Regulation of estrogen receptor signaling in breast and endometrial cancer by the src kinase pathway, the micronutrient selenium, and by novel tamoxifen-regulated biomarkers

Yatrik M. Shah
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Regulation of Estrogen Receptor Signaling in Breast and Endometrial Cancer by the Sre Kinase Pathway, the Micronutrient Selenium, and by Novel Tamoxifen-regulated Biomarkers

Submitted by

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In partial fulfillment of the requirements for the degree of
Doctor of Philosophy in Medical Sciences

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Date of Defense: March 15, 2005
Regulation of Estrogen Receptor Signaling in Breast and Endometrial Cancer by the Src Kinase Pathway, the Micronutrient Selenium, and by Novel Tamoxifen-regulated Biomarkers

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Medical College of Ohio

2005
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INTRODUCTION

Estrogens are important in the growth and maintenance of female reproductive tissues and other non-reproductive tissues such as bone, cardiovascular tissue, and the central nervous system. Estrogen action is mediated through binding to its cognate receptor, estrogen receptor (ER). Two forms of ER have been identified, ERα and ERβ, both the product of separate genes (Kuiper et al., 1996; Mosselman et al., 1996; Tremblay et al., 1997). The ER is a ligand-dependent transcription factor, and upon estrogen binding the receptor either forms heterodimers or homodimers. Upon dimerization, ER binds to the promoter region of target genes where subsequent recruitment of coactivators and local chromatin modifications facilitate gene transcription (Evans, 1988; Klein-Hitpass et al., 1986). In addition ER is activated by ligand-independent mechanisms that involve crosstalk from peptide and growth factor signal transduction pathways (Levin, 2003). In the absence of the physiological ligand 17β-estradiol, cellular kinases can activate ER and modulate ER-regulated gene transcription.

The ER plays a major role in the development and growth of breast cancer. As a treatment for breast cancer, the selective estrogen receptor modifier (SERM) tamoxifen binds with high affinity to the ER and blocks estrogen induced cellular growth (Dutertre et al., 2000). Tamoxifen is the most widely used hormonal therapy for breast cancer and is approved as a chemopreventive agent in high risk women (Jordan, 2000). As an ER antagonist, tamoxifen inhibits estrogen dependent gene transcription that is required for cellular growth in breast tissue and breast-derived cell lines (Dutertre et al., 2000; Jordan, 2000; Shang et al., 2002). Despite the benefits of tamoxifen therapy, almost all
tamoxifen-responsive breast cancer patients develop resistance to therapy (Osborne, 1998). In addition, tamoxifen exhibits ER agonist action in the uterus that manifests in an increase incidence of endometrial cancer (Fornander et al., 1989; Harper et al., 1967; Terenius, 1971).

Several hypotheses are proposed to explain tamoxifen resistance and the estrogen-like activity exhibited in the endometrium. Coregulators are important in the mechanism of tamoxifen resistance. Elevated levels of amplified in breast cancer 1 (AIB-1) (RAC-3/ACTR/SRC-3) in patients receiving tamoxifen treatment are correlated with tamoxifen resistance (Osborne et al., 2003). Elevated steroid receptor coactivator-1 (SRC-1) (NcoA-1), but not glucocorticoid receptor interacting protein-1 (GRIP-1) (TIF-2/NcoA-2/ SRC-2) or AIB-1 correlate with tamoxifen agonist activity in the Ishikawa endometrial cell line (Shang et al., 2002). In addition to coactivators, there is a decrease in nuclear corepressor (N-CoR) levels with the onset of tamoxifen resistance in both human breast cancer samples and in mouse models of breast cancer (Girault et al., 2003; Lavinsky et al., 1998). Also, 4-hydroxytamoxifen acts as a full agonist on ER-dependent transcription in fibroblast cells isolated from the N-CoR null mouse. However, in wild-type mouse embryo fibroblasts containing normal N-CoR levels, 4-hydroxytamoxifen did not activate ER-dependent transcription (Jepsen et al., 2000).

In addition to coregulator protein expression, several deregulated cellular kinase pathways play an important role in tamoxifen resistance and tamoxifen-mediated endometrial proliferation. As mentioned above ligand-dependent or -independent pathways can activate ER and both modes of ER activation are modulated by receptor
phosphorylation. Estrogen receptor is a phosphoprotein and the major phosphorylation sites of ER reside in the N-terminal domain (NTD) at serines 104, 106, 118, and 167 (Arnold et al., 1994b; Kato et al., 1995; Le Goff et al., 1994). Overexpression of erb-2/HER2 an epidermal growth factor receptor (EGFR) family member are associated with tamoxifen resistance (Benz et al., 1993; Liu et al., 1995; Pietras et al., 1995). Erb-2/HER2 are upstream of extracellular regulated kinase (ERK)-1 and ERK-2, which phosphorylate ERα on serine 118 and enhance its transcriptional activity upon estrogen and tamoxifen incubation (Kato et al., 1995). In addition, AKT kinase protects breast cancer cells from tamoxifen-mediated apoptosis, and the consensus AKT site serine 167 on ERα is required (Campbell et al., 2001). MEKK1 increases the agonist activity of tamoxifen in endometrial cells implicating downstream mitogen activated protein kinases (MAPKs) in regulating tamoxifen agonist/antagonist action (Lee et al., 2000). Src kinase, which activates both MAPK and AKT can enhance the transcriptional activity of the tamoxifen in breast and cervical cancer cell lines (Feng et al., 2001).

Coregulator proteins are also substrates for cellular signaling pathways. SRC-1 is phosphorylated on seven consensus sites for proline-directed protein kinases in vivo (Rowan et al., 2000a;b). The GRIP-1 and AIB-1 are phosphorylated in vitro by MAP kinases (Font et al., 2000; Lopez et al., 2001). In breast cancer cells the transcriptional activity of AIB-1 was increased by MAPK phosphorylation that stimulated recruitment of histone acetyltransferases (Font et al., 2000). Recently, it was shown that AIB-1 is phosphorylated on six sites important for its full transcriptional activity (Wu et al., 2004). Furthermore, cellular signaling cascades also phosphorylate corepressors. Interaction of
N-CoR and silencing mediator for retinoid and thyroid hormone (SMRT) with progesterone receptor (PR) decreased after incubation with 8-bromo cAMP (Wagner et al., 1998). *In vitro* phosphorylation by MEKK decreased the interaction of SMRT with thyroid receptor (Hong et al., 1998).

Currently the mechanisms of tamoxifen resistance and tamoxifen agonist actions in the endometrium are undefined. An increased use of tamoxifen for breast cancer therapy and chemoprevention highlights an urgent need to 1) identify reliable biomarkers that can predict with high certainty the response of breast cancer to tamoxifen therapy, 2) understand the precise molecular mechanism which contributes to the development of acquired tamoxifen resistance as well as estrogen-like agonist action in the endometrium and, 3) develop non-cross-resistant therapies designed to deplete ERα and/or disrupt ER signaling that can be used alone or in combination with tamoxifen to alleviate the unwanted side-effects.

The studies described in this dissertation include: the identification of proteins associated with the growth stimulatory effects of tamoxifen in the ERα-positive, tamoxifen resistant Ishikawa endometrial adenocarcinoma cell line using two-dimensional gel electrophoresis; examining the role of non-receptor tyrosine kinase src in promoting the tamoxifen agonist action in the endometrium; assessing the role of the micronutrient selenium in ER signaling and providing a mechanism-based rationale for combining selenium with tamoxifen as a therapeutic strategy to improve the efficacy of tamoxifen in both breast cancer treatment and chemoprevention.
Nuclear Receptor Activation

Nuclear receptors (NR) are a superfamily of liganded transcription factors and are important in a wide variety of cellular processes (Evans, 1988; Tsai et al., 1994). Nuclear receptors can be classified into type 1 and type 2 receptors. Type 1 receptors consist of PR, ER, androgen receptor (AR), glucocorticoid receptor (GR), and mineralocorticoid receptor (MR). Upon specific ligand binding, type 1 receptors dimerize and binds to specific elements termed response elements in the promoter of genes (Nelson et al., 1999a). Subsequent recruitment of coactivator proteins initiates transcription. In contrast type 2 receptors such as thyroid receptor (TR), all-trans retinoic acid receptor (RAR), 9-cis retinoic acid receptor (RXR), and vitamin D₃ receptor (VDR) are already bound to DNA in their unliganded state bound with corepressor proteins that repress basal transcription. Upon ligand binding the corepressor interaction is lost, coactivators are recruited, and transcription is initiated.

Estrogen Receptor

Estrogen is a female sex hormone important in the female reproductive tract. The main source of estrogen produced in the body is from the ovaries through a complex series of hydroxylation, oxidation, and aromatization reactions that convert 21-carbon cholesterol to 18-carbon estrogen. The production of ovarian estrogen is under control of two hormones lutenizing hormone (LH) and follicle stimulating hormone (FSH). The LH stimulates the production of androgens, whereas FSH activates aromatase enzymes that
convert androgens to estrogens. Once formed, estrogen exerts its effects on female reproductive tissues. During the proliferative phase of the menstrual cycle when estrogen concentration is the highest, the endometrium becomes 3 to 5 fold thicker and uterine glands become enlarged. Estrogen stimulates proliferation and keratinization in the vagina. During puberty when estrogen concentrations begin to rise, the mammary gland becomes enlarged due to the increase in ductal size and accumulation of adipose tissue surrounding the ducts (Ryan, 1999).

The specificity of estrogen in tissues is due to the presence of a type 1 NR, ER. Two isoforms of ER have been identified, ERα and ERβ (Kuiper et al., 1996; Mosselman et al., 1996; Tremblay et al., 1997). The ER is part of the nuclear receptor/thyroid superfamily and like all NR, ERα and ERβ contain six modular domains each with a specific functional property (Kumar et al., 1987) (Figure 1). The N-terminal A/B domain (NTD) contains a ligand-independent activation function 1 (AF-1) (Evans, 1988; Tora et al., 1989; Tsai et al., 1994; Webb et al., 1998). A centrally located DNA binding domain (DBD) that contains two zinc fingers motifs is important in binding DNA (Evans, 1988; McKenna et al., 1999; Tsai et al., 1994). A hinge region contains a nuclear localization sequence. The E/F domain or ligand binding domain (LBD) includes sites for hormone binding, ligand dependent activation function 2 (AF-2), and contains the dimerization domain (Evans, 1988; McKenna et al., 1999; Tsai et al., 1994). The ERα and ERβ are 84% to 97% homologous in the DBD, 54% homologous in the LBD but only 28% homologous in the NTD (Enmark et al., 1997; Mosselman et al., 1996) (Figure 1). Targeted deletion of ER in mice demonstrated that ERα was the predominant ER isoform.
**Figure 1.** Schematic Diagram of ERα and ERβ Structure Demonstrating Amino Acid Homology Between Different ER Domains

<table>
<thead>
<tr>
<th>Domains</th>
<th>AF-1</th>
<th>DNA</th>
<th>Ligand/AF-2</th>
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<tbody>
<tr>
<td>A/B</td>
<td>26%</td>
<td>84%</td>
<td>58%</td>
</tr>
<tr>
<td>C</td>
<td>84%</td>
<td>12%</td>
<td>12%</td>
</tr>
<tr>
<td>D</td>
<td>12%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>58%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>12%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ERα:
- Domain A/B: 1-180
- Domain C: 180-263
- Domain D: 263-302
- Domain E: 302-552
- Domain F: 552-593

ERβ:
- Domain A/B: 1-144
- Domain C: 144-227
- Domain D: 227-255
- Domain E: 255-504
- Domain F: 504-530

Amino Acid Homology:
- ERα: 26%
- ERβ: 84%
- ERα: 12%
- ERβ: 12%
in the mammary gland and the uterus. The uterus is comprised of three tissue compartments (myometrium, endometrial stroma, luminal/glandular epithelium) and both ERα and ERβ are expressed in all three compartments (Hiroi et al., 1999). ERα -/- mice are hypoplastic in all three compartments and whole uterine wet weight is half that of wild type mice whereas uteri of the ERβ -/- mice appear similar to wild type mice (Krege et al., 1998). In the mammary gland, ERβ -/- mice do not exhibit abnormalities compared to age matched wild type female mice (Krege et al., 1998). The ERα knockout mice exhibit decreased ductal growth and pregnancy differentiation of the mammary gland (Bocchinfuso et al., 1997). The presence of ERα and ERβ and estrogen synthesis in the fetal ovaries suggests a role for both ERα and ERβ in ovarian development (Sar et al., 1999; Weniger, 1990); however, no histological abnormalities were found with either ERα -/- or ERβ -/- mice. At sexual maturity discrete ovarian abnormalities became apparent. ERα -/- mice were not able to ovulate; whereas, ERβ -/- had considerably lower litter size compared to wild type mice (Krege et al., 1998; Lubahn et al., 1993; Schomberg et al., 1999). Surprisingly, targeted deletions of both ERα and ERβ displayed a different phenotype than the individual ERα -/- or ERβ -/-, clearly demonstrating a role for both ER isoforms in the ovaries (Couse et al., 1999). In addition to estrogen effects on reproductive physiology, estrogens are important in non-classical estrogen target tissues. Estrogens play an important role in brain function. Epidemiological studies demonstrate a protective effect of hormone replacement therapy (HRT) on neurodegenerative diseases such as Alzeiheimer’s disease (Wise et al., 2001). Estrogen-mediated protection of
neurons required ERα and not ERβ (Dubal et al., 2001), although the mechanisms are undefined. Estrogens also exert effects on bone. It has been well characterized that an increase in osteoporosis is correlated with a decrease of estrogen levels during menopause (Watts, 1999). Both ERα and ERβ are expressed in localized areas of the bone (Bord et al., 2001; Braidman et al., 2001; Vidal et al., 1999). Furthermore, both ERα and ERβ are important in bone formation and remodeling (Jessop et al., 2004; Lee et al., 2004). Estrogens are also important in the cardiovascular system (Bakir et al., 2000; Dubey et al., 2000), adipose tissue (Homma et al., 2000), and in male reproduction (Couse et al., 2001) via ERα and/or ERβ.

**Nuclear Receptor Coactivators**

Estrogen Receptor action is potentiated by a group of proteins termed coactivators. Numerous NR coactivator proteins have been identified in recent years. A coactivator is a protein that interacts with NR and potentiates nuclear receptor activity. A coactivator interacts with the NR through an LXXLL motif (where L is leucine and X is any amino acid) termed the NR box or receptor interaction domain (RID) (Heery et al., 1997) (Figure 2). Chromatin structure is a key regulator of basal transcription. Histones repress transcription through tight binding of the negatively charged DNA by positively charged lysine side chains. By acetylation of lysine residues in histones, the interaction is disrupted and the DNA is no longer tightly bound to histone proteins. When the chromatin is in a relaxed conformation it is more conducive to transcription due to accessibility of the transcription machinery. The well characterized p160 family of
**Figure 2.** General Schematic Diagram of p160 Coactivators and N-CoR and SMRT Corepressors.

P160 Coactivator

160 kDa

Corepressor

250-300 kDa

bHLH: helix loop helix region; PAS: Per=ARNT-Sim Domain; S/T: Serine Threonine rich region; Q: Glutamine rich region; RID: Receptor Interaction Domain; RD: Repressor Domain
Coactivators include SRC-1 (Kamei et al., 1996; Onate et al., 1995), GRIP-1 (Hong et al., 1996; Voegel et al., 1996), and AIB-1 (Anzick et al., 1997; Chen et al., 1997; Li et al., 1997; Takeshita et al., 1996; Torchia et al., 1997). The p160 family of coactivators either contain intrinsic histone acetyltransferase (HAT) activity or recruit other HATs such as p300/CBP associated factor (P/CAF) and Creb Binding Protein (CBP) (Chakravarti et al., 1996; Kamei et al., 1996; Spencer et al., 1997). The p160 family of coactivators and CBP function synergistically in enhancing transcription (Chen et al., 1997; Liu et al., 2001; Spencer et al., 1997). However, site-directed mutagenesis targeting the HAT motif on p160 coactivators did not significantly affect its ability to coactivate nuclear receptors (Liu et al., 2001; Spencer et al., 1997), suggesting that p160 family members may be important in the assembly of other transcription factors and coactivators on the promoter. Recent studies demonstrate a dynamic model of coactivator recruitment where ER immediately binds to DNA followed by the sequential recruitment of p160 coactivators, P300, and the TR associated protein (TRAP)/VDR interacting protein (DRIP) complex. Local chromatin structure is modified that facilitates RNA polymerase II binding. Subsequently, CBP acetylation of p160 coactivators leads to coactivator and ER release. Following ER release, CBP and pCAF disassemble and the cycle is repeated (Burakov et al., 2002; Shang et al., 2000).

In addition to histone acetylases, recently identified coactivator-associated arginine methyltransferase 1 (CARM1) and protein arginine N-methyltransferases (PMRTs) provide yet another mechanism of chromatin remodeling. The CARM1 and PRMT bind to the C-terminal region of SRC-1 and methylate histones H3 and H4. The
intrinsic methyltransferase property of these proteins is required for coactivator activity (Chen et al., 1999; Wang et al., 2001b). In contrast the ubiquitin ligase activity of coactivator E6-associated protein (E6-AP) is not required for coactivation of NR \textit{in vitro} and \textit{in vivo} (Nawaz et al., 1999a; Sivaraman et al., 2000; Smith et al., 2002). Another essential group of coactivators are represented by the TRAP/DRIP complex (Burakov et al., 2000; Fondell et al., 1996; Rachez et al., 1998). The TRAP/DRIP complex acts to stabilize the nuclear receptor:coactivator complex at the promoter and bridge the NR complex to the basal transcriptional machinery (Burakov et al., 2000; Gu et al., 1999; Kim et al., 1994).

The importance of each p160 family member on ER action has been demonstrated \textit{in vivo} with targeted deletions of SRC-1, GRIP-1, and AIB-1. SRC-1 \textit{-/-} mice exhibit normal growth and fertility; however, these mice exhibit decreased uterine growth following exposure to estrogen. In addition, there is decreased mammary gland formation and partial resistance to estrogen (Xu et al., 1998). Interestingly, in adult rats an acute knockdown of SRC-1 in the brain results in an increase of GRIP-1 expression (Apostolakis et al., 2002). Similar compensatory expression of GRIP1 was demonstrated in sertoli cells (Mark et al., 2004). Therefore, the modest effects on reproductive phenotypes in SRC-1\textit{-/-} mice may be due to genetic compensation by GRIP-1. The GRIP-1 \textit{-/-} mice display a decrease in male and female fertility (Gehin et al., 2002). In addition, GRIP-1 also affects thermogenesis and energy expenditure (Picard et al., 2002). The AIB-1 \textit{-/-} mice exhibit significant growth retardation and reduced body size. There is a decrease in the estrogen levels of AIB-1 \textit{-/-} mice that delay pubertal development and
mammary gland growth (Wang et al., 2000; Xu et al., 2000). In a mammary gland tumor model, AIB-1-/- mice exhibited reduced tumor formation compared to wild-type mice (Kuang et al., 2004).

**Nuclear Receptor Corepressor**

In contrast to coactivators, corepressors are NR interacting proteins that repress NR activity. Corepressors are associated with unliganded type II receptors or antagonist bound type I receptors. Two well-studied corepressors are N-CoR and SMRT (Chen et al., 1995; Horlein et al., 1995) (Figure 2). Similar to coactivators, corepressors contain RID domains important in interaction with the NR. The RID domain, of corepressors share similar homology to the coactivator-RID domain, and consist of LXXI/HIXXXI/L (where L is leucine, I is isoleucine, histidine, and X is any amino acid). The SMRT and N-CoR mediate transcriptional repression by recruiting histone deacetylase complexes that remove acetyl groups from histone proteins, and thus confer a tight conformation repressing basal transcription (Heinzel et al., 1997; Nagy et al., 1997). Targeted deletion of N-CoR demonstrated the importance of transcriptional repression for mammalian development. The N-CoR deficient embryos died at day 15.5. The N-CoR is needed for active repression of several NR and other transcription factors. In addition, N-CoR was required for the antagonist activity of antiestrogen compounds (Jepsen et al., 2000).

**Classical Ligand-Dependent ER Activation**

Estrogen is a lipophilic molecule that crosses the cell membrane and binds to the LBD of the ER. Upon ligand binding the receptor dissociates from heat shock protein-90 (HSP-90) and other chaperone proteins. The receptor either forms heterodimers
consisting of ER\(\alpha\)-ER\(\beta\) or homodimers of two ER\(\alpha\) or ER\(\beta\) (Cowley et al., 1997; Ogawa et al., 1998; Pettersson et al., 1997). Upon dimerization, ER binds to an estrogen response element (ERE) \(5'\) GGTC\(\alpha\)nnnTGACC \(3'\), a palidromic inverted repeat in the promoters of estrogen dependent genes (Klein-Hitpass et al., 1986). The binding of an agonist to the ER stabilizes helix 12 in the AF-2 region. The conformation allows coactivator proteins to recognize an amphipathic \(\alpha\)-helix on the surface of the agonist-bound LBD formed by helix 3,4,5, and 12 via the NR box (Shang et al., 2000; Steinmetz et al., 2001; Weatherman et al., 1999) (Figure 3).

In addition to activating transcription at an ERE, ER\(\alpha\) and ER\(\beta\) activate transcription at AP1 sites (Figure 3). In contrast to the classical mechanism of ER activation, ER regulation of transcription via AP1 sites does not involve direct binding to DNA, but instead ER binds to jun/fos proteins that, in turn, bind to AP-1 consensus sequences on DNA (Paech et al., 1997b; Webb et al., 1995, 1999). It is hypothesized that ER binds to coactivator proteins recruited by jun/fos and this interaction increases the transactivation capacity of the coactivators. The ER also activates transcription at ERE half sites adjacent to SP1 sites. ER interacts with the c-terminal binding domain of SP1 proteins to transactivate genes (Porter et al., 1997). Additionally, it is found that ER/SP1 complexes activated transcription at GC rich elements independent of ER binding to DNA, similar to what is observed in the ER/AP1 paradigm (Dong et al., 1999; Qin et al., 1999; Samudio et al., 2001; Wang et al., 2001a; Xie et al., 1999). A transgenic mouse model has been developed that does not allow ER\(\alpha\) binding through the
ER is activated by four pathways. 1) Activation of ER at ERE-containing promoters by the classical ligand-dependent pathway. 2) Activation of ER at ERE-containing promoters by ligand-independent pathways. 3) Activation of ERE-independent promoters by either ligand-dependent or independent pathways. 4) Nongenomic signaling by a membrane-associated binding site. \( E_2 \): Estradiol; \( mER \): Membrane ER
classical ERE-mediated mechanism but retains the ability of ERα to bind to AP1 and SP1 proteins. Mice heterozygous for the mutant ERα are infertile preventing generation of homozygous mice. Although more studies are needed, the transgenic model provides strong evidence for ERE-independent mechanisms in vivo. Interestingly, mice heterozygous for ERα +/- are fertile suggesting that the mutant allele act in a dominant negative manner to inhibit estrogen-regulated physiology (Jakacka et al., 2002).

Ligand-Independent/Crosstalk with signaling Pathways

In the absence of estrogen, mitogenic growth factors stimulate ERα transcriptional activity (Figure 3). Both ligand-dependent and -independent ERα activation is modulated by phosphorylation and site-specific phosphorylation of ERα increases its transcriptional activity (Ali et al., 1993; Bunone et al., 1996; El Tanani et al., 1997; Lahooti et al., 1994; Le Goff et al., 1994; Washburn et al., 1991; Weigel et al., 1998). Major phosphorylation sites of ERα are in the NTD at serine 104 (Le Goff et al., 1994), serine 106 (Le Goff et al., 1994), serine 118 (Kato et al., 1995), serine 167 (Arnold et al., 1994). In addition there is one site in the DBD at serine 236, one site in the hinge region at serine 305 (Wang et al., 2002), and two sites in the LBD at threonine 311 (Lee et al., 2002) and tyrosine 537 (Arnold et al., 1997). Numerous signaling pathways phosphorylate ER (REVIEWED IN Coleman et al., 2001; Lannigan, 2003). Mutation of serines 104, 106, 118, and 167 to alanine results in a general decrease in ERα transcriptional activity (Arnold et al., 1995a; Castano et al., 1997; Le Goff et al., 1994; Tzeng et al., 1996). Serine 118 is phosphorylated by MAPK and is important for coactivator binding (Bunone et al., 1996; Dutertre et al., 2003; Endoh et al., 1999; Kato et
Serine 167 is a consensus AKT site and phosphorylation mediates DNA binding (Arnold et al., 1995a; Castano et al., 1997; Martin et al., 2000; Sun et al., 2001b; Tzeng et al., 1996) and promoter interaction. Serine 305 is phosphorylated by protein kinase A (PKA) and p21 activated kinase-1 (PAK1) and is important in the transcriptional activity of ERα (Balasenthil et al., 2004; Michalides et al., 2004). Threonine 311 is downstream of P38 kinase and is important for nuclear export of the receptor (Lee et al., 2002). Tyrosine 537 is phosphorylated by src kinase in vitro (Arnold et al., 1995b) and important in DNA binding (Arnold et al., 1995c). In addition specific amino acid substitutions (Y537S and Y537A) have led to constitutive activation of ERα (Weis et al., 1996).

There is a well-characterized reciprocal crosstalk between ERα and growth factor signaling pathways. Numerous in vitro experiments provide evidence of crosstalk between ER and growth factor signaling (REVIEWED IN Levin, 2003). The best in vivo examples of crosstalk were demonstrated in the uterus. Estrogen and growth factors stimulate proliferation in the endometrium (Nelson et al., 1991). In the insulin-like growth factor-2 (IGF-1) knockout mouse, estrogen-induced uterotrophic effects were significantly retarded in spite of functional ERα present in the uterus (Adesanya et al., 1999). Also in the ERα knockout mice, no uterotrophic effects were detected upon epidermal growth factor (EGF) treatment, even though EGF signaling was intact (Klotz et al., 2002). The role of phosphorylation in ligand-independent crosstalk has been well characterized for ERα. In contrast, phosphorylation and cellular signaling crosstalk is not defined for ERβ; however, ERβ was activated via a ligand-independent pathway and
phosphorylated in the AF-1 region (Chu et al., 2004; Coleman et al., 2003; Tremblay et al., 1999).

In addition to ER, coregulator proteins are targets of signaling pathways. SRC-1 contains seven in vivo phosphorylation sites and all seven sites contain a consensus sequence for proline-directed protein kinases (Rowan et al., 2000b). In addition, the induction of protein kinase A (PKA) enhanced ligand-independent activation of PR through phosphorylation of SRC-1 (Rowan et al., 2000a). In in vitro studies both GRIP-1 and AIB-1 were phosphorylated by MAP kinase, ERK-2 (Font et al., 2000; Lopez et al., 2001). In breast cancer cells the transcriptional activity of AIB-1 increased via MAPK phosphorylation that stimulated the recruitment of other HATs (Font et al., 2000). The AIB-1 contain six phosphorylation sites important for full transcriptional activation (Wu et al., 2004). Also interaction of N-CoR and SMRT with PR decreased after incubation with 8-bromo cAMP (Wagner et al., 1998) and in vitro phosphorylation by MEKK decreased the interaction of SMRT with thyroid receptor (Hong et al., 1998).

