Comparison of the Selective Estrogen Receptor Modulator Arzoxifene (LY353381) with Tamoxifen on Tumor Growth and Biomarker Expression in an MCF-7 Human Breast Cancer Xenograft Model

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ABSTRACT

Arzoxifene (ARZ) is a selective estrogen receptor (ER) modulator with greater bioavailability than raloxifene which is being developed as treatment for breast cancer. We have used an in vivo model of hormone-sensitive breast cancer to study the growth-inhibitory and pharmacodynamic effects of ARZ in comparison with the most widely used antiestrogen, tamoxifen (TAM). We compared the abilities of ARZ and TAM to antagonize the estrogen (E2)-dependent growth of MCF-7 human breast cancer xenografts in oophorectomized athymic mice. At four different time points over 28 days, we studied their time-related pharmacodynamic effects on biomarkers of tumor growth (cell proliferation/death measured by Ki-67 and apoptosis scores), cell cycle activity (cyclin D1 and p27kip1), and hormone-regulated gene expression (ERα, progesterone receptor, and pS2). Although maximal growth inhibition was seen after E2 withdrawal, ARZ and TAM induced significant and similar inhibition of E2-stimulated tumor growth. Inhibition of growth was reflected by changes in the tumor growth index (ratio posttreatment/pretreatment). ARZ and TAM induced significant and similar inhibition of tumor growth. This article must therefore be hereby marked for publication.

INTRODUCTION

The growth of ER3-positive human breast cancer is promoted by estrogen, and consequently, tumor regressions can be achieved by antiestrogenic hormonal therapy. However, the efficacy of antiestrogen therapy in advanced disease can take several months to realize in the clinic. Measurements of pharmacodynamic changes observed within the tumor during specified treatments could provide useful early intermediate biomarker end points of tumor response and may be valuable in assessing the relative merits of any new antiestrogen in development. In clinical studies, significant reductions in cell proliferation were observed after only 14–21 days of TAM therapy given as primary neoadjuvant treatment, and these occurred only in responders to therapy (1). A similar approach has also been used to evaluate the steroidal antiestrogen fulvestrant, where dose-dependent biological effects were seen on inhibition of cell proliferation, together with ER and PgR down-regulation, which was significantly different to either placebo or TAM (2). We have studied these assessments previously in our MCF-7 human breast cancer xenograft model and reported that changes in both hormone and cell growth-related biomarkers may be relevant and reliable time-dependent predictors of tumor response to estrogen deprivation therapy (3).

The benzothiophene LY353381.HCL (ARZ) (Fig. 1) is a SERM that binds to the ER with nanomolar affinity, with an improved oral bioavailability and significantly greater potency compared with the closely related SERM, raloxifene (4, 5). ARZ was developed as an antiestrogen for breast cancer that would have a sustained antiestrogenic effect without any of the agonist effects seen with TAM. Preclinical studies demonstrated that ARZ was devoid of agonist effects in an ovariectomized rat uterotrophic assay in vivo and was able to fully antagonize estrogen-induced elevation of uterine weight (5, 6). ARZ was shown to be a more potent inhibitor of breast cancer cell proliferation in vitro than either TAM or raloxifene without any agonist effects (6) and inhibited the growth of mammary tumor xenografts in vivo when administered at a daily dose of 20 mg/kg (7). It has also been found in vivo that ARZ preserved bone mineral density and reduced serum cholesterol in ovariectomized animals (5, 8). As a SERM, therefore, ARZ possessed several advantages over existing antiestrogens, in particular TAM, in model systems.

In this study, the ability of the novel SERM ARZ to antagonize tumor growth in vivo was compared with TAM in an estrogen-stimulated MCF-7 xenograft model in oophorectomized athymic mice. This is an established model used to investigate the effects of other SERMs and antiestrogens for breast cancer (9, 10), and we previously reported comparative changes in biomarkers seen with the SERMs idoxifene and TAM (11). In this current study, we examined serial time-dependent changes observed in both hormone- and growth-related pharmacodynamic biomarker end points during a 4-week treatment period and evaluated these in relation to the observed rates of tumor regression.

