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INDUCTION OF CYP2B1 AND 3A1, AND ASSOCIATED MONOOXYGENASE ACTIVITIES BY TAMOXIFEN AND CERTAIN ANALOGUES IN THE LIVERS OF FEMALE RATS AND MICE

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Abstract—Previous studies suggest long-term feeding of tamoxifen {*Z*-1-[4-(2-dimethylaminoethoxy)phenyl]1,2-diphenyl-1-butane} to rats gives rise to liver tumours, while mice are resistant. The effects of tamoxifen on cytochrome P450 isoenzymes and associated monooxygenase activities in the livers of female Fischer rats and C57Bl/6 and DBA/2 mice have been compared. Total microsomal cytochrome P450 was not induced in the livers of rats given tamoxifen (45 mg/kg daily for 4 days) and was in fact significantly reduced after 3 days treatment. In contrast, there was a 30–60-fold increase in the metabolism of benzyloxy- and pentoxyresorufins to resorufin. Ethoxyresorufin *O*-deethylase was induced only 2.5-fold. The regio- and stereo-specific hydroxylation of testosterone following tamoxifen pretreatment of rats showed a general time- and dose-dependent induction. 6β- and 16α-hydroxylation of testosterone together with oxidation to androstenedione were increased 2–3-fold while 2β-hydroxylation was induced only marginally, suggesting that tamoxifen produces a mixed pattern of induction with a significant phenobarbitone-like component. No induction of the 2β- or 6β-hydroxylation pathway occurred in either mouse strain. In rats, immunoblotting experiments with polyclonal antibodies raised against CYP2B1 or 3A1 showed that tamoxifen pretreatment resulted in 2–3-fold increases in both CYP2B1, 2B2 and 3A1 proteins, relative to controls. Immunohistochemistry of rat liver sections showed a centrilobular localization of these induced proteins. Similar patterns of induction as measured by immunoblotting experiments and testosterone hydroxylation were seen following the administration of structurally related analogues, toremifene and droloxifene (3-hydroxytamoxifen), thought to be non-carcinogenic in the rat. No induction of these monooxygenase activities was seen in C57Bl/6 mice and only small increases in benzyloxy- and pentoxyresorufin metabolism were in DBA/2 mice. It is suggested that the induction of cytochrome P450-dependent activities by tamoxifen may result in accelerated liver metabolism of this drug with important implications for the disposition of tamoxifen *in vivo* and also for its metabolic conversion to genotoxic metabolite(s). The difference in inducibility of cytochrome P450-dependent monooxygenase activities between rats and mice offers a plausible and testable hypothesis that the difference in tamoxifen metabolism between the two species may contribute to their carcinogenic response to tamoxifen.

The anti-oestrogen tamoxifen {*Z*-1-[4-(2-dimethylaminoethoxy)phenyl]1,2 - diphenyl - 1 - butane} (Fig. 1 I) is currently the drug of choice for the treatment of breast cancer in women [1]. Tamoxifen is being investigated for propyhylactic use in healthy women who might be at greater risk from this disease. Recent life-time feeding of tamoxifen to rats at high dose levels (45 mg/kg/day) results in the formation of liver tumours [2, 3]. Similar studies in mice did not result in the formation of such tumours [4]. Two structurally related analogues, toremifene (Fig. 1 II) and droloxifene (3-hydroxytamoxifen, Fig. 1 III) also failed to cause liver tumours in rats following long-term feeding [2, 5]. It has been suggested that metabolic activation of tamoxifen by monooxygenases results in the formation of reactive intermediates which bind covalently to rat liver microsomal proteins *in vitro* [6]. Administration of tamoxifen to rats *in vivo* can also give rise to ^{32}P-postlabelled DNA adducts in the liver [7].

Extensive hepatic metabolism of tamoxifen by cytochrome P450-dependent monooxygenases plays an important role in modulating the disposition of this compound in humans and in rodents [8, 9]. N-demethylation, a major metabolic pathway, is catalysed by the CYP3A family [10]. It is known that steroid hormones play an important role in the expression and regulation of certain cytochrome P450 isoenzymes [11]. However, the effects of tamoxifen on such systems have been less well studied. Since induction of liver cytochrome P450 activities by tamoxifen may have important implications for the disposition of this drug, as well as its metabolic activation responsible for DNA and protein adducts, we have now investigated the effects of tamoxifen and its analogues on liver microsomal cytochrome P450s and monooxygenase activities. We have used female Fischer rats and the *Ah*-responsive C57Bl/6 and *Ah*-non-responsive DBA/2 mice and measured the regio- and stereo-specific hydroxylation of testosterone and the metabolism of the alkoxyresorufins. Immunoblotting techniques have also been used to determine specific effects of tamoxifen pretreatment on CYP2B1, 2B2 and 3A1 proteins.

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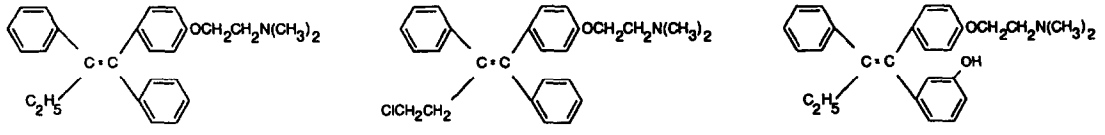


tamoxifen I toremifene II droloxifene III

Fig. 1. Chemical structures.

