THE EFFECTS OF THE SELECTIVE ESTROGEN RECEPTOR MODULATORS MPP AND RALOXIFENE IN NORMAL AND CANCEROUS HUMAN AND MURINE UTERINE TISSUE

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by
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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled
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Angela Marie Davis

Dr. Cheryl S. Rosenfeld and Dr. R. Michael Roberts, Thesis Co-Supervisors

ABSTRACT

The goal of this research was to determine the in vitro and in vivo effects of the Selective Estrogen Receptor Modulators (SERMs), methyl-piperidino-pyrazole (MPP), raloxifene, ICI 182,780 and 17β-estradiol on endometrial carcinoma cells in culture and on murine uterine tissue. These SERMs have been developed to target and understand the role of estrogen receptor action in estrogen-responsive organs. Based on their antagonistic actions, SERMs have both real and potential value in treating estrogen-responsive cancers, including endometrial cancer. The studies described herein verify that the SERMs MPP and raloxifene demonstrate partial agonistic effects in ovariectomized wild-type and ER-β knockout (ERβKO) mice but also induce apoptosis and proliferation in vitro in cultured endometrial cell lines, Ishikawa and RL-95. Thus, MPP and raloxifene exert apparently contrasting in vitro and in vivo effects due to their mixed agonist/antagonist action on murine uterine estrogen receptor in vivo. In addition to these data, I report gene expression changes in the uterus of mice treated individually or with the combination of 17β-estradiol and the SERMs, MPP, raloxifene, ICI 182,780 are
reported herein. A greater number of genes showed up- or down regulation when the mice were co-treated with estrogen and one of the SERMs than when dosed with one of the compounds alone. A combination of a SERM and 17β-estradiol resulted in a combinatorial or synergistic effect. These data may be explained by the fact that dual administration of a SERM with β-estradiol allows the compounds to bind combinatorially to heterodimers as well as homodimers of the two receptors ERα and ERβ. These studies provide a framework of how 17β-estradiol and various SERMs act in the uterus and might result in future therapies for gynecological maladies, including endometrial cancer.
Estrogen is known to have pleiotrophic effects in many organs of the female reproductive system, including the uterus (Schafer et al., 1999; Spencer et al., 1999). Selective Estrogen Receptor Modulators (SERMs) have been used to determine the roles of estrogen receptors, -α and -β in these various organs (Harrington et al., 2003; Sun et al., 2002). These synthetic compounds are capable of binding to one or both receptor forms, and in various reproductive organs. Methyl-piperidino-pyrazole (MPP) is highly selective towards ERα (Davis et al., 2006), but previously our laboratory has demonstrated that MPP shows mixed agonistic and antagonistic actions in normal and cancerous endometrial cells, respectively (Dickson and Stancel, 2000; Siiteri, 1978).

Certain cancers are known to be estrogen-dependent, including Type I endometrial cancer (Curtis et al., 1996; Fisher et al., 1994; Jordan, 1992). Some SERMs, for instance tamoxifen, can increase a woman’s risk of developing endometrial cancer due to its agonistic effects in the uterus (Young, 2007). Other SERMs, including raloxifene, are currently being evaluated for their potential in treating estrogen-responsive breast cancers (Fertuck et al., 2003; Hewitt et al., 2003; Watanabe et al., 2003a; Watanabe et al., 2003b).

A handful of microarray studies have been carried out to test the effects of estradiol on the uterus (Hewitt et al., 2003). However, few global gene expression analyses in the uterus and other organs have been performed in rodents and humans.
treated with SERMs, either individually or in combination with 17β-estradiol. The one study that has examined gene expression in the uterus after the mice were subjected to co-treatment with ICI 182,780 and 17β-estradiol reported that the former abolished the gene expression changes induced by the latter (Moggs and Orphanides, 2001). The studies reported herein describe the phenotypic changes that occur in the uteri of mice treated with MPP, raloxifene, and ICI 182,780 and the gene expression changes these SERMs induce individually and in concert with 17β-estradiol.
CHAPTER II

REVIEW OF LITERATURE

Estrogen history, properties and importance in female reproduction

Estrogen is one of the primary ovarian hormones, and it induces pleiotropic effects in several target organs, including the heart, uterus and bone (Buchanan et al., 1999; Cooke et al., 1997; Couse et al., 1997). Estrogen controls several aspects of female reproduction, including estrus behavior, the increase in serum gonadotropin concentrations and ovulation, uterine proliferation and endometrial gland secretion (Ettinger et al., 1985; Spencer et al., 1999). Estrogen prevents osteopenia (thinning of the bones) and is therefore considered beneficial in postmenopausal women prone to osteoporosis (Ito, 2007). In the cardiovascular system, it is generally presumed that estrogen has protective effects. However, some of the benefits of estrogen replacement therapy (ERT) have been questioned recently due to an increased risk of endometrial carcinoma associated with ERT (Hulley et al., 1998; Manson et al., 2003). The cardio protective advantages of ERT and HRT have also been disputed (Katzenellenbogen et al., 2000; Kuiper, 1996).

Estrogen must bind to its cognate receptor to exert its effects, and in mammals, two forms of the estrogen receptor (ER): alpha (α) and beta (β) exist (Hawkins et al., 2000). Evidence of a third ER, ERγ, has been presented for teleosts (ray-finned) fish (Green et al., 1986; Greene et al., 1986). However, cloning of the human and mouse genomes has failed to reveal a mammalian ERγ ortholog. The human ERα (classical estrogen receptor) was cloned and its cDNA sequenced from MCF-7 cells in 1986 (See
This protein is 595 amino acids long with a DNA coding sequence of 1.8 kb, and is comprised of six functional regions, A through F. The A/B amino-terminal domain is employed during transactivation of gene expression and the functions of A/B (AF-1) and E/F (AF-2) domains are related to cell type and promoter context (Greene and Press, 1986; Moutsatsou and Sekeris, 1997). Region E is responsible for estrogen binding, dimerization of the receptor, interaction with heat-shock protein 90K (hsp90), and activation of transcription (Escande et al., 2006). The C domain contains two zinc fingers, and plays a critical role in specific DNA binding and receptor dimerization (Kuiper, 1996). While attempting to clone an alternative form of the androgen receptor, the cDNA for ERβ was instead identified in a rat prostate cDNA library. Estrogen receptor-β was shown to be expressed in rat prostate and ovary (Mosselman et al., 1996). Estrogen receptor-β was then later cloned from human testes (Ariazi and Jordan, 2006; Bhat et al., 1998; Kuiper, 1996; Leygue et al., 1998; Ogawa et al., 1998). Discrepancy exists in the literature as to the exact cDNA length and full amino acid sequence of ERβ (Cooke et al., 1997; Green et al., 1986; Greene et al., 1986; Kuiper, 1996; Ogawa et al., 1998), but ERβ is shorter than ERα, containing 485 to 530 amino acids. In vitro expression studies have shown that while ERα has a molecular weight of approximately 66,000, ERβ is slightly smaller at 54,200 (Carpenter and Korach, 2006). Estrogen receptors have a DNA-binding domain (DBD or C domain) containing two zinc fingers that binds to the estrogen response element (ERE) on the target genes, and a ligand binding domain (LBD) that binds to estrogen (See Figure 1) (Mosselman et al., 1996). Estrogen receptor-α and -β differ in their A/B, hinge (D), and F-domain regions. Disparity in the A/B domain suggests that there are important differences between
receptor activation function 1 (AF-1) activity, along with interactions between co-
activators and/or repressors (Mosselman et al., 1996). The lack of sequence identity in the
F-domain is intriguing, as this region presumably plays a role in modifying the extent of
agonistic activity of estrogenic compounds that bind to ERα (Gustafsson, 2000). Both
ERs belong to the nuclear hormone receptor family and are widely distributed throughout
the body. Estrogen receptor-α is predominately found in the uterus, breast, ovary and
hypothalamus. In contrast, ERβ is expressed in the kidney, brain, bone, heart, lungs and
prostate (See Figure 2). These receptors bind with equal affinity to 17β-estradiol and
exhibit Kd measurements below the nanomolar range to this ligand (Kuiper et al., 1997;
Levy et al., 2007). However, various subclasses of estrogens and estrogenic compounds
display varying binding affinities to ER-α or –β. For instance, the phytoestrogen genistein
has a much higher relative binding affinity (RBA) for ERβ than ERα (5 and 36 RBA,
respectively) (Giguere et al., 1988).

Estrogen related receptors (ERRs) have sequence identity similar to that of the
estrogen receptor (ERα) (Giguere et al., 1988; Susens et al., 2000). However, ERRs are
considered orphan receptors because, while their sequences closely resemble ERα, they
bind with very low affinity to the estrogen molecule (Giguere et al., 1988). Instead, ERRβ
may bind to compounds such as DES (reviewed in Stein and McDonnell, 2006). Three
receptors exist in this family: ERR-α, -β and -γ. The ERRs and classical ERs share a high
degree of sequence identity in their DBD and LBD. Estrogen related receptor-α shares
about 68% identity with ERα within the DBD (Stein and McDonnell, 2006). The ERRα
and ERα can bind to the same promoter elements. Therefore, ERRα could possibly
substitute for ERα on the ERE or possibly form a heterodimer on the ERE with ERα (Koehler et al., 2005).

When the ER binds to its cognate ligand, it induces a conformational change which allows the receptor to dimerize and bind to promoter sequences of DNA known as hormone response elements (HREs) or ERE in the case of estrogen (Moutsatsou and Sekeris, 1997). This binding, in association with nuclear transcription factors, results in the formation of stable complexes altering RNA polymerase activity (Moutsatsou and Sekeris, 1997). These complexes can then in turn suppress or allow mRNA transcription and protein translation (Cowley et al., 1997; Ogawa et al., 1998; Pettersson et al., 1997). Previously, it was thought that the ER formed only homodimers upon binding to estrogen, but this conclusion is incorrect. Several laboratories have demonstrated that ER-α and -β can form functional heterodimers on DNA, both in vivo and in vitro (Saunders, 1998). It is now universally accepted that estrogen receptor subunits can either form homodimers, i.e. ERα—ERα or ERβ—ERβ or heterodimers, e.g. ERβ—ERα, while associated with their ERE. The various dimer combinations exert contrasting effects on gene transcription (Lubahn et al., 1993).

With the creation of the estrogen receptor alpha knockout (αERKO) mouse (Lubahn et al., 1993) it was demonstrated that animals can develop and survive without a functional ERα, although both the adult male and female are infertile (Lubahn et al., 1993). These αERKO mice were generated by using ES cells (E14TG2a) derived from a 129/J mouse strain. Targeted ES cells were injected into C57BL/6J blastocysts, returned to pseudopregnant C57BL/6J females, and chimeras developed that, when mated, produce animals heterozygous for the ER gene deletion (Lubahn et al., 1993). Subsequent
pairings between these heterozygous phenotypes gave rise to the homozygous ER\(^{-/-}\) mutant mice. These homozygous mutant females are infertile and they display no lordosis posture or interest in males, even after treatment with estrogen (Lubahn et al., 1993). Other aspects of their phenotypes include underdeveloped uteri that are unresponsive to estradiol (Couse and Korach, 1999b; Lubahn et al., 1993), enlarged ovaries containing hemorrhagic cysts, follicles arrested at the pre-antral stage with a dearth of corpora lutea (Korach, 1994; Korach et al., 1996), and under-developed mammary ducts (Couse et al., 1995). The ovaries of αERKO mice produce elevated amounts of estrogen and testosterone, and are not normally capable of ovulation (Rosenfeld et al., 2001). However, superovulation of these mice induces ovulation and corpus luteum development (Korach et al., 1996; Scully et al., 1997). Thus, the infertility in these mice is not due to ovarian defect, but instead due to chronic LH stimulation, caused by the lack of ER\(\alpha\) that would normally control estrogen feedback on the pituitary gland and hypothalamus (Couse et al., 1999). Accordingly, when αERKO adult females with elevated serum LH concentrations are treated with a GnRH antagonist such as Antide at 30 to 60 µg/48 h, the concentrations of serum LH are markedly reduced (Couse et al., 1999). Treatment with a GnRH antagonist at the highest dosage (60 µg/48 h) results in serum LH concentrations identical to those in normal wild-type females and prevents the onset of a polycystic ovarian phenotype (Risma et al., 1995). The αERKO mice are quite similar to a luteinizing hormone β (LH\(\beta\)) transgenic mouse that overexpresses LH (Risma et al., 1995). Such mice, like the ER\(\alpha\) knockout, have enlarged, cystic and occasional tumor-ridden ovaries, elevated serum estradiol and testosterone concentrations, and hyperemia in both cystic and tumor-bearing ovaries (Risma et al., 1995). A unique
feature of the LHβ mouse is enlarged ovaries with increased number of corpora lutea, which is seemingly due to the overstimulation of the LH receptor (Krege et al., 1998).