Nongenomic Estrogen Signaling

Ligand-dependent and -independent ER-regulated actions are considered genomic pathways due to the involvement of ER-mediated gene transcription. A distinct estrogen-mediated pathway referred to as nongenomic signaling exists that does not require mRNA and protein synthesis (Figure 3). A membrane receptor is thought to mediate nongenomic estrogen signaling (Bression et al., 1986; Brubaker et al., 1994; Marquez et al., 2001). However, it is controversial whether a separate membrane receptor that is distinct from the classical ER exists (Filardo et al., 2000; Filardo et al., 2002), or a
receptor that contains homology to the classical ER stimulates the nongenomic estrogen response (Kim et al., 1999; Norfleet et al., 2000). Nongenomic estrogen signaling rapidly (seconds-minutes) activate several signaling pathways that have significant physiological consequences (Filardo et al., 2000; Kahlert et al., 2000; Migliaccio et al., 1996; Sun et al., 2001a). Estrogens rapidly activate the MAPK and AKT pathways that can induce a proliferative (Castoria et al., 1999; Migliaccio et al., 1996, 2000) and an anti-apoptotic response (Castoria et al., 2001). In addition, nongenomic estrogen signaling stimulated nitric oxide release (Stefano et al., 2000a), increased intracellular calcium levels (Stefano et al., 2000b) and enhanced prolactin secretion (Watson et al., 1999).

Breast Cancer

The mammary gland consist of 6 to 10 major ducts that are subdivided into lobules that are considered the functional unit of the mammary gland. Breast carcinomas are divided into noninvasive and invasive carcinomas. Noninvasive carcinomas can be subdivided into two major histological subtypes; ductal carcinoma in situ (DCIS) or lobular carcinoma in situ (LCIS). The DCIS or LCIS do not have the capacity to infiltrate the basement membrane, and therefore do not produce distant metastases (Swain, 1989a,b). However, untreated DCIS and LCIS have an increased risk for development of invasive carcinomas (Mallon et al., 2000; Stallard et al., 2001). Similar to carcinoma in situ, there are two major histological subtypes of invasive carcinomas, ductal and lobular, although other less frequent types exist (Dixon et al., 1982). Epidemiological studies have shown age, diet, and genetic predispositions are risk factors for breast cancer (McPherson et al., 2000).
The best characterized risk factor for breast cancer is hormonal exposure. As early as 1896, it was shown that oophorectomy was a successful treatment for management of breast cancer (Leake, 1996). In addition, early menarche and late menopause that increase the exposure of estrogen in a lifetime can increase the risk of breast cancer (Kelsey et al., 1991). Also multiple pregnancies that interrupt the menstrual cycle and decrease the exposure to estrogen are protective against breast cancer (Hulka et al., 1994; Russo et al., 1982). Women who receive exogenous estrogen have an increased breast cancer risk (1997; Colditz et al., 1995; Magnusson et al., 1999).

The ER\(\alpha\) is expressed in normal breast epithelium, although these cells do not proliferate upon estrogen treatment. It is thought that estrogen induces the secretion of mitogenic growth factors that stimulate adjacent ER-negative cells to proliferate via a paracrine-mediated mechanism (Cunha et al., 1997). In contrast, the majority of premalignant breast cells express ER\(\alpha\) and are sensitive to estrogen-mediated proliferation and regression upon estrogen ablation (Clarke et al., 1993, 1997; Johnston et al., 1994; Jordan et al., 1975). Several animal models demonstrate the importance of ER\(\alpha\) in breast tumorigenesis. Transgenic overexpression of Erb2/Her2 or WNT under control of the mouse mammary tumor virus (MMTV) promoter increased the incidence of mammary tumors (Cardiff et al., 1993). Introduction of the MMTV-erb2/Her2 or MMTV-WNT transgene into an ER\(\alpha\) null background significantly delayed tumor onset indicating that ER\(\alpha\) is important for mammary tumorigenesis (Bocchinfuso et al., 1999; Hewitt et al., 2002).
In contrast to ERα, the role of ERβ in breast cancer remains undefined. The ERβ is expressed in the majority of normal breast epithelial cells and is present as several splice variants that differ in functional properties compared to the full-length ERβ (Speirs et al., 2004). There is limited data on the role of ERβ in breast cancer, but as breast cancers progress from a benign to a more malignant phenotype, the ERα/ERβ is increased with a predominance of ERα expression in the more malignant tumors (Leygue et al., 1998).

Selective Estrogen Receptor Modulators (SERM)

As a treatment for breast cancer SERMs bind with high affinity to ER and act in a tissue specific manner. Tamoxifen is a prototypic SERM and the most widely used hormonal therapy for breast cancer (Jordan, 2000) (Figure 4). Tamoxifen therapy profoundly improved the overall survival of breast cancer patients (Breast Cancer Trialists' Collaborative Group, 1998) without the adverse side-effects associated with cytotoxic chemotherapy (Pritchard et al., 1997). Furthermore, tamoxifen is the only agent approved for breast cancer chemoprevention in high risk women (Jordan, 2000). The major mechanism of action for tamoxifen in breast cancer is as an ER antagonist. Tamoxifen is a competitive inhibitor of estrogen and upon tamoxifen binding, ER recruits a corepressor complex that inhibits estrogen dependent gene transcription (Dutertre et al., 2000; Jordan, 2000; Shang et al., 2002). Tamoxifen is an estrogen antagonist in the breast, but tamoxifen displays uterotropic effects in the endometrium (Harper et al., 1967; Terenius, 1971). Tamoxifen therapy results in a 4-fold increase in the incidence of endometrial cancer in postmenopausal women.
Figure 4. Chemical Structure of SERMs and Aromatase Inhibitors.

Estradiol  Tamoxifen  Raloxifene

EM-652  Anastrozole  Fulvestrant
(Fornander et al., 1989). New generation SERMs are being sought with the same or better efficacy than tamoxifen but with fewer side effects.

Raloxifene, a SERM structurally distinct from tamoxifen, is approved for the treatment and prevention of osteoporosis in postmenopausal women (Figure 4). In addition, raloxifene does not display estrogen-like activity in the endometrium (Boss et al., 1997; Delmas et al., 1997). Although, only modest effects of raloxifene were found in ER-positive breast cancers (Gradishar et al., 2000), an on-going clinical trial is assessing the effects of raloxifene on breast cancer incidence as a beneficial side effect to its anti-osteoporosis activity (Cummings et al., 1999). Acolbifene (EM-652) is also in clinical trial for breast cancer (Figure 4). EM-652 is a potent antiestrogen and does not display estrogen-like action in mouse mammary gland and endometrium. Similar to raloxifene, EM-652 preserves bone density in ovariectomized rats. In addition, EM-652 has beneficial effects on plasma lipid profiles (Labrie et al., 2003) making EM-652 a potentially attractive alternative to tamoxifen therapy. The search for SERMs with an ideal tissue profile has been offset by the development of antiestrogenic agents that inhibit ER action in all tissues. Fulvestrant (faslodex, ICI 182,780) is an ER antagonist with no known agonist effects (Wakeling et al., 1991) (Figure 4). Fulvestrant binds to ER and promotes ER protein degradation (McClelland et al., 1996). Fulvestrant is approved for ER-positive metastatic breast cancer in postmenopausal women with disease progression after tamoxifen therapy (Bross et al., 2003). Aromatase inhibitors have been shown to be effective in inhibiting ER-positive breast cancers. Aromatase inhibitors specifically target the rate-limiting enzyme needed for estrogen production. The
aromatase inhibitor agent anastrozole was more effective than tamoxifen as an adjuvant endocrine treatment for ER-positive breast cancers (Figure 4). However, anastrozole was also associated with an increase in bone fractures in women receiving compared to patients receiving tamoxifen therapy (Howell et al., 2005).

**Mechanisms of Tissue Specific SERM Effects**

Currently there are several proposed mechanisms that explain the tissue specific effects of SERMs. The different complement of coregulator proteins in the uterus vs. the breast has been suggested to underlie tamoxifen agonist activity in the uterus. An increase in corepressors levels decreased the agonist effect of tamoxifen. Whereas, an increase in coactivator levels promoted the agonist activity of tamoxifen (Smith et al., 1997). Shang et al. (2002) have demonstrated a specific increase of SRC-1 expression in tamoxifen resistant Ishikawa endometrial cancer cells compared to tamoxifen sensitive MCF-7 breast cancer cells. Ishikawa cells activated transcription of ER-dependent genes following incubation with estrogen and tamoxifen via recruitment of SRC-1. Tamoxifen treatment repressed transcription in MCF-7 cells via recruitment of corepressors (Shang et al., 2002). It has been hypothesized that expression of AF-1 interacting coactivators may regulate tissue-specificity of SERMs. Synergism between the AF-1 and AF-2 is required for full activity of ERα; however, AF-1 and AF-2 can activate genes to a certain extent independently (Kraus et al., 1995). Tamoxifen inhibits AF-2 but AF-1, a cell and promoter specific activation function, is not blocked (Berry et al., 1990; Tzukerman et al., 1994). It is suggested that if a cell contains a large number of AF-1 specific coactivators
such as p68 helicase and steroid receptor RNA activator (SRA) (Endoh et al., 1999; Lanz et al., 1999), the tamoxifen-ER complex in this context may activate transcription.

Another variable to consider in the tissue specific activity of SERMs is the \( \text{ER}_\alpha/\text{ER}_\beta \) expression ratios. The \( \text{ER}_\alpha \) and \( \text{ER}_\beta \) act in opposite regulatory mode on ER-regulated AP1 elements upon estrogen and tamoxifen treatment (Paech et al., 1997a). Moreover, \( \text{ER}_\beta \) is less transcriptionally active than \( \text{ER}_\alpha \) due to a decrease or absence of AF-1 activity (Delaunay et al., 2000). In addition, \( \text{ER}_\beta \) can reduce the transcriptional activity of \( \text{ER}_\alpha \) through hetreodimerization and/or competitive binding to ER-regulated promoters (Hall et al., 1999). Therefore, tissue specific differences in the \( \text{ER}_\alpha/\text{ER}_\beta \) ratio may potentially alter the pharmological response and modulate the tissue specific activity SERMs.

Differentially activated cellular signaling pathways may regulate tissue specificity of SERMs. As mentioned above, several signaling pathways target ER and coregulators and these pathways can alter the relative antagonist/agonist property of tamoxifen. Activation of PKA in tamoxifen sensitive MCF-7 breast cancer cells increased the transcriptional activity of tamoxifen to levels similar to that of estrogen (Fujimoto et al., 1994). Estrogen phosphorylation at serine 118 enhanced the transcriptional activation in response to estrogen and tamoxifen (Kato et al., 1995). MEKK1 increased the agonistic activity of tamoxifen implicating kinases downstream to be important in the cell specificity of tamoxifen (Lee et al., 2000). Feng et al (2001) demonstrated that the Src/JNK pathway enhanced the transcriptional activity of the tamoxifen-ER complex through the AF-1 domain of the ER. It is doubtful that a single mechanism modulates the
tissue specific effect of SERMs. Rather, it is likely to be multifactorial encompassing several mechanisms described above.

**Tamoxifen Resistance**

Clinical tamoxifen resistance is defined as the inability of tamoxifen to inhibit the growth of hormone dependent breast tissue. Thirty to fifty percent of patients with ERα-positive breast cancer are resistant to tamoxifen therapy (ERα-positive *de novo* resistance) (Osborne, 1998) and those patients that initially respond will eventually become resistant to tamoxifen therapy (acquired resistance) (Osborne, 1998). The majority of breast tumors that acquire tamoxifen resistance are ERα-positive (Johnston et al., 1995) and are sensitive to second line hormonal therapy (Howell et al., 1995; Osborne, 1998). Therefore, loss of ERα is not a major mechanism for acquired tamoxifen resistance. Several ERα mRNA variants exist and many of these variant mRNAs are translated in breast tumors (Murphy et al., 1998). Moreover, there is cell-based evidence that tamoxifen can act as an agonist on specific ERα mutants (Jiang et al., 1992); however, ERα mutations occur at very low frequency in tamoxifen resistant breast tumors (Karnik et al., 1994). Thus it is unlikely that ERα mutations constitute a major mechanism for tamoxifen resistance.

Other mechanisms that modulate ERα function may be important in tamoxifen resistance. Overexpression of AIB-1 is a good prognostic marker of tamoxifen resistance. Samples of 316 frozen breast tumors were assessed for AIB-1 expression. It was found that patients receiving tamoxifen treatment who had elevated AIB-1 levels had a decrease in disease free survival (Osborne et al., 2003). In contrast, low corepressor
levels were associated with tamoxifen resistance. A decrease in N-CoR levels correlated with a shorter relapse free survival in patients undergoing tamoxifen therapy suggesting that N-CoR may be a good independent prognostic marker of tamoxifen resistance (Girault et al., 2003). In addition, a decrease in N-CoR levels correlated with the onset of tamoxifen resistance in a mouse model for human breast cancer (Lavinsky et al., 1998). Finally, 4-hydroxytamoxifen was a full agonist for ER-dependent transcription in fibroblasts isolated from the N-CoR null mice compared to its antagonist activity in fibroblasts from wild type mice (Jepsen et al., 2000).

Several ERβ variants and mutations have been characterized, but the role of ERβ in tamoxifen resistance remains unknown (Reviewed in Speirs et al., 2004). A few reports have shown a decrease in ERβ levels in tamoxifen resistant breast cancers (Esslimani-Sahla et al., 2004; Hopp et al., 2004); however, more studies will be needed to confirm the role of ERβ in tamoxifen resistance.

In addition to ER and coregulator expression, several deregulated cellular kinase pathways were involved in tamoxifen resistance. Overexpression of erb-2/HER2 was associated with tamoxifen resistance (Benz et al., 1993; Liu et al., 1995; Pietras et al., 1995). The HER2 receptor is a member of the EFGR (HER1) family of transmembrane receptor tyrosine kinases that also includes HER3 and HER4. HER2 is recruited as the preferred partner of EGFR, HER3 or HER4 into an active, phosphorylated, heterodimeric complex that activates several signaling pathways involved in the proliferation and enhanced survival of tumor cells (Olayioye et al., 2000; Yarden et al., 2001)
Increased activity of MAPK and PI3K/AKT pathways was associated with tamoxifen resistance. As mentioned earlier, both MAPK and PI3K/AKT pathways phosphorylate and increase the transcriptional activity of ERα. Several studies demonstrated a deregulation of MAPK activity following long-term tamoxifen treatment (Berstein et al., 2003; Donovan et al., 2001; Rabenoelina et al., 2002). Furthermore, breast cancer samples from patients undergoing anti-hormonal therapies that display an increased MAPK activity were associated with decreased survival (Gee et al., 2001). In addition, several studies demonstrated that constitutively active AKT conferred tamoxifen resistance in breast cells (Campbell et al., 2001; Clark et al., 2002). Similar to the tissue specific effects of SERMs, it is likely that several mechanisms contribute to ERα-positive de novo and acquired tamoxifen resistance.

**Therapeutic Role of Signal Transduction Inhibitors (STI)**

In addition to SERMs, new therapies for cancer utilizing inhibitors of signaling pathways are emerging and several agent have been approved for cancer therapy or are currently in clinical trial (Agus et al., 2005; Ardizzoni et al., 2004; Drevs, 2003; Druker et al, 2001a, b; Elsayed et al., 2001; Graham et al., 2004; O'brien et al., 2004; Repp et al., 2003; Slamon et al., 2001; Sridhar et al., 2003a, b; Traxler et al., 2001; Vogel et al., 2002) (Table I). Numerous monoclonal antibodies targeting the extracellular domain of EGFR have been developed (Crombet-Ramos et al., 2002; Overholser et al., 2000; Wallace et al., 2000; Yang et al., 2001). As an alternative approach to antibody inhibitors of cell surface receptors, cell permeable small molecule inhibitor of EGFR such as ZD1839 (Iressa) are currently in phase III.
Table I Signal Transduction Inhibitors Approved or in Clinical Trials.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Company</th>
<th>Tyrosine Kinase Target</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Monoclonal Antibodies</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Herceptin</td>
<td>Genentech</td>
<td>HER2/Neu (Vogel C.L. et al., 2002)</td>
</tr>
<tr>
<td>MDX-H210</td>
<td>Mederex</td>
<td>HER2/Neu (Repp R. et al., 2003)</td>
</tr>
<tr>
<td>2C4</td>
<td>Genentech</td>
<td>HER2/Neu (Agus D.B. et al., 2003)</td>
</tr>
<tr>
<td>C225</td>
<td>Imclone</td>
<td>EGFR (ErbB-1) (Graham J. et al., 2004)</td>
</tr>
<tr>
<td>MDX-447</td>
<td>Mederex</td>
<td>EGFR (ErbB-1) (Sridhar S.S. et al., 2003a)</td>
</tr>
<tr>
<td><strong>Signal Transduction Inhibitor (STI)</strong></td>
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</tr>
<tr>
<td>STI-571 (Gleevec)</td>
<td>Novartis</td>
<td>Abl, c-kit, PDGFR (O’brien S. et al., 2004)</td>
</tr>
<tr>
<td>ZD1839 (Iressa)</td>
<td>AstraZeneca</td>
<td>EGFR (ErbB-1) (Elsayed Y.A. et al., 2001)</td>
</tr>
<tr>
<td>OSI-774</td>
<td>OSI/Genentech</td>
<td>EGFR (ErbB-1) (Ardizzoni A. et al., 2004)</td>
</tr>
<tr>
<td>PKI-166</td>
<td>Novartis</td>
<td>EGFR (ErbB-1); HER2 (Traxler P. et al., 2001)</td>
</tr>
<tr>
<td>PTK-787</td>
<td>Novartis/Schering</td>
<td>VEGFR (Drevs J. 2003)</td>
</tr>
<tr>
<td>SU5416</td>
<td>Sugen/Pharmacia</td>
<td>VEGFR (Sridhar S.S. et al., 2003b)</td>
</tr>
<tr>
<td>SU6668</td>
<td>Sugen/Pharmacia</td>
<td>VEGFR (Sridhar S.S. et al., 2003b)</td>
</tr>
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clinical trials for non-small cell lung carcinomas (NSCLC) and are promising candidates for breast cancer (Elsayed et al., 2001). Herceptin, an approved agent currently used for HER2 over-expressing breast cancer has been shown to be effective as monotherapy for advanced breast cancer (Vogel et al., 2002). Combination of herceptin with several standard chemotherapeutic agents resulted in an ever greater tumor regression and survival (Slamon et al., 2001). Clinical trials are currently assessing combination therapy of STIs with anti-hormonal agents (Reviewed in Ellis, 2004).

Selenium in Cancer Therapy and Chemoprevention

Selenium is a trace and an essential micronutrient shown to have beneficial effects in cancer chemoprevention. Organic selenium agents, methylselenocysteine (MSC) and seleno-L-methionine (Se-Met) used in clinical chemoprevention studies are metabolized in tissues to the active selenium metabolite, methylselenol (Clark et al., 1996; Ip et al., 1999; Wang et al., 2002) (Figure 5). Methylseleninic acid (MSA), developed for assessing organic selenium effects in in vitro cell line experiments, is directly converted to the active metabolite, methylselenol, via a non-enzymatic reaction (Ip et al., 2000a) (Figure 5). Organic selenium compounds inhibit cellular proliferation and induce apoptosis via modulation of cell cycle regulatory proteins and activation of several caspases as well as reduction in bcl-2 (Dong et al., 2002a, b, 2003; Ip et al., 2000b, 2001; Jiang et al., 2001, 2002; Lu et al., 1995; Sinha et al., 1996, 1997, 1999, 2001; Wang et al., 2001c, 2002b; Zhu et al., 2002, 2003). A recent study demonstrated inhibition of AR signaling by MSA that decreases the growth of prostate cancer cell lines (Dong et al., 2004). An additional benefit of organic selenium compounds for chemoprevention is low
or absent toxicity (Clark et al., 1996; Ip et al., 2000a; Marshall, 2001; Nelson et al., 1999b). In contrast, inorganic selenium compounds such as selenite are genotoxic and no longer used for selenium chemoprevention (Ip et al., 1994; Medina et al., 2001; Patterson et al., 1997; Sinha et al., 1996).

In a landmark clinical trial Clark et al. demonstrated the chemopreventative effects of Se-Met against prostate, lung and colon cancer (Clark et al., 1996; Duffield-Lillico et al., 2002). The protective effects of selenium have been demonstrated on several other cancers such as breast and colorectal carcinomas (El Bayoumy et al., 2004; Patrick, 2004). Selenium use has been limited to the chemopreventative setting.

Recently, a synergistic interaction of organic selenium compounds with the topoisomerase 1 inhibitor, irinotecan on in vivo xenograft tumors was demonstrated (Cao et al., 2004). This study provides a strong rationale to utilize selenium as a novel therapy for overt cancer through combination with well-established chemotherapeutic and hormonal agents.
Figure 5. Selenium Activation

Metabolic pathways of organic selenium compounds to its active metabolite methylselenol in cancer chemoprevention.
Selective Estrogen Receptor Modulator Regulated Proteins in Endometrial Cancer Cells

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Abstract

Tamoxifen is the primary hormonal therapy for breast cancer and is also used as a breast cancer chemopreventative agent. A major problem with tamoxifen therapy is undesirable endometrial proliferation. To identify proteins associated with the growth stimulatory effects of tamoxifen in an ER-positive model, the present study profiled total cellular and secreted proteins regulated by estradiol and selective estrogen receptor modifiers (SERMs) in the Ishikawa endometrial adenocarcinoma cell-line using two-dimensional gel electrophoresis. Following 24-hour incubation with $10^{-8}$ M estradiol, $10^{-7}$ M 4-hydroxytamoxifen, or $10^{-7}$ M EM-652 (Acolbifene), nine proteins exhibited significant increase in expression. The proteins identified were heat shock protein 90-α, and -β, heterogeneous nuclear ribonucleoprotein F, RNA polymerase II-mediating protein, cytoskeletal keratin 8, cytoskeletal keratin 18, ubiquitin-conjugating enzyme E2-18kDa and nucleoside diphosphate kinase B. These protein profiles may serve as novel indices of SERM response and may also provide insight into novel mechanisms of SERM-mediated growth.
Introduction

Estrogens are vital regulators of growth and maintenance of female reproductive physiology and other non-reproductive tissues such as bone, cardiovascular tissue, and the central nervous system (Mueller and Korach, 2001). The specificity for estrogen action in tissues requires the expression of the type 1 nuclear receptor, estrogen receptor (ER) (Evans, 1988; Tsai and O'Malley, 1994). Two forms of ER have been identified, ER-α and ER-β, both the product of separate genes (Kuiper et al., 1996; Mosselman et al., 1996; Tremblay et al., 1997). Similar to all nuclear receptors, ER-α and ER-β are comprised of modular domains, each with its own functional properties (Kumar et al., 1987). The N-terminal A/B domain (NTD) contains a ligand-independent activation function 1 (AF-1) (Evans, 1988; Tora et al., 1989; Tsai and O'Malley, 1994; Webb et al., 1998). A centrally located DNA binding domain (DBD), contains two zinc fingers important for DNA binding (McKenna et al., 1999; Tsai and O'Malley, 1994). The hinge region, contains a nuclear localization sequence and the E/F domain (LBD) includes sites for hormone binding and ligand-dependent activation function 2 (AF-2) (McKenna et al., 1999; Tsai and O'Malley, 1994; Webb et al., 1998). ER is a ligand-dependent transcription factor, and upon estrogen binding the receptor either forms heterodimers consisting of ER-α -ER-β (Cowley et al., 1997; Ogawa et al., 1998; Pettersson et al., 1997) or homodimers of two ER-α or ER-β proteins. Upon dimerization, ER binds to the promoter region of target genes where subsequent recruitment of coactivators and local chromatin modifications facilitate gene transcription (Klinge, 2000; Klinge, 2001; McKenna et al., 1999; McKenna and O'Malley, 2000).
Estrogens play a major role in the development and growth of breast cancer and antiestrogen therapies have been devised to oppose estrogen action. Estrogen promotes cell proliferation that increases the chance for random genetic errors to be permanently introduced in the genome of somatic cells. As a treatment for breast cancer, the selective estrogen receptor modifier (SERM) tamoxifen binds with high affinity to the ER and blocks estrogen induced cellular growth (Dutertre and Smith, 2000; Jordan and Koerner, 1975). Tamoxifen is the most widely used hormonal therapy for breast cancer and has been approved as a chemopreventive agent in high risk women (Jordan, 2000). The major mechanism of action for tamoxifen is as an ER antagonist, although receptor-independent mechanisms have been proposed (Mandlekar and Kong, 2001). As an ER antagonist, tamoxifen inhibits estrogen dependent gene transcription that is required for cellular growth in breast tissue and breast-derived cell lines.

Although tamoxifen is an estrogen antagonist in the breast, tamoxifen displays estrogen-like uterotrophic effects in the endometrium (Harper and Walpole, 1967; Terenius, 1971). Tamoxifen therapy results in increased stromal thickening and endometrial hyperplasia and long-term tamoxifen treatment is associated with a 4 fold increase in endometrial cancer (Fisher et al., 1994; Fornander et al., 1989).

Several hypotheses have been proposed to explain the tissue specific actions of tamoxifen and other SERMs. Differential coregulator expression between tissues may account for SERM specificity. In experimental models, elevated corepressors facilitates the antagonist action of tamoxifen whereas an increase in coactivators promotes the tamoxifen agonist activity (Smith et al., 1997). It is also hypothesized that expression of
AF-1 interacting coactivators may drive the tissue-specificity of SERMs. Tamoxifen inhibits AF-2 (Berry et al., 1990), but AF-1 is not blocked suggesting that tamoxifen may display estrogen agonist action in tissues expressing AF-1 specific coactivators such as p68 helicase (Endoh et al., 1999) and steroid receptor RNA activator (SRA) (Lanz et al., 1999). Finally, differentially activated cellular signaling pathways in the uterus vs. the breast may contribute to SERM specificity. (Feng et al., 2001; Kato et al., 1995; Lee et al., 2000).

The problems with tamoxifen therapy have led to the development of new generation SERMs, such as EM-652, that were developed to limit the estrogen-like action of tamoxifen in the uterus. EM-652, the active metabolite of EM-800, does not display estrogen-like action in mouse mammary gland and endometrium (Labrie et al., 1999; Labrie et al., 2001; Luo et al., 1998; Martel et al., 1998; Sourla et al., 1997). In experimental cell culture models, EM-652 inhibits the growth of human breast and endometrial cancer cell lines (Couillard et al., 1998; Labrie et al., 1999; Labrie et al., 2001; Simard et al., 1997). However, unlike other pure antiestrogens, EM-652 preserves bone density in ovariectomized rats (Martel et al., 2000) making EM-652 a potentially attractive alternative to tamoxifen therapy.

Coinciding with the widespread use of tamoxifen therapy and the development of novel SERMs, relatively few SERM-regulated proteins have been identified. We set out to identify novel biomarker proteins that are either direct mediators or surrogate markers of the estrogen agonist action of tamoxifen and the antagonist action of EM-652 in Ishikawa endometrial cancer cells. By focusing on proteins that are regulated by SERMs
of varying estrogenicity using two-dimensional protein electrophoresis and mass spectrometry, we sought to identify proteins that may participate in novel pathways of tamoxifen sensitivity and resistance. Furthermore, these proteins may have predictive value for tamoxifen therapy and may themselves serve as novel tumor targets for therapy and tumor image analysis. By using this approach, we eliminated the problem of discordance between mRNA and protein inherent with the gene array approach.
Material and Methods

**MTT Assay to measure cell proliferation**

Ishikawa cells were plated in 24 well plates (6000 cells/well) and cultured in phenol red free DMEM (Gibco, Grand Island, NY) containing 2% fetal bovine serum that was charcoal stripped to remove endogenous steroids. 24 h after plating, the cells were incubated with vehicle, estradiol (10^{-8} M) (Sigma, St. Louis, MO), 4-hydroxytamoxifen (10^{-7} M and 10^{-10} M) (Sigma) or EM-652 (10^{-7} M and 10^{-10} M). The medium, estrogen and antiestrogens were replaced every two days for a total of 9 days incubation. After 9 days, the medium was replaced with 0.5mL of MTT reagent (ICN, Aurora, OH) (1mg/ml of 2,5-diphenyl tetrazolium bromide in PBS). After 1 h, the MTT reagent was removed and 200 µL of DMSO (Fisher Biotech, Fairlawn, NJ) was added to each well. The plates were read at a wavelength of 595nm.

