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BIOLOGICAL ACTIVITIES OF TAMOXIFEN AZIRIDINE, AN ANTIESTROGEN-BASED AFFINITY LABEL FOR THE ESTROGEN RECEPTOR, *IN VIVO* AND *IN VITRO*

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Summary—Tamoxifen aziridine (TA), an antiestrogen-based affinity label for the estrogen receptor, is highly selective and efficient in its covalent binding to the estrogen receptor (Katzenellenbogen *et al.*, *J. biol. Chem.* **258** (1983) 3487–3495). Thus, it was of interest to investigate the biological character and potency of this compound and, in particular, to determine if the irreversible attachment of this tamoxifen-derived compound to the estrogen receptor would result in enhanced antiestrogenic properties or in unusual biological activity. The effect of tamoxifen aziridine and tamoxifen (Tam), the parent compound which is an antiestrogen that binds reversibly to the estrogen receptor, were compared with respect to their effects on uterine growth, growth of dimethylbenzanthracene (DMBA)-induced mammary tumors in rats, and proliferation and plasminogen activator activity of MCF-7 human breast cancer cells.

In immature (day 20) rats, Tam and TA behaved as weak estrogen agonists and estrogen antagonists in that Tam or TA alone increased uterine weight to levels lower than that evoked by estradiol (E_2), and both were able to suppress the stimulation of uterine weight evoked by E_2. Administration of Tam and TA via Alzet minipumps (25 or 200 μg/rat/day) to mature rats bearing DMBA-induced mammary tumors resulted in marked regression and/or disappearance of most tumors. Uterine weights were also suppressed in these mature rats by Tam and TA. Tam was slightly more potent than TA in evoking tumor regression and in suppressing uterine weights in these *in vivo* studies. In MCF-7 human breast cancer cells in culture, Tam and TA suppressed cell proliferation and evoked no increase in plasminogen activator activity by themselves, while being very effective in preventing plasminogen activator activity stimulation by E_2. Thus, TA displayed a bioactivity profile similar to that of Tam, the reversibly binding ligand, *in vitro* and *in vivo*. The covalent attachment of TA to the receptor does not, therefore, markedly alter the biological character or potency of the antiestrogen receptor complex.

INTRODUCTION

It is well established now that the estrogen receptor is found in about two thirds of human breast cancer specimens and its presence correlates well with the response of breast cancer to endocrine therapy. Thus, quantitation of the estrogen receptor is important clinically in predicting the value of endocrine therapy in breast cancer treatment [1–4].

Normally, the binding of natural and synthetic estrogens to receptor in target tissues is reversible. Upon binding of ligand to receptor, the avidity of receptor for chromatin increases [5]. In estrogen receptor-positive human breast cancer cells, such as MCF-7 cells, estrogen stimulation is associated with an increase in cell proliferation and the induction of several proteins such as the progesterone receptor and plasminogen activator [6–13].

There has been keen interest in the development of affinity labels for the estrogen receptor [14]. Since such ligands would bind covalently to receptor, we were interested in determining if such binding would alter or result in unusual biological activity. In particular, an agent which binds irreversibly to receptor and does not stimulate biological responses would be a most useful therapeutic and anticancer agent. Presently, all known antiestrogens have some estrogen agonist activity.

We have recently reported the highly selective and efficient labeling of estrogen receptor by a tamoxifen-based affinity label, tamoxifen aziridine [15–17]. It is the aim of these studies to investigate the biological consequences that result from irreversible binding of tamoxifen aziridine to receptor. For these studies, we have used 3 systems commonly employed in assessing the effectiveness of estrogens and antiestrogens, namely the immature rat uterus, DMBA-induced mammary tumors in rats, and MCF-7 human breast cancer cells. We have examined the effect of tamoxifen aziridine on uterine growth in the absence and presence of estradiol; on DMBA-induced mammary tumor growth and uterine growth in animals bearing DMBA-induced tumors; and on cell proliferation and plasminogen activator activity of MCF-7 cells. This is the first report on the biological character and potency of tamoxifen aziridine in these estrogen target tissues and cells *in vivo* and *in vitro*.

