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Identification of CYP3A4 as the Principal Enzyme Catalyzing Mifepristone (RU 486) Oxidation in Human Liver Microsomes

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ABSTRACT. Various complementary approaches were used to elucidate the major cytochrome P450 (CYP) enzyme responsible for mifepristone (RU 486) demethylation and hydroxylation in human liver microsomes: chemical and immunoinhibition of specific CYPs; correlation analyses between initial rates of mifepristone metabolism and relative immunodetectable CYP levels and rates of CYP marker substrate metabolism; and evaluation of metabolism by cDNA-expressed CYP3A4. Human liver microsomes catalyzed the demethylation of mifepristone with mean (± SD) apparent K_m and V_{max} values of 10.6 ± 3.8 μM and 4920 ± 1340 pmol/min/mg protein, respectively; the corresponding values for hydroxylation of the compound were 9.9 ± 3.5 μM and 610 ± 260 pmol/min/mg protein. Progesterone and midazolam (CYP3A4 substrates) inhibited metabolite formation by up to 77%. The CYP3A inhibitors gestodene, triacetyloleandomycin, and 17α-ethynylestradiol inhibited mifepristone demethylation and hydroxylation by 70–80%; antibodies to CYP3A4 inhibited these reactions by approximately 82 and 65%, respectively. In a bank of human liver microsomes from 14 donors, rates of mifepristone metabolism correlated significantly with relative immunodetectable CYP3A levels, rates of midazolam 1′- and 4-hydroxylation and rates of erythromycin N-demethylation, marker CYP3A catalytic activities (all $r^2 \geq 0.85$ and $P < 0.001$). No significant correlations were observed for analyses with relative immunoreactive levels or marker catalytic activities of CYP1A2, CYP2C9, CYP2C19, CYP2D6, or CYP2E1. Recombinant CYP3A4 catalyzed mifepristone demethylation and hydroxylation with apparent K_m values 7.4 and 4.1 μM, respectively. Collectively, these data clearly support CYP3A4 as the enzyme primarily responsible for mifepristone demethylation and hydroxylation in human liver microsomes. BIOCHEM PHARMACOL 52;5:753–761, 1996.

KEY WORDS. mifepristone; cytochrome P450; CYP3A4; demethylation; hydroxylation; human liver microsomes

The antiprogestational properties of mifepristone [17β-hydroxy-11β-(4-dimethylaminophenyl)-17α-(1-propynyl)-estra-4,9-dien-3-one, Fig. 1], the first antiprogestin used clinically, were discovered somewhat serendipitously in 1980 by scientists at Roussel Uclaf characterizing a series of antiglucocorticoids [1]. Accordingly, the molecule antagonizes progesterone with more than 2-fold greater binding affinity to the human endometrial progesterone receptor and cortisol with over 10-fold higher affinity to the human placental glucocorticoid receptor [2]. Because of the recognized promise of an antiprogestin in the areas of pregnancy termination and fertility control, the use of mifepristone and newer antiprogestins for these indications has been studied most thoroughly. Currently, mifepristone, in combination with a synthetic prostaglandin analog, is safely and effectively used as an abortifacient in France, Great Britain, Sweden, and China. Numerous other therapeutic uses for antiprogestins have been considered, as reviewed in a 1993 report from the Institute of Medicine [3]. Numerous studies suggest that antiprogestins may be effective as continually, cyclically, or post-coitally administered contraceptive agents [4–7]. Antiprogestins also lend themselves to a great number of other promising, potential uses unrelated to fertility control. Chief among these is antineoplastic agents for certain types of breast cancer [8, 9], prostate cancer [10], meningioma [11, 12], and uterine leiomyoma [13]. Importantly, mifepristone has been found recently to reverse P-glycoprotein-mediated drug resistance *in vitro* [14, 15], a characteristic that could enhance its effectiveness as an anticancer agent alone or in combination therapy. Antiprogestins may also have a role in the treatment of endometriosis [13]. Finally, because of its antiglucocorticoid activity, mifepristone has been studied as a potential treatment for Cushing's syndrome [16].

These probable indications for mifepristone would entail long-term administration of the drug; we thus thought it

