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Evaluation of the Antitumour Activity of the Non-Steroidal Antioestrogen Monohydroxytamoxifen in the DMBA*-induced Rat Mammary Carcinoma Model†

V. C. JORDAN‡ and KAREN E. ALLEN (née NAYLOR)

Department of Pharmacology, Medical and Dental Building, the University, Leeds LS2 9JT, United Kingdom

Abstract—*The antitumour activity of monohydroxytamoxifen has been compared with tamoxifen in the DMBA-induced rat mammary carcinoma model. In general antioestrogen therapy between* 30 *and* 60 days *after DMBA administration was more successful than therapy between* 60 *and* 90 days *after DMBA. Although monohydroxytamoxifen was a more potent antioestrogen than tamoxifen and equivalent dosage regimens were more effective at reducing cytoplasmic oestrogen receptor concentrations in the ovariectomized rat uterus, tamoxifen appeared to be a more potent antitumour agent. The administration of* 4 *weekly courses of tamoxifen* (0.2, 3, 50 *or* 800 μg *daily*, 5 *times a week*) *starting* 30 days *after DMBA produced a dose-related delay in tumour appearance and a decrease in tumour numbers. By contrast, monohydroxytamoxifen* (0.012, 0.2, 3 *or* 50 μg *daily*, 5 *times a week*) *was only weakly active at delaying tumour appearance although tumour numbers were reduced. Most of the tumours that developed in groups previously treated with antioestrogens regressed upon ovariectomy of the host. From studies in ovariectomized rats, tamoxifen was found to have long term effects upon the uterus whereas equivalent doses of monohydroxytamoxifen were only effective for a short period after the cessation of therapy. These data suggest that mammary tumour development is best inhibited in the constant presence of an antioestrogen i.e., biological half-life, as well as potency, is important for antitumour activity. The principle was exemplified by a reduction in the number and sizes of mammary tumours developing during continuous therapy with monohydroxytamoxifen* (3 *or* 50 μg *daily*, 5 *times a week*) *starting* 30 days *after DMBA. Overall it was clear that antioestrogens do not destroy all the foci of hormone dependent tumour cells and long courses of therapy or the use of other antihormonal methods e.g., ovariectomy, are essential to control tumour growth.*

INTRODUCTION

THE INCREASED use of antioestrogens in clinical medicine [1] has stimulated an interest in their mechanism of action. Application of this knowledge may facilitate the introduction of a new generation of therapeutic agents or alternatively suitable compounds can be used in the laboratory as biochemical probes to gain further insight into oestrogen action at the subcellular level. Recently it has been suggested [2] that some antioestrogens are prodrugs; being converted to polar metabolites before exerting their action in oestrogen target tissues [3]. At present therefore, this complex situation questions the validity of comparing results derived from experiments *in vivo* and *in vitro*. Clearly for meaningful studies of the mechanisms of action of antioestrogens, a compound is needed that is either known to be metabolised to much less active derivatives or not metabolised at all.

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*DMBA; 7,12-dimethylbenz(a)anthracene.

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‡Present address and reprint requests: Ludwig Institute for Cancer Research, Inselspital, Bern, Switzerland.

Tamoxifen, a non-steroidal antioestrogen used in the treatment of breast cancer [4], has been shown to have intrinsic activity as an anticancer agent *in vitro* [5] under conditions where metabolic transformations could not be detected [6]. However the situation is more complex *in vivo*. In laboratory animals, tamoxifen is metabolised [7] to the more potent antioestrogen monohydroxytamoxifen and subsequently to the very weak antioestrogen dihydroxytamoxifen [8]. Monohydroxytamoxifen would therefore appear to be of great interest both as a potential agent for breast cancer therapy and as a potent biochemical probe for studying the mechanism of action of antioestrogens.

Since tamoxifen inhibits the initiation [9] and growth [9–11] of DMBA-induced rat mammary carcinomata we have used this model to evaluate the antitumour activity of monohydroxytamoxifen.

MATERIALS AND METHODS

Tamoxifen (*trans* 1-(4-β-dimethylaminoethoxyphenyl)1,2 diphenyl but-1-ene) and monohydroxytamoxifen [1-(4-β-dimethylaminoethoxyphenyl)1, (4-hydroxy-phenyl)-2,phenyl-but-1-ene] were obtained from ICI Ltd (Pharmaceuticals Division). 7,12 Dimethylbenz(a)anthracene (DMBA) was obtained from Sigma Chemicals.

Preparation of solutions

DMBA was dissolved in peanut oil by gentle stirring for 16 hr at room temperature. The final concentration was 10 mg DMBA/ml. Each week a tamoxifen and monohydroxytamoxifen solution were prepared in absolute ethanol and the required volumes were added to peanut oil. The ethanol was evaporated under N_2 on a warm (60°C) water bath. All s.c. injections were made in 0.1 ml peanut oil.

Antitumour activity of antioestrogens

Female rats of the Sprague–Dawley strain were obtained specific pathogen free from the Animal Breeding Unit at ICI Ltd (Pharmaceuticals Division). At 50 days of age each rat was given 20 mg DMBA by gavage. Three experiments were undertaken: (1) Four weeks after DMBA, animals were randomized into nine groups each of 15 rats. Treatments were instituted 30 days after DMBA for four weekly cycles (5 days per week). Four groups were injected s.c. with tamoxifen (0.2, 3, 50, 800 μg daily) and four groups with monohydroxytamoxifen (0.012, 0.2, 3, 50 μg daily). Controls received injections of peanut oil; (2) Eight weeks after DMBA, animals were randomized into five groups each of 15 rats. Treatments were instituted 60 days after DMBA for four weekly cycles (5 days per week). Two groups were injected s.c. with tamoxifen (3, 50 μg daily) and two groups with monohydroxytamoxifen (3, 50 μg daily). Controls received injections of peanut oil; (3) Four weeks after DMBA animals were randomized into 3 groups each of 20 rats. Treatments were instituted 30 days after DMBA, 5 days per week until 200 days after DMBA. Two groups were injected s.c. with monohydroxytamoxifen (3 or 50 μg daily) and controls were injected with peanut oil.

In all experiments animals were palpated weekly for tumours up to 200 days after DMBA. Where necessary animals with large and ulcerated tumours were killed prior to the end of the experiments. In all cases samples of tumour tissue were taken for routine pathological identification at the end of an experiment.

In experiment 1 when a tumour on an animal reached a cross-sectional area of 2–4 cm^2 [measured with calipers and calculated using the formula $\pi \times (\text{length}/2) \times (\text{width}/2)$], the animal was ovariectomized under ether anaesthesia and change in tumour area determined for the next 6 weeks.

In experiment 3 tumour areas were determined every 2 weeks for 6 weeks starting 156 days after DMBA. At 200 days after DMBA all rats previously treated with 3 μg monohydroxytamoxifen daily and some of the animals from the control group were treated for 4 weeks with 100 μg monohydroxytamoxifen daily 5 times per week. A group of controls were injected with peanut oil. Tumour areas were determined at the beginning and at the end of the treatment period.

