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Repurposing Ospemifene for Potentiating an Antigen-Specific Immune Response

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Abstract

Objective—Ospemifene, an estrogen receptor agonist/antagonist approved for treatment of dyspareunia and vaginal dryness in postmenopausal women, has potential new indications as an immune modulator. The overall objective of the present series of preclinical studies was to evaluate the immunomodulatory activity of ospemifene in combination with a peptide cancer vaccine.

Methods—Immune regulating effects, mechanism of action and structure activity relationships of ospemifene and related compounds were evaluated by examining expression of T cell activating cytokines *in vitro*, and antigen-specific immune response and cytotoxic T-lymphocyte activity *in vivo*. The effects of ospemifene (OSP) on the immune response to a peptide cancer vaccine (PV) were evaluated following chronic [control (n=22); OSP 50 mg/kg (n=16); PV (n=6); OSP+PV (n=11)], intermittent [control (n=10); OSP 10 and 50 mg/kg (n=11); PV (n=11); combination treatment (n=11 each dose)] and pretreatment [control; OSP 100 mg/kg; PV 100 µg; combination treatment (n=8 all groups)] ospemifene oral dosing schedules in a total of 317 mixed-sex tumor-bearing and non-tumor-bearing mice.

Results—The results showed that ospemifene induced expression of the key T_H1 cytokines interferon gamma and interleukin-2 *in vitro*, which may be mediated by stimulating T cells through phosphoinositide 3-kinase and calmodulin signaling pathways. In combination with an antigen-specific peptide cancer vaccine, ospemifene increased antigen-specific immune response and increased cytotoxic T-lymphocyte activity in tumor-bearing and non-tumor-bearing mice. The pretreatment, intermittent, and chronic dosing schedules of ospemifene activate naïve T cells, modulate antigen-induced tolerance and reduce tumor-associated, pro-inflammatory cytokines, respectively.

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Conclusions—Taken together, ospemifene’s dose response and schedule-dependent immune modulating activity offers a method of tailoring and augmenting the efficacy of previously failed antigen-specific cancer vaccines for a wide range of malignancies.

Keywords

Ospemifene; Vulvovaginal atrophy; Antigen-specific Immunotherapy; Immunomodulatory activity; Fispemifene

Introduction

It is well known that steroid hormones exert diverse biological effects throughout the entire body. All steroids share a cholesterol skeleton, with their complex and distinct activities stemming in part from various chemical substitutions on the basic structure.¹ Similarly, triphenylethylenes, which are synthetic, non-steroidal hormonal agents including tamoxifen, toremifene and clomiphene, share a common basic structure, with their pharmacological effects being dictated by different chemical substitutions on the basic structure as well as their stereochemistry. Triphenylethylenes were originally synthesized in the 1950s for use as contraceptive agents, but it was discovered that they were actually having the opposite effect, induction of ovulation.² These compounds are commonly referred to as estrogen receptor agonists/antagonists (ERAs) or as SERMs (selective estrogen receptor modulators), owing to their mixed estrogenic/antiestrogenic, tissue-selective activity. Extensive research into structure-activity relationships (SARs) of these compounds in the ensuing decades began to reveal their complex pharmacology, including stimulation of ovulation (clomiphene), antiestrogenic effects (tamoxifen, toremifene), breast cancer chemopreventive effects (tamoxifen) and beneficial effects on bone and serum cholesterol, among others.

The latest triphenylethylene to receive regulatory approval is ospemifene, which is indicated for the treatment of postmenopausal women with dyspareunia and vulvovaginal atrophy (VVA), now referred to as genitourinary syndrome of menopause.³ Early SAR studies of ospemifene and related compounds such as toremifene, of which ospemifene is a principal metabolite, revealed that ospemifene has relatively weak estrogenic/antiestrogenic effects,⁴ and that it is devoid of activity in multidrug-resistant human breast cancer cells.⁵ Preclinically, ospemifene has been shown to prevent breast cancer in various rodent models, similar to tamoxifen.³ Ospemifene’s weak hormonal activity, while still maintaining clinical efficacy, eventually led to its approval by the U.S. Food and Drug Administration (FDA).

The triphenylethylenes have also been studied in regard to their effects on the immune system. For example, tamoxifen treatment induces a shift from cellular T-helper 1 (T_H1) to humoral T_H2 immunity,^{6, 7} which is unfavorable for enhancing T lymphocytes to mount a cellular immune response. Tamoxifen also induces latent transforming growth factor beta (TGFβ) in breast cancer cell lines⁸ and prevents maturation of antigen presenting cells (APCs).⁹ TGFβ can inhibit cytotoxic T lymphocytes (CTLs) and induce T regulatory cells (Tregs), creating an immunosuppressive tumor microenvironment.¹⁰ The aromatase inhibitor anastrozole increased levels of the pro-inflammatory cytokines interferon gamma (IFN-γ) and interleukin (IL)-12, T_H1 cytokines, and decreased levels of the T_H2 cytokines IL-4 and

IL-10 in an experimental polyarthritis study. Anastrozole also suppressed differentiation of naïve T-cells into Tregs, which are known to produce immunosuppressive cytokines in the tumor microenvironment.^{11, 12} These observations suggest that antiestrogenic drugs such as ERAAs and aromatase inhibitors may act as immune modulators. Previously, we have shown that combining tamoxifen with tecemotide, an antigen-specific peptide cancer vaccine, in a double-transgenic breast cancer mouse model resulted in a reduced survival benefit compared to tamoxifen alone, while the combination of the aromatase inhibitor letrozole and tecemotide resulted in an additive survival benefit.¹³

In the present study, we evaluated the effects of ospemifene on T cell activation *in vitro*, the effects of ospemifene on pro-inflammatory serum cytokines *in vivo*, and the potential immunomodulatory effects of ospemifene on the immune response to tecemotide following pretreatment, intermittent and chronic dosing schedules in non-tumor-bearing as well as tumor-bearing mice.¹⁴ Also investigated were the potential mechanism(s) through which ospemifene exerts its effects on immune response and structure activity relationships.

Materials and Methods

Drugs and Chemicals

Urethane, 17 β -estradiol, trifluoperazine and tamoxifen were purchased from Sigma-Aldrich (St. Louis, MO). Dimethyl sulfoxide (DMSO) was purchased from Thermo Fisher Scientific (Waltham, MA). PD98059 and LY294002 were purchased from Selleck Chemicals (Houston, TX). Ospemifene (chemical name Z-2-[4-(4-chloro-1,2-diphenyl-but-1-enyl)phenoxy]ethanol) was manufactured by Lipomed AG (Arlesheim, Switzerland). Ospemifene metabolites (compounds 2–7; see Table 1) were provided by the Orion Corporation, Orion-Pharma (Espoo, Finland). Letrozole was purchased from Toronto Research Chemicals, Inc. (Toronto, Ontario; Canada). Tecemotide and the research peptides used for the enzyme-linked immunosorbent spot (ELISpot) assay were provided by Merck Serono Research, Merck KGaA (Darmstadt, Germany).

