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CHARACTERIZATION OF DEXTROMETHORPHAN N-DEMETHYLATION BY HUMAN LIVER MICROSOMES

CONTRIBUTION OF THE CYTOCHROME P450 3A (CYP3A) SUBFAMILY

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Abstract—In an effort to identify the human cytochromes P450 involved in the N-demethylation of dextromethorphan, the kinetics of 3-methoxymorphinan formation were studied in microsomal enzyme systems. Under initial rate conditions, 3-methoxymorphinan formation demonstrated single enzyme Michaelis–Menten kinetics using microsomes obtained from three human livers (K_m: 0.52–0.71 mM; V_{max}: 375–812 pmol/mg protein/min). B-lymphoblastoid cells expressing CYP3A4 incubated with 0.4 mM dextromethorphan catalyzed the formation of 3-methoxymorphinan at a rate of 22 pmol product/mg protein/min. Midazolam, a prototypic substrate for CYP3A4 and CYP3A5, competitively inhibited dextromethorphan N-demethylation by two human liver microsomal samples with K_i values of 46 ± 10 and 63 ± 8 μM. At a dextromethorphan concentration of 0.4 mM, gestodene (100 μM) inhibited 3-methoxymorphinan formation by approximately 50%. Immunoinhibition of dextromethorphan N-demethylation using rabbit anti-CYP3A4 antibodies resulted in a 60% decrease in 3-methoxymorphinan formation at a dextromethorphan concentration of 0.4 mM. Additional inhibition studies using furafylline, coumarin, sulfaphenazole, mephenytoin, quinidine, and diethyldithiocarbamic acid, which are selective inhibitors of CYP1A2, CYP2A6, CYP2C8/9, CYP2Cmp, CYP2D6, and CYP2E1, respectively, demonstrated no substantial inhibition of dextromethorphan N-demethylation. Correlation analysis was performed using the rate of 3-methoxymorphinan formation at a concentration of 1 mM dextromethorphan and immunoquantified levels of CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP3A5 and their associated characteristic catalytic activities. A significant correlation was observed between dextromethorphan N-demethylase activity and midazolam 1′- and 4-hydroxylase activity ($r^2 = 0.77$ and 0.69 respectively, N = 19, $P < 0.01$); the exclusion of those samples containing both CYP3A4 and CYP3A5 increased the correlation significantly ($r^2 = 0.87$ and 0.91 respectively, N = 12, $P < 0.01$). In the absence of CYP3A5, a significant correlation was observed between 3-methoxymorphinan formation and the sample's erythromycin *N*-demethylase activity ($r^2 = 0.94$, N = 12, $P < 0.01$), testosterone 6 β-hydroxylase activity ($r^2 = 0.96$, N = 7, $P < 0.01$) and relative immunoquantified levels of CYP3A4 ($r^2 = 0.96$, N = 12, $P < 0.01$). Inclusion of those samples expressing CYP3A5 in addition to CYP3A4 reduced the magnitude of the observed correlation. No significant correlation between 3-methoxymorphinan formation and the sample's relative immunoquantified levels of or form-selective activity associated with CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2C19 (or CYP2Cmp), CYP2D6, and CYP2E1 was observed. In conclusion, dextromethorphan N-demethylation appears to be catalyzed primarily by CYP3A4 and to a lesser extent by CYP3A5 *in vitro* in humans. Thus, the administration of dextromethorphan to human volunteers may provide a means of simultaneously phenotyping the *in vivo* activity of CYP2D6 and CYP3A.

Key words: dextromethorphan; human liver microsomes; cytochrome P450; CYP3A; phenotyping

DTM§ is a synthetic analogue of codeine, which is used widely as an over-the-counter antitussive drug [1, 2]. The cytochrome P450-mediated metabolism of DTM results in the formation of DT, via O-demethylation, and 3-MM, via N-demethylation (Fig. 1) [3]. The urinary excretion of DT and 3-MM (conjugated and unconjugated) constitutes 4–60 and 1–8% of a therapeutic dose of DTM, respectively, in humans [4]. At therapeutic doses, the O-demethylation of DTM is mediated by CYP2D6, which is responsible for the metabolism of many other drugs including debrisoquine, bufuralol, metoprolol, sparteine, encainide, propafenone, amitriptyline, and desipramine [3, 5–7]. The ratio of DTM to DT (metabolic ratio) excreted in the urine over 8–24 hr has been used as a phenotyping trait to indicate the presence or absence of CYP2D6 [8–11]

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§ Abbreviations: DTM, dextromethorphan; 3-MM, 3-methoxymorphinan; DT, dextrorphan; 3-OHM, 3-hydroxymorphinan; CYP, cytochrome P450; and V_{max}, maximum rate of metabolism.

