Cancer Chemother Pharmacol (2002) 49: 274–280
DOI 10.1007/s00280-001-0415-1

ORIGINAL ARTICLE

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Effects of paclitaxel, cyclophosphamide, ifosfamide, tamoxifen and cyclosporine on the metabolism of methoxymorpholinodoxorubicin in human liver microsomes

Received: 18 June 2001 / Accepted: 30 November 2001 / Published online: 30 January 2002

Abstract The effects of paclitaxel, cyclosporine, cyclophosphamide, ifosfamide and tamoxifen on the metabolism of methoxymorpholinodoxorubicin (MMDx), a novel anticancer agent, were investigated using human liver microsomes. Paclitaxel, tamoxifen and cyclosporine dramatically inhibited MMDx metabolism, whereas ifosfamide had only a slight effect at high concentrations (200–300 μM) and cyclophosphamide had no effect. The inhibition was dependent on the concentrations of both MMDx and the coincubated drug. Thus, with 1 μM MMDx, paclitaxel (5 μM), tamoxifen (1 μM) and cyclosporine (1 μM) decreased the metabolic rate of MMDx by 36%, 53% and 62%, respectively. At higher concentrations (10, 5 and 5 μM, respectively, with paclitaxel, tamoxifen and cyclosporine) the inhibition was 52%, 91% and 91%, respectively. These three drugs preferentially inhibited the formation of three metabolites (M2, M3 and M6) among eight metabolites produced in liver microsomes. The inhibitory concentrations of paclitaxel, tamoxifen and cyclosporine on MMDx metabolism were in the range of those observed in patients upon administration of these drugs, which are known to be CYP3A4 substrates. These findings suggest that CYP3A4 drug substrates and MMDx in combination must be used with caution, particularly in view of the fact that MMDx is considered as a prodrug whose activation is entirely dependent upon metabolic transformation by CYP3A4.

Keywords Methoxymorpholino doxorubicin · Metabolism · Drug interaction

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Introduction

Anthracyclines are a group of drugs currently used for the treatment of a wide range of malignancies. The toxicity of these compounds and the emergence of multidrug resistance (MDR) after treatment with anthracyclines prompted the search for more-potent and less-toxic analogues. Morpholinylanthracyclines are an interesting new class of compounds, being active in vitro and in vivo against MDR tumours [7, 29].

PNU152243 (3′-deamino-3′-(2(*S*)-methoxy-4-morpholinyl) doxorubicin, MMDx) is a new highly lipophilic doxorubicin (DOX) derivative possessing a methoxymorpholinyl group at the 3′ position of the sugar moiety (Fig. 1). This compound, currently undergoing phase I/II clinical trials [1, 12, 30], has shown activity in sensitive and MDR models. The in vitro cytotoxicity of MMDx is markedly enhanced by preincubating the drug with liver microsomes and NADPH [24], a metabolic process inhibited by cyclosporine and erythromycin, both of which are substrates for cytochromes P450 (CYPs) of the 3A family. These findings suggest that active metabolite(s) biosynthesized via CYP3A mediate MMDx cytotoxicity in vivo [24].

Because cancer patients are often treated with multiple drug regimens, evaluation of potential drug interactions that might compromise therapeutic efficacy are of considerable interest with regard to the optimization of cancer chemotherapy. Metabolism represents a major factor in the variability in drug response and toxicity, particularly for anticancer agents that exhibit a narrow therapeutic index. CYP3A4 is involved in the metabolism of several chemotherapeutic agents, including epipodophyllotoxins, tamoxifen, ifosfamide, docetaxel,

