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DOI: 10.1002/cmdc.200700104

Structure–Activity Relationships for a Family of Benzothiophene Selective Estrogen Receptor Modulators Including Raloxifene and Arzoxifene

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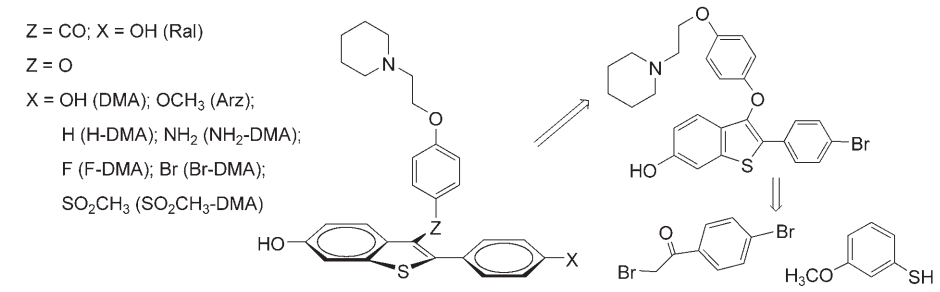
The search for the "ideal" selective estrogen receptor modulator (SERM) as a substitute for hormone replacement therapy (HRT) or use in cancer chemoprevention has focused on optimization of estrogen receptor (ER) ligand binding. Based on the clinical and preclinical benzothiophene SERMs, raloxifene and arzoxifene, a family of SERMs has been developed to modulate activity and oxidative lability. Antiestrogenic potency measured in human endometrial and breast cancer cells, and ER ligand binding data were correlated and seen to provide a guide to SERM design only when viewed in toto. The in vitro studies were extended to the juvenile rat model, in which the desired antiestrogenic profile and putative cardiovascular benefits of SERMs were observed.

Introduction

Development of the "ideal" selective estrogen receptor modulator (SERM) is of importance in postmenopausal women's health. This SERM would be estrogenic in bones to prevent osteoporosis, in the heart to prevent cardiovascular disease, and in the CNS to prevent hot flashes and neurological disorders such as Alzheimer's disease. Conversely, this SERM would be an antiestrogen in hormone sensitive tissues such as the breast and the endometrium to prevent hormone dependent cancers. Tamoxifen, the archetype SERM, remains of use in all stages of hormone-dependent breast cancer, including in chemoprevention,[1] despite increased risk of endometrial cancer, which has been linked to estrogenic activity in the uterus and endometrium.[2–4] An alternative chemical carcinogenesis mechanism is based upon the oxidative lability of tamoxifen and its active metabolite, 4-hydroxytamoxifen (HOT), which is oxidatively bioactivated to genotoxic quinoid metabolites.[5,6] HOT, is susceptible to bioactivation, in common with many SERMs, because it is a polyaromatic phenol.

Optimism has surrounded the newer benzothiophene SERMs, raloxifene (Ral) and arzoxifene (Arz) which appear devoid of agonist activity in the endometrium, whilst being potent antiestrogens in the breast and agonists in bone.[1,7] Nevertheless, Ral and Arz are also readily bioactivated to quinoid metabolites, although with different reactivity to the quinoids derived from tamoxifen.[8–11] A family of benzothiophene SERMs that includes Arz and its active metabolite desmethylarzoxifene (DMA) has been developed to gain a deeper understanding of both ER-dependent and ER-independent relationships between structure and activity (Scheme 1).[12] The 4′-OH group of DMA facilitates quinoid formation (Scheme 2); one important question to answer is how replacement of the 4′-OH with other functional groups will be tolerated by the ER and influence ER-dependent activity.



Scheme 1. Benzothiophene SERMs used in this study: synthetic disconnection.

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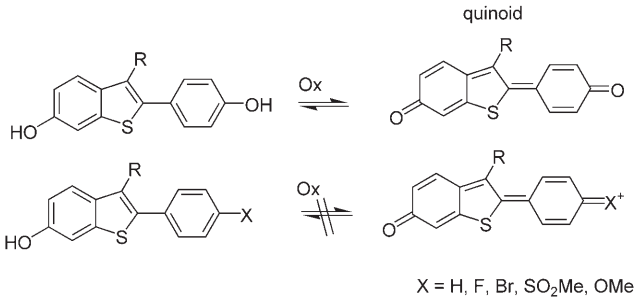

Scheme 2. Substitution of the 4′-OH blocks oxidative bioactivation to a quinoid.