MATERIALS AND METHODS

Chemicals and Drug Delivery. TAM [Z-trans-1-{4-[2-(dimethlamino)ethoxy]phenyl}-1,2-diphenyl-1-buten] was obtained from Sigma Chemical Co. (Poole, United Kingdom), and ARZ [2-(methyleneoxophenyl)-3-[4-[2-(1-piperidinyl)ethoxy]benzo[b]thiophene-6-ol hydrochloride] was provided by Eli Lilly Research Centre (Erl Wood, Surrey, United Kingdom). Stock solutions of both drugs were prepared by dissolving them into a solution of 50% polyethylene glycol (PEG300)/50% sterile water. These constituents were vortexed and then subjected to three to four bursts of low frequency sonication. Each solution was administered daily by oral gavage in a total volume of 0.25 ml/mouse.

MCF-7 Xenograft Model. Transplantable MCF-7 human breast cancer xenografts were initiated from a parent tumor that had been established previously in oophorectomized estrogen conditioned athymic nude mice (3, 11). The hormone dependence of this tumor was continuously monitored by withdrawal of estrogen from a set of mice. All procedures were approved by the Institute of Cancer Research Ethics Committee and were covered by a Home Office project license for these specific studies.

A total of 126 randomly bred female athymic mice of 6–8 weeks of age was

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3 The abbreviations used are: ER, estrogen receptor; TAM, tamoxifen; PgR, progesterone receptor; ARZ, arzoxifene; SERM, selective estrogen receptor modulator; DMT, N-desmethyltamoxifen; OBT, trans-4-hydroxytamoxifen; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labelling; DDW, double-distilled water.
bilaterally ovarioctomized and allowed a 2 week recovery period before the next surgical procedure. MCF-7 tumor was obtained from the viable edge of a transplantable donor tumor between 1 and 1.5 cm in diameter and cut into 1-mm³ pieces in sterile medium. Under anesthesia, a 1.7-ng 60-day release 1β-estradiol pellet (Innovative Research, Sarasota, FL) was implanted into the interscapular region of each mouse. At the same time, a piece of tumor was implanted s.c. under the mammary fat pad area of each mouse. The tumor sites were monitored twice a week and when palpable, bidimensional diameters were measured using vernier calipers at weekly intervals. Tumor volume in cubic mm was estimated using the formula: tumor volume = [width² x length]/2.

After 3 weeks when the hormone-supplemented tumors had grown to 0.7–0.8 cm in diameter (180–250 mm³), the mice were randomized into five treatment groups of 24 mice each. The remaining 6 mice were sacrificed as baseline controls for E2 alone with sham operation (day 0 control). Sham operation involved an incision of the skin at the pellet site similar to that conducted for removal and was carried out in all of the treatment groups. The treatment groups were as follows: (a) E2 support with 1.7 mg 60-day release pellet + daily vehicle oral gavage; (b) withdrawal of E2 support + daily vehicle oral gavage; (c) E2 support + TAM 20 mg/kg/day (TAM20) by daily oral gavage; (d) E2 support + ARZ 20 mg/kg/day (ARZ20) by daily oral gavage; and (e) E2 support + ARZ 2 mg/kg/day (ARZ2) by daily oral gavage.

The number of animals was determined by power calculations relating to a primary comparison of ARZ20 versus TAM20. Comparisons of ARZ2 with ARZ20 were considered secondary analyses. An additional cohort of lower dose TAM was not considered justifiable because TAM20 was deemed the standard arm from previous studies, and the question of a lower biologically effective dose related only to the novel SERM being studied.

Treatment was continued in the five groups for a total of 4 weeks, and tumor volume was recorded weekly. Six mice from each treatment group were sacrificed on day 3, 7, 14, and 28 to study pharmacodynamic endpoints in the treated tumor. In all of the sacrificed mice, the tumor was dissected out and immediately placed in 10% neutral buffered formal saline. The animals were sacrificed 24 h after the last oral gavage (i.e., there was no dosing done on the day of sacrifice). Therefore, all drug levels were trough values to minimize any variability in the pharmacokinetic values obtained.