MATERIALS AND METHODS

Chemicals. Androstenedione, corticosterone, 16α-hydroxytestosterone NADPH and tamoxifen were from the Sigma Chemical Co. (Poole, U.K.). 16β-Hydroxytestosterone, 7α-hydroxytestosterone and 6β-hydroxytestosterone were from Steraloids (Croydon, U.K.). 2β-Hydroxytestosterone was from the Steroid Reference Centre, Queen Mary's College, London, U.K. Resorufin, 7-benzyloxy- and 7-ethoxy- and 7-pentoxyresorufins were from the Boehringer Corp. (Lewes, U.K.). The following were generously supplied as gifts: droloxifene citrate, from Dr B. Rattel, Klinge Pharma, GmbH (Munich, Germany); pyrrolidinotamoxifen, Dr M. Jarman, Institute of Cancer Research (Sutton, Surrey, U.K.) and toremifene citrate, Dr Y. Hirsimaki, Orion Corp., Farmos (Turku, Finland). Polyclonal antibodies raised in rabbits to rat CYP2B1, 2B2 were gifts from Dr A. J. Paine, DHSS Dept. of Toxicology, London, U.K., and CYP3A1 from Dr D. J. Waxman, Harvard Medical School, Boston, U.S.A.

Animals and dosing. Female Fischer F344/N rats, 6 weeks old (120–130 g) and C57Bl/6J mice (20–25 g) were bred on site. Female DBA/2 (20–25 g) mice were from Harlan Olac (Oxford, U.K.). Tamoxifen and pyrrolidinotamoxifen were dissolved in tricaprylin; toremifene and droloxifene citrates in propylene glycol. For dosing up to 7 days, compounds were administered by oral gavage, except where indicated, at the doses indicated in volumes of 0.1–0.15 mL. Controls received tricaprylin vehicle only. Four animals were in each dose group. For 30 days administration, tamoxifen was made up in powdered diet (0.45 g/kg diet) and animals allowed food and water *ad lib.*

Preparation of liver microsomal fractions. Livers were homogenized in ice-cold 0.25 M sucrose using a Teflon in glass homogenizer and washed microsomal fractions were prepared by differential centrifugation as described by Lake [12]. Protein concentrations were determined by the method of Lowry *et al.* [13] using bovine serum albumin as standard.

Testosterone hydroxylation. Reaction mixtures were essentially those described by van der Hoeven [14]. To an incubation mixture of 2 mL volume in 0.05 M Hepes/NaOH buffer pH 7.4 containing $MgCl_2$ (10 mM) and NADPH (1 mM) was added the liver microsomal preparation (1–2 mg protein). Following equilibration to 37°, reactions were started by the addition of testosterone (1 mM final concentration dissolved in 20 μL methanol). Flasks were shaken for 5 min in air at 37°. Reactions were stopped by adding 1 mL of the mixture to 0.3 mL of ice-cold methanol containing 23 μM corticosterone as internal standard. Metabolites were extracted into CH_2Cl_2 (5 mL), washed and subjected to reverse phase HPLC using a methanol/tetrahydrofuran/water gradient system as described [14]. Peaks were detected at 240 nm using a Kontron 432 HPLC detector and quantitated using a Kontron PC Integration Pak (Kontron Instruments, Watford, U.K.). Concentrations were determined relative to the internal standard.

Alkyloxyresorufin dealkylation. The dealkylation of three alkyloxyresorufins by control and tamoxifen-treated rat liver microsomal preparations was studied using a method derived from Lubet *et al.* [15]. Incubations were carried out in 50 mM Tris buffer, pH 7.5, containing $MgCl_2$ (25 mM) at 28°. The final microsomal protein concentration was 44 μg/mL using ethoxy- and pentoxyresorufin. The substrate concentrations were 2 and 10 μM, respectively. For the benzyloxyresorufin assays, a microsomal protein concentration of 9 μg/mL was employed and a substrate concentration of 5 μM. All three substrates were dissolved in dimethyl sulphoxide (10 μL).

The assays were started by the addition of NADPH to a final concentration of 80 μM. The resulting fluorescence was monitored using a Perkin–Elmer LS50 luminescence spectrophotometer with an excitation wavelength of 522 nm and an emission wavelength of 586 nm.

Determination of cytochromes b_5 *and P450.* The concentrations of total cytochrome b_5 and P450 in washed liver microsomal preparations were determined spectrophotometrically: cytochrome b_5 from the NADH reduced vs oxidized difference spectra using an absorbance coefficient of 185 $L \cdot mmol^{-1} \cdot cm^{-1}$ for the difference in absorbance between 424 and 409 nm; cytochrome P450 from the CO-dithionite reduced vs dithionite reduced difference spectra using an absorption coefficient of 91 $L \cdot mmol^{-1} \cdot cm^{-1}$ [16].

Quantitative estimates of individual P450 isoenzyme concentrations in liver microsomal fractions were performed by western blotting. Protein samples (25 μg) were run on 9% polyacrylamide gels. Following transfer to nitrocellulose sheets, immunoreactive bands were detected by incubation first with the appropriate polyclonal rabbit anti-P450 (1:750 dilution) and then with an anti-rabbit alkaline phosphatase-conjugated second antibody (1:1000 dilution, Sigma Chemical Co., Poole, U.K.). Incubation of the sheets was carried out at room

