ABSTRACT:

Cytochrome P450 3A4 is known to catalyze the metabolism of both endogenous substrates (such as the 6β-hydroxylation of testosterone) and numerous therapeutic agents, including the N-demethylation of erythromycin. However, erythromycin and testosterone have been reported to have little or no effect on the metabolism of each other by recombinant CYP3A4. In an effort to understand the basis of these observations, we studied the N-demethylation of erythromycin and the 6β-hydroxylation of testosterone in human liver microsomes and in microsomes from cells containing recombinant human CYP3A4 and P450 reductase under a variety of experimental conditions. In both human liver microsomal and recombinant CYP3A4 systems, erythromycin inhibited testosterone 6β-hydroxylation in a concentration dependent manner, and vice versa. However, the inhibition mechanism was complex. At low substrate concentrations, testosterone and erythromycin acted as competitive inhibitors to each other. Under these experimental conditions, an apparent competitive inhibition of testosterone 6β-hydroxylation by erythromycin was observed, with Ki values similar to that of the Kₘ values for erythromycin. When the rates of testosterone 6β-hydroxylation and erythromycin N-demethylation were determined in microsomal incubations containing both substrates at lower concentrations, the observed rates for each reaction were in good agreement with the calculated rates based on the rate equation describing simultaneous metabolism of two substrates by a single enzyme. However, at high substrate concentrations, the kinetic results could be best explained by a mechanism involving partial competitive inhibition. We conclude from these studies that testosterone and erythromycin mutually inhibit the metabolism of each other, consistent with the fact that CYP 3A4 catalyzes the metabolism of both substrates.
Materials and Methods

Materials. Testosterone, 6β-hydroxytestosterone, corticosterone, erythromycin, glucose 6-phosphate, NADP, and glucose 6-phosphate dehydrogenase were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents and solvents were of high analytical grade supplied by Fisher Scientific (Fair Lawn, NJ).

Human liver microsomal preparations kindly provided by Dr. Judy Raucy (Agouron Institute, La Jolla, CA) were used in this study. The organ donors were a 38-yr-old male with no known drug history (HL 24493) and a 34-yr-old male with a history of alcohol and cocaine use (HL 3926). Microsomes were prepared as described elsewhere (16). The pyrophosphate-washed microsomes were resuspended at a protein concentration of 10–15 mg/ml in 10 mM potassium phosphate buffer, pH 7.4 with 1 mM EDTA, 6 mM MgCl₂, and an NADPH-generating system consisting of 10 mM glucose 6-phosphate, 1 mM NADP, and 0.35 units glucose 6-phosphate dehydrogenase in a total volume of 0.5 ml. Reactions were performed at 37°C for 10 min with 0.125 mg (HL 3926) or 0.25 mg (HL 24493) of human liver microsomes and at 37°C for 20 min with 0.25 mg of microsomes prepared from human B-lymphoblast cells. After stopping reactions with 5 ml of CH₃Cl, the samples were spiked with 25 µl of 1 mM corticosterone as internal standard, vortexed, and centrifuged at 3,000 × g for 10 min. The organic layer was removed and evaporated to dryness under nitrogen stream. Samples were dissolved in 0.25 ml of methanol and analyzed by HPLC. Aliquots of 50 µl samples were injected directly onto a Zorbax ODS C18 column (4.6 mm × 250 mm, 5 µm, Sigma-Aldrich, Milwaukie, WI) and eluted with methanol (7.5% tetrahydrofuran):H₂O (92.5%) by a linear gradient from 35% to 60% in 35 min at a flow rate of 1 ml/min. Chromatographic peaks were monitored with a UV detector at 254 nm. The retention times for 6β-hydroxytestosterone, corticosterone, and testosterone were 8.9, 17.5, and 25.2 min, respectively.

Erythromycin N-demethylation was determined by the method of Tu and Yang (21) with modifications. Erythromycin was incubated at 37°C for 15 min with 0.5 mg (HL 3926) or 1 mg (HL 24493) of human liver microsomes for 30 min with 1 mg of microsomes from human B-lymphoblast cells in the presence of a NADPH-generating system as described above. Reactions were quenched with 0.05 ml of 25% ZnSO₄ and 0.05 ml of 0.3 N Ba(OH)₂. After the samples were vortexed and centrifuged at 14,000 × g for 10 min, 0.35 ml of supernatant was transferred to another tube and then mixed with 0.15 ml of a concentrated Nas reagent (15 g of ammonium acetate and 0.2 ml of acetylatec in 18 ml of 3% acetic acid). The mixture was incubated at 56°C for 30 min and transferred to a 96-well plate. Samples were analyzed by measuring absorbance at 405 nm with Ceres UV 900 HDI spectrophotometer to determine the formation of formaldehyde (22).

