ABSTRACT

New tamoxifen analogues were tested for their antiproliferative activity both in vitro and in vivo. Binding studies showed that both 4-iodotamoxifen and pyrrolidino-4-iodotamoxifen and 2.5-fold higher affinities for the estrogen receptor compared with tamoxifen. Pyrrolidino-4-iodotamoxifen was also 1.5-fold more effective in causing inhibition of estrogen-induced growth of MCF-7 cells compared with tamoxifen. 4-iodotamoxifen analogue was similar to tamoxifen in its inhibitory action at 10^-6 M. Antiproliferative activities of these drugs were tested using the nitrosomethylurea-induced rat mammary tumor model. Pyrrolidino-4-iodotamoxifen caused regression in 92% of rats, whereas tamoxifen caused regression in 75% of rats. The agonist activity of the analogues was determined using the immature rat and mouse uterotrophic assays. Both tamoxifen and 4-iodotamoxifen had similar partial agonist activity, and this was greater than that seen with pyrrolidino-4-iodotamoxifen. Furthermore, pyrrolidino-4-iodotamoxifen caused a dose-dependent inhibition of estrogen-induced vaginal cornification, whereas tamoxifen and 4-iodotamoxifen did not. These studies demonstrate that pyrrolidino-4-iodotamoxifen is more effective than tamoxifen in inhibiting tumor regression and that its reduced uterotrophic activity and increased estrogen receptor binding may give it significant clinical advantages over the parent compound.

INTRODUCTION

The use of the antiestrogen, tamoxifen, is well established for the treatment of breast cancer in both advanced disease (1) and adjuvant treatment of primary breast cancer (2-4). The major advantage of tamoxifen over other antiestrogens has been its low incidence of side effects (5). However, there are a number of serious drawbacks with the use of tamoxifen and other antiestrogens, such as clomiphene and nafodixodine; (a) not all estrogen-responsive tumors respond; and (b) duration of action is limited to an average of about 18 mo, and this may be related to the partial agonist activity of the drugs. Clinical evidence of an estrogen-like stimulation of tumors by tamoxifen has been observed in some breast cancer patients starting therapy (6-8). This tumor "flare" seen with tamoxifen appears to be transient and is indicative of the estrogen sensitivity of the tumor; however, tumor regression subsequently occurs in most cases. Furthermore, patients who fail to respond to tamoxifen therapy or gain resistance often subsequently respond to other agents directed at estrogen deprivation, such as aromatase inhibitors (9-11). Tamoxifen-stimulated growth could occur in patients as one form of drug resistance that results in therapeutic failure.

The search for nonsteroidal antiestrogens which lack significant agonist activity has been only partially successful. Those antiestrogens which display weak or no agonist activity, for example, keoxifene and the related compound LY117018, are rapidly conjugated, metabolized, and excreted, which may explain their low potency as antitumor agents in animals (12-14). The antiestrogen MER-25 displays no agonist activity, but the use of this compound has been limited by its low potency and relatively high toxicity (15).

The precise mode of action of antiestrogens in breast cancer is not clearly resolved. The relative importance of estrogenic and antiestrogenic mechanisms in inducing tumor regression remains uncertain (16). Nonetheless, the development of pure antiestrogens which display greater ER binding affinity than present compounds may be valuable in eliminating many of the problems associated with drugs exhibiting partial agonist properties. Comparative studies of a pure antiestrogen and tamoxifen may also provide a clearer understanding of the mode of action of the latter compound in breast cancer patients.

In this paper, we describe the properties of a novel tamoxifen analogue which has improved ER binding compared with that of tamoxifen and has a greater antiproliferative activity both in vitro and in vivo. Furthermore, it appears to show reduced agonist activity in the immature rat and mouse uterotrophic assays.

MATERIALS AND METHODS

Animals

Inbred virgin female (Ludwig/Wistar/Olac) rats bearing tumors induced with NMU were supplied by Olac, Oxon, United Kingdom.

