THE INTERPLAY OF GENOMIC AND NON-GENOMIC ESTROGEN RECEPTOR SIGNALING PATHWAYS IN MEDIATING CELLULAR REPSONSES TO XENOESTROGENS

BY

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To my family

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LIST OF ABBREVIATIONS

BPA	Bisphenol-A
DPN	ESR2 Specific Agonist
E_2	Endogenous Estrogen, 17β-Estradiol
ERE	Estrogen Response Element
ESR	Nuclear Estrogen Receptors
G-1	GPER Specific Agonist
G-15	GPER Specific Antagonist
GPER	G Protein-Coupled Estrogen Receptor
MCF-7	ESR positive, GPER positive human breast cancer cell line
NDRG2	N-Myc Downstream Regulated Gene 2
PPT	ESR1 Specific Agonist

Abstract of Thesis Presented to the Graduate School
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Estrogen can exert cellular effects through both nuclear (ESR1 and ESR2) and membrane-bound receptors (GPER). It is unclear if these receptors act independently or engage in crosstalk to influence hormonal responses. To investigate each receptor's role in proliferation, activation of reporter genes and protein phosphorylation in breast cancer cells (MCF-7), we employed selective agonists for ESR1 (PPT), ESR2 (DPN) and GPER (G-1). We also determined the impact of the xenoestrogens BPA and genistein, on these cellular effects. As anticipated, 17β-estradiol (E_2), PPT, DPN, BPA, and genistein each independently enhanced cell proliferation and activation of an estrogen response element (ERE) driven reporter gene when compared to control cells, whereas G-1 had no significant impact. However, G-1 inhibited E_2, PPT-, DPN-, BPA, and genistein-induced proliferation and ERE activation at doses greater than 500 nM. Furthermore, inhibition of ESR activity was not receptor isotype specific and preliminary studies using the GPER antagonist (G-15) suggests these observations are GPER independent. As membrane receptors initiate a cascade of phosphorylation events, we performed a global phosphoproteomic analysis on cells exposed to E_2 or G-1. Of the

238 phosphorylated proteins identified only 10 sites were similarly phosphorylated between E_2 and G-1 whereas 31 and 13 sites were specifically modified by each ligand, respectively. Using pathway analysis we identified ‘central nodes’ (i.e. NDRG2) phosphorylated by G-1 as putative proteins that modulate the inhibition of E_2- and xenoestrogen-mediated ESR activity and cell proliferation. Taken together, these results reveal a novel role for G-1 in interfering with nuclear receptor activity driven by E_2 and xenoestrogens.

CHAPTER 1
LITERATURE REVIEW

Endocrine Disruption

Endocrine disruptors are environmental contaminants that can interfere with normal hormonal signaling by disrupting the tightly regulated synthesis, structure or function of hormones and contribute to adverse effects in human and wildlife populations (Colborn *et al.*, 1993; Kavlock *et al.*, 1996). The idea that exogenous chemicals could bind to hormone receptors and cause adverse effects was motivated by the observation of impaired reproductive performance in sheep grazing on red clover pastures in Australia (Morley *et al.*, 1964). This concept was translated to humans when it was noticed that a rare form of vaginal cancer occurred in daughters of women who had been administered a synthetic estrogen, Diethylstilbestrol (DES), during pregnancy to prevent miscarriages (Herbst *et al.*, 1970; Herbst *et al.*, 1999). The unfortunate consequences of DES exposure and subsequent research solidified the 'endocrine disruptor hypothesis' which states that both naturally occurring and synthetic chemicals may disrupt the normal functions of the endocrine system and its hormones in organisms (Patisaul and Adewale, 2009). Since then, the most widely studied endocrine disruptors are those that interfere with signaling by the endogenous estrogen, 17β-estradiol (E_2), which is necessitated by the drastic increase in number of chemicals identified with estrogenic activity in the past few decades (Thomas and Dong, 2006). These xenoestrogens can be naturally occurring, such as the soy-based phytoestrogens (i.e. genistein), or are anthropogenic and encompass a diverse array of chemicals that are ubiquitously used in pesticides, plastics, and pharmaceuticals. Many of these compounds are suspected to contribute to a myriad of disorders including

cardiovascular disease, hypertension, metabolic diseases, immune disorders, and reproductive cancers (Deroo and Korach, 2006).

The classical mechanism of action of xenoestrogens is well established to be mediated by ligand-activated transcription factors known as the nuclear estrogen receptors (ESR1 and ESR2). Once engaged by ligand the ESRs adopt a conformational change that promotes dimerization and recruitment of various co-regulatory proteins to the transcriptional complex (McKenna and O'Malley, 2001). This ESR receptosome then interacts with *cis*-acting estrogen response elements in the promoters of estrogen-responsive genes by either direct DNA binding to classical estrogen response elements (ERE) or through alternate elements (AP-1, Sp-1) via tethering mechanisms thereby initiating aberrant gene expression (Hall *et al.*, 2001).

GPER

Recently, a putative membrane-bound estrogen receptor, G-protein coupled estrogen receptor (GPER), has been implicated in mediating the rapid, non-genomic effects of E_2 by influencing cell adhesion, migration, survival, and proliferation (Revankar *et al.*, 2005c). The GPER is a member of the seven transmembrane domain G protein-coupled receptor (GPCR) superfamily which represents the largest class of signaling molecules in the human genome (Venter *et al.*, 2001). Studies to date support a number of theories regarding a functional role for GPER and not all studies are in agreement. For example, the subcellular location of GPER is debated as multiple studies have indicated expression at either the plasma membrane or endoplasmic reticulum (Revankar *et al.*, 2005b; Thomas *et al.*, 2005a; Filardo *et al.*, 2007) and still others suggest the GPER cycles between these two cellular compartments (Cheng *et al.*, 2011). Initially, GPER was linked to E_2 signaling when it was found to be required for

E_2-induced rapid activation of the mitogen activated protein kinase (MAPK), extracellular signal-regulated kinase-1 and -2 (ERK-1/-2, p42/44) (Filardo *et al.*, 2000). Activation of ERK-1/-2 occurs via a Gβγ-subunit protein complex-dependent pertussis toxin-sensitive signaling mechanism that requires both Src and Shc (Filardo *et al.*, 2000) which supports a GPCR initiated mechanism opposed to a nuclear ESR-dependent mechanism that was suggested by previous studies (Improta-Brears *et al.*, 1999; Pedram *et al.*, 2006; Madak-Erdogan *et al.*, 2008). Since this initial observation, a number of studies collectively support a proposed GPER-mediated signaling pathway that is depicted in Figure 1.1. This diagram shows that once activated by various ligands (E_2, xenoestrogens, tamoxifen, G-1), GPER phosphorylates Shc which results in matrix metalloproteinase (MMP)-mediated cleavage and release of heparin-bound epidermal growth factor (proHB-EGF) from the cell surface. EGF transactivates the epidermal growth factor receptor (EGFR) which ultimately leads to activation of MAPK (Filardo *et al.*, 2000) and phosphoinositide 3-kinase (PI3K) (Revankar *et al.*, 2005b; Ariazi *et al.*, 2010) which play a role in influencing cell growth through modulation of downstream genes (c-fos, cyclins). Nuclear ESRs may also feed into this pathway. Aside from this proposed model, E_2 -activated GPER stimulates adenyl cyclase and intracellular cAMP production to promote the activation of Protein Kinase A (PKA) and subsequent suppression of EGF-induced ERK-1/-2. This forms a regulatory loop that returns GPER induced signaling back to baseline levels (Filardo *et al.*, 2002).

GPER Ligands

Significant overlap exists in the ligand specificity of the ESRs and GPER as multiple compounds in addition to the natural ligand E_2, such as the ESR antagonists ICI 182,780 and tamoxifen (TAM) also display binding affinities for GPER (Hall *et al.*,

2001; Korach *et al.*, 2003; Revankar *et al.*, 2005c). These ESR antagonists surprisingly act as GPER agonists, suggesting crosstalk may occur between nuclear and membrane receptor pathways that may even lead to opposing control of cellular effects. Efforts to investigate the consequences of select activation of the GPER have been made possible with the development of a specific receptor agonist. Through a combination of virtual and bioactivity screenings aimed at sifting through vast numbers of molecules with similar structure to E_2, a substituted dihydroquinoline was discovered to competitively inhibit binding of E_2 to GPER and is known as G-1 (Bologa *et al.*, 2006). Binding assays yielded high affinity for GPER (K_i of 11 nM) compared to E_2 (K_i of 5.7-6.6 nM) whereas negligible binding of G-1 to the ESRs up to 10uM was observed (Bologa *et al.*, 2006). Experiments to assay the intracellular effects of G-1 revealed that GPER activation by this compound caused increases in intracellular calcium mobilization, accumulation of PIP3 indicative of PI3K activation, and cell migration which were all consistent with E_2 induced GPER activation (Bologa *et al.*, 2006), thereby satisfying the requirement for a high-affinity, selective GPER agonist.

The same method was then utilized to search for a GPER-selective antagonist. Results revealed a compound related in structure to G-1 that binds GPER and prevents both E_2- and G-1 mediated mobilization of intracellular calcium and PI3K activation in breast cancer cells lacking ESRs (Dennis *et al.*, 2009). In murine epithelial uterine cells, G-15 was able to block E_2-induced proliferation (Dennis *et al.*, 2009) and has also been shown to block G-1 induced ERK-1/-2 activation and ESR1 phosphorylation at serine 118 (Lucki and Sewer, 2011). However, these results are complicated by the ability of G-15 to weakly bind and activate ESRs at high doses (10 μM) that were employed in

these studies (Dennis *et al.*, 2011). Overall, development of these GPER-specific ligands can be valuable tools for answering questions aimed at teasing apart non-genomic and genomic E_2- and xenoestrogen-mediated cellular responses.

GPER-Mediated Transcriptional Modulation

Before the identification of selective ligands, the mechanisms by which GPER modulates kinase activity had been documented, but little was known about the downstream transcriptional effects as a consequence of activation. A few recent studies that have employed the selective agonist G-1 and a series of select kinase inhibitors show GPER up-regulates the 'immediate early' proto-oncogene, c-*fos* in an EGFR-dependent manner via rapid ERK-1/-2 phosphorylation in breast cancer cell lines. Studies using a series of ESR positive (MCF-7) and ESR negative (SKBR3) cell lines confirmed this effect was GPER-mediated (Maggiolini *et al.*, 2004). In addition, GPER has been found to up-regulate expression of connective tissue growth factor (CTGF) and Bcl-2 by E_2 (Kanda and Watanabe, 2003; Pandey *et al.*, 2009). Other genes that are down-regulated by G-1 include those involved in regulating the cell cycle (cyclins, p21) (Lubig *et al.*, 2012).

Cell Type-Specific GPER-Mediated Responses

Studies to elucidate the cellular impacts of GPER signaling pathways have yielded complex, and in some cases, controversial results. It has been proposed that GPER activation leading to proliferative effects depends on the cell type and corresponding ESR expression profile. For example, in SKBR3 cells which express GPER but not ESRs, exposure to either 100 nM E_2 or 100 nM G-1 increased proliferation which suggests that GPER signaling is sufficient to stimulate cell growth independent of the ESRs (Albanito *et al.*, 2007; Albanito *et al.*, 2008). The increased

proliferation in SKBR3 cells coincides with up-regulation the CTGF gene suggesting that modulation of this mRNA may influence proliferation in the absence of ESRs (Pandey *et al.*, 2009). Likewise, GPER knockdown inhibits the E_2 induced proliferative effects in SKRB3 cells. However, other studies have indicated that GPER activation by G-1 (100 nM – 1 μM) inhibits proliferation of the same cell line (Lubig *et al.*, 2012). Further, GPER knockdown potentiates E_2 induced proliferation of breast cancer cells that express GPER and ESRs (MCF-7) suggesting an inhibitory role (Ariazi *et al.*, 2010). These results collectively indicate that the ESR expression profile may influence the pro- or anti-proliferative properties of GPER activation and suggest a compensatory mechanism may exist between the ESRs and GPER.

Dose-Dependent GPER-Mediated Cellular Proliferation

The proliferative effects of GPER signaling are also dependent on dose of ligand. Low dose (10 nM) exposure to G-1 has been shown to stimulate proliferation of MCF-7 cells through up-regulation of acid ceramidase (ASAH1) (Lucki and Sewer, 2011) while a high dose (1 μM) inhibits E_2 induced proliferation of MCF-7 cells (Ariazi *et al.*, 2010). Collectively, these results indicate that activation of GPER with high doses of G-1 has an inhibitory effect on proliferation whereas low dose exposure has a stimulatory effect. Attempts to explain this phenomenon have yielded a current hypothesis that suggests G-1 signals through a pathway independent of the GPER at high doses. This notion is supported by the failure of pretreatment of cells with G-15 to rescue the effects of G-1 at high doses, namely inhibition of breast cancer cell growth (Wang *et al.*, 2012). Furthermore, knockdown of GPER using siRNA strategies also failed to rescue G-1 mediated effects. Finally, it has been suggested that the membrane-bound ER splice

variant, ERα-36 may mediate the rapid, non-genomic effects of E_2 and G-1 at high doses (Kang *et al.*, 2010).

