In Vivo Antitumor Activity and Host Toxicity of Methoxymorpholinyl Doxorubicin: Role of Cytochrome P450 3A1

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ABSTRACT

Methoxymorpholinyl doxorubicin (MMDX; PNU 152243) is a promising doxorubicin derivative currently undergoing clinical evaluation. Previous in vitro studies suggested that the compound undergoes hepatic biotransformation by cytochrome P450 (CYP) 3A into a more cytotoxic metabolite(s). The present study examined the role of CYP3A-mediated metabolism in the in vivo antitumor activity and host toxicity of MMDX in the mouse model and investigated the potential for increasing the therapeutic effectiveness of the drug by inducing its hepatic CYP-catalyzed activation. We found that MMDX cytotoxicity for cultured M5076 tumor cells was potentiated 22-fold by preincubating the drug with NADPH-supplemented liver microsomes from untreated C57BL/6 female mice. A greater (50-fold) potentiation of MMDX cytotoxicity was observed after its preincubation with liver microsomes isolated from animals pretreated with the prototypical CYP3A inducer pregnenolone-16a-carbonitrile. In contrast, in vivo administration of the selective CYP3A inhibitor troglitazone (TAO) reduced both potentiation of MMDX cytotoxicity and the rate of CYP3A-catalyzed N-demethylation of erythromycin by isolated liver microsomes (55.5 and 49% reduction, respectively). In vivo antitumor activity experiments revealed that TAO completely suppressed the ability of 90 μg/kg MMDX i.v., a dose close to the LD₅₀, to delay growth of s.c. M5076 tumors in C57BL/6 mice and to prolong survival of DBA/2 mice with disseminated L1210 leukemia. Moreover, TAO administration markedly inhibited the therapeutic efficacy of 90 μg/kg MMDX i.v. in mice bearing experimental M5076 liver metastases; a complete loss of MMDX activity was observed in liver metastases-bearing animals receiving 40 μg/kg MMDX i.v. plus TAO. However, pregnenolone-16α-carbonitrile pretreatment failed to enhance MMDX activity in mice bearing either s.c. M5076 tumors or experimental M5076 liver metastases. Additional experiments carried out in healthy C57BL/6 mice showed that TAO markedly inhibited MMDX-induced myelosuppression and protected the animals against lethal doses of MMDX. Taken together, these findings demonstrate that an active metabolite(s) of MMDX synthesized by CYP3A contributes significantly to its in vivo antitumor activity and host toxicity.

INTRODUCTION

DX is a clinically effective, wide-spectrum antitumor agent, but its use is limited by the emergence of drug resistance and dose-related cardiomyopathy (1); consequently, several DX analogues have been synthesized. Among these, MMDX, a DX derivative bearing a methoxymorpholinyl group at position 3 of the sugar moiety, has been selected for clinical evaluation in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. 3 The abbreviations used are: DX, doxorubicin; MMDX, 3′-deaminooxido-3′-(12S)-methoxy-4- morpholinyl(4- doxorubicin; MMDX; PNU 152243); CYP, cytochrome P450; PCN, pregnenolone-16a-carbonitrite; TAO, troglitazone; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MST, median survival time; ILS, increase in life span.

MMDX is 80–150-fold more potent than DX when administered in vivo to mice, as revealed by both tumor growth delay and survival time assays (2, 3). Furthermore, its maximum tolerated dose, as defined on the basis of drug-induced myelosuppression in Phase I trials, is 50-fold lower than that of DX (5). In contrast, MMDX is only 2–10-fold more potent than DX in vitro against both tumor and hematopoietic cells (2, 6, 7). This discrepancy between MMDX cytotoxicity in vitro and in vivo suggests the generation of a more potent metabolite(s) in vivo. Accordingly, it was shown that preincubation of MMDX with human liver microsomes or rat liver S9 (9000 × g supernatant) fraction in the presence of NADPH markedly enhanced its cytotoxicity for cultured tumor cells (8–10); this metabolic process is antagonized by cyclosporin A and erythromycin, both of which are substrates/inhibitors of CYP enzymes belonging to the 3A subfamily (9, 10). A CYP3A-mediated liver microsomal potentiation was also demonstrated for morpholinyl DX, a closely related analogue of MMDX, but not for DX (11). Moreover, enhancement of both MMDX and morpholinyl DX in vitro cytotoxicity by NADPH-supplemented liver microsomes or liver S9 fraction was shown to be associated with the formation of DNA interstrand crosslinks (8, 10–12). To date, two hepatic MMDX metabolites exhibiting increased potency compared to the parent compound in in vitro and in vivo tumor growth inhibition assays have been identified; however, neither possesses DNA-alkylating activity (13).