A knockout mouse model for ERβ, βERKO, has been established (Krege et al., 1998). While mutant mice lacking the ERα gene are completely infertile, male mice lacking the ERβ gene exhibit normal fertility, but the females are sub-fertile. For example, ERβ−/− females exhibit a reduction in number and size of litters, as well as low numbers of CL and partially arrested follicular development (Krege et al., 1998). The ovarian pathology most likely arises as a result of improper granulosa cell function, as these cells are those primarily expressing ERβ in the ovary (Carpenter and Korach, 2006). These βERKO females remain sub-fertile with age, but retain the ability to carry pregnancies to term, signifying that they possess sufficient uterine function to support a litter of pups despite their deficiency in ERβ (Couse and Korach, 1999b).

Double knockout mice (αβERKO) that lack both ER-α and -β were established by breeding male ERα heterozygous mice to female βERKO mice (Couse and Korach, 1999b). These mice have abnormal reproductive phenotypes that are similar to the αERKO. Males are infertile but exhibit a normal reproductive tract with testes that display various stages of spermatogenesis, although sperm volume and motility are decreased by 80 % and 5 %, respectively (Couse and Korach, 2001). Female αβERKO mice demonstrate normal differentiation of Müllerian-derived reproductive organs (uterus, cervix, and upper vagina). The ovaries of adult double knockout females exhibit normal ovarian structures, including abnormally high numbers of primordial and growing follicles (Couse and Korach, 2001). These mice possess cystic follicles similar to those found in the αERKO ovary, although they are neither as large nor as hemorrhagic as
those of αERKO females. Several larger follicles comprised of a thecal shell and basal lamina frequently contain a degenerating oocyte (Couse et al., 1999). A unique feature of these females is the transformation of granulosa cells into cells resembling Sertoli cells of the testis, which are not present in prepubertal αβERKO, αERKO or βERKO models (Dupont et al., 2000). Sertoli and granulosa cells are derived from a similar precursor ectodermal cell, and these results thus indicate both ER forms must be present for this common progenitor cell to differentiate into granulosa cells. The default pathway appears to be formation of Sertoli cells.

In 2000, Dupont et al. generated their own ERα, ERβ, and ERαβ knockout mice (ERαKO, ERβKO, and ERαβKO, respectively) to help illustrate the individual role of the ligand-independent transactivation function 1 (AF1) and ligand-dependent transactivation function 2 (AF2, ligand binding) of ERα and ERβ, in regards to their estrogen and anti-estrogen dependence, target genes, phosphorylation and function (Krege et al., 1998; Lubahn et al., 1993). These mice differ from those created previously (Dupont et al., 2000) in that the mutation was generated in exon 3 rather than exon 2 (Krege et al., 1998). As predicted, their ERαKO and ERαβKO males are infertile. In accordance with earlier βERKO male mutants (Dupont et al., 2000), ERβKO males show normal fertility (Dupont et al., 2000). These ERαKO females are infertile, whereas some ERβKO females are infertile and others have reduced fertility. In this study all ERβKO females acquired vaginal plugs, signifying normal sexual behavior, yet nine out of 12 females gave birth to far fewer pups than an average wild-type counterpart would. Three females exhibited complete infertility, presumably due to inability to ovulate (Dupont et al., 2000). A unique phenotype of ERβKO mutants, not seen in the βERKO model, is the lack of
atretic follicles in the ovary. Ovaries from ERαKO mice demonstrated large, hemorrhagic cysts similar to those seen in both α/βERKO mice previously generated by Lubahn et al. (1993) and Krege et al. (1998) (Fisher et al., 1998; Toda et al., 2001).

To understand estrogen function better, transgenic mice lacking the aromatase gene (ArKO) have been created by two research laboratories (Toda et al., 2001). Aromatase cytochrome P450 (P450arom) is the enzyme responsible for the production of estrogens from C₁₉ steroids, and when disrupted causes lack of aromatase activity in the ovaries of mutant females leading to an absence of measurable estrogen concentrations in blood plasma. In contrast, testosterone is strikingly elevated, particularly in female homozygous mutant mice whose serum concentrations are ten-fold higher than wild-type females (wt = 132 ± 38.5 pg/ml, ArKO = 1381 ± 498 pg/ml) (Jones et al., 2000). Testosterone is presumably elevated in these mice because of the mutation in the Cyp19 gene that catalyzes the last step in biosynthesis of C₁₈ hormones (E₂) from C₁₉ steroids (testosterone) (Britt et al., 2001; Fisher et al., 1998). Without this enzyme, ArKO mice are incapable of converting the testosterone into estrogen, and thus it is likely that excessively elevated concentrations of testosterone accumulate. LH and FSH are also elevated in ArKO mice compared to wild-type (LH: 3.38 and 1.26 ng/ml, respectively; FSH: 11.16 and 3.4 ng/ml, respectively). These mice develop hemorrhagic cysts similar to those noted in αERKO and LHβ transgenic mice (Fisher et al., 1998). These findings further demonstrate that estrogen is required to inhibit gonadotropin secretion (Fisher et al., 1998). ArKO mice are infertile, show lack of sexual behavior, and are incapable of proper ovarian folliculogenesis and spermatogenesis (Simpson et al., 2005). Both male and female ArKO mice display osteopenia (thinning of the long bones) and a metabolic
syndrome with insulin resistance known as truncal obesity, where mice show increased adiposity in their gonadal and visceral fat pads (Jones et al., 2000; reviewed in Simpson et al., 2005).

The advent of murine ER knockout models suggests that ERα is more important in terms of uterine biology and function than ERβ (Carpenter and Korach, 2006). Uterine tissue expresses mainly ERα both in the stroma and epithelium, and mitogenic effects of estradiol on uterine epithelium seem to be mediated by ERα (Buchanan et al., 1999; reviewed in Cooke et al., 1997). However, ERβ might act in the uterus to suppress ERα expression (Pole et al., 2005; Sun et al., 2002).

**Selective Estrogen Receptor Modulators (SERMs)**

Selective Estrogen Receptor Modulators (SERMs) are estrogenic compounds that have the ability to exert both antagonist and agonist effects on the ER of different tissues throughout the body, and can dominate transcriptional activity through the ERE within a given gene. Many families of SERMs exist, including the triphenylethylene derivatives (tamoxifen, toremifene, clomiphene, and idoxifene), the benzothiophene derivatives (raloxifene), and basic side-chain pyrazoles (BSC-pyrazoles and triarylpyrazoles; MPP and PPT) (reviewed in Kamada et al., 2004). Tamoxifen was the first SERM to be discovered in the 1960’s. This drug was consequently marketed as an anti-breast cancer drug because of its antagonistic effects on breast tissue (Cox and Helvering, 2006). Molecular structures commonly found among these SERMs are a non-steroidal inner core containing an aromatic ring with a phenolic-OH, connected to another ring by a spacer group and a basic side chain that normally consists of an aryl group and an alkyl amine
(See Figure 3) (Kamada et al., 2004). Current clinical SERMs include toremifene for breast cancer, clomiphene for inducing ovulation, and raloxifene for treatment and prevention of osteoporosis (Barkhem et al., 1998; Escande et al., 2006). Raloxifene has recently been cleared by an FDA panel for use as a preventative medication for ER-positive breast cancer (reviewed in Young, 2007). Raloxifene is postulated to be a better breast cancer treatment SERM than tamoxifen because raloxifene has lower toxicity risk and is not agonistic in the uterus (Young, 2007).

A feature of some SERMs is that they have the ability to bind with high affinity to both ER-α and -β, while others are more selective. The SERM methyl-piperidino-pyrazole (MPP) is considered to be primarily an ERα-selective antagonist because it has an approximate 200-fold higher selectivity for ERα than ERβ (Sun et al., 2002). In contrast, propylpyrazole triol PPT (4-propyl-1,3,5-tris(4hydroxy- phenyl) pyrazole) has predominantly agonistic affinity for ERα (>1000-fold selectivity for ERα) (Sun et al., 2002). Raloxifene displays high binding affinity to ERα compared to other antagonists including 4-hydroxytamoxifen (4OH-Tam), ICI 182,780 and RU486 (anti-progesterone) (Levy et al., 2007; Weatherman et al., 2001). Other studies however have found that raloxifene causes more gene changes and binds more effectively in ERβ than ERα (Cameron et al., 1996; Escande et al., 2006). The compound RU486 is selective towards progesterone receptor (PR) and ERβ (Lemieux et al., 2005). Acolbifene (ACOL) is a SERM that binds to ER-α and -β with an affinity much higher than that of estrogen (Labrie et al., 2001). This binding selectivity may be beneficial in cancer treatment since ACOL is a pure antiestrogen in both the breast and the uterus (McDonnell et al., 2002; Wu et al., 2005).
Many SERMs exert agonist and/or antagonist or partial agonist/antagonist properties that depend upon the type of tissue in which they are acting. Tamoxifen is an estrogen antagonist in breast tissue, but an estrogen agonist in skeletal tissue and the cardiovascular system, (reviewed in Jordan, 2004). Additionally, tamoxifen is a partial estrogen agonist in the rodent uterus (Dhingra, 2001; Jordan, 1998). Raloxifene is an estrogen agonist in bone and is used to prevent fractures and retain bone density in postmenopausal women, yet raloxifene acts as an antagonist in the breast and uterus (Dhingra, 2001; Komm and Lyttle, 2001).

Research has focused on finding the ideal SERM or SERM/estrogen combination that does not result in agonistic effects in the uterus and breast, but maintains the beneficial characteristics in the bone, heart, and adipose tissue (Buzdar, 2005; Yamamoto et al., 2005). Recently, researchers have found several novel SERMs that seemingly show greater promise than tamoxifen and raloxifene. Many of these compounds demonstrate reduced agonistic properties in tissues including the uterus, as is the case for the experimental SERM, GW5638. Unlike tamoxifen, in this SERM the dimethylaminoethyloxy group is exchanged with an acrylate side chain, resulting in GW5638 displaying more potent antagonistic characteristics in breast cancer cells and no uterotrophic side-effects (Wu et al., 2005). The SERM TAS-108 is a pure ERα antagonist and a partial ERβ agonist in the breast and uterus, and this compound demonstrates equal binding affinity for both ERs. This SERM recruits coactivator transcriptional intermediary factor 2 to ERβ, which may result in beneficial effects on bone (Geiser et al., 2005). Another compound that is currently being tested in clinical trials as an ideal SERM is LY353381 (Arzoxifene). Arzoxifene is an analog of raloxifene that lacks any
intrinsinc estrogen-agonist effects on the breast cancer cell line MCF-7 and in the rat uterus, where it appears not to induce endometrial stimulation. Its side effects, such as hot flashes, have been minimal (Dhingra, 2001). The SERM EM800 exerts estrogen-antagonistic activity on the hypothalamic-pituitary axis, benign and malignant mammary tissue, and in the uterus (Dhingra, 2001). The novel SERM LY2066948, developed in 2005, shows powerful uterine antagonist activity, minimally stimulates the ovary and might be rotated into a SERM drug regimen used to combat uterine fibroids and other estrogen-dependent gynecological maladies (Sonoda and Barakat, 2006). These and many other SERMs will potentially provide novel treatment regimens for estrogen-dependent cancers in the future.

Endometrial cancer and the role of estrogen and SERMs

Estradiol (E2) stimulates proliferation of uterine epithelial cells in vivo and is necessary for proper uterine epithelial secretory activity, morphogenesis and cytodifferentiation. However, under certain circumstances the mitogenic effects of estrogen may become unrestrained, resulting in endometrial cancer. There are two types of endometrial cancer, Type I and II (Schneider, 2002; Weiderpass et al., 1999). Type I is the most common (comprising 75 to 80 % of cases) but less aggressive form and is more prevalent in younger women who exhibit higher concentrations of circulating estrogen. Type II is more aggressive and develops spontaneously (Sonoda and Barakat, 2006). The chance that a woman will develop endometrial cancer (Type I) increases dramatically (as much as 4- to 8-fold) after being subjected to hormone replacement therapy (HRT) using unopposed estrogens (Creasman, 2002; Feeley and Wells, 2001). For this reason, most
current HRT regimens include progestin to counteract the effects of estrogen (Grady et al., 1995). A meta-analysis of published reports looking at dosage, period of use, type of estrogen used, and length of time since termination of treatment in unopposed estrogen therapy revealed that the relative risk (RR) of developing endometrial cancer increases as estrogen exposure increases (Barkhem et al., 1998; Maturana et al., 2007). The RR for endometrial cancer also increases when women are subjected to excessively elevated doses (0.625 to ≥ 1.25 mg) of conjugated rather than synthetic estrogens. After cessation of estrogen therapy, women demonstrate up to a 2-fold risk for up to five years (Grady et al., 1995). Many postmenopausal women undergo HRT to reduce the risk of developing osteoporosis from estrogen deficiency. During menopause, a woman’s endogenous estrogen production decreases dramatically, leading to a decline in bone mass and increased risk for osteoporosis (Fornander et al., 1989).