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EXPERIMENTAL

17β-Estradiol, insulin, and hydrocortisone were purchased from Sigma Chemical Co. (St Louis, Missouri). *Trans*-tamoxifen was a gift from ICI Americas (Wilmington, Delaware) and dimethylbenzanthracene (DMBA) a gift from the Upjohn Co. (Kalamazoo, Michigan). All media, sera, and antibiotics used in cell culture were obtained from GIBCO (Grand Island, New York). *Bis*(Cbz–Ile–Pro–Arg)-rhodamine was synthesized and purified according to the procedure of Leytus *et al.* [18]. Plasminogen was purified from fresh dog plasma as described in Leytus *et al.* [19]. Tamoxifen aziridine (1-[4-(2-*N*-aziridinyl-ethoxy)phenyl]-1,2-diphenylbut-l(z)-ene) was synthesized and generously provided by Drs David Robertson and John Katzenellenbogen, University of Illinois, Urbana. Details of the synthesis and purification of this compound have been reported [15].

Animals

Immature Sprague–Dawley female rats, 20–24 days old (Holtzman, Madison, Wisconsin) were used in the uterotrophic/antiuterotrophic studies. Virgin female Sprague–Dawley rats (Holtzman; 47–50 days old) were used in the DMBA-induced mammary tumor studies. All animals receiving treatment were housed separately and kept under standard lighting conditions (lights on 0500–1900 h). Purina rat chow and water were available at all times.

Uterotrophic and antiuterotrophic assays

Stock solutions of tamoxifen, tamoxifen aziridine or estradiol prepared in ethanol were diluted with sesame oil immediately prior to injection such that the desired concentration of drug would be delivered in 0.1–0.2 ml of oil containing 10% ethanol. Immature rats (20–22 days) were injected subcutaneously with the compound ± 1 μg E_2 for 3 days. At 24 h after the last injection, animals were sacrificed. Uteri were excised, drained of luminal fluid and weighed using a torsion balance.

Mammary tumor regression studies

Forty-five day old virgin female Sprague–Dawley rats were obtained from the Holtzman Co. (Madison, Wisconsin). At 47–50 days of age, the rats received a single injection of 1 ml of a lipid emulsion containing 5 mg dimethylbenzanthracene (DMBA) into the jugular vein [20]. Beginning 3 weeks after injection, animals were palpated biweekly for the appearance of mammary tumors. Tumors were measured along the two longest dimensions (length × width) using calipers; number of tumors, dimensions of tumors, and date of appearance were recorded. After a sufficient number of rats bearing tumors larger than 0.36 cm^2 tumor area were available (i.e. tumors with a minimum of 0.6 cm in each dimension), animals were divided into groups of 7–8 rats with similar tumor size and number.

Tamoxifen or tamoxifen aziridine was administered using Alzet osmotic pumps, model 2001 (Palo Alto, California). The drugs were freshly dissolved in ethanol, then mixed with propylene glycol to give a final concentration of ethanol no more than 5%. Pumps were filled with 219.2 μl such that 25 or 200 μg of drug was delivered per day. The rate of delivery is 1.02 μl/h with a standard deviation of 0.15 μl/h. The pumps were placed subcutaneously at the back of the neck while the animal was under ether anesthesia. Pumps were changed every 8 days; treatment was continued for 38 days, after which time animals were decapitated, uteri removed, tumors collected, and all tissues frozen on dry ice. Control animals received pumps containing the vehicle, 5% ethanol in propylene glycol; pumps were changed as in treated animals.

At the end of the study, tumors were categorized as growing—continual increase in tumor area over the treatment period; stable—area of tumor remaining approximately the same size as at the initiation of the study; regressing—a decrease in size by 50% or more from the original tumor area; or disappeared—total regression, no longer palpable.

Cell culture

MCF-7 cells were originally obtained from the Michigan Cancer Foundation (Detroit, Michigan). Cells were maintained in closed 150 cm^2 Corning T-flasks incubated at 37°C. The cells were grown in Eagle's minimal essential media (MEM) containing Hank's salts supplemented with 1% nonessential amino acids, 4 mM L-glutamine, and gentamicin (50 μg/ml). Bovine insulin (6 ng/ml), 5% calf serum, 0.01 M 4-(2-hydroxyethyl)-1-piperazine-ethane sulfonic acid (HEPES) buffer, penicillin (100 U/ml), streptomycin (100 μg/ml), and hydrocortisone (3.75 ng/ml) were added to the media.

