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IN VITRO AND *IN VIVO* BINDING OF TOREMIFENE AND ITS METABOLITES IN RAT UTERUS

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Summary—The *in vitro* binding affinities of toremifene (TOR), 4-hydroxy toremifene (4-OH-TOR) and several other metabolites for the rat uterine cytosolic estrogen receptor were compared with those of tamoxifen (TAM) and 4-hydroxy tamoxifen (4-OH-TAM). Only small differences were observed and the binding affinities of both 4-hydroxy metabolites were similar to that of estradiol (E2). Uterine uptake and subcellular distribution of [^{3}H]TOR and [^{3}H]TAM were then compared at 1, 8 and 72 h after administration to castrated rats. The uptake and retention of both antiestrogens were similar at all times. In each case the amount of nuclear bound radioactivity declined to low levels at 8 and 72 h but the ratios of 4-OH-TAM/TAM and 4-OH-TOR/TOR determined by HPLC analysis increased dramatically at 72 h. The level of radioactivity in both plasma and uterine cytsol at 72 h was significantly higher following [^{3}H]TAM administration. However, most of the radioactivity appeared to be in a conjugated form since it was not extractable with solvent. Finally, the ability of prior administration of each antiestrogen (100 mg/kg) to block uterine [^{3}H]estradiol uptake was examined at 3 and 7 days. It was found that uterine wet weights were higher than control one week after administration of both compounds. Prior administration of TOR increased nuclear uptake of [^{3}H]E2 whereas TAM had no effect. The results of these experiments suggest that toremifene and tamoxifen have very similar *in vitro* and *in vivo* binding properties but differences in metabolism exist that may be important.

INTRODUCTION

The well established antiestrogenic properties of triphenylethylene drugs such as clomiphene and TAM have led to their widespread use in clinical medicine. Although discordant observations such as marked species differences in agonist/antagonist potency exist [1–4], it is generally believed that these compounds compete with E_2 for binding to the estrogen receptor in target tissues and form inactive complexes (for review, see [5]). Many studies have demonstrated that antiestrogens compete with E2 for binding to the estrogen receptor in broken cell preparations of target tissues, such as the uterus. However, the estrogen antagonism expressed *in vivo* is complicated by the extensive metabolism to both more active and less active metabolites [6].

The present studies were undertaken to compare both *in vitro* and *in vivo* binding in the rat uterus of a standard antiestrogen that has found clinical acceptance, TAM, with a closely related compound TOR that has recently become available.

MATERIALS AND METHODS

Reagents and animals

Both unlabeled TOR and [^{3}H]toremifene (sp. act. 23 Ci/mmol) were provided to us by Farmos Ltd, Turku, Finland. [^{3}H]TAM (sp. act. 82 Ci/mmol) was purchased from New England Nuclear (Boston, Mass) and [^{3}H]estradiol (sp. act. 140 Ci/mmol) from Amersham Corp. (Arlington Heights, Ill). Six metabolites of toremifene were also provided to us by Famos: *N*-monomethyl-toremifene (Fc-1200), *N*-desmethyl-toremifene (Fc-1270), 4-hydroxy-toremifene (Fc-1263), the deaminated primary alcohol of toremifene (Fc-1271), the 4-hydroxy primary alcohol of toremifene (Fc-1272) and 4-hydroxy carboxyl toremifene (Fc-1330). Scintiverse II (Fisher Scientific Chemicals; Fair Lawn, N.J.) was used for liquid scintillation counting. All other chemicals were of highest purity commercially available. Female Sprague–Dawley rats weighing approximately 250–300 g were used in all experiments. The rats were ovariectomized 3–5 days prior to the start of each experiment.

