Eur J Cancer Clin Oncol, Vol. 24, No. 12, pp. 1817–1821, 1988.
Printed in Great Britain

0277-5379/88$3.00 + 0.00

Antitumor Actions of Toremifene in the 7,12-Dimethylbenzanthracene (DMBA)-induced Rat Mammary Tumor Model*

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Abstract—*Toremifene* (200 *and* 800 μg/*day*) *or tamoxifen* (200 μg) *were effective in preventing the development of* 7,12-*dimethylbenzanthracene-induced rat mammary tumors when given p.o. from day* 28 *after carcinogen administration. This antitumor action was completely reversed if the toremifene or tamoxifen treatment was stopped or partially reversed by coadministration of progesterone* (4 mg/*day*). *Large doses of toremifene* (4000 *and* 8000 μg/*day*) *for* 10 *days produced very high circulating levels of both the parent compound* (282 ± 49 *and* 1002 ± 224 ng/ml, *respectively*) *and* N-*desmethyltoremifene* (2631 ± 449 *and* 6999 ± 1308 ng/ml, *respectively*). *However, even these very high levels did not reduce the number of animals ultimately developing tumors or the number of tumors each animal developed following cessation of toremifene administration. These findings indicate toremifene has a tumoristatic rather than tumoricidal action in this tumor model.*

INTRODUCTION

THE ANTIESTROGEN, tamoxifen, is currently the endocrine therapy of choice for hormone-dependent breast cancer in the postmenopausal woman. However, tamoxifen has been shown, in a variety of models, to have a tumoristatic rather than a tumoricidal action and cessation of therapy results in renewed tumor development [1, 2]. Based on these findings, continuous adjuvant tamoxifen therapy has been suggested to be the best strategy for clinical evaluation [3]. The validity of this approach has been supported by results from recent clinical trials [4–6].

Toremifene (Fc-1157a) is a new antiestrogen to treat breast cancer. It competes with estrogen binding to the estrogen receptor [7] and inhibits the growth of established DMBA-induced rat mammary tumors [8]. Interestingly, toremifene has also been reported to slow the progression of an estrogen-receptor negative tumor (murine uterine sarcoma) when administered in massive doses (100 and 200 mg/kg/day) for 5 days [8] and be cytolytic to MCF-7 cells at 5×10^{-6} M in tissue culture [8]. This suggests it may be possible to produce a tumoricidal action and influence hormone-independent tumor cells with toremifene treatment although high doses may be needed. Toremifene is presently being used in clinical trials at higher doses (60 mg/day) [9] than are usually recommended for tamoxifen (20 or 40 mg/day) [10]. Whether this will provide a greater therapeutic benefit is yet to be established. The present study assesses the effectiveness of toremifene treatment and the action of high dose therapy in preventing the development of mammary tumors in the DMBA-treated Sprague–Dawley rat.

MATERIALS AND METHODS

DMBA, progesterone and tamoxifen were obtained from the Sigma Chemical Company (St. Louis, MO). Toremifene and metabolite standards were a gift from Adria Laboratories (Columbus, OH). Progesterone solution was prepared by adding a small volume of ethanol to crystalline material and then stirring with peanut oil. The ethanol (3–5%) was required in the progesterone solution to prevent crytallization. Toremifene and tamoxifen solutions were prepared by mixing with peanut oil. At high concentrations a crystalline suspension was used by vigorously mixing before administration. Toremifene and tamoxifen was administered orally (0.2 ml) and progesterone by s.c. injection (0.1 ml)

Accepted 4 July 1988.
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*These studies supported by Adria Laboratories, Columbus, Ohio.

into the loose fold of skin on the back of the neck. DMBA, dissolved in peanut oil by stirring overnight, was administered (20 mg in 2 ml) by gavage to 50-day-old Sprague–Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) as previously described [11].