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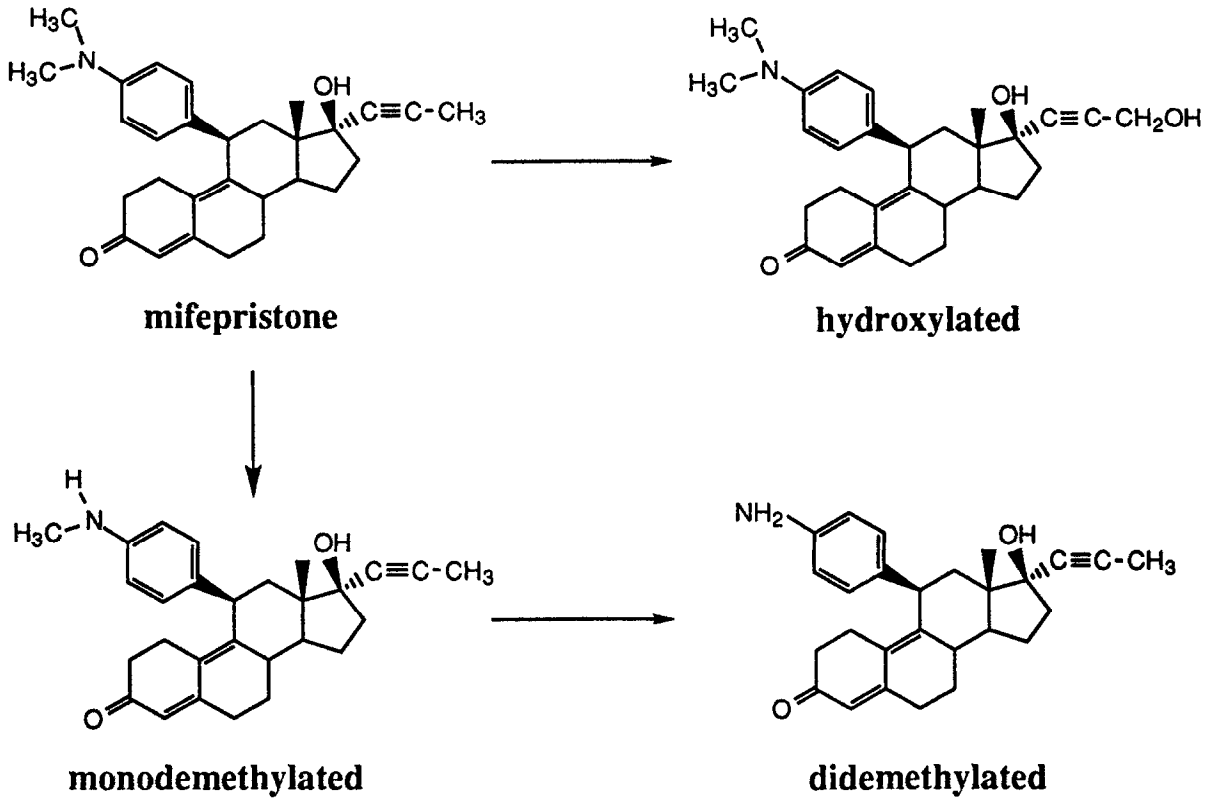

FIG. 1. Mifepristone and its three major metabolites in humans, produced via successive demethylations of the 11β-dimethylaminophenyl and hydroxylation of the 17α-propynyl groups.

important to determine which CYP§ isoform is primarily responsible for its metabolism in humans, hypothesizing that it is CYP3A4. This enzyme and others of the CYP3A subfamily are known to catalyze steroid oxidations in humans [17, 18] as well as the metabolism of a great number of structurally diverse xenobiotics including nifedipine [19], the immunosuppressants cyclosporine [20] and tacrolimus [21], midazolam and triazolam [22], the antiarrhythmic agents lidocaine [23], amiodarone [24, 25] and quinidine [26], taxol [27], etoposide [28], vinblastine and other vinca alkaloids [29, 30]. Thus, CYP3A4 involvement in the metabolism of mifepristone could have important implications for potential drug–drug interactions.

In humans, mifepristone is metabolized to three major metabolites through successive demethylations of the 11β-dimethylaminophenyl group and hydroxylation of the 17α-propynyl moiety (Fig. 1). To date, the CYPs involved in the formation of these metabolites have only been investigated in the rat. Using inducers of various CYP isoforms, the involvement of members of the 2B, 2C, and 3A subfamilies was suggested [31], while immunoinhibition experiments in a subsequent work implicated a major role for CYP2B1 [32]. The related human isoform CYP2B6 comprises only ~0.2% of expressed liver CYPs [33], and its role in xenobiotic metabolism may be very limited [34]. Further studies in rat hepatoma variants support the ability of all three implicated rat subfamilies to catalyze mifepristone oxidations [35, 36]. CYP-mediated steroid metabolism in the rat is known to involve multiple subfamilies [18], perhaps making this species an inappropriate model for the study of synthetic (or endogenous) steroid metabolism when extrapolations to humans are to be made.

For the present work, a variety of approaches were employed to determine the CYP primarily responsible for mifepristone metabolism in human liver microsomes: chemical and immunoinhibition of specific isoforms; correlation analyses between initial rates of mifepristone metabolite formation and relative immunodetectable CYP levels and rates of CYP isoform marker substrate metabolism; and evaluation of metabolism by cDNA-expressed CYP3A4.

MATERIALS AND METHODS

Chemicals and Specimens

Mifepristone and its monodemethylated and hydroxylated metabolites were gifts from Roussel Uclaf (Romainville, France). Didemethylated mifepristone and gestodene were supplied by Schering AG (Berlin, Germany). Midazolam was a gift from Hoffmann–LaRoche (Nutley, NJ, U.S.A.). 7,8-Benzoflavone, quinidine, sulfinpyrazone, 17EE, TAO, disulfiram, progesterone, deoxycorticosterone, NADPH, and sodium phosphate were purchased from the Sigma

§ *Abbreviations:* CYP, cytochrome P450; 17EE, 17α-ethynylestradiol; and TAO, triacetyloleandomycin.