Potential for Receptor Crosstalk

GPER and ESRs may participate in crosstalk at some level as both receptors regulate distinct and overlapping transcriptional profiles (Notas *et al.*, 2011). As previously mentioned, many cellular responses to E_2 and the GPER-selective agonist, G-1, are dependent on the ESR (1/2) expression profile which suggests the presence of certain receptor types influences the downstream responses that are cell type specific. As evidence for convergent signaling pathways between ESR and GPER, E_2- and TAM-induced activation of ERK-1/-2 via GPER enhances ESR1 transcriptional activity by phosphorylating serine 118 within the transcriptional activation function I (AF-I) domain (Kato *et al.*, 1995). In addition, both the ESR1 and GPER are able to stimulate PI3K which results in the accumulation of PIP3 (Revankar *et al.*, 2005b). It has also been suggested that extranuclear and nuclear signaling inputs converge on similar target genes. For example, GPER and ESR knockdown experiments revealed a requirement for both receptors in maximal induction of the c-*fos* gene. This study concluded that both ESRs and GPER signal through overlapping signal transduction pathways in ovarian cancer cells (Albanito *et al.*, 2007).

In support of divergent signaling pathways, knockdown of GPER in breast cancer cells lacking ESRs is sufficient to block the E_2- and G-1-induced growth stimulation and c-*fos* induction, suggesting ESRs are not required for these effects (Albanito *et al.*, 2007). Further, G-1 had no effect on the expression of the ESR target gene, progesterone receptor (Albanito *et al.*, 2007). Most striking however, is the ability of the ESR antagonist ICI 182,780 and partial antagonist TAM to act as GPER agonists

and activate ERK-1/-2 by GPER mediated transactivation of EGFR (Filardo *et al.*, 2000; Revankar *et al.*, 2005b).

GPER and Xenoestrogens

The GPER represents a potential target for interference by endocrine disrupting chemicals as it has been shown that several xenoestrogens are capable of binding to this receptor with low affinity, up-regulating adenyl cyclase activity and consequently increasing intracellular levels of cAMP (Thomas and Dong, 2006). The soy-based phytoestrogen genistein and the plasticizer bisphenol-A (BPA) bind to GPER with relative binding affinities of 13.41% and 2.83% compared to E_2 (100%), respectively, (Thomas and Dong, 2006) and have both been shown to activate ERK-1/-2 signaling via a GPER dependent mechanism(Maggiolini *et al.*, 2004; Dong *et al.*, 2011). Exposure to BPA has been linked to impaired reproductive performance (Mlynarčíková *et al.*, 2005; Sugiura-Ogasawara *et al.*, 2005; Rasier *et al.*, 2006) and Genistein exposure has been shown to promote proliferation of estrogen-dependent breast and thyroid cancer cells (Allred *et al.*, 2001; Vivacqua *et al.*, 2006) which necessitates the need to elucidate the complex molecular mechanisms of action of EDCs.

Cell Line

MCF-7 human breast cancer cells are an ideal model to study the interaction between the ESRs and the GPER as they are known to express each receptor (ESR1, ESR2, GPER) and are very well characterized with respect to E_2 action. Further, the effects of Genistein and BPA exposure on MCF-7 proliferation are well known, thus rendering them an appropriate system in which to study the effect of GPER and ESR interplay on established cellular endpoints in response to xenoestrogens (Hsieh *et al.*, 1998; Pupo *et al.*, 2012).

Research Objectives

The interplay of GPER and ESR activation and its estrogen/xenoestrogen-dependence in controlling downstream cellular responses is currently unknown. Studies aimed at elucidating the mechanisms of xenoestrogen action have been primarily limited to probes of their interaction with ESRs. Current knowledge suggests that the GPER may represent an alternate and highly relevant (e.g. fast and sensitive) mechanism by which environmental contaminants exert their effects on biological systems.

Based on these notions, the objectives of this research are two-fold; (1) to elucidate the impact of low versus high doses of G-1-activated GPER on ESR-mediated function and cellular responses; (2) to identify downstream targets phosphorylated by G-1 as a means to uncover novel pathways modulated both dependently and independently of GPER and potentially xenoestrogens. To perform these studies we employed a series of cell-based assays coupled with phosphoproteome analysis. Overall, these studies provide a groundwork for elucidating novel mechanisms of xenoestrogen actions.

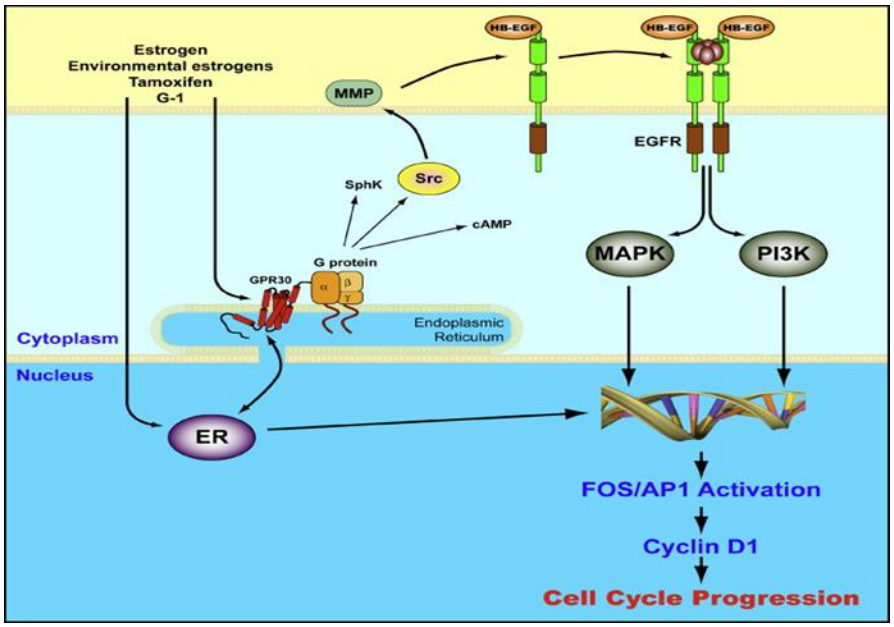

Figure 1-1. Signaling pathways activated by GPER. E_2, xenoestrogens, and G-1 are able to bind to GPER, shown here localized on the endoplasmic reticulum, and transactivate EGFR through matrix metalloproteinase mediated cleavage and release of proHP-EGF. EGFR activation leads to multiple downstream signaling events including activation of MAPKs and PI3K which results in the expression of transcription factors (*c-fos*) which ultimate leads to cell cycle progression. Adapted from Prossnitz, E. R. and M. Maggiolini (2009). Molecular and cellular endocrinololgy **308**(1): 32-38.

CHAPTER 2
GPER SPECIFIC AGONIST G-1 INHIBITS ESR MEDIATED CELLUAR RESPONSES TO XENOESTROGENS

Introduction

Endocrine disruptors are environmental contaminants that can interfere with normal hormonal signaling by either disrupting the tightly regulated synthesis, structure or function of hormones, thereby causing adverse effects in human and wildlife populations (Colborn *et al.*, 1993; Kavlock *et al.*, 1996). The most widely studied endocrine disruptors are those that interfere with signaling by the endogenous estrogen, 17β-estradiol (E_2), which is necessitated by the drastic increase in number of chemicals identified with estrogenic activity in the past decade (Thomas and Dong, 2006).

Xenoestrogens can be naturally occurring, such as the soy-based phytoestrogens (i.e. Genistein), or they can be anthropogenic and include a diverse array of chemicals that are ubiquitously used in pesticides, plastics, and pharmaceuticals. Many of these compounds are suspected to contribute to a myriad of disorders including reproductive abnormalities, cardiovascular disease, hypertension, metabolic diseases, immune disorders, and reproductive cancers (Deroo and Korach, 2006).

A classical mode of action of xenoestrogens is well established to be mediated by ligand-activated transcription factors known as the nuclear estrogen receptors (ESR1 and ESR2). Once engaged by ligand, the ESRs adopt a conformational change that promotes dimerization and recruitment of various co-regulatory proteins to the transcriptional complex (McKenna and O'Malley, 2001). This ESR receptosome then interacts with *cis*-acting estrogen response elements in the promoters of estrogen-responsive genes by either direct DNA binding to classical estrogen response elements

(ERE) or through alternate elements (AP-1, Sp-1) via tethering mechanisms thereby initiating aberrant gene expression (Hall *et al.*, 2001). Xenoestrogens such as bisphenol-A (BPA) and Genistein have been shown to weakly bind and modulate ESR activity. The consequences of these actions are vast and are cell and tissue-type specific. In breast cancer cells (MCF-7) activation of ESRs by E_2 and xenoestrogens is a major driver of proliferation.

Recently, a putative membrane-bound estrogen receptor, G-protein coupled estrogen receptor (GPER), has been implicated in mediating the rapid, non-genomic effects of E_2 (Revankar *et al.*, 2005c). The GPER is a member of the seven transmembrane domain, G protein-coupled receptor superfamily and is required for E_2-induced rapid responses such as activation of ERK-1/-2, mobilization of intracellular calcium stores, and stimulation of intracellular cAMP production (Revankar *et al.*, 2005b; Prossnitz *et al.*, 2008). The role of GPER in mediating cell responses to E_2 is a source of controversy as the subcellular location has been extensively debated and has been suggested to signal from both the endoplasmic reticulum and plasma membranes (Revankar *et al.*, 2005c; Thomas *et al.*, 2005a; Filardo *et al.*, 2007) while other studies have indicated that it shuttles between these two compartments (Cheng *et al.*, 2011). The fact that xenoestrogens have been shown to have weak affinity for GPER suggests modulation of this receptor may represent a potential novel mechanism whereby EDCs exert biological effects.

Evaluating ESR- and GPER-specific actions is complicated due to significant overlap in ligand specificity. Multiple well-established ESR ligands, such as E_2, ICI 182,780 and tamoxifen (TAM) display similar binding affinities (Hall *et al.*, 2001; Korach

et al., 2003; Revankar *et al.*, 2005c) for both receptors, the latter compounds acting as antagonists for ESR while agonizing the GPER. This has led to the idea that ESR and GPER likely engage in crosstalk, and may even modulate compensatory or opposing cell signaling pathways as it has been shown that GPER is able to mediate proliferative effects of E_2 in breast cancer cells lacking ESRs (Pandey *et al.*, 2009).

Sorting cellular signaling networks specifically modulated by GPER and ESRs required identification of GPER specific agonists and antagonists. A combination of virtual and bioactivity screenings revealed a substituted dihydroquinoline, termed G-1, that is a selective GPER agonist (Bologa *et al.*, 2006). Availability of this ligand has allowed for studies aimed at investigating a GPER-specific role in non-genomic biological responses to E_2. However, a number of studies that have employed G-1, primarily in cell-based experiments, have produced mixed results. For example, in assessing proliferation, some studies show G-1 enhances cell growth while others find it inhibits proliferation in breast cancer cells both expressing and lacking ESRs (Albanito *et al.*, 2007; Albanito *et al.*, 2008; Ariazi *et al.*, 2010; Lucki and Sewer, 2011; Lubig *et al.*, 2012). Interestingly, it has been suggested that G-1 may signal independently of GPER at high doses (Wang *et al.*, 2012).

Based on the current body of knowledge, the goals of this research were (1) to determine the impact of GPER activation on breast cancer cell growth and ESR activity in response to E_2 and the xenoestrogens BPA and Genisten and (2) to identify novel pathways modulated by E_2 and G-1 using a global phosphoproteomic approach. While we originally set out to look at GPER activation by G-1 and xenoestrogens, our results defined a role for G-1 in suppressing E_2- and xenoestrogen-driven cellular proliferation

and ESR activity. We further show that this inhibition of ESRs is not isotype specific and likely occurs independently of GPER. Finally, through phosphoproteomics analysis we present plausible protein targets, such as NDRG2, that are selectively phosphorylated by G-1 and may contribute to the observed inhibition of cell growth and ESR activity. These results are significant in that they suggest that high doses of G-1 may act through pathways independent of GPER to modulate ESR-driven cell growth by E_2 and the xenoestrogens BPA and Genistein.