Antitumor activity of moderate dose toremifene and tamoxifen treatment

Twenty-eight days after DMBA administration, rats were randomized into groups of 20 animals and treated daily with (a) peanut oil; (b) 50 μg toremifene; (c) 200 μg toremifene; (d) 800 μg toremifene; (e) 200 μg toremifene + 4 mg progesterone; (f) 200 μg tamoxifen; (g) 200 μg tamoxifen + 4 mg progesterone. After 4 months of treatment, groups (a), (b), (e) and (g) were sacrificed. Treatment was stopped in groups (c), (d) and (f) at this time and the subsequent tumor occurrence was recorded. Tumors were detected by palpation at regular intervals. Student's *t*-tests were used to compare the tumor burden between groups.

Antitumor activity of high dose toremifene treatment

Twenty-eight days after DMBA administration rats were randomized into groups of 24 animals and treated daily for 10 days with (a) peanut oil; (b) 800 μg toremifene; (c) 4000 μg toremifene; and (d) 8000 μg toremifene. Blood samples were taken from five representative animals from each group approx. 24 h after the last dose by light ether anesthesia and bleeding from the eye orbit. These animals all recovered and continued in their respective groups. Animals were weighed and palpated to detect tumors at weekly intervals for the duration of the experiment.

Toremifene analysis

Blood samples were allowed to clot overnight at 4°C and centrifuged at 2000 ***g*** to prepare serum. Samples were stored at −20°C until analyzed.

Serum samples (300 μl) were spiked with 100 ng of enclomiphene (Merrel Dow, Cincinnati, OH) in 10 μl ethanol and extracted three times with 1 ml of hexane:amyl alcohol (98:2) by vigorous mixing for 5 min. The aqueous layer was frozen in an acetone/dry ice mixture before removal of the organic extract. The three extracts were combined by evaporating to dryness in a conical 1.1 ml capacity vial (Rainin Instrument Co., Woburn, MA). Extracts were subsequently resuspended in 100 μl of mobile phase (see below) and analyzed by injection (Gilson Model 231 autosampler; Gilson Medical Electronics, Inc., Middleton, WI) into a Gilson Series 4000 chromatograph and metabolites separated on a Silica, 5 μm particle size, column (100 × 4 mm, Scientific Glass Engineering, Austin, TX) using an iso-octane:ethanol:isopropanol:diethylamine:acetic acid (75:23.5:1.5:0.05:0.05) mobile phase at a 1 ml/min flow rate. Toremifene and metabolites underwent post-column on line u.v. activation from the triphenylethylene to the fluorescent phenanthrene derivative as previously described for tamoxifen [12]. Compounds were detected with a Shimadzu 351 fluorimeter (excitation 257 nm and re-emission 383 nm). Quantitation was performed using pure metabolite standards spiked into control rat serum and extracted as described. Serum concentrations were calculated from the standard curves using the enclomiphene internal standard to correct for loss.

RESULTS

The administration of 20 mg DMBA to 50-day-old Sprague–Dawley rats resulted in palpable mammary tumors starting to appear after 30 days. By 100 days post carcinogen, approx. 75% of control animals had developed tumors. Many of these animals had more than one tumor and several had to be sacrificed before the end of the treatment period because of very large tumors. The daily oral administration of 50 μg of toremifene was insufficient to significantly reduce the number of animals developing tumors or the number of tumors per animal. However, 200 μg and 800 μg of toremifene per day or 200 μg of tamoxifen per day were all very effective doses with less than 20% of the animals developing any tumors and only single tumors occuring on these animals during the treatment period (see Fig. 1).

Cessation of toremifene or tamoxifen after 4 months of treatment resulted in a loss of the protective antitumor action and the gradual development of tumors over the next 4 1/2 months. By the end of this period, about 70% of rats in the 200 and 800 μg toremifene-treated groups and the 200 μg tamoxifen treated group had developed tumors and a similar number of tumors were found per group as detected in the control group 100 days after DMBA (see Fig. 1). The finding that neither toremifene (200 or 800 μg/day) nor tamoxifen (200 μg/day) treatment for 4 months was capable of reducing the number of rats ultimately developing tumors or the number of tumors per rat suggests a tumoristatic rather than a tumoricidal action for these agents.