Two weeks prior to the assay, cells were transferred to medium containing 5% charcoal–dextran treated calf serum (CDCS). Serum was stripped of endogenous steroid hormones by a 45-min incubation at 50°C with 0.50% Norit A, 0.05% dextran in 0.14 M NaCl. Charcoal was removed by centrifugation, twice at 800 *g* (3000 rpm) for 20 min, and the serum stored at −20°C [11, 13].

Cell proliferation studies

Cells were grown in media containing 5% charcoal–dextran treated calf serum for at least 2 weeks prior to being seeded into T-25 flasks. At 2 days after seeding, several flasks were harvested and counted using a Coulter Counter (Day 0 cell count). Compounds to be tested were dissolved in ethanol and then added to the media. Controls were exposed to the 0.1% ethanol vehicle. At several time points during the proliferation study, cells from duplicate sets of flasks were counted.

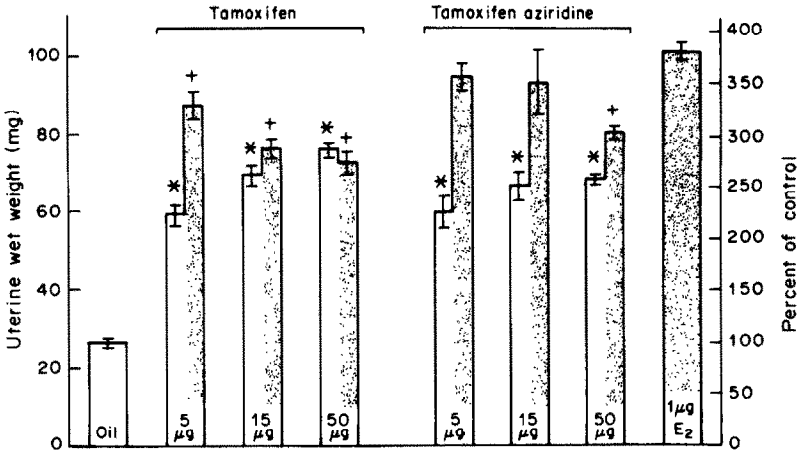

Fig. 1. The effect of administration of tamoxifen or tamoxifen aziridine alone, or with E_2 (shaded) on uterine weights in immature rats. Twenty-day-old female rats received E_2 (1 μg), TAM/TA (5, 15 or 50 μg), or both compounds together. Compounds were injected sc in oil once daily at 24 h intervals for 3 days and uterine wet weight was determined 24 h after the third injection. The control (oil vehicle) uterine weights are shown in the far left bar. Values represent the mean ± SEM of four uteri. $*P < 0.05$ for TAM or TA alone vs control; $+P < 0.05$ for TAM + E_2 or TA + E_2 vs 1 μg E_2.

Assay for plasminogen activator activity

Plasminogen activator activity of MCF-7 cells was determined by a 2-step assay using the rhodamine-based compound Bis (Cbz–Ile–Pro–Arg)-rhodamine, abbreviated BZIPAR, as a substrate for plasmin as described previously [13]. In brief, 25–200 × 10^3 cells were seeded into microwells (Costar, 96-well tissue culture cluster plates) and incubated in a sealed humidified jar at 37°C for 24 h. Cells were rinsed twice with HEPES-buffered saline prior to addition of 50 μl dog plasminogen (1 mg/ml). The amount of plasmin formed was measured by removing a 40 μl aliquot and adding it to 960 μl of 50 μM BZIPAR dissolved in 0.01 M HEPES, pH 7.2, containing 15% (v/v) ethanol and 5% (v/v) dimethylformamide. After 15 min, the fluoresence was measured in a Perkin–Elmer MPF-44A or a 650-40 fluorescence spectrophotometer as detailed previously [13].

