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Colchicine Biotransformation by Human Liver Microsomes

IDENTIFICATION OF CYP3A4 AS THE MAJOR ISOFORM RESPONSIBLE FOR COLCHICINE DEMETHYLATION

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ABSTRACT. Colchicine disposition involves both active biliary and renal excretion of parent drug, and at least in mammals a substantial fraction undergoes hepatic demethylation prior to excretion. We investigated the biotransformation of [^{3}H]colchicine in a panel of microsomal preparations obtained from sixteen human liver samples. The production rate of the main metabolites of colchicine's 3-demethylcolchicine (3DMC) and 2-demethylcolchicine (2DMC), was linear in relation to incubation time, cytochrome (P450) content, and substrate concentration. Following the incubation of colchicine (5 nM) with microsomes in the presence of an NADPH-generating system for 60 min, 9.8% and 5.5% of the substrate were metabolized to 3DMC and 2DMC, respectively. The formation rate of colchicine metabolites exhibited a marked variation between the different microsomal preparations. The formation rates of both colchicine metabolites were correlated significantly with nifedipine oxidase activity, a marker of CYP3A4 activity ($r = 0.96$, $P < 0.001$), but not with the metabolic markers of CYP2A6, CYP2C19, CYP2C9, CYP2D6, and CYP2E1 activities. Chemical inhibition of CYP3A4 by preincubation with gestodene (40 μM) or troleandomycin (40 μM) reduced the formation of 3DMC and 2DMC by 70 and 80%, respectively, whereas quinidine, diethyldithiocarbamate, and sulfaphenazole had no inhibitory effect. Similarly, antibodies raised against CYP3A4 almost completely abolished colchicine demethylation and nifedipine oxidase activity, but preimmune IgG had no effect. In conclusion, colchicine was metabolized to 3DMC and 2DMC by human liver microsomes. The production of colchicine metabolites was mediated by CYP3A4, and its rate varied greatly between microsomal preparations obtained from different liver samples. The coadministration of colchicine with known inhibitors or substrates of CYP3A4 may inhibit colchicine metabolism, resulting in concentration-related toxicity. *Copyright © 1996 Elsevier Science Inc.*, BIOCHEM PHARMACOL 53;1:111–116, 1997.

KEY WORDS. colchicine; CYP3A4; cytochrome P450; drug metabolism; drug interaction

Colchicine is an alkaloid that has been used to treat gout from as early as the sixth century AD [1]. More recently, its beneficial effect in the treatment and the prevention of Familial Mediterranean Fever attacks has been established [2]. Additionally, colchicine treatment has been tried with variable success in several disease states including Behçet's disease [3], cirrhosis [4, 5], progressive systemic scleroderma [6], and amyloidosis [7, 8].

Despite colchicine's prolonged therapeutic use, only limited information regarding its metabolism is currently available. Animal studies have indicated that active biliary secretion of colchicine and its demethylated metabolites is a major route of elimination, but the importance of this pathway to overall colchicine disposition varies greatly among different species [9]. Thus, the fraction of the parent drug undergoing oxidation to 2DMC and 3DMC (Fig. 1) prior to biliary excretion is species dependent and ranges from 10 to 50% [9]. Colchicine metabolites have never been identified in plasma of human subjects, but 2DMC and 3DMC accounted for 3.5 to 14.5% of the colchicine urinary concentrations (expressed as colchicine equivalents, measured by radioimmunoassay and validated by HPLC) in samples obtained from six patients who were receiving colchicine