Methods for Measuring Plasma and Intratumoral Drug Concentrations. Samples of plasma (0.5 ml) from TAM-treated mice were mixed with 0.5 ml of acetone/tritile, and the precipitated protein was removed by centrifugation (13,000 rpm for 2 min). The supernatant was extracted with 6 ml of hexane:butanol (98:2) and the extracts evaporated to dryness at 40°C under nitrogen and reconstituted in 200 μl of eluent. Tumor samples were homogenized, and samples of plasma and tumor extract were then subjected to high-pressure liquid chromatography for TAM and its major metabolites DMT and OHT according to our previous published methodology (12). The detection limits for the plasma assay were 1 ng/ml for TAM/DMT and 25 ng/ml for OHT, whereas for the tumor assay, detection limits were 0.02 μg/g for TAM/DMT and 0.5 μg/g for OHT.

The plasma samples for LY353381 and its major desmethyl metabolite (LY335562) were analyzed by Eli Lilly Laboratories for Bioanalytical Research (Scarborough, Ontario, Canada) using a validated liquid chromatography mass spectrometer methodology. Tumor samples were analyzed by high-pressure liquid chromatography/mass spectrometry methodology on a Micromass Platform LCZ mass spectrometer by Dr. Mary-Pat Knadler at Eli Lilly and Company (Indianapolis, IN).

Immunohistochemistry for Ki-67, Apoptosis, ER, PgR, pS2, Cyclin D1, and p21

General reagents were purchased from Sigma Chemical Co., and unless otherwise specified, primary antibodies and avidin-biotin complex were bought from Dako (Cambridge, United Kingdom). Sections of 3 μm were cut from the formalin-fixed, paraffin wax-embedded MCF-7 tumor xenografts and dried onto charged microscope slides.

For each analyte, sections were stained in one batch for each time point plus the appropriate known positive controls. With the exception of apoptosis (TUNEL protocol) and pS2 (no antigen retrieval), the following standard staining procedure was used. Sections were dewaxed in xylene, taken to water, and endogenous peroxidase activity was blocked. Antigen retrieval was performed by microwaving the sections at 750 W in citrate buffer at pH 6.0 for 10 min, after which, they were cooled in the buffer to room temperature, and a blocking antibody was applied in PBS (pH 7.4) before primary antibody. The sections were incubated for 45 min in 1/200 biotinylated antimonoe immunoglobulins and rinsed, and avidin-biotin complex was applied for 30 min. After washing in PBS, the peroxidase anti-peroxidase reaction was developed to a brown stain by 0.05% 3,3′-diaminobenzidine enhanced with 0.07% imidazole and hydrogen peroxide. Antigen retrieval, as described above, was not used for sections undergoing the TUNEL protocol (13). Instead, at this stage of the procedure, the water-washed sections had their nuclei stripped of proteins by incubation with 0.5% pepsin, pH2, (Sigma Chemical Co.) for 30 min at 37°C under humid conditions. The sections were washed five times in DDW to remove all traces of pepsin. Each section was incubated for 5 min in Tris buffer (pH 7.6). Then, the sections were incubated for 1 h at 37°C in a humidified chamber in 100 μl of reaction mixture. This mixture consisted of 15 units terminal deoxynucleotidyltransferase fast protein liquid chromatography pure (Pharmacia, Windsor, Berkshire, United Kingdom), 0.5 nmol of biotin-16-dUTP (Boehringer Mannheim, Mannheim, Germany), 5 nmol chloroacetate, 25 μM Tris HCI (pH 6.6), and 0.25 mg/ml BSA, fraction V dissolved in DDW. After extensive washing in DDW, the sections were incubated for 20 min at room temperature in 1/400 dilution of horseradish peroxidase conjugated to streptavidin (Dako, High Wycombe, Bucks, United Kingdom) in PBS supplemented with 1% BSA and 0.5% Tween 20. After washing, the color development procedure, dehydrating, counterstaining, and mounting of the sections was as described above.

