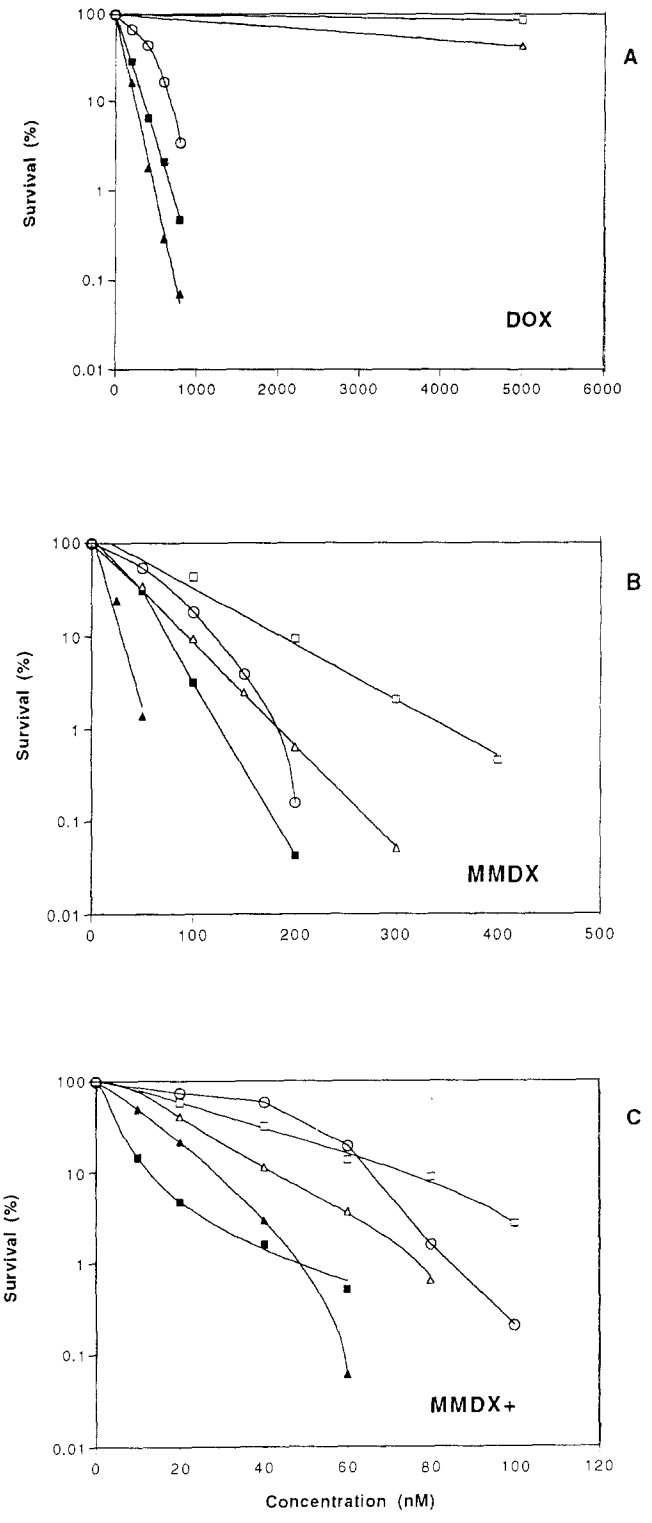


**Fig. 1 A–C.** Inhibition of colony formation of CEM (▲) and K562 (■) cells as well as their respective MDR variants CEM/VLB100 (△) and R7 (□) as compared with normal human granulocyte-macrophage progenitors (○) after incubation with drug for 2 h at 37°C. **A** DOX. **B** MMDX. **C** MMDX bioactivated by human liver microsomes in the presence of NADPH. Points represent mean values ($n = 3-4$)

MMDX by human liver microsomes enhanced the cytotoxic effect on the resistant variants (Fig. 1C). CEM/VLB100 cells were significantly more sensitive to MMDX+ than were CFU-GMs, and R7 cells displayed a low level of