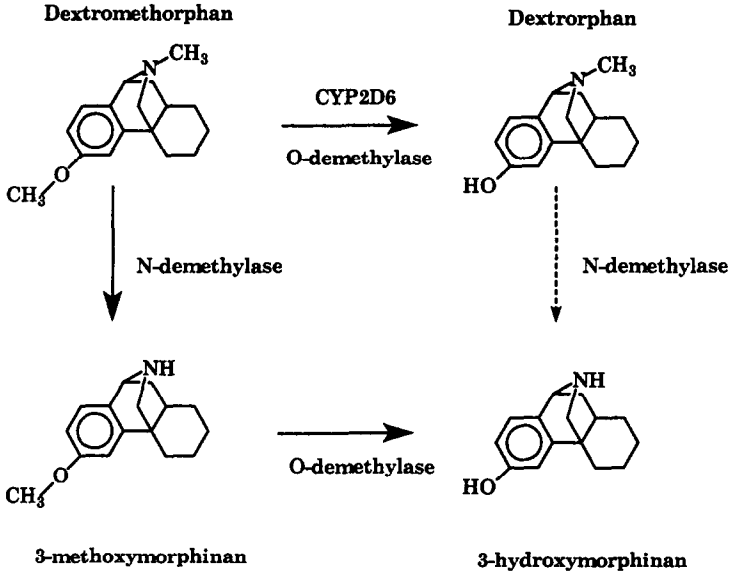

Fig. 1. Biotransformation of dextromethorphan.

and thus define poor and extensive metabolizer phenotypes.

In contrast to the O-demethylation pathway, the CYPs involved in the formation of 3-MM have not been characterized completely. Initial studies by Ladona and co-workers [12] indicate that fetal liver microsomes are capable of N-demethylating DTM and that the ability of the sample to form 3-MM correlated with immunoquantified levels of CYP3A7. However, CYP3A7 is found primarily in human fetal tissue, and therefore the role in adults of the CYP3A subfamily in this biotransformation is unclear. In a preliminary study, Jacqz–Aigrain and colleagues [13] indicated that antibodies raised against CYP3A4 apparently reduce 3-MM formation by 50–60 and 20–40% in adult and fetal liver samples, respectively. In view of the incomplete characterization of the CYP involved in DTM N-demethylation, the contribution that human CYP3A and other CYP make to this biotransformation remains to be determined completely.

The CYP3A subfamily is a major contributor to the human metabolism of a wide variety of compounds including calcium channel antagonists, immunosuppressives, vinca alkaloids, macrolide antibiotics, benzodiazepines, and antihistamines. In adult humans, CYP3A4* is universally expressed in hepatic tissue, but a closely related enzyme, CYP3A5 (84% similarity in amino acid sequence with CYP3A4), is expressed in only 20–30% of tissue samples [14, 15]. Substantial inter-individual variability in the relative levels of CYP3A enzymes exists [16, 17], which is thought to result in a corresponding inter-individual variability in the pharmacokinetics of the substrates for these enzymes. The development of a method to predict this variability in CYP3A activity is of considerable interest due to the widespread use and limited therapeutic safety of some substrates such as cyclosporine A and terfenadine [18, 19]. *In vivo* methods that have been discussed in this context include 6β-hydroxycortisol excretion and the radiolabeled erythromycin breath test [20–22]. However, the former test lacks predictive capability, and the latter test is not suited to widespread use. DTM is already used widely to phenotype subjects for CYP2D6 activity, and therefore the possibility that an index of CYP3A activity could be obtained simultaneously by phenotyping via 3-MM formation is very attractive. With this in mind, DTM N-demethylase activity was evaluated in a group of human liver microsomes as an initial step in assessing the potential of DTM as an *in vivo* probe for CYP3A activity.

* The independent confirmation of the expression of CYP3A3 has not occurred; in fact, CYP3A3 was not detected in 10 liver samples using the polymerase chain reaction with primers specific for CYP3A3. Watkins PB, University of Michigan, personal communication (8/4/93); cited with permission.

MATERIALS AND METHODS

Materials. Midazolam, 1′-hydroxymidazolam, 4-hydroxymidazolam, DT, 3-MM, and 3-OHM were gifts of Hoffmann-La Roche (Nutley, NJ, and Basel, Switzerland). Furafylline was a gift from Dr. K. Kunz at the University of Washington. Gestodene was a gift of Dr. F. P. Guengerich of Vanderbilt University. Sulfaphenazole and (±)-mephenytoin were gifts from Ciba-Geigy (Summit, NJ) and Sandoz (East Hanover, NJ), respectively. All other supplies were of the highest grades available from standard commercial sources.

Specimens. Human adult liver specimens were obtained at surgery in accordance with protocols

approved by the appropriate Committee for the Conduct of Human Research (The Medical College of Virginia, Richmond, VA; The Medical College of Wisconsin, Milwaukee, WI; The University of Michigan, Ann Arbor, MI; and Indiana University, Indianapolis, IN). All specimens received from the Medical Colleges of Wisconsin and Virginia and the University of Michigan were from patients who had normal bilirubin and transaminase levels. Specimens from the Medical College of Virginia are identified with patient code numbers of HL-30, HL-34, HL-35 and HL-O. Individual liver specimens received from the Medical College of Wisconsin are coded with letters A through N (e.g. HL-A). One specimen was obtained from The University of Michigan and is coded as UM-11. The ages, genders, smoking habits, drug histories, relative immunoquantified CYP contents, and form-selective catalytic activities for samples received from the Medical Colleges of Wisconsin and Virginia and the University of Michigan have been reported previously [23, 24]. A specimen from a 36-year-old male with a history of alcoholic cirrhosis and hepatitis was obtained at Indiana University Medical Center and is coded IUL-1. This patient received no drugs known to induce CYPs. Microsomes from a B-lymphoblastoid cell line expressing CYP3A4 (CYP3A5 not available) and control microsomes were obtained from Gentest (Woburn, MA).

