Arzoxifene, a New Selective Estrogen Receptor Modulator for Chemoprevention of Experimental Breast Cancer

Nanjoo Suh,1 Andrew L. Glasebrook,1 Alan D. Palkowitz, Henry U. Bryant, Lorris L. Burris, James J. Starling, Homer L. Pearce, Charlotte Williams, Christopher Peer, Yongping Wang, and Michael B. Sporn


Abstract
Arzoxifene ([6-hydroxy-3-[4-[2-(1-piperidinyl)-ethoxy]phenoxy]-2-(4-methoxyphenyl)benzo[b]thiophene] is a selective estrogen receptor modulator (SERM) that is a potent estrogen antagonist in mammary and uterine tissue while acting as an estrogen agonist to maintain bone density and lower serum cholesterol. Arzoxifene is a highly effective agent for prevention of mammary cancer induced in the rat by the carcinogen nitrosomethylurea and is significantly more potent than raloxifene in this regard. Arzoxifene is devoid of the uterotrophic effects of tamoxifen, suggesting that, in contrast to tamoxifen, it is unlikely that the clinical use of arzoxifene will increase the risk of developing endometrial carcinoma.

Introduction
Optimal prevention of all breast cancer in women at high risk has not yet been achieved. Although prophylactic bilateral mastectomy is clearly efficacious (1–4), it does not guarantee prevention of disease in all women (2, 3). Furthermore, this procedure subjects many women who would not develop invasive disease to surgery that is disfiguring and psychologically damaging (2). A clear alternative is to devise chemopreventive strategies that would safely achieve prevention without drastic surgical intervention (2). The SERM4 concept provides an ideal framework for development of new agents for chemoprevention of estrogen receptor-positive breast cancer (5–7), and during the past 3 years, important clinical advances have occurred in the use of SERMs for this purpose. Data have been published from large clinical trials, indicating that two SERMs, i.e., the triphenylethylene Tam and the benzothiophene Ral, have significant benefit in lowering the risk of developing invasive disease in women (8–10). However, neither Tam nor Ral is an ultimate agent for prevention of all breast cancer, either in terms of efficacy or total freedom from undesirable side effects. There is special concern about enhanced risk of endometrial cancer after prolonged use of Tam (11, 12). Thus, there is a continuing need for development of new and better chemopreventive agents.

In this report, we describe one such molecule, the new SERM Arz (Fig. 1). We show that Arz is a potent antagonist of estrogen-induced stimulation of both mammary and uterine tissues, in contrast to the uterotrophic effects of Tam that may result infrequently in endometrial carcinoma. A primary goal of our efforts was to increase the efficacy of Ral while maintaining its desirable SERM profile. During the course of structure-activity relationship studies of Ral derivatives, we found that replacement of the Ral carbonyl group with oxygen yielded a new agent, DMA (Fig. 1), which in vitro was an estrogen antagonist more potent than any compound made previously (13). However, when administered p.o., DMA had suboptimal in vivo efficacy because of inadequate bioavailability. Subsequent structure-activity relationship studies showed that methylation of the 4′-hydroxyl group of DMA gave a compound optimized for activity in vivo, i.e., Arz. In turn, given the known metabolic pathways associated with demethylation of arylmethylethers, there is potential for the in vivo conversion of Arz to its more potent parent, DMA.

The present article describes the following properties of Arz: its high affinity for the estrogen receptor, its ability to antagonize the growth of human breast cancer cells (MCF-7) stimulated by E, as well as its superiority to Tam in antagonizing uterine hypertrophy stimulated by estrogen. A previous communication has shown Arz to be superior to Ral in rat models, in terms of prevention of bone loss in the absence of estrogen, as well as in lowering cholesterol levels in the blood (14). Finally, we report the marked activity of Arz as a chemopreventive agent in a series of experiments involving >300 rats. We have used a standard model of mammary carcinogenesis with NMU as the carcinogen. We have compared the relative efficacies of Arz and Tam in this model, with the practical goal of developing a potent new SERM that would be devoid of the undesirable uterotrophic activity of Tam.

Materials and Methods
Reagents. Reagents were from Sigma Chemical Co. unless otherwise noted. The radiochemicals [3H]E (specific activity, 114–162 Ci/mmol) and [3H]thymidine (specific activity, 6.7 Ci/mmol) were purchased from DuPont/ NEN. Arz, DMA, and Ral were synthesized at Lilly Research Labs; in all experiments in the present study, the SERMs were used as their hydrochloride salts.
Assays for ER Binding, Proliferation of MCF-7 Cells, and Effects on Uterine Growth in Vivo. These methods have been described in detail (13, 14).
Prevention of Mammary Carcinogenesis in Rats. Procedures used here for evaluation of chemopreventive agents in the standard rat model which uses NMU as carcinogen have been described in numerous previous publications (15–17).