Many SERMs that are currently used in cancer treatment regimens have varying degrees of confounding side-effects. For example, the SERM tamoxifen, currently being employed in breast cancer treatment, is an estrogen antagonist in the breast and an estrogen agonist in the uterus. Consequently, women receiving tamoxifen demonstrate an increased risk of developing endometrial cancer, where the compound promotes rather than inhibits cellular proliferation of targeted cells (Fornander et al., 1989; Goldstein, 2001). Data collected from one study suggested a 7.5-fold increase in the risk of endometrial carcinoma in women who had been taking tamoxifen for breast cancer for more than two years (Zhang et al., 2005). Tamoxifen induces luminal and glandular epithelial cell proliferation similar to E₂, although it does not result in full estrogenic response; these results suggest that tamoxifen operates similar to a classical impeded
estrogen (reviewed in Hibner et al., 2004). Treatment with tamoxifen causes the recruitment of cyclin D1 into the nucleus of uterine epithelial cells, which ultimately results in cyclin E/CDK2 activation, induction of cyclin A, elevation of CDK2 activity, and nuclear localization of PCNA staining (Zhang et al., 2005). These combined results support the hypothesis that tamoxifen stimulates endometrial proliferation.

Raloxifene is an estrogen antagonist in the breast and endometrium, and this compound prevents estrogen-induced transcription (Watanabe et al., 2006). When Ishikawa endometrial carcinoma cells are subjected to raloxifene at high concentrations (10 or 100 ng/ml), however, its administration speeds up cell growth by 10 to 15 % (Hibner et al., 2004). A possible reason for this contradictory finding is that the ER properties of malignant endometrial cells might render raloxifene a mitogenic stimulator rather than an antagonist (Hibner et al., 2004). Future studies with these and other endometrial carcinoma cell lines might help determine how SERMs act on their target cells and tissues and why they might have therapeutic value under some circumstances but not others.

Endometrial cancer cell lines, including Ishikawa and Hec1a, express both ERα and even greater ERRα mRNA (Watanabe et al., 2006). The ERRα might suppress ERα expression or transcriptional activity in these cancerous cells. Accordingly, cancer cells that have marked expression of ERRα grow slower than those that do not (Fertuck et al., 2003; Watanabe et al., 2003b). The importance of this finding would be that if cancer cells express ERRα, and investigators were able to identify its binding partners, then chemotherapy regimens might be developed to target this pathway.
Introduction to microarray technology

Microarray analysis is a relatively new tool used to detect gene expression changes in a multitude of diverse systems (Carter et al., 2005). The two most popular types of microarrays used in research today are oligonucleotide and cDNA microarrays. DNA microarrays (otherwise known as oligonucleotide microarrays) are usually glass slides that have been spotted with hundreds to thousands of gene sequences, also called probes, e.g. those from Affymetrix, Inc. These microarrays will be discussed to a greater extent in Chapter 3. The probes can number to up to 40,000, and a single array can represent most of the transcribed genome of a particular organism, including many of the more common splice variants (Ryan et al., 2005; Sonoda and Barakat, 2006). The mRNA from the tissue to be studied is isolated, labeled with a fluorescent dye, and hybridized to the DNA microarray. The mRNA binds to its complementary DNA (in the case of both cDNA and oligonucleotide arrays) resulting in a fluorescent signal, which is detected by a customized array scanner. Genes whose transcription is up- or down-regulated in various disease states, such as cancer, or in response to a pharmacological treatment can be analyzed relative to normal control tissues. Any differences in gene expression might then be correlated with cancerous transformation or pharmacological treatment.

Use of microarray technology to study estrogen and SERM-induced gene changes in normal or cancerous endometrium

Endometrial cancer is the fourth most common cancer found in women in developed countries, and the most common type of gynecologic cancer (Hewitt et al.,
My thesis studies (described in Chapters 2 and 3) focus on examining the effects of estrogen and SERMs in normal mouse uterus as well as in human endometrial cancer cells. Thus, included below is a review of the current literature on estrogen and SERM-induced gene changes in normal and cancerous endometrium, as determined in many cases by microarray analyses.

Estrogenic actions and acute responses in uterine tissue from ERKO and wild-type mice have been annotated via microarray technology (Hewitt et al., 2003). Prior to ovulation, the level of estrogen in the body surges and uterine tissues demonstrate well-characterized physiological and biochemical responses that can be separated into early (within a few hours of E2 surge) and late (up to 24 h after administration) responses (Hewitt et al., 2003). More genes are regulated at 2 h (early) and 24 h (late) in WT and βERKO samples than in αERKO samples, and the intensity of gene responsiveness is diminished in αERKO, signifying the need for ERα to yield a full E2 response (Hewitt et al., 2003). Estrogen regulates many genes involved in cell cycle progression including \( p21 \), a gene causing delay of the S phase, and this up-regulation is observed 30 minutes after E2 administration. Another important S phase gene, \( cyclin G1 \), is up-regulated after 12 h. Mitotic arrest-deficient homolog-like 2 (\( MAD2 \)), which controls mitotic spindle assembly, was robustly up-regulated (20-fold induction at 2 h), and c-\( Fos \) was induced at 30 min. \( Lactotransferrin \) was present in the late treatment clusters, after being induced 24 h after E2 injection (Hewitt et al., 2003). Similar to my own findings (presented in Chapter 3), several genes in the Wnt signaling pathway were also E2 regulated, including Casein kinase 1 and Axin2 (Hewitt et al., 2003). Treatment with ICI 182,780 prior to E2 injection inhibited genomic responses at early and late phases, and was most exaggerated
in WT and βERKO samples (Watanabe et al., 2003b). Another genomic study that analyzed multiple time points (1 h, 2 h, 6 h, 12 h, 24 h, and 48 h) found that expression of regulated genes was maximized between 6 and 12 h after E2 treatment, but an E2 dosage of 5 µg/kg BW can influence gene expression even up to 12 to 24 h following administration (Hewitt et al., 2003). A few of the same genes found in the previously mentioned study (Watanabe et al., 2003b) were also discovered in the second study, including MAD2 (up-regulated 2 h post-E2) and c-fos (induced 1 to 2 h post-E2). Clustering analysis reveals that several genes are repressed by E2 including cyclin G2, which is also consistent with the conclusion that E2 advances progression through the cell cycle (Pole et al., 2004).

The gene expression profiles of human endometrial carcinoma cells during treatment with estrogen and SERMs have also been evaluated. Glandular and stromal epithelial carcinoma cell types originating from primary cultures were treated with 0.1 µM of β-estradiol, tamoxifen or raloxifene (a concentration previously found to influence cell homeostasis) and a number of genes associated with cancer development were identified. Cyclooxegenase-2 (COX-2) and several transcripts (α-A1-adrenergic receptor, ER gene and the androgen receptor, AR) related to hormonal responses were detected (Pole et al., 2004). Tamoxifen and raloxifene up-regulated expression of ER in cancerous endometrium compared to normal endometrial cells (Gielen et al., 2005; Takai et al., 2005). Stable endometrial cancer cell lines are also routinely employed for many genomic studies, and similar responses are observed in these cell lines and in vivo (Inoue et al., 2007).
Microarray analysis has contributed to the discovery and classification of estrogen-responsive genes potentially involved in endometrial cancer (Hong et al., 2006). In one study, the gene expression profiles of Ishikawa human endometrial carcinoma cells were analyzed after cells were treated with estrogen, diethylstilbestrol (DES), or bisphenol A (BPA) (Inoue et al., 2007). The compound DES is a synthetic estrogen, while bisphenol A is a hormone disrupting chemical compound normally found in plastics (Inoue et al., 2007). Diethylstilbestrol has a relative binding affinity (RBA) of 468 and 295 for ERα and ERβ, respectively; bisphenol A shows a RBA of 0.05 and 0.33, respectively (Kuiper et al., 1997). Ishikawa cells treated with estrogen (1 µM), DES (10 nM), or BPA (10 µM) showed low to moderate degrees of correlation ($R = 0.26, 0.36,$ and 0.54, respectively) in their gene expression profiles as determined by microarray. These data indicate that these compounds might induce different sets of genes in such cells (Inoue et al., 2007). The E2-treated Ishikawa endometrial cells had a gene expression profile similar to that of E2-treated MCF-7 breast cancer cells, with 120 genes sharing expression profiles in both cell lines (Baker et al., 2000; Duax et al., 1985; Ho, 2004). This discovery shows promise in terms of elucidating common gene pathways that result in various gynecological cancers, and thereby might aid in future genetic screening methods.

The Ishikawa, HEC-1B, RL95-2, KLE, AN3CA, and HEC59 cell lines were used in a recent study that analyzed genes silenced by DNA methylation and histone deacetylation (HDAC) in endometrial carcinoma (Takai et al., 2005). Out of more than 22,000 genes, two were studied closely: tazarotene-induced gene 1 ($Tig1$) and CCAAT/enhancer binding protein-α ($C/ebpα$). Previously, some investigators have
proposed that \textit{C/ebpa} is an important regulator of cellular differentiation in leukemia, and loss of \textit{Tig1} expression has been documented in cases of endometrial cancer (Takai et al., 2005). The expression of \textit{Tig1} was silenced in all six cell lines, and then subsequently induced when the cells were treated with a DNA methylation blocking agent (5-aza-2′-deoxycytidine) in combination with an HDAC inhibitor (suberoylanilide bishydroxamide; SAHA). In contrast, \textit{C/ebp}α was up-regulated by these treatments. Findings from this study suggest that these two genes act as tumor suppressors in endometrial cancer and DNA methylation inhibitors may be useful for treating endometrial cancer.

In summary, the steroid hormone estradiol is important in mediating several reproductive processes, and it exerts its actions via two receptors ERα and -β. Creation and experimental analysis of estrogen knockout mice models have enhanced our knowledge of the interactions between estrogen and its receptors. Various compounds bind to ERs in different ways, and this can be especially important where hormone replacement therapy (HRT) is concerned. Selective estrogen receptor modulators (SERMs) are estrogenic compounds that have the ability to bind to ERs, and are currently being used to treat diverse disorders, including osteoporosis and breast cancer. The search for an ideal SERM that can be used either in an HRT regimen or to treat other types of cancer, including endometrial cancer, continues, however, there is currently no “perfect SERM” that lacks confounding side effects. Microarray analysis has revealed many differentially expressed estrogen-responsive genes that can be targeted via estrogen or SERMs to incite or suppress key pathways regulating cell cycle progression, apoptosis, etc., and therefore, these compounds might one day be useful in treating cancers, such as endometrial carcinoma. My experiments, explained in Chapters 2 and 3 of this thesis, relate to finding
this “ideal SERM”; one that is capable of not only helping the symptoms of
postmenopausal women, but also treating any number of gynecological maladies,
including endometrial carcinoma.
CHAPTER III

THE EFFECTS OF THE SELECTIVE ESTROGEN RECEPTOR MODULATORS, METHYL-PIPERIDINO PYRAZOLE (MPP), AND RALOXIFENE IN NORMAL AND CANCEROUS ENDOMETRIAL CELL LINES AND IN THE MURINE UTERUS

ABSTRACT

Since estrogens have vital functions in the uterus but might also contribute to endometrial cancer, we sought to determine the in vitro effects of methyl-piperidino-pyrazole (MPP), raloxifene, and β-estradiol on Ishikawa and RL-95 endometrial cancer and ovine luminal endometrial (oLE) cell lines and the in vivo effects of these compounds in the rodent uterus. The SERMs MPP and raloxifene (1 nM) induced significant apoptosis in the endometrial cancer and oLE cell lines compared to β-estradiol treated and control cells (P ≤ 0.0001). To determine the in vivo uterine effects of these compounds, ovariectomized wild-type (WT) and estrogen receptor-β knockout (ERβKO) mice were treated with 25, 50, 100, or 150 μg of each compound. Although raloxifene caused no significant increase in uterine weight, the presumptive ERα antagonist, MPP (25 to 150 μg) increased uterine weight and cell proliferation significantly relative to vehicle control in WT and ERβKO mice (P ≤ 0.001). However, MPP did not increase uterine wet weight as effectively as β-estradiol (P ≤ 0.0001), and administration of either
50 μg of MPP or raloxifene effectively reversed the positive stimulation by 50 and 100 μg β-estradiol. Unexpectedly, in view of the in vitro studies, MPP and raloxifene treatment of ovariectomized mice did not induce apoptosis of the luminal epithelial cells but rather these compounds induced apoptosis of the underlying uterine stromal cells. These results demonstrate that MPP and raloxifene can exert apparently contrasting in vitro versus in vivo effects and that they have mixed agonist/antagonist action on murine uterine ERα in vivo.

**INTRODUCTION**

Selective estrogen receptor modulators (SERMs) have been created to target and decipher the roles of estrogen in responsive organs and for potentially treating estrogen-responsive cancers. These SERMs demonstrate tissue specific ER agonistic/antagonistic activity by binding to ER-α and/or –β. Other compounds, such as diethylstilbestrol and genistein, exert only ER agonistic activity (Campen et al., 1985; Carthew et al., 1999). In contrast, tamoxifen exerts full agonistic activity in the mouse uterus, partial agonistic activity in rat uterus, and antagonistic activity in the breast (Dukes et al., 1993; Howell et al., 2000), and thus, this compound has been useful in treating breast but not endometrial cancer. Other compounds that bind to ER, such as ICI 182,780 (Falsodex or Fulvestrant) and raloxifene, which is a benzothiophene derivative, have estrogenic antagonistic activity in the uterus, and the former is thought to bind ER competitively in every estrogen-responsive organ tested to date (Harrington et al., 2003; Harris et al., 2002; Sun et al., 2002). These compounds do not discriminate between ERα and ERβ, and thus, the
individual ER effects cannot be discerned with raloxifene or ICI 182,780. The creation of selective ER\(\alpha\) and ER\(\beta\) agonists and antagonists has permitted further dissection and targeting of ER-\(\alpha\) versus -\(\beta\) actions (Lindberg et al., 2002). These SERMs include methyl-piperidino-pyrazole (MPP), which, based on co-transfection assays and \textit{in vitro} binding studies, is presumed to be a selective ER\(\alpha\) antagonist (Harrington et al., 2003; Sun et al., 2002).

