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Brief communication

Tamoxifen metabolism is altered by simultaneous administration of medroxyprogesterone acetate in breast cancer patients

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Key words: endocrinology, medroxyprogesterone acetate, metabolism, tamoxifen

Summary

We have measured the levels of tamoxifen and three of its metabolites in the blood of patients receiving tamoxifen alone or combination therapy with tamoxifen and medroxyprogesterone acetate. Our results indicate that addition of the progestogen significantly alters the metabolism of tamoxifen over a six-month period. We suggest that the interaction between these drugs may involve additional sites (probably hepatic) besides the desired target tumour.

Introduction

Endocrine manipulation with the anti-oestrogen tamoxifen is capable of causing beneficial effects in at least one third of breast cancer cases [1, 2]. Its safety and efficacy have made it a widely used first-line therapy for locally advanced and disseminated breast cancer in post-menopausal women. The mode of action of tamoxifen is still not entirely clear; some of its effects are oestrogenic. For example, it causes induction of the progesterone receptor in breast tumours [3, 4]. Since progestogens, particularly in high doses, can inhibit the growth of breast cancers, the possibility arises of a synergistic effect between these agents and tamoxifen.

To test this possibility, a trial of tamoxifen alone versus combined tamoxifen/medroxyprogesterone acetate (MPA) therapy has been set up in our hospital. Tamoxifen is extensively metabolized, and demethylated and hydroxylated derivatives can be detected in the blood of treated individuals [5, 6]. As their potency may differ from that of the parent compound [5], we set out to study the effect of the simultaneous administration of MPA on the pattern of tamoxifen metabolism in our trial patients.

Although it is too early to say whether combined therapy has any clinical advantages, it is already clear that MPA perturbs the metabolism of tamoxifen. In other words the two drugs are indeed interacting, but at other sites in addition to the tumour target.

Methods

Patients

20 post-menopausal women presenting with stage III or IV disease and ineligible for surgery were entered in the trial, for which consent had been obtained from the local Ethics Committee. All received tamoxifen (Nolvadex 20mg bd) for two weeks before being randomised to receive tamoxi-

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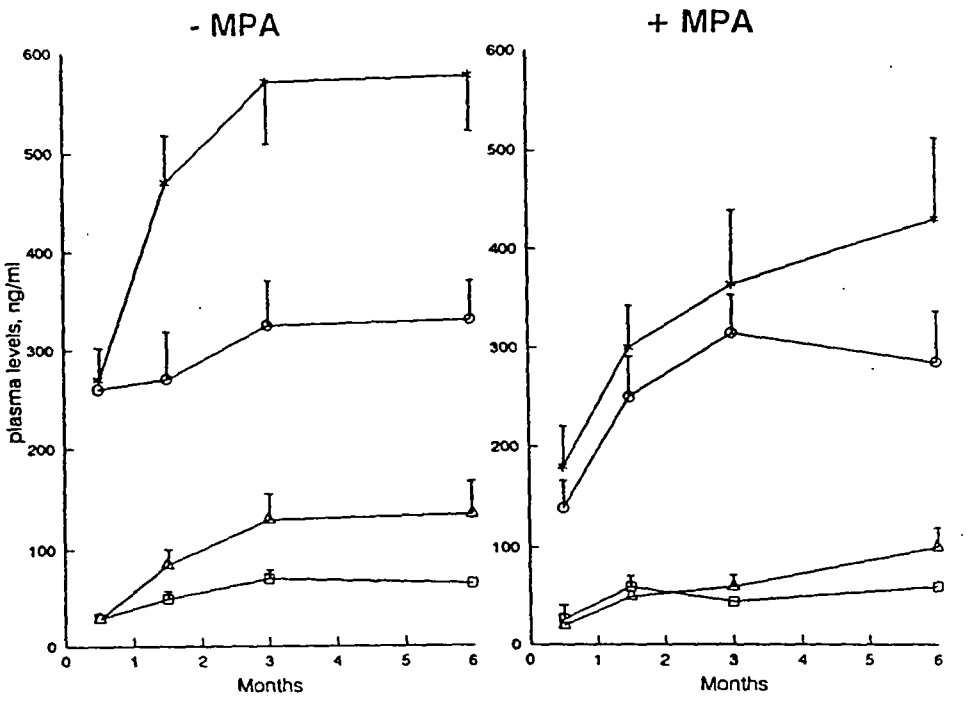

Fig. 1. Serum levels of tamoxifen and its metabolites. O—O tamoxifen, *—* desmethyltamoxifen, Δ—Δ desdimethyltamoxifen, □—□ metabolite Y. For the TAM/PLA group (−MPA), eight or more data values were available at each time point. For the TAM/MPA group (+MPA), four values were available at the 2 week time point and six or more at the others. The bars represent the standard errors of the means. For clarity, only one half of each bar is shown, and where the bar was smaller than the symbol it has been omitted completely.

fen plus placebo (TAM/PLA) or tamoxifen plus MPA (Farlutal 500 mg bd) (TAM/MPA).

There were no significant differences in age distribution between the two groups (TAM/PLA mean age 70 y, range 57–79; TAM/MPA mean age 74 y, range 58–87). Two of the TAM/PLA and 4 of the TAM/MPA patients had stage III disease, the remainder having stage IV cancer.

Drug analysis

Blood samples were taken at 2 weeks (i.e. immediately before randomisation), 6 weeks, 3 months, and 6 months. Serum was separated and stored at −70°C before assay. For tamoxifen analysis, 100 µl was mixed with 100 µl acetonitrile containing clomiphene as an internal standard, centrifuged, and injected onto the HPLC column. HPLC was carried out on a Waters Resolve C_8 radial compression cartridge eluted with 50 mM octane sulphonic acid in a mixture of 85% acetonitrile and 15% 10 mM potassium phosphate pH 3.0 at a flow rate of 2 ml min^{-1}. Fluorimetric detection after post-column UV photocyclisation [7] was employed.

Levels of tamoxifen, metabolite Y, desmethyltamoxifen, and desdimethyltamoxifen were calculated with the aid of computerised data collection and peak integration. Levels of 4-hydroxytamoxifen, although detectable, were too small for any meaningful comparisons to be carried out. Results are expressed as mean values, and differences have been analysed for statistical significance using Wilcoxon's rank sum test.

Results

In the TAM/PLA group, levels of the unmetabolized drug had clearly stabilized after 2 weeks of administration (Fig. 1). However, the metabolites

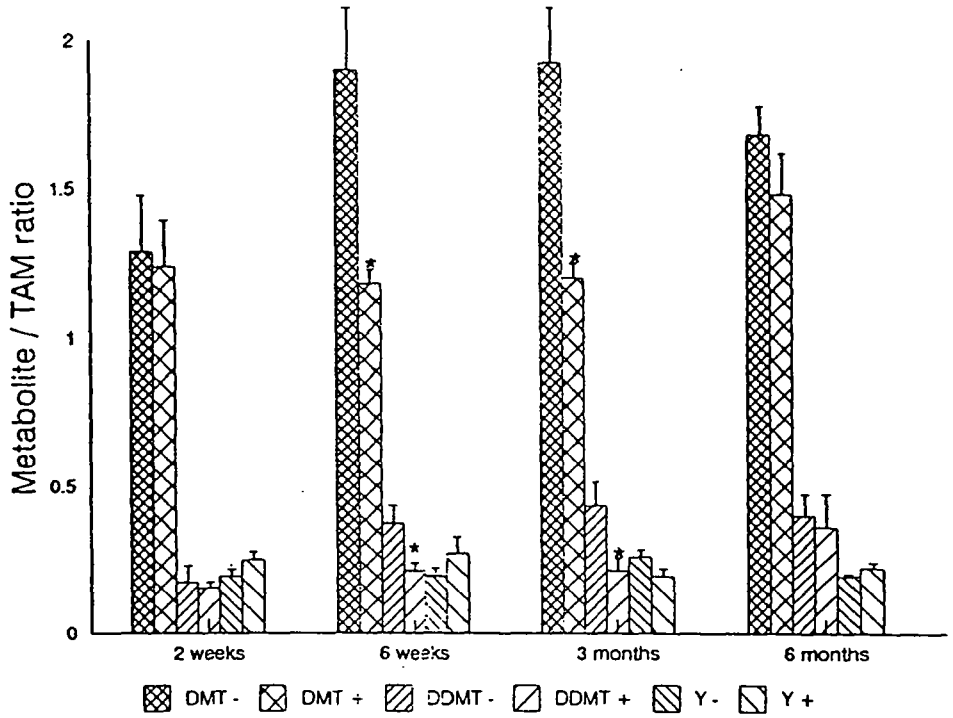

Fig. 2. Metabolite/tamoxifen ratios. The levels of the three metabolites measured have been divided by the level of parent tamoxifen at each time point and for each patient. Each pair of bars represents the mean values in the TAM/PLA (–) and TAM/MPA (+) groups (error bars represent the standard errors of the means). Asterixes denote values in the TAM/MPA group that are significantly lower than the corresponding values in the TAM/PLA group ($p < 0.05$, Wilcoxon rank sum test). DMT, desmethyltamoxifen; DDMT, desdimethyltamoxifen; Y, metabolite Y.

continued to accumulate, only reaching plateau values after 3 to 6 months. The demethylated components were the major species (Fig. 1).

A qualitatively similar picture was observed in the TAM/MPA group, though the magnitudes of the changes were considerably reduced (Fig. 1). However, the starting levels of tamoxifen (i.e. before the randomisation to receive MPA) were also lower in this group, making direct comparison difficult. To correct for this inter-group difference, the metabolite levels were recalculated as a ratio to the tamoxifen level at the same date for each patient. These data are shown in Fig. 2. It is clear that desmethyltamoxifen levels are significantly higher at 6 weeks and three months in the TAM-PLA group than in the TAM/MPA group. The difference in desdimethyltamoxifen levels also reaches statistical significance at 3 months. At 2 weeks (before randomisation) and at 6 months, there are no significant differences in tamoxifen metabolites between the two groups (Fig. 2).

For four patients in the TAM/PLA group and two in the TAM/MPA group, the complete time series of metabolite levels was available. We calculated the areas under the concentration curves for each metabolite and patient. In each case, the values in the TAM/MPA group fell within the corresponding range of values in the TAM/PLA group (data not shown). The number of patients is too small to allow any definitive conclusion. However, there is no clear difference between the two groups in cumulated amounts of metabolites over the 6 month period.

Discussion

Although serum levels of tamoxifen itself appear to reach a steady state within 2 weeks of starting treatment, it is clear that its metabolism continues to evolve over the next 6 months. In particular, the demethylating pathway only reaches peak activity

after 3 months administration. The simultaneous administration of a structurally unrelated compound, MPA, inhibits the rate of plasma accumulation of some tamoxifen metabolites, though after 6 months a new steady state appears to be reached in which MPA no longer has this effect.

The starting levels of tamoxifen were lower in the TAM/MPA group, even though this was before administration of the progestogen. It is possible that this arises from this group having more patients with stage IV disease, but further investigation will be required to resolve this issue.

