Eur J Cancer Clin Oncol, Vol. 20, No. 1, pp. 137–143, 1984.
Printed in Great Britain.

0277-5379/84$3.00+0.00

The Measurement of Tamoxifen and Metabolites in the Rat and Relationship to the Response of DMBA-induced Mammary Tumours

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Abstract—*The concentrations of tamoxifen and two of its metabolites, N-desmethyltamoxifen and 4'-hydroxytamoxifen (metabolite B), have been measured in rat plasma and DMBA-induced tumours using gas chromatography–mass spectrometry. At a dose of* 100 μg/day *all three compounds produced tumour regression and, in the case of tamoxifen, the number and extent of regressions and the inhibition of new tumours were dependent upon dosage. No correlation was observed, however, between tumour regression and the concentrations of tamoxifen or N-desmethyltamoxifen in the plasma of individual animals. When tamoxifen, N-desmethyltamoxifen and metabolite B were measured in oestrogen-receptor-positive tumours a correlation was found between reduction in tumour and the tamoxifen concentration in cytosol fractions. The concentrations of all three compounds in both nuclear and cytosol fractions were higher than could be accounted for by binding to the oestrogen receptor. The mechanistic significance of these high values is, at present, unclear.*

INTRODUCTION

THE REQUIREMENT of some mammary tumours for hormones is well documented and forms the basis upon which the treatment of breast cancer by endocrine manipulation is founded [1]. One form which such endocrine therapy may take is the administration of the antioestrogenic drug tamoxifen, *trans*-1-(4-β-dimethylaminoethoxyphenyl)-1,2-diphenylbut-1-ene. As with other forms of endocrine treatment, tamoxifen (formulated as tamoxifen citrate; Nolvadex®) causes objective remission in about 30% of patients and is primarily effective against tumours which contain specific receptors for oestradiol [2–4]. It is believed that the drug may act primarily via its association with the oestrogen receptor [5, 6], although almost half of the tumours containing measurable concentrations of this protein (ER+) fail to respond to tamoxifen therapy [2–4]. The reasons for these failures are, at present, unclear, but might include the inability of some patients or tumours to absorb sufficient amounts of the drug or its active metabolites to suppress completely the action of endogenous oestrogens.

The concentration of tamoxifen has been measured in plasma [7–9] and in tumour tissue [10] from treated patients and steady-state levels reached after 4–6 weeks of continuous treatment have generally been found to be high in comparison with that of endogenous oestradiol, the concentration of which was not affected [9]. In addition, it has been established that the drug is extensively metabolised by laboratory animals and human volunteers [11, 12], and two metabolites, *N*-desmethyltamoxifen and 4'-hydroxytamoxifen (metabolite B), have been detected at relatively high concentrations in the plasma of women administered the antioestrogen [7]. As yet, however, no correlation between these concentrations and the response of individual tumours to the drug has been reported in either experimental or human neoplasms. The present study therefore examines the relationship between tissue and plasma levels of tamoxifen and response to the drug in an animal model for breast cancer, the dimethylbenzanthracene (DMBA)-induced mammary tumour of the rat.

Accepted 15 June 1983
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MATERIALS AND METHODS

Chemicals

Tamoxifen, *N*-desmethyltamoxifen and 4'-hydroxytamoxifen were gifts from ICI Ltd, Pharmaceuticals Division, Macclesfield, U.K. Sephadex LH-20 was bought from Pharmacia Fine Chemicals, Uppsala, Sweden. The preparation of sulphoethyl Sephadex LH-20 was as described by Setchell *et al.* [13]. [2,4,6,7-^{3}H]-Oestradiol (specific activity 93 Ci/mmol) was obtained from Amersham International Ltd, Amersham, Bucks, U.K. Diethylstilboestrol (DES) was supplied by Koch-Light Laboratories Ltd, Colnbrook, Bucks, U.K. *N-O*-Bis-(trimethylsilyl)trifluoroacetamide (BSTFA) and heptafluorobutyric anhydride (HFBA) were purchased from the Pierce Chemical company, Rockford, IL, U.S.A. All solvents used in the preparation of samples for analysis were of analytical grade and distilled before use, with the exception of diethyl ether which was laboratory grade and was washed with sodium hydroxide (1 M) before distillation.