Estrogen induces pleiotrophic effects within the uteri of various species. These effects include an increase in uterine wet weight, proliferation and differentiation of the luminal and glandular epithelium, stimulation of transcription and translation of a variety of gene products that regulate cell-cycle progression, hyperemia, stromal edema and water imbibition, angiogenesis, recruitment of immune cells, and endometrial morphology (Dickson and Stancel, 2000; Rosenfeld et al., 2001). The predominant estrogen-induced effects in the uterus, including those that result in an increase in uterine wet weight, are mediated by ER\(\alpha\) as opposed to ER\(\beta\) (Sakamoto et al., 2002).

In women, several endometrial cancers are estrogen-induced (Dickson and Stancel, 2000; Siiteri, 1978). Women who receive tamoxifen treatment for breast cancer are at increased risk of developing endometrial cancer because of the agonistic effects of tamoxifen in the uterus (Curtis et al., 1996; Fisher et al., 1994; Jordan, 1992). Tamoxifen stimulates proliferation of Ishikawa 3H-12 endometrial cancer cells and constitutively activated mitogen-activated protein (MAP) kinase-signaling pathway enhances the transcriptional activity of ER\(\alpha\) in these cells (Ho, 2004; Pole et al., 2004; Watanabe et al., 2003b).
Administration of SERMs in combination with microarray technology has been useful in determining the wide assortment of estrogen-induced uterine genes (Ho, 2004). As evidenced by these studies, β-estradiol and SERMs induce elaborate and in some instances contrasting effects. Laser capture microdissection (LCM) and cDNA microarrays from estradiol-treated mice reveal that estrogen induces a varying complement of genes in different endometrial cells (Helvering et al., 2005). Microarray comparison of rats treated with either estradiol or raloxifene demonstrates that estradiol stimulates genes that increase metabolic activity and promote matrix breakdown. In contrast, raloxifene stimulates pro-apoptotic and anti-proliferative genes (Farnell and Ing, 2003; Jazaeri et al., 1999; Way et al., 1983).

Based on these past studies, we sought to determine the effects of the presumptive ER antagonists, raloxifene and MPP, on human cancerous and ovine endometrial cell lines and the in vivo uterine effects of these compounds in ovariectomized WT and estrogen receptor-β knockout (ERβKO) mice.

**MATERIALS AND METHODS**

*Animals*

Wild-type CF1 and ERβKO female mice were maintained at the University of Missouri Animal Sciences Research Center. All of the experiments were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the University of Missouri Office of Animal Resources. The mice were housed under a 12 hours Light:12 hours Dark photoperiod and controlled temperature (72
± 2°F) and ad libitum fed Purina 5001 chow-based diet (Purina, St. Louis, MO) and water. Mice were ovariectomized at 7 to 9 weeks of age. One week after the surgical procedure, the CF1 mice were injected intraperitoneally (I.P.) with two dosages 24 h apart of 25 µg (n = 3), 50 µg (n = 11) or 100 µg (n = 5), or 150 µg (n = 3) of MPP (Tocris Cookson, Inc., Ellisville, MO) 25 µg (n = 3), 50 µg (n = 10) or 100 µg (n = 5), 150 µg (n = 3) of raloxifene (NCI Chemical Carcinogen Repository, Midwest Research Institute, Kansas City, MO) or 25 µg (n = 3), 50 µg (n = 10) or 100 µg (n = 4), 150 µg (n = 3), or β-estradiol (Sigma Chemical, Saint Louis, MO) or vehicle alone (n = 20). To study the potential competitive antagonism of MPP and raloxifene, additional ovariectomized mice were co-treated with 50 µg of MPP and 50 µg β-estradiol (n = 3), 50 µg MPP and 100 µg β-estradiol (n = 3), 50 µg raloxifene and 50 µg β-estradiol (n = 3), or 50 µg raloxifene and 100 µg β-estradiol (n = 3). ERβKO mice were also treated with two dosages of 50 µg MPP (n = 6), β-estradiol (n = 9) or DMSO vehicle control (n = 7). On the third day, mice were humanely sacrificed with CO2 inhalation and the uteri collected, weighed, and fixed in 10% Neutral Buffered Formalin (NBF).

**Uterine Histology**

The tissues were paraffin embedded and 3 to 4 µm thick sections were stained with hematoxylin and eosin (H & E). The slides were visualized and images acquired by using an Olympus Provis Microscope (Olympus Corp., New Hyde Park, NY) with an attached Spot 2 camera (Diagnostic Instruments, Inc., Sterling Heights, MI).

**Immunohistochemistry**

Slides containing unstained uterine tissues were washed with Clear Rite 3 (Richard-Allen Scientific, Kalamazoo, MI), and re-hydrated with a graded (100% to
75%) series of 10 min ethanol washes. Slides were then washed 3X for 5 min each in 1X PBS and microwaved in 10 mM Citrate Buffer for 5 min. Ten percent H₂O₂ was incubated on the tissues for 10 min at room temperature (RT) followed by three 5 min washes with 1X PBS. The sections were immersed with goat serum for 30 to 60 min at RT to prevent non-specific protein binding. Primary rabbit polyclonal antibody of 1:25 Ki67 (Abcam, Cambridge, MA) was incubated on the tissues for either 1 h at RT or overnight at 4°C. Slides were washed with 1X PBS (3X, 5 min each) and treated with biotin solution (LSAB2 Biotinylated Link for HRP/AP kit, DAKO Cytomation, Carpinteria, CA) for 10 min at RT. After three 5 min 1X PBS wash steps, the tissues were incubated in streptavidin peroxidase solution (LSAB2 Peroxidase Conjugated Streptavidin kit, DAKO) for 10 min at RT. Slides were then washed in 1X PBS for 5 min followed by incubation with DAB + substrate solution (Liquid DAB + Substrate Chromogen System, DAKO). Once a brownish hue was detected, the DAB + solution was immediately poured off and the reaction stopped with 1X PBS. Samples were counterstained with Harris Hematoxylin (StatLab, Lewisville, TX) for 30 sec to 1 min and cover slipped with permount. Slides were visualized under the Olympus Provis Microscope (Olympus America Inc., Center Valley, PA) and digital images acquired by using the Spot 2 camera (Diagnostic Instruments, Inc., Sterling Heights, MI). For each treatment group, 10 μm uterine sections and eight slides from each mouse were tested.

**Endometrial Cancer Cell Lines**

The estrogen receptor-positive endometrial cancer cell lines, RL-95 and Ishikawa (donated by Dr. Steve Young, Univ. of N. Carolina, Chapel Hill), were employed for these experiments (Johnson et al., 1999; Stewart et al., 2001). The cells were cultured in
Dulbecco’s Modified Eagle Medium with 4 mM L-glutamine, 2 g/L sodium bicarbonate, 4.5 g/L glucose, 1 mM sodium pyruvate, 10 mM HEPES, 5 μg/ml bovine insulin and F-12K medium with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, and 10% FBS in 75 cm³ flasks (BD Biosciences, Bedford, MA) and incubated at 37°C with 5% CO₂.

**Ovine Luminal Endometrial Cell Line**

Ovine luminal endometrial (oLE) cell line (which are primarily from the intercaruncular region, donated by Dr. Tom Spencer, Texas A&M University) were cultured in Dulbecco’s Modified Eagle Medium/ F12-K and 10 % fetal bovine serum with gentamicin in 75 cm³ flasks (BD Biosciences) until the cells had undergone 30 to 40 passages (Dardes et al., 2002; Geiser et al., 2005; Taylor et al., 2006). Cells were divided (1:2) every 2 days to maintain the cultures below 75% confluency.

**Immunofluorescence for Estrogen Receptor-α and –β in oLE Cells**

Ovine LE cells for immunofluorescence were cultured for 24 h on glass coverslips in 6-well plates (Nalge Nunc, Rochester, NY). The cells were fixed with 2% paraformaldehyde in 100 mM glycine for 20 min at RT. The plate containing the cells on the coverslips was rinsed 3X for 5 min each with 1X PBS and was permeabilized by using 1% Triton X-100, upon which the plates were micro-waved in 1X PBS for 30 sec to facilitate heat-induced antigen retrieval. To prevent non-specific binding, 5% goat serum and 5% bovine serum albumin in 1X PBS was added to each of the wells, and the cells were immersed in the solution for 30 min at 37°C. Dual-labeling immunofluorescence for ER-α and -β was performed. The polyclonal ERβ antibody from Santa Cruz Biotechnology (Santa Cruz, CA) was diluted 1:50 with 1X PBS and 0.1% Triton X-100 and incubated on the cells for 1 h at RT. The negative control wells were
incubated with 1X PBS and 0.1% Triton X-100 without the primary antibodies. The cells were rinsed three times with 1X PBS for 5 min each, and the secondary antibody, Alexa Fluor 568 goat anti-rabbit IgG (Molecular Probes, Eugene, OR) at a 1:500 dilution was incubated on the cells for 45 min at RT, and the cells were then rinsed with 1X PBS as above. A mouse monoclonal IgG antibody against ERα from Santa Cruz Biotechnology was diluted 1:50, as above and added to the wells overnight in a humidified chamber at 4°C. The cells were washed with 1X PBS as above, and the secondary antibody, Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes) at a 1:500 dilution was added to the wells for 45 min at RT. After washing with 1X PBS, the coverslips containing the cells were removed from the wells and placed on positively charged slides containing 5 μl of Vectashield Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA). The coverslips were sealed onto the slides with a thin coat of clear nail polish and stored in a darkened chamber at 4°C. Slides were visualized under the Olympus Provis Microscope (Olympus Corp) and digital images acquired by using a Spot 2 camera (Diagnostic Instruments, Inc.).

*TUNEL Assay*

Cells were cultured in the presence of 1 nM concentrations of MPP, raloxifene, β-estradiol, or DMSO (vehicle control) for 24 h in 8-well BD Falcon CultureSlides (BD Biosciences, Bedford, MA). Previous concentrations that have examined the apoptotic or cell proliferation effects of SERM and β-estradiol on various cell lines have employed similar or even greater concentrations (as high as 10 nM to 15 μM) of these compounds (Helvering et al., 2005). Thus, the concentration of SERM and β-estradiol that we have chosen to employ is in line with previous studies and should be sufficient to elicit an
effect without inducing toxic effects within the cells. To verify further that the potential anti-apoptotic effects are due to genomic actions rather than toxicity, additional cells were cultured in the presence of 1 nM MPP + 10 nM β-estradiol or 1 nM raloxifene + 10 nM β-estradiol. The 10-fold excess of β-estradiol should presumably squelch the genomic effects of the SERM. If, however, the putative anti-apoptotic effects are attributed to toxicity of the compounds, the excess β-estradiol would not be able to rescue the cells. The cells were then fixed with 4% paraformaldehyde and incubated for 25 min at 4°C. The cells were permeabilized by using 0.2% Triton X-100 in 1X PBS for 5 min. The Dead End Fluorimetric TUNEL Assay kit and protocol (Promega, Madison, WI) were used to determine the number of apoptotic endometrial cancer or oLE cells. Briefly, 100 μl of Equilibration Buffer from the TUNEL kit was added to each of the eight wells for 10 min. Subsequently, the cells were incubated with the nucleotide mix and rTdT enzymes provided with the kit and incubated in a humidified, light-sealed chamber at 37°C for 60 min. The reactions were then terminated by adding 500 μl of the 2X SSC to each well for 15 min at room temperature. The nuclei of the cells were labeled with 0.5 mM of propidium iodide (PI) in 1X PBS solution. Coverslips were mounted onto the slides with MOWIOL medium (Calbiochem, San Diego, CA) and nail polish. The number of apoptotic cells was determined by using an Olympus IX70 inverted microscope (Olympus Corp.) coupled to a BioRad Radiance 2000 confocal system (Carl Zeiss Microimaging Inc., Thornwood, NY) and the Metamorph Software Program (Universal Imaging Corporation, Downingtown, PA). Three to four sections was examined for each treatment group. The number of replicates for the cell lines included the following:
Ishikawa: 50 MPP, 44 Raloxifene, 54 1 nM β-estradiol, 12 10 nM β-estradiol, 18 MPP + 10 nM β-estradiol, 18 Raloxifene + 10 nM β-estradiol, and 25 DMSO vehicle control.