Effect of antioestrogens in ovariectomized rats

Two experiments were undertaken: (1) Female Sprague–Dawley rats (100 days old) were ovariectomized under ether anaesthesia and 14 days later were randomized into 25 groups each of five rats. Four major treatment groups each containing five of the groups of rats were selected for treatment with 4 cycles (5 days per week) of either tamoxifen (0.2 or 800 μg daily) or monohydroxytamoxifen

(0.012 or 50 μg daily). Controls (5 groups) were injected s.c. with peanut oil. One of each of the groups was killed after 1 week of treatment, on the last day of treatment and 1, 3 and 5 weeks after the last day of treatment. Uteri were dissected out, cleared of adhering tissue and weighed wet on a torsion balance before the determination of [^{3}H] oestradiol-17β and [^{3}H] R5020 binding (see below); (2) Female Sprague–Dawley rats (50 days old) were ovariectomized under ether anaesthesia and 14 days later randomized into groups of 5 rats. Groups were injected s.c. with tamoxifen (3, 50 or 800 μg daily) 5 days per week for 4 weeks. Controls were injected s.c. with peanut oil. Groups were killed on the last day of treatment and at 21, 42, 63 and 84 days after the end of treatment. Uteri were dissected out, cleaned of adhering tissue and weighed wet on a torsion balance. The determination of [^{3}H] oestradiol and [^{3}H] R5020 binding was undertaken only with uteri obtained on the last day of antioestrogen treatment.

Determination of [^{3}H] *oestradiol and* [^{3}H] *R5020 binding*

Each uterus was homogenized with ice/water cooling in 2 ml TED buffer (Tris 0.01 mole/l; EDTA 0.0015 mole/l or dithiothreitol 0.0005 mole/l, pH 7.4) using 2 × 10 sec bursts of an Ultraturrax tissue homogenizer. Homogenates were centrifuged (4°C) at 2000 ***g*** for 30 min. Supernatants were used to determine the binding of [6, 7-^{3}H] oestradiol-17β (42 Ci/mmole, Amersham) and [^{3}H] R5020 (dimethyl-19-norpregna-4,9-diene-3,20 dione, 17α-[17α-methyl ^{3}H] (87 Ci/mmole, New England Nuclear.) For [^{3}H] oestradiol binding, uterine supernatants (150 μl) were added to 50 μl TED buffer or 50 μl TED buffer containing 5×10^{-6} mole/l diethylstilboestrol. Fifty microlitres TED buffer containing 2.5×10^{-8} mole/l [^{3}H] oestradiol was added to each tube and the mixtures incubated at 0–4°C for 18 hr. All samples were assayed in duplicate. Four hundred microlitres of a Dextran coated charcoal suspension (1%) in TED was added to each tube and allowed to stand with occasional shaking for 20 min at 0–4°C. Tubes were centrifuged at 2000 ***g*** (4°C) for 10 min and 400 μl samples of the supernatant counted in 10 ml tritium scintillator (6 g butyl PBD [2(4′-*t*-butylphenyl)-5-(4″ biphenylyl)-1, 3, 4 oxadiazole], 100 g naphthalene, 135 ml toluene, 720 ml dioxan and 45 ml methanol) for 10 mins in a Beckman LS-3133T liquid scintillation spectrometer.

To determine the binding of [^{3}H] R5020, 100 μl supernatant was added to either 100 μl TED containing 30% glycerol or 100 μl TED with 30% glycerol containing 3×10^{-6} mole/l norethindrone (Sigma Chemicals). One hundred microlitres TED containing 3×10^{-8} mole/l [^{3}H] R5020 was added to each tube and incubated at 0°C for 1 hr. Four hundred microlitres of a 0.25% dextran coated charcoal suspension in TED buffer was added to each tube and incubated at 0°C for 5 min. Tubes were centrifuged at 2000 ***g*** (4°C) for 3 min and 400 μl of supernatants were taken to determine the levels of radioactivity as described above.

RESULTS

Antitumour activity of antioestrogens

The administration of tamoxifen (0.2, 3, 50 or 800 μg daily) between 30 and 60 days after DMBA resulted in a dose-related delay in the percentage of rats in groups without tumours (Fig. 1a). By contrast, treatment with monohydroxytamoxifen (0.012, 0.2, 3 or 50 μg daily) did not produce a clear dose-related inhibition of tumour appearance (Fig. 1b). In fact, the group treated with 0.2 μg monohydroxytamoxifen daily was tumour free for longer than the group treated with 50 μg monohydroxytamoxifen daily. However, treatment of animals with 50 μg monohydroxytamoxifen daily between 60 and 90 days after DMBA (Fig. 2) effectively kept the animals tumour-free for 40 days. The lowest dose of monohydroxytamoxifen (0.012 μg daily) was without effect. Tamoxifen treatment (50 or 3 μg daily) between 60 and 90 days after DMBA did not initially delay tumour appearance but tumours appeared more slowly than controls during the post-therapy period.

Although the various therapies delayed the initial appearance of tumours, the majority of animals had at least one palpable mammary tumour at 200 days after DMBA. This effect was most pronounced in the group of animals treated with 800 μg tamoxifen daily between 30 and 60 days after DMBA (Fig. 1a). The rats were completely tumour free up to 135 days after DMBA but by 200 days after DMBA 80% of animals had tumours. However, the cumulative number of tumours in the group was only about 30% of controls (Fig. 3). This effect of tamoxifen on the total numbers of tumours in the treatment groups was again dose related but it was also dependent upon the time of antioestrogen ad-

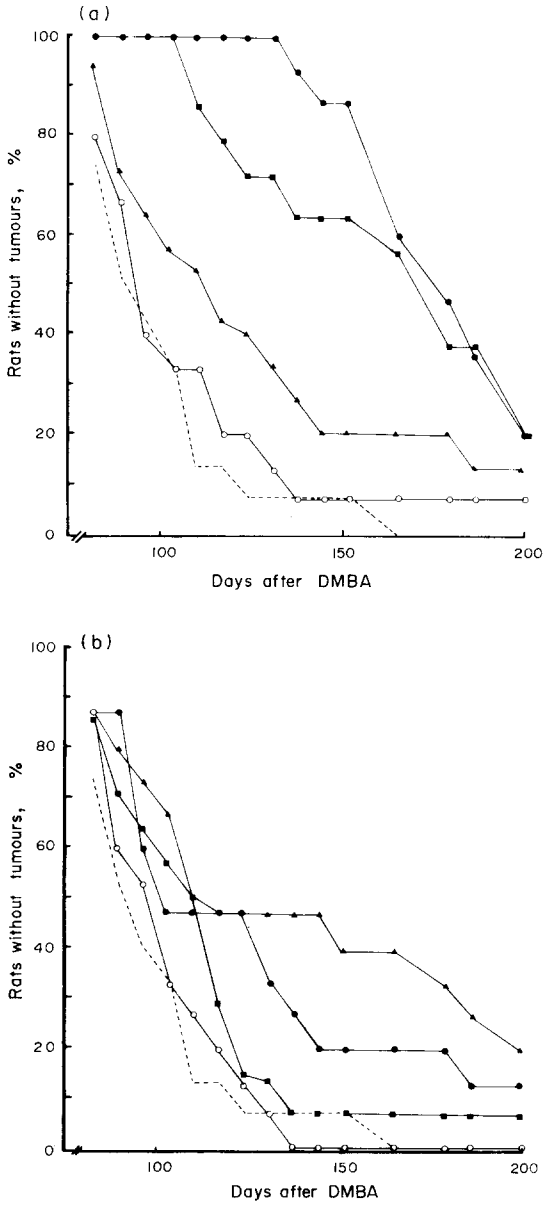


Fig. 1. Effect of the administration of (a) tamoxifen (800 μg ●; 50 μg ■; 3μg ▲; 0.2 μg ○; daily) or (b) monohydroxytamoxifen (50 μg ●; 3 μg ■; 0.2 μg ▲; 0.012 μg ○; daily) between 30 and 60 days (5 times per week) after DMBA on the percentage of rats in groups without mammary tumours. Controls (----) were injected with peanut oil. Fifteen rats per group.