Internal standards

The internal standards employed in the preparation of samples and their subsequent analysis by gas chromatography–high-resolution mass spectrometry (GC-HRMS) were the pentene homologues of their respective analytes, *viz.* a mixture of *cis* and *trans* isomers of 1-(4-β-dimethylaminoethoxyphenyl)-1,2-diphenylpent-1-ene (I) for tamoxifen analyses, *trans*-1-(4-β-dimethylaminoethoxyphenyl)-1-(4-hydroxyphenyl)-2-phenylpent-1-ene (II) for 4'-hydroxytamoxifen analyses and *cis* and *trans*-1-(4-β-methylaminoethoxyphenyl)-1,2-diphenylpent-1-ene (III) for *N*-desmethyltamoxifen analyses. I and II were gifts from ICI Pharmaceuticals Division. III was prepared by demethylation of I as described previously [10]. Internal standards (10–100 ng) were added to plasma (1 ml) or tissue extracts in 100 μl ethanol, mixed and allowed to equilibrate before preparation of the samples for GC-HRMS.

DMBA-induced mammary tumours

Tumours were induced in virgin female Sprague–Dawley rats by a single gavage of 20 mg DMBA dissolved in 1 ml sesame oil. Tumours were subsequently located, 2–4 months after induction, by palpation, and two perpendicular diameters measured with calipers. The spherical volume was calculated from measurements of the average diameter. The average diameter of tumours before the start of treatment was not less than 10 mm.

Tissue samples for oestrogen receptor analysis were obtained by biopsy of tumours while rats were under anaesthesia. Blood samples were also obtained under ether anaesthesia by exsanguination via the abdominal aorta.

Tumour-bearing animals were injected subcutaneously with various doses of tamoxifen, with 4'-hydroxytamoxifen or *N*-desmethyltamoxifen dissolved in sesame oil (100 μl) or with sesame oil alone.

Preparation of samples for analysis by GC-HRMS

Plasma. The methods of extraction and purification for rat plasma were similar to those previously described for samples of human plasma [7,10]. In summary, after equilibration with internal standard, the plasma (1 ml) was made alkaline and extracted with diethyl ether (2 × 2.5 ml). Extracts were then purified by cation exchange chromatography on sulphoethyl Sephadex LH-20 before GC-HRMS analysis.

Tumour tissue. Samples of tumour tissue (about 200 mg) were minced, and homogenised in 10 mM Tris–sucrose buffer (10 mM Tris, 0.25 M sucrose, 1 mM EDTA, 0.5 mM dithiothreitol, pH 7.4) using a motor-driven, glass-on-Teflon homogeniser (6 passes at 4000 rpm). The homogenate was then centrifuged at 800 ***g*** (av.) for 10 min to yield a supernatant and a crude nuclear pellet. These fractions were extracted separately.

Supernatants were equilibrated with internal standards I, II and III (10–100 ng dissolved in 100 μl ethanol) for 1 hr, made alkaline, extracted with diethyl ether (2 × 2.5 ml) and subjected to sulphoethyl Sephadex LH-20 chromatography in the manner described above for plasma. Protein concentration was measured in a portion of the supernatant by the method of Lowry *et al.* [14].

The nuclear pellets were washed twice with Tris–sucrose buffer containing 0.1% (v/v) Triton X-100 (1 ml) followed by a further wash with Tris–sucrose buffer alone (1 ml). The final nuclear pellet was resuspended in 10 mM Tris buffer and internal standards (10–100 ng) added in 100 μl ethanol. After equilibration for 1 hr the nuclear preparations were extracted with 5 ml acetone:ethanol (4:1 v/v) and the extracts evaporated to minimum volume at 50°C under a stream of nitrogen. Residues were made alkaline, re-extracted with diethyl ether and chromatographed on sulphoethyl Sephadex LH-20 as described previously [10] for extracts of human tumour homogenates. DNA content of the nuclear pellets was measured by the method of Burton [15].