Microsomes were prepared by differential centrifugation and stored at −70° in a 100 mM potassium phosphate buffer (pH 7.25) containing 1 mM EDTA, 20% glycerol, 20 μM butylated hydroxytoluene, and 100 μM phenylmethylsulfonyl fluoride until used [25]. Protein concentrations were determined colorimetrically by the method of Lowry *et al.* [26]. The method of Omuro and Sato [27] was used to determine the total CYP content, using an extinction coefficient of 91 mM cm^{-1}. Rabbit anti-CYP3A4 antibodies were prepared as described elsewhere [15].

The relative levels of CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A, and CYP3A5 in the hepatic samples were determined using immunoblot analysis as previously described [23, 28, 29]. The catalytic activities of CYP1A2, CYP2A6, CYP2Cmp,* CYP2D6, CYP2E1, and CYP3A were determined by monitoring ethoxyresorufin *O*-deethylase, coumarin 7-hydroxylase, *S*-mephenytoin 4′-hydroxylase, bufuralol 1′-hydroxylase, *N*-nitroso dimethylamine *N*-demethylase, and erythromycin *N*-demethylase activity, respectively, as described elsewhere [23].

Dextromethorphan N-demethylase and other assays. All incubations consisted of the following except where noted: 50–100 μg microsomal protein, 100 mM sodium phosphate buffer (pH 7.4) containing 5 mM magnesium chloride, 5 mM isocitrate, 1 U isocitrate dehydrogenase, 1 mM β-NADP, and up to 2 mM DTM in a final volume of 200 μL. The mixture was incubated at 37° for 30 min except when time was varied, the reaction was terminated by adding 200 μL of acetonitrile containing internal standard (codeine), and the samples were stored at −70° until analysis. In the case of midazolam hydroxylation, microsomes were incubated under initial rate conditions [30] at 37° for 10 min with a substrate concentration of 25 μM and terminated with 200 μL acetonitrile containing internal standard (flunitrazepam). The effects of putative inhibitors on 3-MM formation were determined at a DTM concentration of 0.4 mM. Each inhibitor was investigated using at least four concentrations. The inhibitors studied and the range of concentrations used were: furafylline (1–100 μM) [31], coumarin (1–1000 μM) [32], sulfaphenazole (1–50 μM) [33], mephenytoin (1–50 μM) [34], quinidine (0.1–5.0 μM) [35], diethyldithiocarbamic acid (1–1000 μM) [36], gestodene (12.5–100 μM) [37], and midazolam (100–800 μM) [30, 38]. In the case of furafylline and gestodene, the inhibitors were incubated with microsomal protein and an NADPH-regenerating system in a small volume (100 μL) for 10 and 30 min, respectively, prior to the addition of 0.4 mM DTM as previously described [31, 37]. Immunoinhibition studies were conducted by incubating various amounts of sera or control pre-immune sera with 50 μg microsomal protein for 30 min at room temperature prior to the addition of NADPH and 0.4 mM DTM.

For correlation analyses, 19 human liver microsomes were incubated with 1 mM DTM for 30 min to assess the N- and O-demethylating activity or 25 μM midazolam for 10 min to assess midazolam 1′- and 4-hydroxylase activity. For comparative purposes, the ability of gestodene to inhibit the 1′-hydroxylation of midazolam was investigated in the bank of 19 adult liver microsomal samples. Microsomal protein was incubated with gestodene and NADPH (*supra vida*) for 30 min at room temperature. The reaction was initiated by the addition of midazolam (final concentration 25 μM). The incubation proceeded for 10 min and then was terminated by the addition of 200 μL acetonitrile containing flunitrazepam as the internal standard. In all cases, the total organic solvent concentration in the incubation was less than or equal to 2% of the total incubation volume.

HPLC determination of dextromethorphan metabolites. DTM, 3-MM, 3-OHM, DT and codeine (internal standard) were separated using an HPLC equipped with a 4-μm cyano radial compression column (8 mm × 100 mm; Novapak®, Waters Associates, Milford, MA) as described previously [11]. The mobile phase consisted of acetonitrile: 100 mM sodium acetate (15:85) containing 0.6 mL triethylamine per liter, which was adjusted to pH 3 with phosphoric acid. Quantification was achieved from peak height ratios using a fluorescent detector (No. 890, Applied Biosystems Inc., Foster City, CA) with excitation at 195 nm and no emission filter.

HPLC determination of midazolam metabolites. Midazolam, 1′-hydroxymidazolam, 4-hydroxymidazolam and flunitrazepam (internal standard) were separated using a 5-μm octadecylsilane column (4.6 mm i.d. ×250 mm; Ultrasphere, Beckman, San

* CYP2Cmp is used to designate the cytochrome P450 responsible for *S*-mephenytoin 4′-hydroxylation. The high correlation between CYP2C19 levels and *S*-mephenytoin 4′-hydroxylase activity suggests that CYP2C19 and CYP2Cmp are the same enzyme [29].

Table 1. Estimated Michaelis–Menten parameters for the formation of 3-methoxymorphinan by human liver samples

	V_{max}* (pmol/mg protein/min)	K_m* (mM)	CL_{int} (μL/min)
HL-I	812 ± 111	0.5 ± 0.2	1.6
HL-J	658 ± 39	0.7 ± 0.1	0.9
IUL-1	375 ± 16	0.7 ± 0.1	0.5

* Values represent estimate ± SE. Standard error represents the asymptotic standard error of the parameter estimated by nonlinear regression.

