Cross-Resistance of Triphenylethylene-type Antiestrogens but not ICI 182,780 in Tamoxifen-stimulated Breast Tumors Grown in Athymic Mice

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ABSTRACT

The triphenylethylene antiestrogens, idoxifene (Idox) and toremifene (Tor), are structurally related analogues of tamoxifen (Tam) and were developed to improve the therapeutic index for advanced breast cancer patients. However, the issue of cross-resistance with Tam for these new agents is critical for clinical testing because the majority of breast cancer patients have already received or failed adjuvant Tam. The goal of this study was to determine the effectiveness of Idox as an antitumor agent in three models of Tam-stimulated breast cancer in athymic mice and compare the results with the actions of Tor and ICI 182,780 in a Tam-stimulated MCF-7 tumor model. We first compared the activities of Tam and Idox in the 17β-estradiol (E2)-stimulated MCF-7 tumor line MT2:E2. Tam and Idox reduced E2-stimulated growth by 65–70% (week 9: P = 0.0009 for Tam, P = 0.0005 for Idox versus E2 alone). However, Tam (1.5 mg daily) and Idox (1.0 mg daily) both produced T47D breast tumors in athymic mice during 23 weeks of treatment (12 tumors/22 sites and 15 tumors/18 sites, respectively). Tam and Idox stimulated tumor growth equally in two different Tam-stimulated MCF-7 models and in a T47D model. Tor was completely cross-resistant with Tam in the MCF-7 tumor model, which implied that neither Idox nor Tor would be effective as a second-line endocrine therapy after Tam failure and may offer no therapeutic advantages over Tam as adjuvant therapies. In contrast, ICI 182,780, a pure antiestrogen currently being tested as a treatment for breast cancer after Tam failure, had no growth-stimulatory effect on the MCF-7 Tam-stimulated breast tumor line. This agent may provide an advantage as an adjuvant therapy and increase the time to treatment failure.

INTRODUCTION

Tam5 is the endocrine treatment of choice for all stages of ER-positive breast cancer (1, 2) and the first agent used to reduce the incidence of breast cancer in high-risk women (3). Although Tam is proven to provide survival advantages after adjuvant therapy, the development of drug resistance is a potentially limiting factor to extending therapy and to enhancing the beneficial actions of the drug (4).

 Clearly, any new antiestrogen that is developed for breast cancer therapy must not produce premature drug resistance and must have a better toxicological profile than Tam. A new antiestrogen that has the advantage of being used for extended therapy (>5 years) and has fewer serious side effects than Tam would have broad applications as a treatment and preventative. Thus, a new antiestrogen must be proven to be superior to Tam in the laboratory before a commitment can be made for widespread clinical testing. However, any new agent must be tested clinically in a population of patients with recurrent breast cancer who may already have been exposed to Tam during adjuvant therapy. As a result, there is a high probability that the disease will already be resistant to Tam. The issue of cross-resistance for the new agent will be critical for the success of clinical testing and ultimately the advancement of the drug for testing as an adjuvant therapy.

The triphenylethylene derivatives, Idox and Tor, are structurally related analogues of Tam and have been studied to improve the therapeutic index (5). Idox differs from Tam in that it contains an iodine atom located at position 4. This substitution affords this compound a higher affinity for the ER and higher metabolic stability than Tam (6, 7). There is evidence that Idox is more effective than Tam as adjuvant therapies. In contrast, ICI 182,780, a pure antiestrogen currently being tested as a treatment for breast cancer after Tam failure, had no growth-stimulatory effect on the MCF-7 Tam-stimulated breast tumor line. This agent may provide an advantage as an adjuvant therapy and increase the time to treatment failure.

5 The abbreviations used are: Tam, tamoxifen; ER, estrogen receptor; E2, 17β-estradiol; Idox, idoxifene; Tor, toremifene.
analouges of Tam with less agonist effect are effective at inhibiting the growth of Tam-resistant tumors.