Cells were examined under a standard light microscope using ×40 objective and 10 × 10 eyepiece incorporating a graticule. Ki-67-positive nuclear staining cells were recorded as percentages assessed from scoring 10 high power fields. The apoptotic index was expressed as a percentage that was calculated from the number of brown staining cells displaying apoptotic bodies out of 3000 tumor cells/seciton counted under high power, excluding any necrotic areas. The scoring was conducted by one analyst (S. D.) and was audited by another (J. S.). The brown nuclear staining resulting from all of the other biomarkers' immunohistochemistry staining was scored as 0–6 staining, with 0 representing no staining; 1 weak; 2 moderate; 3 moderate-strong; 4 strong; 5 strong-very strong; and 6 100%, thereby estimating the percentage of positively staining epithelial cancer cells. The intensity of staining was scored as 0 = no staining; 1 = weak; 2 = moderate; and 3 = strong. The product of proportion and intensity could give a quickscore ranging between 0 and 18.

Growth Index. The geometric means of the ratios of Ki-67 over apoptotic index were computed. The growth index was calculated according to the following formula: (Ki-67/Al) posttreatment / (Ki-67/Al) pretreatment.

Statistics. Growth curves were plotted from the arithmetic means of the volumes ± SE for each time point. The linear fit for individual tumors was calculated from the regression coefficient of log (volume) versus time, under the assumption of constant growth. To adjust for the reduction in sample size over time because of the harvesting of tumors for the biomarker studies, we used linear regression analysis for the statistical analysis of the growth curves. Linear regression analysis tests for trends and calculates the growth rate, allowing for the fact that a variable number of tumors may contribute at each time point. The growth rates were compared between the groups using the Kruskal-Wallis ANOVA, and between-group comparisons were undertaken using the Mann-Whitney U test.

For each biomarker, the arithmetic mean was calculated from each of the immunohistochemical scores, and the results were expressed as a standardized score, which was calculated from the mean percentage log transformed (bi-

Fig. 1. Chemical structure of LY353381.HCL (arzoxifene).
RESULTS

Tumor Growth Inhibition. The tumor take-rate after implantation ranged from 95 to 100%, and after 3 weeks of E2 support, the mean tumor size and animal weight were similar in the five groups, which were randomized. Tumor growth continued in control animals that were randomized. Tumors in each group were harvested after 0, 3, 7, 14, and 28 days.

The 10-fold lower dose of ARZ2 was also associated with significantly reduced tumor volumes (184 ± 42 mm³) compared with E2-treated controls. Fig. 2 shows that tumor growth inhibition was slower to occur than the 20 mg/kg dose: it was not until after 2 weeks of ARZ2 treatment that the slope of the growth curve declined. However, between weeks 2 and 4, the slope of the tumor regression curves was parallel to the growth curves resulting after TAM20 and ARZ20 treatment.

Table 1 Tumor and plasma concentration of TAM and ARZ at each time point in the three treatment groups
Values are expressed as ng/ml (plasma), ng/g (tumor), and as a plasma:tumor ratio. The animals were sacrificed 24 h after the last oral gavage.

<table>
<thead>
<tr>
<th></th>
<th>TAM20</th>
<th>Day 3</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>27 ± 23</td>
<td>16 ± 6</td>
<td>39 ± 23</td>
<td>22 ± 10</td>
<td></td>
</tr>
<tr>
<td>Tumor</td>
<td>580 ± 380</td>
<td>350 ± 200</td>
<td>520 ± 130</td>
<td>390 ± 120</td>
<td></td>
</tr>
<tr>
<td>Plasma/tumor</td>
<td>21.48</td>
<td>21.87</td>
<td>13.33</td>
<td>17.72</td>
<td></td>
</tr>
<tr>
<td>ARZ20</td>
<td>137 ± 18</td>
<td>147 ± 9.8</td>
<td>184 ± 24</td>
<td>149 ± 12</td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td>3279 ± 523</td>
<td>1619 ± 227</td>
<td>2388 ± 1187</td>
<td>3261 ± 776</td>
<td></td>
</tr>
<tr>
<td>Tumor</td>
<td>23.93</td>
<td>11.01</td>
<td>12.98</td>
<td>21.88</td>
<td></td>
</tr>
<tr>
<td>Plasma/tumor</td>
<td>22 ± 4</td>
<td>25 ± 3</td>
<td>26 ± 4</td>
<td>25 ± 2</td>
<td></td>
</tr>
<tr>
<td>ARZ2</td>
<td>250 ± 17</td>
<td>319 ± 40</td>
<td>312 ± 78</td>
<td>489 ± 54</td>
<td></td>
</tr>
<tr>
<td>Plasma/tumor</td>
<td>11.36</td>
<td>12.76</td>
<td>12.00</td>
<td>19.56</td>
<td></td>
</tr>
</tbody>
</table>
both metabolites to TAM were similar, demonstrating rapid achievement of steady state with daily oral gavage. Intratumoral concentrations of TAM were less variable (coefficient of variation, 39–80%) than plasma concentrations but again were not time dependent (Table 1). The mean tumor/plasma ratio ranged between 13–21 after 3–28 days of treatment.