ministration. Treatment with 50 μg tamoxifen daily between 30 and 60 days after DMBA reduced tumour numbers from 36 in controls at 200 days after DMBA to 12 in the treatment group whilst the administration of the same regimen between 60 and 90 days after DMBA only reduced the tumour numbers from a control value of 33 to 20 (Fig. 3). Similarly monohydroxytamoxifen was effective at reducing tumour numbers and, again, was more active if administered earlier. The principle was illustrated with the 0.2 μg daily dose of monohydroxytamoxifen which appeared to be more active during the early therapy period i.e., 30–60 days after DMBA (Fig. 3) and inactive if administered between 60 and 90 days after DMBA.

In the first experiment, the effect of host ovariectomy on the growth of tumours that reached a cross-sectional area of 2–4 cm^2 in control and treatment groups was determined. For convenience, the tumours were arbitrarily divided into those that had reached the required area between 85 and 140 days after

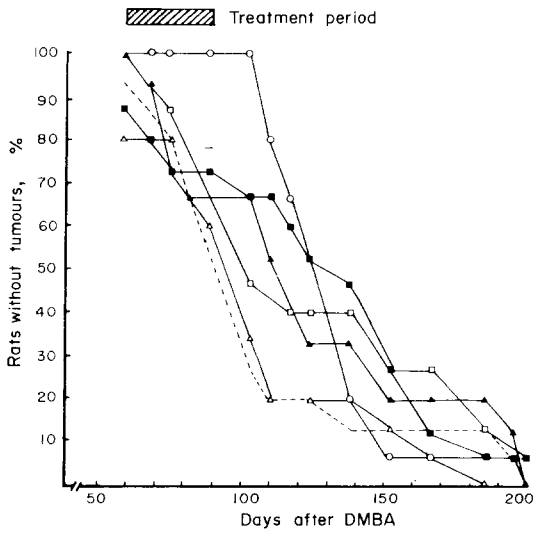


Fig. 2. Effect of the administration of tamoxifen (50 μg ■; 3 μg ▲; daily) or monohydroxytamoxifen (50 μg ○; 3 μg □; 0.2 μg △; daily) between 60 and 90 days (5 times per week) after DMBA on the percentage of rats in groups without mammary tumours. Controls (----) were injected with peanut oil. Fifteen rats per group.

DMBA (Group I) and 140 and 190 days after DMBA (Group II) (Fig. 4). Tumours from control animals in Group I uniformally regressed (6/6) over 6 weeks whereas the single tumour in Group II regressed and then regrew. In the group treated with 0.2 μg tamoxifen daily, 5/6 tumours regressed whilst 1/6 grew in Group I. However in Group II, 12/13 tumours regressed and 1/13 grew and then regressed. Similarly in the 3 (1/5), 50 (1/3) and 800 (1/3) μg tamoxifen groups only single tumours did not regress in response to host ovariectomy (Fig. 4b). Overall, 28–32 tumours regressed to <50% of their original size from tamoxifen treated groups whereas 4/32 tumours either did not regress or regrow after an initial regression.

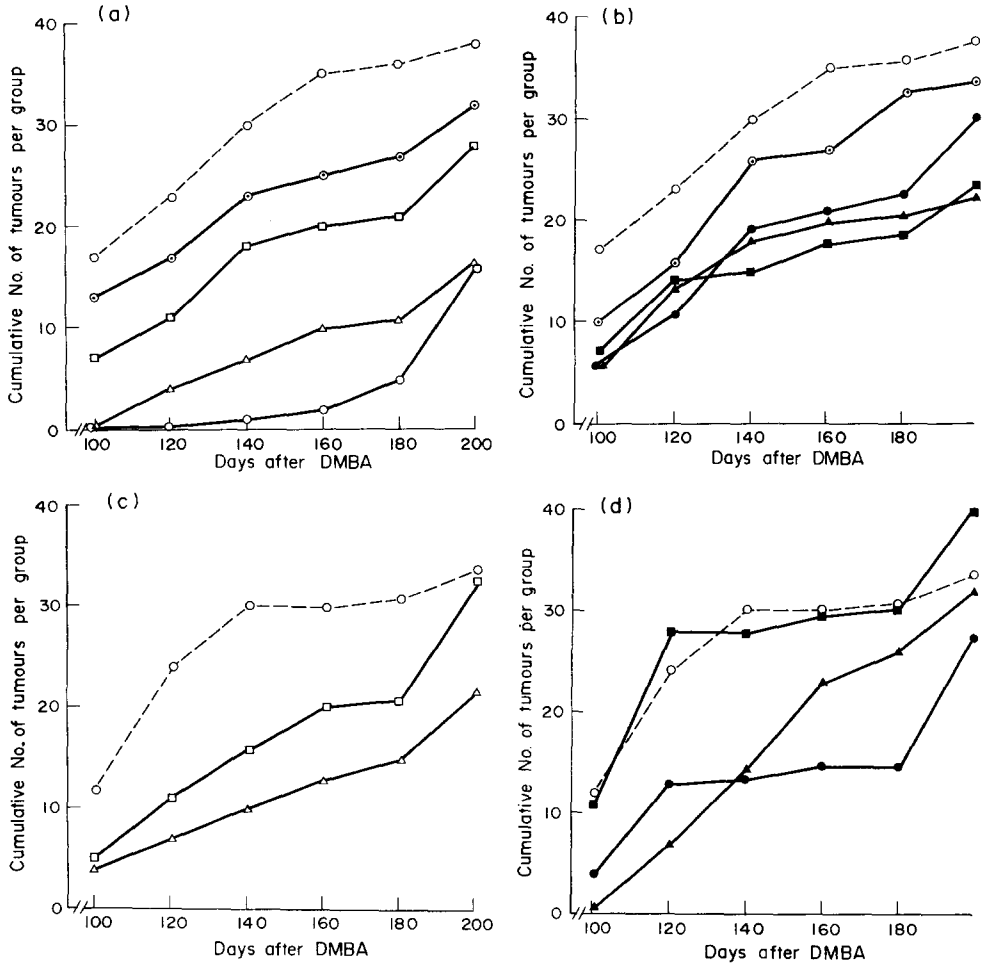


Fig. 3. Cumulative numbers of mammary tumours after the administration of (A) tamoxifen (800 μg ○; 50 μg △; 3 μg □; 0.2 μg ⊙ daily) or (B) monohydroxytamoxifen (50 μg ▲; 3 μg ●; 0.2 μg ■; 0.012 μg ⊙ daily) between 30 and 60 days after DMBA or (C) tamoxifen (50 μg △; 3 μg □; daily) or (D) monohydroxytamoxifen (50 μg ▲; 3 μg ●; 0.2 μg ■; daily) between 60 and 90 days after DMBA. Controls (○ - - - ○) received injections of peanut oil. Fifteen rats per group.

