0026-895X/94/020352-07$03.00/0

MOLECULAR PHARMACOLOGY, 45:352-358



# O-Demethylation of Epipodophyllotoxins Is Catalyzed by Human Cytochrome P450 3A4

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Received July 1, 1993; Accepted November 15, 1993

## SUMMARY

We previously demonstrated that *O*-demethylation of the pendant dimethoxyphenol ring of epipodophyllotoxins to produce their respective catechol metabolites is catalyzed by cytochrome(s) P450 in human liver microsomes. Our objective was to identify the specific human cytochrome(s) P450 responsible for catechol formation. Using a panel of prototypical substrates and inhibitors for specific cytochromes P450, we identified substrates for CYP3A4 (midazolam, erythromycin, cyclosporin, and dexamethasone) as inhibitors of catechol formation from both etoposide and teniposide. Dexamethasone inhibition was competitive, with $K_i$ values of 60 and 45 $\mu$M for etoposide and teniposide, respectively. In 58 human livers, the correlation coefficients for teniposide catechol formation versus 1′- and 4-hydroxymidazolam formation were 80% and 85%, respectively; for etoposide catechol formation versus 1′- and 4-hydroxymidazolam formation $r^2$ was 83% and 79%, respectively. Teniposide and etoposide catechol formation rates were also significantly correlated with immunodetectable CYP3A ($r^2$ = 49% and 51%, respectively) and not with immunodetectable CYP1A2, 2E1, or 2C8. Finally, cDNAs for human CYP3A4, 3A5, 2A6, 2B6, 2C8, and 2C9 were functionally expressed in HepG2 cells, using a vaccinia viral vector. Teniposide and etoposide catechol formation was catalyzed primarily by 3A4 (15.4 and 40.9 pmol/pmol/hr, respectively) and to a lesser degree by 3A5 (1.94 and 11.3 pmol/pmol/hr, respectively), whereas there was no detectable *O*-demethylation of epipodophyllotoxins by 2A6, 2B6, 2C8, 2C9, or the control virus alone. Moreover, the relative activities of midazolam hydroxylation, compared with *O*-demethylation of epipodophyllotoxins, were similar for heterologously expressed 3A4 and for human liver microsomes. We conclude that catechol formation from teniposide and etoposide is primarily mediated by human CYP3A4, making these reactions susceptible to inhibition by prototypical 3A substrates and inhibitors.

The epipodophyllotoxins VM26 and VP16 are commonly used anticancer agents with a broad range of antitumor activity (1). Although clinical pharmacokinetic studies demonstrate that nonrenal clearance mechanisms account for the majority of epipodophyllotoxin elimination (1), the precise metabolic fate of these agents remains largely undefined. We have recently demonstrated that *O*-demethylation of the pendant dimethoxyphenol ring to produce relatively stable catechol metabolites is catalyzed by P450 *in vitro* with human liver microsomes (2).

This work was supported by National Institutes of Health Grant R29 CA51001, Cancer Center CORE Grant CA21765, a Center of Excellence grant from the State of Tennessee, American Lebanese Syrian Associated Charities, and National Institutes of Health Grant NO1-CO-74102 with PRI/DynCorp. The contents of this publication do not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the United States Government.

The identification of specific P450s responsible for catalysis of drug oxidation is useful for several reasons, particularly if it can be demonstrated that a reaction is primarily mediated by a specific P450. This may allow prediction of the metabolic fate of the compound based on an assessment of the constitutive activity of that P450 in individuals, particularly for P450s whose activity exhibits genetic polymorphism. Moreover, there are data available for a number of substrates identifying the primary P450 involved in their metabolism (3, 4) and for a number of compounds that inhibit (5) or induce specific P450s (6). Therefore, it is now possible to predict many *in vivo* drug interactions based on *in vitro* identification of substrates for P450 enzymes (7). With these potential applications in mind, our objective was to identify the human P450(s) responsible for *O*-demethylation of VP16 and VM26 to their respective catechol metabolites.

**ABBREVIATIONS:** VM26, teniposide; VP16, etoposide; P450 or CYP, cytochrome P450; HPLC, high performance liquid chromatography; MAb, monoclonal antibody.