Chemicals

The antiestrogens used in this study were tamoxifen [111-trans-1-[4-(2-dimethylamino)ethoxy]phenyl]-1,2-diphenyl-buten-1], 4-iodotamoxifen [4-iodophenyl)-1-[4-(2-pyrrolidinoethoxy)phenyl]-2-phenyl-1-buten-1], and pyrrolidino-4-iodotamoxifen [E-1-[4-(4-iodophenyl)-1-[4-(2-pyrrolidinoethoxy)phenyl]-2-phenyl-1-buten-1]. Structures are illustrated in Fig. 1.

Estrogen Receptor Binding Assay

Competitive binding assays to measure the relative affinity of antiestrogens for rat uterine ER followed the method described by Wakeling (19). All stock antiestrogens were prepared in absolute ethanol, and 10-fold dilutions were made in 10 mM phosphate buffer, pH 7.4, containing 1.5 mM EDTA, 2.0 mM 2-mercaptoethanol, and 0.1% bovine serum albumin. Cytosols were prepared from mature rat uterus by homogenization in the above-described 10 mM phosphate buffer, pH 7.4, and centrifuged at 100,000 × g for 1 h at 4°C. Aliquots of cytosol (100 μl) were incubated with 50 μl of 10 nM 17β-[2,4,6,7-3H]estradiol (85 to 110 Ci/mmol; Amersham, United Kingdom) in the absence or presence of an increasing concentration of drug (in 50 μl) for 16 h at 4°C. After incubation, a suspension (200 μl) of dextran-coated charcoal [0.5% dextran T-70:5% Norit A charcoal (w/v) in 10 mM phosphate buffer, pH 7.4, without 0.1% bovine serum albumin] was added, and incubation was continued for 15 min at 4°C. After removal of charcoal by centrifugation, the supernatant was extracted with n-butanol, and radioactivity was determined by liquid scintillation counting.

The abbreviations used are: ER, estrogen receptor; NMU, nitrosomethylurea; PIT, pyrrolidino-4-iodotamoxifen; 4-IT, 4-iodotamoxifen; i.m., intramuscular; E2B, estradiol benzoate.

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3 The abbreviations used are: ER, estrogen receptor; NMU, nitrosomethylurea; PIT, pyrrolidino-4-iodotamoxifen; 4-IT, 4-iodotamoxifen; i.m., intramuscular; E2B, estradiol benzoate.
Cell Growth Studies

MCF-7 cells were obtained from P. Dabre (Imperial Cancer Research Fund, London, England), and MDA-MB-231 cells were obtained from M. O'Hare (Institute of Cancer Research, Sutton, Surrey, United Kingdom). All stock cells were grown in Dulbecco's modified Eagle's medium with phenol red (Gibco), 2 mM L-glutamine (Sigma, Poole, United Kingdom), 10 nM estradiol (Sigma), 10 µg/ml of insulin (Sigma), plus 5% charcoal-stripped fetal calf serum for 7 days. In all experiments, 3 x 10^5 cells/well (unless otherwise stated) were seeded into 24-well plates and left to settle for 24 h. The medium was removed and replaced with fresh medium containing the appropriate drug at the stated concentration. Cells were treated with drug for 7 days, with medium (containing appropriate drug) changed every 2 days. Cells were harvested by trypsinization (1% trypsin/versene solution) and counted using trypan blue (Sigma) on Days 3 and 7. Data were tested for statistical significance using Student's t test.

In Vivo Studies

The NMU-induced rat mammary tumor model was used as described previously (20). In all studies, adult rats bearing tumors between 10 and 20 mm in diameter were randomized. All rats were treated with daily i.m. injections of drugs or vehicle 5 days/wk for 4 wk. Tumor measurements were made weekly for 4 wk by measuring two diameters at right angles with Vernier callipers. Tumor volume was estimated using the following formula

\[
\frac{\pi}{6} d_1 \cdot d_2 \cdot d_3 \sqrt{3}
\]

At the end of the 4-wk period, all palpable lesions were removed from the mammary area and stored in liquid nitrogen.