Gas chromatography–high resolution mass spectrometry (GC-HRMS). Tamoxifen was analysed by GC-HRMS without derivatisation. The trimethylsilyl ether derivatives of 4'-hydroxytamoxifen and its internal standard

[7] and the heptafluorobutyrate of *N*-desmethyltamoxifen [10] were prepared as described previously. Instrumental conditions during GC/HRMS/selected ion monitoring of the analytes using a Varian 2700 gas chromatograph interfaced to a Varian MAT 731 mass spectrometer have also been previously described [7, 10].

Oestrogen receptor determination

Oestrogen receptors were measured in DMBA-induced mammary tumours using a single-point competitive binding assay [16]. Portions of mammary tumours (approx. 100 mg) were homogenised in Tris–sucrose buffer as described above and centrifuged at 800 *g* (av.) for 10 min. The supernatant was subjected to a further centrifugation at 105,000 *g* (av.) for 1 hr and aliquots (200 μl) of the cytosol fraction incubated for 18 hr at 4°C with 10 nM [^{3}H]-oestradiol (100 μl) in the presence or absence of 10 μM diethylstilboestrol (DES, 100 μl). A portion of the cytosol was reserved for the estimation of protein concentration by the method of Lowry *et al.* [14].

After incubation, tubes were maintained at 4°C and 400 μl of charcoal suspension (0.5% Norit A charcoal, 0.05% dextran and 0.1% gelatin) in Tris buffer added. The suspension was incubated for 30 min at 4°C and charcoal removed by centrifugation at 800 *g* (av.) for 10 min. Aliquots (500 μl) of the supernatant containing protein-bound [^{3}H]-oestradiol were removed for the measurement of radioactivity by liquid scintillation counting. Specifically bound [^{3}H]-oestradiol was calculated by subtraction of non-specifically bound radioactivity (obtained from incubations in the presence of DES) from the total binding. Tumours were assessed as oestrogen-receptor-positive (ER+) if the receptor concentration exceeded 5 fmol/mg protein.

RESULTS

Response of DMBA-induced tumours to tamoxifen, N-desmethyltamoxifen and 4′-hydroxytamoxifen

Figure 1a shows the effect on ER+ DMBA-induced tumours of the administration of tamoxifen (100 μg/day) to tumour-bearing animals for 3 weeks. Five out of the 9 tumours regressed to less than 50% of their initial volumes, while smaller regressions were seen in the remaining tumours. Tumour regression was also induced by the administration of the same dose of either 4′-hydroxytamoxifen (Fig. 1b) or *N*-desmethyltamoxifen (Fig. 1c), although in the latter case 3 out of 9 ER+ tumours failed to respond to treatment. In animals treated with sesame oil only most tumours continued to grow over the 3-week period (Fig. 1d).