## Experimental Procedures

**Materials.** VM26 and VP16 were gifts of Bristol (Syracuse, NY). VP16 and VM26 were purified by HPLC before use as substrates in incubations. The catechols of VM26 and VP16 were synthesized from VM26 and VP16 as described previously (2). Midazolam, 1′-hydroxymidazolam, 4-hydroxymidazolam, and desmethylmidazolam were gifts of Hoffmann-LaRoche (Nutley, NJ). Cyclosporin was a gift of Sandoz Research (East Hanover, NJ). Mephenytoin was provided by Drs. A. Küpfer and G. Karlaganis (University of Berne, Switzerland). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO) or Boeringher-Mannheim (Indianapolis, IN).

Human liver samples were obtained through the Liver Tissue Procurement and Distribution System (University of Minnesota), the National Disease Research Interchange (Philadelphia, PA), the University of Tennessee (Memphis, TN), the Medical College of Virginia (Richmond, VA) and from Dr. Urs A. Meyer (University of Basel, Switzerland) (KDL 21, 18, and 33) and were stored frozen at −80° until the time of microsome preparation. All samples were surgical (not autopsy) specimens and were frozen in liquid nitrogen within 1 hr of removal of tissue. Appropriate consent was obtained from the Institutional Review Board for use of human tissues.

**Liver microsomal activities.** Microsomes were prepared from human liver specimens as described previously (8). The average yield was 2.5% of liver wet weight recovered as microsomal protein; protein concentrations were measured as described by Bradford (9).

All incubations were performed in duplicate by addition of the appropriate volume of a methanolic solution of substrate to individual tubes and evaporation of the methanol under nitrogen at room temperature. Microsomes (0.05–0.2 mg/incubate) in 0.1 M potassium phosphate buffer, pH 7.4, were added, and all samples were preincubated for 15 min (VM26 and VP16) at room temperature or for 3 min (midazolam) at 37°. Reactions were initiated by addition of 1/10th volume of a concentrated aliquot of an NADPH-regenerating system (10 units/ml isocitrate dehydrogenase, 50 mM isocitrate, 10 mM sodium NADP, and 50 mM magnesium chloride) and vortex mixing. Samples were incubated at 37° for either 30 min (VM26 and VP16) or 10 min (midazolam). Incubations of epipodophyllotoxins were terminated by freezing of reaction mixtures in an acetone/dry ice bath; midazolam reaction mixtures were stopped by addition of 100 μl of cold methanol. Incubates were extracted for metabolite quantitation as outlined below. Enzyme activities are expressed as nmol of product/mg of microsomal protein/hr.

**Effects of P450 substrates on VP16 and VM26 *O*-demethylation.** All inhibition experiments were performed at least in duplicate using microsomes from the same liver, LB108. VM26 and VP16 kinetic parameters were determined using conditions described previously (2); the $K_m$ values were 33.2 and 132 μM and the $V_{max}$ values were 1.14 and 2.47 nmol/mg/hr for VM26 and VP16, respectively. Potential inhibitors were added as methanolic solutions to tubes, evaporated to dryness under nitrogen, and incubated for 30 min with 0.2 mg of microsomal protein, 25 μl of NADPH, and 0.1 M potassium phosphate buffer, pH 7.4, in a total volume of 250 μl. Samples were extracted and checked for chromatographic interferences in the assay for catechols of epipodophyllotoxins. Those compounds without chromatographic interferences were added at 500 μM to either VM26 or VP16 (75 μM) and incubated for 30 min as described above. The average catechol formation rates in the presence of the putative inhibitors were divided by the mean of the formation rates obtained in control incubates without inhibitor. For two of the inhibitors, Dixon plots were constructed using two different substrate concentrations and several inhibitor concentrations; experiments were performed in duplicate.

**Catalytic activity of heterologously expressed P450s.** Recombinant wild-type vaccinia virus or virus containing cDNA encoding either CYP3A4,[1] 3A5, 2C8, 2C9, 2A6, or 2B6 was used to infect HepG2 cells to obtain catalytically active P450 expression, as described previously (10). Cells were disrupted by sonication, and crude cell homogenates were incubated with NADPH and either midazolam, VM26, or VP16. Metabolite production was determined as described above. Cellular protein amounts per incubation were 3.2–8 mg for midazolam and 7–16 mg for VM26 and VP16. Enzyme activities are expressed as nmol of product/nmol of spectrophotometrically quantitated P450/hr.