**Table 2.** Dose-response data from a colony-forming assay for DOX, MMDX, and bioactivated MMDX

| Cell line | DOX | | MMDX | | MMDX+[a] | |
|---|---|---|---|---|---|---|
| | $IC_{50}$ (n*M*) | $IC_{90}$ (n*M*) | $IC_{50}$ (n*M*) | $IC_{90}$ (n*M*) | $IC_{50}$ (n*M*) | $IC_{90}$ (n*M*) |
| CFU-GM | 347 | 624 | 55 | 108 | 43 | 65 |
| K562 | 136 | 338 | 39 | 70 | 6.1 | 14 |
| R7 | 25,500 | >50,000[b] | 92 | 198 | 26 | 69 |
| CEM | 118 | 243 | 17 | 32 | 9.6 | 27 |
| CEM/VLB100 | 4,476 | ≈27,000[b] | 35 | 98 | 18 | 41 |
| Range | 118–25,500 | 243–>50,000 | 17–92 | 32–198 | 6.1–43 | 14–41 |

[a] MMDX activated in the presence of human liver microsomes and NADPH, filtered sterile (numbers not corrected for an estimated 50%–75% loss of filtration

[b] Estimated numbers: range, minimal and maximal $IC_{50}$ or $IC_{90}$ values

**Table 3.** Comparison between DOX, MMDX, and bioactivated MMDX based on colony-forming assay data

| Cell line | Potency ratio[a] | | Therapeutic ratio[b] | | |
|---|---|---|---|---|---|
| | DOX/MMDX | MMDX/MMDX+ | DOX | MMDX | MMDX+ |
| CFU-GM | 6.3/5.8 | 1.3/1.7 | 1.0/1.0 | 1.0/1.0 | 1.0/1.0 |
| K562 | 3.5/4.8 | 6.4/5.0 | 2.6/1.8 | 1.4/1.5 | 7.0/4.6 |
| R7 | 277/>250 | 7.6/2.7 | 0.01/<0.01 | 0.6/0.5 | 1.7/0.9 |
| CEM | 6.9/7.6 | 1.8/1.2 | 2.9/2.6 | 3.2/3.4 | 4.5/2.4 |
| CEM/VLB100 | 128/≈275 | 1.9/2.4 | 0.08/0.02 | 1.6/1.1 | 2.4/1.6 |

[a] Ratio of $IC_{50}$ and $IC_{90}$ values for the respective drugs

[b] Ratio of CFU-GM and L-CFU compared for $IC_{50}$ and $IC_{90}$ values, respectively

resistance at drug concentrations of >60 n*M*. Moreover, the tumor specificity of MMDX+ in the sensitive parental cells surpassed that of DOX. The actual cytotoxic potency of MMDX+ in comparison with MMDX and DOX on a molar basis is greater than that indicated in Table 2. Activity was lost by sterile filtration after incubation with liver microsomes. On the basis of a comparison between the cytotoxicities of filtered and nonfiltered MMDX (data not shown), we estimate that about 50%–75% of the activated metabolite was bound to the filter.

### *Effect of MMDX and bioactivated MMDX in comparison with DOX on leukemia and lymphoma cells*

In MTT assays a panel of 14 human leukemia and lymphoma cell lines was tested for sensitivity to the 3 respective drugs. As summarized in Table 4, the response to DOX was heterogeneous among these cell lines, with $IC_{50}$ values ranging from 4.4 n*M* for HSB cells to 34.0 μ*M* for the resistant R7 line. Even after the exclusion of both MDR variants, the $IC_{50}$ values varied 339-fold. For MMDX and MMDX+, however, both the $IC_{50}$ values and the heterogeneity of the response were decreased. The variation between the most sensitive and the most resistant MDR-negative cell line was reduced to 14-fold for MMDX and to 4-fold for MMDX+. As illustrated in Fig. 2, the $IC_{50}$ values indicate a relatively close relationship between sensitivity to DOX and to MMDX ($r^2 = 0.71$; MDR variants excluded). In contrast, the correlation between DOX and MMDX+ ($r^2 = 0.57$) as well as between MMDX and MMDX+ ($r^2 = 0.41$; not illustrated) was poor.