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Relative binding affinities of toremifene, tamoxifen and their metabolites

Uterine cytosol from castrated untreated rats was prepared as described below. The relative binding affinities of metabolites were determined using the centrifugal ultrafiltration dialysis method previously described [7]. Increasing amounts of [^{3}H]E2 were added to a series of 10 tubes (1–100 nM) in the presence or absence of a constant amount of non-radioactive competitors: E2, TAM, TOR, 4-OH-TAM/4-OH-TOR, *N*-monomethyl TOR and the primary alcohol of TOR. the organic solvent was evaporated and 0.5 ml of the cytosol added followed by approximately 5000 cpm of [^{14}C]glucose. After a 2 h incubation at 4 , 200 μl of the incubation mixture was placed in duplicate capsules and centrifuged for 16 h at 4 C at 300 g. The samples were processed through the remainder of the assay as previously described [8] and the percent free E2 determined. Bound E2 was calculated (total-free) and Lineweaver–Burke plots used to determine the inhibition constant (K_i) for the competitors.

Distribution of [^{3}H]antiestrogen in rat uterus subcellular organelles

Tritiated antiestrogens were dissolved in 0.5 ml of a solution containing 0.15 M saline and ethanol (9:1) and injected into rats intraperitoneally. The mean dose for tamoxifen was 0.7 nmol and for toremifene 1.7 nmol, both giving approximately 35×10^6 cpm/rat. After 1, 8 or 72 h the rats were anesthetized with pentobarbital (Nembutal; Abbot Laboratories), and blood was withdrawn by cardiac puncture with a heparinized syringe.

The animals were sacrificed by exsanguination, the uterus excised, rinsed with saline and frozen immediately in liquid nitrogen. The uteri were pulverized and suspended in 2 ml of 10 mM Tris, 1 mM EDTA buffer, pH 7.4 at 4 C and homogenized with two 10 s bursts of a Polytron homogenizer. The homogenate was centrifuged at 800 g for 10 min yielding a crude nuclear pellet and crude supernatant. The crude nuclear pellet was washed 3 times with 1.5 ml of Tris buffer and suspended in 1 ml of the buffer. The crude supernatant was centrifuged at 10,000 g for 20 min to yield a "mitochondrial" pellet that was suspended in 1 ml of the buffer while the supernatant was centrifuged a third time at 150,000 g for 60 min to separate "microsomes" and cytosol. The microsomal pellet was also suspended in 1 ml of buffer. Aliquots of all the fractions were taken for liquid scintillation counting. Subcellular fractions as well as plasma (1 ml) were extracted twice with 5 ml of diethyl ether:ethyl acetate (9:1). The combined organic phases were evaporated under a stream of nitrogen and the metabolites dissolved in 2 ml of toluene. Aliquots of both the extractable (organic phase) as well as from the non-extractable (aqueous) phase were taken for liquid scintillation counting.

HPLC separation of toremifene and tamoxifen metabolites

To identify TOR and TAM metabolites, aliquots of the nuclear and plasma solvent extracts were applied to a HPLC column. The column was a 10 μ Vydac C18 reverse phase (4.6 × 250 mm) run at 45 C with a C18 guard column. Mobile phase A was 0.01 M $NaH_2PO_4 \cdot H_2O$:acetonitrile:isopropranol: triethylamine 640:300:60:2, pH 3.9, and mobile phase B was isopropanol. An isocratic gradient program was used with a flow rate of 2 ml/min with 100% mobile phase A between 0 and 24 min, and a linear program where mobile phase A decreased from 100 to 70% while mobile phase B was increased correspondingly between 24 and 44 min. Available non-radioactive standards were added to extracts, and detected with a u.v.-detector set at 254 nM. Fractions from each column were collected (0.5 ml) and the radioactivity determined by liquid scintillation counting.

RESULTS

The relative binding affinities (K_i) of TAM and TOR and selected metabolites are shown in Figs 1 and 2. The value of the dissociation constant (K_d) for E2 binding to the estrogen receptor determined from measurements of free E2 in the same series of experiments was 5×10^{-11} M in agreement with our earlier results. When nonradioactive E2 was added as a competitor, the value of K_i was 1.2. As can be seen, both TOR and 4-OH-TOR have about the same affinity for the receptor as TAM, and 4-OH-TAM. All of the other toremifene metabolites studied have considerably lower affinity than 4-OH-TOR. It should be noted that the K_i values shown in Fig. 2 are