Kinetic studies for testosterone 6β-hydroxylation and erythromycin N-demethylation by microsomes were carried out at testosterone or erythromycin concentrations ranging from 10–1000 µM. Experiments were performed under initial rate conditions. Kinetic parameters were obtained by hyperbolic saturation curves by least squares fit of the data to the Michaelis-Menten equation by nonlinear regression analysis, using K. cat computer software program (BioMetallics, Inc., Princeton, NJ). For inhibition studies, testosterone and erythromycin were incubated simultaneously with microsomes in the presence of a NADPH-generating system. Kᵣ values were determined using the Dixon plot (23).

Results

Kinetic Parameters. Two human liver microsomal preparations, one high (HL 3926) and one low (HL 24493) in CYP3A4 activity, and microsomes from cells containing recombinant human CYP3A4/OR were used to determine kinetic parameters for testosterone 6β-hydroxylation and erythromycin N-demethylation (table 1). The Kᵣ values ranged from 53 to 128 µM for testosterone and 44 to 78 µM for erythromycin. The Vₘ values were considerably higher for testosterone 6β-hydroxylation (2 to 20 nmol/min/mg) than for erythromycin N-demethylation (from 0.22 to 2 nmol/min/mg).

Mutual Inhibition. The Kᵣ for testosterone and erythromycin are not very different; therefore, one should expect mutual inhibition since both substrates are metabolized by CYP3A4. If this is the case, then the reaction rates of each substrate in the presence of another substrate can be predicted from the following equations (24):

\[ V = \frac{V_m \theta_i s_i}{K_i (1 + s_i/K_i) + s_i} \]

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where Vᵣ and Vₑ are the rates for testosterone 6β-hydroxylation and erythromycin N-demethylation, respectively, in the presence of the other substrate; Vᵣₘₑ and Kᵣ are the maximum velocity and the Michaelis-Menten constant for testosterone; Vₑₘₑ and Kₑ are the maximal velocity and Michaelis-Menten constant for erythromycin; sᵣ and sₑ are the testosterone and erythromycin concentration used in the incubation mixture.

In this study, four concentrations (125, 250, 500, and 1000 µM) of erythromycin were incubated either in the absence or in the presence of 50 and 250 µM of testosterone. After incubation, the rates of metabolite formation were independently measured for each substrate. These rates were also calculated from the equations based on the known Kᵣ and Vᵣₑ, and the concentration of each substrate used in the incubations. As shown in table 2, the observed experimental rates were generally in good agreement with the calculated rates when 125 and 250 µM of erythromycin were used in the incubations. However, at higher substrate concentrations (500 and 1000 µM erythromycin and 250 µM testosterone), the observed reaction rates were substantially higher than the calculated rates, particularly for testosterone 6β-hydroxylation. These results suggest that at lower substrate concentrations testosterone and erythromycin behave as mutual inhibitors in CYP3A4-catalyzed reactions, whereas at higher substrate concentrations additional factor(s) may contribute to the kinetic mechanism involving both substrates.

To further explore the nature of interaction between erythromycin and testosterone during catalysis, two types of experiments were conducted to investigate the effect of erythromycin on CYP3A4-catalyzed testosterone 6β-hydroxylation. First, inhibition kinetics of testosterone 6β-hydroxylation by erythromycin were studied at lower concentrations for both substrates (erythromycin, up to 80 µM in one experiment, 800 µM in another experiment; testosterone, up to 40 µM in one experiment, 800 µM in another experiment). Dixon plots indicate that erythromycin competitively inhibited testosterone 6β-hydroxylation with Kᵣ values of 80 µM in HL 3926 (fig. 1A) and 30 µM in CYP3A4/OR (fig. 1B). These Kᵣ values ranged from 53 to 128 µM for testosterone and 44 to 78 µM for erythromycin.
values were within the range of $K_m$ for erythromycin determined in separate experiments (see table 1), consistent with the notion that testosterone and erythromycin are alternative competing substrates for CYP3A4 at these lower substrate concentrations. In the second approach, experiments were designed to determine the effect of erythromycin on the rate of testosterone 6β-hydroxylation; attention was paid particularly at higher concentrations of erythromycin and testosterone. Fig. 2 shows that in both human liver microsomal (figs. 2A and 2B) and recombinant CYP3A4 (fig. 2C) systems, testosterone 6β-hydroxylation was inhibited by erythromycin in a concentration-dependent manner. At a fixed concentration of 60 μM testosterone, the rate of 6β-hydroxylation could be greatly reduced by high concentrations of erythromycin. However, at higher fixed amounts of testosterone, particularly at 250 and 500 μM concentrations, only partial inhibition of 6β-hydroxylation was observed even at high erythromycin concentrations. These data confirm the results in table 2, i.e., at high concentrations for both substrates, the calculated rates for testosterone 6β-hydroxylation were considerably lower than the observed rates due to partial inhibition by erythromycin.

