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## APPROVAL PACKAGE FOR:

## APPLICATION NUMBER
## 17-970/S-046

# Clinical Pharmacology and Biopharmaceutics Review

# CLINICAL PHARMACOLOGY AND BIOPHARMACEUTICS REVIEW

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**sNDA 17-970/046**

**Submission Date:** December 28, 1999
June 22, 2000

**Drug and Formulation:** **NOLVADEX** (tamoxifen citrate) 10 and 20 mg tablets

**Sponsor:** AstraZeneca Pharmaceuticals
1800 Concord Pike, P.O. Box 15437
Wilmington, DE 19850-5437

**Reviewers:** Brian Booth, Ph.D.

**Type of Submission:** Supplemental NDA

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## I. SYNOPSIS

The applicant is seeking supplemental marketing approval for the use of NOLVADEX in treating Ductal Carcinoma In Situ in women following breast surgery and radiation. According to prior agreements with the FDA and the Division of Oncology Drug Products, the basis of the current submission is the safety and efficacy data from the B-24 trial of the National Surgical Adjuvant Breast and Bowel Project. The applicant requested and received a biowaver for Clinical Pharmacology and Biopharmaceutics studies because of the similarity in the dosing regimen and the similarity between the current patient population and the patient population for which NOLVADEX is currently approved.

The Nolvadex labeling was amended by the sponsor to include the DCIS data. FDA updated the CLINICAL PHARMACOLOGY and Drug-Drug Interactions paragraph of the PRECAUTIONS sections. FDA included the following information regarding tamoxifen metabolism and tamoxifen-drug interactions in the labeling.

- **Tamoxifen is a cytochrome P-450 3A substrate.** (Desai PB, Duan JZ, Zhu YW, Kouzi S. European Journal of Drug Metabolism and Pharmacokinetics, 23, 417-424, 1998. Dehal SS, Kupfer D. Cytochrome P-450 3A and 2D6 catalyze ortho hydroxylation of 4-hydroxytamoxifen and 3-hydroxytamoxifen (droloxifene) yielding tamoxifen catechol; involvement of catechols in covalent binding to hepatic proteins. Drug metabolism and Dispostion 27, 681-688, 1999).
- **Tamoxifen is a P-glycoprotein inhibitor.** (Gottesman MM, Pastan I. Biochemistry of multidrug resistance mediated by the multidrug transporter. Annual Review in Biochemistry 62, 385-427, 1993. Leveille-webster CR, Arias IM. The biology of P-glycoproteins. Journal of Membrane Biology, 143, 89-102, 1995).
- **Tamoxifen reduced the plasma concentration of letrozole by 37%.** (Dowsett M, Pfister C, Miles DW, Houston SJ, Verbeek JA, Gundacker H, Sioufi A, Smith IE. Clinical Cancer Research 5, 2338-2343, 1999).

- **Rifampin, a CYP 3A4 inducer, reduced tamoxifen AUC and $C_{max}$ by 856 and 55 %, respectively.** (Kivisto KT, Villikka K, Nyman L, Anttila M, Neuvonen PJ Clinical Pharmacology and Therapeutics, 64, 648-654, 1998)
- **Medroxyprogesterone reduces tamoxifen and N-desmethyltamoxidfen plasma concentrations.** (Reid AD, Horobin JM, Newman EL, Preece PE. Breast Cancer Research and Treatment 22, 153-156, 1992).
- **Aminogluthimide reduces tamoxifen and N-desmethyltamoxidfen plasma concentrations.** (Lien E, Anker G, Lonning PE, Solheim E, Ueland PM Decreased serum concentrations of tamoxifen and its metabolites induced by aminogluthimide. Cancer Research 50, 5851-5857, 1990).
- **In vitro studies showed that erythromycin, cyclosporin, nifedipine, and diltiazem competitively inhibited formation of N-desmethyl tamoxifen with apparent $K_I$ of 20,1, 45 and 30 μM, respectively.** (Jacolot F, Simon I, Dreano Y, Beaune P, Riche C, Berthou F Biochemical Pharmacology 41 (12) 1911-1919, 1991).

These publications are attached as Appendix 3.

## II. Labeling Comments

### FDA Labeling

Italics indicate a rearrangement of paragraphs and bold indicates FDA-added information.

**CLINICAL PHARMACOLOGY**

Draft Labeling

1 pages redacted from this section of the approval package consisted of draft labeling

## III. Recommendations

The NOLVADEX labeling should be altered according to the review comment to provide updated information regarding the metabolism and drug-drug interactions of tamoxifen. Please update the NOLVADEX labeling according to the labeling comments provided in this review.

/S/

Brian Booth, Ph.D.
Pharmacokinetic Reviewer
Division of Pharmaceutical Evaluation I

/S/ 6/28/00

N.A.M. Atiqur Rahman, Ph.D.
Team Leader
Division of Pharmaceutical Evaluation I

cc. NDA 17-970 (original file)
HFD-150/Division File
HFD-150 ~~GWilson~~ A. Baird
HFD-150 SHonig, GWilliams
HFD-860 BBooth, ARahman, Csahajwalla, MMehta
CDR Biopharm

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# APPENDIX 1

## AstraZeneca Request for a Clinical Pharmacology & Biopharmaceutics Biowaiver

AstraZeneca

COPY 1

SENT VIA RAPIFAX AND
UPS NEXT DAY AIR

FEB 17 2000

Dr. Brian P. Booth
Division of Biopharmaceutical Evaluation I
Food and Drug Administration
HFD No. 860, Room No. 2077
Woodmont II Building
1451 Rockville Pike
Rockville, MD 20852-1448

Dear Dr. Booth:

Re: NOLVADEX® (tamoxifen citrate)
NDA 17-970/S-046
Waiver Request

AstraZeneca Pharmaceuticals (formerly Zeneca Pharmaceuticals) requests a biopharmaceutics waiver for the NOLVADEX® (tamoxifen citrate) supplemental New Drug Application to support the treatment of women with ducal carcinoma in situ (DCIS), NDA 17-970/S-046. The tablets used in NSABP's B-24 clinical trial were the commercial formulation for NOLVADEX 10 mg Tablets as specified in NDA 17-970 with the exception that the tablets were not intagliated to permit blinding of the drug treatment groups. The supplemental application is based solely on the clinical data from the B-24 trial.

Please do not hesitate to contact me if you have any questions.

Sincerely,

Gary M. Cooper
Regulatory Project Group Leader
Regulatory Affairs Department
(302) 886-5132
(302) 886-2822 (fax)

GMC/gem

Desk Copy: Ms. Amy Chapman, HFD No. 150, Room No. 2106

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**APPENDIX 2**
**Applicant-Proposed NOLVADEX Labeling**

29 pages redacted from this section of the approval package consisted of draft labeling

## Appendix 3: Published Literature

EUROPEAN JOURNAL OF DRUG METABOLISM AND PHARMACOKINETICS 1998, Vol. 23, No. 3, pp. 417–424

# Human liver microsomal metabolism of paclitaxel and drug interactions

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[1]*Division of Pharmaceutical Sciences, College of Pharmacy, University of Cincinnati Medical Center, Cincinnati, Ohio, USA*

[2]*Division of Basic Pharmaceutical Sciences, School of Pharmacy, Northeast Louisiana University, Monroe, Louisiana, USA*

*Received for publication* : September 1, 1998

*Keywords* : Paclitaxel, human microsomes, hepatic metabolism, drug interactions, tamoxifen, R-verapamil

## SUMMARY

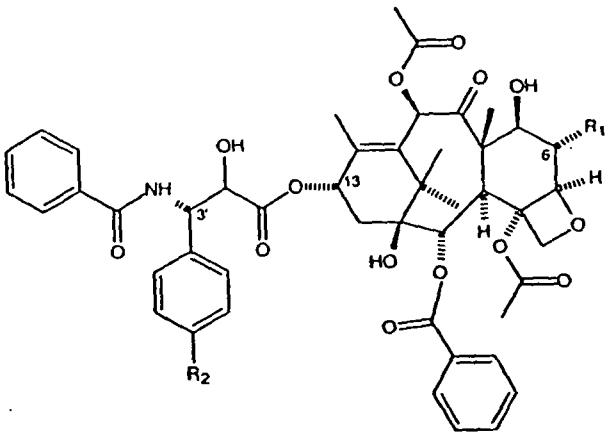
The aim of this study was to investigate the influence of several anticancer drugs and investigational multidrug resistance (MDR) reversing agents on the hepatic metabolism of paclitaxel (Taxol) to its primary metabolites, 6 α-hydroxypaclitaxel (metabolite, $M_A$) and 3′-p-hydroxypaclitaxel (metabolite, $M_B$). There is significant inter-individual variability associated with the levels of these two metabolites. In many cases, 6α-hydroxypaclitaxel has been observed to be the predominant metabolite, in others, 3 ′-p-hydroxypaclitaxel has been the principal metabolite. The formation of 6α-hydroxypaclitaxel and 3′-*p*-hydroxypaclitaxel is catalyzed by cytochrome P450 isozymes CYP2C8 and CYP3A4, respectively. A number of factors, including co-administration of drugs and adjuvants, are known to influence the activity of these isozymes. Therefore, the influence of MDR reversing agents, R-verapamil, cyclosporin A (CsA) and tamoxifen and anti-cancer drugs doxorubicin, etoposide (VP-16) and cisplatin on paclitaxel metabolism was assessed employing human liver microsomes in vitro. Paclitaxel (10 μM) was incubated with human liver microsomes (1 mg protein, ~0.34 nmol CYP) in the presence of a NADPH generating system at 37°C for 1 h, with and without the presence of interacting drug. Controls included incubations with quercetin and ketoconazole, known inhibitors of 6α-hydroxypaclitaxel and 3′-*p*-hydroxypaclitaxel formation, respectively. At the end of the incubation period, paclitaxel and the metabolites were extracted in ethyl acetate and analyzed employing an HPLC method. Significant inhibition of paclitaxel conversion to 6α-hydroxypaclitaxel and 3′-*p*-hydroxypaclitaxel was observed in the presence of R-verapamil, tamoxifen and VP-16 ($P$ 0.005). Doxorubicin significantly inhibited the formation of 3′-*p*-hydroxypaclitaxel and CsA inhibited the formation of 6α-hydroxypaclitaxel ($P$ 0.005). This study demonstrates that co-administration of several of the above listed compounds could lead to significant changes in the pharmacokinetics of paclitaxel.

