Biochemical Pharmacology, Vol. 38, No. 1, pp. 195–199, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

IN VITRO INHIBITION OF HEPATIC STEROID HYDROXYLATION BY TAMOXIFEN, A SERIES OF TAMOXIFEN ANALOGUES AND RELATED COMPOUNDS

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(*Received* 29 *June* 1988; *accepted* 16 *August* 1988)

Abstract—The *in vitro* inhibition of the cytochrome P-450 (P-450) isozyme specific positional hydroxylation of androst-4-ene-3,17-dione (androstenedione) by the alkylamino containing compounds *trans*- and *cis*-tamoxifen, 4-hydroxytamoxifen, *N*-desmethyltamoxifen, SKF 525-A and the non-alkylamino containing compounds tamoxifen metabolite E, and tamoxifen analogue U-23469 was assessed in pooled hepatic microsomes isolated from untreated male rats. P-450 IIA 1-mediated androstenedione 7α-hydroxylation appeared refractory to inhibition, with the lowest I_{50}s being approximately 200 μM (*cis*- and *trans*-tamoxifen, 4-hydroxytamoxifen). (According to the recently recommended nomenclature for cytochromes P-450 (Nebert DW and Gonżalez FJ, *Ann Rev Biochem* **56**: 945–993, 1987), rat hepatic cytochromes P-450 UT-A, PB-B, PCN-E and UT-F are encoded by genes IIC 11, IIB 1, IIIA 1/2 and IIA 1, respectively.

I_{50}s toward the P-450 IIC 11-, IIB 1-, and IIIA 1/2-catalysed reactions, androstenedione 16α-, 16β- and 6β-hydroxylations, respectively, were generally in the range 70–190 μM. However, metabolite E exhibited a rather specific and potent capacity to inhibit androstenedione 16α-hydroxylase activity (I_{50} = 18 μM). Since a number of alkylamine compounds have been shown to sequester microsomal P-450 as an inactive metabolite intermediate (MI), the tamoxifen analogues were investigated for their *in vitro* MI complexation capacity. However, spectral binding studies revealed that the incubation of these compounds with NADPH-fortified microsomal fractions did not result in MI complex formation. In binding experiments conducted with oxidised microsomal fractions it was apparent that most of the tamoxifen analogues are type I ligands of quite high affinity for ferric P-450 (K_s range 10–60 μM). It seems unlikely that MI formation is involved in the observed inhibition of androstenedione hydroxylation by tamoxifen and congeners. Instead, and in contrast to the situation observed with SKF 525-A, it would appear that the inhibitory capacity of the tamoxifen analogues is more closely related to type I binding capacity with ferric P-450. A finding of particular interest is that metabolite E, in which the alkylamino side-chain is absent, elicited a type I interaction of high capacity. The maximal absorbance change of the type I interaction of this compound with microsomal P-450 was about three-fold greater than the other compounds. It therefore appears likely that removal of the tamoxifen side-chain results in quite potent inhibition of P-450 IIC 11-mediated monooxygenase activity and an enhanced binding interaction with the cytochrome.

Many studies have documented that compounds containing the alkylamino functional group, such as SKF 525-A and the macrolide antibiotics, are metabolised by hepatic microsomal cytochrome P-450 (P-450) to form inhibitory metabolite intermediate (MI) complexes [1]. The complex effectively sequesters P-450 in a catalytically inactive state and results in inhibition of the metabolism of exogenous and endogenous substrates, such as carcinogens and sex steroids.

Tamoxifen (*trans*-1-[4-[2-(dimethylamino ethoxy]-phenyl]-1,2-diphenyl-1-butene; Fig. 1) is an antineoplastic agent used in the treatment of oestrogen-dependent breast cancer [2]. The therapeutic effects of tamoxifen are generally thought to be mediated, at least in part, via competition with oestradiol for the oestrogen receptor [3]. In mammals, including humans, tamoxifen is extensively metabolised by the hepatic P-450s [2]. This agent has also been shown to be an inhibitor of the P-450 system [2, 4], but the possible involvement of the dimethylamino side chain of tamoxifen in MI complex formation has not been considered.

The present study was undertaken to determine the capacity of tamoxifen and a series of metabolites including *N*-desmethyltamoxifen, 4-hydroxytamoxifen and *trans*-1-(4-hydroxyphenyl)-1,2-diphenyl-1-butene (metabolite E), as well as *cis*-tamoxifen and the fused-ring analogue U-23469 (Fig. 1), to generate MI complexes with ferrous P-450. Additional studies determined the interaction of these compounds with ferric P-450 in rat hepatic microsomes as well as the potencies of the analogues as inhibitors of P-450 isozyme-specific pathways of drug metabolism. Thus, the steroid androst-4-ene-3,17-dione (androstenedione) was selected for study since, in untreated male rat hepatic microsomes, the formation of 6β-, 7α, 16α- and 16β-hydroxy-metabolites are specific for the P-450s IIIA 1/2, IIA 1, IIC 11 and IIB 1, respectively [5].

