Novel roles of prolactin and estrogens in breast cancer: resistance to chemotherapy

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Abstract

Resistance to chemotherapy is a major complication in the treatment of advanced breast cancer. Estrogens and prolactin (PRL) are implicated in the pathogenesis of breast cancer but their roles in chemoresistance have been overlooked. A common feature to the two hormones is activation of their receptors by diverse compounds, which mimic or antagonize their actions. The PRL receptor is activated by lactogens (PRL, GH, or placental lactogen) originating from the pituitary, breast, adipose tissue, or the placenta. Estrogen receptors exist in multiple membrane-associated and cytoplasmic forms that can be activated by endogenous estrogens, man-made chemicals, and phytoestrogens. Here, we review evidence that low doses of PRL, estradiol (E_2), and bisphenol A (BPA) antagonize multiple anticancer drugs that induce cell death by different mechanisms. Focusing on cisplatin, a DNA-damaging drug which is effective in the treatment of many cancer types but not breast cancer, we compare the abilities of PRL, E_2 , and BPA to antagonize its cytotoxicity. Whereas PRL acts by activating the glutathione-S-transferase detoxification enzyme, E_2 and BPA act by inducing the antiapoptotic protein Bcl-2. The implications of these findings to patients undergoing chemotherapy are discussed.

Endocrine-Related Cancer (2010) 17 R91–R107

Introduction

Each year, over a million women worldwide are diagnosed with breast cancer, accounting for 25% of all female cancers. Treatments include surgery, radiation therapy, chemotherapy, or their combinations. Neoadjuvant chemotherapy is used to reduce tumor size before surgery, while adjuvant chemotherapy is used after tumor excision. Chemotherapy is the mainstay treatment for patients with triple negative tumors (estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (Her-2)) who are resistant to hormone or targeted therapy, and for those with advanced metastatic disease (Coley 2008). Dozens of anticancer drugs have been developed, with treatment options taking into account tumor grade and histology and whether the desired outcome is curative or palliative. Most regimens combine drugs that act by different mechanisms aimed at improving the odds of suppressing tumor growth (Ocana & Pandiella 2008).

While both the selection and success of chemotherapeutic agents have increased, tumor resistance remains a major obstacle, which results in treatment failure. Some tumors are intrinsically resistant to

certain drugs, while others acquire resistance following treatment. Resistance can result from drug efflux by transporters, inactivation by detoxification enzymes, altered expression of pro/antiapoptotic proteins, changes in tumor suppressor genes, and increased DNA repair mechanisms (Coley 2008). Whereas hormones such as prolactin (PRL) and estradiol (E_2) have long been implicated in the pathogenesis of breast cancer, their involvement in chemoresistance has been overlooked. The objective of this review is to evaluate emerging evidence that these hormones confer resistance against a variety of chemotherapeutic agents that kill breast cancer cells by different mechanisms and discuss the clinical implications.

PRL and estrogens are dissimilar in chemical structure, receptor characteristics, and signaling mechanisms. Whereas estrogens can bind to several classical ($ER\alpha$ and $ER\beta$) and nonclassical (G protein-coupled receptor 30, GPR30) receptors (Manavathi & Kumar 2006), there is only one receptor (PRLR) for PRL, albeit it exists in several isoforms which couple to different signaling pathways (Swaminathan *et al.* 2008). Yet, there is crosstalk between the two hormones, with PRL

increasing the expression and phosphorylation of ER α (Carver *et al.* 2009), and E₂ inducing the transcription of both PRL (Duan *et al.* 2008) and the PRLR (Swaminathan *et al.* 2008). Such interactions can result in augmentation, or synergism, between the two hormones.

Several features that are common to PRL and E₂ confound the understanding of their roles in breast cancer. One is the multiple sites of origin, with both hormones reaching the breast from the systemic circulation as well as from local sources (Ben-Jonathan *et al.* 2002, Foster 2008). Thus, blood levels of PRL or E₂ do not reveal the full extent of breast exposure to these hormones. Another is a variable expression of their receptors in tumors, which often depends upon the luminal or basal origin of the tumor. Thus, neither hormone affects tumors that do not express its receptor. Importantly, 80–90% of breast carcinomas express the PRLR (Touraine *et al.* 1998), while ~75% contain ER α (Karayiannakis *et al.* 1996). This indicates that

most breast tumors express both receptors, reinforcing their importance as therapeutic targets. Finally is the issue of receptor promiscuity, with ERs capable of binding steroidal and nonsteroidal compounds (Bai & Gust 2009), and the PRLR capable of binding other lactogens (Goffin *et al.* 2005). Thus, xenoestrogens can mimic or antagonize endogenous estrogens, while GH and placental lactogen (PL) can augment or interfere with PRL actions. Figure 1 illustrates the various compounds that affect breast cancer via their interactions with either the PRLR or ERs.

Properties of selected chemotherapeutic drugs

Over the past 20–30 years, treatment of metastatic breast cancer has evolved from the anthracyclines to taxanes to hormonal and targeted therapy and their combinations (Coley 2008). Here, we focus on those drugs that are affected by PRL and/or estrogens.

Figure 1 Diagram of the different compounds and their sites of origin which affect breast cancer by activating either the prolactin (PRL) receptor (PRLR) or estrogen receptors (ERs). The right side of the illustration depicts the three structurally related lactogens, prolactin (PRL), GH, and placental lactogen (PL), which bind to the PRLR. The left side shows the variety of compounds which activate either classical or nonclassical ERs. These include natural estrogens: estradiol (E₂) and estril (E₃); man-made chemicals, diethylstilbestrol (DES), ethinylestradiol (EE₂), and bisphenol A (BPA); and phytoestrogens. Adipose tissue represents the stromal compartment within the breast as well as abdominal and subcutaneous depots. Both PRL and estrogens (likely via aromatization) are also produced within tumor cells themselves, where they act as autocrine/paracrine agents.