*Please send reprint request to* : Dr Pankaj B. Desai, Division of Pharmaceutical Sciences, College of Pharmacy, University of Cincinnati Medical Center, 3223 Eden Avenue, Cincinnati, OH 45267-0004, USA

*Present address* : Dr John Z. Duan, Division of Pharmaceutical Evaluation, Center for Drug Evaluation and Research, US Food and Drug Administration, Rockville, Maryland, USA

## INTRODUCTION

Paclitaxel, an anti-microtubule agent with a unique mechanism of cytotoxic action, is the most promising anti-cancer drug introduced in the past two decades (1). While it is currently approved for use in the treatment of ovarian and breast carcinoma, paclitaxel is in advanced stages of clinical studies for the treatment of




| | | |
|---|---|---|
| Paclitaxel | $R_1 = H$ | $R_2 = H$ |
| $M_A$ | $R_1 = OH$ | $R_2 = H$ |
| $M_B$ | $R_1 = H$ | $R_2 = OH$ |
| $M_C$ | $R_1 = OH$ | $R_2 = OH$ |

*Fig. 1* : Chemical structure of paclitaxel and its primary human metabolites.

malignant melanomas, non-small cell lung carcinoma and head and neck cancer. In the clinical setting, paclitaxel is frequently employed in combination with drugs such as cisplatin, carboplatin, VP-16 and doxorubicin (2–4).

Recent efforts have focused on the biological fate of paclitaxel and its implications for therapeutic action and toxic effects. Paclitaxel is primarily eliminated via the hepatobiliary route (5–9). Approximately, 20% of the unchanged drug is excreted in bile, less than 10% in urine and the remaining fraction is likely eliminated via metabolism. The three main paclitaxel metabolites detected in human bile and human liver microsomal incubations are 6α-hydroxypaclitaxel ($M_A$), 3′-*p*-hydroxypaclitaxel ($M_B$) and 6α-3′-*p*-dihydroxy paclitaxel (metabolite $M_C$) (Fig. 1). It has been suggested that CYP2C8 is the primary enzyme involved in the formation of 6α-hydroxypaclitaxel and CYP3A4 in the biotransformation of paclitaxel to 3′-p-hydroxypaclitaxel and 6α-3′-*p*-dihydroxypaclitaxel (5–8).

Several studies have shown that metabolite 6α-hydroxypaclitaxel is the principal metabolite accounting for over 26% of the administered dose of paclitaxel (5,9). However, there is significant inter-individual variability in paclitaxel metabolism. In some individuals, 3′-*p*-hydroxypaclitaxel may be the predominant metabolite (10). Clearly, factors affecting the catalytic activities of CYP2C8 and CYP3A4, including enzyme inhibition due to co-administration of drugs, could have significant influence on paclitaxel pharmacokinetics. This is especially important since paclitaxel follows saturable, non-linear pharmacokinetics (11). Even relatively minor quantitative variations in paclitaxel metabolism may lead to non-proportional increases in the area under the plasma concentration curve and $C_{max}$ of paclitaxel, resulting in unpredicted changes in the pharmacological drug effects (11,12). In this regard, anti-cancer agent cisplatin and multidrug resistance (MDR) reversing agent R-verapamil have been shown to significantly reduce paclitaxel clearance in humans (2,13). The purpose of this investigation was to examine the influence of several MDR reversing agents including R-verapamil, tamoxifen, CsA and anti-cancer drugs cisplatin, doxorubicin and VP-16, on paclitaxel metabolism in vitro.

## MATERIALS AND METHODS

### Materials

Human liver microsomes (HepatoSomes), were purchased from Human Biologics, Inc. (Phoenix, AZ, USA). Paclitaxel was obtained from the Developmental Therapeutics Program, National Cancer Institute (Bethesda, MD, USA).

Doxorubicin.HCl was a gift from Adria Laboratories Inc. (Columbus, OH, USA), etoposide from Bristol Myers Co., (Wallingford, CT, USA), R-verapamil from BASF Bioresearch Corporation (Cambridge, MA, USA), and cyclosporin A from Sandoz (Basel, Switzerland). *Cis*-platinum(II)diamine dichloride (cisplatin), NADP, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were purchased from Sigma Chemical Co. (St Louis, MO, USA). Other chemicals and solvents were obtained from commercial sources. Dr Robert Discordia (Bristol Myers Squibb Pharmaceutical Research Institute) kindly supplied reference standard 3′-*p*-hydroxypaclitaxel, and 6α-hydroxypaclitaxel was purchased from Gentest Corporation (Woburn, MA, USA).

### Incubation of paclitaxel with human liver microsomes

The metabolism of paclitaxel by human liver microsomes was carried out according to a published procedure (5). Microsomal protein (1 mg protein, ~0.34 nmol) was suspended in a final volume of 1 ml of sodium phosphate buffer (100 mM, pH = 7.4), $MgCl_2$ (10 mM), glycerol (20%), containing NADP (0.1 mM) and glucose 6-phosphate (1 mM). Paclitaxel was added to the incubation mixture to yield a range of

paclitaxel concentrations (5–100 μM). Paclitaxel stock solution was prepared in methanol. The reaction was initiated by the addition of glucose 6-phosphate dehydrogenase (1 unit/ml). The reaction mixtures were incubated at 37°C in a water bath with gentle agitation. In initial studies, replicate samples were used for several time points, and at 15 min time intervals, 2.5 ml ethyl acetate was added in each individual tube to extract unchanged paclitaxel and its metabolites. The concentration of the parent compound and the metabolites were analyzed using HPLC. Reaction velocity was determined for several paclitaxel concentrations. Employing Michaelis-Menten kinetics, $K_M$ values for the formation of each of the two metabolites were generated. Preliminary experiments showed that the concentrations of 6α-hydroxypaclitaxel and 3′-*p*-hydroxypaclitaxel increased linearly for a period of 1 h. Therefore, a time point of 1 h was chosen in our subsequent studies.

## Influence of other compounds on paclitaxel metabolism

The effects of pre-treatment and co-incubation of human liver microsomes with cisplatin on paclitaxel biotransformation were investigated. 10 μM paclitaxel was employed in all the inhibition experiments. In one set of experiments, cisplatin was incubated with the microsomes as described above in a final volume of 1 ml in the presence of the NADPH generating system for 15–30 min prior to the addition of paclitaxel. In the second set of experiments, cisplatin was added to the reaction buffer simultaneously with paclitaxel. The reaction was initiated by the addition of the NADPH-generating system in a final volume of 1 ml.

Similarly, the effect of simultaneous addition of R-verapamil, tamoxifen, CsA, doxorubicin and VP-16 was investigated. Quercetin and ketoconazole, which are known inhibitors of 6α-hydroxypaclitaxel and 3′-*p*-hydroxypaclitaxel, respectively, were included as controls. Stock solutions of tamoxifen citrate, and doxorubicin.HCl were prepared in PBS (pH 7.4). The stock solution of VP-16, R-verapamil, CsA, quercetin and ketoconazole were prepared in methanol.

## Sample preparation and quantitation of paclitaxel metabolites

Tubes containing aqueous microsomal suspensions and ethyl acetate were vortexed and centrifuged. The supernatant was collected and dried under a nitrogen stream. Residue was then dissolved in 250 μl of a mixture of methanol and water (ratio, 65:35%, v/v). Analysis of unchanged paclitaxel and its metabolites was carried out using a modification of a previously reported method (14). The HPLC system comprised a Waters model 510 pump, U6K injector, a Model 486 UV/VIS variable wavelength detector, and Millennium 2010 software. Injection of 100 μl was made onto a $C_{18}$ ODS analytical column (mBondapak, 100 × 300 mm, particle size 5 μm). Methanol-water combination (65:35%, v/v) was used as the solvent system at a flow rate of 1 ml/min. Paclitaxel and its metabolites were detected at a wavelength of 230 nm. The calibration curve for paclitaxel was constructed by adding known quantities of paclitaxel to human liver microsomal pellets followed by the above extraction procedure. The extraction efficiency of paclitaxel and its metabolites ranged from 87–89%. Due to lack of sufficient quantities of paclitaxel metabolites as reference standards, the two metabolites were quantitated using the standard curve for paclitaxel. As shown in earlier studies, extraction efficiencies and the molar extinction at 230 nm of the metabolites are nearly identical to that of pacitaxel (7,14). The HPLC method was found to be highly sensitive (detection limit of paclitaxel, 20 pmoles), precise and reproducible (co-efficients of intra-day and inter-day variation were less than 5%).

## Statistical analysis

Two-tailed Student's t-test for paired data was performed to calculate $P$ values; $P$ 0.05 was considered significant.

# RESULTS

## Microsomal metabolism of paclitaxel

A typical HPLC profile of paclitaxel and its metabolites extracted from microsomal incubations is shown in Figure 2. Using reference standards we confirmed that the two major metabolites with retention times of 9.8 and 7.2 min were 6α-hydroxypaclitaxel and 3′-*p*-hydroxypaclitaxel, respectively. The analysis of paclitaxel metabolites in microsomal incubations and in plasma samples from cancer patients is now well documented (5–10). The pattern of paclitaxel metabolism shown here is similar to those reported in several in vitro studies using human liver slices and microsomes. Analysis of plasma and bile from cancer patients have confirmed the presence of these two major metabolites (9,14).