The demethylated metabolites of tamoxifen are generally accepted to be of lower pharmacological potency than the parent drug. Levels of tamoxifen itself were not changed by the addition of MPA, suggesting that overall elimination of the compound had been diverted into another pathway than demethylation, rather than merely reduced. Data on the effects, if any, of this temporary diversion on clinical outcome are awaited. However, the possibility that drugs may interact at sites other than and in addition to their presumed target appears to be real, and must be taken into account in any similar study.

Acknowledgements

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Decreased Serum Concentrations of Tamoxifen and Its Metabolites Induced by Aminoglutethimide[1]

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ABSTRACT

The antiestrogen tamoxifen and the aromatase inhibitor aminoglutethimide show similar response rates when used in the endocrine management of advanced breast cancer. However, numerous clinical trials have demonstrated no increase in response rate from treatment with the drug combination of tamoxifen plus aminoglutethimide. We investigated the possibility of a pharmacokinetic interaction between these two drugs in six menopausal woman with breast cancer. All patients were investigated under three different conditions (termed phases A, B, and C). The steady state kinetics of tamoxifen were determined when administered alone (phase A) and after coadministration of aminoglutethimide for 6 weeks (phase B). In phase B, the pharmacokinetics for aminoglutethimide were determined and compared with these parameters after a tamoxifen washout of 6 weeks (phase C). The serum concentration of tamoxifen and most of its metabolites {[*trans*-1(4-β-hydroxy-ethoxyphenyl)-1,2-diphenylbut-1-ene], 4-hydroxytamoxifen, 4-hydroxy-*N*-desmethyltamoxifen, *N*-desmethyltamoxifen, and *N*-desdimethyltamoxifen} were markedly reduced following aminoglutethimide administration, corresponding to an increase in tamoxifen clearance from 189–608 ml/min. The amount of most metabolites in serum increased relative to the amount of parent tamoxifen. These data are consistent with induction of tamoxifen metabolism during aminoglutethimide exposure. We found no effect of tamoxifen on aminoglutethimide pharmacokinetics or acetylation. We conclude that this aminoglutethimide-tamoxifen interaction should be taken into account when evaluating the clinical effect of this drug combination relative to monotherapy.

INTRODUCTION

The growth of human breast cancer is supported by endogenous estrogens (1, 2). Tamoxifen and aminoglutethimide are drugs currently used in the endocrine management of breast cancer, and they probably act by suppressing the growth-stimulating effect of estrogens (2–4).

Tamoxifen [*trans*-1-(4-β-dimethylaminoethoxyphenyl)-1,2-diphenylbut-1-ene] is a nonsteroidal antiestrogen which is effective against breast cancer in both pre- and postmenopausal women. It is assumed to exert its main effects by blocking the action of estrogens at the receptor site (4). Tamoxifen undergoes extensive hepatic metabolism, and in man metabolites formed by *N*-demethylation are the main circulating species. Significant amounts of hydroxylated metabolites, including the primary alcohol, 4-hydroxytamoxifen, (4) and 4-hydroxy-*N*-desmethyltamoxifen (5) have also been demonstrated in serum. This may be important since some hydroxylated metabolites have higher affinity *in vitro* toward the estrogen receptor than the parent drug, tamoxifen (6–9). Thus, biotransformation of tamoxifen may be an important determinant of drug action. Known metabolites of tamoxifen formed through demethylation and hydroxylation are depicted in Fig. 1.

Aminoglutethimide inhibits the enzyme aromatase, which converts androgens to estrogens in peripheral fat tissue (3). This conversion is the main estrogen source in postmenopausal women. In addition, aminoglutethimide may reduce the concentration of plasma estrogens by enhancement of estrogen metabolism (10, 11). Aminoglutethimide causes response rates in postmenopausal breast cancer patients similar to those of tamoxifen, but because of more frequent side effects aminoglutethimide is generally used after tamoxifen as a second line endocrine treatment (12).

Combination therapy with tamoxifen plus aminoglutethimide should afford both estrogen receptor blockade and reduced plasma estrogen levels, and because of different targeting of these drugs the combination is expected to be more effective than monotherapy. This possibility is supported by studies on human breast carcinoma transplanted into nude mice (13), but the results from clinical trials have been disappointing (14–19) since they all show that the response to tamoxifen is not augmented by adding aminoglutethimide (Table 1).

The reason why the response rate is not increased with combination therapy has not been evaluated. A pharmacokinetic interaction should be considered, especially because aminoglutethimide is a potent inducer of certain hepatic mixed function oxidases and enhances the metabolism of several drugs and steroids (10, 20, 21). In addition, tamoxifen might influence the disposition of aminoglutethimide. Tamoxifen is a potent inhibitor of some mixed function oxidases *in vitro* (22) and may inhibit its own metabolism (23–25) as well as the metabolism of other drugs (26–28).

In the present paper we describe the effect of aminoglutethimide on the disposition of tamoxifen in patients receiving steady state tamoxifen treatment. We also report that tamoxifen does not affect aminoglutethimide disposition. The investigation was motivated by the large number of clinical studies of the combination therapy (Table 1) and also by preliminary findings suggesting that aminoglutethimide alters serum levels of tamoxifen and its metabolites.[3]

MATERIALS AND METHODS

Patients. All patients gave their informed consent to participate in the study. Six postmenopausal women were enrolled. All of them had advanced breast cancer relapsing during tamoxifen therapy and were, therefore, transferred to an aminoglutethimide regimen. Patient characteristics are given in Table 2. All patients had normal liver and renal function tests. One patient (K. N.) did not enter the final part of the study (phase C) because of rapidly progressing disease.

Chemicals. Tamoxifen, metabolite B, and metabolite X were obtained from Pharmachemie B.V. (Haarlem, Holland) and metabolites Y, BX, and Z were gifts from Imperial Chemical Industries, PLC, Pharmaceuticals Division (Macclesfield, United Kingdom). Aminoglutethimide and *N*-acetylaminoglutethimide were gifts from Ciba-Geigy (Basel, Switzerland).

Study Protocol. The study protocol was approved by the regional ethical committee.

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[3] C. Rose and E. A. Lien, unpublished data.

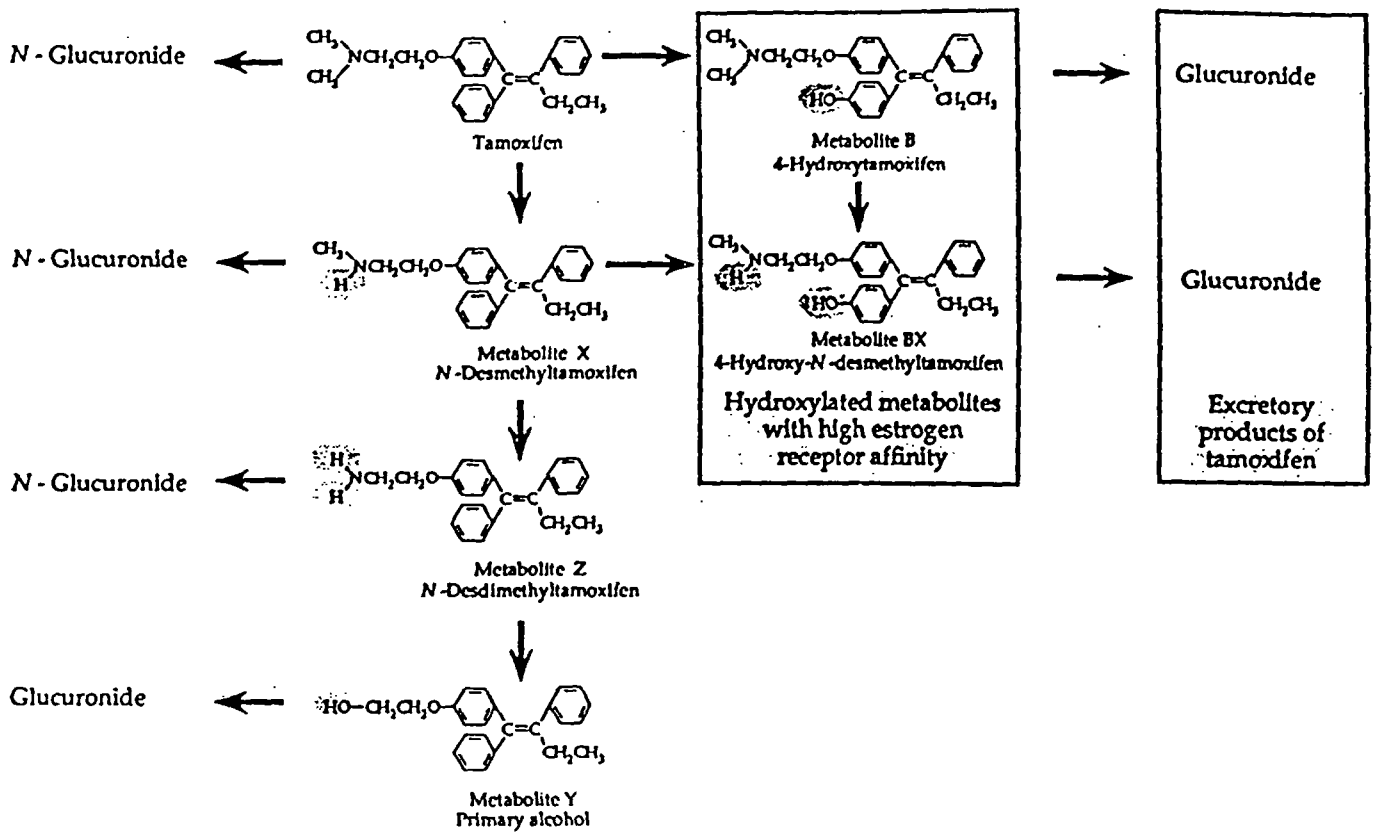

Fig. 1. Proposed metabolic pathways of tamoxifen.

Table 1 *Trials comparing tamoxifen monotherapy with the combination of tamoxifen and aminoglutethimide in breast cancer patients*

Drug[a]	Dose (mg)	Response rate CR + PR *n*[a]	%	Ref.
TAM	10 b.i.d.	18/60	30	14
TAM AG H	10 b.i.d. 250 q.i.d. 20 b.i.d.	23/62	37	
TAM	10 b.i.d.	3/9	33	15
TAM AG H	10 b.i.d. 250 q.i.d. 20 b.i.d.	4/11	36	
TAM	10 b.i.d.	5/26	19	16
TAM AG H	10 b.i.d. 250 q.i.d. 10 b.i.d.	6/26	23	
TAM	10 b.i.d.	21/49	43	17
TAM AG H	10 b.i.d. 250 q.i.d. 10 + 10 + 20	25/51	49	
TAM	10 t.i.d.	32/94	34	18
TAM AG H	10 t.i.d. 250 q.i.d. 20 t.i.d.	24/83	29	
TAM	20 b.i.d.	18/34	53	19
TAM AG H	20 b.i.d. 250 q.i.d. 10 + 10 + 20	11/29	38	

[a] CR, complete response; PR, partial response; *n*, number of patients; TAM, tamoxifen; AG, aminoglutethimide; H, hydrocortisone.