Experimental Animals

These studies included 317 mixed-sex wild type and human mucin 1 (MUC1) transgenic (hMUC1.Tg) C57BL/6 mice supplied by our breeding colony maintained by the University of California, Davis Mouse Biology Program and housed in a UC Davis Center for Laboratory Animal Science vivarium in Sacramento, CA. The hMUC1.Tg founder mice were purchased from Mayo Clinic (Scottsdale, AZ). All animal studies were conducted under protocols approved by the UC Davis Institutional Animal Care and Use Committee. UC Davis is an Association for Assessment and Accreditation of Laboratory Animal Care accredited institution. Mice were housed in cages of four with free access to food and water and maintained in a room with constant temperature and humidity on a 12h/12h light/dark schedule. For all mouse studies, the week number refers to age of the mice.

Terminal Procedures

At the conclusion of each study, all mice were euthanized by carbon dioxide asphyxiation. Whole blood was collected by cardiac puncture, allowed to clot for 30 min at room

temperature, and then centrifuged at $10,000 \times g$ for 10 min at 4°C to isolate serum, which was then flash frozen in liquid nitrogen and stored at -80°C until analyzed as described below. Following blood collection, spleens, where applicable, were removed and processed as described below to isolate lymphocytes for ELISpot, immunostaining and CTL assays.

Tissue Culture

Human Jurkat acute T cell leukemia cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA). Cells were cultured using RPMI 1640 growth medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT). Mouse mammary tumor cells (MMT-494) were derived in-house from a human MUC1-expressing mammary tumor (MMT) mouse model.¹³ Breast tumor cells were cultured using improved minimum essential medium (IMEM; Life Technologies) supplemented with 10% FBS (HyClone). Cultures were maintained in a water-jacketed incubator at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air.

Quantitative Real-Time PCR (qRT-PCR)

Total cellular RNAs were isolated using TriZol LS reagent (Life Technologies) according to the manufacturer's instructions. Total RNA concentration was quantified by absorbance at 260 nm using a NanoDrop^{TM} 2000 UV-Vis spectrophotometer (Thermo Fisher Scientific). Total RNA (2.5 μg) was then subjected to reverse transcription using SuperScript VILO MasterMix (Life Technologies). cDNA was quantified by the QuantStudio 6 Flex Real Time polymerase chain reaction (PCR) system (Life Technologies) using PowerUp SYBR Green Master Mix (Life Technologies) with specific primers (Table 2). The expression levels were quantified using the comparative C_t method, and normalized against the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or mouse $\beta 2$ -microglobulin (B2M).

Multiplex Cytokine Assays

For the study examining the effects of ospemifene on pro-inflammatory serum cytokine levels, a Mouse Cytokine 20-plex Panel (Life Technologies) was utilized. The 20-plex panel consisted of IL-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17, IFN- γ , IFN- γ -induced protein-10 (IP-10), monokine induced by IFN- γ (MIG), keratinocyte-derived cytokine (KC), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1 alpha (MIP-1 α), granulocyte/macrophage colony stimulating factor (GM-CSF), vascular endothelial growth factor (VEGF), tumor necrosis factor alpha (TNF- α) and basic fibroblast growth factor (FGF-basic). The assay was performed according to manufacturer's instructions. For all other studies, a custom 6-plex panel consisting of IL-6, IL-1 β , IFN- γ , MCP-1, IP-10 and MIP-1 α was utilized. All analyses were performed on a Luminex® 100/200 system running xPonent software version 3.1 (Luminex Corporation, Austin, TX). Serum cytokine concentrations were determined using Milliplex^{TM} Analyst version 5.1 software (EMD Millipore, Billerica, MA). Serum samples collected from wild type C57BL/6 mice challenged with 200 μg of lipopolysaccharide (LPS) from *Escherichia coli* serotype O111:B4 (Sigma-Aldrich) were used as in-house controls for the multiplex assays.

ELISpot Assay

A Mouse IFN- γ /IL-4 dual color ELISpot Kit (R&D Systems, Minneapolis, MN) or eBioscience (San Diego, CA) Mouse IFN- γ ELISpot Ready-SET-Go kit were used to perform the analysis according to the manufacturer's instructions. Spleens were removed aseptically and processed through 100- μ m nylon mesh cell strainers (BD Biosciences, San Jose, CA) into phosphate buffered saline (PBS), and lymphocytes were isolated by layering the cell suspension over lymphocyte separation medium (Lonza, Walkersville, MD) and centrifuging at $600 \times g$ for 15 min at room temperature. Following a PBS wash, lymphocytes isolated from spleens were resuspended in RPMI 1640 culture medium (Life Technologies) containing 10% FBS, 50 μ g/ml streptomycin and 50 U/ml penicillin (Life Technologies) before a cell count a viability assessment using an Auto T4 Cellometer (Nexcelom Bioscience, Lawrence, MA). Lymphocytes were seeded into 96-well microplates and incubated overnight at 37°C with medium only, BP25 target peptide, or scrambled BP1–424 peptide (5 μ g/ml) in triplicate.

Flow Cytometry Analysis

Lymphocytes were stained with phycoerythrin (PE)-Cy5 anti-mouse CD4 and CD8, PE-anti-CD27 and PE-anti-CD69 (eBioscience) in staining buffer (2% FBS in PBS) for 30 min at 4°C. For intracellular FoxP3 staining, cells were resuspended in freshly prepared Fixation/Permeabilization solution (eBioscience) and then incubated with PE anti-mouse FoxP3 antibody (eBioscience) for 30 min at 4°C. Data acquisition was performed using a MUSE cell analyzer (EMD Millipore).

CTL Assay

The CD8⁺ lymphocytes isolated from spleens were purified utilizing the Life Technologies DynaBeads FlowComp Mouse CD8 Kit according to the protocol provided by the manufacturer. The isolated CD8⁺ effector cells (5×10^5 /well) were incubated with target MMT-494 cells (5×10^4 /well) in a 24-well plate overnight at 37°C. The apoptosis of target cells was analyzed on a MUSE cell analyzer with an Annexin V kit (EMD Millipore) according to the manufacturer's protocol.

