Biochemical Pharmacology, Vol. 29, pp. 1583–1587.

0006-2952/80/0601-1583 $02.00/0

# INHIBITION OF RABBIT LIVER MICROSOMAL OXIDATIVE METABOLISM AND SUBSTRATE BINDING BY TAMOXIFEN AND THE GEOMETRIC ISOMERS OF CLOMIPHENE*

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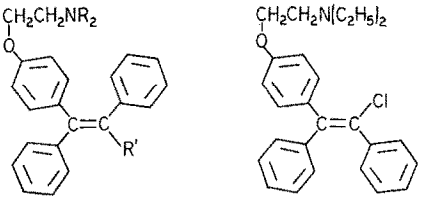
(*Received* 19 *March* 1979; *accepted* 26 *December* 1979)

**Abstract**—The inhibitory effects of the triarylethylene antiestrogens tamoxifen (TAM) and enclomiphene (EC), and of the estrogenic geometric isomer of the latter compound (zuclomiphene, ZC) on the binding and metabolism of standard substrates in the presence of rabbit liver cytochrome P-450 have been studied. In the presence of microsomes at pH 7.4, the triarylethylenes interfered with the binding of β-diethylaminoethyl diphenylpropylacetate (SKF 525-A) and with the *N*-demethylation of ethylmorphine. Their inhibitory effects were less pronounced on the binding and metabolism (hydroxylation) of aniline. Kinetic studies showed that EC and TAM interfered with SKF 525-A binding by a mixed mechanism. These results suggest that the triarylethylenes affect preferentially the binding of 'Type I' substrates with cytochrome P-450.

Clinically useful estrogen antagonist activity has been found in both steroidal and nonsteroidal estrogen analogues. The most significant group of nonsteroidal antiestrogens is the triarylethylenes. This group includes tamoxifen and clomiphene, which have been used, respectively, in cancer chemotherapy and treatment of infertility in women [1, 2].

Results of both clinical and whole animal studies of the metabolism of tamoxifen (TAM) have indicated that aromatic ring hydroxylation is a major metabolic route [3, 4]. The resulting metabolite, hydroxytamoxifen, was postulated to account, in part, for the extended duration of antiestrogenic activity, by virtue of its sequestration in the enterohepatic circulation. Several other metabolites resulting from oxidative alteration of TAM were reported in these studies and in a later clinical study [5]. Less information is available regarding the metabolism and disposition of clomiphene. This drug is a mixture of two geometric isomers, enclomiphene (EC) and zuclomiphene (ZC). It was reported to be eliminated primarily via the feces and to be subject to enterohepatic recycling [6], characteristics which suggest that it may undergo oxidative metabolism in a manner similar to that of TAM.

Enzymes responsible for oxidative drug biotransformations are generally found at highest levels in the liver, with activity concentrated in microsomal subcellular fractions [7]. These oxidations have been shown to involve a family of enzymes with cytochrome P-450 acting as terminal oxidase. The *in vivo* studies of the fate of TAM and the clomiphene isomers (see above) had suggested an interaction of these with the cytochrome P-450 system. Thus, the present study was aimed at characterizing these interactions in rabbit liver microsomes.




| | R | R' |
|---|---|---|
| EC, | $C_2H_5$ | Cl |
| TAM, | $CH_3$ | $C_2H_5$ |

## EXPERIMENTAL

### *Chemicals*

All biochemicals used in this study were purchased from the Sigma Chemical Co., St. Louis, MO. β-Diethylaminoethyl diphenylpropylacetate (SKF 525-A) was a gift from Smith, Kline, & French Laboratories, Philadelphia, PA. Tamoxifen citrate (Nolvadex) was a gift from Stuart Pharmaceuticals Division of ICI Americas, Wilmington, DE: gas–liquid chromatographic analysis, performed by adaption of a previous method [3, 4], showed it to be one-peak material. The isomers of clomiphene citrate (Clomid) were gifts from Merrell-National Laboratories Division of Richardson-Merrell, Cincinnati, OH. Gas–liquid chromatographic analysis of these isomers, under reported conditions [8, 9], showed the E-isomer (EC) to be one-peak material,while the Z-isomer (ZC) contained about 5 per cent of an impurity which had a retention time equal to that of EC.