Chemical Co. (St. Louis, MO, U.S.A.). Furafylline was obtained from Research Biomedicals International (Natick, MA, U.S.A.). HPLC grade methanol and acetonitrile were from Fisher Scientific (Pittsburgh, PA, U.S.A.).

Human liver specimens were obtained from organ donors, all of whom had died as a result of head trauma, under a protocol approved by the Committee on Human Research of the University of California at San Francisco. Microsomes were prepared by homogenization and differential centrifugation, following established methods [37], of non-transplantable liver from a 53-year-old male (HL-01), a 5-year-old male (HL-02) and a 36-year-old female (HL-03). The microsomes were stored until used at −80° in 10 mM Tris acetate (pH 7.4) containing 1 mM EDTA and 20% (w/v) glycerol. Protein and CYP concentrations were determined by the Pierce bicinchoninic assay (Pierce Chemical Co., Rockford, IL, U.S.A.) and Fe^{2+} vs Fe^{+2}-CO difference spectra [38], respectively.

The bank of human liver microsomes from 14 donors, used for correlation analyses (designated HL-A through N), has been previously described and characterized for relative immunoreactive CYP levels and for initial rates of CYP isoform marker substrate metabolism [39–41]. Rabbit antibodies used in immunoinhibition experiments were produced as previously described [40]. Microsomes from a human β-lymphoblastoid cell line stably transfected to coexpress CYP3A4 and NADPH-CYP reductase were obtained from the Gentest Corp. (Woburn, MA, U.S.A.).

Assay for Mifepristone and Metabolites

A published HPLC assay for the determination of mifepristone and its three major metabolites in serum [42] was modified for measuring levels in microsomal incubations. Briefly, the mobile phase was methanol:acetonitrile:water (35:30:35) at a flow rate of 1.4 mL/min through a Beckman Ultrasphere C-18 column (5 μm × 4.6 mm i.d. × 250 mm) with UV monitoring (304 nm). The autoinjector, pump, and detector were Shimadzu models SIL-9A, LC-600, and SPD-6A, respectively. A Hewlett Packard 3392A integrator was used. Quantitation was done with extinction coefficients from authentic standards.

Incubation Conditions

In general, incubations consisted of 60 μg microsomal protein (or 200 μg protein for microsomes containing cDNA-expressed CYP3A4) in 0.1 M Na_2HPO_4 buffer (pH 7.4) at 37° with substrate (mifepristone or its monodemethylated metabolite in the absence or presence of inhibitors) added in methanol (final concentration ≤2%, v/v). Reactions were initiated by adding NADPH in buffer (to 1 mM, total volume 200 μL) after a 5-min preincubation period, stopped after 2 min by adding a 2-fold volume of acetonitrile containing deoxycorticosterone as internal standard, and vortexed. Precipitated proteins were pelleted by centrifugation (5 min at 11,000 g), and 100–150 μL of the supernatant was subjected to HPLC.

For mechanism-based inhibitors, catalysis-dependent inactivation was initiated by the addition of NADPH (using HL-03 microsomes) and carried out for 30 min, followed by 10-fold dilution of the microsomes with buffer containing mifepristone and NADPH. Thereafter, reactions were stopped at 2 min and samples processed as described above. In some experiments, inhibition of the second demethylation was evaluated using the monodemethylated metabolite (synthetic standard) as substrate.

In immunoinhibition experiments, various amounts of sera from pre-immune and immunized rabbits (to CYP2C9 and CYP3A4) were incubated with HL-02 microsomes at 24° for 30 min before the addition of substrate and the assay of catalytic activity. The antisera to CYP2C9 was found to be maximally inhibitory (by approximately 75%) of tolbutamide hydroxylation at 75 μL/mg protein (data not shown).

Data Analysis

For characterization of metabolite formation, substrate concentration was varied up to 200 μM, and kinetic parameters were estimated by non-linear regression analyses (with Minim 1.8a) assuming single enzyme Michaelis–Menten kinetics with a weighting factor equal to the reciprocal of the observed initial rate. No evidence of biphasic kinetics was observed in Eadie–Hofstee plots. Correlation analyses were performed by linear regression using a commercially available statistics program (Statworks 1.2). All results are presented as the means of duplicate determinations.

RESULTS

Kinetics of Metabolite Formation

Initial incubation conditions were developed with HL-02. As expected from metabolite formation observed *in vivo*, monodemethylated metabolite formed most quickly and extensively; levels of hydroxylated and didemethylated metabolites remained lower throughout the observed incubation periods. Product formation was linear up to approximately 0.4 mg protein/mL and 3 min and was not affected by substitution of an NADPH-generating system or NADPH concentrations greater than 1 mM. Thus, a protein concentration of 0.3 mg/mL, an incubation period of 2 min, and 1 mM NADPH were used routinely for initial rate conditions.