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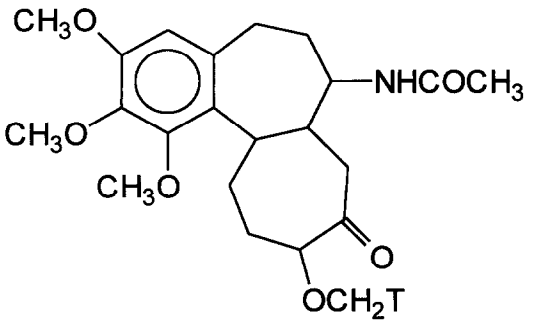
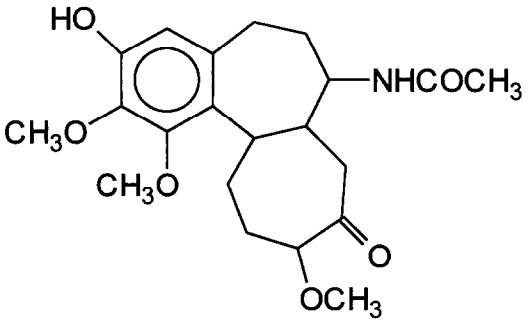
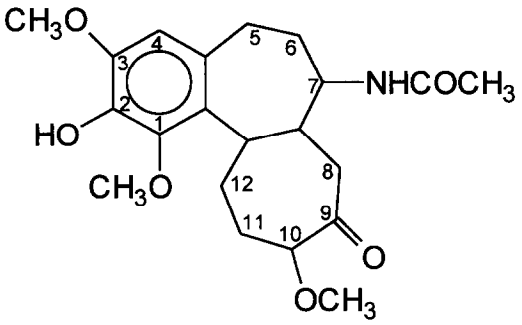
‖ *Abbreviations:* 2DMC, 2-demethylcolchicine; 3DMC, 3-demethylcolchicine; 10DMC, 10-demethylcolchicine.



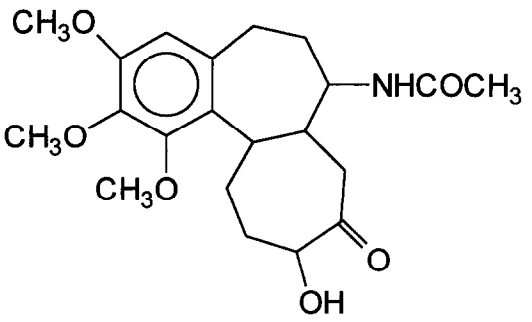
Colchicine



3-demethylcolchicine



2-demethylcolchicine



Colchiceine

FIG. 1. Chemical structure of colchicine, 2DMC, 3DMC, and 10DMC (colchiceine).

regularly [10]. The extent of colchicine oxidation in humans has not been defined, but the possibility that it may play an important role in colchicine biotransformation has been suggested by a recent report describing a near fatal colchicine intoxication in a patient who simultaneously received erythromycin [11]. The purpose of the present study was to investigate the biotransformation of colchicine in human microsomes and to identify the specific P450 responsible for the formation of its demethylated metabolites.

MATERIALS AND METHODS

Chemical

[^{3}H]Colchicine (73 Ci/mmol) was purchased from New England Nuclear, Dupont Medical Products (Wilmington, DE, U.S.A.). The radiochemical purity of [^{3}H]colchicine was 99% (evaluated by TLC). Colchicine metabolites, 2DMC, 3DMC, and 10DMC (colchiceine) were gifts from Dr. Arnold Brossi (Natural Products Section, Laboratory of Structural Biology, NIH, Bethesda, MD, U.S.A.). Gestodene was provided by Professor H. Kuhl (University of Frankfurt, Frankfurt, Germany) and sulfaphenazole was a gift from the Meiji Yakuhim Co., Ltd. (Tokyo, Japan). Colchicine, troleandomycin, and erythromycin were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals were obtained from various commercial sources and were of the highest analytical grade.