MATERIALS AND METHODS

Chemicals. [4-^{14}C]-Androst-4-ene,3,17-dione (androstenedione; spec. activity 59 mCi/mmol) was obtained from Amersham Australia (Sydney,

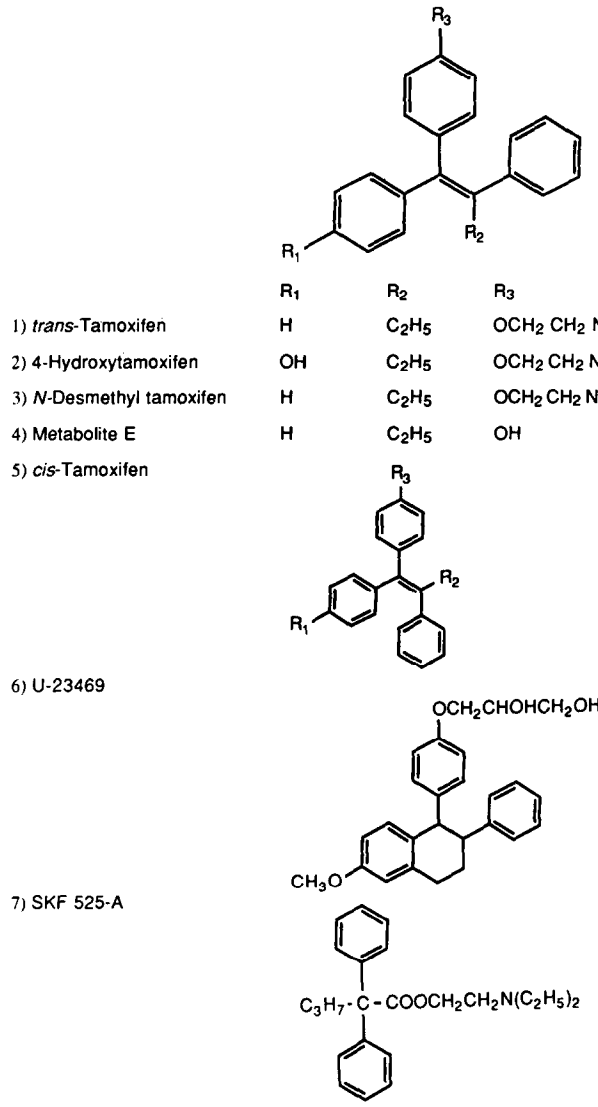

Fig. 1. Structure of tamoxifen and related compounds.

NSW). Testosterone, unlabelled androstenedione, 6β-hydroxy- and 16α-hydroxyandrostenedione, as well as all biochemicals, were purchased from Sigma Chemical Company (St. Louis, MO). 7α-Hydroxyandrostenedione was obtained from Professor D. N. Kirk of the MRC Steroid Reference Collection, Queen Mary's College (London, U.K.). 16β-Hydroxyandrostenedione was prepared enzymatically by the action of 3β-hydroxysteroid dehydrogenase (Sigma Chemical Co.) on 3β-, 16β-dihydroxyandrost-5-ene-17-one (MRC Collection) as described by Talalay and Dobson [6]. Other steroid standards were obtained from either Sigma, MRC Collection or Steraloids, Inc. (Wilton, NH).

trans- and *cis*-Tamoxifen, *N*-desmethyltamoxifen and 4-hydroxytamoxifen were the generous gifts of ICI (Cheshire, U.K.). Metabolite E and U-23469 were kindly donated by Dr. Colin Watts, Garvan Institute of Medical Research, Sydney, Australia. SKF 525-A (*N*,*N*-diethylaminoethyl-2,2-diphenyl valerate hydrochloride) was kindly donated by Smith, Kline and French Pty Ltd., Sydney, Australia. Solvents and miscellaneous chemicals were from Ajax Chemicals (Sydney, Australia) and were at least analytical reagent grade.

Animals. Male Wistar rats (200–250 g) were obtained from the animal house of the Institute of Clinical Pathology and Medical Research at Westmead Hospital. Animals were held under conditions of constant temperature (22°), humidity and lighting (12 hr dark-light cycle). Livers from eight rats were pooled and microsomal fractions were prepared as described elsewhere [7].