### *Preparation of microsomes*

Microsomes were prepared from the livers of male New Zealand white rabbits (1.5 to 2.0 kg) by methods reported previously [10, 11]. Protein concentra-

* This research was supported in part by research grant HL 22873-01 from the National Institutes of Health.

tion was determined by the method of Lowry *et al.* [12]. Cytochrome P-450 concentration was determined by the method of Omura and Sato [13].

*Binding studies*

Spectral recordings were made with an Aminco DW-2 dual wavelength scanning spectophotometer operated in the split beam mode at 25°. Solutions of EC or ZC were prepared by dissolving 1 mg in 0.5 ml of 0.2 M phosphate buffer, pH 6.1, containing 1 per cent bovine serum albumin. Solutions of TAM were prepared by dissolving 1 mg in 0.5 ml acetone. (The final concentration of acetone present in microsomal mixtures to which the tamoxifen solution was added was less than 8% in all experiments.) Aliquots of the triarylethylene solutions (< 20 μl) were added to microsomal suspensions (2–3 mg protein/ml) in 0.2 M phosphate buffer, pH 7.4, containing 10 per cent glycerol. The suspension was then divided equally between two 1 cm cells, and the spectral baseline was recorded. Increasing amounts of SKF 525-A or aniline in distilled water were added to the sample cell. Equal amounts of water were added to the reference cell. Spectra were recorded from 360 to 460 nm. Control experiments were done by observing the spectral change caused by addition of SKF 525-A or aniline in the presence of solvents only (i.e. 1 per cent BSA in 0.2 M phosphate buffer, pH 6.1; acetone). Spectral dissociation constants ($K_s$) were calculated by the method of Estabrook *et al.* [14].

*Metabolism studies*

*Incubation mixtures.* The standard incubation mixture contained microsomal suspension equivalent to 10 mg protein, 300 μmoles KCl, 200 μmoles phosphate buffer, pH 7.4, and a cofactor mixture comprised of 2 μmoles NADP, 40 μmoles glucose-6-phosphate, 3 units glucose-6-phosphate dehydrogenase and 25 μmoles $MgCl_2$ in a volume of 4 ml. After a 3-min preincubation at 37°, 1 ml of 10 mM ethylmorphine HCl or aniline HCl in water was added. Then 25 μl of a 0.1 M solution of the triarylethylene in acetone–ethanol 1 : 1) were added, and the mixture was shaken at 70 cycles/min for 15 min. Control incubations were carried out in which 25 μl of solvent alone was added.

*Analysis of incubation mixtures.* The formaldehyde produced in mixtures which contained ethylmorphine was determined colorimetrically [15] as was the *p*-aminophenol produced in mixtures which contained aniline [16]. Conceivably, *N*-demethylation of TAM could have contributed to the formaldehyde produced in the former mixtures to which TAM had been added. However, experiments in which TAM alone was incubated under the above conditions indicated negligible formaldehyde production.

## RESULTS

The extent of microsomal binding of SKF 525-A was inhibited significantly by EC, as shown in Table 1. Although TAM, and to a lesser extent ZC, also appeared to inhibit SKF 525-A binding, the respective $K_s$ values were not significantly different from those of controls. This was due to large standard errors associated with calculation of $K_s$ values, which reflect difficulties in quantitation of absorbance changes accompanying addition of SKF 525-A to microsomes.

Table 1. Effect of the triarylethylenes on the kinetics of SKF 525-A spectral changes*

| Additive | $A_{385}$–$A_{420}$/mg protein × $10^2$ | $K_s$ (μM) |
|---|---|---|
| EC† | 0.61 ± 0.16 | 4.82 ± 0.06 |
| ZC† | 0.51 ± 0.15 | 1.78 ± 0.91 |
| Solvent only‡ | 0.66 ± 0.14 | 1.41 ± 0.55 |
| TAM† | 0.59 ± 0.35 | 1.53 ± 0.46 |
| Solvent only§ | 0.60 ± 0.21 | 0.87 ± 0.32 |

* Concentrations of SKF 525-A were varied from 1.54 to 30 μM. Each value represents the mean ± S.E.M. determined from six experiments, except controls where these values were determined from two experiments.