The antitumor action produced by the daily administration of 200 μg toremifene was partially reversible by the simultaneous administration of 4 mg progesterone. The coadministration of progesterone for 4 months results in 15% more rats developing tumors and a significantly ($P < 0.05$) higher mean tumor burden per rat than the toremifene-alone group. A similar result was observed when progesterone was combined with tamoxifen (200 μg/day) treatment (see Fig. 2).

The use of very large doses of toremifene were

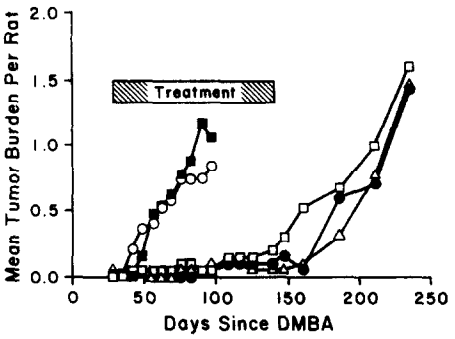

Fig. 1. Effect of toremifene and tamoxifen treatment on the occurrence of rat mammary tumors induced by DMBA. DMBA was administered to rats 28 days prior to drug treatment. Groups of 20 animals were dosed (p.o.) daily with peanut oil (■—■), 50 μg toremifene (○—○), 200 μg (●—●), 800 μg toremifene (□—□) or 200 μg tamoxifen (△—△). Tumor burden was calculated as the number of tumors per group divided by the number of rats. The treatment period is indicated by the hatched box.

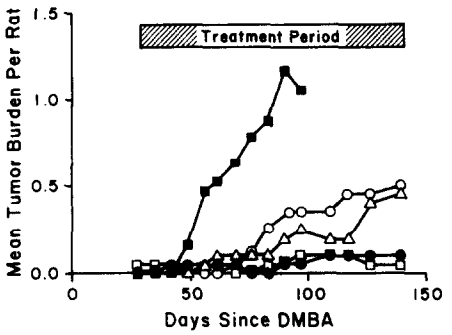

Fig. 2. Effect of toremifene and tamoxifen treatment alone or in combination with progesterone on the occurrence of rat mammary tumors induced by DMBA. DMBA was administered to rats 28 days prior to drug treatment. Groups of 20 animals were treated daily with either peanut oil (■—■), toremifene (200 μg, ●—●), toremifene (200 μg) and progesterone (4 mg, △—△) tamoxifen (200 μg, □—□) or tamoxifen (200 μg) and progesterone (4 mg, ○—○). Toremifene and tamoxifen were administered orally (0.2 ml) and progesterone s.c. (0.1 ml) in peanut oil. Tumor burden was calculated as the number of tumors per group divided by the number of rats. Treatment period is indicated by the hatched box.

then studied to determine the possible tumoricidal action of the antiestrogen. Rats treated with DMBA 28 days previously, were given 8000, 4000 or 800 μg of toremifene each day for 10 days. At the end of the treatment period, measurable levels of parent compound, 4-hydroxytoremifene and *N*-desmethyltoremifene (see Fig. 3) were detected in the serum (see Table 1). The 8000 μg/day dose of toremifene produced not only very high circulating levels of these metabolites but also several additional peaks of fluorescence (see Fig. 4). The retention times of these peaks did not correspond to any standards available to us. However, even the 8000 μg dose of toremifene did not reduce the percentage of rats ultimately developing tumors (about 90% in each group by 150 days) or the mean number of tumors per rat (see Fig. 5). There was a slight increase in latent period to tumor development produced by the high dose which corresponded approximately with the length of the treatment period. Although the regimen used did not produce a tumoricidal action, it did cause a dose-related decrease in body weight (see Fig. 6). The effect was very pronounced with the highest dose of toremifene. Noticeable lethargy and malaise were also seen in animals receiving the 8000 μg/day dose of toremifene.