Ral is in clinical use for postmenopausal osteoporosis and is currently in the breast cancer chemoprevention studies on tamoxifen and raloxifene trial (STAR);[13] recent results indicating that Ral has a superior risk–benefit ratio to tamoxifen. Early results from the raloxifene use for the heart trial (RUTH) show that Ral use is not associated with the increased risk of coronary events associated with HRT,[14] but data supporting the significant cardiovascular benefits indicated by animal models has not yet been presented. The low bioavailabilty of Ral stimulated the discovery of Arz, a next-generation SERM in late-stage clinical trials which has shown additional promise in endometrial cancer therapy.[15,16] In inhibition of estrogen-mediated proliferation in MCF-7 human breast cancer cells, Arz ($IC_{50}=0.4$ nM) was reported to be more potent that tamoxifen, but less potent that its metabolite, DMA ($IC_{50}=0.05$ nM).[17]

The polyaromatic phenolic cores of SERMs mimic estradiol (E_2) binding at the ER ligand binding domain (LBD). Ral and DMA both contain the 2-phenyl-benzothiophene core linked to an amine side arm that confers antiestrogenic activity; the only difference is in the use of an ether versus a ketone "hinge" between the core and the side arm, which in ER-bound structures are aligned orthogonally (Scheme 1).[18] In the development of Ral, Griese and co-workers reported almost 100 modifications of the 2-phenyl-benzothiophene core, but reported poor correlations of inhibition of proliferation in MCF-7 cell culture with competitive ligand binding to the isolated ER, suggesting that the high structural variability was partly to blame.[19] To systematically probe structure–activity relationships, a small, homologous family of 4′-X-substituted-4′-desmethoxyarzoxifenes (X-DMA) was prepared using Br-DMA as a common synthon.[12]

The agonist/antagonist activity at the ER of X-DMA SERMs was compared with Ral, HOT, and the pure antiestrogen ICI 182780 (ICI) in MCF-7 cells and Ishikawa human endometrial cells, for correlation with ER-ligand binding data. For the X-DMA SERMs, Arz, DMA, and F-DMA, the study was extended in vivo to measure uterotrophic and serum lipid biomarkers of estrogenic and antiestrogenic activity.

Results and Discussion

Chemistry

The X-DMA SERMs were synthesized by an efficient, improved synthesis using the Br-DMA synthon (Scheme 1).[12]

In vitro Biology

Estrogenic and antiestrogenic activity in MCF-7 cells positive for either ERα or ERβ was measured using transient transfection with an ERE-luciferase reporter. Estrogenic activity was not observed, but compounds antagonized the effects of estradiol (E_2; 1 nM). Luciferase induction was measured at four concentrations straddling the IC_{50} for each compound to quantify IC_{50} values (Table 1; Figure 1). DMA was the most potent compound with an estimated IC_{50} in ERα(+) and ERβ(+) cells of 1.3 nM and <1 nM, respectively. Antiestrogenic activity was also measured in the ERα(+) Ishikawa cell line using an ERE-alkaline phosphatase (ALP) reporter (Table 1). Again, DMA was the most potent antiestrogen (IC_{50} = 0.1 nM).

The collected measures of antiestrogenic potency were compared with IC_{50} values for displacement of E_2 from the pure, isolated full-length ER protein, reported previously (Table 1).[12] SO_2CH_3-DMA was a very poor ligand for isolated ERβ, with predicted selectivity for ERα/ERβ of 70-fold, however this selectivity did not translate into the cell-based assays. Other clear disparities included the problematic correlation between potency in the two ERα(+) cell lines. Correlations across the family of compounds were explored graphically. Interestingly, a good correlation was seen for ERα versus ERβ ligand binding affinity (Figure 2a), with the two outliers being SO_2CH_3-DMA and Ral; the latter understandable because Ral is not part of the DMA family. Consistent with this observation, in the correlations of

Table 1. IC_{50} values (nM) for SERM-ER interactions in competitive receptor binding, ERE-luciferase inhibition, and alkaline phosphatase inhibition assays.

SERM[a]	Competitive binding[a,b]		MCF-7 ERE-luc[c]		Ishikawa ALP[b]
	ERα	ERβ	ERα	ERβ	ERα
Ral	21 ± 2.7	560 ± 150	6.2	3.3	2.9 ± 1.6
Arz	22 ± 6.5	66 ± 3.1	4.5	2.9	1.3 ± 0.3
DMA	7.8 ± 1.9	9.6 ± 1.9	0.7	0.5	0.1 ± 0.1
H-DMA	11 ± 1.5	16 ± 0.8	3.4	0.82	3.0 ± 0.7
F-DMA	17 ± 0.6	28 ± 11	3.8	2.5	1.4 ± 0.4
NH_2-DMA	19 ± 1.7	49 ± 1.9	8.1	58	3.9 ± 0.7
Br-DMA	27 ± 4.6	67 ± 8.5	21	200	5.4 ± 1.4
SO_2CH_3-DMA	27 ± 4.8	1800 ± 100	110	280	4.6 ± 0.8
HOT	1.0 ± 0.3	3.2 ± 0.4	6.4	15	0.5 ± 0.1
ICI			4.2	0.7	0.4 ± 0.2

[a] From ref. [12]. [b] Mean IC_{50} with S.D. from at least five concentrations tested at least three times independently in triplicate. [c] Estimated mean IC_{50} from triplicate measures of at least four concentrations in two separate cell passages with errors <10% (see Figure 1).