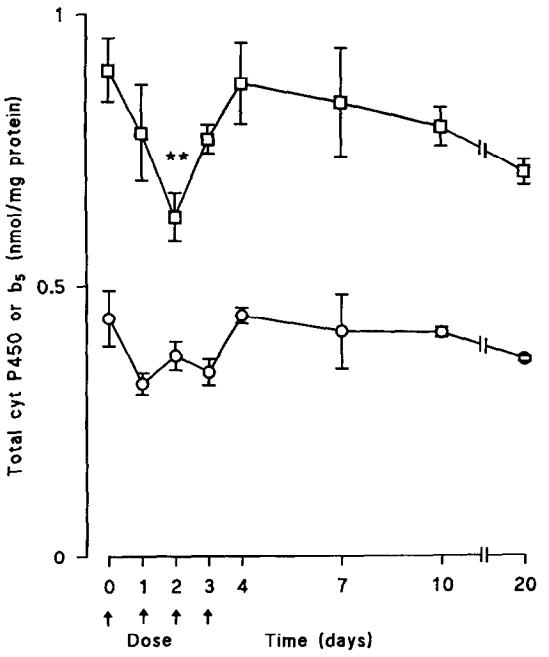


Fig. 2. Effects of repeated daily dosing with tamoxifen on total liver cytochrome P450 and b_5 in the livers of rats. Female rats were given daily doses of tamoxifen (0.12 mmol/kg i.p.) as indicated (↑) for up to 4 days and killed 24 hr later. In a separate experiment, rats were fed tamoxifen in the diet (0.12 mmol/kg/day) continuously for 30 days (solid symbols). Cytochrome P450 (□) and cytochrome b_5 (○) were estimated spectrophotometrically as described in Materials and Methods. Control values for cytochrome P450 and b_5 were determined on day 0 only. Results represent the means ± SE for at least four experiments. Probability of significance of differences between dosed and control animals: $^*P < 0.05$, $^{**}P < 0.01$.

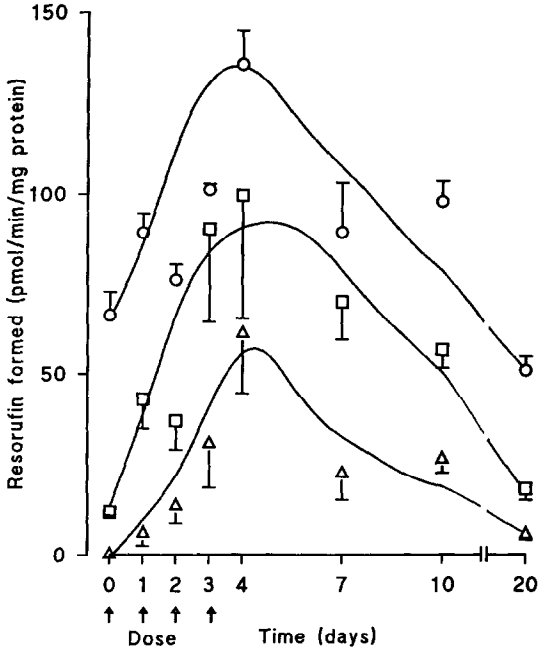


Fig. 3. Effects of time of pretreatment of rats with tamoxifen on the metabolism of alkoxyresorufins. Female rats were given daily doses of tamoxifen (0.12 mmol/kg, i.p.) as indicated for up to 4 days and killed 24 hr later. In a separate experiment, animals were fed tamoxifen in the diet (0.12 mmol/kg) continuously for 30 days (solid symbols). The metabolism of ethoxy (○), benzyloxy (□) and pentoxy (△) -resofurin to resofurin was estimated in liver microsomal preparations spectrofluorometrically as described in Materials and Methods. Results represent the means ± SE for four animals.

temperature with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (both 0.4 mM) dissolved in 0.1 M Tris–HCl buffer, pH 9.5, containing 0.1 M NaCl and 5 mM $MgCl_2$ [17]. Reactions were stopped by rinsing the sheets in 0.1 M citrate/phosphate buffer pH 7.0 containing 20 mM EDTA. Bands were quantitated using a LKB soft laser scanning densitometer (LKB Biochrom., Cambridge, U.K.). As the cytochrome P450 isoenzyme protein standards were not available, concentrations were expressed relative to microsomal preparations from vehicle-dosed controls processed at the same time. There was a linear correlation between the apparent concentration of CYP2B1 and 3A1 and the amount of protein applied to the SDS gel over the range 10–50 μg microsomal protein.

Immunohistochemical localization of cytochrome P450 *in liver sections.* Livers from control rats or those that had been pretreated with tamoxifen (0.12 mmol/kg p.o. for 4 days) were fixed in acetone and 5 μm paraffin sections prepared by standard procedures. Following dewaxing, hydration and inactivation of endogenous peroxidases, sections were incubated with the appropriate anti-cytochrome P450 antibody (1:200 dilution). Binding of the antibody was visualized by standard procedures using peroxidase–antiperoxidase staining with 3,3'-diaminobenzidine (0.5 mg/mL) and H_2O_2 (0.01%) in phosphate-buffered saline, pH 7.4, as substrate. Sections were lightly counterstained with Gill's haematoxylin. All steps were carried out at room temperature.

RESULTS

Effects of tamoxifen on total liver microsomal cytochrome b_5 *and P450*

Figure 2 shows that when tamoxifen was given at a daily dose of 45 mg/kg (0.12 mmol/kg) i.p. for 4 days there was little overall change in the concentration of cytochrome b_5 in liver microsomal preparations of female rats relative to controls. The concentration of cytochrome P450 however was significantly decreased at day 3. This effect was transitory and the cytochrome P450 concentration had returned to control levels by day 5. Results are shown relative to control microsomal preparation at day 0 (Fig. 2). Age-matched control values for each of the remaining time points were not determined, with the exception of the 30 days groups, where the following values for cytochrome P450 (nmol/mg

Table 1. Effects of pretreatment of female rats or mice with tamoxifen or its analogues on the metabolism of alkoxyresorufins by liver microsomal preparations

Inducer	Resorufin homologue metabolism (pmol/min/mg microsomal protein)		
	Ethoxy-	Benzyloxy-	Pentoxy-
Rats			
Controls	66 ± 6	12 ± 1	1 ± 0.2
Tamoxifen	147 ± 14†	316 ± 56†	170 ± 25*
Toremifene	97 ± 10‡	154 ± 10*	79 ± 10*
Droloxifene	74 ± 13 NS	40 ± 17 NS	14 ± 8 NS
Pyrrolidinotamoxifen	63 ± 4 NS	21 ± 6 NS	19 ± 5‡
Mice			
C57BL/6			
Controls	107 ± 8	196 ± 6	49 ± 6
Tamoxifen	53 ± 9†	89 ± 10*	24 ± 1†
DBA/2			
Controls	54 ± 19	125 ± 28	25 ± 4
Tamoxifen	47 ± 4 NS	190 ± 45 NS	43 ± 4‡

Female Fischer rats or C57Bl/6 or DBA/2 mice were given daily doses of tamoxifen or analogues (0.12 mmol/kg by gavage except for pyrrolidinotamoxifen which was administered at a dose of 0.06 mmol/kg) for 4 days and killed 24 hr later. The metabolism of ethoxy-, benzyloxy- and pentoxyresorufin was estimated spectrofluorometrically in liver microsomal preparations as described in Materials and Methods.