DISCUSSION

These studies demonstrate the efficacy of long term toremifene therapy in the DMBA-induced rat mammary carcinoma model. The prevention of tumor occurence is consistent with the antitumor action of the agent on established tumors [8]. A marked similarity is seen between the actions of toremifene and those of tamoxifen in both inhibition of tumor development and the reversal of this antitumor action. We reported previously, a low dose of progesterone (4 mg/day s.c.) is capable of reversing the antitumor action of tamoxifen (50 μg/day s.c.) in the DMBA-induced rat mammary tumor model via a progesterone receptor-mediated mechanism [13]. A similar but less dramatic effect is now demonstrated when progresterone (4 mg/day s.c.) is combined with a higher dose of tamoxifen (200 μg p.o.) or with toremifene (200 μg p.o.). The finding that toremifene has a tumoristatic rather than tumoricidal action concurs with our findings for tamoxifen in the present and previous [1] studies and is consistent with tamoxifen's action in other models [2, 14]. Therefore, if toremifene is used clinically in the same way as tamoxifen to treat a low tumor burden (adjuvant therapy of Stage I/II disease) it seems logical that a strategy of continuous therapy should be used.

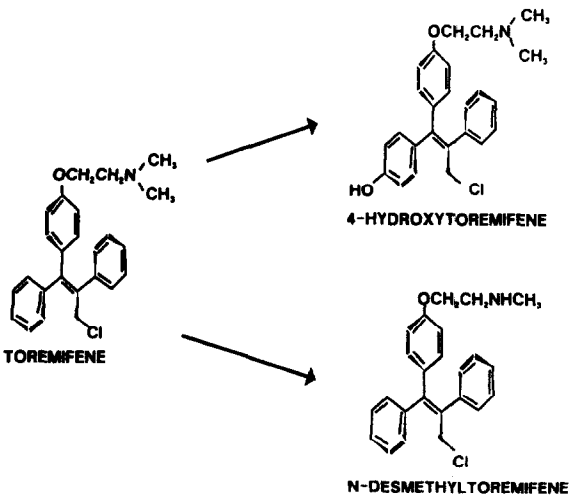

Fig. 3. The structures of toremifene and two metabolites identified in rat serum (see Fig. 4 and Materials and Methods).

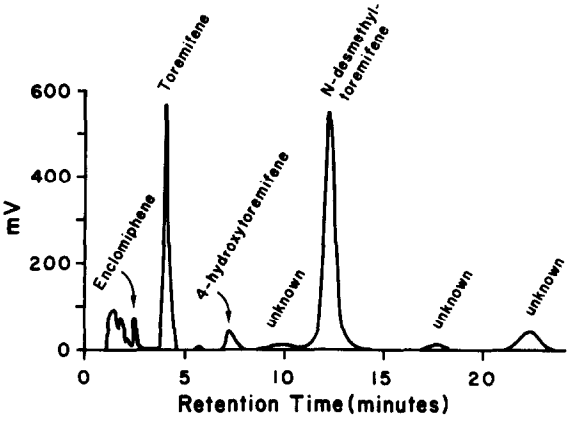

Fig. 4. Chromatograms of toremifene and metabolites extracted from rat serum following the daily administration of 8000 μg *of toremifene p.o. for* 10 *days. Metabolites were separated by HPLC on a Silica column and then converted to the phenanthracene derivative by exposure to u.v. light. The phenanthrene derivatives were subsequently activated and their fluorescence detected. The retention times of toremifene* (4.05 *min*), 4-*hydroxytoremifene* (7.1 *min*), *and* N-*desmethyltoremifene* (12.3 *min*) *were determined using pure standards and corresponding peaks are labeled. Enclomiphene was used as an internal standard. Additional unidentified peaks as shown were consistently seen in serum of rats treated with large doses of toremifene.*

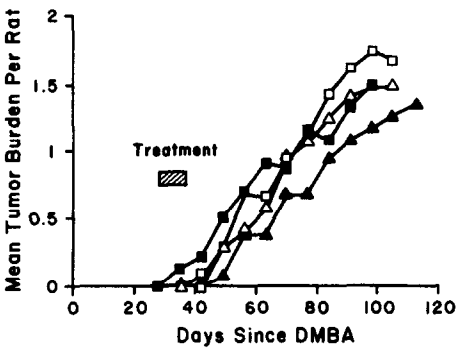

Fig. 5. Effect of high dose toremifene treatment on the occurrence of rat mammary tumors induced by DMBA. Groups of 25 *animals were dosed (p.o.) daily with* 8000 μg (▲—▲), 4000 μg (△—△) *or* 800 μg (□—□) *of toremifene in peanut oil* (0.2 ml). *Control* (■—■) *animals received peanut oil alone. Tumor burden was calculated as the number of tumors per group divided by the number of rats. Treatment period is indicated by the hatched box.*