The majority of tumours arising on animals previously treated with monohydroxytamoxifen regressed in response to ovariectomy of the host. Overall, 31/38 tumours regressed to $<50\%$ of their original size, 3/38 tumours continued to grow whilst 4/36 tumours remained static or regressed to $>50\%$.

Continuous cycles of treatment with 3 μg monohydroxytamoxifen daily starting 30 days after DMBA resulted in a decrease in the number of rats developing tumours (Fig. 5a), the cumulative number of tumours (Fig. 5b) and the individual tumour sizes measured at 163, 178 and 192 days after DMBA (Fig. 6). The larger daily dose of monohydroxytamoxifen (50 μg daily) was more effective in each of the recorded parameters (Figs. 5a and b, 6) however a single tumour (FIL2) in this group grew very rapidly during therapy. Ovariectomy of the host resulted in a progressive regression of the tumour over 6 weeks.

Treatment of rats (200 days after DMBA) with established mammary tumours with 5 day cycles of 100 μg monohydroxytamoxifen daily did not produce a consistent tumour regression. As a result, the response of individual tumours are shown in Fig. 7. In general the tumours on control animals grew and did not undergo any dramatic reductions in tumour area. Treatment with monohydroxytamoxifen did cause some tumours to undergo regression whilst others remained static or

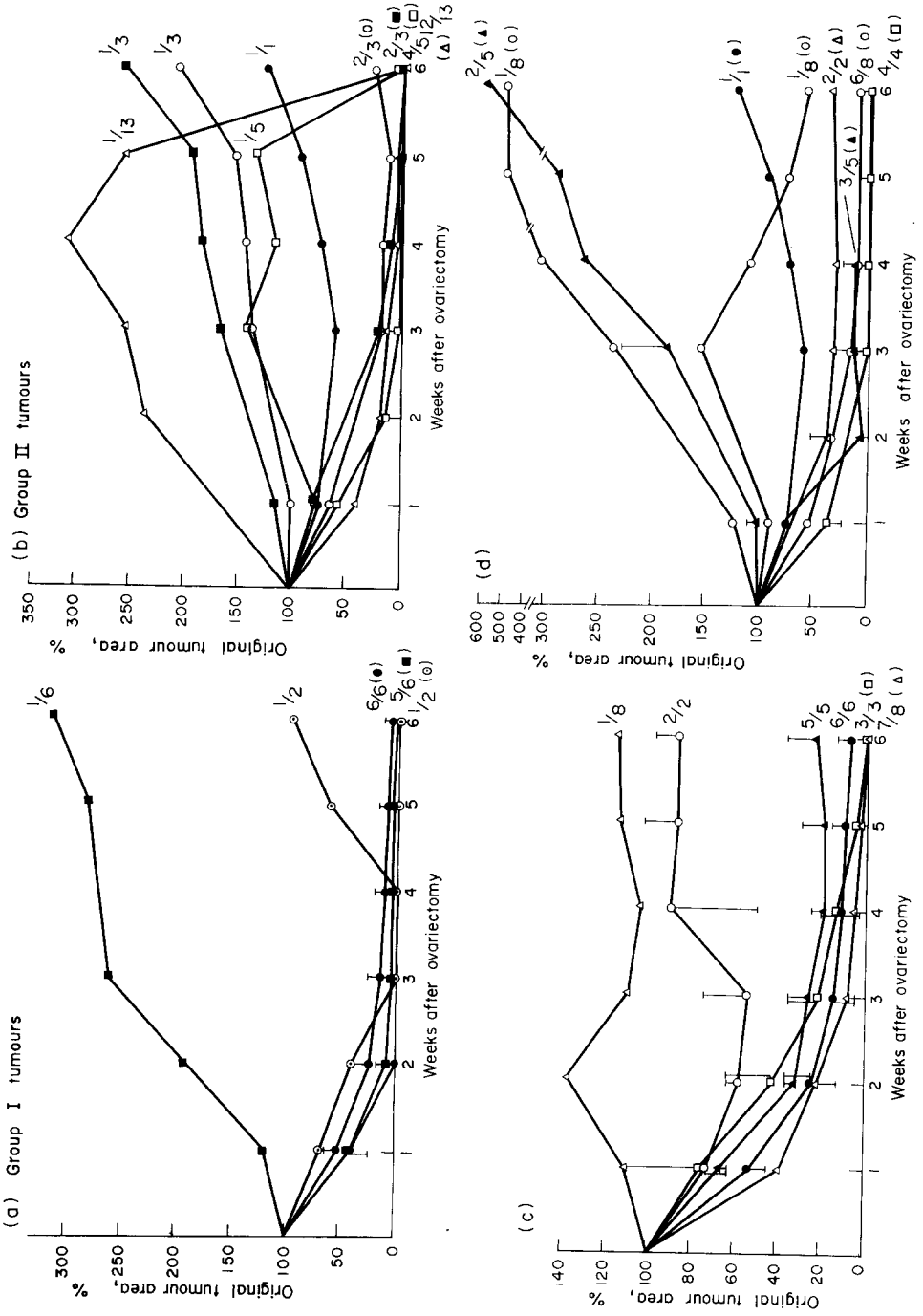


Fig. 4. Effect of ovariectomy on the percentage change in DMBA-induced mammary tumour area. Groups had been treated between 30 and 60 days after DMBA with tamoxifen (a), 0.2 μg ■; 3 μg ⊙ daily (b), 800 μg○; 50 μg ■; 3 μg □; 0.2 μg △; daily of monohydroxytamoxifen (c), 50 μg □; 3 μg ○; 0.2 μg △; 0.012 μg ▲ daily (d), 50 μg □; 3 μg ○; 0.2 μg △; 0.012 μg ▲; daily). Controls (●) were injected with oil. Group I tumours were from animals ovariectomized 85–140 days after DMBA and Group II tumours from animals ovariectomized 140–190 days after DMBA. The proportion of tumours in each group that responded similarly shown.