Tor, a chlorinated derivative of Tam, is approved for the treatment of advanced breast cancer in postmenopausal women (12) and has been evaluated as an adjuvant therapy (13). Tor has a similar pharmacological profile to Tam, but it is less potent. Tor is recommended at a dose of 60 mg daily, whereas Tam is recommended at a dose of 20 mg daily. The primary reason for developing Tor was to assuage concerns about rat liver carcinogenesis with Tam (14). An investigation of the mechanism of carcinogenesis suggests that the metabolic pathways necessary to induce DNA adducts are specific to the rat (15); therefore, the results from these laboratory studies may have little relevance for humans.

We have developed a series of human models of drug resistance to Tam in the laboratory that may mimic drug resistance to Tam in the clinic (16–19). The initial goal of this study was to compare and contrast the effectiveness of Idox as an antitumor agent in the MCF-7 and T47D models of Tam-stimulated breast cancer. Our aim was not only to test the veracity of the view (10) that Idox is not cross-resistant with Tam in the clinic (16–19). The initial goal of this study was to test the veracity of the view (10) that Idox is not cross-resistant with Tam so that clinical testing could proceed but also to build on our experience in predicting clinical outcomes. Where possible, we have compared our findings with Tor and the pure antiestrogen ICI 182,780. The pure antiestrogen is not cross-resistant with Tam (20) and is showing promise as a new treatment after the development of drug resistance to Tam-stimulated cancers (21).

**MATERIALS AND METHODS**

**Athymic Mouse Model.** The MCF-7 and T47D tumors used in these experiments originated from an inoculation of MCF-7 or T47D cells (American Type Culture Collection) into the mammary fat pads of old ovariectomized BALB/c nu/nu athymic mice (Harlan Sprague Dawley, Madison, WI), 4–6 weeks of age, as described previously (16, 19). The MT2 tumors were derived from MCF-7 breast cancer cells that had been injected into athymic mice and serially passaged with E$_2$ or Tam capsule. The tumors contain a point mutation in the ligand domain of the ER, which results in a tyrosine for aspartate substitution at amino acid 351 (17). The characteristics of the individual tumors are summarized in Table 1.

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Derived from</th>
<th>E$_2$-stimulated</th>
<th>Tam-stimulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7/Tam</td>
<td>MCF-7 cells</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>MT2:E$_2$</td>
<td>MCF-7 cells</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>MT2/Tam</td>
<td>MCF-7 cells</td>
<td>Yes</td>
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<td>T47D:E$_2$</td>
<td>T47D cells</td>
<td>Yes</td>
<td>No</td>
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<tr>
<td>T47D/Tam</td>
<td>T47D cells</td>
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Hormone and Drug Treatments. Mice were divided into groups of 10 and were treated with E$_2$, antiestrogen, or combinations. Silastic E$_2$ capsules (0.3 or 1 cm in length) were made as described previously (22), implanted s.c., and replaced after 8–10 weeks of treatment. The 0.3-cm E$_2$ capsule produced a mean of 83.8 pg/ml of serum E$_2$, whereas the 1.0-cm capsule produced 379.5 pg/ml of serum E$_2$ (23). Each was designed to represent the E$_2$ levels observed in post- or premenopausal women, respectively. Tam (Sigma Chemical Co., St. Louis, MO), Tor (a gift from Orion Pharmaceutical, Oula, Finland), or Idox (a gift from Smith Kline Beecham, Philadelphia, PA) were first dissolved in ethanol and suspended in a solution of 90% carboxymethylcellulose (1% carboxymethylcellulose in double-distilled water) and 10% polyethylene glycol 400/Tween 80 (99.5% polyethylene glycol and 0.5% Tween 80). Ethanol was evaporated under nitrogen before use. Tam, Tor, or Idox was administered p.o. at various doses: 0.5, 1.0, or 1.5 mg/mouse/day 5 days/week. ICI 182,780, a generous gift from Zeneca Pharmaceuticals, was dissolved in ethanol and suspended in peanut oil to a final concentration of 50 mg/ml. Ethanol was evaporated under nitrogen. ICI 182,780 was injected s.c. at a dose of 5 mg/mouse once a week.

**Tumor Measurements.** Tumor measurements were performed weekly using Vernier calipers. The cross-sectional area was calculated using the formula: length $\times$ width/4 $\times$ $\pi$.

**Statistical Analysis.** Comparisons in mean tumor between the animal groups were analyzed by ANOVA at each week and followed by unpaired Student’s t test. The two tailed $P$ of the last week of each experiment was reported using StatMost 2.5 (Datamost Corp., Salt Lake City, UT).