† The final cuvette concentration was 27 μM.

‡ Phosphate buffer (0.2 M, pH 6.1) containing 1% bovine serum albumin.

§ Acetone.

Kinetic studies resulted in further characterization of the interference of EC and TAM with microsomal binding of SKF 525-A. As shown in Fig. 1 and 2, both of these triarylethylenes inhibited binding by a mixed mechanism, with TAM having the greater effect. The triarylethylenes had no effect on the microsomal binding spectrum of aniline at respective concentrations of 27 μM. At much higher concentrations (200 μM), significant inhibition of binding in the presence of the clomiphene isomers was seen, accompanied by increases in absorption amplitudes ($A_{430}$–$A_{390}$) in the corresponding spectra (Table 2). Tamoxifen had no significant effects on the binding spectrum of aniline under these conditions.

Each of the triarylethylenes inhibited aniline hydroxylation by about 20 per cent (Table 3). More pronounced effects on ethylmorphine metabolism were observed in their presence; both EC and ZC inhibited its demethylation by about 60 per cent, and TAM inhibited this by 77 per cent. The greater inhibitory effect of TAM with respect to EC at

Table 2. Effect of the triarylethylenes on the kinetics of aniline spectral changes*

| Additive | $A_{430}$–$A_{390}$/mg protein × $10^2$ | $K_s$ (mM) |
|---|---|---|
| EC† | 5.00 ± 0.20 | 1.81 ± 0.09 |
| ZC† | 5.60 ± 0.28 | 1.89 ± 0.19 |
| Solvent only‡ | 4.20 ± 0.15 | 1.40 ± 0.03 |
| TAM† | 3.40 ± 0.38 | 4.85 ± 0.85 |
| Solvent only§ | 3.13 ± 0.24 | 3.85 ± 0.65 |

* Concentrations of aniline were varied from 0.15 to 3 μM. Each value represents the mean ±S.E.M. determined from six experiments, except controls where these values were determined from two experiments.

† The final cuvette concentration was 220 μM.

‡ Phosphate buffer (0.2 M, pH 6.1) containing 1 per cent bovine serum albumin.

§ Acetone.