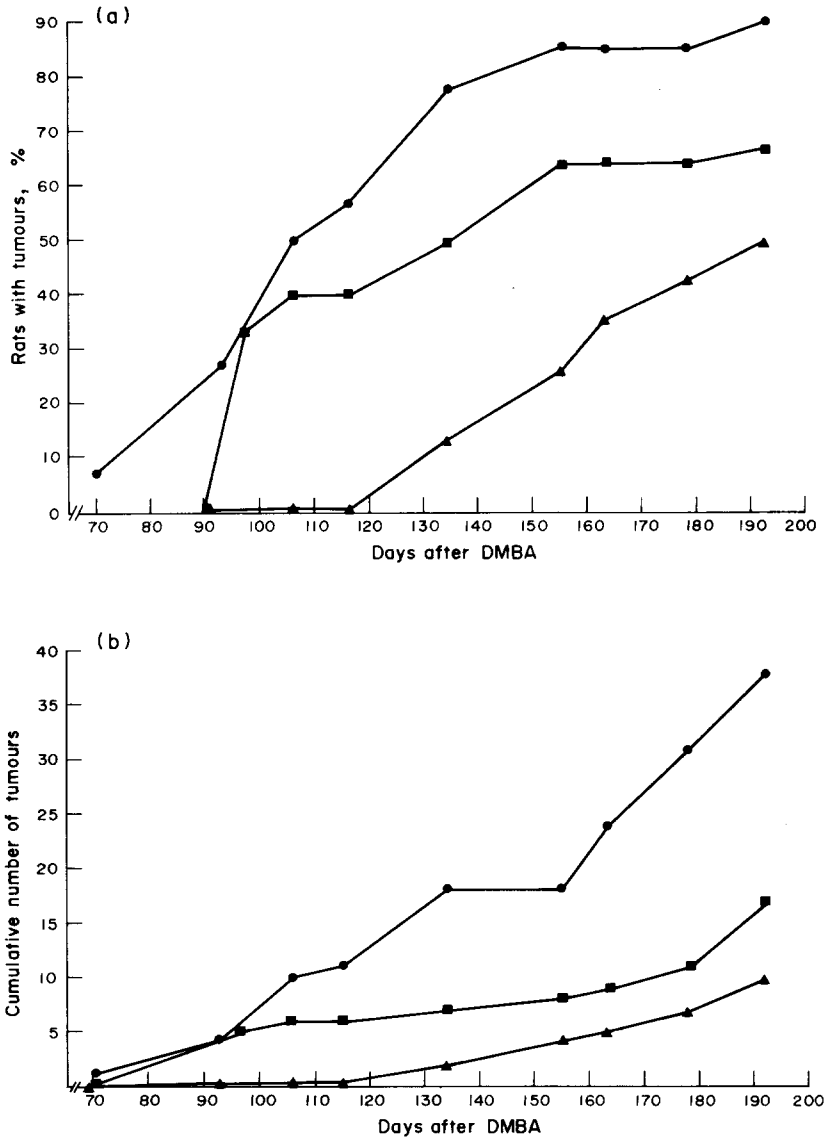


Fig. 5. Effect of continuous (5 times per week starting 30 days after DMBA) treatment with monohydroxytamoxifen (3 μg ■; 50 μg ▲; daily) on (a), the percent of animals in groups with tumours and (b), the cumulative number of DMBA-induced mammary tumours. Controls (●) were injected with peanut oil. Twenty rats per group.

continued to grow. Again some of the tumours growing on rats previously treated with 3 μg monohydroxytamoxifen daily (Fig. 7, treatment B) regressed in response to the larger dose of monohydroxytamoxifen whilst others continued to grow.

Effects in ovariectomized rats

A comparison of the uterine effects of tamoxifen (800 or 0.2 μg daily) and monohydroxytamoxifen (50 or 0.012 μg daily) showed that only the higher dose of each compound was biologically active and the effects of tamoxifen were more long-term than the effects of monohydroxytamoxifen. During therapy tamoxifen (800 μg) and monohydroxytamoxifen (50 μg) both increased uterine wet weight (Fig. 8a) and the uterine binding of [^{3}H] R5020 (Fig. 8b) and completely reduced the uterine binding of [^{3}H] oestradiol (Fig. 8c). However uterine wet weight and [^{3}H] R5020 binding rapidly decreased during the 2 weeks after the last injection of monohydroxytamoxifen and the binding of [^{3}H] oestradiol increased. Five weeks after the end of therapy

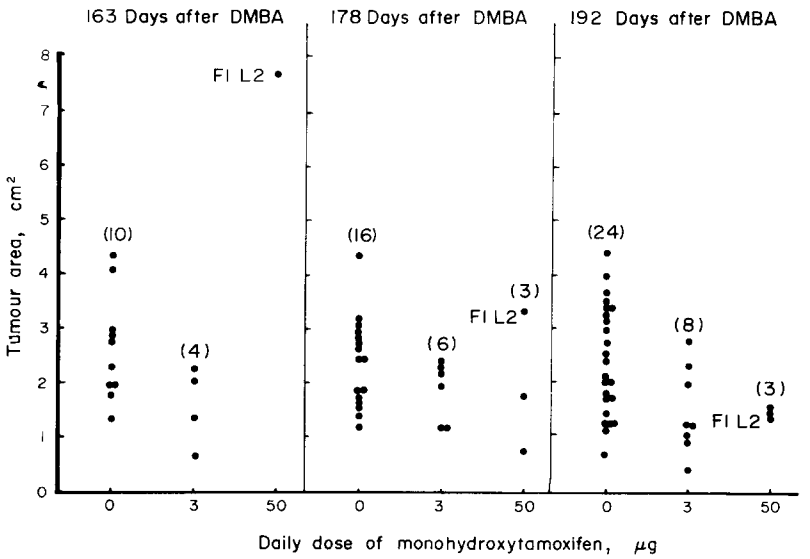


Fig. 6. Areas of mammary tumours measured in controls or in groups treated continuously with either 3 μg or 50 μg monohydroxytamoxifen daily (5 times per week). The animal with tumour FIL2 was ovariectomized 163 days after DMBA. The numbers of tumours measured are shown in parentheses. Twenty rats per group.

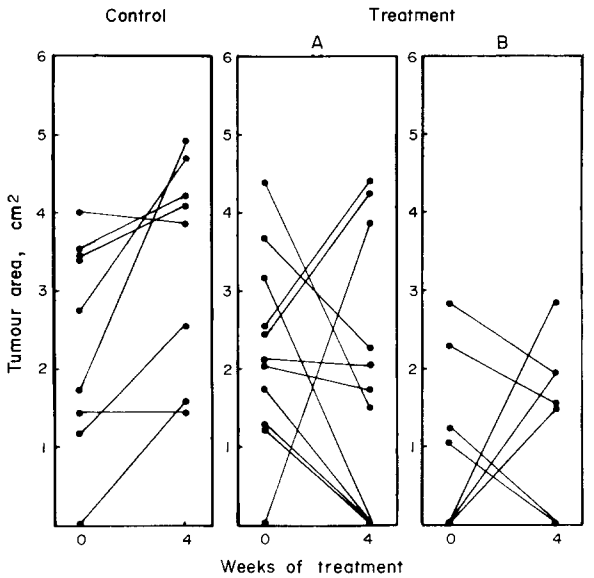


Fig. 7. Effect of monohydroxytamoxifen (100 μg daily) administration for 4 cycles of therapy (5 times per week) on the area of DMBA-induced rat mammary tumours. Experiments were started 200 days after DMBA. Treatment A animals had not previously received any other treatments whereas treatment B animals had been injected with 3 μg monohydroxytamoxifen (5 times per week) from 30 days after DMBA administration. Control animals received injections of peanut oil.