Table 2 *Patient characteristics and drug treatment*

		Treatment		
		Tamoxifen[a]		
Patient	Age (yr)	Duration of treatment before entrance (mo)	Dose (mg)	Aminoglutethimide[b] dose (mg)
A. K.	66	66	30 q.d.	250 q.i.d.
I. L.	60	30	30 q.d.	250 t.i.d.
M. H.	60	41	20 q.d.	250 q.i.d.
B. H.	62	6	30 q.d.	250 q.i.d.
K. N.	47	31	30 t.i.d.	250 q.i.d.
M. F.	60	18	80 q.d.	250 q.i.d.

[a] Phases A and B.
[b] Phases B and C.

Tamoxifen and aminoglutethimide pharmacokinetics were evaluated under three different conditions, termed phases A, B, and C. Drug doses are given in Table 2.

Phase A refers to chronic (>6 months) treatment with tamoxifen given as a single agent. Tamoxifen kinetics and serum levels of its metabolites were determined. For the last 3 days prior to sampling, tamoxifen was given daily at 8 a.m. to all patients after overnight fasting except patient K. N. who received 30 mg t.i.d.[4] at strict 8-h intervals. On the day of investigation, tamoxifen was given at 8 a.m. Blood samples were drawn 0, 0.5, 1, 1.5, 2, 3, 4, 6, 9, 12, 15, and 24 h after the last dose.

Phase B is after treatment with the combination of tamoxifen plus aminoglutethimide and cortisone acetate at fixed doses for 6 weeks. Each patient received the same dose of tamoxifen as during phase A. Aminoglutethimide (250 mg q.i.d) was given with cortisone acetate (50 mg b.i.d for 2 weeks; thereafter 25 mg b.i.d.) as recommended (29). Cortisone acetate is combined with aminoglutethimide treatment because aminoglutethimide blocks the adrenal steroid synthesis (20). During the last 3 days before sampling, tamoxifen was given as in phase A. Aminoglutethimide and cortisone acetate were given at strict 6- and 12-h intervals, respectively. On the day of blood sampling, all drugs were given at 8 a.m. after overnight fasting. Then, cortisone acetate was given after 12 h, and tamoxifen was given after 24 h, but aminoglutethimide was withheld for 48 h. The sampling schedule was as described for phase A with additional samples obtained at 36 and 48 h to allow for determinations of aminoglutethimide half-life.

Phase C is 6 weeks after cessation of tamoxifen therapy. During this period the patients were treated with aminoglutethimide and cortisone acetate only. The kinetics of aminoglutethimide were determined as in phase B.

Blood samples were obtained by venous puncture. Each sample was allowed to clot for 30–60 min prior to centrifugation. Serum was removed and stored at −20°C until analysis. To eliminate between-day variations in the analysis, all samples from each patient were analyzed in the same run.

Determination of Tamoxifen and Its Metabolites. We used a modification of a high performance liquid chromatography assay described previously (30). The method and the modifications are as follows. Samples of 250 μl of serum deproteinized with acetonitrile were post-column on column concentrated on a small precolumn (0.21 x 3 cm), packed with 5 μm ODS material. The analytes were then directed into an analytical ODS Hypersil column (0.21 x 10 cm) by elution and column switching. The mobile phases and other details have been described previously (5, 30). Tamoxifen and its metabolites were post-column converted to fluorophors by UV illumination while passing through a quartz tube and then monitored by fluorescence detection (30).

The within-day precision (CV) of the assay for tamoxifen and its metabolites Y, B; X, and Z were 0.6–5.6% for serum levels between 10 and 800 ng/ml. Because our standard for metabolite BX is a mixture of the *cis* and *trans* isomers (5), the CV was not determined for this metabolite.

Determination of Aminoglutethimide and *N*-Acetylaminoglutethimide. Serum was deproteinized using a mixture of acetonitrile and perchloric acid. The samples were chromatographed on a 3-μm ODS Hypersil column, which was eluted isocratically as described previously (31). The absorbance was routinely recorded at 242 nm.

The CVs for aminoglutethimide and *N*-acetylaminoglutethimide at a concentration of 0.5 μg/ml are 3.9 and 2.6%, respectively.

Identification of Metabolite BX by LC/MS. For patient K. N., all serum samples from phase A and all samples from phase B were pooled in separate tubes. Ten ml from each pool was extracted with 10 volumes of hexane/butanol (98/2, v/v). The supernatant was evaporated in plastic beakers at 55°C under nitrogen, redissolved in 1 ml 50% acetonitrile, and centrifuged. The supernatant was transferred to sample vials, capped, and analyzed. The analytical column was connected to a LC/MS thermospray system (model 201; Vestec, Houston, TX). Before entering the thermospray, the effluent from the column was mixed with 0.1 M ammonium acetate, delivered at a rate of 0.3 ml/min via a zero dead volume T-connector. The flow rate of the HPLC system was 0.7 ml/min.

Pharmacokinetic Calculations. The area under drug concentration-time curve during steady state corresponding to one dose interval was calculated, using the trapezoidal rule (32). Clearance was calculated by the formula:

$$Cl = \frac{F \cdot D}{AUC_{ss}^{\tau}} \qquad (A)$$

where F is the fraction of the dose (D) absorbed, and AUC_{ss}^{τ} is the area under the concentration-time curve corresponding to one dosing interval during steady state treatment (33). For aminoglutethimide, F is close to 1 (34). For tamoxifen, the value for F is unknown in humans, but F is close to 1 in animals (35). Because there is indirect evidence of good absorption in man (24), we assumed an F value equal to 1 in all patients under all conditions investigated (phases A and B).

The fraction of drug converted to the metabolite (f_m) is given by the equation (32):

$$f_m = \frac{AUC_{met} \cdot Cl_{met}}{AUC_{drug} \cdot Cl_{drug}} \qquad (B)$$

where AUC_{met} and AUC_{drug} are the area under the serum concentration-time curve for the metabolite and drug, respectively. Cl_{met} is the clearance for metabolite, and Cl_{drug} is the clearance for the parent drug.

Rearrangement of equation B gives:

$$\frac{AUC_{met}}{AUC_{drug}} = f_m \cdot \frac{Cl_{drug}}{Cl_{met}} \qquad (C)$$

A formula expressing the relationship between AUC_{met} and f_m and Cl_{met} was obtained by combining equations A and B:

$$AUC_{met} = f_m \cdot \frac{F \cdot D}{Cl_{met}} \qquad (D)$$

Statistical Methods. The Wilcoxon signed rank test for paired data was used to compare the tamoxifen pharmacokinetic parameters obtained in phases A and B and aminoglutethimide parameters in phases B and C. P values were always expressed as two tailed.

RESULTS

Effect of Aminoglutethimide on Tamoxifen Kinetics and Metabolism. We compared the steady state pharmacokinetics and serum metabolite concentrations of tamoxifen given as a single

[4] The abbreviations used are: t.i.d., 3 times/day; q.i.d., 4 times/day; b.i.d, 2 times/day; q.d., 1 time/day; CV, coefficient of variation; ODS, octadecylsilane; metabolite Y, [*trans*-1(4-β-hydroxyethoxyphenyl)-1,2-diphenylbut-1-ene]; metabolite B, 4-hydroxytamoxifen; metabolite BX, 4-hydroxy-*N*-desmethyltamoxifen; metabolite X, *N*-desmethyltamoxifen; metabolite Z, *N*-desdimethyltamoxifen; LC/MS, liquid chromatography/mass spectrometry; HPLC, high performance liquid chromatography; CL, total body clearance; AUC, area under the concentration-time curve; C_{max}, maximum concentration during one dosing interval; C_{min}, minimum concentration during one dosing interval; M, the molecular ion; m/z, the mass to charge ratio.

Table 3 *Effect of aminoglutethimide treatment on tamoxifen pharmacokinetics*

Patient	Aminoglutethimide[a]	Tamoxifen			Metabolite Y			Metabolite B			Metabolite BX			Metabolite X			Metabolite Z		
		C_{max}[b]	C_{min}[c]	AUC[d]	C_{max}	C_{min}	AUC	C_{max}	C_{min}	AUC	C_{max}	C_{min}	AUC	C_{max}	C_{min}	AUC	C_{max}	C_{min}	AUC
A. K.	−	113	76	1761	24	13	314	4	3	62	11	6	192	207	141	3804	23	12	370
	+	50	23	767	19	7	271	3	1	41	9	2	117	121	78	2061	18	8	271
	−/+	2.3	2.9	2.3	1.3	1.9	1.2	1.3	3.0	1.5	1.2	3.0	1.6	1.7	1.8	1.8	1.3	1.5	1.4
I. L.	−	160	93	2647	18.1	6	194	10	4	129	8	3	119	264	170	4545	40	20	630
	+	65	24	770	13	4	119	6	2	63	0	0	0	95	64	1909	15	5	238
	−/+	2.5	3.9	3.4	1.4	1.5	1.6	1.7	2.0	2.0				2.8	2.7	2.4	2.7	4.0	2.6
M. H.	−	229	104	2929	8	1	42	18	10	312	55	15	500	308	135	3793	32	12	613
	+	81	36	1052	7	1	53	14	10	248	0	0	0	117	77	2078	13	7	230
	−/+	2.8	2.9	2.8	1.1	1.0	0.8	1.3	1.0	1.3				2.6	1.8	1.8	2.5	1.7	2.7
B. H.	−	433	212	7775	37	16	478	2	0	11	62	33	1166	379	268	7578	62	38	1204
	+	124	44	1494	35	6	315	0	0	0	3	0	2	160	86	2432	33	9	387
	−/+	3.5	4.8	5.2	1.1	2.7	1.5				20.7		583	2.4	3.1	3.1	1.9	4.2	3.1
K. N.[e]	−	356	279	7515	159	112	3208	21	12	409	64	45	1302	1100	860	23277	231	164	4627
	+	93	63	1792	83	54	1531	5	4	94	9	0	150	333	240	6731	100	62	1849
	−/+	3.8	4.4	4.2	1.9	2.1	2.1	4.2	3.0	4.4	7.1		8.7	3.3	3.6	3.5	2.3	2.6	2.5
M. F.	−	323	143	4728	160	61	2171	3	0	35	74	13	770	647	424	12588	109	63	1832
	+	162	31	1558	75	22	845	3	0	39	0	0	0	361	178	5484	78	28	1096
	−/+	2.0	4.6	3.0	2.1	2.8	2.6	1.0		0.9				1.8	2.4	2.3	1.4	2.3	1.7
Mean	−	269	150	4559	68	35	1068	10	5	160	46	19	675	484	333	9264	83	52	1546
Mean	+	96	37	1239	39	16	522	5	3	81	4	0.3	45	198	121	3449	43	20	679
	−/+	2.8	4.1	3.7	1.7	2.2	2.0	2.0	1.7	2.0	11.5	63.3	15.0	2.4	2.8	2.7	1.9	2.6	2.3
Significance[f]	(P) − vs. +			0.032			0.063			0.063			0.032			0.032			0.032

[a] −, without aminoglutethimide treatment; +, during aminoglutethimide treatment; −/+, ratio.
[b] ng/ml.
[c] ng/ml.
[d] ng·h·ml^{-1}.
[e] Patient K. N. used tamoxifen three times daily. *AUC* in this patient is estimated during 8 h and normalized to 24 h.
[f] Wilcoxon signed rank test for paired data.

agent (phase A) with these parameters in the same patients when they were given the drug combination of tamoxifen plus aminoglutethimide for 6 weeks (phase B).