RESULTS

Uterotrophic and antiuterotrophic activity of tamoxifen aziridine and tamoxifen in immature rats

Immature rats (20-days old) were injected with 5, 15, or 50 μg of tamoxifen or tamoxifen aziridine or with these compounds plus 1 μg E_2 for 3 days and uterine weights were then determined (Fig. 1). Both tamoxifen and tamoxifen aziridine were capable of stimulating uterine weight gain over that of control. When given in conjunction with 1 μg E_2, a 50 μg dose of tamoxifen aziridine resulted in a suppression of estrogen-stimulated uterine growth, while tamoxifen suppressed the E_2-stimulated growth at the three doses tested (Fig. 1).

Effects of tamoxifen aziridine and tamoxifen on the growth of dimethylbenzanthracene-induced mammary tumors and on uterine weights in these mature rats

Forty-seven to 50-day old female Sprague–Dawley rats were injected intravenously with 1 ml of emulsion containing 5 mg of dimethylbenzanthracene. Three weeks later, animals were palpated along the neck and sides of the body for the appearance of mammary tumors. Thereafter tumor growth was monitored twice per week. After a sufficient number of animals had developed tumors, treatment began and proceeded for 38 days, during which time the tumors were continuously exposed to 25 or 200 μg of TAM or TA per day. Compounds were administered via Alzet minipumps so as to assure a continuous exposure to fresh compound throughout the treatment period.

The effects of these compounds on tumor growth are shown in Fig. 2. Data are expressed as average tumor area per rat (% of change from day 1) for each treatment. Twenty-five μg TA inhibited tumor growth compared to controls but did not fully suppress growth. Treatment with 25 μg TAM resulted in a decline in tumor area following an initial period of growth. With 200 μg TAM or TA, treatment resulted in an immediate decline in average tumor area/rat. By day 38 of treatment, average tumor area had decreased by 74% for tamoxifen-treated animals and by 65% for tamoxifen aziridine-treated animals.

After the treatment period, tumors were categorized as growing, stable, regressing or disappeared (Table 1), as defined in the Experimental section. Table 1 shows that in all treatment groups, tumor growth was reduced compared to that of the control group. In the control group, 63% of the tumors continued to grow, whereas only 0–18% of the total number of tumors present grew in the treated animals. Tamoxifen and tamoxifen aziridine evoked the regression or disappearance of more than 50% (53–84%) of the treated tumors. Table 1 also gives absolute values of average tumor area/rat both before and after each treatment, from which it is seen that

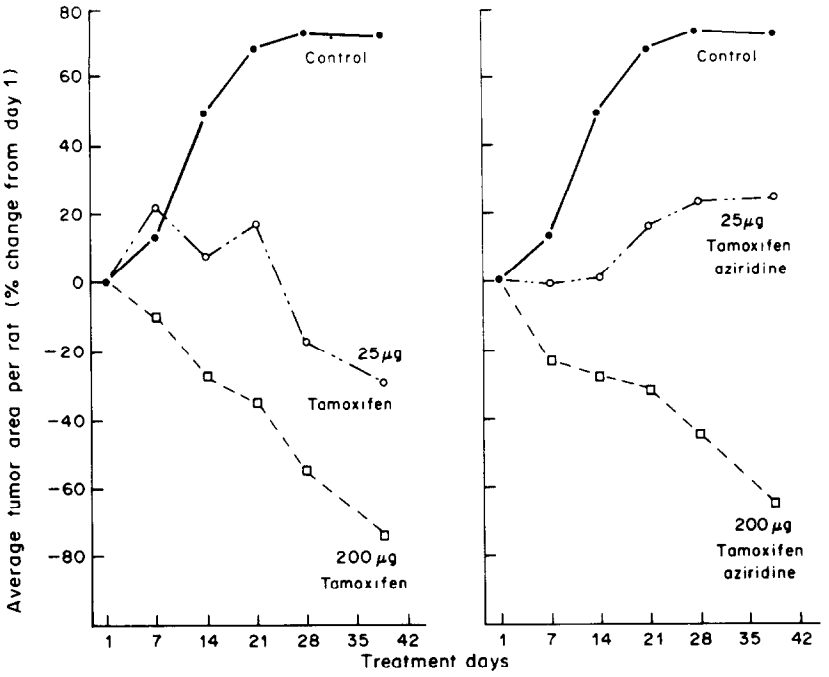

Fig. 2. The effect of tamoxifen or tamoxifen aziridine on the growth of DMBA-induced rat mammary tumors. Virgin female Sprague–Dawley rats received DMBA at 47–50 days of age. Approximately 60 days after DMBA injection, rats received subcutaneous implants of Alza osmotic pumps containing tamoxifen, tamoxifen aziridine, or vehicle. Pumps were changed every 8 days. Data are expressed as percent change from day 1 of treatment. Average tumor area/rat at the initial time of treatment was the following for each of the groups. Control, 3.04 cm^2/rat; 25 μg TAM, 4.14; 200 μg TAM, 3.67; 25 μg TA, 5.45; 200 μg TA, 4.71.