The mean plasma and intratumoral concentrations of ARZ at the two doses used in this study are also shown in Table 1. ARZ20 plasma concentrations were similar at each time point. The desmethyl metabolite LY335562 was also detected in plasma but at much lower levels (mean, 5 ± 1 ng/ml). Plasma concentrations of ARZ2 were correspondingly ~8–10 fold lower than those observed with the higher ARZ20 dose, and the metabolite LY335562 was undetectable at the lower 2 mg/kg daily dose. There was no significant difference in the plasma concentrations with time. The mean tumor/plasma ratio of ARZ20 was 23.9, 11.0, 12.9, and 21.8 after 3, 7, 14, and 28 days of treatment, respectively. There was a suggestion that the tumor ARZ concentrations after the lower ARZ2 dose increased over time, reflected by the gradual increase in mean tumor/plasma ratio with time. In addition, these ratios were lower than those in the 20 mg/kg dose, consistent with an increased accumulation of drug over time in tumors treated with the lower dose.

**ER Expression.** The ER quickscore was 1.6 ± 0.2 for the untreated E2 controls, and an average of all time points in the E2 sham-operated controls was 2.33 ± 0.22 (range of 1.4–5). At days 3, 7, and 28 in the sham-operated E2 control group, there was an increase in ER with time, which could be attributable to the cumulative effects of treatment stress (Fig. 3). With the exception of the day 28 time point, this increase in ER within that group was still significantly lower than that observed after administration of the other treatments.

Estrogen deprivation caused a significant increase in ER by day 14 (Fig. 3; P < 0.001 versus E2 sham-operated controls at days 3, 7, and 14 after treatment), which reduced by the end of the experiment. TAM treatment caused an earlier elevation of ER, and the decline that followed was evident sooner than with the E2 withdrawal group. Both of the doses of ARZ resulted in an early, less substantial but maintained increase in ER (P < 0.001 versus E2-treated controls) through the course of the experiment. This increase was significantly higher than the E2 sham-operated controls but only during the first three time points. There was no significant difference between ARZ20 and TAM20 at the 2-week time point, and the difference between the higher and lower dose of ARZ was also not significant.

**PgR Expression.** There was a significant increase in PgR scores in the sham-operated E2 controls with time (Fig. 3; P < 0.01, day 28 versus 0). After the withdrawal of E2 support, there was a highly significant drop in PgR expression (Fig. 3, P < 0.001 versus E2 control) within these tumors at all time points. The 20 mg/kg/day dose of TAM and ARZ also significantly lowered PgR expression, but it took over 2 weeks before the PgR levels attained those measured in the E2-deprived tumors. Compared with the E2 controls, the 10-fold lower dose of ARZ did not result in significant reduction in PgR levels until day 28 (Fig. 3).

**pS2 Expression.** pS2 expression in the E2 controls remained constant throughout the experiment. By day 28, E2 deprivation resulted in 30% reduction in pS2 expression, and both TAM20 and ARZ20 led to a 50% reduction (P < 0.001). In contrast, ARZ2 only diminished pS2 by 10%. Overall, the treatment effects on this biomarker appeared to be much delayed compared with all of the other biomarkers (Fig. 3).