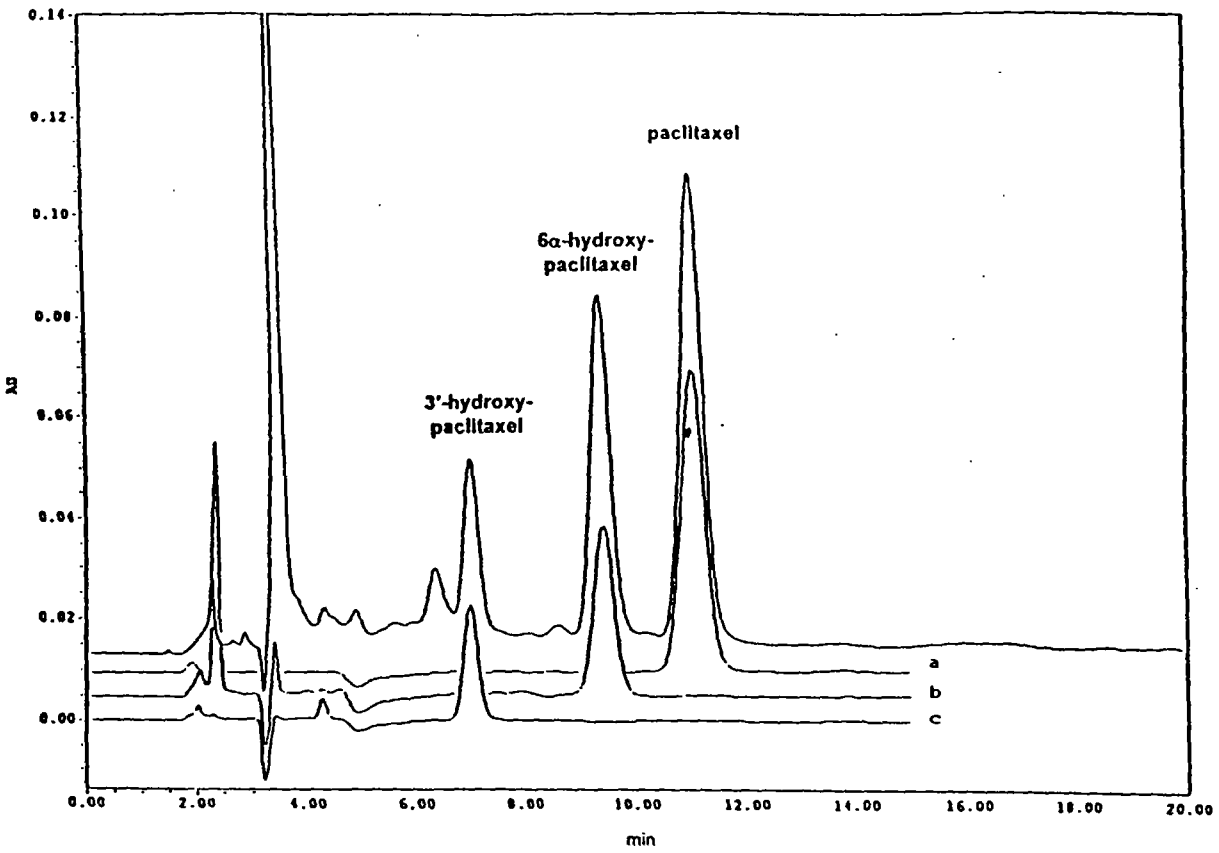



*Fig. 2* : Typical reversed-phase HPLC chromatogram of paclitaxel and its metabolites extracted from microsomal incubation. Chromatograms of reference standards are also shown: (a) paclitaxel; (b) 6α-hydroxypaclitaxel; and (c) 3'-*p*-hydroxypaclitaxel.

## Kinetic characterization of metabolism

Preliminary studies were conducted to determine the influence of the incubation period and the substrate concentration on paclitaxel biotransformation. The rates of formation of 6α-hydroxypaclitaxel and 3'-*p*-hydroxypaclitaxel were found to be linear up to 60 min. Therefore, an incubation period of 60 min was employed in all our subsequent studies. A Lineweaver-Burk plot of the formation of 6α-hydroxypaclitaxel and 3'-*p*-hydroxypaclitaxel (shown in Fig. 3) indicated that the metabolism could be characterized employing Michaelis-Menten kinetics. The range of substrate (paclitaxel) concentration employed was 5–100 mM. The calculated $K_M$ values were 26 and 21 μM for 6α-hydroxypaclitaxel and 3'-*p*-hydroxypaclitaxel, respectively. In order to maximize the chances of observing drug interactions, 10 μM paclitaxel, which is lower than the calculated $K_M$ values, was employed in further experiments.

## Studies on drug interaction

In Figure 4, the influence of several MDR reversing agents on the levels of 6α-hydroxypaclitaxel and 3'-*p*-hydroxypaclitaxel is shown. R-verapamil markedly inhibited the formation of both metabolites. For instance, the levels of 6α-hydroxypaclitaxel were 75 and 63% of the control, in the presence of 10 and 50 μM R-verapamil, respectively. Similarly, the levels of 3'-*p*-hydroxypaclitaxel were 70 and 21% of the controls in the presence of 10 and 50 μM R-verapamil, respectively. Tamoxifen lowered the levels of 3'-*p*-hydroxypaclitaxel to 85 and 48% of the control at 10 and 50 μM concentrations and at a concentration of 50 μM lowered the 6α-hydroxypaclitaxel levels to 55% of the control. CsA (5 μM) significantly ($P$ 0.005) inhibited the formation of 6α-hydroxypaclitaxel. The levels of 3'-*p*-hydroxypaclitaxel were not significantly (P 0.05) modulated in the presence of the indicated concentrations of CsA.

The influence of anti-cancer drugs is shown in Figure 5. First, the effect of simultaneous addition of cisplatin to the incubation mixture as well as pre-treatment with cisplatin was examined. As shown, simultaneous presence of cisplatin did not significantly modulate the levels of 6α-hydroxypaclitaxel or 3′-p-hydroxypaclitaxel. Similarly, addition of cisplatin up to 30 min prior to the addition of paclitaxel also did not alter the levels of the two metabolites (data not shown). Doxorubicin and VP-16 exhibited variable effects on the two metabolites. Doxorubicin (50 μM) significantly lowered the levels of 3′-*p*-hydroxypaclitaxel, but had no significant influence on 6α-hydroxypaclitaxel ($P$ 0.05). VP-16 (10 and 50 μM) lowered the levels of 6α-hydroxypaclitaxel to 86 and 84% (relative to controls), respectively. While 50 μM VP-16 lowered the levels of 3′-p-hydroxypaclitaxel to 70%, the influence at 10 μM was found to be statistically insignificant (P 0.05).

## DISCUSSION

Recent studies have elucidated the role of CYP2C8 and CYP3A4 in the enzymatic pathways of paclitaxel biotransformation (5–8). Cytotoxicity studies employing human myeloid leukemia HL-60 cells have revealed that the metabolism of paclitaxel results in loss of anti-cancer activity (15). Several pharmacokinetic and pharmacodynamic studies have linked paclitaxel plasma concentrations and AUC to toxicities such as neutropenia and neurotoxicity (11,12). Thus, drug interactions resulting from the inhibition of cytochrome P450 enzymes can potentially result in severe consequences, such as increased toxicities

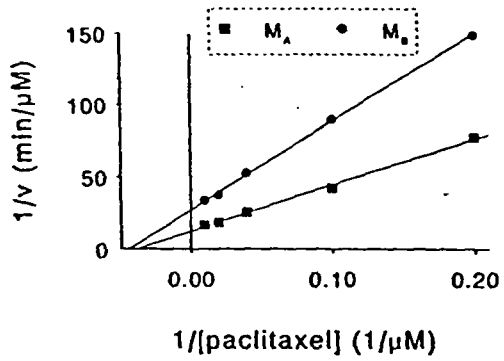



*Fig. 3* : Lineweaver Burk plot of paclitaxel metabolism by human liver microsomes. Microsomal protein (equivalent to 0.34 nmol CYP) was incubated with increasing concentrations of paclitaxel for 1 h at 37°C. Results shown are from a typical experiment performed employing duplicate samples.

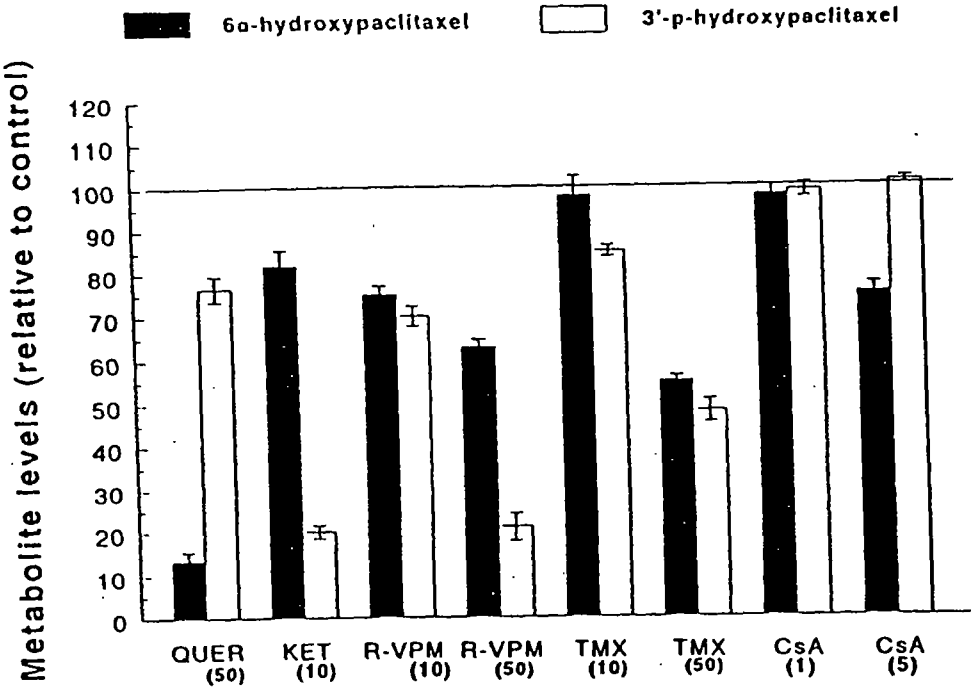



*Fig. 4* : Percent inhibition (relative to control) of paclitaxel conversion to 6α-hydroxypaclitaxel and 3′-*p*-hydroxypaclitaxel in the presence of MDR reversing agents. Duplicate incubations of paclitaxel (10 μM) with or without R-verapamil (R-VPM, 10 and 50 μM), tamoxifen (TMX, 10 and 50 μM) and cyclosporin A (CsA 1 and 5 μM) were performed. Incubations with quercetin (quer, 50 μM) and ketoconazole (ket, 10 μM), known inhibitors of 6α-hydroxypaclitaxel and 3′-p-hydroxypaclitaxel, respectively, were included as controls.

