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Activity of the Antioestrogen Trioxifene Against *N*-Nitrosomethylurea-induced Rat Mammary Carcinomas*

DAVID P. ROSE,† ANDREW H. FISCHER and V. CRAIG JORDAN

Division of Clinical Oncology, Wisconsin Clinical Cancer Center, University of Wisconsin, Madison, Wisconsin 53792, *U.S.A.*

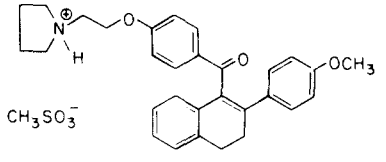
Abstract—*The therapeutic efficacy of a new antioestrogen, trioxifene, was compared with that of tamoxifen and ovariectomy against rat mammary tumours induced by* N*-nitrosomethylurea. In control animals, all but* 1/59 *tumours either progressed* (52) *or remained static* (6) *and* 31 *new tumours appeared. All* 3 *modes of therapy were highly effective. Trioxifene mesylate,* (300 μg *s.c. daily) induced regressions in* 26/61 (42.6%) *tumours, and stasis in* 13 (21.3%). *Nine new tumours appeared during trioxifene treatment. Tamoxifen* (200 μg *s.c. daily), produced regression of* 22/50 *tumours* (44.0%), *while* 19 (38.0%) *remained static and* 2 *new tumours developed. Ovariectomy caused regression of* 35/56 (62.5%) *tumours, growth was inhibited in another* 8 (14.3%) *and* 2 *new tumours developed. Regression after ovariectomy was greater compared with trioxifene therapy* ($P < 0.04$), *but no different for ovariectomy and tamoxifen, or tamoxifen and trioxifene.*

The relative binding affinity (*RBA*, $OE_2 \equiv 100$) *of trioxifene* (8.8–11) *for NMU-induced mammary tumour oestrogen receptors was consistently* 4–5× *greater than that of tamoxifen. The comparative study* in vitro *did not parallel the finding* in vivo. *However, the RBA of monohydroxytamoxifen, a metabolite of tamoxifen, was the same or* 2× *greater than that of oestradiol, suggesting that tamoxifen's action may be facilitated by conversion to active metabolites. Further study of trioxifene's metabolites may increase our understanding of its mechanism of action* in vivo.

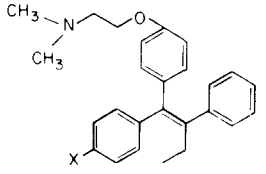
INTRODUCTION

THE SUCCESSFUL introduction of the non-steroidal antioestrogen tamoxifen [1] for the treatment of advanced breast cancer [2] has stimulated interest in the development of structurally related drugs. One such compound, trioxifene (Fig. 1) is antioestrogenic in immature rats and mice [3, 4], and preliminary studies have been completed to evaluate the toxicity, effects on the endocrine system, and efficacy in patients with breast cancer [5].

While clinical trials are being expanded, no studies have been published concerning the antitumour properties of trioxifene under laboratory conditions. This information would not only provide data of value in planning future clinical studies, but also establish model systems to evaluate trioxifene as a potential research tool.



TRIOXIFENE MESYLATE



TAMOXIFEN X = H
MONOHYDROXYTAMOXIFEN X = OH

Fig. 1. *Structural formulae of antioestrogens used in the study.*

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†To whom requests for reprints should be sent.

The majority of rat mammary carcinomas induced by *N*-nitrosomethylurea (NMU) are hormone dependent, and regress after

ovariectomy or treatment with tamoxifen [6, 7]. These tumours offer some advantages over the more widely used DMBA-induced rat mammary tumour model. Virtually all of them are carcinomas, spontaneous regressions are uncommon, they are locally invasive, and may metastasize to distant sites [6, 7].

This report describes the ability of trioxifene to cause regression of NMU-induced mammary carcinomas, and compares the efficacy of this compound with ovariectomy and tamoxifen therapy. In addition, the inhibition of oestradiol binding to the oestrogen receptor of these tumours *in vitro* is discussed.