**Luciferase assay to measure gene transcription.**

Ishikawa cells were plated in 6-well plates as described above. The cells were transfected with 500 ng of EREelb-luciferase reporter using Fugene transfection reagent (Roche, Madison, WI). 24 h post transfection, the cells were incubated with vehicle, estradiol (10^{-8} M), 4-hydroxytamoxifen (10^{-7} M) or EM-652 (10^{-7} M) for 24 h. The cells were scraped from the plates and centrifuged at 500 x g for 4 min and the resulting cell pellet was lysed with luciferase cell lysis buffer (Promega, Indianapolis, IN). Following centrifugation at 20,000 x g for 5 min to remove cellular debris, the supernatant was used for standard luciferase assay measured using a Lumat luminometer (Perkin Elmer,
Downers Grove, IL). The luciferase values were normalized to protein measured by Bradford assay (BioRad, Hercules, CA).

*RNA extraction and cDNA synthesis.*

Ishikawa cells were plated in 100 mm plates (2x10^6 cell/plate) in DMEM medium as described above. The cells were incubated with vehicle, estradiol (10^{-8} M), 4-hydroxytamoxifen (10^{-7} M) or EM-652 (10^{-7} M) for 2 hours. Cells were pelleted by centrifugation at 500 x g for 4 min and total RNA was extracted using Trizol (Invitrogen). Briefly, 300 µL of Trizol was added to the cell pellets and vortexed for 1 min. After incubation at room temperature for 10 min, 100 µL of 100% chloroform was added and the solution was vortexed for 15 seconds. The solution was incubated at room temperature for 3 min and then centrifuged for 10 min at 20,000 x g. The top aqueous layer was removed and 600 µL of 100% isopropanol was added. After vortexing, the solution was incubated at room temperature for 10 min followed by centrifugation at 20,000 x g for 10 min. The supernatant was removed and the RNA pellet was washed with 300 µL of 70% ethanol in DEPC water. The pellet was resuspended in DEPC water and the RNA concentration was quantified using a DU 640 spectrophotometer (Beckman Coulter, Palo Alto, CA). RNA was diluted to 52 ng/µL and reverse transcribed using TaqMan Kit reagents (Applied Biosystems, Foster City, CA). Briefly, 1 µL 10X Buffer, 2.2 µL MgCl₂, 2 µL dNTPs, 0.5 µL random hexamers, 0.2 µL RNase inhibitor, and 0.25 µL reverse transcriptase were added to 200 ng of diluted RNA for a final volume of 10 µL. Reverse transcription was performed using a PTC-0150 MiniCycler (MJ Research, Waltham, MA) using conditions described in the TaqMan Kit.
Real time RT-PCR.

Primers and probes were designed using Primer Express Version 2.0 (Applied Biosystems). All primers and probes, except GAPDH, were manufactured by Integrated DNA Technologies, Inc. (Coralville, IA) Primers and probes for GAPDH were designed and manufactured by Applied Biosystems. The probes were dual-labeled with the fluorescent dye 56-FAM at the 5’ end and 3BHQ-1 at the 3’ end.

**Progesterone Receptor (PR):**

FWD-5’-CTATGCAGGACATGACAACAAA-3’

REV-5’-TGCCTCTCGCTAGTTGATTAAG-3’

Probe-5’-56FAM CCTGACACCTCCAGTTTGTGCTGACAAG/3BHQ-1/-3’

**Lactoferrin (LF):**

FWD-5’-GACGAGCAGGGTGAGATTAAGTG-3’

REV-5’-ACCGAAGAACCCCCAGTGTA-3’

Probe-5’-/56FAM/TGCCCAACAGCAACGAGATACGTACGG/3BHQ-1/-3’

Fwd primer, rev primer and probe for PR, LF, or GAPDH and Taqman Universal Master Mix (Applied Biosystems) were added to 200ng of cDNA. Real-time RT-PCR was performed using a GeneAmp 5700 Sequence Detection System (Perkin Elmer). PCR conditions were as follows: samples were heated at 50° C for 2 min, then 95° C for 10 min, followed by 40 cycles of 95° C for 15 sec and 60° C for 1 min. Values were quantified using the Comparative CT method. All samples were normalized to GAPDH and calibrated to vehicle treated samples.
Two-dimensional gel electrophoresis

Ishikawa cells were plated in 150 mm plates (5 x 10^6 cells/plate) in DMEM containing 2% charcoal stripped serum for 48 h. After incubation the media was replaced with DMEM containing no serum and the cells were incubated with vehicle, estradiol (10^{-8} M), 4-hydroxytamoxifen (10^{-7} M) or EM-652 (10^{-7} M) for an additional 24 h. For the secreted proteins, the serum-free DMEM conditioned medium was removed following the 24 h incubation with estrogen and antiestrogens. The medium was dialyzed against 25mM ammonium bicarbonate, and then lyophilized. The lyophilized proteins were resuspended in 2D buffer (8 M urea, 2% chaps, 2% 3-10 non-linear carrier ampholytes, 60 mM DTT and a trace of bromophenol blue). To prepare proteins for the total cellular fraction, the medium was removed and the cells were washed with 1X PBS. Cells were scraped from the plates and centrifuged at 500 x g for 4 min. The resulting cell pellet was resuspended in 2D buffer and the cell extract was centrifuged at 100,000 x g for 30 min to remove cellular debris. Following protein determination by the Bradford assay, equal amounts of protein from each drug incubation were used to rehydrate the 3-10 non-linear immobilized pH gradient (IPG) strips overnight. The IPG strips were electrophoresed in the first dimension using a gradient program increasing to 3500V, for a total of 19.5 h using the Multiphor II Isoelectric Focusing System (Amersham, Uppsala, Sweden). Following first dimension electrophoresis, the IPG strips were equilibrated for 10 min in SDS PAGE equilibration buffer (50 mM tris-HCL, 6 M urea, 30% glycerol, 2% SDS, 1% DTT, and a trace of bromophenol blue). The equilibrated IPG strips were electrophoresed
in the 2\textsuperscript{nd} dimension on a 12% SDS PAGE gel for 14 h at 10 mA per gel. Proteins were visualized by either silver-stain or Colloidal Coomassie blue stain (Invitrogen).

\textit{Protein identification by mass spectrometry.}

From the 2D gels, protein spots that exhibited ligand-induced change in expression from at least three separate experiments were excised using a clean surgical steel blade. The gel piece was diced into 1 mm cubes and rehydrated in 0.1 M ammonium bicarbonate buffer containing 0.4 µg sequencing grade-modified trypsin at 37°C for 14 h. The tryptic peptides were extracted from the gel slices using 60% acetonitrile, 0.1% trifluoroacetic acid for 30 minutes at 30°C. The peptides were pooled and concentrated in a vacufuge to about 20 µl volume. Two µls of the digest was separated on a reverse phase column (Aquasil C18, 15 µm tip x 75 µm id x 5 cm Picofrit column, New Objectives, Woburn, MA) using acetonitrile/1% acetic acid gradient system (5-75% acetonitrile over 35 min followed by 95% acetonitrile wash for 5 min) at a flow rate of 250nl/min). The eluent (peptides) was directly introduced into an ion-trap mass spectrometer (LCQ-Deca XP Plus, ThermoFinnigan) equipped with a nano-spray source. The mass spectrometer was set for analyzing the positive ions and operated on double play mode in which the instrument was set to acquire a full MS scan and a collision induced dissociation (CID) spectrum on the most abundant ion from the full MS scan (relative collision energy \~30%). Dynamic exclusion was set to collect two CID spectra on the most abundant ion and then exclude it for 2 min. An initial database search of CID spectra was carried out against an indexed human database using TurboSEQUEST (BioworksBrowser v 3.0,
ThermoFinnigan). Peptide CIDS which showed Xcorr and ΔCn (delta Cn) of >2 and >0.2 respectively were considered positive and manually verified. Any un-interpreted CID spectra were searched using the MS-Tag provision of Protein Prospector (http://prospector.ucsf.edu), with default search settings, against human/mouse proteins in the non-redundant and/or SwissProt databases.

**Western Blot Analysis.**

Ishikawa cells were incubated with agents and protein extract was prepared as described above. 50 µg of each protein extract was separated by electrophoresis on an SDS-PAGE gel. The gel was transferred to nitrocellulose membrane, and blocked in 5% nonfat milk in tris-buffered saline/Tween 20 (TBST) (10 mM tris, pH 8, 150 mM NaCl, 0.1% Tween-20) for 1h at room temperature, followed by washes with 1x TBST. The membranes were incubated with primary antibodies in 5% dried milk/TBST at 4º C for 16 hours. Primary antibodies used were cofilin non-muscle isoform, cytokeratin 8/18, hnRNP F, UBCh7, NDPK B (Santa Cruz Biotechnology INC, Santa Cruz, CA) and HSP90 (Upstate Charlottesville, VA). All antibodies were diluted 1:1000 in 5% dried milk/TBST except cofilin and UBCh7, which were diluted 1:150 and 1:500, respectively. Following primary antibody incubation, the membranes were washed with TBST and incubated for 1 hour at room temperature with secondary biotinylated anti-rabbit, goat, or mouse at 1:2000 depending on the primary antibody used. Following TBST washes, the membranes were incubated with horseradish peroxidase streptavidin (1 µg/ml) for 1 hour at room temperature and washed with TBST. The signal was developed with addition of
enhanced chemiluminescence (ECL) solution (1:1 mixture of solution A+B (A: 9.9 ml of 0.1 M Tris pH 8.5 + 100 µl luminol + 44 µl P-coumaric, B: 10 ml of 0.1 M tris pH 8.5 + 6 µl hydrogen peroxide). The membranes were exposed to Kodak XOMAT film, and the signal was quantitated using a Kodak Digital Science 1D image analysis software station 440 system.

Data Analysis

All data are expressed as ± S.D. P values were calculated using one-way anova Dunnett’s T Test. $P<.05$ was considered significant.
Results

Estrogen receptor-regulated gene expression and cell growth in Ishikawa cells.

The Ishikawa cell line is a well-characterized, endometrial adenocarcinoma cell line in which tamoxifen exhibits similar agonist properties as estrogen. To validate earlier published observations on tamoxifen and estrogen effects in Ishikawa cells and to investigate the role of the SERM EM-652, Ishikawa cells were incubated with estradiol, 4-hydroxytamoxifen, EM-652 and ER-target gene expression and cell proliferation was assessed. Estradiol and 4-hydroxytamoxifen, activated the ERE2e1b-luciferase reporter but EM-652 antagonized the reporter in transfected cells (Fig.1 panel A). Similar results were observed using real-time RT PCR to measure endogenous ER-dependent gene expression, estradiol and 4-hydroxytamoxifen, but not EM-652, induced the lactoferrin and progesterone receptor genes in non-transfected cells (Fig. 1 panel B). When cell proliferation was assessed, estradiol and 4-hydroxytamoxifen, but not EM-652, induced Ishikawa cell proliferation (Fig. 1, panel C). These results confirm the relative agonist and antagonist actions of estradiol, 4-hydroxytamoxifen and EM-652 in the Ishikawa cell line providing an adequate model to correlate the relative agonist activity of these SERMs with the profile of proteins that are regulated following treatment with these agents.

Identification of proteins regulated by estrogen and SERMs in Ishikawa cells.
A two-dimensional electrophoresis/mass spectrometry proteomic approach was used to identify changes in protein expression following incubation with estradiol and SERMs in Ishikawa cells. All drug treatments were performed in duplicates and experiments were repeated at least three times. Only those protein spots that exhibited consistent change in expression over all experiments were excised and sequence identified by mass spectrometry. Inconsistently regulated protein spots were excluded from further analysis. Several proteins in the whole cell extract were down regulated following incubation of cells with estradiol and SERMs. However, there were inconsistencies in the magnitude of reduced expression over several independent experiments and these proteins were not pursued for further analysis. For this reason, only proteins that were induced by estradiol and SERMs were sequence identified since these proteins exhibited the greatest change in expression.

500 µg of whole cell lysate or 100 µg of proteins secreted into serum-free medium (Figs. 2 and 4, respectively) were separated by two-dimensional gel electrophoresis followed by visualization with colloidal Coomassie or silver staining. Visual analysis of the gels identified fourteen proteins that were differentially regulated by estradiol and SERMs in whole cell lysate and the secreted fraction. LC-Tandem-MS was used to sequence identify eight regulated proteins from whole cell lysate and one protein from the secreted fraction (data summarized in Table 1). In the whole cell lysate, three proteins were induced by both estradiol and 4-hydroxytamoxifen (Keratin type I cytoskeletal 18 (ck18), Heterogeneous nuclear ribonucleoprotein F (hnRNP F), and Keratin type II cytoskeletal 8 (ck 8)) (Fig. 3 panel B and C). Three proteins were induced by EM-652
alone: ubiquitin-conjugating enzyme E2-18kDa (UbcH7), RNA polymerase II subunit 5-mediating protein (RMP) and cofilin, non-muscle isoform (Fig. 3 panel D). Two proteins were induced by estrogen and EM-652: heat shock protein 90-α (HSP90-α) and heat shock protein 90-β (HSP90-β) (Fig. 3 panel A). Based on molecular weight and isoelectric point (pI) only the N-terminal region of RMP was identified possibly because of degradation. The c-terminal region of RMP was not detected possibly because of an acidic pI that was outside the pH range of the isoelectric focussing step.

In the secreted fraction, a total of six proteins were observed to be differentially regulated by estradiol and SERMs. Five proteins were induced by estradiol and 4-hydroxytamoxifen and one protein was downregulated by EM-652 alone. Only one protein could be identified because of the low protein present in the secreted fraction (required silver staining to visualize) and poor recovery of tryptic peptides due to silver stain. Several attempts were made at mass spectrometric identification, but only one of the protein spots was identified as nucleoside diphosphate kinase B (NDKB). NDKB was induced by estradiol and 4-hydroxytamoxifen (Fig. 4 panel E).

*Confirmation of estrogen and SERM regulated proteins by Western blot analysis.*

Western blot analysis and enhanced chemiluminescence quantitation was used to confirm the proteins identified by mass spectrometry sequencing. Ishikawa cells were incubated with estradiol and SERMs for 24 h. The cell extract and secreted proteins were prepared as described above. All drug treatments were performed in duplicates and experiments were repeated at least three times. The Western blot results were consistent
with the two-dimensional electrophoresis/mass spectrometry proteomic approach. In the whole cell lysate Ck8, Ck18, and hnRNP F were induced by estradiol and 4-hydroxytamoxifen (Fig 5 panel A and B). HSP90 was induced by estradiol and EM652 treatment (Fig 5 panel A) and UBCh7 and coflin were induced by EM652 alone (Fig 5 panel A and B). In the secreted fraction NDPK B was induced by estradiol and 4-hydroxytamoxifen (Fig 5 panel C). NDPK B was also induced in the whole cell lysate by estradiol and 4-hydroxytamoxifen (Fig 5 panel A and B.). RMP expression could not be confirmed by Western blot analysis because antibody to this protein is not currently available. Because of the increased sensitivity of Western blot analysis compared to Coomassie stained gels, the expression of Ck8, Ck18, HSP90 and coflin was observed in all treatment conditions. (Fig 5 panel A). Regardless, the relative induction of these proteins by estradiol and SERMs as measured by Western blot analysis was identical to that observed with Coomassie stained gels confirming the regulation of these proteins.
Discussion

Tamoxifen-induced growth of the endometrium is a major clinical problem for women undergoing tamoxifen therapy for breast cancer. We sought to identify proteins that were regulated by estrogen, tamoxifen (partial agonist), and EM-652 (antagonist) in the Ishikawa endometrial adenocarcinoma cell line. Using two-dimensional proteomic technology, nine estradiol- and SERM-regulated proteins were identified from whole cell lysate and the secreted fraction of Ishikawa cells. Regulation of eight of these proteins were confirmed by Western blot analysis (antibody to RMP was not available for Western blot analysis). Among the identified proteins were chaperones proteins, RNA and RNA polymerase binding proteins, cytoskeletal proteins, a ubiquitin ligase and a multi-functional kinase. With the exception of HSP90, Ck 8, and Ck 18, all of the proteins are novel estradiol and/or SERM regulated proteins. HSP90 has been shown to be induced by estrogen in the endometrium and in several uterine-derived cell lines (Ramachandran et al., 1988; Shyamala et al., 1989; Tang et al., 1995; Wu et al., 1996). Although Ck 8 and Ck 18 were shown to be regulated by estrogen and tamoxifen in a breast cancer cell line (Coutts et al., 1996), this is the first study to show estrogen and tamoxifen regulation of Ck 8 and Ck 18 in an endometrial-derived cell line.

HSP 90 α and β are chaperone proteins important in the proper folding of numerous proteins and important in nuclear receptor signaling (for review see ref. (Pratt and Toft, 1997; Walter and Buchner, 2002)). The present finding of estradiol-dependent induction of HSP 90 α and β in Ishikawa cells is consistent with the findings of several other reports providing an internal control for estrogenic response (Ramachandran et al.,
1988; Shyamala et al., 1989; Tang et al., 1995; Wu et al., 1996). Remarkably, we also found induction of HSP-90 α and β with EM-652, an ER antagonist in Ishikawa cells. This is the first indication that ER ligands that exhibit opposite agonist and antagonist properties in the same cell line can induce expression of the same chaperone protein. The function of the estrogenic regulation on HSP90 is still unknown but it may serve as a negative feedback to down regulate estrogen signaling in the endometrium. In addition, induction of HSP90 by EM-652 could attenuate estrogen signaling in the endometrium. Further studies will explore the function for HSP90 induction by these two ligands.

Estradiol and 4-hydroxytamoxifen, unlike EM-652, exhibit agonist properties in Ishikawa cells and three proteins (Ck 8, Ck 18 and hnRNP F) were induced with these agonist ER ligands. Cytokeratins are structural proteins involved in cellular organization (for review see ref. (Kirfel et al., 2003)). There is also evidence that cytokeratins are important in modulating external stimuli and signal cascades (for review see ref. (Paramio and Jorcano, 2002)). The related protein, cytokeratin 19 (Ck 19), is regulated by estrogen in breast cancer cell lines (Choi et al., 2000). Ck 8 and Ck 18 were shown to be regulated by estrogen in T47D cells, a breast cancer cell line in which tamoxifen is an ER antagonist unlike its action in Ishikawa cells. When T47D cells were grown in estrogen-depleted medium, expression of Ck 8 and Ck 18 was lost but expression was restored when exogenous estrogen was added to the medium. Interestingly, incubation of T47D cells with tamoxifen caused a reduction of Ck 8 and Ck 18 protein expression (Coutts et al., 1996). Taken together with the results of the present study in Ishikawa cells, Ck 8 and
Ck 18 could serve as prospective biomarkers for the mixed agonist/antagonist action of tamoxifen.

hnRNP F was also induced by both estradiol and 4-hydroxytamoxifen in Ishikawa cells. Over twenty members of the hnRNP family have been identified. These proteins are important in gene expression, pre-mRNA splicing, silencing, packaging and transport (Carson et al., 1998; Grabowski, 1998; Mayeda and Krainer, 1992; Nakielny and Dreyfuss, 1997; Ostareck et al., 1997). hnRNP proteins contain RNA binding motifs with different substrate binding specificities (Matunis et al., 1993). hnRNP F binds with high affinity to polyG sequences (Matunis et al., 1994). At this time, a role for hnRNP F in ER signaling has not been identified, although other hnRNP family members are regulated by estrogen and may bind to other nuclear receptors (Arao et al., 2002; Powers et al., 1998). Recently, Auboeuf et al. found that steroid receptors mediate RNA processing from steroid response elements (Auboeuf et al., 2002), suggesting that nuclear receptors may recruit hnRNP-containing protein complexes involved in both transcription and RNA processing.

Three proteins (UbcH7, RMP, and Cofilin, non-muscle isoform) were induced only with the antagonist EM-652. Our finding of induction of UbcH7 is the first report of a protein involved in the ubiquitin pathway that is specifically regulated by a SERM. UbcH7 is an E2-type ubiquitin carrier protein involved in the ubiquitin-mediated proteolytic pathway (for review see ref. (Weissman, 2001)). Several proteins involved in the ubiquitin-mediated proteolytic pathway have also been shown to be important in estrogen signaling (Lonard et al., 2000; Nawaz et al., 1999a). A functional disruption of
the ubiquitin ligase E6-associated protein (E6-AP) in mice reduced estrogen mediated growth of the uterus (Smith et al., 2002). UbcH7 is a functional binding partner of E6-AP (Nuber et al., 1996) and is also important in the degradation of E6-AP and transcriptional intermediary factor-2 (TIF-2), a nuclear receptor coactivator (Yan et al., 2003). These findings taken together with results from the present study suggest that UbcH7 may be an important mediator of estrogen and SERM signaling in endometrial cells.

RMP was induced only with EM-652 although the result could not be confirmed by Western blot analysis. Interestingly, RMP binds to subunit 5 of RNA polymerase and inhibits RNA polymerase II function (Dorjsuren et al., 1998). It is possible that induction of RMP by EM-652 could participate in the antagonism of ER dependent gene expression by EM-652. Cofilin was also selectively induced by EM-652. Cofilin is a multifunctional protein important in actin depolymerization and in cell locomotion (Chen et al., 2001; Maciver and Weeds, 1994; Nagaoka et al., 1995). The increase of cofilin expression may be followed by changes in actin organization required for EM652-mediated repression of cell growth.

In the secreted fraction of Ishikawa cells, NDPK B was induced by estradiol and 4-hydroxytamoxifen. Previous studies have identified NDPK B as a secreted protein (Chopra et al., 2003; Donaldson et al., 2002; Willems et al., 2002) although it is possible that induction of the cytoplasmic levels (Fig 5 panel C.) led to increased extracellular shedding. NDPK B is encoded by the NM23-h2 gene. Currently, eight separate genes have been cloned which belong to the NM23 family. NDPK is involved in the transfer of phosphates from NTPs to NDPs (Walinder, 1968). Several studies have confirmed the
metastatic-suppressing property of NDPK A and NDPK B (Baba et al., 1995; Leone et al., 1991; Leone et al., 1993; Nakamori et al., 1993; Steeg et al., 1988). In addition, NDPK B has also been shown to increase cell proliferation (Caligo et al., 1995; Caligo et al., 1996). The NDPK A gene is directly regulated by estrogen (Lin et al., 2002) but the present study is the first report to find the NDPK B protein induced by both estrogen and tamoxifen. Since estrogen and tamoxifen increase cell proliferation in Ishikawa cells, it will be interesting to assess whether NDPK B participates in estrogen- and tamoxifen-dependent proliferation in Ishikawa and other endometrium-derived cell lines. Future studies may determine whether NDPK B could serve as a biomarker for the tamoxifen resistant phenotype. Since NDPK B is a secreted protein it could easily be assayed by non-invasive procedures.

In summary, using two-dimensional proteomic technology we have identified nine estradiol and SERM regulated proteins from the whole cell lysate and secreted fraction in the Ishikawa cell line. It is likely that other proteins are regulated by estrogen and SERMs but are not detected by the proteomic approach. A disadvantage of this approach is visualization and identification of low abundance proteins. However, a proteomic-based approach allows direct analysis of gene expression at the protein level. By correlating the estradiol and SERM-induced proteins to effects of these ligands on growth stimulation and gene expression in Ishikawa cells, we may identify proteins involved in novel mechanisms of SERM action. These proteins may further be useful as biomarkers for the development of SERM resistance. Further studies will be performed to
elucidate the roles of these proteins in estrogen and/or SERM-regulated gene expression and cellular growth.

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Figure legends

Figure 1. The effect of estradiol and SERMs on gene expression and proliferation in Ishikawa cells. (A) Ishikawa cells (2 x 10^5 cells/well) were transfected with 500 ng of ERE2e1b-luciferase reporter and 50 ng of expression vector for hERα. 24 hours after transfection, cells were incubated with vehicle (lane 1, Veh) 10^{-8} M estradiol (lane 2, E_2), 10^{-7} M 4-hydroxytamoxifen (lane 3, 4-OHT) or 10^{-7} M EM652 (lane 4) for 24 hours. Standard luciferase assays were performed on cell extracts as described in the Materials and Methods and the mean value of triplicate samples ± S.D. are plotted. (*) = P<.05. (B) The effect of estradiol and SERMs on endogenous ER-dependent genes. Ishikawa cells (2 x 10^6 cells/plate) were incubated with vehicle (lane 1) 10^{-8} M estradiol (lane 2), 10^{-7} M 4-hydroxytamoxifen (lane 3) or 10^{-7} M EM-652 (lane 4) for 4 hours. Expression of the lactoferrin and progesterone receptor genes was measured by real time RT-PCR as described under Materials and Methods. Expression was normalized to GAPDH, and values were plotted relative to vehicle treated samples (set to 1). Each bar represents the mean value ± S.D. (•): P<.05. (C) Growth response of Ishikawa cells following incubation with estradiol and SERMs. Ishikawa cells incubated with vehicle (lane 1), 10^{-8} M estradiol (lane 2), 10^{-7} M 4-hydroxytamoxifen (lane 3), 10^{-10} M 4-hydroxytamoxifen (lane 4), 10^{-7} M EM-652 (lane 5), and 10^{-10} M EM-652 (lane 6) for 9 days as described in the Materials and Methods. Cell growth was assessed by the MTT assay. Each bar represents the mean value ± S.D. (•) = P<.05.

Figure 2. Two-dimensional gel images of total cellular proteins from Ishikawa cells incubated with estradiol and SERMs. Ishikawa cells (5 x 10^6 cell/plate) were incubated
for 24 hours with (A) vehicle, (B) $10^{-8}$ M estradiol, (C) $10^{-7}$ M 4-hydroxytamoxifen or (D) $10^{-7}$ M EM-652. 500 µg of total cellular protein was separated in the first dimension by isoelectric focussing using IPG pH 3-10 NL 2D PAGE (12.5% T), and the 2nd dimension by 12% SDS-PAGE. The proteins were visualized by colloidal Coomassie blue stain. The regions enlarged in Fig. 3 are boxed and numbered 1-4.

Figure 3. Enlarged regions of the two-dimensional gel images of total cellular lysate from Figure 2. The dotted boxes indicate no change in protein expression relative to vehicle; solid boxes indicate a change in protein expression relative to vehicle (A) Enlarged gel image corresponding to region 1 in Figure 2. Induction of HSP 90 α & β following 24 hour incubation with $10^{-8}$ M estradiol and $10^{-7}$ M EM-652. (B) Enlarged gel image corresponding to region 2 in Figure 2. Induction of CK18 and hnRNP F after 24 hour incubation with $10^{-8}$ M estradiol and $10^{-7}$ M 4-hydroxytamoxifen. (C) Enlarged gel image corresponding to region 3 in Figure 2. Induction of CK8 after 24 hour incubation with $10^{-8}$ M estradiol and $10^{-7}$ M 4-hydroxytamoxifen. (D) Enlarged gel image corresponding to region 1 in Figure 2. Induction of cofilin, UBCh7, and RBP5-mediating protein after 24 incubation with $10^{-7}$ M EM-652.

Figure 4. Two-dimensional gel images of secreted proteins from Ishikawa cells. Ishikawa cells ($5 \times 10^6$ cell/plate) were cultured in serum-free DMEM and incubated for 24 hours with (A) vehicle, (B) $10^{-8}$ M estradiol, (C) $10^{-7}$ M 4-hydroxytamoxifen, or (D) $10^{-7}$ M EM-652. The secreted proteins were isolated as described under Materials and Methods. 100 µg of protein was separated in the first dimension by isoelectric focussing using IPG pH 3-10 NL 2D PAGE (12.5% T) and in the 2nd dimension by 12% SDS-
PAGE. Proteins were visualized by silver stain. The region enlarged in panel E is boxed and numbered 1. (E) Enlarged gel image corresponding to region 1 in Figure 2 Panel A, B C, D. Induction of NDPK B after 24 hour incubation with $10^{-8}$ M estradiol and $10^{-7}$ M 4-hydroxytamoxifen.