Liver Microsomes and Incubation Conditions

Liver samples were obtained from sixteen liver donors through the Tennessee Donor Service (Nashville, TN, U.S.A.). Microsomes were prepared as described elsewhere [12], and the P450 content was determined by Fe^{2+}-CO versus Fe^{2+} difference spectroscopy [13]. Incubation conditions were determined through preliminary studies that demonstrated linear formation rates of the major colchicine metabolites, 2DMC and 3DMC, at incubation durations ranging from 15 to 60 min (Fig. 2) and P450 concentrations ranging from 50 to 200 pmol per incubation vial (Fig. 3). Colchicine concentration was, unless stated otherwise, 5 nM, simulating the *in vivo* plasma concentration when given in the usual 1 mg daily dose [8, 10]. Thus, a mixture of [^{3}H]colchicine (5 nM), microsomes (200 pmol), and potassium phosphate buffer (0.1 mM, pH 7.4) was preincubated for 3 min in open glass vials immersed in a shaking bath at a constant temperature of 37°. The reaction was initiated by the addition of an NADPH-generating system consisting of 10 mM glucose-6-phosphate, 0.5 mM $NADP^+$ and 0.5 IU of glucose-6-phosphate dehydrogenase. The reaction was terminated 60 min later by the addition of 5 mL dichloromethane.

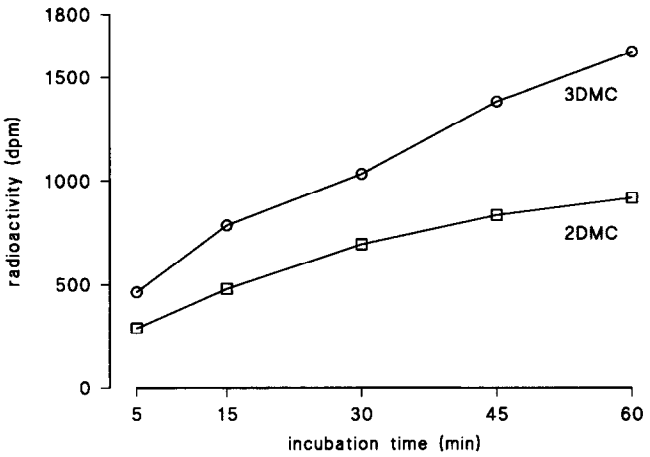


FIG. 2. Formation rate of 2DMC and 3DMC by human microsomes (200 pmol/vial) in relation to incubation time. Data are means of duplicates.

Inhibition Studies

The formation rate of demethylated colchicine metabolites by microsomal preparations from a single liver was defined under the incubation conditions described above and in the presence and absence of various concentrations of known specific P450 inhibitors such as gestodene (P4503A4), troleandomycin (P4503A4), diethyldithiocarbamate (P4502E1), sulfaphenazole (P4502C9 and 2C10), and quinidine (P4502D6). Except for quinidine, the chemical inhibition was evaluated at the following four different inhibitor concentrations: 0, 20, 40, and 100 μM. Quinidine inhibition was evaluated at 0, 1, 2, and 5 μM. The inhibition produced by gestodene, troleandomycin, and diethyldithiocarbamate was measured by preincubating these inhibitors with microsomes and NADPH-generating system at 37° for 20 min before adding colchicine [14]. Immunoinhibition was examined by preincubating microsomes (50 pmol P450/incubation vial) with preimmune globulin or specific antibodies raised to P4503A4 (10 mg/nmol P450)

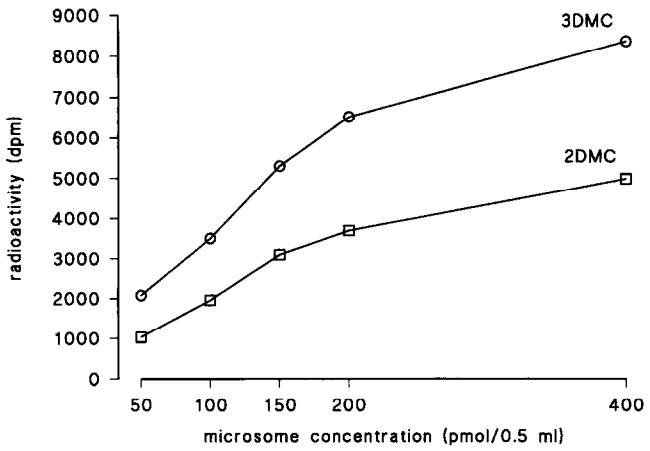


FIG. 3. Formation rate of 2DMC and 3DMC by human microsomes related to P450 concentration. Data are means of duplicates. Incubation time was 60 min.

in 0.1 mM potassium phosphate buffer (pH 7.4) for 20 min at 23°. Colchicine (5 nM) and the components of the NADPH-generating system were then added, and the reaction was allowed to continue for an additional 60 min as described above.