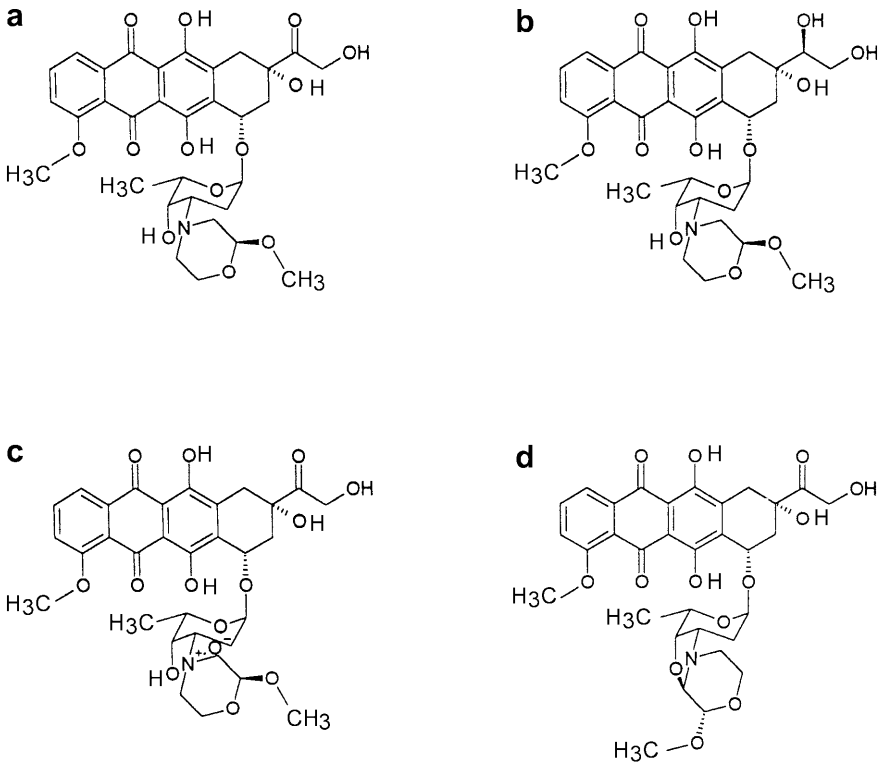

Fig. 1a–d Chemical structure of methoxymorpholino doxorubicin (PNU152243) (**a**) and its known metabolites MMDx-ol (**b**), PNU156686 (**c**) and PNU159682 (**d**)

paclitaxel and vinca alkaloids, and in the metabolism of MMDx [9, 10, 18, 21]. In addition, CYP3A4 substrates may also modulate MDR in cancer chemotherapy [19] because several inhibitors and substrates of CYP3A4 are also inhibitors of and substrates for P glycoprotein (P-gp) [11]. Concomitant treatment of anthracyclines with inhibitors of P-gp such as verapamil and cyclosporine could, therefore, interfere with drug disposition. Cyclosporine, in particular, is known to increase the AUC of DOX by 55% [2].

Since MMDx may potentially be coadministered with cyclosporine, we tested this compound for its capacity to alter MMDx metabolism in human liver microsomes. We also investigated the effects of paclitaxel, cyclophosphamide, ifosfamide and tamoxifen on the metabolism of MMDx in order to evaluate potential metabolic anticancer drug interactions between MMDx and these compounds.

Materials and methods

Chemicals

Pure standards of MMDx (PNU152243) and its metabolites 13-dihydro-MMDx (MMDx-ol), PNU156686 and PNU159682 were generous gifts from Pharmacia & Upjohn (Milan, Italy). These compounds were dissolved in methanol as stock solutions and stored at –80ºC until use. NADPH, dexamethasone, tamoxifen and bovine serum albumin were provided by Sigma (Saint Louis, Mo.). Methanol and ammonia solution (25%) were purchased from Merck (Darmstadt, Germany). Acetonitrile was from SDS (Peypin, France). Formic acid was purchased from Prolabo (Fontenay-sous-Bois, France). Paclitaxel (Taxol) in ready-for-use solution (30 mg/5 ml) was purchased from Bristol-Myers Squibb (Paris La Défense, France). Cyclophosphamide (Endoxan) and ifosfamide (Holoxan) were supplied by Asta Medica (Mérignac, France). Cyclosporine (Sandimmun) was purchased from Novartis (France).

Liver microsomes

Human livers were obtained from tissue removed at surgery. The protocol for obtaining and using human liver microsomes was approved by the local ethical committee. Human liver microsomes were prepared according to the method of Berthou et al. [3]. Microsomes were stored in liquid nitrogen until use.

Microsomal protein content was measured according to the method of Bradford [4] using bovine serum albumin as a standard.

Standard incubation with MMDx

Drugs were dissolved either in dimethylsulfoxide (DMSO) or in methanol. The concentration of organic solvent in reaction incubations did not exceed 0.1%. Preliminary studies with different incubation times (10 to 60 min) and with various microsomal protein concentrations (0.5 to 2 mg/ml) were performed. The results showed that the optimal conditions of incubation were as follows: 0.5 mg protein/ml and 30 min incubation time.