**Figure 5.** Western blot analysis of estradiol and SERM regulated proteins. (A) Following a 24 incubation with vehicle, $10^{-8}$ M estradiol, $10^{-7}$ M 4-hydroxytamoxifen or $10^{-7}$ M EM-652, total cellular protein extract was prepared from Ishikawa cells for Western blotting analysis membrane as described in the Material and Methods. The membranes were probed with antibodies against hnRNP F, NDPK B, Ck8/18, cofilin, HSP90 and UBCh7. Representative Western blots from one of three separate experiments. (B). Quantitation of the Western blot signal normalized to β-actin. The graphs indicate the fold difference of each protein expression versus vehicle (set at a value of 1) following measurement of Western blot signal intensity by Kodak Image Station 440CF and 1D Image Analysis software. In each independent experiment, duplicate samples for each treatment condition were normalized to the β-actin levels. The experiment was performed three times and the mean values from these experiments ± S.D. is reported in the graphs. Asterisk indicates statistically significant difference from vehicle treatment analyzed by one-way ANOVA Dunnett’s T Test. (*p<0.05).
Table 1.
Identification of proteins regulated by estradiol and SERMs in whole cell lysate

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<th>Con</th>
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Identification of proteins regulated by estradiol and SERMs in the secreted fraction

| Nucleoside Diphosphate Kinase B | 17.3 | 8.52 | ↑   | ↑   | —   | 2    | P22392 |

Est = estradiol
Tam = 4-hydroxytamoxifen
Accession# according to SwissProt database
↑ = Increase in expression relative to control
— = No change relative to control
Fig. 1
Fig. 2 SDS-PAGE

A. Vehicle  B. Estradiol

C. Tamoxifen  D. EM-652

pH 3 → 10
Fig. 3
Fig. 4

A. Vehicle  B. Estradiol

C. Tamoxifen  D. EM652

E. Veh  E₂  4-OHT  EM652
Fig. 5
C.

Fig. 5
Title: The Src Kinase Pathway Promotes Tamoxifen Agonist Action in Ishikawa Endometrial Cells through Phosphorylation-Dependent Stabilization of Estrogen Receptor α Promoter Interaction and Elevated SRC-1 Activity.

Abbreviated Title: Src Kinase Potentiation of Tamoxifen Agonist Action

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Abstract

Tamoxifen is the most widely used selective estrogen receptor modulator (SERM) for breast cancer in clinical use today. However, tamoxifen agonist action in endometrium remains a major hurdle for tamoxifen therapy. Activation of the non-receptor tyrosine kinase src promotes tamoxifen agonist action, although the mechanisms remain unclear. To examine these mechanisms, the effect of src kinase on estrogen and tamoxifen signaling in tamoxifen-resistant Ishikawa endometrial adenocarcinoma cells was assessed. A novel connection was identified between src kinase and serine 167 phosphorylation in estrogen receptor-α (ER) via activation of AKT kinase. Serine 167 phosphorylation stabilized ER interaction with endogenous ER-dependent promoters. Src kinase exhibited the additional function of potentiating the transcriptional activity of Gal-SRC-1 and Gal-CREB binding protein (CBP) in endometrial cancer cells while having no effect on Gal-PCAF and Gal fusions of the other p160 coactivators GRIP1 (TIF-2/NcoA-2/SRC-2) and AIB-1 (RAC-3/ACTR/SRC-3). Src effects on ER phosphorylation and SRC-1 activity both contributed to tamoxifen agonist action on ER-dependent gene expression in Ishikawa cells. Taken together, these data demonstrate that src kinase potentiates tamoxifen agonist action through serine 167-dependent stabilization of ER promoter interaction and through elevation of SRC-1 and CBP coactivation of ER.
Introduction

Estrogen Receptor-α (referred to as ER) is a member of the nuclear receptor superfamily of transcription factors that is activated by ligand binding of estrogenic and other ligands. ER is also activated by ligand-independent mechanisms that involve crosstalk from peptide and growth factor signal transduction pathways. In the ligand-dependent pathway, estrogen crosses the cell membrane and binds to the ligand-binding domain (LBD) of the ER. Upon ligand binding, receptor interaction with heat shock protein-90 (HSP-90) and other chaperone proteins is lost and the ER dimerizes and binds to estrogen responsive elements (ERE) in the promoters of ER-dependent genes (1;2). ER activation results in recruitment of coactivators of the p160 family (SRC-1 (NcoA-1) (3;4), GRIP1 (TIF-2/NcoA-2/SRC-2) (5;6), AIB-1 (RAC-3/ACTR/SRC-3) (7-11)) and CREB binding protein (CBP) (12) that modify chromatin structure and facilitate mRNA transcription (13).

Both ligand-dependent and ligand-independent activation of ER is modulated by receptor phosphorylation. ER is a phosphoprotein and upon ligand binding receptor phosphorylation is enhanced (14-16). The major phosphorylation sites of ER reside in the N-terminal domain (NTD) at serines 104, 106, 118, and 167. Mutation of serines 104, 106, and 118 to alanine results in a general decrease in ER transcriptional activity (14). Phosphorylation at serine 167 was shown to be important in DNA binding by the receptor (17-19). Numerous signaling pathways regulate ER phosphorylation. Serines 104/106/118 are targets of the mitogen activated protein kinases (MAPK) family (20-22), and serine 167 has been shown to be phosphorylated by AKT (22-25).
In addition to ER, cellular signaling pathways phosphorylate coregulator proteins. SRC-1 is phosphorylated on seven sites in vivo all of which contain a consensus sequence for proline-directed protein kinases (26). In addition, protein kinase A (PKA) enhanced ligand-independent activation of PR through phosphorylation of SRC-1 (27). GRIP-1 and AIB-1 are phosphorylated in vitro by MAP kinases (28;29). In breast cancer cells the transcriptional activity of AIB-1 is increased by MAPK phosphorylation which stimulates the recruitment of histone acetyltransferases (28). In addition to coactivators, cellular signaling cascades also phosphorylate corepressors. Interaction of N-CoR and SMRT with PR decreased after incubation with 8-bromo cAMP (30). In vitro phosphorylation by MEKK decreased the interaction of SMRT with thyroid receptor (31). Interaction of the corepressor N-CoR with the ER is decreased by treatment with forskolin and epidermal growth factor (32).

Coregulator interactions with ER are important mechanisms mediating selective estrogen receptor modulator (SERM) action. Tamoxifen inhibits coactivator recruitment and promotes corepressor association with ER to inhibit estrogen-dependent gene transcription in tamoxifen-sensitive breast cancer cells (33). With regard to this mechanism, elevation of coactivator proteins and reduction in corepressor proteins may be associated with the progression of breast cancer to a more malignant and tamoxifen-resistant phenotype. In a mouse model for human breast cancer, decreased levels of N-CoR correlated with the onset of resistance to tamoxifen therapy (32). Jepsen et. al. (34) showed that the absence of N-CoR in mouse embryo fibroblasts isolated from the N-CoR null mouse allowed 4-hydroxytamoxifen to act as a full agonist for ER-dependent
4-hydroxytamoxifen did not activate ER-dependent transcription in wild-type mouse embryo fibroblasts containing normal N-CoR levels. The hypothesis that elevated coactivator levels promote tamoxifen action may be relevant in the uterus since tamoxifen is an agonist in normal uterus (35;36) and endometrial-derived cell lines (37-39). However in contrast to normal versus malignant breast, we did not detect any distinct differences between coactivator and corepressor expression patterns in endometrium with both coactivator and corepressor levels increased during the progression from normal to malignant endometrium (40).

Several reports suggest activated cellular signaling pathways in the uterus may promote the tissue specific agonist effects of tamoxifen. Phosphorylation at serine 118 in ER increased transcriptional activation in response to estrogen and tamoxifen (21). MEKK1 increased the agonistic activity of tamoxifen implicating downstream MAPKs in regulating tamoxifen agonist/antagonist action (41). Src kinase can enhance the transcriptional activity of the tamoxifen-ER complex through the MAPK pathway (42). Finally, although elevated Src kinase activity is detected in late stage steroid resistant breast cancer (43) we recently found that src kinase activity is higher in normal compared to malignant endometrium (44). This may indicate a role for activated Src kinase in promoting tamoxifen agonist action in normal endometrium.

Src kinase is a non-receptor tyrosine kinase and a proto-oncogene that is deregulated in several different cancers (45). Src kinase activates several signaling cascades including the MAPK and AKT pathways (46-48) both of which impact on ER-dependent signaling. Feng et al. (42) reported two independent pathways for src kinase
potentiation of estrogen and tamoxifen signaling in HeLa cells: 1) an ER-dependent pathway that is partially dependent on phosphorylation of serine 118 and; 2) an ER-independent pathway implicating src kinase effects on coregulators. However, the precise mechanisms and specific phosphorylation sites through which src kinase modulates ER signaling are incompletely defined, most likely because multiple proteins important for ER signaling are targeted by src. For a more thorough, mechanistic understanding of the role of src kinase in tamoxifen agonist action in the endometrium, the effect of src on both ER and coactivator activity and phosphorylation was investigated. This report describes src kinase induced phosphorylation of ER serine 167 via AKT kinase as a major mechanism for src potentiation of ER signaling through stabilization of ER promoter interaction. Src kinase also specifically elevated SRC-1 activity through multiple phosphorylation sites. Finally, cell-type specific targeting of CBP by src kinase was demonstrated.
Results

Src kinase modulates estradiol and 4-hydroxytamoxifen action on ERE<sub>2</sub>e1b-luciferase reporter in an endometrial-derived cell line. Interest in src kinase potentiation of tamoxifen agonist activity in endometrium stemmed from our observation that tamoxifen-resistant endometrial cancer cell lines (Ishikawa and HEC1A) exhibited elevated active src compared to tamoxifen-sensitive breast cancer cell lines (MCF-7 and T47D) (Fig. 1A). To further confirm the role of endometrial src kinase in tamoxifen agonism, Ishikawa cells were transfected with an ERE<sub>2</sub>e1b-luciferase reporter and either incubated with SU6656, a specific src kinase inhibitor (Fig. 1B) or cotransfected with a dominant active mutant of src kinase (Fig. 1C) followed by measurement of luciferase expression. Incubation of Ishikawa cells with 4-hydroxytamoxifen resulted in similar activation of the reporter (1.5 –2 fold relative to vehicle) as occurred following incubation of cells with estradiol (3-4 fold relative to vehicle) (Fig. 1B). Both 4-hydroxytamoxifen and estradiol activation of the reporter was significantly reduced in cells incubated with SU6656 (Fig. 1B, lanes 4 and 6) These data coincided with reduced src kinase activation in cells incubated with SU6656 as measured by Western blot analysis using a phospho-specific src kinase antibody (data not shown). Transfection with dominant active src kinase increased basal luciferase expression almost two fold indicating ligand-independent activation of ER (Fig.1C, lane 2). Dominant active src also potentiated estradiol and 4-hydroxytamoxifen stimulated luciferase expression 4-5 fold and 3 fold relative to vehicle, respectively (Fig 1C, lanes 4 and 6)). To confirm that the inhibition of src kinase downregulates estradiol and 4-hydroxytamoxifen activation of the
ERE<sub>e1b</sub>-luciferase reporter, siRNA was used to reduce src protein levels. Western blot analysis confirmed that src protein levels were reduced more than 65% in Ishikawa cells transfected with siRNA for src kinase (siRNA-src) (Fig. 1D). When siRNA-src was cotransfected with ERE<sub>e1b</sub>-luciferase reporter, the basal activity was reduced by more than 50% (Fig. 1E, lane 2) and both the estradiol and 4-hydroxytamoxifen induction of the reporter was decreased to levels equivalent to vehicle alone (Fig. 1E, lanes 4 and 6 vs. lane 1). To rule out nonspecific effects of SU6656, siRNA-src and dominant active c-src on general transcription, these agents were incubated with or cotransfected into cells with a constitutively active RSV-luciferase reporter (Fig. 1F). These agents had no significant effect on the RSV-luciferase reporter suggesting that src kinase specifically modulates estradiol and 4-hydroxytamoxifen signaling. The requirement of ER for the src kinase effects on estradiol and 4-hydroxytamoxifen signaling was demonstrated by incubation of Ishikawa cells with the pure antiestrogen ICI 182,780. ICI 182,780 blocked dominant active src potentiation of estradiol and 4-hydroxytamoxifen activation of the reporter (data not shown). Furthermore as previously reported (42), dominant active src potentiated estradiol and 4-hydroxytamoxifen activation of ERE<sub>e1b</sub>-luciferase only in cells cotransfected with ER (data not shown).

**Src kinase modulates estradiol and 4-hydroxytamoxifen action on endogenous ER-regulated genes.** Real time RT-PCR was used to measure expression of two well-characterized ER-regulated genes, c-myc and pS2. The c-myc gene is regulated by ER through both a consensus ERE as well as a nonconsensus sequence hypothesized to be an
AP1 site. ER indirectly associates with the AP-1 site through direct binding with AP1 proteins (33;49). The pS2 gene contains consensus EREs in the 5’ flanking region of the gene (50). C-myc and pS2 gene expression were measured following incubation with estradiol and 4-hydroxytamoxifen alone or in combination with the src kinase inhibitor SU6656. Both estradiol and 4-hydroxytamoxifen induced c-myc gene expression 2-3 fold and 4-5 fold relative to vehicle, respectively (Fig. 2A, lanes 3 and 5). The pS2 gene was regulated by ligands in a similar manner (Fig. 2B). Ligand dependent induction of c-myc and pS2 was reduced more than 50% by coincubation of cells with SU6656 (Fig. 2A, B lanes 4 and 6).

Cells that exhibit high transfection efficiency were needed to measure the role of dominant active src on endogenous ER-regulated genes. Although the Ishikawa cells were not appropriate for these experiments due to low transfection efficiency, the well-characterized ER-negative HeLa cells exhibited more than 90% transfection efficiency (data not shown) and were therefore useful to measure effects of both dominant active src and various ER\(\alpha\) mutants. It should be noted that unlike Ishikawa cells, HeLa cells transfected with ER expression plasmid do not exhibit tamoxifen agonist activity. High efficiency transfection of HeLa cells with ER was sufficient to initiate estradiol, but not 4-hydroxytamoxifen, induction of endogenous c-myc and pS2 gene expression 6-7 fold and 4-5 fold relative to vehicle, respectively (supplemental data Fig. S-1A, S-1B). Similar to results in Ishikawa cells, cotransfection of HeLa cells with dominant active src potentiated basal, and estradiol-regulated c-myc and pS2 expression. Remarkably, although 4-hydroxytamoxifen alone had no effect on gene expression and dominant
active src alone resulted in only 1.5 fold induction over vector transfected cells, the combination of tamoxifen and dominant active src induced c-myc and pS2 gene expression to a level comparable to estradiol alone (supplemental data Fig. S-1). These data demonstrate that src kinase promotes tamoxifen agonist action on endogenous genes in a cellular context in which tamoxifen is an antagonist. Although, HeLa and Ishikawa cells differ with respect to tamoxifen agonism/antagonism, ER\textsubscript{α} negative Ishikawa variants transfected with ER do not exhibit tamoxifen agonist activity and in this regard are similar to HeLa cells (51). Although tamoxifen agonism/antagonism cannot be directly compared in HeLa and Ishikawa cells, it is clear that for each line activation of the src pathway potentiates tamoxifen agonist activity above control.

**Src kinase partially mediates its effect on ER-dependent transcription through phosphorylation at both serine 118 and serine 167.** As previously reported (42), src kinase-mediated effects on ER-regulated gene expression was localized to the NTD of ER (data not shown). Consequently, phosphorylation within this domain was measured using phospho-specific antibodies for serine 104/106, 118, and 167. In ER-transfected HeLa cells, estradiol increased phosphorylation at serines 118 and 167, an effect that was inhibited by co-incubation with SU6656 (Fig. 3A). A band was not detected when Western blots were probed with the antibody specific for phospho-104/106 on ER (data not shown). Similar results were found in Ishikawa cells in which both estradiol and 4-hydroxytamoxifen consistently increased serine 118 and serine 167 phosphorylation, an effect that was blocked by co-incubation with SU6656 (data not shown). To further
confirm these results, point mutations of each phosphorylation site were prepared and
cotransfected with ERE\textsubscript{2e1b}-luciferase into ER-negative HeLa cells to assess whether
dominant active src could potentiate estradiol-mediated induction of the reporter. As
previously described (14), mutation of the ER NTD phosphorylation sites decreased basal
and estradiol-stimulated reporter activation (Fig. 3B) although the mutations did not
affect ER protein levels (Fig. 3C). Dominant active src potentiated estradiol-stimulated
luciferase expression by wild type ER, ER/S104A, and ER/S106A mutants, but not the
ER/S118A and ER/S167A mutants (Fig. 3B). S118A and ER/S167A also blocked the
ability of dominant active src to induce tamoxifen activation of ERE\textsubscript{2e1b}-luciferase in
HeLa cells (data not shown). Taken together, these data demonstrate that src kinase can
mediate effects on ER-dependent gene transcription through phosphorylation of serine
118 as previously described (42) and also through phosphorylation at serine 167.

**Inhibition of src kinase disrupts interaction of ER with endogenous promoters.**
Previous work has established that src kinase increases serine 118 phosphorylation
through activation of the ERK1/2 pathway in HeLa cells (42). However, there are no
reports of src kinase modulating phosphorylation of ER serine 167 and no studies have
examined regulation of ER signaling in endometrial-derived, tamoxifen resistant cells.
Because serine 167 is reported to regulate *in vitro* DNA binding by ER (17-19), the role
of src kinase in ER interaction with endogenous promoters was assessed by ChIP assays.
To assess several paradigms of ER-dependent gene expression, three promoter elements
were examined: 5’ consensus ERE on the pS2 promoter, the consensus ERE on the c-myc
promoter (52), and the nonconsensus ERE on the c-myc promoter (33). In Ishikawa cells, estradiol and 4-hydroxytamoxifen increased ER recruitment to the ERE sequence in the pS2 promoter and this ligand-dependent recruitment was blocked by co-incubation with src kinase inhibitor SU6656 (Fig. 4, left panel). Conversely, SU6656 did not affect the well-characterized interaction of SP1 with the hTERT promoter (53) demonstrating that the effect of SU6656 on ER was not a result of general inhibition of transcription factor binding to DNA (data not shown). Quantitative ChIP assays verified the increased promoter recruitment of ER by estradiol and 4-hydroxytamoxifen and the inhibition of this recruitment by SU6656 (Fig. 4, right panel). Identical results to those described in Figure 4 were found for the consensus ERE and the nonconsensus ERE on the c-myc promoter (Supplemental data, Fig. S-2). These data indicate that src kinase modulates ligand-dependent ER promoter recruitment. Interestingly, estradiol resulted in quantitatively greater promoter recruitment of ER than 4-hydroxytamoxifen, an observation that has not previously been reported.

**Src kinase partially mediates its effect on ER-dependent transcription through PI3K/AKT kinase. Inhibition of AKT disrupts ER promoter interaction.** Since src mediates its effects on ER through serine 167 phosphorylation, it was important to identify the downstream effectors of src that lead to altered serine 167 phosphorylation. Although src has been shown to activate AKT pathways (48) and *in vitro* phosphorylation of serine 167 by AKT has been reported (23-25), the connection between src kinase and serine 167 phosphorylation has not been demonstrated.
Consequently it was important to assess whether AKT could mediate src effects on ER-dependent gene transcription in Ishikawa cells. Western blot analysis revealed an increase in phospho-AKT when cells were incubated with estradiol and 4-hydroxytamoxifen (Fig. 5A left and right panel, lanes 3 and 5) and this induction was blocked by co-incubation with SU6656 (Fig. 5A, left and right panel, lanes 4 and 6). To determine whether AKT inhibition would block the dominant active src potentiation of estradiol- and 4-hydroxytamoxifen-mediated gene transcription, Ishikawa cells were incubated with either a specific AKT inhibitor or an inhibitor of phosphoinositide 3-OH kinase (PI3K), a kinase upstream of AKT. Ishikawa cells were cotransfected with ERE2e1b-luciferase and dominant active src and incubated with wortmannin (PI3K inhibitor) or an AKT inhibitor alone or in combination with estradiol or 4-hydroxytamoxifen. Incubation with wortmannin or the AKT inhibitor completely blocked dominant active src potentiation of the estradiol and 4-hydroxytamoxifen stimulated ERE2e1b-luciferase (Fig. 5B and C, lanes 8 and 12) suggesting that src kinase signals to ER through AKT. Control Western blots indicated that AKT activity was inhibited following incubation with PI3K inhibitor or the AKT inhibitor and neither agent affected the constitutively active RSV-luciferase reporter in transfected Ishikawa cells (data not shown). To determine if inhibition of AKT alters ER interaction with endogenous promoters, ChIP assays were performed in Ishikawa cells on the three promoter elements described in Figure 4. The estradiol- and 4-hydroxytamoxifen-induced recruitment of ER to the ERE sequence in the pS2 was blocked by co-incubation with wortmannin (Fig. 5D). Conversely, wortmannin did not affect SP1 interaction with the
hTERT promoter (data not shown). Identical results to those described in Figure 5 were found for the consensus ERE and the nonconsensus ERE on the c-myc promoter (Supplemental data, Fig. S-3). Taken together, these data demonstrate that src kinase potentiates estradiol and 4-hydroxytamoxifen action by modulating ER promoter interaction via the PI3K/AKT pathway.

**Serine 167 of the ER regulates promoter interaction by the receptor.** To directly investigate the role of ER phosphorylation in promoter interaction, quantitative ChIP assays were performed in ER-negative HeLa cells transfected with ER phosphorylation site mutants. HeLa cells were cotransfected with dominant active src and either wild type ER, ER/S118A or ER/S167A and promoter interaction was measured as described above. There was a decrease in basal and estradiol-stimulated recruitment of ER/S167A to the pS2 promoter compared to wild type ER and ER/S118A (Fig. 6). A previous report described a similar result for ER/S167A using *in vitro* interaction with a synthetic ERE sequence (19). Dominant active src increased basal level and estradiol-stimulated recruitment of wild type ER (Fig. 6 lanes 2, 4) and ER/S118A (Fig. 6 lanes 6, 8) to the pS2 promoter but had no effect on ER/S167A recruitment (Fig. 6 lanes 10, 12). Similar results were found with the c-myc promoter (Supplemental data Fig. S-4). These results demonstrate that src kinase modulates ER promoter interaction through phosphorylation of serine 167. Interestingly, mutation of serine 118 to alanine had no effect on ER promoter interaction suggesting that mechanisms other than DNA binding are responsible for regulation of ER signaling by serine 118 phosphorylation.
Src kinase potentiates SRC-1 and CBP transactivation in endometrial-derived cell lines. To measure the effects of src kinase on coactivator activity, SRC-1, SRC-2 (TIF-2/GRIP-1), SRC-3 (AIB-1/RAC-3/ACTR), PCAF, and CBP were fused to the GAL4-DBD expression plasmid and cotransfected with a Gal response element reporter (GalRE-luciferase) into Ishikawa and HEC-1A cells. All GAL constructs except GAL-PCAF activated GalRE-luciferase above activity of the GAL-DBD alone. When dominant active src or siRNA-src was cotransfected with the GAL-coactivator constructs, only GAL-SRC-1 and GAL-CBP transactivation function was both potentiated by dominant active src and inhibited by siRNA-src in Ishikawa cells (Fig. 7A). The effect of the src kinase inhibitor SU6656 on the GAL-coactivator activities was also assessed. The src inhibitor reduced GAL-SRC-1 and GAL-CBP activity while having no effect on GAL-SRC-2, GAL-SRC-3, and GAL-PCAF activity (Fig. 7B). These assays were performed in an additional endometrial-derived cell line, HEC1A. When the GAL-coactivator constructs were cotransfected with either the dominant active src or siRNA-src, only SRC-1 activity was potentiated with the dominant active src and repressed with the siRNA-src (Fig 7C). Src kinase inhibitor SU6656 inhibited the activities of all the GAL-coactivator constructs, possibly through nonspecific effects since the GAL-DBD alone was also inhibited (Fig. 7C). Because src kinase selectively activated the p160 family coactivator SRC-1, it was of interest to determine whether specific SRC-1 phosphorylation sites were mediating the increased SRC-1 activity. Seven SRC-1 phosphorylation sites (26) were mutated to alanine (S372A, S395A, S517A, S569A,
S1033A and T1179A/S1185A) and each single phosphorylation site mutant was fused to the GAL4-DBD. Dominant active src kinase potentiated activity of each single site SRC-1 phosphorylation mutant in both Ishikawa and HEC1A cells (data not shown). These findings argue against the notion that a single SRC-1 phosphorylation site mediates src kinase potentiation of SRC-1 activity although the possibility of different combinations of sites was not examined. The preceding data confirm that src kinase specifically modulates the activity of SRC-1 and CBP in Ishikawa cells. Interestingly, CBP activity was not altered by src kinase in HEC1A cells suggesting cell specific targeting by src kinase (Fig. 7C).

**Dominant active src kinase potentiates the coactivation function of SRC-1 on ER-dependent gene transcription.** The experiments measuring the effect of src kinase on GAL-SRC-1 activity were performed in the absence of ER (as shown in Fig. 7). It was of interest to determine whether the src potentiation of SRC-1 activity contributed to the overall potentiation of ER-dependent transcription by src. To assess the SRC-1 contribution, SRC-1, ER, and ERE2e1b-luciferase were cotransfected in HeLa cells and luciferase activity was measured. To specifically assess the contribution of SRC-1, the ER/S167A mutant, and not wt ER, was used since dominant active src does not potentiate the activity of this mutant in HeLa cells (see Fig. 3B). Therefore, any potentiation by dominant active src would be the result of SRC-1 coactivation and could not be attributed to ER. Although estradiol induced ER/S167A-dependent luciferase expression 2-3 fold relative to vehicle (Fig. 8A, lane 2), dominant active src kinase failed to potentiate the
estradiol-stimulated luciferase expression (Fig 8A, lane 4). HeLa cells express low levels of endogenous SRC-1 when compared to Ishikawa cells (data not shown). It was therefore likely that restoring SRC-1 levels in HeLa cells comparable to levels detected in Ishikawa cells might uncover a src kinase contribution to ER/S167A activity that occurred specifically through SRC-1. High efficiency transfection in HeLa cells resulted in SRC-1 levels comparable to levels detected in Ishikawa cells (data not shown). Basal level luciferase activity was increased two fold over vector-transfected cells and SRC-1 coactivated estradiol-dependent reporter activation 5-6 fold over control (Fig. 8A lanes 5-6). Only under conditions where SRC-1 was expressed did dominant active src potentiate reporter activation under both basal conditions (10-12 fold over control) and estradiol treatment (20 fold over control) (Fig. 8, lanes 7 and 8). These data confirm that src kinase elevation of SRC-1 activity contributes to the overall potentiation of ER-dependent gene transcription by specifically enhancing SRC-1 coactivation of ER.
A major concern with tamoxifen therapy for breast cancer is acquired resistance as well as estrogen-like agonist action in the endometrium resulting in an increased risk for development of endometrial cancer (35;36;54;55). Tamoxifen resistance and tamoxifen agonist activity may result from the altered activity of cellular kinases that change the phosphorylation status of ER and coregulators. This study describes a mechanistic role of the non-receptor tyrosine kinase, src, in promoting the agonist action of tamoxifen in the endometrium. In endometrial-derived Ishikawa cells, tamoxifen-dependent activation of reporter gene (ERE\textsubscript{2}e1b-luciferase) and endogenous gene (pS2, c-myc) transcription was inhibited or activated by using agents that reduce or elevate src kinase activity, respectively. The data demonstrated that src kinase modulates ER-dependent transcription by altering the phosphorylation of serines 118 and 167 of ER. Phosphorylation at serine 167 occurred via src-induced activation of the PI3K/AKT pathway, that in turn regulated promoter interaction at both consensus and nonconsensus EREs. Phosphorylation at serine 118 had no effect on ER promoter interaction. To our knowledge, this is the first report of the connection between activated src kinase and serine 167 phosphorylation of ER. This phosphorylation underlies a major mechanism for src kinase regulation of ER action by modulating ER interaction with endogenous promoters. Src kinase also elevated SRC-1 activity that was sufficient to enhance coactivation of the ER. Taken together, this report finds that src kinase potentiation of
tamoxifen agonist action results from multiple phosphorylation and activity changes in receptor and coactivators rather than a single phosphorylation event in one protein.