Results represent the means ± SE for four experiments. Probability of significance of difference between dosed and controls: *$P < 0.001$; †$P < 0.01$; ‡$P < 0.05$; NS, not significant.

protein) were found: 0.54 ± 0.04 (N = 4) for the control group and 0.68 ± 0.06 (N = 4) for rats given tamoxifen continuously in the diet ($P > 0.05$). None of the tamoxifen treatments caused any significant change in liver weight, expressed as a percentage of body weight, relative to the controls. Up to 30 days exposure to tamoxifen, treated rats gained weight more slowly than controls (results not shown).

Induction of alkoxyresorufin metabolism by tamoxifen

Figure 3 shows that the *in vitro* metabolism of the alkoxyresorufin homologues to resorufin by rat liver microsomes was induced by tamoxifen given by the intraperitoneal route. Maximal levels of induction occurred at day 4 when the metabolism of benzyloxy- and pentoxyresorufin was increased 30–60-fold, while ethoxyresorufin *O*-deethylase was increased only about 2.5-fold. On cessation of dosing, activity slowly decreased to values close to control levels by day 20. When the drug was given continuously in the diet for 30 days (compared to matched controls of the same age), these activities were also increased but not to a greater extent than observed on repeat dosing (results not shown).

Dosing of rats with tamoxifen and toremifine caused a significant increase in pentoxyresorufin metabolism; droloxifene produced a weaker response (Table 1). Only with tamoxifen itself and toremifene was there significant induction of ethoxy-, pentoxy- and benzyloxyresorufin metabolism in the liver microsomal fractions, relative to controls. Administration of tamoxifen by oral gavage consistently gave higher levels of induction of alkoxyresorufin metabolism, particularly pentoxy- and benzyloxyresorufin, than intraperitoneal administration (cf. Fig. 3 and Table 1). This could be due to a more rapid first pass metabolism of tamoxifen following intraperitoneal dosing.

Control mice of both the C57Bl/6 and DBA/2 strains had significantly greater activities of benzyloxy and pentoxyresorufin dealkylation than control rats. After treatment with tamoxifen a small increase in pentoxy- and benzyloxyresorufin metabolism, relative to vehicle-dosed controls, was seen only in DBA/2 mice (Table 1), whereas a significant loss of all three activities was seen in C57Bl/6 mice.

Regio- and stereo-specific hydroxylation of testosterone by liver microsomal preparations

Figure 4 shows the effect of four daily doses of tamoxifen (0.12 mmol/kg i.p.) on the regio- and stereo-specific hydroxylation of the model substrate testosterone. After four doses and cessation of dosing, maximum induction occurred between days 3 and 7, depending on the hydroxylation reaction, followed by a slow decrease over the next 13 days. The majority of the regio- and stereo-specific hydroxylations were induced by tamoxifen pretreatment, but to different degrees, e.g. 6β- and 16α-hydroxylation of testosterone together with oxidation to its androstenedione were elevated 2–3-fold, while 7α- and 2β-hydroxylation of testosterone were increased only marginally.

The extent of induction of testosterone hydroxylation was dependent on the dose administered up to 0.12 mmol/kg (45 mg/kg) (Fig. 5). The lowest dose tested, 13 μmol/kg (5 mg/kg) i.p. for 4 days, still caused significant increases in 6β-hydroxylation and androstenedione formation.

In contrast, administration of tamoxifen (0.12 mmol/kg) to female *Ah* responsive C57Bl/6 or *Ah* non-responsive DBA/2 mice caused little increase

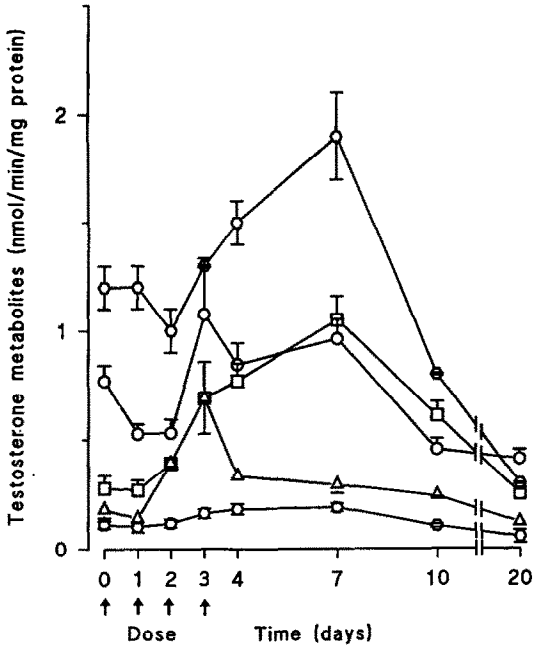


Fig. 4. Dependence on time of exposure to tamoxifen of the regio- and stereo-specific hydroxylation of testosterone in rat liver microsomal preparations. Female rats were dosed daily with tamoxifen (0.12 mmol/kg i.p.) as indicated (↑) for up to 4 days and killed 24 hr after doxing. In a separate experiment rats were fed tamoxifen in the diet (0.12 mmol/kg/day) for 30 days (solid symbols). Testosterone and its metabolites were estimated by reverse phase HPLC. These were: androstenedione (○); 6β-hydroxy (□); 7α-hydroxy (○); 16α-hydroxy (△) and 2β-hydroxy (▽) -testosterone. Results represent the means ± SE for four animals.

