

Antitumor Activity of Methoxymorpholinyl Doxorubicin: Potentiation by Cytochrome P450 3A Metabolism

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ABSTRACT

Methoxymorpholinyl doxorubicin (MMDX) is a novel liver cytochrome P450 (P450)-activated anticancer prodrug whose toxicity toward cultured tumor cells can be potentiated up to 100-fold by incubation with liver microsomes and NADPH. In the present study, a panel of human liver microsomes activated MMDX with potentiation ratios directly correlated to the CYP3A-dependent testosterone 6 β -hydroxylase activity of each liver sample. Microsome-activated MMDX exhibited nanomolar IC₅₀ values in growth-inhibition assays of human tumor cell lines representing multiple tissues of origin: lung (A549 cells), brain (U251 cells), colon (LS180 cells), and breast (MCF-7 cells). Analysis of individual cDNA-expressed CYP3A enzymes revealed that rat CYP3A1 and human CYP3A4 activated MMDX more efficiently than rat CYP3A2 and that human P450s 3A5 and 3A7 displayed little or no activity. MMDX cyto-

toxicity was substantially increased in Chinese hamster ovary cells after stable expression of CYP3A4 in combination with P450 reductase. CYP3A activation of MMDX abolished the parent drug's residual cross-resistance in a doxorubicin-resistant MCF-7 cell line that overexpresses P-glycoprotein. CYP3A-activated MMDX displayed a comparatively high intrinsic stability, with a $t_{1/2}$ of ~5.5 h at 37°C. MMDX was rapidly activated by CYP3A at low (~1–5 nM) prodrug concentrations, with 100% tumor cell kill obtained after as short as a 2-h exposure to the activated metabolite. These findings demonstrate that MMDX can be activated by CYP3A metabolism to a potent, long-lived, and cell-permeable cytotoxic metabolite and suggest that this anthracycline prodrug may be used in combination with CYP3A4 in a P450 prodrug activation-based gene therapy for cancer treatment.

Anthracyclines, such as daunorubicin and doxorubicin, are used to treat a wide range of malignancies, but myelosuppression, cardiotoxicity, and the emergence of multidrug resistance limit their effectiveness in the clinic. Novel anthracyclines that show improved activity, lower toxicity, and the ability to circumvent drug resistance are therefore sought. These efforts have led to the discovery of morpholinyl anthracyclines such as MMDX (PNU-152243), a particularly promising candidate that is presently under clinical evaluation (Suarato et al., 1999; Sun et al., 2003). MMDX contains a methoxymorpholinyl group at the 3' position of the sugar moiety and is highly lipophilic. Unlike doxorubicin, MMDX is not cardiotoxic at optimal antitumor doses (Danesi et al., 1993) and shows substantially reduced cross-resistance in tumor cell lines highly resistant to doxorubicin (Ripamonti et

al., 1992; Kuhl et al., 1993; van der Graaf et al., 1995; Bakker et al., 1997). MMDX toxicity is associated with induction of DNA strand breaks primarily through topoisomerase I cleavage, whereas doxorubicin induces DNA lesions through topoisomerase II cleavage (Duran et al., 1996).

MMDX exhibits up to ~8-fold greater toxicity than doxorubicin toward drug-sensitive tumor cells *in vitro* (Kuhl et al., 1993) but 80-fold greater potency *in vivo* (Ripamonti et al., 1992). This difference in activity reflects the fact that MMDX is a prodrug that is activated *in vivo* in a liver cytochrome P450 (P450)-catalyzed reaction. Thus, the cytotoxicity of MMDX in cell culture can be markedly enhanced by preincubation with liver microsomes and NADPH in a metabolic process that is inhibited by the cytochrome P450 3A (CYP3A)-selective inhibitors triacetyloleandomycin and ketoconazole (Lewis et al., 1992; Quintieri et al., 2000; Baldwin et al., 2003). P450-activated MMDX induces DNA-DNA interstrand cross-links, in contrast to MMDX, which primarily induces protein-associated DNA single-strand breaks (Lau et al., 1989, 1994). Other studies suggest that activated MMDX

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ABBREVIATIONS: MMDX, methoxymorpholinyl doxorubicin; P450, cytochrome P450; P450R, cytochrome P450 reductase; CHO, Chinese hamster ovary; MDR, multidrug resistance; P-gp, P-glycoprotein; A, absorbance; HLM, human liver microsome; DMEM, Dulbecco's modified Eagle's medium; MEM, minimal essential medium; FBS, fetal bovine serum; Dex, dexamethasone; PNU-152243, 3'-deamino-3'-[2(S)-methoxy-4-morpholinyl]doxorubicin; PNU-155051, 13-dihydro-3'-deamino-3'-[2(S)-methoxy-4-morpholinyl]doxorubicin; SD2 PSC 833, Cyclosporin D, 6-[[2S,4R,6E]-4-methyl-2-methylamino]-3-oxo-6-octenoic acid].

differs from the parent drug in terms of its spectrum of antitumor activity and pattern of resistance (Kuhl et al., 1993; Geroni et al., 1994; van der Graaf et al., 1995).

In the present report, we investigate the role of specific CYP3A enzymes in the metabolic activation of MMDX and we evaluate the cytotoxicity of P450-activated MMDX to a range of human tumor cells. We characterize the intrinsic stability of activated MMDX and its cytotoxicity toward tumor cells resistant to doxorubicin and partially cross-resistant to MMDX. Finally, we evaluate the impact of tumor cell expression of CYP3A on MMDX toxicity. Overall, our findings indicate that MMDX has several properties that make it an ideal candidate for combination with CYP3A4 in a P450 prodrug-activation gene-therapy strategy (Chen and Waxman, 2002; Jounaidi, 2002).

Materials and Methods

Chemicals. NADPH (tetrasodium salt) was purchased from Sigma-Aldrich (St. Louis, MO). MMDX hydrochloride (PNU-152243) was a gift from Pfizer, Inc. (Milan, Italy). MMDX was prepared as a 10 mM (6.8 mg/ml) stock solution in double-distilled H₂O and then diluted to 10 μ M and stored in aliquots at -20°C, which retained activity for at least 3 months. NADPH was prepared fresh for each experiment as a 120 mM stock solution in double-distilled H₂O and kept on ice until use. Aqueous solutions of MMDX and NADPH were filter-sterilized immediately after preparation by passage through a 0.22 μ M syringe filter (Pall Corporation, East Hills, NY). Except for the initial weighing of chemicals, all solutions used for cell-culture studies were prepared under sterile conditions. Crystal violet solution for cell staining was prepared by dissolving 1.25 g of crystal violet (Sigma-Aldrich) in 50 ml of 37% formaldehyde and 450 ml of methanol and then stored at room temperature.