Since normal human bone-marrow progenitor cells cannot be used in the MTT assay, we chose K562 cells as a drug-sensitive reference line for further analysis of the MTT data. This cell line was identified as being sensitive in clonogenic assays (therapeutic ratio >1; Table 3). Cell lines that were as sensitive as K562 cells in MTT assays were considered to be more sensitive than normal human bone marrow progenitors. Thus, the antitumor activity of DOX, MMDX, and activated MMDX was calculated relative to the respective effect on K562 cells (Table 5). When both MDR sublines were included in the analysis, 6/16 cell lines (4/14 for MDR-negative cells) had $IC_{50}$ values for DOX that were greater than or equal to that obtained for K562, with a maximal resistance factor of 135 (5.9 for MDR-negative cells) being recorded. The corresponding numbers for MMDX were 4/16 (2/14) lines with up to 5.8-fold (1.1-fold) resistance. However, the extreme sensitivity to DOX observed in three cell lines (Reh, Lac-1, and HSB) was significantly diminished for MMDX. Due to the very uniform antitumor effect of MMDX+, the cytotoxic activity relative to that determined in K562 cells varied between only 0.2 (maximal resistance; 0.7 in MDR-negative cell lines) and 2.7 (maximal sensitivity). However, 8/16 cell lines (6/14 without MDR variants) were found to be equally or less sensitive to MMDX+ than were K562 cells. The potency ratios listed in Table 5 indicate the relative efficacy of MMDX and MMDX+ in cell lines that were DOX-resistant.

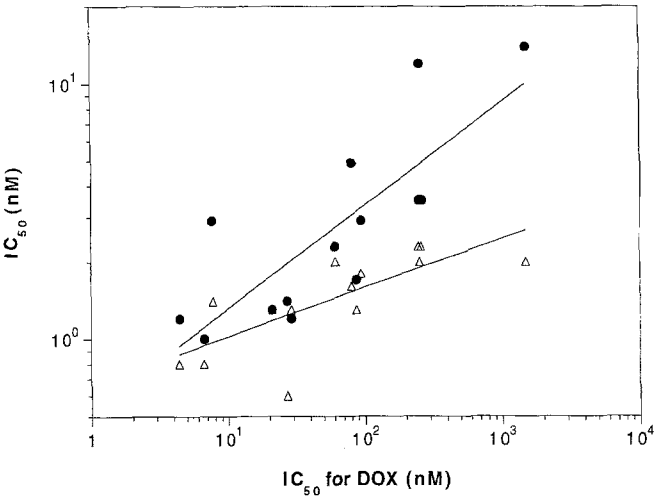


**Fig. 2.** Correlation of sensitivity between DOX and MMDX versus bioactivated MMDX. Data from MTT assays were fitted by exponential regression ($y = a\ x^b$), resulting in $r^2 = 0.71$ for DOX/MMDX (●) and $r^2 = 0.57$ for DOX/MMDX+ (△). MDR cell lines, i.e., R7 and CEM/VLB100, were excluded from this analysis

*Effect of CSA on the residual resistance of MDR-positive cell lines to MMDX*

With regard to residual cross-resistance and mechanism of action, there are more similiarities between MMDX and DOX than between activated MMDX and DOX. We therefore tested the possibility that MMDX might be a substrate for the MDR transporter P-gp. Modulation experiments were performed using coincubation with CSA in the MTT assay. As shown in Table 6, the nontoxic concentration of 1 μ*M* CSA dramatically sensitized both R7 and CEM/VLB100 cells to DOX, restoring almost complete sensitivity in the latter cell line. Despite the observation of some nonspecific toxicity in the parental lines (sensitization, 1.2-fold for K562 cells and 2.2-fold for CEM cells), the effect was relatively specific for the MDR sublines (specific modulation factor, 30.5 for R7 and 8.8 for CEM/VLB100). In contrast, it was impossible to abrogate the residual resistance to MMDX in these sublines by the addition of CSA. Instead, CSA treatment resulted in a nonspecific 2- to 3-fold sensitization in all cell lines.