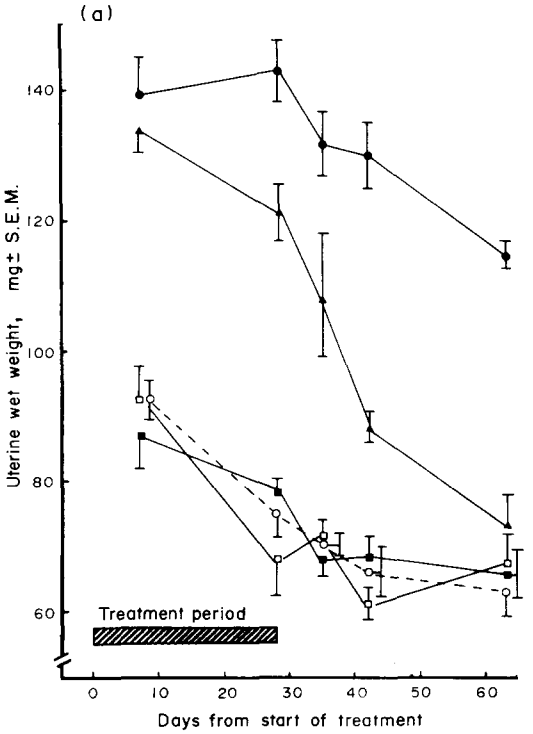
with monohydroxytamoxifen there was no significant difference between the treatment and control groups in all parameters studied. In contrast the uterine effects of tamoxifen (800 μg daily) were maintained until the end of the experiment i.e., 5 weeks after therapy was finished.

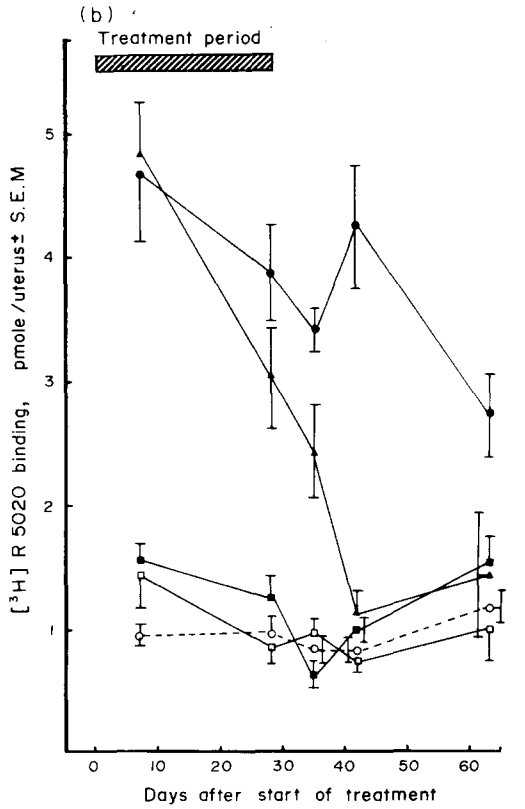
In a separate experiment, the dose-related effect of tamoxifen on the ovariectomized rat uterus was compared. After four 5-day cycles of tamoxifen therapy (3, 50 or 800 μg daily) there was a dose-related decrease in the uterine binding of [^{3}H] oestradiol and a dose-related increase in the uterine binding of [^{3}H] R5020 (Fig. 9). During the 84 days after the end of therapy the dose-related increase in uterine wet weight (Fig. 10) gradually returned towards control values however even at 84 days after therapy the uterine wet weight of the groups previously treated with 800 μg tamoxifen daily and 50 μg tamoxifen daily were still significantly ($P<0.001$) higher than controls.

DISCUSSION

The primary aim of the present study was to establish a suitable assay system to evaluate the antitumour potential of the potent anti-oestrogen monohydroxytamoxifen. The anti-oestrogen tamoxifen was selected for comparative purposes since it is already established as a therapy in advanced breast cancer [4]. Ideally a transplantable mammary tumour system would provide the most accurate assay method, however the lack of hormone responsiveness in the tumour system previously evaluated in this laboratory [12] directed our attention to the DMBA-induced rat mammary carcinoma model [13]. Although tam-

oxifen inhibits the growth of the majority of established tumours [14] the system is difficult to use as an assay because of the different growth rates of the tumours and the heterogeneous hormone responsiveness. We have therefore concentrated upon the antitumour activity of antioestrogens in the early stages of tumour development.





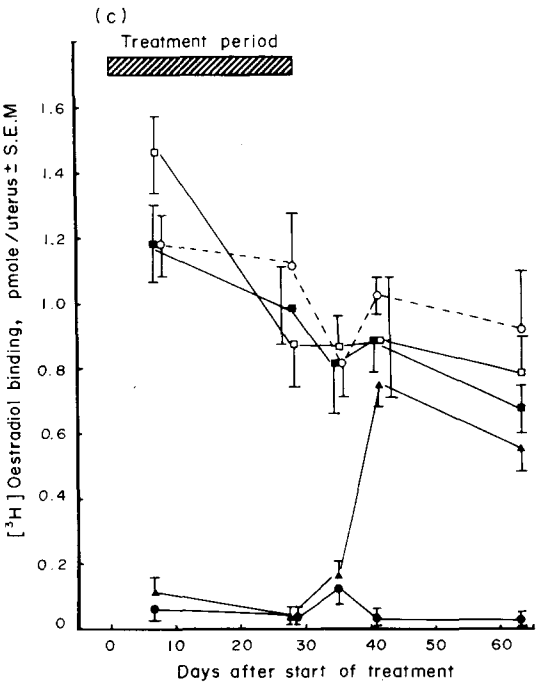


Fig. 8. Effect of tamoxifen (800 μg ●; 0.2 μg ■ *daily*) *or monohydroxytamoxifen* (50 μg ▲; 0.012 μg □; *daily*) *administration, for 4 cycles of therapy 5 times per week, on ovariectomized rat* (*a*) *uterine wet weight,* (*b*) *uterine* [^{3}H] *R5020 binding,* (*c*) *uterine* [^{3}H] *oestradiol binding. Controls* (○ - - - ○) *received injections of peanut oil. Five rats in each group.*

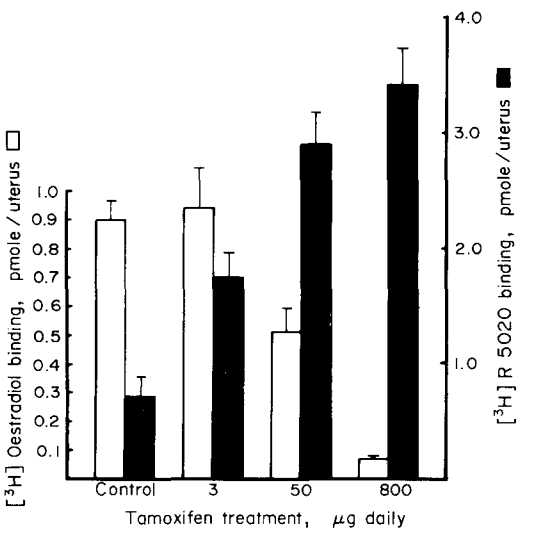


Fig. 9. The level of [^{3}H] *oestradiol binding and* [^{3}H] *R5020 binding in ovariectomized rat uteri on the last day of therapy after 4 cycles of tamoxifen treatment* (3, 50 *or* 800 μg *daily*) *administered 5 times per week. Controls received injections of peanut oil. Five rats in each group.*