Materials and Methods

Chemicals

All chemicals used in the studies were dissolved in DMSO and included**;** 17β-estradiol (Sigma Aldrich, St. Louis, MO), bisphenol-A (BPA, supplied by NIEHS), Genistein (Acros Organics, Morris Plains, NJ), (propyl-pyrazole-triol (PPT, Tocris, Ellisville, MO), diarylpropionitrile (DPN, Tocris), (±)-1-[(3a*R**,4*S**,9b*S**)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-tetrahydro-3*H*-cyclopenta[*c*]quinolin-8-yl]- ethanone (G-1, Santa Cruz Biotechnology, Santa Cruz, CA), and (3a*S**,4*R**,9b*R**)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3*H*-cyclopenta[*c*]quinolone (G-15, Tocris).

Cell Culture

Human breast cancer cells (MCF-7, purchased from American Type Tissue Collection) were cultured in Minimum Essential Medium (MEM) without phenol red (Cellgro 17-305-CV, Manassas, VA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, Penstrep, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, and 1.5 g/L sodium bicarbonate. Cells were maintained at 37°C in humidified 5% CO_2 atmosphere. All exposures were performed in phenol red free MEM with 10% charcoal-stripped FBS and the supplements listed above.

Proliferation Assays

MCF-7 cells were seeded at a concentration of 4 X 10^3 cells per well of a 96 well Tissue Culture Treated Microplate (Costar® Corning Incorporated) in 100ul cell culture medium. The cells were allowed to adhere overnight then were switched to cell culture medium containing 10% charcoal-stripped FBS for 24 hours. Cells were then exposed to various concentrations of chemicals dissolved in DMSO (final DMSO ≤ 0.2%) or vehicle control for 24, 48, 72 and 96 hours. Proliferation was measured by modified MTT assay (Promega Corp., WI, USA). Briefly, proliferation was measured every 24 hours by incubating cells in 15 µl Dye Solution for 4 hours followed by addition of 100µL Solubilization/Stop buffer. After 1 hour, absorbance measurements (570 nm) were acquired on a BioTek Synergy H1 plate reader. Exposure media was changed every 48 hours throughout the time-course.

Reporter Gene Activity Assays

MCF-7 cells were seeded at a concentration of 2 X 10^5 cells per well of a 24 well Tissue Culture Treated Plates (Multiwell™, Falcon) in 1.0 ml of complete cell culture medium. The cells were allowed to adhere overnight then were switched to 10% charcoal-stripped FBS-containing medium without Penstrep. Cells were transiently transfected with 500 ng reporter plasmid containing a 2X estrogen response element (ERE) upstream of the luciferase gene and 50 ng Renilla pRL-TK using Lipofectamine 2000 (Invitrogen). After 24 hours, cells were exposed to chemicals dissolved in DMSO for 24 hours. Cells were washed with 500 µl 1X Phosphate Buffered Saline without Magnesium and Calcium (Corning cellgro, Manassas, VA) and collected in 100 µl 1X Passive Lysis Buffer (Promega). To facilitate lysis, cells were rocked on a shaking platform at 50 rpm for 20 minutes at room temperature. Cell lysates were collected and

placed in 1.5 ml microcentrifuge tubes and centrifuged at 12,000 x g at 4°C. Thereafter, luciferase activity was measured using the Dual Luciferase Reporter Kit (Promega Corp., WI, USA) on a BioTek Synergy H1 plate reader. To each well of a 96-well LUMITRAC 200 white immunology plate (USA Scientific), 20 µl of each lysate was added followed by 50 µl of the Firefly Luciferase Reagent. The activity in each well was read, followed by the addition of 50 µl of Stop & Glo Substrate. Firefly luciferase luminescence was normalized to Renilla luminescence and reported as either fold change over control or percent maximal response.

Quantitative Real-Time PCR

MCF-7 cells were seeded at a concentration of 2 X 10^5 cells per well of a 24 well Tissue Culture Treated Plate (Multiwell™, Falcon) in 1.0 ml of complete cell culture medium. The cells were allowed to adhere overnight then were switched to complete cell culture medium containing 10% charcoal-stripped FBS. After 24 hours, cells were exposed to chemicals dissolved in DMSO or vehicle control for 24 hours. Cells were lysed and mRNA isolated per the manufacturer's protocol (RNeasy Mini kit, Qiagen) with the optional on-column DNase treatment (Qiagen). Thereafter, total RNA was quantified on a Biotek Syngery H1 plate reader and 1 µg was reverse transcribed per the manufacturer's protocol using random hexamers (Reverse Transcription System, Promega). TaqMan probes specific to TFF1/pS2 (Hs00170216_m1), NDRG2 (Hs01045114_g1), FOS (Hs04194186_s1), and the housekeeping gene, GAPDH (Hs02758991_g1) were purchased from Applied Biosystems (Carlsbad, CA). Amplification was performed in duplicate in TaqMan Universal PCR Master Mix (Applied Biosystems) using the standard run time on the 7500 Fast Real-Time PCR System

(Applied Biosystems). Target gene expression was normalized to GAPDH and relative quantitation was calculated using the Delta Delta CT method.

Protein Isolation

MCF-7 cells were seeded at a concentration of 3 X 10^5 cells per ml on 60 mm suspension culture plates (Multiwell^{TM}, Falcon) coated with Bactogelatin and in 5 ml complete phenol red free MEM. The cells were allowed to adhere overnight then switched to MEM with 10% charcoal-stripped FBS. After 24 hours, cells were exposed to chemicals dissolved in DMSO or vehicle control for 30 minutes. Cells were then washed three times with 2 ml ice-cold PBS and harvested in 100 ul ice-cold whole cell lysis buffer (20 mM Tris pH 7.6, 1% Triton-X 100, 137 mM NaCl, 0.1 mM EDTA, 1X EDTA Free Protease Inhibitor Tablet (Pierce 88661)). Lysates were passed through a 26 gauge needle and incubated on ice for 30 minutes, then centrifuged at 14,000 g for 15 min at 4°C. Supernatant was collected and quantified by the Bradford Protein Assay (BioRad). For Alkaline Phosphatase treatment, 80 µg protein was incubated with 80 units (1 unit/µg protein) of Calf Intestinal Alkaline Phosphatase (Promega) at 37°C for 2 hours.

Phosphoproteomic Analysis

MCF-7 cells were exposed to 10 nM E_2, 1 µM G-1 or vehicle control for 30 minutes and total protein was isolated from MCF-7 cells as previously described. Protein phosphorylation was assessed using a quantitative, label-free approach at Duke University within the Proteomics Core Facility (Soderblom *et al.*, 2011). Briefly, protein extracts were digested with trypsin and subjected to phospho-peptide enrichment using TiO_2-packed spin columns. After elution, phospho-peptides were identified using DIA MS^E HPLC-MS/MS with the addition of phosphorylation as a considered modification on

serine, tyrosine, and threonine residues. Proteins were identified using MASCOT, and phosphorylation sites were mapped via a combination of automated database searching and *de novo* (manual) sequencing. Analysis of the phosphoproteome was performed using Scaffold (Searle, 2010) and pathway analysis was performed using Pathway Studio Version 9.0 (Elsevier).

Western Blots for NDRG2

Total protein was isolated as previously described and 80 µg protein was diluted in Laemmli Sample buffer and separated on 16.5% acrylamide Mini-PROTEAN Tris-Tricine Precast Gels (BioRad). Protein was transferred to Nitrobind Cast 0.45 micron Pure Nitrocellulose membrane (GE Water & Process Technologies) at 35 volts overnight at 4°C in Towbin buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol (pH 8.3)) using a wet transfer apparatus (BioRad). Thereafter, the blot was blocked for one hour in 5% Blotto and then probed with rabbit antibody specific to human NDRG2 (Novus Biologicals, Littleton, CO). The blot was probed overnight with primary antibody at 4°C at a dilution of 1:300 in Tris-buffered saline with Tween-20 (pH 7.6). After washing, the blot was incubated with the secondary antibody, ECL™ anti-rabbit IgG, Horseradish Peroxidase Linked (GE Healthcare, UK) at a dilution of 1:5000 for 1 hour at room temperature then incubated with NOVEX® ECL HRP Chemiluminescent Substrate Reagent kit (Invitrogen, Carlsbad, CA) for 1 minute at room temperature. The blot was imaged using the ChemiDoc XRS (BioRad).

Statistical Analysis

Data obtained from the gene reporter and qRT-PCR assays were analyzed by one-way ANOVA followed by Dunnett's test for post-hoc multiple comparisons to

determine statistical differences between treatments. Differences were considered significant with a p-value < 0.05.

Results

G-1 Suppresses Nuclear ER-driven Cell Proliferation

To explore the effect of GPER activation by the selective agonist, G-1, (Bologa *et al.*, 2006) on the proliferation of breast cancer cells we utilized a modified MMT assay. MCF-7 cells were exposed to 10 nM E_2 alone and in combination with 1 µM G-1 and proliferation was measured every 24 hours for either 72 or 96 hours total. As expected, E_2 increased cellular proliferation compared to the untreated control group over a period of 96 hours (Figure 2.1). Interestingly, exposure to 10 nM E_2 in the presence of 1 µM G-1 abolished proliferation which was undistinguishable from the control cells. Of note, individual exposures to low (10 nM) and high (1 µM) doses of G-1 individually had no effect on proliferation.

In order to determine the ER isoform specificity of the inhibitory effect of G-1 on MCF-7 proliferation, cells were exposed to 100 nM PPT (ESR1 specific agonist), or 100 nM DPN (ESR2 specific agonist) individually and in combination with 1 µM G-1. While exposure of MCF-7 cells to both 100 nM PPT and 100nM DPN increased proliferation over the untreated control group, co-exposure to 1 µM G-1 decreased proliferation to the level of control cells for both treatments (Figure 2.1).

G-1 Inhibits Xenoestrogen-Induced Cellular Proliferation

To determine whether G-1 could also suppress xenoestrogen-driven cellular proliferation, MCF-7 cells were exposed to 10 µM BPA or 1 µM Genistein individually and in combination with a low or high dose of G-1 (10 nM or 1 µM, respectively). Cell proliferation was assessed by modified MTT assay every 24 hours for 96 hours. In

agreement with previous reports (Hsieh *et al.*, 1998; Schafer *et al.*, 1999; Nakaya *et al.*, 2007), exposure to BPA and Genistein increased proliferation of MCF-7 cells compared to the untreated control group over the course of 96 hours (Figure 2.2). Similar to results obtained with E_2, co-exposure with the high dose of G-1 (1 µM) decreased BPA- and Genistein-driven proliferation to the level of the untreated control group over the course of 96 hours (Figure 2.2). This inhibition was not observed when the low dose (10 nM) of G-1 was used.

G-1 Suppresses ER-Mediated Transcriptional Activation

To further examine a plausible mechanism for the G-1 mediated inhibition of E_2-driven cell proliferation, we examined the ability of G-1 to modulate ESR activity by utilizing a 2XERE-driven luciferase reporter gene assay. In addition to E_2, ESR isoform specific agonists (PPT, DPN) were used individually and in combination with G-1 to also examine effects on ESR isoform specific activity. We also employed the GPER antagonist G-15 in these studies. MCF-7 cells were transfected with a 2XERE-Luciferase reporter gene and exposed to E_2 or the ESR isoform specific agonists individually and in combination with 1 µM G-1 for 24 hours. Data from these experiments revealed that individual exposures to 10 nM E_2, 100 nM DPN and 100 nM PPT significantly increased ERE activation 12, 10 and 14 fold, respectively, over the untreated control group (Figure 2.3). These results indicated that both ESRs activated transcription via 2XERE interaction in our model cell line. Exposing MCF-7 cells to 1 µM G-1 did not have any effect on ERE activation. In agreement with results from the proliferation assays, exposure to 10 nM E_2, 100 nM DPN or 100 nM PPT in combination with 1 µM G-1 significantly decreased ERE activation compared to individual exposures with 10 nM E_2, 100 nM DPN and 100 nM PPT (Figure 2.3). Surprisingly, attempts to

rescue this inhibition by G-1 in E_2 and DPN exposed cells by pretreating cells with 20 µM G-15 were unsuccessful.