Assay and Kinetic Analysis

One milliliter of 1 M bicarbonate buffer (pH 10) and 8 mL of dichloromethane containing unlabeled colchicine metabolites (2DMC and 3DMC, 2 μg each) were added to each incubation vial. The mixture was shaken for 10 min, and then the organic phase was separated from proteins and other debris by centrifugation at 1000 *g* for 5 min and concentrated to dryness under a gentle stream of N_2 at 25°. Each sample was reconstituted in the mobile phase prior to injection onto the HPLC system. Colchicine and its demethylated metabolites were analyzed by a previously published HPLC method with minor modifications [15]. The HPLC system consisted of a pump (Waters, Marlborough, MA, U.S.A.), an AS-100 automatic injector (Bio–Rad, Richmond, CA, U.S.A.), a 3.9 × 300 mm μBondapak ODS column (Waters), an L-4000 UV detector (Hitachi, Tokyo, Japan), a 746 recorder (Waters), and a 680 controller (Waters). The mobile phase consisted of 10% acetonitrile in 10 mM sodium phosphate (pH 6.0), and the HPLC eluate was monitored at 340 nm. Under these chromatographic conditions, the retention times of 3DMC, 2DMC, 10DMC, and colchicine were 9.8, 11.6, 18.1, and 26.1 min, respectively. The effluent fraction coinciding with colchicine metabolites was collected and counted by liquid scintillation (Ecolume scintillation liquid, ICN Pharmaceuticals, Costa Mesa, CA, U.S.A.; Racbeta 1219 scintillation counter, Wallac Oy, Turku, Finland). Estimates of K_m and V_{max} Michaelis–Menten parameters for 2-demethylation and 3-demethylation of colchicine were calculated from a transformation of product formation rates at selected substrate concentrations (from 5 μM to 5 mM) using a nonlinear regression program ("k · cat," Bio-Metallics, Princeton, NJ, U.S.A.).

Statistical Analysis

The association between the formation rate of colchicine metabolites and the activity of specific P450s was examined by linear regression analysis. A *P* value of less than 0.05 was considered to be statistically significant.

RESULTS

Following the incubation of 5 nM [^{3}H]colchicine with 200 pmol of P450 for 60 min, two peaks in addition to colchicine were noted. The retention times of these peaks corresponded to 3DMC and 2DMC and accounted for 9.8 and 5.5% of the substrate, respectively. The apparent K_m values for 3DMC and 2DMC were 5.6 ± 0.8 and 8.1 ± 1.1 mM, respectively, and the apparent V_{max} values were 1.4 ± 0.1

and 4.5 ± 0.4 nmol metabolite formed/min/nmol P450, respectively.

The formation rate of 3DMC by microsomal preparations obtained from sixteen liver samples exhibited a 7-fold variation (at the substrate concentration used) ranging from 0.64 to 4.65 [pmol 3DMC formed (nmol P450)$^{-1}$ · min^{-1} [mean ± SD, 1.46 ± 1.23]. Similarly, a 10-fold variation was noted in the production rate of 2DMC, which ranged from 0.26 to 2.61 [pmol 2DMC formed (nmol P450)$^{-1}$ · min^{-1}] [mean ± SD, 0.76 ± 0.71]. A significant correlation was noted between the formation rate of 3DMC and 2DMC ($r = 0.99$, $P < 0.001$), and both were correlated significantly with nifedipine oxidase activity ($r = 0.96$, $P < 0.001$), a marker of P4503A4 activity (Fig. 4). No significant correlations were noted between the formation rate of 3DMC or 2DMC and coumarin hydroxylation, ethoxyresorufin O-deethylation, (S)-mephenytoin 4′-hydroxylation, bufuralol 1-hydroxylation, and chlorzoxazone 6-hydroxylation (Table 1).