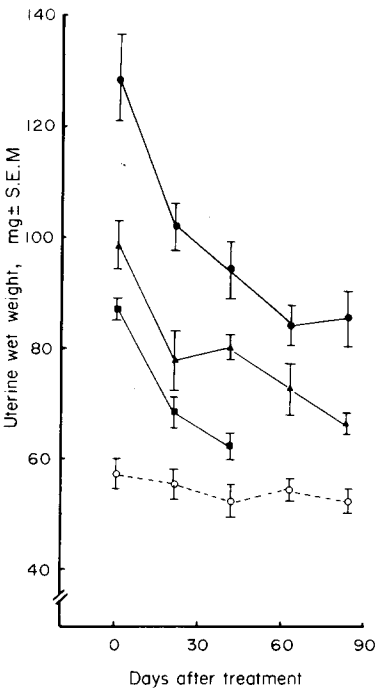


Fig. 10. Effect of tamoxifen administration (800 μg ●; 50 μg ▲; 3 μg ■; *daily*), *for 4 cycles of therapy 5 times per week, on ovariectomized rat uterine wet weight. Controls* (○----○) *received injections of peanut oil. Five rats in each group.*

Carcinogenesis in breast tissue with DMBA is critically dependent upon the age of the animals [13] and the correct hormonal environment [15]. At the subcellular level, prolactin-stimulated thymidine incorporation has been correlated with DMBA-induced carcinogenesis [16] which suggests that both effective levels of circulating prolactin and target tissue DNA synthesis are prerequisites for carcinogenesis. Although it is known [9] that the simultaneous administration of tamoxifen and DMBA results in a reduction in tumour numbers this is a potentially poor assay for evaluating antitumour activity. Tamoxifen inhibits oestrogen, stimulating rises in circulating prolactin levels [17] and it is possible that breast tissue DNA synthesis may be inhibited since antioestrogens have been found to inhibit cell division in other oestrogen target tissues of the rat [18]. Therefore under these conditions the process of carcinogenesis may be inhibited rather than malignant cells destroyed. Furthermore besides any direct effects upon the breast tissue, it is not known whether antioestrogens alter the metabolic transformations normally undergone by the DMBA molecule [19] that may, in turn, be fundamental to carcinogenesis. After consideration of these factors, all therapies were instituted 28 days after DMBA administration when it was assumed that carcinogenesis had occurred and microfoci of malignant cells were present.

In the experiments to compare the antitumour properties of tamoxifen and monohydroxytamoxifen, smaller daily doses of monohydroxytamoxifen were selected because of its higher antioestrogenic potency [8]. Furthermore at the same dose level, monohydroxytamoxifen was more effective than tamoxifen at reducing the cytoplasmic oestrogen receptor concentration in the ovariectomized rat uterus (Figs. 8 and 9). However, in complete contrast, the administration of tamoxifen between 30 and 60 days after DMBA was much more effective than monohydroxytamoxifen at inhibiting the rate of tumour appearance and reducing the number of tumours. In general similar doses of tamoxifen and monohydroxytamoxifen were ultimately less effective when administered between 60 and 90 days after DMBA which suggests that early therapy with antioestrogens may be an advantage because of the smaller tumour burden. Of interest though was the observation that during the 60–90 day treatment period monohydroxytamoxifen produced a rapid effect whilst tamoxifen was more slowly acting. It is possible that the increased hydrophilic nature of the monohydroxytamoxifen molecule coupled with increased affinity for the oestrogen receptor [8] may result in immediate high concentrations at the site of action. This effect has been noted earlier [20] since monohydroxytamoxifen increases ovariectomized rat uterine progesterone receptor content more rapidly than the same dose of tamoxifen.

The finding that an early, short course of monohydroxytamoxifen only reduced the numbers of tumours rather than cured the animals made it important to determine whether monohydroxytamoxifen could exert a sustained antitumour action. Monohydroxytamoxifen, like other non-steroidal antioestrogens [20–24], inhibited the growth of established DMBA-induced mammary carcinomata. However the tumour responses to monohydroxytamoxifen was very heterogeneous (Fig. 7) which probably reflects the lower hormone dependency of older tumours. Treatment with monohydroxytamoxifen on a continuous basis starting 28 days after DMBA was successful in controlling the development of the majority of tumours. Therefore it appears that the maintenance of high blood levels of a short acting antioestrogen can inhibit hormone dependent gro-

wth. Clearly the prolonged biological activity of a compound like tamoxifen appears to be more important than antioestrogenic potency as a criterion for antitumour action.

The principle that a metabolite of an antioestrogen has antioestrogenic and antitumour activity has important implications for the biochemical interpretation of data derived *in vivo*. We have demonstrated (Allen, Clark and Jordan, unpublished observation) that for antioestrogenic activity it is an advantage, but not a requirement, for tamoxifen to have the opportunity to undergo metabolic *para* hydroxylation. Similarly the fact that monohydroxytamoxifen has antitumour properties suggests that the antitumour activity of tamoxifen is the net result of a complex interaction of the parent compound and its primary non-conjugated metabolite with a tumour target site.

In spite of the fact that there are difficulties in being able to precisely describe the molecular events involved in the antitumour activity of non-steroidal antioestrogens it is important to note that irrespective of whether the parent compound or a metabolite is involved, microfoci of hormone dependent malignant cells can survive courses of antioestrogen therapy *in vivo*. This conclusion is based on two observations. Although tumour development was initially inhibited by very large doses of tamoxifen eventually tumours occurred that responded favourably to a second anti-hormonal therapy i.e., ovariectomy (Figs. 3 and 4). Similarly, during the continuous administration of monohydroxytamoxifen (50 μg daily, 5 times per week), a single tumour grew very rapidly and regressed rapidly following ovariectomy of the host (Fig. 6).

The results of the present study are in marked contrast to the published reports [5, 6] that human breast cancer cells maintained in long term tissue culture are eventually destroyed by high concentrations of antioestrogens. However there are several important differences between the DMBA-induced carcinoma model and the long term tissue cell system that might explain the unusual sensitivity of cancer cells to antioestrogens *in vitro*. The antitumour action of antioestrogens in the rat model is probably at a variety of sites e.g., inhibition of oestrogen-stimulated prolactin release [17, 25], inhibition of ovarian function [26, 27] or a direct action on the tumour cells via the oestrogen receptor system [11, 28, 29]. It is clear that only the last mechanism is possible in cell culture. In the whole animal a host of humoral, biochemical and physiological factors may protect the hormone dependent malignant cells from antioestrogen action. As one possible example; minor fluctuations in prolactin secretion may secure tumour survival since it is known that increases in prolactin levels induced by perphenazine can reverse tamoxifen-induced tumour regression [30]. Clearly, with breast cancer cells growing under the restricted conditions of long term culture, no such countermeasures are available as an alternative to cell death. Whether human breast cancer cells are protected from antioestrogen action *in vivo* must await the completion of clinical trial with tamoxifen as an adjuvant therapy following mastectomy.