the 200 μg/day doses of TAM and TA were considerably more effective than the 25 μg/day doses.

Examination of uterine weights from these animals (Fig. 3) demonstrated that 200 μg TAM or TA and 25 μg TAM depressed uterine weights significantly from control using Duncan's test ($P < 0.05$). Animals treated with 200 μg TAM or TA showed uterine weights significantly lower than those treated with 25 μg TAM or TA, indicating a dose–response effect ($P < 0.05$). At a given dose, there was no significant difference in the uterine weights from animals treated with the two compounds.

Effects of tamoxifen aziridine and tamoxifen on the proliferation of MCF-7 cells

Tamoxifen and tamoxifen aziridine both inhibited the proliferation of MCF-7 cells (Fig. 4). As shown in this Figure, 10^{-6} M concentrations of Tam or TA markedly suppressed cell proliferation. Although TA appeared to be slightly more effective than Tam in this experiment, in two other repeat growth studies, TA and Tam appeared to be equally effective growth inhibitors.

Effects of tamoxifen aziridine on plasminogen activator activity of MCF-7 cells

Plasminogen activator activity is known to be markedly stimulated by low concentrations of estradiol in MCF-7 cells [9, 10, 13]. Estradiol, at 10^{-9} M gave marked stimulation of plasminogen activator activity while a 4-day exposure of the cells to tamoxifen aziridine alone did not enhance or

Table 1. Effect of tamoxifen vs tamoxifen aziridine on the growth of DMBA-induced mammary tumors

Treatment	No. of rats/group	Response after 38 days treatment§					Avg. tumor area/rat (cm^2/rat)‖		
		Grow	Stable	Regress	Disappear	Regress + disappear	Before	After	After / Before (%)
Control	6	5/8* (63)†	1/8 (13)	2/8 (25)	0/8 (0)	25‡	3.04	5.22	172
25 μg TAM	8	2/15 (13)	5/15 (33)	4/15 (27)	4/15 (27)	53	4.14	2.96	71
200 μg TAM	7	0/11 (0)	2/11 (18)	6/11 (55)	3/11 (27)	82	3.67	0.97	26
25 μg TA	7	2/11 (18)	3/11 (27)	5/11 (45)	1/11 (9)	54	5.45	6.78	124
200 μg TA	8	2/16 (13)	4/16 (25)	5/16 (31)	5/16 (31)	63	4.71	1.64	35

*Rats were given injections of DMBA at 45–50 days of age. Animals bearing tumors with a minimum size of 0.6 cm in each dimension (length and width) received Alzet osmotic pumps with tamoxifen, tamoxifen aziridine, or vehicle every eight days and followed for 38 days.

†Number in parentheses, percentage of tumors showing each response.

‡Combined percentage of tumors showing regression or disappearance.

§Based on tumors > 0.36 cm^2 on day 7 of treatment.

‖Based on tumors > 0.36 cm^2 on day 1 of treatment.