Fig. 2 shows the steady state serum profiles for tamoxifen and its metabolites in patient M. H. during one dosing interval in the absence and presence of aminoglutethimide. In agreement with earlier results (36), the serum levels of tamoxifen and metabolites Y, BX, X, and Z reached a maximum concentration (C_{max}) about 2 h after drug intake (data not shown). The difference between C_{max} and the lowest level during one dosing interval (C_{min}) was reduced for tamoxifen and its metabolites during aminoglutethimide treatment (Fig. 2 and Table 3). Notably, the concentrations of metabolite BX were reduced to near the detection limit during the combination therapy. The results for all 6 patients are summarized in Table 3.

The marked reduction in the amount of metabolite BX in serum during aminoglutethimide treatment (Table 3), was confirmed by mass spectrometry analysis. The LC/MS traces for the $(M + 1)^+$ ion show that this metabolite nearly disappeared in serum during aminoglutethimide treatment (Fig. 3).

Aminoglutethimide caused a significant decrease in *AUC* ($P = 0.032$) for tamoxifen (mean reduction, 73%; range, 80–56%), corresponding to a mean increase in tamoxifen clearance of 222% (Table 4). *AUC* for most metabolites was reduced (mean reduction, about 50%) (Table 3).

The ratio AUC_{met}/AUC_{drug} increased 35–80% during aminoglutethimide treatment for all metabolites, except metabolite BX (Table 5).

Aminoglutethimide Pharmacokinetics and Acetylation. The pharmacokinetics of aminoglutethimide and its metabolite, *N*-acetylaminoglutethimide, were determined in patients receiving chronic treatment with the drug combination of tamoxifen plus aminoglutethimide (phase B) and after tamoxifen was withdrawn for 6 weeks (phase C). In phase C neither tamoxifen nor

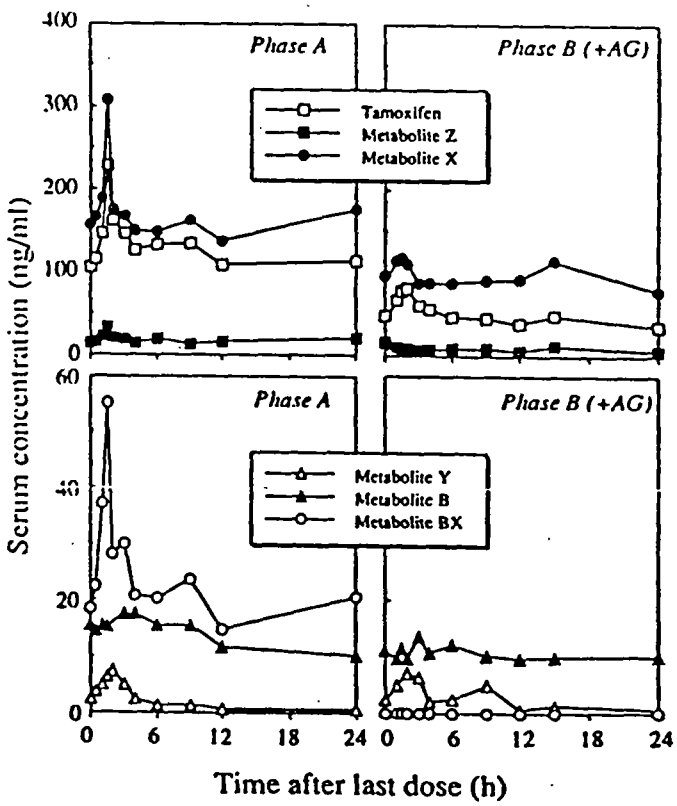

Fig. 2. Serum concentrations curves for tamoxifen and metabolites in patient M. H. during one dosing interval. Phase A is steady state tamoxifen treatment. Phase B is after 6 weeks of combination therapy with tamoxifen and aminoglutethimide. The tamoxifen dose was 30 mg once daily in both phases.

its metabolites were detected in patient sera, with the exception of metabolite X which was found in low concentrations (<1 ng/ml) in sera from three patients (A. K., M. H., and M. F.). The

results from a single patient (B. H.) are shown in Fig. 4. Data from all patients are summarized in Tables 4 and 6.

Tamoxifen did not affect the pharmacokinetics of aminoglutethimide or its conversion to *N*-acetylaminoglutethimide (Fig. 4 and Tables 4 and 6).

DISCUSSION

This study demonstrates a pronounced reduction in the serum concentrations of tamoxifen and most of its serum metabolites during aminoglutethimide treatment (Table 3). Several explanations should be considered. Aminoglutethimide may decrease the serum concentration of tamoxifen and its metabolites by reducing the absorption of tamoxifen, reducing tamoxifen protein binding, or by enhancement of tamoxifen metabolism.

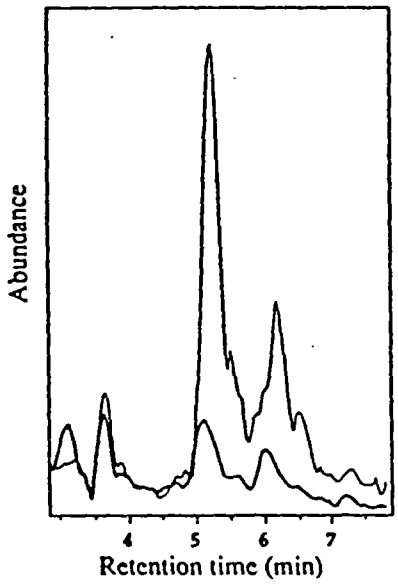

Fig. 3. Chromatography of extracts from pooled sera from phase A and phase B (patient K. N.). Reversed phase LC/MS and sample preparation were performed as described in the text. *Top trace*, selected ion-monitoring trace for the (M + 1)$^+$ ion for metabolite BX (374 m/z) from phase A (tamoxifen as single drug); *bottom trace*, phase B (tamoxifen combined with aminoglutethimide). The second peak eluting after 6 min is due to interference from the tamoxifen peak (372 m/z).

Aminoglutethimide is not known to influence the growth of intestinal bacteria or drug uptake. Thus, there are no data to suggest that aminoglutethimide may impair tamoxifen absorption.

Tamoxifen is highly (>98%) bound by protein in serum (30) and alterations in protein binding may affect the metabolism and distribution of this drug. Because aminoglutethimide is only moderately protein bound (about 25%) (34), it is unlikely that aminoglutethimide can displace tamoxifen from its binding sites.

Our data show that aminoglutethimide reduces the serum level and enhances the elimination of tamoxifen, corresponding to an increase in tamoxifen clearance from 189–608 ml/min (Table 4). This effect from aminoglutethimide is probably due to induction of tamoxifen metabolism, because there is ample evidence that aminoglutethimide may stimulate metabolic processes important in tamoxifen biotransformation.

Tamoxifen is metabolized by hydroxylations and demethylations followed by glucuronidation of the different metabolites as well as of tamoxifen itself (Fig. 1) (4, 35, 37). Aminoglutethimide is an efficient inducer of cytochrome P450 mixed function oxidases (10, 20, 21, 38, 39), and it shows similarities with phenobarbital in this respect (40). Treatment of rats with

Table 4 *Interaction between aminoglutethimide and tamoxifen*

Patient	Clearance of tamoxifen (ml/min) −AG[a]	Clearance of tamoxifen (ml/min) +AG[b]	Clearance of aminoglutethimide (ml/min) −TAM[c]	Clearance of aminoglutethimide (ml/min) +TAM[d]
A. K.	284	652	105	84
I. L.	189	649	113	87
M. H.	114	317	175	235
B. H.	64	335	111	107
K. N.[e]	200	837		
M. F.	282	856	71	86
Mean	189	608	115	120
Significance (P)[f]	0.032		>0.20	

[a] No aminoglutethimide, phase A.
[b] Aminoglutethimide treatment, phase B.
[c] No tamoxifen, phase C.
[d] Tamoxifen treatment, phase B.
[e] K. N. did not enter the final part of the study because of rapidly progressing disease.
[f] Wilcoxon signed rank test for paired data.

Table 5 *Effect of steady state aminoglutethimide treatment on the amount of tamoxifen metabolites relative to parent drug in serum*

Patient	Aminoglutethimide	AUC^{ss} for metabolite/AUC^{ss} for tamoxifen Y	B	BX	X	Z
A. K.	−	0.18	0.04	0.11	2.16	0.21
	+	0.35	0.05	0.15	2.69	0.35
I. L.	−	0.07	0.05	0.04	1.72	0.24
	+	0.15	0.08	<0.001[b]	2.48	0.31
M. H.	−	0.01	0.11	0.17	1.29	0.21
	+	0.05	0.24	<0.001[b]	1.98	0.22
B. H.	−	0.06	0.001	0.15	0.97	0.15
	+	0.21	0.00	0.001	1.63	0.26
K. N.	−	0.43	0.05	0.17	3.10	0.62
	+	0.85	0.05	0.08	3.76	1.03
M. F.	−	0.46	0.01	0.16	2.66	0.39
	+	0.54	0.03	<0.001[b]	3.52	0.70
Mean	−	0.20	0.04	0.13	1.98	0.30
	+	0.36	0.07	0.04	2.68	0.47
P − vs. +[c]		0.032	>0.10	0.032	0.032	0.032

[a] AUC^{ss}, AUC in steady state during one dosing interval.
[b] BX not detectable during aminoglutethimide therapy.
[c] Wilcoxon signed rank test for paired data.

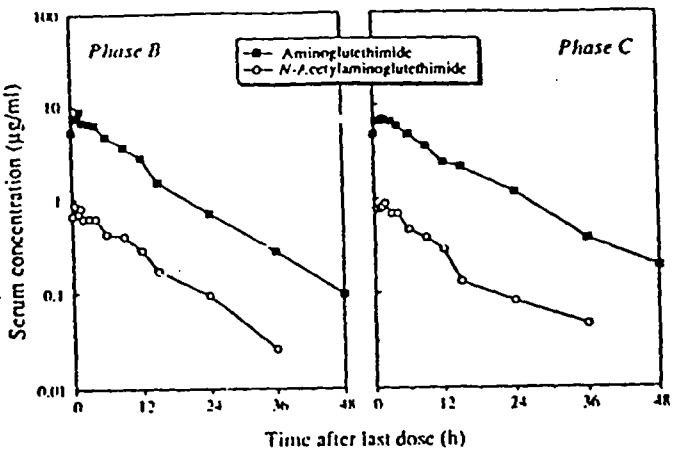

Fig. 4. Serum concentration curves for aminoglutethimide and N-acetylaminoglutethimide in patient B. II. Phase B is after 6 weeks of combination therapy with tamoxifen and aminoglutethimide. Phase C is during administration of aminoglutethimide as a single agent 6 weeks after cessation of tamoxifen treatment. In both phases the kinetics of aminoglutethimide were recorded during a period of 48 h of withdrawal of this drug.

barbiturate increases demethylation of tamoxifen in liver microsomes *in vitro* (41).