**Cell Proliferation.** Fig. 4 illustrates that estrogen withdrawal resulted in a rapid fall in percentage Ki-67-immunostaining ratio and that this occurred after only 3 days; thereafter, cell proliferation declined to <10% of the untreated controls (P < 0.001). The 20 mg/kg/day dose of TAM and ARZ resulted in a similar but delayed pattern of reduced proliferation: from day 7 onward, these drug treatments significantly reduced Ki-67 compared with equivalent E2 controls (P < 0.001, Fig. 4). With ARZ2, there was a slower onset of the reduction in Ki-67-positive cells, which reached significance by day 14 (P < 0.002 versus E2-treated controls).

**Apoptosis Index (TUNEL).** The apoptotic index remained steady throughout the time course in the E2-bearing sham-operated control tumors (Fig. 4). Within 3 days, there was a significant induction of apoptosis in tumors deprived of E2 and this peaked on day 14 (P < 0.001; E2-deprived versus E2-supplemented controls; Fig. 4). Both TAM and ARZ induced a significant 183 and 166% elevation in apoptosis, respectively, after 3 days, and this elevation was maintained to day 28 (P < 0.001 versus E2-treated controls; Fig. 4). The lower dose of ARZ initially had no effect on apoptosis but, by the end of the experiment, was also associated with a significant ~2-fold induction of apoptosis (P < 0.001 versus E2-treated controls; Fig. 4).

The percentage of TUNEL-positive cells measured 14 days after treatment with the lower dose of ARZ was significantly less than in the tumors deprived of estrogen (P < 0.001).

**Growth Index.** The growth indices, which are calculated from the posttreatment/pretreatment proliferation:apoptosis ratios, are summa-

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Fig. 3. Time course of effects of antiestrogen treatments on the hormone-related biomarkers ER, PgR, and pS2. Standardized score: mean % log transformed biomarker score/day of untreated control score in E2-supplemented tumors. Data expressed in log mean standardized score ±/− SE for each time point. TAM20, 20 mg/kg tamoxifen. ARZ20, 20 mg/kg arzoxifene. ARZ2, 2 mg/kg arzoxifene.
rized in Table 2. These data demonstrate the differences between the
treatment groups, as well as the effect of time. There was a marked
drop in growth index evident in all treatment groups, which was
maximal in the E2-deprived treated tumors. There was no difference
in the growth indices between the TAM20 and ARZ 20 groups,
whereas the ARZ2 treatment had a slower but gradual reduction in
growth index from 0.78 on day 3 to 0.19 by day 28. Changes in the
growth indices were closely related to the tumor growth curves for
each of the therapies (compare data in Table 2 with Fig. 2). This
suggests that the biomarker data on tumor growth derived from
the Ki-67 and apoptosis scores can provide an accurate reflection of
tumor growth as measured by change in tumor volume in vivo.

Cyclin D1 Expression. The sham-operated E2 control group
showed a slight but insignificant increase in cyclin D1 with time. E2
deprivation, TAM, and ARZ resulted in a rapid and significant fall in
cyclin D1 (\( P = 0.001 \)), which persisted throughout the period examined.
The 10-fold lower dose of ARZ was also suppressive of cyclin
D1 but was not as effective as the higher dose (Fig. 5).

p27kip1 Expression. p27kip1 remained constant throughout the
course of the experiment in the E2-supplemented control tumors. E2
deprivation gave an early 3–4-fold significant increase in expression
of this biomarker compared with the E2 controls, and this remained
constant throughout the experiment. An increase comparable with the

Figs. 4 and 5. Time course of effects of antiestrogen treatments on cycle-dependent biomarkers cyclin D1 and p27 KIP1. Standardized score, mean % log transformed biomarker score/day of untreated control score in E2-supplemented tumors. Data expressed in log mean standardized score +/− SE for each time point. TAM20, 20 mg/kg tamoxifen. ARZ20, 20 mg/kg arzoxifene. ARZ2, 2 mg/kg arzoxifene.