**Analytical methods.** VM26 catechol and VP16 catechol were assayed by HPLC as described previously (2). 4-Hydroxymidazolam, 1′-hydroxymidazolam, and desmethylmidazolam were assayed by a modification of previous assays (11, 12). After addition of cold methanol, proteins were removed by centrifugation at 10,000 × *g* and 50 μl of supernatant were directly injected onto the isocratic reverse phase HPLC system [mobile phase, acetonitrile/methanol/0.05 M potassium phosphate buffer, pH 7.4/tetrahydrofuran, 28:30:40:2, v/v/v/v, at 1 ml/min; Hypersil BDS C18, 5 μm, 250- × 4.6-mm column (Keystone, Inc); UV detection at 254 nm]. Run times were <15 min, with 4-hydroxymidazolam, 1′-hydroxymidazolam, desmethylmidazolam, and midazolam retention times of 5.8, 6.4, 7.3, and 8.5 min, respectively. Interday coefficients of variation were as follows: 4-hydroxymidazolam, 4.9% and 4.0% at 2.44 and 24.4 μM; 1′-hydroxymidazolam, 5.3% and 4.9% at 9.77 and 97.7 μM; and desmethylmidazolam, 5.0% and 4.9% at 1.34 and 13.4 μM, respectively. The lower limit of detection was approximately 20 pmol on the column for each metabolite.

HPLC standards were prepared exactly as the human liver incubations (microsomes, potassium phosphate buffer, substrate, and NADPH-regenerating system), except that rat microsomes were used instead of human, samples were spiked with known quantities of metabolites, and no incubation took place before sample extraction.

**Immunoquantitation.** Quantitative immunoblots were performed as described (13). Briefly, appropriate quantities (5–25 μg) of microsomal protein from a subset of livers determined to give an immunoreactive response in a linear range were resolved on 12.5% sodium dodecyl sulfate-polyacrylamide gels, electroblotted to nitrocellulose membranes, and incubated with a MAb to human CYP3A (MAb K03, also known as MAb 13-7-10, directed against P-450 HL5, which was shown to be identical to CYP3A3/4) (14), which was generously provided by Dr. Philippe Beaune (CHU Necker, Paris, France). Filters were exposed to peroxidase-conjugated antibodies and visualized by reaction with 3,3′-diaminobenzidine tetrahydrochloride and hydrogen peroxide. Alternatively, the primary MAb was developed using the Bethesda Research Laboratories (Gaithersburg, MD) anti-mouse streptavidin-biotin development system. The integrated absorbance of the 3A bands was quantitated using a Zeiss integrated scanning densitometer, and the amounts are expressed as the nmol of CYP3A/mg of microsomal protein (15). Quantitative immunoblot analyses of 2E1, 1A2, and 2C8 in livers from the Medical College of Virginia have been published previously (16–19); immunoquantified values for 2E1 (18), 1A2 (16), and 2C8 (17) were expressed relative to the densitometric value obtained for HL14, which was arbitrarily set at 100%.

**Numerical analysis.** Linear regression was used to determine the relationships of ratios of metabolite peak height to internal standard peak height versus known metabolite concentrations. Nonlinear least squares regression, as implemented in MINSQ (MicroMath, Salt Lake City, UT), was used to estimate kinetic parameters by fitting the untransformed Michaelis-Menten equation, i.e., rate = $[(V_{max} \cdot [S])/(K_m + [S])]$, to the metabolite formation rate data for each substrate. Correlations of activities among livers and correlations between activities and immunoquantitated amounts of P450s were estimated using linear regression.

## Results

**Screen of inhibitors.** Percentages of control VM26 and VP16 catechol formation in the presence of prototypical P450 substrates and inhibitors are depicted in Fig. 1. The most potent inhibitors were erythromycin, cyclosporin, dexamethasone,

[1] The nomenclature used in this report is that described in Ref. 46.