When serine 118 or serine 167 was mutated to alanine, the transcriptional activity of ER was severely abrogated and no src kinase-mediated potentiation of estradiol action was detected. However when these same ER mutants were assayed for promoter interaction by ChIP, only the serine 167 mutant exhibited a decrease in promoter interaction. These results indicate that although dephosphorylation at either site results in decreased ER-dependent transcription, the mechanisms through which each site regulates transcription are quite different. Previous reports and the present study have shown that phosphorylation of serine 167 promotes DNA binding (17-19), whereas serine 118 may have a role in coactivator binding. Dutertre et al. (56) have shown that interaction of ER with SRC-1 or CBP is decreased when serines 104/106/118 are mutated to alanine. In addition Endoh et al. (57) has shown that phosphorylation at serine 118 increases the interaction of ER with p68 RNA helicase providing direct evidence for serine 118 in coactivator binding. Thus, src kinase may affect two distinct modes of ER action; promoter recruitment and coactivator recruitment.

There is increasing evidence that bidirectional crosstalk between ER and cellular kinase pathways may lead to tamoxifen resistance (58;59). We found an increased expression of activated src in tamoxifen resistant endometrial cell lines when compared to tamoxifen sensitive breast cancer cell lines (Fig 1A). Furthermore, in HeLa cells in
which tamoxifen is an antagonist (60;61), increasing src activity promoted tamoxifen agonist action on gene transcription to a similar extent as estrogen treatment (supplemental data Fig. S-2). Although c-myc and pS2 genes were not induced by tamoxifen, transfection with a dominant active src promoted tamoxifen agonist action above that of the dominant active src alone. By comparison, incubation with src kinase inhibitor SU6656 further potentiated the antiestrogen effect of tamoxifen by reducing basal gene transcription. These findings suggest that src kinase may play an important role in the development of tamoxifen resistance.

In addition to receptor phosphorylation, src kinase impacts coactivator activity and increases SRC-1 coactivation of ER-dependent gene transcription (Fig. 7 and 8). In Ishikawa cells, src kinase specifically enhanced SRC-1 and CBP activity but not the activities of SRC-2 (TIF-2/GRIP-1), SRC-3 (AIB-1/RAC-3/ACTR), and PCAF (Fig. 7A and B). Src kinase selective modulation of SRC-1 activity is interesting in light of a recent report by Shang et al. (33) indicating that elevated SRC-1 in endometrial cancer cell lines was correlated with tamoxifen agonist activity when compared to breast cancer cell lines that express lower levels of SRC-1 and exhibit tamoxifen antagonist action. The authors further demonstrated that SRC-1, but not SRC-2 or SRC-3, was important for the tamoxifen agonist activity in Ishikawa cells. This report by Shang et al (33) and the present study suggest that SRC-1 is a key protein involved in tamoxifen agonist action in endometrium not only through elevated expression levels, but also as a selective target of src kinase pathways. These results, taken together with our recent finding of
elevated src kinase activity in endometrium (44) suggest that SRC-1 may be a preferential substrate of src kinase in the endometrium possibly because of higher SRC-1 expression in this tissue.

CBP activity was also elevated by src kinase in a cell specific manner. In Ishikawa cells, src kinase increased CBP activity (Fig 7A and B) whereas in HEC1A endometrial adenocarcinoma cells, CBP activity was not altered by src kinase (Fig 7C). A number of proteins have been reported as substrates of src kinase (62;63). Substrate specificity by src could simply be related to the relative expression levels of these substrates in a given tissue/cell line. It is likely that src kinase targets a distinct repertoire of coregulators in different tissues and src effects on these coregulators could influence estrogen and tamoxifen signaling. This may also explain the differences between the present study examining coactivator activity in endometrial-derived cells and a previous report by Feng et al (42) in which it was reported that src kinase increased the activities of SRC-2 (TIF-2/GRIP-1) and CBP in ER-negative HeLa cells.

Although src kinase increased the activity of SRC-1 and enhanced its coactivation function for the ER, no single SRC-1 phosphorylation site was responsible for the src potentiation of SRC-1 activity. Several possibilities may explain these results. Since all seven SRC-1 phosphorylation sites contain consensus sequences for serine/threonine-proline directed kinases (26) it is possible that only one or few kinases contribute to steady state SRC-1 phosphorylation. In addition, six of seven SRC-1 sites can be
phosphorylated in-vitro by ERK-2 (26) a known downstream effector of src kinase (64). In this regard, it is likely that src kinase increases phosphorylation at multiple SRC-1 sites and that no single phosphorylation event is sufficient to reproduce the src kinase potentiation of ER-dependent gene transcription. Another possibility is that activation of src kinase mediates a dephosphorylation event on SRC-1 that is required for SRC-1 potentiation of tamoxifen agonist action. Finally, a different protein in the SRC-1 complex may be a direct or indirect substrate of src kinase. These possibilities are currently being assessed.

In summary, this report has demonstrated that multiple proteins and phosphorylation sites are substrates of src kinase during potentiation of tamoxifen agonist activity in endometrial-derived cells. The major mechanism by which src potentiates tamoxifen agonist action is likely through phosphorylation at serine 167 that stabilized interaction of ER with promoters of ER-dependent genes. Phosphorylation at ER serine 118 and phosphorylation of SRC-1 and possibly CBP also contribute to src potentiation of ER-dependent gene transcription (Fig. 9A). In the absence of src kinase activation, serine 167 phosphorylation is decreased resulting in reduced ER promoter interaction. Dephosphorylation at serine 118 may lead to loss of other downstream transcriptional effects. Inhibition of src kinase also decreased the intrinsic activities of SRC-1 and CBP further contributing to the reduction in ER-dependent gene transcription (Fig. 9B). Future work will focus on identifying the ER and coregulator phosphorylation fingerprints associated with tamoxifen agonist/antagonist effects on specific genes.
These studies may provide the mechanistic groundwork for rational design of preclinical therapeutic strategies designed to inhibit src kinase to circumvent tamoxifen resistant breast cancer and relieve the deleterious estrogen-like side effects of tamoxifen and other SERMs in non-target tissues.
Materials and Methods

Cell lines: Ishikawa and HEC1A were grown at 37°C with 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM) (Cellgro) supplemented with 5% fetal bovine serum (FBS) (Gibco) and 1% penicillin-streptomycin (Gibco). MCF-7 were grown at 37°C with 5% CO₂ in DMEM supplemented with 10% FBS, 4 mM of glutamine (Gibco), and 1% penicillin-streptomycin. HeLa cells were grown at 37°C with 5% CO₂ in DMEM supplemented with 10% FBS, 4 mM of glutamine and 1% penicillin-streptomycin. T47D were grown at 37°C with 5% CO₂ in RPMI 1640 (Gibco) supplemented with 10% FBS, 1% penicillin-streptomycin, and 5µg/mL of insulin (Sigma).

Plasmids: Mutagenesis of ER/S104A, ER/S106A, ER/S118A, and ER/S167A was performed using the Stratagene Quick Change Kit. ER sequence and mutations were confirmed by DNA sequencing. ER-ABCD and ER-CDEF domain mutants were constructed by PCR amplifying nucleotides, which correspond to amino acids 1-302 for ABCD and 180-593 for CDEF. The resulting amplicons were subcloned into pCR3.1 vector. pBABE dominant active src (src Y527F) was a gift from Dr. Kavita Shah (Genomics Institute of the Novartis Research Foundation). SRC-1 and Gal-SRC-1 were constructed as previously described (27). Gal-SRC-2 and Gal-SRC-3 were gifts from Dr. Carolyn Smith (Baylor College of Medicine). Gal-SRC-1/S372A, Gal-SRC-1/S395A, Gal-SRC-1/S517A, Gal-SRC-1/S569A, Gal-SRC-1/S1033A, and Gal-SRC-
1/T1179/S1185A were constructed by digesting pCR3.1-SRC-1/S372A, -SRC-1/S395A, -SRC-1/S517A, -SRC-1/S569A, -SRC-1/S1033A, and -SRC-1/T1179/S1185A at AccI sites and subcloning into Gal-SRC-1 expression vector. siRNA for c-src was constructed by annealing the sense oligo 5′GCAGACATA
GAAGAGCCAATTCAAGAGATGTGGCTC TTCTATGTCTGCTTTTTT 3′ to the antisense oligo 5′ AATTAAAAAGCAGACATAGAAGAGCCAATCTCTTGGAA
TTGGCTCTTTCTATGTCTGCCGCGCC 3′ and ligating the annealed oligos into the pSilencer 1.0 U6 vector (Ambion).

**Luciferase assay:** Ishikawa, HEC1A or HeLa cells were plated in 6-well plates (2×10^5 cells/well) and cultured in phenol red free DMEM containing 2% FBS that was charcoal stripped to remove endogenous steroids. The cells were transfected with expression vector as indicated in the figure legends using Fugene transfection reagent (Roche). 24 h posttransfection, the cells were incubated with vehicle, estradiol (10^{-8} M) (Sigma), or 4-hydroxytamoxifen (10^{-7} M) (Sigma) for 24 h. In experiments in which 1µM wortmannin (Upstate Biotechnology), 1µM SU6656 (Calbiochem) or 10µM AKT inhibitor III (Calbiochem) was used, the cells were incubated for 2 h with the inhibitors followed by incubation with estradiol (10^{-8} M) and 4-hydroxytamoxifen (10^{-7} M) for 24 h. Luciferase expression was measured and normalized as described previously (65).

**Real-Time RT-PCR:** Ishikawa and HeLa cells were plated in 100mm plates (2×10^6 cells/plates) in 2% stripped FBS in DMEM. Ishikawa cells were maintained in the
stripped media for 3 days until 90% confluency. For HeLa cells, 24h post-plating the cells were transfected with expression vector for hER (500ng/plate), and dominant active src (3µg/plate) or empty vector (3µg/plate) and maintained in 2% stripped FBS in DMEM for an additional 48h. The cells were incubated with vehicle, estradiol (10^{-8} M), or 4-hydroxytamoxifen (10^{-7} M) for 6h. In experiments in which 1µM SU6656 was used, the cells were incubated for 2 h with the inhibitor followed by incubation with estradiol (10^{-8} M) and 4-hydroxytamoxifen (10^{-7} M) for 6 h. Total mRNA was extracted from the cell pellet, reverse transcribed and gene expression was measured by real-time RT-PCR as described previously (40). Briefly, total RNA was extracted using Trizol (Invitrogen) according to the manufacturer instructions. 200ng of mRNA was reverse transcribed using Taqman Kit reagents (Applied Biosystems). Primers and probes for GAPDH were designed and manufactured by Applied Biosystems and primers and probes for c-myc and pS2 were synthesized by Integrated DNA Technologies, Inc.

C-myc:
FWD-5’-CGTCTCCACACATCAGCACAA -3’
REV-5’-TGTTGGCAGCAGGATAGTCCTT -3’
Probe-5’-56FAM/ACGCAGCCTCCCTCCACTC/3BHQ-1/-3’

pS2:
FWD-5’-CGTGAAAGACAGAATTGTGGTTTT-3’
REV-5’-CGTCGAAACAGCAGCCCTTA-3’
Fwd primer, rev primer, and probe for c-myc, pS2, or GAPDH and Taqman Universal Master Mix (Applied Biosystems) were added to 200ng of cDNA. Real-time RT-PCR was performed using a GeneAmp 5700 Sequence Detection System (Perkin Elmer).

**Chromatin Immunoprecipitation (ChIP) Assay:** Ishikawa and HeLa cells were plated in 100mm plates (2×10^6 cells/plates) in 2% stripped FBS in DMEM. Ishikawa cells were maintained in the stripped media for 3 days until 90% confluent. Ishikawa cells were incubated with vehicle, estradiol (10^{-8} M), or 4-hydroxytamoxifen (10^{-7} M) for 2.5 h. In experiments in which 1µM wortmannin or 1µM SU6656 was used, the cells were incubated for 2 h with the inhibitor followed by incubation with estradiol (10^{-8} M) or 4-hydroxytamoxifen (10^{-7} M) for 2.5 h. Ishikawa cells were washed twice with ice cold phosphate buffered saline (PBS) and fixed with formaldehyde (1% final concentration) for 10 min at room temperature. The cells were washed twice with ice cold PBS and then collected in 100mM Tris-HCl (pH 9.0) and 10mM dithiothreitol (DTT). Ishikawa cells were incubated at 30°C for 15 min. Cells were rinsed twice with ice cold PBS, and a nuclear extraction was performed by swelling the cells on ice in 20 volumes of nuclei extraction buffer (5 mM piperazine-N, N’-bis 2-ethanesulfonic acid (pH 8.0) (PIPES), 85 mM KCl, 0.5% Nonidet P-40, and protease inhibitor cocktail (Sigma)) for 30 min. Nuclei were collected by centrifugation at 250 × g for 10 min. The nuclear pellet was washed once with nuclei extraction buffer and resuspended in 200 µl of ChIP lysis buffer (1%
SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1, and protease inhibitor cocktail). For HeLa cells, 24h post-plating the cells were transfected with expression vector for either ER or ER phosphorylation mutants (500ng/plate), and either dominant active src (3µg/plate) or empty vector (3µg/plate) and maintained in 2% stripped FBS in DMEM for an additional 48h. The cells were incubated with vehicle, estradiol (10^{-8} M), or 4-hydroxytamoxifen (10^{-7} M) for 2.5h. In experiments in which 1µM SU6656 was used, the cells were incubated for 2 h with the inhibitor followed by incubation with estradiol (10^{-8} M) or 4-hydroxytamoxifen (10^{-7} M) for 2.5 h. HeLa cells were washed twice with ice cold PBS and fixed with formaldehyde (1% final concentration) for 10 min at room temperature. The cells were washed twice with ice cold PBS. The cells were then collected in 100mM Tris-HCl (pH 9.0) and 10mM DTT and incubated at 30°C for 15 min. Following the 15 min incubation, the cells were rinsed twice with ice cold PBS, and lysed in ChIP lysis buffer. The soluble chromatin for Ishikawa and HeLa cells were sonicated three times for 10 sec at setting 4 (Fisher Sonic Dismembrator, Model 550) and centrifuged at 20,000 × g for 15 min to remove cellular debris. The supernatant was diluted 10 fold in ChIP dilution buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.1 and protease cocktail inhibitor) followed by preclearing with 10µL of normal mouse IgG (Santa Cruz) and 60µL of a 50% gel slurry of protein A-sepharose beads (Amersham Biosciences). The beads were prepared by reswelling in PBS and then blocking overnight in 0.1% bovine serum albumin (BSA) in PBS. To 1.5 mL of packed beads, 600µg of sonicated salmon sperm DNA and 1.5 mg of BSA was added to a final volume of 3mL in 10mM Tris-HCl (pH 8.0), 1mM EDTA, and .05%
sodium azide giving a final 50% gel slurry. After preclearing, the beads were microfuged for 1 min at 4000 × g and 20µL of supernatant was set aside for input. 10µL of anti-ERα D-12 antibody or SP1 antibody (Santa Cruz) was added to the supernatant and the samples were immunoprecipitated overnight at 4°C. Precipitates were washed for 5 min each wash first in low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), then in high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl), and finally in LiCl buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1). Precipitates were then washed 3 × 5 min with TE buffer (10mM Tris-HCl (pH 8.0), 1mM EDTA). Precipitated chromatin complexes were removed from the beads and formaldehyde cross-linking was reversed by incubation for 6h at 65°C in 100 µL of 1% SDS, 0.1 M NaHCO₃ with vortexing several times throughout the incubation period. DNA was purified with a QIAquick Spin Kit (Qiagen) according to manufacturer’s instructions. 10µL of extracted DNA was amplified using Accuprime taq polymerase (Invitrogen), and visualized by ethidium bromide stained 1.5% agarose gels.

c-myc nonconsensus ERE region:
FWD-5’- AGGCGCGCGTAGTTAATTCAT-3’
REV-5’- CGCCCTCTGCTTTGGGA-3’

c-myc ERE region:
FWD-5’- GATCCTCTCTCGCTAATCTCC-3’
REV-5’- CGCTGAAATTACTACAGCGAG-3’

pS2 ERE region:
FWD-5’-GGCCATCTCTCACTATGAAATCACTTCT -3’
REV-5’- GGCAGGCTCTGTGGTCTTTAAAGAGCG-3’

HTERT SP1 region:
FWD-5’-TGCCCCTTCACCTTCCAG-3'
REV-5’-CAGCGCTGCTTAAAGAGCG-3’

Quantitative ChIP Assays: Chromatin was prepared as described above. 10µL of extracted DNA was used in a real-time RT-PCR reaction for the detection of c-myc and pS2 promoters.

c-myc nonconsensus ERE region:
FWD-5’- GGTAGGCGCGCTAGT TAAT -3’
REV-5’-GGCGAGCCGACTCTCT -3’
Probe-5’-56FAM/ ATGCGGCTCTTTACTCAATCATCCTAGAC/3BHQ-1/-3’

c-myc ERE region:
FWD-5’- GGCTGGCGGGAAAAAGA -3’
REV-5’-GGCAGCGCGCAGCCT -3’
Probe-5’-56FAM/ATGCGGCTCTTTACTGTTTACATCCTAGA3/3BHQ-1/-3’

pS2 ERE region:
FWD-5’-TCAGATCCCTCAGCCAAGATG -3’
REV-5’- TGGTCAAGCTACATGGAAGGATT-3’
Probe-5’-56FAM/CCTCACCACATGTCGTCTCTGTCT/3BHQ-1/-3’

Values were normalized to input samples.

**Western Blot Analysis:** Ishikawa and HeLa cells were plated and transfected as described above. For the detection of phospho-ER and phospho-AKT, Ishikawa cells were serum deprived for 24 hours. Following serum deprivation, the cells were stimulated by incubation with vehicle, estradiol (10^{-8} M), or 4-hydroxytamoxifen (10^{-7} M) for 30 min. In experiments in which 1 \mu M SU6656 was used, the cells were incubated for 2 h with the inhibitor followed by incubation with estradiol (10^{-8} M) and 4-hydroxytamoxifen (10^{-7} M) for 30 min. In experiments in which kinase inhibitor efficacy was assessed, Ishikawa cells were serum deprived for 24 hours. Following serum deprivation, cells were incubated with either vehicle, 1 \mu M SU6656, 1 \mu M wortmannin, or 10 \mu M AKT inhibitor, and then stimulated by incubation with FBS (20% final concentration) for 30 min. The cells were lysed in high salt extraction buffer (10 mM Tris-HCl pH 8, 0.4 M NaCl, 2 mM EDTA, 2 mM EGTA, 50 mM potassium phosphate, 50 mM sodium fluoride, 10 mM \beta-mercaptoethanol, 0.1% triton X-100, 0.2% protease inhibitor cocktail, and 0.1% PMSF). For detection of phospho-AKT and SRC-1 in
Ishikawa cells, ER and SRC-1 in HeLa cells and active src in MCF-7, T47D, HEC1A, and Ishikawa cells, whole cell lysate was used by lysing the cells in high salt extraction buffer. 50 µg of whole cell or nuclear lysate was prepared for Western blotting as previously described (44). Briefly, proteins were separated by electrophoresis on a SDS-PAGE gel. The gel was transferred to nitrocellulose membrane, and the membrane was blocked in 5% nonfat milk in tris-buffered saline/Tween 20 (TBST) (10 mM tris, pH 8, 150 mM NaCl, 0.1% Tween-20) for 1h at room temperature, followed by washes with 1x TBST. The membranes were incubated with primary antibodies against ER (Novacastra), phospho-ER 104/106, phospho-ER 118, phospho-ER 167, non-phospho-527 src, phospho-AKT, AKT, (Cell Signaling Technology) src (Upstate Biotechnology), and SRC-1 and β-actin (Santa Cruz). Following the primary antibody incubation, horseradish peroxidase conjugated secondary antibody (Vector) was incubated for 1h at room temperature in 5% nonfat milk in TBST. The signal was developed by addition of enhanced chemiluminescence (ECL) solution. The membranes were exposed to Kodak XOMAT film, and/or the signal from the membrane was quantitated using a Kodak Digital Science 1D image analysis software station 440 system.

Validation of siRNA-src: Ishikawa cells were plated in 100mm plates (2×10^6 cells/plates) in 5% FBS in DMEM. Ishikawa cells were cultured until cells reached 90% confluency. Ishikawa were transfected with either siRNA-src or vector (20µg/plate) using Lipofectamine 2000 (Invitrogen) according to manufacturer’s
instructions. 72 hours posttransfection the cells were harvested for Western Blot analysis measuring src levels as described above.

**Data Analysis**: All data are expressed as ± S.D. P values were calculated using Anova Dunnett’s T Test and Independent T Test. P<.05 was considered significant.

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Figure Legends

Fig. 1. Src kinase regulates ER-dependent transcription of ERE$_2$e1b-luciferase in Ishikawa cells. (A) Increased expression of activated src kinase in endometrial- versus breast-derived cell lines. MCF-7, T47D, HEC1A and Ishikawa cells (2 x 10$^6$ cells/plate) were plated in 2% charcoal stripped serum for 3 days. Protein extract was prepared from the cells and used for Western blot analysis to measure levels of activated src kinase as described in the Material and Methods. Quantitation of the Western blot signal was normalized to the Western blot signal for total src and β-actin levels (right panel). Each bar represents the mean value ± S.D. (§) = $P<.05$ compared to MCF-7 and T47D cells. (B and C) Ishikawa cells (2 x 10$^5$ cells/well) were transfected with ERE$_2$e1b-luciferase reporter (0.5µg/well). 24 hours posttransfection cells were incubated with (A) 1µM SU6656, a specific src kinase inhibitor, for 2 h or (B) cells were cotransfected with dominant active src kinase (Dom) (0.5µg/well) or empty vector pBABE (0.5µg/well). Following incubation with inhibitor or 24 hours posttransfection, cells were incubated with 10$^{-8}$ M estradiol (E$_2$) or 10$^{-7}$ M 4-hydroxytamoxifen (Tam) for 24 hours. Standard luciferase assays were performed on cell extracts in triplicate as described in the Materials and Methods. Each bar represents the mean value ± S.D. (§) = $P<.05$ compared to estradiol or tamoxifen incubated samples alone. (†) = $P<.05$ compared to untreated (Con). (D) Western blot analysis of src kinase levels following transfection of a siRNA specific for src kinase (siRNA-src). The siRNA-src was constructed as described in the Material and Methods. Ishikawa cells (2 x 10$^6$ cells/plate) were transfected with siRNA-
src (20µg/Plate). 72 h posttransfection, protein extract was prepared from Ishikawa cells and used for Western blot analysis to measure expression levels of src kinase as described in the Material and Methods. Quantitation of the Western blot signal was normalized to β-actin (right panel). Each bar represents the mean value ± S.D. (*) = P<.05. (E) siRNA-src decreases estradiol and 4-hydroxytamoxifen activation of ERE2e1b-luciferase reporter. Ishikawa cells were plated as described above and cotransfected with ERE2e1b-luciferase reporter (0.5µg/well) and siRNA-src (0.5µg/well) or empty vector (0.5µg/well). 72 hours posttransfection, cells were incubated with 10⁻⁸ M estradiol (E₂) or 10⁻⁷ M 4-hydroxytamoxifen (Tam) for 24 hours. Standard luciferase assays were performed on cell extracts in triplicate as described in the Materials and Methods. Each bar represents the mean value ± S.D. (*) = P<.05 compared to estradiol or tamoxifen incubated samples alone. (†) = P<.05 compared to untreated (Con) samples. (F) SU6656, dominant active src, or siRNA-src had no effect on a constitutively active RSV-luciferase reporter. Ishikawa cells were plated as described above and transfected with a RSV-luciferase reporter (0.5µg/well) and incubated with 1µM SU6656 or cotransfected with either siRNA-src (0.5µg/well) or dominant active src (Dom). 24 hours posttransfection or treatment, standard luciferase assays were performed on cell extracts in triplicate as described in the Materials and Methods.

Fig. 2. Activation or inhibition of src kinase regulates estradiol- and 4-hydroxytamoxifen-dependent c-myc and pS2 gene expression. Ishikawa cells (2 x 10⁶ cells/plate) were incubated with 1µM SU6656 for 2 h. Following incubation with the
inhibitor, cells were incubated with $10^{-8}$ M estradiol ($E_2$) or $10^{-7}$ M 4-hydroxytamoxifen (Tam) for 6 h. Expression of (A) c-myc and, (B) pS2 genes was measured by real time RT-PCR as described in the Materials and Methods. Expression was normalized to GAPDH and each bar represents the mean value ± S.D. ($\ast$) = $P<.05$ compared to estradiol and 4-hydroxytamoxifen incubated samples alone. ($\dagger$) = $P<.05$ compared to untreated (Con) samples.

Fig. 3. Src kinase induces phosphorylation of serines 118 and 167 of ER. (A) HeLa cells ($2 \times 10^6$ cells/plate) were transfected with ER (0.5µg/plate). 24 h posttransfection the HeLa were incubated with 1µM SU6656 for 2 h. Following incubation with the inhibitor the cells were incubated with $10^{-8}$ M estradiol ($E_2$) for 30 min. Protein extract was prepared from HeLa cells and used for Western blot analysis to measure levels of phosphorylated serines 104/106, 118 and 167 on ER as described in the Material and Methods. Protein levels of total ER and β-actin were used as loading controls. (B) Mutation of serine 118 and serine 167 to alanine on ER inhibits dominant active src potentiation. HeLa cells ($2 \times 10^5$ cells/well) were cotransfected with ERE$_2$e1b-luciferase reporter (0.5µg/well), either dominant active src (0.5µg/well) (Dom) or empty vector pBABE (0.5µg/well), and either wild type ER or phosphorylation mutants ER/S104A, ER/S106A, ER/S118A, or ER/S167A (0.05µg/well). 24 hours posttransfection cells were incubated with $10^{-8}$ M estradiol ($E_2$) for 24 hours. Standard luciferase assays were performed on cell extracts in triplicate as described in the Materials and Methods. Each bar represents the mean value ± S.D. ($\ast$) = $P<.05$ compared to estradiol incubated
samples alone. (C) The decrease in transcriptional activity of ER phosphorylation mutants was not due to ER expression levels. A portion of the extract used in part B above was prepared for Western blot analysis for the detection of ER as described in the Material and Methods.

Fig. 4. The src kinase inhibitor SU6656 disrupts ER promoter interaction measured by ChIP. Ishikawa cells (2 x 10^6 cells/plate) were incubated with 1µM SU6656 for 2 h. Following incubation with the inhibitor the cells were incubated with 10^{-8} M estradiol (E_2) or 10^{-7} M 4-hydroxytamoxifen (Tam) for 2.5 h. Chromatin was prepared and immunoprecipitated with antibody against ER. The purified DNA was amplified by PCR and visualized by ethidium bromide staining (left panel). Relative levels of promoter interaction were measured by real time RT-PCR (right panel) using primers that span the ERE region of the pS2 gene as described in Material and Methods. For quantitative ChIP assays, relative levels of promoter interaction were normalized to input and each bar represents the mean value ± S.D. (*) = P<.05 compared to estradiol and 4-hydroxytamoxifen incubated samples alone. (†) = P<.05 compared to untreated (Con) samples.