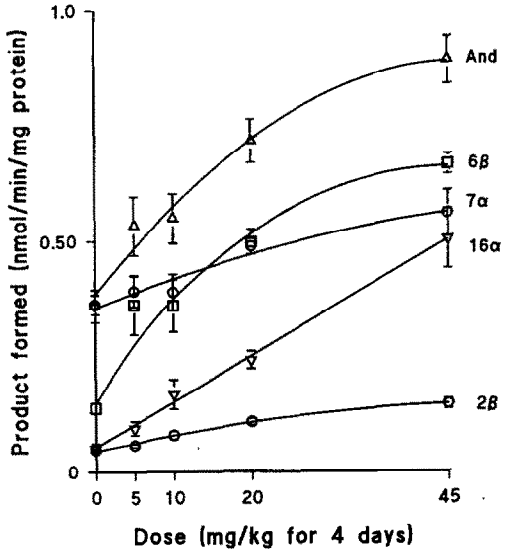


Fig. 5. Dependence on dose of tamoxifen pretreatment of the regio- and stereo-specific induction of testosterone metabolism. Female rats were pretreated with tamoxifen administered i.p. at the doses indicated daily for 4 days and killed 24 hr later. Testosterone metabolism in liver microsomal preparations was estimated by reverse phase HPLC as described in Materials and Methods. Androstenedione (△); 6β-hydroxy (□); 7α-hydroxy (○); 16α-hydroxy (▽) and 2β-hydroxy (○) -testosterone. Results represent the means ± SE for four animals.

in testosterone hydroxylation (Fig. 6). In control mice, rates of oxidation of testosterone, particularly with regard to androstenedione and 7α-hydroxylation formation were 2–4-fold higher than in control female rats (cf. Fig. 5). The same applied to the metabolism of the alkoxyresorufins in control mice (compared to control rats) where activities were 1.6–49-fold higher (Table 1).

Following exposure to tamoxifen analogues (0.12 mmol/kg, p.o. for 4 days), the induction of testosterone hydroxylation in female rat liver microsomal preparations was broadly similar to that by tamoxifen itself (Fig. 7). 2β-Hydroxylation showed little induction while 6β-hydroxylation and androstenedione formation were increased 5–8-fold. Because of the greater acute toxicity of pyrrolidinotamoxifen, causing the animals to become moribund after 3 days dosing of 0.12 mmol/kg, this compound was given at a dose of 0.06 mmol/kg and microsomes prepared from animals given this smaller dose. None of the analogues tested caused a significant change in liver weight, expressed as a percentage of body weight.

Induction of CYP2B and 3A families by tamoxifen

Immunoblotting experiments (Fig. 8A) showed that tamoxifen, pyrrolidinotamoxifen, toremifene and droloxifene pretreatments (0.12 mmol/kg p.o. for 4 days) resulted in only modest 2–3-fold increases in the concentration of CYP2B1 and 2B2 proteins in rat liver microsomal preparations. CYP3A1 was also induced 3-fold by tamoxifen; the remaining analogues caused lower levels of induction.

In female mice, only tamoxifen was investigated for its possible inductive effect. Results show (Fig. 8B) that in DBA/2 mice, tamoxifen (0.12 mmol/kg p.o. for 4 days) caused a <2-fold increase in the concentration of CYP2B1 while the induction was not significant in female C57Bl/6 mice. In this strain of mice there was no induction of CYP3A1 but in C57Bl/6 mice a small induction (<2-fold) was observed.

Localization of induced cytochrome P450 isoenzymes in the liver lobule

Immunohistochemistry of liver sections from tamoxifen-pretreated female rats (0.12 mmol/kg daily by gavage for 4 days) showed CYP2B1, 2B2 to be localized mainly in zone 3 (centrilobular) of the liver, while CYP3A showed a more diffuse centrilobular to midzonal induction in the liver lobule (Fig. 9).

DISCUSSION

The doses of tamoxifen used in the present study (45 mg/kg daily) are very much higher than those

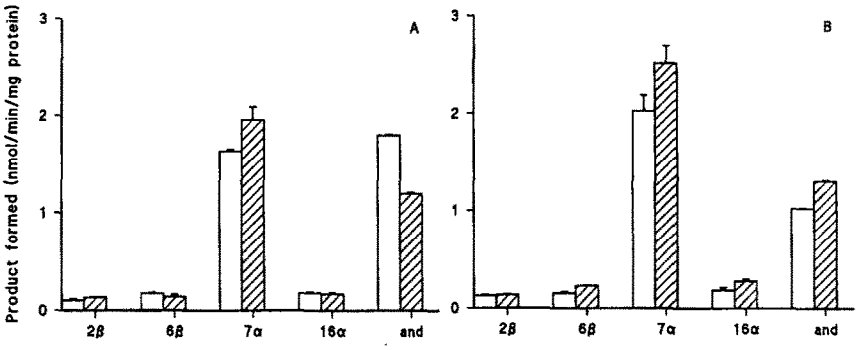


Fig. 6. Comparisons of the effects of pretreating female C57Bl/6 or DBA/2 mice with tamoxifen on the regio- and stereo-specific hydroxylation of testosterone. Mice were given tamoxifen (0.12 mmol/kg p.o.) daily for 4 days and killed 24 hr later. Testosterone metabolism in liver microsomal preparations was estimated by reverse phase HPLC. (a) C57Bl/6 mice; (b) DBA/2 mice. Control, vehicle only (□); tamoxifen-dosed (▨). Results represent the means ± SE for four experiments.