Results
Arz Binding to the hER. The binding affinity of Arz for hER was determined using a competitive inhibition binding assay, with whole cell lysates from MCF-7 cells as the source of hER. Serial dilutions of Arz or other competitors were mixed with hER and a fixed concentration of [3H]E, and after incubation to allow for equilibrium binding, the amount of bound radioligand was determined. Typically, such...
human MCF-7 breast cancer cells, and a fixed concentration of [3H]E. For full details of estrogen antagonists for their antiproliferative activity (19), MCF-7 cells have been useful for comparative analysis of E and to inhibition of this stimulation by estrogen antagonists. His-absence of E.

MCF-7 cells are exquisitely sensitive to stimulation by E.

Binding studies to hER are conducted at 4°C and reported as the RBA compared with E (IC50 E/IC50 test compound). At 4°C, Arz inhibited the binding of [3H]E to hER with an RBA of 0.08 ± 0.03 in seven independent assays. Moreover, when the assay was conducted at 37°C, the RBA of Arz increased 4-fold to 0.32 ± 0.08, as shown in Fig. 2. As observed previously (13), we have confirmed that DMA, the demethylated metabolite of Arz, binds to hER at 37°C with an affinity almost identical to that of E (data not shown).

Because MCF-7 cell lysates may contain a mixture of ER isoforms (18), we determined the ability of Arz to inhibit binding of [3H]E to recombinantly expressed hERα and rERα. Arz bound to hERα at 4°C or 37°C with an RBA of 0.55 or 0.60, respectively. In comparison, Arz bound to rERβ at 4°C or 37°C with an RBA of 0.28 or 0.24, respectively, suggesting a slightly greater affinity for binding hERα. The parent compound, DMA, bound to hERα at 4°C or 37°C with an RBA of 0.40 or 0.92, respectively, and to rERβ at 4°C or 37°C with an RBA of 0.68 or 0.86, respectively. These data demonstrate that Arz and DMA bind to hER with an affinity similar to or greater than that for other SERMs, such as Ral or Tam. In particular, the affinity of DMA for either ERα or ERβ at physiological temperature is comparable with that of E itself.

Effects of Arz on MCF-7 Proliferation in the Presence or Absence of E. MCF-7 cells are exquisitely sensitive to stimulation by E and to inhibition of this stimulation by estrogen antagonists. Historically, MCF-7 cells have been useful for comparative analysis of estrogen antagonists for their antiproliferative activity (19–22). When assayed for inhibition of proliferation of MCF-7 cells stimulated by E (10 pm), Arz was more potent (IC50, 0.4 nm) than either Tam (IC50, 480 nm) or its active metabolite, 4-OH-Tam (IC50, 1.2 nm). Confirming previously reported results (13), we found DMA to be the most potent of all agents we have tested in this assay (IC50, 0.05 nm), whereas Ral was equivalent to Arz. Interestingly, in the absence of added E, Arz and DMA, at concentrations >1 nm, still inhibited basal proliferation of MCF-7 cells, whereas Tam (10 nm to 1 μM) reproducibly stimulated basal proliferation (data not shown).

Effects on Uterine Growth. Estrogen antagonism by SERMs in the uterus was evaluated in immature female rats 21 days of age. The uterus at this age is fully responsive to E, although this hormone is not yet produced by the ovaries. Our assay was developed to permit maximal uterine stimulation by exogenous EE, a p.o. bioavailable estrogen; EE induces a 3-fold increase in uterine weight over a 3-day period (13). SERMs were dosed daily, p.o., following dosing with EE. Twenty-four h after the last dose, the animals were euthanized, and the uteri were removed and weighed. As shown in Fig. 3A, Arz (1 mg/kg) completely blocked the effects of EE, with an ED50 of ~0.01 mg/kg. The ED50 for Tam was 10-fold greater than the ED50 for Arz. The complete antagonism of EE by Arz is notably different from that of Tam, which caused only a 50% inhibition of uterine growth, even at doses as high as 10 mg/kg (Fig. 3A). This inadequacy of Tam may be the result of its partial estrogen agonist activity in the uterus.

Studies were also conducted in adult female OVX rats to evaluate potential estrogenic effects of Arz on uterine tissue. In this model, Arz did not stimulate statistically significant increases in uterine weight

A

B
after 35 days of oral dosing (Fig. 3B). In contrast, Tam had definite uterotrophic activity in this assay, as shown in Fig. 3B.