Microsomal proteins (0.5 mg/ml) were preincubated in phosphate buffer (0.1 *M*, pH 7.4) at 37ºC, 5 min with MMDx at concentrations ranging between 1 and 25 μ*M*, in a total volume of 500 μl. The reaction was started by adding NADPH (1 m*M*). After 30 min incubation, the reaction was stopped by adding ethanol (500 μl). The samples were centrifuged and the supernatant was evaporated to dryness under a stream of nitrogen. The dried residue was dissolved in 200 μl formate buffer (pH 4.0). Control incubations received the same volume of solvent and were run as described above except that microsomal proteins or NADPH were omitted. The presence of 0.1% DMSO or 0.1% methanol in the reaction incubations did not significantly modify MMDx metabolism in liver microsomes.

Table 1 Effect of tamoxifen on the formation of various MMDx metabolites in human liver microsomes. MMDx was incubated with 0.5 mg protein/ml for 30 min in a final volume of 500 µl. The results are presented as picomoles per minute per milligram protein and as means ± SD of two experiments carried out in duplicate (*n.d.* not detectable)

MMDx (µ*M*)	Tamoxifen (µ*M*)	M1	M2	M3	M5	M6	M7
1	0	n.d.	9.2 ± 0.7	17.4 ± 0.3	n.d.	21.1 ± 0.4	5.3 ± 1.5
	1	n.d.	1.9 ± 2.7	5.2 ± 0.4	n.d.	3.4 ± 0.5	14.4 ± 5.2
	5	n.d.	n.d.	2.3 ± 0.1	n.d.	n.d.	2.6 ± 0.3
5	0	4.2 ± 0.3	11.8 ± 0.1	20.4 ± 0.6	4.2 ± 0.3	20.6 ± 0.5	11.0 ± 2.4
	1	4.3 ± 0.3	3.5 ± 0.1	7.0 ± 0.6	1.7 ± 0.3	3.3 ± 0.5	34.9 ± 2.4
	5	4.3 ± 0.4	0.9 ± 0.1	3.8 ± 0.1	n.d.	n.d.	6.6 ± 0.8
10	0	8.5 ± 0.4	10.0 ± 0.1	16.3 ± 0.1	7.1 ± 0.1	15.7 ± 0.4	5.5 ± 0.7
	1	6.7 ± 1.0	2.8 ± 0.3	6.3 ± 0.1	2.8 ± 0.3	2.5 ± 0.1	31.6 ± 13.9
	5	7.6 ± 0.8	n.d.	4.1 ± 0.2	2.0 ± 0.7	0.5 ± 0.7	2.9 ± 1.4

Table 2 Effect of cyclosporine on the formation of various MMDx metabolites in human liver microsomes. MMDx was incubated with 0.5 mg protein/ml for 30 min in a final volume of 500 µl. The results are presented as picomoles per minute per milligram protein and as means ± SD of two experiments carried out in duplicate (*n.d.* not detectable)

MMDx (µ*M*)	Cyclosporine (µ*M*)	M1	M2	M3	M5	M6	M7
1	0	n.d.	9.5 ± 1.1	19.2 ± 1.5	n.d.	22.3 ± 2.1	4.3 ± 0.02
	1	n.d.	7.2 ± 1.6	0.8 ± 1.1	n.d.	7.1 ± 0.9	6.1 ± 0.4
	5	n.d.	n.d.	n.d.	n.d.	n.d.	4.4 ± 2.0
5	0	3.3 ± 0.7	12.9 ± 1.3	22.4 ± 0.2	4.8 ± 0.1	22.4 ± 0.9	9.2 ± 0.2
	1	3.7 ± 0.1	5.9 ± 0.6	11.5 ± 0.2	4.1 ± 0.1	8.3 ± 0.4	7.8 ± 1.6
	5	3.9 ± 0.3	1.9 ± 0.1	3.2 ± 0.3	1.8 ± 0.1	1.6 ± 0.1	11.8 ± 2.3
10	0	7.1 ± 0.5	12.0 ± 0.7	18.2 ± 0.5	6.6 ± 0.2	15.9 ± 1.2	7.5 ± 0.2
	1	8.7 ± 2.2	5.1 ± 0.8	10.8 ± 0.4	5.8 ± 0.5	7.8 ± 0.3	13.3 ± 3.1
	5	8.3 ± 0.2	1.6 ± 0.7	4.4 ± 0.3	2.9 ± 0.4	1.4 ± 0.1	22.8 ± 2.5