Statistical Analyses

Serum cytokine concentrations, CTL activity, Treg and RNA expression were analyzed using an unpaired, two-tailed Student's t-test or one-way analysis of variance (ANOVA) with Bonferroni's multiple comparisons post-test as indicated. ELISpot data were analyzed using either a one-way or two-way ANOVA with Bonferroni's multiple comparisons post-test as indicated. All statistical analyses were performed using GraphPad® Prism software version 5 (GraphPad Software, Inc., La Jolla, CA). All results are expressed as mean $\pm$ standard error of the mean (SEM) or standard deviation (SD) as indicated, and p-values <0.05 were considered statistically significant.

Immunomodulatory Effect of Ospemifene Compared to Estradiol and Letrozole

To verify the antiestrogenic effects of high-dose ospemifene, 32 female hMUC1.Tg C57BL/6 mice, starting at approximately six weeks of age, were administered 10 weekly

doses of urethane (0.75 mg/g) by intraperitoneal (i.p.) injection (100 μ l) to induce lung cancer. Two weeks following the final dose of urethane (Week 17), the mice were randomized into four treatment groups of approximately equal average weight (n=8, all groups): control (no treatment); ospemifene 100 mg/kg; estradiol 25 μ g/kg; and letrozole 0.8 mg/kg. To prepare oral dosing solutions, ospemifene and letrozole were dissolved in DMSO and then brought up to volume in peanut oil yielding final concentrations that would deliver the desired doses in a volume of 100 μ l. The final concentration of DMSO in all solutions was 2%. Solutions were orally administered daily for 14 days by oral gavage (100 μ l) using 20-gauge stainless steel gavage needles (Popper & Sons, New Hyde Park, NY) fitted to a 1-ml syringe. Estradiol was dissolved in 100% ethanol, diluted in PBS to achieve the desired concentration, and then administered daily for 14 days by subcutaneous (s.c.) injection at rotating sites. In Week 19, the study was terminated, 24 h after the last treatments, and serum and spleens were collected for IFN- γ and Treg analyses.

Effects of Ospemifene on Treg Expression Following Prolonged Dosing

This experiment was performed as previously described.¹⁵ Briefly, 4–6-week-old female polyomavirus middle-T transgenic (MTag.Tg) C57BL/6 mice were assigned to four treatment groups: control (n=7) and ospemifene 5 mg/kg (n=7), 25 mg/kg (n=8), and 50 mg/kg (n=8). Breast tumor specimens from this study were fixed in formalin and then stored in 70% ethanol prior to performing immunohistochemistry (IHC) for Tregs. Immunohistochemical staining was performed by the streptavidin-biotin-peroxidase method. Formalin-fixed, paraffin-embedded mouse breast tumor specimens were cut in 5- μ m sections, which were air-dried overnight in a chemical fume hood. The sections were deparaffinized in xylene for 10 min and rehydrated through graded alcohols (100%, 90%, and 70%) to water (5 min each). Antigen epitopes were unmasked by boiling in sodium citrate solution (10 mM, pH 6.0) for 10 min. Sections were treated with 3% H₂O₂ for 5 min to block endogenous peroxidase activity and then incubated in blocking buffer containing 10% horse serum for 30 min. The sections were incubated with rat anti-mouse FoxP3 (1:100; eBioscience) at room temperature for 2 h. Signals were developed using the Rat Vecta Stain ABC standard kit with DAB substrate (Vector Labs, Burlingame, CA) according to manufacturer's instructions. Lastly, the sections were counterstained with hematoxylin (Thermo Fisher Scientific) and evaluated under a microscope. Images were captured by a Leica Biosystems Digital Pathology Whole Slide Scanner (Buffalo, IL). At least two cross-sections per mouse were analyzed.

Effects of Ospemifene on Pro-inflammatory Cytokines During Tumorigenesis

A total of 48 wild type female mice, starting at approximately five weeks of age, received 10 weekly doses of urethane (0.75 mg/g) to induce lung cancer. Following the final dose of urethane, mice were randomized into four groups of approximately equal average weight (n=12, all groups): control and 5, 25, and 50-mg/kg ospemifene. Starting in Week 15, mice were treated daily according to treatment group assignment. Oral dosing solutions were prepared and administered as described above. The control solution (diluent) was composed of 2% DMSO in peanut oil.

After one month of treatment (Week 20), a total of 16 mice, four from each treatment group, were euthanized for the collection of serum for multiplex cytokine analysis. The remaining mice continued daily ospemifene treatments according to the outlined schedule. An additional 16 mice were euthanized following two (Week 24) and three (Week 28) months of treatment.

Effects of Ospemifene on Tecemotide-Induced Immune Response: Chronic Dosing

Fifty-five mixed-sex, five-week old, hMUC1.Tg, urethane-induced, tumor-bearing mice were used. Approximately six weeks after the last dose of urethane, mice were randomized into four treatment groups: control (n=22); ospemifene 50 mg/kg (n=16); tecemotide (n=6); and ospemifene + tecemotide (n=11). Mice assigned to tecemotide groups received two cycles of eight weekly 10- μ g doses each delivered by s.c. injection (100 μ l), with approximately four weeks between cycles. Cycle 1 was administered during Weeks 21–28, and Cycle 2 from Weeks 32–39. Mice in ospemifene groups began daily oral treatment in Week 31, which continued through the end of the study (approximately eight weeks of dosing). Mice not assigned to ospemifene treatment received daily oral doses of control solution. All mice were euthanized 48 h after the final dose of tecemotide and 24 h after the last dose of ospemifene.

Effects of Ospemifene on Tecemotide-Induced Immune Response: Intermittent Treatment

Six weeks after the last dose of urethane (Week 20), 65 male hMUC1.Tg urethane-induced, tumor-bearing mice were randomized into six treatment groups of approximately equal average weight: control (n=10); ospemifene 10 and 50 mg/kg (n=11 each dose); tecemotide alone (n=11); and ospemifene 10 and 50 mg/kg in combination with tecemotide (n=11 each dose). All mice in tecemotide treatment groups received eight weekly 10- μ g doses. For the last 16 days of the study, mice assigned to ospemifene treatment were administered daily oral doses. All other mice received daily oral doses of control solution. All mice were euthanized 24 h after the final dose of ospemifene and 48 h after the last dose of tecemotide (Week 27).

To evaluate the effects of ospemifene on the immune response to tecemotide following an intermittent dosing schedule in non-tumor-bearing mice, 43 female wild type mice (Week 7) began treatment with eight weekly 10- μ g doses of tecemotide. Prior to starting ospemifene dosing, mice were randomized into four treatment groups of approximately equal average weight: control (tecemotide alone; n=10) and tecemotide + ospemifene 10, 50, and 100 mg/kg (n=11 all groups). All mice in the ospemifene groups were dosed daily according to treatment group assignment for the last 16 days of the study. In Week 14, 24 h after the final dose of ospemifene and 48 h after the last dose of tecemotide, all mice were euthanized.