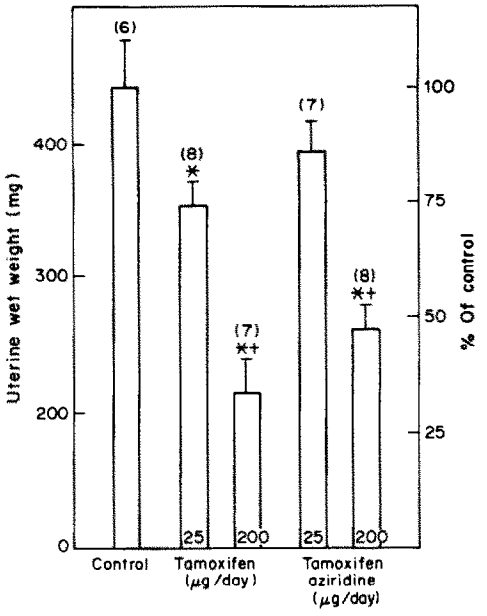

Fig. 3. Uterine weights in female tumor-bearing rats treated with the indicated doses of TAM/TA for 38 days. Uteri were excised at 12 h following the last day of treatment and weighed. The number of rats in each treatment group is indicated in parentheses. Values represent the mean + SEM. $^{*}P < 0.05$ vs control, and $+P < 0.05$ vs 25 μg TAM/TA.

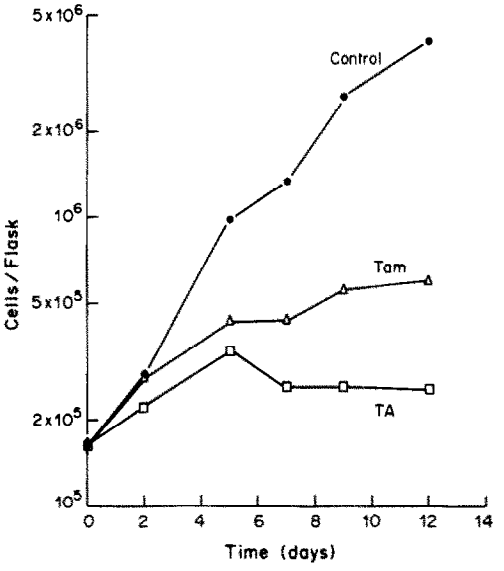

Fig. 4. Effect of tamoxifen and tamoxifen aziridine on the growth of MCF-7 cells. Cells were grown in T-25 flasks in the presence of 1×10^{-6} M tamoxifen or tamoxifen aziridine with media and compound changed daily. Duplicate flasks of cells were counted at each time point.

suppress the plasminogen activator activity of control cells (Fig. 5). However, when administered with 10^{-9} M E_2, tamoxifen aziridine (at 10^{-7} M and 10^{-6} M) acted as a potent antiestrogen, suppressing plasminogen activator activity to 10–25% of the activity evoked by 10^{-9} M estradiol (Fig. 5). Tamoxifen alone also does not stimulate plasminogen activator activity of these cells, and it was found to effectively suppress the plasminogen activator activity evoked by estradiol, as reported previously [13].

DISCUSSION

Our results demonstrate that irreversible binding of tamoxifen aziridine (TA) to the estrogen receptor neither eliminates the biological activity of the receptor complex nor enhances the antiestrogenic properties of the complex. Thus, the bioactivity of tamoxifen aziridine was similar to that of tamoxifen, the reversibly binding parent compound in the several systems evaluated. Thus, in the immature rat uterus, tamoxifen aziridine displayed weak estrogen agonist activity as well as antiestrogenic activity, as does tamoxifen, while in mature rats it evoked uterine weight reduction and mammary tumor regression; likewise it suppressed cell proliferation and behaved as an antiestrogen in terms of plasminogen activator activity in MCF-7 breast cancer cells in culture, as does tamoxifen [13].

While tamoxifen and tamoxifen aziridine showed similar potencies *in vitro*, tamoxifen aziridine was less potent than tamoxifen *in vivo*. This may reflect a more rapid clearance of tamoxifen aziridine *in vivo* or may be due to metabolism of tamoxifen aziridine to a form or forms which are less active or more rapidly cleared than tamoxifen or its metabolic products. In view of our only limited amounts of tritiated-TA and the difficulties in analyzing metabolites that are irreversibly bound to proteins, we have not analyzed the metabolic fate of TA *in vivo*.