G-1 Mediated Suppression of Xenoestrogen-Driven Transcriptional Activation is Dose-Dependent

After establishing that a high dose of G-1 inhibited ESR-mediated transcriptional activation at an ERE, we next sought to determine whether the inhibition was dose-dependent. For these experiments, MCF-7 cells were transfected with a 2XERE-Luciferase reporter gene and exposed to 10 nM E_2 individually and in combination with increasing concentrations of G-1 (10 nM, 100 nM, 500 nM or 1 µM) for 24 hours. Results revealed a dose-dependent decrease in ERE activation with a downward trend in ERE activation starting at 500 nM G-1 that resulted in a significant reduction (60%) for the highest dose of G-1 tested (1 µM) compared to cells exposed only to 10 nM E_2 (Figure 2.4). Because 1 µM G-1 also inhibited xenoestrogen-induced proliferation, we next examined the potential inhibitory effects of low and high doses of G-1 on xenoestrogen-induced ERE activation. For these experiments, EC_{50} values for BPA and Genistein were calculated based on a dose-response assessment of ERE activity using the pharmacology function in SigmaPlot (Systat Software, San Jose, CA). The calculated EC_{50} values for BPA and Genistein were 640 nM and 284 nM, respectively. Transfected MCF-7 cells were exposed to these concentrations individually and in combination with increasing concentrations of G-1 (10 nM, 100 nM, 500 nM, or 1 µM) for 24 hours. Similar to results observed with E_2, low doses of G-1 (10 nM – 100 nM) had no effect on Genistein-induced ERE activation whereas co-exposure to 500 nM G-1 and 1 µM G-1 resulted in a 40% reduction in ERE activation compared to cells exposed to Genistein individually (Figure 2.4). Contrary to results observed in the E_2 and

Genistein co-exposure experiments, a downward trend in BPA (640 nM)-induced activation began at doses as low as 10 nM G-1 with the greatest reduction of BPA-driven ERE activation occurring at 1 µM G-1 (40% of individual exposure). Additional experiments with more doses are required to delineate if significant differences exist in the inhibitory dose-response between BPA and Genistein.

G-1 Does Not Affect pS2 Gene Expression

Next, we sought to examine the effect of G-1 on transcription of the well described E_2 responsive gene pS2 (TFF1). In order to verify that this was a good candidate gene for co-exposure experiments, we first examined pS2 responsiveness to an array of G-1 doses. For these experiments, MCF-7 cells were exposed to E_2 (10 nM), DPN (100 nM), PPT (100 nM) or various doses of G-1 (10 nM, 100 nM, 500 nM, 1 µM) for 24 hours and pS2 expression was measured by qRT-PCR. Results revealed that exposure to 10 nM E_2, 100 nM DPN, and 100 nM PPT increased pS2 gene expression 12, 16, and 2 fold, respectively (Figure 2.5). Exposure to any of the doses of G-1 (10 nM – 1 µM) did not have any effect on pS2 expression (Figure 2.5).

Xenoestrogen-Induced pS2 Expression is not Sensitive to G-1

After validating pS2 as an acceptable gene to study G-1 mediated inhibition of ESR-driven pS2 expression, we next sought to determine the effect of co-exposure to low and high dose G-1 on E_2, BPA, and Genistein-induced pS2 expression. For these studies MCF-7 cells were exposed to 10 nM E_2, 10 µM BPA, or 1 µM Genistein individually and in combination with 10 nM G-1 or 1 µM G-1 for 24 hours and pS2 expression was measured by qRT-PCR. Results revealed a trend of increased expression after exposure to (xeno)estrogens individually and a trend of pS2 expression potentiation after co-exposure to 10 nM G-1 and 1 µM G-1 (Figure 2.6). These results

revealed that E_2 and xenoestrogen-induced pS2 expression is not sensitive to the inhibitory effects of G-1.

G-1 and E_2 Exhibit Differential Phosphorylation Patterns

In order to identify alternate pathways of G-1 mediated inhibition of ESR function, we utilized global phosphoproteomic strategies. MCF-7 cells were exposed to vehicle control, 10 nM E_2 or 1 µM G-1 and total protein was isolated after 30 minutes in order to identify early targets of G-1 and E_2-induced non-genomic signaling pathways. Total protein was digested with trypsin and enriched for phosphorylated peptides using a TiO_2-based spin method (Soderblom *et al.*, 2011). Phospho-peptides were identified using LC-MS/MS strategies and matched to proteins using MASCOT. Results revealed 238 proteins that were phosphorylated on specific serine, threonine, or tyrosine residues. These identified peptides were organized into a Venn diagram based on treatment group (Figure 2.7). Overall, 13 and 37 phospho-peptides were differentially phosphorylated by G-1 and E_2, respectively, while 10 peptides revealed phosphorylation by both compounds but at specific sites. The proteins among the 238 identified were further grouped according to associated biological processes using the Gene Ontology (GO) Consortium (Figure 2.8). A large number of proteins were assigned to the categories of development, localization and response to stimulus. Interestingly, of those differentially phosphorylated by G-1, a number were sorted into the biological process of reproduction and these 27 proteins are listed in Table 2.1. A number of distinct phosphorylation trends are evident from the data. For example, a number of proteins were phosphorylated only by G-1 (NDRG2, RS6, SRPK1, WAPL) whereas others were dephosphorylated by G-1 (JIP4, PEBP1, UBR4, UNG, BAG6, IF4G1, RLA2) compared

to control and E_2 treatments. Other trends included proteins that were phosphorylated only by E_2 or showed shared phosphorylation patterns by E_2 and G-1.

Robust pathway analysis (Pathway Studio, Elsevier) of proteins differentially phosphorylated by G-1 revealed N-myc downstream regulated gene 2 (NDRG2) and serine/threonine protein kinase (SRPK1) as central 'nodes' that interconnect with numerous other proteins, in particular kinases, that have been established to be activated by G-1 (i.e. MAPK, AKT) (Bologa *et al.*, 2006) (Figure 2.9). While GPER could possibly be connected to these 'nodes' as shown in the hypothetical pathway, modulation of these proteins by G-1 may occur independently of the GPER.

NDRG2 is a Plausible Novel Target of G-1 Mediated Signaling

In order to validate the results of the phosphoproteomic analysis we attempted to examine differential phosphorylation of NDRG2 on Serine-332 by E_2 and G-1 by Western blot analysis. The addition of phosphates alters the migration pattern of the protein in a polyacrylamide gel which can be visualized as a higher molecular weight band compared to the native unphosphorylated form. Results from the Western blot showed the presence of NDRG2 as a series of bands which likely indicate multiple differentially phosphorylated sites on the protein (Figure 2.10). It is difficult to discern whether there were any noticeable differences between treatments as the banding, particularly for E_2 and G-1, occurred in a diffuse pattern. However, evidence that these bands represented phosphorylated forms of the protein was supported by the presence of discrete and intense lower molecular weight bands when protein fractions were treated with alkaline phosphatase.

NDRG2 mRNA Expression is Downregulated by ESR Activation

In order to characterize the transcriptional regulation of NDRG2, we examined the effect of E_2 and G-1 treatment on NDRG2 mRNA expression by qRT-PCR. For this experiment, MCF-7 cells were exposed to 10 nM E_2, 1 µM G-1, 10 µM G-15, 100 nM PPT, or 100 nM DPN for 24 hours. Results revealed that NDRG2 expression was not affected by 1 µM G-1 while exposure to 10 nM E_2, 100 nM PPT, and 100 nM DPN significantly decreased NDRG2 expression compared to control cells. Surprisingly, a trend of decreased NDRG2 expression was observed in cells treated with G-15.

Discussion

The biochemistry and molecular biology of nuclear ESRs has been a focus of study for decades, but a newly discovered (Revankar *et al.*, 2005a; Thomas *et al.*, 2005b) putative membrane receptor for estrogens, GPER (Valdes and Weeks, 2009), has been found to contribute to rapid non-genomic actions of E_2. However, the precise role of GPER in modulating E_2-signaling and activity is controversial (reviewed in Langer et al.) (Langer *et al.*, 2010) and its function as an estrogen receptor is heavily debated. It is possible that GPER and ESRs are capable of signaling through both convergent and divergent pathways which complicates attempts to fully understand their role in E_2 signaling and downstream consequences. In addition, the clear findings that xenoestrogens, such as BPA and Genistein bind and modulate both ESR and GPER activity highlights the need to more thoroughly examine genomic and non-genomic-driven cellular processes as an alternate and highly relevant mode of endocrine disruption in the context of environmental exposures.

We began investigations to assess the role of GPER in mediating E_2 and xenoestrogen-driven cellular responses in MCF-7 cells. We also wanted to specifically

determine the role of each ESR isoform (ESR1, ESR2) in these endpoints. Based on results from our proliferation studies we can draw a number of conclusions. First G-1 inhibits E_2-driven proliferation but only at high doses $\geq$ 500 nM. This is in agreement with other reports that show G-1 (100 nM - 1 µM) inhibits proliferation of breast (MDA-MB-231) and ovarian granulosa (KGN) cancer cells (Ariazi *et al.*, 2010; Wang *et al.*, 2012). Recent studies have suggested this inhibitory effect occurs independently of GPER as G-15, the GPER antagonist, and siRNA to knock down GPER expression, both failed to rescue G-1 inhibited proliferation of ovarian cells. A few other studies have shown similar effects by G-1 on proliferation in cells that do not express GPER. (Wang *et al.*, 2012). These studies, including ours, all utilized G-1 at relatively high doses (500 nM – 1 µM), suggesting the observed reduction in cellular proliferation has a dose threshold.

While some reports have indicated that low doses of G-1 (10 nM – 100 nM) stimulate the proliferation of breast cancer cells (Lucki and Sewer, 2011), our results contradict this observation as 10 nM G-1 did not stimulate growth compared to the untreated control group. The reasons for these discordant observations are not clear but could be a result of variable cell lines and experimental conditions. For example, in some studies, proliferation was observed in cells exposed to G-1 that expressed GPER but not ESRs (SKBR3) (Albanito *et al.*, 2007; Albanito *et al.*, 2008; Pandey *et al.*, 2009) which implies GPER function largely relies on the ESR expression profile of the cell. However, other studies have indicated that GPER activation by G-1 inhibits proliferation in the same cell line (Lubig *et al.*, 2012). Disparate results have also been observed in cells that express both types of receptors (GPER and ESRs). For example, one study

using MCF-7 cells showed that a low dose exposure to G-1 increased proliferation (10 nM) (Lucki and Sewer, 2011) while another study reported a high dose (1 μM) was inhibitory (Ariazi *et al.*, 2010). It should be noted that in the former study these cells were manipulated to overexpress proteins of interest (i.e. ASAH1) which may change the proliferative capacity of G-1 (Lucki and Sewer, 2011). Future experiments include examining the specific role of GPER in mediating the inhibitory effects of high doses of G-1 on xenoestrogen-induced MCF-7 proliferation by utilizing GPER blocking experiments (utilizing G-15 and siRNA) as it has been reported that signaling by high doses of G-1 (500 nM-1 μM) may occur independently of GPER (Wang *et al.*, 2012).

A second conclusion that emerged from the proliferation studies is the novel observation that the inhibitory effect of G-1 is not ESR isotype specific. Proliferation induced by both ESR specific agonists PPT (ESR1-specific agonist) and DPN (ESR-2 specific agonist) was inhibited in the presence of 1 μM G-1 revealing that the mechanism of inhibition was not specific to either receptor subtype. Although further studies are required to elucidate the specific mechanisms of action of G-1, a few studies suggest that a sustained increase in cytosolic Ca^{2+} concentrations that is dependent on GPER but independent of E_2 (Ariazi *et al.*, 2010). Furthermore, these studies revealed that G-1 blocks cell cycle progression and modulates the expression of relevant genes (p53, p21, cyclin D1). Whether these effects contribute to our observed results remains to be determined.

Third, G-1 (at 1 μM) inhibited proliferation stimulated by BPA and Genistein. Because previous reports indicated that the environmentally-relevant xenoestrogens BPA and Genistein show binding affinities for the ESRs (Kuiper *et al.*, 1997) and more

recently GPER with weak affinity (Thomas and Dong, 2006) and are able to stimulate proliferation of MCF-7 cells (Hsieh *et al.*, 1998; Schafer *et al.*, 1999; Recchia *et al.*, 2004; Nakaya *et al.*, 2007), we examined the ability of G-1 to inhibit xenoestrogen-induced cellular proliferation. High doses of BPA and Gen (10 µM and 1 µM, respectively) were used because they are known to significantly increase proliferation of MCF-7 cells at these concentrations. By utilizing co-exposure experiments, we showed that while 1 µM G-1 does in fact inhibit proliferation stimulated by BPA and Genistein, 10 nM G-1 was insufficient (Figure 2.2). Because these results paralleled those observed in the co-exposure experiments utilizing the ESR agonists, they suggest that BPA and Genistein largely promote proliferation of MCF-7 cells through a genomic mechanism of ESR action and provide additional support for the existence of divergent signaling pathways activated by a high dose of G-1 in which non-genomic signaling opposes genomic actions of the ESRs.