Liver Microsomes. Liver microsomes prepared from dexamethasone-induced, phenobarbital-induced, and uninduced male Sprague-Dawley rats were purchased from In Vitro Technologies, Inc. (Baltimore, MD) and used in the experiments shown in Figs. 1 and 5. Dexamethasone-induced male Fischer 344 rat liver microsomes prepared in this laboratory by differential centrifugation were used in all other experiments. Microsomes prepared from individual human liver donors and microsomes from baculovirus-infected insect cells expressing individual P450 cDNAs (Supersomes) were purchased from BD Gentest (Woburn, MA). Donor information was provided by the supplier for three of the liver donors, human liver microsome (HLM) samples 42, 89, and 112, which were from female donors aged 48, 71, and 2 years old and had CYP3A4 protein contents of 306, 177, and 331 pmol of P450/mg of protein, respectively. Donor 112 had been treated with phenobarbital and several other medications during hospitalization. The Supersomes used contained the following P450s and related enzymes: human CYP3A4, in combination with human P450 reductase (P450R) and cytochrome b₅; rat CYP3A1 with rat P450R and cytochrome b₅; rat CYP3A2 with rat P450R and cytochrome b₅; human CYP3A5 with human P450R; human CYP3A7 with human P450R and cytochrome b₅; and control Supersomes, which are devoid of P450, P450R, and cytochrome b₅ (all from BD Gentest). All microsomes were stored in aliquots at -80°C.

Cell Culture. The rat gliosarcoma cell line 9L is a brain tumor-derived adherent cell line and was cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μ g/ml streptomycin (Invitrogen). A549, a human lung carcinoma cell line, and U251, a human glioma cell line, were cultured in RPMI 1640 medium containing 5% FBS and penicillin/streptomycin antibiotic mixture (1:100; Invitrogen). LS180, a human colon adenocarcinoma cell line, was grown as a monolayer in 10% minimal essential

medium (MEM) containing penicillin/streptomycin. The human breast carcinoma cell line MCF7 and its doxorubicin-resistant subline, MCF7-ADR, were cultured in RPMI 1640 medium containing 5% FBS plus penicillin/streptomycin. CHO cell lines expressing human P450R alone or P450R in combination with CYP3A4 (~8 pmol P450/mg, and CYP3A4-dependent testosterone 6 β -hydroxylase activity of 572 pmol/min/mg) were kindly provided by Dr. T. Friedberg (University of Dundee, Dundee, Scotland) (Ding et al., 1997). CHO-hP450R cells were cultured in MEM plus 10% FBS, 1:100 diluted penicillin/streptomycin mixture, and hypoxanthine/thymidine supplement (Invitrogen). CHO-3A4-hP450R cells were cultured in α -MEM containing 10% dialyzed fetal bovine serum (Invitrogen) and penicillin/streptomycin (Ding et al., 1997).

Microsomes Prodrug Coculture Assay. Tumor cell lines were seeded in 96-well microplates at a density of 1000 cells/well (9L, A549, U251, CHO-hP450R, and CHO-3A4-hP450R cells) or 3000 cells/well (MCF7, MCF7-ADR, and LS180 cells) and were allowed to attach for 24 h. MMDX diluted in fresh culture medium was then added to each well at concentrations specified in each experiment. Each well also contained 0.3 mM NADPH (final concentration) and microsomal protein (rat or human liver microsomes; 2 μ g/well) or insect cell microsomes containing cDNA-expressed P450s (0.2 pmol P450/well), as indicated. The final volume of each well was 200 μ l. Cells were routinely cultured for 4 d after the addition of MMDX,

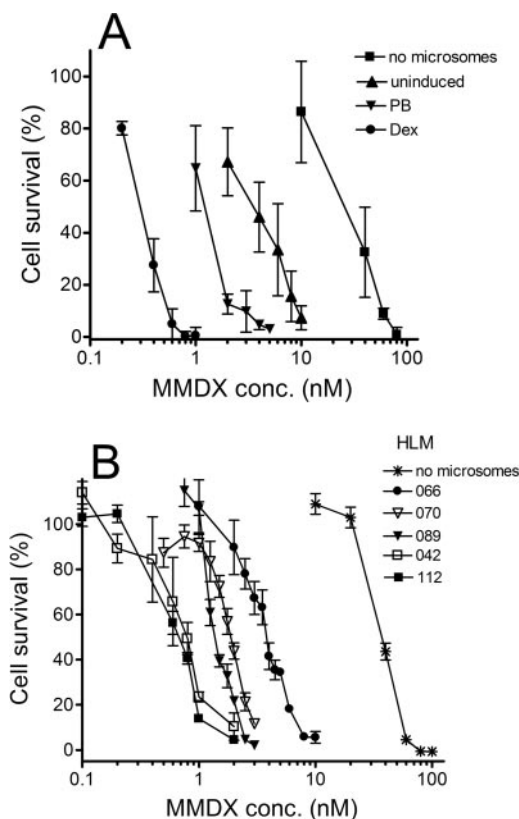


Fig. 1. Activation of MMDX by rat and human liver microsomes. A, growth-inhibition assay of 9L cells treated with MMDX alone ("no microsomes") or with MMDX in the presence of drug-induced rat liver microsomes and NADPH. Liver microsomes were prepared from dexamethasone-induced rats (Dex), phenobarbital-induced rats (PB), or uninduced rats. Each cell culture well contained 200 μ l of cell culture medium, 300 μ M NADPH, 2 μ g of rat liver microsomal protein, and MMDX at the indicated concentrations. Data were obtained in a 4-day growth-inhibition assay, as described under *Materials and Methods*. Each data point is the mean \pm S.D. values based on $n = 3$ independent samples. B, 9L growth-inhibition assay carried out as in A, except that human liver microsomes (HLM) from individual donors (0.5 μ g of microsomal protein/well) were used in place of rat liver microsomes. IC₅₀ values derived from the data illustrated in B are shown in Table 1. Data shown are mean values \pm S.D. ($n = 3$).

NADPH, and microsomes. The culture medium was then removed, and the wells were gently washed with phosphate-buffered saline. Crystal violet staining solution (100 μ l; see above) was added to each well, and the plates were shaken gently for 20 min on an orbital shaker. Excess stain was then removed, and the plates were washed by immersion in water and air-dried. The cell-adhering crystal violet stain was suspended in 100 μ l of 70% ethanol over 20 min with gentle shaking. A_{595} values were determined using an SLT SPECTRA Shell Reader (SLT Lab Instruments, Salzburg, Austria). Background A_{595} values, derived from an average of triplicate cell-free wells on each plate, were subtracted from the A_{595} value of each experimental sample and determined in triplicate. Relative cell survival, expressed as a percentage of the corresponding drug-free control, was calculated as follows: cell survival (%) = $[(A_{595} \text{ drug-treated} - A_{595} \text{ background}) / (A_{595} \text{ drug-free control} - A_{595} \text{ background})] \times 100$. IC_{50} values were determined from a semilogarithmic graph of the data points.

To evaluate the time course for MMDX activation, 9L cells were seeded in 96-well tissue-culture microtiter plates at a density of 1000 cells/well and allowed to attach for 24 h. MMDX at 0.5, 1, or 5 nM and dexamethasone-induced rat liver microsomes (2 μ g/well) plus NADPH were then added to each well. Cells were cultured for different time periods, after which the culture medium containing drugs, microsomes, and NADPH was removed and replaced with fresh DMEM plus 10% FBS. Cells were then cultured in the absence of the microsomal activation system for the remainder of the experiment (i.e., for a total of 4 days after beginning MMDX treatment), at which time the cells were stained with crystal violet. Relative cell survival as a function of time of incubation with MMDX was calculated as described above, with the drug-free control taken as the zero time point.