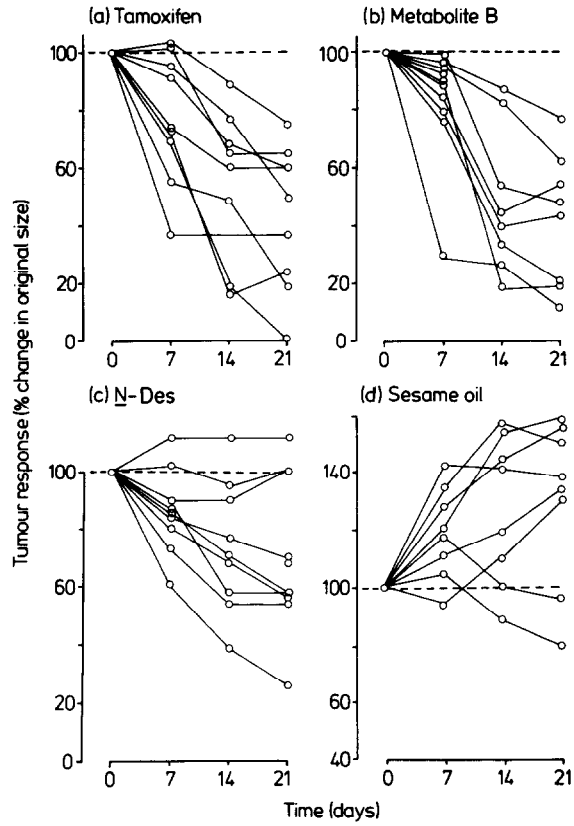

Fig. 1. The effect of (a) tamoxifen, (b) metabolite B, (c) N-desmethyltamoxifen (100 μg/day s.c.) or (d) sesame oil on the growth of DMBA-induced mammary tumours in the rat.

Response of DMBA-induced tumours and plasma concentrations of tamoxifen and N-desmethyltamoxifen after various doses of tamoxifen

The data presented in Table 1 show that a clear relationship exists between the dose of tamoxifen used during a 5-week treatment period and the number of tumours responding to treatment, the extent of regression produced and the appearance of new tumours. Thus in animals injected with 300 μg tamoxifen per day, 8 out of 12 (67%) tumours regressed to less than 50% of their original volume with no new tumours being observed, while the daily administration of 25 μg per day produced similar regressions in only 2 out of 9 (22%) tumours and 4 new tumours were

Table 1. Effect of the administration of various doses of tamoxifen on the growth of DMBA-induced tumours

Tumour volume (% of initial vol.)	Dose (μg) 300	100	50	25
<50	8	3	3	2
50–99	1	3	1	2
≥100	3	4	4	5
New tumours	0	0	2	4
% regressing	75	60	50	44

detected. All the tumours subsequently regressed in response to ovariectomy performed at the conclusion of tamoxifen treatment.

The concentrations of tamoxifen and *N*-desmethyltamoxifen were measured in plasma from treated animals at the end of the 5-week period (Fig. 2). At a dose of 25 μg tamoxifen/day the average concentration of the drug was 6.1 ng/ml and that of its *N*-desmethyl metabolite 1 ng/ml. With increasing dosages these concentrations became progressively greater, so that in plasma from animals injected with 300 μg tamoxifen/day the levels of the parent compound and its metabolite were 31.4 and 9.7 ng/ml respectively. The proportion of metabolite relative to parent drug also increased with the dose.

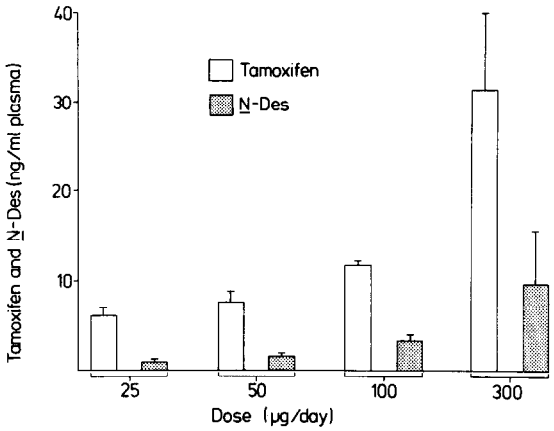

Fig. 2. Concentration of tamoxifen and N-*desmethyltamoxifen* (N-*Des*) *in plasma from tumour-bearing rats after daily administration* (25, 50, 100 *or* 300 μg *s.c.*) *of tamoxifen for* 35 days (*mean* ± *S.D.*, n = 5).

The greatest variation in the concentration of the compounds was seen at the highest dose level used. The plasma concentration within this group, however, did not appear to be related to tumour response. Thus in a rat with a plasma concentration of 27 ng/ml tamoxifen and 6.9 ng/ml *N*-desmethyltamoxifen, one tumour regressed to 5% of its initial volume while another increased in size by 300% (Table 2). When linear regression analysis was performed on the pooled data from all four dosage groups no significant correlation was found between the plasma concentration of tamoxifen and the extent of the regression produced in 39 individual tumours ($r = -0.19$). Nor was any correlation found between tumour response and *N*-desmethyltamoxifen concentration ($r = -0.17$), or response and total concentration of tamoxifen plus *N*-desmethyltamoxifen ($r = -0.19$).