Cisplatin is a platinum-based drug that is highly effective against testicular, ovarian, and lung cancers but has limited efficacy as monotherapy in breast cancer patients (Decatris *et al.* 2004). Following uptake into the nucleus, cisplatin interacts with DNA and forms adducts via intrastrand cross-links that induce cell cycle arrest. The DNA can either be repaired by the nucleotide excision pathway, or the cell is destined to die (Kelland 2007). The caspase 3-deficient MCF7 cells are not killed by cisplatin (Blanc *et al.* 2000), although cisplatin can induce apoptosis via a caspase 3-independent mechanism in ovarian cancer cells (Henkels & Turchi 1999).

Doxorubicin (adriamycin) is an anthracycline antibiotics used in multiple cancers, and is considered the standard treatment in breast cancer. Upon entering the nucleus, doxorubicin inhibits topoisomerase II and helicase activities, and interferes with DNA double helix religation. This stops DNA replication and induces apoptosis (Rabbani *et al.* 2005). Apoptosis may occur via activation of pro-apoptotic proteins, since antisense against Bcl-2 and Bcl-xL sensitizes breast cancer cells to this drug (Simoes-Wust *et al.* 2002). Doxorubicin can also cause replicative senescence, as evident by micronuclei formation and senescence-associated β -galactosidase staining (Chang *et al.* 2002).

Taxol (paclitaxel) has been highly successful in treating breast and ovarian cancer, especially in combination with anthracyclines. Taxol targets the microtubules, which mediate alignment of the chromosomes along the equatorial plane prior to segregation to daughter cells. It binds to polymerized tubulin and inhibits microtubule disassembly, thereby suppressing both microtubule treadmilling and dynamic instability (Zhou & Giannakakou 2005). Taxol-induced apoptosis often correlates with phosphorylation of Bcl-2 (Ferlini *et al.* 2003). Apoptosis can occur via caspase 3-dependent or -independent mechanisms (Friedrich *et al.* 2001).

Vinblastine, a vinca alkaloid, is another microtubule-altering drug. Unlike taxol, it binds to monomeric tubulin and prevents its polymerization. Mitosis is blocked at the metaphase/anaphase transition, and the prolonged arrest leads to cell death (Zhou & Giannakakou 2005). Vinblastine also interferes with amino acid, cAMP, and glutathione metabolism, and can induce apoptosis through the nuclear factor κ B/inhibitor of κ B (NF- κ B/I κ B) pathway (Huang *et al.* 2004). Apparently, c-Jun protects T47D cells from vinblastine-induced cell death, although it does not prevent the mitotic block (Duan *et al.* 2007). At nontoxic doses, vinblastine inhibits chemotaxis

and endothelial cell proliferation, highlighting its antiangiogenic properties (Vacca *et al.* 1999).

Apoptotic signals are also mediated via the tumor necrosis factor (TNF) family of death receptors. The TNF-related apoptosis-inducing ligand (TRAIL) induces oligomerization of the intracellular domains (ICDs) of the death receptors and causes apoptosis in many cancer cell types without killing normal cells (Wang & El Deiry 2003). In recent clinical trials, TRAIL agonists showed no major toxicity, but therapy is limited to patient with TRAIL-sensitive tumors (Bellail *et al.* 2009).

The dependence of tumor expansion and metastasis on angiogenesis has lead to the development of angiogenesis blockers, which inhibit the release of pro-angiogenic proteins such as VEGF, block mitogenic/survival pathways of endothelial cells, or prevent extracellular matrix breakdown (Sessa *et al.* 2008). Over 300 angiogenic inhibitors have been developed, and dozens are in various phases of clinical trials. Avastin, an anti-VEGF monoclonal antibody, was the first antiangiogenic drug shown to prolong patient survival.

Mechanisms of chemoresistance

Resistance to chemotherapy results from many causes, including drug extrusion by transporters, drug metabolism, increased antiapoptotic proteins, decreased pro-apoptotic proteins, and enhanced DNA damage repair. A major problem is that tumors often exhibit resistance to a diversity of chemotherapeutic agents, which act by different mechanisms.

Multidrug resistance transporters, which target structurally diverse drugs, are major contributors to drug resistance. The best characterized are members of the ATP-binding cassette transporters, which include P-glycoprotein, multidrug resistance protein 1 (MRP1), and breast cancer resistance protein (BCRP; Higgins 2007). P-glycoprotein confers resistance against taxol, vinblastine, doxorubicin, and etoposide, with over 40% of breast tumors expressing this transporter (Trock *et al.* 1997). MRP1 confers resistance against anthracyclines, antifolates, and vinca alkaloids, but not against taxanes or cisplatin (Trock *et al.* 1997). Although originally isolated from a drug-resistant MCF7 subline, the BCRP protein is rarely found in E₂-responsive cells, because of its downregulation by estrogens (Imai *et al.* 2005).

The glutathione metabolic pathway confers resistance against environmental insults and drugs. Glutathione-S-transferases (GSTs) are phase II detoxification enzymes that catalyze the conjugation of

glutathione to electrophilic compounds, resulting in easily extruded products (Townsend & Tew 2003, McIlwain *et al.* 2006). They inactivate platinum drugs, doxorubicin, cyclophosphamide, and etoposide, but not antimicrotubule drugs. Some GST isozymes inhibit JNK1 via protein:protein interactions and inactivate drugs that act via the mitogen activated protein (MAP) kinase pathway even when they are not subject to conjugation with glutathione. GSTs are inducible enzymes classified by substrate specificity and intracellular distribution into several families, e.g. α , μ , π and θ subtypes. Overexpression of GST π is associated with drug resistance and poor patient survival, while mutations in GST μ and GST π predispose the affected individuals to environmental carcinogens (Shiga *et al.* 1999).