Optical difference spectroscopy. Cytochrome P-450 was measured according to the method of Omura and Sato [8], using an Aminco-Chance DW-2a

spectrophotometer and employing an extinction coefficient of 91 mM^{-1} cm^{-1} for the ferrous P-450/carbonyl spectral complex.

Difference spectral interactions between ferric P-450 and the test compounds were measured at 37° in an Aminco-Chance DW-2a spectrophotometer using 1 cm cuvettes containing 1 ml aliquots of microsomal suspensions (2 mg microsomal protein/ml) in potassium phosphate buffer (0.1 M pH 7.4). Test compounds were added to the sample cuvette in microlitre quantities in ethanol and the difference spectra were recorded between 380 and 500 nm; an equal volume of solvent was added to the reference cuvette. Spectral dissociation constants (K_s) and maximal spectral changes (ΔA_{max}) were determined from double reciprocal plots of the data according to established procedures [9]. MI-Complex formation was monitored at 37° in a Aminco-Chance DW-2a spectrophotometer as described for difference spectra measurements. Metabolism was initiated by addition of NADPH (final concentration 1 mM) to both reference and sample cuvettes. Repetitive scans over the 380–500 nm region of the spectrum were made, for at least 15 min, after NADPH addition.

Androstenedione hydroxylase activity. Microsomal androstenedione hydroxylase activity was assayed essentially as described previously [10]. Each incubation (4.0 ml final volume) contained 3.0 mg microsomal protein, an NADPH-generating system (4 mM glucose 6-phosphate, 1 mM NADP and 1 unit glucose 6-phosphate dehydrogenase) and androstenedione (final concentration 175 μM). Reactions were initiated by addition of the generating system and terminated after 10 min by the addition of 2 ml of 5.5% zinc sulphate. The chloroform extract of the resultant supernatant was applied to TLC plates (silica gel 60, F_{254} type, 20 × 20 cm × 0.25 mm thickness, and activated at 100° for 15 min, before use; E. Merck, Darmstadt, F.R.G.). Plates were developed twice in the solvent system ($CHCl_3$:ethyl acetate 1:2, v/v) as described by Waxman *et al.* [5]. Zones corresponding to hydroxylated androstenedione standards were visualised under UV light and scraped into vials for scintillation spectrometry.

Inhibitors were introduced into the reaction mixtures in 50 μl of ethanol (final concentration 1.25%); solvent alone was added to the control incubations. Plots of log inhibitor concentration vs per cent of control activity were constructed from mean per cent inhibition data at four different inhibitor concentrations (10, 50, 100, 300 μM). Each point was the mean of three individual estimates from separate incubations.

Protein assay. Microsomal protein concentration was estimated using the modified Lowry method of Chaykin [11].

RESULTS AND DISCUSSION

The data presented in Table 1 clearly demonstrate that tamoxifen was similarly effective as an inhibitor of four microsomal androstenedione hydroxylase pathways *in vitro*. With the exception of steroid 16β-hydroxylation, which is mediated by the minor P-450 form IIB 1 [5], I_{50}s were in the range 100–200 μM. The data obtained in the present study therefore indicate that the concentration of tamoxifen to produce 50% inhibition was quite similar to that of the steroid substrate used in this assay (175 μM). Very similar findings were noted for *cis*-tamoxifen, the geometric isomer of the antineoplastic agent, and for one of the principal phase 1 metabolites, 4-hydroxytamoxifen. Another major metabolite, the *N*-desmethyl compound, appeared quite similar to tamoxifen with regard to inhibition of androstenedione 6β-, 16α- and 16β-hydroxylation whereas 7α-hydroxylation was refractory to inhibition. Table 1 also reveals that the tamoxifen analogue U-23469 and the alklyamino containing compound, SKF 525-A, appeared quite similar to most of the tamoxifen derivatives in that the 16α-, 16β- and 6β-hydroxylation pathways were similarly susceptible to inhibition. As with *N*-desmethyltamoxifen 7α-hydroxylase activity was refractory to inhibition by these compounds. The most interesting finding, however, was that metabolite E, in which the amine-bearing side-chain is absent, was a specific and potent inhibitor of P-450 IIC 11-mediated steroid 16α-hydroxylase activity. The I_{50} of 18 μM obtained indicates that metabolite E inhibits steroid 16α-hydroxylation at about one-tenth the usual substrate concentration.