RL-95: 36 MPP, 30 Raloxifene, 38 1 nM β-estradiol, 15 each MPP + 10 nM β-estradiol, and Raloxifene + 10 nM β-estradiol, 10 nM β-estradiol, and 18 DMSO vehicle control.

oLE: 28 MPP, 28 Raloxifene, 28 1 nM β-estradiol, 14 MPP + 10 nM β-estradiol, 16 Raloxifene + 10 nM β-estradiol, 8 10 nM β-estradiol, and 14 DMSO vehicle control.

The TUNEL assay was also performed on uteri sections from the MPP, raloxifene, β-estradiol, and control treated ovariectomized mice. The unstained uteri sections were first re-hydrated through decreasing series of ethanol concentrations and then the TUNEL procedure was performed as described above. Four replicates per treatment group were done.

Statistics

An ANOVA was performed for each cell line. The data were analyzed as a Completely Randomized Design (CRD) which included eight treatments as described previously. The treatment variances proved to be heterogeneous, thus a log10 transformation was applied to stabilize the variances. Differences between treatment means were determined by using Fisher’s Least Significant Difference (LSD). All data were analyzed by using the SAS software analysis program (SAS/STAT User's Guide, 1988).
RESULTS

Since SERMs, including raloxifene, have been previously demonstrated to increase transcription of pro-apoptotic genes in endometrial cells (Hewitt et al., 2003; McDougall et al., 2003), the compounds were tested for their abilities to induce apoptosis in the human endometrial cancer and oLE cells. The 1 nM concentrations of MPP and raloxifene induced significant apoptosis in Ishikawa cells, with 5.83 ± 0.07 % and 6.83 ± 0.07 %, respectively, of cells staining positively in the TUNEL assay relative to Ishikawa cells treated with β-estradiol (2.77 ± 0.02 %) or vehicle alone (1.43 ± 0.02 %) (P ≤ 0.001) (Figures 4 and 5). MPP and raloxifene also induced significant apoptosis (P ≤ 0.001) in RL-95 cells with 16.89 ± 0.12 % and 18.08 ± 0.12 % apoptotic cells, respectively. In contrast, β-estradiol and vehicle control induced 7.76 ± 0.07 % and 0.43 ± 0.01 % apoptotic RL-95 cells, respectively (Figures 6 and 7).

We also tested these SERMs and β-estradiol in the stable ovine uterine luminal epithelial (oLE) cell line, which expresses both ER-α and –β (Figure 8). Ovine LE cells treated with MPP and raloxifene had 13.86 ± 0.11 % and 15.57 ± 0.15 % apoptotic cells, respectively, while β-estradiol and vehicle-treated cells had 3.30 ± 0.04 % and 1.68 ± 0.04 % apoptotic cells, respectively (P ≤ 0.001 to 0.0001) (Figures 9 and 10). To verify that the pro-apoptotic effects observed with MPP and raloxifene were genomic in origin rather than due to some unforeseen toxicity, additional Ishikawa, RL-95, and oLE cells were cultured in the presence of 1 nM concentrations MPP or raloxifene along with a 10-
fold excess of β-estradiol. Estradiol treatment decreased the SERM-induced apoptosis within Ishikawa, RL-95, and oLE cells (Figures 4 to 7 and 9 to 10).

To determine whether the \textit{in vivo} responses of these SERMs and β-estradiol mirror the \textit{in vitro} responses, ovariectomized mice were treated with MPP, raloxifene, β-estradiol, or vehicle control. The dosages of these compounds ranged from 25 to 150 μg (similar to previous doses used (Yamamoto et al., 2005)). The non-significant interaction indicated that all of the treatments were running in a similar fashion across dosages, although different dosages yielded different responses. Treatment of ovariectomized mice with 25, 50 or 100 μg of β-estradiol induced a significant increase in average uterine wet weight/body weight ratio relative to the SERM-treated and vehicle control groups (P \leq 0.0001) (Figures 11 and 12) and luminal endometrial proliferation (Figure 13). Unexpectedly, 150 μg of β-estradiol resulted in a decrease in average uterine weight/body weight ratio compared to the other tested concentrations of β-estradiol.

Ovariectomized mice treated with 25, 50 or 100 μg of MPP, the presumed ERα antagonist, had a significant increase in average uterine wet weight/body weight ratio relative to vehicle control (P \leq 0.001) (Figures 11 and 12). As with β-estradiol, the 150 μg dosage of MPP resulted in a decrease in average uterine weight/body weight ratio relative to other tested concentrations of MPP. Proliferation also increased in ovariectomized mice treated with MPP, as evidenced by an increase in endometrial cells staining positively for the proliferation marker, Ki67 (P \leq 0.05) (Figures 13 and 14).

A slight but insignificant increase in average uterine wet weight/body weight ratio was observed in ovariectomized mice treated with 50, 100, or 150 μg of raloxifene. Ovariectomized mice treated with MPP and to a lesser extent raloxifene caused
hyperplasia of the uterine luminal epithelium with several areas transitioning from a simple columnar to stratified columnar epithelium (Figure 15).

To test the potential competitive antagonism, as measured by a change in uterine weight/body weight ratio, between these SERMs and β-estradiol, ovariectomized mice were treated with combinations of the compounds. Both MPP and raloxifene (50 and 100 μg/ml) were able to reverse the effects of β-estradiol (P ≤ 0.001) (Figure 16).

In contrast to the in vitro effects observed with these compounds, the SERMs did not induce significant apoptosis in luminal endometrial cells. Instead, ovariectomized mice treated with MPP and raloxifene had a significant increase in apoptotic cells within the underlying uterine stromal cells relative to ovariectomized mice treated with β-estradiol or DMSO vehicle control (Figure 17).

Another ERα antagonist, TAS-108, has been shown to have partial ERβ agonistic activity (Harrington et al., 2003; Sun et al., 2002), which might also be the case for MPP. To determine whether MPP is exerting its uterotrophic agonistic effects through ERβ, 50 μg of MPP was tested in ovariectomized ERβKO mice. As with WT mice, MPP led to an increase in average uterine weight/body weight ratio and endometrial proliferation in the uteri from treated ovariectomized ERβKO females relative to those treated with DMSO vehicle control alone (0.14 % ± 0.04 g) (P ≤ 0.005) (Figure 18). ERβKO mice treated with 50 μg of β-estradiol had a greater increase in uterine weight to body weight ratio than MPP or vehicle control ERβKO mice (P ≤ 0.001) (Figure 18). Nonetheless, these results suggest that ERβ is not essential for mediating the uterine agonist effects of MPP.
DISCUSSION

The results herein demonstrate that the SERMs, MPP and raloxifene, potentially exert differential \textit{in vivo} versus \textit{in vitro} endometrial responses. Past studies have only examined the \textit{in vitro} effects of MPP, which appeared to act as a selective ER$\alpha$ antagonist (Cooke et al., 1997; Cunha et al., 2004; Stewart et al., 2001). In our experiments, while MPP induced apoptosis of the cancerous human endometrial and ovine endometrial cell lines tested, in ovariectomized mice it caused proliferation of the epithelium and an increase in uterine wet weight.

There are several possible explanations that might account for these conflicting \textit{in vitro} versus \textit{in vivo} results. Significant estrogen-induced cross-talk occurs between the luminal epithelium and underlying stroma in the uterus. In particular, stromal ER mediates luminal epithelial mitosis, proliferation, and release of factors such as tumor necrosis factor-\(\alpha\) (TNF$\alpha$) (Hom et al., 1998; Sato et al., 2002). Insulin-like growth factor-1 (IGF-1) and epidermal growth factor (EGF)-receptor signaling may be the means whereby stromal cells regulate luminal epithelial proliferation (Campen et al., 1985; Jordan and Robinson, 1987; Jordan et al., 1978). The activation of MPP to stromal ER and subsequent IGF and EGF signaling pathways might, therefore, underpin the increase in uterine weight and luminal epithelial proliferation that occurred in our experiments. The oLE and cancerous endometrial cells used for the \textit{in vitro} studies were devoid of stromal companion cells. Consequently, the apoptotic action of MPP is direct and mediated through its ability to bind ER within the epithelial cells. It will be of interest to
culture uterine epithelium with stromal cells, and determine whether the outcome is survival and proliferation rather than apoptosis of the epithelial cells.

An alternative explanation for the conflicting *in vitro* versus *in vivo* results is that the *in vitro* concentrations selected might not mirror what the cells would be exposed to *in vivo*. However, due to the intricate pharmacokinetics and metabolism of steroidogenic compounds, it is impossible to replicate conditions that occur *in vivo* within the culture dish.

Other studies that have examined the *in utero* effects of tamoxifen and raloxifene have yielded apparent conflicting results. Part of this disparity might be attributed to the species-specific uterine effects elicited by some SERMs, such as tamoxifen. In the mouse, tamoxifen acts as a full agonist, while in the rat it is only a partial agonist (Stygar et al., 2003). Ovariectomized rats treated with 500 μg of tamoxifen had a dramatic increase in IGF-1 expression and uterine weight but no corresponding increase in uterine cell proliferation (Karlsson et al., 1998). In contrast, 500 μg of raloxifene failed to alter the uterine weight or IGF-1 expression. Tamoxifen or toremifene (a SERM similar to tamoxifen) treatment of rats induced focal multi-layered hyperplasia within the uterus (Zheng et al., 2004), which is similar to what we observed with MPP and to a lesser extent raloxifene-treated mice in the experiments herein.

Immature rats treated with three dosages of 0.1 mg/kg of raloxifene had a 1.7-fold increase in the height of the uterine epithelium that was accompanied with increased vacuolation of these cells (Ashby et al., 1997). Another study demonstrated that while 3 mg/kg of raloxifene did not increase uterine weight in ovariectomized mice, this compound increased DNA synthesis and proliferation of the uterine luminal epithelium,
which peaked 16 h post-treatment (Sakamoto et al., 2002; Watanabe et al., 2003b). However, when 3 mg/kg raloxifene and 50 μg/kg of estradiol were administered together, raloxifene antagonized estrogen-stimulation of uterine DNA synthesis. These combined studies further suggest that raloxifene might exert mixed agonistic/antagonistic effects within the rodent uterus.

The SERMs MPP and raloxifene induced significant in vitro apoptosis of the endometrial cancer cell lines and the oLE cells. However, in vivo, these SERMs exclusively induced apoptosis of the underlying uterine stromal cells. Microarray studies have revealed that β-estradiol and SERMs induce several uterine apoptotic factors, including cytochrome C, sphingosine-1-phosphate phosphatase 1, programmed cell death six interacting protein, BAD, IkBa, AKT-1, and several caspases (Fuchs-Young et al., 1996; Palomba et al., 2005). Conceivably, the two SERMs induced similar gene products in uterine epithelial cells grown in vitro. In vivo, their actions might again be indirect through the stimulation of pro-apoptotic factors in the uterine stromal cells. On the other hand, raloxifene is known to increase apoptosis of uterine leiomyoma cells but not in normal myometrial cells (Ashby et al., 1997). Thus, the cultured cell lines might be sensitive to raloxifene and MPP because they are transformed.

In order to discern the selective activities of one ER-α versus -β, a great deal of focus has been placed on deriving selective ER-α and -β antagonists (Harris et al., 2002; Sun et al., 2002). Past in vitro studies have suggested that MPP is a selective ERα antagonist (Harrington et al., 2003; Sun et al., 2002). However, our current in vivo data suggest that MPP has agonistic effects in the mouse uterus. Another potential ERα antagonist, TAS-108, has been shown to have partial ERβ agonistic activity (Yamamoto
et al., 2005). A reasonable hypothesis is that MPP, like TAS-108, is a partial ERβ agonistic, thereby accounting for some of the in vivo results described here. However, ERα is the dominant ER in the uterus (Lindberg et al., 2002), and thus, the proliferative response that occurred in the ovariectomized mice most likely involves MPP binding and activation of ERα. Moreover, MPP-treated ERβKO mice exhibited a similar increase in uterine weight as mice WT for ERβ. Therefore, the in vivo uterotrophic effects induced by MPP are unlikely to involve ERβ but instead are presumably due to potential agonistic interaction with ERα.

While MPP and to a lesser extent raloxifene can induces agonistic effects in the mouse uterus, as evidenced by an increase in uterine weight, these compounds also can act as competitive antagonist in the mice that are co-treated with β-estradiol (Figure 16). Such an outcome is consistent with other studies that have examined the combined effects of raloxifene and β-estradiol (Liu et al., 2005). In addition, MPP has recently been shown to partially but not completely antagonize the in vitro protective effects of estrogen on cultured neurons (Galand et al., 1971; Lee, 1980; Yamashita et al., 1990). The authors interpreted their results to mean that the neuroprotective effects of estrogen are mediated by both ER-α and –β. However, an alternative explanation is that MPP exerts mixed partial agonistic or antagonistic activity on binding to ERα.