Detailed inhibition kinetic studies (e.g., determination of $K_i$ for testosterone) for erythromycin N-demethylation were not attempted since the rates of this reaction were low and the colorimetric assay method was not precise enough to measure even lower rates in the presence of testosterone. However, studies were carried out to investigate the effect of testosterone on the rate of erythromycin N-demethylation, including high concentrations of both substrates. Fig. 3 shows that in both human liver microsomal (figs. 3A and 3B) and recombinant CYP 3A4 (fig. 3C) systems, erythromycin N-demethylation was inhibited by testosterone in a concentration-dependent manner. Erythromycin N-demethylation appeared to be more sensitive to testosterone inhibition even when 1000 μM of erythromycin was used.

**Discussion**

Using a reconstituted system containing purified recombinant CYP3A4 and NADPH-cytochrome P450 reductase fusion protein and lipid, Shet et al. (7) recently reported that although both testosterone and erythromycin were metabolized by CYP3A4, they did not significantly inhibit the metabolism of each other when equimolar concentrations (200 μM) of each compound were used in the incubation mixtures. Interestingly, the metabolism of testosterone and erythromycin at the same concentration was strongly inhibited by 200 μM of nifedipine, another substrate of CYP3A4. One would expect mutual inhibition if testosterone and erythromycin were metabolized by the same enzyme. This observation may be explained as follows: (a) It may not always be possible to show the mutual effect of two compounds when substrates are tested at a single concentration. In this respect, experiments conducted at a wide range of concentrations involving both compounds should give more definitive results. (b) Testosterone and erythromycin may have different binding sites on CYP3A4; thus, the presence of one substrate does not significantly affect the metabolism of the other substrate. However, these binding sites cannot be remotely separated on CYP3A4 since both substrates must be close enough to the active oxygen on the heme for oxygenation. Evidence presented by Shou et al. (12) indicates that both phenanthrene and 7,8-benzoflavone can be present simultaneously in the CYP3A4 active site. (c) The active site topology of the fused protein may not be totally identical to that of the single protein; therefore, the lack of a significant mutual effect may be unique only for the fused CYP3A4.

In an effort to address the question of whether testosterone and erythromycin mutually affect the metabolism of each other by CYP3A4, the metabolism of both compounds was examined under a variety of conditions. Both human liver microsomes and microsomes prepared from human lymphoblastoid cell line containing recombinant CYP3A4 and P450 reductase were used as the enzyme source so that results obtained from one system can be compared with a different system. Our data indicate that in the microsomal preparations used in this study testosterone and erythromycin mutually inhibit the metabolism of each other consistent with the fact that CYP3A4 catalyzes both testosterone 6β-hydroxylation and erythromycin N-demethylation. However, the inhibition mechanism is not purely competitive. Additional mechanisms may contribute to the interaction between these two substrates with CYP3A4, particularly at high concentrations. Although further studies will be required to investigate the nature of testosterone and erythromycin interaction with CYP3A4, we propose the following mechanism, based on partial competitive inhibition (25), to explain our data:

![Chemical Reaction Diagram]

Using a reconstituted system containing purified recombinant CYP3A4 and NADPH-cytochrome P450 reductase fusion protein and lipid, Shet et al. (7) recently reported that although both testosterone and erythromycin were metabolized by CYP3A4, they did not significantly inhibit the metabolism of each other when equimolar concentrations (200 μM) of each compound were used in the incubation mixtures. Interest-

### TABLE 2

<table>
<thead>
<tr>
<th>Erythromycin (μM)</th>
<th>Testosterone (μM)</th>
<th>Testosterone 6β-Hydroxylation (nmol/min/mg)</th>
<th>Erythromycin N-Demethylation (nmol/min/mg)</th>
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Incubations were performed with 0.5 mg human liver microsome (HL 3926) at 37°C for 15 min, and metabolite formation independently measured for each substrate.