Fig. 5. Src kinase potentiation of ER-dependent transcription and promoter interaction is partially mediated via the PI3K/AKT pathway. (A) Ishikawa cells (2 x 10^6 cells/plate) were incubated with 1µM SU6656 for 2 h. Following incubation with the inhibitor the cells were incubated with 10^{-8} M estradiol (E_2) or 10^{-7} M 4-hydroxytamoxifen (Tam) for
Protein extract was prepared from Ishikawa cells and used for Western blot analysis to measure levels of activated AKT as described in the Material and Methods. Quantitation of the Western blot signal was normalized to the Western blot signal for total AKT and β-actin levels (right panel). Each bar represents the mean value ± S.D. (*) = \( P<.05 \) compared to estradiol and 4-hydroxytamoxifen incubated samples alone. (†) = \( P<.05 \) compared to untreated (Con) samples. (B) PI3K or (C) AKT inhibition blocks the dominant active src potentiation of estradiol- and 4-hydroxytamoxifen-mediated gene transcription. Ishikawa cells (2 x 10^5 cells/well) were cotransfected with ER\(_E2\)e1b-luciferase reporter (0.5µg/well) and either dominant active src (0.5µg/well) (Dom) or empty vector pBABE (0.5µg/well). 24 hours posttransfection the cells were incubated with 10^{-8} M estradiol (E\(_2\)) or 10^{-7} M 4-hydroxytamoxifen (Tam). For the samples in which 1µM wortmannin (WORT) or 10µM AKT inhibitor (AKT INH) was used, the cells were incubated for 2 h with the inhibitor followed by incubation with 10^{-8} M estradiol (E\(_2\)) or 10^{-7} M 4-hydroxytamoxifen (Tam). Standard luciferase assays were performed on cell extracts in triplicate as described in the Materials and Methods. Each bar represents the mean value ± S.D. (∗) = \( P<.05 \) compared to estradiol and 4-hydroxytamoxifen incubated samples alone. (†) = \( P<.05 \) compared to estradiol and 4-hydroxytamoxifen in combination with dominant active src. (D) Wortmannin disrupts ER promoter interaction. Ishikawa cells (2 x 10^6 cells/plate) were incubated with 1µM wortmannin (WORT) for 2 h. Following incubation with the inhibitor the cells were incubated with 10^{-8} M estradiol (E\(_2\)) or 10^{-7} M 4-hydroxytamoxifen (Tam) for 2.5 h. Chromatin was prepared and immunoprecipitated with antibody against ER. The purified
DNA was amplified by PCR and visualized by ethidium bromide staining (left panel) or relative levels of promoter interaction were measured by real time RT-PCR (right panel) using primers that span the ERE region of the pS2 gene, (as described in the Material and Methods. For quantitative ChIP assays, relative levels of promoter interaction were normalized to input and each bar represents the mean value ± S.D. (**) = P<.05 compared to estradiol and 4-hydroxytamoxifen incubated samples alone. (**†) = P<.05 compared to untreated (Con) samples.

Fig. 6. Src kinase modulates promoter interaction through serine 167 phosphorylation of ER. HeLa cells (2 x 10^6 cells/plates) were cotransfected with either dominant active src (0.5µg/well) (Dom) or empty vector pBABE (3µg/well) and either wild type ER or phosphorylation mutants ER/S118A or ER/S167A (0.5µg/well). 24 hours posttransfection the cells were incubated with 10^-8 M estradiol (E2) for 2.5 h. Chromatin was prepared and immunoprecipitated with antibody against ER. The purified DNA was amplified by real time RT-PCR using primers that span the ERE region of the pS2 gene, as described in the Material and Methods. Relative levels of promoter interaction were normalized to input and each bar represents the mean value ± S.D. (**) = P<.05 compared to estradiol incubated samples alone. (**†) = P<.05 compared to untreated (Con) samples.

Fig. 7. Src kinase increases the activity of SRC-1 and CBP. (A and B) Ishikawa cells (2 x 10^5 cells/well) were cotransfected with GalRE-luciferase (0.5µg/well), dominant active src (Dom) (0.5µg/well), or siRNA-src (0.5µg/well) and Gal-SRC-1, Gal-SRC-2, Gal-
SRC-3, Gal-PCAF, Gal-CBP, or GAL-DBD (0.5µg/well). 48 hours posttransfection standard luciferase assays were performed on cell extracts in triplicate as described in the Materials and Methods. Each bar represents the mean value ± S.D. (*) = P<.05 compared to GAL-DBD. (B) The samples receiving src kinase inhibitor 1µM SU6656 (INH) were transfected with GalRE-luciferase and Gal-SRC-1, Gal-SRC-2, Gal-SRC-3, Gal-PCAF, Gal-CBP, or GAL-DBD (0.5µg/well). 24 hours posttransfection the cells were incubated with 1µM SU6656 (INH) for 24 hours. Standard luciferase assays were performed on cell extracts in triplicate as described in the Materials and Methods. Each bar represents the mean value ± S.D. (*) = P<.05 compared to the GAL-DBD. (C) Src kinase increases SRC-1 activity in HEC1A cells. HEC1A cells (2 x 10^5 cells/well) were cotransfected with GalRE-luciferase (0.5µg/well), either dominant active src (Dom) (0.5µg/well) or empty vector pBABE (0.5µg/well), either siRNA-src (0.5µg/well) or empty vector (0.5µg/well), and either Gal-SRC-1, Gal-SRC-2, Gal-SRC-3, Gal-PCAF, Gal-CBP, or GAL-DBD (0.5µg/well). For samples receiving the inhibitor, 24 hours posttransfection the cells were incubated with 1µM SU6656 (INH) for 24 hours. Standard luciferase assays were performed on cell extracts in triplicate as described in the Materials and Methods. Each bar represents the mean value ± S.D. (*) = P<.05 compared to the GAL-coactivator constructs alone.

Fig. 8. Src kinase increases SRC-1 coactivation on ER-dependent gene transcription. HeLa cells (2 x 10^5 cells/well) were cotransfected with ER/S167A (0.05µg/well), either
dominant active src (Dom) (0.5µg/well) or empty vector pBABE (0.5µg/well), and either
SRC-1 (0.5µg/well) or empty vector pCR3.1 (0.5µg/well). 24 hours posttransfection,
cells were incubated with 10⁻⁸ M estradiol (E₂) for 24 h. Standard luciferase assays were
performed on cell extracts in triplicate as described in the Materials and Methods. Each
bar represents the mean value ± S.D. (*) = P<.05 compared to cells transfected with
ER/S167A, dominant active src and SRC-1. (†) = P<.05 compared to cells transfected
with ER/S167A and SRC-1.

Fig. 9. Model of src kinase potentiation of estradiol- and 4-hydroxytamoxifen-dependent
gene transcription. (A) Activation of src kinase increases ERK1/2 and AKT activity that
results in phosphorylation of ER on serines 118 and 167, respectively. Src kinase
activation also increases the activity of coactivators SRC-1 and CBP through unknown
pathways. (B) In the absence of src kinase activation, serine 118 and 167
phosphorylation is decreased resulting in reduced promoter interaction and transcriptional
activity for ER. Inhibition of src kinase also decreases SRC-1 and CBP activity that is
important for coactivation of estrogen- and tamoxifen-dependent gene transcription.
Fig 1

A

Active src
Total src
β-actin

B

RLU

Con
INH
E2
E2+SU6656
Tam
Tam+SU6656

C

RLU

Con
Dom
E2
E2+Dom
Tam
Tam+Dom

Active src/total src/β-actin

MCF-7
T47D
HEC1A
Ishikawa

*
Fig 1

D

Vector
-srC

Con

siRNA-src

Tam

SU6656

Dom

E

F
**Fig 2**

(A) C-MYC

(B) pS2
Fig 3
**Fig 4**
Fig 5
Fig 6
**Fig 7**

A

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C-myc non-
Consensus ERE

B  
C-myc consensus
ERE

ER wt  ER S118A  ER S167A

Con  Dom  E2  E2-Dom  Con  Dom  E2  E2-Dom  Con  Dom  E2  E2-Dom

**Fig S-4**
**Title:** Attenuation of Estrogen Receptor α (ERα) Signaling by Selenium in Breast Cancer Cells via Downregulation of ERα Gene Expression.

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**Running Title:** Selenium Inhibits ER-α Signaling in MCF-7 Cells. 

**Keywords:** Breast cancer, Estrogen Receptor, Selenium, MCF-7

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Abstract

Numerous studies have shown that selenium provides beneficial effects as a cancer chemoprevention agent. Although long-term intervention trials failed to confirm selenium protection against breast cancer in humans because of insufficient cases, the evidence of effective selenium chemoprevention in animal mammary tumor models or human breast cancer cells is substantial and convincing. The present study demonstrates that the selenium compound methylseleninic acid (MSA) inhibits estrogen receptor α (ERα) signaling in ER-positive MCF-7 breast cancer cells as evidenced by decreased estradiol-dependent cell growth and gene expression. MSA diminishes estradiol induction of endogenous ER-regulated pS2 and c-myc genes as well as the expression of an ER-regulated reporter gene. A major mode of MSA action on ER signaling is through a downregulation of ERα gene expression that precedes a decrease in ERα protein level. This study provides a mechanism driven rationale for using selenium as a chemopreventive agent for women at high risk for developing breast cancer or as a therapeutic strategy for ER-positive breast cancer.
Introduction

Breast cancer is the most common female neoplasia in the western world with one in eight women developing breast cancer in the United States [1]. Estrogens promote the growth of breast cancer and a strong correlation exists between prolonged estrogen exposure and breast cancer risk [2]. The major mechanism of estrogen action in breast tissues is through binding to a specific nuclear estrogen receptor (ER) [3,4]. Two isoforms of ER have been identified. The role of the major isoform, ER\(\alpha\), in breast cancer has been clearly defined although the role of the recently discovered ER\(\beta\) remains unclear [5-7]. ER is a ligand-activated transcription factor that upon estrogen stimulation binds to promoter regions of ER-regulated genes to modulate the expression of genes important for estrogen-regulated cell proliferation [4].

The micronutrient selenium has beneficial effects in inhibiting cancer growth. In a clinical trial Clark et al. demonstrated the protective effects of selenium against prostate, lung and colon cancer [8,9]. In addition, selenium inhibits mammary tumorigenesis [10] and breast-derived cell growth [11]. A recent study demonstrated that selenium interferes with androgen receptor (AR) signaling to decrease prostate cancer cell growth [12]. Since the ER belongs to the same superfamily of ligand-activated nuclear receptors as does the AR, it was of interest to determine whether selenium compounds could impact ER signaling in breast cancer cells.
The present study used methylseleninic acid (MSA), developed specifically for testing selenium effects in *in-vitro* cell line experiments [13]. MSA is a monomethylated selenium compound that bypasses the need for metabolic enzymes required to convert other selenium species to the active metabolite, methylselenol. This allows MSA, as opposed to other selenium species to act rapidly in cell lines. Selenomethionine, currently used in the SELECT clinical trail of prostate cancer, cannot be used in cell culture studies because most epithelial cell lines lack enzymes needed for metabolism to the active methylselenol [14]. Our results in the estrogen-dependent MCF-7 breast cancer cell line demonstrate that MSA inhibits ERα-dependent gene transcription by decreasing ERα mRNA levels and resulting protein levels. Attenuation of ER signaling in breast cancer cells is likely a major mechanism contributing to the growth inhibitory effect of MSA.
Material and Methods

MTT Assay

MCF-7 cells were plated in 24 well plates (20,000 cells/well) and cultured in phenol red-free DMEM (Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS). Cells were incubated with 1, 5, or 10 µM of MSA at 24 h after plating. An aliquot of 125 µL of MTT reagent (5 mg/ml of 2,5-diphenyl tetrazolium bromide in PBS) was pipetted into each well after 24, 48, or 72 h of exposure to MSA. The media with the MTT reagent was removed after 15-30 min and 300 µL of DMSO (Fisher Biotech, Fairlawn, NJ) was added to each well. The plates were read at a wavelength of 570 nm.

BrdU Labeling Assay.

Cells were seeded in T75 culture flasks at a density designed to reach 70-80% confluency at the time of assay. At 48 hr after seeding, cells were exposed to 10 µM MSA for 16 or 24 hr. During the last 30 min of MSA treatment, cells were labeled with 10 µM of bromodeoxyuridine (10 µl of 1 mM BrdU was added to each ml of culture media). BrdU-labeled cells were trypsinized, fixed, treated with DNase I, and stained with fluorescein isothiocyanate (FITC)-conjugated anti-BrdU antibody using the BrdU Flow Kit from BD Pharmigen (San Diego, CA). Stained cells were then quantified by flow cytometry, and the data were analyzed with the WinList software (Variety Software House, Topsham, ME).
Detection of Apoptosis.

Cells were seeded in triplicate in a 96-well microtiter plate at a density designed to reach ~10^4 cells per well at the time of assay. At 48 hr after seeding, cells were exposed to either 5 or 10 µM MSA for 24 hr. Detached cells were pulled with attached cells by centrifugation. Cytoplasmic histone-associated DNA fragments were quantified using the Cell Death Detection ELISA<sup>PLUS</sup> Kit (Roche Applied Science, Indianapolis, IN) as per manufacturer’s protocol. The absorbance measured at 405 nm (with reference wavelength 492 nm) was normalized by the protein concentration of the samples.

Quantitation of Propidium-Iodide-Stained Cells by Flow Cytometry.

MCF-7 cells were seeded in T75 culture flasks at a density designed to reach 70-80% confluency at the time of assay. At 48 hr after seeding, cells were exposed to either 5 or 10 µM MSA for 6, 16 or 24 hr. Adherent cells harvested by mild trypsinization were pooled together with detached cells (if any). Cells were incubated with 1 µg/ml of propidium iodide (PI) for 5 min on ice. PI-stained cells were subsequently quantified by flow cytometry, and the data were analyzed with the WinList software (Variety Software House, Topsham, ME).

Luciferase Assay

MCF-7 and HeLa cells were plated in 6-well plates (2 ×10^5 cell/well) and cultured in phenol red-free DMEM containing 2% FBS that had been charcoal stripped to remove
endogenous steroids. At 24 h after plating, the MCF-7 cells were transfected with 500 ng of EREe1b-luciferase reporter, and the HeLa cells were cotransfected with 50 ng of ERα and 500 ng EREe1b-luciferase reporter using Fugene transfection reagent (Roche, Madison, WI). At 24 h post transfection, the cells were incubated with either vehicle, estradiol (10^{-8} M) (Sigma, St. Louis, MO), MSA (1, 5 or 10 µM), or a combination of estradiol and MSA for 24 h. Luciferase expression was measured and normalized as previously described [15].

**Western blot analysis**

MCF-7 and HeLa cells were plated in 100 mm dishes (3 ×10^6 cell/plate), and cultured in 2% charcoal-stripped FBS in DMEM. MCF-7 cells were maintained in the stripped media for 3 days until 90% confluency. HeLa cells were transfected at 24h post-plating with the expression vector for hERα (0.5 µg/plate) or the empty vector (0.5 µg/plate) and maintained in 2% stripped FBS in DMEM for an additional 48 h. Both MCF-7 and HeLa cells were incubated with either vehicle, estradiol, MSA or a combination of estradiol and MSA at varying concentrations and times as indicated in the figure legends. The cells were lysed and prepared for Western blotting as previously described [15]. The membranes were incubated with an antibody against ERα (Novacastra, Newcastle on Tyne, UK), and normalized to β-actin (Santa Cruz Biotechnology INC, Santa Cruz, CA).

**Real-Time RT-PCR**
The culture and/or transfection conditions for MCF-7 and HeLa cells were identical to that described above for Western blot analysis. Total mRNA was extracted from the cell pellet, reverse transcribed and gene expression was measured by real-time RT-PCR as described previously [15].

C-myc:
FWD-5’- CGTCTCCACACATCAGCACAA -3’
REV-5’- TGTTGGCAGCAGGATAGTCCTT -3’
Probe-5’-56FAM/ACGCAGCGCTCCCTCCACTC/3BHQ-1/-3’

pS2:
FWD-5’- CGTGAAAGACAGAATTGTGGTTT-3’
REV-5’- CGTCGAAACAGCAGCCCTTA-3’
Probe-5’-/56FAM/ TGTCACGCCCCTCCCAGTG/3BHQ-1/-3’

ERα
FWD-5’- AGACGGACCAAAGCCACTTG -3’
REV-5’- CCCCGTGATGTAATACTTTGCA -3’
Probe-5’-/56FAM/ TGCAGCCTCTCCAGTG/3BHQ-1/-3’

ERβ
FWD-5’- CCCAGTGCGCCCTCAC-3’
REV-5’- CAACTCCTTGTCGGCCAACT -3’
Probe-5’-/56FAM/ AGGCCTCCATGATGTCCCTGA /3BHQ-1/-3’

Cofilin
FWD-5’- TGTGCCGGCTGGTTCCCT-3’
REV-5’- CTTACTGGTCCTGCTTCCATGAG -3’
Probe-5’-/56FAM/ CTATTCCCCCTGTCACGCT /3BHQ-1/-3’

CRABPII
FWD-5’- TTCTCTGGCAACTGGAAAATCA -3’
REV-5’- CATTCACCCCCCAGCACTTTG -3’
Probe-5’-/56FAM/ CGGATCGGAAAAACTTTCGAGGAATTGC /3BHQ-1/-3’

Smooth Muscle Actin
FWD-5’- TCCTCCCTTGAGAAGAGTTACGA -3’
REV-5’- GGCAGCGGAACGTTTACCA -3’
Probe-5’-/56FAM/ TGCCTGATGGGCAAGTGATCA /3BHQ-1/-3’

Data Analysis

Results are expressed as mean ± S.D. P values were calculated using Anova Dunnett’s T Test and Independent T Test. P<.05 was considered significant.
Results

**MSA inhibits MCF-7 cell growth via inhibition of proliferation and induction of apoptosis.**

MCF-7 is an ER positive breast cancer cell line that is sensitive to estradiol-induced cell growth and ER-regulated gene expression. MCF-7 cells were incubated with MSA and cell growth was measured by the MTT assay. MSA at 5 or 10 µM inhibited cell growth by approximately 50% at 24, 48, and 72 h (Fig. 1A). To assess the contribution of apoptosis and anti-proliferation to MSA growth inhibition in MCF-7 cells, BrdU incorporation and DNA fragmentation were assessed. 10µM MSA decreased BrdU incorporation by 50% and 70% at 16h and 24h, respectively (Fig 1B). In the same time course, MSA increased apoptosis at 24 h by 8 and 15 fold at 5µM and 10uM MSA, respectively (Fig. 1C). MSA had no effect on cell toxicity up to 24h as assessed by trypan blue staining and only very modest effects at 24h as assessed by propidium iodine (PI) staining (Fig. 1D, top and bottom panel)

**MSA inhibits estradiol-dependent induction of estrogen response element (ERE)-luciferase reporter gene (ERE_{2e1b-luciferase}) and endogenous ERα-regulated genes.**

To assess whether MSA could alter ER signaling, MCF-7 cells were transfected with the ERE_{2e1b-luciferase} reporter gene and incubated with estradiol or MSA alone, or co-incubated with estradiol and MSA. MSA alone decreased basal luciferase expression and inhibited estradiol-dependent stimulation of the ERE_{2e1b-luciferase} reporter presence of
1, 5, or 10 µM MSA (Fig. 2A). Since MSA inhibited estradiol-dependent induction of the ERE\_2\_e1b-luciferase reporter, it was of interest to determine whether MSA could inhibit two well-characterized estrogen-regulated genes, c-myc, and pS2. Real-time RT PCR was used to measure c-myc and pS2 expression after incubation with estradiol alone or in combination with MSA for 6 h. As expected, estradiol induced pS2 and c-myc expression and this induction was inhibited in the presence of 1, 2.5, 5, or 10 µM MSA in a dose-dependent manner (Fig. 2B and C). Additional time course experiments revealed that estradiol induction of pS2 and c-myc occurred at 2 h and 1 h, respectively (Fig. 2D, 2E). A period of 4 h co-incubation with MSA was required to block the response to estradiol induction (Fig. 2D and E).

**MSA specifically inhibits estradiol-dependent signaling.**

In order to rule out the nonspecific effects of MSA on general transcription, MCF-7 cells were transfected with constitutively active RSV-luciferase reporter and incubated with 1, 5, or 10 µM MSA. No effect on the RSV-luciferase reporter was observed at any MSA concentration (Fig. 3A). In addition to assess the specificity of MSA towards ER signaling, MSA effects on several genes that are expressed in MCF-7 cells but unrelated to estrogen signaling were examined. Cellular retinoic acid binding protein II (CRABPII) is expressed in MCF-7 cells but is not modulated by estradiol [16]. Cofilin [17] and smooth muscle actin [18] are expressed in breast cancer cell lines but not regulated by estradiol (data not shown). MSA had no effect on CRABPII, cofilin, or smooth muscle
actin mRNA expression (Fig. 3B). These data demonstrate a specificity of MSA towards estradiol-dependent signaling.

**MSA reduces ERα protein expression in MCF-7 cells.**

Since MSA inhibited ER signaling, we proceeded to determine if MSA altered ERα protein levels. MCF-7 cells were incubated with estradiol, MSA, or estradiol + MSA for 6 h, and ERα levels were assessed by Western blot analysis. MSA alone at 5 or 10 µM reduced ERα level significantly compared to vehicle-incubated samples. Estradiol treatment alone reduced ERα protein level (Fig 4A, lane 6), most likely due to ubiquitin-mediated downregulation as reported previously (15). MSA at 5 or 10 µM further reduced ERα levels when co-incubated with estradiol (Fig 4A, lanes 9 and 10). To measure time-dependent decreases of ER by MSA, MCF-7 cells were incubated with 10 µM of MSA for 2, 4, 6, or 12 h. A significant reduction in ERα protein level was detected after 4 h of incubation with MSA (Fig. 4B); this time frame coincided with the time it took MSA to inhibit estradiol-dependent induction of pS2 and C-myc (Fig 2D and E). The effect of MSA on ERβ protein level was also assessed. However, Western blotting of ERβ in MCF-7 cells generated very weak signals, so the result was inconclusive (data not shown). To determine whether ERα protein downregulation was a major mechanism by which MSA inhibits ER signaling, we constitutively expressed ERα protein in the well-characterized, ER-negative HeLa cell line by transfection with a
constitutively expressed ERα expression plasmid. HeLa cells are cervical carcinoma cells in which reexpression of ERα results in robust estradiol-dependent reporter activation. Incubation of transfected cells with 10 µM MSA did not affect ERα protein level (Fig. 4C inset). Under these conditions in which ERα protein levels remained constant, MSA did not inhibit estradiol induction of the ERE<sub>2e1b</sub>-luciferase reporter (Fig 3C), nor endogenous pS2 (Fig 4D) or c-myc (Fig. 4E). These results suggest that a major mechanism contributing to MSA inhibition of estrogen signaling is through reduction in ERα protein. One caveat to this finding is that HeLa is a cervical carcinoma and not a breast cancer cell line. Although similar experiments were attempted in two ER-negative breast cancer cell lines (MDA-MB-231, MDA-MB-468) transfected with ERα, results were inconclusive since neither cell line displayed estradiol activation of cotransfected ERE<sub>2e1b</sub>-luciferase (data not shown).

**MSA reduces ERα mRNA.**

To determine whether the effect of MSA on ERα was at the transcriptional level, ERα mRNA in MCF-7 cells was measured by real time RT-PCR following incubation of cells with estradiol, MSA, or estradiol + MSA. MSA at 5 or 10 µM inhibited ERα gene expression (Fig. 5A) but had no effect on ERβ gene expression (Fig. 5B). Time course studies revealed that ERα mRNA was inhibited by 10 µM MSA within 2 h (Fig. 5C), thereby preceding the decrease in protein level that was not detected until after 4 h, (see
Fig. 5B), suggesting that the mechanism by which MSA reduces ERα protein level is through decreased ERα mRNA transcription.
Discussion

ER is a major therapeutic target for hormone-dependent, ER-positive breast cancers. Antiestrogen therapies designed to compete with estrogen for binding to ER have proven effective in reducing estrogen-dependent tumor growth. In addition to targeting ER function, strategies designed to lower ER protein level are also useful in the management of hormone-dependent, ER-positive tumors. The present study is the first to describe the disruption of ER signaling in breast cancer cells by a selenium compound. We found that MSA blocked estradiol-dependent activation of a reporter gene (ERE\textsubscript{2e1b-luciferase}) as well as the transcription of endogenous ER-regulated genes. A major underlying mechanism by which MSA interferes with ER signaling was through a decrease in ER\textalpha gene transcription and the subsequent reduction of ER\textalpha protein.

Several mechanisms may contribute to the growth inhibitory effects of MSA including antioxidant properties and alteration of redox reactions that may subsequently induce apoptosis [14,19]. Several independent studies have demonstrated MSA-induced growth inhibition in \textit{in-vitro} cell lines via induction of apoptosis. In hyperplastic mammary epithelium cells TM12 and TM2H [13] and in premalignant human breast cells MCF10AT1 and MCF10AT3B [20], MSA inhibited cell proliferation and induced apoptosis. MSA also demonstrated similar effects in human lung cancer cell lines [21] and human prostate carcinoma cell lines [22,23]. Moreover, MSA effects on cell growth were not mediated by selenium related toxicity [13] as is detected with inorganic
selenium compounds such as sodium selenite and sodium selenide [24]. In addition, based on our characterization of MSA action in MCF-7 cells, the growth inhibitory effects of MSA occur through both apoptosis and antiproliferation mechanisms (Fig. 1B and C). Comprehensive analysis of these mechanisms in a variety of hormone-dependent and hormone-independent breast cancer cell lines is currently being assessed. What is demonstrated here for the first time in estrogen-dependent breast cancer cells is a novel component of the overall antiproliferative effect of MSA; the inhibition of ER signaling. Estrogens are important mitogenic signals for the growth of breast cancers, and have been shown to induce G\textsubscript{1}/S transition in breast cancer cells [25] by control of several key cell-cycle regulators (for ref see [26]). In addition to inducing cellular proliferation, estrogens increase cell survival by upregulating the antiapoptotic factor bcl-2 [27] and downregulating several proapoptotic factors [28]. The overall growth inhibitory effects of MSA in estrogen dependent breast cancer cells are likely attributed to MSA disruption of ER signaling and non-ER antiproliferative and apoptotic effects of MSA in hormone dependent breast cancer. Indeed, MSA also inhibited growth of an ER\textsubscript{α}-negative breast cancer cell line (MDA-MB-231, data not shown). Future work is being performed to assess the relative contribution of loss of ER-signaling to the overall growth inhibitory effect of MSA in ER\textsubscript{α} positive breast cancer cells.

Low concentrations of MSA (1 or 2.5 µM) had no significant effect on ER\textsubscript{α} protein levels although both concentrations were capable of inhibiting estradiol-dependent reporter gene activation (Fig. 2A) and endogenous pS2 and c-myc genes (Fig.

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2B and C). This apparent discrepancy may be attributed to MSA effects on other proteins important for ERα action. Dong et al. [20] demonstrated that MSA regulates expression of several proteins shown to be key mediators of ER signaling. For example, MSA decreased expression of cyclin D1, a known coregulator for ER action [29]. In addition, MSA decreased the expression of AKT2, a kinase known to phosphorylate ERα resulting in increased transcriptional activity [30] and stabilization of ERα binding to the pS2 and c-myc promoters (Shah and Rowan, *Molecular Endocrinology, in press*).

Although both ERα (present study) and AR [12] gene expression and protein levels were reduced by MSA, ERβ mRNA expression was unaltered. The lack of unanimity suggests a specificity of MSA for some, but not all, nuclear receptors. Future studies will examine the promoter regions of ERα and AR genes to assess whether homologous transcription factor binding sites are present, and to delineate the promoter regions required for MSA downregulation of ERα and AR transcription.