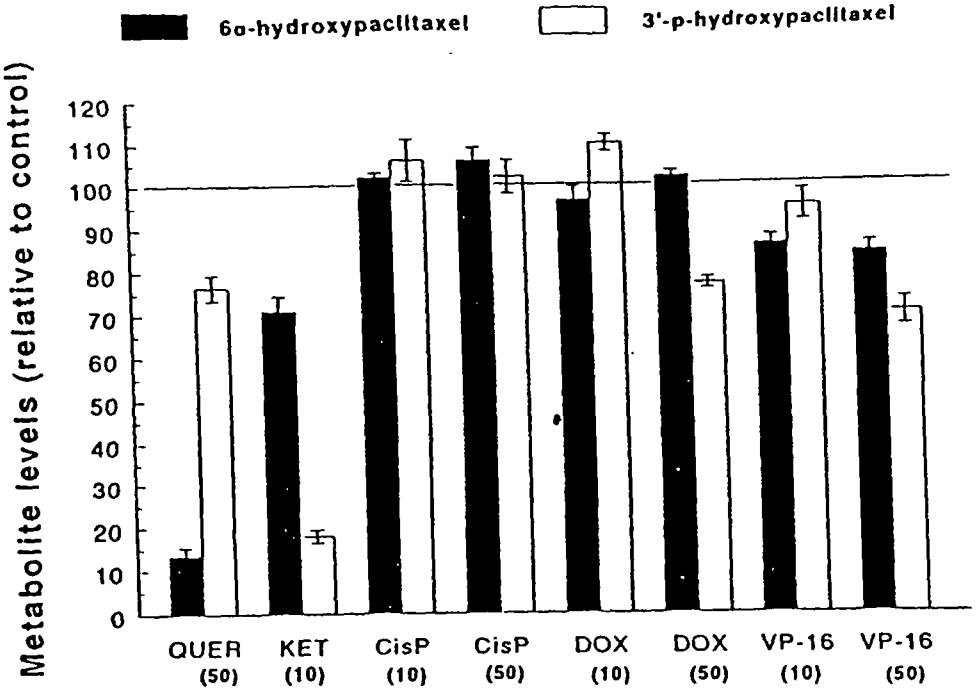



*Fig. 5* : Percent inhibition (relative to control) of paclitaxel conversion to 6α-hydroxypaclitaxel and 3′-*p*-hydroxypaclitaxel by various anticancer drugs. Duplicate incubations of paclitaxel (10 μM) with or without cisplatin (CisP, 10 and 50 μM), doxorubicin (DOX, 10 and 50 μM), and etoposide (VP-16, 10 and 50 μM) were performed. Incubations with quercetin (quer, 50 μM) and ketoconazole (ket, 10 μM), known inhibitors of 6α-hydroxypaclitaxel and 3′-*p*-hydroxypaclitaxel, respectively, were included as controls.

associated with paclitaxel therapy. In this study, we investigated if any of the compounds currently being used or investigated in combination therapy with paclitaxel can alter its metabolism.

The novel finding of this study is the observed influence of MDR reversing agents tamoxifen, R-verapamil and CsA on paclitaxel metabolism. One of the major mechanisms of MDR is the over-expression of a transport protein called P-glycoprotein, which participates in active cellular drug efflux (16). Paclitaxel is a known substrate of P-glycoprotein (17). A number of studies have indicated that R-verapamil, tamoxifen and CsA inhibit P-glycoprotein mediated drug efflux, and may be clinically useful in reversing resistance to several anti-cancer drugs including paclitaxel (13,18,19).

Studies on the influence of tamoxifen on paclitaxel metabolism have not been reported. Tamoxifen is widely used as an adjuvant in the treatment and prevention of breast cancer. In this study, it was observed that tamoxifen inhibited the formation of both paclitaxel metabolites. Tamoxifen has a long elimination half-life (7 days) and persists in the body for an extended period of time (20). Therefore, the potential influence of tamoxifen on paclitaxel clearance in breast cancer patients deserves further consideration.

Although verapamil has been shown to inhibit paclitaxel metabolism in earlier studies, the influence of the R-enantiomer has not been reported (6,21). In a clinical study by Berg et al., it was observed that R-verapamil significantly reduced paclitaxel clearance (13). The mechanism of this interaction is not understood. Based on our studies, it is likely that the inhibition of paclitaxel conversion to 6α-hydroxypaclitaxel and 3′-*p*-hydroxypaclitaxel may result in reduced paclitaxel clearance in vivo.

Although studies on the influence of CsA on paclitaxel metabolism have been reported earlier, there is significant variability in the reported findings. Harris et al. observed that CsA concentrations in the range of 2–100 μM significantly reduced the levels of 3′-*p*-hydroxypaclitaxel in microsomal incubations, but caused a modest decrease in the 6α-hydroxypaclitaxel levels only at high concentrations of 50 and 100 μM (7). Kumar et al. did not observe a significant influence of CsA (50 μM) on paclitaxel metabolism (6). Contrary to these findings, Sonnichsen et al. observed that CsA (50 μM) profoundly inhibited formation of 6α-hydroxypaclitaxel and 3′-*p*-hydroxypaclitaxel (10). In our study, we utilized significantly

lower concentrations of CsA (1–5 μM). While at these concentrations we did not observe a significant inhibition of 3′-*p*-hydroxylation, 5 μM CsA reduced 6α-hydroxypaclitaxel levels to a significant extent. It is important to note that previous studies have shown that CsA inhibits in vitro metabolism and also reduces the in vivo clearance of VP-16, a CYP3A4 substrate (22). It is feasible that such interactions may also occur with paclitaxel. It is important to note that, although the three MDR modulators used in this study are known CYP3A4 substrates, a significant influence on the 6α-hydroxypaclitaxel, a CYP2C8 metabolite, was observed (23–25).

Paclitaxel is routinely used with other anti-cancer agents such as cisplatin, doxorubicin and VP-16. Rowinsky et al. have reported a sequence-dependent influence of cisplatin on paclitaxel (2). The administration of cisplatin before paclitaxel was observed to induce more profound neutropenia than that observed with the alternate sequence. This was related to a significant decrease in paclitaxel clearance when patients were pre-treated with cisplatin. Due to this interaction, the clinically recommended sequence is paclitaxel infusion followed by cisplatin. It was hypothesized that this interaction may entail inhibition of paclitaxel metabolism. Our study shows that, even at fairly high concentrations, cisplatin did not competitively inhibit the biotransformation of paclitaxel in the microsomal system. However, since cisplatin is known to modulate the expression of isozymes CYP2C11 and CYP2C12 in rats (26), the possibility that the clinically observed effect of cisplatin pre-treatment on paclitaxel clearance may entail indirect suppression of CYP2C8 expression, cannot be ruled out.

Doxorubicin and etoposide were included in our studies since isozymes of CYP2C and CYP3A subfamilies are reportedly involved in the metabolism of these agents. Relling et al. have reported that catechol formation by O-demethylation of VP-16 is primarily mediated by CYP3A4 (27). Doxorubicin metabolism is complex but studies have suggested that CYP3A plays a role in the biotransformation (28). In agreement with studies reported by Sonnichsen et al. and Royer et al., we observed that doxorubicin (50 μM each) significantly inhibited paclitaxel metabolism to 3′-*p*-hydroxypaclitaxel (10,21). Although Royer et al. observed a profound decrease in 6α-hydroxypaclitaxel levels in the presence of doxorubicin, this was not noticed in our studies (21). The observed effect of VP-16 appears to be consistent with the earlier study where Sonnichsen et al. reported considerable inhibition of paclitaxel metabolism by VP-16 when employed at a concentration of 500 μM (10). In comparison, at significantly lower levels (10 and 50 μM) we noted a modest, but statistically significant, influence of VP-16 on the formation of 6α-hydroxypaclitaxel and 3′-*p*-hydroxypaclitaxel.

The above mentioned differences in findings of this study and those reported by other investigators could be related to differences in the catalytic activity of the microsomal preparations and the concentrations of the interacting compounds. The concentration of compounds used in our study were considerably lower than those employed by the other investigators. Nonetheless, the concentrations we used are in excess of normally achieved serum levels. This was done with two considerations. First, the hepatic concentrations of compounds such as tamoxifen, R-verapamil and paclitaxel are markedly higher than the respective plasma levels (29–31). For instance, tamoxifen levels in the human liver may be as much as 60-fold higher than the serum levels (29) and paclitaxel levels in rat liver tissue were found to be 160-fold higher than in plasma (31). Therefore, consideration of only the plasma/serum drug concentrations could markedly underestimate the potential for drug interactions. Also, the catalytic activity of microsomes employed in our study was lower compared to other reports. The reported $K_M$ values for 6α-hydroxypaclitaxel range from 4–21 μM (5–9) and the reported $K_M$ for 3′-*p*-hydroxypaclitaxel is 15 μM (5). Since there is significant inter-individual variability associated with paclitaxel metabolism, it is possible that individuals with higher catalytic activity may exhibit more pronounced drug interactions at lower concentrations of the interacting drugs.