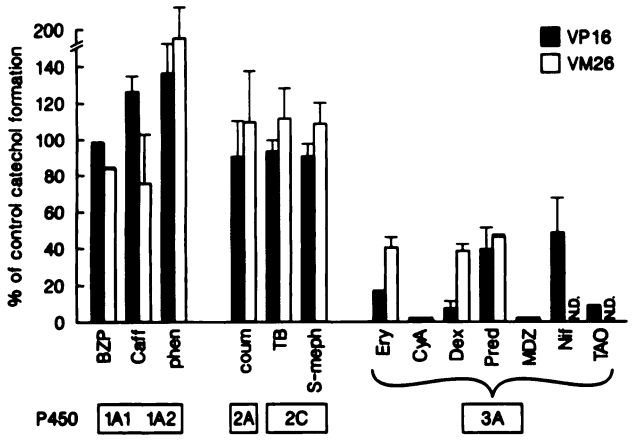


**Fig. 1.** Percentage of control activities for VP16 and VM26 catechol formation in the presence of benzo(*a*)pyrene (*BZP*), caffeine (*Caff*), phenacetin (*phen*), coumarin (*coum*), tolbutamide (*TB*), (*S*)-mephenytoin (*S-meph*), erythromycin (*Ery*), cyclosporin (*CyA*), dexamethasone (*Dex*), prednisone (*Pred*), midazolam (*MDZ*), nifedipine (*Nif*), or troleandomycin (*TAO*). Inhibitor concentrations were 500 μM and substrate concentrations were 75 μM. Results are expressed as the mean + standard error of two to four replicates. P450 families and subfamilies are indicated; *N.D.*, not done because of chromatographic interference.

prednisone, midazolam, nifedipine, and troleandomycin. Inhibition of VM26 and VP16 catechol formation by dexamethasone was competitive, with $K_i$ values of 45 and 60 μM, respectively (Fig. 2). Inhibition by erythromycin was not purely competitive (Fig. 2), and thus estimation of a $K_i$ was not possible.

**Correlation of midazolam, VM26, and VP16 oxidative activities.** VM26 and VP16 catechol formation rates at 500 μM substrate concentrations were compared with midazolam 4- and 1′-hydroxylations (as markers of CYP3A activities) in 58 human livers (11). VM26 catechol formation was correlated with midazolam 4-hydroxylation ($r^2$ = 85%) and 1′-hydroxylation ($r^2$ = 80%) (both with $p < 0.001$) (Fig. 3). VP16 catechol formation was also correlated with midazolam 4-hydroxylation ($r^2$ = 79%) and with 1′-hydroxylation ($r^2$ = 83%) (both with $p < 0.001$) (Fig. 4). Moreover, in all cases the *y*-intercept was essentially zero. As reported previously (2), VM26 catechol and VP16 catechol formation rates were highly correlated with each other ($r^2$ = 87%, $p < 0.001$, $n$ = 58).

**Immunoquantitation of CYP3A.** Immunoquantitation of P450 isoforms or subfamily members was available for a subset of livers in which catalytic activities had also been measured. There was a significant positive correlation between VM26 catechol formation and immunoquantitation of CYP3A content in 33 liver microsomal preparations ($r^2$ = 49%, $p < 0.001$) and between VP16 catechol activity and CYP3A content in the 32 livers for which both measurements were available ($r^2$ = 51%, $p < 0.001$) (Fig. 5). Midazolam 4- and 1′-hydroxylations were similarly correlated with CYP3A content, whereas there were no significant correlations between catalytic activities and immunoquantified 1A2, 2E1, or 2C8 (Table 1).

**Catalytic activity of expressed P450s.** Homogenates from HepG2 cells infected with either 3A4- or 3A5-containing recombinant virus possessed significant midazolam hydroxylase activity (Table 2), whereas cells infected with 2A6-, 2B6-, 2C8-, or 2C9-containing or wild-type virus had no detectable activity. Catalytic activity of the 2C8 and 2C9 preparations was demonstrated for tolbutamide hydroxylation (data not shown) (20), and catalytic activities of 2A6 and 2B6 using the same expression strategy have been demonstrated previously (21–23) with a number of substrates. The identical preparations of