Further studies are clearly necessary to decide whether MPP has a different mode of action in vitro than in vivo or whether the effects in vivo are an outcome of the complex nature of the cellular interactions that occur. The fact that different target cells can also respond quite differently to SERMs (Geiser et al., 2005) indicates that it is
difficult to draw general conclusions or make predictions about how any specific SERM will behave in any particular situation, whether in vivo or in vitro.
CHAPTER IV

THE EFFECTS OF ESTRADIOL, METHYL-PIPERIDINO-PYRAZOLE (MPP), RALOXIFENE, AND ICI 182,780 ON GENE EXPRESSION IN THE MURINE UTERUS

ABSTRACT

Selective estrogen receptor modulators (SERMs) may be used to treat various endometrial disorders, including endometrial cancer. However, a better understanding of the gene expression patterns that occur in the uterus following treatment by these compounds is needed. Microarray analyses with the Affymetrix Mouse Genome 430 2.0 short oligomer arrays were employed here to determine the gene expression changes in uteri of ovariectomized mice treated with estradiol, methyl-piperidino-pyrazole (MPP), ICI 182,780, and raloxifene or the combination of estradiol with one of the SERMs. The nine treatments organized into two major clusters with MPP, raloxifene and the high dose of estradiol grouping together. The second cluster included the low dose of estradiol, ICI 182,780 + estradiol, ICI 182,780, MPP + estradiol, and raloxifene + estradiol. Based on this arrangement, it seems the combination of the high dose of estradiol and one of the SERMs, in particular ICI 182,780, induces gene changes similar to those of the low dose of estradiol treatment. Combination of estradiol and MPP, raloxifene or ICI 182,780 had a combinatorial effect and markedly increased the number of genes regulated compared to the individual treatments. The expression patterns for selected genes identified as
altered in the microarray analyses were examined in the uteri of estradiol and SERM-treated mice by immunohistochemistry and results were consistent with the microarray data. These results provide a framework for the genetic actions of estradiol, MPP, raloxifene, and ICI 182,780 in the uterus.

INTRODUCTION

Estrogen action has pleiotrophic effects in the uterus (Krege et al., 1998; Lubahn et al., 1993). In species that have an invasive form of placentation, estrogen functions to prepare the uterus for conceptus implantation by inducing proliferation of the endometrial lining and endometrial glands, uterine edema and hyperemia, angiogenesis, and remodeling the endometrium in preparation for conceptus penetration into the uterine wall (Yamashita et al., 1990). This hormone also induces uterine estrogen receptor expression (Pentecost and Teng, 1987), and uterine epithelial secretory proteins, such as lactoferrin (Sundstrom et al., 1989), complement component 3 (C3) (Surveyor et al., 1995), and Mucin-1 (MUC1) (Fertuck et al., 2003; Hewitt et al., 2003; Khalyfa et al., 2003; Watanabe et al., 2004; Yanaihara et al., 2005). Previous microarray studies have revealed that estrogen-induced gene changes in the uterus are involved with transcription regulation, proteolysis, regulation of cell cycle and proliferation, tissue remodeling (including endometrial basement membrane breakdown), immunological control, metabolism, detoxification and stress responses (Schafer et al., 1999; Spencer et al., 1999).
Selective estrogen receptor modulators (SERMs) have been employed to further delineate the effects of estrogen acting through ER-α or -β (Harrington et al., 2003; Sun et al., 2002). Additionally, these SERMs might hold therapeutic promise in treating various uterine pathologies, including endometrial cancer. While some SERMs such as tamoxifen, raloxifene, and ICI 182,780 bind to both ER-α and –β, others are more selective and only bind in an agonistic or antagonistic manner to one ER form. Methyl-piperidino-pyrazole (MPP) is presumed to show greater selectivity for ER-α versus ER-β (Davis et al., 2006). However, we have previously demonstrated that this compound exhibits both agonistic and antagonistic actions in normal and cancerous endometrial cells, respectively (Dukes et al., 1993; Howell et al., 2000). The antiestrogenic compound ICI 182,780 is considered to be a pure estrogen receptor antagonist (Fertuck et al., 2003; Hewitt et al., 2003; Khalyfa et al., 2003; Watanabe et al., 2004; Yanaihara et al., 2005).

Numerous microarray studies have been performed to test the effects of estradiol in the rodent and human uterus (Hewitt et al., 2003), but few have reported on global gene expression changes in the uterus induced by SERMs, either alone or in combination with 17β-estradiol, particularly MPP, whose actions are still not well understood. Only one study to date has examined gene expression changes in the uteri of mice co-treated with 17β-estradiol + ICI 182,780 (Diel et al., 2001; Kaye et al., 2001; Tanos et al., 2002; Wong et al., 2007), and this study reported that co-treatment of wild-type and estrogen receptor-β knockout mice resulted in ICI 182,780 abolishing the gene expression changes that would have otherwise occurred in the uteri in response to estradiol alone. By combining 17β-estradiol and one of the SERMs, we hypothesized that either the SERM would negate the estrogen-induced gene expression changes or a combinatorial effect...
might result, as has been described previously when estrogen was used in association with a SERM or phytoestrogen (Chin et al., 2005; Davis et al., 2006; Gutman et al., 2002; Hewitt et al., 2003). Consequently, we sought to perform a comprehensive analysis of chronic gene changes in the uteri of mice treated with MPP, raloxifene, ICI 182,780, low dose and high doses of 17β-estradiol and the combination of a SERM + β-estradiol.

MATERIALS AND METHODS

Animals

All of the animal experiments were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals [1996 (7th ed.) Washington, DC: National Academy Press, aka National Research Council Guide.], and were approved by the University of Missouri Animal Care and Use Committee (ACUC). Wild-type CF-1 mice approximately five to nine weeks old were employed for these studies. They were ovariectomized at five to seven weeks of age, and a week later they were injected two times, 24 h apart, with one of the following treatments: 1 µg β-estradiol (n = 3), 50 µg β-estradiol (n = 5), 50 µg MPP (n = 3), 50 µg raloxifene (n = 3), 50 µg ICI 182,780 (n = 3), 50 µg β-estradiol + 50 µg MPP (n = 2), 50 µg β-estradiol + 50 µg raloxifene (n = 2), 50 µg β-estradiol + 50 µg ICI 182,780 (n = 5), and DMSO vehicle control (n = 3). These doses were chosen based on past studies that tested the effects of β-estradiol and SERMs in mice (Keisler et al., 1995). On day three, the mice were sacrificed and their uteri harvested and stored either in RNA later (Ambion, Austin, TX) or in 10% Neutral
Buffered Formalin (NBF) for future experiments and the serum collected via cardiac puncture for the determination of estradiol concentrations (detailed below).

**RNA isolation and analysis:**

RNA was isolated from uterine tissue samples (30 to 50 mg) that were homogenized by using an Ultra-Turrax T25 basic homogenizer for 30 to 45 sec (IKA-Werke, Germany). Tissues previously stored in RNA*later* solution (Ambion) were isolated by using either TRI Reagent® (Sigma, St. Louis, MO) or the RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. The concentration of the RNA was determined on a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE) and the quality of the RNA assessed by using the Experion Automated Electrophoresis System (Bio-Rad Laboratories, Hercules, CA) at the University of Missouri’s DNA Core Facility. Once the RNA was evaluated as suitable for analysis, it was subsequently used for microarray hybridization.

**RNA Quality Control:**

Immediately before cDNA synthesis, the concentration and purity of RNA samples were determined from OD_{260/280} readings by using a dual beam UV Spectrophotometer. RNA consistency was determined by capillary electrophoresis by using the RNA 6000 Nano Lab-on-a-Chip kit and the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) as per the manufacturer’s instructions.

**cRNA Synthesis and Labeling:**

RNA was processed and labeled according to standard RT-IVT methods. First and second-strand cDNA were generated from 2.0 µg of total RNA by using oligo-dT_{24}-T7 (5’-GGG CAG TGA ATT GTA ATA CGA CTC ACT ATA GGG AGG AGG CGG-3’) as
the primer and the Bioarray™ cDNA Synthesis Kit (ENZO Diagnostics Inc., Farmingdale, NY) according to the manufacturer’s instructions. Complementary RNA (cRNA) was synthesized and labeled with biotinylated UTP and CTP by in vitro transcription for 16 h at 37°C with T7 promoter-coupled double-stranded cDNA as template and the Bioarray™ HighYield™ RNA Transcript Labeling Kit (ENZO Diagnostics Inc). Labeled cRNA was separated from unincorporated ribonucleotides by passing through a CHROMA SPIN-100 column (Clonetech, Mountain View, CA) and ethanol precipitated at -20°C 1 h to overnight.

**Oligonucleotide array hybridization and analysis:**

Labeled cRNA was re-suspended in RNase-free H2O, and 15 µg fragmented by ion-mediated hydrolysis at 95°C for 35 min. in 200 mM Tris-acetate (pH 8.1), 500 mM potassium acetate, and 150 mM magnesium acetate. The fragmented cRNA was hybridized for 16 h at 45°C to Affymetrix Mouse Genome 430 2.0 short oligomer arrays, which detect approximately 44,000 mouse transcripts representing over 34,000 well-characterized mouse genes (Affymetrix, Santa Clara, CA). Arrays were washed and stained by using a Fluidics Station 450 (Affymetrix) according to the manufacturer’s instructions. The arrays were stained with phycoerythrin-conjugated streptavidin (Invitrogen, Carlsbad, CA) and the fluorescence intensities determined by using a GCS 3000 7G high-resolution confocal laser scanner (Affymetrix). The scanned images were analyzed by using programs available through the GeneChip Operating System v1.4 (GCOS; Affymetrix).
**Immunohistochemistry:**

Uterine tissue that had been fixed in 10% NBF at the time of collection was paraffin-embedded and sectioned for use in immunohistochemistry. Slides containing unstained tissues were washed in Clear-Rite 3 (Richard-Allen Scientific, Kalamazoo, MI) and re-hydrated with an ethanol series (100% to 50%). Slides were washed in 1X PBS (X 3) for 5 min and then incubated in a 50 μg pepsin solution in 0.01N HCl (modified from Davis et al., 2006) for 3 to 5 min at 37°C. Slides were then marked with a Pap pen (Electron Microscopy Sciences, Hatfield, PA) and washed three times for 5 min in 1X SSC and two times for 5 min in DDH₂O. Next, slides were heated in either Antigen Unmasking Solution (Vector Laboratories, Burlingame, CA) for 5 min in the microwave or in Target Retrieval Solution (DAKO North America, Carpinteria, CA) in a pressure cooker for 30 to 40 min (heat-inactivated antigen retrieval step). Another 1X PBS wash series was performed, followed by incubation in 0.01% Tween-20, 5% Non-Fat Dried Milk (NFDM) and fish gelatin blocking solution for 30 min at room temperature. A 5% goat serum blocking step followed, for 45 min at room temperature, and then the primary antibody was added and incubated for one hour at room temperature. Gene-specific antibodies for C3 (Abcam, Cambridge, MA), Klk1 (Valdez), MUC1 (Abcam) and IHH (R&D Systems, Minneapolis, MN; Santa Cruz) were used for each treatment group; normal rabbit serum was used as the negative control. After being washed in 1X PBS, the slides were then incubated in ImmPress Reagent (Vector) for 30 min at room temperature and washed again in 1X PBS. Slides were then stained by using ImmPact DAB + chromogen solution (Vector Laboratories) for 2 to 10 min, then washed in DDH₂O 2X for 5 min. Mowiol (Calbiochem, Switzerland) was then used to mount slides,
and 22 x 50 cm coverslips used to cover tissues. Coverslips were fixed to the slides by using a thin layer of clear nail polish, and then the sections imaged on an Olympus Provis bright field microscope at 20X magnification. The procedure was repeated six times for MUC1 and C3 and seven times for KLK1.

**Serum Estradiol Assays**

Serum samples (up to 200 μl) from each mouse were extracted twice with a fresh mixture of 80% ethyl acetate and 20% chloroform (1.5 ml), as described previously (SAS/STAT User's Guide, 1988). After the samples had been dried down under air at room temperature, they were re-constituted in 1% BSA/1X PBS solution. The resulting extracted samples were assayed by using the Ultra-Sensitive Estradiol CELISA kit (Calbiotech, Spring Valley, CA) according to the manufacturer’s instructions. The standards were first added to the 96-well plate, then the extracted serum samples and controls. Rabbit anti-estradiol and Estradiol Enzyme Conjugate were added to each well, and then the plate was covered and incubated for 3 h at room temperature with vigorous shaking on a Boekel Orbitron Rotator III (Boekel Scientific, Feasterville, PA). After 3 h, the plate was washed 3X with wash buffer, luminol substrate added, and the absorption of the colored product of the reaction read immediately on a luminometer at 420 nm (Veritas Microplate Luminometer, Turner Biosystems, Sunnyvale, CA). This CELISA kit has a minimal detection limit for 17β-estradiol of 0.4 pg/ml. The intra- and inter-assay coefficients of variation were 7.1 % and 15.3 %, respectively.

**Statistical Analysis**

Dependent variables, fold changes for microarray gene expression results and serum estradiol concentrations were analyzed for normality by using the Wilk-Shapiro
test (SAS/STAT User's Guide, 1988). Fold changes of gene expression were logarithmically transformed to approach a normal distribution and data analyzed by using the general linear model (GLM) tests of SAS (Davis et al., 2006; Gutman et al., 2002; Hewitt et al., 2003; Yamamoto et al., 2006). Differences in gene expression changes and serum estradiol concentrations among all treatment groups were determined by using Fisher’s Least Significant Difference, with P < 0.05 considered statistically significant.