MATERIALS AND METHODS

Trioxifene mesylate was a gift from Eli Lilly and Co., Indianapolis, Indiana; tamoxifen and monohydroxytamoxifen were gifts from Stuart Pharmaceuticals, Division of ICI Americas, Inc., Wilmington, Delaware. NMU was purchased from ICN Pharmaceuticals Inc., Plainview, New York, oestradiol-17β from Sigma Chemicals Co., St. Louis, Missouri, and diethylstilboestrol from Nutritional Biochemicals Corp., Cleveland, Ohio. [6, 7^{3}H] Oestradiol-17β (60 Ci/mmol) was obtained >96% pure from the Radiochemical Centre, Amersham, Buckinghamshire, England, and was used without further purification. TEM buffer (60 mM Tris; 1.5 mM EDTA; 0.5 mM α monothioglycerol, pH 7.4) was used for studies *in vitro*. Dextran-coated charcoal was prepared for use by diluting a 2.5% charcoal and 0.25% dextran T70 suspension in 100 mM Na phosphate buffer (pH 7.4) obtained from Wein Laboratories, Inc., Succasunna, New Jersey 1:10 with TEM buffer.

Mammary tumour induction and endocrine therapies

Carcinomas were induced in female Sprague–Dawley rats (King Laboratories, Oregon, Wisconsin) by administering NMU in 3 i.v. injections of 5 mg/100 g body weight at 4 weekly intervals, the first being given when the animals were 50–55 days old [6, 7]. Tumour size was measured at weekly intervals with a caliper, and the surface area calculated as previously described [7].

Animals were allocated to 1 of 4 groups, which were matched as closely as possible for average tumour size, and duration of the latent period. Treatment was commenced when at least 1 tumour had a maximum diameter of approximately 2.0 cm. The 10 rats, with a total of 28 tumours, in the control group were given daily s.c. injections of 0.2 ml corn oil. Fourteen rats, initially bearing 54 tumours, were subjected to bilateral ovariectomy. Fourteen rats, with a total of 48 tumours, received tamoxifen, and 14, bearing 52 tumours, were treated with trioxifene. Tamoxifen was administered s.c. in a dose of 200 μg/day dissolved in 0.2 ml of corn oil. Trioxifene was given as the mesylate in a daily dose of 300 μg (equivalent to 247 μg of the free base) by s.c. injection. Because of poor solubility the drug had to be given as a suspension in corn oil. The amount of free base equivalent was therefore increased by approximately 25% relative to the dose of tamoxifen.

Administration of the antioestrogens, and evaluation of responses to endocrine therapy, were continued for 4 weeks. Tumour responses were classified as: regression, a decrease in surface area of 50% or more; progression, an increase of 50% or more; or static.

Inhibition of [^{3}H] *oestradiol binding* in vitro

Tissue from an NMU-induced tumour was rinsed in ice-cold TEM buffer, and homogenized in the same buffer (1:10 w/v) using 3 × 5 sec bursts of a Polytron PT10-35 tissue homogenizer (Brinkmann Instruments, Inc., Westbury, New York) with 30 sec cooling intervals in an ice/water mixture. Homogenates were centrifuged at 120,000 ***g*** for 1 hr to obtain the cytosol. Incubation mixtures were set up by adding 480 μl of cytosol to each assay tube in an ice/water bath. Ethanol (20 μl) containing [^{3}H] oestradiol to give a final concentration of 3.7 nM was added, alone or with a competitor, to each tube. The ranges of the concentrations of the competitors in the incubation mixture were: oestradiol and monohydroxytamoxifen, 3.3×10^{-10} to 1.0×10^{-7} M; tamoxifen, 3.3×10^{-8} to 1.0×10^{-5} M; trioxifene, 3.3×10^{-9} to 1.0×10^{-6} M. All tubes were set up in duplicate, together with a set of tubes containing [^{3}H] oestradiol and 1×10^{-5} M diethylstilboestrol to determine non-specific binding of oestradiol.