Half-Life of CPA3A-Activated MMDX. MMDX at 0.5 and 1 nM was preincubated in DMEM (without FBS) and dexamethasone-induced rat liver microsomes (10 μ g/ml) plus NADPH for 30 min at 37°C. The reaction was terminated by spinning down microsomal proteins at 12,000 g for 5 min. The supernatant was filtered through a 0.22- μ m syringe filter, and the filtrates were divided into aliquots. One aliquot was immediately removed, and a portion was diluted 2-fold into fresh culture medium. The diluted and undiluted filtrates were then added to 9L cells as a zero time point. The remaining aliquots were incubated at 37°C for times up to 12 h, at which point the samples and 2-fold dilutions prepared from each sample were added to 9L cells, which were cultured for 4 days. Cell survival was determined by crystal violet staining as described above. Data were graphed as the log of percentage of cell kill (i.e., 100% - cell survival percentage) versus time of incubation at 37°C, and $t_{1/2}$ values were calculated from the log-linear decay portion of each curve.

Statistical Analysis. IC_{50} values and 95% confidence intervals were calculated from semilogarithmic dose-response curves using a variable slope sigmoid equation (Prism 4; GraphPad Software Inc., San Diego, CA). Values differed significantly at $P < 0.05$ when their 95% confidence intervals did not overlap. The relationship between IC_{50} values and testosterone 6 β -hydroxylase activities was analyzed by Pearson correlation.

Results

Cytotoxicity of MMDX Activated by Rat and Human Liver Microsomes. The cytotoxicity of MMDX toward 9L gliosarcoma cells can be increased by incubation with dexamethasone-induced rat liver microsomes in an NADPH-dependent manner (Baldwin et al., 2003). In agreement with this finding, the cytotoxicity of MMDX toward 9L cells was increased in a 4-day growth-inhibition assay carried out in the presence of NADPH and either uninduced, phenobarbital-induced, or dexamethasone-induced rat liver microsomes and NADPH (Fig. 1A). The potentiation ratio (ratio of IC_{50} of

MMDX in the absence of microsomes to IC_{50} of MMDX incubated with microsomes) was 87 for dexamethasone-induced liver microsomes, 22 for phenobarbital-induced microsomes, and 8 for uninduced liver microsomes. Strong increases in MMDX cytotoxicity were also obtained using a panel of microsomes prepared from individual human liver donors (Fig. 1B). These microsomes varied in cytochrome P450 protein content and activity profiles, reflecting individual differences caused by factors such as age, gender, and drug exposure of the liver donors. Potentiation ratios varied from ~10 (HLM-066) to ~60 (HLM-112), and the IC_{50} values ranged from 0.66 to 3.8 nM (Table 1). Several of the HLM samples were similar in activity to the highly active dexamethasone-induced rat liver microsomes (Fig. 1A). Moreover, the capacity of each HLM to activate MMDX correlated directly with its CYP3A activity, assayed by testosterone 6 β -hydroxylation, a CYP3A-selective liver microsomal activity [$r = 0.945$ for IC_{50} (MMDX) versus testosterone 6 β -hydroxylase activity for $n = 5$ HLM samples; $P < 0.05$].

Activation of MMDX by cDNA-Expressed CYP3A Enzymes. We next evaluated a panel of cDNA-expressed CYP3A enzymes (Supersomes) to determine the potential of these enzymes to activate MMDX (Fig. 2). CYP3A4, a human P450 enzyme, exhibited the highest activity ($IC_{50} = 1.4$ nM) when tested in a 4-day 9L growth-inhibition assay. The rat enzymes CYP3A1 and CYP3A2 also exhibited significant activity ($IC_{50} = 2.5$ and 5.4 nM, respectively). Two other human P450 3A enzymes, CYP3A5 and CYP3A7, exhibited little or no MMDX activation compared with the P450-deficient insect microsome control. Thus, CYP3A4, the major CYP3A enzyme in human liver, is the most active catalyst of MMDX activation.

Experiments were carried out using CHO cells that stably express CYP3A4 and P450R (Ding et al., 1997) to determine whether MMDX cytotoxicity can also be increased when CYP3A4 is expressed intracellularly. A potentiation ratio of 22 was obtained for MMDX incubated with CHO-3A4-hP450R cells compared with the CYP3A4-deficient control cell line CHO-hP450R (Fig. 3). This MMDX potentiation ratio was 2.8-fold higher than when CHO-hP450R cells were incubated with MMDX in the presence of dexamethasone-induced microsomes (potentiation ratio = 8) (Fig. 3 and Table 2). On the basis of the measured IC_{50} of 0.8 nM MMDX for the CHO-3A4-hP450R cells (Table 2), a cellular CYP3A4 content of 8 pmol P450/mg protein (Ding et al., 1997), and an estimated content of ~1 to 2 μ g of protein/well (0.008–0.016

TABLE 1
 IC_{50} values and potentiation ratios for MMDX incubated with 9L cells and a panel of human liver microsomes

IC_{50} data are derived from triplicate samples analyzed as shown in Fig. 1B. The potentiation ratio is IC_{50} of MMDX incubated with control microsomes/ IC_{50} of MMDX incubated with HLM. Testosterone 6 β -hydroxylase data, provided by BD Gentest, exhibited a significant Pearson correlation; the potentiation ratio values are shown ($r = 0.9452$, $P < 0.05$).

HLM No. (Liver Donor Number)	IC_{50}	Potentiation Ratio	Testosterone 6 β -Hydroxylase Activity
	<i>nM</i>		<i>pmol/min/mg</i>
No microsomes	38.75	1	
66	3.84	10	5800
70	1.86	21	8960
89	1.46	27	11,730
42	0.74	52	13,150
112	0.66	59	17,520

pmol CYP3A4 protein/well), compared with an IC_{50} of 1.4 nM for MMDX activation by 0.2 pmol of extracellular CYP3A4 (Fig. 2), we estimate that the cells are 20- to 40-fold more sensitive to MMDX when the prodrug is activated intracellularly.

Sensitivity of Human Tumor Cell Lines to Activated MMDX. We next investigated whether the strong potentiation of MMDX cytotoxicity seen with dexamethasone-induced microsomes and rat 9L cells is also observed with human tumor cells. MMDX activated by dexamethasone-induced microsomes was highly toxic toward the human tumor cell lines A549 (lung), U251 (brain), MCF-7 (breast), and LS180 (colon) (Fig. 4), with potentiation ratios ranging from 11 to 43 (Table 2). A 6-fold difference in sensitivity to unactivated MMDX was seen when the most sensitive tumor cell line (U251) was compared with the least sensitive cell line (LS180). Cell line-based differences in the IC_{50} values of activated MMDX were

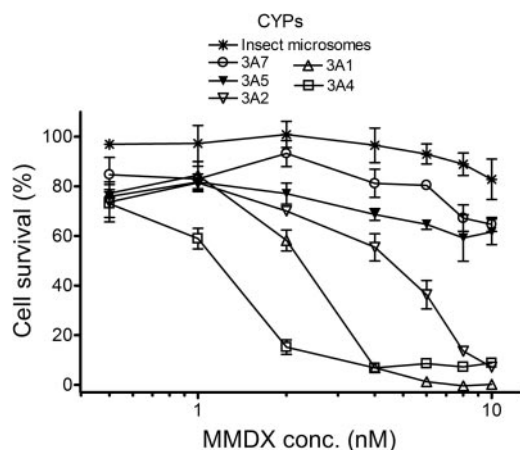


Fig. 2. Activation of MMDX by cDNA-expressed CYP3A enzymes. 9L growth-inhibition assays were carried out as a function of MMDX concentration, as described in Fig. 1, using insect cell microsomes containing individual cDNA-expressed human or rat CYP3A proteins, as indicated, with coexpressed P450R and cytochrome b_5 (Supersomes; 0.2 pmol P450 protein/well) in place of liver microsomes. Insect cell microsomes not containing P450 served as a negative control. Data shown are mean values \pm S.D. ($n = 3$).