Measurement of tamoxifen and metabolites in DMBA-induced mammary tumours

Biopsies of tumour tissue were taken for GC-HRMS analysis after 1, 7 or 14 days administration of tamoxifen (300 μg/day) to tumour-bearing animals. The results of these analyses are shown in Fig. 3. The concentration of both tamoxifen and its *N*-desmethyl metabolite rose over the 14-day experimental period in both the supernatant fraction (Fig. 3a) and also in extracts of pellets (Fig. 3b). This was particularly evident for *N*-desmethyltamoxifen in the supernatant fractions, where the concentration of the metabolite, originally below 1 ng/mg protein 24 hr after the first injection, rose to a value slightly in excess of that of the parent compound after 14 days administration.

Levels of 4'-hydroxytamoxifen were below detection limits (<0.25 ng/mg of protein, <0.7 ng/mg DNA) in tumour tissue after a single injection of tamoxifen but, following 7 days administration of the drug, rose to an average of 0.48 ng/mg of protein (± 0.13, $n = 7$) in supernatant fractions and 1.5 ng/mg DNA (± 1.64, $n = 7$) in nuclear pellets (not illustrated). *N*-Desmethyltamoxifen was therefore the more abundant metabolite in both fractions after these treatment periods. Oestrogen receptor status was not measured in these tumours.

Table 3 shows the response of ER+ DMBA-induced tumours to treatment with tamoxifen (300 μg/day) for 35 days. Tumour biopsies were taken before the start of treatment for receptor assay and again after 7 days of tamoxifen administration for GC-HRMS analysis. There were significant correlations ($P < 0.05$) between

Table 2. Concentration of tamoxifen and N-*desmethyltamoxifen in tumour-bearing rat plasma and relation to the response of tumours to tamoxifen treatment*

	% initial tumour vol. after treatment for 35 days Tumour				Concentration (ng/ml plasma)	
Animal	A	B	C	D	Tamoxifen	*N*-Desmethyltamoxifen
1	6	0	0	0	19	4.3
2	0	0	130		42	19
3	49				33	6.6
4	62	159			36	11.8
5	206	5			27	6.9

the extent of regressions produced and the concentration of tamoxifen in supernatant fractions, and between regression and the total concentration of tamoxifen plus *N*-desmethyltamoxifen in the supernatant. The best correlation, however, was observed between the extent of regression and the oestrogen receptor content of the tumour before treatment ($r = -0.90$).

No correlation was apparent between regression and the concentration of any of the antioestrogens in nuclear pellets.

The average concentrations of tamoxifen, *N*-desmethyltamoxifen and 4'-hydroxytamoxifen in supernatant fractions were 7.8, 5.0 and 1.2 pmol/mg protein respectively, while in nuclear pellets their average concentrations were 35, 22 and 3.6 pmol/mg DNA. While these figures cannot be compared directly with the mean concentration of ER measured before treatment (27.6 fmol/mg protein), it is clear that on a molar basis tissue levels of the antioestrogens greatly exceed those of ER.