Tumors can acquire drug resistance by overexpressing antiapoptotic proteins (e.g. Bcl-2 and Bcl-xL) or downregulating pro-apoptotic proteins (e.g. Bax). MCF7 cells, which express high levels of Bcl-2, are less responsive to cisplatin, but become sensitized to the drug upon Bcl-2 downregulation by RNA interference (Yde & Issinger 2006). Breast cancer cells that overexpress Bcl-xL are less sensitive to paclitaxel, which correlates with failure of the drug to activate caspase 9 (Wang *et al.* 2005). In addition, Bax overexpression in MCF7 cells restores their sensitivity to various apoptotic agents. Higher Bax expression levels are detected in normal breast epithelium than in adjacent tumors (Bargou *et al.* 1996).

Mutations in tumor suppressors strongly influence cancer cells sensitivity to anticancer drugs. Alterations in p53 are the most common genetic changes in breast cancer, with specific mutations associated with resistance to doxorubicin (Aas *et al.* 1996). The tumor suppressor gene BRCA1 is also frequently mutated in breast cancer. It responds to DNA damage by affecting DNA damage repair. Loss of BRCA1 confers sensitivity to many DNA-damaging agents (Kennedy *et al.* 2004), and its knockdown results in a twofold increase in cell sensitivity to irrofulven (Wiltshire *et al.* 2007).

Characteristics of PRL and PRLR

PRL is a 23-kDa hormone of pituitary origin whose main target is the breast, where it stimulates proliferation, differentiation, survival, and secretory activity (Ben-Jonathan *et al.* 2008). Depending upon the cell context and physiological conditions, PRL can exert opposite actions such as proliferation versus differentiation in both malignant and nonmalignant cells. PRL belongs to a family of proteins, named lactogens, which share structural homology and some

overlapping functions. The most prominent members are PRL, GH, and PL, which are made of a single polypeptide chain with 2–3 intramolecular disulfide bridges. Lactogens have a high homology in their primary amino acids and a similar tertiary structure, composed of four antiparallel, up–up, down–down helical bundle (Teilum *et al.* 2005).

Unique to humans, PRL is also produced in multiple nonpituitary sites, including the decidua, myometrium, breast, and prostate (Ben-Jonathan *et al.* 1996). Whereas pituitary PRL is controlled by a proximal promoter which requires the pituitary-specific Pit-1 transcription factor for transactivation, expression of extrapituitary PRL is driven by a superdistal promoter (Gerlo *et al.* 2006). The insensitivity of the superdistal promoter to dopamine explains the failure of dopamine agonists, such as bromocriptine, to suppress breast PRL and affect PRL-dependent tumors in patients (Ben-Jonathan *et al.* 2008). Within the normal breast, PRL is produced at much larger quantities by stromal adipocytes than by the epithelium (Zinger *et al.* 2003) and is up-regulated in carcinomas as compared with benign breast epithelium (McHale *et al.* 2008).

The PRLR is a member of the cytokine receptor superfamily, which is nontyrosine kinase, single-pass membrane receptor. It has a three-domain organization: an extracellular ligand-binding domain, which confers specificity, a short transmembrane domain, and an ICD (Swaminathan *et al.* 2008, Clevenger *et al.* 2009). In addition to the most abundant 80 kDa long isoform, shorter variants that couple to different signaling pathways are detectable in breast cancer. Among cell lines, T47D cells express the highest PRLR levels, followed by MCF7, BT483, MDA-MB-468, and BT474 (Peirce *et al.* 2001). The human PRLR is indiscriminate in its binding preferences, with GH binding not only to its receptor (GHR) but also to the PRLR. In contrast, nonprimate GH binds only to the GHR, while PRL binds only to the PRLR but not to GHR in any species (Ben-Jonathan *et al.* 2008). PL does not have a receptor of its own and binds only to the PRLR (see Fig. 1).

Two binding sites on the ligand are required for PRLR activation. One receptor binds to a high affinity site 1, while a second receptor binds to a lower affinity site. This forms an active ternary complex composed of one hormone molecule and receptor homodimer (Teilum *et al.* 2005). The existence of preformed, inactive dimers without a ligand suggests that receptor dimerization is necessary but insufficient for its activation (Clevenger *et al.* 2009). Ligand binding induces relative rotations of the two units, resulting in allosteric reorganization of the ICD. This brings the

ICD and Jak2 kinase into close proximity, enabling their phosphorylation. The lactogens, which differ in critical interacting residues, do not induce identical conformational changes in the receptor (Gertler *et al.* 1996). Instead, each imposes a different stability on the active complex, thereby affecting its dynamics and binding parameters of the associated partners.

Several PRLR antagonists, made by modifications of the PRL molecule, have been generated, which block PRL actions *in vitro* and in experimental animals (Goffin *et al.* 2005). However, their use as an effective treatment in breast cancer patients is uncertain because of their short half-life and the necessity for their administration by injection. Efforts are underway to find small molecules that selectively block the PRLR and can be delivered orally.

Binding of PRL to its receptor activates several signaling pathways, of which the Jak2–Stat5 pathway is the best understood. Jak2 is rapidly activated by PRL and phosphorylates Stat proteins. These dimerize and translocate to the nucleus, where they bind to GAS elements within the promoters of target genes. Stat5a/b mediate many of the PRL actions in normal and malignant breast cells (Clevenger *et al.* 2009). PRL-responsive genes that are involved, directly or indirectly, in cell cycle regulation include cyclin D1, AP-1, c-Myc, and heat shock protein α (Brockman *et al.* 2002, Acosta *et al.* 2003, Gutzman *et al.* 2005, Perotti *et al.* 2008).