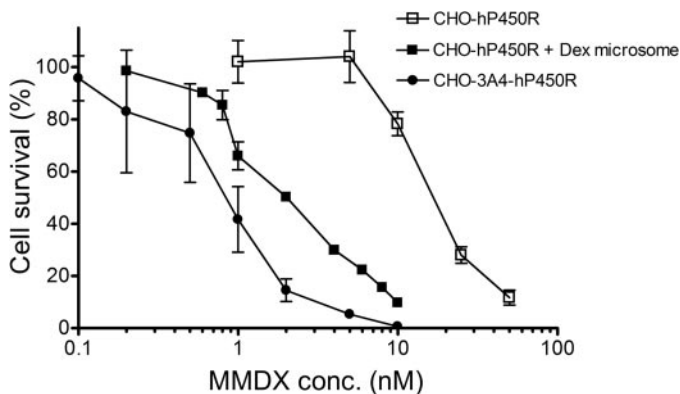


Fig. 3. Impact of intracellular CYP3A4 expression on MMDX cytotoxicity. MMDX toxicity to CHO cells expressing human P450R alone (CHO-hp450R cells) or P450R in combination with CYP3A4 (CHO-3A4-hp450R cells) was determined in a 4-day growth-inhibition assay. The cytotoxicity of MMDX to CHO-hp450R cells incubated in the presence of dexamethasone-induced rat liver microsomes (0.5 μ g of protein/well) and NADPH is shown for comparison. Data shown are mean values \pm S.D. ($n = 3$). IC_{50} values and potentiation ratios derived from these data are shown in Table 2.

more narrow, only \sim 2.3 fold, and did not correlate with the sensitivity of each cell line to MMDX itself (Table 2). This finding is consistent with earlier findings that MMDX and activated MMDX kill tumor cells by distinct mechanisms (Lau et al., 1989, 1994).

Evaluation of Cross-Resistance of MCF7-ADR Cells to MMDX and CYP3A-Activated MMDX. MCF7-ADR is an MCF-7 cell line derivative that overexpresses the drug efflux pump P-glycoprotein and is 500-fold resistant to doxorubicin (Table 3) (Fairchild et al., 1987; Chen and Waxman, 1995). MCF7-ADR cells are cross-resistant to many other structurally distinct drugs, which are also substrates for P-glycoprotein (P-gp) and are efficiently pumped out of the cells. Previous reports indicate that MMDX is active against cultured tumor cell lines that overexpress P-glycoprotein and are highly resistant to doxorubicin; however, some cross-resistance to MMDX is apparent (Kuhl et al., 1993; van der Graaf et al., 1995; Bakker et al., 1997). In contrast, *in vivo* studies indicate that MMDX retains substantial activity against doxorubicin-resistant tumor cells (Ripamonti et al.,

TABLE 2

IC_{50} values and potentiation ratios of MMDX or MMDX incubated with dexamethasone-induced rat liver microsomes plus NADPH on human tumor cell lines and CHO cell lines that express either human P450 reductase or CYP3A4 supplemented with human P450 reductase. Each IC_{50} value is derived from triplicate samples analyzed as shown in Figs. 3 and 4. Potentiation ratio is the IC_{50} of MMDX/ IC_{50} of (MMDX + dexamethasone-induced liver microsomes).

Cell Line	IC_{50}		Potentiation Ratio
	MMDX	MMDX + Dexamethasone Microsomes	
	<i>nM</i>		
CHO-hP450R	17.6	2.2	8
CHO-3A4-hP450R	0.8	N.D.	
U251	11.8	1.1	11
A549	24.2	0.99	24
MCF7	36.6	2.3	16
LS180	73.6	1.7	43

N.D., not determined.

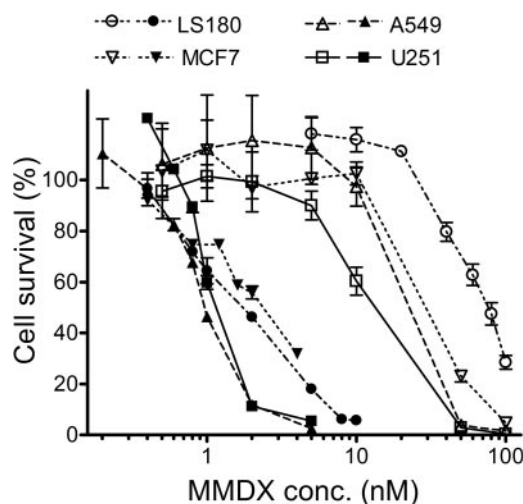


Fig. 4. Sensitivity of human tumor cell lines from multiple tissues of origin to microsomes-activated MMDX. Data shown are taken from a 4-day growth-inhibition assay similar to Fig. 1A in the absence of NADPH and dexamethasone-induced rat liver microsomes (2 μ g of protein/well) (open symbols) or in the presence of NADPH and liver microsomes (filled symbols). Data shown are mean values \pm S.D. ($n = 3$). IC_{50} values and potentiation ratios derived from these data are shown in Table 2.

1992). To clarify this issue, we evaluated the cytotoxicity of MMDX and of activated MMDX toward MCF-7 cells and toward the P-glycoprotein-overexpressing subline MCF7-ADR. As shown in Fig. 5 and Table 3, MCF7-ADR cells were ~5.2-fold more resistant to MMDX compared with MCF-7 cells. The cytotoxicity of MMDX toward MCF7-ADR cells was substantially improved in the presence of dexamethasone-induced liver microsomes (potentiation ratio = 15), such that activated MMDX was equally active against MCF-7 and MCF7-ADR cells (resistance factor = 1). In the case of doxorubicin, however, incubation with microsomes led to a ~7-fold decrease in activity toward MCF-7 cells (potentiation ratio = 0.14), indicating that doxorubicin is inactivated by microsomal metabolism. The cytotoxicity of doxorubicin toward MCF7-ADR cells was not significantly affected by microsomal metabolism. We conclude that CYP3A activation of MMDX, but not doxorubicin, fully reverses the resistance phenotype of MCF7-ADR cells.

Intrinsic Activity and Stability of CYP3A-Activated MMDX. In the growth-inhibition assays described above, tumor cells were cultured with liver microsomes and MMDX for a total of 96 h, at which time drug-induced tumor cell kill was evaluated. Experiments were therefore carried out to determine whether strong cytotoxic responses could be achieved after shorter times of incubation and microsomal metabolism. Figure 6 shows that a 2-h incubation of MMDX (5 nM) with dexamethasone-induced rat liver microsomes generated sufficient active metabolite to kill $\geq 95\%$ of the 9L tumor cells by the end of the 4-day assay period. Thus, the MMDX activation reaction is rapid and does not require the full 96-h period to generate activated, cytotoxic metabolites. Even at a 5-fold lower MMDX concentration, ~85% tumor cell kill was achieved within a 6-h incubation period. It is interesting that under these latter conditions, the formation of cytotoxic microsomal metabolites was not detectable during the initial 1-h period. This apparent lag period suggests that the concentration of activated MMDX may need to reach a minimum threshold level, perhaps reflecting saturation of cellular binding sites not linked to the cytotoxic response.