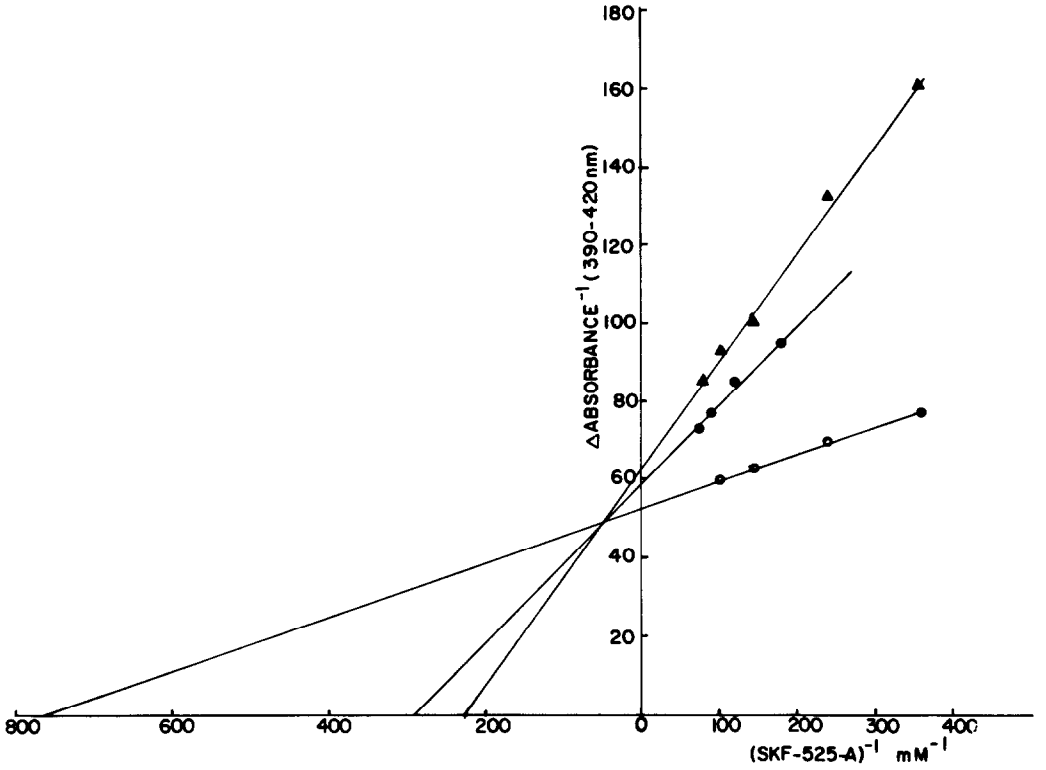



Fig. 1. Kinetics of inhibition of microsomal binding of SKF 525-A by EC. The protein concentration was 3 mg/ml in 0.2 M phosphate buffer, pH 7.4, containing 10 per cent glycerol. The concentrations of EC were: 0 mM (○), 0.139 mM (●) and 0.279 mM (▲).

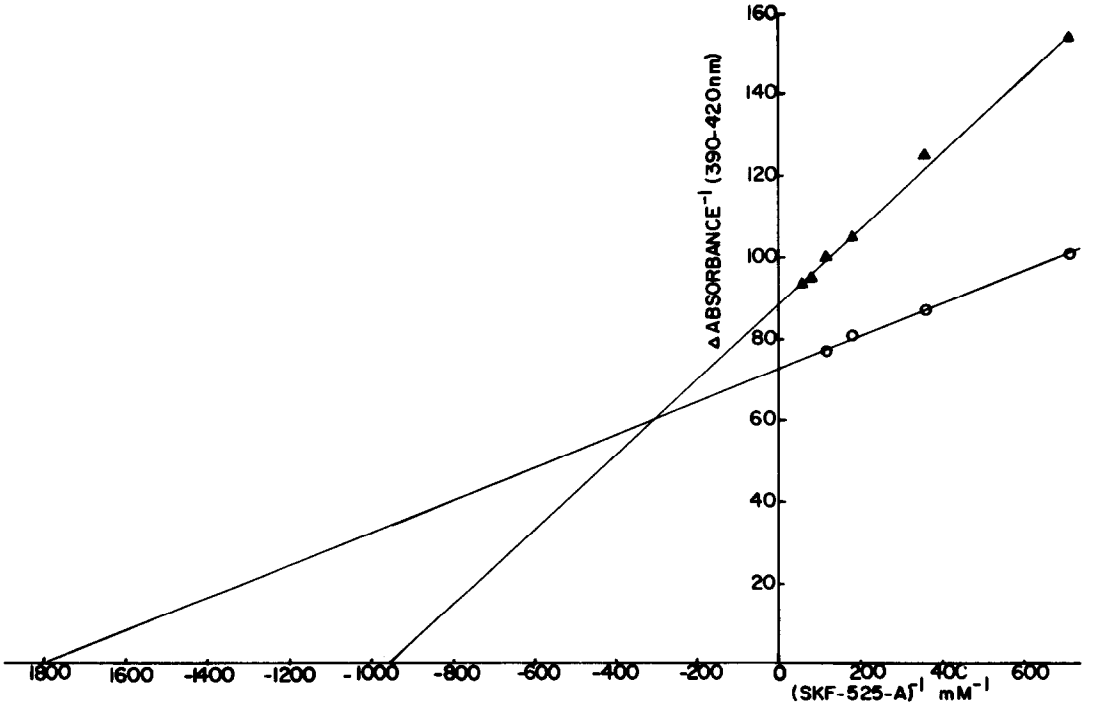



Fig. 2. Kinetics of inhibition of microsomal binding of SKF 525-A by TAM. Microsomal protein concentration was as stated in Fig. 1. The concentrations of TAM were: 0 mM (○) and 0.028 mM (▲).