Although activation of the Jak2–Stat pathway is critical for lobuloalveolar development and lactation in the normal breast, other PRL-induced pathways are important in breast cancer. One is the Ras–Raf–MAPK pathway, with ERK1/2 and *c-jun* N-terminal kinase being its primary mediators. PRL induces phosphorylation of ERK1/2 in both T47D and MCF7 cells, and can synergize with epidermal growth factor (EGF) to induce ERK (Acosta *et al.* 2003). Activation of the phosphoinositide-3-kinase (PI3K)–Akt survival pathway by PRL has been implicated in cell migration (Maus *et al.* 1999). Crosstalk between PRLR and ER α occurs at several levels. For example, PRL and E₂ cooperatively enhance AP-1 activity (Gutzman *et al.* 2005), and E₂ rapidly phosphorylates Stat5 (Fox *et al.* 2009), while PRL activates the unliganded ER (Gonzalez *et al.* 2009).

Role of PRL in carcinogenesis

The role of PRL in mammary tumorigenesis in rodents has long been recognized, while its involvement in breast cancer only recently became accepted. Prospective studies found a modest association between higher

serum PRL levels and cancer risk in both premenopausal and postmenopausal women, primarily those with ER+ tumors (Tworoger & Hankinson 2008). However, shortcomings of epidemiological studies include single blood sample determination and assay standardization. Most importantly, they do not take into account the local production of PRL by the breast or the status of PRLR expression in the tumors.

PRL exerts multiple actions in breast cancer cells, including increased proliferation, enhanced motility, and prolonged survival. Suppression of T47D cell proliferation by PRL antisense oligos, anti-PRL antibodies, and PRLR antagonists served as the evidence for the mitogenic activity of autocrine PRL (Chen *et al.* 1999, Vonderhaar 1999, Llovera *et al.* 2000). The role of autocrine/paracrine PRL is supported by studies with nude mice, where growth of tumors derived from T47D cells is inhibited by treatment with the hPRL antagonist G129R (Chen *et al.* 2002), while PRL overexpressing MDA-MB-435 cells form faster growing tumors (Liby *et al.* 2003).

PRL also affects cytoskeleton modulation, as reveals by its enhancement of breast cancer cell migration and induction of PI3K-dependent membrane ruffling and stress fibers (Maus *et al.* 1999). PRL and its cleaved fragment 16K PRL can stimulate and inhibit angiogenesis respectively, suggesting an indirect role for PRL in carcinogenesis via alterations in tumor blood supply (Clapp *et al.* 2008). Of great importance is a recent report that mouse PRL does not activate the human PRLR (Utama *et al.* 2006), raising issues of interpretation of drug responsiveness of human xenografts in mice, which are unaffected by circulating PRL.

Unlike estrogen, PRL is only a modest mitogen in breast cancer. In fact, Stat5 activation by PRL may be linked to induction of differentiation and suppression of invasion rather than to proliferation (Sultan *et al.* 2005). This is supported by a lower expression of activated Stat5 in node-positive breast cancer than in normal breast or less advanced tumors (Nevalainen *et al.* 2004). Yet, the argument that PRL acts solely as an antimetastatic factor is overreaching, since signaling pathways other than Stat5 are activated by PRL. PRL could serve as a suppressor of metastasis in advanced tumors but as a promoter of cell growth in early tumors. A switch between tumor promotion to suppression is exemplified by transforming growth factor- β (TGF β), which inhibits the growth of normal epithelial cells but accelerates the malignant process of late-stage tumors (Bachman & Park 2005). Estrogen represents another case of contrasting actions, since in addition to its mitogenic actions it can induce apoptosis under some conditions (Lewis-Wambi & Jordan 2009).

PRL and chemoresistance

Accumulating evidence suggests that PRL opposes cytotoxicity by a wide variety of anticancer drugs. In PC3 prostate cancer cells, TRAIL-induced apoptosis is partially inhibited by PRL, which by itself has no effect on cell proliferation (Ruffion *et al.* 2003). Another group reported that pretreatment of ovarian carcinoma cells with PRL inhibits cisplatin-induced cell death (Asai-Sato *et al.* 2005). PRL also antagonizes apoptosis caused by methotrexate, an antifolate agent, in human promyelocytic leukemia HL-60 cells (Hsu *et al.* 2006).

Epidemiological data suggest that women with elevated blood PRL levels have increased treatment failure and worse survival (Tworoger & Hankinson 2008). Indeed, hyperprolactinemic patients with metastatic breast cancer are less responsive to taxol than those with normal serum PRL levels (Lissoni *et al.* 2001). A small clinical trial revealed better responsiveness in patients treated with a combination of taxol and bromocriptine, compared to those receiving only taxol (Lissoni *et al.* 2002). These data should be replicated in larger trials with PRL inhibitors (i.e. bromocriptine and cabergoline) together with various anticancer drugs. However, an effective blockade of the PRLR would likely be more effective in sensitizing tumors to anticancer drugs than the suppression of pituitary PRL release.

Several studies have focused on PRL as an anti-cytotoxic factor in breast cancer cells. Ramamoorthy *et al.* (2001) found that induction of apoptosis by cisplatin in T47D cells is enhanced by co-treatment with the hPRL antagonist G129R, suggesting that endogenous PRL is protective. Another antagonist, $\Delta 1$ -9-G129R-hPRL, potentiates the effects of paclitaxel and doxorubicin in breast cancer cells (Howell *et al.* 2008). In addition, cells that produce PRL, e.g. T47D and MCF7, are more resistant to ceramide-induced apoptosis than those with low or no PRL (Perks *et al.* 2004). PRL can also overcome growth arrest caused by γ -irradiation (Chakravarti *et al.* 2005). None of the above studies, however, have resolved the mechanisms underlying the protective effects of PRL.