**Prevention of Mammary Cancer by Arz.** Arz was markedly more active than either DMA or Ral in preventing breast cancer in rats. In these experiments, animals received a single i.v. injection of NMU and 1 week later were randomly assigned to experimental groups. They were then fed diets containing either control vehicle, Arz, DMA, or Ral, (each of the three drugs at either 60 or 20 mg/kg diet, comparable with a dosage of 3 or 1 mg/kg body weight, respectively). Rats were autopsied after 10 weeks, and tumors were counted and weighed. Rats treated with Arz had significant decreases in tumor incidence (reduced 91%), average number of tumors/rat (reduced 97%), and average tumor burden/rat (reduced 99%) compared with controls (Table 1). Nearly equivalent results were obtained with both doses, suggesting the potency had been maximized. In contrast to the results obtained with Arz, DMA was markedly less effective, reflecting its inadequate bioavailability when given p.o. (13). Table 1 also shows that Arz is markedly more potent than Ral in this rat assay.

The chemopreventive activity of Arz was next compared with that of Tam over a wide dose range. Tam has been shown previously to be highly active in this NMU model (16, 23). The results in Table 2 confirm the high potency of Arz at 20 mg/kg diet shown above in Table 1 and further show that even at doses as low as 0.6 mg/kg diet (0.03 mg/kg body weight), Arz significantly decreased the average number of tumors/rat and average tumor burden. At the two lowest doses tested, Arz and Tam were approximately equivalent; both agents have a broad plateau of activity at low doses. We conclude from these studies that Arz is an effective agent for prevention of rat mammary tumors induced by NMU; it has a potency comparable with that of Tam. Finally, in another set of experiments involving 120 rats, we have found that Arz still retains potency for prevention of breast cancer, even when its administration is delayed until 5 weeks after injection of the carcinogen, NMU (data not shown). Average tumor burden in 24 rats at autopsy was reduced 20-fold from controls (24 in injection of the carcinogen, NMU (data not shown). Average tumor burden in 24 rats at autopsy was reduced 20-fold from controls (24).

### Table 1 Prevention of breast cancer by Arz, DMA, and Ral

<table>
<thead>
<tr>
<th>Treatmenta</th>
<th>No. of tumor-free rats/total No. of rats (P)b</th>
<th>Average No. of tumors (P)b</th>
<th>ATB (P)b</th>
<th>Rats with 3 or more tumors (P)b</th>
<th>Rats with tumor burden &gt;5 g (P)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (vehicle)</td>
<td>3/24</td>
<td>2.5</td>
<td>11.0</td>
<td>8/24</td>
<td>13/24</td>
</tr>
<tr>
<td>Arz, 60 mg/kg diet</td>
<td>11/12 (&lt;0.0001)</td>
<td>0.1 (0.004)</td>
<td>0.1 (0.002)</td>
<td>0/12 (0.03)</td>
<td>0/12 (0.002)</td>
</tr>
<tr>
<td>Arz, 30 mg/kg diet</td>
<td>11/12 (&lt;0.0001)</td>
<td>0.1 (0.002)</td>
<td>&lt;0.1 (0.002)</td>
<td>0/12 (0.03)</td>
<td>0/12 (0.002)</td>
</tr>
<tr>
<td>DMA, 60 mg/kg diet</td>
<td>7/12 (0.007)</td>
<td>0.9 (0.05)</td>
<td>1.6 (0.02)</td>
<td>2/12</td>
<td>2/12 (0.04)</td>
</tr>
<tr>
<td>DMA, 30 mg/kg diet</td>
<td>7/12 (0.007)</td>
<td>0.8 (0.03)</td>
<td>3.3 (0.04)</td>
<td>2/12</td>
<td>3/12</td>
</tr>
<tr>
<td>Ral, 60 mg/kg diet</td>
<td>8/12 (0.002)</td>
<td>1.3 (0.03)</td>
<td>1.5 (0.002)</td>
<td>2/12</td>
<td>2/12 (0.04)</td>
</tr>
</tbody>
</table>

a All rats (55 days of age) were given an i.v. injection of 50 mg of NMU per kg body weight 1 week before starting the feeding of chemopreventive agents. Rats were autopsied 10 weeks later.

b P is the value for the comparison of rats treated with chemopreventive agents with control rats treated with vehicle alone.

c ATB, average tumor burden; average weight in grams of a rat’s tumor at autopsy.

### Discussion

Our data show clearly that Arz is superior to either Tam or Ral in a variety of *in vitro* and *in vivo* experimental assays targeted toward development of a practical agent for clinical chemoprevention of breast cancer. Both Tam and Ral have already proven to be efficacious for clinical chemoprevention of breast cancer (8–10, 24); there are extensive data from basic science studies to document the rationale for their clinical use. However, more potent and safer agents are needed, which is the basis for the present studies on Arz.