High-performance liquid chromatographic analysis

HPLC analysis was performed with a quaternary pump (Spectra System P1000 XR) on a Lichrospher column 100 RP-18 end-capped, 5 µ*M* particle diameter, 125×4 mm (Merck, Darmstadt, Germany). The mobile phase consisted of a mixture of ammonium formate buffer (0.05 *M*, pH 4) and acetonitrile as described by Israel et al. [16] with slight modifications. In this study, a gradient mode was used. The buffer/acetonitrile ratio (80/20 v/v) was maintained for 5 min and then raised linearly to 65/35 (v/v) over 15 min. This proportion was maintained for 5 min at a flow rate of 0.9 ml/min.

Metabolite peaks were detected by fluorescence (excitation 486 nm, emission 550 nm) using a spectrofluorometer FL3000 from Thermo Separation Products (Les Ulis, France). Sample (50 µl) was injected by an autosampler (Thermo Separation Products, model AS 3000) and metabolites were identified by their retention times. The coefficients of variation of the measurements were between 6% and 12% in all experiments.

Data treatment

Because of the lack of definitive identification of the metabolites and their specific fluorescence, metabolic rates were determined by considering the ratio of each metabolite peak area to the sum of the peak areas of all metabolites and unchanged drug. Total metabolic rates were determined by considering the ratio of the sum of all metabolite peak areas to the sum of the peak areas of all metabolites and unchanged drug.

These ratios were multiplied by (substrate concentration)× (incubation volume)/(incubation times)×(mg protein). The final results were expressed as picomoles per minute per milligram protein, assuming therefore identical specific fluorescence yields for all metabolites and parent drug.

Results and discussion

MMDx metabolism

When MMDx was incubated with human liver microsomes in the presence of NADPH, four to eight metabolites were formed, depending on the MMDx concentration. In this study the metabolites were named according to their chromatographic retention times: MMDx-ol or M1 (RT14), M2 (RT14.3), M3 (RT15.1), PNU156686 or M4 (RT19.8), M5 (RT21.1), M6 (RT21.9), M7 (RT22.9) and PNU159682 or M8 (RT26.3). At all concentrations of MMDx, PNU156686 and PNU159682 were minor metabolites and were not considered further in this study. At the lowest MMDx concentration (1 µ*M*), MMDx-ol and metabolite M5 were not detected in human liver microsomes (Tables 1 and 2).

As shown in Fig. 2, the total metabolic rate of MMDx rapidly reached a plateau (at 2.5 µ*M*) and decreased at the highest concentration used (25 µ*M*).

Interaction MMDx and paclitaxel

When MMDx was coincubated with paclitaxel, total MMDx metabolism was decreased in proportion to the concentration of paclitaxel (Fig. 2). This is commonly

observed when both drugs are metabolized by the same CYP450. In previous studies, it has been shown that MMDx is at least partly metabolized by CYP3A4 (Beulz-Riché, unpublished results). In addition, paclitaxel has been shown to be metabolized in human liver into two primary metabolites, 6β-hydroxypaclitaxel and 3-*p*-hydroxypaclitaxel, by CYP2C8 and CYP3A4, respectively [14, 25]. Sonnichsen et al. [28] found that 6β-hydroxypaclitaxel was the major metabolite in 75% of human liver microsomes preparations, but 3-*p*-hydroxypaclitaxel was more abundant in 25% of human microsomal preparations.

The inhibiting effect of paclitaxel on MMDx metabolism was dependent on MMDx concentration. At the highest MMDx concentration (25 μM), paclitaxel had no significant effect on MMDx metabolism. These results can be explained by the fact that MMDx may be simultaneously a substrate and an inhibitor of CYP3A4, as is commonly observed for CYP3A substrates [13].

Analysis of the MMDx metabolic profile (Fig. 2) showed that paclitaxel mainly decreased the formation of metabolites M2, M3 and M6 in dose-dependent manner. This suggests that the metabolic pathway leading to the formation of these metabolites is directly mediated by CYP3A4. These results confirm our previous findings that CYP3A4 is responsible for the formation of these three metabolites (Beulz-Riché, unpublished results).