Table 2. Tumor growth indices for each treatment group
The growth index was calculated from the geometric means of the posttreatment ratios of Ki-67/apoptosis over the pretreatment ratio of Ki-67/apoptosis.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 3</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2</td>
<td>1.05</td>
<td>1.04</td>
<td>1.26</td>
<td>0.88</td>
</tr>
<tr>
<td>TAM20</td>
<td>1.50</td>
<td>1.13</td>
<td>0.04</td>
<td>0.05</td>
</tr>
<tr>
<td>ARZ20</td>
<td>0.54</td>
<td>0.16</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>ARZ2</td>
<td>0.78</td>
<td>0.66</td>
<td>0.43</td>
<td>0.19</td>
</tr>
<tr>
<td>V without E2</td>
<td>0.17</td>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
</tr>
</tbody>
</table>

\( ^* \) Day 0 sham-operated control ratio Ki-67/apo = 27.2. V, vehicle.
with the degree of change in tumor volume observed after 3 months of
treatment (15). Large randomized Phase III trials with the aromatase
inhibitors letrozole and anastrozole confirm these drugs to be superior
clinically to TAM in the advanced (20, 21), neo-adjuvant (22, 23), and
adjunctive settings (24). Thus, changes in biomarkers in experimental
models may provide comparative data between different systemic
drug treatments, and similar clinical pharmacodynamic studies can
be undertaken in the preoperative neoadjuvant setting. As such an
approach is not feasible in the adjuvant setting, this may be attractive
to assess biological efficacy of new therapies in early-stage primary
breast cancer.

ARZ appeared equally effective at inhibiting E2-dependent MCF-7
breast tumor growth compared with TAM in this study. Analysis of
the tumors revealed similar quantitative and time-dependent effects on
all of the cell growth and hormone-related biomarkers studied. These
data are in contrast with in vitro comparisons in MCF-7 cells, which
suggested that ARZ was significantly more potent than TAM at
inhibiting estrogen-dependent growth (6). In that system, the most
potent antiestrogen was the metabolite desmethyl-ARZ, although we
found levels of this metabolite to be very low in vivo. A similar
discrepancy between the in vitro and in vivo antiestrogenic effects of
ARZ and TAM was reported in experimental models of human
endometrial cancer (25). In vitro ARZ was significantly more potent
than TAM at inhibiting estrogen-stimulated growth of ECC-1 TAM-
naive human endometrial cancer cells, but in vivo, both drugs were
equally effective at antagonizing estrogen-stimulated growth. How-
ever, one of the major reasons for developing ARZ was the potential
for reduced agonist effects in comparison with TAM on target organs,
including the uterus, endometrium, and breast. In vitro, neither ARZ
nor its metabolite showed any stimulation of basal MCF-7 breast
cancer cell growth in the absence of estrogen (6). However, mixed
effects have been reported in in vivo models of TAM-stimulated
growth, with growth stimulation by ARZ reported in both EnCa101-
endometrial tumors (25) and MCF-7/Tam breast tumors (26).

These effects may be model specific because lack of stimulation by
ARZ and, hence, noncross-resistance with TAM was found in a
TAM-stimulated T47-D xenograft model (26). Thus, although triphen-
ylethylenic structures such as idoxifene are completely cross-resis-
tant with TAM in these antiestrogen-stimulated models (27), struc-
turally different antiestrogens such as the steroidal compound
fulvestrant and, to a lesser extent the benzothiophene ARZ, may be
partially noncross-resistant (26, 27).

Previous experimental data suggesting differences with TAM en-
couraged clinical trials of ARZ to be undertaken both in patients with
TAM-sensitive and TAM-resistant advanced breast cancer. In a Phase
I study of 32 patients with advanced breast cancer, no change in
endometrial thickness was seen over 12 weeks therapy, confirming a
lack of agonist effect (28). Two Phase II studies have compared two
different doses (20 or 50 mg daily) of ARZ, and although response
rates of 30–36% were seen in hormone-sensitive ER+ advanced
breast cancer, efficacy in TAM-resistant patients was low with a
response rate of <10% (29, 30). A multicenter Phase III trial of 20 mg
of ARZ versus TAM as first-line therapy in postmenopausal women
with ER+ advanced breast cancer was started, although it remains to
be seen whether ARZ will offer any significant clinical advantage
over TAM in this setting. In clinical trials with triphenylethylenic-like
SERMs (e.g., idoxifene, toremifene, and droloxifene), no difference in
clinical activity was found compared with TAM, with little or no
activity in the setting of TAM resistance (31). The evidence from our
 xenograft model would suggest there may be no difference in initial
clinical response rates between ARZ and TAM in hormone-sensitive
breast cancer. However, an antiestrogen with reduced agonist prop-
erties might be expected to prevent or delay the emergence of anties-
trogen-stimulated resistance, and our short-term study cannot rule out
clinical differences that may exist between ARZ and TAM in long-
term control rates measured by time to disease progression.