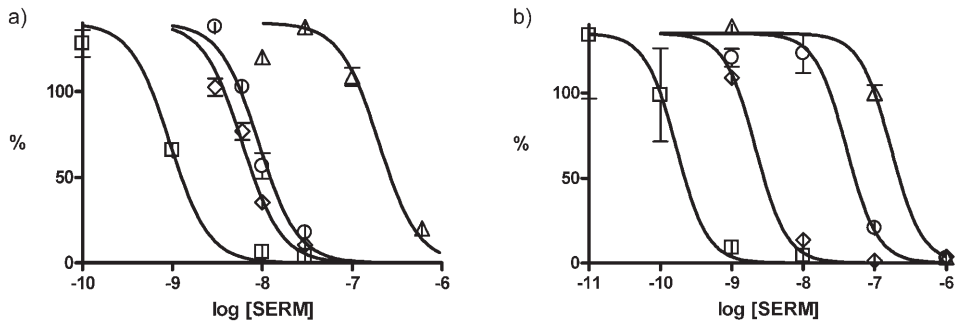

Figure 1. Representative concentration-response curves for ERE-luciferase induction in a) ERα(+) and b) ERβ(+) MCF-7 cells showing antiestrogenic activity of X-DMA SERMs: DMA (square); F-DMA (diamond); NH_2-DMA (circle); SO_2CH_3-DMA (triangle). SERM concentration is given in M.

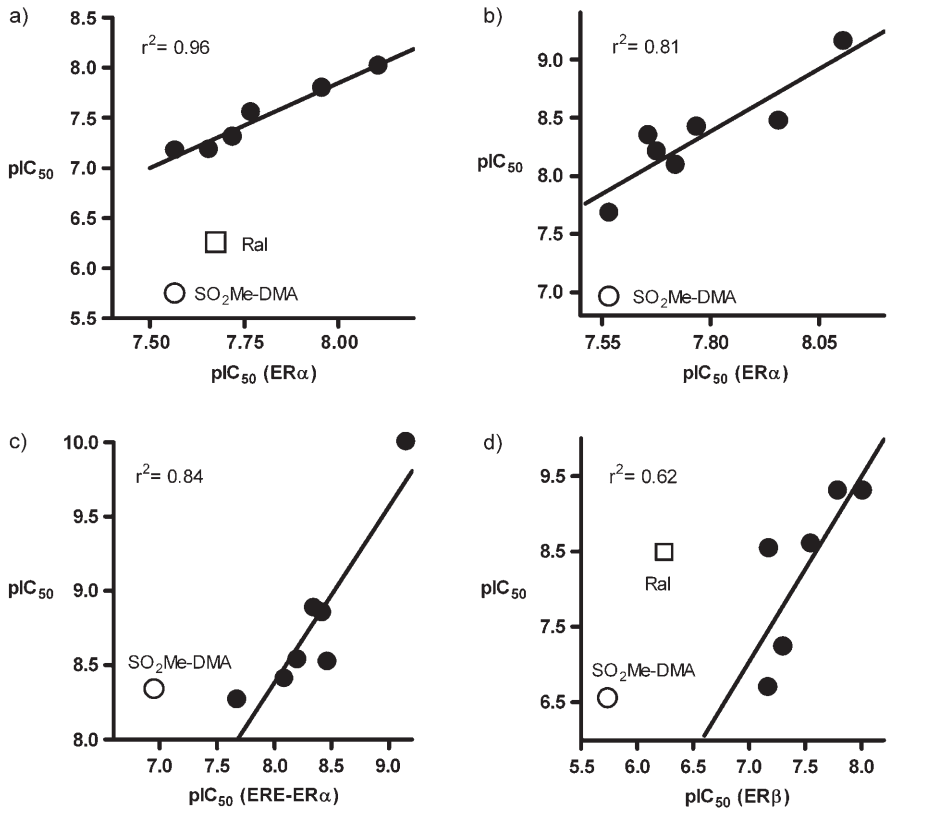

Figure 2. Correlations of observed data for benzothiophene SERMs presented in Table 1: a) ERα versus ERβ ligand competitive binding; b) ERα ligand binding versus ERα-MCF-7 ERE-luc; c) ERα-MCF-7 ERE-luc versus ERα-Ishikawa ALP; d) ERβ ligand binding versus ERβ-MCF-7 ERE-luc. SERMs shown by solid symbols are included in linear correlation analysis. $pIC_{50} = -\log(IC_{50})$, where IC_{50} is in M.

ligand binding with ERE-luciferase induction, either SO_2CH_3-DMA or both SO_2CH_3-DMA and Ral were outliers for ERα and ERβ, respectively (Figure 2b and d). Given these exclusions, modest correlations were observed, a situation mirrored in the relationship observed between the two cell-based assays of ERα antagonist activity (Figure 2d).