Ramon, CA) using a previously published method [30, 39]. A mobile phase of methanol:potassium phosphate (dibasic), pH 7.4:tetrahydrofuran (52:46:2) was delivered at a flow of 1 mL/min, and the eluate was monitored using ultraviolet absorbance at 230 nm. Quantification was achieved using peak height ratios.

Analysis of kinetic data and statistics. The data represent the mean of duplicate assays for every experiment. Untransformed kinetic data were analyzed by weighted nonlinear regression (PCNONLIN v.4.2, SCI Software, Lexington, KY) assuming single enzyme Michaelis–Menten kinetics and various models of inhibition when applicable. The appropriateness of the fit was determined by visual inspection of residual patterns, residual sums of squares, and precision of the parameter estimates. A weighting factor equal to the reciprocal of the observed data was found to be most appropriate. The coefficient of determination and its corresponding statistical significance were determined by conventional methods [40].

RESULTS

The formation of 3-MM at 0.4 mM DTM was linear for 60 min in the presence of 100 μg of microsomal protein (HL-I). Therefore, an incubation time of 30 min was employed routinely to ensure initial rate conditions for the formation of 3-MM. The rate of DT formation was also linear for 30 min in the same system. The formation of 3-MM exhibited simple single enzyme Michaelis–Menten kinetics resulting in the parameter estimates presented in Table 1, using three human liver samples (HL-I, HL-J, and IUL-1). Eadie–Hofstee plots indicated that a single enzyme or a group of enzymes with similar substrate affinities were responsible for the biotransformation of DTM to 3-MM (not shown).

To identify the enzyme responsible for the N-demethylation of DTM, selective inhibitors of specific CYPs were used. Inhibition studies using a range of concentrations of midazolam, a well characterized CYP3A substrate [30, 38], at various DTM concentrations indicated that the N-demethylation of DTM was inhibited competitively by midazolam (Fig. 2). Based upon estimates of V_{max} and K_m (data not shown) for midazolam in these microsomal preparations, depletion of inhibitor during these incubations was less than 10% under the conditions studied, resulting in minor deviations from initial rate conditions. Consistent with a previous report [41], K_i values of 63 ± 8 and 46 ± 10 μM for midazolam were estimated using nonlinear kinetics with two liver samples, HL-J and HL-I, respectively. Other models of inhibition, such as uncompetitive, noncompetitive, and two enzyme sites with one site inhibited did not fit the data as well as simple competitive inhibition. This finding suggests that CYP3A may be the enzyme catalyzing the N-demethylation of DTM.

One method of investigating the capability of a CYP to perform a particular biotransformation is to use purified or isolated protein. Thus, microsomes from B-lymphoblastoid cells expressing CYP3A4 and control cells were incubated with 0.4 mM DTM. In support of the above observation, B-lymphoblastoid cells expressing CYP3A4 catalyzed the formation of 3-MM at a rate of 22 pmol/mg protein/min, with the control microsomes catalyzing the same reaction at a rate of less than 1 pmol/mg protein/min. In addition, DT formation was not observed in these same samples.

To confirm the role of CYP3A in the metabolism of DTM, the effect of gestodene, a mechanistic inhibitor of the CYP3A subfamily [37] and rabbit anti-human CYP3A antibodies [15], on the N-demethylation of DTM was investigated using HL-I and HL-J, respectively. The anti-CYP3A antibodies maximally inhibited 3-MM formation by 60% (Fig. 3) and have been shown previously to inhibit midazolam 1′-hydroxylation by approximately 85%*. The formation of 3-MM was also inhibited by gestodene by approximately 50% in HL-I (Fig. 3), with a maximal effect at 50–100 μM. However, in an analogous experiment using midazolam (25 μM) as the substrate, gestodene (100 μM) showed a widely variable effect on the 1′-hydroxylation of midazolam in a panel of 19 adult livers with the average inhibition being 63 ± 14% (mean ± SD) with a range of 48 to 89% (HL-I; 80% inhibition). Antibodies and gestodene (<15% inhibition) had little effect on DT formation by HL-J and HL-I.

The incomplete inhibition of DTM N-demethylation by polyclonal antibodies and gestodene suggests that other forms of CYP may be involved. This possibility was addressed by inhibition studies using relatively selective inhibitors of other well characterized CYPs. Little inhibition (<10%) of DTM N-demethylation by relatively selective inhibitors of CYP1A2 (furafylline) [31], CYP2A6 (coumarin) [32], CYP2C8 and CYP2C9 (sulfaphenazole) [33], CYP2D6 (quinidine) [35], and CYP2E1 (diethyldithiocarbamic acid) [36] was observed (Fig. 3). In the case of mephenytoin (CYP2Cmp) [34], a 10–15% inhibition of DTM *N*-demethylase activity was seen.

To understand further the possible contribution of human CYPs to the N-demethylation of DTM, correlation analyses were performed using the formation of 3-MM and DT at a DTM concentration of 1 mM in nineteen adult liver samples (Table 2). These human liver samples were characterized previously for relative amounts of CYP1A2,

* Wrighton SA, unpublished observation.