Using the equation:

$$1/v = (1/Vm + Km/Vm + (S)) + (Km/Vm * (S) \times (I)/Ki)$$

where (S) is substrate (MMDx) and I is inhibitor (paclitaxel), Ki values were calculated and results showed that paclitaxel inhibited the formation of M2, M3 and M6 metabolites with apparent Ki values ranging from 5 to 10 μM (Fig. 3). These values are close to the concentrations found in the plasma of patients treated with this drug. According to the literature, the mean peak plasma concentration (C_{max}) of paclitaxel varies from 3.4 μmol/l to 4.3 μmol/l after administration of 175 mg/m^2 and 200 mg/m^2, respectively [23]. These results suggest that paclitaxel may potentially interact with MMDx in the clinical situation.

The effect of paclitaxel on the formation of metabolite M7 was more complex. At low MMDx concentration (1 μM), the formation of metabolite M7 decreased in the presence of paclitaxel (results not shown). At higher MMDx concentrations (5 to 25 μM), paclitaxel had rather a stimulating effect on the formation of this metabolite. Paclitaxel had no significant effect on the formation of MMDx-ol and metabolite M5 except at a high paclitaxel concentration (50 μM), with which the formation of MMDx-ol and M5 was decreased.

Effect of tamoxifen

Tamoxifen, a nonsteroidal agent often used in the treatment of metastatic breast cancer in patients with oestrogen receptor-positive tumours, is also known to be metabolized by CYP3A [8, 17, 20]. When tamoxifen was coincubated with 1 μM MMDx, total MMDx metabolism was decreased and this was dependent on tamoxifen

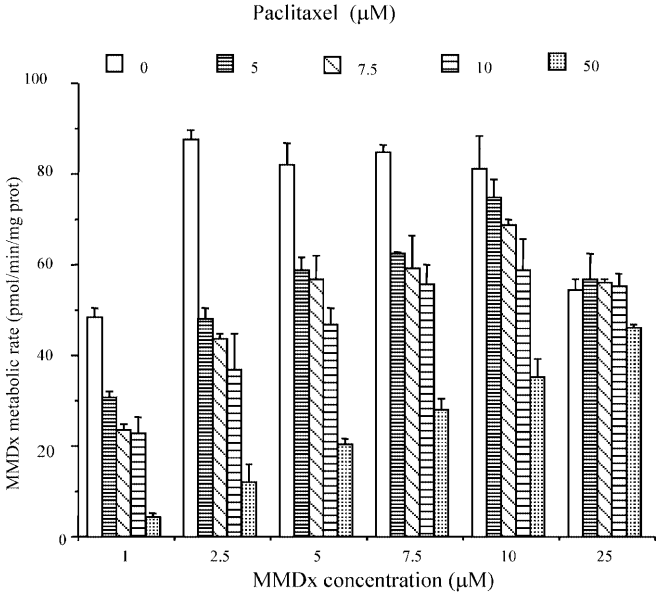

Fig. 2 Effect of paclitaxel (0 to 50 μM) on methoxymorpholino doxorubicin metabolism in human liver microsomes. MMDx was incubated with 0.5 mg protein/ml for 30 min in a final volume of 500 μl. The results are presented as the means ± SD of two experiments carried out in triplicate