As proliferation of MCF-7 cells by E_2 is known to be controlled via nuclear ESRs, we performed reporter gene assays to measure the ability of these receptors to transcriptionally activate an ERE-luciferase gene. Our results revealed that G-1 inhibited ERE activation by E_2, PPT and DPN which paralleled results from the proliferation assays (Figure 2.3). These results suggest that G-1 is influencing ESR activity in a non-isoform specific manner. While the specific mechanism of action is not known, these results suggest that a number of mechanisms are involved which may be at the level of DNA interaction through modulation of ESR transcriptional co-regulatory proteins (Hall and McDonnell, 2005), increased ESR degradation (Nawaz *et al.*, 1999), or inducing ESR aggregation in the nucleus (Hung, 2004). Interestingly, attempts to block G-1-

mediated inhibition of ESR-driven ERE activation by pretreatment with G-15 failed to rescue these effects (Figure 2.3) which suggests that transcriptional inhibition by 1 µM G-1 may occur independently of GPER. This observation, while not supported by all studies, has been recently corroborated by another group (Wang *et al.*, 2012). Our data however should be interpreted with caution as G-15 has been shown to weakly bind nuclear ESRs at the doses used in our study (Dennis *et al.*, 2011). Future studies to employ knockdown strategies, similar to those observed by Wang et al., (Wang *et al.*, 2012) are planned to more carefully address the role of GPER in the observed effects.

With the exception of BPA, where a trend in decreased activation was seen at low doses, G-1 only inhibited ERE activity at doses $\geq$ 500 nM (Figure 2.4). These results coincide with our observation that a low dose (10 nM) of G-1 had no effect on ligand-induced proliferation and correlated with inhibition of cell growth at doses above 500 nM. Of note, low doses of G-1 (10 nM – 100 nM) also did not inhibit ERE activation driven by lower doses of E_2 (10 pM – 100 pM) which refutes the possibility that the inability of low doses of G-1 to inhibit ESR function is due to saturation of the ESRs (data not shown). These results lend additional support to the notion that high doses of G-1 may signal independently of GPER to inhibit ESR function by estrogenic ligands.

While reporter assays allow for examining ESR activity of a specific ERE, promoters of E_2-responsive endogenous genes are much more complex and are typically regulated by more than just a consensus response element. To that end, we examined the capability of G-1 to inhibit the expression of a known ESR-driven gene. The pS2 (TFF1) gene is well established to be inducible by E_2 via ESRs in MCF-7 cells (Jakowlew *et al.*, 1984), and therefore was chosen as a candidate gene with which to

study the effects of low and high doses of G-1. Our results substantiated previous results of pS2 induction by E_2 and select xenoestrogens (Jakowlew *et al.*, 1984; Recchia *et al.*, 2004) and revealed that expression is not modulated by G-1 alone (Figure 2.5). Interestingly, results revealed that E_2- and xenoestrogen-induced pS2 expression is not sensitive to low or high doses of G-1 in co-exposure conditions (Figure 2.6). Although this may suggest that G-1 mediated inhibition of proliferation occurs farther upstream of transcriptional activation, it is more likely due to the complexity of the pS2 promoter. It has been shown that the pS2 promoter contains only a half ERE and is regulated by additional transcription factors such as AP-1 (Barkhem *et al.*, 2002). Taken together, our results indicate that the inhibition of ESR activity is not at the level of degrading the ESRs but interfering with interactions of the receptors at select response elements (ERE). Further studies are required to define these mechanisms.

Only a handful of studies have begun to investigate the precise mechanisms involved in G-1-mediated inhibition of proliferation. To date, these investigations have primarily focused on a few select kinases (MAPK, etc.) and downstream genes involved in cell cycle progression (cyclins etc.) (Lubig *et al.*, 2012). To our knowledge, no studies have assessed the global proteome to begin to identify alternate pathways that are modulated by G-1. The propagation of signaling pathways largely relies on reversible post-translation modifications such as phosphorylation (Metodiev and Alldridge, 2008), and as such, global phosphoproteomic strategies are emerging as a powerful tool to decipher complex signaling networks (Harsha and Pandey, 2010; Sudhir *et al.*, 2011). Although it is well established that G-1 activates various signaling cascades including the PI3K pathway through modulation of the GPER (Bologa *et al.*, 2006), our results

and those of others have indicated that the inhibitory effects of G-1 at high doses may occur either independently of GPER (Wang *et al.*, 2012) or modulate alternate pathways driven in some yet unknown way by GPER. As an initial attempt to identify novel targets of G-1 mediated signaling in order to uncover pathways by which G-1 inhibits ESR functions, we performed a global phosphoproteome analysis on cells exposed to E_2 or G-1 for 30 minutes. Results of these studies revealed 238 peptides in total that were phosphorylated on specific serine, threonine, and tyrosine residues. A number of the peptides that were differentially phosphorylated by G-1 compared to controls are involved in reproduction as determined by GO analysis (Figure 2.8) and are annotated in greater detail in Table 2.1. Our observation that a high dose of G-1 and E_2 exhibited differential phosphorylation patterns (Figure 2.7) is intriguing because it substantiates the notion that G-1 may signal independently of GPER at high doses as previous studies have indicated that low doses of G-1 activates kinases analogous to E_2 (Kato *et al.*, 1995; Revankar *et al.*, 2005c; Albanito *et al.*, 2007).

Of particular interest is the G-1 specific phosphorylation of the candidate tumor suppressor protein NDRG2 which was one of many differentially phosphorylated proteins that were associated with reproduction and cell growth. NDRG2 has been shown to play a role in cell growth inhibition of colon cancer cells when phosphorylated at the particular residue (S332) identified in our phosphoproteomic analysis (Kim *et al.*, 2009). Neither the control nor E_2 treatments resulted in S332 phosphorylation. We attempted to validate the G-1 specific phosphorylation of NDRG2 by Western blot and results support variable phosphorylation patterns between E_2 and G-1 however; we were unable to discern the precise phosphorylation status of S332 using this method

(Figure 2.10). A number of reports reveal numerous phosphorylation sites on NDRG2 that have been linked with various functions. For example, phosphorylation of S332 inhibits the insulin-induced phosphorylation of T348 resulting in inhibition of muscle growth and metabolism of muscle cells (Burchfield *et al.*, 2004). These studies highlight the complexity and multiple phosphorylation sites that are important in governing its function. We have recently obtained a plasmid encoding NDRG2 (kindly supplied by Dr. Lim, Sookmyung Women's University, Seoul Korea) which has been mutated at S332 (to alanine) rendering it incapable of being phosphorylated. This tool will allow us to perform additional studies to address the role of S332 in G-1 inhibitory effects on cell proliferation. Regardless, the results of our phosphoproteomic analysis provided a useful first-pass screening of the phosphoproteome and have laid a framework on which to build future experiments to address G-1 modulated cell signaling pathways.

Based on reports that the NDRG2 promoter contains a response element shown to interact with ESR2 and its expression is modulated by E_2 in several cancer lines through promoter methylation, mutation, and genomic deletion (Liu *et al.*, 2007), we performed experiments to assess the impact of ESR-specific ligands and G-1 on NDRG2 mRNA levels. Here we report that E_2, PPT and DPN decrease NDRG2 expression at 24 hours post exposure whereas G-1 had no effect compared to control cells (Figure 2.11). These results suggest that while ESRs decrease NDRG2 expression thereby allowing cell growth to occur, G-1 does not alter expression levels, but may phosphorylate the protein at select residues such as S332 which may contribute to cell cycle inhibition. While NDRG2 has been shown to act as an inhibitor of cell proliferation in other cells (Kim *et al.*, 2009) one study showed that ESR2 increases transcriptional

activation of the NDRG2 promoter using a reporter assay after 12 hours in MCF-7 cells (Li *et al.*, 2011). Although we observed an opposite effect, it is difficult to directly compare results as the published study only assessed a portion of the NDRG2 promoter. It is also possible that differences are the result of periodicity and further time-points should be evaluated. Additional studies to discern the impact of xenoestrogens and the role of GPER on NDRG2 phosphorylation are planned. Collectively, results of the present work indicate that doses of the GPER agonist G-1 > 500 nM inhibit E_2 and xenoestrogen-induced, ESR-mediated proliferation of human breast cancer cells possibly by interference with ESR-mediated transcriptional activation. These data support the possibility that G-1 may be acting independently of GPER through selective phosphorylation and activation of the tumor suppressor protein NDRG2 (Lubig *et al.*, 2012). These collective results are significant because they highlight a novel role for G-1 in inhibiting E_2 and xenoestrogen-dependent proliferation. While these data do not necessarily support a role for xenoestrogens in activating GPER, elucidating pathways that inhibit their effects (through ESRs) have enormous implications for adjuvant therapies in the treatment of estrogen receptor-positive breast cancers.

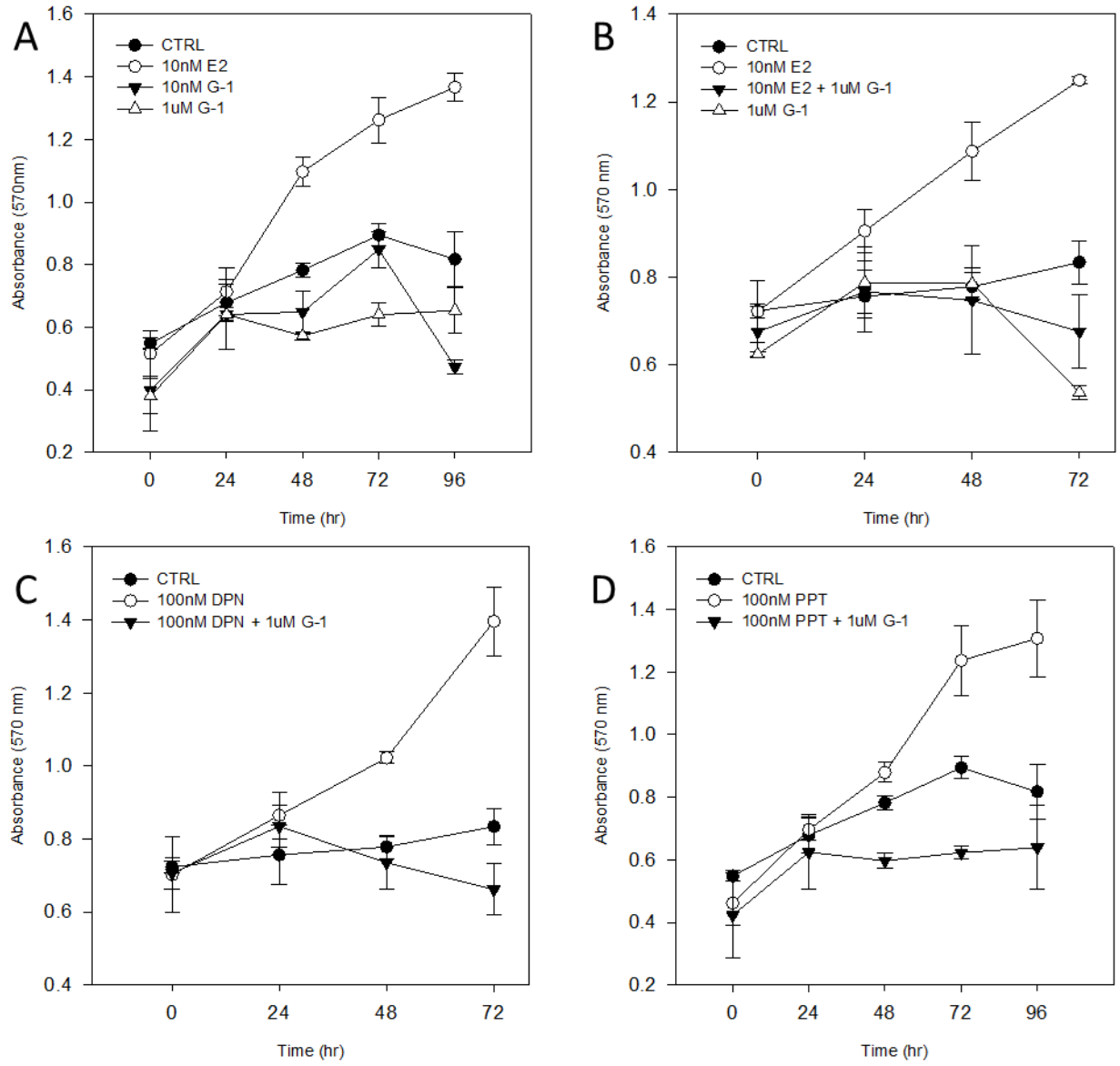

Figure 2-1. Co-exposure to 1 μM G-1 suppresses nuclear ER-driven proliferation of MCF-7 cells. Cells were seeded in 96-well cell culture plates and exposed to chemicals dissolved in DMSO for 72 or 96 hours. Proliferation was determined by a modified MTT assay and graphed as 570 nm absorbance over time. A) Exposure to 10 nM E2, 10 nM G-1 or 1 μM G-1. B) Exposure to 10 nM E_2 in combination with 1 μM G-1. C) Exposure to DPN (ESR2 agonist) with and without 1 μM G-1. D) Exposure to the PPT (ESR1 agonist) with and without 1 μM G-1.