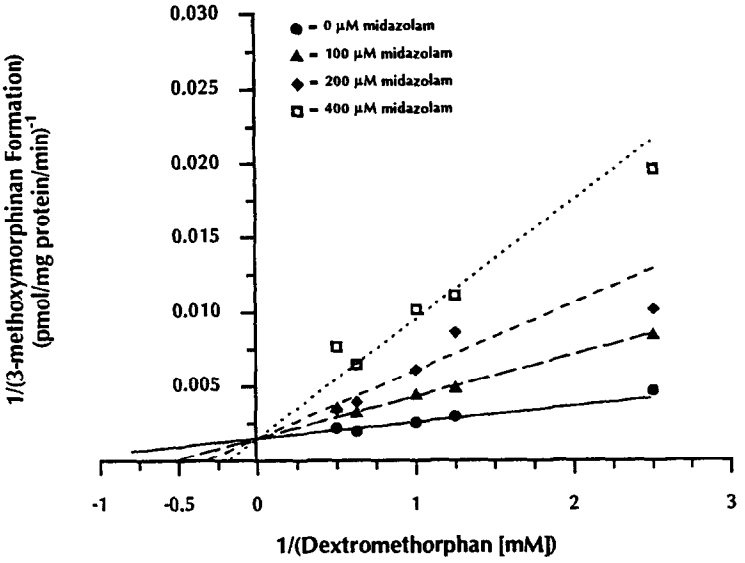

Fig. 2. Double-reciprocal plot of the inhibition of dextromethorphan N-demethylation by midazolam in human liver microsomes (HL-J). Lines represent best fit of the data to the Michaelis–Menten equation with competitive inhibition and were determined by nonlinear regression of untransformed data.

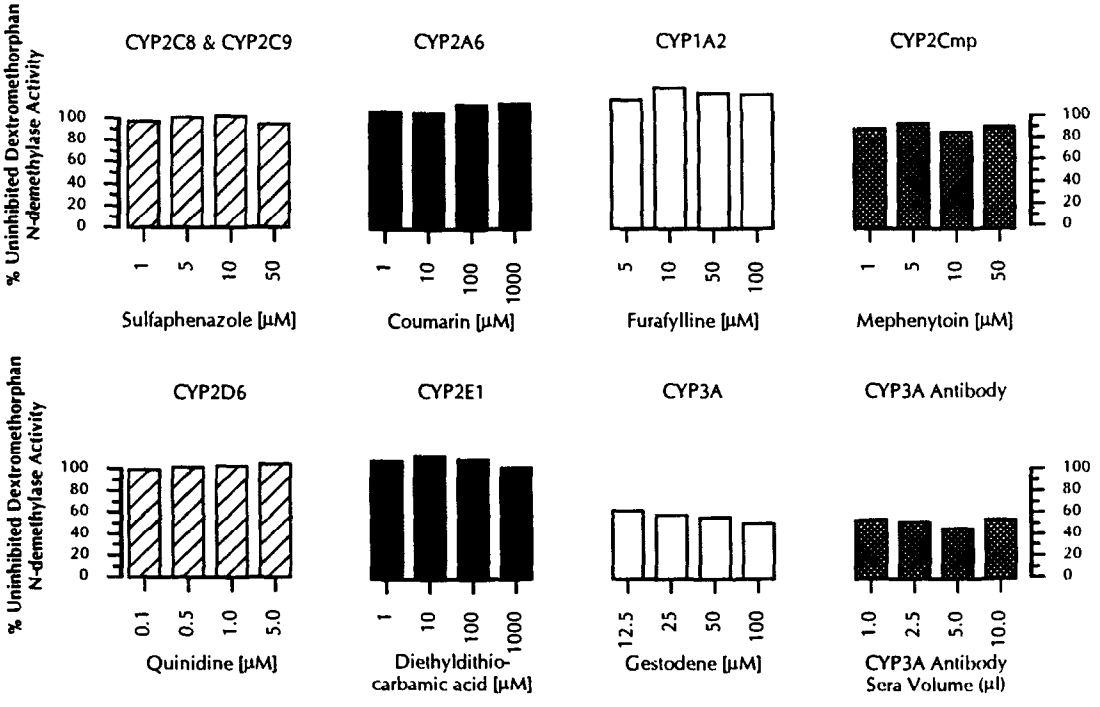

Fig. 3. Inhibitory effects of various compounds and rabbit anti-CYP3A antibodies on dextromethorphan *N*-demethylase activity at a substrate concentration of 0.4 mM. Final inhibitor concentrations and volume of sera used are indicated. Sulfaphenazole, coumarin, furafylline, mephenytoin, quinidine, diethyldithiocarbamic acid, and gestodene were used as selective inhibitors of CYP2C8 and CYP2C9, CYP2A6, CYP1A2, CYP2Cmp, CYP2D6, CYP2E1, and CYP3A, respectively. Each bar represents the mean of duplicate measurements. All experiments were conducted in microsomes from HL-J except for gestodene inhibition, which was conducted in microsomes from HL-I.

Table 2. Rates of erythromycin N-demethylation, bufuralol 1′-hydroxylation, dextromethorphan O- and N-demethylation, midazolam 1′- and 4-hydroxylation, and the relative levels of three cytochromes P450 in a bank of microsomes from 20 human livers