Next, we investigated the intrinsic stability of activated MMDX. Initial studies showed that a protein-free filtrate, prepared after preincubation of MMDX with dexamethasone-induced microsomes and NADPH for 30 min, killed $>90\%$ of 9L tumor cells in a 4-day growth-inhibition assay, but that a 12-h preincubated MMDX sample was totally inactive. This loss of activity after 12-h incubation with microsomes could

TABLE 3

IC₅₀ values, potentiation ratios, and resistance factors for MMDX and doxorubicin toward MCF-7 and MCF7-ADR cells incubated in the presence or absence of dexamethasone-induced rat liver microsomes and NADPH

IC₅₀ values include data derived from triplicate samples as shown in Fig. 5 and from data not shown. Potentiation ratios are IC₅₀ values of drug/IC₅₀ of drug + dexamethasone-induced liver microsomes. Resistance factor is IC₅₀ for MCF7-ADR/IC₅₀ for MCF-7.

	IC ₅₀ (Potentiation Ratios)		Resistance Factor
	MCF7	MCF7-ADR	
	<i>nM</i>		
Doxorubicin	12.6	6600	524
Doxorubicin + Dex microsomes	86.6 (0.14)	10,300 (0.64)	119
MMDX	31	161	5.2
MMDX + Dex microsomes	10.2 (3.0)	10.7 (15.0)	1.0

reflect the intrinsic (chemical) instability of MMDX or, alternatively, may be the result of further microsomal metabolism of activated MMDX to yield inactive, secondary metabolites. To distinguish these possibilities, we evaluated the intrinsic stability of activated MMDX incubated at 37°C in the absence of liver microsomes. Protein-free filtrates were pre-

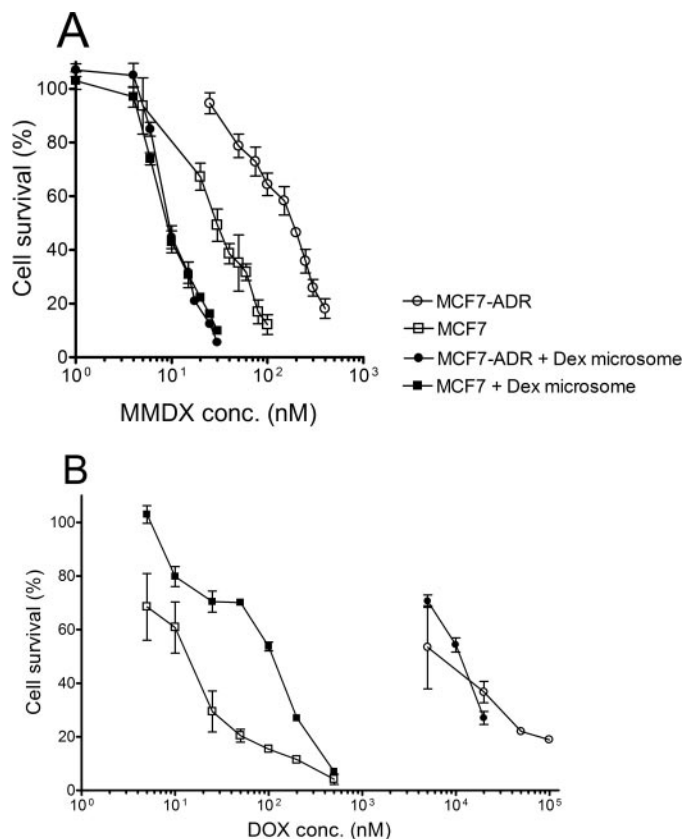


Fig. 5. Sensitivity of MCF7 and MCF7-ADR cells to MMDX (A) or doxorubicin (B) in the absence (open symbols) or presence (filled symbols) of microsomal activation system. Four-day growth-inhibition assay was carried out in the presence or absence of dexamethasone-induced rat liver microsomes, as described in Fig. 1A. Data shown are mean values \pm S.D. ($n = 3$). IC₅₀ values based on these data are shown in Table 3.

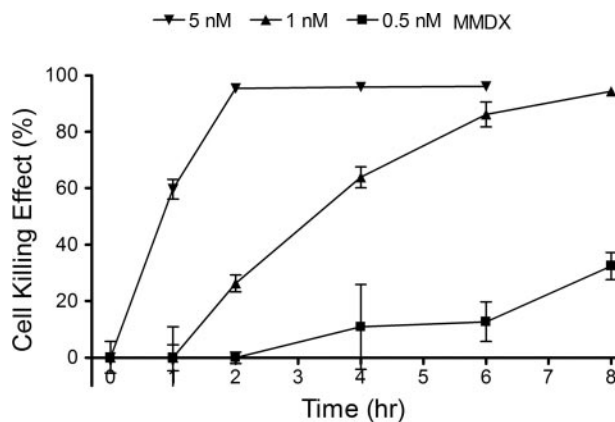


Fig. 6. MMDX growth inhibition as a function of time of incubation with dexamethasone-induced liver microsomes. 9L cells were incubated with MMDX (0.5–5 nM), 2 μ g of dexamethasone-induced rat liver microsomes/well, and NADPH (0.3 mM) for the indicated periods of time, at which point the cells were washed and placed in drug- and microsome-free culture medium for a 4-day culture period as described under *Materials and Methods*. Data shown are the mean values \pm S.D. ($n = 3$).

pared from samples of MMDX preincubated with dexamethasone-induced liver microsomes and NADPH for 30 min. The protein-free filtrates, containing activated MMDX, were then incubated at 37°C for times ranging from 2 to 12 h, at which point aliquots were added to 9L cells, which were then cultured for 4 days in a growth-inhibition assay. Figure 7 shows that the growth-inhibitory activity of CYP3A-activated MMDX was retained for an extended period of time. The length of this "plateau" period of stability was directly related to the extent of MMDX activation during the 30-min preincubation period, as indicated by the cytotoxic activity at time 0 (y-intercept value). At the end of each plateau period, the cytotoxic activity of each activated MMDX sample declined in a log-linear fashion, giving a series of parallel lines whose slopes indicate first-order kinetics for decomposition of activated MMDX with a $t_{1/2}$ (apparent) of 5.5 ± 1.6 h.