Effects of Ospemifene on Tecemotide-Induced Immune Response: Pretreatment

A total of 32 three-month old female wild type mice were assigned to four treatment groups (n=8) of approximately equal average weight: control; ospemifene 100 mg/kg; tecemotide 100 μ g; and ospemifene combined with tecemotide. On Day 0 of the study, all 32 mice were implanted with 1.0×10^6 mouse breast cancer cells by s.c. injection on the right front flank behind the axilla. Two days later, mice began a three-day regimen of daily oral ospemifene

treatment. On Day 5, mice were administered their first weekly dose of tecemotide. This treatment schedule was repeated for a total of four weeks. Tumor growth was assessed using calipers twice weekly. All mice were euthanized on Day 28 to assess immune response to treatment.

Dose and Schedule-Dependent Activity of Ospemifene on Key Markers of Immune Response

To optimize the immunomodulatory effect of ospemifene on tecemotide immune response, a total of 42 mixed-sex wild type mice (10–11 weeks of age) were assigned to seven treatment groups (n=6; 3/3 male/female) such that average weights of the males and females in each group were approximately equal: control (no treatment); tecemotide + ospemifene 25 mg/kg (1d, 2d and 3d pretreatment); and tecemotide + ospemifene 50 mg/kg (1d, 2d and 3d pretreatment). Daily oral dosing of ospemifene commenced on Day 0 (3d pretreatment), Day 1 (3d and 2d pretreatment) and Day 2 (3d, 2d and 1d pretreatment), and the first weekly 10- μ g dose of tecemotide was administered 4 h after ospemifene treatment on Day 2. This treatment schedule was repeated for a total of three weeks. Approximately 48 h after the third and final dose of tecemotide, all mice were euthanized for the assessment of immune response.

Results

Ospemifene-Induced Expression of IFN- γ and IL-2

The immune regulating effect of ospemifene was first evaluated in Jurkat cells by examining the expression of the T cell activating cytokines. IFN- γ and IL-2 are early response genes expressed by T cells upon activation, and they are also T_H1 cytokines, which enhance cellular immune response. After 24 h treatment, ospemifene significantly induced both IFN- γ ($p < 0.01$; Fig. 1A) and IL-2 ($p < 0.0001$; Fig. 1B) mRNA expression in a dose-dependent fashion in Jurkat cells. These results suggest that ospemifene has potential immunomodulatory effects by stimulating the expression of key T cell-activating cytokines.

Triphenylethylene-Mediated T Cell Activation: Structure Activity Relationships

The structure activity relationship of the triphenylethylenes in regard to T-cell activation was assessed by comparing the expression of IFN- γ and IL-2 mRNA in Jurkat cells. As shown in Table 1, ospemifene (Compound 1) and a hydroxylated form (Compound 2) strongly increased the expression of both IFN- γ and IL-2 relative to the other compounds. Substitution at the R¹ position appears to be critical with respect to T cell activation: replacement of the ethanol side chain present in Compounds 1 and 2 with a carboxylic acid (Compounds 4 and 5) or an amine (Compounds 6 and 7) results in no meaningful effect on the expression of IFN- γ and IL-2. Removal of the ethanol side chain (Compound 3) resulted in only a weak increase of IFN- γ expression.

Ospemifene-Induced Expression of IFN- γ and IL-2 via Calmodulin and PI3K Signaling Pathways

T cell activation requires multiple signals including phosphoinositide 3-kinase (PI3K), mitogen-activated protein kinase (MAPK) and calcium signaling pathways.¹⁶ To identify the

potential mechanism through which ospemifene affects immune activation, Jurkat cells were pretreated with inhibitors for PI3K (LY294002), MAPK (PD98059) and calmodulin (trifluoperazine) prior to ospemifene treatment *in vitro*. As shown in Fig. 1, trifluoperazine ($p<0.001$) and LY294002 ($p<0.0001$) significantly decreased ospemifene-induced expression of IFN- γ (Fig. 1C) and IL-2 (Fig. 1D) mRNA, whereas PD98059 did not affect the induction of IFN- γ or IL-2 mRNA expression by ospemifene. Both LY294002 and trifluoperazine exhibited dose-dependent inhibition of ospemifene-induced IFN- γ and IL-2 expression (Fig. 2). These results suggest that ospemifene stimulates T cells through PI3K and calmodulin signaling pathways, which in turn activate the transcription factors, nuclear factor of activated T cells (NFAT) and nuclear factor kappa B (NF- κ B), for both IFN- γ and IL-2.

Negligible Effect of Tamoxifen on Ospemifene-Induced IFN- γ Expression

Although tamoxifen is in the same chemical class as ospemifene, it has been shown that tamoxifen induces a T_H2 immune response. To determine whether tamoxifen is able to interfere with the immune modulating effect of ospemifene, IFN- γ expression was analyzed in Jurkat cells treated with control, ospemifene (2.5 μ M), tamoxifen (2.5 μ M), or a combination of ospemifene and tamoxifen (Fig. 3A). Ospemifene alone ($p<0.01$) and in combination with tamoxifen ($p<0.001$) induced significantly increased expression of IFN- γ compared to control, whereas tamoxifen alone did not increase IFN- γ expression. These results demonstrated that tamoxifen did not affect the activity of ospemifene on T cell activation.

Immunomodulatory Effect of Ospemifene Compared to Estradiol and Letrozole

To verify the immune activity of ospemifene, a total of 32 urethane-induced, lung tumor-bearing mice were randomized into four groups: control, ospemifene 100 mg/kg, estradiol 25 μ g/kg, and letrozole 0.8 mg/kg ($n=8$, all groups). After two weeks of daily treatment, serum was collected and lymphocytes were isolated from spleen. Analysis of serum IFN- γ showed that both ospemifene and letrozole-treated animals were not significantly different compared to estradiol-treated animals (data not shown). In order to determine whether ospemifene was inducing an immunosuppressive tumor microenvironment, similar to tamoxifen, the percentage of Tregs (FoxP3+/CD4+) in all treatment groups was analyzed (Fig. 3B). Our data showed that neither ospemifene nor letrozole treatment increased Tregs compared to estradiol treatment.

Preventing Breast Tumor Growth and Suppressing Treg Expression

In a previous study, ospemifene effectively prevented breast cancer tumor growth following chronic dosing with 5, 25 and 50 mg/kg/day for more than four months.¹⁵ Here, tumor specimens from each treatment group were stained for Tregs using FoxP3 antibody. As seen in Fig. 4, Tregs were strongly expressed in control (A, B), whereas expression decreased with increasing doses of ospemifene [5 mg/kg (C, D); 25 mg/kg (E, F); 50 mg/kg (G, H)]. These results illustrate a dose response effect of ospemifene in decreasing Treg expression associated with tumor suppression.