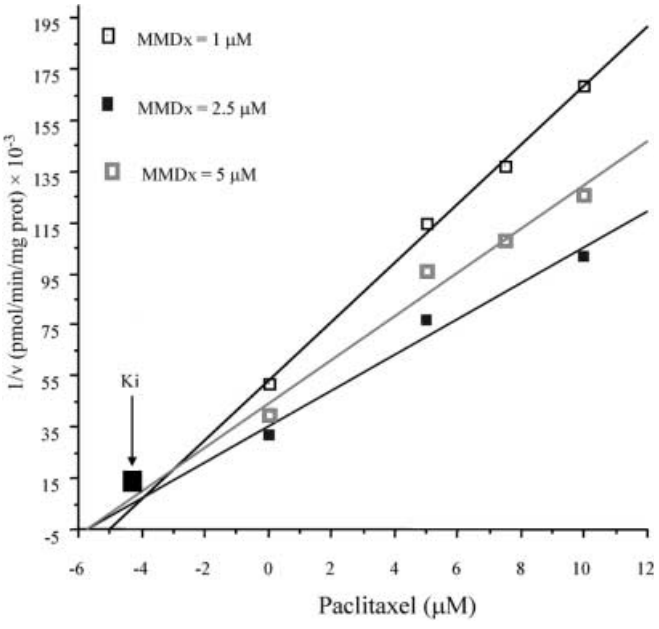

Fig. 3 Competitive inhibition of M6 metabolite formation by paclitaxel. Liver microsomes was assayed for MMDx metabolism (1, 2.5 and 5 μM respectively). The data were analysed using the Dixon plot

concentration (Fig. 4A): tamoxifen at 1 and 5 μ*M* decreased total MMDx metabolism by 53% and 91% respectively. At higher MMDx concentrations (5 and 10 μ*M*), a significant inhibition of total MMDx metabolism was observed only with tamoxifen at 5 μ*M*, which is far below the apparent Ki value of tamoxifen on CYP3A inhibition in vitro (100 μ*M*) [17]. Furthermore, at the therapeutic dose, the mean plasma concentrations of tamoxifen are about 0.1 μ*M*, ten times less than the lowest concentration used in this study. This suggests that tamoxifen may only weakly inhibit the metabolism of MMDx in the clinical situation.

As found with paclitaxel, tamoxifen particularly decreased the formation of metabolites M2, M3 and M6. The inhibition of metabolite M5 formation was only observed at the highest tamoxifen concentration (Table 1).

Effect of cyclosporine

This drug has been coadministered with anthracyclines in MDR reversal clinical trials [2] and is known to be a substrate of both CYP3A4 and P-gp [13]. At all the concentrations of MMDx used (1 to 10 μ*M*), cyclosporine inhibited MMDx metabolism by human liver microsomes. The decrease was dependent upon cyclosporine and MMDx concentration (Fig. 4B). With 1 μ*M* and 5 μ*M* MMDx, 1 μ*M* cyclosporine decreased MMDx metabolic rate in liver microsomes by 62% and 45%, respectively. This inhibitory concentration is five times the therapeutic concentration of cyclosporine which is about 0.2 μ*M*. These results also suggest that cyclosporine may potentially interact with MMDx in the clinical situation.

Analysis of the MMDx metabolic profile showed that cyclosporine particularly decreased the formation of metabolites M2, M3 and M6 (Table 2) and had no effect on MMDx-ol formation.

Effect of cyclophosphamide and ifosfamide

When MMDx was coincubated with cyclophosphamide and ifosfamide, no significant modification of total MMDx metabolism was observed (Fig. 5). Analysis of

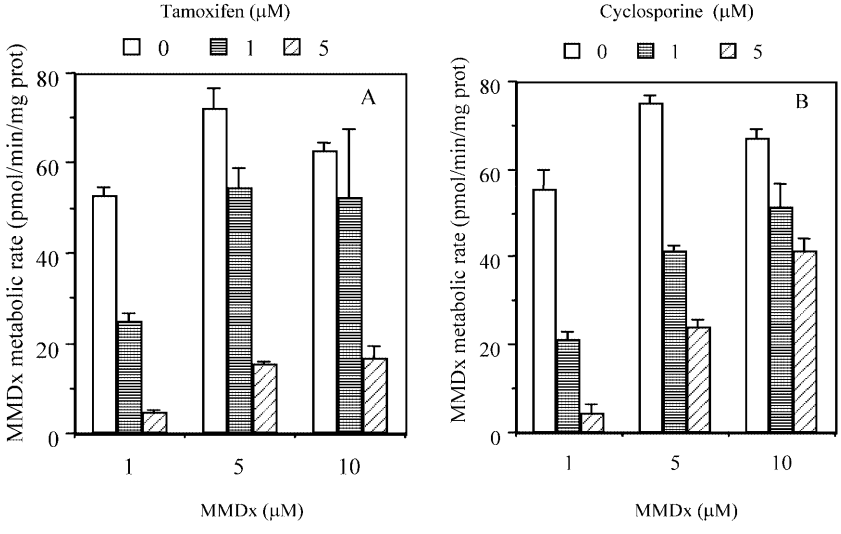

Fig. 4a, b Effect of tamoxifen (**a**) and cyclosporine (**b**) on methoxymorpholino doxorubicin metabolism in human liver microsomes. MMDx was incubated with 0.5 mg protein/ml for 30 min in a final volume of 500 μl. The results are presented as the means ± SD of two experiments carried out in duplicate