**RESULTS**

An initial study was carried out to determine the dose of Idox equivalent to that of Tam, which has been well established in our athymic nude mice model. Because Idox is metabolized at a 2–3-fold slower rate than Tam in the rat (6, 7), the same dose of Idox, such as 0.5 or 1.5 mg/day, is more than enough to compare with these doses of Tam for effectiveness. However, our early experiments performed with 1.5 mg of Idox showed that this high dose is lethally toxic to the mice. Thus, we treated mice with 0.5 mg/day for low-dose Idox or 1.0 mg/day for high-dose Idox.

To determine whether Idox inhibits the tumor growth more effectively than Tam, we implanted 50 mice with MT2:E$_2$ tumors that grew with E$_2$ and were inhibited by Tam. The animals were divided into a control (no drug) and four treated groups. The four treated groups of mice were implanted with 0.3-cm E$_2$ capsules and treated with 0.5 mg Tam, 0.5 mg Idox, 1.0 mg Idox, or E$_2$ capsules alone for 9 weeks. As shown in Fig. 1, the abilities of Tam and Idox to inhibit tumor growth were almost identical. Both drugs reduced 65–70% of tumor growth at a 2–3-fold slower rate than Tam in the rat (6, 7), the same dose of Idox, such as 0.5 or 1.5 mg/day, is more than enough to compare with these doses of Tam for effectiveness. However, our early experiments performed with 1.5 mg of Idox showed that this high dose is lethally toxic to the mice. Thus, we treated mice with 0.5 mg/day for low-dose Idox or 1.0 mg/day for high-dose Idox.

In the next experiment, we determined whether high-dose Idox is capable of stimulating tumor growth to the same extent as high-dose Tam in our previous Tam-stimulated T47D tumors (19). T47D:E$_2$ tumors were transplanted into 40 mice, which
were divided into four groups: control, 1-cm E2 capsule, 1.5-mg Tam, or 1.0-mg Idox (Fig. 2). Over the course of 8 weeks, the E2 group grew robustly, whereas neither Tam nor Idox groups grew. The experiment was extended to 23 weeks, where 7 and 4 large tumors (>0.5 cm^2) grew in the Tam (12 of 22 sites) and Idox (15 of 18 sites) groups, respectively. There was no significant difference between the mean tumor size or tumor incidence (P = 0.3662).

To verify whether Idox is effective after Tam failure in advanced breast cancer, we tested the cross-resistance between Idox and Tam in our Tam-stimulated tumor models: MT2:Tam, MCF-7:Tam, and T47D:Tam. Then, we extended this cross-resistance experiment with Tor or with the pure antiestrogen ICI 182,780.

For the evaluation of the impact of Idox on the growth of Tam-stimulated breast tumors, all three tumors were tested. In the first experiment, 40 athymic mice were implanted with MT2:Tam and treated with 0.5 mg Tam, 0.5 mg Idox, or 1.0 mg Idox, or control (no drug). As expected, Tam resulted in rapid growth of these tumors, which reached an average tumor area of 1 cm^2 by 9 weeks (Fig. 3A). Interestingly, 0.5 mg (P = 0.4346) or 1.0 mg (P = 0.8947) Idox produced a similar growth rate compared with Tam. The same experiment was performed using the MCF-7:Tam tumors implanted into 30 athymic mice (Fig. 3B). High-dose Tam or Idox stimulated growth of MCF-7:Tam tumors at a similar rate by 12 weeks (mean tumor area ± SE: Tam, 0.93 ± 0.12 versus Idox, 0.73 ± 0.08 P = 0.23), whereas tumors in the control group did not grow. Experiments performed with 1.0 mg Idox showed no difference compared with 0.5 mg Idox in the MT2:Tam tumors; therefore, a lower dose (0.5 mg Idox) was chosen for the experiment using T47D:Tam. In this experiment, 30 mice were transplanted with T47D:TAM tumors and separated into three groups: control, 1.5 mg Tam, and 0.5 mg Idox (Fig. 3C). As expected, the tumors in Tam treated mice grew, whereas the tumors in controls did not. The Idox-treated mice produced only three larger tumors (>0.5 cm^2), suggesting that there is partial cross-resistance for Idox with Tam in the T47D:Tam tumor.