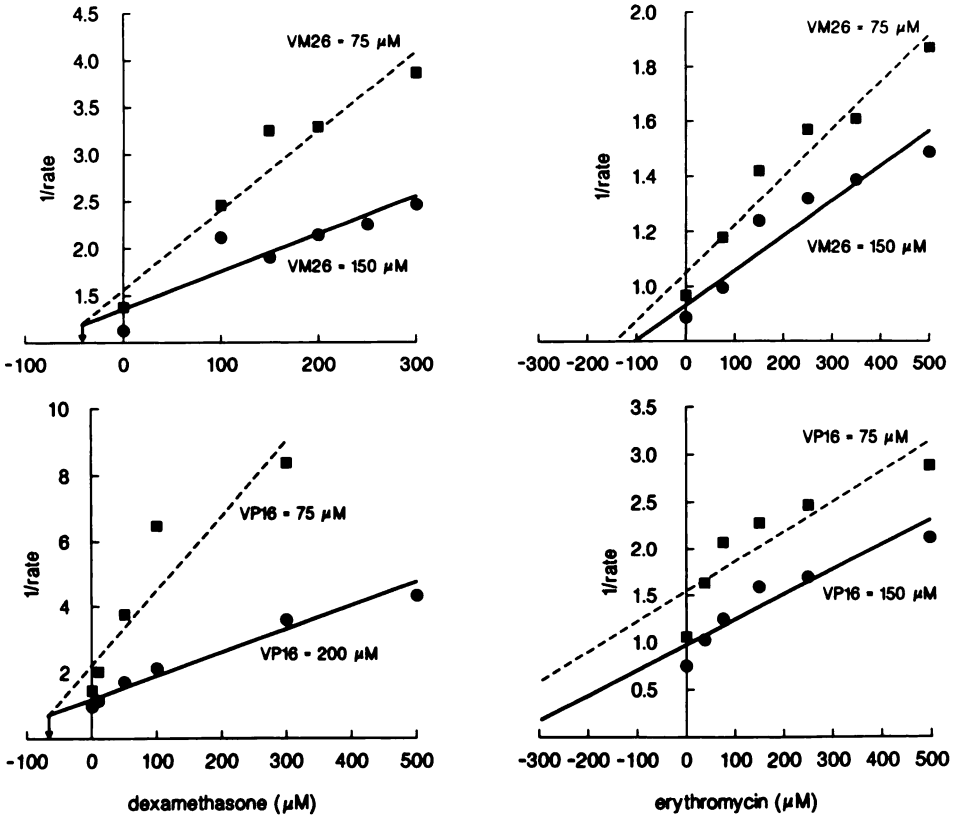


**Fig. 2.** Dixon plots for dexamethasone inhibition (*left*) of VM26 (*upper*) and VP16 (*lower*) catechol formation and for erythromycin (*right*) inhibition of VM26 (*upper*) and VP16 (*lower*) catechol formation. Results are plotted as the mean of duplicate determinations.

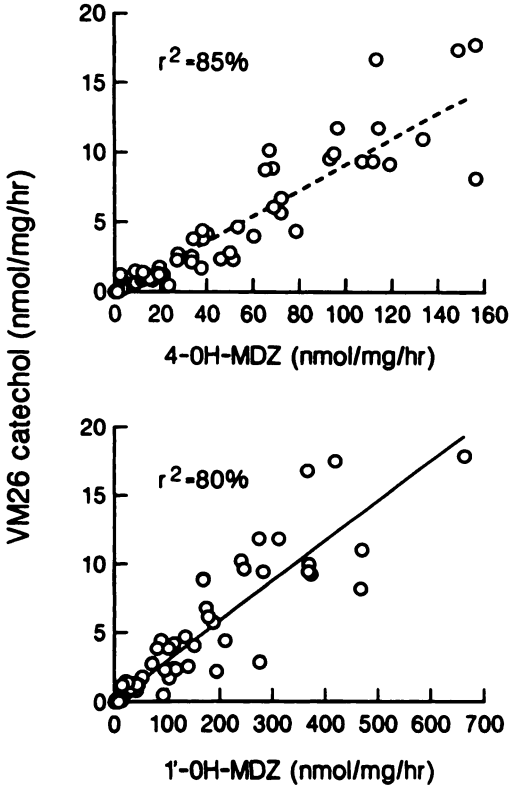


Fig. 3. Correlations between VM26 catechol formation and midazolam (*MDZ*) 4-hydroxylation (*upper*) and 1′-hydroxylation (*lower*) in 58 human liver microsomal preparations. Results are plotted as the mean of duplicate determinations.

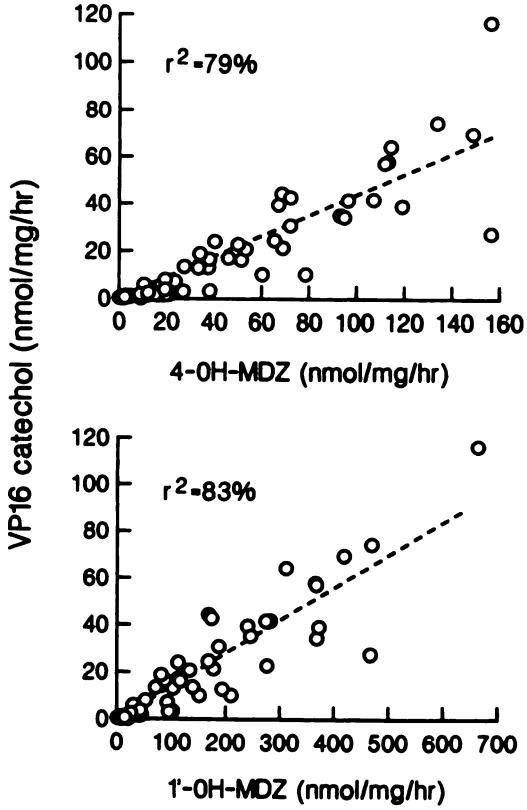


Fig. 4. Correlations between VP16 catechol formation and midazolam (*MDZ*) 4-hydroxylation (*upper*) and 1′-hydroxylation (*lower*) in 58 human liver microsomal preparations. Results are plotted as the mean of duplicate determinations.