Computational docking of the X-DMA SERMs to the relaxed ERα ligand binding site was observed to quantitatively correlate with experimental ligand binding affinity for all X-DMA SERMs studied herein, except for SO_2CH_3-DMA.[12] Hence, the results have reasonable internal consistency, suggesting that SO_2CH_3-DMA exerts antiestrogenic activity by interactions with ERα and ERβ in a mode differing from the other X-DMA SERMs. This binding mode is effective, as the observed potency of SO_2CH_3-DMA is nanomolar and submicromolar in ERα(+) and ERβ(+) cells, respectively.

To confirm the antiestrogenic activity of the X-DMA SERMs in regulating endogenous ER-mediated gene transcription,[20] PTGES (prostaglandin E synthase gene) was measured by real time PCR after incubation of MCF-7 cells with E_2 (10 nM) in the absence or presence of SERM (1 μM) for 2 h. All X-DMA compounds tested were full antagonists of estrogen action under these conditions (data not shown).

In vivo Biology

Three X-DMA SERMs were selected for in vivo testing, to compare Arz with the putative active metabolite, DMA, and the metabolically inactive analogue, F-DMA. The juvenile rat model (employing 19–21 day-old female Sprague-Dawley rats) commonly used in the study of estrogenic activity, was selected.[21] The organs of rats at this age are fully estrogen responsive, but the hormone is not yet being produced in the ovaries, so this rat model can offer a clear hormone background when studying SERM activity.[22] Eight groups of immature female rats were treated daily with E_2 (0.1 mg kg^{-1}) or vehicle control with or without X-DMA SERMs (10 mg kg^{-1}) for 3 days. Animals were sacrificed on the fourth day, and uterine weight was measured for the excised organ (Figure 3). None of the X-DMA compounds were uterotrophic at the dose tested, indicating the lack of the detrimental estrogenic effect on the uterus as seen for HOT. In contrast, all three SERMs showed significant antiestrogenic activity in blocking the estrogen-induced uterine weight gain ($P < 0.01$). Similar results were obtained regardless of correction for overall weight changes to the animals (Figure 3b). Although no significant difference was

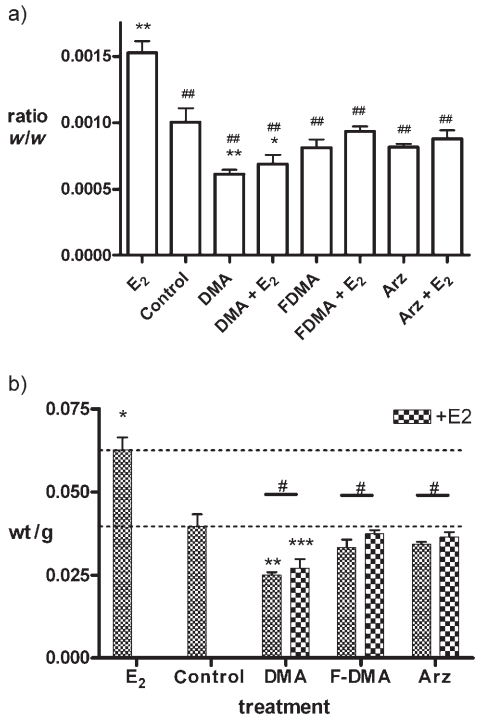

Figure 3. Uterine weights measured after 3 day drug treatment of female, juvenile rats with SERMs (10 mg kg^{-1}) either alone or challenged with E_2 (0.1 mg kg^{-1}). a) Uterine weights corrected for body weights (uterine weight/body weight): ** $P < 0.01$ compared with control; * $P < 0.05$ compared with control. ## $P < 0.01$ compared with E_2. b) Uterine weights. *** $P < 0.05$ compared to control; ** $P < 0.01$ compared with control; * $P < 0.001$ compared to all other groups; # $P > 0.05$ comparison of each SERM with and without E_2. Data show mean ± SD analyzed by one-way ANOVA with Tukey or Dunnett's test.