Three incubation conditions were used for each experiment: 30° for 30 min, 20° for 4 hr, and 0–4°C for 22 hr. At the end of the incubation period the tubes were allowed to cool for 5 min in ice-water, and 1 ml of dextran-coated charcoal suspension was added. Incubation was continued at 0–4°C for 20 min with occasional shaking. The tubes were then centrifuged at 1500 ***g*** for 5 min, and the supernatants decanted into 10 ml of ACS scintillant (Amersham Corp., Arlington Heights, Illinois) and the radioactivity counted for 10 min in a Tricor Analytic Mark III liquid scintillation spec-

trometer. Results were converted to dpm by an automatic standardization technique. The inhibition curves for each competitor were plotted, and the RBAs calculated from the following relationship.

$$\text{RBA} = 100 \times \frac{\text{molar concentration of oestradiol for 50\% inhibition of specific binding}}{\text{molar concentration of competitor for 50\% inhibition of specific binding}}.$$

RESULTS

Tumour responses to endocrine therapies

The responses of tumours in the control and three treatment groups are summarized in Table 1. The posttreatment surface area refers to the measurement after 4 weeks of observation, or the last measurement before death. There were no significant differences by the Krustal–Wallis test between the surface areas of the controls and the animals allocated to the treatment groups before starting therapy. The mean total tumour area of the controls increased approximately 4-fold during the 4 week observation period (Wilcoxon paired rank test, $P < 0.01$). Table 2 shows that all but one of 59 tumours either progressed, or remained static, and 31 new tumours (average 3.1/rat) developed in the untreated group. Three rats died during the 4 week study.

In the ovariectomized group, there was no significant difference in the mean total surface areas before and after 4 weeks of evaluation, but the posttreatment difference from the controls was highly significant ($P < 0.001$). Regressions occurred in 35 of 56 tumours (62.5%), while 8 remained static and only 2 new tumours (average 0.1/rat) appeared after castration (Table 2). There were no deaths in this treatment group.

Tamoxifen and trioxifene were both effective in preventing tumour progressions (Table 1 and 2). Regressions occurred in 22 of 50 tumours (44.0%) treated with tamoxifen, and 19 (38.0%) remained static. Only 2 new tumours

Table 1. The mean tumour surface areas (±SD) of controls, and before and after treatment with ovariectomy, tamoxifen or trioxifene

Group	Mean tumour area (cm^2)		Body weight (g)	
	Pretreatment	Posttreatment†	Pretreatment	Posttreatment
Controls (10)*	5.88 ± 3.26	21.99 ± 8.81	224 ± 12	243 ± 41
Ovariectomy (14)	7.26 ± 4.37	5.33 ± 5.16‡	231 ± 15	275 ± 15‖
Tamoxifen¶ (14)	6.61 ± 2.69	6.58 ± 3.80‡	238 ± 14‖	248 ± 17
Trioxifene** (14)	6.66 ± 3.30	9.28 ± 9.21§	228 ± 14	238 ± 14

*Numbers in parentheses, number of animals in each group.
†The observation/treatment period was of 4 weeks duration, or until death.
‡Significantly different from the control group, $P < 0.001$.
§Significantly different from the control group, $P < 0.003$.
‖Significantly different from the control group, $P < 0.02$.
¶200 μg s.c. once daily.
**300 μg of the mesylate s.c. once daily.

Table 2. Responses of individual tumours and deaths in controls and rats treated with overiectomy, tamoxifen or trioxifene

Group	Initial tumours	New tumours	Regression	Static	Progression	Died*
Controls	28	31	1	6	52	3
Ovariectomy	54	2	35†	8	13	0
Tamoxifen	48	2	22†	19	9	0
Trioxifene	52	9	26†‡	13	22	2

*All deaths occurred at least two weeks after commencing treatment.
†Significantly different from the control group, $P < 0.0001$ (Fisher's exact test).
‡Significantly different from the ovariectomized group, $P < 0.04$ (χ^2 test).

(average 0.1/rat) developed during tamoxifen therapy, and there were no deaths. Trioxifene induced regressions in 26 of 61 tumours (42.6%), and inhibited further growth in 13 (21.3%) others. Nine new tumours appeared (average 0.6/rat), and 2 animals died.

The Kruskal–Wallis test was applied to the data to provide a comparison between more than 2 groups. The difference in overall growth rates, based on the changes in total tumour areas, between the controls and the three treatment groups was highly significant ($P < 0.001$). There was no significant difference in the extent of the responses between the 3 modes of therapy, as judged by the tumour total surface areas, although the trend was for ovariectomy to be more effective. Similarly, there was only a marginal difference in the frequency of regressions between the ovariectomy and trioxifene-treated groups ($P < 0.04$), and none between castration and tamoxifen, or tamoxifen and trioxifene (Table 2).