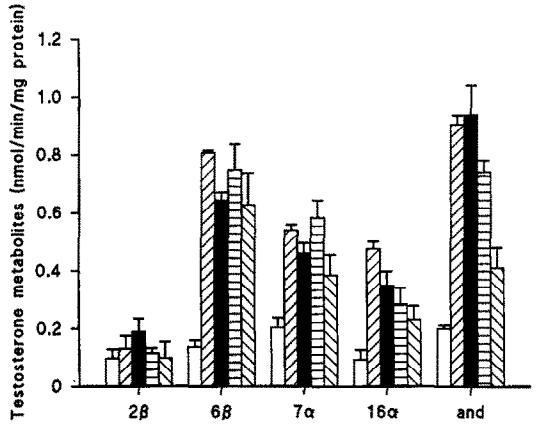


Fig. 7. Comparison of the regio- and stereo-specific induction of the metabolism of testosterone caused by pretreating rats with tamoxifen and structurally related analogues. Rats were dosed with tamoxifen and analogues at a dose of 0.12 mmol/kg by gavage except for pyrrolidinotamoxifen where animals received 0.06 mmol/kg. Animals were given four daily doses and killed 24 hr later. Testosterone metabolism in liver microsomal preparations was estimated by reverse phase HPLC. Histograms represent: controls (vehicle only, □); tamoxifen (▨); toremifene (■); droloxifene (▤) and pyrrolidinotamoxifen (▧). Abscissa represents hydroxylation of testosterone at the 2β, 6β, 7α and 16α positions, and = oxidation of testosterone to androstenedione.

used by women (20 mg/day), although women may take this compound for many years. The administration of tamoxifen at this dose level (45 mg/kg) to female rats for up to 30 days did not result in a general induction of hepatic cytochrome P450 and there was no hyperplastic response in the liver as judged by the absence of a change in liver weight as a percentage of body weight. The cause of the initial transient decrease in cytochrome P450 after 3 days of dosing (Fig. 2), while the monooxygenase activities investigated were increasing, is not known. No accumulation of *N*-alkyl porphyrins (an index of cytochrome P450 haem destruction) could be detected (I. N. H. White, unpublished observations), as has been shown to occur with certain contraceptive steroids [18]. It is known that tamoxifen can be activated by cytochrome P450 to form reactive intermediates which covalently bind to microsomal protein [5], but it is not known if this affects the stability of the relevant cytochrome P450 enzyme(s). Pretreatment of female rats with synthetic oestrogens, such as diethylstilbestrol, for 7 days results in significant increases in liver weight and hepatic cytochrome P450 concentrations [19], an effect quite different from that seen in the present study with tamoxifen.

Effect of tamoxifen pretreatment on alkoxyresorufin metabolism in liver microsomal systems

The relative rates of metabolism of ethoxy-, pentoxy- and benzyloxyresorufins provide a good indication of the relative induction of the isoenzymic forms of cytochrome P450 by phenobarbitone or 3-methylcholanthrene [20]. The present results show (Fig. 3) a 30–60-fold increase in the metabolism of pentoxy- and benzyloxyresorufins and only a slight increase in ethoxyresorufin *O*-deethylase. This suggests the induction of CYP2B1, 2B2. The extent of conversion of pentoxyresorufin to resorufin was lower, however, than the 200-fold increase caused by phenobarbitone pretreatment [20]. It has been reported that any induction of the steroid inducible CYP3A family cannot be detected by increased metabolism of alkoxyresorufin homologues [20]. For this reason, testosterone was chosen as an alternative model substrate.

Regio- and stereo-specific hydroxylation of testosterone

Purified cytochrome P450 isoenzymes show characteristic patterns in the oxidation of testosterone and this has proved useful in distinguishing the regulation and induction of multiple isoenzymic forms [11, 21]. We wished to investigate the effects

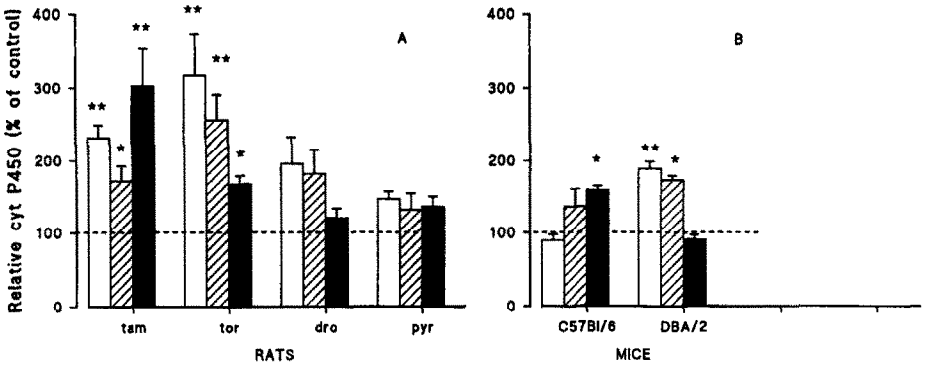


Fig. 8. Induction of CYP2B1, 2B2 and 3A protein in liver microsomal preparations from rats and mice following pretreatment with tamoxifen. Rats and mice were dosed with tamoxifen (0.12 mmol/kg by oral gavage) for 4 days and killed 24 hr later. Where indicated, rats were dosed with tamoxifen or its analogues: tam = tamoxifen, tor = toremifene, dro = droloxifene and pyr = pyrrolidinotamoxifen, at the same doses except for pyrrolidinotamoxifen where animals received 0.06 mmol/kg. Liver microsomal proteins (25 μg) were separated on 9% SDS–polyacrylamide gels, blotted onto nitrocellulose sheets and the cytochrome P450 proteins detected by the use of the appropriate antibodies. Results are expressed as percentages of concentrations in vehicle-dosed controls and represent the means ± SE for four experiments. Significance of difference between controls and dosed animals: $^{*}P < 0.05$; $^{**}P < 0.01$. (a) Female Fischer rats; (b) female C57BL/6 or DBA/2 mice. Histograms represent: CYP2B1 (□); 2B2 (□); 3A (■).