Inhibition of Pro-inflammatory Cytokines by Ospemifene During Tumorigenesis

To examine the potential immunomodulatory effects of ospemifene during tumorigenesis as well as to assess treatment dose and duration, a total of 48 urethane-induced, tumor-bearing mice were treated with 5, 25, or 50 mg/kg ospemifene. Four mice from each treatment group were euthanized following 30, 60 and 90 days of daily oral dosing, and terminal serum cytokines were analyzed 24 h after the last dose at each time point. Noticeable differences were found in pro-inflammatory cytokine levels of IL-6, IL-1 β , MIG, KC and IP-10 from the control group compared to the treated groups (Fig. 5B–F). Among these cytokines, MIG was significantly lower at the 25- and 50-mg/kg ospemifene dose levels after 90 days of treatment compared to control ($p < 0.05$; Fig. 5D). Pro-inflammatory cytokine levels in the control group trended upwards and exhibited elevated concentrations, while ospemifene-treated groups trended downwards and exhibited lower cytokine concentrations in a dose-dependent fashion. Increasing pro-inflammatory serum cytokine levels in the control mice may be a result of an inflammatory response associated with tumor progression resulting from urethane treatment.¹⁴ In contrast, decreasing levels of pro-inflammatory serum cytokines in ospemifene-treated mice may suggest an anti-inflammatory effect. With respect to T_H1 and T_H2 cytokines, no apparent polarization was observed. However, a major T_H1 cytokine, IFN- γ , was elevated after four weeks of treatment at the lowest dose of ospemifene, but decreased dramatically by eight weeks (Fig. 5A).

Lack of Efficacy of Chronic Ospemifene on Tecemotide-Induced Tolerance

Tecemotide is a liposome-based cancer vaccine designed to elicit a cellular immune response against the cell surface glycoprotein MUC1, which is overexpressed and aberrantly glycosylated in many adenocarcinomas, including breast and non-small cell lung cancer (NSCLC). Early clinical trials demonstrated a survival benefit of tecemotide in patients with inoperable stage III NSCLC who had received primary chemoradiotherapy,¹⁷ but a phase III clinical trial showed that this benefit might be limited.¹⁸ Previously, we found that administering two cycles of eight weekly doses of tecemotide was effective in the urethane-induced lung cancer mouse model.¹⁴ In this study, ospemifene was administered to hMUC1.Tg mice starting approximately one week before the second cycle and continued until the end of the study. Lymphocytes from both tecemotide-treated groups showed a significant and specific IFN- γ immune response to the MUC1 peptide compared to the control group ($p < 0.01$, $p < 0.0001$; Fig. 6A). Surprisingly, the tecemotide-induced, MUC1-specific immune response was significantly decreased in the combination treatment group compared to tecemotide alone ($p < 0.0001$; Fig. 6A). In addition, CTL activity was not increased with combination therapy (Fig. 6B). These results suggest that chronic dosing of ospemifene created an immunosuppressive environment.

Ospemifene Dosing Schedule: Intermittent Treatment

We then elected to modify the timing of administration and duration to begin after five tecemotide vaccinations, with a total of 16 daily doses of ospemifene given with the final three weekly tecemotide treatments to complete the full course of eight weekly vaccinations. Unlike the chronic dosing schedule, the tecemotide-induced, MUC1-specific immune response was significantly increased following intermittent ospemifene treatments ($p < 0.05$;

Fig. 6C). This finding suggests that intermittent dosing of ospemifene may be necessary for activating a robust immune response when used in combination with antigen-specific immunotherapy.

To confirm these observations, a second intermittent ospemifene treatment study with a similar design was conducted, and it showed that the combination of tecemotide with ospemifene at 50 mg/kg significantly increased induction of CTL activity compared to ospemifene ($p<0.01$) or tecemotide alone ($p<0.05$) (Fig. 6D). The results from both studies suggest that intermittent dosing of ospemifene enhances antigen immune response following chronic vaccination and may be a method for preventing immune tolerance.

Ospemifene Dosing Schedule: Pretreatment

To further examine the effect of ospemifene on the immune response to tecemotide in tumor-bearing mice, ospemifene was given for three days prior to each of four weekly tecemotide treatments, similar to the pretreatment schedule of cyclophosphamide (CPA) used clinically.¹⁸ As shown in Fig. 7A, treatment with tecemotide alone and in combination with ospemifene reduced tumor volume compared to the control group as measured on Days 22 and 25. Serum IFN- γ immune response on Day 28, 48 hours following the fourth and final dose of tecemotide, is shown in Fig. 7B. Both vaccinated groups demonstrated significantly increased levels of serum IFN- γ compared to the control and ospemifene alone treatment groups ($p<0.05$). As shown in Fig. 7C, when the T cell immune response was assessed by ELISpot, both groups treated with tecemotide showed specific IFN- γ responses to the target antigen (BP25); however, mice treated with tecemotide in combination with ospemifene showed an increased immune response compared to mice treated with tecemotide alone. This study demonstrated that ospemifene short-course pretreatment primes the tecemotide-induced, antigen-specific T cell immune response.

Dose and Schedule-Dependent Activity of Ospemifene on Key Markers of Immune Response

In order to determine the optimal pretreatment schedule of ospemifene, T cell immune response was measured following three dose schedules and two dose levels (25 mg/kg or 50 mg/kg) of ospemifene treatment in 42 mice. Each week for three weeks, mice received 1d, 2d or 3d of daily ospemifene treatment followed by tecemotide vaccination given 4 h after the last ospemifene dose.

Fig. 8A shows that serum IFN- γ increased, with the highest levels observed following 3d treatment with ospemifene at both 25 and 50 mg/kg. Due to variation in responses, only 50-mg/kg 2d pretreatment IFN- γ levels reached statistical significance compared to control ($p<0.05$). In general, serum IFN- γ increased with dose and number of ospemifene treatments. We next examined the antigen-specific immune response by ELISpot and found a clear and significant increase at the 50-mg/kg dose compared to the 25-mg/kg dose after 2d ($p<0.001$) and 3d pretreatment ($p<0.05$), with the most pronounced response seen following 3d treatment with 50 mg/kg (Fig. 8B).