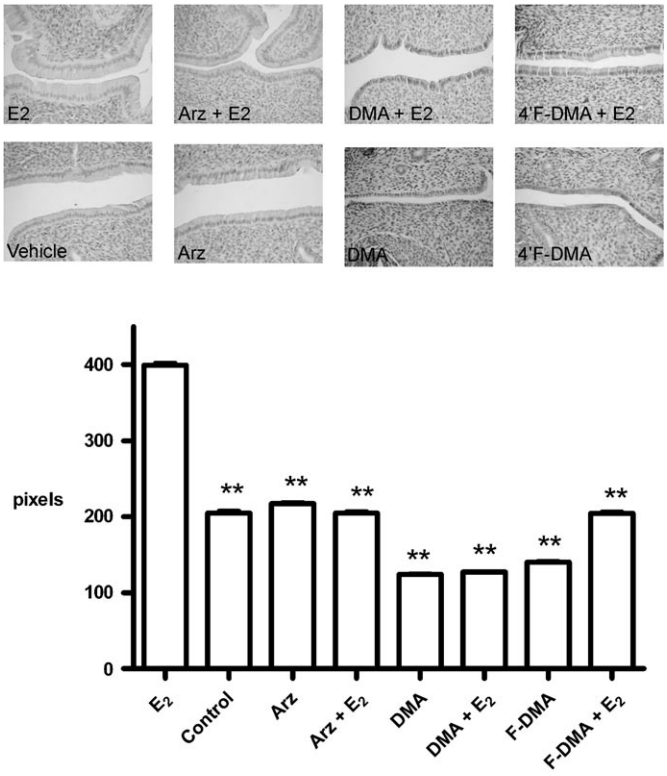

Figure 4. Visualization and quantitation of epithelial cell height. A section from each uterus was analyzed after 3 day drug treatment of female, juvenile rats with SERMs (10 mg kg^{-1}) either alone or challenged with E_2 (0.1 mg kg^{-1}) by measuring the height (in pixels) of 30 epithelial cells at 400 × magnification. ** p value < 0.01 compared to E_2. Data show mean ± SD analyzed by one-way ANOVA with Dunnett's test.

observed in the SERM administered groups between the E_2-treated and untreated arms, the effect of DMA was to significantly reduce uterine weight over the vehicle control arm (Figure 3b). The likely explanation is that the control group is influenced by low endogenous estrogen production that is totally antagonized by DMA, the most potent antiestrogen studied (Table 1).[10,23]

Uteri were processed for hemotoxylin and eosin staining to visualize drug effects on the luminal epithelial cells, known to be a sensitive biomarker for hormonal uterine stimulation (Figure 4). E_2 treatment increased epithelial cell height growth up to twofold compared to control. The SERMs showed no estrogenic activity and when co-administered with E_2, dramatically blocked the estrogenic effect. In accord with the uterine weight data, the effect of DMA was significant relative to vehicle control ($P < 0.01$), and in this assay, F-DMA also reached significance ($P < 0.05$) (Figure 4).

Early studies on Arz (0.1–10 mg kg^{-1} day^{-1} for 5 weeks) reported reduction in total serum cholesterol after treatment of adult rats, in both ovariectomized and sham-operated groups.[24] A significant reduction in serum triglycerides was also reported in ovariectomized and sham adult mice administered Arz for 12 weeks.[25] Postmenopausal elevation of serum lipids causing increased risk of cardiovascular disease is a prime target for SERM therapy which is routinely modeled in ovariectomized rodent models. The juvenile rat model is rarely used in this respect, presumably because of the short period of drug treatment. The estrogen-mediated elevation of serum lipids was not observed in this model, however significantly lowered triglyceride levels were observed in response to SERM treatment (Figure 5a). Furthermore, Arz administration also reached significance in reduction of plasma cholesterol (Figure 5b).

Benzothiophene SERM Design

Early hypotheses for SERM design required a planar molecule with hydroxyl groups to hydrogen bond to both His524 and Glu353/Arg394 at either end of the E_2-bonding pocket of ERα-LBD, mimicking the binding of E_2. In the case of Ral and DMA, the 7-OH and the 4′-OH would perform these roles, and a salt bridge between the side-arm ammonium and Asp351 would provide the antiestrogenic action.[26] The antiestrogenic activity of the X-DMA SERMs is in accord with the published literature, that the His524 interaction will tolerate replacement of a hydroxyl by other groups that poorly participate in hydrogen bonds (Table 1).

Differential ligand binding for ERβ over ERα has been difficult to achieve by modifications to the planar core of SERMs because both receptors bind estrogen as the natural ligand;

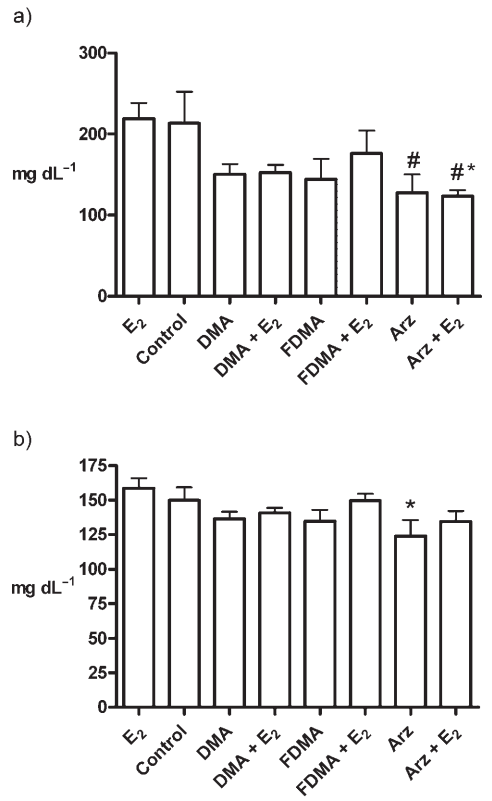

Figure 5. Serum lipid measurements after 3 day drug treatment of female, juvenile rats with SERMs (10 mg kg^{-1}) either alone or challenged with E_2 (0.1 mg kg^{-1}). a) Triglyceride levels (reported triglyceride ranges for rats are 0–200 mg dL^{-1}): # $P < 0.05$ compared to E_2; * $P < 0.05$ compared to control. b) Total cholesterol levels: * $P < 0.05$ compared to E_2. Data show mean $\pm$ SD analyzed by one-way ANOVA with Dunnett's test.