The CYP enzymes constitute a large superfamily of heme-containing proteins that play a central role in the metabolism of a wide variety of endogenous compounds and foreign chemicals, including drugs (14). In mammals, the main drug-metabolizing families of CYP (CYP1, CYP2, and CYP3) are primarily expressed in the liver, although specific isoforms are present in some extrhepatic tissues (15). Members of the CYP3A subfamily are found in both experimental animals and humans and show similar molecular weight, immunological reactivity, and substrate specificity (14, 16). CYP3A4, the most abundantly expressed CYP enzyme in adult human liver, contributes to the oxidative metabolism of more than 60% of all clinically used drugs, including anticancer agents, such as cyclophosphamide, ifosfamide, paclitaxel, vinblastine, and eптоподфиллютоксин (17–19). Moreover, CYP3A enzymes are expressed at different levels in human tumors (20–23), exhibit a highly variable hepatic expression (17), and can be inhibited or induced by a number of common drugs (24, 25); these characteristics may profoundly affect the activity and/or the host toxicity of antitumor agents that are substrates of these enzymes.

The aim of this study was to elucidate the contribution of CYP3A-mediated drug metabolism to the overall in vivo cytotoxicity of MMDX and to explore the potential for increasing MMDX activity by inducing its hepatic CYP3A-dependent bioactivation. Our findings in tumor-bearing and healthy mice strongly suggest that the MMDX active metabolite(s) synthesized by CYP3A contributes significantly to MMDX in vivo antitumor activity and host toxicity.

MATERIALS AND METHODS

Chemicals

MMDX was supplied as hydrochloride salt by Pharmacia & Upjohn (Nerviano, Italy). One mM stock solutions were prepared in sterile bidistilled water.
and stored at –20°C. Unless indicated otherwise, all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Animals and Tumor Cell Lines

Seven- to nine-week-old inbred female C57BL/6 and DBA/2 mice (Charles River, Calco, Italy), were used throughout this study. The mice were fed standard mouse chow, had free access to water, and were age-matched in individual experiments.

M5076, a spontaneously metastasizing reticulosarcoma cell line, was maintained in vivo as ascitic tumor in syngeneic C57BL/6 mice. The leukemia cell line L1210 was maintained by in vitro passages in DMEM supplemented with 5% FCS, 2 mM glutamine, 25 mM HEPES (all from Life Technologies, Ltd., Paisley, Scotland), and antibiotics.

Monitoring of Animals during in Vivo Experiments

Procedures involving animals and their care were in conformity with institutional guidelines that comply with national and international laws and policies (European Economic Community Directive 86/609, OJ L 358, 1. Dec. 12, 1987; NIH Guide for the Care and Use of Laboratory Animals, NIH Publication no. 85-23, 1985). During in vivo survival experiments, animals in all experimental groups were examined daily for a decrease in physical activity and other signs of disease to identify those expected to become moribund within a short time. Severely ill animals were euthanized by ethyl ether overdose. Survival time of each animal was calculated as the number of days elapsed between tumor inoculation and euthanization.

Preparation of Mouse Liver Microsomes

Microsomal fractions were prepared from pooled livers (n = 4–5) recovered from untreated, PCN (50 mg/kg i.p. in corn oil for 4 days), TAO (100 mg/kg i.p. in corn oil), or corn oil-treated C57BL/6 mice by conventional techniques as described previously (26). Total CYP content was measured spectrophotometrically by the method of Omura and Sato (27), and microsomal protein was determined by the method of Lowry et al. (28) with BSA as the standard.

Determination of CYP Marker Activities

To monitor the CYP1A and CYP2B subfamilies, microsomal ethoxyresorufin and pentoxysorulin O-dealkylation, respectively, were assayed according to the fluorometric method of Burke et al. (29) at a substrate concentration of 5 μM.

Erythromycin N-demethylation activity, a functional marker of CYP3A, was determined at 37°C in an incubation mixture containing 100 mM Tris-HCl (pH 7.4), 5 mM substrate, 1 mg of microsomal protein, 5 mM KCl, 3 mM MgCl₂, and 5 mM semicarbazide hydrochloride (final volume, 1 ml); the reaction was initiated by adding 0.5 mM NADPH and conducted for 20 min, and the rate of formaldehyde formation was measured according to the method of Nash (30) as modified by McLean and Day (31). These experimental conditions were chosen on the basis of preliminary kinetics experiments showing that the rate of erythromycin demethylation linearly increased with increasing concentration of the substrate from 0.5 to 5 mM. Data were expressed as mean ± SE and analyzed using the Student-Newman-Keuls test.