Induction of glucuronidation has been reported in man after treatment with other well-known enzyme inducers such as phenytoin, phenobarbital, and rifampicin (42), and recently rat liver glucuronidation was found to be enhanced by aminoglutethimide (43). The two hydroxylated metabolites, B and BX, are excreted in bile (5). Their biliary excretion as glucuronides may significantly contribute to their total clearance, and induction of glucuronidation of hydroxylated tamoxifen metabolites by aminoglutethimide may decrease serum levels of these species.

N-Glucuronidation of tertiary amines has been demonstrated only in higher primates (42), suggesting a metabolic pathway for tamoxifen in man, not existing in most experimental animals. Stimulation of tamoxifen *N*-glucuronidation (Fig. 1) by aminoglutethimide would enhance the metabolic clearance of the drug but cannot explain the altered ratio between *AUC* for a metabolite relative to that of the parent drug.

The observation that *AUC* for tamoxifen metabolites is reduced (Tables 3 and 5) also agrees with the idea that aminoglutethimide affects tamoxifen metabolism. Our study does not allow delineation of the kinetics behind the reduction in metabolite *AUC*. According to equation D, AUC_{met} depends on the fraction of tamoxifen converted into the metabolite as well as on the metabolite clearance. These (f_m and Cl_{met}) are parameters not accounted for by the present study design. However, reduction of *AUC* for tamoxifen metabolites may be due to reduced f_m or increased Cl_{met}. Reduction in f_m may result if aminoglutethimide stimulates the formation of metabolites not detected by our HPLC system, which was optimized for the analysis of triphenylethylenes present in human serum during monotherapy (30). Increased metabolite clearance may occur following enhancement of metabolic glucuronidation.

Our patients were given cortisone acetate as a glucocorticoid substitution during aminoglutethimide treatment. There is evidence that corticosteroids may affect the metabolism of some drugs (40, 44). We do not consider cortisone acetate responsible for the observed alteration in tamoxifen metabolism for two reasons. First, aminoglutethimide is an inhibitor of adrenal cortisol synthesis, and the cortisone acetate substitution does not increase plasma cortisol above physiological levels (34). Second, aminoglutethimide is an enzyme inducer also in the absence of glucocorticoid substitution (39).

The effect of aminoglutethimide on tamoxifen metabolism has important implications. Obviously, lowering the serum concentration of tamoxifen and its active metabolites reduces their effects. In addition, aminoglutethimide increases the relative amount in serum of most metabolites compared with the parent drug (Table 5). This also suggests that the tamoxifen-aminoglutethimide interaction is due to increased metabolism and not decreased gastrointestinal absorption (see above). An increased ratio AUC_{met}/AUC_{drug} is observed for the hydroxylated metabolite B, whereas the ratio decreases for metabolite BX, another hydroxylated metabolite. These metabolites have considerably higher affinity for the estrogen receptor than tamoxifen itself (6–9). It has recently been demonstrated that the inhibition of growth of the estrogen receptor-positive MCF-7 cells in the presence of tamoxifen and metabolites Y, B, X, and Z parallels the relative affinity of these agents for the estrogen receptor (45). Effects of higher doses of tamoxifen given in combination with aminoglutethimide may therefore be influenced by the altered metabolite profile of tamoxifen.

Tamoxifen is a weak estrogen agonist and strong antagonist, and tamoxifen metabolites may also have agonistic or antagonistic properties (46). Thus, alterations in tamoxifen metabolism induced by aminoglutethimide may increase the amount of estrogen agonists at the expense of estrogen antagonists. Such a metabolic effect would counteract the biological effect of aminoglutethimide thought to be mediated by estrogen depletion, and a decreased additive effect of the drug combination of tamoxifen and aminoglutethimide would ensue. This could explain the negative results from the clinical trials of this drug combination.

Our results also show major variations in ratios of tamoxifen to its metabolites in the absence of aminoglutethimide therapy (Table 3). This raises the question that breast cancer patients who respond to tamoxifen therapy may have a tamoxifen metabolism different from that of the nonresponders.

There are occasional reports that tamoxifen interacts with other drugs (26–28). We observed no effect of tamoxifen ad-

Table 6 *Effect of tamoxifen treatment on aminoglutethimide pharmacokinetics and acetylation*

Tamoxifen[a]		Aminoglutethimide			*N*-Acetylaminoglutethimide
		AUC_{ss}[b] (μg·h·ml^{-1})	$T_{\frac{1}{2}}$ (h)	V_z[c] (liter)	AUC_{ss} (μg·h·ml^{-1})
+	Mean[d]	40.5	7.4	76.1	4.2
	SD	13.5	1.4	42.1	2.4
−	Mean	39.3	7.2	75.8	3.8
	SD	12.4	1.8	38.7	1.6

[a] +, during tamoxifen treatment (phase B); −, without tamoxifen (phase C).
[b] AUC in steady state during one dosing interval.
[c] Pharmacokinetic volume of distribution during terminal phase.
[d] $n = 5$.

ministration on the disposition of aminoglutethimide (Tables 4 and 6).

In conclusion, the present report demonstrates that aminoglutethimide markedly reduces serum concentrations of tamoxifen and its metabolites, probably by inducing tamoxifen metabolism. Our findings suggest that clinical trials performed on tamoxifen plus aminoglutethimide combined therapy (14–19) may be biased by low tamoxifen serum levels and change in its metabolite profile. This may explain why combination therapy did not result in significantly higher response rates than tamoxifen monotherapy (Table 1). Future clinical trials of the combination therapy should therefore include serum concentration monitoring and tamoxifen doses should possibly be increased to compensate for decreased bioavailability of tamoxifen and its metabolites.

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IDENTIFICATION OF THE CYTOCHROME P450 IIIA FAMILY AS THE ENZYMES INVOLVED IN THE N-DEMETHYLATION OF TAMOXIFEN IN HUMAN LIVER MICROSOMES

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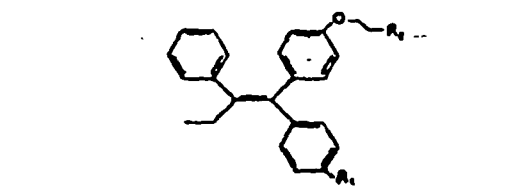
Abstract—The antiestrogen tamoxifen (Tam or Nolvadex, ICI)—Z-1-[4-[2-(dimethylamino) ethoxy]phenyl]-1,2-diphenyl-1-butene is widely used in treatment of hormone-dependent breast cancer. The drug is extensively metabolized by cytochrome P450 dependent hepatic mixed function oxidase in man, yielding mainly the *N*-desmethyl metabolite (DMT). This study has been carried out to determine the P450 enzyme involved in the *N*-oxidative demethylation of Tam in microsomal samples from 25 human livers (23 adults, two children). This metabolic step was inhibited by carbon monoxide up to 75%. Tam was demethylated into DMT with an apparent K_m of 98 ± 10 μM; rates varied between 37 and 446 pmol/min/mg microsomal protein. These metabolic rates were strongly correlated with 6β-hydroxylation of testosterone ($r = 0.83$) and erythromycin *N*-demethylase ($r = 0.75$), both activities known to be associated with P450 IIIA enzyme. To further assess whether or not the Tam demethylation pathway is catalysed by the same P450, the inhibitory effect of TST on this reaction was determined. The competitive inhibition had an apparent K_i of 100 ± 10 μM. Drugs such as erythromycin, cyclosporin, nifedipine and diltiazem were shown to inhibit *in vitro* the metabolism of tamoxifen. Furthermore the P450 IIIA content of liver microsomal samples, measured by Western blot technique using a monoclonal P450NF (nifedipine) antibody, was strongly correlated with DMT formation ($r = 0.87$). Tam *N*-demethylase activity was inhibited by more than 65% with polyclonal anti-human anti-P450NF. All these *in vitro* observations establish that a P450 enzyme of the IIIA sub-family is involved in the oxidative demethylation of tamoxifen in human liver.

Tamoxifen‖ [compound 1 (see Fig. 1), Nolvadex from ICI [46, 474] is a non-steroidal anti-estrogen which is currently used for the treatment of human hormono-dependant breast cancer [1]. Metabolism may play an important role in modulating the biological activity of the drug in so far as it is thought to act by competing with cytoplasmic estradiol receptor by means of its metabolites, especially 4-hydroxy-metabolite (compound 2). The drug is extensively metabolized by hepatic cytochrome P450 dependent mixed function oxidase in man [2] and various other mammalian species [3–9]. Originally the 4-OH-T was the only serum metabolite detected in human serum [2], but later Adam *et al.* [10], showed the major serum metabolite to be the *N*-desmethyl derivative, DMT (compound 3). Up to now five metabolites have been identified in human serum: DMT, 4-OH-T, 4-OH-DMT, compounds Z and Y [11–14]. *In vitro* studies with rat [5, 7], rabbit [6] liver microsomes or isolated rat hepatocytes [3, 4] identified other metabolites such as TNO (compound 4) and derivatives. All these metabolites may together with the parent drug contribute to the observed clinical response. Thus, the present study was undertaken to determine the metabolic pathways of Tam in human liver microsomes. In addition previous studies suggest the involvement of the microsomal cytochrome P450 superfamily in Tam metabolism by rodents [6, 9]. So in order to identify the P450 enzyme involved in the metabolism of Tam, a thorough investigation was carried out with human liver microsomes. In this paper evidence is presented



Compound 1	TAM	$R_1 = N\text{-}(CH_3)_2$	$R_2 = H$
Compound 2	4-OH-TAM	$R_1 = N\text{-}(CH_3)_2$	$R_2 = OH$
Compound 3	DMT	$R_1 = NH\text{-}CH_3$	$R_2 = H$
Compound 4	TNO	$R_1 = N \rightarrow O$ $\lvert CH_3 \rvert$	$R_2 = H$

Fig. 1. Chemical structure of tamoxifen and its major metabolites.

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‖ Abbreviations: Tam, tamoxifen, Z-1-[4-[2-(dimethylamino) ethoxy]phenyl]-1,2-diphenyl-1-butene; DMT, *N*-desmethyltamoxifen; TNO, tamoxifen *N*-oxide; 4-OH-4-hydroxy tamoxifen; 4-OH-DMT, 4-hydroxy-*N*-methyl tamoxifen; Z, *N,N*-didesmethyltamoxifen; Y, 1[4-[2-(hydroxy)ethoxy]phenyl]-1,2-diphenyl-1-butene; ER, estrogen receptor; PBS, phosphate buffer saline; TST, testosterone.

that human P450 IIIA* enzymes family, previously identified as nifedipine, cyclosporine, erythromycin oxidases [15–17] are the major enzymes involved in the N-demethylation of Tam.