Uterotrophic Studies

The method used has been previously described in detail (21). In brief, immature female Wistar rats (19 to 21 days, 40 to 50 g) were obtained from the breeding unit at St George's Hospital Medical School and randomized into 5 rats/treatment group. All drugs used in the study were dissolved in peanut oil (Sigma, United Kingdom) prior to injection. Rats were dosed s.c. daily for 4 days. On Day 5 rats were sacrificed, the uteri were removed and dissected free of extraneous tissue, fluid content was expelled, tissues were blotted, and the wet weight was recorded.

The effect of tamoxifen, 4-IT, and PIT on uterine weight in immature mice was also investigated as previously described (22). Immature female MF-1 mice (21 days, 10 to 11 g) were obtained from the breeding unit at St George's Hospital Medical School. Animals were randomized into 5 mice/treatment group. All drugs used in the study were dissolved in peanut oil and dosed s.c. daily for 3 days. On Day 4, mice were sacrificed, and the uteri were removed and dissected free of extraneous tissue, fluid content was expelled, tissues were blotted, and the wet weight was recorded.

Vaginal Cornification Studies

The vaginal cornification assay has been described by Harper and Walpole (23). In brief, adult female Wistar rats were ovariectomized 14 days prior to the start of the experiment. On Day 15, rats were randomized into 2 experimental groups for the assessment of (a) estrogenic activity and (b) antiestrogenic activity.

Estrogenic Activity. Rats were randomized into 5 animals/group and dosed s.c. for 3 days. Vaginal cornification was assessed by taking smears each day from each rat for 5 days. Smears were classified as cornified when composed of cornified and/or nucleated epithelial cells with no more than the occasional leukocyte.

Antiestrogenic Activity. Rats were randomized into 5 animals/group. Each group received 1.25 µg/kg of estradiol plus appropriate drug s.c. Dosing was carried out for 7 days, and vaginal smears were examined daily from the first to eighth day of the experiment. Control animals received 1.25 µg/kg of estradiol only. The appearance, during this time, of leukocytes in the smears from animals given the analogues was taken as an index of their antiestrogenic effect.

RESULTS

Estrogen Receptor Binding Assays. Estrogen receptor binding affinities of the new antiestrogens were compared to that of estradiol (•), tamoxifen (A), 4-IT (V), and PIT (♦). Each point represents the mean of 3 determinations. Bars have not been included because they were smaller than the data symbols.
Fig. 3. Effect of antiestrogens on the growth of MCF-7 cells. A total of 3 x 10^4 MCF-7 cells/well were treated with an increasing concentration of drugs ranging from 10^-8 M to 10^-4 M. a, tamoxifen; b, 4-IT; c, PIT. d shows the effect of estradiol (10^-8 M) and control (0.1% ethanol) in the absence of added drug. Medium was changed every 2 days. Cell counts were made on 3 days (□) and 7 days (■). Columns, mean of triplicate determinations; bars, SEM.

Fig. 4. Effect of antiestrogens on the estrogen-stimulated growth of MCF-7 cells. A total of 3 x 10^4 MCF-7 cells/well were treated with medium containing 10^-8 M estradiol and a increasing concentration of drug ranging from 10^-8 M to 10^-4 M. a, tamoxifen; b, 4-IT; c, PIT; d, estradiol alone (10^-8 M) and control (0.1% ethanol) in the absence of added drug. Medium was changed every 2 days. Cell counts were made on 3 days (□) and 7 days (■). Columns, mean of triplicate determinations; bars, SEM.
Table 1 Effect of antiestrogens on the growth of MDA-MB-231 cells