In order to further characterize the effect of ospemifene on immune activation, we evaluated IL-6 and CD27 and CD69 expression in spleen-derived lymphocytes. CD69 is a well-known

early activation marker for natural killer (NK) cells and T cells,¹⁹ while CD27 is a co-stimulatory molecule expressed on NK and B, naïve CD4 and CD8 T cells.²⁰ Fig. 8C shows that the intracellular expression of IL-6 was significantly increased ($p<0.001$) compared to control in most treatment groups, which is indicative of a T cell response. As shown in Fig. 8D, CD69 expression significantly increased after 3d ospemifene pretreatment ($p<0.01$), while CD27 expression significantly decreased (Fig. 8E) after 3d ($p<0.01$) and 2d pretreatment ($p<0.05$). A loss of CD27 expression is associated with high levels of effector function, promotes development of CTLs and enhances T cell survival.^{21, 22}

Discussion

Estradiol is known to have effects on the immune system,²³ and elevated levels of estrogen seen in women of childbearing age have been associated with the greatly increased incidence of autoimmune diseases compared to men of the same age.²⁴ At the same time, sustained higher levels of circulating estrogen seen during pregnancy seem to have a protective, immunosuppressive effect. Indeed, symptoms of experimental autoimmune encephalomyelitis and multiple sclerosis improve during pregnancy but tend to recur following childbirth.^{25, 26} Estrogen has also been shown to affect the inflammatory microenvironment.²³ Thus, it would appear that estrogen is exerting multiple effects on the immune system, although the overall picture remains very complex. The synthetic estrogen diethylstilbestrol has been shown to have effects similar to estradiol on T-lymphocyte cytokine production,²⁷ while the potent antiestrogen acolbifene has been shown to antagonize this effect.²⁸ In addition, as ERAAs are known to exert both estrogenic and antiestrogenic effects,² it is reasonable to conclude that these compounds may also affect the immune response.

Compared to other mixed ERAAs such as tamoxifen, toremifene and raloxifene, ospemifene has relatively weak hormonal activity. This weak activity led to the hypothesis that ospemifene should have fewer hormone-related side effects, which was proven to be correct and helped lead to its eventual approval for the treatment of postmenopausal women with VVA symptoms. Other SAR studies of toremifene and its metabolites, which include ospemifene, showed that only those compounds containing a protonated amino group in the side chain were effective in reversing the doxorubicin accumulation defect in multidrug resistant (MDR) human breast cancer cells.⁵ Compounds such as ospemifene, which has an ethanol side chain, had no activity in reversing MDR. The triphenylethylenes' MDR-reversing activity may be regulated through the inhibition of calmodulin-dependent pathways.²⁹ Interestingly, when evaluating SARs for potential immunomodulatory activity in the present study, the same compounds found to be effective in MDR were inactive with respect to stimulation of IFN- γ and IL-2 expression, while the compounds lacking amino side chains, e.g. ospemifene and fispemifene, a prodrug of ospemifene³⁰, were effective in stimulating expression of IFN- γ and IL-2.

The activation of naïve T-cells requires calcium signaling initiated through binding of the T cell receptor (TCR) with antigen/major histocompatibility complex (MHC) and CD28 co-stimulation by APCs.^{31, 32} Upon binding calcium, calmodulin undergoes a conformational change that promotes its ability to activate the protein phosphatase calcineurin, resulting in

translocation of NFAT to the nucleus, where NFAT participates in the transcription of IL-2 and other key cytokines.^{31, 33} Other TCR signaling pathways include PI3K activation of NF- κ B and MAPK activation of activator protein 1 (AP-1).¹⁶ Our hypothesis was that ospemifene activates T-cells through these pathways.

Furthermore, it was previously shown that estrogen induces a transcription-independent increase in intracellular calcium and cyclic adenosine monophosphate (cAMP), thereby activating MAPK, leading to promotion of cell proliferation.³⁴ Similarly, the antitumor effect of tamoxifen is believed to involve a combination of both estrogen receptor (ER)-mediated and non-genomic non-ER-mediated mechanisms.^{35–38} It has been shown that tamoxifen and its active metabolites hydroxytamoxifen and desmethyltamoxifen can bind to and act as antagonists of calmodulin, thus inhibiting calmodulin-dependent cAMP phosphodiesterase and subsequent signaling pathways.^{39, 40} Therefore, it was logical to assess whether tamoxifen would interfere with ospemifene or have similar activity with respect to calmodulin-dependent signaling. In the present study, tamoxifen had no discernable effect on ospemifene-induced IFN- γ expression, which suggests that the binding affinity of ospemifene for calmodulin may be greater than that of tamoxifen or that the contribution of PI3K signaling plays a dominant role in the induction of IFN- γ . Overall, these results suggest that ospemifene activates T cells through PI3K and calmodulin signaling pathways, which may be important for priming T cell activation for an antigen-specific immunotherapy such as a cancer vaccine.

There have been a number of initially promising therapeutic cancer vaccines that ultimately failed in clinical trials such as GTOP99 (MyVax),⁴¹ belagenpumatucel-L (Lucanix),⁴² dasiprotimut-T (BioVaxID)⁴³ and sialyl-Tn (Theratope).⁴⁴ To date, only one cancer vaccine, sipuleucel-T (Provenge) for prostate cancer, has been approved. As with tecemotide, there were subgroups of patients who responded better to these vaccines in the various clinical trials compared to the overall population. We believe that patients' immune status prior to and during treatment with an antigen-specific immunotherapy is critical for understanding the transient nature of the immune system when inducing an immune response.⁴⁵ One approach that has been adopted is to pre-treat patients with low-dose CPA for reducing Tregs prior to vaccination.¹⁸ Our results suggest that pretreatment with ospemifene increases T cell activation, CTL activity and antigen-specific immune response to tecemotide, suggesting that ospemifene could be used to prime the immune environment in order to maximize a T cell response to an antigen-specific immunotherapy. Based on mass to body surface area ratios,⁴⁶ the ospemifene dose levels employed in the present studies are equivalent to doses that have been evaluated clinically.