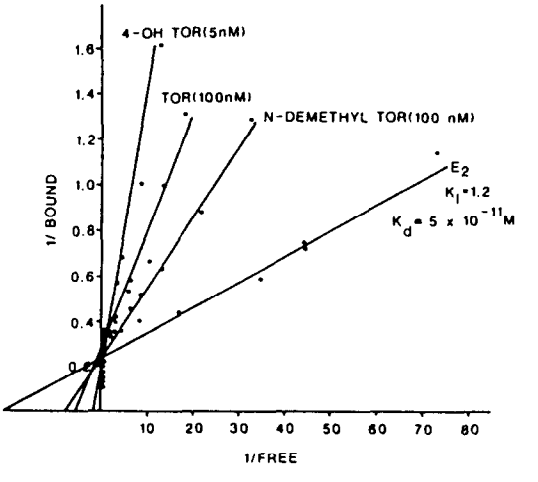
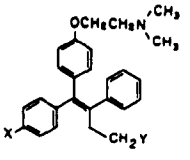


Fig. 1. Lineweaver-Burke plots for the determination of inhibition constants (K_i) of toremifene metabolites. Rat uterine cystosol was prepared and competition by toremifene metabolites for [^{3}H]estradiol binding to the estrogen receptor was determined as described in the text. The concentrations of competitors are shown in the figure and the plots were determined with the aid of a least squares linear regression program.

approximations since the free concentrations of the metabolites were not measured. Attempts to measure directly the binding affinity of [^{3}H]TOR and [^{3}H]TAM using centrifugal ultrafiltration were unsuccessful due to extensive nonspecific binding.

Plasma radioactivity following administration of [^{3}H]TAM or [^{3}H]TOR

Figure 3 illustrates the distribution of solvent extractable and water soluble radioactive metabolites at 1, 8 and 72 h following administration of [^{3}H]TAM or [^{3}H]TOR. The results, normalized to the same dose of radioactivity, indicate that the clearance of the two compounds is quite different. Surprisingly, the total plasma radioactivity following [^{3}H]TAM at 72 h was equal to or greater than that found at 1 h whereas the plasma level of [^{3}H]TOR and metabolites declined about 80% over this period. The difference in total tritium levels at 72 h was highly significant. However, virtually all of the [^{3}H]TAM derived plasma radioactivity was water soluble at 72 h. On the other hand, only 25–30% of [^{3}H]TOR derived radioactivity was solvent extractable and this difference was also highly significant. The identity of the water soluble plasma radioactive metabolites was not investigated although preliminary experiments using limpet aryl sulphatase and beta-glucuronidase indicated that most of the [^{3}H]TAM metabolites were conjugated with glucuronic acid (data not shown).



		K_i
X,Y=H	TAMOXIFEN (TAM)	21
X-OH,Y=H	4-HYDROXY-TAM	1.7
X=H,Y=CL	TOREMIFENE(TOR)	14
X-OH,Y=CL	4-HYDROXY-TOR	0.8
-O-CH_2CH_2N<$^{CH3}_{H}$	TOR	41
-OCH_2CH_2-NH_2	TOR	22
-O-CH_2CH_2OH	TOR	326
-OCH_2CH_2OH	HO-TOR	14
-O-CH_2-COOH	HO-TOR	38

Fig. 2. Inhibition constants of toremifene and tamoxifen metabolites. The K_i's of various antiestrogen metabolites were determined as described in the text and Fig. 1.