Incubation of MMDX with Mouse Liver Microsomes

Fifty μM MMDX was incubated with 0.5 mg/ml mouse liver microsomes and 0.45 mM NADPH in 0.3 M Tris-HCl (pH 7.4) for a final volume of 2.5 ml in a 10 ml-Erlenmeyer flask. In control incubation, NADPH or NADPH plus microsomes was omitted. The incubation mixture was shaken at 37°C for 30 min, rapidly chilled to 4°C, and centrifuged at 105,000 × g for 20 min (4°C). The resulting supernatant was stored at –20°C and assayed for in vitro cytotoxicity within 7 days; these storage conditions were chosen on the basis of preliminary experiments showing no difference between fresh supernatants and supernatants that had been stored at –20°C for 3 months. Appropriate dilutions of the supernatants were prepared in complete medium consisting of RPMI 1640 supplemented with 10% (v/v) horse serum, 2 mM glutamine, 1 mM sodium pyruvate (all from Life Technologies, Ltd.) and antibiotics, and filtered through a Minisart 0.2 μm filter (Sartorius, Goettingen, Germany) immediately before each MTT assay. In a separate set of experiments, the CYP3A selective inhibitor TAO (100 μM; Refs. 32 and 33) was preincubated with 0.5 mg/ml mouse liver microsomes and 0.45 mM NADPH in 0.3 M Tris-HCl (pH 7.4), at 37°C for 20 min. Thereafter, 50 μM MMDX and an additional 0.45 mM NADPH were added, and the reaction was allowed to proceed for 30 min. As formaldehyde was produced during the metabolism of TAO, control incubations without MMDX were also carried out. The incubation mixtures were then processed as described above.

MTT Assay

The in vitro cytotoxicity of hepatic microsome-treated and untreated MMDX was studied in M5076 cells using a slightly modified MTT assay (34). M5076 cells, suspended in complete medium, were seeded into 96-well round-bottomed microtiter plates (2 × 10⁵ well) and cultured (37°C, 5% CO₂) in the presence of various MMDX concentrations for 78 h (final volume, 200 μl). Forty μl of an MTT solution (2.5 mg/ml in PBS) were then added to each well. After 3 h of incubation, the supernatant containing the unreacted dye was replaced with DMSO (100 μl/well), plates were vigorously shaken, and absorbance at 540 nm was measured by a Titertek Multiscan (MCC) reader within 1 h. Within each experiment, determinations were performed in quadruplicate, and experiments were repeated at least three times. The percentage of cell survival was calculated from the absorbance values as follows: (Δabsorbance of treated – Δabsorbance of untreated) × (100/Δabsorbance of control), with Δabsorbance referring to the absorbance of wells that contained only medium and MTT. IC₅₀ values were calculated from semilogarithmic dose-response curves by linear interpolation. Data were expressed as mean ± SE and analyzed by the Mann-Whitney test. Potentiation ratio was defined as the ratio of the IC₅₀ of MMDX incubated in Tris buffer without microsomes and NADPH (termed “untreated MMDX”) below to the IC₅₀ of the microsome and NADPH-treated drug.

In Vivo Tumor Models and Treatments

**M5076 Hepatic Metastases.** To obtain experimental liver metastases, C57BL/6 mice were i.v. injected with 5 × 10⁵ ascitic M5076 cells. Two days later, the animals were randomly assigned to an experimental group (n = 7–8/group) and received either a single i.v. injection of MMDX in saline plus corn oil (10 ml/kg i.p., 2 h before and 2 h after the MMDX dose), MMDX plus TAO (100 mg/kg in corn oil i.p., 2 h before and 2 h after the MMDX dose), or TAO (two doses, 4 h apart) plus saline i.v.; control mice received only drug vehicles. The mice were sacrificed 20 days after the tumor cell injection, livers were harvested and fixed in Bouin’s solution, and surface metastatic colonies counted with the aid of a dissecting microscope. In a separate experiment, mice received an i.p. injection of PCN (50 mg/kg/day in corn oil) or corn oil on days 0, 1 and 2. On day 1, animals were injected i.v. with 5 × 10⁵ ascitic M5076 cells. Two days after tumor cell injection (day 3), the animals were randomly assigned to an experimental group (n = 7–8) and received an i.v. injection of MMDX or saline. Mice were sacrificed 20 days after tumor cell injection, and liver tumor nodules were evaluated as described above. Results were analyzed by the Mann-Whitney test.