Arz is a SERM that is a potent estrogen antagonist in mammary and uterine tissue while it acts as an estrogen agonist to maintain bone density and to lower serum cholesterol. We have used MCF-7 cells, a human breast cancer line known to be responsive to estrogen and antiestrogens (19–22, 25), as a primary assay for evaluating potency of various SERMs. Arz inhibited proliferation of estrogen-stimulated MCF-7 cells, with an average IC₅₀ of 0.4 nM, whereas its parent compound and metabolite, DMA, was 8-fold more potent. In contrast, the active metabolite of Tam, 4-OH-Tam, was 3-fold less potent than Arz in this assay.

Most notably, both Arz and DMA also inhibited basal proliferation of MCF-7 cells in the absence of exogenous E, whereas in contrast to the results obtained with Arz, Tam stimulated MCF-7 proliferation under these conditions. Others have reported similar effects of Tam (21, 22). Furthermore, we have shown clearly that Arz is superior to Tam in blocking trophic effects of exogenous estrogen in the uterus, as well as being devoid of the uterotrophic effects of Tam in OVX rats.

Mammary tumorigenesis in rats induced by the carcinogen NMU is a well-accepted model of breast cancer (26, 27), suitable for analyzing the activity of chemopreventive agents (16, 17, 23). Essentially all of the mammary tumors observed in this model are carcinomas. Using this model, we found Arz to be a highly effective and potent chemopreventive agent and to be clearly superior to Ral. Arz had significant preventive activity at doses as low as 0.6 mg/kg diet (comparable with 0.03 mg/kg body weight), and its efficacy was comparable with that of Arz.

### Table 2 Dose response with Arz and Tam in the prevention of breast cancer

<table>
<thead>
<tr>
<th>Treatmenta</th>
<th>No. of tumor-free rats/total no. of rats (P)b</th>
<th>Average no. of tumors (P)b</th>
<th>ATB (P)b</th>
<th>Rats with 3 or more tumors (P)b</th>
<th>Rats with tumor burden &gt;5 g (P)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (vehicle)</td>
<td>3/24</td>
<td>3.2</td>
<td>10.5</td>
<td>8/24</td>
<td>13/24</td>
</tr>
<tr>
<td>Arz, 20 mg/kg diet</td>
<td>8/12 (0.002)</td>
<td>0.5 (0.01)</td>
<td>1.5 (0.005)</td>
<td>1/12 (0.01)</td>
<td>1/12 (0.004)</td>
</tr>
<tr>
<td>Arz, 6 mg/kg diet</td>
<td>4/12</td>
<td>1.4</td>
<td>3.1 (0.03)</td>
<td>3/12</td>
<td>3/12</td>
</tr>
<tr>
<td>Arz, 2 mg/kg diet</td>
<td>6/12 (0.04)</td>
<td>0.9 (0.03)</td>
<td>2.7 (0.02)</td>
<td>1/12 (0.01)</td>
<td>3/12</td>
</tr>
<tr>
<td>Arz, 0.6 mg/kg diet</td>
<td>3/12</td>
<td>1.3 (0.02)</td>
<td>2.9 (0.01)</td>
<td>3/12</td>
<td>3/12</td>
</tr>
<tr>
<td>Arz, 0.2 mg/kg diet</td>
<td>3/12</td>
<td>1.8</td>
<td>7.8</td>
<td>4/12</td>
<td>6/12</td>
</tr>
<tr>
<td>Tam, 6 mg/kg diet</td>
<td>9/12 (0.004)</td>
<td>0.3 (0.03)</td>
<td>1.7 (0.002)</td>
<td>0/12 (0.002)</td>
<td>1/12 (0.004)</td>
</tr>
<tr>
<td>Tam, 2 mg/kg diet</td>
<td>6/12 (0.04)</td>
<td>0.6 (0.01)</td>
<td>1.4 (0.02)</td>
<td>0/12 (0.002)</td>
<td>1/12 (0.004)</td>
</tr>
<tr>
<td>Tam, 0.6 mg/kg diet</td>
<td>8/12 (0.002)</td>
<td>0.8 (0.01)</td>
<td>1.2 (0.002)</td>
<td>1/12 (0.01)</td>
<td>1/12 (0.004)</td>
</tr>
<tr>
<td>Tam, 0.2 mg/kg diet</td>
<td>3/12</td>
<td>3.8</td>
<td>9.8</td>
<td>7/12</td>
<td>6/12</td>
</tr>
</tbody>
</table>

a All rats (55 days of age) received NMU (50 mg/kg, i.p.) 1 week before starting the feeding of chemopreventive agents and were autopsied 10 weeks later.

b P is the value for the comparison of rats treated with chemopreventive agents with control rats treated with vehicle alone.

c ATB, average tumor burden; average weight in grams of a rat’s tumor at autopsy.
efficacy are not yet available. Although SERMs are now recognized as important drugs for both prevention and treatment of breast cancer, as single agents they do not appear to be useful for ER-negative breast cancer, either preventively or therapeutically. Development of new agents to control ER-negative breast cancer remains a major challenge.

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References

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