Although our study demonstrates a strong MSA inhibition of estrogen signaling and cell growth in MCF-7 breast cancer cells *in vitro*, selenium compounds appear to have negligible effects on proliferation of normal rodent mammary gland [31-33]. This discrepancy may be due to differences in ERα expression in the normal versus malignant mammary gland. A very small percentage of epithelial cells in the normal, adult mammary gland are proliferating and these proliferating cells express very little or no ERα. In contrast, proliferating, epithelial breast cancer cells are ERα positive [34] and
these cells are strong candidates for growth inhibition by antiestrogens [35-37]. If
disruption of ER signaling is one of the major mechanisms for selenium-mediated growth
inhibition, then the normal mammary epithelium may only be modestly affected due to
absence of ERα in the proliferating epithelial cells. These possibilities remain under
intense investigation by our laboratories.

Lastly, the present study presents the possibility that selenium compounds may
not only be useful as a chemopreventative, but may also be efficacious as a therapy for
existing tumors. Several studies have shown growth inhibition of established tumors by
selenium compounds in in-vivo models [33,38-41]. However these studies used inorganic
selenium compounds that are genotoxic and no longer used for selenium
chemoprevention and/or chemotherapy studies. In addition, Yan et al. demonstrated that
supplementation with dietary selenium and magnesium had no effect on HTB123 human
mammary cancer cells inoculated in athymic nude mice [42]. To our knowledge no study
has examined the effects of MSA on established tumors in an in-vivo model. However,
Cao et al. have shown a synergistic interaction of organic selenium compounds with the
anticancer drug irinotecan [43]. Mice bearing squamous cell carcinoma of the head/neck
and colon carcinoma xenografts were given selenium in form of 5-methylselenocysteine
and seleno-l-methionine orally seven days prior to intravenous injection of irinotecan.
Combination treatment of irinotecan + selenium decreased the toxicity of the
chemotherapeutic agent and increased the cure rate of the tumor bearing mice inoculated
with cancer cells sensitive and resistant to irinotecan [43]. Although it is unknown
whether selenium monotherapy would be efficacious against MCF-7 xenografts, these tumors are tamoxifen sensitive [44] suggesting the possibility of combination therapy of tamoxifen with selenium compounds to improve efficacy.

Tamoxifen is currently the major antiestrogen used for breast cancer therapy, even though tamoxifen resistance and tamoxifen uterotropism represent significant drawbacks of this modality. ER signaling remains functional in the majority of breast cancers that exhibit tamoxifen resistance [45]. This suggests that any strategy designed to disrupt ER signaling or remove ERα protein in tamoxifen-resistant cells may provide some measure of efficacy. For example, the antiestrogen fulvestrant results in ERα protein degradation and apoptotic cell death. Fulvestrant is recommended for postmenopausal women who exhibit breast cancer progression following tamoxifen therapy (for a review see ref. [46]). We are currently assessing whether MSA may be useful in a similar manner as fulvestrant since MSA potentiates the growth inhibitory effects of tamoxifen in tamoxifen-sensitive breast cancer cells and resensitizes tamoxifen-resistant breast cancer and endometrial cancer cells in vitro (submitted for publication).

An important goal in the endocrine management of hormone-dependent breast cancer is to increase the proportion of cells undergoing apoptotic cell death relative to cell cycle arrest. Increasing the proportion of cells undergoing apoptosis would prevent cells from reentering the cell cycle once tamoxifen-resistant mechanisms are acquired resulting in disease recurrence. MSA induces significant apoptosis in MCF-7 cells. Preclinical therapeutic regimens combining tamoxifen with selenium are being
explored for efficacy in preventing or delaying tamoxifen resistance and/or reversing tamoxifen resistance.

**Acknowledgements**

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Reference List


Figure Legends

Fig. 1. MSA inhibits MCF-7 cell growth through altering proliferation and apoptosis. (A) Cell growth analysis by MTT assay of MCF-7 cells incubated with vehicle (Veh) or 1, 5, and 10 µM of MSA for 24, 48, and 72 h. Each bar represents the mean value ± S.D. Vehicle treated samples were set at 100%. (✱) = P<.05 compared to vehicle. (B) MCF-7 cell proliferation was measured by BrdU incorporation. MCF-7 cells were incubated with 10µM MSA for 16h and 24 h. BrdU incorporation was measured as described in Material and Methods, each bar represents the mean value ± S.D. (✱) = P<.05 compared to vehicle (Veh) incubated samples. (C) MCF-7 cells were incubated with 5µM and 10µM MSA for 24h. Following incubation with MSA DNA fragmentation was assessed as described in Material and Methods, each bar represents the mean value ± S.D. (✱) = P<.05 compared to vehicle (Veh) incubated samples. (D) MCF-7 cells were incubated with 5µM and 10µM MSA for 6, 16, and 24 h. Cell toxicity was measured by PI staining (top panel) and by trypan blue staining (bottom panel) as described in Material and Methods, each bar represents the mean value ± S.D. (✱) = P<.05 compared to vehicle (Veh) incubated samples.

Fig. 2. MSA inhibits estradiol-dependent activation of an EREe1b-luciferase reporter, pS2, and c-myc gene expression. (A) MCF-7 cells were transfected with ERE2e1b-luciferase reporter (0.5 µg/well). 24 hours posttransfection cells were incubated with vehicle (Veh), 10^{-8} M estradiol (E2) or MSA alone or co-incubated with 10^{-8} M estradiol and MSA. Standard luciferase assays were performed on cell extracts in triplicate as
described in the Materials and Methods. Each bar represents the mean value ± S.D. (•) = $P<.05$ compared to estradiol incubated samples alone. Dose response of MSA on (B) pS2 or (C) c-myc gene expression. MCF-7 cells (2 x 10^6 cells/plate) were incubated with vehicle (Veh), 10^{-8} M estradiol (E2) or MSA alone or co-incubated with 10^{-8} M estradiol and MSA for 6 h. Time titration of MSA on (D) pS2 or (E) c-myc gene expression. MCF-7 cells (2 x 10^6 cells/plate) were incubated with vehicle (Veh), 10^{-8} M estradiol (E2) or 10 μM MSA alone or co-incubated with 10^{-8} M estradiol (E2) and 10μM MSA. Expression of (B and D) pS2 and, (C and E) c-myc genes was measured by real time RT-PCR as described in the Materials and Methods. Expression was normalized to GAPDH and each bar represents the mean value ± S.D. (•)= $P<.05$ compared to vehicle incubated samples (‡) = $P<.05$ compared to estradiol (E2) incubated samples.

Fig. 3. MSA specifically inhibits estradiol-dependent signaling. (A) MCF-7 cells were transfected with RSV-luciferase reporter (0.5 μg/well). 24 hours posttransfection cells were incubated with vehicle 1, 5, or 10μM MSA. Standard luciferase assays were performed on cell extracts in triplicate as described in the Materials and Methods. Each bar represents the mean value ± S.D. (•) = $P<.05$ compared to estradiol incubated samples alone. (B) MCF-7 cells (2 x 10^6 cells/plate) were incubated with vehicle (Veh) or 10 μM MSA for 2 h. Smooth muscle actin, cofilin and CRABPII genes were measured by real time RT-PCR as described in the Materials and Methods. Expression was normalized to GAPDH and each bar represents the mean value ± S.D. (•)= $P<.05$ compared to vehicle incubated samples.
Fig. 4. MSA inhibits ERα protein expression, which is required for its effect on ER-dependent gene expression. (A) Dose response of MSA on ERα protein expression. MCF-7 cells (2 x 10^6 cells/plate) were incubated with vehicle (Veh), 10^{-8} M estradiol (E2) or MSA alone or co-incubated with 10^{-8} M estradiol and MSA for 6 h and Western blot analysis was performed. (B) Time titration of MSA on ERα protein expression. MCF-7 cells (2 x 10^6 cells/plate) were incubated with vehicle (Veh) or 10 Ì M MSA and Western blot analysis was performed. Quantitation of the Western blot signal for ERα was normalized to the Western blot signal for β-actin levels (Fig 3A and B, bottom panels), and each bar represents the mean value ± S.D. (×) = P<.05 compared to vehicle incubated samples (‡) = P<.05 compared to estradiol (E2) incubated samples. (C) HeLa cell were transfected with 500 ng of constitutively expressed ER and incubated with vehicle or 10 Ì M MSA for 6 h and Western blot analysis was performed for ERα (Inset) or transfected with 50 ng of constitutively expressed ERα and 500 ng ERE_E2e1b-luciferase reporter and incubated with (Veh), 10^{-8} M estradiol (E2) or 10μM MSA alone or co-incubated with 10^{-8} M estradiol (E2) and 10μM MSA. Standard luciferase assays were performed on cell extracts in triplicate as described in the Materials and Methods. (×) = P<.05 compared to vehicle incubated samples (‡) = P<.05 compared to estradiol (E2) incubated samples. (D and E) HeLa cells were transfected with 500 ng of constitutively expressed ERα and incubated with (Veh), 10^{-8} M estradiol (E2) or 10 Ì M MSA alone or co-incubated with 10^{-8} M estradiol (E2) and 10μM MSA for 6 h. Expression of (D) pS2 and, (E) c-myc genes was measured by real time RT-PCR as described in the Materials.
and Methods. Expression was normalized to GAPDH and each bar represents the mean value ± S.D. (•) = $P<.05$ compared to vehicle incubated samples (‡) = $P<.05$ compared to estradiol (E2) incubated samples.

Fig. 5. MSA inhibits ERα gene expression. Dose response of MSA on (A) ERα or (B) ERβ gene expression. MCF-7 cells (2 x 10^6 cells/plate) were incubated with vehicle (Veh), 10^{-8} M estradiol (E2) or MSA alone or co-incubated with 10^{-8} M estradiol and MSA for 6 h. (C) Time titration of MSA on ERα gene expression. MCF-7 cells (2 x 10^6 cells/plate) were incubated with vehicle (Veh) or 10 µM MSA. ERα and ERβ genes were measured by real time RT-PCR as described in the Materials and Methods. Expression was normalized to GAPDH and each bar represents the mean value ± S.D. (•) = $P<.05$ compared to vehicle incubated samples.
Fig 1
Fig 1
Fig 2
Fig 2
Fig 3
Fig 4

A

Veh  MSA 10M  MSA 2.5mM  MSA 5M  E2  E2+MSA 10M  E2+MSA 2.5mM  E2+MSA 5M  E2+Sel 10M  Pos

**ER**

β-actin

Veh 2h 4h 6h 12h

β-actin

ER-α

B

Veh 2a 4h 6h 12h

ER-α

β-actin

ER-α/β-actin

ER-α/β-actin
Fig 4

C

D

E

pS2

C-myc
**Fig 5**
Fig 5
Title: Selenium Disrupts Estrogen Receptor α Signaling and Restores Tamoxifen Sensitivity in Tamoxifen Resistant Breast and Endometrial Cancer Cells.

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Running Title: MSA Reverses Tamoxifen Resistance in ERα-positive Cells.

Keywords: Breast cancer, Endometrial cancer, Tamoxifen resistance, Estrogen Receptor, Selenium

Abbreviation list: Estrogen receptor; ER, Estrogen responsive elements; ERE, Selective estrogen receptor modulator; SERM, Methylselenocysteine; MSC, Seleno-L-methionine; Se-Met, Methylseleninic acid; MSA, Dulbecco’s modified Eagle’s medium; DMEM, Fetal bovine serum; FBS, Fetal calf serum; FCS, Amplified in breast cancer-1; AIB1, Steroid receptor coactivator-1; SRC-1, Transcriptional intermediary factor-2; TIF2, Nuclear corepressor; N-CoR.

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Abstract
As a treatment for breast cancer, tamoxifen a selective estrogen receptor modulator (SERM) is the most widely prescribed hormonal therapy for breast cancer. Despite the benefits of tamoxifen therapy, almost all tamoxifen-responsive breast cancer patients develop resistance to therapy. In addition, tamoxifen displays estrogen-like effects in the endometrium increasing the incidence of endometrial cancer. New therapeutic strategies are needed to circumvent tamoxifen resistance in breast cancer as well as tamoxifen toxicity in endometrium. Organic selenium compounds are highly effective chemopreventative agents with well-documented benefits in reducing total cancer incidence and mortality rates for a number of cancers. The present study demonstrates that the organic selenium compound methylseleninic acid (MSA, 2.5 µM) can potentiate growth inhibition of 4-hydroxytamoxifen (10⁻⁷ M) in tamoxifen sensitive MCF-7 and T47D breast cancer cell lines. Remarkably, in tamoxifen resistant MCF-7-LCC2, HEC1A, and Ishikawa cells, co-incubation of 4-hydroxytamoxifen with MSA resulted in a marked growth inhibition that was substantially greater than MSA alone. Growth inhibition by MSA and MSA + 4-hydroxytamoxifen in all cell lines was preceded by a specific decrease in ERα mRNA and protein without an effect on ERβ levels. Estradiol and 4-hydroxytamoxifen induction of endogenous ER-dependent gene expression (pS2, c-myc) as well as ER-dependent reporter gene expression (ERE2ε1b-luciferase) was also attenuated by MSA in all cell lines prior to effect on growth inhibition. Taken together these data strongly suggest that specific decrease in ERα levels by MSA is required for both MSA potentiation of the growth inhibitory affects of 4-hydroxytamoxifen and resensitization of tamoxifen resistant cell lines.
Introduction

Estrogens are key hormones for the growth and maintenance of female mammary gland and reproductive tract and critical for reproduction and fertility. Estrogen binds to its cognate receptor, the estrogen receptor (ER) belonging to the nuclear receptor superfamily of ligand dependent transcription factors (1,2). Two different forms of ER have been characterized, ERα and ERβ, which share high sequence homology (3-5). Upon ligand binding, the receptor undergoes conformational changes that release ER from chaperone proteins. Following dimerization, ER binds to estrogen responsive elements (ERE) in the promoters of ER-dependent genes, and subsequent recruitment of coactivators initiates ER-dependent gene transcription (6,7).

In addition to maintaining normal reproductive physiology estrogens are important mitogenic signals in the breast and endometrium, thus implicating the hormone in breast and endometrial tumorigenesis. As a treatment for breast cancer, tamoxifen a selective estrogen receptor modulator (SERM), binds to ER and blocks estrogen mediated breast cancer cell growth (8,9). Tamoxifen is the most widely prescribed endocrine therapy for breast cancer, and the only agent approved for breast cancer chemoprevention (10). However, tamoxifen therapy has two major drawbacks. Most tamoxifen responsive breast cancer patients succumb to tamoxifen resistance (11) in which tumors do not respond to the growth inhibitory properties of tamoxifen. In addition, tamoxifen displays estrogen-like effects in the endometrium increasing the incidence of endometrial cancer (12). Alternative therapeutic strategies that can be used alone or in combination with
tamoxifen in ERα positive breast cancers may prove useful in combating tamoxifen resistance in breast and estrogenic activities in other tissues.

Selenium is an essential micronutrient shown to inhibit cancer growth. Organic selenium compounds are the agents of choice for chemopreventative studies. These compounds have fewer side effects and lack the genotoxic action of inorganic selenium compounds such as selenite (13). Organic selenium agents used in chemoprevention trials such as methylselenocysteine (MSC) and seleno-L-methionine (Se-Met) are water-soluble compounds that are metabolized in tissues to the active selenium metabolite, methylselenol (14-16). The clinical usefulness of MSC and Se-Met are in the ability of these compounds to inhibit DNA synthesis and cell doubling and induce apoptotic cell death. One of the greatest benefits of organic selenium compounds for chemoprevention is the very low or absent toxicity (16,17).

In a double-blind placebo-controlled clinical trial Clark et al. demonstrated the protective effects of selenium-enriched yeast against prostate, lung and colon cancer (16,18). Although, the study failed to show statistical significance in breast cancer risk due to insufficient cases there is extensive data demonstrating the growth inhibitory properties of selenium in breast cancer cell lines and mammary tumor models. Our previous study using methylseleninic acid (MSA), a rapidly metabolized selenium compound useful in cell cultures studies (19), demonstrated that MSA inhibits estradiol induced cell growth and ERα-mediated gene transcription in the ERα-positive MCF-7 breast cancer cell line with no significant toxicity (revised manuscript submitted to Breast
The major mechanism by which MSA attenuates ER signaling was through decrease in ERα mRNA levels and subsequent protein levels with no effect on ERβ levels. These data suggested a novel mechanism of growth inhibition by MSA through disruption of estrogen signaling.

Since breast cancers vary widely with regard to ERα expression and de novo tamoxifen resistance, the present study examined the growth inhibitory mechanisms of MSA in cell lines that represent different paradigms of ERα expression and tamoxifen sensitivity/resistance. We show that MSA can inhibit ER signaling and potentiate the antiestrogen activity of tamoxifen via downregulation of ERα mRNA and protein levels. MSA in combination with tamoxifen potentiated growth inhibitory properties when compared to either agent alone in tamoxifen sensitive breast cancer cell lines, and tamoxifen resistant breast and endometrial cell lines.
Material and Methods

Cell lines

MCF-7 and MDA-MB-468 were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Cellgro, Herndon, VA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY), 4 mM of glutamine (Gibco), and 1% penicillin-streptomycin (Gibco) at 37°C with 5% CO₂. MCF-7-LCC2 were maintained in IMEM (Biosource, Rockville, MD) supplemented with 5% fetal calf serum (FCS) (Gibco) that had been charcoal stripped to remove endogenous steroids and 1% penicillin-streptomycin at 37°C with 5% CO₂. Ishikawa and HEC1A were maintained in DMEM supplemented with 5% FBS and 1% penicillin-streptomycin at 37°C with 5% CO₂.

MTT Assay

MCF-7, MCF-7-LCC2, MDA-MB-468, Ishikawa, and HEC1A cells were plated in 24 well plates (20,000 cells/well) and cultured in medium described above. Cells were incubated with MSA (2.5 µM), 4-hydroxytamoxifen (10⁻⁷ M) (Sigma, St. Louis, MO) or combination of 4-hydroxytamoxifen and MSA at 24 h after plating. An aliquot of 125 µL of MTT reagent (ICN, Aurora, OH) (5 mg/ml of 2,5-diphenyl tetrazolium bromide in PBS) was pipetted into each well after 24, 48, 72, or 96 h post treatment. The media with the MTT reagent was removed after 30 min-1 h and 300 µL of DMSO (Fisher Biotech, Fairlawn, NJ) was added to each well. The plates were read at a wavelength of 570 nm.
Luciferase Assay

MCF-7, Ishikawa, and HEC1A were plated in 6-well plates (2 × 10^5 cell/well) and cultured in phenol red-free DMEM containing 2% charcoal-stripped FBS. MCF-7-LCC2 were plated in 6-well plates (2 × 10^5 cell/well) cultured in phenol red-free IMEM containing 2% charcoal-stripped FCS. 24 h after plating, MCF-7, MCF-7-LCC2, Ishikawa, and HEC1A cells were transfected with 500 ng of EREe1b-luciferase reporter using Fugene transfection reagent (Roche, Madison, WI). 24 h post transfection MCF-7, Ishikawa, and HEC1A cells were incubated with vehicle, estradiol (10^{-8} M) (Sigma, St. Louis, MO), 4-hydroxytamoxifen (10^{-7} M), MSA (10 µM), or a combination of either estradiol, tamoxifen, and MSA for 24 h. MCF-7-LCC2 were incubated with vehicle, estradiol (10^{-9} M), 4-hydroxytamoxifen (10^{-8} M), MSA (10 µM), or a combination of estradiol, tamoxifen and MSA for 24 h. Luciferase expression was measured and normalized as previously described (20).

Western blot analysis

MCF-7, MCF-7-LCC2, and Ishikawa were plated in 100 mm dishes (3 × 10^6 cell/plate), and cultured in 2% charcoal-stripped FBS in DMEM. MCF-7-LCC2 were plated in 100 mm dishes (3 × 10^6 cell/plate) and cultured in 2% charcoal-stripped FCS. The cells were maintained in the stripped media for 3 days until 90% confluency. MCF-7, MCF-7-LCC2, Ishikawa, and HEC1A were incubated with vehicle, estradiol (10^{-8} M), 4-hydroxytamoxifen (10^{-7} M), MSA (10 µM), or a combination of estradiol or 4-
hydroxytamoxifen and MSA for 6 h. The cells were lysed and prepared for Western blotting as previously described (20). The membranes were incubated with an antibody against ERα (Novacastra, Newcastle on Tyne, UK), and normalized to β-actin (Santa Cruz Biotechnology INC, Santa Cruz, CA).

**Real-Time RT-PCR**

The culture conditions for MCF-7, MCF-7-LCC2, Ishikawa, and HEC1A were identical to that described above for Western blot analysis. The cells were incubated with vehicle, estradiol (10⁻⁸ M), 4-hydroxytamoxifen (10⁻⁷ M), MSA (10 μM), or a combination of estradiol or tamoxifen and MSA for 2 h when measuring ERα and ERβ mRNA expression and 6 h when measuring c-myc and pS2 mRNA expression. Total mRNA was extracted from the cell pellet, reverse transcribed and gene expression was measured by real-time RT-PCR as described previously (20).

**C-myc:**

FWD-5’-CGTCTCCACACATCAGCACAA -3’
REV-5’-TGTTGGCAGCAGGATAGTCTTTT -3’
Probe-5’-56FAM/ACGCAGCGTCCGCTCCCTAC/3BHQ-1/-3’

**pS2:**

FWD-5’-CGTGAAAGACAGAATTGTGGTTTT-3’
REV-5’- CGTCGAAACAGCAGCCTTA-3’
Probe-5’-/56FAM/ TGTCACGCCCTCCCAGTGTGCA/3BHQ-1/-3’

ERα

FWD-5’- AGACGGACCAAAGCCACTTG -3’
REV-5’- CCCCGTGATGTAATACTTTTGCA -3’
Probe-5’-/56FAM/ TGCGGGCTCTACTTCATCGCATTCC /3BHQ-1/-3’

ERβ

FWD-5’- CCCAGTGCGCCCTTCAC-3’
REV-5’- CAACTCCTTGTCGGCCAACT -3’
Probe-5’-/56FAM/ AGGCCTCCATGATGTCCCTGA /3BHQ-1/-3’

Ligand Binding Assay

MCF-7 cells were plated in 6-well plates (2 × 10^5 cell/plate), and cultured in 2% charcoal stripped FBS in DMEM. The cells were maintained in the stripped media for 3 days until 90% confluent. MCF-7 cells were incubated with MSA (10µM) for 1 h. Following 1 h incubation 10nM [^3H] E2 in presence or absence 500 fold excess cold estradiol was added to each well and incubated for 1 h. Following 1 h incubation, the cells were washed 3X with cold 1X PBS. 700µL of ethanol was added to each well and incubated at room temperature for 30 min. 500µL of ethanolic extract was counted using liquid scintillation. The relative binding affinity (RBA) was calculated by (Specific
binding – nonspecific binding/ specific binding) × 100. Vehicle treated samples were set as 1

Data Analysis

Results are expressed as mean ± S.D. P values were calculated using Anova Dunnett’s T Test and Independent T Test. P<.05 was considered significant.
Results

MSA inhibits estradiol- and tamoxifen-dependent activation of an estrogen response element (ERE)-luciferase reporter gene (ERE2e1b-luciferase).

The effects of MSA on ER signaling were assessed in MCF-7 cells, a well-characterized ER-positive, tamoxifen sensitive human breast cancer cell, and in MCF-7-LCC2 cells, an ER-positive tamoxifen resistant variant of the MCF-7 parental line. Two tamoxifen resistant human endometrial cancer cell lines, Ishikawa and HEC1A, were also assessed for MSA effects on the ERE2e1b-luciferase. MCF-7, HEC1A, and Ishikawa cells were transfected with the ERE2e1b-luciferase reporter gene and incubated with estradiol (10^{-8} M), 4-hydroxytamoxifen (10^{-7} M), or MSA (10\mu M) alone, or co-incubated with combinations of estradiol, 4-hydroxytamoxifen and MSA as indicated (Fig. 1A, C, and D). MCF-7-LCC2 cells were transfected with the ERE2e1b-luciferase reporter gene and incubated with estradiol (10^{-9} M), 4-hydroxytamoxifen (10^{-8} M), or MSA (10\mu M) alone, or co-incubated with combinations of estradiol, 4-hydroxytamoxifen and MSA as indicated (Fig. 1B). Although MCF-7-LCC2 cells are resistant to tamoxifen growth inhibition, the cells do not display a true tamoxifen agonist activation of the ERE2e1b-luciferase reporter (Fig 1B, Bar 5), an activation that is observed in the endometrial cancer cell lines HEC1A and Ishikawa (Fig 1C-D, Bar 5) as we and others have previously reported (20-22). Tamoxifen resistance in MCF-7-LCC2 cells is manifested by the inability of 4-hydroxytamoxifen to reverse estradiol activation of the reporter (Fig. 1B, Bar 7), which is observed at 10^{-9} M estradiol + 10^{-8} M 4-hydroxytamoxifen. In the
tamoxifen-sensitive MCF-7 parental cells a higher concentration of estradiol (10^-8 M) was required to observe estradiol activation of the reporter. Consequently, a higher concentration of 4-hydroxytamoxifen (10^-7 M) was used for reversing estradiol activation in MCF-7 cells (Fig. 1A, Bar 7).

To determine whether MSA might impact reporter activation by estradiol and tamoxifen, cells were co-incubated with estradiol, 4-hydroxytamoxifen, and MSA. MSA alone decreased basal ERE<sub>e1b</sub>-luciferase expression in MCF-7 and MCF-7-LCC2 cells (Fig. 1A-B, bar 2) and inhibited estradiol-dependent stimulation of the reporter in all cell lines (Fig. 1A-D, bars 3 and 4). Although MSA did not significantly impact 4-hydroxytamoxifen action in either breast cancer cell line (Fig 1A and B, bar 6), MSA completely blocked 4-hydroxytamoxifen activation of the reporter in both endometrial cell lines (Fig. 1C and D, bar 6). Co-treatment with MSA potentiated the inhibitory effect of 4-hydroxytamoxifen on estradiol activation of the reporter in MCF-7 cells (Fig 1A, compare bars 7,8). In MCF-7-LCC2 cells, MSA combined with tamoxifen reversed tamoxifen resistance by blocking estradiol activation of the reporter greater than the effect of MSA alone (Fig 1B, compare bars 4,7,8). Note that co-incubation with 4-hydroxytamoxifen + estradiol + MSA resulted in greater inhibition of reporter activation than estradiol + MSA (Fig 1A-B, compare bars 4, 8) or estradiol + 4-hydroxytamoxifen (Fig 1A-B, compare bars 6, 8). These data demonstrate the MSA inhibits estradiol activation of the ERE<sub>e1b</sub>-luciferase reporter in breast and endometrial cancer cells, potentiates the antiestrogen effects of 4-hydroxytamoxifen in MCF-7-LCC2 cells, and blocks 4-hydroxytamoxifen agonist action in endometrial cancer cell lines.
MSA inhibits the endogenous ERα-regulated gene expression.

To determine whether MSA affected endogenous ER-regulated genes, the well-characterized estrogen-regulated gene c-myc was assessed. MCF-7, MCF-7-LCC2, HEC1A, and Ishikawa were incubated with either vehicle, estradiol (10⁻⁸ M), 4-hydroxytamoxifen (10⁻⁷ M) or MSA (10μM) alone or a combination of estradiol or 4-hydroxytamoxifen with MSA for 6 h (Fig. 2A-D). MSA had no effect on basal c-myc gene expression with the exception of HEC1A cells (fig 2A-D, bar 2). However, MSA inhibited estradiol-induced gene expression of c-myc in all cell lines (fig 2A-D, bars 3 and 4). 4-hydroxytamoxifen had no effect on basal c-myc gene expression in MCF-7 cells, and MSA in combination with 4-hydroxytamoxifen also had no effect when compared to vehicle or 4-hydroxytamoxifen incubated samples alone (Fig 2A, bars 5 and 6). In contrast to the inability of 4-hydroxytamoxifen to activate the ERE₂e1b-luciferase reporter in MCF-7-LCC2 cells, 4-hydroxytamoxifen displayed true agonist activation of endogenous c-myc in these cells (Fig. 2B, bar 5) that was also evident in both endometrial cell lines HEC1A and Ishikawa (Fig 2C-D, bar 5). MSA blocked 4-hydroxytamoxifen activation of c-myc in MCF-7-LCC2, Ishikawa, and HEC1A cells (Fig 2B-D, bar 6). Similar results to those described in Figure 2 were found for the ER-dependent pS2 gene (data not shown).