ND: not detectable, limit of detection is <0.01 nmol/min/mg.
where $P_T$ is the metabolite from testosterone ($S_T$); $P_E$ the metabolite from erythromycin ($S_E$).

The $K_m$ values for testosterone and erythromycin are very similar (50 to 128 $\mu$M, see table 1). Therefore, at lower substrate concentrations, e.g. less than 250 $\mu$M, testosterone and erythromycin act as competitive inhibitors in the CYP3A4-catalyzed reaction. This conclusion is supported by the following observations. When the rates of testosterone 6β-hydroxylation and erythromycin N-demethylation were determined in microsomal incubations containing both substrates at lower concentrations, the observed rates for each reaction were in close agreement to the calculated rates based on the rate equation describing simultaneous metabolism of two substrates by a single enzyme. Inhibition kinetics of human liver microsomal testosterone 6β-hydroxylation by erythromycin was also analyzed at these concentration ranges. An apparent competitive inhibition was observed with a $K_i$ value of 80 $\mu$M. This value corresponded with the $K_m$ of erythromycin (78 $\mu$M) determined with the same microsomal incubation.

At higher substrate concentrations, e.g. greater than 250 $\mu$M, testosterone can bind to $E_S$ and erythromycin can bind to $E_T$ to form the $E_T S_E$ complex. This could happen if both testosterone and erythromycin can be present simultaneously in the CYP3A4 active site, and if $K_{s(T)}$ and $K_{s(E)}$ are substantially higher than $K_{s(T)}$ and $K_{s(E)}$. Shou et al. (12) have presented evidence that both phenanthrene and 7,8-benzoflavone can simultaneously be at the active site of CYP3A4. Thus, for testosterone 6β-hydroxylation, high concentrations of both substrates will drive the $E_T S_E$ formation which can then produce $P_T$ and $P_E$. Since $k_1$ is substantially greater than $k_2$, one could assume that $k_1$ is also greater than $k_2$. Therefore, at high concentrations inhibition of testosterone 6β-hydroxylation by erythromycin will be limited. This could explain why in the presence of high substrate concentrations the observed reaction rates for 6β-hydroxylation were substantially higher than the calculated rates (table 2) and why high erythromycin concentrations only showed partial inhibition of 6β-hydroxylation at 500 $\mu$M testosterone (fig. 2). For erythromycin

![Graph 1](image1.png)

**Fig. 1.** Dixon Plot for the inhibition of testosterone 6β-hydroxylation by erythromycin.

A. Microsome HL 3926 was incubated with 30, 40, and 80 $\mu$M of testosterone and 100, 200, 400, and 600 $\mu$M of erythromycin. The estimated $K_i$ value was 80 $\mu$M. B. Microsome CYP3A4/OR was incubated with 20, 30, and 40 $\mu$M of testosterone and 10, 30, 50, and 80 $\mu$M of erythromycin. The estimated $K_i$ value was 30 $\mu$M.

![Graph 2](image2.png)

**Fig. 2.** Inhibition of testosterone 6β-hydroxylation by erythromycin in microsomes.

All microsomes were incubated with the indicated testosterone concentrations and various concentrations of erythromycin. A. Microsome HL 3926 was incubated with 60, 125, and 500 $\mu$M of testosterone. The corresponding control activities were 3.66, 13.06, and 11.96 nmol/min/mg, respectively. B. Microsome HL 24493 was incubated with 60, 125, and 250 $\mu$M of testosterone. The corresponding control activities were 2.79, 4.58, and 4.97 nmol/min/mg, respectively. C. Microsome CYP3A4/OR was incubated with 60 and 250 $\mu$M of testosterone. The corresponding control activities were 0.54 and 0.86 nmol/min/mg, respectively.
N-demethylation, greater inhibition was observed by high concentrations of testosterone since ES₂S₉ is favored to produce P₂ rather than P₁.

In summary, evidence is presented that testosterone and erythromycin mutually inhibited the metabolism of each other by CYP3A4 through competitive inhibition at lower substrate concentrations and likely partial competitive inhibition at higher substrate concentrations. Additional experiments will be conducted to test this hypothesis.

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References