s.c. M5076 Tumors. To induce s.c. tumors (extrathoracic primary tumor model), 5 × 10⁵ ascitic M5076 cells were injected s.c. into the right flank of C57BL/6 mice. One day later, the animals were randomly assigned to an experimental group (n = 8–9) and received MMDX and/or TAO as described above. The growth of each resulting tumor was evaluated once or twice a week using vernier calipers and expressed as the product of the two largest diameters (in mm) measured at a 90-degree angle to each other. Mice were sacrificed on day 28, and lungs, liver, spleen, kidneys, and ovaries were examined for the presence of gross metastases. Results were analyzed by the Mann-Whitney test.

**Disseminated L1210 Leukemia.** Disseminated neoplasia was induced in DBA/2 mice by i.v. injection of 10⁶ L1210 leukemia cells. One day later, the animals were randomly assigned to an experimental group (n = 10) and treated as described above. Treatment efficacy was evaluated by comparing the MST in the treated and control groups, and expressed as ILS as follows: %
RESULTS

Potentiation of MMDX Cytotoxicity by NADPH-supplemented Mouse Liver Microsomes: Effect of CYP3A Modulators. A first set of experiments was carried out in vitro to study the effect of MMDX preincubation with mouse liver microsomes, in the presence or absence of NADPH, on MMDX cytotoxicity for M5076 tumor cells. The IC_{50} of MMDX incubated with liver microsomes in the absence of NADPH was essentially similar to that of MMDX incubated in Tris buffer in the absence of both liver microsomes and NADPH (untreated MMDX: 205 ± 46 versus 220 ± 65 nm, respectively; P = 0.72). The IC_{50} of microsome and NADPH-treated MMDX was 10.0 ± 0.8 nm (P < 0.0001 versus untreated MMDX), which correspond to a cytotoxic potentiation of 22-fold (ratio of the IC_{50} of untreated MMDX to IC_{50} of microsome and NADPH-treated drug; potentiation ratio).

It was shown that the CYP3A substrates/inhibitors cyclosporin A and erythromycin strongly inhibit the potentiation of MMDX cytotoxicity by human liver microsomes in the presence of NADPH (9, 10). To confirm CYP3A involvement in the potentiation of MMDX cytotoxicity by NADPH-fortified mouse liver microsomes and evaluate the possibility of influencing MMDX hepatic bioactivation in vivo by using modulators of CYP3A activity, we compared the ability of liver microsomes from both PCN and TAO-treated mice to increase MMDX cytotoxicity versus that of microsomes from corn oil (vehicle)-treated animals. Results of representative experiments are shown in Fig. 1.

Microsomes prepared from the livers of mice treated with the prototypic CYP3A inducer PCN (50 mg/kg/day i.p. for 4 days) displayed a 2.5-fold greater ability than control microsomes to potentiate MMDX cytotoxicity in the presence of NADPH (IC_{50} = 4.4 ± 0.1 versus 11.0 ± 0.7 nm, respectively; P < 0.05; potentiation ratio, 50 versus 20, respectively; Fig. 1A). As expected, PCN treatment was also associated with a significant induction (6.7-fold; P < 0.001) of liver microsomal erythromycin-N-demethylase activity, a specific probe for members of the CYP3A subfamily (Ref. 16; Table 1). PCN slightly increased the rate of liver microsomal ethoxyresorufin O-dealkylation, a marker of CYP1A (35); although statistically significant (P < 0.05), this increase is not likely to be biologically relevant (Table 1).

Table 1. Total CYP content and metabolism of isozyme-selective CYP substrates by hepatic microsomes from C57BL/6 female mice treated with corn oil, PCN, or TAO

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CYP Content (nmol/mg protein)</th>
<th>Ethoxyresorufin-O-dealkylation (pmol/min/mg protein)</th>
<th>Pentoxysorufin-O-dealkylation (pmol/min/mg protein)</th>
<th>Erythromycin-N-demethylation (pmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn oil (10 ml/kg i.p. for 4 days)</td>
<td>0.309 ± 0.011</td>
<td>105.42 ± 0.53</td>
<td>22.14 ± 2.60</td>
<td>2.15 ± 0.09</td>
</tr>
<tr>
<td>PCN (50 mg/kg i.p. for 4 days)</td>
<td>0.440 ± 0.024*</td>
<td>120.16 ± 1.04*</td>
<td>29.81 ± 3.21</td>
<td>14.45 ± 0.60*</td>
</tr>
<tr>
<td>TAO (100 mg/kg)</td>
<td>0.335 ± 0.018</td>
<td>103.60 ± 0.87</td>
<td>18.48 ± 1.54</td>
<td>1.10 ± 0.03*</td>
</tr>
</tbody>
</table>

* No significant differences in CYP content and CYP-mediated enzymatic activities were observed following a single injection or one injection daily for 4 days of corn oil.