MATERIALS AND METHODS

Chemicals

Tam was obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.) and its metabolites are a gift of Dr McCague (Institute of Cancer Research, Sutton, U.K.). Erythromycin, cyclosporin and diltiazem were supplied by Sigma, Sandoz (Rueil-Malmaison, France) and Synthelabo-LERS (Meudon la Forêt, France) respectively. Testosterone and its metabolites were from Steraloids (Wilton, NH, U.S.A.). [4-^{14}C]Testosterone (sp. act. 57 mCi/mmol) was from Amersham (Aimersham, U.K.).

Human liver samples and microsomes preparation

Human liver samples were obtained from 23 adult organ donors immediately after death (19 males, four females, mean age: 37 ± 12 years) and two children: 5 months old (Br023) and 2 years old (Br025). Sampling was made in accordance with French legal considerations. Ethical Committee approval was obtained prior to this study. Pre-death dietary habits and exposure to environmental chemicals were not known. Liver fragments were frozen immediately and stored in liquid nitrogen. Microsomal fractions were prepared as previously described [18] and stored at −80° until use.

Determination of monooxygenase activities

Protein content of microsomal preparations was determined by the method of Lowry *et al.* [19]. Total cytochrome P450 levels were measured according to Omura and Sato [20], with molecular extinction coefficient of 91 $mM^{-1} cm^{-1}$.

Tamoxifen metabolism. Incubations were run in polypropylen test tubes or siliconized glass tubes in the dark. The standard incubation mixture contained, in a final volume of 0.5 mL, 40 mM potassium phosphate buffer pH 7.4, 120 mM potassium chloride, 5 mM magnesium chloride, 0.5 mM tamoxifen and 2.4 mM NADPH. After 2 min of pre-incubation at 37°, the reaction was started by addition of 1 mg microsomal proteins. After 15 min shaking at 37°, the reaction was stopped by addition of 5 mL chloroform. The mixture was vortexed and re-extracted at pH 9.0 with 5 mL chloroform. The organic extracts were pooled and dried at 40° under nitrogen stream. To the drug residue was added 0.5 mL methanol–water (90/10; v/v) mixture for HPLC analysis. Control incubations were run as described except that microsomal proteins or NADPH were omitted. Tam metabolites were analysed by HPLC with a Lichrosorb C-18 RP-Select B column, 250 × 4 mm, from Merck (Darmstadt, F.R.G.) eluted by a mobile phase consisting of methanol, water, triethylamine (90/10/0.1; v/v/v) with a flow rate of 0.8 mL/min. Eluates were detected by UV at 238 nm with a sensitivity of 0.005 AUFS. Peaks were identified by their retention time and spectral characteristics in comparison with standard compounds. Peaks were quantified by an integrator-calculator SP-4100 from Spectra-Physics (Santa Clara, CA, U.S.A.). Overall biotransformation was expressed as the percentage of substrate transformed into known metabolites relative to the untransformed drug.

Testosterone metabolism. The standard incubation mixture contained, in a final volume of 1 mL, 100 mM potassium phosphate buffer pH 7.4, 0.05 mM EDTA, 0.5% glycerol, 25 μM [4-^{14}C]testosterone (sp. act. 5 mCi/mmol) and 0.2 mg microsomal proteins. After pre-incubation at 37°, the reaction was started by addition of 2.4 mM NADPH. After 15 min shaking at 37°, the reaction was stopped by addition of 5 mL methylene chloride. The organic phase was taken to dryness under nitrogen stream at 45°. Testosterone metabolites were analysed either by thin-layer chromatography according to Waxman *et al.* [21] or by HPLC according to Sonderfan *et al.* [22]. In all cases, 6β-hydroxy-testosterone was identified according to its chromatographic behavior and its radioactivity was counted by liquid scintillation spectrometry.

Erythromycin demethylase. Erythromycin demethylation activity was determined at 37° in an incubation mixture containing 1 mM erythromycin, 1 mg of microsomal protein, 2.5 mM hydrochloride semicarbazide in a final volume of 1 mL of 0.1 mM potassium phosphate buffer pH 7.4. The reaction was initiated by the addition of 2 mM NADPH, proceeded for 15 min, and formaldehyde formation was measured according to Werringloer [23].

Inhibition of Tam metabolism by different compounds

Incubations were performed as above described with 0.1 mM tamoxifen, except that testosterone, erythromycin, cyclosporin, nifedipine, estradiol or diltiazem were added at concentrations ranging between 25 and 500 μM. Control experiments were conducted in the same conditions with the same amount of organic solvent, acetone or methanol, needed for solubilization of drugs. Three liver samples were used for these experiments: FH-2A, Br022 and Br024 on the basis of their high tamoxifen-metabolizing activity.

Inhibition of Tam metabolism by anti-P450 IIIA antibody

Diluted microsomes from FH-2A sample

* P450 nomenclature. The new recommended nomenclature for cytochrome P450 (Nebert DW, Nelson DR, Adesnik M, Coon MJ, Estabrook RW, Gonzalez FP, Guengerich FP, Gunsalus IC, Johnson EF, Kemper B, Levin W, Philpot IR, Sato R and Waterman MR. The P450 superfamily: updated listing of all genes and recommended nomenclature for the chromosomal loci. *DNA* 8: 1–13, 1989) is used throughout this paper. The human P450 IIIA subfamily appears to have at least four distinct genes. As these genes are encoding proteins whose primary sequences are at least 85% homologous, polyclonal antibodies against any P450 IIIA form are expected to cross-react with all other proteins of the subfamily. So, it is not possible to decide which form(s) of this subfamily is the one involved in Tam, nifedipine or cyclosporin metabolism. We shall accordingly only use the term P450 IIIA to designate the P450 IIIA involved in these activities.

0.50 nmole cytochrome P450/mL) in 40 mM potassium phosphate buffer pH 7.4 were incubated at room temperature for 20 min in the absence or in the presence of increasing amounts of polyclonal P450-NF antibody [24] or non-immune rabbit IgG. Tam and NADPH were added and the reaction was allowed to proceed as indicated above.

Immunoblot analysis

Protein samples (20 μg) were separated by electrophoresis on 9% sodium dodecyl sulphate (SDS)-polyacrylamide gel according to Laemmli [25], and transferred electrophoretically to a nitrocellulose sheet [26]. After incubation at 37° for 30 min by 3% bovine serum albumin and 10% newborn calf serum in PBS, the nitrocellulose sheet was sequentially treated with monoclonal anti-P450-NF human [24] overnight at 4°, washed with PBS then PBS containing rabbit anti-mouse immunoglobulins conjugated to peroxidase (Dako, Versailles, France). Finally the sheet was washed with PBS and the peroxidase activity was detected with 4-chloronaphtol and H_2O_2. The quantification of P450 IIIA was performed by densitometry. The integrated peak area of the various microsomal preparations was expressed as arbitrary units relatively to the amount of proteins.

Data analysis

The values are means ± SD from 24 livers; the sample from the 5-month-old subject Br023 was not included. Correlation coefficients were calculated using an ANOVA table by the least-squares regression analysis from the raw data. As a normal gaussian distribution in the population was observed, correlation coefficients were determined with 25 samples.

RESULTS

Metabolites of tamoxifen

By using the optimized HPLC conditions, eight metabolites were totally separated within 35 min (Fig. 2). In order to reduce analysis time, the mobile phase was modified as indicated in Materials and Methods. These modified conditions did not allow separation of α-hydroxy-Tam-*N*-oxide from metabolite Y. Since these two compounds were not detected in biological samples from human microsomes, these analytical HPLC conditions were used. Figure 3 shows metabolic profiles of Tam incubated with human and rat microsomal samples in comparison with control incubation. DMT, 4-OH-Tam and TNO metabolites were identified by their chromatographic behavior and their UV-spectra. Furthermore, DMT peak was shown to be homogeneous by its constant absorbance ratio 240/260 nm during its elution. Whereas 4-OH-Tam was formed at a very low level (about 1.6 ± 1.3 pmol/min/mg protein) which is close to the minimum amount detectable, TNO was produced in detectable amounts, however very similar in control and human microsomal samples (Fig. 3A and B). It is worth noting that TNO was formed in amounts greater when using rat microsomes than human microsomes. Accordingly TNO was not taken into account in calculating the metabolic rate of Tam in human microsomes.

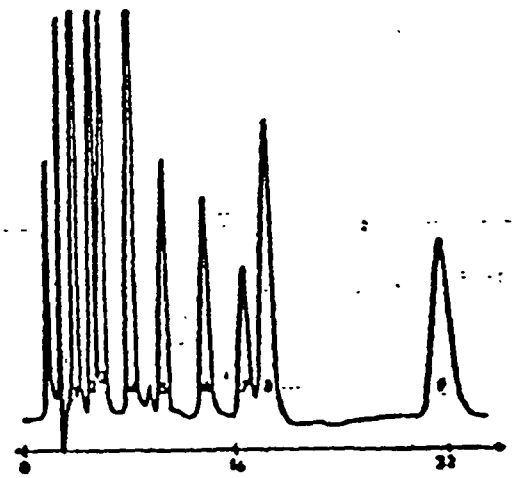

Fig. 2. HPLC chromatogram of Tam metabolites. Analysis was carried out on a Lichrosorb RP-select B, 5 μm column (25 × 0.4 cm); mobile phase was methanol/water/triethylamine (80/20/0.1, v/v/v) at 0.8 mL/min. Eluates were detected at 238 nm with 0.005 AUFS sensitivity. For identification of peaks, see Fig. 1. Tam metabolites (100 ng each) are numbered as follows: 1: metabolite F or Z-1-[4-(2-hydroxyethoxy)phenyl]-1-[(4-hydroxy)phenyl]-2-diphenyl-1-butene; 2: α-hydroxy-tamoxifen-*N*-oxide; 3: metabolite Y or Z-1-[4-(2-hydroxyethoxy)phenyl]-1,2-diphenyl-1-butene; 4: 4-hydroxytamoxifen; 5: tamoxifen-*N*-oxide; 6, 7: Z and E-1-[4-(2-methylamino)ethoxy)phenyl]-1-[4-hydroxyphenyl]-1,2-diphenyl-1-butene; 8: tamoxifen; 9: desmethyl-tamoxifen.

Cytochrome P450 dependence of tamoxifen metabolism

Preliminary experiments suggested the involvement of cytochrome P450 in the Tam metabolism by human liver microsomal samples. This evidence included localization of activity in the microsomal subcellular fraction, heat lability, absolute dependence upon the presence of NADPH for catalytic activity. Furthermore, the binding of Tam to human liver microsomes was determined by differential spectroscopy. Type I difference spectra with minima at 420 nm and maxima at 390 nm were obtained upon addition of increasing amounts of Tam in liver microsomes.