Three x 10^4 MDA-MB-231 cells/well were treated with medium containing vehicle alone (control, 0.1% ethanol), estradiol (10^-8 M), or estradiol plus analogues at 10^-8 M. Medium was changed every 2 days.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of initial tumors (≥10 mm)</th>
<th>No. of new tumors</th>
<th>No. of rats showing total tumor progression &gt;50%</th>
<th>% of rats showing any tumor regression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>18</td>
<td>12</td>
<td>1</td>
<td>16.7 (58.3)</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>12</td>
<td>12</td>
<td>1</td>
<td>16.7 (58.3)</td>
</tr>
<tr>
<td>4-IT</td>
<td>12</td>
<td>12</td>
<td>1</td>
<td>16.7 (58.3)</td>
</tr>
<tr>
<td>PIT</td>
<td>12</td>
<td>12</td>
<td>1</td>
<td>16.7 (58.3)</td>
</tr>
</tbody>
</table>

Table 2 Dose-response of PIT on the growth of NMU-induced rat tumors

All rats were given daily i.m. doses of 1.0 mg/kg of drug dissolved in peanut oil or vehicle (control) for 5 days/wk for 4 wk. Tumor growth was recorded every wk for the 4-wk period, by measuring two diameters at right angles with Vernier calipers. Tumor volume was estimated using the following formula

\[
\text{Tumor volume (cm}^3\text{)} = \left[\left(\frac{d_1}{2}\right) \times \left(\frac{d_2}{2}\right) \times \pi\right]
\]

Each of the animals was categorized into one of three groups: (a) those with 50% or greater inhibition; (b) those with 0 to 50% inhibition; or (c) those with no increase.

Table 3 Comparison of tumor response rates with tamoxifen, 4-IT, and PIT using the NMU-induced rat mammary tumor model

All rats were given daily i.m. doses of 1.0 mg/kg of drug dissolved in peanut oil or vehicle (control) for 5 days/wk for 4 wk. Tumor growth was recorded every wk for the 4-wk period, by measuring two diameters at right angles with Vernier calipers. Tumor volume was estimated using the following formula

\[
\text{Tumor volume (cm}^3\text{)} = \left[\left(\frac{d_1}{2}\right) \times \left(\frac{d_2}{2}\right) \times \pi\right]
\]

Each of the animals was categorized into one of three groups: (a) those with 50% or greater regression; (b) those with 0 to 50% regression; or (c) those with no increase.

Reversal of Antiestrogen Inhibition by Estradiol. The inhibitory effects of the antiestrogens on the estrogen-stimulated growth of MCF-7 cells is shown in Fig. 4, a to c. Again, a dose of 10^-5 M was cytotoxic after 3 days of treatment. Maximal growth inhibition was seen at 10^-6 M after 7 days of treatment. At this dose, tamoxifen and 4IT produced 44% and 48% inhibition, respectively, of the estradiol-induced proliferation; PIT produced 65% inhibition. There was no significant difference between tamoxifen and 4IT in their levels of inhibition at a dose of 10^-6 M (P = 0.155). However, a comparison between tamoxifen and PIT showed that the latter was a significantly more effective inhibitor (by 1.5-fold) than tamoxifen (P = 0.006). At doses of 10^-7 M and 10^-8 M, tamoxifen and 4-IT did not have much effect on the estradiol-stimulated growth of MCF-7 cells. However, PIT was still effective and produced 51% inhibition at 10^-7 M but was without effect at 10^-8 M.

Reversal of Antiestrogen Inhibition by Estradiol. The inhibitory effects of the antiestrogens on the estrogen-stimulated growth of MCF-7 cells after 3 and 7 days. At 10^-5 M, all analogues were completely cytotoxic compared with the control. Receptor binding curves for the various agents are shown in Fig. 2. These were used to calculate the relative binding affinities. Tamoxifen, 4IT and PIT had ER binding affinities of 5, 12.5, and 12.5, respectively, compared with that of estradiol. The analogues 4-IT and PIT both showed 2.5-fold greater affinity for ER compared with tamoxifen.