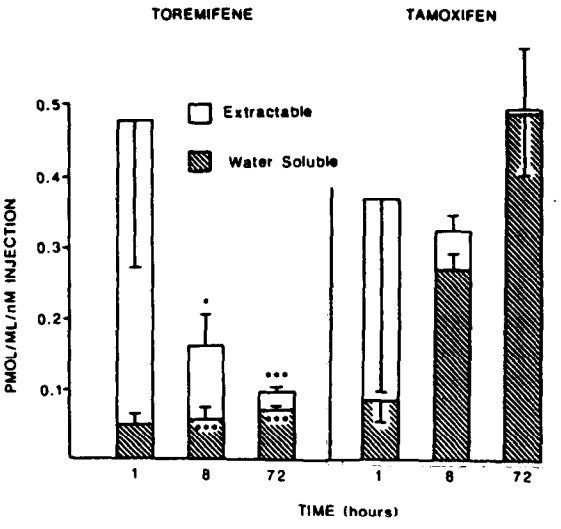


Fig. 3. Plasma distribution of antiestrogens injected into rats. [^{3}H]Antiestrogens (35 × 10^6 cpm/rat) were injected into rats and the plasma distribution of metabolites between ether:ethyl acetate (9:1) extractable and non-extractable were determined at 3 time periods. Data were normalized to account for the different specific activities of the two antiestrogens. Each bar represents the mean ± SEM, $n = 3$. Significance levels: $^*P < 0.05$; $^{***}P < 0.001$.

Uterine uptake and distribution of toremifene and tamoxifen

The subcellular distribution of radioactivity in the uterus 1 h following injection of both antiestrogens is shown in Fig. 4. In each case 75–80% of the radioactivity was present in the nuclear extracts whereas only 5–8% was associated with the mitochondrial and microsomal fractions. About 10% the total radioactivity was recovered in the cytosol fraction after 1 h. Clearly, the major portion of the radioactivity

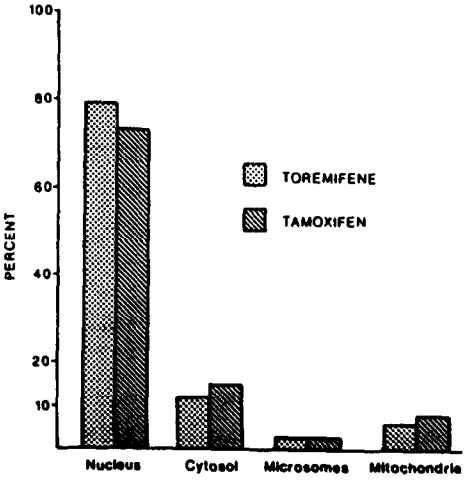


Fig. 4. Subcellular distribution of injected [^{3}H]antiestrogens. [^{3}H]Antiestrogens (35 × 10^6 cpm/rat) were injected into rats and the amount of [^{3}H]antiestrogen bound to uterine subcellular fractions 1 h after injection was determined. Each bar represents an average of 3 experiments.

derived from both antiestrogens in the uterus was associated with the nuclear fraction. As determined by HPLC most of the radioactivity present in the nuclear fraction at 1 h could be accounted for by unmetabolized [^{3}H]TAM or [^{3}H]TOR. Figure 5 compares the relative amounts of [^{3}H]TOR and [^{3}H]TAM and their 4-hydroxy metabolites recovered from the nuclear fractions after 1, 8 and 72 h. Although there appeared to be more [^{3}H]TOR than [^{3}H]TAM at 1 h this difference was not statistically significant. Also shown in the inset of Fig. 5 are the ratios of the parent compounds to their 4-hydroxy metabolites. After 72 h, the amount of [^{3}H]OH-TAM exceeded that of [^{3}H]TAM whereas the amount of [^{3}H]TOR recovered was still about twice the amount of [^{3}H]4-OH-TOR.

Antiestrogen blockade of [^{3}H]estradiol uptake by the uterus

In another series of experiments nonradioactive antiestrogens (100 mg/kg) were administered to ovariectomized female rats by a gastric tube. Three or seven days later, [^{3}H]estradiol (2×10^7 cpm, i.p.) was injected 1 h before sacrifice. Uterine weights were determined and total plasma and uterine radioactivity was measured. The results were compared with those obtained with rats-receiving vehicle (5% methylcellulose) alone. The results shown in Fig. 6 indicate that uterine weights were above control values 3 and 7 days after administration of both antiestrogens. Indeed, the increase over control was greater (45–50%) at 7 days. Both total uterine content of [^{3}H]E2 and the amount extractable from the uterine nuclei measured 1 h after [^{3}H]E2 injection were reduced by administration of both antiestrogens