Table 1 summarizes the Michaelis–Menten parameter estimates for mifepristone demethylation and hydroxylation in microsomes from HL-01, HL-02, HL-03, and β-lymphoblastoid cells expressing CYP3A4. For the microsomes from the three human livers, the mean (±SD) apparent K_m and V_{max} values for demethylation were 10.6 ± 3.8 μM and 4920 ± 1340 pmol/min/mg protein, respectively; the corresponding values for hydroxylation were 9.9 ± 3.5 μM and 610 ± 260 pmol/min/mg protein. The microsomes contain-

TABLE 1. Estimated Michaelis–Menten parameters for mifepristone demethylation and hydroxylation in human liver microsomes 01–03 and in microsomes containing recombinant CYP3A4 and NADPH-CYP reductase*

	Demethylation			Hydroxylation			V_{max}/K_m ratio
	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m	Demethylation/ Hydroxylation
HL-01	14.5	3370	232	13.3	310	23	10.1
HL-02	10.3	5750	558	9.9	800	81	6.9
HL-03	6.9	5640	817	6.4	720	112	7.3
CYP3A4	7.4	1140	154	4.1	110	26	5.9

* Apparent K_m, V_{max} and V_{max}/K_m values are expressed in μM, pmol/min/mg protein, and μL/min/mg protein, respectively.

ing cDNA-expressed CYP3A4 catalyzed the two oxidations with similar apparent K_m but lower V_{max} values. A comparison of the relative ratio of V_{max}/K_m for the two metabolic pathways revealed a consistent 6- to 10-fold greater rate of elimination via demethylation.

Effects of Chemical Inhibitors on Metabolite Formation

The following competitive inhibitors were tested (with their CYP isoform specificities): sulfinpyrazone (CYP2C9), quinidine (CYP2D6), progesterone and midazolam (CYP3A4/5). Sulfinpyrazone and quinidine up to concentrations of 100 μM did not inhibit mifepristone demethylation, while progesterone and midazolam over the same concentration range did so by 77 and 66%, respectively (Fig. 2). We also attempted to evaluate the effects of 7,8-benzoflavone (up to 100 μM), which resulted in concentration-dependent inhibition of metabolism (up to 78%, data not shown). This compound is less selective for CYP1A2 than furafylline, has been reported to inhibit CYP2C9 [43], and has been found to activate or inhibit some CYP3A4 reactions [43–45]. Importantly, while the flavone more selectively and potently (by ~90%) inhibits CYP1A2 at low (<10 μM) concentrations [43], little inhibition (<18%) was observed in our studies at these concentrations (data not shown). The result is therefore more consistent with inhibition of CYP3A4 than of CYP1A2. This was confirmed subsequently using furafylline (see below).

The effects of the following quasi-irreversible (TAO) or mechanism-based inhibitors were evaluated: furafylline (CYP1A2), disulfiram (CYPs 2A6, 2B6, and 2E1), gestodene (CYP3A4/5), TAO (CYP3A4/5), and 17EE (CYP3A4). The compounds specific to CYP3A enzymes markedly inhibited both demethylation and hydroxylation reactions by 70–80% (Fig. 3). Moreover, 17EE and TAO inhibited the second demethylation to the same extent ob-

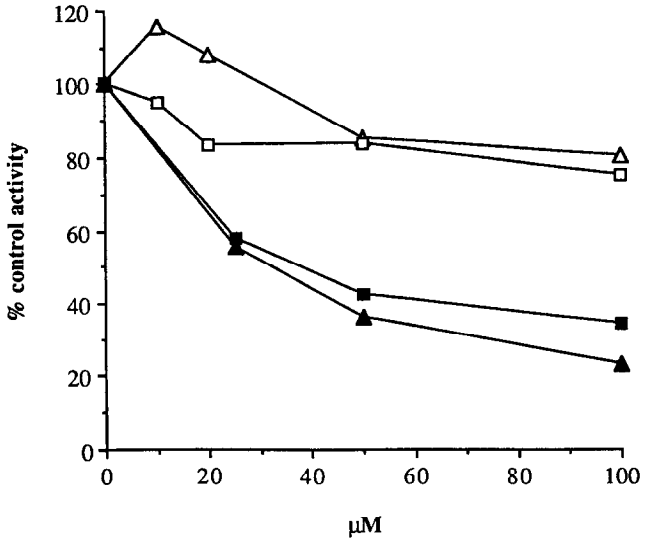

FIG. 2. Effects of midazolam (■), progesterone (▲), quinidine (□), and sulfinpyrazone (△) on mifepristone demethylation. Each data point represents the mean of duplicate determinations. Control activity was 1950 pmol/min/mg protein.