Effect of Analogues on the Growth of Breast Cancer Cells in Vitro. Fig. 3, A to C, shows the effect of varying concentrations of the analogues alone on the growth of MCF-7 cells after 3 and 7 days. At 10^-5 M, all analogues were completely cytotoxic after 3 days of treatment. The highest level of inhibition, compared with that of the control, for all the analogues was observed at 10^-6 M after 3 days of treatment (tamoxifen, 38.7%; 4-IT, 35.2%; PIT, 58.7%). After 7 days of treatment at 10^-6 M there was a reduced but significant inhibition produced by all the analogues, compared with the control (P = >0.001). At doses of 10^-6 M and 10^-7 M after both 3 and 7 days of treatment there was reduced by significant inhibition of growth. Although PIT showed a reduction in the level of inhibition at 10^-7 and 10^-8 M after 3 and 7 days, these levels were significantly different from those of tamoxifen and 4-IT (P = >0.001). With estradiol alone (Fig. 3d), a 2.4-fold increase in cell number was observed compared with the control.
NEW ANTIESTROGENS FOR THE TREATMENT OF BREAST CANCER

91.7% of rats at both 1-mg and 2.0-mg/kg doses. This was significantly different from the control (1.0 mg, \( P = 0.006; 2.0 \text{ mg}, \ P = 0.0007\)). In a third comparative study among tamoxifen, 4-IT, and PIT (Table 3) at doses of 1 mg/kg all three drugs inhibited tumor progression compared with the control at 28 days (Kruskal-Wallis = \( H = 8.61\), \( P < 0.02\)). The greatest percentage of reduction in tumor volume was achieved in the order: PIT > tamoxifen > 4-IT. Although there was no significant difference between tamoxifen and PIT in decreasing total tumor volume, in this particular study PIT was effective in causing some degree of tumor regression in all rats, whereas tamoxifen caused tumor regression in only 75% of animals; the remaining 25% of rats showed tumor progression. Overall in the three studies, PIT caused regression in 92% of rats, which was significantly greater than that caused by tamoxifen.

Uterotrophic and Antiuterotrophic Study. The effect of the various agents, either alone or together with E2B, on uterine weight in immature rats is illustrated in Fig. 6. E2B alone produced a 3.7-fold increase in uterine weight. Tamoxifen given alone s.c. at doses up to 10 mg/kg stimulated an average uterine growth of 123 mg/100 g of body weight compared with the control of 65.2 mg/100 g of body weight (1.9-fold increase). When administered together with E2B it failed to inhibit completely the uterotrophic action of E2B. The 4-IT analogue also resulted in partial agonist effects, as an increase in uterine weight of 108 mg/100 g of body weight was recorded at the highest dose compared with the control 1.7-fold increase). However, the increase in weight with 4-IT was less than that observed with tamoxifen. Doses of 1 and 10 mg/kg of PIT alone both resulted in a 1.3-fold increase in uterine weight compared with the control (1 mg/kg, \( P = 0.018; 10 \text{ mg/kg}, \ P = 0.006\)). When given with E2B it produced a dose-dependent

Table 4 Effect of 4-IT and PIT on tamoxifen uterotrophic activity in immature rats