Our exploration of the mechanism by which PRL antagonizes anticancer drugs was inspired by the finding that PRL overexpression in MDA-MB-435 cells enhanced tumor growth and up-regulated Bcl-2 (Liby *et al.* 2003). Pretreatment of breast cancer cells with low doses of PRL antagonizes cytotoxicity by taxol, vinblastine, doxorubicin, and cisplatin, albeit at different efficacies (LaPensee *et al.* 2009b). We were especially interested in the mechanism by which PRL opposed cisplatin, which has shown only little

effectiveness in breast cancer patients. Unlike its strong apoptotic effects in MDA-MB-468 cells, cisplatin is only moderately effective in T47D cells. Reasoning that the resistance of T47D cells may be due to their high endogenous PRL levels, the mechanistic studies were conducted with MDA-MB-468 cells.

Measurement of platinum in nuclear extract by mass spectroscopy reveals that PRL reduces the amount of cisplatin bound to DNA. Lower entry of cisplatin into the nucleus could be due to transporters such as MRP that extrude the drug, or to detoxification enzymes such as GST that inactivate cisplatin (Siddik 2003). Previous work showed that PRL increases hepatic GST activity (Luquita *et al.* 1999). Using inhibitors of the two potential targets, we discovered that GST, but not MRP, accounts for the suppression of cisplatin entry to the nucleus by PRL. This action is mediated by the Jak-Stat and MAPK pathways, but not by PI3K pathway. Subsequent studies show that PRL induces the expression of the GST μ isoform and increases GST enzyme activity in MDA-MB-468 cells (LaPensee *et al.* 2009b). The GST μ - and θ -null genotypes are associated with increased survival in women with advanced breast cancer that were treated with chemotherapy (Ambrosone *et al.* 2001). Future studies should determine whether knockdown of specific GST isozymes abrogates the protective effects of PRL.

A model which conceptualizes the mechanism by which PRL confers resistance against cisplatin is presented in Fig. 2. After diffusing into the cell, cisplatin enters the nucleus and binds to DNA, with the ensuing cell cycle arrests leading to apoptosis. Binding of PRL to its receptor induces the activation of Jak-Stat and MAPK pathways, which separately or in concert increase the expression and activity of GST. GST conjugates cisplatin to glutathione, leading to its extrusion from the cell. Consequently, less cisplatin is available for entering the nucleus and inflicting DNA damage. The overall effect of PRL is a marked reduction in cisplatin-induced cell death. In addition to cisplatin, GST confers resistance to doxorubicin but not to the microtubule-altering drugs (L'Ecuyer *et al.* 2004). Thus, the mechanism by which PRL antagonizes drugs which are not substrates for GST may involve alterations in Bcl-2 family proteins.

Estrogens: multiple ligands and diverse receptors

E₂, estriol, and estrone are naturally occurring estrogens, which differ in affinity for the various ERs. They have dissimilar bioactivities, with E₂ being the most potent. From menarche to menopause, the ovaries

Figure 2 Proposed mechanism by which prolactin (PRL) antagonizes cisplatin-induced apoptosis in breast cancer cells. Cisplatin (Pt) diffuses into the cell and enters the nucleus, where it binds to DNA, causes cell cycle arrest, and induces apoptosis. Binding of PRL to the receptor enables its association with Jak2 and Shc, and the subsequent signaling via Stat5a/b and ERK1/2 pathways. It is through either, or both, of these pathways that PRL increases transcription of the detoxification enzyme glutathione-S-transferase (GST). Increased GST activity promotes conjugation of glutathione (GSH) to cisplatin, followed by extrusion of the conjugate from the cell via transporters. Consequently, less cisplatin is available for entry into the nucleus, resulting in decreased apoptosis.

are the primary source of estrogens. After menopause, estrogens can be generated through the conversion of androgens secreted by the adrenals and the ovaries. This process is carried out in sites such as the skin and adipose tissue by the aromatase enzyme complex (Jongen *et al.* 2006; see Fig. 1).

Breast cancer expresses several sex steroid-producing enzymes, including aromatase, 17 β -hydroxysteroid dehydrogenase, which catalyzes interconversion among estrogens, and steroid sulfatase, which hydrolyzes sulfated steroids to their bioactive forms (Suzuki *et al.* 2005). Although serum E₂ levels in postmenopausal women are only 5–10% of those before menopause, their tumors are exposed to comparable levels of active estrogens. Indeed, the tumor/plasma ratio of E₂ is >20 in breast carcinomas from postmenopausal women but only 5 in those from premenopausal women (Pasqualini *et al.* 1996). Aromatase inhibitors are effective in blocking the growth of early ER+ tumors (Nabholtz *et al.* 2009). Similar to the higher production of PRL by breast

stroma (Zinger *et al.* 2003), estrogen synthesis is higher in the stroma than in epithelial cells due to higher expression of aromatase (Santen *et al.* 1997).

As schematically illustrated in Fig. 1, endocrine disruptors that mimic or antagonize endogenous estrogens are relevant to breast cancer. Estrogen-like compounds include pesticides, industrial chemicals, pharmaceuticals, and plant-derived compounds, all of which can expose humans through food or water supply (Gray *et al.* 2009). Many are lipophilic and can be stored in adipose tissue. The most widely studied are components of plastics, e.g. bisphenol A (BPA; discussed in the next chapter), and detergents such as octyl and nonyl phenols. Chlorinated insecticides, e.g. kepone, dichloro diphenyl trichloroethane (DDT), dieldrin, and methoxychlor, also possess estrogen-like properties. Two unresolved issues are whether early exposure to endocrine disruptors increases the risk of developing breast cancer, and what is the effect of interactions between chemicals in mixtures (Birnbaum & Fenton 2003, Gray *et al.* 2009).