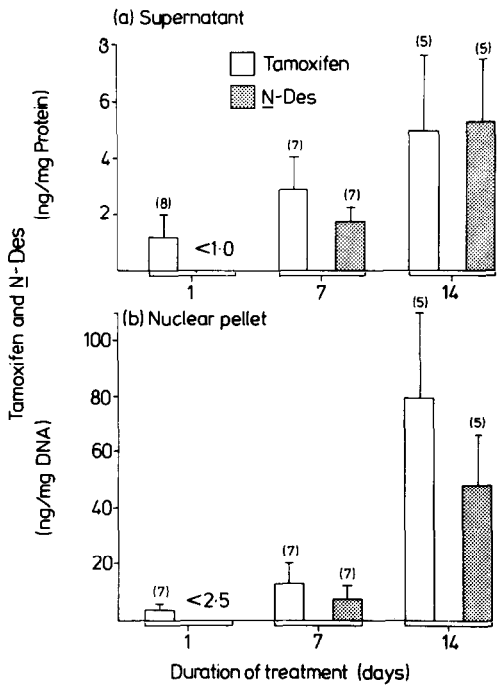

Fig. 3. Concentration of tamoxifen and N-*desmethyltamoxifen* (N-*Des*) *in DMBA-induced tumours after daily administration* (300 μg *s.c.*) *of tamoxifen to tumour-bearing animals* (*mean* ± *S.D.; figures in parentheses indicate* n).

DISCUSSION

The DMBA-induced mammary tumour of the rat is commonly used as a model for breast cancer because, as in the human disease, the growth of many tumours may be arrested or reversed by a wide range of endocrine manipulations [1,17]. Administration of tamoxifen is one such treatment which is effective against both human [2–4] and DMBA-induced tumours [5, 18]. In this experiment, however, ER+ rat mammary tumour regressions were induced both by tamoxifen and by equal doses (100 μg/day) of two of its metabolites, 4'-hydroxytamoxifen and *N*-desmethyltamoxifen (Fig. 1). These metabolites have previously been measured in the plasma [7, 10] and tumour tissue [10] from tamoxifen-treated patients, where in most cases the concentration of *N*-desmethyltamoxifen exceeded that of the parent drug by a factor of 1.5–2. In addition, GC-HRMS analysis demonstrated that the metabolites were present in supernatant and nuclear pellet fractions of regressing rat mammary tumours after tamoxifen administration (Table 3), although, in contrast to human studies, the concentration of *N*-desmethyltamoxifen was lower than that of the parent drug. Taken together, these observations indicate that 4'-hydroxytamoxifen and *N*-desmethyltamoxifen may be active with the parent drug in controlling tumour growth after tamoxifen administration.

Table 3. Concentration of tamoxifen, N-*desmethyltamoxifen and 4'-hydroxytamoxifen in ER+ DMBA-induced tumours in relation to the response of tumours to tamoxifen treatment*

	Concentration: ng/mg protein in supernatant (S) or ng/mg in DNA in pellet (P)								
% initial tumour vol.	Tamoxifen		*N*-desmethyltamoxifen		4'-Hydroxytamoxifen		Tamoxifen + *N*-desmethyltamoxifen		ER concentration (fmol/mg protein)
	S	P	S	P	S	P	S	P	
61	1.2	7.3	1.4	4.5	0.3	1.2	2.6	11.8	11.0
57	1.8	29.1	1.2	17.5	0.5	5.0	3.0	46.6	16.5
57	2.4	10.2	1.3	7.4	0.5	1.9	3.7	17.6	10.2
30	4.2	11.7	2.1	8.6	0.5	1.0	6.3	20.3	41.3
27	4.1	9.8	2.7	5.5	0.7	<0.4	6.8	15.3	22.8
19	3.3	10.9	1.8	7.2	0.5	<0.4	5.1	18.1	45.6
	3.3	12.2	2.0	5.0	0.5	0.8	5.3	17.2	45.6
correlation coefficient (r)	−0.755*	0.243	−0.697	0.386	−0.444	-	−0.760*	0.30	−0.90*

*$P < 0.05$.

A daily dose of tamoxifen of 100 μg caused regression of 60% (6/10) of DMBA-induced tumours over a 5-week treatment period (Table 1), a rate of response similar to that previously reported [5]. Elevation of the dose to 300 μg/day increased the proportion of regressing tumours to 75%—a response rate almost as high as that induced by ovariectomy [10]. In contrast, when the dose was reduced to 50 or 25 μg/day lower response rates were observed and the treatment, unlike that at higher doses, did not prevent the appearance of new tumours (Table 1). Both the regression of established tumours and the inhibition of new ones, therefore, are clearly dependent on the dose of the drug administered within the range examined.