In conclusion, it seems that the short acting antioestrogen monohydroxytamoxifen might be impractical as a therapy for breast cancer in its present form although a sustained-release or depôt preparation may be clinically useful. However monohydroxytamoxifen does not appear, at this stage, to offer any therapeutic advantages over tamoxifen. By contrast, the finding that monohydroxytamoxifen has antitumour activity *in vivo* without the need for metabolic intervention, has confirmed that this compound is a potent pharmacological tool for the investigation and comparison of oestrogenic, antioestrogenic and antitumour mechanisms *in vivo* and *in vitro*.

REFERENCES

1. C. B. Lunan and A. Klopper, Antioestrogens: a review. *Clin. Endocr.* **4,** 551 (1975).
2. B. S. Katzenellenbogen, E. R. Ferguson and N. C. Lan, Fundamental differences in the action of estrogens and antiestrogens on the uterus: comparison between compounds with similar duration of action. *Endocrinology* **100,** 1252 (1977).
3. B. S. Katzenellenbogen, J. A. Katzenellenbogen, E. R. Ferguson and N. Kranthammer, Antiestrogen interaction with uterine estrogen receptors. *J. biol. Chem.* **253,** 697 (1978).

4. H. T. Mouridson, T. Palshof, J. Patterson and L. Battersby, Tamoxifen in advanced breast cancer. *Cancer Treat. Rev.* **5,** 131 (1978).
5. M. E. Lippman, G. Bolan and K. Huff, Interactions of antiestrogens with human breast cancer in long term tissue culture. *Cancer Treat. Rep.* **60,** 1421 (1976).
6. K. B. Horwitz, Y. Koseki and W. L. McGuire, Estrogen control of progesterone receptor in human breast cancer: role of estradiol and antiestrogen. *Endocrinology* **103,** 1742 (1978).
7. J. M. Fromson, S. Pearson and S. Bramah, The metabolism of tamoxifen (ICI 46,474). I. In laboratory animals. *Xenobiotica* **3,** 693 (1973).
8. V. C. Jordan, M. M. Collins, L. Rowsby and G. Prestwich, A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. *J. Endocr.* **75,** 305 (1977).
9. V. C. Jordan, Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA-induced rat mammary carcinomata. *Europ. J. Cancer* **12,** 419 (1976).
10. R. I. Nicholson and M. P. Golder, The effect of synthetic anti-oestrogens on the growth and biochemistry of rat mammary tumours. *Europ. J. Cancer* **11,** 571 (1975).
11. V. C. Jordan and L. J. Dowse, Tamoxifen as an antitumour agent: effect on oestrogen binding. *J. Endocr.* **68,** 297 (1976).
12. V. C. Jordan, B. Dixon, G. Prestwich and B. J. Furr, The mode of action of the antitumour agent GP 48989 in the rat. *Europ. J. Cancer* **15,** 755 (1979).
13. C. Huggins, L. C. Grand and P. Brillantes, Mammary cancer induced by a single feeding of polynuclear hydrocarbons, and its suppression. *Nature (Lond.)* **189,** 204 (1961).
14. V. C. Jordan, Antiestrogenic and antitumor properties of tamoxifen in laboratory animals. *Cancer Treat. Rep.* **60,** 1409 (1976).
15. T. L. Dao, The role of ovarian hormones in initiating the induction of mammary cancer in rats by polynuclear hydrocarbons. *Cancer Res.* **22,** 973 (1962).
16. H. Nagasawa, R. Yanai and H. Taniguchi, Importance of mammary gland DNA synthesis on carcinogen induced mammary tumorigenesis in rats. *Cancer Res.* **36,** 2223 (1976).
17. V. C. Jordan and S. Koerner, Tamoxifen as an antitumour agent: role of oestradiol and prolactin. *J. Endocr.* **68,** 305 (1976).
18. V. C. Jordan and C. J. Dix, Effect of oestradiol benzoate, tamoxifen and monohydroxytamoxifen on immature rat uterine progesterone receptor synthesis and endometrial cell division. *J. Steroid. Biochem.* **11,** 285 (1979).
19. T. S. Tamulski, C. E. Morreal and T. L. Dao, Comparative metabolism of 7,12-dimethylbenz(a)anthracene in liver and mammary tissue. *Cancer Res.* **33,** 3117 (1973).
20. V. C. Jordan and G. Prestwich, Effect of non-steroidal antioestrogens on the concentration of rat uterine progesterone receptors. *J. Endocr.* **76,** 363 (1978).
21. L. Terenius, Antioestrogens and breast cancer. *Europ. J. Cancer* **7,** 57 (1971).
22. E. R. DeSombre and L. Y. Arbogast, Effect of the antiestrogen CI628 on the growth of rat mammary tumors. *Cancer Res.* **34,** 1971 (1974).
23. K. D. Schultz, B. Haselmayer and F. Hölzel, The influence of clomid and its isomers on dimethylbenzanthracene induced rat mammary tumours. In *Basic Action of Sex Steroids in Target Organs.* (Edited by P. O. Hubinot, F. Leroy and P. Galand) p. 274, Karger, Basel (1971).
24. T. L. S. Tsai and B. S. Katzenellenbogen, Antagonism of development and growth of 7,12-dimethylbenz(a)anthracene-induced rat mammary tumours by the antiestrogen U23, 469 and effects on estrogen and progesterone receptors. *Cancer Res.* **37,** 1537 (1977).
25. J. C. Heuson, C. Waelbroeck, N. Legros, G. Gallez, C. Robyn and M. L'Hermite, Inhibition of DMBA-induced mammary carcinogenesis in the rat by 2-Br-α ergokryptine (CB154), an inhibitor of prolactin secretion and nafoxidine (U-11,100A), an oestrogen antagonist. *Gynecol. Invest.* **2,** 130 (1971/72).
26. J. Watson, F. B. Anderson, M. Alam, J. E. O'Grady and P. J. Heald, Plasma hormones and pituitary luteinizing hormone in the rat during the early stages of pregnancy and after post-coital treatment with tamoxifen (ICI 46,474). *J. Endocr.* **65,** 7 (1975).

27. J. WATSON and J. W. H. HOWSON, Inhibition by tamoxifen of the stimulatory action of FSH on oestradiol-17β synthesis by rat ovaries *in vitro*. *J. Reprod. Fertil.* **49,** 375 (1977).
28. R. I. NICHOLSON, M. P. GOLDER, P. DAVIS and K. GRIFFITHS, Effects of oestradiol-17β and tamoxifen on total and accessible cytoplasmic oestradiol-17β receptors in DMBA-induced rat mammary tumours. *Europ. J. Cancer* **12,** 711 (1976).
29. R. I. NICHOLSON, P. DAVIS and K. GRIFFITHS, Effects of oestradiol and tamoxifen on nuclear oestradiol-17β receptors in DMBA-induced rat mammary tumours. *Europ. J. Cancer* **13,** 201 (1977).
30. A. MANNI, J. E. TRUJILLO and O. H. PEARSON, Predominant role of prolactin in stimulating the growth of 7,12-dimethylbenz(a)anthracene-induced rat mammary tumor. *Cancer Res.* **37,** 1216 (1977).