[^{3}H] *Oestradiol binding* in vitro

In preliminary experiments (not shown) each of the incubation conditions was found to produce equilibrium binding of [^{3}H] oestradiol. For each of the incubation conditions, oestradiol and the antioestrogens inhibited the binding of [^{3}H] oestradiol in the cytosol in a concentration-related manner. The data obtained using 20°C for 4 hr are shown in Fig. 2. Trioxifene was 4 to 5 times more active than tamoxifen, and each produced a consistent RBA regardless of the procedure used (Table 3). Similarly, oestradiol produced 50% inhibition of [^{3}H] oestradiol binding at 3–4 nM in each assay. In contrast, monohydroxytamoxifen has a consistently higher RBA than oestradiol at 20 and 30°C.

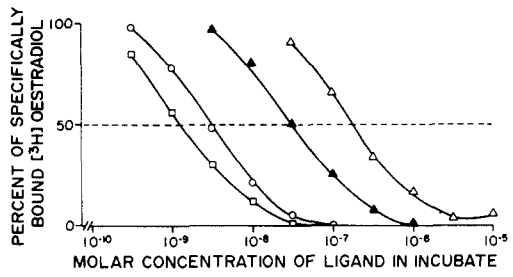

Fig. 2. Effect of increasing concentrations of monohydroxytamoxifen (□), oestradiol (○), trioxifene (▲) and tamoxifen (△) on the specific binding of [^{3}H] oestradiol (3.7 nM) in NMU-induced rat mammary tumour cytosol. Duplicate incubations were at 20°C for 4 hr. The concentration of unlabelled oestradiol to give 50% inhibition was 3.9 nM. Specific [^{3}H] oestradiol binding (100%) in control was 13,631 dpm. For further details see Materials and Methods.

DISCUSSION

Trioxifene, in a dose of 247 μg free base daily, produced regression, or inhibited further growth, in the majority of NMU-induced rat mammary tumours. Although ovariectomy appeared to be somewhat more effective, no difference was found in the efficacy of trioxifene and tamoxifen at the doses employed. These results do not, of course, exclude the possibility that differences in antitumour activity of the two antioestrogens might emerge using other dosage schedules, but they are consistent with the similar oestrogenic potencies of tamoxifen and trioxifen in the ovariectomized mouse [4].

Inhibition of [^{3}H] oestradiol binding to oestrogen receptors derived from NMU-induced tumours by tamoxifen and trioxifene was used to quantify the relative activities of the antioestrogens. The use of this assay does not imply a belief that antioestrogens only have a direct antitumour action via the oestrogen receptor. In the DMBA-induced rat mammary carcinoma model, antioestrogens interact with the tumour oestrogen receptor system *in vivo* [8, 9] and *in vitro* [10], but overall the antitumour effects are probably facilitated by interaction with oestrogen receptor systems in other sites,

Table 3. Binding affinities of monohydroxytamoxifen, trioxifene, and tamoxifen relative to oestradiol-17β (100) data calculated for the 50% inhibition of the specific binding of [^{3}H] oestradiol-17β (3.7 nM) in an NMU-induced rat mammary tumour cytosol under various incubation conditions

Compound	Incubation conditions		
	30°C, 0.5 hr.	20°C, 4 hr.	4°C, 22 hr.
Monohydroxytamoxifen	238	230	117
Trioxifene	11	8.8	9.5
Tamoxifen	2.1	1.7	2.3

for example the hypothalamo-pituitary axis [11]. Similarly, in the NMU-induced tumours direct and indirect mechanisms may be involved in antioestrogen-mediated regressions. Nevertheless, trioxifene was consistently 4 to 5 times more active than tamoxifen *in vitro*; a result at variance with the findings in *vivo*.

It has been suggested that some antioestrogens are pro-drugs [12], being converted to polar metabolites before binding in oestrogen target tissues [13]. In the rat tamoxifen is metabolized to monohydroxytamoxifen (Fig. 1), a compound with a high affinity for the oestrogen receptor (Fig. 2), potent antioestrogenic activity [14], and antitumour activity in the DMBA-induced rat mammary carcinoma model [15]. [^{3}H] Monohydroxytamoxifen has been isolated from rat uterine nuclei after the administration of [^{3}H] tamoxifen [16], and the antioestrogenic activity of tamoxifen *in vivo* has been suggested to be the result of the combined effects of the parent compound and its metabolites [17]. The antitumour effects of tamoxifen in the rat probably also represent the combined activities of the parent compound, and its major non-conjugated metabolite monohydroxytamoxifen.