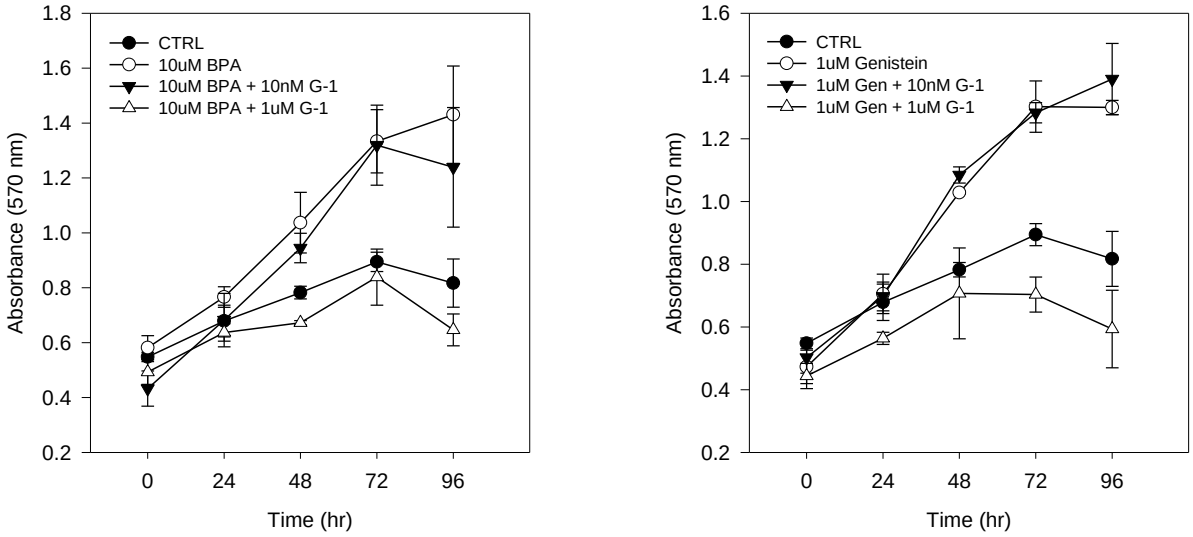

Figure 2-2. Co-exposure to 1 µM G-1 suppresses xenoestrogen-induced proliferation of MCF-7 cells. Cells were seeded in 96-well cell culture plates and exposed to chemicals dissolved in DMSO for 96 hours. Proliferation was determined by a modified MTT assay and graphed as 570 nm absorbance over time. A) Exposure to 10 µM BPA with and without 10 nM G-1 or 1 µM G-1. B) Exposure to 1 µM genistein with and without 10 nM G-1 or 1 µM G-1.

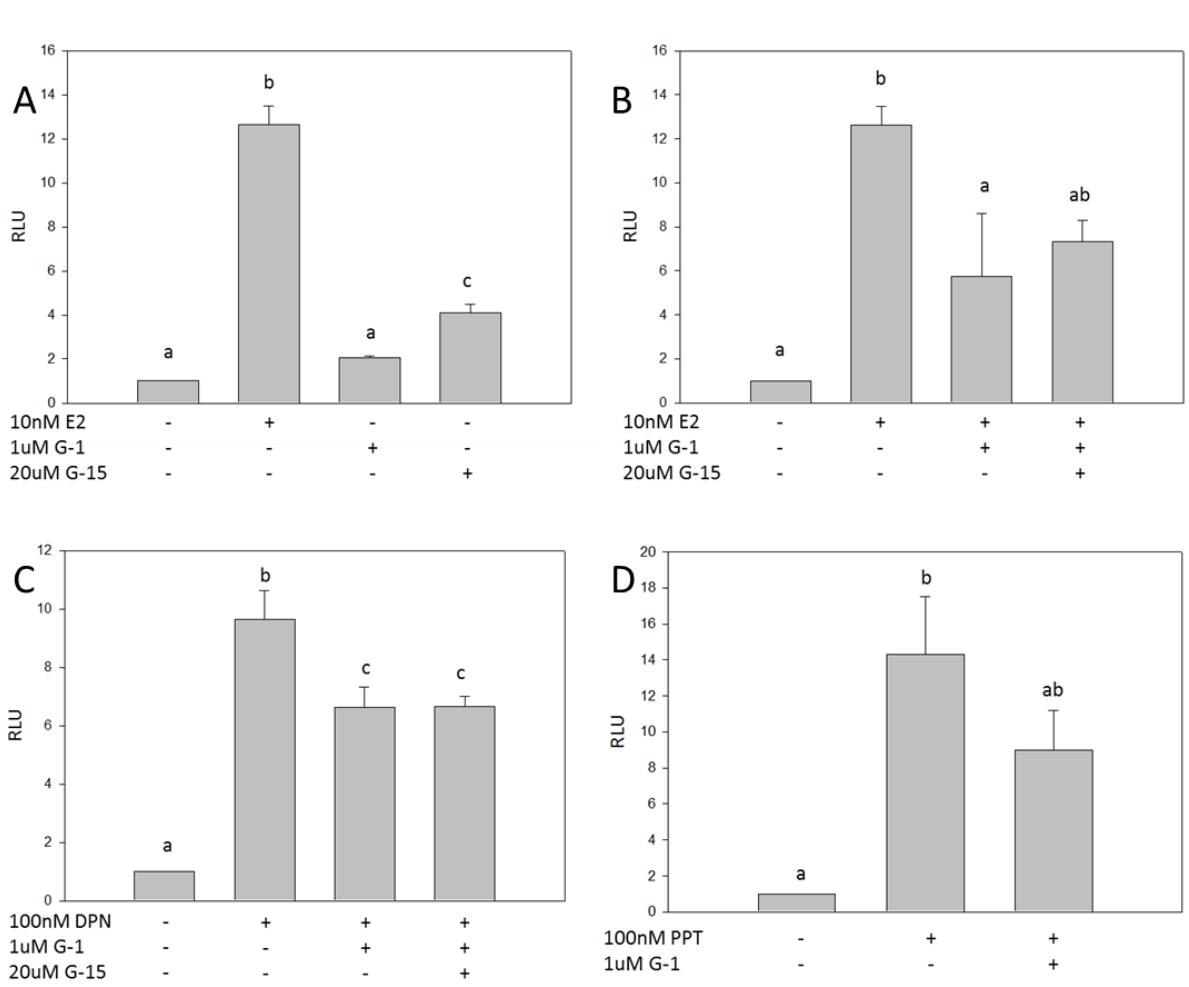

Figure 2-3. Co-exposure to 1 µM G-1 suppresses ESR-mediated activation at an ERE. MCF-7 cells were transfected with a 2XERE-Luciferase reporter construct for 24 hours and exposed to chemicals dissolved in DMSO for 24 hours thereafter. A) Exposure to 10 nM E_2, 1 µM G-1 and 20 µM G-15. B) Exposure to 10 nM E_2 and co-exposures with 1 µM G-1 and 20 µM G-15. C) Exposure to 100 nM DPN and co-exposures with 1 µM G-1 and 20 µM G-15. D) Exposure to 100 nM PPT and co-exposures with 1 µM G-1. Results are represented as the mean ± SEM of three experiments. Letters denote significance at $p < 0.05$.

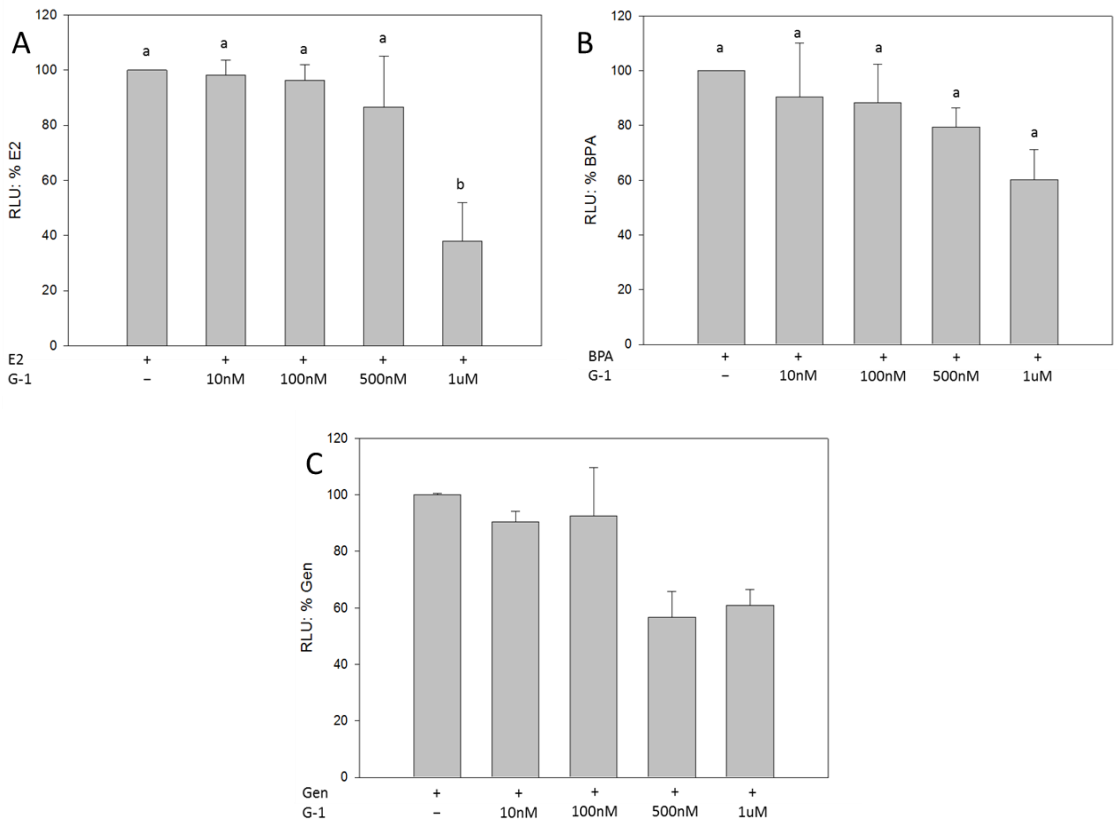

Figure 2-4. GPER-mediated suppression of ERE activation is dose-dependent. MCF-7 cells were transfected with 2XERE-Luciferase reporter gene and exposed to chemicals dissolved in DMSO for 24 hours. Bars represent percent of maximal activity (RLU) for E_2, BPA, or genistein. A) Exposure to 10 nM E_2 with increasing concentrations of G-1. B) Exposure to 640 nM BPA with increasing concentrations of G-1. C) Exposure to 284 nM genistein with increasing concentrations of G-1. Results are represented as mean ± SD. Letters are significant at $p < 0.05$. Note; results presented in panel 3 represent data from one experiment so statistics could not be performed.

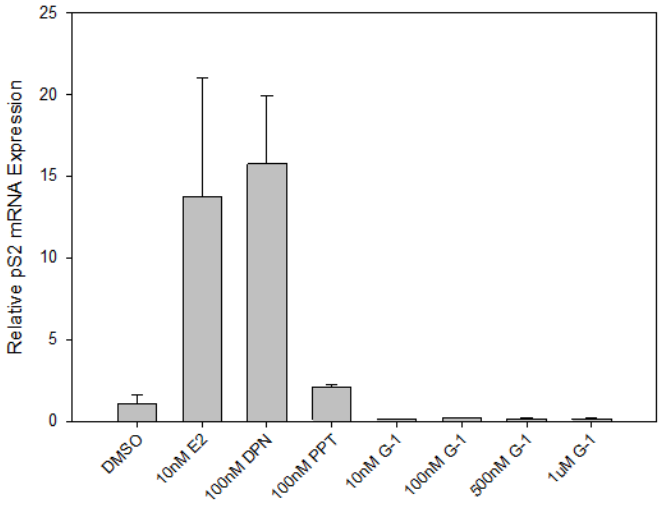

Figure 2-5. pS2 expression is responsive to nuclear ER agonists but not G-1. MCF-7 cells were exposed to 10 nM E_2, 100 nM DPN, 100 nM PPT, G-1 (10 nM, 100 nM, 500 nM, 1 µM) for 24 hours. Results are represented as mean ± SD of one experiment.