Following injection of [^{3}H]tamoxifen into immature female rats, tamoxifen is converted to several polar metabolites. Hydroxytamoxifen has been identified as the first formed and least polar of these

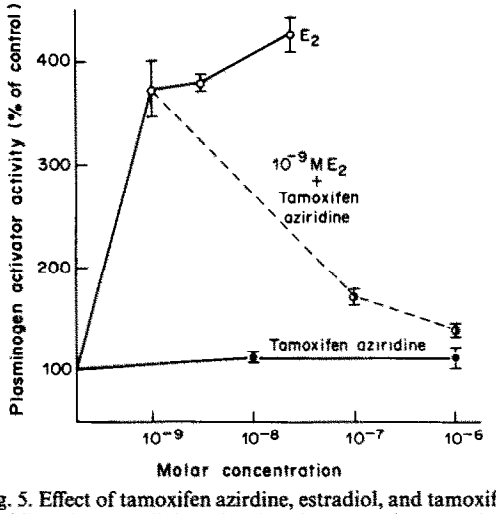

Fig. 5. Effect of tamoxifen azirdine, estradiol, and tamoxifen aziridine plus estradiol on the plasminogen activator activity of MCF-7 cells. Cells were incubated with the indicated concentration of compound for 4 days, with fresh media and compounds renewed every 48 h. Cells were then harvested, distributed into microwells, and assayed for plasminogen activator activity as described in the Experimental section. Values represent the mean ± SE of triplicate determinations.

metabolites [21–22]. The nature of the other metabolites in rats has not yet been established definitively. *N*-desmethyltamoxifen and 4-hydroxytamoxifen are the two major metabolites formed in humans following chronic administration of tamoxifen (20 mg/day). The predominant constituent in plasma is *N*-desmethyltamoxifen which is present at concentrations approximately twice that of tamoxifen [23]. 4-Hydroxytamoxifen is present at concentrations only about 2.5% of tamoxifen [24]. Determinations of the relative binding affinities of these three forms for receptor indicate that the affinities of *N*-desmethyltamoxifen and tamoxifen are comparable (*ca* 2–3% that of estradiol) while that of 4-hydroxytamoxifen is 50–100 times greater [13, 21, 25–27]. Hence, while these forms may all contribute to the activity of TAM *in vivo*, of interest is the observation that 4-hydroxytamoxifen is a less potent antitumor agent compared to TAM *in vivo*, possibly reflecting a more rapid clearance and/or metabolism [28]. Whether tamoxifen aziridine is metabolized in a similar manner *in vivo* has yet to be investigated.

MCF-7 human breast cancer cells are a useful *in vitro* system for examining the activity of antiestrogens in target cells. Moreover, the MCF-7 cells do not metabolize tamoxifen and hydroxytamoxifen [11–13, 27], and thus it seems likely that tamoxifen aziridine would not be extensively metabolized by these cells. In addition, *in situ* labeling of estrogen receptor in MCF-7 cells using [^{3}H]tamoxifen aziridine followed by SDS polyacrylamide gel electrophoresis of the salt extractable nuclear estrogen receptor verifies that tamoxifen aziridine binds irreversibly to the receptor [17] in intact cells. It is also unlikely that the biological activity reported here is due to the interaction between tamoxifen aziridine and the microsomal antiestrogen binding sites [8, 29–30]. Tamoxifen aziridine has low affinity for these antiestrogen binding sites (8% that of tamoxifen) and binds reversibly, rather than irreversibly, to these sites [17].

The biological activity of another compound, 11β-chloromethylestradiol, which binds in an apparently irreversible fashion to the estrogen receptor has been reported recently [31]. Interestingly, this compound acts as a potent estrogen. It stimulates cell proliferation, progesterone receptor content and plasminogen activator activity of MCF-7 cells and it was at least as potent as estradiol by the criteria examined.

Related studies on the bioactivity of dexamethasone-21-mesylate, an electrophilic affinity labeling agent for the glucocorticoid receptor have shown that this compound behaves as a partial agonist as well as an antagonist of glucocorticoid action in HTC hepatoma cells *in vitro* [32] and as a long-acting glucocorticoid with little anti-glucocorticoid activity in Syrian hamster melanoma cells [33]. Hence while the biological character of dexamethasone mesylate appears to differ in these hepatoma and melanoma cells, based upon the endpoints of bioactivity that have been examined, the receptor–ligand complexes display significant biological activity.

The studies we report here clearly show that covalent attachment of a ligand to the estrogen receptor does not substantially alter the biological activity of the receptor, so that in the case of tamoxifen aziridine, its binding does not enhance nor markedly diminish the antiestrogenic activity compared to the reversibly binding parent compound, tamoxifen.

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