The reciprocal plot of the absorbance change at 390 minus 420 nm against Tam concentration (Fig. 4) allowed determination of an apparent dissociation constant K_s of 9 μM and a maximal absorbance change $\Delta A_{max} = 0.0064$. Moreover, DMT formation from Tam was inhibited up to 75% when the incubation mixture was bubbled with CO before addition of microsomal sample.

Kinetic parameters

Figure 5 shows the linear kinetics of DMT formation from Tam by microsomes from human liver FH-2A. K_m was determined as 96 ± 10 μM and V_{max} as 550 pmol/min/mg microsomal protein.

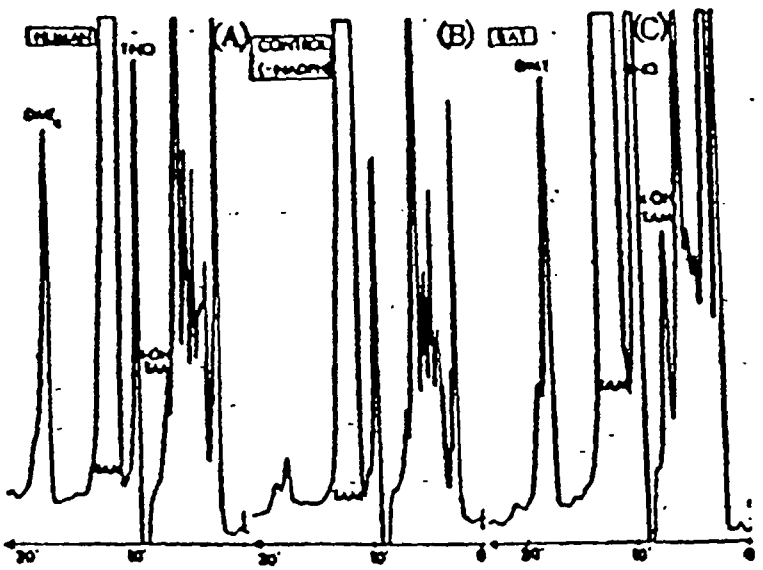

Fig. 3. Typical HPLC chromatograms of incubation medium of 0.5 mM Tam with microsomal samples from FH-2A human liver containing NADPH (A) and not containing NADPH (B = control) and from rat liver (C). For HPLC conditions, see Fig. 2. Mobile phase consisted on methanol/water/triethylamine (90/10/0.1; v/v/v) at 0.8 mL/min.

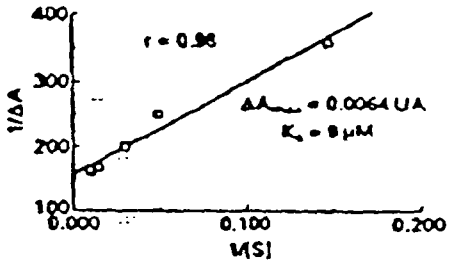

Fig. 4. Reciprocal plot of A ($A_{390} - A_{430}$) against Tam concentration. Microsomal sample from FH-2A liver was diluted at 1.6 mg protein/mL or 0.8 nmol cytochrome P450/mL. Increasing amounts of Tam were added to the sample cuvette and the same amount of ethanol to the reference cuvette. After zero recording, differential spectra were recorded.

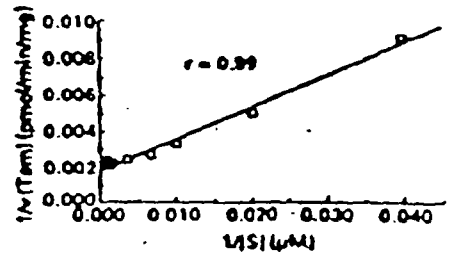

Fig. 5. Reciprocal plot of v (pmol/min/mg protein) against Tam concentration (μM) according to Lineweaver and Burk. Microsomal sample from FH-2A liver was used.

Correlation studies between Tam metabolism and different monooxygenase activities in human liver microsomes

Table 1 shows the rate of biotransformation of Tam and testosterone by 24 adult human and one newborn liver microsomes. The rate of N-oxidative demethylation of Tam ranged from 37 to 446 pmol/min/mg whereas the rate of 6β-hydroxylation of TST varied between 165 to 4075 pmol/min/mg. An excellent correlation between these two activities was observed (Fig. 6B) ($r = 0.83$; $N = 25$; $P < 0.001$). Similarly, Tam demethylation was significantly correlated with erythromycin *N*-demethylase activity (Fig. 6A) ($r = 0.75$; $N = 24$; $P < 0.001$).

Immunoblot analysis of microsomal proteins

Immunoblot analysis of isolated human liver microsomes using a monoclonal antibody reactive with P450-NF revealed a band of 52,000 Daltons as the major reactive polypeptide (Fig. 7). In two of the liver samples (Br015 and Br017) shown in the insert of figure, however, a band of 52,500 Daltons was also detected with the monoclonal antibody.

Correlation of monooxygenase activities with immunoquantitated P450 IIIA

N-Oxidative demethylation of tamoxifen as well as 6β-hydroxylation of testosterone in 25 microsomal samples was highly significantly correlated with the amount of P450 IIIA immunodetected ($r = 0.87$, $r = 0.90$ respectively) (Fig. 8).

Inhibition studies

To further assess whether or not the N-demethylation of Tam is catalysed by the P450 enzyme involved in 6β-hydroxylation of TST (i.e.

Table 1. Cytochrome P450-dependent activities in microsomal sample from 25 human livers

Subjects	Sex, age (years)	Total P450 (pmol/mg)	Tam *	TST *	ER †	P450 IIIA Δ
FH11	M,18	269	79	815	1.14	1.21
FH2A	M,41	527	446	4075	2.58	5.61
FH3	M,47	470	151	3420	1.86	3.65
Br015	M,43	414	86	985	0.85	2.81
Br016	M,44	369	123	1850	1.15	2.89
Br017	M,26	593	164	1795	1.42	2.58
Br018	F,45	334	93	950	1.00	1.80
Br019A	M,45	241	37	740	0.83	1.07
Br021	M,49	257	104	1105	1.06	1.23
Br022	M,23	286	225	3650	1.90	3.34
Br024	F,15	505	299	3935	2.12	4.49
Br025	M,2	110	128	1085	1.00	1.20
Br027	M,21	100	54	435	0.76	1.02
Br028	M,27	165	100	845	0.50	1.90
Br029	M,36	77	66	165	0.35	0.71
Br031	M,23	154	51	430	0.17	0.40
Br032	M,36	286	134	2455	1.66	2.95
Br033	M,44	363	92	1626	0.74	1.46
Br034	M,32	286	72	747	0.44	1.26
Br035	M,35	252	172	2120	1.57	2.36
Br036	M,57	153	147	1310	0.64	1.12
Br037	M,56	230	117	279	0.32	0.91
Br038	F,51	120	44	324	0.24	1.00
Br039	F,46	538	328	2450	0.98	3.88
Mean		296	138	1566	1.05	2.12
± SD		150	98	1200	0.63	1.34
Br023	F,5 months	228	97	375	ND	2.04

* pmol/mg/min.
† nmol/mg/min.
Δ, expressed as relative arbitrary relative unit.
Tam, N-oxidative demethylation; TST: 6β-hydroxylation.
ER, erythromycin *N*-demethylase.
ND, not determined.

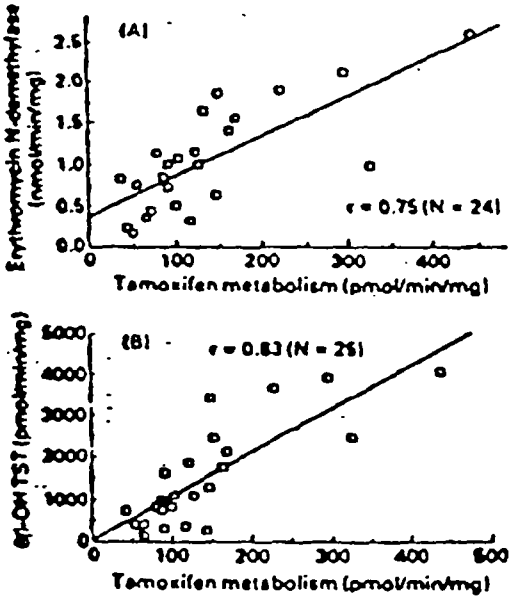

Fig. 6. Correlation between *N*-oxidative demethylation of Tam and erythromycin *N*-demethylase (A) and 6β-hydroxylation of TST (B). The correlation coefficient (*r*) as determined by the least-squares linear regression analysis.

P450 IIIA), the effect of TST on this reaction was determined. Figure 9 illustrates the competitive inhibition of TST on Tam demethylation with an apparent K_i of 100 ± 10 μM and the same effect of Tam on 6β-hydroxylation of TST with an apparent K_i of 10 μM.

Immunoinhibition of Tam demethylase in human liver microsomes

In order to confirm the role of P450 IIIA as the major enzyme involved in the N-demethylation of Tam, immunoinhibition experiments were carried out on microsomal preparation from FH-2A liver. Results are reported in Fig. 10.

Tam *N*-demethylase activity was inhibited by more than 65% of control activity with 5 mg immune Ig G per nmole P450.

Tamoxifen drug interactions

In order to assess drugs that interfere with the hepatic metabolism of Tam, erythromycin, cyclosporin A, nifedipine, diltiazem and estradiol were tested. Table 2 reports the results.

Clearly erythromycin, cyclosporin, nifedipine and diltiazem competitively inhibited Tam *N*-demethylase activity with apparent K_i of 20, 1, 45 and 30 μM, respectively. However, estradiol did not significantly inhibit Tam metabolism.

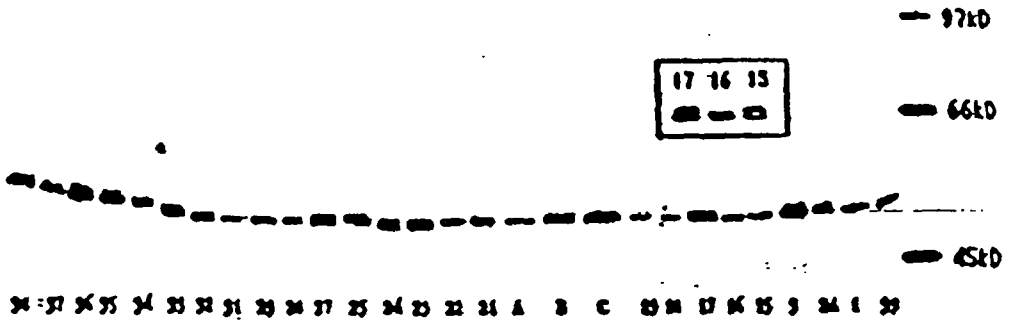

Fig. 7. Immunoblot of P450 IIIA protein. Microsomal proteins (20 μg) were probed with monoclonal anti-human P450 NF. Lanes are numbered according to human samples of Table 1. Lanes A, B, C: purified human cytochrome P450IIIA (1, 2 and 4 pmoles respectively). Right lane: molecular weight markers silver-stained. Insert: lanes 15, 16 and 17 developed on another electrophoretic gel.