HepG2 cells were incubated with VM26 and VP16; catechol formation was greater with 3A4 than with 3A5, and activity from the 2A6-, 2B6-, 2C8-, and 2C9-containing and wild-type virus-infected cells was undetectable (Table 2).

The median ratios of either 4- or 1′-hydroxylation of midazolam to either VM26 or VP16 catechol formation in 58 human liver microsomal preparations were closer to the ratios of activities obtained using expressed 3A4 than they were to the ratios of activities obtained using expressed 3A5 (Table 3). The VM26 and VP16 *O*-demethylation values obtained with expressed 3A4 were within a 3–4-fold range of the values predicted from the regression equation relating activity to nmol of P450 3A in human liver microsomes (Fig. 5), as were the midazolam 4-hydroxylation (409, 206, and 277 pmol/pmol/hr) and 1′-hydroxylation values (1509, 1540, and 4532 pmol/pmol/hr) for microsomes, expressed 3A4, and expressed 3A5, respectively.

## Discussion

Results of the current study indicate that CYP3A4 is the predominant human P450 responsible for *O*-demethylation of VM26 and VP16 to their respective catechol metabolites. Activity measurements using expressed 3A4 and 3A5 enzymes demonstrated that 3A4 rather than 3A5 is the primary P450 catalyzing *O*-demethylation of epipodophyllotoxins, whereas other P450s (2C8, 2C9, 2B6, and 2A6) lacked activity. The role of the CYP3A forms was further demonstrated by the ratios of hydroxylation of midazolam to that of the epipodophyllotoxins in 58 human liver microsomal preparations, which were closer to the ratios obtained with expressed 3A4 than they were to the ratios obtained with expressed 3A5. It is not surprising that 3A4 has greater VM26 and VP16 *O*-demethylation activity than does 3A5, given that for most substrates tested (21–25) 3A4 exhibits greater catalytic activity than does 3A5. One of the few exceptions in which 3A5 has been shown to have significant activity is 1′-hydroxylation of midazolam, which was greater for expressed 3A5 than 3A4, making the ratio of 1′-hydroxylation to 4-hydroxylation of midazolam a putative discriminatory activity to identify livers expressing 3A5 (26).

Confirmation that 3A4 is the predominant P450 involved in human microsomal *O*-demethylation is provided by several lines of evidence. Firstly, the *y*-intercepts for the correlations between VM26 *O*-demethylation or VP16 *O*-demethylation and midazolam 4- or 1′-hydroxylation are close to zero, indicating that in the absence of midazolam hydroxylation there is little or no VM26 or VP16 *O*-demethylation. Secondly, two prototypical 3A substrates, midazolam and cyclosporin (7, 11), were able to completely inhibit VM26 and VP16 *O*-demethylation in human liver microsomes. Thirdly, several other major drug-metabolizing P450s lacked catalytic activity towards both VM26 and VP16 *O*-demethylation. Finally, immunoquantified 3A significantly positively correlated with VM26 and VP16 catechol formation, whereas immunoquantified 1A2, 2C8, and 2E1 did not. However, it must be acknowledged that the correlation between catalytic activity and immunoquantified 3A was not as predictive as the correlations among midazolam and VM26 or VP16 *O*-demethylation activities, which may be explained partly by greater precision in HPLC analytical techniques, compared with densitometric scanning, and also by