Table 3. Effect of the triarylethylenes on rabbit liver microsomal ethylmorphine demethylase and aniline hydroxylase activities*

| Inhibitor† | Per cent inhibited activity | |
|---|---|---|
| | Ethylmorphine demethylase‡ | Aniline hydroxylase‡ |
| Enclomiphene (EC) | 59 (51–68) | 10 (3–17) |
| Zuclomiphene (ZC) | 56 (45–67) | 19 (15–23) |
| Tamoxifen (TAM) | 77 (75–79) | 23 (17–29) |

* The data shown are averages of at least four experiments with different animals. Standard deviations are enclosed in parentheses.
† The incubation mixture concentration was 0.5 mM.
‡ The initial incubation mixture concentration of substrate was 2 mM.

various ethylmorphine concentrations is illustrated in Fig. 3. The effect of ZC was similar to that of EC and is not shown.

## DISCUSSION

Numerous endogenous and exogenous chemicals have been shown to interact with cytochrome P-450 [17]. Considered in general, conformational changes in the hemoprotein resulting from such interactions produce two types of characteristic spectral changes, referred to as "Type I" and "Type II" [18–21]. Type I compounds, such as ethylmorphine and SKF 525-A, produce difference spectra with $\lambda_{max}$ between 385 and 390 nm and $\lambda_{min}$ between 418 and 427 nm. Type II compounds, such as aniline, produce spectra with $\lambda_{min}$ and $\lambda_{max}$ between 390 and 405 nm and 425 and 435 nm, respectively [22]. Both Type I and Type II binding sites have been shown to reside in the same molecule, with the binding properties of one site influenced by the interaction of substrates or inhibitors with the other [23].

An extensive study of factors affecting the appearance of cytochrome P-450 difference spectra in the presence of various compounds has shown that binding of Type I compounds was inhibited noncompetitively by other Type I compounds of dissimilar structure, and by a mixed mechanism by other Type I compounds of similar structure [21]. Furthermore, the amplitude of the difference spectra of Type II compounds was often increased in the presence of Type I compounds. As shown in Figs. 1 and 2, the triarylethylenes inhibited binding of the structurally similar SKF 525-A by a mixed mechanism. Also, each of these compounds caused an increase in the absorption amplitude in the spectrum of aniline, a Type II compound.

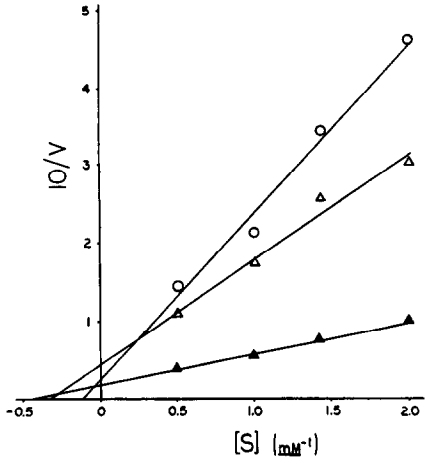



Fig. 3. Kinetics of inhibition of rabbit liver microsomal ethylmorphine demethylase by EC and TAM. Incubation mixtures contained substrate plus 0.5 mM TAM (○), substrate plus 0.5 mM EC (△), and substrate only (▲). Reaction velocity (V) was expressed in nmoles of formaldehyde found per mg of protein per 10 min. This figure represents averages of four experiments with different animals.

The inhibition of SKF 525-A binding by the triarylethylenes, and the relative lack of such an effect on the binding of aniline, prompted the study of their effects on aniline and ethylmorphine metabolism. The data shown in Table 3 indicated that the triarylethylenes inhibited significantly the metabolism of the Type I substrate with lesser inhibition of the Type II substrate. Kinetic data for the inhibition of metabolism of the former substrate suggest a mixed mechanism of inhibition (Fig. 3), with TAM having the greatest inhibitory effect. Thus, results of the metabolism kinetic studies were generally consistent with those from the binding inhibition studies and suggest a direct interaction of the triarylethylenes with cytochrome P-450.

The triarylethylene antiestrogens contain a variation of the diphenylmethane moiety, suggested to be a primary structural requirement for inhibitory activity in the cytochrome P-450 system [24]. These compounds may be serving as alternate substrates for the system, as do a number of other structurally related inhibitors [15, 25]. Experiments carried out prior to commencement of the metabolism studies indicated that no detectable *N*-demethylation of TAM was occurring. The extent and specific structural nature of biotransformation products resulting from the interaction of TAM, EC and ZC with rabbit hepatic cytochrome P-450 are currently being studied.

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