Diethylstilbestrol (DES) and ethinylestradiol are potent pharmaceuticals used to treat symptoms of menopause, as contraceptives and as palliative therapy in advanced prostatic cancer. Given the millions of users, home toilets are the major source of these compounds in wastewater (Falconer 2006). Although excreted into urine as inactive glucuronides or sulfates, some can be degraded in sewage treatment plants and release the active compounds.

Resveratrol, daidzein, quercetin, and genistein represent the most commonly ingested and intensely studied plant-derived phytoestrogens (Martin *et al.* 2007, Mense *et al.* 2008). They show differential binding to ER α and ER β , exert nongenomic actions, and also affect estrogen biosynthesis and metabolism. Although the general belief is that long-term consumption of phytoestrogens (i.e. soy products) helps in reducing a woman's risk of breast cancer, this notion is controversial (Martin *et al.* 2007, Gray *et al.* 2009).

Estrogens bind to multiple receptors of diverse structure that can be localized in the membrane, cytoplasm, and nucleus. ER α and ER β differ in their ligand-binding domain, underlying the dissimilar binding affinities of the various estrogenic ligands to the two receptors. ER α is expressed at low levels in the normal breast epithelium (Ricketts *et al.* 1991), but increases in *in situ* carcinomas (Karayiannakis *et al.* 1996). The expression pattern of ER β is opposite that of ER α , suggesting that loss of ER β expression indicates breast cancer development and/or progression (Shaaban *et al.* 2003). Following ligand binding, classical ERs dimerize and bind to estrogen response elements in the promoters of target genes. Recruitment of co-regulators results in the formation of complexes that mediate transcription (Nilsson *et al.* 2001). The plethora of cell-specific co-activators and co-repressors account, in part, for the partial agonist versus antagonist activities of tamoxifen in the uterus, breast, bone, and cardiovascular system.

Similar to the differential binding dynamics of the three lactogens to the PRLR (Gertler *et al.* 1996), the various estrogenic ligands can induce distinct changes in ER conformation, thereby altering co-factor recruitment and receptor stability (Bai & Gust 2009). This is exemplified by an induction of rapid ER α degradation by the pure ER antagonist ICI 162 780, but not by E₂ or tamoxifen (Van Den Bemd *et al.* 1999). The ERs also regulate transcription via protein–protein interactions with transcription factors such as the Fos–Jun complex (Normanno *et al.* 2005).

Estrogens can rapidly activate the MAPK and PI3K/Akt signaling pathways, traditionally associated with membrane receptors (Bjornstrom & Sjoberg

2005), but the nature of the receptor(s) involved is controversial. In neurons, pituitary and endothelial cells, G-proteins, ion channels, cytoplasmic protein kinases, and adaptor proteins have been implicated (Manavathi & Kumar 2006, Fox *et al.* 2009). In breast cancer cells, one model stipulates that a subpopulation of ERs is localized to the cell membrane. Steroid receptors do not have transmembrane or kinase domains and thus are unlikely to be incorporated to the cell membrane as integral proteins. Instead, they may interact via palmitoylation of membrane-associated proteins such as caveolin, striatin, and Sch (Song *et al.* 2006). Both IGF1 and EGF receptors are involved in tethering ER α to the membrane and in initiating MAPK and PI3K activation. Although the above model provides a plausible explanation for nongenomic actions of E₂, it does not explain the rapid actions of some xenoestrogens, which have much lower affinities to ER α and ER β , and yet are active at subnanomolar doses (Watson *et al.* 2007).

GPR30, a 7-transmembrane domain receptor that signals through trimeric G-proteins, represents a different model by virtue of its direct binding to estrogens (Filardo & Thomas 2005, Prossnitz *et al.* 2008). Its actions have mostly been studied in SKBr3 breast cancer cells, which express GPR30 but not classical ERs (Filardo *et al.* 2000). Estrogen signaling can be restored in the ER-negative MDA-MB-231 cells by transfection with GPR30. Binding of E₂ to GPR30 stimulates the cAMP pathways through G α s, and Src through G $\beta\gamma$. Subsequently, heparan-bound EGF is released, activates the EGF receptor and its downstream signaling that include MAPK, PI3K, and phospholipase C (PLC) (Filardo & Thomas 2005). Both tamoxifen and ICI act as agonists, rather than antagonists, of GPR30. Expression of GPR30 is higher in invasive carcinoma and is associated with larger tumor size, suggesting that it may be a predictor of aggressive disease (Filardo *et al.* 2006). The relatively high binding affinity of GPR30 to E₂ (K_d of 3 nM) makes this receptor a likely mediator of estrogen actions in ER-negative breast cancer cells (Thomas *et al.* 2005), but its relative role in cells that also express ER α and ER β is unclear.

Chemoresistance by estrogens

In spite of the abundance of man-made or plant-derived estrogen mimetics which can impact on breast cancer, little is known about their potential interactions with anticancer drugs. In addition, only few studies have examined the role of endogenous estrogens in chemoresistance. This oversight is enigmatic because

stimulation of tumor growth by estrogens involves not only increased cell proliferation but also reduced cell death. This is exemplified by the activation of both the PI3K/Akt survival pathway and Bcl-2 antiapoptotic proteins in breast cancer by estrogens (Huang *et al.* 1997, Rodrik *et al.* 2005). Perhaps research on anticytotoxic effects of estrogens has been hampered by an adherence to the classification of breast cancer cells into those that express classical ERs (estrogen-responsive) and those that do not (estrogen-unresponsive), leading many researchers to ignore cells that do not express ER α or ER β .