Table 1. *In vitro* inhibition of androstenedione hydroxylation in microsomes isolated from untreated male rats by tamoxifen and tamoxifen congeners

Compound	I_{50} μM Hydroxylation pathway			
	16α	16β	6β	7α
trans-Tamoxifen	173*	69	104	192
cis-Tamoxifen	189	160	98	191
N-Desmethyltamoxifen	97	197	69	—†
Metabolite E	18	128	86	257
U-23469	168	181	133	—†
4-Hydroxytamoxifen	173	171	116	194
SKF 525-A	180	110	130	—†

* I_{50} values were determined after three separate experiments from plots of log (inhibitor) concentration versus percent inhibition, using four concentrations of drug. Microsomes prepared from the pooled livers of eight rats, were used as the enzyme source. In all cases $r > 0.90$. Control activities for 16α, 16β, 6β and 7α-androstenedione hydroxylation were 2.40 ± 0.04, 0.19 ± 0.03, 2.10 ± 0.04, 0.35 ± 0.02 nmol/min/mg protein, respectively.

† I_{50} not able to be determined.

These results are consistent with the findings of other studies which have shown tamoxifen to be an inhibitor of cytochrome P-450 mediated monooxygenation. Thus, Meltzer *et al.* [2] showed tamoxifen, 4-hydroxytamoxifen and *N*-desmethyltamoxifen to effectively inhibit both ethylmorphine and aminopyrine *N*-demethylation *in vitro* and *in vivo*. Al-Turk *et al.* [12] demonstrated a decrease in aryl hydrocarbon hydroxylase and 7-ethoxycoumarin-*O*-deethylase activities in male rats after tamoxifen administration. Further studies [13, 14]

Table 2. Parameters of the Type 1 interaction with tamoxifen and tamoxifen congeners in microsomes isolated from untreated male rats*

Compound	K_s (μM)	ΔA_{max} (abs. unit/nmol P-450)	MI-complex (%P-450 complexed)
trans-Tamoxifen	27.0 ± 4.0	$1.8 \pm 0.1 \times 10^{-2}$	
cis-Tamoxifen	23.7 ± 3.0	$1.7 \pm 0.2 \times 10^{-2}$	
N-Desmethyltamoxifen	53.6 ± 5.0	$1.8 \pm 0.2 \times 10^{-2}$	
Metabolite E	42.6 ± 3.7	$4.5 \pm 0.5 \times 10^{-2}$	
U-23469	9.15 ± 0.2	$1.6 \pm 0.1 \times 10^{-2}$	
SKF 525-A	1.28 ± 0.1	$1.8 \pm 0.1 \times 10^{-2}$	19.0‡
4-Hydroxytamoxifen	—†	—†	

* Values are means ± SD of three separate experiments using microsomes isolated from the pooled livers of eight rats.

† This compound elicited a mixed Type 1/reverse Type 1 spectral change of inconsistent magnitude.

‡ Calculated using an extinction coefficient of 65 $mM^{-1} cm^{-1}$ [1].

have shown that tamoxifen, *N*-desmethyltamoxifen and 4-hydroxytamoxifen have long elimination half-lives (>7 days) in humans and that this parameter is increased after a multiple dosage regimen. The results of the present investigation reveal that tamoxifen and its metabolites are inhibitors of relatively uniform effect against several rat hepatic P-450s (IIC 11, IIIA 1/2 and IIB 1) whereas the P-450 IIA 1 isozyme appears relatively resistant to inhibition. Taken together these data strongly suggest that tamoxifen or its metabolites may be active inhibitors of P-450-mediated drug metabolism *in vivo*.

Alkylamino-containing compounds such as SKF 525-A have been shown to inhibit drug metabolism *in vivo* via the formation of MI complexes. However, when tamoxifen or analogues were incubated with NADPH and hepatic microsomes from untreated male rats, complex formation was not observed. In contrast, incubation with SKF 525-A resulted in the formation of a MI complex, with a maximum absorbance at 456 nm (Table 2). From these results it would appear that both the *in vitro* and *in vivo* inhibition of P-450 monooxygenation by tamoxifen, observed here and in other studies [2, 12], is not related to MI complex formation. Our results also suggest that MI complex formation does not necessarily lead to potent inhibition of steroid hydroxylase activity. Hence, SKF 525-A, which forms an MI complex, exhibited I_{50}s of the same order of magnitude as tamoxifen and related compounds, which do not form complexes (Table 2).

Recent work [15] has suggested that the anti-oestrogen binding sites (AEBS), to which tamoxifen and related have been shown to bind, may be P-450 isozymes. In these studies the basic, aminoalkyl side-chain of tamoxifen was shown to be required for binding. However, as we have observed, the absence of the aminoalkyl side-chain (metabolite E) actually enhanced the degree of inhibition of P-450 IIC 11, mediated steroid oxidation. Further, as shown in Table 2, removal of the side-chain actually produced a compound with a 3-fold greater binding capacity (ΔA_{max}) with ferric P-450 in hepatic microsomes. This observation suggests either that the side-chain may not necessarily be required for tamoxifen binding or that the AEBS and P-450 are distinct sites.