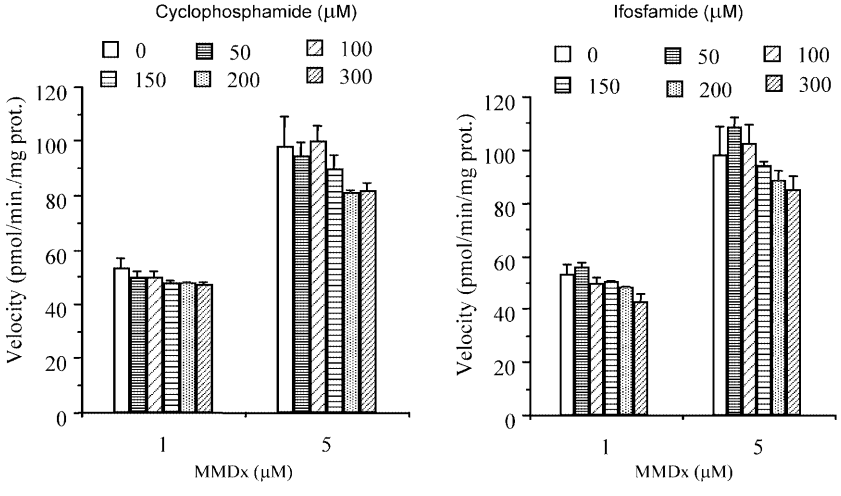

Fig. 5a, b Effect of cyclophosphamide (**a**) and ifosfamide (**b**) on methoxymorpholino doxorubicin metabolism in human liver microsomes. MMDx was incubated with 0.5 mg protein/ml for 30 min in a final volume of 500 μl. The results are presented as the means ± SD of one experiment carried out in duplicate

the MMDx metabolic profile showed that only ifosfamide at the highest concentrations used (200–300 μ*M*) decreased slightly (about 25%) but significantly the formation of M2 and M3 metabolites. These two compounds are metabolized by 4-hydroxylation, primarily catalysed by CYP2B6 in the case of cyclophosphamide and by CYP3A4 for ifosfamide [5, 27]. The same group of authors have reported that CYP3A4 catalyses 95% and 70% of liver microsomal *N*-dechloroethylation with cyclophosphamide and ifosfamide as substrates, respectively [6, 15, 26]. Furthermore, cyclophosphamide and ifosfamide have been reported to competitively inhibit CYP3A4 in human liver microsomes, but with relatively high Ki values of 510 μ*M* and 490 μ*M*, respectively [22]. The concentrations of the two agents used in this study were far below these Ki values. This may explain why there was no effect of cyclophosphamide and ifosfamide on MMDx metabolism. It appears doubtful that a significant interaction between oxazaphosphorines and MMDx metabolism occurs in the clinical setting.

In conclusion, three compounds known to undergo an important CYP3A4-mediated metabolism have a significant effect on the formation of three major metabolites of MMDx (M2, M3 and M6). These compounds are in the process of being identified using LC-MS, and their purification for NMR studies is in progress. No effect of the compounds tested was detected on the formation of MMDx-ol, which is produced by an aldo-keto reductase without any CYP intervention. Concerning metabolites M5 and M7, the effect of the drugs tested was mild and no consistent inhibition of their formation could be found. These metabolites are not likely to be produced by CYP3A4, and the identification of the pathway involved in their synthesis remains to be done.

The results of this study showed that paclitaxel, tamoxifen and cyclosporine, at concentrations close to those reached in the clinical situation, especially paclitaxel, may potentially interact with MMDx metabolism. The alteration of MMDx biotransformation was specifically related to the compounds already known to be specific substrates or inhibitors of CYP3A4. CYP3A4-metabolized drug in combination with MMDx must be used with caution. This is especially important in view of the fact that MMDx is considered as a prodrug whose activation is entirely dependent upon metabolic activation by CYP3A4. Any change in metabolic rates may considerably alter drug activity.

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