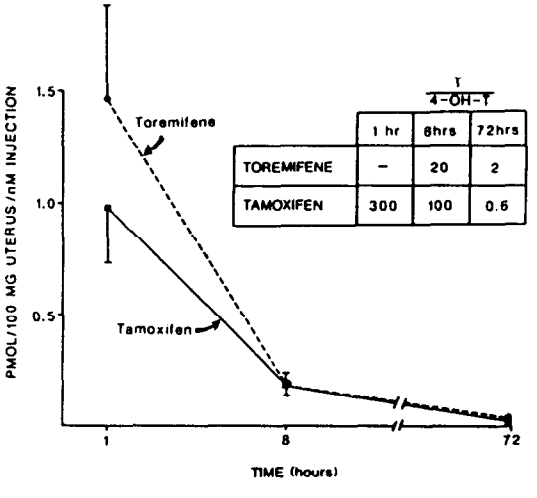


Fig. 5. Nuclear retention of antiestrogens. [^{3}H]antiestrogens were injected into rats as described in Fig. 3 and crude nuclei isolated (see Methods). The radioactivity was extracted with ether:ethyl acetate (9:1) and an aliquot counted for recovery. The remaining extract was analyzed by HPLC for metabolites. Inset shows the ratio of antiestrogen to its 4-OH metabolite. Each point represents mean ± SEM, $n = 3$.

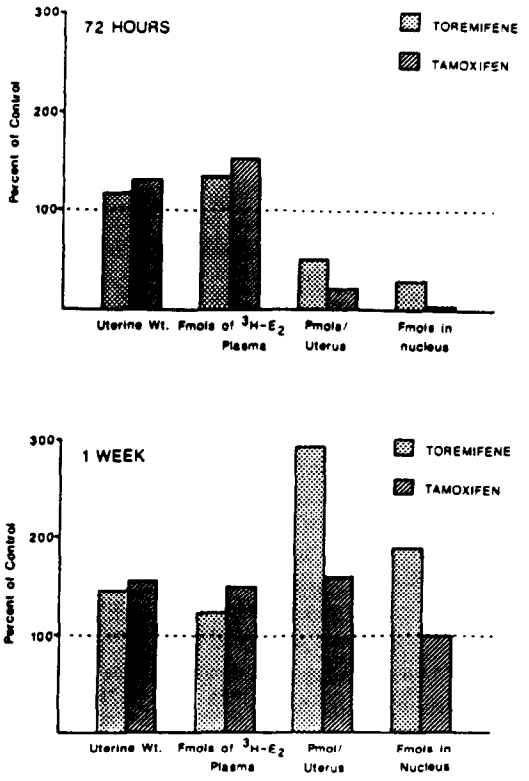


Fig. 6. Antiestrogen blockade of [^{3}H]E2 uptake antiestrogens (100 mg/kg) were administered to rats and 3 or 7 days later [^{3}H]estradiol uptake at 1 h was measured as described in text. Uterine weights as well as amount of [^{3}H]E2 in plasma, uterus and uterine nuclei were determined. Each bar represents the mean average of 3 experiments.

72 h earlier. Nuclear [^{3}H]E2 was suppressed 70 and 90% by administration of TOR and TAM, respectively. However, after one week had elapsed from the time of TAM administration no effect on nuclear uptake of [^{3}H]E2 was observed. In contrast 3-fold more [^{3}H]E2 was found in the uterus and nuclear [^{3}H]E2 content was increased nearly 2-fold above control levels by administration of TOR 7 days earlier.

DISCUSSION

It is generally agreed that the triphenylethylene type of antiestrogens exert their effects by direct competition with estradiol for binding to the estrogen receptor [9, 10]. Abundant evidence exists suggesting that the resulting antiestrogen–receptor complex does not have the proper conformation required for interaction with the genomic regulatory binding sites occupied by the normal E2–receptor complex [11]. The fact that antiestrogenic potency correlates at least roughly with binding affinity for the estrogen receptor supports this view [12]. The results of our binding studies with TAM and 4-OH-TAM are in agreement with the results of others although we find that 4-OH-TAM only has about a 10–12-fold higher affinity than TAM [12, 13]. A similar relationship was

observed with TOR and 4-OH-TOR and therefore it is to be expected that following prolonged administration of the parent compounds the higher affinity metabolites will occupy more of the receptor. Indeed this is what was observed in the present experiments (Fig. 6). However, caution must be exercised in extrapolating *in vitro* estimates of binding affinity to *in vivo* results. As pointed out earlier [7] large errors are possible, indeed, likely with lipophilic ligands, when binding affinities are estimated without knowledge of free ligand concentrations. Nonetheless, it seems likely that metabolism of the diethylaminohydroxy side chain reduces the binding affinity of TOR metabolites (Fig. 2). The failure to detect significant amounts of [^{3}H]metabolites of TOR other than 4-OH-TOR is consistent with the *in vitro* binding results.