Preincubation of 40 μM gestodene or 40 μM troleandomycin in the presence of an NADPH-generating system inhibited the production of 3DMC and 2DMC by approximately 70 and 80%, respectively (Fig. 5). In contrast, the presence of sulfaphenazole, diethyldithiocarbamate, or quinidine in the incubation medium had only a negligible effect on the formation rate of 3DMC and 2DMC (Fig. 5). Preincubation with antibodies raised against P4503A4 almost completely abolished the formation of both 2DMC and 3DMC (Fig. 6). A similar effect was observed on nifedipine oxidase activity, whereas preincubation with preimmune IgG had no significant effect (Fig. 6).

TABLE 1. Coefficient of variation and statistical significance for the correlations between the production rate of 3 DMC and 2DMC and the microsomal activity of different CYP isoforms

Substrate	P450 isoform	3DMC production		2DMC production	
		r	*P*	*r*	*P*
Ethoxyresorufin	P4501A2	−0.10	0.71	−0.10	0.70
Coumarin	P4502A6	−0.23	0.39	−0.23	0.40
Nifedipine	P4503A4	0.96	< 0.001	0.96	< 0.001
Mephenytoin	P4502C19	0.16	0.55	0.15	0.58
Bufuralol	P4502D6	−0.28	0.28	−0.28	0.30
Chlorzoxazone	P4502E1	−0.41	0.11	−0.44	0.09

DISCUSSION

Active biliary excretion of colchicine and, to a lesser extent, its urinary excretion are considered the main elimination pathways of colchicine [16]. In rats, the disposition of the parent compound through the biliary system and the kidney has been estimated recently to account for approximately 30 and 16%, respectively, of total colchicine clearance [17, 18]. Data regarding human subjects are sparse, and extrapolation from animal studies may be misleading since colchicine elimination through different routes has been shown to be species specific. Recently, Rudi *et al.* have shown that in two patients suffering from extrahepatic biliary obstruction, colchicine biliary clearance is approximately 20% of total body clearance. Furthermore, although *in vivo* and *in vitro* studies have indicated that in mammals a substantial fraction of colchicine is metabolized to 3DMC and 2DMC, hepatic demethylation of colchicine in humans prior to renal or biliary elimination has not been demonstrated previously [9, 20]. We have shown in the present study that colchicine is metabolized by human microsomal preparations through demethylation to yield 2DMC and 3DMC. The possible production of colchicine (10DMC) could not be shown in the present study due to the removal of the tritium from colchicine in the course of colchicine demethylation. However, following the incubation with microsomes, mass balance seems to be maintained so that the combined radioactivity of colchicine, 3DMC, and 2DMC did not differ significantly from the total preincubation radioactivity of the substrate, colchicine, implying that under these conditions the production of 10DMC