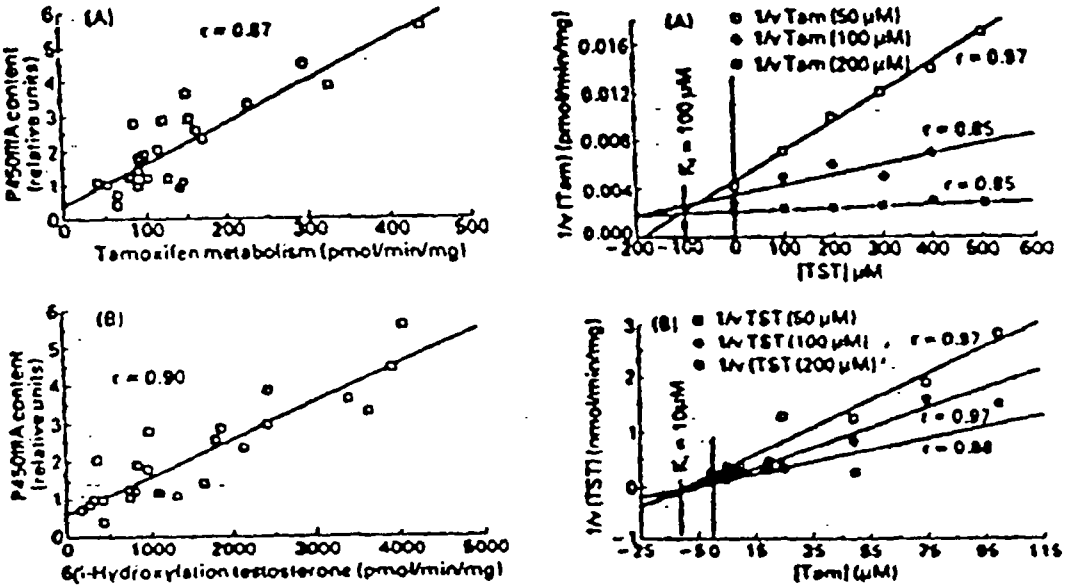

Fig. 8. Correlation between Tam-*N*-demethylase activity (A), 6β-hydroxylation of TST (B) and immunoquantitated P450 IIIA in human liver microsomes. Microsomal preparation from 25 human livers were analysed for Tam biotransformation and 6β-hydroxylation of TST. P450 IIIA content determined by immunoblot analysis of 20 μg of microsomal protein using monoclonal anti-human P450 NF. Intensity units are relative arbitrary units determined by densitometry. Correlation coefficient (*r*) was calculated by the least-squares linear regression method ($P < 0.001$).

Fig. 9. Competitive inhibition of Tam demethylation by testosterone (A) and of testosterone-6β-hydroxylation by Tam (B). Upper plot: liver microsomal preparation from FH-2A donor was assayed for Tam (50, 100 and 200 μM demethylase activity in the presence of increasing amounts of TST. Lower plot: liver microsomal sample preparation from Br039 liver was assayed for 6β-hydroxylation TST (50, 100 and 200 μM) activity in the presence of increasing amounts of Tam. The data were analysed according to the Dixon plot.

DISCUSSION

Metabolism of tamoxifen in mammalian species appears to have been subject to controversy. While Fromson *et al.* [2] detected the 4-OH-T as the only Tam metabolite in human serum, Adam *et al.* [10] showed the major serum metabolite to be DMT. Since these earlier works, it has been clearly established that this N-demethylated derivative is the most abundant metabolite detected in plasma [13, 14, 27, 28]. This is consistent with our observation that this metabolite was the major compound produced by human microsomes. N-Demethylation of Tam varied between 37 and 446 with a mean of 138 ± 98 pmol/min/mg protein. No significant difference was observed according to gender or age of liver donors. The 4-OH-Tam was produced at a very low level, 1.6 ± 1.3 pmol/min/mg protein. It represented about 1% of DMT; this is in agreement with the DMT/4-OH-Tam ratio measured in the

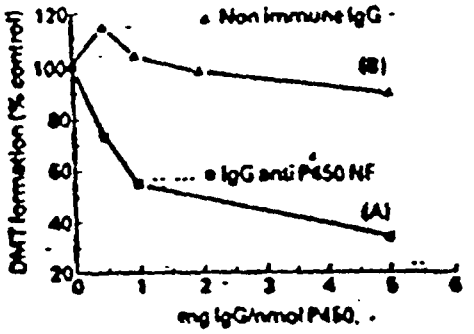

Fig. 10. Inhibition of Tam *N*-demethylase activity in microsomal preparation by anti-P450 IIIA antibody. Liver microsomes (diluted to 0.5 nmole P450/mL) from FH-2A liver was incubated 20 min at room temperature in the presence of increasing amounts of immune Ig G anti-P450 NF (A) or non-immune Ig G (B). Uninhibited activity was 275 pmol/min/mg.

Table 2. Inhibition of Tam *N*-demethylase activity in human liver microsomes by different compounds

Inhibitor (μM) concentration	25	50	100	200	250	500
Erythromycin	—	—	83	—	92	95
Cyclosporin	95	97	100	—	—	—
Nifedipine	—	16	38	66	75	81
Estradiol	0	0	0	—	15	21
Diltiazem	51	60	76	82	—	—

Values are expressed as percentage of inhibition of Tam metabolism by the Br022 hepatic microsomal sample in presence of an associate drug. Control value (0% inhibition) was 292 pmol/min/mg protein. Tam concentration was 100 μM.

serum of patients [13, 14]. The metabolic profile of Tam was quite different in rodents. For example, the 4-OH-Tam was formed at the metabolic rate of 250, 230 and 290 pmol/min/mg in microsomal preparations from rat, mouse and rabbit respectively, i.e. at a level close to that of DMT (results not shown). Such a result is in agreement with a previous study conducted on rabbit and rat liver microsomes [6]. Amounts of TNO were also detected in incubation medium but they did not differ significantly from those found in incubation where NADPH was not added. Thus, TNO may be considered as an artefact of *in vitro* experiments in so far as N-oxidation of tamoxifen was shown to be strongly dependent on oxygen availability in isolated rat hepatocytes [4].

N-Demethylation of tamoxifen was shown to be cytochrome P450 dependent. This enzymatic reaction was inhibited by carbon monooxide and needed NADPH. Spectra binding studies indicated a very high affinity of Tam for the type I binding site of P450 with a K_s of 9 μM. Such binding type was described using rat [9] and rabbit liver microsomes [8]. Furthermore, Tam did not inactivate Ph50 enzyme (results not shown). It should be noted that the Michaelis constant K_m and apparent spectral dissociation constant K_s are in disagreement in one order of magnitude: 100 vs 9 μM. Such a result was not surprising since K_s is obtained from spectral changes which are determined for the ferric protein in the absence of reducing equivalents while K_m is obtained from an actively metabolizing system [29]. However, Tam *N*-demethylase activity was poorly correlated with the total P450 amount ($r = 0.66$; $N = 24$). These results suggest that the variations observed are due to the content of specific P450 isoform(s). All the results reported in this paper demonstrated clearly that cytochromes P450 from the P450 IIIA subfamily are the major enzymes involved in the N-demethylation of Tam in human liver.

This conclusion was derived from the following observations:

1. Correlation of Tam *N*-demethylase activity with TST 6β-hydroxylation ($r = 0.83$; $P < 0.001$), erythromycin *N*-demethylase ($r = 0.75$; $P < 0.001$) and nifedipine oxidase ($r = 0.75$; $P < 0.001$, results not shown), all these activities known to be supported by P450 IIIA [21, 30, 31];
2. Correlation of Tam *N*-demethylase activity with P450 IIIA level determined by Western blot ($r = 0.87$; $P < 0.001$), but no significant correlation with P450 1A ($r = 0.38$; $N = 25$) and P450 IIE1 ($r = 0.11$; $N = 25$) determined by immunoblotting [32];
3. Inhibition of Tam *N*-demethylase activity by anti-P450 NF (IIIA) antibody;
4. Competitive inhibition between Tam and TST, cyclosporin, erythromycin, nifedipine and diltiazem, all compounds known to be metabolized by P450 IIIA enzymes [15–17, 21, 30, 33].

The human P450 IIIA gene subfamily [34] appears to contain at least four members, HLp or IIIA3 [35], P450 NF or IIIA4 [31], hPCN3 or IIIA5 [36] and HFL 33 or IIIA6 [37], the latter being expressed in the fetus. These forms are at least 82% homologous in terms of amino acid sequences. Thus antibodies against any P450 IIIA isoforms are expected to cross-react with all other proteins of the subfamily. So, it is not yet known whether only or several of these isoform(s) is (are) involved in cyclosporin, nifedipine, diltiazem monooxygenase activities. Furthermore, enzymatic analyses of the P450 IIIA purified forms have generally yielded very low catalytic activities. Thus, experiments reported here do not allow to distinguish between closely related P450 III family forms involved in the metabolism of tamoxifen. However, the P450 IIIA4 isoform seems to be the most important member of the family, while the P450 IIIA5 or hPCN3 is a minor form expressed in about 15% of the general population [36], probably in the Br015 and Br017 samples in this study. Concerning the other isoform P450 IIIA3, it constitutes a very minor form in human liver [38]. Accordingly, knowing that 6β-hydroxylation of testosterone is mainly mediated by hPCN1 or P450 IIIA4 [36], our results may suggest that this P450 IIIA isoform is probably involved in the N-demethylation of tamoxifen.

The list of drugs and endogeneous compounds which have been characterized as specific substrates

of human P450 IIIA includes erythromycin [30], nifedipine [15], cyclosporin [16, 17], testosterone [21], diltiazem [33] and cortisol [39]. It can be therefore anticipated that any of these drugs should lead to interaction with tamoxifen when given in association. This hypothesis was confirmed by our study *in vitro*. Thus, erythromycin, cyclosporin, and diltiazem at 100 μM concentration inhibited the biotransformation of Tam to DMT by more than 75%. In opposite, estradiol significantly inhibited N-demethylation of Tam only at concentrations greater than 500 μM. This is consistent with the observation that estradiol was mainly metabolized by a cytochrome P450 IA and to a lesser extent by P450 IIIA4 [40]. In theory, metabolism of tamoxifen should be decreased during concomitant administration of one of these drugs. However, it should be kept in mind that the extent of the effect is likely to be modulated by various factors such as dose, biodisponibility and relative K_m of both drugs. Up to now, a severe interaction of tamoxifen with warfarin was described [41]; the investigation pointed out that inhibition of cytochromes P450 enzymes by tamoxifen may be the basis for the interaction between tamoxifen and warfarin. As warfarin is known to be mainly metabolized by the cytochrome P450IIIA enzymes family (named cytochrome P450-S in Ref. 42) this drug interaction could be due to a competitive inhibition. In this respect, it has to be emphasized that tamoxifen therapy may be complicated when given in association with drugs known to be inducers or inhibitors of P450 IIIA enzymes.

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