Repurposing old drugs for new uses continues to be a research focus of many investigators and companies. For example, thalidomide, introduced in the 1950s as a treatment for morning sickness in pregnant women, was withdrawn from the market in the early 1960s due to severe teratogenic side effects. It was later discovered that thalidomide and related compounds possessed anti-angiogenic,⁴⁷ anti-inflammatory and immunomodulatory properties,⁴⁸ and thalidomide received FDA approval in 2006 for use in the treatment of multiple myeloma.⁴⁹ Triphenylethylenes are also a class of drugs commonly being examined for new clinical indications. Interestingly, tamoxifen was recently reported as having a new

potential for enhancing the immune function of neutrophils through a ceramide-mediated pathway.⁵⁰

The biphasic action of ospemifene on immune function, i.e. activation of T cells following pretreatment and anti-inflammatory activity following prolonged dosing, comes as no surprise for scientists who have spent their careers studying hormonal action.⁵¹ More recently, as reported by Katzenellenbogens' group, the biphasic action of ERAAs may be important when treating endometriosis.⁵² In general, as shown in Fig. 9, the pharmacodynamics of the ERAAs class of compounds is dependent on dose and time. Research into each target area such as reversal of MDR, cytotoxicity, anti-inflammatory activity, cholesterol lowering, antiestrogenicity, osteoporosis, VVA, and immune activation has shown that these effects are dose and schedule dependent.^{2, 53} Taken together, in this age of precision medicine, the one-size-fits-all concept of dosing ERAAs should be reconsidered. For example, tamoxifen is dosed at 20 mg/day independently of body surface area or weight considerations when treating patients for breast cancer, while ospemifene is dosed at 60 mg/day in all patients for the treatment of VVA. Finally, the present study illustrated how integrating SARs, dose response and specific biologic activity led to discovering ospemifene's immunomodulatory actions.⁵⁴

Conclusions

In the present study, ospemifene, a drug currently approved for the treatment of dyspareunia and vaginal dryness associated with genitourinary syndrome of menopause, was found to possess immunomodulatory activity. The pretreatment, intermittent and chronic dosing schedules of ospemifene activate naïve T cells, modulate antigen-induced tolerance and reduce tumor-associated, pro-inflammatory cytokines, respectively. Taken together, ospemifene's dose response and schedule-dependent immune modulating activity offers a method of tailoring and augmenting the efficacy of previously failed antigen-specific cancer vaccines for a variety of malignancies.

Potential Clinical Value

Postmenopausal women receiving chemotherapy or endocrine therapy for cancer will almost universally experience symptoms of VVA. It is possible that the biphasic action of ospemifene (augmentation of an antigen-specific immune response while decreasing inflammation) may also help alleviate the VVA symptoms associated with chemotherapy. For these reasons, we believe ospemifene should be clinically developed as an immune modulator in postmenopausal women with cancer.

Of the hundreds of cancer vaccine projects, only the prostate cancer vaccine sipuleucel-T has received FDA approval. Multiple explanations including empiric dose schedules, immune tolerance, population genetics and stage of disease may explain the lack of consistent efficacy seen in numerous other cancer vaccine trials. One approach for improving cancer vaccine effectiveness has been to administer low-dose cyclophosphamide, for the purpose of reducing Tregs, prior to vaccination. The results of the present study suggest that tailoring ospemifene dosing is effective in maximizing an antigen-specific T cell

response to a peptide cancer vaccine. Repurposing the relatively inexpensive agent, ospemifene, as an immunomodulator must include a new paradigm of dosing that is based on biologic activity, for example monitoring tolerance following repeated vaccinations, as a guide for when to use intermittent ospemifene therapy. Tailoring ospemifene dosing may represent an effective and inexpensive means of improving the clinical efficacy of previously failed cancer vaccines for a variety of malignancies.

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Fig. 1.

IFN- γ and IL-2 expression in human Jurkat cells following exposure to ospemifene (OSP) with and without PD98059, trifluoperazine (TFP) and LY294002 *in vitro*. Jurkat cells (2.0×10^6) were treated with different concentrations of ospemifene as indicated for 24 h, and mean (+SD) IFN- γ (A) and IL-2 (B) mRNA expression levels (n=4) were detected by qRT-PCR and normalized with expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (*p<0.05, **p<0.01, ****p<0.0001 compared to control; two-tailed Student's t-test). (C, D) Jurkat cells (2.0×10^6) were pretreated with PD98059 (50 μ M), TFP (10 μ M) and LY294002 (25 μ M) for 1 h, then incubated with medium containing 2.5 μ M OSP for 24 h. Mean fold changes (+SEM) in IFN- γ (C) and IL-2 (D) mRNA expression levels (n=4) were detected by qRT-PCR and normalized with GAPDH (**p<0.01, ***p<0.001, ****p<0.0001 compared to OSP alone unless indicated otherwise; one-way ANOVA).

Fig. 2.

Effects of ospemifene (OSP) alone and in combination with trifluoperazine (TFP) or LY294002 (LY) on IFN- γ and IL-2 expression in human Jurkat cells *in vitro*. Jurkat cells (2.0×10^6) were pretreated with different concentrations of (A, B) TFP (0, 0.1, 1.0, 5.0, and 10.0 μ M diluted in medium from a 1000- μ M concentrated stock made in 0.9% sterile saline) and (C, D) LY (0, 1, 5, 10, and 25 μ M diluted in medium from a 25-mM concentrated stock made in DMSO) for 1 h, then incubated with medium containing 2.5 μ M OSP for 24 h. (A, C) IFN- γ and (B, D) IL-2 mRNA expression levels (mean+SD) were detected by qRT-PCR and normalized with the expression of GAPDH (n=3–5). *p<0.05, **p<0.01, ***p<0.001 compared to OSP alone (one-way ANOVA).

Fig. 3.

Effects of ospemifene (OSP), tamoxifen, letrozole and estrogen (E2) on immune response *in vitro* and *in vivo*. (A) Effects of tamoxifen on OSP-induced IFN- γ expression *in vitro*.

Jurkat cells (2.0×10^6) were treated with OSP (2.5 μ M) and/or tamoxifen (2.5 μ M) for 24 h.

The expression levels (mean+SD) of IFN- γ were analyzed by qRT-PCR and normalized

with GAPDH (n=3) (**p<0.01, ***p<0.001 compared to control; one-way ANOVA). (B)

Immunomodulatory effect of OSP versus E2 and letrozole. Average proportions (+SEM) of

CD4+FoxP3+ cells (Tregs) among total CD4+ cells from untreated control, OSP (100 mg/

kg), E2 (25 µg/kg), and letrozole (0.8 mg/kg) groups were analyzed following daily treatment for 14 days (n=8 all groups) (*p<0.05; two-tailed Student's t-test).

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Fig. 4.

Expression of Tregs in breast tumors following chronic ospemifene dosing. MTag.Tg mice were treated with control (A, B) or ospemifene 5 (C, D), 25 (E, F) or 50 mg/kg (G, H) daily for more than four months. Sections were analyzed for Treg expression by IHC. At least two sections per mouse were analyzed, and each pair of panels represents sections taken from different tumors. Images were captured by a Leica Biosystems Digital Pathology Whole Slide Scanner.

Fig. 5.