P < 0.05 versus corn oil.

P < 0.001 versus corn oil.

Bone Marrow Toxicity. Groups of healthy C57BL/6 mice (n = 5/group) were treated on day 0 with either a single therapeutic dose of MMDX (90 µg/kg) plus corn oil (10 ml/kg i.p., 2 h before and 2 h after the MMDX dose), MMDX plus TAO (100 mg/kg i.p., 2 h before and 2 h after the MMDX dose), TAO (two doses, 4 h apart) plus saline i.v., or drug vehicles. On days 7, 11, and 17, retro-orbital sinus blood samples were collected, diluted with 10 ml EDTA, and counted with a hemocytometer after lysis of RBCs with acidified methyl violet solution. Results were analyzed by the Mann-Whitney test.

Host Toxicity Experiments

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Table 2  Effect of TAO on MMDX activity against M5076 liver metastases

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment†</th>
<th>MMDX dose (µg/kg)</th>
<th>Liver metastasis-bearing mice (day 20)</th>
<th>Median no. (range) of liver metastases (day 20)</th>
<th>Statistical comparison of metastasis no. with group‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Control</td>
<td>8/8</td>
<td>247 (169–&gt;300)</td>
<td>B (P = 0.86), C (P = 0.28), D (P = 0.0002)</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>TAO</td>
<td>7/7</td>
<td>251 (199–271)</td>
<td>C (P = 0.29), D (P = 0.0003)</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>MMDX + TAO</td>
<td>40</td>
<td>268 (194–&gt;300)</td>
<td>D (P = 0.0002)</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>MMDX</td>
<td>40</td>
<td>76.5 (48–119)</td>
<td></td>
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</table>

† On day 0, C57BL/6 mice were injected i.v. with 5 × 10⁶ ascitic M5076 cells. Two days later, the animals were randomly assigned to an experimental group (n = 7–8) and received either a single i.v. injection of MMDX in saline plus corn oil i.p. (2 h before and 2 h after the MMDX dose), MMDX plus TAO (100 mg/kg in corn oil i.p., 2 h before and 2 h after the MMDX dose), or TAO (two doses, 4 h apart) plus saline i.v.; control mice received only drug vehicles. All mice were sacrificed on day 20; livers were harvested and fixed in Bouin’s solution, and surface metastatic colonies were counted with the aid of a dissecting microscope.

‡ Two-tailed Mann-Whitney test.

TAO, like other macrolide antibiotics, is oxidized by CYP3A, leading to the formation of a stable and enzymatically inactive TAO metabolite-CYP3A complex that is still present in liver microsomal preparations after its in vivo administration (36, 37). NADPH-supplemented liver microsomes from TAO-treated mice (100 mg/kg i.p. 2 h before liver harvesting) showed a reduced ability to potentiate MMDX cytotoxicity, compared with control microsomes (IC₅₀ = 24.7 ± 1.5 versus 11.0 ± 1.0 nm; P < 0.01; potentiation ratio, 8.9 versus 20; Fig. 1B); interestingly, this reduction of the microsomal potentiation ratio after TAO administration in vivo was similar to the decrease observed at the same time point in the rate of CYP3A-catalyzed N-demethylation of erythromycin (55.5 and 49% reduction, respectively; Table 1). In contrast, TAO administration did not significantly affect the rate of microsomal dealkylation of either ethoxyresorufin or penoxyresorufin mainly associated with CYP1A and CYP2B, respectively (Table 1). Experiments were therefore performed in which TAO was preincubated with NADPH-fortified isolated liver microsomes prior to incubation with additional NADPH and MMDX. Potentiation of MMDX cytotoxicity by liver microsomes from both PCN- and corn oil-treated animals was strongly inhibited by 100 µM TAO (77 and 86% reduction of potentiation ratio, respectively; data not shown).