MSA reduces ERα protein levels in tamoxifen-sensitive and –resistant cell lines.
We previously demonstrated that ERα protein and mRNA downregulation was likely a major mechanism by which MSA inhibited ER signaling in MCF-7 cells; MSA had no effect on ERβ mRNA (revised manuscript submitted to *Breast Cancer Research and Treatment*). These experiments were extended to determine whether MSA altered ERα protein in both tamoxifen-sensitive and resistant cells. In addition, the effect of estradiol or 4-hydroxytamoxifen alone or in combination with MSA on ERα protein expression was also assessed. Due to very low expression of ERα in HEC1A cells, the Western blot analysis was inconclusive (data not shown). MCF-7, MCF-7-LCC2 and Ishikawa cells were incubated with estradiol (10^-8 M), 4-hydroxytamoxifen (10^-7 M), or MSA (10µM) alone, or estradiol or 4-hydroxytamoxifen in combination with MSA for 6 h and ERα levels were assessed by Western blot analysis. Estradiol treatment alone reduced ERα protein levels only in the MCF-7 cells (Fig 3A, bar 3) that is likely mediated through the ubiquitin proteosome pathway (23). In contrast to estradiol, MSA alone significantly reduced ERα protein in all cell lines (Fig 3A-C, bar 2). MSA further reduced ERα levels when co-incubated with estradiol in MCF-7 cells (Fig 3A, bars 3 and 4) as we have previously demonstrated (revised manuscript submitted to *Breast Cancer Research and Treatment*). Remarkably, in MCF-7-LCC2 and Ishikawa cells estradiol had no effect on ERα protein expression, a previously unreported observation for estradiol regulation of ERα (Fig. 3B and C, bar 3). Estradiol + MSA reduced ERα protein expression to the level detected with MSA treatment alone (Fig 3B and C, bars 3 and 4).
4-hydroxytamoxifen stabilized ERα protein expression in MCF-7 cells (Fig. 3A, bar 5) as previously reported (24). 4-hydroxytamoxifen also stabilized ERα expression in Ishikawa cells but had no effect on ERα levels in MCF-7-LCC2 cells (Fig 3B and C, bar5). Co-incubation of 4-hydroxytamoxifen with MSA decreased ERα expression when compared to either vehicle or 4-hydroxytamoxifen alone in all cell lines (Fig. 3A-C, bars 5 and 6).

Taken together, these data demonstrate that MSA downregulation of ERα is not restricted to tamoxifen sensitive MCF-7 cells and occurs in the presence or absence of ligand. Two unrelated findings from these experiments were that estradiol had no effect on ERα protein expression in MCF-7-LCC2 and Ishikawa cells, and that 4-hydroxytamoxifen stabilized ERα expression in Ishikawa cells (Fig 3C, bar 5), but had no effect on ERα levels in MCF-7-LCC2 cells (Fig 3B, bar 5). The molecular mechanisms of cell-specific and ligand-dependent receptor turnover are currently being investigated.

**MSA reduces ERα mRNA but has no affect on ERβ.**

Our previous study found that MSA decreased ERα mRNA in MCF-7 cells and the decrease in mRNA preceded a decrease in ERα protein (revised manuscript submitted to *Breast Cancer Research and Treatment*). We extended these studies to tamoxifen resistant cell lines and also included assessment of 4-hydroxytamoxifen + MSA. ERα mRNA in MCF-7, MCF-7-LCC2, HEC1A and Ishikawa cells was measured by real-time
RT-PCR following incubation of cells with estradiol (10^{-8} M), 4-hydroxytamoxifen (10^{-7} M) or MSA (10\mu M) alone or estradiol or 4-hydroxytamoxifen + MSA for 2 h. As previously shown, MSA reduced ER\alpha expression in MCF-7 cells (Fig. 4A, bar 2). MSA also decreased ER\alpha mRNA in MCF-7-LCC2, HEC1A, and Ishikawa cells (Fig. 4B-C, bar 2). Estradiol or 4-hydroxytamoxifen had no effect on ER\alpha gene expression in MCF-7, MCF-7-LCC2, and HEC1A (Fig. 4A-C, bars 3 and 5), although 4-hydroxytamoxifen significantly decreased ER\alpha mRNA in Ishikawa cells (Fig 4D bar 5). Co-incubation of estradiol or 4-hydroxytamoxifen with MSA decreased ER\alpha mRNA to levels observed with MSA alone (Fig. 4A-D, bars 4 and 6). MSA, estradiol, or 4-hydroxytamoxifen alone or in combination had no effect on ER\beta mRNA expression in all cell lines (Fig 5A-D) suggesting selective effects of MSA on ER\alpha.

At two hours incubation with MSA, ER\alpha mRNA was reduced with no observed effects on ER\alpha protein levels (data not shown). This suggests that the MSA-dependent decrease in ER\alpha mRNA may account for subsequent decrease in ER\alpha protein.

Remarkably, although 4-hydroxytamoxifen decreased ER\alpha mRNA in Ishikawa cells (Fig. 4D, bar 5), ER\alpha protein actually increased with treatment (Fig. 3D, bar 5). The mechanisms underlying this novel observation are under investigation.

**MSA potentiates the growth inhibitory properties of 4-hydroxytamoxifen.**

Our previous study found that MSA decreased the growth of MCF-7 cells through a combination of decreased DNA synthesis and elevated apoptosis (revised manuscript)
submitted to *Breast Cancer Research and Treatment*). MCF-7 cells are also sensitive to growth inhibition by tamoxifen (25). It was of interest to determine whether co-incubation of 4-hydroxytamoxifen with MSA could further potentiate growth inhibition compared to either agent alone. Furthermore, since long-term tamoxifen treatment is associated with tamoxifen resistance and endometrial proliferation, it was desirable to know whether co-incubation of MSA + 4-hydroxytamoxifen could reverse tamoxifen resistance in breast cancer cells and inhibit tamoxifen-induced endometrial cell proliferation. MCF-7, MCF-7-LCC2, HEC1A, Ishikawa and an additional tamoxifen-sensitive ER-positive breast cancer cell line, T47D, were incubated with 4-hydroxytamoxifen (10^{-7} M), MSA (2.5\mu M) or both agents for 24, 48, 72 and 96 h and growth was assessed by the MTT assay. Only tamoxifen-sensitive MCF-7 and T47D cells were growth inhibited by 4-hydroxytamoxifen whereas no effect was observed in the tamoxifen-resistant MCF-7-LCC2, HEC1A, and Ishikawa cells (Fig. 6A). Increasing incubation time with 4-hydroxytamoxifen to 9 days resulted in proliferation of HEC1A and Ishikawa cells (20). In all cell lines, MSA decreased cell growth by 96 hours incubation (Fig. 6B-F). In tamoxifen sensitive MCF-7 and T47D cells, MSA potentiated 4-hydroxytamoxifen effects on reducing cell growth with the combined treatment more effective than either agent alone (Fig. 6B and C). Remarkably, in tamoxifen resistant MCF-7-LCC2, HEC1A and Ishikawa cells in which 4-hydroxytamoxifen alone has no effect on cell growth, co-incubation of 4-hydroxytamoxifen with MSA resulted in a marked decrease in cell growth that was substantially greater than MSA treatment alone (Fig. 6D-F). These results demonstrate that MSA not only potentiates the antiestrogen
effect of 4-hydroxytamoxifen in tamoxifen-sensitive cells, but MSA also resensitizes tamoxifen-resistant cells to the growth inhibitory properties of 4-hydroxytamoxifen.

**Tamoxifen and MSA do not synergize for growth inhibition in ERα negative cell lines.**

MDA-MB-468 cells, an ERα-negative (26), ERβ-positive (27) human breast cancer cell line, were incubated with 4-hydroxytamoxifen (10⁻⁸ M) for 24, 48, 72, and 96 h and growth was assessed by the MTT assay. No growth inhibitory effects were observed after incubation with 4-hydroxytamoxifen (Fig. 7A) as previously reported (28). Although MSA (1µM) alone decreased cell growth (Fig. 7A), co-incubation of 4-hydroxytamoxifen with MSA did not further decrease cell growth (Fig. 7A) suggesting that ERα may be required for the additive and/or synergistic effect of tamoxifen + MSA on cell growth. Similar results were found with the ERα-negative MDA-MB-231 cell line (data not shown).

Inorganic selenite is reported to interact with the ligand binding domain of ERα and activate ERα transcriptional activity (29). To determine whether MSA altered estradiol binding to ERα, whole cell ligand binding assays were performed in MCF-7 cells. One hour incubation of MCF-7 cells with MSA (10µM) did not alter [³H] estradiol binding to ERα (Fig. 7B).
Discussion

Tamoxifen resistance and tamoxifen-induced endometrial proliferation are major limitations of tamoxifen therapy and chemoprevention. The present study demonstrates that MSA inhibition of ERα signaling is not restricted to tamoxifen sensitive MCF-7 cells. MSA antagonism of estradiol-dependent ERE2e1b-luciferase and endogenous c-myc and pS2 gene expression was demonstrated in MCF-7-LCC2 cells, an ERα-positive, tamoxifen resistant variant of the MCF-7 parental line and in two ERα-positive human endometrial cancer cell lines, Ishikawa and HEC1A (Fig. 1 and 2). In addition, MSA also blocked tamoxifen activation of these genes in endometrial Ishikawa and HEC1A cells (Fig. 1 and 2 C-D, bar 6). The major mechanism for MSA disruption of ER signaling in all ERα positive cells lines was via rapid decrease of ERα mRNA and protein that preceded disruption of ERα-regulated gene expression (Fig. 3 and 4). MSA alone inhibited the growth of tamoxifen-sensitive and tamoxifen-resistant cells. MSA also potentiated tamoxifen growth inhibition of ERα-positive, tamoxifen-sensitive cells (MCF-7 and T47D) and tamoxifen-resistant cells (MCF-7-LCC2, Ishikawa, and HEC1A) (Fig. 6) but not ERα-negative, tamoxifen resistant cells (MDA-MB-468) (Fig. 7A) suggesting that ERα is required for the additive and/or synergistic effect on cell growth inhibition when MSA is combined with tamoxifen.

Interestingly our studies demonstrated that sensitivity to MSA growth inhibition is cell line specific. HEC1A exhibited a marked decrease in cell growth after treatment with 2.5 µM MSA for 48 h, whereas MCF-7-LCC2 cells do not show a significant
decrease in cell growth until 72 h incubation with MSA (Fig. 6 D and E). In addition, 1µM MSA reduced growth of MDA-MB-468 at 72 h (Fig. 7A), whereas the same concentration did not affect growth of MCF-7, MCF-7-LCC2, HEC1A, and Ishikawa cells (data not shown). Currently the cell specific sensitivity of MSA is under investigation.

A review of the literature from cancer cell lines and in vivo tumors reveals that MSA and tamoxifen exhibit similarities in growth inhibitory mechanisms. Both agents induce G1 arrest that is associated with a similar profile of changes in cell cycle regulatory proteins (19,30-32). Both agents induce a dose dependent apoptosis that is p53 independent and may involve activation of the same caspases as well as reduction in bcl-2 (14,15,33-35). This suggests that the added efficacy observed with combination of selenium with tamoxifen is the result of more pronounced perturbations in several common regulatory proteins resulting in elevated apoptosis and reduced proliferation when compared to effects of either agent alone.

As we have previously demonstrated, low concentrations of MSA (1-2.5 µM) had no effect on ERα mRNA and protein expression while still capable of inhibiting estradiol-dependent gene expression (revised manuscript submitted to Breast Cancer Research and Treatment). Therefore at low MSA concentrations, disruption of ER signaling occurred via mechanisms independent of ERα depletion suggesting that MSA also affected ERα function. Dong et al. (35) demonstrated that MSA regulates
expression of several proteins known to be important mediators of ERα action. In this study it was shown that MSA decreased cyclin D1 levels in premalignant human breast cancer cells. In addition to its functions as a cell cycle regulator, cyclin D1 is also an ERα coactivator (36) and its overexpression is correlated with tamoxifen resistance in ERα-positive postmenopausal breast cancer (37,38). Exogenous expression of cyclin D1 in tamoxifen sensitive breast cancer cells reverse the growth inhibitory properties of tamoxifen (39). In addition to cyclin D1, other coactivators are important in the mechanism of tamoxifen resistance. Overexpression of AIB1 (amplified in breast cancer-1 (SRC-3/RAC-3/ACTR)) in patients receiving tamoxifen was correlated with tamoxifen resistance (40). Elevated SRC-1 (steroid receptor coactivator-1 (NcoA-1)), but not TIF-2 (transcriptional intermediary factor-2 (SRC-2/NcoA-2/GRIP1)) or AIB1 was correlated with tamoxifen agonist activity in Ishikawa cells (41). Corepressor levels have been associated with tamoxifen resistance. A decrease in nuclear corepressor (N-CoR) levels have been associated with a shorter relapse free survival in tamoxifen treated patients, suggesting that N-CoR may be a good independent prognostic marker of tamoxifen resistance (42). Dong et al. (35) reported that MSA reduced AKT levels, which is interesting in light of several reports demonstrating increased AKT activity in tamoxifen resistant breast cancer cells (43,44). Future studies will elucidate the molecular mechanisms underlying the ability of MSA to restore tamoxifen sensitivity in resistant cells.
Although clinical trials with selenium are currently limited to chemoprevention, recent evidence now strongly demonstrate the potential of utilizing selenium in a new way; as a novel therapy for overt cancer through combination with well-established chemotherapeutic and hormonal agents. Several studies have demonstrated growth inhibition of established tumors by selenium in in vivo models (45-49). However these studies used inorganic selenium compounds that are genotoxic and no longer used for selenium chemoprevention. More recently, Cao et al. demonstrated a synergistic interaction of organic selenium compounds with the topoisomerase 1 poison irinotecan (50). Xenograft mice bearing squamous cell carcinomas of the head/neck and colon were given selenium in the form of MSC and Se-Met orally seven days prior to intravenous injection of irinotecan. Combination treatment of irinotecan + selenium decreased the toxicity of the chemotherapeutic agent and increased the cure rate of the tumor-bearing mice inoculated with cancer cells sensitive and resistant to irinotecan (50). The present study provides a compelling rationale to explore therapeutic regimens combining tamoxifen with organic selenium compounds for hormone dependent breast cancer.
Figure Legends

Fig. 1. **MSA inhibits estrogen and tamoxifen activated ERE_{2}e1b-luciferase reporter.** (A) MCF-7, (B) MCF-7-LCC2, (C) HEC1A, and (D) Ishikawa cells (2 x 10^5 cells/well) were transfected with ERE_{2}e1b-luciferase reporter (0.5 µg/well). 24 hours posttransfection cells were incubated with vehicle (Veh), 10^{-8} M estradiol (E2) for MCF-7, HEC1A and Ishikawa or 10^{-9} M E2 for MCF-7-LCC2 cells, 10^{-7} M 4-hydroxytamoxifen (Tam) for MCF-7, HEC1A and Ishikawa or 10^{-8} M Tam for MCF-7-LCC2 cells or 10µM MSA alone or co-incubated with E2 and MSA, Tam and MSA, or E2, Tam and MSA for 24 h. Standard luciferase assays were performed on cell extracts in triplicate as described in the Materials and Methods. Each bar represents the mean value ± S.D. (*) = P<.05 compared to vehicle incubated samples. (†) = P<.05 compared to estradiol or 4-hydroxytamoxifen treated samples. (‡) = P<.05 compared to compared to estradiol and 4-hydroxotamoxifen co-incubated samples.

Fig. 2. **MSA inhibits estradiol and tamoxifen-dependent activation of c-myc gene expression.** (A) MCF-7, (B) MCF-7-LCC2, (C) HEC1A, and (D) Ishikawa cells (2 x 10^6 cells/plate) were incubated with vehicle (Veh), 10^{-8} M estradiol (E2) 10^{-7} M 4-hydroxytamoxifen (Tam) or 10µM MSA alone or co-incubated with E2 and MSA or Tam and MSA for 6 h. C-myc mRNA was measured by real time RT-PCR as described in the Materials and Methods. Expression was normalized to GAPDH and each bar represents
the mean value ± S.D. (⁎)= P<.05 compared to vehicle incubated samples (†) = P<.05 compared to estradiol or 4-hydroxytamoxifen incubated samples.

Fig. 3. **MSA reduces ERα protein.** (A) MCF-7, (B) MCF-7-LCC2, and (C) Ishikawa cells (2 x 10^6 cells/plate) were incubated with vehicle (Veh), 10^{-8} M estradiol (E2) 10^{-7} M 4-hydroxytamoxifen (Tam) or 10μM MSA alone or co-incubated with E2 and MSA or Tam and MSA for 6 h. Western blot analysis was performed. Quantitation of the Western blot signal for ERα was normalized to the Western blot signal for β-actin levels (Fig 3A,B, and C, Right panels), and each bar represents the mean value ± S.D. (⁎)= P<.05 compared to vehicle incubated samples (†) = P<.05 compared to estradiol or 4-hydroxytamoxifen incubated samples.

Fig. 4. **MSA reduces ERα mRNA.** (A) MCF-7, (B) MCF-7-LCC2, (C) HEC1A, and (D) Ishikawa cells (2 x 10^6 cells/plate) were incubated with vehicle (Veh), 10^{-8} M estradiol (E2), 10^{-7} M 4-hydroxytamoxifen (Tam), or 10μM MSA alone or co-incubated with E2 and MSA or Tam and MSA for 2 h. ERα mRNA was measured by real time RT-PCR as described in the Materials and Methods. Expression was normalized to GAPDH and each bar represents the mean value ± S.D. (⁎)= P<.05 compared to vehicle incubated samples (†) = P<.05 compared to estradiol or 4-hydroxytamoxifen incubated samples.

Fig. 5. **MSA does not alter ERβ mRNA.** (A) MCF-7, (B) MCF-7-LCC2, (C) HEC1A, and (D) Ishikawa cells (2 x 10^6 cells/plate) were incubated with vehicle (Veh), 10^{-8} M
estradiol (E2), 10^-7 M 4-hydroxytamoxifen (Tam), or 10µM MSA alone or co-incubated with E2 and MSA or Tam and MSA for 2 h. ERβ mRNA was measured by real time RT-PCR as described in the Materials and Methods. Expression was normalized to GAPDH and each bar represents the mean value ± S.D.

Fig. 6. **MSA increases the growth inhibitory properties of 4-hydroxytamoxifen.** (A) Growth inhibitory effects of 4-hydroxytamoxifen as measured by MTT assay. (A) MCF-7, T47D, MCF-7-LCC2, HEC1A, and Ishikawa cells (2 x 10^4 cells/well) were incubated with vehicle or 10^-7 M 4-hydroxytamoxifen for 24, 48, 72, and 96 h. Each bar represents the mean value ± S.D. The graph is expressed as % vehicle, where vehicle incubated samples are set as 100% (●) = P<.05 compared to vehicle. (B) MCF-7, (C) T47D, (D) MCF-7-LCC2, (E) HEC1A, and (F) Ishikawa cells (2 x 10^4 cells/well) were incubated with vehicle, 10^-7 M 4-hydroxytamoxifen (Tam) or 2.5µM MSA or co-incubated with Tam and MSA for 24, 48, 72, and 96 h and MTT assays were performed. The graph is expressed as % vehicle, where vehicle incubated samples are set as 100% (●) = P<.05 compared to 4-hydroxytamoxifen or MSA incubated samples alone.

Fig. 7. **Tamoxifen and MSA do not synergize for growth inhibition in ERα negative cell lines.** (A) MDA-MB-468 cells (2 x 10^4 cells/well) were incubated with vehicle, 10^-7 M 4-hydroxytamoxifen (Tam) or 1µM MSA or co-incubated with Tam and MSA for 24, 48, 72, and 96 h and MTT assays were performed. The graph is expressed as % vehicle, where vehicle incubated samples are set as 100% (●) = P<.05 compared to 4-hydroxytamoxifen or MSA incubated samples alone.
binding. MCF-7 cells (4 x 10⁵ cells/well) were incubated with vehicle (Veh) or 10μM MSA for 1 h. Following 1 h incubation 10nM [%H] E₂ in presence or absence 500 fold excess cold estradiol was added to each well and incubated for 1 h. Relative binding affinity was measured as described in Material and Methods, and vehicle incubated samples were set as 1.
Reference List


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**Fig 2**

C. HEC1A

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D. Ishikawa

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SUMMARY

The research projects in this dissertation focus on the molecular mechanisms of tamoxifen sensitivity and resistance with the goal of improving tamoxifen efficacy in breast and endometrial cancer. These studies have provided protein profiles that may serve as novel indices of SERM response and may also provide insight into novel mechanisms of SERM-mediated growth. Following 24-hour incubation with $10^{-8}$ M estradiol, $10^{-7}$ M 4-hydroxytamoxifen, or $10^{-7}$ M EM-652 (Acolbifene), nine proteins exhibited significant increase in expression. The proteins identified were heat shock protein 90 (HSP90)-α, and –β, heterogeneous nuclear ribonucleoprotein F (hnRNP F), RNA polymerase II-mediating protein (RMP), cytoskeletal keratin 8 (Ck 8), cytoskeletal keratin 18 (Ck 18), ubiquitin-conjugating enzyme E2-18kDa (UbcH7), and nucleoside diphosphate kinase B (NDPK B). Interestingly, we have shown NDPK B to be induced by estradiol and tamoxifen in both the secreted and cytoplasmic fraction of Ishikawa cells. Previous studies have shown ectopic expression of NDPK B to increase cellular proliferation (Caligo et al., 1995, 1996). Therefore, future studies will assess whether NDPK B participates in estrogen- and tamoxifen-dependent proliferation in Ishikawa and other endometrium-derived cell lines. The NDPK B may serve as a biomarker for the tamoxifen resistant phenotype, and since NDPK B is a secreted protein it could easily be assayed by non-invasive procedures.

In addition, these studies present a mechanistic groundwork for rational design of preclinical therapeutic strategies designed to inhibit src kinase and combine tamoxifen with organic selenium compounds to circumvent tamoxifen resistant breast cancer and
relieve the deleterious estrogen-like side effects of tamoxifen and other SERMs in non-target tissues. It was found that src kinase inhibitors and MSA disrupted ERα signaling and potentiated the antagonist action of tamoxifen in sensitive and resistant breast and endometrial cell lines via separate mechanisms. Src kinase inhibitors blocked ERα promoter interaction through decrease phosphorylation at serine 167 and inhibited SRC-1 transactivation. Seven phosphorylation sites have been identified on SRC-1 (Rowan et al., 2000b), although no single site was responsible for src kinase mediated potentiation of SRC-1. Several possibilities will be assessed in the future: 1) Since all seven SRC-1 phosphorylation sites contain consensus sequences for serine/threonine-proline directed kinases it is possible that only one or few kinases contribute to steady state SRC-1 phosphorylation; 2) Src kinase increases phosphorylation at multiple sites; 3) Activation of src kinase mediates a dephosphorylation event on SRC-1; 4) A different protein in the SRC-1 complex may be a direct or indirect substrate of src kinase; and 5) A novel phosphorylation site may be involved in src kinase dependent increase in SRC-1 activity.

The MSA decreased ERα mRNA and protein levels in tamoxifen-sensitive and resistant breast and endometrial cancer cell lines in vitro. Future experiments will delineate molecular mechanisms modulating MSA decrease of ERα levels by assessing ERα transcription rates, hnRNA and cytosolic mRNA stability, nuclear mRNA export, translation rates and protein turnover. A decrease in ERα expression is thought to be the major mechanism in disruption of ERα-regulated gene expression and potentiation of tamoxifen-mediated growth inhibition. However, low concentrations of MSA (1-2.5 µM) had no effect on ERα mRNA and protein expression while still capable of inhibiting
estradiol-dependent gene transcription. Therefore at low MSA concentrations, disruption of ER signaling occurred via mechanisms independent of ERα depletion suggesting that MSA also affects other parameters of ERα function. The MSA inhibits in vitro DNA binding by AR (Dong et al., 2004). Dong et al. (2002a) demonstrated that MSA decreased cyclin D1 levels in premalignant breast neoplasms. In addition to its functions as a cell cycle regulator, cyclin D1 is also an ERα coactivator (Neuman et al., 1997) and its overexpression is correlated with tamoxifen resistance in ERα-positive postmenopausal breast cancer (Kenny et al., 1999; Stendahl et al., 2004). Dong et al. (Dong et al., 2002a) reported that MSA reduced AKT levels, which have been shown to be important in tamoxifen resistant breast cancer cells (Campbell et al., 2001; Clark et al., 2002). Future experiments will assess the effects of MSA on ERα promoter interaction as well as expression levels of other coregulators and kinases.

Future studies will utilize animal models to further validate the SERM-regulated proteins identified in this dissertation and for the development of non-cross-resistant therapies utilizing inhibitors of src kinase or selenium in combination with tamoxifen. The studies presented in the dissertation are particularly important in the prevention and therapy of breast cancer. By understanding the precise molecular mechanisms better therapeutic strategies can be developed to inhibit tamoxifen resistant breast cancer and relieve the deleterious estrogen-like side effects of tamoxifen and other SERMs in non-target tissues.


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ABSTRACT

Tamoxifen is the most widely prescribed selective estrogen receptor modulator (SERM) for breast cancer. Despite the benefits of tamoxifen therapy, almost all tamoxifen-responsive breast cancer patients develop resistance to therapy and tamoxifen exhibits ER agonist action in the uterus that is associated with an increased incidence of endometrial cancer. These problems with tamoxifen therapy indicate a need to identify new biomarkers for tamoxifen therapy that are predictive of tamoxifen resistance and/or the agonist effects associated in the endometrium. Protein expression profiles associated with a graded estrogen response were generated in an ER-positive and tamoxifen resistant Ishikawa endometrial adenocarcinoma cell-line using two-dimensional gel electrophoresis and mass spectrometry sequencing. Among the identified proteins were chaperones proteins, RNA and RNA polymerase binding proteins, cytoskeletal proteins, an ubiquitin ligase, and a multi-functional kinase. The proteins identified may serve as useful biomarkers for development of tamoxifen resistance or serve as novel tumor targets.

In related projects designed to identify novel mechanisms to increase tamoxifen efficacy, it was found that src kinase inhibitors and the micronutrient selenium modulated ERα signaling and potentiated the antagonist action of tamoxifen in sensitive and resistant breast and endometrial cell lines through discrete mechanisms. Src kinase induced ERα phosphorylation at serine 167 via the PI3K/AKT pathway that was shown to be critical for ERα promoter interaction. In addition, src specifically enhanced the
transcriptional activity of steroid receptor coactivator-1 (SRC-1), which was sufficient to enhance its coactivation of ERα. Inhibition of src kinase blocked these mechanisms and prevented tamoxifen agonist action in tamoxifen resistant cell lines.

Methylseleninic acid (MSA) is a monomethylated selenium compound that inhibits growth of cancer cells in vitro. MSA decreased ERα mRNA and protein levels in tamoxifen-sensitive and -resistant breast and endometrial cancer cell lines in vitro that resulted in subsequent antagonism of estradiol-dependent ERE2-e1b-luciferase and endogenous c-myc and pS2 gene expression. MSA also reversed tamoxifen activation of these genes in endometrial Ishikawa and HEC-1A cells. In addition, MSA potentiated the growth inhibitory affects of tamoxifen in tamoxifen-sensitive and -resistant breast and endometrial cancer cells.