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Uterine wt (mg/100 g of body wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>66.7 ± 3.5*</td>
</tr>
<tr>
<td>Estradiol (0.5 μg/kg)</td>
<td>204.8 ± 5.8</td>
</tr>
<tr>
<td>Drugs alone (mg/kg)</td>
<td></td>
</tr>
<tr>
<td>Tamoxifen</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>126.8 ± 1.6</td>
</tr>
<tr>
<td>10</td>
<td>130.7 ± 2.2</td>
</tr>
<tr>
<td>4-IT</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>103.9 ± 3.1</td>
</tr>
<tr>
<td>10</td>
<td>115.5 ± 3.5</td>
</tr>
<tr>
<td>PIT</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>97.0 ± 0.6</td>
</tr>
<tr>
<td>10</td>
<td>96.1 ± 1.1</td>
</tr>
<tr>
<td>Tamoxifen (1 mg/kg) + 4-IT (mg/kg) at doses given below</td>
<td></td>
</tr>
<tr>
<td>0.01</td>
<td>120.1 ± 5.8</td>
</tr>
<tr>
<td>0.1</td>
<td>105.4 ± 1.9</td>
</tr>
<tr>
<td>1.0</td>
<td>103.9 ± 4.3</td>
</tr>
<tr>
<td>10.0</td>
<td>107.8 ± 5.6</td>
</tr>
<tr>
<td>Tamoxifen (1 mg/kg) + PIT (mg/kg) at doses given below</td>
<td></td>
</tr>
<tr>
<td>0.01</td>
<td>120.5 ± 4.0</td>
</tr>
<tr>
<td>0.1</td>
<td>107.5 ± 4.1</td>
</tr>
<tr>
<td>1.0</td>
<td>103.6 ± 5.9</td>
</tr>
<tr>
<td>10.0</td>
<td>100.2 ± 1.8</td>
</tr>
</tbody>
</table>

* Mean ± SEM of 5 determinations.

growth could be reversed by removing the drug after 3 days (Fig. 5) and treating with \( 10^{-8} \) M estradiol alone for 4 days. This indicated that the inhibitory effect of the antiestrogens was reversible.

Effect of Antiestrogens on MDA-MB-231 Cells. The effect of the various drugs on the growth of the ER-negative cell line MDA-MB-231 is illustrated in Table 1. None of the compounds had any effect on cell numbers after 7 days compared with untreated controls. No growth stimulation of MDA-MB-231 was observed with estradiol.

In Vivo Studies. The NMU-induced rat mammary tumor model was used to investigate the antiproliferative activities of the various antiestrogens. In a preliminary single-dose study, PIT produced tumor regression in 83% of rats. In a subsequent dose-response experiment (Table 2), PIT caused regression in

![Fig. 6. Uterotrophic/antiuterotrophic responses in immature rats. Rats received 4 daily s.c. doses of vehicle alone (lower open bar), 0.5 μg/kg of E2B (upper hatched bar), or increasing doses of drug alone (broken line) or together with E2B (solid line). Each point is the mean of 5 determinations. a. tamoxifen; b. 4-IT; c. PIT.](image-url)
inhibition of the uterotrophic action of E\textsubscript{2}B. At 10 mg/kg, PIT completely blocked the effect of E\textsubscript{2}B. This study showed that PIT had the lowest uterotrophic activity, which was significantly lower by 1.4- and 1.27-fold compared with tamoxifen and 4-IT, respectively (\(P = 0.030\)).

The ability of 4-IT and PIT to inhibit tamoxifen uterotrophic activity was investigated, and the results are shown in Table 4. Although both PIT and 4-IT showed some effect on inhibiting tamoxifen-stimulated uterine weight, this was not significant (\(P = 0.3\) to 0.9 between 1 and 10 mg/kg, respectively).

Uterotrophic Activity in Immature Mice. The uterotrophic activities of the various antiestrogens are illustrated in Fig. 7. Tamoxifen alone was highly uterotrophic at 0.1 mg/kg, whereas PIT and 4-IT showed minimal uterotrophic activity at this dose but were highly uterotrophic at 10 mg/kg. Both tamoxifen and 4-IT were unable to block the E\textsubscript{2}B effect. PIT, however, showed a dose-dependent inhibition of the E\textsubscript{2}B stimulation but was unable to block the effect completely at the highest dose.