the major difference being the smaller size of the ERβ cavity. The modest fourfold selectivity of H-DMA in MCF-7 cells for ERβ is thus consistent. Across the X-DMA series, as the size of the 4′-substituent was varied, a concomitant loss of binding affinity to both ERα and ERβ was observed, until the bulk of the methylsulfonyl substituent blocked binding to the ERβ-LBD receptor by SO_2CH_3-DMA (Figure 2 A). However, as the results from the cell culture assays demonstrate (Table 1), even this modification of the 4′-position is sufficiently tolerated to result in an antiestrogen of submicromolar potency for both ERα and ERβ.

Based upon studies largely on E_2, HOT, ICI, and Ral, a theory for antiestrogenic mechanisms continues to evolve from the original "H12 molecular switch" postulate.[27,28] Helix 12 (H12), the C-terminal ER-LBD, contains a ligand-responsive transcriptional activation function (AF-2) that is located adjacent to the ER DNA-binding domain. E_2 and other agonists bind at the ligand receptor site of the ER-LBD allowing helix 12 (H12) of AF-2 to lie across and cap the opening to the ligand binding pocket. This liganded-ER is both stabilized and able to interact with transcriptional co-activator proteins that recognize a binding motif formed by H3-5 and H12. The role of AF-2, for which H12 is crucial, is recruitment of these coactivators that are required for transcriptional activation by liganded-ER bound at the ERE.

The SERMs HOT and Ral are postulated to act as ER antagonists because the amine side chain prevents H12 from capping the ligand pocket and in crystal structures H12 is seen to fold into the H3-H5 grove blocking co-activator recruitment. A modification of this H12 molecular switch postulate recognizes the "coregulator context"; that is, the identity of the protein co-activator or co-repressor, which will be cell specific. This modification recognizes the observation that an effect of antiestrogen perturbation of ER conformation is to enhance recruitment of co-repressors that suppress AF-1 transcriptional activation. The coregulator context importantly provides a mechanistic basis for the observed tissue agonist/antagonist selectivity of SERMs that is central to their action.

Pure antiestrogens, such as ICI, are able to reduce levels of cellular ER, either by enhancing ubiquitination which signals proteasomal degradation or by destabilization of the ER–ligand complex. Recent elegant studies have emphasized the importance of hydrophobic residues in the conformationally mobile H12, which function as recognition elements for binding to H3-H5, ubiquitin ligase, and coregulator proteins; and which when exposed to an aqueous environment by antiestrogen binding can lead to insoluble intracellular ER aggregates.[27,28] The antiestrogenic activity of SERMs appears to include a SERM-mediated reduction in functional ER, to variable degrees, by both ER degradation and ER aggregation. The cellular context determines the protein partners of ER, including co-regulators, chaperones, immunophilins, and ubiquitination machinery. The modest correlations between antiestrogenic activity in different cell cultures and ligand binding, even in the structurally conservative family of X-DMA SERMs studied herein, must be viewed in this context.

Conclusions

The observed tolerance for modifications at the 4′-position of benzothiophene SERMs opens the possibility to fully study the influence of oxidative bioactivation on SERM function and toxicity, as bioactivation can be structurally modulated with no significant perturbation of ER-dependent activity in vitro or in vivo. Furthermore, it seems likely that the beneficial mix of estrogenic and antiestrogenic SERM activity can be maintained with submicromolar potency whilst modifications at the 4′-position can be utilized to modulate bioavailability and tissue selectivity. One such opportunity would be exploitation of the enhanced passive transport of lipophilic SERMs such as F-DMA.

Experimental Section

Chemicals and reagents

All chemicals and reagents were purchased from Fisher (Hanover Park, IL) or Sigma–Aldrich (St. Louis, MO) unless otherwise indicated. All media for cell culture and human recombinant ERα and ERβ were purchased from Invitrogen (Grand Island, NY). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Norcross, GA). Raloxifene was synthesized as described.[9] The X-DMA SERM synthesis and characterization was recently reported.[12]