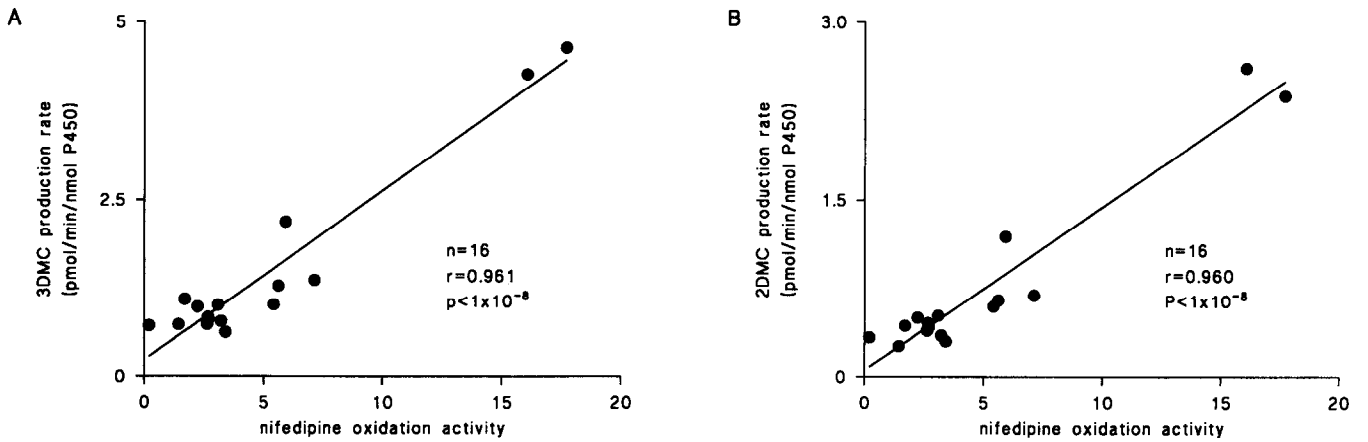


FIG. 4. Relationship between 3DMC (A) and 2DMC (B) production rate and nifedipine oxidase activity (a marker of P4503A4 activity) in sixteen microsomal preparations. Data are means of duplicates.

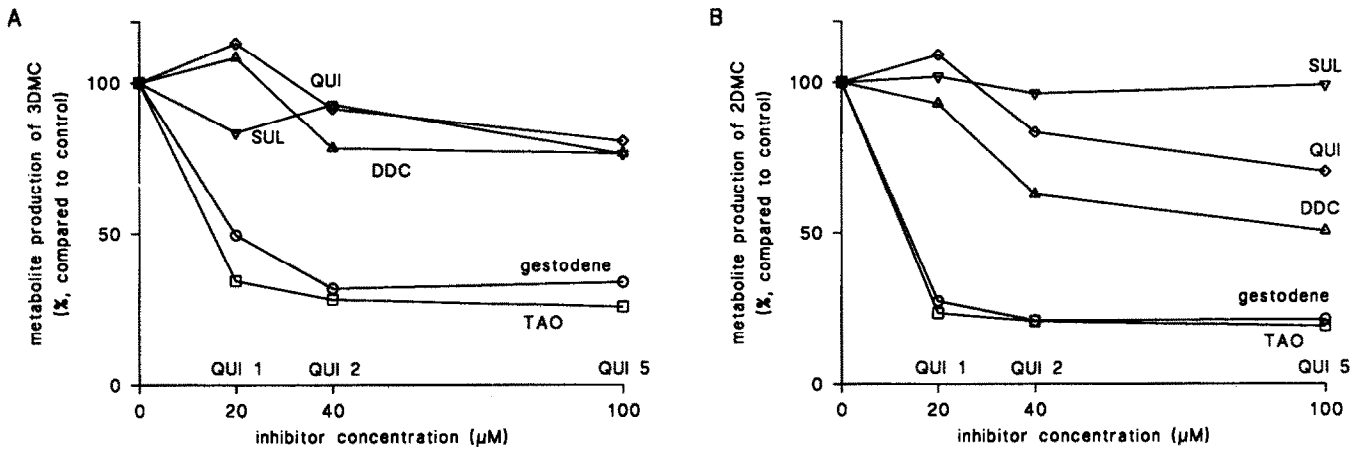


FIG. 5. Production rate of 3DMC (A) and 2DMC (B) relative to control level (3.54 and 2.30 pmol formed/nmol P450/min) with increasing concentrations of gestodene (open circles), troleandomycin (TAO, open squares), diethyldithiocarbamate (DDC, open triangles), quinidine (QUI, open diamonds) and sulfaphenazole (SUL, open inverted triangles). Data are means of duplicates.

or any other unidentified metabolites is probably negligible. Our findings support a recent report in which 2DMC and 3DMC were identified by specific HPLC assay in urine obtained from six patients treated with colchicine [10].