Vaginal Cornification in Adult Ovariectomized Rats. Tables 5 and 6 display the results of the vaginal cornification studies. Tamoxifen displayed estrogenic activity as indicated by vaginal cornification at 0.5 mg/kg and was completely cornifying at 20 mg/kg. However, in contrast, 4-IT and PIT did not show any estrogenic activity at 0.5 mg/kg but did so at 5 mg/kg. Full cornification was observed at 50 mg/kg with 4-IT and PIT.

Control animals, given estradiol only, showed cornified smears from the fourth to the eighth day inclusive. At doses much below those required to induce vaginal cornification, tamoxifen and 4-IT both displayed antiestrogenic activity. They produced a dose-dependent inhibition of estradiol stimulation but were unable to completely block the effect at 10 mg/kg. However, PIT was able to completely block the estradiol-induced cornification in a dose-dependent manner. Inhibition caused by PIT at 10 mg/kg was significantly different from tamoxifen and 4-IT (\(P = 0.0009\)).

DISCUSSION

The agents described here all belong to the triphenylethylene group of antiestrogens. The data presented in this study indicate

}\textbf{Table 5 Estrogenic activity of antiestrogens using the rat vaginal cornification assay}

<table>
<thead>
<tr>
<th>Drug dose (mg/kg/day)</th>
<th>No. of smears showing cornified epithelial cells/rat group from Days 3 to 5 inclusive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tamoxifen</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>5/15 (33.3%)(^{a,b})</td>
</tr>
<tr>
<td>5.0</td>
<td>8/15 (53.3%)(^{c})</td>
</tr>
<tr>
<td>20.0</td>
<td>15/15 (100)(^{c})</td>
</tr>
<tr>
<td>50.0</td>
<td>15/15 (100)</td>
</tr>
<tr>
<td>4-IT</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>0/15 (0)(^{c})</td>
</tr>
<tr>
<td>5.0</td>
<td>4/25 (16)(^{c})</td>
</tr>
<tr>
<td>20.0</td>
<td>10/15 (66.6)%(^{c})</td>
</tr>
<tr>
<td>50.0</td>
<td>15/15 (100)</td>
</tr>
<tr>
<td>PIT</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>0/15 (0)(^{c})</td>
</tr>
<tr>
<td>5.0</td>
<td>5/15 (33.3%)(^{c})</td>
</tr>
<tr>
<td>20.0</td>
<td>10/15 (66.6)%(^{c})</td>
</tr>
<tr>
<td>50.0</td>
<td>15/15 (100)</td>
</tr>
<tr>
<td>Control Estradiol (1.25 (\mu)g/kg/day)</td>
<td>0/15 (0)</td>
</tr>
<tr>
<td></td>
<td>15/15 (100)</td>
</tr>
</tbody>
</table>

\(^{a}\) Numbers in parentheses, percentage.

\(^{b}\) 4-IT and PIT are different from tamoxifen at 0.5 mg/kg (\(P = 0.014\)).

\(^{c}\) No difference between any analogue at 5 mg/kg (\(P = 0.27\)).

\(^{d}\) 4-IT and PIT are different from tamoxifen at 20 mg/kg (\(P = 0.014\)).

\(5856\)
that binding to the ER can be increased by modifying the parent tamoxifen molecule. The two analogues tested both included an iodine atom at position 4 which has been shown to increase the affinity for the ER (17). This modification was designed to produce a tamoxifen analogue which would have similar properties to those of 4-hydroxytamoxifen. This is a metabolite of tamoxifen which has about 100 times greater potency in vitro than tamoxifen but is rapidly inactivated in vivo by glucuronidation of the hydroxyl group (24, 25). It was expected that the iodine would improve the potency of tamoxifen in vitro because of an increased resistance to metabolism, since the iodine-containing compound cannot undergo glucuronidation at position 4. An additional theoretical advantage of this modification is that the isomerization of trans-4-hydroxytamoxifen to the cis-form, which is associated with the estrogen agonist property of tamoxifen, cannot occur in the iodine-substituted molecule.