Discussion

CYP3A-selective inhibitors, such as troleandomycin, markedly decrease the therapeutic efficacy of MMDX in mice bearing M5076 liver metastases (Quintieri et al., 2000) and substantially inhibit liver microsome-catalyzed activation of MMDX to tumor cell cytotoxic species in vitro (Lewis et al., 1992; Baldwin et al., 2003). These findings were extended by the present study, which revealed a strong correlation between CYP3A-catalyzed testosterone 6 β -hydroxylase activity and the potentiation of MMDX cytotoxicity in a panel of human liver microsomes and demonstrated that the human enzyme CYP3A4 and its rodent counterparts CYP3A1 and CYP3A2 are active catalysts of the prodrug activation reaction. These findings are consistent with the observation that the metabolism of MMDX by human liver microsomes can be

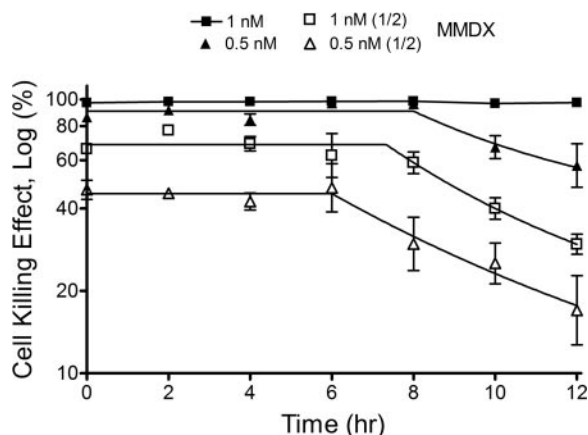


Fig. 7. Intrinsic stability of CYP3A-activated MMDX. MMDX (0.5 or 1 nM) was preincubated for 30 min with dexamethasone-induced rat liver microsomes (10 μ g of protein/ml culture medium) and NADPH (0.3 mM). The samples were then filtered, and the protein-free filtrate (diluted 2-fold into fresh culture medium where indicated; 1/2) was added to 9L cells to assay for cytotoxic activity in a 4-day growth-inhibition assay. Cell killing was calculated as described under *Materials and Methods*. A half-life value of 5.5 ± 1.6 h was calculated for activated MMDX from the portions of each curve showing a time-dependent, log-linear decline in cell-killing activity (mean \pm S.D., $n = 3$ curves). No decline in cytotoxicity was observed for the 1 nM MMDX sample over the 12-h incubation period, indicating that the amount of activated MMDX initially present in this sample was at least in 4-fold excess over that required to kill all of the 9L cells [i.e., no decline in activity was apparent after incubation at 37°C for 12 h (~ 2 half-lives) before addition to the cells].

competitively inhibited by the CYP3A4 substrates paclitaxel, tamoxifen, and cyclosporin (Beulz-Riche et al., 2002).

CYP3A metabolism resulted in a striking potentiation of MMDX cytotoxicity at nanomolar prodrug concentrations, both when the P450 enzyme was present extracellularly and when it was expressed endogenously in CHO cells stably transfected with CYP3A4. Comparison of the increase in MMDX cytotoxicity conferred by intracellular versus extracellular CYP3A4 expression suggested that intracellular MMDX activation is at least 20-fold more potent than the extracellular activation system, a phenomenon that has been seen with other anticancer prodrugs (Lawrence et al., 1998). Extratumoral (i.e., hepatic) activation of MMDX is nevertheless likely to be the major site of prodrug activation in vivo, given the very low level of CYP3A4 expression and activity found in human tumors (Kivisto et al., 1996; Knupfer et al., 1999; Murray et al., 1999; Iscan et al., 2001; Martinez et al., 2002). These findings point to the potential advantages of using gene-therapy vectors to increase the content of CYP3A4 within tumor cells and thereby augment the toxicity, and the tumor-cell selectivity, of MMDX. A P450 prodrug activation-based gene therapy designed to achieve this goal has been developed recently with CYP2B6 for the anticancer prodrug cyclophosphamide and has shown promise in pre-clinical and in initial phase I/II clinical trials (Chen and Waxman, 2002; Jounaidi, 2002).

The precise nature of the active, cytotoxic metabolite of MMDX is not presently known. P450-activated MMDX is highly cytotoxic and induces DNA-DNA cross-links (Lau et al., 1994), in contrast to MMDX itself, which, like doxorubicin, induces DNA strand breaks (Ross et al., 1979). Six to eight distinct metabolites are formed when MMDX is incubated with liver microsomes, depending on the species and the MMDX concentration (Beulz-Riche et al., 2001). Among these, the only characterized metabolite detected both in human patients and in animals is MMDX-ol (PNU-155051). However, this metabolite is formed by an aldo-keto reductase without the involvement of cytochrome P450 and is less cytotoxic than MMDX (Breda et al., 2000). Several other characterized and uncharacterized MMDX metabolites are formed by human liver microsomes in a reaction that can be inhibited by CYP3A4 substrates (Beulz-Riche et al., 2001, 2002). However, the potential role of these metabolites in MMDX cytotoxicity remains to be determined.

Analysis of four human tumor cell lines revealed a 6-fold range in sensitivity to MMDX, with IC_{50} values ranging from 12 nM MMDX for U251 brain cancer cells to 74 nM for LS180 colon cancer cells. It is noteworthy that both the IC_{50} values and the cell-line-to-cell-line differences in drug sensitivity were decreased for CYP3A-activated MMDX. The more uniform response of the tumor cells to P450-activated MMDX may reflect differences in the mechanism of cell killing, with activated MMDX able to induce a direct, and rapid, alkylation of tumor cell DNA (Lau et al., 1992). P450-activated MMDX may not display improved tumor selectivity compared with MMDX, however, given its comparable cytotoxicity against tumor cells and hematopoietic progenitors (Kuhl et al., 1993; Ghilmini et al., 1998). Consistent with this observation, the CYP3A inhibitor troleandomycin markedly inhibits MMDX induced myelosuppression in vivo (Quintieri et al., 2000). Strategies to reduce systemic metabolism and increase intratumoral MMDX metabolism (e.g., using a tu-

mor-targeted gene-therapy vector delivering CYP3A4) may therefore be useful in increasing the therapeutic index of MMDX.

In addition to the differences in mechanism of action noted above, activated MMDX differs from MMDX in its pattern of drug resistance. The doxorubicin-resistant MCF-7 subline MCF7-ADR displays 500-fold resistance to doxorubicin associated with overexpression of the drug efflux pump P-gp (Fairchild et al., 1987). Other factors, including a decrease in topoisomerase activity, may also contribute to the drug resistance of this cell line (Sinha et al., 1988). MCF7-ADR cells displayed a much lower degree of resistance (~5-fold) to MMDX than to doxorubicin (Fig. 5), which may reflect the higher lipophilicity of MMDX, a lower affinity for P-gp, or perhaps the formation of DNA strand breaks through topoisomerase I, rather than topoisomerase II (Duran et al., 1996). The cyclosporin derivative and P-gp inhibitor SDZ-PSC833 partially reverses resistance to MMDX in four models of multidrug resistance involving P-gp overexpression (Michieli et al., 1996). Moreover, intracellular levels of MMDX are reduced 2-fold in a doxorubicin-resistant, P-gp-overexpressing cell line compared with those in the drug-sensitive parent cell line (Bakker et al., 1997). These results suggest that P-gp may prevent MMDX from reaching its nuclear target, particularly when the drug concentration is low. The elevated glutathione peroxidase activity in MCF7-ADR cells (Chen and Waxman, 1995) could also contribute to the residual MMDX cross-resistance, as suggested by the correlation between increased glutathione peroxidase activity and decreased MMDX activity in a panel of human melanoma cell lines (Alvino et al., 1997). Alterations in topoisomerase I activity in MCF7-ADR cells could also contribute to the cross-resistance to MMDX. It is interesting that although cross-resistance to MMDX is also seen in a doxorubicin-resistant cell line that over-expresses MDR-associated protein (van der Graaf et al., 1995), MMDX is not a substrate of the drug transporter MDR-associated protein (Bakker et al., 1997), suggesting that other mechanisms are responsible for the cross-resistance seen in P-gp-negative MDR cell lines. However, in contrast to the cross-resistance of MCF7-ADR cells to MMDX, no cross-resistance to P450-activated MMDX was seen in the present study. This finding is consistent with the report that a cyanomorpholino derivative of doxorubicin that has alkylating properties similar to the active metabolites of MMDX is not cross-resistant with doxorubicin in MDR cells (Scudder et al., 1988). Together, these findings suggest that P450-activated MMDX is not a substrate for the drug-efflux pump P-gp and may be effective against tumors that exhibit a multidrug-resistance phenotype.