Cell Culture Conditions

The Ishikawa cell line was provided by R. B. Hochberg (Yale University, New Haven, CT) and was maintained in Dulbecco's Modified Eagle medium (DMEM/F12) containing sodium pyruvate (1%), nonessential amino acids (NEAA, 1%), glutamax-1 (1%), insulin (0.05%), and heat-inactivated FBS (10%). MCF-7 WS8 cells were provided by V. C. Jordan (currently Fox Chase Cancer Center) and were grown in RPMI 1640 media containing glutamax-1 (1%), NEAA (1%), insulin (0.05%), and heat-inactivated FBS (10%). The MCF-7 C4-12-5 ERβ positive stable cell line (referred to as MCF-7 ERβ)[29] was provided by D. B. Lubahn (University of Missouri) and was grown in MEM (catalogue number 3024) supplemented with stripped CBS (5%), Pen/Strep (2%), insulin (6 ng mL^{-1}), sodium carbonate (2.2 g L^{-1}), HEPES (1.25 M, 8 mL), glutamax (1%), and G418S (50 mg mL^{-1} stock, 6 mL). Stripped serum was prepared by incubating the serum with acetone-washed activated charcoal (100 mg mL^{-1}) at 4 °C for 30 min, and centrifuged at 4000 RPM for 15 min at 4 °C. This step was repeated in triplicate. DMSO concentrations for all cell culture assays were below 0.1%.

ERα and ERβ competitive binding assays

The competitive ERα and ERβ binding assays were used with tritiated estradiol based on the method of Obourn et al.,[30] with minor modifications,[31] to determine in vitro binding affinities of the substrates with the receptors. The reaction mixture consisted of sample in DMSO (5 μL), pure human recombinant diluted ERα and ERβ (0.5 pmol, 5 μL) in ER binding buffer, "Hot Mix" [400 nM, 5 μL prepared fresh using 95 Ci/mmol [^{3}H] estradiol, diluted 1:1 ethanol:ER binding buffer; obtained from NEN Life Science Products (Boston, MA)], and ER binding buffer (85 μL). The incubation was carried out at room temperature for 2 h before a hydroxyappatite slurry (HAPs, 50%, 100 μL) was added. The tubes were incubated on ice for 15 min with vortexing every 5 min. The appropriate ER wash buffer was added (1 mL), and the tubes were vortexed before centrifuging at 10000 × g for 1 min. The supernatant was discarded, and this wash step was repeated three times. The HAPs pellet containing the ligand–receptor complex was resuspended in ethanol (200 μL) and transferred to scintillation vials. An additional volume of ethanol (200 μL) was used to rinse the centrifuge tube. Cytoscint [4 mL/vial; ICN (Costa Mesa, CA)] was added, and the radioactivity was counted using a Beckman LS 5801 liquid scintillation counter (Schaumburg, IL). The percent inhibition of [^{3}H] estradiol binding to each ER was determined using Equation (1). The binding capability (percent) of the sample was calculated in comparison with that of estradiol (50 nM, 90%).

$$[1-(\mathrm{dpm_{sample}}-\mathrm{dpm_{blank}})/(\mathrm{dpm_{DMSO}}-\mathrm{dpm_{blank}})] \times 100 = \%\ \text{sample binding} \quad (1)$$

Measure of ERE activation

The Dual-Luciferase Reporter Assay System from Promega (Madison, WI) was used to evaluate the functional formation of the ER–ERE complex and luciferase protein expression. Both MCF-7 WS8 and MCF-7 ERβ cell lines were cultured in phenol red free, estrogen-stripped media 96 h before transfection. The cells were transfected with the pERE-luciferase plasmid (2 μg), which contains three copies of the Xenopus laevis vitellogenin A2 ERE upstream of fire fly luciferase (a gift from Dr. V. C. Jordan). To normalize transfection efficiency, pRL-TK plasmid (1 μg, Promega) was co-transfected. Cells (5×10^6) in serum-free media were transfected by electroporation in a 0.4 cm cuvette (Bio-Rad Laboratories) at a voltage (0.320 kV) and a high capacitance (950 μF) in a GenePulser X-cell (Bio-Rad Laboratories). The cells were resuspended in estrogen-free media, transferred to 12-well plates immediately after electroporation, and incubated overnight. The cells were treated with the appropriate compounds for 18 h in the presence of E_2, 1 nM). The luciferase activities in the cell lysates were measured using the dual-luciferase reporter assay system from Promega (Madison, WI) with a FLUOstar OPTIMA (BMG LABTECH, Durham, NC). Data are reported as estimated IC_{50} values based on four concentration points that are distributed above and below 50%. IC_{50} values were estimated using Graphpad Prism using nonlinear fit. For ERα, the curve was constrained at the maximal activity, and the minimal activity was constrained to zero and therefore the IC_{50} is half of the maximal activity. For ERβ there was clearer consensus between the unconstrained and the values when the curve was constrained such that the minimal activity was zero and maximal activity was 100%. Therefore the IC_{50} value was the average of the two nonlinear regressions. Relative luciferase activity was the fire fly luciferase reading divided by the Renilla luciferase reading.