As a follow-up in our studies on antagonism of cisplatin by PRL (LaPensee *et al.* 2009b), we ask whether E₂ acts similarly and if so, by what mechanism. Low doses of E₂ (0.01–10 nM) abrogate cisplatin toxicity in T47D and MDA-MBA-468 cells by increasing cell proliferation and decreasing apoptosis (LaPensee *et al.* 2009a). Protection by estrogen occurs in the presence of ER α and ER β antagonists, in ER α -negative MDA-MB-468 cells, and in T47D cells with ER β knockdown, indicating independence of classical ERs. Since both cell types express GPR30 (LaPensee *et al.* 2009c), this receptor is a plausible candidate for transducing survival signals by E₂. Future studies should determine whether GPR30 knockdown abrogates the protective effect of E₂.

Unlike PRL, E₂ does not alter entry of cisplatin into the nucleus, suggesting that its protective effects occur downstream of DNA damage (LaPensee *et al.* 2009a). Because previous reports implicated Bcl-2 in estrogen-induced chemoresistance (Teixeira *et al.* 1995, Huang *et al.* 1997), we focused on this antiapoptotic protein. Indeed, E₂ increases Bcl-2 expression in T47D cells, both in the presence and absence of cisplatin, but does not alter Bcl-xL or Bax. A Bcl-2 inhibitor partially abrogates the protection by E₂, indicating that alterations in Bcl-2 may be only part of its mechanism of actions (LaPensee *et al.* 2009a).

Other data support the concept that estrogens confer chemoresistance. For example, MCF7 cells depleted of estrogen are twice as sensitive to doxorubicin than estrogen-treated cells (Teixeira *et al.* 1995). Estrogen depletion is accompanied by decreased Bcl-2 expression, and Bcl-2 reconstitution restores resistance to doxorubicin. Others reported that modulation of Bcl-2 levels affects cell sensitivity to taxol (Huang *et al.* 1997). Also, E₂ reduced taxol cytotoxicity in cells overexpressing ER α , with the cells sensitized to taxol by treatment with the ER α antagonist ICI (Sui *et al.* 2007). Estrogen also antagonizes taxol- and radiation-induced apoptosis by altering JNK activity (Razandi *et al.* 2000). A combination of tamoxifen and TRAIL is

more effective than each alone in inducing apoptosis in the ER α -negative MDA-MB-231 cells, and in arresting tumor growth in xenografts (Lagadec *et al.* 2008). This sensitization was associated with decreased Bcl-2 and increased Bax levels. Another mechanism by which estrogens can increase chemoresistance is by affecting drug transporters. This was revealed by estrogen-induced increase in cytoplasmic p-glycoprotein in MCF7 cells, which are resistant to doxorubicin cytotoxicity, but not in T47D cells, which are sensitive to the drug (Zampieri *et al.* 2002).

BPA, breast cancer, and chemoresistance

BPA is used in the manufacture of polycarbonate plastics and is the constituent of a wide array of consumer products, including plastic food containers, baby bottles, and the lining of metal food cans (Welshons *et al.* 2006). Migration of BPA into food or water from plastic containers is influenced by the manufacturing process, storage conditions, and heating by users (Kang *et al.* 2006, Le *et al.* 2008). Human exposure to BPA is well documented, with BPA detectable at 0.2–10 ng/ml in serum from most individuals tested (Welshons *et al.* 2006). Being lipophilic, BPA can accumulate in breast adipose tissue (Fernandez *et al.* 2007).

The mechanism by which BPA exerts its actions is enigmatic, since its binding affinity for ER α or ER β is 10 000- and 1000-fold lower than that of E₂ respectively (Kuiper *et al.* 1998). Yet, BPA at low nanomolar or subnanomolar doses often elicits activities that are similar to those of E₂ (Watson *et al.* 2005, Hugo *et al.* 2008). It has been suggested that BPA binds differentially within the ligand-binding domain of the ERs or recruits a different set of co-activators (Safe *et al.* 2002). In addition, estrogen-related receptors (ERRs) may serve as alternative receptors for transmitting BPA signals (Ariazi & Jordan 2006). Although ERRs do not bind estrogen, ERR γ binds BPA with high affinity (K_d of 5.5 nM; Okada *et al.* 2008). ERR γ is overexpressed in 75% of breast tumors compared to the normal epithelium (Ariazi *et al.* 2002). Phytoestrogens have also been identified as ERRs ligands (Ariazi & Jordan 2006).

BPA rapidly activates nongenomic signaling in many cell types. In MCF7 cells, MAPK and Akt are phosphorylated within 10 min of BPA exposure, similar to that seen with E₂ and other xenoestrogens (Li *et al.* 2006). BPA at low doses induces rapid influx of calcium in breast cancer cells (Walsh *et al.* 2005) and rat hippocampal neurons (Tanabe *et al.* 2006). In cerebellar neurons, Belcher *et al.* (2005) observed a

rapid BPA-induced activation of ERK1/2, PKA, and Src family kinases but not PI3K/Akt pathways. BPA at very low doses (0.01–1 nM) rapidly activates cAMP- and cGMP-dependent protein kinase and triggers rapid phosphorylation of CREB in human testicular seminoma cells (Bouskine *et al.* 2009). These BPA actions are neither reversed by ICI 182 780 nor reproduced by E₂ or DES, leading the authors to conclude that classical ERs are not involved.

The estrogenic activity of BPA was discovered upon noticing that BPA leaching from autoclaved plastic containers increases growth of MCF7 cells (Krishnan *et al.* 1993). In spite of its striking structural resemblance to DES, BPA exhibits a much lower binding affinity to either ER α or ER β (Ben-Jonathan & Steinmetz 1998). The low binding affinity of BPA to classical ERs explains its inability to exert a strong mitogenic activity in MCF7 cells. One study found increased cell proliferation in response to BPA only at 60 000 times higher concentrations than E₂ (Olsen *et al.* 2003). Another group confirmed that BPA at a relatively high dose (1 μ M) showed a modest stimulation of proliferation of MCF7 cells cultured in estrogen-depleted medium, while E₂ was effective at subnanomolar concentrations (Hess-Wilson *et al.* 2006).