The dose-dependent variation in the response of tumours to tamoxifen treatment was accompanied by a similar variation in the concentration of the drug and its *N*-desmethyl metabolite in plasma (Fig. 2). In the case of the metabolite the increase in concentration was linear with dose. The apparent lack of linear dose to concentration relationship for tamoxifen itself might not be significant in view of the variation in plasma concentration at the highest dose administered. It has been reported, however, that tamoxifen has a biphasic serum half-life in the rat [11].

Unfortunately, while gross alterations in the plasma concentrations of the compound were apparently related to response rate, no clear correlation was observed between the extent of decrease in tumour volume and the concentration of tamoxifen or *N*-desmethyltamoxifen in the plasma of individual animals (Table 2). Individual plasma concentrations alone, therefore, were not indicative of tumour response.

When the concentrations of tamoxifen, *N*-desmethyltamoxifen and 4′-hydroxytamoxifen were measured in ER+ tumour tissue no obvious relationship between the levels of the compounds in extracts of crude nuclear pellets and the extent of tumour regression was found (Table 3). In the supernatant fractions, however, significant correlations ($P < 0.05$) were observed between response and tamoxifen concentrations, and response and total concentration of tamoxifen plus *N*-desmethyltamoxifen. It has previously been demonstrated [16] that the degree of regression of DMBA-induced tumours produced in response to tamoxifen therapy can also be correlated with the concentration of displaceable binding sites for both [^{3}H]-oestradiol and [^{3}H]-tamoxifen. This finding was confirmed in the case of [^{3}H]-oestradiol in this experiment (Table 3). The concentration of tamoxifen and its metabolites in both supernatant and pellet extracts, however, greatly exceeded that of the oestrogen receptor measured in whole homogenates of tumour tissue before tamoxifen treatment. Therefore only a very small proportion of the antioestrogen present in these tumours could be bound to the oestrogen receptor. It is possible, however, that a large excess of antioestrogen is necessary in order to maintain a constant high concentration of the nuclear antioestrogen–receptor complex since the rate of dissociation of tamoxifen from the receptor is relatively rapid [19].

The high concentrations of tamoxifen and *N*-desmethyltamoxifen in the supernatant fractions (Fig. 3) might partly be accounted for by the possible existence of an antioestrogen receptor described by Sutherland *et al.* [20]. The presence of this moiety, which apparently binds specifically to compounds of the triphenylethylene type, has not, however, been reported in nuclear preparations, where the concentration of antioestrogen was also found to be high (Fig. 3).

It was noticeable that the concentration of the hydroxy metabolite in tumour tissue was considerably lower than that of either the parent drug or *N*-desmethyltamoxifen (Table 3). In the rat tumour, therefore, as in the human patient [7,10], this compound apparently constitutes only a minor metabolite of tamoxifen. The relative affinity of this hydroxy metabolite for the oestrogen receptor is approximately 10 times higher than that of the parent drug [19] and, indeed, it has been reported that 4′-hydroxytamoxifen was the major labelled species bound to uterine oestradiol receptor after rats had been injected with [^{3}H]-tamoxifen [21]. It is possible, therefore, that the hydroxy metabolite has a greater influence on the oestrogen receptor mechanism than its relative concentration in tumours might otherwise suggest.

These observations illustrate the fundamental importance of pharmacodynamic data in the interpretation of drug mechanism of action. In this study it has been demonstrated that there is an overall correlation between the effect of tamoxifen on tumour growth in the rat and the concentration of the drug and a major metabolite in plasma (Table 1, Fig. 1). Similar correlations were demonstrated between the concentrations of antioestrogens in supernatant fractions of DMBA-induced tumours and the extent of response to tamoxifen administration. These relationships exist despite the apparent high concentration of the drug and at least one of its metabolites relative to the oestrogen receptor in tumour biopsies (Table 3). The nature of the interaction between these very high concentrations of antioestrogens and the oestrogen receptor remains to be fully elucidated.

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