Phase I and phase II clinical trials and pharmacokinetic studies of MMDX showed a terminal half-life of 40 h after a rapid distribution phase (Vasey et al., 1995; Bakker et al., 1998). The area under the plasma concentration-time curve calculated up to 24 h after dosing at 1.25 mg/m² MMDX was ~20 ng/h/ml, and the C_{max} ranged from 2 to 6 ng/ml (3–9 nM) (Vasey et al., 1995; Bakker et al., 1998). These latter concentrations are greater than or similar to the IC₅₀ values presently reported for MMDX toxicity to human tumor cells after incubation with liver microsomes and NADPH (Table 2). Investigation of the time course of MMDX activation in vitro revealed that CYP3A-activated MMDX metabolites accumulate within 2 h at 5 nM prodrug to levels that are sufficient to

induce maximum cytotoxicity, suggesting that strong MMDX antitumor activity may be readily achieved in vivo. Although the plasma MMDX concentration during the terminal elimination phase is less than 1 ng/ml (1.5 nM) (Vasey et al., 1995; Bakker et al., 1998), active metabolites generated by liver CYP3A enzymes during this time period could still be highly toxic, given the very slow elimination of MMDX, which at 40 h is seven times longer than the 6 h presently required for efficient activation of 1 nM MMDX by liver microsomes in vitro (Fig. 6). Furthermore, we observed that activated MMDX generated during a 30-min liver microsomal activation reaction maintained its initial, maximal level of cytotoxicity for as long as 3 to 6 h when incubated in the presence of cells, after which activity declined with first-order kinetics and a half-life of 5.5 h. Thus, CYP3A-activated MMDX can be formed rapidly and decays relatively slowly, suggesting that sufficient levels of active drug may accumulate in vivo to sustain antitumor activity. However, the highly toxic nature of liver CYP3A-activated MMDX is probably related to the dose-limiting hematotoxicity (Sessa et al., 1999; Fokkema et al., 2000). This problem may be minimized in the case of tumors containing high CYP3A4 expression and activity, whether expressed endogenously or achieved using gene-therapy vectors that augment intratumoral CYP3A4 expression.

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References

- Alvino E, Gilberti S, Cantagallo D, Massoud R, Gatteschi A, Tentori L, Bonmassar E, and D'Atri S (1997) In vitro antitumor activity of 3'-desamino-3'-(2-methoxy-4-morpholinyl) doxorubicin on human melanoma cells sensitive or resistant to triazine compounds. *Cancer Chemother Pharmacol* **40**:180–184.
- Bakker M, Droz JP, Hanauske AR, Verweij J, van Oosterom AT, Groen HJ, Pacciarini MA, Domenigoni L, van Weissenbruch F, Pianezzola E, et al. (1998) Broad phase II and pharmacokinetic study of methoxy-morpholino doxorubicin (FCE 23762-MMRDX) in non-small-cell lung cancer, renal cancer and other solid tumour patients. *Br J Cancer* **77**:139–146.
- Bakker M, Renes J, Groenhuijzen A, Visser P, Timmer-Bosscha H, Muller M, Groen HJ, Smit EF, and de Vries EG (1997) Mechanisms for high methoxymorpholino doxorubicin cytotoxicity in doxorubicin-resistant tumor cell lines. *Int J Cancer* **73**:362–366.
- Baldwin A, Huang Z, Jounaidi Y, and Waxman DJ (2003) Identification of novel enzyme-prodrug combinations for use in cytochrome P450-based gene therapy for cancer. *Arch Biochem Biophys* **409**:197–206.
- Beulz-Riche D, Robert J, Menard C, and Ratanasavanh D (2001) Metabolism of methoxymorpholino-doxorubicin in rat, dog and monkey liver microsomes: comparison with human microsomes. *Fundam Clin Pharmacol* **15**:373–378.
- Beulz-Riche D, Robert J, Riche C, and Ratanasavanh D (2002) Effects of paclitaxel, cyclophosphamide, ifosfamide, tamoxifen and cyclosporine on the metabolism of methoxymorpholinodoxorubicin in human liver microsomes. *Cancer Chemother Pharmacol* **49**:274–280.
- Breda M, Benedetti MS, Battaglia R, Castelli MG, Poggiesi I, Spinelli R, Hackett AM, and Dostert P (2000) Species-differences in disposition and reductive metabolism of methoxymorpholinodoxorubicin (PNU 152243), a new potential anticancer agent. *Pharmacol Res* **41**:239–248.
- Chen G and Waxman DJ (1995) Complete reversal by thaliblastine of 490-fold adriamycin resistance in multidrug-resistant (MDR) human breast cancer cells. Evidence that multiple biochemical changes in MDR cells need not correspond to multiple functional determinants for drug resistance. *J Pharmacol Exp Ther* **274**:1271–1277.
- Chen L and Waxman DJ (2002) Cytochrome P450 gene-directed enzyme prodrug therapy (GDEPT) for cancer. *Curr Pharm Des* **8**:1405–1416.
- Danesi R, Agen C, Grandi M, Nardini V, Bevilacqua G, and Del Tacca M (1993) 3'-Deamino-3'-(2-methoxy-4-morpholinyl)-doxorubicin (FCE 23762): a new anthracycline derivative with enhanced cytotoxicity and reduced cardiotoxicity. *Eur J Cancer* **29A**:1560–1565.
- Ding S, Yao D, Burchell B, Wolf CR, and Friedberg T (1997) High levels of recombinant CYP3A4 expression in Chinese hamster ovary cells are modulated by coexpressed human P450 reductase and hemin supplementation. *Arch Biochem Biophys* **348**:403–410.
- Duran GE, Lau DH, Lewis AD, Kuhl JS, Bammler TK, and Sikic BI (1996) Differential single- versus double-strand DNA breakage produced by doxorubicin and its morpholinyl analogues. *Cancer Chemother Pharmacol* **38**:210–216.