In summary, our studies emphasize the possibility of drug interaction that may result from inhibition of paclitaxel metabolism. Previous studies on inter-individual variability in paclitaxel metabolism have suggested that 6α-hydroxypaclitaxel, catalyzed by CYP2C8, is not the predominant metabolite in all individuals and that other CYP3A4 catalyzed hydroxylated products also significantly contribute to overall paclitaxel biotransformation in vivo. Therefore, the influence of factors affecting the activity of both isozymes on paclitaxel metabolism should be carefully considered. Since hepatic elimination is a principal route of systemic elimination of paclitaxel, some of the compounds used in combination therapy may alter the pharmacokinetics of paclitaxel and, potentially, its pharmacodynamics.

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# CYTOCHROME P-450 3A AND 2D6 CATALYZE *ORTHO* HYDROXYLATION OF 4-HYDROXYTAMOXIFEN AND 3-HYDROXYTAMOXIFEN (DROLOXIFENE) YIELDING TAMOXIFEN CATECHOL: INVOLVEMENT OF CATECHOLS IN COVALENT BINDING TO HEPATIC PROTEINS

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**ABSTRACT:**

**Earlier study suggested that 3,4-dihydroxytamoxifen (tam catechol), a tamoxifen metabolite, is proximate to the reactive intermediate that binds covalently to proteins and possibly to DNA (Dehal and Kupfer, 1996). The current study demonstrates that rat and human hepatic cytochrome P-450s (CYPs) catalyze tam catechol formation from tamoxifen (tam), 3-hydroxy-tam (Droloxifene), and 4-hydroxy-tam (4-OH-tam). Higher levels of catechol were formed from 4-OH-tam and 3-hydroxy-tam than from tam. Evidence that human hepatic CYP3A4 and 2D6 catalyze the formation of tam catechol from 4-OH-tam and supportive data that the catechol is proximate to the reactive intermediate, was obtained: 1) There was a good correlation ($r = 0.82$; $p \leq .0004$) between steroidal 6β-hydroxylase (CYP3A activity) and *ortho* hydroxylation of 4-OH-tam in human liver microsomes; 2) monospecific antibodies against CYP3A4 strongly inhibited catechol formation from 4-OH-tam and its covalent binding to proteins in human liver microsomes; 3) low levels of ketoconazole inhibited catechol tam accumulation and covalent binding of 4-OH-tam to human liver proteins; 4) among human P-450s expressed in insect cells (supersomes), only CYP3A4 and 2D6 noticeably catalyzed catechol formation, and cytochrome $b_5$ markedly stimulated the CYP3A4 catalysis; and 5) human livers with high CYP3A and low or high CYP2D6 activity exhibited high catechol formation and those with low 3A and 2D6 activities formed only little catechol. These findings demonstrate that CYP3A4 and to a lesser extent 2D6 catalyze tam catechol formation and support the participation of tam catechol in covalent binding to proteins.**

Tamoxifen (tam)[1], a member of the triphenylethylene (TPE) class of compounds that exhibits antiestrogenic activity, is the current endocrine therapeutic agent of choice for all stages of breast cancer (Jordan, 1993). Recent large scale clinical trials of tam as a chemopreventive prophylactic agent in women considered at risk of breast cancer demonstrated protection in 45% of the subjects (Fisher et al., 1998) and resulted in its approval by the Food and Drug Administration for that purpose. Of concern, however, are the findings that tam increases the incidence of human endometrial cancer and causes hepatocellular carcinoma and a high abundance of *p53* mutations in rats (Killackey et al., 1985; Williams et al., 1993; Vancutsem et al., 1994). Tam is not mutagenic in microbial systems and the mechanism of its carcinogenic activity is not understood; nevertheless, the possibility that tam metabolites are involved in carcinogenesis has been considered (King, 1995; Dehal and Kupfer, 1996). In view of these concerns, several TPE derivatives (possibly devoid of uterine carcinogenic activity) are being developed as therapeutic agents against breast cancer, among these 3-hydroxy-tamoxifen (3-OH-tam; Droloxifene).

The major metabolites of tam in mammalian liver are tam-*N*-oxide, mono-*N*-desmethyl-tam and 4-hydroxy-tam (4-OH-tam; Foster et al., 1980; Reunitz et al., 1984; McCague and Seago, 1986; Fig. 1). Whereas tam-N-oxidation is catalyzed primarily by the flavin-containing monooxygenase (Mani et al., 1993a), certain cytochrome P-450s (CYPs) also catalyze that reaction (Dehal and Kupfer, 1997). Tam N-demethylation is primarily catalyzed by hepatic CYP3A (Jacolot et al., 1991; Mani et al., 1993b) and although the mammalian enzyme catalyzing tam-4-hydroxylation resisted identification, $P\text{-}450_{TCDD}$ isolated from livers of TCDD-treated chick embryos effectively catalyzed that reaction (Kupfer et al., 1994). Recently, it was suggested that that human hepatic CYP2D6, 2C9, and 3A4 catalyze tam-4-hydroxylation (Crewe et al., 1997). By contrast, our studies

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[1] Abbreviations used are: tam, tamoxifen [Z-1-(4-(2-dimethylaminoethoxy)-phenyl]-1,2-diphenyl-1-butene]; 4-OH-tam, 4-hydroxy-tamoxifen; 3,4-di-OH-tam, 3,4-dihydroxy-tamoxifen (tam catechol); 3-OH-tam, 3-hydroxy-tamoxifen (Droloxifene); CYP, cytochrome P-450; TPE, triphenylethylene; SAM, *S*-adenosyl-L-methionine iodide salt; COMT, catechol-*O*-methyltransferase; PB, phenobarbital; TAO, troleandomycin; Dex, dexamethasone; KZ, ketoconazole; RI, reactive intermediate; DTT, dithiothreitol; [$^3$H]-SAM, [$^3$H-methyl]-*S*-adenosyl-L-methionine; TLC, thin-layer chromatography; IIAM, International Institute for the Advancement of Medicine.

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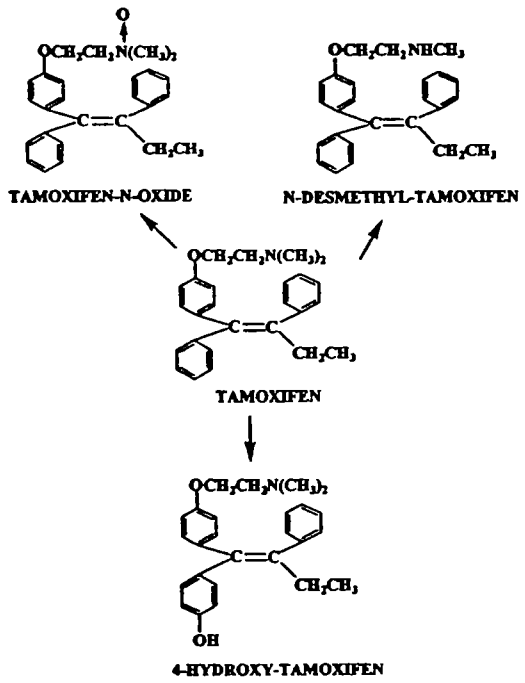


FIG. 1. *Major pathways of tam metabolism.*

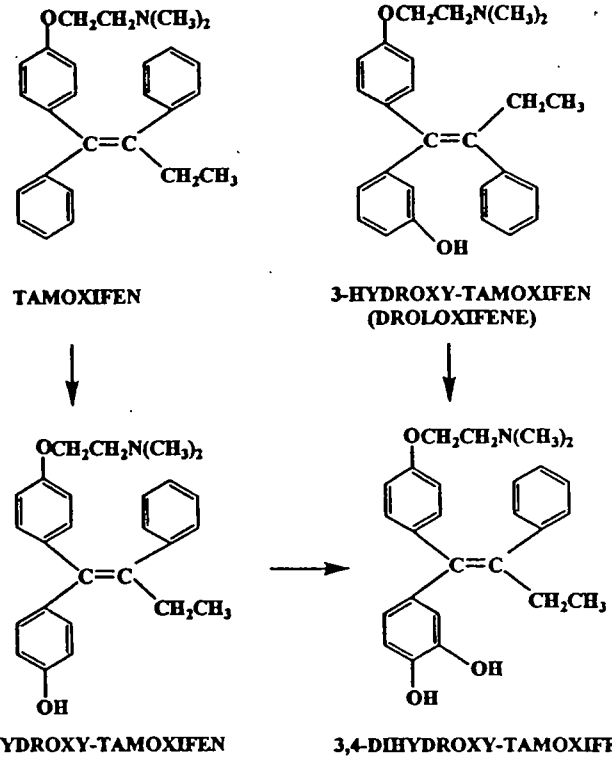


FIG. 2. *Pathway of 3,4-di-OH-tam (catechol) formation from tam, 4-OH-tam, and 3-OH-tam (Droloxifene).*

with several systems (human liver microsomes and cDNA-expressed human P-450s) demonstrated that tam-4-hydroxylation is catalyzed by CYP2D6 and that the other CYPs were inactive (Dehal and Kupfer, 1997).