An increase in ER levels occurs in breast cancer cells in vitro after
estrogen deprivation because under normal circumstances, in the
presence of estrogen, there is enhanced ubiquitin-proteasomal degra-
deration of ER (32). A rise in ER levels was seen in this study after E2
withdrawal (Fig. 3), again consistent with our previous observations
(3, 33). Likewise, estrogen deprivation resulted in down-regulation of
ER-regulated genes, as measured by a fall in PgR expression. We
observed greater quantitative changes in ER expression after anties-
trogen therapy with TAM but less reduction in progesterone expres-
sion, which has been attributed in the past to TAM’s partial agonist
activity (34). The effects of ARZ20 on ER were intermediate, an
observation consistent with those reported for ARZ20 in endometrial
cancer cells in comparison with TAM, estrogen, and estrogen with-
drawal (25) and supportive of ARZ having less agonist activity than
TAM in MCF-7 tumors.

The putative E2-regulated pS2 protein was only reduced by 20% of
the sham-operated controls in the E2-deprived group, and the fact that
pS2 was only diminished by 30% in the treated groups indicates that
other factors regulate its expression (35). Ki-67 and cyclin D1 mir-
rored the considerable and consistent decline seen in the PgR stan-
dardized score. The biomarkers cyclin D1 and p27Kip1 were measured
due to their cell cycle and/or ER association, and because in a
previous E2 deprivation study (3), the early change in these two
biomarkers heralded the onset of tumor volume regression. Recently,
it has been postulated that cyclin D1 in breast cancer is not associated
with Ki-67 but is associated with ER (36). Our findings suggest a
relationship between cyclin D1 and estrogen function. In this study,
the reduction in cyclin D1 was accompanied by an expected increase
in p27Kip1 expression, consistent with our previous findings (3).

There was a dose effect for ARZ on inhibition of all biomarkers,
which was parallel to the delayed effect of the 10-fold lower dose of
growth and the slower intratumoral accumulation of sufficient drug.
However, others have confirmed in breast cancer chemoprevention
models that doses as low as 0.03 mg/kg ARZ still had a significant
effect on the prevention of N-nitroso-N-methyleurea-induced rat mam-
mary tumors (6). Our data suggest that in the long term, similar
biological effects may be seen with lower doses of ARZ, which in
the chemoprevention setting may allow selection of a dose below that
was used therapeutically, and this would be expected to reduce any sys-
temic toxicity yet still allow the desired end organ biological effect to
be seen. More recently, the same group has reported synergy between
ARZ and a novel renoxinoid LG 100268 (37). Thus, as a SERM, which
is as effective as TAM without the agonist effects, ARZ may be very
suitable for additional studies in the prevention setting.

In conclusion, tumor biomarker analysis of growth- and hormone-
dependent gene expression can be a useful tool in evaluation of novel
doncendocrine therapies for breast cancer. We have shown in an MCF-7
breast cancer xenograft model that growth inhibition is matched by
significant reduction in cell proliferation and induction of apoptosis
and that the growth index may be a useful predictor of posttreatment
changes occurring in the tumor. In our system, ARZ, a benzothio-
ephene with reduced agonist properties, showed no significant differ-
ences to TAM and was less effective than estrogen withdrawal.

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Comparison of the Selective Estrogen Receptor Modulator Arzoxifene (LY353381) with Tamoxifen on Tumor Growth and Biomarker Expression in an MCF-7 Human Breast Cancer Xenograft Model

Simone Detre, Sharon Riddler, Janine Salter, et al.


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