- Fairchild CR, Ivy SP, Kao-Shan CS, Whang-Peng J, Rosen N, Israel MA, Melera PW, Cowan KH, and Goldsmith ME (1987) Isolation of amplified and overexpressed DNA sequences from adriamycin-resistant human breast cancer cells. *Cancer Res* **47**:5141–5148.
- Fokkema E, Verweij J, van Oosterom AT, Uges DR, Spinelli R, Valota O, de Vries EG, and Groen HJ (2000) A prolonged methoxymorpholino doxorubicin (PNU-152243 or MMRDX) infusion schedule in patients with solid tumours: a phase 1 and pharmacokinetic study. *Br J Cancer* **82**:767–771.
- Geroni C, Pesenti E, Broggin M, Belvedere G, Tagliabue G, D'Incalci M, Pennella G, and Grandi M (1994) L1210 cells selected for resistance to methoxymorpholinyl doxorubicin appear specifically resistant to this class of morpholinyl derivatives. *Br J Cancer* **69**:315–319.
- Ghielmini M, Colli E, Bosshard G, Pennella G, Geroni C, Torri V, D'Incalci M, Cavalli F, and Sessa C (1998) Hematotoxicity on human bone marrow- and umbilical cord blood-derived progenitor cells and in vitro therapeutic index of methoxymorpholinyl-doxorubicin and its metabolites. *Cancer Chemother Pharmacol* **42**:235–240.
- Iscan M, Kilaavuniemi T, Coban T, Kapucuoğlu N, Pelkonen O, and Raunio H (2001) The expression of cytochrome P450 enzymes in human breast tumours and normal breast tissue. *Breast Cancer Res Treat* **70**:47–54.
- Jounaidi Y (2002) Cytochrome P450-based gene therapy for cancer treatment: from concept to the clinic. *Curr Drug Metab* **3**:609–622.
- Kivisto KT, Griese EU, Fritz P, Linder A, Hakkola J, Raunio H, Beaune P, and Kroemer HK (1996) Expression of cytochrome P 450 3A enzymes in human lung: a combined RT-PCR and immunohistochemical analysis of normal tissue and lung tumours. *Naunyn Schmiedeberg's Arch Pharmacol* **353**:207–212.
- Knupfer H, Knupfer MM, Hotfilder M, and Preiss R (1999) P450-expression in brain tumors. *Oncol Res* **11**:523–528.
- Kuhl JS, Duran GE, Chao NJ, and Sikic BI (1993) Effects of the methoxymorpholino derivative of doxorubicin and its bioactivated form versus doxorubicin on human leukemia and lymphoma cell lines and normal bone marrow. *Cancer Chemother Pharmacol* **33**:10–16.
- Lau DH, Duran GE, Lewis AD, and Sikic BI (1994) Metabolic conversion of methoxymorpholinyl doxorubicin: from a DNA strand breaker to a DNA cross-linker. *Br J Cancer* **70**:79–84.
- Lau DH, Duran GE, and Sikic BI (1992) Characterization of covalent DNA binding of morpholino and cyanomorpholino derivatives of doxorubicin. *J Natl Cancer Inst* **84**:1587–1592.
- Lau DH, Lewis AD, and Sikic BI (1989) Association of DNA cross-linking with potentiation of the morpholino derivative of doxorubicin by human liver microsomes. *J Natl Cancer Inst* **81**:1034–1038.
- Lawrence TS, Rehemtulla A, Ng EY, Wilson M, Trosko JE, and Stetson PL (1998) Preferential cytotoxicity of cells transfected with cytosine deaminase compared to bystander cells after treatment with 5-fluorocytosine. *Cancer Res* **58**:2588–2593.
- Lewis AD, Lau DH, Duran GE, Wolf CR, and Sikic BI (1992) Role of cytochrome P-450 from the human CYP3A gene family in the potentiation of morpholino doxorubicin by human liver microsomes. *Cancer Res* **52**:4379–4384.
- Martinez C, Garcia-Martin E, Pizarro RM, Garcia-Gamito FJ, and Agundez JA (2002) Expression of paclitaxel-inactivating CYP3A activity in human colorectal cancer: implications for drug therapy. *Br J Cancer* **87**:681–686.
- Michieli M, Damiani D, Michelutti A, Melli C, Ermacora A, Geromin A, Fanin R, Russo D, and Baccarani M (1996) Overcoming PGP-related multidrug resistance. The cyclosporine derivative SDZ PSC 833 can abolish the resistance to methoxymorpholinyl-doxorubicin. *Haematologica* **81**:295–301.
- Murray GI, McFadyen MC, Mitchell RT, Cheung YL, Kerr AC, and Melvin WT (1999) Cytochrome P450 CYP3A in human renal cell cancer. *Br J Cancer* **79**:1836–1842.
- Quintieri L, Rosato A, Napoli E, Sola F, Geroni C, Floreani M, and Zanovello P (2000) In vivo antitumor activity and host toxicity of methoxymorpholinyl doxorubicin: role of cytochrome P450 3A. *Cancer Res* **60**:3232–3238.
- Ripamonti M, Pezzoni G, Pesenti E, Pastori A, Farao M, Bargiotti A, Suarato A, Spreafico F, and Grandi M (1992) In vivo anti-tumour activity of FCE 23762, a methoxymorpholinyl derivative of doxorubicin active on doxorubicin-resistant tumour cells. *Br J Cancer* **65**:703–707.
- Ross WE, Glaubiger D, and Kohn KW (1979) Qualitative and quantitative aspects of intercalator-induced DNA strand breaks. *Biochim Biophys Acta* **562**:41–50.
- Scudder SA, Brown JM, and Sikic BI (1988) DNA cross-linking and cytotoxicity of the alkylating cyanomorpholino derivative of doxorubicin in multidrug-resistant cells. *J Natl Cancer Inst* **80**:1294–1298.
- Sessa C, Zucchetti M, Ghielmini M, Bauer J, D'Incalci M, de Jong J, Naegle H, Rossi S, Pacciarini MA, Domenigoni L, et al. (1999) Phase I clinical and pharmacological study of oral methoxymorpholinyl doxorubicin (PNU 152243). *Cancer Chemother Pharmacol* **44**:403–410.
- Sinha BK, Haim N, Dusre L, Kerrigan D, and Pommier Y (1988) DNA strand breaks produced by etoposide (VP-16,213) in sensitive and resistant human breast tumor cells: implications for the mechanism of action. *Cancer Res* **48**:5096–5100.
- Suarato A, Angelucci F, and Geroni C (1999) Ring-B modified anthracyclines. *Curr Pharm Des* **5**:217–227.
- Sun Y, Li H, Lin Z, Sun L, Valota O, Battaglia R, and Pacciarini M (2003) Phase I and pharmacokinetic study of nemorubicin hydrochloride (methoxymorpholino doxorubicin; PNU-152243) administered with iodinated oil via hepatic artery (IHA) to patients (pt) with unresectable hepatocellular carcinoma (HCC). *Proc Am Soc Clin Oncol* **22**:361.
- van der Graaf WT, Mulder NH, Meijer C, and de Vries EG (1995) The role of methoxymorpholino anthracycline and cyanomorpholino anthracycline in a sensitive small-cell lung-cancer cell line and its multidrug-resistant but P-glycoprotein-negative and cisplatin-resistant counterparts. *Cancer Chemother Pharmacol* **35**:345–348.
- Vasey PA, Bissett D, Strolin-Benedetti M, Poggesi I, Breda M, Adams L, Wilson P, Pacciarini MA, Kaye SB, and Cassidy J (1995) Phase I clinical and pharmacokinetic study of 3'-deamino-3'-(2-methoxy-4-morpholinyl)doxorubicin (FCE 23762). *Cancer Res* **55**:2090–2096.

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