Differential gene profiling in response to BPA has also been reported. In one study, BPA induces a different set of genes than E₂ in MCF7 cells, and about 15 genes in ER-null MCF7 cells, leading the authors to conclude that at least some of its actions are independent of ER α (Singleton *et al.* 2004). Another group added BPA to cultures of breast tissue aspirates (Dairkee *et al.* 2008). Expression profiling revealed that BPA is associated with high grade tumors and decreased patient survival. These data suggest that exposure to BPA may contribute to the establishment and/or maintenance of breast tumors. In both studies, however, the BPA doses were at the upper nanomolar to micromolar levels. A major criticism of studies using BPA at very high doses is that they do not reflect human exposure levels to this compound. Since BPA exhibits a ‘U’-shaped dose-dependent curve in MCF7 cells (Samuelsen *et al.* 2001), extrapolation from its action, or lack of action, at high doses to its presumed activity at low doses can be misleading.

Our study was the first to report that BPA at environmentally relevant concentrations confers chemoresistance (LaPensee *et al.* 2009c). Similar to the actions of PRL and E₂, BPA antagonizes multiple anticancer drugs, showing equimolar potency with E₂ in opposing cisplatin toxicity. These BPA actions do not appear to be mediated via ER α and ER β , but the

receptor involved was not identified (LaPensee *et al.* 2009c). In addition to GPR30, we found that both MDA-MB-468 and T47D cells express ERR γ . Given its high binding affinity for ERR γ , BPA may exert its chemoprotective effects via this receptor. ERR γ has been implicated in tamoxifen resistance in a cell line derived from invasive breast carcinoma (Riggins *et al.* 2008). Studies are underway with siRNA directed against ERR γ to examine its involvement in BPA-induced chemoresistance. However, we cannot rule out involvement of as yet unidentified receptor. We are also examining if BPA actions are mediated via genomic versus nongenomic mechanisms.

BPA alone, or in combination with doxorubicin (LaPensee *et al.* 2009c) or cisplatin (LaPensee *et al.* 2009a) increases Bcl-2 expression. Treatment with a Bcl-2 inhibitor completely blocks the BPA-induced antagonism of cisplatin, whereas it only partially abrogates protection by E₂. This suggests that BPA and estrogen may exert protection against cytotoxicity by somewhat different mechanisms, i.e. antiapoptosis versus mitogenesis. This notion is supported by flow cytometry and BrdU incorporation showing that BPA alone increases cell survival, while estrogen alone increased cell proliferation. Figure 3 schematically illustrates the role of Bcl-2 antiapoptotic protein in mediating chemoresistance by BPA. Note that the mechanism by which BPA exerts chemoresistance against cisplatin differs from that caused by PRL (see Fig. 2).

Summary and perspectives

Hormones have long been implicated in the pathogenesis of breast cancer, but only a few studies have addressed their role in chemoresistance. Mounting evidence indicates that low doses of PRL, E₂, and BPA antagonize multiple anticancer drugs that induce cell death by different mechanisms. PRL opposes cisplatin by increasing GST activity, while E₂ and BPA act by increasing Bcl-2 expression. This serves as an excellent example of why targeting one mechanism of resistance may not be sufficient for slowing down tumor growth or eliminating metastases. Future studies should examine in more detail the potential crosstalk between PRL and E₂ in conferring resistance, and expand *in vitro* studies to pre-clinical models. FDA-approved inhibitors of PRL or E₂, e.g. bromocriptine and tamoxifen, should provide for an easy transition from animal models to clinical trials. As for PRL, blockade of the receptor should be more effective than attempting to reduce the hormone itself.

Figure 3 Proposed mechanism by which bisphenol A (BPA) antagonizes cisplatin-induced apoptosis in breast cancer cells. Cisplatin (Pt) diffuses into the cell and enters the nucleus, where it binds to DNA. The ensuing cell cycle arrest leads to the release of mitochondrial cytochrome *C* (Cyt *C*), activation of caspases and apoptosis. BPA binds either to a membrane receptor or diffuses into the cell and binds to a cytoplasmic/nuclear receptor. This results in increased expression of the antiapoptotic protein Bcl-2, which blocks Cyt *C* release and apoptosis. Estradiol (E_2) also antagonizes cisplatin-induced apoptosis by activating similar or different mechanisms. Neither the identity of the receptor(s), which mediate the anticytotoxic actions of BPA/ E_2 , nor the pathway(s) underlying Bcl-2 activation is known.

A reduction in the ability of PRL and estrogens to confer chemoresistance should have several benefits to breast cancer patients, including an increase in the number as well as efficacy of valuable drugs. For example, drugs such as cisplatin, which has shown success in treating many other types of cancers, could be introduced into breast cancer regimens, while the efficacy of already successful anticancer drugs such as taxol could increase. Many treatment regimens use drugs that act by different mechanisms to improve the chances of suppressing tumor growth. Hence, having more options for combination therapy should especially benefit those patients who undergo second- or third-line anticancer treatment. Furthermore, increased efficacy should enable the use of lower drug doses, thereby reducing the toxicity and side effects associated with high dose therapy and improving the quality of life. Finally, because the actions of E_2 , BPA, and possibly other endocrine disruptors may

be independent of $ER\alpha$ and $ER\beta$, patients with ER-negative tumors could benefit from the blockade of E_2 and estrogen-like compounds.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by NIH grants ES012212 and CA096613, Center for Environmental Genetics P30 ES06096, Department of Defense grant BC05725, Susan G Komen Breast Cancer Foundation grant BCRT87406 (to N B-J), and NIH training grant 5T32ES007250 (to E W L).

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