Studies of uterine uptake and subcellular distribution revealed little difference in the behavior of TOR and TAM. Both compounds were accumulated by uterine nuclei to about the same extent when the results were normalized to the same dose of tracer. Although direct comparisons with retention of [^{3}H]E2 were not made it is apparent that both antiestrogens are lost from the nucleus more rapidly than is estradiol. This is to be expected at early times (1–8 h) since the parent compounds TAM and TOR, which have much lower affinity for ER than E2, are the predominant species. As mentioned earlier, however, it is of interest that the proportion of both 4-hydroxy metabolites increases with time reflecting the slow rate of production but higher affinity for the estrogen receptor. In order to obtain results more relevant to the administration of therapeutic levels of antiestrogens, we used an approach similar to that described by Jordan and his associates [14]. When the ability of the antiestrogens to block uterine accumulation of [^{3}H]E2 was examined, the results (Fig. 6) indicated that both TOR and TAM at a dose of 100 mg/kg block uterine uptake of [^{3}H]E2 given 72 h later. Tamoxifen was more effective in this regard particularly in preventing binding of [^{3}H]E2 to nuclear receptors. Presumably the inhibition of [^{3}H]E2 uptake resulted from occupation of the receptors by the 4-hydroxy metabolites of the antiestrogens. The results obtained one week after administration of the drugs were somewhat surprising. Both antiestrogens still appeared to exert an estrogenic effect on the uterus since uterine weights were 45–50% above control. Furthermore, total-uterine uptake of [^{3}H]E2 was enhanced by both compounds although the effect of TOR was much larger (300 vs 150%). Also, TOR administration one week earlier nearly doubled the amount of nuclear bound [^{3}H]E2 at 1 h whereas TAM had no effect. Since uterine weights were stimulated to the same extent these results could suggest that TOR has a greater stimulatory effect on the level of uterine estrogen receptors than does TAM. This could reflect differences in metabolism resulting in greater agonist activity during long-term administration of TOR. However, much more data is required to evaluate E2 uptake and effects when studied against the pharmacokinetic background of both antiestrogens.

The patterns of radioactivity found in plasma showed marked differences following administration of [^{3}H]TAM and [^{3}H]TOR (Fig. 3). After 1 h the concentrations of radioactivity were similar for both compounds. However, after 72 h, the plasma level of [^{3}H]TOR derived material had declined about 5-fold whereas little change was found with [^{3}H]TAM. This difference in behavior of the two antiestrogens was clearly evident 8 h after administration. These patterns may reflect important differences in metabolism between the two compounds since a much greater proportion of TAM associated tritium was found to be water soluble. This was particularly evident at 72 h when very little TAM-derived radioactivity could be extracted with solvent. Previous studies in the rat demonstrating extensive enterohepatic recycling of TAM metabolities are in accord with this observation. It is possible that the presence of the chlorine atom in TOR reduces the rate of hepatic metabolism and/or conjugation to water soluble metabolites. Further investigation of this point seems warranted since similar results were found in uterine cytosol and it is reasonable to assume that glucuronic acid conjugates of these antiestrogens are inactive as is the case with natural steroid hormone metabolites.

In summary, the results demonstrate that the new antiestrogen toremifene is very similar to tamoxifen with respect to *in vitro* binding to the estrogen receptor, metabolism and *in vivo* uptake. Clearly the relative merits of the two compounds as therapy for human breast cancer cannot be assessed by such experiments. Differences in tolerance, pharmacokinetics or even mechanism of action in humans may prove to be important in selecting one or the other compound for therapeutic use.

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