of tamoxifen on the CYP3A family since these cytochromes are induced by steroids such as dexamethasone and pregnenolone 16α-carbonitrile [21, 22]. In the female rat, CYP3A mediates > 85% of microsomal testosterone 6β-hydroxylase activity [23]. In addition, CYP3A has been identified as being responsible for the N-demethylation of tamoxifen, a major metabolic pathway in the rat [10, 24].

The present results suggest tamoxifen is a weaker inducer of CYP3A1 than dexamethasone or pregnenolone 16α-carbonitrile. Induction of CYP3A1 is associated with an induction of both 2β- and 6β-hydroxylation of testosterone [23, 25–27]. Tamoxifen pretreatment of rats results in induction of testosterone 6β-hydroxylation but only marginal increases in 2β-hydroxylation (Fig. 5). Elevation of 6β-hydroxylation, without induction of 2β-hydroxylation, may indicate that the induction of CYP2B1, 2B2 contributes significantly, since phenobarbitone pretreatment of rats can also lead to increased 6β-hydroxylation of testosterone. Such phenobarbitone pretreatment stimulates 16α-hydroxylation of testosterone and oxidation of this steroid to androstenedione [27]. This was the metabolic pattern observed in the present study following tamoxifen pretreatment of rats. This suggests that induction of CYP2B1, 2B2 by tamoxifen plays a more important role than CYP3A1 in the overall metabolism of testosterone although we cannot exclude that other isoenzymic forms may also be involved since maximal induction of pentoxyresorufin metabolism catalysed by CYP2B1 occurred at 3 days (Fig. 3), while 6β-hydroxylation of testosterone and oxidation to androstenedione occurred at day 7 (Fig. 4). For both testosterone and the alkoxyresorufin substrates, on cessation of dosing with tamoxifen, the time required for nearly complete loss of induced activity was similar, namely 13–16 days after treatment. This is considerably longer than the 6 hr plasma half-life of tamoxifen in the rat, although liver levels of this compound are up to two orders of magnitude higher than in plasma and persist much longer [9].

Cytochrome P450 isoenzyme induction determined by western blotting

The extent of the induction of the different cytochrome P450 isoenzymic proteins was investigated by immunoblotting with antibodies which recognise CYP2B1, 2B2 and 3A, and the results are shown in Fig. 8. As has been suggested by the induction of pentoxyresorufin metabolism by tamoxifen pretreatment, the level of induction by CYP2B1 and 2B2 proteins (2–3-fold) was very much lower than the 40–50-fold increases in these isoenzymes seen following pretreatment with phenobarbitone [28, 29]. In a recent abstract, an increase in immunoreactive CYP2B1 and 2B2 has also been reported in livers of rats given tamoxifen [30]. Similarly, the induction of CYP3A was more modest than the 15-fold increase caused by dexamethasone pretreatment of female rats [20]. The polyclonal antibody used in the present experiments recognises a single band of molecular mass 51 kDa in tamoxifen-pretreated rat liver microsomes, similar in molecular mass and immunoreactivity to that induced in rats pretreated with dexamethasone [22]. Although the different CYP3A isoenzymes have slightly different molecular masses [22] we cannot exclude the possibility that tamoxifen may induce more than one member of the CYP3A family.

The small increases in CYP2B1, 2B2 protein concentrations seen in the livers of DBA/2 but not