## Discussion

Morpholino derivatives of anthracyclines were synthesized to circumvent both cardiotoxicity and *mdr1*-related drug resistance in cancer chemotherapy. Among these substances, MMDX has displayed promising in vitro and in vivo activity [10] and has therefore entered phase I clinical trials in Europe [18].

We studied the cytotoxic effect of MMDX and MMDX+ in a panel of 14 different human leukemia and lymphoma cell lines, including two P-gp-positive, highly DOX-resistant sublines. The microsomal activation of MMDX in vivo results in a 10–100 times more active metabolite with alkylating properties similar to those of cyanomorpholino DOX [13, 14]. To assess the properties of bioactivated MMDX in vitro, we also incubated MMDX with human liver microsomes in the presence of NADPH.

The finding of an $IC_{50}$ value of 0.35 μ*M* for CFU-GM after a 2-h incubation with DOX correlates with the clinical finding that the standard application of DOX (60 mg/m² i.v.) resulting in peak plasma levels of 1 μ*M* is associated with moderate reversible myelosuppression [4]. The limited tumor selectivity of DOX with a 2-fold therapeutic ratio in leukemia cell lines such as K562 and CEM has been

**Table 4.** Dose-response data from an MTT assay for DOX, MMDX, and bioactivated MMDX

| Cell line | $IC_{50}$ values with 95% confidence intervals (n*M*) | | | | | |
|---|---|---|---|---|---|---|
| | DOX | | MMDX | | MMDX+[a] | |
| HPB-ALL | 1,493 | (1,378–1,617) | 14 | (11–17) | 2.0 | (1.6–2.4) |
| HL60 | 260 | (190–356) | 3.5 | (2.9–4.1) | 2.3 | (2.0–2.7) |
| K562 | 252 | (188–336) | 12 | (9–15) | 2.0 | (0.9–4.5) |
| R7[b] | 34,041 | (27,389–42,309) | 70 | (54–90) | 8.1 | (6.9–9.6) |
| DHL4 | 248 | (183–336) | 3.5 | (1.6–7.6) | 2.3 | (2.0–2.6) |
| CEM | 94 | (80–112) | 2.9 | (2.2–3.9) | 0.8 | (0.5–1.1) |
| CEM/VLB100[b] | 2,844 | (1,612–5,018) | 11 | (8–13) | 1.8 | (1.4–2.5) |
| U937 | 87 | (75–100) | 1.7 | (0.7–4.1) | 1.3 | (1.0–1.5) |
| Jurkat | 80 | (70–90) | 4.9 | (3.8–6.4) | 1.6 | (0.9–2.7) |
| Daudi | 61 | (54–70) | 2.3 | (1.8–2.8) | 2.0 | (1.4–2.9) |
| Molt3 | 29 | (26–32) | 1.2 | (0.7–1.8) | 1.3 | (0.8–2.1) |
| 697 | 27 | (14–50) | 1.4 | (0.7–2.6) | 0.6 | (0.4–0.9) |
| Molt4 | 21 | (19–25) | 1.3 | (0.8–2.2) | 1.3 | (1.0–1.8) |
| Reh | 7.7 | (6.4–9.3) | 2.9 | (2.3–3.8) | 1.4 | (1.1–1.8) |
| Lac-1 | 6.7 | (5.9–7.5) | 1.0 | (0.7–1.3) | 0.8 | (0.6–1.1) |
| HSB | 4.4 | (4.0–4.9) | 1.2 | (0.7–2.2) | 0.8 | (0.7–1.0) |
| Range | | 4.4–34,041 | | 1.0–70 | | 0.6–8.1 |
| (without MDR lines) | | 4.4–1,493 | | 1.0–14 | | 0.6–2.3 |