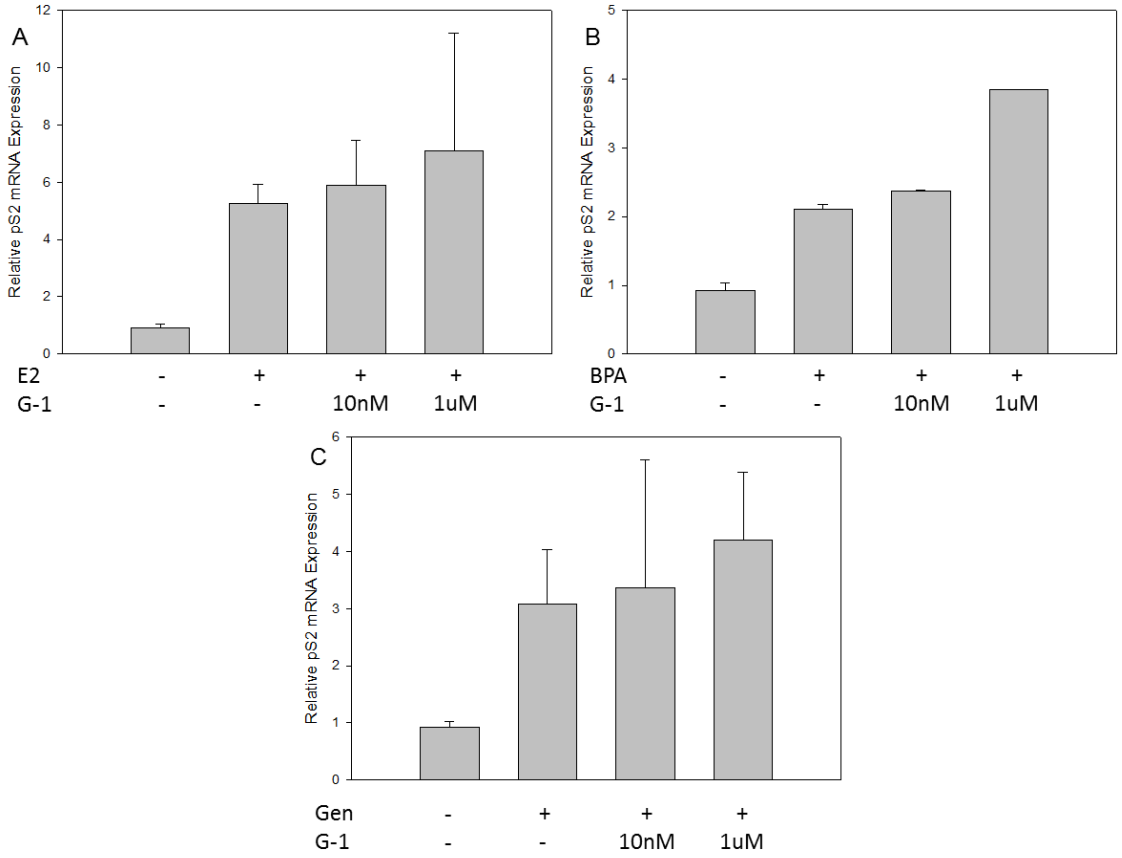

Figure 2-6. Xenoestrogen-induced pS2 expression is not responsive to G-1. MCF-7 cells were exposed to chemicals dissolved in DMSO for 24 hours. A) Exposure to 10 nM E_2 or 1 µM G-1. B) Exposure to 10 µM BPA. C) Exposure to 1 µM genistein. Results are represented as mean ± SD of one experiment.

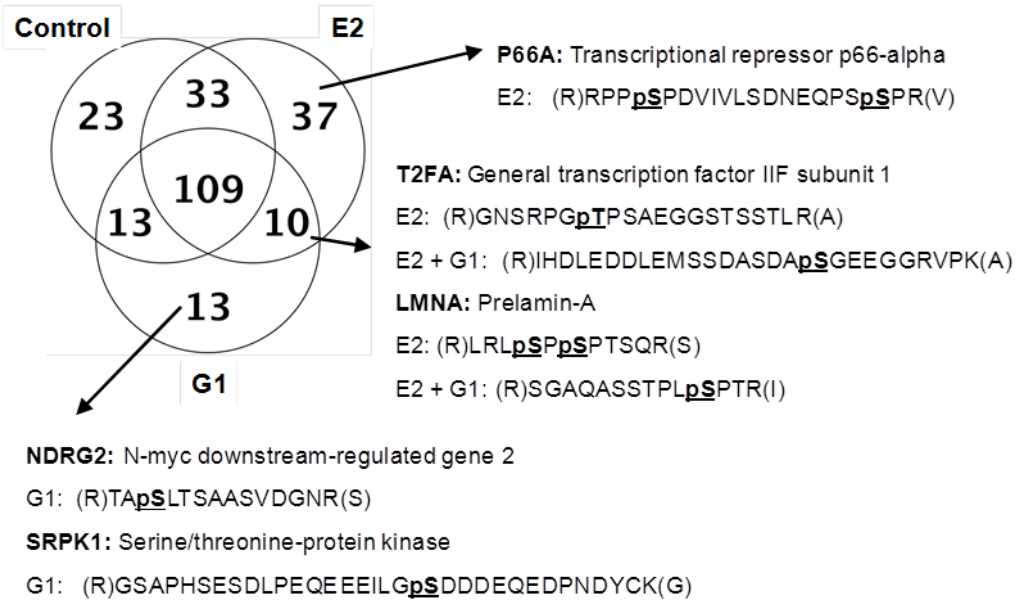

Figure 2-7. Distribution of the number of phosphorylated proteins identified in MCF-7 cells exposed to E_2 or G-1 compared to control cells. The Venn diagram depicts the distribution of peptides phosphorylated for each treatment after exposure for 30 minutes. Representative peptides are shown for specific, relevant proteins identified within discrete treatments, with phosphorylation sites noted as bold underlined **pS** or **pT** in each sequence.

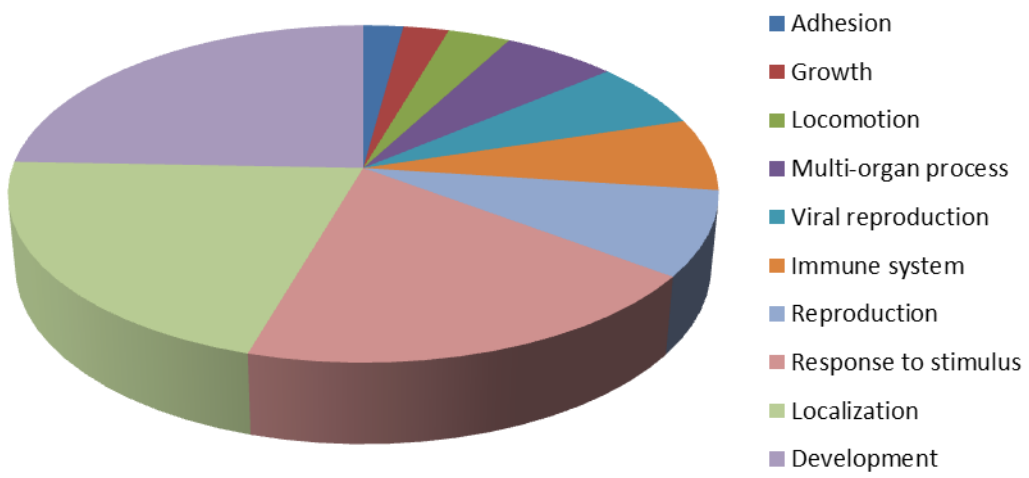

Figure 2-8. Phosphorylated peptides organized by biological process. Pie chart showing the representative biological processes determined by GO analysis of the 238 proteins phosphorylated by E_2 and/or G-1 after 30 minutes of exposure.

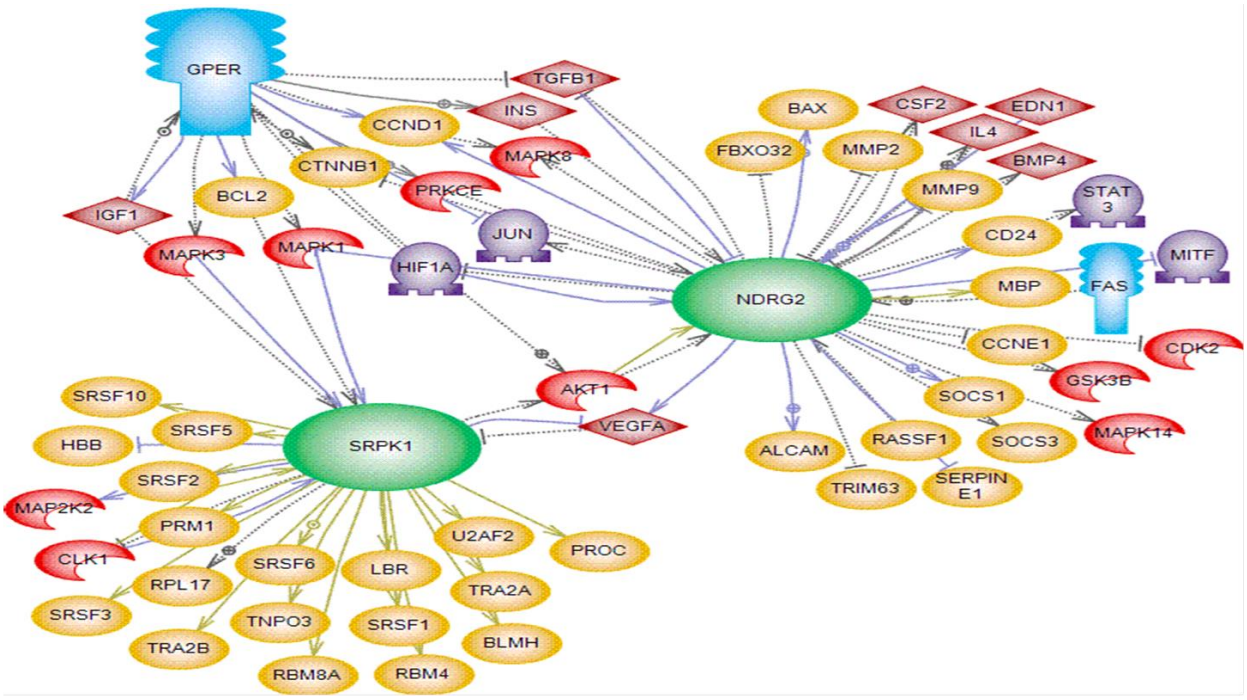

Figure 2-9. Pathway showing relationship between proteins phosphorylated specifically by G-1 in MCF-7 cells. The larger central circular entities (NDRG2 and SRPK1) are phosphorylated proteins identified in the phosphor-proteomic analysis. This simplified pathway depicts these proteins as 'hubs' that may play a role in G-1 signaling through GPER. The shapes represent the following; diamonds – growth factors/peptides; semi circles – kinases; circles – proteins; others – receptors and transcription factors. Lines represent various relationships between entities including post-translational modifications, expression, and general relationships.

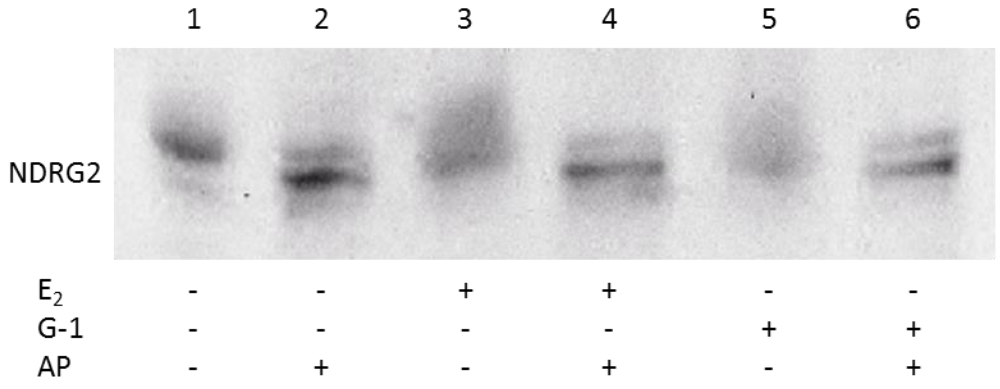

Figure 2-10. Phosphorylation of NDRG2. MCF-7 cells were exposed to DMSO, 10 nM E_2, or 1 µM G-1 for 30 minutes and total protein was separated on Tris-Tricine polyacrylamide gel and probled with anti-NDRG2. Lanes 2, 4, and 6 represent protein fractions treated with alkaline phosphatase (AP).