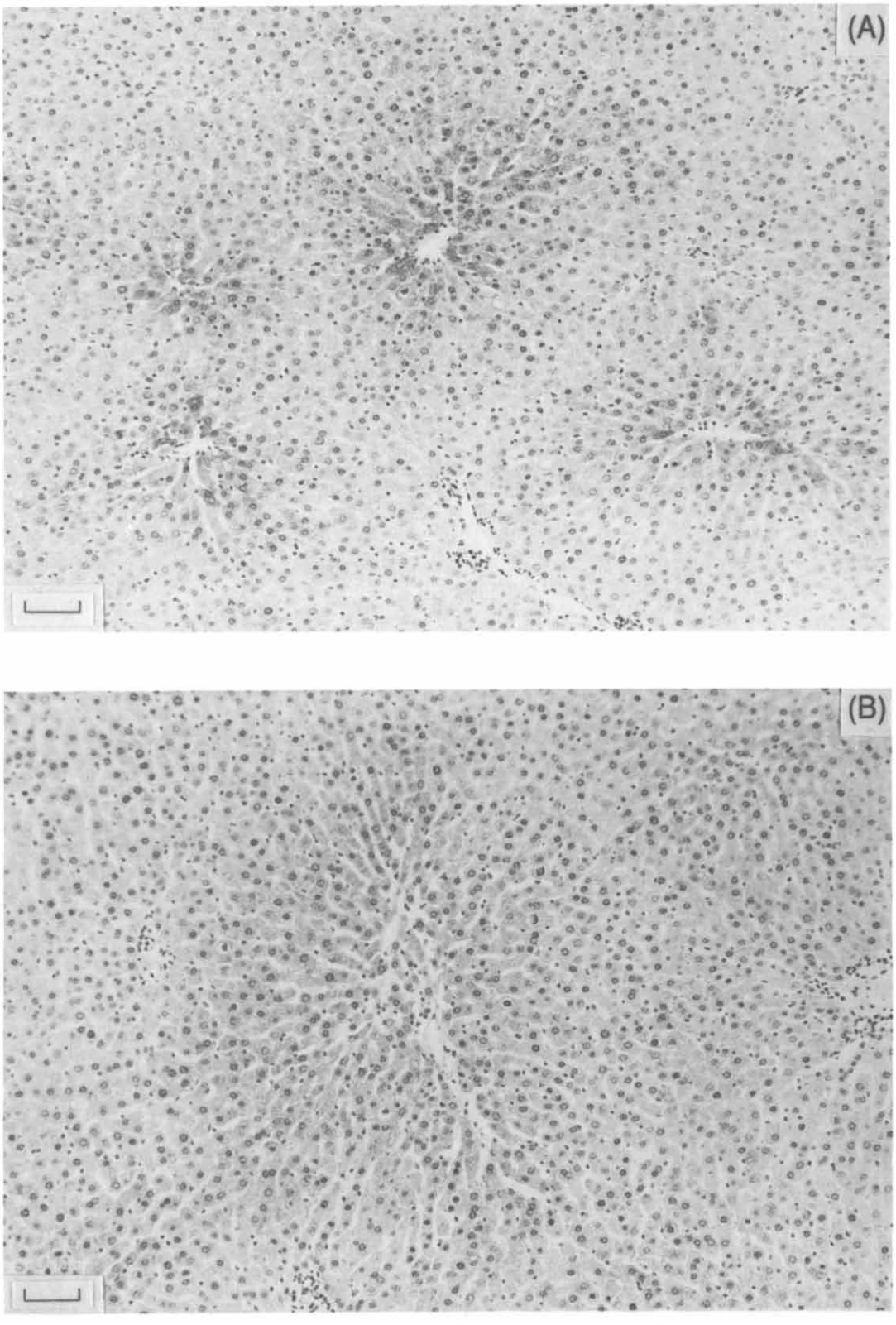
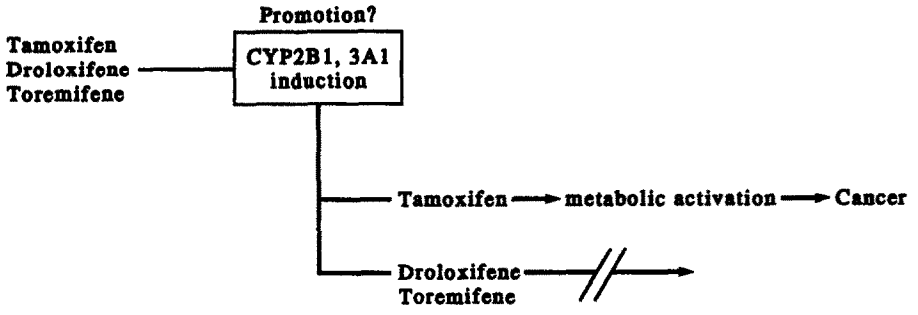


Fig. 9. Immunohistochemical localization of CYP2B1, 2B2 or 3A in the livers of rats following pretreatment with tamoxifen. Rats were dosed with tamoxifen (0.12 mmol/kg by gavage) for 4 days and killed 24 hr later. Liver sections were fixed in acetone and 5 μm paraffin sections prepared. Following dewaxing and incubation with the appropriate polyclonal anti-P450 antibody, cytochrome P450 protein was visualized by peroxidase–antiperoxidase staining. Bar represents 100 μm. (a) Rat liver stained for CYP2B1, 2B2; (b) rat liver stained for CYP3A.



Scheme 1.

C57Bl/6 mice (Fig. 8b) caused by tamoxifen pretreatment agree well with the low induction of pentoxyresorufin metabolism seen in the former strain (Table 1).

Immunohistochemistry of liver sections of rats pretreated with tamoxifen showed that for both the CYP2B1, 2B2 and 3A families, induction tended to be concentrated in the centrilobular (zone 3) area (Fig. 9). This is similar to the zonal induction found following pretreatment of rats with phenobarbitone [31].

Induction of cytochrome P450 isoenzymes by tamoxifen analogues: comparisons between female rats and mice

The present results suggest, as judged from testosterone hydroxylation and immunoblotting experiments, that the induction of cytochrome P450 isoenzymes by tamoxifen analogues investigated in this study is broadly similar to that by tamoxifen itself. The mechanism by which tamoxifen induces liver cancer in the rat is not yet known. Dragan *et al.* [32] have concluded from their experiments that tamoxifen may act as a pure promoter, inducing the development of spontaneously initiated liver lesions. Assuming that the induction of cytochrome P450 isoenzymes is a good indicator of promotion, our findings comparing rats and mice would be compatible with this model. However, promotion alone cannot explain the carcinogenicity of tamoxifen on this basis, since the non-carcinogenic analogues toremifene and droloxifene are also cytochrome P450 isoenzyme inducers. We cannot eliminate the importance of metabolic activation of tamoxifen in the mechanism of carcinogenicity, as the small differences in molecular structure which exist between tamoxifen and its analogues may well account for the generation of metabolites of very different genotoxic potential. Also, in agreement with this, we found that in ^{32}P-postlabelling experiments, tamoxifen produced a significant increase in modified DNA bases, whereas droloxifene and toremifene failed to do so (Scheme 1) [33].

Accordingly, the induction of cytochrome P450 activities by tamoxifen may result in accelerated activation of the drug to genotoxic metabolites and in this way contribute to the mechanism of tamoxifen hepatocarcinogenesis. In addition, the difference in inducibility of the cytochrome P450-dependent monooxygenase activities between rats and mice may offer a plausible and testable hypothesis that the difference in tamoxifen metabolism between the two species may account for differences in carcinogenic response.

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