The significant correlation noted in the present study between the formation rate of 3DMC and 2DMC and nifedipine oxidase activity, but not with any of the other specific metabolic markers, implies that the demethylation of colchicine in positions 3 and 2 is mediated mainly through CYP3A4. This conclusion is further supported by the significant inhibition exerted by specific inhibitors of CYP3A4 such as gestodene, troleandomycin, and anti-CYP3A4 on the formation of colchicine-demethylated metabolites.

The identification of a specific P450 as the major enzyme responsible for drug biotransformation is clinically important, especially for drugs with a narrow therapeutic window. The coadministration of a known inhibitor of this specific enzyme is likely to cause toxicity and may necessitate dose reduction and careful monitoring. Furthermore, the combined administration of two substrates of the same enzyme may produce competitive inhibition and excessive plasma concentrations. Caraco *et al.* [11] have described recently a patient with Familial Mediterranean Fever who developed near fatal colchicine intoxication while being treated concurrently with erythromycin for suspected pneumonia. Hence, colchicine toxicity, which may be fatal, may occur when inhibitors or other substrates of CYP3A4 are given concomitantly with colchicine [21]. The use of such drugs in combination with colchicine should be avoided, but in case their administration is unavoidable, extra care should be exercised and the patients should be followed closely for the development of excessive colchicine effects.

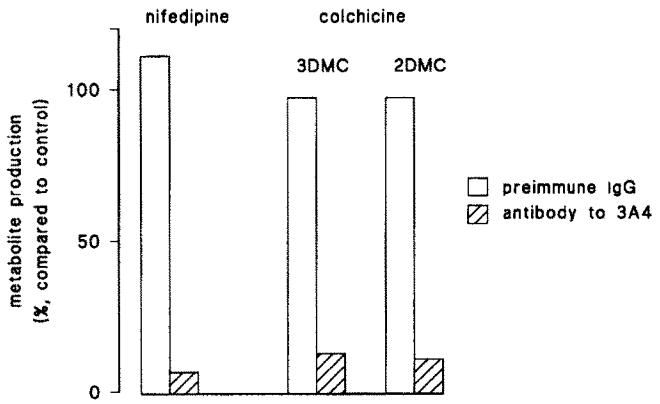


FIG. 6. Effect of anti-P4503A4 and preimmune IgG on the production rate of 3DMC, 2DMC, and nifedipine oxidase activity relative to control values (4.01 and 2.01 pmol formed/nmol P450/min and 16 nmol formed/nmol P450/min, respectively). Data are means of duplicates.

P4503A4 activity is characterized by significant interindividual variability. In the present study, the formation rate of 2DMC and 3DMC varied 10- and 7-fold, respectively. The biological *in vivo* activity of these metabolites is not clear, but although 2DMC and, to a lesser extent, 3DMC have been shown to bind to tubulin, they do so with an affinity lower than the parent compound, colchicine [22, 23]. Thus, if this reduced *in vitro* activity is associated with a lower anti-mitotic effect of colchicine metabolites, subjects with accelerated demethylation will exhibit lower plasma colchicine concentrations and a reduced pharmacodynamic effect. Interestingly, about 10% of Familial Mediterranean Fever patients do not respond to usual doses of colchicine [24, 25], raising the possibility that enhanced P4503A4 activity in these patients results in lower colchicine concentrations and a reduced therapeutic effect.

In summary, we have shown that P4503A4 is the major enzyme responsible for colchicine demethylation. Clinically important drug interactions resulting in life-threatening colchicine toxicity may be expected when potent inhibitors or other substrates of P4503A4 are coadministered with colchicine. The marked interindividual

difference in P4503A4 activity, as demonstrated in the current study for the formation of colchicine metabolites, may account for the known variation in its therapeutic effect.

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