Tam undergoes metabolic activation by hepatic P-450 enzymes, resulting in reactive intermediates (RIs) that bind covalently to microsomal proteins (Mani and Kupfer, 1991; Kupfer, 1996). Although the ultimate RI has not been identified, it appears that **4-OH-tam** is on the path of formation of the RI that binds covalently to proteins (Dehal and Kupfer, 1996) and possibly to DNA (Randerath et al., 1994; Pathak et al., 1995). Recently, we demonstrated that **4-OH-tam** undergoes *ortho* hydroxylation catalyzed by hepatic microsomes, yielding 3,4-dihydroxy-tamoxifen (3,4-di-OH-tam) catechol that appears proximate to the RI that binds covalently to proteins (Dehal and Kupfer, 1996). Our earlier observations that CYP3A catalyzes hydroxylation *ortho* to the phenolic moiety of several compounds (Stresser and Kupfer, 1997) and that CYP3A is involved in covalent binding of tam to proteins (Mani et al., 1994) suggested that this enzyme could transform **4-OH-tam** and 3-OH-tam into tam catechol (Fig. 2) and catalyze subsequent covalent binding.

The current study demonstrates that CYP3A enzymes (human and rat) and human CYP2D6 catalyze the hydroxylation *ortho* to the phenolic moieties of TPEs, exemplified by the hydroxylation of **4-OH-tam** and 3-OH-tam. The catalytic activity of CYP3A toward certain substrates is often increased by the presence of cytochrome $b_5$ (Yamazaki et al., 1996) and we observed that cytochrome $b_5$ markedly stimulates the CYP3A4 catechol formation. Additionally, indirect evidence is provided that the catechols are proximate metabolites to the RI that binds covalently to proteins.

## Materials and Methods

NADPH, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, EDTA, dexamethasone (Dex), catechol-*O*-methyltransferase (COMT), troleandomycin (TAO), and *S*-adenosyl-L-methionine iodide salt (SAM) were purchased from Sigma Chemical Co. (St. Louis, MO). Dithiothreitol (DTT) was obtained from Calbiochem (La Jolla, CA). [$^{14}$C-Ring-labeled]tamoxifen citrate (21.1 mCi/mmol; currently available only through custom synthesis) and [$^{3}$H-methyl]-*S*-adenosyl-L-methionine ([$^{3}$H]-SAM, 15 Ci/mmol) was obtained from DuPont-NEN (Boston, MA). Droloxifene was purchased from Research Biochemicals International (Natick, MA). Phenobarbital (PB) sodium salt was obtained from Mallinckrodt (St. Louis, MO). cDNA-expressed human P-450s in baculovirus-infected insect cell line (supersomes) and in lymphoblasts were purchased from Gentest Corporation (Woburn, MA). Human liver microsomes were obtained from the International Institute for the Advancement of Medicine (IIAM; Exton, PA). Ultima-Gold biodegradable scintillation fluid was obtained from Packard Instrument Co., Inc. (Downers Grove, IL). Normal phase thin-layer chromatography (TLC) plates, containing fluorescent indicator and preadsorbent strip, were purchased from Whatman, Inc. (Clifton, NJ). All other chemicals were of reagent grade quality and were used without further purification.

**Animals and Treatment.** Sprague-Dawley CD rats (90–100 g), from Charles River Breeding Laboratories (Wilmington, MA), were housed under controlled temperature (22°C) and light (12-h light/dark cycle; lights off at 7:00 PM.). Rats were injected with PB (37.5 mg/kg i.p. in 0.2 ml water, twice daily) for 4 days and liver microsomes (PB-microsomes) were prepared 12 h after the last dose. A second group of rats was treated with Dex (50 mg/kg in 1.0 ml corn oil daily for 3 days) and liver microsomes (Dex-microsomes) were prepared 24 h after the last injection. Control animals from each treatment group received the same regimen of the respective vehicle only.

**Preparation of Liver Microsomes.** Rat livers were homogenized in 0.25 M sucrose (5 ml/g liver) at 4°C and microsomes were prepared by differential centrifugation and, unless noted otherwise, represent a pool of four to eight livers (Burstein and Kupfer, 1971; Dehal and Kupfer, 1996).

Human liver microsomes, prepared and characterized with respect to their P-450 isoform enzymatic activities, were purchased from IIAM and were used as such.

**Incubations: Covalent Binding Assay.** Rat liver microsomes suspended in fresh 1.15% KCl solution were incubated with [$^{14}$C]-tam or [$^{14}$C]-4-OH-tam as described previously (Dehal and Kupfer, 1996; Kupfer and Dehal, 1996). The enzyme-catalyzed reaction was terminated by adding 10 ml of ethanol. The aqueous ethanolic solution was filtered through a 2.4-cm Whatman GF/C glass microfiber filter (Whatman, Ltd., Maidstone, Kent, England) in a filter holder (Schleicher & Schuell, Inc., Keene, NH) attached to a vacuum filter flask. The

filter containing trapped protein precipitate was rinsed with ethanol (20 ml) and methanol (10 ml) followed by various other organic solvents to remove the loosely bound tam metabolites, as described previously (Mani et al., 1993b; Kupfer and Dehal, 1996). To elute the proteins, the filter was placed in a 20-ml scintillation vial containing 2 ml of 2% aqueous SDS solution and incubated at 37°C for 2 h. The solution was transferred into a 12- × 75-mm glass culture tube and the vial containing the filter paper was rinsed with an additional 1 ml of 2% aqueous SDS solution. The combined SDS solution was processed as illustrated previously (Dehal and Kupfer, 1996). An aliquot of the SDS solution was analyzed for radioactivity by scintillation spectrometry and the rest was used for protein determination. The covalent binding of activated tam metabolites is expressed as picomoles tam equivalents bound per milligram of protein in the SDS solution.

Human liver microsomes from HAM were thawed and used as such in incubations described above for rat liver microsomes.

**Analysis of Tamoxifen Metabolites.** The combined alcoholic filtrate from above was evaporated to dryness under a stream of nitrogen at ambient temperature. The residue was taken up in 2.0 ml ethanol and the radioactivity of an aliquot (10 μl) in duplicate was determined in a Packard Tri-Carb 460 CD liquid scintillation spectrometer using an automatic quench correction curve previously generated with a series of quenched $^{14}C$ and $^{3}H$ standards. Routinely, 10 to 20% of the ethanolic sample was used for chromatographic separation and quantification of metabolites on TLC and the rest of the sample was stored at 0–4°C under argon for repetition of TLC or for future needs. Chromatographic separation was performed on Whatman silica gel TLC plates and developed in $CHCl_3/CH_3OH/NH_4OH$ (80:20:0.5 v/v/v), slightly modified from the previously described system (Reunitz et al., 1984). Radiolabeled metabolites on TLC were quantified with a System 2000 Imaging Scanner (Bioscan, Inc., Washington, DC).

**Preparation of Radiolabeled 4-OH-Tam.** [$^{14}C$]-Tam (200,000 dpm, 100 nmol) was incubated as described above with liver microsomes from untreated adult chickens in the presence of NADPH-regenerating system for 60 min (Mani et al., 1994). The radiolabeled 4-OH-tam, the major metabolite formed by chicken liver microsomes corresponding chromatographically to authentic radioinert 4-OH-tam, was eluted off a TLC plate with ethanol and purified further on TLC using the above solvent system. Because of the photo-lability of 4-OH-tam, the experiments were carried out in subdued light.

**Assay of 3,4-Di-OH-tam.**[2] Microsomal suspension (1.0 mg protein or as indicated) was added to 0.6 ml of sodium phosphate buffer (pH 7.4; 60 μmol) containing EDTA (0.1 μmol); 0.1 ml aqueous solution of $MgCl_2$ (10 μmol); radioinert tam, 4-OH-tam (25 or 100 nmol), or 3-OH-tam (25 nmol) in 10 μl of ethanol; DTT (50 nmol in 10 μl); (in rat liver microsomes there appears sufficient COMT to yield optimal methylation, however, with human microsomes, additional COMT was necessary, hence 150 IU in 15 μl was added); [$^{3}H$]-SAM (1 μCi, 200 nmol in 12 μl $H_2O$) and water to a final volume of 1.0 ml (or as stated in *Results*). After preincubation at 37°C for 2 min, the reaction was initiated by adding the NADPH-regenerating system (as above in *Incubations: Covalent Binding Assay*) in 0.1 ml of sodium phosphate buffer (pH 7.4; 10 μmol) and the vials were placed at 37°C in a water bath shaker for 30 min. To terminate the reaction the incubation mixture was placed on ice. The aqueous phase was extracted with ice-cold hexane (2 × 3 ml) by thorough mixing with a vortex. The resulting mixture was centrifuged and the hexane phase was removed. The combined hexane phase was back-washed with 2 ml of water, and after centrifugation the aqueous phase was discarded and an aliquot of the hexane phase containing the monomethylated catechol was taken for radioactivity determination in a scintillation spectrometer (Hoffman et al., 1980; Kupfer et al., 1990; Dehal and Kupfer, 1996).

[2] Because 3,4-di-OH-tam catechol is not an end product of tam metabolism, but is further transformed and thus cannot be accurately assessed by chemico/physical means, we used a method that permits methylation of the formed catechol in situ, using endogenous or exogenous COMT and $^{3}H$-SAM, and the resulting radiolabeled catechol is quantified by scintillation spectroscopy. The value for catechol formation may represent an underestimation, because of competing oxidative reaction resulting in formation of quinones. However, if the rate of catechol oxidation is much slower than the COMT-mediated methylation, then catechol underestimation would be insignificant.

TABLE 1

*Formation of catechol products from tam, 4-OH-tam, and 3-OH-tam (Droloxifene) catalyzed by liver microsomes from PB- and Dex-treated rats*

| | Microsomes | Time | Substrate | Methylated catechol |
|---|---|---|---|---|
| | | *min* | | *pmol/mg protein* |
| Expt. 1 | PB | 15 | tam | 100 ± 7 |
| | PB | 30 | tam | 194 ± 7 |
| | PB | 15 | 4-OH-tam | 707 ± 55 |
| | PB | 30 | 4-OH-tam | 925 ± 128 |
| | PB | 15 | 3-OH-tam | 866 ± 70 |
| | PB | 30 | 3-OH-tam | 954 ± 114 |
| Expt. 2 | Dex | 30 | tam | 226 |
| | Dex | 30 | 4-OH-tam | 1357 |
| | Control | 30 | 4-OH-tam | 842 |
| | PB | 30 | tam | 288 |
| | PB | 30 | 4-OH-tam | 1159 |

Rat liver microsomes (1.0 mg) containing different substrates (25 μM) were incubated in th presence of [$^{3}H$]SAM (1.0 μCi/200 nmol), DTT (50 μM), and NADPH-regenerating system i a final volume of 1.0 ml, at 37°C. Values represent a mean ± S.D. of triplicate (Expt. 1) and mean of duplicate (Expt. 2) measurements.