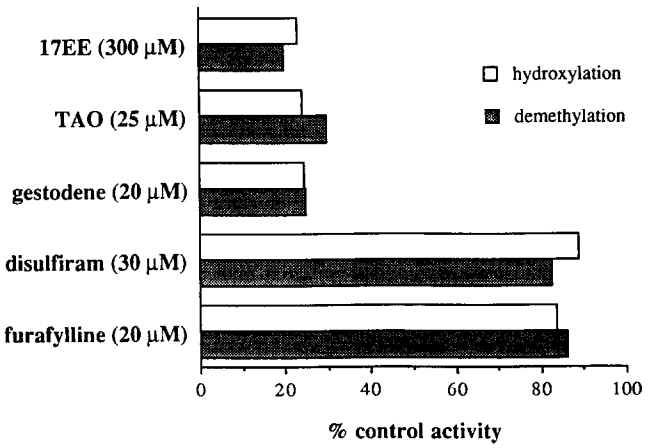

FIG. 3. Maximal concentration-dependent inhibition of mifepristone demethylation and hydroxylation by quasi-irreversible and mechanism-based chemical inhibitors of CYP3A4/5 (TAO and gestodene), CYP3A4 (17EE), CYPs 2A6, 2B6, and 2E1 (disulfiram) and CYP1A2 (furafylline). Each bar represents the mean of duplicate measurements. Control activities for demethylation and hydroxylation were 3400 and 530 pmol/min/mg protein, respectively.

served for the other two oxidations (data not shown). Disulfiram and furafylline did not inhibit demethylation or hydroxylation significantly (Fig. 3). The minor (11–17%) inhibition observed with these compounds was likely due to slight inhibition of CYP3A4 at these concentrations [43, 46].

Immunoinhibition Experiments

Antibodies to CYP3A4 strongly inhibited both mifepristone demethylation (~82%) and hydroxylation (~65%), as shown in Fig. 4A. We assessed the effects of antibodies to CYP2C9 because previous work in the rat [31, 32, 35, 36] had implicated CYP2C enzymes. These antibodies, as well as pre-immune sera, had no effect on either biotransformation (Fig. 4B).

Correlation Analyses with Relative CYP Levels and Rates of Marker Substrate Metabolism

Initial rates of mifepristone first and second demethylations and hydroxylation in human liver microsomes HL-A through N correlated very well with relative CYP3A levels (Fig. 5A), with rates of midazolam 4-hydroxylation (Fig. 5B) and 1′-hydroxylation (Fig. 5C), with rates of erythromycin N-demethylation (Fig. 5D), and with each other (Fig. 6). The correlation analyses with rates of midazolam hydroxylation depicted in panels B and C of Fig. 5 were carried out excluding the samples known to contain CYP3A5 in addition to CYP3A4 (HL-E, F, and G). CYP3A5, which is polymorphically expressed in only ~20–30% of adult human livers [47, 48], is known to have marked regioselectivity for hydroxylation of midazolam at the 1′-position relative to the 4-position [49]. For the analyses with midazolam 4-hydroxylation, inclusion of these samples only slightly lowered correlation coefficients for the first and second demethylations and hydroxylation to 0.97, 0.90, and 0.93 (all $P < 0.001$), respectively. Inclusion of the microsomal samples containing CYP3A5 in the analyses with 1′-hydroxylation lowered the respective coefficients more noticeably to 0.83, 0.77, and 0.76 (all $P < 0.001$). This reflects the regioselectivity of CYP3A5 for midazolam hydroxylations and an apparent lack of similar regioselectivity for oxidations of mifepristone.

No significant correlations were observed between metabolite formation rates and relative immunodetectable levels of CYPs 1A2, 2D6, and 2E1 (r^2 range 0.00 to 0.21, mean ± SD = 0.10 ± 0.08, all $P > 0.05$, data not shown). Additionally, no correlations were observed between initial rates of metabolite formation and rates of ethoxyresorufin O-deethylation (CYP1A2), coumarin 7-hydroxylation (CYP2A6), S-warfarin 7-hydroxylation (CYP2C9), S-mephenytoin 4′-hydroxylation (CYP2C19), bufuralol 1′-hydroxylation (CYP2D6), and N-nitrosodimethylamine N-demethylation (CYP2E1) (r^2 range 0.00 to 0.28, mean ± SD = 0.11 ± 0.09, all $P > 0.05$, data not shown).