**TAO Administration Markedly Inhibits MMDX Activity in Tumor-bearing Mice.** The role of CYP3A-mediated drug metabolism in the in vivo antitumor activity of MMDX was studied in M5076 tumor-bearing mice. M5076 tumor cells preferentially metastasize to the liver after both i.v. and s.c. injection into syngeneic C57BL/6 mice (38). As described previously by others, selective inhibition of murine hepatic CYP3A activity could be obtained by injecting 100 mg/kg TAO in corn oil i.p. 2 h before and 2 h after a single dose of the investigated compound (39, 40). In mice bearing 2-day established M5076 liver metastases, we observed that treatment with TAO completely suppressed the growth inhibitory effect of 40 µg/kg MMDX (Table 2, Experiment I); in our previous studies, this dosage showed a strong therapeutic effect in terms of tumor reduction (i.e., about 70% decrease in the number of liver tumor colonies on day 20) but was unable to grant a survival benefit in this tumor model (7). Furthermore, TAO administration markedly decreased the ability of 90 µg/kg MMDX, a dose close to the LD₅₀ (3, 7) to inhibit M5076 tumor growth in the liver (Table 2, Experiment II). No modulation of tumor growth was observed in mice receiving TAO alone. In a similar manner, TAO treatment completely suppressed the ability of 90 µg/kg MMDX to delay the growth of 1-day-established s.c. M5076 tumors in C57BL/6 mice (Fig. 2). Moreover, on day 28, autopsy of mice receiving TAO plus MMDX revealed the presence of macroscopic tumor nodules in the liver and/or spleen of 100% of the animals. In contrast, at the same time point, more than 40% of the animals receiving MMDX alone were free of gross metastases (Fig. 2, inset). To examine the effect of increased liver CYP3A-mediated MMDX bioactivation on the cytotoxicity of this drug in vivo, the antitumor activity of MMDX was assessed in mice pretreated with PCN (50 mg/kg/day i.p. for 3 or 4 days). Despite the inducibility of hepatic CYP3A-mediated erythromycin-N-demethylase activity by PCN (Table 1), as well as the greater ability of liver microsomes from PCN-treated mice to increase MMDX cytotoxicity compared to control microsomes in vitro (Fig. 1A), PCN pretreatment did not appear to modify MMDX antitumor activity against experimental M5076 liver metastases or against s.c. M5076 tumors (data not shown and Fig. 3). The effect of TAO administration on MMDX antitumor activity in
versus PCN, WBC counts were observed in mice receiving TAO alone compared versus MMDX bone marrow toxicity, peripheral WBC counts were taken in MMDX (5). To determine the effect of TAO coadministration on is a major dose-limiting toxicity of many anticancer agents, including Healthy Mice against Lethal Doses of the Drug.

TAO Attenuates MMDX-induced Myelotoxicity and Protects Healthy Mice against Lethal Doses of the Drug. Myelosuppression was further studied in a model of disseminated neoplasia in DBA/2 mice established by i.v. transfer of syngeneic L1210 leukemia cells. Treatment was started 1 day after tumor cell injection, and the therapeutic effect was evaluated in terms of ILS of tumor-bearing animals. Survival time was significantly prolonged in mice receiving MMDX alone (% ILS = 83; P < 0.0001); in contrast, no ILS was observed in animals receiving both MMDX and TAO (Table 3).

DISCUSSION

Previous in vitro studies showed that unlike DX, both MMDX and its closely related analogue morpholinyl DX are biotransformed by CYP3A to a more cytotoxic metabolite(s) endowed with DNA cross-linking activity (8, 10–12). This phenomenon could at least partially explain why these novel anthacyclines possess a much higher potency in vivo but not in vitro compared to DX, both in experimental animals and in humans (3, 5, 41). Ongoing studies suggest that both rat and mouse liver tissue is able to synthesize highly cytotoxic metabolites of MMDX, such as 3′-deamino-3′-4-anhydro-[2′-(S)-methoxy-3′(R)-hydroxy-4′-morpholinyl]DX, the biological activity of which is still under active evaluation (13). However, preliminary data indicate that none of these active metabolites is detectable in extrahepatic tissues after MMDX administration to mice or possesses DNA alkylating activity (13), thus suggesting the existence of at least a third, as yet unidentified, highly cytotoxic hepatic metabolite of MMDX.