**Immunoinhibition Studies.** To determine whether *ortho* hydroxylation an covalent binding of 4-OH-tam are catalyzed by P-4503A enzymes, variou amounts of serum from preimmune and from immunized rabbits against CYI 3A4 (containing characterized monospecific anti-CYP3A4 antibodies) wer preincubated with human liver microsomes at room temperature (~25°C) fo 30 min and after incubation for 30 min at 37°C the enzymatic activity wa determined as above.

## Results

The broad specificity of hepatic microsomal COMT toward a variety of catechol substrates (Hoffman et al., 1980; Kupfer et al., 1990; Dehal and Kupfer, 1996) suggested that it would be possible to detect formation of catechol-TPEs in situ by the COMT-catalyzed radiolabeled methylation, using [$^{3}H$]-SAM. Indeed, incubation of 4-OH-tam or 3-OH-tam with PB- and Dex-rat liver microsomes and NADPH in the presence of [$^{3}H$]-SAM generated the radiolabeled monomethylated tam catechol (Table 1). The monomethylated catechol isolated from incubations of tam, 3-OH-tam, or 4-OH-tam with liver microsomes was chromatographically identical on TLC (not shown), suggesting that the three compounds form the same 3,4-tam catechol. Furthermore, mass spectrometric analysis (electrospray) of the monomethylated catechol [$M^+ - 31(OCH_3) + 1 = 387$] and the GC/MS/EI of the trimethylsilyl ether of the monomethylated catechol [$M^+ - 31(OCH_3) = 459$; $M^+ - 103(tms + OCH_3) = 387$] supported the assignment of a monomethyl ether of tam-catechol. However, the analysis did not distinguish between the sites of methylation. Catechol formation from tam, 3-OH-tam, and 4-OH-tam by PB-microsomes was linear as a function of microsomal protein concentration; however, it was not directly proportional (not shown). The finding that SKF 525A, metyrapone, and benzylimidazole (inhibitors of P-450) blocked the formation of tam catechols from 4-OH-tam (Table 2) indicated that the *ortho* hydroxylation of 4-OH-tam is catalyzed by P-450 enzyme(s).

An increase in the formation of tam catechol from 4-OH-tam over that obtained with tam by liver microsomes from Dex-treated rats and the findings that CYP3A enzymes effectively catalyze the *ortho* hydroxylation of phenolic compounds (Stresser and Kupfer, 1997) suggested that CYP3A enzymes are involved in tam catechol formation. To obtain further evidence for CYP3A participation in tam catechol formation, troleandomycin (TAO), a mechanism based inhibitor of CYP3A, was incorporated in the incubations. Although TAO inhibited the 6β-hydroxylation of testosterone, there was only a

TABLE 2

*Inhibition by P-450 inhibitors of catechol formation from 4-OH-tam using liver microsomes from PB-treated male rats*

| Additions | Methylated catechol | % Inhibition |
|---|---|---|
| | *pmol/mg protein* | |
| | 1339 ± 36 | |
| SKF525A (1.0mM) | 540 ± 21* | 60 |
| Metyrapone (0.5mM) | 306 ± 28* | 77 |
| Benzylimidazole (0.1mM) | 935 ± 55* | 30 |

Liver microsomes (1.0 mg protein) were incubated with 4-OH-tam (10 nmol) in the presence of [$^3$H]SAM (1.0 μCi/200 nmol), DTT (50 μM), and NADPH-regenerating system in a final volume of 1.0 ml, for 30 min at 37°C. Values represent a mean ± S.D. of triplicate measurements.

* $p \leq .0001$.

minimal inhibition of tam catechol formation (not shown). The possibility that the lack of inhibition by TAO was due to the dissociation of the CYP3A-TAO complex by the catechol was considered. The unavailability of a sufficient amount of 3,4-di-OH-tam led to experiments with another catechol (1,2-dihydroxybenzene) as a model. However, 1,2-dihydroxybenzene did not diminish the TAO-mediated inhibition of 6β-hydroxylation of testosterone, indicating that the benzene catechol does not split the complex. Thus, the evidence for CYP3A involvement in tam catechol formation in rats has remained equivocal. A more direct method to demonstrate rat CYP3A involvement in catechol formation would require the use of purified and reconstituted CYP3A. However, the reconstitution of rat CYP3As has been problematic (Eberhart and Parkinson, 1991). Consequently, we resorted to the use of human CYP3A isoforms, which are available in stable and active form, expressed with or without cytochrome $b_5$, in mammalian or insect cell lines.

The above findings raised the possibility that human livers could catalyze tam catechol formation from phenolic tam derivatives (e.g., 3-OH-tam and 4-OH-tam). Incubations of human liver microsomes (from 14 donors) with 4-OH-tam generated tam catechol (Fig. 3A and B). Human liver microsomes demonstrated a good correlation ($r$ = 0.82, $p \leq .0004$) between 6β-hydroxylation of testosterone (CYP3A activity) and tam catechol formation (Fig. 3B), suggesting CYP3A4 and/or 3A5 involvement. By contrast, the correlation of dextromorphan *O*-demethylation (2D6 activity) and tam catechol formation by human liver microsomes was not significant ($r$ = 0.05) .Additionally, liver microsomes with high CYP3A activity exhibited high catechol formation from 4-OH-tam (Table 3); a notable exception was donor #80, that had low 3A activity but high 2D6 activity, suggesting the involvement of 2D6 in the catalysis (for further evidence see below). Of interest is the observation that human liver microsomes converted 3-OH-tam into tam catechol (Table 3). The formation of tam catechol from 3-OH-tam (approximately 8 nmol/mg protein) was higher than from 4-OH-tam (1.7–3.2 nmol/mg protein), indicating that 3-OH-tam is a better substrate than 4-OH-tam. Because the experiment used liver microsomes with high 3A4 and low 2D6 activity and liver microsomes with low 3A4 and high 2D6, it appears that 3-OH-tam is a better substrate for both enzymes.

To further ascertain that in human liver CYP3A4 catalyzes the *ortho* hydroxylation of 4-OH-tam, the effect of monospecific anti-CYP3A4 antibodies on tam catechol formation was examined using liver microsomes with high CYP3A activity (Fig. 4). The anti-CYP3A4 antibodies displayed strong inhibition (>80%) of the transformation of 4-OH-tam into tam catechol, indicating that 3A4 catalyzes the major portion of the *ortho* hydroxylation in this preparation of human liver.

To delineate whether other P-450 isoforms in addition to CYP3A4 exhibit *ortho* hydroxylation of 4-OH-tam, individual cDNA-expressed human P-450s in baculovirus-infected insect cell line (supersomes) were examined. CYP3A4 and to a lesser extent 2D6 were found to be active (Fig. 5A), however, little or no activity was observed with CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C19, 3A5, or 4A11. Furthermore, cytochrome $b_5$ (coexpressed with 3A4) markedly stimulated CYP3A4 activity and had only little or no quantitative effect on other CYPs examined (Fig. 5B). The unavailability of 2D6 supersomes, with coexpressed $b_5$, precluded the determination of whether coexpressed $b_5$ would stimulate 2D6 activity. The examination of cDNA-expressed P-450s in lymphoblasts toward tam catechol formation from 4-OH-tam demonstrated that CYP3A4 was by far the most active and 2D6 had some activity; however, other P-450s exhibited little or no activity (not shown), providing further support for the above conclusion.

Because CYP2D6 catalyzes the 4-hydroxylation of tam (Dehal and Kupfer, 1997; Crewe et al., 1997) and the 3-hydroxylation of 4-OH-tam (this study), it seemed conceivable that the second hydroxylation could occur sequentially while the newly formed 4-OH-tam resides at the enzyme-active site. This suggested that 2D6 would form tam catechol from tam. However, there was no detectable formation of the catechol from tam in supersomes by either 2D6 alone or by a mixture of 2D6 and 3A4 (not shown). This indicated that the amount of

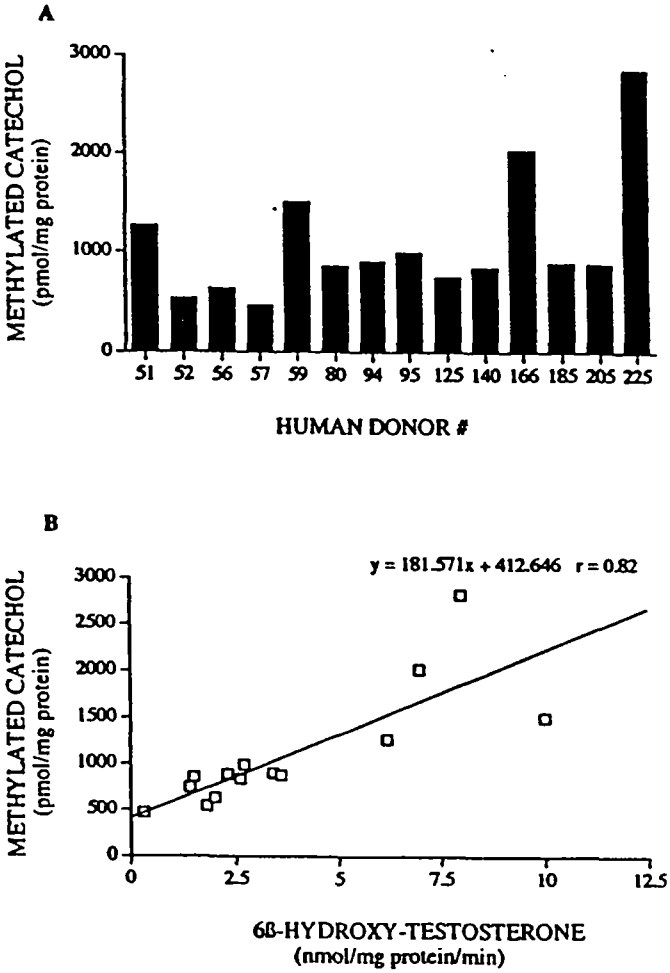


FIG. 3. *A, catechol formation from 4-OH-tam by human liver microsomes.*

Human liver microsomes (10 μg protein) were incubated with 4-OH-tam (25 μM) in the presence of [$^3$H]SAM (1 μCi/200 μM), DTT (50 μM), COMT (120 U), and NADPH-regenerating system in a final volume of 0.4 ml, at 37°C for 30 min. Values represent a mean of duplicate measurements. B, correlation of tam-catechol formation with testosterone 6β-hydroxylation in human liver microsomes.