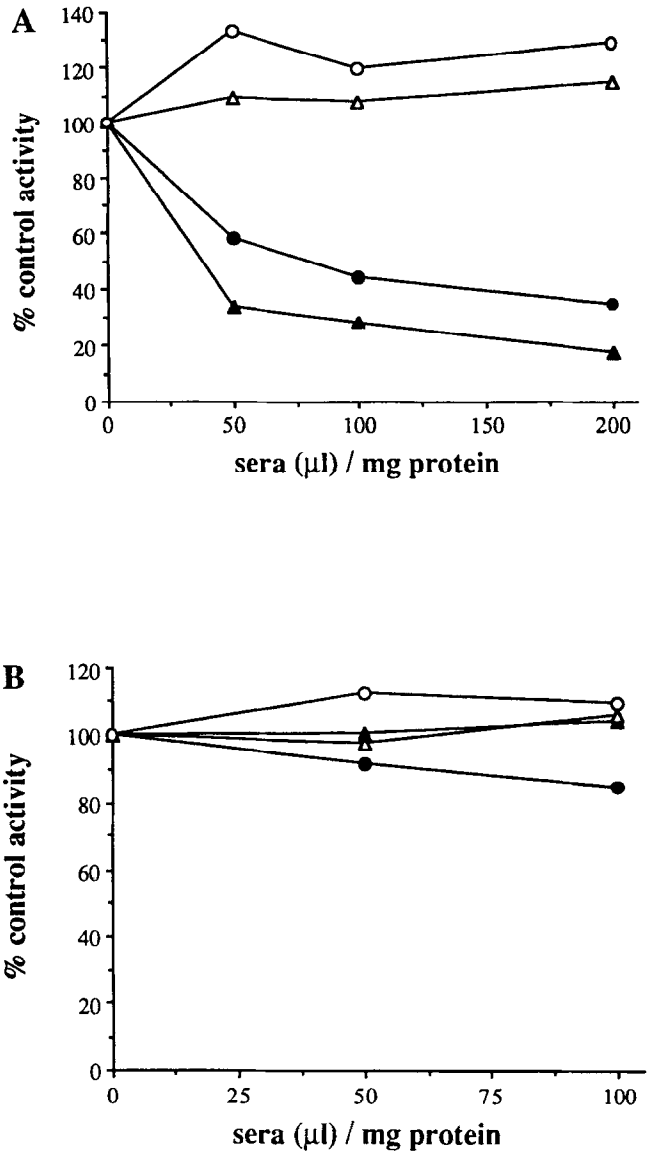

FIG. 4. (A) Inhibition of mifepristone hydroxylation (●, ○) and demethylation (▲, △) by antibodies to CYP3A4 (solid symbols) and lack of inhibition by pre-immune IgG (open symbols). (B) Lack of inhibition by antibodies to CYP2C9 (solid symbols) and lack of inhibition by pre-immune IgG (open symbols). Each data point is the mean of duplicate determinations; control activities for demethylation and hydroxylation were 2180 and 240 pmol/min/mg protein, respectively.

We should note that correlation analyses of rates of first and second demethylations and hydroxylation with relative immunoreactive CYP2A6 levels determined previously [39–41] resulted in r^2 values of 0.33, 0.45, and 0.32, respectively. The correlations for the two demethylations were significant ($P < 0.05$) but that for the hydroxylation was not ($P = 0.06$). It is likely that these correlations stem from an inherent reciprocity between relative levels of CYP3A and CYP2A6 in this bank of human liver microsomes ($r^2 = 0.41$, $P < 0.02$). With this in mind, any CYP3A4-catalyzed