The present study addressed the impact of CYP3A-mediated bio- transformation of MMDX on its in vivo activity in tumor-bearing and healthy mice, without reference to the chemical structure of the metabolite(s) formed via this metabolic pathway, which is still unknown. A first set of experiments showed that the potentiation of MMDX cytotoxicity for cultured tumor cells by NADPH-supplemented liver microsomes could be modified by administering the CYP3A activity modulators PCN and TAO to mice. Indeed, pretreatment of C57BL/6 female mice with PCN, a strong inducer of CYP3A subfamily members in several species, including mice (16, 40), markedly increased both the rate of CYP3A-catalyzed erythromycin-N-demethylation (Table 1) and the extent of potentiation of MMDX cytotoxicity for cultured M5076 tumor cells isolated from tumor-bearing mice (Fig. 1A); in contrast, administration of TAO, a macrolide antibiotic that has already found widespread use in rodents as well as in humans as a CYP3A selective inhibitor both in vitro and in vivo (40, 42–44), led to a similar decrease (approximately 50%) in both liver microsomal erythromycin-N-demethylase activity and potentiation of MMDX cytotoxicity (Table 1 and Fig. 1B, respectively). Moreover, the addition of TAO to isolated liver microsomes from either PCN or corn oil-treated animals strongly inhibited the microsomal potentiation of MMDX cytotoxicity (77 and 86% inhibition, respectively; data not shown). Taken together, these results corroborate previous in vitro studies suggesting a major involvement of CYP3A enzymes in the metabolic potentiation of MMDX cytotoxicity (10–12) and indicate the feasibility of modulating the extent of MMDX metabolic conversion into more cytotoxic species in vivo in mice through the use of appropriate CYP3A inducers or inhibitors.

**Table 3. Effect of TAO on MMDX activity against disseminated L1210 leukemia**

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment§</th>
<th>MMDX dose (µg/kg)</th>
<th>MST (days)</th>
<th>% ILS</th>
<th>Statistical comparison with group§</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Control</td>
<td>90</td>
<td>6</td>
<td>0</td>
<td>B (P = 0.73), C (P = 0.31), D (P &lt; 0.0001)</td>
</tr>
<tr>
<td>B</td>
<td>TAO</td>
<td>90</td>
<td>6</td>
<td>0</td>
<td>C (P = 0.19), D (P &lt; 0.0001)</td>
</tr>
<tr>
<td>C</td>
<td>MMDX + TAO</td>
<td>90</td>
<td>6</td>
<td>0</td>
<td>D (P &lt; 0.0001)</td>
</tr>
<tr>
<td>D</td>
<td>MMDX</td>
<td>90</td>
<td>83</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

§ On day 0, DBA/2 mice were injected i.p. with 10⁷ L1210 leukemia cells. One day later, the animals were randomly assigned to an experimental group (n = 10) and received either a single i.v. injection of MMDX in saline plus corn oil i.p. (2 h before and 2 h after the MMDX dose), MMDX and TAO (100 mg/kg in corn oil i.p., 2 h before and 2 h after the MMDX dose), or TAO (two doses, 4 h apart) plus saline i.v.; control mice received only drug vehicles. Treatment efficacy was evaluated by comparing MST in the treated and control groups, and expressed as ILS as follows: % ILS = (100 × MST of treated mice/MST of control mice) – 100.

Two tailed Mann-Whitney test.
The subsequent experiments, therefore, were designed to obtain information about the effect of the CYP3A-mediated biotransformation of MMDX on the activity of this drug in vivo. Findings in different murine tumor models demonstrated that MMDX activity was markedly compromised by administration of the CYP3A inhibitor TAO to tumor-bearing animals (Tables 2 and 3 and Fig. 2). Indeed, after TAO administration, the antitumor activity of 90 μg/kg MMDX was completely lost both in C57BL/6 mice bearing s.c. M5076 tumors and in DBA/2 mice with disseminated L1210 leukemia (Fig. 2 and Table 3, respectively). Interestingly, MMDX antitumor activity was also completely lost in mice bearing experimental M5076 hepatic metastases that were given 40 μg/kg MMDX plus TAO but not in animals receiving 90 μg/kg MMDX plus TAO (Table 2). That MMDX antitumor activity at the dosage of 90 μg/kg was completely inhibited by TAO in mice with s.c. M5076 tumors but not in mice bearing M5076 hepatic metastases might be caused by a residual CYP3A activity in the liver of these animals and the subsequent achievement of therapeutic concentrations of a cytotoxic metabolite(s) of the drug in this organ, but not at extrahepatic sites. This discrepancy could also be caused by a selective generation in the liver of highly cytotoxic MMDX metabolites, synthesized via an alternative metabolic pathway to that involving CYP3A; these metabolites might contribute to MMDX activity against liver-infiltrating tumors but might not be sufficiently long-lived to reach target tumor cells growing at extrahepatic sites. To test these possibilities, MMDX metabolite(s) synthesized via CYP3A must be identified, and the pharmacokinetic profiles in mice receiving MMDX alone or MMDX plus TAO must be compared. Additional experiments in M5076 tumor-bearing animals showed that PCN pretreatment did not improve MMDX activity (Fig. 3 and data not shown) despite its effects on liver microsomal bioactivation of MMDX in vitro. Accordingly, pretreatment with dexamethasone (100 mg/kg for 4 days) also failed to improve MMDX effectiveness in BDF1 mice bearing DX-resistant P388 leukemia. Enhancement of drug bioactivation in vitro but no significant improvement in antitumor activity in vivo was also observed with cyclophosphamide, a liver CYP-activated anticancer agent, after administration of phenobarbital, a CYP inducer, to tumor-bearing mice or rats (42, 45, 46). Acceleration of the rate but no increase in the net extent of cyclophosphamide activation (i.e., unchanged plasma area under the curve of the active metabolite of cyclophosphamide, 4-hydroxy-cyclophosphamide) by phenobarbital pretreatment may explain this inconsistency (42). A similar explanation might be offered for our finding that PCN pretreatment did not increase MMDX cytotoxicity in vivo.