TABLE 3

*Formation of catechol products from 4-OH-tam and 3-OH-tam (Droloxifene) catalyzed by human liver microsomes*

| Donor # | Liver Microsomes | | Substrate | Methylated Catechol |
|---|---|---|---|---|
| | CYP3A Activity[a] | CYP2D6 Activity[b] | | |
| | | | | *nmol/mg protein* |
| 166 | 7.0 | 763 | 4-OH-tam | 3.16 ± 0.04 |
| 51 | 6.2 | 507 | 4-OH-tam | 1.42 ± 0.09 |
| 225 | 8.0 | 261 | 4-OH-tam | 3.15 ± 0.60 |
| 59 | 10.0 | 234 | 4-OH-tam | 1.62 ± 0.13 |
| 80 | 1.5 | 856 | 4-OH-tam | 1.67 ± 0.11 |
| 57 | 0.3 | 568 | 4-OH-tam | 0.37 ± 0.18 |
| 56 | 2.0 | 181 | 4-OH-tam | 0.71 ± 0.12 |
| 185 | 2.3 | 180 | 4-OH-tam | 0.99 ± 0.12 |
| 94 | 3.4 | 186 | 4-OH-tam | 0.60 ± 0.25 |
| 80 | 1.5 | 856 | 3-OH-tam | 8.63 ± 0.34 |
| 225 | 8.0 | 261 | 3-OH-tam | 8.22 ± 0.72 |

Human liver microsomes (50 μg), 3-OH-tam, or 4-OH-tam (25 μM), DTT (50 μM), COMT (100 U), SAM (200 μM/1 μCi), and NADPH-regenerating system were incubated in a final volume of 0.4 ml for 30 min at 37°C. Values represent a mean ± S.D. of triplicate measurements.

[a] Catalytic activity (mean ± S.D.) of 135 human livers = 2.5 ± 2.1 nmol 6β-hydroxytestosterone/mg protein/min.

[b] Catalytic activity (mean ± S.D.) of 135 human livers = 273.6 ± 179.8 pmol dextromethorphan-*O*-demethylation/mg protein/min).

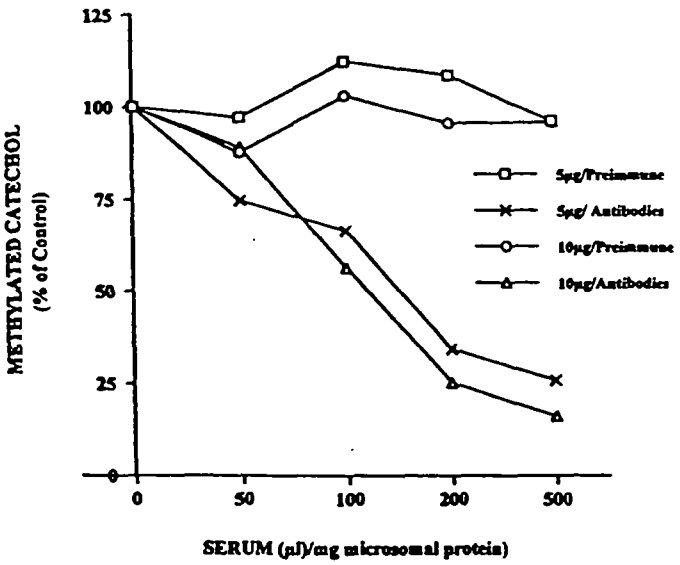


FIG. 4. *Effect of polyclonal antibodies against CYP3A4 on catechol formation from 4-OH-tam by human liver microsomes.*

Human liver microsomes (5 μg; □, x and 10 μg; ○, Δ protein; IIAM human donor #225 that contained high level of CYP3A and low level of 2D6 activities) were preincubated with various amounts of preimmune serum (IgG) and polyclonal antibodies against CYP3A4 at room temperature for 30 min. This mixture was incubated with 4-OH-tam (25 μM) in the presence of [$^3$H]SAM (1 μCi/200 μM), DTT (50 μM), COMT (150 U), and NADPH-regenerating system in a final volume of 0.4 ml at 37°C for 30 min. The concentration of protein was almost identical in preimmune serum and serum containing antibodies. Values represent a mean of duplicate measurements.

4-OH-tam formed by 2D6 was insufficient to sustain the second hydroxylation by either 2D6 or 3A4. Surprisingly, low levels of quinidine (up to 1 μM), a specific competitive inhibitor of 2D6 activity (Grace et al., 1994), did not inhibit the 2D6-mediated catechol formation from 4-OH-tam in supersomes. Similarly, quinidine (up to 5 μM) did not inhibit catechol formation in liver microsomes with high 2D6 and low 3A4 activity. The lack of inhibition by quinidine is not understood. Possibly the $K_m$ of 4-OH-tam and 3-OH-tam is extremely low, hence the competitive quinidine interference is obviated.

It was reasoned that if the tam catechol is proximate to the tam-RI

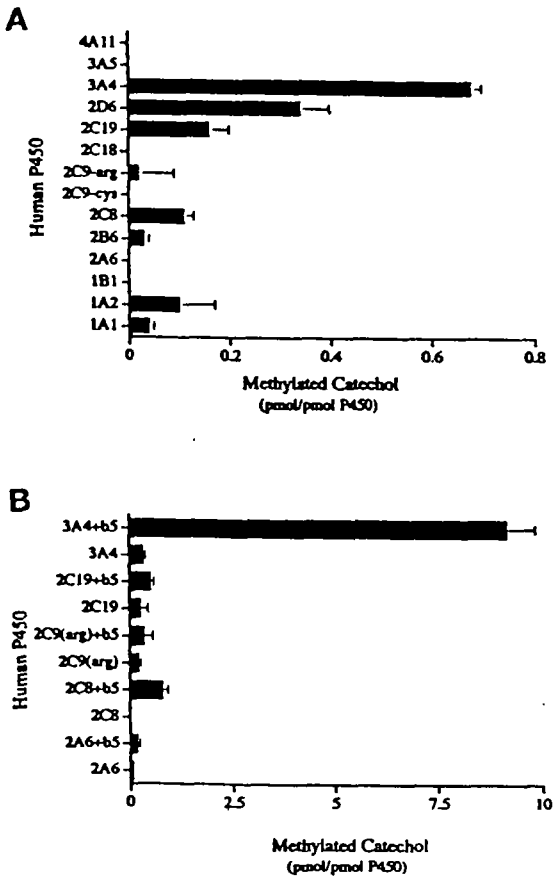


FIG. 5. *A, catechol formation from 4-OH-tam by cDNA-expressed human P-450s in baculovirus-infected insect cell line (supersomes).*

Supersomes containing P-450s (8 pmols) with coexpressed NADPH-P-450 oxidoreductase were incubated with 4-OH-tam (10 nmol) in the presence of COMT (100 U), [$^3$H]-SAM (80 nmol/0.4 μCi), DTT (20 nmol), and NADPH-regenerating system in a final volume of 0.4 ml at 37°C for 30 min. Values represent a mean ± S.D. of triplicate measurements. B, effect of coexpressed cytochrome $b_5$ on catechol formation from 4-OH-tam by cDNA-expressed human P-450s in supersomes. Incubation conditions were as above in Fig. 5A.

involved in covalent binding, then diminution of catechol formation should decrease the covalent binding. Indeed, an inhibitor of CYP3A enzymes, ketoconazole (KZ) at 20 μM, significantly inhibited both catechol formation (from 4-OH-tam) by 42% and its covalent binding by 88% in Dex-microsomes (not shown). Furthermore, in human liver microsomes, KZ inhibited the covalent binding of metabolically activated radiolabeled 4-OH-tam (Fig. 6). The inclusion of low levels of KZ (0.1–0.5 μM) in the incubations of 4-OH-tam with human liver microsomes resulted in 20 to 25% inhibition of tam catechol accumulation; however, surprisingly, at higher levels of KZ (1–5 μM) there was a marked increase in accumulation of the catechol (Table 4). Catechol accumulation may have been due to KZ inhibition of the further transformation of the catechol and/or of the *O*-demethylation of the [$^3$H]-monomethylated catechol. The latter possibility was excluded because incubation of the [$^3$H]-monomethylated tam catechol with various cDNA-expressed human P-450s did not display *O* demethylation (not shown). Additionally, this finding demonstrates that the extremely low or lack of *ortho*-hydroxylase activity by various P-450s other than 3A4 or 2D6 was not a mere artifact of the loss of radioactivity due to the enzymatic demethylation of the [$^3$H] methylated catechol by these P-450s. The opposing effects of low versus high KZ concentrations could have contributed to the incomplete inhibition of tam catechol accumulation by the low levels of KZ