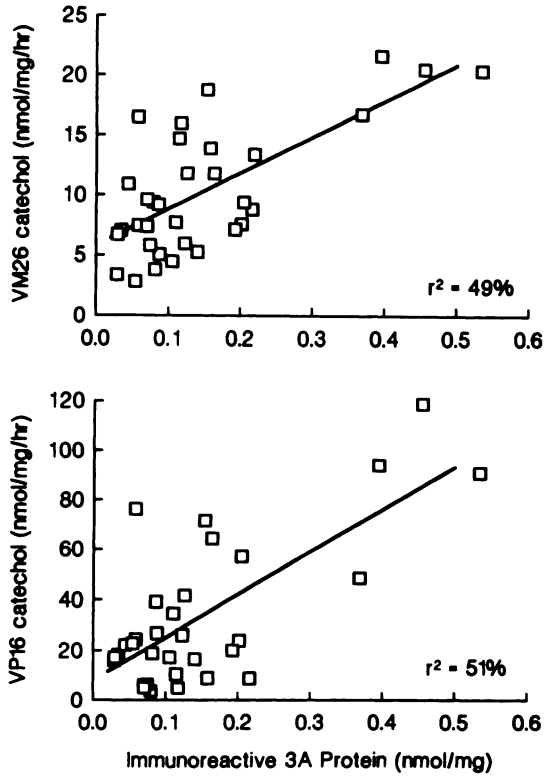


**Fig. 5.** Correlations between VP16 ($n$ = 32) (*lower*) and VM26 ($n$ = 33) (*upper*) catechol formation and immunoquantitated CYP3A in human liver microsomal preparations. Activities are depicted as the mean of duplicate determinations.

cross-reactivity of the 3A antibody with 3A isoforms other than 3A4.

The extent to which *O*-demethylation accounts for elimination of VM26 and VP16 *in vivo* is unknown. Quantitative assessment of these pathways in patient tissue is complicated by the high reactivity of the catechols and the related quinone and semiquinone species (27). If *O*-demethylation (or other 3A-mediated pathways) were to account for a significant percentage of VM26 and VP16 elimination, typical CYP3A-mediated drug interactions would be anticipated to affect the disposition of parent compounds. For example, the induction of VM26 and VP16 metabolism by rifampin, phenobarbital, phenytoin, and possibly glucocorticoids could be anticipated; interestingly, we have observed a significant increase in VM26 clearance with concurrent anticonvulsant therapy *in vivo* (28). Moreover, one could anticipate inhibition of *O*-demethylation by concurrent administration of 3A substrates (erythromycin, cyclosporin, or quinidine) or inhibitors. One intriguing implication of these potential drug interactions is the fact that both VM26 and VP16 (or their metabolites) have been shown to be substrates for a transmembrane glycoprotein ("P-glycoprotein") (29, 30) that causes classical multidrug resistance on the basis of enhancing anticancer drug efflux from tumor cells. Interestingly, many of the compounds that reverse multidrug resistance *in vitro* and have been tested as putative resistance-reversing agents in humans are CYP3A substrates (cyclosporin, quinidine, and nifedipine) (7, 31, 32). Moreover, several clinical trials of P-glycoprotein inhibitors have demonstrated decreased systemic clearance of the concurrently administered anticancer drug, including VP16 (33–34). Such pharmacokinetic interactions might be anticipated if it is demonstrated that the anticancer drug metabolism is mediated primarily by P450s (e.g., 3A) that also catalyze the metabolism of the P-glycoprotein inhibitors (e.g., cyclosporin and quinidine). Thus, increased cytotoxicity with the coadministration of reversing agents and anticancer drugs may be due to decreased elimination of the antineoplastic agents, in addition to or instead of modulation of P-glycoprotein-mediated efflux.

We observed that erythromycin inhibition of both VM26 and VP16 catechol formation was not characteristic of purely competitive inhibition, as would be predicted from its known irreversible binding to and partial inactivation of CYP3A, combined with its competitive effect being based on erythromycin itself being metabolized by CYP3A (35, 36). These results, however, are in contrast to the competitive inhibition reported previously for erythromycin and cyclosporin (37) and for midazolam (38). One difference between our experiments and those cited is that we used a longer time period for incubation with NADPH, which could have provided more permissive conditions for formation of the reactive erythromycin nitroso derivative that forms a stable complex with the hemoporphyrin of 3A, thereby causing 3A inactivation (35–36).

Interestingly, we found that phenacetin caused an increase in catechol formation rate, particularly for VM26. The reason for this enhanced activity is under investigation. Others have reported the enhancement of 3A catalytic activity when microsomes (39) or expressed 3A (40) is incubated in the presence of $\alpha$-naphthoflavone.