A second explanation for the absence of improved MMDX antitumor activity after PCN treatment might relate to a lack of CYP3A induction in the tumor cell. In this regard, data addressing the expression and functional regulation of the CYP3A proteins in M5076 and L1210 murine tumor cells are not yet available. Thus, the possibility that MMDX cytotoxic metabolites generated intratumorally play a significant role in its activity against M5076 and L1210 tumors in vivo cannot be ruled out. Moreover, evaluation of the impact of TAO administration on MMDX toxicity in healthy mice revealed that drug-induced myelosuppression was markedly inhibited and that TAO protected the animals against lethal doses of the drug (Fig. 4 and data not shown).

Although MMDX in itself possess remarkable antitumor activity in vitro (2, 7, 10), the present results indicate that the active metabolite(s) synthesized via CYP3A contributes significantly to its overall cytotoxicity in vivo in mice.

Cytotoxic agents used for cancer treatment are typically administered in combination regimens that include antibiotics, antiemetics, and other drugs, several of which are CYP3A substrates. Therefore, clinicians should weigh the results of this study when evaluating MMDX. In particular, the administration of drugs that are potent inhibitors of CYP3A enzymes, such as some macrolide antibiotics (e.g., erythromycin) orazole antifungals (e.g., ketoconazole; Ref. 25), should be avoided during therapy with MMDX to reduce the risk of misinterpreting the efficacy of this drug. Potential host toxicity attributable to MMDX inhibition of CYP3A-mediated metabolic inactivation of a co-administered drug should also be considered, because some hazardous drug-drug interactions involving CYP3A have already been described (25).

Although early data from chemical carcinogenesis models in experimental animals suggested that CYP enzymes are down-regulated in tumor tissue (47), more recent analyses have demonstrated that significant levels of CYP3A proteins persist in some human tumors arising from tissues that constitutively express these enzymes, such as liver and colon (20, 23). CYP3A expression has also been detected in a high percentage of biopsy samples of tumors, such as prostate carcinomas and soft tissue sarcomas, that arise from tissues that normally do not express proteins belonging to this CYP subfamily (21, 22). The existence of a CYP3A-mediated metabolic activation pathway for MMDX might conceivably render this drug highly effective against tumors expressing CYP3A. Accordingly, a Phase I clinical study reported regressions in patients with liver metastases from colorectal cancer (5). Moreover, as demonstrated for the CYP-activated anticancer agents cyclophosphamide and ifosfamide (48), the therapeutic efficacy of MMDX might be enhanced by transfer of an opportune drug-activating CYP gene into tumor cells. Further studies are needed to evaluate these possibilities.

In conclusion, our findings indicate a major role of CYP3A-mediated drug metabolism in the in vivo activity of MMDX; identification of the cytotoxic metabolite(s) synthesized from MMDX by the CYP3A metabolic pathway, and evaluation of the potential therapeutic

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tic advantage of MDMX intratumor activation are prerequisites for the full exploitation of this anticancer agent.

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In Vivo Antitumor Activity and Host Toxicity of Methoxymorpholinyl Doxorubicin: Role of Cytochrome P450 3A

Luigi Quintieri, Antonio Rosato, Eleonora Napoli, et al.


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