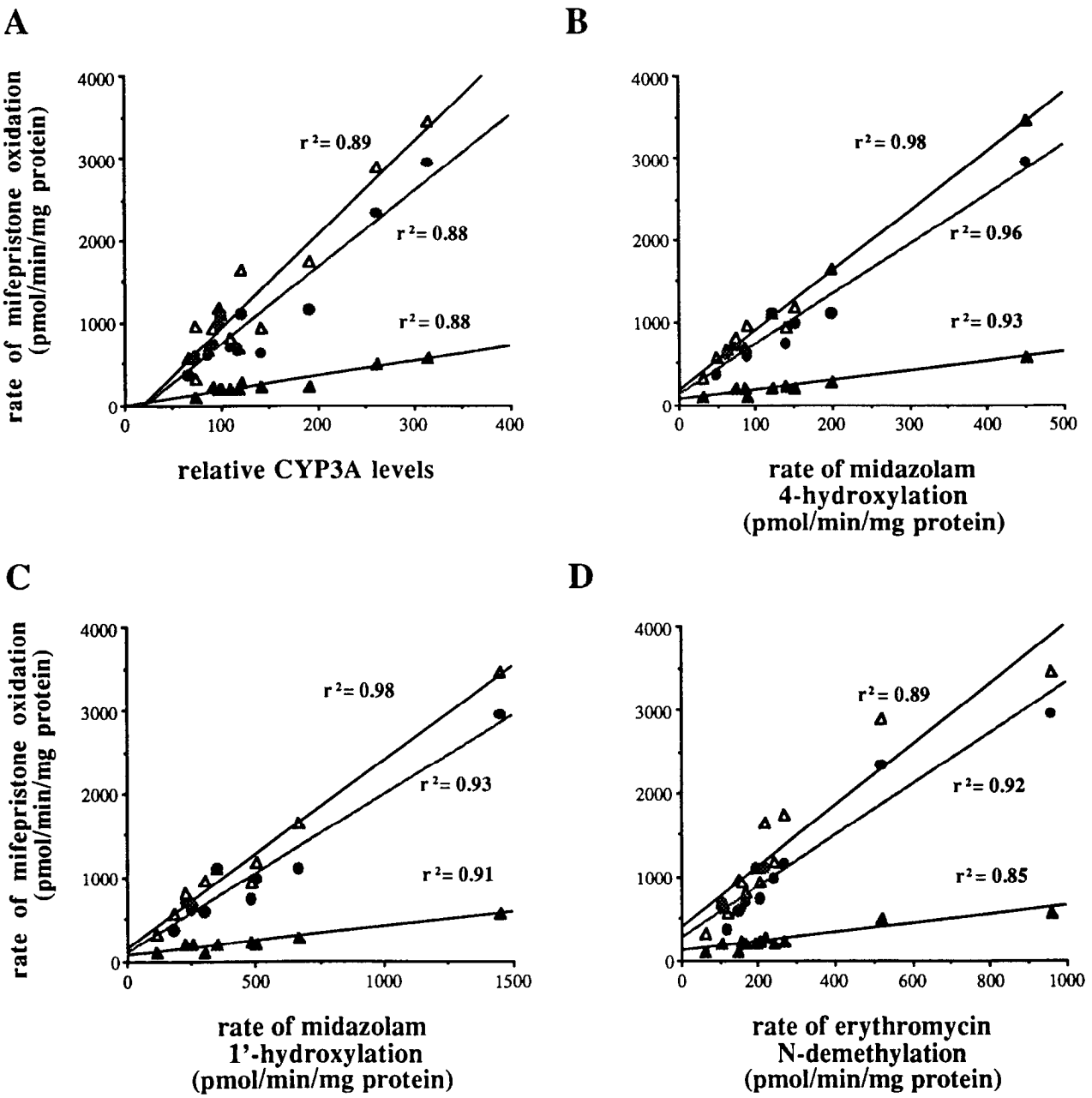

FIG. 5. Correlations between mifepristone first (△) and second (●) demethylations and hydroxylation (▲) and (A) relative immunodetectable CYP3A levels, (B and C) rates of midazolam 4- and 1'-hydroxylation, and (D) rates of erythromycin N-demethylation in human liver microsomes (all $P < 0.001$). All initial rates are the means of duplicate determinations.

reaction in these microsomes would be expected to correlate weakly with CYP2A6 levels. Indeed, similar weak but significant correlations with CYP2A6 were also observed for initial rates of erythromycin N-demethylation (r^2 = 0.46, $P < 0.01$) and midazolam 4-hydroxylation (r^2 = 0.35, $P < 0.03$), both well established marker activities of CYP3A4. Furthermore, the weak correlations for mifepristone demethylations were inconsistent with the lack of correlation observed with rates of coumarin 7-hydroxylation (all $P > 0.05$ as noted above) and the lack of inhibition by disulfiram at concentrations that have been shown to inhibit CYP2A6 by >70% [46].

DISCUSSION

In this work, complementary lines of evidence were obtained that collectively support CYP3A4 as the major CYP catalyzing mifepristone demethylations and hydroxylation in human liver microsomes. Chemical and immunoinhibition of CYP3A4 resulted in significant inhibition of mifepristone metabolism, which was further confirmed through correlation analyses. Furthermore, a recombinant form of CYP3A4, like the metabolizing microsomal enzyme, appeared to oxidize preferentially to the demethylated derivative (as evidenced by a higher V_{max}/K_m relative to