Data represent mean values ($n = 4–6$)

[a] MMDX+ filtered after microsomal activation

[b] MDR cell lines; range, minimal and maximal $IC_{50}$ value

**Table 5.** Comparison between DOX, MMDX, and bioactivated MMDX based on MTT-assay data

| Cell line | Potency ratio[a] | | | Relative activity[b] | | |
|---|---|---|---|---|---|---|
| | DOX/MMDX | MMDX-/MMDX+[c] | DOX/MMDX+[d] | DOX | MMDX | MMDX+ |
| HPB-ALL | 110 | 9.2 | 1,016 | 0.17 | 0.9 | 1.0 |
| HL60 | 75 | 5.6 | 417 | 1.0 | 3.6 | 0.7 |
| K562 | 20 | 10 | 211 | 1.0 | 1.0 | 1.0 |
| R7[e] | 494 | 18 | 8,851 | 0.01 | 0.2 | 0.2 |
| DHL4 | 71 | 5.2 | 364 | 1.0 | 3.6 | 0.7 |
| CEM | 35 | 7.0 | 243 | 2.6 | 4.5 | 2.1 |
| CEM/VLB100[e] | 240 | 11 | 2,523 | 0.1 | 1.1 | 0.9 |
| U937 | 51 | 5.7 | 289 | 3.0 | 7.4 | 1.3 |
| Jurkat | 16 | 6.7 | 107 | 3.2 | 2.6 | 1.0 |
| Daudi | 27 | 5.3 | 45 | 4.2 | 5.6 | 0.8 |
| Molt3 | 25 | 3.0 | 74 | 8.9 | 11.0 | 1.3 |
| 697 | 19 | 5.1 | 96 | 9.6 | 9.1 | 2.7 |
| Molt4 | 16 | 3.6 | 57 | 12 | 9.4 | 1.3 |
| Reh | 2.7 | 3.7 | 10 | 33 | 4.3 | 1.2 |
| Lac-1 | 7.0 | 4.0 | 28 | 38 | 13.0 | 2.1 |
| HSB | 3.6 | 5.7 | 21 | 58 | 10.0 | 2.0 |

[a] Ratio of $IC_{50}$ values for the respective drugs
[b] Ratio of $IC_{50}$ values for the respective cell lines versus K562
[c] Potency ratio of MMDX after microsomal activation in the presence (+) or absence (–) of NADPH, both filtered
[d] Product of prior potency ratios
[e] MDR cell lines

**Table 6.** Modulation of resistance to DOX versus MMDX by the addition of cyclosporine: MTT-assay data

| Cell line | DOX | | | | | MMDX | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | –CSA | +CSA | RF | MF | SMF | –CSA | +CSA | RF | MF | SMF |
| K562 | 252 | 209 | 1 | 1.2 | 1.0 | 11.5 | 5.0 | 1.0 | 2.3 | 1.0 |
| R7[a] | 34,068 | 930 | 135 | 36.6 | 30.5 | 69.7 | 35.0 | 6.0 | 2.0 | 0.9 |
| CEM | 94 | 43 | 1 | 2.2 | 1.0 | 2.9 | 1.2 | 1.0 | 2.4 | 1.0 |
| CEM/VLB100[a] | 2,844 | 147 | 30 | 19.3 | 8.8 | 10.5 | 3.6 | 3.6 | 2.9 | 1.2 |

[a] MDR cell lines
–/+ CSA, $IC_{50}$ values (n*M*) in the absence or presence of coincubation with 1 μ*M* CSA (means, $n = 3-6$); RF, resistance factor ($IC_{50}$ value for the MDR subline versus $IC_{50}$ value for the parental line); MF, modulation factor ($IC_{50}$ value in the absence of CSA versus $IC_{50}$ value in the presence of CSA); SMF, specific modulation factor (MF for the resistant line versus MF for the parental line)

confirmed by other studies [3, 22]. On a molar basis, MMDX was approximately 5–7 times more potent than DOX, and activated MMDX was at least 20–30 times more potent than DOX. In contrast to earlier mouse experiments, MMDX did not display an improved tumor specificity over DOX in sensitive human leukemia lines, whereas bioactivated MMDX did show a somewhat enhanced selectivity in these experiments.