Microarray MAS 5.0-generated data was analyzed for expression changes by a one-way ANOVA test followed by independent t-tests for each treatment group (versus DMSO control group). Significant changes in gene expression were determined by a ≥1.5-fold change in expression for at least one treatment group (versus DMSO control), an ANOVA P-value ≤ 0.02, a t-test P-value ≤ 0.02 for at least one treatment group, and MAS 5.0 Detection P-values ≤ 0.065 (M) for all samples within at least one treatment group. Prior to heat map generation and clustering analysis, probe set signal values were standardized by row mean centering and scaled by standard deviation. Unsupervised hierarchical clustering was performed in GeneMaths XT (Applied Maths, St. Martens-Latem, Belgium) by UPGMA (Unweighted Pair-Group Method using Arithmetic averages) with Pearson correlation distance for array clustering and Euclidean distance for gene clustering. Gene annotation, gene ontology, and biochemical pathway information were obtained by using the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov), NetAffx (www.affymetrix.com), Gene Ontology Consortium (http://amigo.geneontology.org), the Kyoto Encyclopedia of Genes and Genomes (www.genome.jp/kegg), and WebGestalt (http://bioinfo.vanderbilt.edu/webgestalt).
Significant enrichment of specific gene ontology categories or KEGG pathways were estimated by hypergeometric tests ($P \leq 0.01$).

RESULTS

**Microarray Analyses:** A total of 765 probe sets, representing 747 different gene transcripts, exhibited significant differential expression (fold change $\geq 1.5$, $P$-value $\leq 0.02$) between at least one treatment group and the vehicle control group. Unsupervised hierarchical clustering (Figure 19) and principal component analysis (data not shown) showed that samples within each treatment group had the greatest similarity with replicate samples from the same group. The nine treatments grouped into two main clusters with MPP, raloxifene, and the high dose of estrogen clustering together. The second cluster included the low dose of estrogen, ICI 182,780 + 17β-estradiol, ICI 182,780, MPP + 17β-estradiol and raloxifene + 17β-estradiol with the low dose of 17β-estradiol and the ICI 182,780 + 17β-estradiol correlating most closely. Based on this arrangement, it seems as if when the high 17β-estradiol concentration is combined with one of the SERMs, in particular ICI 182,780, the gene expression changes in the uterus mirror those observed in low dose 17β-estradiol treatment (Figure 19).

To analyze gene expression changes in more detail, we constructed several Venn diagrams to compare the overlap in up- and down-regulated genes in the various groups. Surprisingly, little overlap in up and down-regulated genes was evident in the single treatments (Figure 20). However, when 17β-estradiol was combined with one of the SERMs (in particular raloxifene), it resulted in a combinatorial or synergistic effect, with
more alterations in gene expression than with the individual treatments, particularly when 17β-estradiol was combined with raloxifene (629 genes regulated). Similar gene expression was affected in the low dose 17β-estradiol group and ICI 182,780 + high dose 17β-estradiol treatment groups. The predominant genes up-regulated by these treatments included those that control cellular metabolism and synthesis (including purine and pyruvate metabolism and folate and vitamin K biosynthesis), JNK/p38 signaling pathway, glycan structure degradation, apoptosis, and reactive oxygen species. Genes inhibited by these treatments include those that regulate cell cycle and DNA replication, focal adhesion, actin cytoskeletal organization, MAPK, Wnt, and tyrosine kinase signaling pathways, and stress response genes (e.g. heat shock proteins). The combination of MPP + high dose of 17β-estradiol produced a similar expression pattern as raloxifene + high dose of 17β-estradiol, where genes such as eIF2a/4a1 and Hsp40 subunits were up-regulated, while genes involved in DNA replication, mitosis, glycolysis and gluconeogenesis were down-regulated.

The high dose of 17β-estradiol down-regulated genes mediating several cell differentiation and growth control signaling pathways, including the Hedgehog or Smoothened pathway (Ptch2, Gli1, Sufu), and the TGF-beta pathway (ID3, ID4), several ECM components (Lamc3, Spon2, Timp3), a number of genes that maintain actin cytoskeletal structures and cell morphology (Ang1, Cdgap, Dbn1, Rdx). In contrast, several genes that stimulate actin depolymerization (Capg, Cdc42ep5) are up-regulated in response to high dose of 17β-estradiol, suggesting it might induce mobilization of cytoskeletal structures. While this treatment induces several genes involved in glycolysis (Aldoa, Gba, Nagk, Tpi1), 17β-estradiol suppresses several genes that utilize glucose in
synthetic reactions (Gn2, Ugcg). Overall, a high dose of 17β-estradiol presumably prepares the uterus to enter a growth-like state, but concomitantly it might attenuate cell cycle progression by down regulating several genes involved in cell cycle machinery, including CycD1, Skp2, 14-3-3 (Ywhae).

While the individual SERM treatments did not yield much overlap in gene expression changes, some of the common genes induced by MPP, raloxifene, and ICI 182,780 are involved in transcription regulation, intracellular transport/GTPase-mediated signaling, and DNA methylation. Additionally, raloxifene up-regulates the IL-7 receptor. Genes that were down-regulated by these individual treatments are involved in cell cycle regulation, cell adhesion, microtubule cytoskeletal organization, signal transduction, and protein transport. In line with its antagonistic actions, ICI 182,780 suppressed more genes involved in mitosis and cytokinesis compared to MPP and raloxifene treatments.

**Immunohistochemistry:** To determine whether 17β-estradiol and the SERMs MPP, raloxifene, and ICI 182,780 induced any changes in the protein expression for MUC1, KLK1, and C3, immunohistochemistry was performed (Figure 21). We additionally wished to test for changes in IHH, but our studies with two different commercial IHH antibodies (Santa Cruz, Santa Cruz, CA) and (R&D Systems, Minneapolis, MN) failed to yield any specific staining, even in the uteri of DMSO control mice. These results may suggest that either the antibodies are incapable of binding to the mouse IHH gene, or that IHH protein expression is low, even in the controls. MUC1, KLK1, and C3 were strongly expressed in the luminal and glandular epithelium in the uteri of the 17β-estradiol, MPP, and raloxifene treated mice. The uteri of the ICI treated mice expressed KLK1 and C3 in the glandular epithelium but scant expression for MUC1
was detected in these uterine samples. The DMSO control uterine samples expressed MUC1 and C3 in the glandular epithelium, indicating that there is some constitutive expression of these proteins.

Serum estrogen concentrations: Many different concentrations of 17β-estradiol have been tested in mice, and no consensus has been reached as to what provides a maximal response (Couse and Korach, 1999a). Therefore, we sought to examine the serum estradiol concentrations in all of the treated mice three days after injection of the compounds to determine if any of the treatments overwhelmed the normal metabolic pathways for 17β-estradiol resulting in elevated concentrations of this hormone in the blood. Analysis with the Ultra-Sensitive Estradiol CELISA kit (Calbiotech, Spring Valley, CA), which has a minimal detection limit of 0.4 pg/ml, detected average serum 17β-estradiol concentrations between 1.5 and 3.5 pg/ml (for each group) but none of the differences were statistically significant (Figure 22). Other commercially available test kits were not sensitive enough to detect such low concentrations. These serum estradiol concentrations (for all the groups) ranked far below the range of serum estradiol concentrations (20 to 80 pg/ml) previously reported for wild-type and various transgenic mice, including ER-α KO mice (Harrington et al., 2003; Sun et al., 2002).

**DISCUSSION**

We have performed a comparison of the global chronic gene expression changes in the uterus in response to individual and combined treatments of 17β-estradiol and the SERMs MPP, raloxifene, and ICI 182,780, which is considered to be a pure estrogen
receptor antagonist. However, the microarray gene expression changes in response to ICI 182,780 were similar enough to those caused by the low dose of 17β-estradiol to indicate that this supposed anti-estrogen might have partial estrogenic effects. For example, the clustering analysis placed ICI 182,780 closer to 17β-estradiol than the other two SERMs. Additionally, the gene expression changes induced by MPP and raloxifene in the uterus cluster separately from those of the 17β-estradiol and ICI 182,780 groups. Importantly, gene changes in response to MPP and raloxifene were somewhat similar and clearly distinct from the 17β-estradiol and ICI 182,780 groups. MPP is presumed to be a selective ER-α antagonist (Schafer et al., 1999; Spencer et al., 1999), while raloxifene acts as an ER-α and –β antagonist on the endometrium (Dupont et al., 2000; Hewitt et al., 2003; Lubahn et al., 1993; Shughrue et al., 1998). Importantly, estrogen receptor-α is considered to be the dominating ER in the uterus (Weihua et al., 2000), however, ER-β has some effects in the uterus including possible modulation of ER-α expression (Andrade et al., 2002; Davis et al., 2006; Zheng et al., 2004). Therefore, the gene expression changes observed in the uterus in response to MPP and raloxifene treatment would be expected to be governed primarily by their actions through ER-α.

The overall gene expression changes induced by the high dose of 17β-estradiol are consistent with the general function of this hormone to prepare the uterus for implantation by the conceptus by stimulating genes involved in glycolysis, such as Aldoa, Gba, Nagk, Tpi1, and inducing actin depolymerization (Capg, Cdc42ep5), which would result in mobilization of cytoskeletal structures. The gene expression changes in response to the individual SERM treatments are consistent with their ability to inhibit cellular
proliferation by down-regulating genes that control cell cycle and mitosis, signal
transduction pathways, and oxidative phosphorylation/electron transport.

The guiding hypothesis of this work was that when 17β-estradiol was combined
with one of the SERMs, the latter would largely eliminate the 17β-estradiol effects, as
was observed in WT and βERKO mice co-treated with 17β-estradiol and ICI 182,780
(Hewitt et al., 2003). Additionally, other studies suggest that these SERMs antagonize the
effects of 17β-estradiol (Diel et al., 2001; Kaye et al., 2001; Tanos et al., 2002; Wong et
al., 2007). Instead, the combination of 17β-estradiol and a SERM resulted in a
combinatorial effect on gene expression. Although somewhat surprising, these data are
consistent with the results of several other groups, who also showed that the combination
of estrogenic and anti-estrogenic compounds can lead to an increased number of gene
expression changes when compared to the individual treatments (Cowley et al., 1997;
Forman et al., 1995; Kuiper and Gustafsson, 1997; Leung et al., 2006; Pettersson et al.,
1997). However, our study is the first to test several SERMs in combination with 17β-
estradiol and to observe a similar combinatorial effect in all cases, which provides robust
evidence for the existence of SERMs to induce synergistic effects in combination with
17β-estradiol. The dimerization of these ligands with ER might account for this
synergistic effect. The combination of ER and ligands might account for this effect. Once
ER-α and -β are ligand-bound, the receptors can form homo- and heterodimers, which
bind to the estrogen response elements (ERE) in their target genes (Cowley et al., 1997;
Li et al., 2004; Matthews and Gustafsson, 2003; Pettersson et al., 1997). While it is clear
that the combination of ER homo- and heterodimers can yield contrasting gene
expression changes (Kaye et al., 2001), which depend upon the nature of the ligand
bound, elucidating exactly what blend of ligands bind to these dimers and their molar proportions present a challenge. It has been postulated that the synergistic effects of the estrogen and SERMs are contingent on the combination of homo- and heterodimers of ER-bound estrogen and SERM-bound ER forms (Kim et al., 2005). In addition, coactivator recruitment might only occur when both monomer receptors are bound by an agonistic ligand (Levy et al., 2007). The arrangement of two receptor forms that can form hetero- and homodimers and two ligands that can act through both receptor forms would theoretically yield 12 combinations of regulatory complexes, each of which might favor distinct sets of genes. Thus, the ligand-bound permutations in homo/heterodimers of the ER might account for the synergistic effects we observed in the uteri of 17β-estradiol + SERM-treated mice. Consistent with this interpretation, a recent report showed that estradiol, tamoxifen, and raloxifene might differentially regulate native ER regulatory elements, with raloxifene primarily stimulating regulatory elements when bound to ERβ. In contrast, tamoxifen activates more response elements in concert with ERα (Carthew et al., 1999; Davis et al., 2006; Hewitt et al., 2003; Jones and Bern, 1977; Yamamoto et al., 2006).

The doses of 17β-estradiol and the SERMs we opted to test were based on several previously published reports and our own studies where we observed a phenotypic effect in the uterus of treated mice. However, controversy exists as to what is an acceptable range to test in rodents (Carpenter and Korach, 2006). To determine if the doses we employed dramatically and persistently altered serum estradiol concentrations by overwhelming metabolic enzymes, we measured 17β-estradiol in cardiac blood from all of the treated and control mice 24 h after their last treatment. The absence of any
significant differences in 17β-estradiol in any of the groups suggests that the dosages we employed were not excessive to the point of inducing pathological or toxicological changes. Our average concentration of ~3 pg/ml in the various groups is well below the serum estradiol concentrations measured in ER-α KO and WT mice (20 and 80 pg/ml), respectively.