Our receptor binding studies confirm earlier results of increased ER binding of 4-iodo-substituted derivatives of tamoxifen (17). The difference between 4-IT and PIT is that the latter agent has a pyrrolidino group instead of the dimethylamino group found in the parent compound. The original aim of this replacement was to prevent the conversion by the liver of the dimethylamino group to give desmethyl metabolite, which results in the release of formaldehyde, which cannot occur with the pyrrolidino group. It is interesting to note that the LY117018 which has low agonist activity also contains the pyrrolidino substituent (26).

Effects on breast cancer cells in vitro indicated that PIT was at least 1.5-fold more effective in causing inhibition of estrogen-induced cell growth compared with tamoxifen at a dose of 1 μM. The 4-IT analogue at 1 μM had no additional advantage over tamoxifen in this respect. Having demonstrated that the new analogues bound to the ER and inhibited growth of breast cancer cells, it was important to establish their effects in vivo. These studies showed that 4-IT produced the lowest level of regression of endocrine-dependent rat mammary tumors. There was no statistical difference between tamoxifen and PIT in their ability to cause regression of tumors. However, in one particular study, it was observed that tamoxifen caused only 75% of rats to show tumor regression, whereas PIT caused some tumor regression in 100% of the rats. It is possible that the ability of this drug to cause regression of all the animal tumors could give it an important advantage in the future use of the compound in the clinical management of breast cancer.

The uterotrophic studies indicated that both tamoxifen and 4-IT had partial agonist activity. The interesting finding from this study was that PIT showed reduced agonist activity compared with that of tamoxifen and 4-IT. This may be at least partly responsible for its apparent improved efficacy in the animal studies. Uterotrophic studies in immature mice demonstrated that both 4-IT and PIT were only uterotrophic at a dose which was 10 times higher than for tamoxifen. However, 4-IT was unable to block the E2B effect, but PIT in this respect showed some antiuterotrophic activity. The reduced agonist activity of 4-IT and PIT was further demonstrated in the vaginal cornification studies. In this case 4-IT and PIT had agonist effects at a dose 10 times higher than with tamoxifen. In the antiuterotrophic study, PIT produced a dose-dependent inhibition of estradiol stimulation, whereas tamoxifen and 4-IT were unable to block the estradiol-stimulated vaginal cornification.

Our results are consistent with those of Allen et al. (27), who showed that para-halogenated derivatives of tamoxifen show reduced uterotrophic activity compared with tamoxifen. The only antiestrogen so far described which lacks agonist activity is the steroidal antiestrogen ICI 164 384 (28). Previous comparative studies of the uterotrophic and antiuterotrophic action of antiestrogens with different degrees of intrinsic estrogenic activity have clearly shown that their gross effect on the uterus represents a balance between estrogenic and antiestrogenic activity (29, 30). A compound devoid of estrogenic activity should thus be capable of complete antagonism of estrogen action. This appears to be the case with ICI 164 384. The advantage of the tamoxifen analogue, PIT, over LY117018 and nafoxidine, which also contain the pyrrolidino group, is that the latter are rapidly metabolized (31). It has recently been shown that both 4-IT and PIT are metabolized 4 times more slowly than tamoxifen by rat hepatocytes.4 PIT may also have some advantages over the ICI 164 384 compound in terms of slow metabolic removal. Although PIT did show some agonist activity, its marked inhibitory effect on tumor growth shows favorable comparison with the ICI 164 384 compound (32).

The precise mode of action of pure antiestrogens remains to be determined but probably involves the blocking of the TAF2 transcriptional activation function of the ER (33; for review, see Ref. 34). Since PIT binds to the ER with greater affinity than tamoxifen, there should be a greater reduction in ER transcriptional activation than is seen with tamoxifen. Studies are currently in progress with PIT to assess its effects on the levels of estrogen-induced transcripts.

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