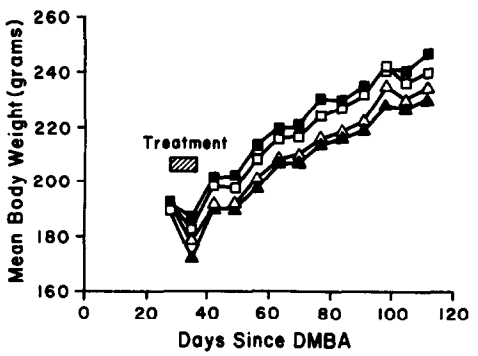

Fig. 6. Rats treated with toremifene 8000 μg (▲—▲), 4000 μg (△—△), 800 μg (□—□) *or peanut oil* (■—■) *as described in Fig.* 5 *were weighed at weekly intervals from the start of treatment.*

The possibility that treatment with high doses of toremifene could produce tumoricidal actions and also influence hormone-independent tumors would be an important advance in antiestrogen therapy. However, our findings that a short course of massive doses of toremifene were ineffective in reducing the total number of DMBA-induced tumors ultimately developing does not support this hypothesis. Furthermore, the loss in body weight and toxicity observed prevented us from using doses higher than 8000 μg/day or treatment periods longer than 10 days. The serum concentrations of toremifene and *N*-desmethyltoremifene with the highest dose were approx. 2.5×10^{-6} and 1.8×10^{-5} M, respectively. These levels are equivalent to the levels of toremifene shown to be cytolytic (5×10^{-6} M) in culture to MCF-7 cells [8]. The toxic effects observed in the rat may be because the high circulating levels of toremifene and metabolites have a nonspecific cytolytic action. The metabolites detected in this study support previous reports [15] that *N*-desmethylation is the major route of toremifene metabolism, and the handling of large oral doses of toremifene by the rat is very similar to that of tamoxifen [16].

The need for continuous application of both tamoxifen and toremifene for antitumor action

Table 1. *Metabolites* (ng/ml ± S.E.M.)

	Dose	Toremifene	4-Hydroxytoremifene	*N*-Desmethyltoremifene
8000 μg	(n = 4)	1002 ± 224	84 ± 14	6999 ± 1308
4000 μg	(n = 5)	282 ± 49	30 ± 5	2631 ± 449
800 μg	(n = 5)	21 ± 6	0.8 ± 0.6	158 ± 61
Control	(n = 5)	0	0	0

Serum levels of toremifene and metabolites in rats treated for 10 days with different doses of toremifene. Blood samples were taken from the eye orbit 24 h after the last dose. Serum (0.3 ml) was extracted 3 times with 1 ml hexane:amyl alcohol (98:2) and the extract analyzed by HPLC separation, u.v. activation and fluorescence detection as described in Materials and Methods. Quantitation was from standard curves of pure metabolites spiked into control rat serum. Enclomiphene was used as an internal extraction standard to correct for loss. The number of estimations (n) are shown in parentheses.

makes relative toxicology of importance. The report that tamoxifen produces hyperplasia or neoplastic nodules in the livers of rats treated with high doses, whereas toremifene does not [8], could become relevant to future clinical applications. If toremifene is found to be at least equally as effective as tamoxifen in the treatment of breast cancer, then reduced toxicity may make it a more desirable agent for the prolonged adjuvant therapy of Stage I disease or for an application as a preventative agent in postmenopausal women only at risk for breast cancer.

Acknowledgements—We wish to thank Sue Langan for technical assistance with toremifene measurements and Adria Laboratories for supporting these studies.

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