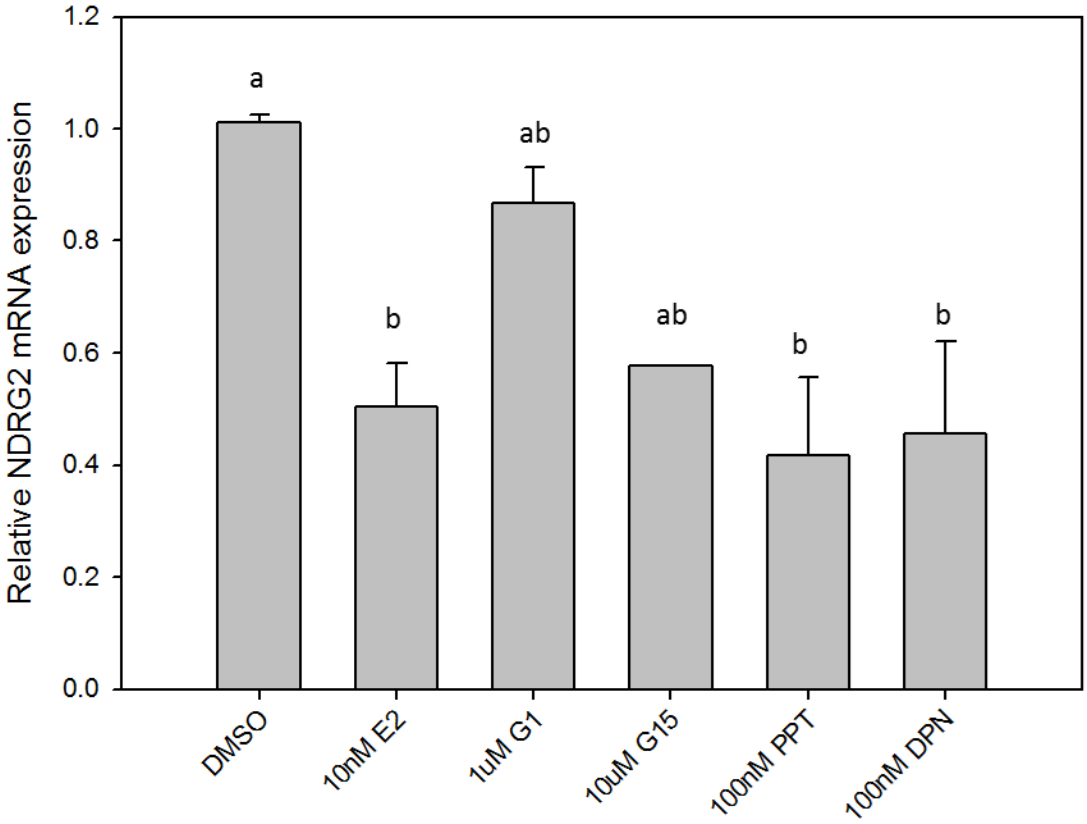

Figure 2.11. Nuclear ERs suppress the expression of NDRG2 mRNA. MCF-7 cells were exposed to 10 nM E_2, 100 nM PPT, 100 nM DPN, 1 µM G-1, or 10 µM G-15. Bars represent mean ± SD of two experiments Letters are significant at $p < 0.05$.

Table 2-1. Peptides differentially phosphorylated by G-1 and sorted into biological process 'reproduction' by GO analysis.

Protein ID	Name	Phosphorylation by Treatment			Phospho Site(s)	Sequence	Protein Probability (%)	MASCOT ID Score
		Control	E2	G1				
NDRG2	NMYC downstream regulated gene 2			1	S332	(R)TA**S**LTSAASVDGNR(S)	88%	28.6
RS6	40S ribosomal protein S6			1	S235, S236	(R)RL**SS**LR(A)	88%	25.0
SRPK1	Serine/threonine-protein kinase			1	S51	(R)GSAPHSESDLPEQEEEILG**S**DDDEQEDPNDYcK(G)	88%	32.3
WAPL	Wings apart-like protein			1,2	S77, S221, S226	(K)RPE**S**PSEI**S**PIKGSVR(T); (K)VEEESTGDPFGFD**S**DDESLPVSSK(N)	100%	31.6
JIP4	C-Jun-amino-terminal kinase-interacting protein 4	1,2	1,2		S730, S733	(R)SA**S**QS**S**LDKLDQELKEQQK(E)	70-100%	32.1
PEBP1	Phosphatidylethanolamine-binding protein 1	1	1		S52	(K)NRPT**S**ISWDGLDSGK(L)	89-90%	30.3
UBR4	E3 ubiquitin-protein ligase	1	1		S2719	(R)HVTLPS**S**PR(S)	68-76%	26.3
UNG	Uracil-DNA glycosylase	1	1,2,3, 4		S23, T60, S63, S64	(R)HAP**S**PEPAVQGTGVAGVPEESGDAAAIPAK(K); (K)KAPAGQEEPG**T**PP**SS**PLSAEQLDR(I)	85-100%	32.8
BAG6	Large proline-rich protein	1,2	1,2	1	S113, S1117	(R)APPQTHLPSGASSGTGSASATHGGG**S**PPGTR(G); (R)LQEDPNY**S**PQRFPNAQR(A)	74-100%	33.2

IF4G1	Eukaryotic translation initiation factor 4 gamma 1	1,2,3,4	1,2,3, 4	1,2	S1185, S1187. T205, T207	(R)**S**F**S**KEVEER(S); (R)TAS**T**P**T**PPQTGGGLEPQANG ETPQVAVIVRPDDR(S)	88-100%	34.1
RLA2	60S acidic ribosomal protein P2	1,2	1,2	2	S102, S105	(K)DEKKEE**S**EE**S**DDDMGFGLFD (-)	89-100%	29.5
AGFG1	Afr-GAP domain	1		1	S181	(K)GTPSQ**S**PVVGR	95%	27.4
NPM	Nucleophosmin	1		1	S125	(K)cGSGPVHISGqHLVAVEEDAE SEDEEEEDVK(L)	90-100%	33.6
PACS1	Phosphofurin acidic cluster sorting protein 1	1,2,3	2,3	1,2,3	S495, S529, S531	(K)TDLQGSA**S**PSKVEGVHTPR(Q); (R)TNS**S**D**S**ERSPDLGHSTQIPR(K)	89-100%	31.6
PARN	Poly(A)-specific ribonuclease P	1,2	3,4	1,2	S572, S583, T589, T594	(R)NLSP**S**QEEAGLEDGV**S**GEIS D**T**ELEQ**T**DScAEPLSEGR(K)	88-89%	34.1
PSA3	Proteasome subunit alpha type-3	1,2	1	1,2	S243, S250	(K)YAKE**S**LKEEDE**S**DDDNM(-)	89-100%	28.5
SCRIB	Scribble		1		S1316, S1319	(K)mAESPcSPSGQQPP**S**PP**S**PD ELPANVK(Q)	89%	32.7
UBP2L	Ubiquitin-associated protein 2-like	4	1,2,3, 4	4	S454, S462, S604, S609	(K)SPAVATSTAAPPPPS**S**PLPSK (S); (K)ST**S**APQMSPGSSDNQSSSP QPAQQK(L); (R)RYP**S**SISS**S**PQKDLTQAK(N)	88-100%	30.6
LMNA	Prelamin A/C		1	1	S22	(R)SGAQASSTPL**S**PTR(I)	100%	28.6

RD23A	UV excision repair protein RAD23 homolog A		1	1	S128	(R)EDKSPSEE**S**APTTSPESVSG SVPSSGSSGR(E)	88-89%	33.4
TRI25	E3 ubiquitin/ISG15 ligase		1		S100	(R)ASAP**S**PNAQVAcDHcLK(E)	100%	32.1
AKT1	Serine/Threonine protein kinase	1,2	1,2	1,2	S124, S129	(R)SG**S**PSDN**S**GAEEMEVSLAKP K(H)	88-90%	31.5
CTNA1	Catenin alpha-1	1	1	1	S641	(R)TPEELDD**S**DFETEDFDVR(S)	88-90%	29.4
HMCS1	Hydroxymethylglutaryl-CoA synthase, cytoplasmic	1	1	1	S516	(R)LPATAAEPEAAVI**S**nGEH(-)	88-90%	30.6
HNRPK	Heterogeneous nuclear ribonucleoprotein K	1	1	1	S284	(R)DYDDM**S**PR(R)	76-100%	25.0
IMA2	Importin subunit alpha-2	1	1	1	S62	(R)NVSSFPDDAT**S**PLQENR(N)	88-90%	30.3
NACA	Nascent polypeptide-associated complex subunit alpha	1	1	1	S166	(K)VQGEAVSNIQENTQTPTVQE E**S**EEEEVDETGVEVK(D)	88-90%	34.9
XRN2	5'-3' exoribonuclease 2	1,2	1,2	1,2	S499, S501	(R)KAED**S**D**S**EPEPEDNVR(L)	88-90%	26.7

CHAPTER 3

GENERAL CONCLUSIONS

The initial goal of this research was to decipher the potential interplay between genomic and non-genomic signaling pathways initiated by the GPER and ESRs in response to xenoestrogens. Uncovering the complex cellular responses to xenoestrogen exposure offers valuable information regarding their contribution to disease. However, our investigations led us in a different direction as it was revealed that the putative GPER specific agonist (G-1) is able to inhibit ESR function initiated by E_2 and xenoestrogens and that these mechanisms of inhibition may occur independently of GPER. These results reveled for the first time, a novel role for G-1 in modulating the actions of E_2 and xenoestrogens that may have value in the development of therapeutics in the treatment of endocrine disease. This could be of particular importance in the treatment of estrogen receptor-positive breast cancer which proliferates in response to ESR agonists. In the US, approximately 1 in 8 women will develop breast cancer in her lifetime and ESR positive breast cancers are the most common type, constituting 70% of diagnoses. Remarkably, 30% of women who develop ESR positive breast cancer are or become endocrine resistant and do not respond to antihormone therapeutics such as the selective estrogen receptor modulator (SERM), tamoxifen (García-Becerra *et al.*, 2012). These staggering statistics necessitate the identification of a more comprehensive treatment to mitigate ESR mediated proliferation.

Although it is still unknown whether exposure to xenoestrogens can impact cancer risk, experimental evidence is mounting. For example, dietary exposure to Genistein is of particular concern in estrogen responsive breast cancers as the

estrogenic properties of Genistein are largely determined by the levels of circulating E_2 and ESR expression profile. In the absence of circulating E_2, Genistein has been found to increase the growth of ESR positive breast cancer cells (Chen *et al.*, 2003). In light of this, G-1 may prove to be a beneficial therapeutic in the treatment of postmenopausal women diagnosed with estrogen-responsive breast cancer. Further, BPA has been shown to increase proliferation of breast cancer cells .,(Schafer *et al.*, 1999; Recchia *et al.*, 2004) and even decrease the efficacy of chemotherapeutic drugs by antagonizing their cytotoxic properties independent of ESRs *in vitro* (LaPensee *et al.*, 2009). Based on our results, G-1 may present a potential alternative to chemotherapeutics.

The fact that G-1 mediated inhibitory pathways of ESR function may occur independently of GPER requires uncovering novel targets of G-1 in order to construct a plausible cellular signaling network to determine the mechanism by which G-1 functions at high doses. Elucidating the complex mechanism of G-1 action may further substantiate its use as an adjuvant therapeutic in the treatment of xenoestrogen mediated disease, particular estrogen-responsive breast cancer.

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BIOGRAPHICAL SKETCH

Ley Cody Smith was born on August 1, 1988 in Orlando, FL. After graduating from William R. Boone High School in 2006, he enrolled at the University of Florida where he received his Bachelor of Science in integrative biology in 2010. Mr. Smith then worked as a research associate in the lab of Dr. David Barber in the Center for Environmental and Human Toxicology for the summer after completing his bachelor's. Thereafter, he enrolled in the University of Florida graduate school in August 2011 and received his Master of Science in the spring of 2013 under the direction of Dr. Tara Sabo-Attwood. He was awarded a Graduate School Fellowship form the University of Florida in the spring of 2013 and will begin his Doctor of Philosophy program studying interdisciplinary toxicology in the fall of 2013 also under the direction of Dr. Tara Sabo-Attwood.