In conclusion, concordant results from microarray and immunohistochemical analysis have revealed that the SERMs MPP, raloxifene, and ICI 182,780 differentially regulate gene expression in the uteri of treated mice. While ICI 182,780 is considered a pure ER antagonist, the gene expression changes that occurred in the uteri of mice treated with this compound closely resembles those treated with a high dose of 17β-estradiol. The combination of 17β-estradiol + SERM resulted in a synergistic effect with a much greater increase in gene expression changes compared to each of the individual treatments, and this pattern is particularly evident in the 17β-estradiol + raloxifene treatment group. These results support the view that the expression of estrogen receptor target genes might be determined by specific combinations of ER forms and their various interacting ligands.
Figure 1: Estrogen receptor-α and β structure; ERα and ERβ are shown including A-F domains and sequence identity between the two. Three alternative translational start sites for ERβ (M1, M2, and M3) are also depicted (Carpenter and Korach, 2006).
Figure 2: Distribution of estrogen receptors -α and -β within the body and reproductive tissues (target organs). (Health Orchid Online; K.V.H.S. Online)
**Figure 3:** SERMs with their molecular structure, relative binding affinity (RBA) to ER-α and –β (if known), and classification.

Images and RBA’s adapted from (Kuiper et al., 1997; Sun et al., 2002; Spencer et al., 1999; Katzenellenbogen et al., 2003; Lerner and Jordan, 1990; Martel et al., 2000; Dardes et al., 1995; Yamamoto et al., 2005; Geiser et al., 2005; Labrie et al., 2001; MacGregor and Jordan, 1998; Sato et al., 1998; Tocris Online).
Figure 4: TUNEL Assay in SERM and β-estradiol-treated Ishikawa cells. A) MPP (1 nM); B) MPP (1 nM) + β-estradiol (10 nM); C) Raloxifene (1 nM) D) Raloxifene (1 nM) + β-estradiol (10 nM), E) β-estradiol (1 nM) and F) DMSO vehicle control. Apoptotic cells are demarcated by a green fluorescence (white arrows). Nuclei are stained with propidium iodide. MPP and raloxifene induced significant apoptosis relative to β-estradiol and vehicle alone treated cells. The addition of 10-fold excess β-estradiol reduced the percentage of apoptosis, thereby indicating that the SERM-induced apoptosis is attributed to genomic actions rather than toxicity. Scale bar = 50 microns.
Figure 5: Graph of the average percentage of apoptotic (TUNEL +) cells in SERM and β-estradiol-treated Ishikawa cells. MPP and raloxifene induced significant apoptosis relative to β-estradiol and vehicle alone treated cells. The addition of 10-fold excess β-estradiol reduced the percentage of apoptosis, thereby indicating that the SERM-induced apoptosis is attributed to genomic actions rather than toxicity.

a Significantly different from b (P ≤ 0.001).

a Significantly different from c and d (P ≤ 0.0001).
Figure 6: TUNEL Assay for SERM and β-estradiol-treated RL-95 cells. A) MPP treatment (1 nM); B) MPP (1 nM) + β-estradiol (10 nM); C) Raloxifene (1 nM) D) Raloxifene (1 nM) + β-estradiol (10 nM), E) β-estradiol (1 nM) and F) DMSO vehicle control. Apoptotic cells are demarcated by a green fluorescence (white arrows). Nuclei are stained with propidium iodide. MPP and raloxifene induced significant apoptosis relative to β-estradiol and vehicle alone treated cells. The addition of 10-fold excess β-estradiol reduced the percentage of apoptosis, thereby indicating that the SERM-induced apoptosis is attributed to genomic actions rather than toxicity. Scale bar = 50 microns.
Figure 7: Graph of the average percentage of apoptotic (TUNEL +) cells in SERM and β-estradiol-treated RL-95 cells. MPP and raloxifene induced significant apoptosis relative to β-estradiol and vehicle alone treated cells. The addition of 10-fold excess β-estradiol reduced the percentage of apoptosis, thereby indicating that the SERM-induced apoptosis is attributed to genomic actions rather than toxicity.

a Significantly different from b (P ≤ 0.001).

a Significantly different from c and d (P ≤ 0.0001).
**Figure 8:** Immunofluorescence for ERα and ERβ in oLE cells. A) ER-α and -β as well as nuclear (DAPI) stain in oLE cells; B) Cells treated with pre-immune serum (negative control) showing only nuclear (DAPI) staining. The oLE cells express ERα (green) and ERβ (red) (positively staining cells are indicated by white arrows). Nuclear and cytoplasmic staining was evident for ERα and ERβ, respectively. DAPI (blue) was used to demarcate the nuclei. Scale bar = 20 microns.
Figure 9: TUNEL Assay for SERM-treated oLE cells. A) MPP treatment (1 nM); B) MPP (1 nM) + β-estradiol (10 nM); C) Raloxifene (1 nM) D) Raloxifene (1 nM) + β-estradiol (10 nM), E) β-estradiol (1 nM) and F) DMSO vehicle control. Apoptotic cells are demarcated by a green fluorescence (white arrows). Nuclei are stained with propidium iodide. MPP and raloxifene induced significant apoptosis relative to β-estradiol and vehicle alone treated cells. The addition of 10-fold excess β-estradiol reduced the percentage of apoptosis, thereby indicating that the SERM-induced apoptosis is attributed to genomic actions rather than toxicity. Scale bar = 50 microns.
Figure 10: Graph of the average percentage of apoptotic (TUNEL +) cells in SERM-treated and control oLE cells. MPP and raloxifene induced significant apoptosis relative to β-estradiol and vehicle alone treated cells. The addition of 10-fold excess β-estradiol reduced the percentage of apoptosis, thereby indicating that the SERM-induced apoptosis is attributed to genomic actions rather than toxicity.

a Significantly different from b (P ≤ 0.001).

a Significantly different from c and d (P ≤ 0.0001).
Figure 11: Sub-gross uterine photomicrographs from the SERM-treated and control ovariectomized mice.
A) 50 µg MPP; B) 50 µg β-estradiol; C) 50 µg raloxifene; D) DMSO vehicle control; E) 50 µg ICI 182,780. Scale bar = 5 mm. β-estradiol, MPP, and to a lesser extent raloxifene were uterotrophic by inducing an increase in uterine wet weight and size when compared to vehicle control and ICI 182,780 treated mice, whose uteri were thread-like and often difficult to see and dissect from the surrounding mesometrial adipose tissue.
Uterine Weight/Body Weight Ratio for the SERM-Treated and Control Ovariectomized Mice

Figure 12: Graph of uterine wet weight/body weight (BW) ratio (%) in SERM-treated and control ovariectomized mice.

Average uterine weight/body weight ± SEM (%): Vehicle control (25 µg dosage) = 0.115 ± 0.002, Vehicle control (50 µg dosage) = 0.162 ± 0.01, Vehicle control (100 µg dosage) = 0.173 ± 0.01, Vehicle control (150 µg dosage) = 0.98 ± 0.0, Raloxifene (25 µg dosage) = 0.12 ± 0.0, Raloxifene (50 µg dosage) = 0.218 ± 0.016, Raloxifene (100 µg dosage) = 0.213 ± 0.011, Raloxifene (150 µg dosage) = 0.21 ± 0.005, MPP (25 µg dosage) = 0.2 ± 0.004, MPP (50 µg dosage) = 0.238 ± 0.014, MPP (100 µg dosage) = 0.271 ± 0.001, MPP (150 µg dosage) = 0.171 ± 0.01, β-estradiol (25 µg dosage) = 0.345 ± 0.019, β-estradiol (50 µg dosage) = 0.405 ± 0.0234, β-estradiol (100 µg dosage) = 0.447 ± 0.129, and β-estradiol (150 µg dosage) = 0.258 ± 0.007.

a Significantly different from b, c and d (P ≤ 0.0001).
b Significantly different from d (P ≤ 0.001).
Figure 13: Immunohistochemistry for Ki67 in uteri from SERM-treated ovariectomized mice. To determine the degree of uterine proliferation in response to these SERMs, immunohistochemistry for the cell proliferation marker Ki67 was performed. A) Ki67 staining in uterus of β-estradiol treated mouse; B) Pre-immune negative control for same uterus of β-estradiol treated mouse as in (A); C) Ki67 staining in uterus of MPP treated mouse D) Pre-immune negative control for uterus of same MPP treated mouse as in (C); E) Ki67 staining in uterus of raloxifene treated mouse; and F) Pre-immune negative control for uterus of same raloxifene treated mouse as in (E). Black arrows denote DAB+ positive brown-stained nuclei. Scale bar =10 microns.
Figure 14: Graph of quantitation for Ki67 positive-staining cells in uteri from SERM-treated ovariectomized mice.

*Significantly different from \( p \leq 0.05 \).
**Figure 15:** Uterine histology for SERM-treated and control ovariectomized mice. H&E stained uterine sections from the different treatment groups and control mice. A) MPP; B) Raloxifene; C) β-estradiol; D) DMSO vehicle control. Black arrows denote areas where epithelium transitions into a thickened stratified columnar epithelium. The normal uterine epithelial lining is a simple columnar epithelium (black arrows). Scale bar = 10 microns.
Table 16: Graph of uterine wet weight/body weight (BW) ratio (%) in SERM and β-estradiol-treated ovariectomized CF1 mice. Co-treatment of MPP or Raloxifene plus 50 or 100 μg E2 resulted in competitive antagonism between the SERMs and β-estradiol, as evidenced by a decrease in uterine weight/body weight ratio in these groups. Average uterine weight/body weight ± SEM (%) for the combined treatments: MPP (50 μg dosage) + β-estradiol (50 μg dosage) = 0.207 ± 0.006, MPP (50 μg dosage) + β-estradiol (100 μg dosage) = 0.223 ± 0.007, Raloxifene (50 μg dosage) + β-estradiol (50 μg dosage) = 0.186 ± 0.004, and Raloxifene (50 μg dosage) + β-estradiol (100 μg dosage) = 0.197 ± 0.022.

\(^a\) Significantly different from \(^b\), \(^b'\) and \(^c\) (P ≤ 0.001).

\(^b'\) Significantly different from \(^c\) (P ≤ 0.05).
**Figure 17:** TUNEL Assay with uteri from SERM-treated ovariectomized mice. A) MPP-treated mouse; B) Raloxifene-treated mouse; C) β-estradiol-treated mouse; D) Negative control mouse. LE = luminal epithelium. Most of the apoptotic cells (white arrows) were present in the underlying stroma, particularly in the case of raloxifene.
Figure 18: Uterine histology for MPP-treated and negative control ERβKO ovariectomized mice. Uteri from MPP-treated ERβKO exhibited similar increase in luminal epithelial thickening (black arrows) as the uteri from MPP-treated control mice. No increase in luminal epithelial thickness was observed in the uteri from the negative control ovariectomized ERβKO mice. Scale bar = 20 microns. The average uterine weight/body weight ratio (%) was significantly different in the β-estradiol and MPP-treated ERβKO mice versus the vehicle alone treated ERβKO mice. Average uterine weight/body weight ± SEM (%): Vehicle control 0.137 ± 0.011, MPP (50 μg) 0.218 ± 0.007, and β-estradiol (50 μg) 0.236 ± 0.015.

a Significantly different from b (P ≤ 0.0001).
b Significantly different from c (P ≤ 0.005).
Figure 19: Cluster heatmap of microarray analysis. Cluster heatmap diagram from microarray analysis. Probes on the map represent genes regulated at least 1.5-fold vs. DMSO vehicle control. Red = up-regulated, green = down-regulated (key in upper left corner). Treatments are color-coded and listed below the map. Boxed in areas represent the divergence between overall induced genes found in the low and high dose 17β-estradiol treatments. In general, the high dose 17β-estradiol group clustered more closely with the MPP and Raloxifene single treatments; whereas the low dose 17β-estradiol group clustered with ICI and combination treatments.
Figure 20: Venn diagrams from microarray analysis comparing up- and down-regulated genes in several treatment groups. The charts compare the up and down-regulated genes in several of the nine different treatment groups. Intersected areas represent genes that overlap two or three of the groups. In general, scant gene overlap existed in the individual treatment groups. However, the combination treatment groups yielded substantially more gene alterations than the individual treatments.
Figure 21: Immunohistochemistry in the uteri of treated and control mice for MUC1, KLK1 and C3. Immunohistochemistry was performed for MUC1, KLK1, and C3 in the uteri of the treated and control mice. Mice treated with 17β-estradiol, MPP, and raloxifene demonstrated an increase in protein expression for these three genes predominantly in the luminal and glandular epithelium (arrows). In the ICI 182,780 treated mice, the endometrial glands expressed KLK1 and C3 but only background expression for MUC1. In the control DMSO group, some constitutive expression for MUC1 and C3 was expressed in the endometrial glands.
Figure 22: Serum estradiol concentrations from CF1 wild-type mice. Serum estradiol concentrations were measured in the 17β-estradiol, SERM and 17β-estradiol + one of the SERM treatment groups to verify that these treatments did not induce any striking increases in this hormone. No significant differences in serum estradiol concentrations were detected in any of the groups.
LITERATURE CITED


Tocris Online. 2007. “MPP dihydrochloride”