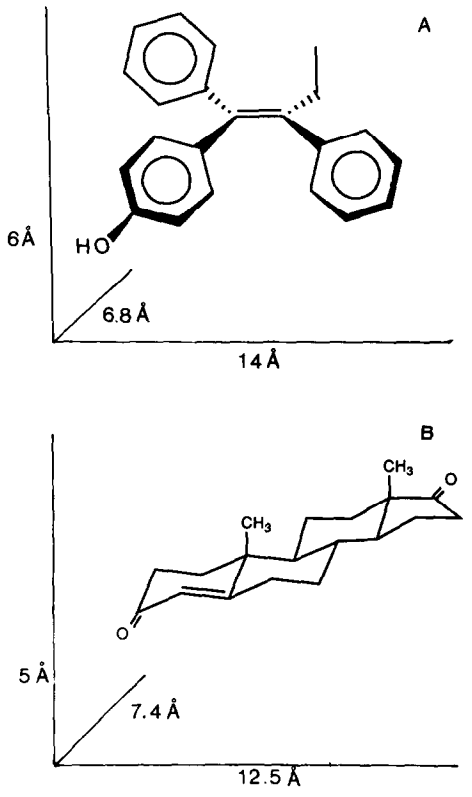

Fig. 2. Dimensions of (A) metabolite E and (B) androstenedione.

The data in Table 2 also show that the binding affinities (K_s) for *cis*- and *trans*-tamoxifen were quite similar (27 vs 24 μM) and that both isomers appeared to interact to a similar extent with ferric P-450 (ΔA_{max}). Indeed, the similarity in binding parameters appears reflected in the androstenedione inhibition data. This apparent equipotency of the isomers with regard to P-450 binding and inhibition of steroid hydroxylation is in contrast to the marked differences in oestrogen antagonism and oestrogen

receptor binding noted for *cis*- and *trans*-tamoxifen [16].

Both *N*-desmethyltamoxifen and metabolite E elicited spectral binding interactions of lower affinity than either tamoxifen isomer. In contrast, the tamoxifen analogue U-23469 elicited a spectral change with oxidised microsomes of higher affinity. Of particular interest in the present study was the finding that addition of the ring hydroxylated metabolite (4-hydroxytamoxifen) to oxidised microsomal fractions produced a mixed type I/reverse type I spectral interaction of inconsistent magnitude. Thus, 4-hydroxytamoxifen apparently binds to both low spin (giving rise to the type I interaction) and high spin (reverse type I interaction) P-450, in contrast to the other compounds which apparently only interact with low spin P-450.

A recent study [17] has shown that groups capable of forming hydrogen bonds, such as ketones and hydroxyls, influence the orientation, binding and hydroxylation of steroid substrates in the active site of P-450. It is interesting then to consider the interaction between metabolite E and P-450. The presence of a hydroxyl in the R_3 position of tamoxifen (metabolite E) results in preferential and potent inhibition of P-450 IIC 11 mediated, androstenedione 16α-hydroxylation. Comparison of metabolite E with androstenedione (Fig. 2) shows that these molecules have similar dimensions (androstenedione $5 \times 12.5 \times 7.4$ Å and metabolite E $6 \times 14 \times 6.8$ Å, depth/length/width, respectively). Superimposition of these two molecules leads to optimal overlap if the hydroxyl group of metabolite E is orientated over the 3-ketone group of androstenedione. In the context of the capacity of metabolite E to inhibit P-450 IIC 11 mediated 16α-hydroxylation it is possible that hydrogen bonding at the 3 ketone position of androstenedione with an amino acid residue of this enzyme co-ordinates 16α-hydroxylation.

Several points have emerged from the present study. First, it should not be assumed that the alkylamino structural feature, in a particular drug, is contiguous with MI complex formation with P-450 isozymes. Second, MI complexation does not necessarily result in increased inhibition. Finally, some drug metabolites may be more effective inhibitors of subsequent drug oxidation than the parent drugs (viz. metabolite E of the present investigation).

Studies of this type, in which drugs and potential metabolites are assessed as inhibitory molecules, will ultimately provide insights into the mechanisms of drug interactions of therapeutic significance. Indeed, with regard to tamoxifen, it is now clear that a multiple dosage regimen leads to the further oxidation of this drug. One consequence of this is that tamoxifen metabolites with enhanced inhibitory strength may accumulate in the liver.

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