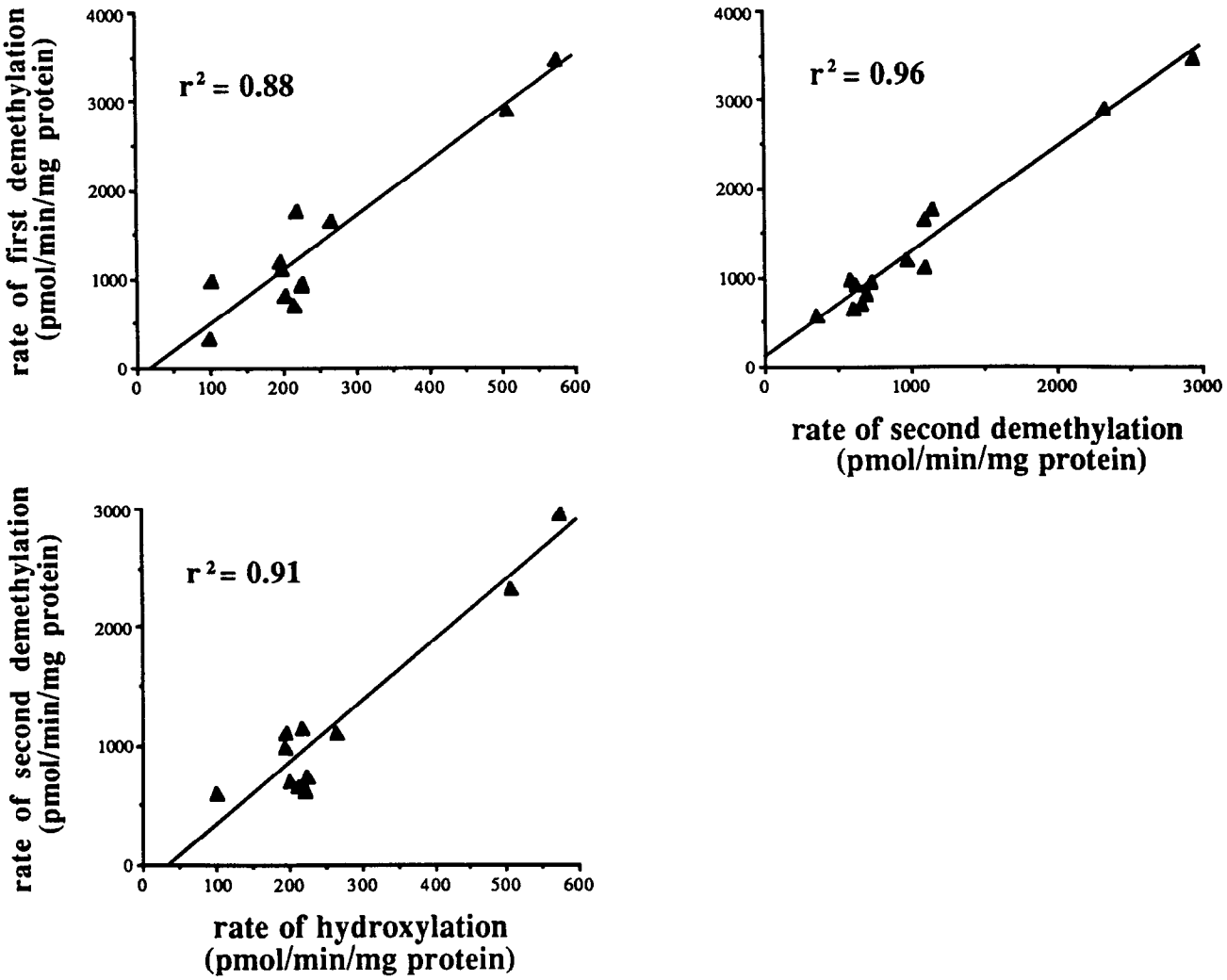

FIG. 6. Respective correlations between initial rates of mifepristone metabolite formation (reported as the means of duplicate determinations) in human liver microsomes (all $P < 0.001$).

that of hydroxylation). Conversely, inhibition of other CYP isoforms had no effect on mifepristone metabolism, which was again consistent with the results of correlation analyses. Thus, unlike that reported in the rat [31, 32, 35, 36], enzymes of the CYP2C and CYP2B subfamilies do not appear to be involved in mifepristone metabolism in humans.

The weak but significant correlations observed between rates of mifepristone demethylation and relative immunoreactive CYP2A6 levels illustrate the need to supplement data from correlation analyses with that from other lines of experimentation. An inherent weakness in performing correlation analyses is the possibility of apparent but likely artifactual relationships in a particular bank of microsomes. A very small role of CYP2A6 in mifepristone metabolism cannot be ruled out completely, but is refuted by the observed lack of biphasic kinetics (at concentrations up to 50-fold those observed *in vivo*), lack of correlation with CYP2A6 marker activity, and a lack of inhibition by disulfiram. Moreover, the very high levels of significance of correlations with CYP3A4 levels and activity probes, integrated with the inhibition results, argue clearly for a principal (and thus clinically important) role of CYP3A4. This demonstrates the critical importance of evaluating the entire body of evidence to reach a conclusion about the principal CYP catalyzing the metabolism of a drug.

It was reported recently that CYP3A7, heretofore considered fetal liver specific, was detected at the protein and mRNA levels in endometrium (of pregnant and nonpregnant women) and placenta [50]. CYP3A4 and CYP3A5 were not detected in these tissues. CYP3A7 shares some substrate specificity with CYP3A4 and is known to oxidize one steroid, dehydroepiandrosterone 3-sulfate, at an apparently greater rate [34, 51, 52]. The expression of CYP3A7 in these extrahepatic tissues was variable, but seemed to increase during the menstrual cycle and with gestation length. When used as an abortifacient, mifepristone derives its effect primarily through antagonism of receptors in the endo- and myometrium. We hypothesize that instances of non-response to mifepristone when used in this capacity

could be related in part to CYP3A7-mediated, target tissue metabolism of the compound. Such differences in response to mifepristone could not be attributed to differences in drug or metabolite plasma levels or levels of α_1-acid glycoprotein (to which it is highly bound) [42]. It seems plausible to also suggest that this isoform may influence the efficacy of antiprogestins when they are used as contraceptives or for endometriosis.

Given the numerous and promising potential uses of mifepristone (and other antiprogestins), the finding that CYP3A4 is its major metabolizing enzyme in human liver suggests the likelihood of drug–drug interactions subsequent to long-term administration of the compound. This is notably exemplified by the implications for its potential anticancer uses, since several current antineoplastic agents are also CYP3A4 substrates. Knowledge of this, combined with its potential for inhibiting P-glycoprotein *in vivo*, could lead to more rational and effective use of this compound.

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