Human liver sample	Total CYP3A*	% Total CYP3A5	Total CYP2D6*	Erythromycin *N*-demethylase activity†	Bufuralol 1′-hydroxylase activity†	Dextromethorphan		Midazolam	
						O-Demethylase activity†	*N*-Demethylase activity†	4-Hydroxylase activity†	1′-Hydroxylase activity†
A	100		100	193	29	143	398	122	351
B	110		371	168	42	208	366	75	229
C	118		338	106	35	144	234	87	257
D	73		431	63	75	386	338	32	114
E‡§	262	26	724	520	88	396	2577	455	2221
F	192	28	264	268	37	170	853	238	1020
G	142	32	361	156	56	317	432	119	541
H	73		184	149	32	155	278	89	305
I‡	315		302	960	42	185	1816	451	1453
J	121		291	218	47	242	585	200	667
K	92		0	205	19	58	331	140	484
L	66		73	119	20	65	179	49	185
M	86		325	112	57	314	311	62	250
N	98		0	243	22	90	511	151	502
O‡	364		ND‖	1390	ND	473	1946	802	2899
30	212	15	ND	ND	ND	628	380	282	901
34	291	24	ND	ND	ND	544	346	166	805
35	204	18	ND	ND	ND	544	277	273	1029
UM-11	129	21	ND	ND	ND	387	60	124	417
IUL-1¶	ND	ND	ND	ND	ND	986	102	ND	ND

* Immunoquantification of total CYP3A-related protein and CYP2D6 protein in the microsomal sample. The densitometric value of HL-A was arbitrarily set at 100% and was used to determine the relative CYP3A or CYP2D6 content in subsequent samples. The amount of CYP3A5 is given as a percentage of the total CYP3A content.

† Activity is expressed in pmol product/mg protein/min.

‡ Indicates patients whose drug histories indicated exposure to barbiturates.

§ Indicates patient whose drug history indicated exposure to phenytoin. Additional patient information can be found in Refs. 18, 21, and 27.

‖ Not determined.

¶ Indicates patient who had liver cirrhosis and hepatitis secondary to alcoholism.

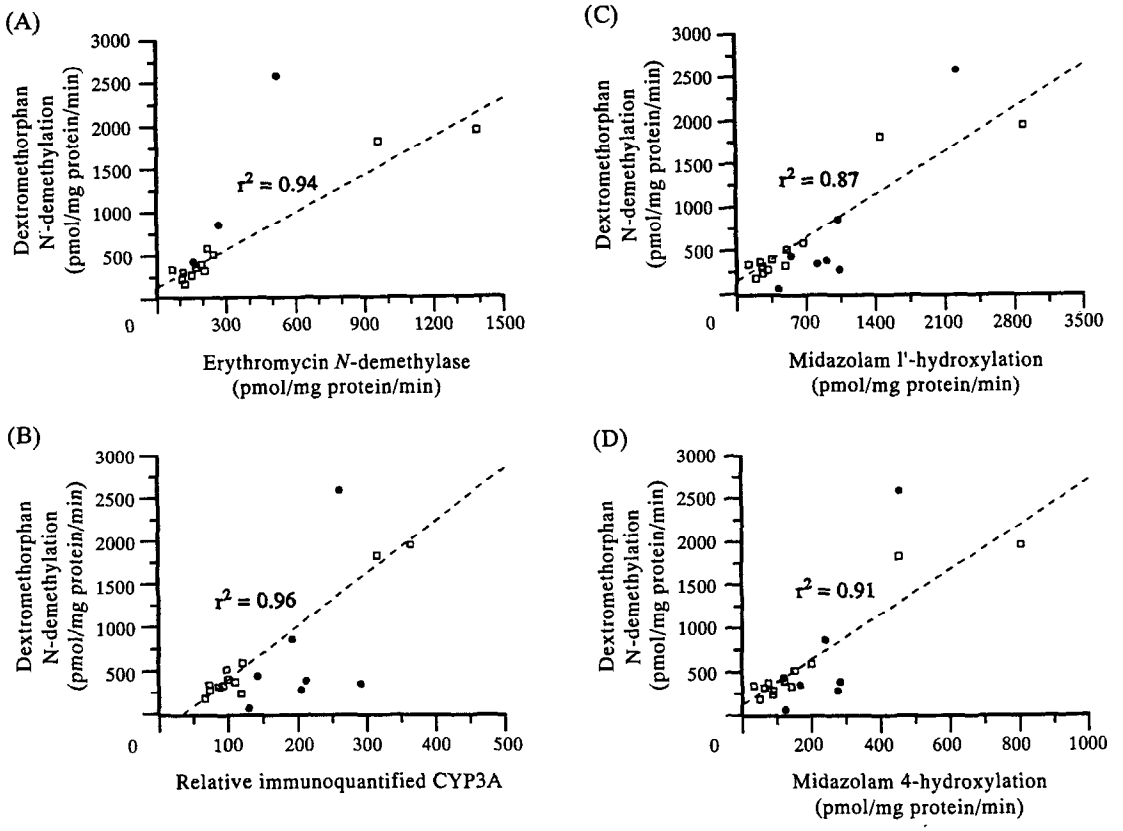

Fig. 4. Correlation between 3-methoxymorphinan formation and erythromycin *N*-demethylase activity (N = 12; A), immunoquantified CYP3A (N = 12; B), midazolam 1′-hydroxylation activity (N = 12; C), and midazolam 4-hydroxylation activity (N = 12; D) in human liver microsomal samples containing only CYP3A4 (□) and samples co-expressing CYP3A4 and CYP3A5 (●). Lines of best fit and coefficients of determination (r^2) are given for samples containing only CYP3A4 by a dashed line.

CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP3A5 and their form-specific activities [15, 23, 24, 29]. The formation of 3-MM was correlated significantly with the erythromycin *N*-demethylase activity ($r^2 = 0.62$, N = 15, $P < 0.01$) and immunoquantified CYP3A levels ($r^2 = 0.48$, N = 19, $P < 0.01$) of the samples. These correlations increased significantly ($r^2 = 0.94$, N = 12, $P < 0.01$; and $r^2 = 0.96$ N = 12, $P < 0.01$, respectively; Fig. 4A and 4B) when samples co-expressing CYP3A5 and CYP3A4 were excluded from the analysis. The DTM *N*-demethylase activity of the samples correlated well with the rate of midazolam (25 μM) 1′-hydroxylation and 4-hydroxylation ($r^2 = 0.77$, N = 19, $P < 0.01$ and $r^2 = 0.69$, N = 19, $P < 0.01$, respectively). These correlations increased slightly to $r^2 = 0.87$, (N = 12, $P < 0.01$; Fig. 4C) and $r^2 = 0.91$ (N = 12, $P < 0.01$; Fig. 4D) with the removal of those samples containing CYP3A4 and CYP3A5 from the analysis. Ten of the samples (HL-A through HL-J) have been characterized previously for their ability to 6β-hydroxylate testosterone [24], a characteristic biotransformation of CYP3A [42, 43]. In these samples, a significant correlation was observed between the rate of 3-MM formation and the rate of testosterone 6β-hydroxylation ($r^2 = 0.77$, N = 10, $P < 0.01$), which improved significantly when samples containing CYP3A5 were excluded from the analysis ($r^2 = 0.96$, N = 7, $P < 0.01$).

No significant correlation ($r^2 > 0.36$, $P < 0.05$) between relative immunoquantified levels of CYPs and DTM N-demethylation was identified in 14 human liver samples (HL-A through HL-N). Furthermore, there was an absence of a significant correlation between the rate of 3-MM formation and previously reported rates of ethoxyresorufin O-deethylation, coumarin 7-hydroxylation, *S*-mephenytoin 4′-hydroxylation, bufuralol 1′-hydroxylation, and *N*-nitroso dimethylamine N-demethylation [23], which primarily reflect the activities of CYP1A2, CYP2A6, CYP2Cmp, CYP2D6, and CYP2E1, respectively. In good agreement with previous reports, the rate of O-demethylation of DTM (Table 2) in these samples was correlated significantly with the rate of bufuralol 1′-hydroxylation, a prototypic substrate for CYP2D6 ($r^2 = 0.95$, N = 14, $P < 0.01$) and immunoquantified levels of CYP2D6 ($r^2 = 0.75$, N = 14, $P < 0.01$) [3, 5].

DISCUSSION

The O-demethylation of DTM to give DT has

been widely used as a probe to phenotype humans for their ability to perform CYP2D6-mediated biotransformations. The broad acceptance of this phenotyping procedure stems from its use of a globally available non-prescription drug that can be given orally and requires only an 8- to 24-hr post-dose urine collection for the determination of phenotype [8–11]. This approach has been used not only to segregate people between extensive and poor metabolizer phenotypes but also to provide a quantitative measure of CYP2D6 catalytic activity within the extensive metabolizer group [9]. In addition to O-demethylation, DTM is also subject to N-demethylation in humans to give 3-MM. However, the CYP family (or families) responsible for this biotransformation has not been well defined. Ladona and colleagues [12] have demonstrated that a member of the CYP3A subfamily, probably CYP3A7, contributes to the N-demethylation of DTM by fetal liver microsomes. The number of CYPs present in human fetal liver has not been determined, but there are clear differences between fetal and adult liver in terms of both the enzymes expressed and their substrate selectivities. In a preliminary report, Jacqz-Aigrain and co-workers [13] have also suggested, on the basis of immunoinhibition studies, the involvement of CYP3A subfamily enzymes in the N-demethylation of DTM but did not indicate whether other CYPs may make a significant contribution to this biotransformation in adult humans. To evaluate the potential of DTM to be used *in vivo* to quantify both CYP2D6 and CYP3A subfamily activity, we examined the nature of its N-demethylation *in vitro*.

It is clear that CYP3A4 metabolizes DTM as demonstrated by the activity of the expressed enzyme system and the inhibition of microsomal formation of 3-MM formation by anti-human CYP3A antibodies. However, for DTM to be useful as an *in vivo* probe for CYP3A, this enzyme group should be the major if not the only one involved. Proving that no other enzymes catalyze this reaction is more difficult, if not impossible, given our current knowledge. However, several lines of evidence indicate that CYP3A is the major enzyme involved.

In some cases, the contribution of more than one enzyme in a biotransformation can be detected from the multi-phasic nature of product formation rate versus substrate concentration data particularly when transformed into an Eadie–Hofstee plot. This approach has been employed successfully to illustrate that omeprazole hydroxylation was catalyzed by CYP3A4 and CYP3A5 at high substrate concentrations but by *S*-mephenytoin hydroxylase at low substrate concentrations [34, 44]. Our data give no indication of multiple enzyme involvement in DTM N-demethylation given the complete description of untransformed and Eadie–Hofstee transformed product formation versus substrate concentration data by simple single-site Michaelis–Menten kinetics. However, the possibility remains that multiple enzymes with similar substrate affinities are involved in this oxidation. In contrast to the N-demethylation pathway, the O-demethylation of DTM is clearly catalyzed by multiple enzymes as reflected in biphasic Eadie–Hofstee plots (data not shown) and confirmed by the incomplete inhibition of this pathway by quinidine at high substrate concentrations [4, 35]. Further kinetic evidence suggesting that only CYP3A4 and CYP3A5 are involved in the formation of 3-MM is provided by the inhibition of this pathway by midazolam. This well characterized CYP3A substrate [30, 38] competitively inhibited DTM N-demethylation with a K_i of 46–63 μM, suggesting that only CYP3A4 and CYP3A5 catalyzed this reaction. Our estimated K_i is consistent with the value of 40 μM reported by Pichard *et al.* [41] using midazolam to competitively inhibit cyclosporin A metabolism by human liver microsomes over short incubation times. Furthermore, this estimate is similar in magnitude to the K_m values of midazolam *per se* via 1′-hydroxylation (2–5 μM) and 4-hydroxylation (65–120 μM) [30]. There is no indication that midazolam interacts with any CYP other than the CYP3A subfamily, but this possibility remains. Examples of drugs that competitively inhibit CYPs other than those that oxidize them include quinidine and fluoxetine [35, 45, 46].