To confirm that the triphenylethylene derivatives have similar effects on Tam-stimulated breast tumor growth, we tested the effect of Tor on MT2:Tam tumors (Fig. 4). Forty athymic mice were implanted with MT2:Tam tumors and treated with 0.5 mg Tam, 0.5 mg Tor, or 1.5 mg Tor daily. A control group of mice received vehicle alone. Similar growth rates were seen in the 0.5 mg Tam- and 1.5 mg Tor-treated mice (1.00 ± 0.16 versus 1.46 ± 0.14; P = 0.73).

As shown previously, the pure antiestrogen ICI 182,780 can inhibit Tam-stimulated tumor growth (20); therefore, we confirmed that ICI 182,780 had no growth-stimulatory effect on another type of Tam-stimulated breast tumor. Thirty mice were implanted with MT2:Tam tumors and treated with 0.5 mg Tam, 0.5 mg Tor, or 1.5 mg Tor daily. A control group of mice received vehicle alone. Similar growth rates were seen in the 0.5 mg Tam- and 1.5 mg Tor-treated mice (1.00 ± 0.16 versus 1.46 ± 0.14; P = 0.73).

As shown previously, the pure antiestrogen ICI 182,780 can inhibit Tam-stimulated tumor growth (20); therefore, we confirmed that ICI 182,780 had no growth-stimulatory effect on another type of Tam-stimulated breast tumor. Thirty mice were implanted with MT2:Tam tumors and treated with 0.5 mg Tam, 0.5 mg Tor, or 1.5 mg Tor daily. A control group of mice received vehicle alone. Similar growth rates were seen in the 0.5 mg Tam- and 1.5 mg Tor-treated mice (1.00 ± 0.16 versus 1.46 ± 0.14; P = 0.73).

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DISCUSSION

Although Tam is the endocrine therapy of choice for all stages of breast cancer (2), most tumors that initially respond to Tam eventually develop acquired resistance and regrow (24, 25). However, at least two-thirds of the tumors that become resistant to Tam continue to express ER (26–28), and many of these respond to a second-line endocrine therapy.

In recent years, a number of new compounds have been synthesized in an attempt to overcome the drawbacks of Tam or to improve therapeutic efficacy (5). In the present study, we have used the athymic mouse breast tumor xenograft model to evaluate the potential of Idox, Tor, and ICI 182,780 for clinical use and to build our understanding of the mechanism of drug resistance to Tam in vivo (16, 22, 29). The comparative studies of cross-resistance in three novel models of drug resistance to Tam were based on prior experience with Tam in this setting. In an earlier study (23), we examined the circulating levels of Tam and found that a dose of 0.5 and 1.5 mg Tam/day produced serum levels of $58 \pm 7$ ng/ml and $203 \pm 100$ ng/ml, respec-

Fig. 3 Stimulatory effect of Tam or Idox on Tam-stimulated MT2:Tam (A), MCF-7:Tam (B), and T47D:Tam (C) tumor growth in ovariectomized athymic mice (data are means; bars, SE). Mice were implanted with MT2:Tam, MCF-7:Tam, or T47D:Tam tumors and treated with Tam or Idox. The magnitude of growth stimulation was the same as that of Tam in the MCF-7 model, MT2:Tam ($P = 0.4346$ for 0.5 mg, $P = 0.8947$ for 1.0 mg) and MCF-7:Tam ($P = 0.23$). Although the magnitude was ~50% less than Tam in T47D:Tam, Idox had significant growth-stimulatory effect on tumor growth compared with control group ($P = 0.0055$), which had no tumors at all.
tively. Because the metabolism is different between the two species, it is difficult to relate administered doses between humans and mice. Nevertheless, the animal model has been validated because the level of circulating Tam in the mouse is equivalent to that observed in patients receiving 20 mg daily (30). Thus, the experimental doses of Idox or Tor were selected for evaluation based on the dose ratio proposed for patients.