Induction of alkaline phosphatase in cultured Ishikawa cells

The procedure of Liu et al. was used as described previously.[31] Ishikawa cells (1.5×10^4 cells/190 μL/well) were preincubated in 96-well plates overnight in estrogen-free medium. Test samples (10 μL at varying concentrations in DMSO) were added to determine EC_{50} values, and the cells in a total volume (200 μL media/well) were incubated at 37 °C for 4 days. For the determination of antiestrogenic activity, estradiol (2 nM) was added to the 10 mL of media used to dilute the test samples. The induction plates were processed by washing the plates with PBS and adding Triton x 100 (0.01%, 50 μL) in Tris buffer (pH 9.8, 0.1 M). Plates were subjected to a freeze thaw (−80 °C for at least 24 h before warmed on a plate warmer to 37 °C). An aliquot (150 μL) of *p*-nitrophenylphosphate (phosphatase substrate, 1 mg mL^{-1}) in Tris buffer (pH 9.8, 0.1 M) was added to each well. The enzyme activity was measured by reading the release of *p*-nitrophenol at 405 nm every 15 s with a 10 s shake between readings for 16 readings using a Power Wave 200 microplate scanning spectrophotometer (Bio-Tek Instruments, Winooski, VT). The maximal slopes of the lines generated by the kinetic readings were calculated. For antiestrogenic determination, the percent induction as compared with the background induction control was calculated using Equation (2).

$$[1-((\mathrm{slope_{sample}}-\mathrm{slope_{cells}})/(\mathrm{slope_{estrogen}}-\mathrm{slope_{cells}}))] \times 100 = \%\ \text{antiestrogenic induction} \quad (2)$$

Animals

Guidelines established by our institutional animal care and use committee and state and federal regulations were followed for all procedures. The protocol complied with the guide for the care and use of laboratory animals and the facilities are association for the assessment and accreditation of laboratory animals care approved. Female juvenile Spraque-Dawley rats were received at 12 d of age from Harlan (Indianapolis, IN) and housed 11 pups to a cage with a nursing dam. Animals had free access to rat chow and water and were allowed one week to acclimate. The animals were randomized into groups ($n=6$) to receive either Arz, DMA, or 4-FDMA (sc; 10 mg kg^{-1}) for 3 days either alone or with 17β estradiol-3-ben-

zoate (E_2 as benzoate; 0.1 mg kg^{-1}). Approximately 24 h after the last injection the animals were sacrificed by bottled gas CO_2. The blood and uterus were collected. The uterus was trimmed of fat and connective tissue, and blotted with filter paper, and weighed. Blood (1 mL) was collected using syringe needles and stored on ice before centrifuging and being stored at −80 °C.

Histology

The uterus from animals were processed for paraffin embedding. The uterus was arranged horizontally and sectioned (4 μm increments). One slide per animal was stained using hemotoxylin and eosin as follows: xylene (3×3 min); EtOH (10%, 2×2 min); EtOH (95%, 2×2 min); water (2×2 min); Harris hemotoxylin (1×8 min); running water (1×10 min); HCl (1%, 4×0.5 s); water (1×2 min); lithium carbonate (1%, 4×1 s); water (55 °C , 1×10 min); eosin (0.5% in 70% EtOH and acid 1×1.5 min); EtOH (95%, 2×30 sec); EtOH (100%, 2×2 min); xylene (3×2 min). Slides were coversliped using paramount and representative pictures of the luminal epithelium were acquired (400x). Representative epithelial cell height was measured in pixels using MetaValue (Universal Imaging) software (N = 30 per picture). Data were combined using GraphPad Prism and average height in pixels was calculated.

Measurement of LDL, cholesterol, and triglyceride levels

Measurements were performed at the Biological Resources Laboratory as a technical service using LDL-c plus (Roche Diagnostics).

Measurement of PTGES gene transcription

To study endogenous gene regulation by estrogen and X-DMA compounds, MCF-7 cells were cultured for 4 days in phenol-red free MEM supplemented with charcoal-dextran stripped calf serum (5%). Cells were treated with E_2 (10 nM) in the presence or absence of each X-DMA compound (1 uM) for 2 h. RNA was isolated and real-time Q-PCR was carried out as previously described.[20] Primers used for PTGES were F 5′-CTTCCTTTTCCTGGGCTTCG and R 5′-GAAGACCAGGAAGTGCATCCA and for the internal control 36B4 were F 5′-GTGTTCGACAATGGCAGCAT and R 5′-GACACCCTCCAGGAAGCGA.

Acknowledgements

This work was supported by NIH Grants CA102590 and CA79870. Edward M. Curran, Dennis B. Lubahn, Wade V. Welshons, and Xiaohui Yuan are thanked for provision of cell lines. Ping Yao, Qi Shen, and Qing Ling are acknowledged for technical assistance.

Keywords: cancer · chemoprevention · estradiol · estrogen receptor · hormone replacement therapy · SERM

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Received: May 3, 2007
Revised: June 15, 2007
Published online on July 26, 2007