Clinical pharmacodynamic relationships between parent VM26 and VP16 and both anticancer activity (41) and toxicity (42–44) have been demonstrated. Given that *O*-demethylated VM26 and VP16 metabolites, including the measured catechols and the more reactive quinone and semiquinone moieties, which presumably are also formed, possess some cytotoxicity (45), the clinical relevance of any potential drug interactions remains to be demonstrated. If *O*-demethylated metabolites contribute more to adverse effects than to therapeutic activity, then inhi-

TABLE 1

**Correlations between catalytic activities and immunoquantified P450s in human liver microsomes**

All assessments of catalytic activity were analyzed as the mean of duplicate determinations. Immunoquantification of P450s was as described in Experimental Procedures.

| Product | CYP3A | | CYP2C8 | | CYP1A2 | | CYP2E1 | |
|---|---|---|---|---|---|---|---|---|
| | $r^2$ ($n$) | p value[a] | $r^2$ ($n$) | p value | $r^2$ ($n$) | p value | $r^2$ ($n$) | p value |
| | % | | % | | % | | % | |
| VM26 catechol | 49 (33) | <0.001 | 6.6 (30) | 0.17 | 7.3 (23) | 0.21 | 0.0 (23) | 0.96 |
| VP16 catechol | 51 (32) | <0.001 | 7.3 (29) | 0.16 | 1.1 (22) | 0.65 | 2.4 (22) | 0.49 |
| 4-Hydroxymidazolam | 51 (32) | <0.001 | 5.8 (29) | 0.21 | 3.4 (22) | 0.41 | 2.8 (22) | 0.45 |
| 1′-Hydroxymidazolam | 41 (32) | <0.001 | 9.5 (29) | 0.10 | 0.0 (22) | 0.98 | 1.5 (22) | 0.58 |

[a] All significant correlations had positive slopes.

TABLE 2

**Vaccinia-expressed P450 catalytic activities**

Values are expressed as the mean ± standard error of quadruplicate (VM26 and VP16 catechol formation) or duplicate (midazolam hydroxylation) determinations.

| Reaction | Activity | | | | | | |
|---|---|---|---|---|---|---|---|
| | 3A4 | 3A5 | 2B6 | 2A6 | 2C8 | 2C9 | Wild-type[a] |
| | pmol/pmol of P450/hr | | | | | | |
| VM26 catechol formation | 15.4 ± 2.6 | 1.94 ± 0.16 | ND[b] | ND | ND | ND | ND |
| VP16 catechol formation | 40.9 ± 12.9 | 11.3 ± 2.4 | ND | ND | ND | ND | ND |
| Midazolam 4-hydroxylation | 206 ± 7.2 | 277 ± 4.6 | ND | ND | ND | ND | ND |
| Midazolam 1′-hydroxylation | 1541 ± 53.2 | 4532 ± 34.3 | ND | ND | ND | ND | ND |

[a] Wild-type vaccinia virus without P450 cDNA insert.
[b] ND, not detected.

TABLE 3

**Ratios of midazolam hydroxylation to VM26 and VP16 O-demethylation activities**

| | Human liver microsomes[a] | | 3A4 ratio[b] | 3A5 ratio[b] |
|---|---|---|---|---|
| | Median ratio | Slope | | |
| 4-Hydroxymidazolam/VM26 catechol | 12 | 8.7 | 13.4 | 143 |
| 4-Hydroxymidazolam/VP16 catechol | 2.7 | 1.7 | 5.0 | 25 |
| 1′-Hydroxymidazolam/VM26 catechol | 34 | 28.1 | 100 | 2336 |
| 1′-Hydroxymidazolam/VP16 catechol | 9.8 | 5.7 | 38 | 401 |

[a] Median ratio, median ratio of midazolam hydroxylation to VM26 or VP16 catechol formation measured in 58 liver microsomal preparations assayed in duplicate. Slope, slope of regression line of midazolam hydroxylation versus VM26 or VP16 catechol formation rates.
[b] Ratio of average catalytic activities from vaccinia-expressed P450 catalytic activities (see Table 2).

bition of their formation would be desirable. Although catechol formation appears to account for a relatively small percentage of the metabolism of the epipodophyllotoxins (2), the significance of these reactive metabolites has not been assessed in definitive clinical studies. Whether through effects on disposition of parent compound or effects on production of reactive metabolites, inhibition and induction of VM26 and VP16 metabolism may have clinical importance. The current study indicates that modulation of CYP3A4 activity, whether by drug interactions, disease effects, or other regulation, would have a significant influence on the *O*-demethylation of VP16 and VM26.

**Acknowledgments**

We gratefully acknowledge the technical expertise of Ken Cox, Pamela McGill, and Doug Byrd.

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