Average pro-inflammatory serum cytokine concentrations after 30, 60, and 90 days of ospemifene (OSP) treatment at 5, 25, and 50 mg/kg. Terminal serum specimens were collected following 30, 60, and 90 days of treatment. (A) IFN- γ , (B) IL-6, (C) IL-1 β , (D) MIG, (E) KC, and (F) IP-10 cytokines were analyzed by multiplex, and concentrations represent the mean of individual samples (n= 3–4). In some cases only one mouse at a particular dose level and time point had measurable concentrations of IL-1 β or KC.

Concentrations below detection limits were counted as zero. * $p < 0.05$ compared to control (one-way ANOVA).

Fig. 6.

Effects of ospemifene (OSP) on IFN- γ immune response and cytotoxic T lymphocyte activity following chronic and intermittent dosing schedules. Antigen-specific IFN- γ immune response was assessed by ELISpot 48 h following the final dose of tecemotide peptide vaccine (PV). (A) Average spot forming cells (SFC/ 3.0×10^5 cells + SEM) and (B) average target cell apoptosis percentages (+SEM) following nine weeks of chronic daily OSP (50 mg/kg; n=16) compared to control (n=22), two cycles of PV alone (n=6) and OSP + PV (n=11) (**p<0.01, ****p<0.0001 compared to control unless indicated otherwise; two-way ANOVA). (C) Average SFC/ 5.0×10^5 cells + SEM following treatment with one cycle of PV (n=10) or PV combined with intermittent (16 days) OSP 10, 50, or 100 mg/kg (n=11 each combination) (*p<0.05, two-way ANOVA). (D) Average target cell apoptosis percentages (+SEM) were assessed in control (n=10), one cycle of PV (n=11), short-course (16 days) OSP at 10 and 50 mg/kg (n=11), and OSP + PV combination (n=11) treatment groups (*p<0.05, **p<0.01; one-way ANOVA).

Fig. 7. Antitumor effects and serum IFN- γ immune response to tecemotide peptide vaccine (PV) immunotherapy following treatment with control, PV alone, ospemifene (OSP) 100 mg/kg alone or OSP + PV. Each of four weekly 100- μ g s.c. injections of PV was preceded by three daily oral doses of OSP (100 mg/kg). All mice were implanted with breast cancer allografts on Day 0, and serum and spleens were collected on Day 28 following four weeks of treatment. Tumor volumes were measured twice each week. (A) Average tumor volumes (+SEM) are shown for Days 22 and 25 along with (B) serum IFN- γ levels as assessed on

Day 28 (n=8, all groups) (*p<0.05 compared to both control and OSP 100 mg/kg; two-tailed Student's t-test). (C) Representative ELISpot results are displayed for the scrambled and BP25 peptides for each treatment group along with the range of spot forming cells (SFC)/ 5.0×10^5 total cells and the number of subjects (n=5–6) with more than 300 and 1000 SFCs, respectively, in response to the BP25 peptide.

Fig. 8.

Dose and schedule-dependent effects of ospemifene (OSP) on serum IFN- γ , lymphocyte IL-6 expression, antigen-specific immune response and T cell activation. Mice were treated with OSP at 25 or 50 mg/kg for 1, 2 or 3 days prior to tecemotide peptide vaccine (PV) administration each week for 3 weeks ($n=6$, all groups). (A) Average serum IFN- γ ($\pm$ SEM) was analyzed by multiplex (* $p<0.05$ compared to control; two-tailed Student's t-test). (B) Average spot-forming cells (SFC/ 1.0×10^6 cells + SEM) following ELISpot analysis (* $p<0.05$, *** $p<0.001$; two-tailed student's t-test). (C) Mean intracellular IL-6 (+SEM) in

spleen-derived lymphocytes was analyzed by qRT-PCR and normalized against the expression of mouse β 2-microglobulin (B2M) (** $p < 0.001$, *** $p < 0.0001$ compared to control; one-way ANOVA). Average expression (+SEM) of CD69 (D) and CD27 (E) in spleen-derived lymphocytes as assessed by flow cytometry (* $p < 0.05$, ** $p < 0.01$ compared to control; one-way ANOVA).

Fig. 9.

Dose response relationship and biological activity of the triphenylethylene (TRIP) estrogen receptor agonists/antagonists. VVA (vulvovaginal atrophy); MDR (multidrug resistance).

This Figure is intended to illustrate the need for a new treatment paradigm for this class of drugs given their dose-response relationships.

Table 1 Effects of different chemical substitutions on triphenylethylene-mediated IFN- γ and IL-2 expression.

R Substitution	IFN- γ Expression ^a	IL-2 Expression ^a	
R ₁ =CH ₂ CH ₂ OH; R ₂ =H; R ₃ =H (1)	+++	+++	
R ₁ =CH ₂ CH ₂ OH; R ₂ =OH; R ₃ =H (2)	+++	+++	
R ₁ =H; R ₂ =H; R ₃ =H (3)	+	–	
R ₁ =CH ₂ COOH; R ₂ =OH; R ₃ =H (4)	–	–	
R ₁ =CH ₂ COOH; R ₂ =H; R ₃ =H (5)	–	–	
R ₁ =CH ₂ CH ₂ NHCH ₃ ; R ₂ =OH; R ₃ =H (6)	–	–	
R ₁ =CH ₂ CH ₂ NH ₂ ; R ₂ =H; R ₃ =H (7)	–	–	

^a +++ (strong increase); ++ (moderate increase); + (weak increase); – (no effect).

Jurkat cells were plated in 6-well culture dishes at 1.0×10^6 cells/well, then incubated with medium containing 0.1–10 μ M of each respective compound for 24 h. Concentrated stock solutions (200X) of each compound were prepared in a solution consisting of 2.9% DMSO and 60% ethanol in sterile 0.9% saline, which was then diluted in medium to achieve the desired final concentrations. Expression levels of IFN- γ and IL-2 (n=10) were detected by qRT-PCR and normalized with the expression of GAPDH.

Table 2

Sequences of qRT-PCR primers

Gene Name	Sequence
hGAPDH	F: 5'-AATCCCATCACCATCTTCCAG-3' R: 5'-AAATGAGCCCCAGCCTTC-3'
hIFN- γ	F: 5'-GCATCGTTTTGGGTCTCTTG-3' R: 5'-AGTTCCATTATCCGCTACATCTG-3'
hIL-2	F: 5'-AAAGAAGGCACAGCTACAAGTGG-3' R: 5'-GAAGATGTTTCAGTTCTGTGGC-3'
mB2M	F: 5'-TGGTCTTTCTGGTGCTTGTC-3' R: 5'-GGGTGGAAGTGTGTACGTAG-3'
mIL-6	F: 5'-CAAAGCCAGAGTCCTTCAGAG-3' R: 5'-GTCCTTAGCCACTCCTTCTG-3'