MMDX and MMDX+ were relatively active in MDR sublines highly resistant to DOX, confirming similar reports by other authors [9, 10, 18]. Moreover, MMDX is also highly active in cell lines displaying an atypical MDR phenotype due to a decreased expression or activity of topoisomerase II ([9]; Kühl, unpublished data). However, the mechanism of a residual resistance to MMDX in MDR cell lines could not be elucidated. CSA, an efficient competitive inhibitor of the P-gp pump, did not sensitize MDR cells to MMDX. This observation is in agreement with the finding of a low affinity of MMDX for P-gp in membrane studies (Lau, manuscript in preparation).

It appears that when it is present, MDR plays a dominant role in determining DOX resistance relative to MMDX. To explore further the relationship between DOX and MMDX cytotoxicity in the absence of MDR, we disregarded the two MDR variant cell lines in further analyses. The absence of any detectable *mdr1* expression in cell lines displaying an $IC_{50}$ value for DOX of >50 n*M* was confirmed by a highly sensitive PCR technique. In contrast to a similar study by Ishida et al. [11], our U937 cells were found to be MDR-negative.

We observed a relatively close relationship between sensitivity to MMDX and to DOX, which was absent between DOX and MMDX+ as well as between MMDX and MMDX+. Moreover, the potency ratio determined for MMDX+ versus DOX correlated with the $IC_{50}$ value obtained for DOX, suggesting a lack of cross-resistance between the two substances. These findings probably reflect the mechanism of action of these three compounds. Like DOX, MMDX produces extensive protein-associated DNA single-strand breaks and stabilizes cleavable com-

plexes ([14]; Sikic, unpublished data). Unlike DOX, MMDX produces relatively few DNA double-strand breaks. One possible explanation for this difference is that MMDX acts primarily via topoisomerase I rather than topoisomerase II. In contrast to both DOX and MMDX, activated MMDX with its presumed open morpholino ring possesses alkylating properties causing DNA interstrand cross-links [8, 14].

We found a striking heterogeneity in the sensitivity of the tested cell lines to DOX in contrast to a surprisingly uniform response to MMDX+. The response to MMDX was also less heterogeneous than that to DOX. The inability to test human bone-marrow progenitor cells in the MTT assay did not allow us to derive therapeutic ratios (i.e., ratio of the myelotoxicity to the antileukemia effect, Table 3) for the analysis of MTT data. Instead, we calculated the antitumor effects of the cytotoxins on each cell line relative to that on K562 cells (relative activity, Table 5). If one considers K562 to be a standard drug-sensitive cell line, MMDX appeared to have an overall greater activity in this leukemic cell line panel than did DOX. Although 3/13 cell lines (5/15 with the MDR lines included) were less sensitive to DOX than were K562 cells, none of the 13 cell lines (only 2/15 with MDR variants included) was less sensitive to MMDX than were K562 cells. However, 5/13 cell lines (5/15 with the MDR lines included) were less sensitive to MMDX+ than were K562 cells.

It is not yet clear whether the uniform cytotoxic effect of MMDX+ in vitro will translate into an overall increased therapeutic efficacy in vivo rather than in a nonspecific toxicity to both tumors and normal tissues. The wider spectrum of responsiveness to DOX observed in vitro may reflect the presence of some extremely sensitive tumors that would also go into remission in vivo. The current clinical trials of MMDX will hopefully address these questions.

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