To investigate the putative role of other CYPs, form-selective inhibitors were used to identify additional mediators of DTM N-demethylation. 3-MM formation was not inhibited by form-selective inhibitors of other CYPs (Fig. 3). Mephenytoin was the sole exception to this observation, with the observed inhibition being approximately 15%. Initially, this finding suggested that CYP2Cmp may play a small role in DTM N-demethylation. However, a lack of correlation between 3-MM formation and immunoquantified levels of CYP2C19 and *S*-mephenytoin 4′-hydroxylase activities argues against this conclusion. Although CYP2Cmp appears to be the predominant CYP in the catalysis of *S*-mephenytoin by 4′-hydroxylation in humans, the other pathways of mephenytoin metabolism, *R*- and *S*-mephenytoin N-demethylation and *R*-mephenytoin 4′-hydroxylation, appear to correlate with immunoquantified CYP3A [17]. Thus, the observed inhibition may reflect competition for CYP3A rather than competition for CYP2Cmp.

The formation of 3-MM correlated significantly with erythromycin *N*-demethylase, midazolam 1′- and 4-hydroxylase, and testosterone 6β-hydroxylase activities and immunoquantified CYP3A. The high correlation between these indices of CYP3A activity and DTM N-demethylation are also consistent with the hypothesis that this is the only enzyme system involved, particularly in view of the kinetic data (*vide supra*). In each case, the correlation was strengthened with the removal of samples co-expressing CYP3A4 and CYP3A5. In addition, 3-MM formation did not correlate with other form-selective activities or immunoquantified levels of CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP2E1. These findings are in agreement with previous reports using fetal samples expressing CYP3A7 [12] and adult samples [13, 47], and confirm the role of CYP3A in DTM N-demethylation.

The role of CYP3A5 in the N-demethylation of DTM is unclear, but some insight can be gained by comparing microsomal samples that contain CYP3A5

with those that do not. DTM N-demethylation was typically greater in those samples co-expressing CYP3A4 and CYP3A5 when compared with the corresponding erythromycin *N*-demethylase activity (Fig. 4A). Erythromycin is not a good substrate for CYP3A5 [15], and therefore the latter observation suggests that CYP3A5 does catalyze DTM N-demethylation. Furthermore, DTM *N*-demethylase activity was lower in samples co-expressing CYP3A4 and CYP3A5 when compared with their midazolam 1′-hydroxylase activity. In light of the ability of CYP3A5 to catalyze the formation of 1′-hydroxymidazolam with a greater V_{max} than CYP3A4 [30], the latter observation is also consistent with the formation of 3-MM by CYP3A5. Therefore, it appears that both CYP3A4 and to a lesser extent CYP3A5 contribute to the formation of 3-MM and that *in vivo* formation of this metabolite may reflect the activity of both members of the CYP3A subfamily expressed in adults. Further study with purified or expressed enzyme is necessary to definitively address this issue.

Overall, the evidence accumulated in this study indicates that CYP3A4 and CYP3A5 are the principle enzymes involved in the N-demethylation of DTM. The capability of polyclonal rabbit anti-human CYP3A antibodies to inhibit this reaction by 60% is also consistent with this conclusion. However, under comparable conditions midazolam 1′-hydroxylase was inhibited by 85%, suggesting that other enzymes may also make a relatively small contribution to the N-demethylation of DTM in human liver. In an attempt to further address this issue, gestodene was examined as a form-specific inhibitor of the CYP3A subfamily but resulted in slightly less inhibition than noted with the antibody, despite 80% inhibition of midazolam 1′-hydroxylation and previous reports of complete inhibition of nifedipine oxidase [37]. However, the latter observations do not necessarily implicate non-CYP3A subfamily enzymes in DTM N-demethylation. Yun and associates [48] have reported previously the incomplete inhibition of terfenadine oxidation by gestodene but complete inhibition by polyclonal antibodies. Furthermore, the inhibitory effect of gestodene is often difficult to interpret. For example, Kerlan *et al.* [47] noted that the 2- and 4-hydroxylation of estradiol were both CYP3A mediated but were differentially inhibited by gestodene.

In conclusion, it is apparent that CYP3A4 and potentially to a lesser extent CYP3A5 are primarily responsible for the N-demethylation of DTM by adult human liver. This raises the possibility that DTM, a drug already in widespread use as a phenotypic probe for CYP2D6, may find an additional role in the quantification of CYP3A subfamily activity *in vivo*. The prospective evaluation of this possibility in controlled human studies using selective inhibition and induction of CYP3A subfamily is clearly indicated.

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