Idox is more metabolically stable than Tam and binds the ER with a higher affinity (6, 7). However, the fact that Tam is converted to 4-hydroxytamoxifen in athymic mice (31), which has high affinity for the ER, implies that higher doses of Idox should be tested. We initially compared the magnitude of growth inhibition on Tam-sensitive tumors treated with the same dose of 1.5 mg/day Idox as that of Tam. Unfortunately, this dose was lethal to mice; therefore, the dose was reduced to 1.0 mg/day. Because Idox has an approximately 2–3-fold longer half-life (32), we determined that the 1.0 mg dose was sufficient to compare the efficacy of Idox with Tam. In fact, Tam and Idox produced comparable antiestrogenic effects on the growth of the MT2:E2 tumor (Fig. 1).

The acquisition of resistance during prolonged therapy of Tam can be explained by the estrogenic activities of Tam. Tam-stimulated, ER-positive tumors are selected by the weak estrogenic effect of Tam, which may be amplified by an alteration in the complement of coactivators and corepressors present in the cells (33, 34). Johnston et al. (11) have shown that Idox is unlikely to produce an Idox-stimulated tumor because the drug is less estrogenic and produces sustained apoptosis in the MCF-7 xenograft. It has also been shown that Idox is less estrogenic in rat uterus (8). To address the question of whether Idox will result in development of an Idox-stimulated tumor; we compared and contrasted the activities of Tam and Idox using the T47D tumor line transplanted into athymic mice (Fig. 2). There was no difference between the rate of drug resistance demonstrated by Tam and Idox. Naturally, we believed that different tumor models could explain the contradictory result with the report by Johnston et al. (11); therefore, we decided to evaluate the cross-resistance between the Tam and Idox in both MCF-7 and T47D models. The evaluation of Idox in the two Tam-stimulated models would then test the hypothesis of whether the modest decrease in estrogenic properties of Idox is relevant to neutralize the drug-resistance mechanism.

Idox stimulated tumor growth in both Tam-stimulated MCF-7 and T47D tumors (Fig. 3). The magnitude of growth stimulation was the same as that with Tam in two MCF-7 models, MT2:Tam and MCF-7:Tam. Although the magnitude of tumor growth was around half that of Tam in the T47D:Tam model, Idox had a significant growth-stimulatory effect on tumor growth compared with the control group (P = 0.0055). Additionally, we believe that the action of triphenylethylene-type antiestrogens to stimulate tumor growth is dose related. We have shown this previously with the MCF-7 (16) and T47D (19) models. The effect is illustrated with different doses of Tor in the MCF-7 tumor model (Fig. 4). Although using higher doses of Idox in the T47D model would test our hypothesis, we believe that a dose-related principal is already established, and no further useful information about Idox would be obtained. Idox appears to offer no therapeutic advantages over Tam, and
we predict that the drug would be unsuitable for testing after Tam failure. Idox has recently been withdrawn from clinical testing.

To confirm and validate our models with clinical outcome, a cross-resistance experiment was designed using MT2:Tam tumor treated with another Tam analogue, Tor, and the pure antiestrogen ICI 182,780. Tor is known to be cross-resistant with Tam in clinical trials (35, 36) and produce Tor-stimulated tumors in animal models (37). The laboratory studies showed clearly that Tor and Tam are cross-resistant, but ICI 182,780 is not. These data suggest that in women who fail Tam neither Idox nor Tor would be effective as a second-line endocrine therapy. ICI 182,780 has shown promising results clinically, with high response rates of ~70% after Tam failure in advanced breast cancer (21). It has also been shown in the laboratory that ICI 182,780 does not stimulate the growth of Tam-resistant breast and endometrial tumors (20, 23).

These laboratory models of drug resistance to Tam have extended previous studies with Idox (10, 11) and shown that a triphenylethylene-like antiestrogen is unlikely to reduce the risk of premature drug resistance compared with Tam. A goal of our laboratory is to build a database of model systems with a clinical correlation. The array of models of drug resistance to Tam we have used show cross-resistance with Idox and Tor but not ICI 182,780, which parallels clinical experience. Although these model systems may prove to be useful for testing any new selective ER modulator, an understanding of the mechanism of drug resistance must remain a priority for further study. A systematic investigation of drug mechanisms in model systems will potentially provide valuable clues to improve the efficacy of breast cancer therapy.

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