There is no information concerning the metabolism of trioxifene in animals or man. A precise study of the pharmacological properties of trioxifene metabolities is clearly mandatory in order to understand the mechanism of action of the drug in animal and human cancers.

REFERENCES

1. HARPER MJK, WALPOLE AL. A new derivative of triphenylethylene: Effect on implantation and mode of action in rats. *J Reprod Fertil* 1967; **13**: 101–119.
2. HEEL RC, BROGDEN RN, SPEIGHT RM, AVERY GS. Tamoxifen: A review of its pharmacological properties and therapeutic use in the treatment of breast cancer. *Drugs* 1978; **16**: 1–24.
3. JONES CD, SUAREZ T, MASSEY EH, BLACK LJ, TINSLEY J. Synthesis and antiestrogenic activity of [3,4-dihydro-2-(4-methoxyphenyl)-1-naphthalenyl](4-(1-pyrrolidinyl) ethoxyphenyl methanone, methanesulfonic acid salt. *J Med Chem* 1979; **22**: 962–966.
4. BLACK LJ, GOODE RL. Uterine bioassay of tamoxifen, trioxifene and a new estrogen antagonist (LY117018) in rats and mice. *Life Sci* 1980; **26**: 1453–1458.
5. MANNI A, ARAFAH B, PEARSON OH. Changes in endocrine status following antioestrogen administration to pre- and post-menopausal women. In SUTHERLAND RL, JORDAN VC, eds. *Non-Steroidal Antioestrogens.* Sydney: Academic Press, in press.
6. GUILLINO PM, PETTIGREW HM, GRANTHAM FH. *N*-Nitrosomethylurea as a mammary gland carcinogen in rats. *J. Natl Cancer Inst* 1975; **54**: 401–414.
7. ROSE DP, PRUITT B, STAUBER P, ERTÜRKE, BRYAN GT. Influence of dosage schedule on the biological characteristics of *N*-nitrosomethylurea-induced rat mammary tumors. *Cancer Res* 1980; **40**: 235–239.
8. JORDAN VC, DOWSE LJ. Tamoxifen as an antitumour agent: Effect on oestrogen binding. *J Endocrinol* 1976; **68**: 297–303.
9. NICHOLSON RI, GOLDER MP, DAVIS P, GRIFFITHS K. Effects of oestradiol-17β and tamoxifen on total and accessible cytoplasmic oestradiol-17β receptors in DMBA-induced rat mammary tumours. *Eur J Cancer* 1976; **12**: 711–717.
10. TERENIUS L. Antioestrogens and breast cancer. *Eur J Cancer* 1971; **7**: 57–64.
11. JORDAN VC, ALLEN KE, DIX CJ. The pharmacology of tamoxifen in laboratory animals. *Cancer Treat Rep* 1980; **64**: 745–759.
12. FERGUSON ER, KATZENELLENBOGEN BS. A comparative study of antiestrogen action: Temporal patterns of antagonism of estrogen stimulated uterine growth and effects on estrogen receptor levels. *Endocrinology* 1977; **100**: 1242–1251.
13. KATZENELLENBOGEN BS, KATZENELLENBOGEN JA, FERGUSON ER, KRANTHAMMER N. Antiestrogen interaction with uterine estrogen receptors. *J Biol Chem* 1978; **253**: 697–707.
14. JORDAN VC, COLLINS MM, ROWSBY L, PRESTWICH G. A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. *J Endocr* 1977; **75**: 305–316.
15. JORDAN VC, ALLEN KE. Evaluation of the antitumour activity of the non-steroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. *Eur J Cancer* 1980; **16**: 239–251.

16. BORGNA JL, ROCHEFORT H. Occupation *in vivo* des recepteurs oestrogène par des métabolites hydroxylés du tamoxifène. *C.R. Acad Sci* 1979; **289**: 114–117.
17. ALLEN KE, CLARK ET, JORDAN VC. Evidence for the metabolic activation of non-steroidal antiestrogens: a study of structure activity relationships. *Br J Pharmacol* 1980; **71**: 83–91.