Published in final edited form as:

Steroids. 2019 February ; 142: 84–93. doi:10.1016/j.steroids.2018.06.014.

Xenoestrogen interference with nongenomic signaling actions of physiological estrogens in endocrine cancer cells

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Abstract

Rapid nongenomic signaling by estrogens (Es), initiated near the cell membrane, provides new explanations for the potent actions of environmental chemicals that imperfectly mimic physiological Es. These pathways can affect tumor growth, stabilization, or shrinkage via a number of signaling streams such as activation/inactivation of mitogen-activated protein kinases and caspases, generation of second messengers, and phospho-triggering of cyclin instability. Though prostate cancers are better known for their responsiveness to androgen deprivation, ~17% of late stage tumors regress in response to high dose natural or pharmaceutical Es; however, the mechanisms at the cellular level are not understood. More accurate recent measurements show that estradiol (E₂) levels decline in aging men, leading to the hypothesis that maintaining young male levels of E₂ may prevent the growth of prostate cancers. Major contributions to reducing prostate cancer cell numbers included low E₂ concentrations producing sustained ERK phospho-activation correlated with generation of reactive oxygen species causing cancer cell death, and phospho-activation of cyclin D1 triggering its rapid degradation by interrupting cell cycle progression. These therapeutic actions were stronger in early stage tumor cells (with higher membrane estrogen receptor levels), and E₂ was far more effective compared to diethylstilbestrol (the most frequently prescribed E treatment). Xenoestrogens (XEs) exacerbated the growth of prostate cancer cells, and as we know from previous studies in pituitary cancer cells, can interfere with the nongenomic signaling actions of endogenous Es. Therefore, nongenomic actions of physiological levels of E₂ may be important deterrents to the growth of prostate cancers, which could be undermined by the actions of XEs.

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Keywords

ERK activation; JNK activation; membrane estrogen receptors; signaling; cyclin D1; ROS

Introduction

Estrogens (Es) of all types (physiological, pharmaceutical, dietary, environmental contaminant) operate via multiple receptors, in multiple cellular locations, and via diverse cellular signaling pathways [1–3]. Tissues vary in their expression of estrogen receptor (ER) subtypes (α , β , or GPR30) residing in different cellular compartments, where they are associated with alternative types of machinery to operate different signaling mechanisms. We need to identify these players and pathways for each functional consequence. In addition, some Es have differential binding affinities for receptor subtypes, a characteristic which might be used to either apply them as treatments or avoid harmful consequences from exposure.

The functional actions of steroids on the growth of endocrine cancers is well known and the basis of many of our current therapies. The incidence of prostate cancer has steadily risen with the current likelihood being one in seven men will be diagnosed with prostate cancer in his lifetime [4]. Though androgens are well known for driving cell proliferation in many tumors that arise from male reproductive tissues, later-stage prostate tumors become much less responsive to androgen deprivation of various types [5]. However, some late-stage tumors (~17%) regress in response to a high doses of the pharmaceutical E, diethylstilbestrol (DES). The mechanistic basis for this is unclear [6] and could be selectively exploited if we knew more about the signaling pathways and ER subtypes involved in this tumor cell death/growth-stopping response, including those mediators employed in non-classical nongenomic (other than the classical transcription regulation) pathways which are currently not considered.

Little attention has been given to the physiological Es in adult men or the nongenomic pathways of estrogenic signaling through which such low levels of male Es may operate. We must also evaluate the actions of xenoestrogens (XEs) on the background of already present physiological concentrations of Es to see how they may interfere. Men normally produce from 60–200 pM of estradiol (E_2), which is ~20 times lower compared to the E_2 concentration in women [7–13]. Though E blood levels over the lifetime of men have been debated, much of the controversy stems from the methods used in measurements. Recent techniques (ultrasensitive immunoassays, chromatography/mass spectrometry) have improved the accuracy of measuring low hormonal levels, and the majority of such studies now report that men's E levels decline with age [9, 10, 12–14]. In addition, many investigators have in the past reported E_2 /testosterone ratios, rather than the absolute levels of each hormone, and because T declines more as men age, the ratio of E to T rises. However, it must be recognized that Es and T do not merely oppose each other, but have entirely separate mechanistic and functional outcomes, so each class of hormones should be considered for actions on their own. The declining E_2 levels in aging men are correlated with an increased death rate most closely associated with age-dependent cancers [6, 15] like

prostate cancer. Men with the highest E₂ levels have the lowest rates of prostate cancer [8], These findings suggest that physiological Es at youthful concentrations may limit the growth of prostate cancers [8, 12, 16–18], We are now trying to elucidate the mechanisms that may be involved so we can better prevent loss of protection by physiological Es and potential harm from interfering XEs (including replacement of XE compounds currently in consumer products) [19],

A common explanation for late-stage androgen-resistant prostate tumors responding to DES has been feedback inhibition on endogenous androgen synthesis at the level of the hypothalamic-pituitary-testis axis. However, Es also alter the growth of human prostate cancer cells in culture [20] (and data shown here) so mechanisms acting directly at the level of the cancer cell must also be considered. Others attribute the growth inhibitory actions of Es to their binding to mutant androgen receptors, yet this can only explain the growth of a small fraction of tumors. Prostate cancer cell lines to examine actions of Es through wild type ERs (and not mutant ARs) are readily available and have been chosen for our studies.

Our studies focus on the tumor progression-promoting effects of Es and XEs on already established cancers, rather than the more commonly discussed carcinogenic effects leading to the establishment of new cancers, including the perinatal exposure origins of adult disease [21–24], The tumor-promoting effects are highly relevant considerations as most men develop prostate cancer which slowly develops throughout their adult lives (as shown by autopsies on men dying of other causes) [25], Therefore, controlling the growth rate of these cancers would have a large impact on the development of a debilitating stage of the disease.

Key to understanding the mechanisms by which typical male physiological E₂ levels may keep prostate cancer cell growth in check is understanding via which estrogen receptor (ER) subsets these actions may be occurring. We know from our previous work that there are different patterns of signaling responses to specific Es/XEs and in particular tissues [26–34] that can host different types or ratios of ERs. Therapeutic or anti-therapeutic tumor responses to specific Es may act via specific ER subtypes, in different subcellular locations, and via alternative signaling pathways. For instance, several phytoestrogens thought to be therapeutic or to prevent cancers [35–39] preferentially bind to ERβ [40], which in some tissues opposes the actions of ERα [41], Other Es (like E₂ and DES) can bind relatively equally to all three subtypes of ERs but may mediate different effects through each subtype, in a tissue-dependent manner. Determining relative expression levels of ER subtypes in different tumor cell subcellular locations could allow selection of patients most likely to respond favorably to a given therapeutic E or be the basis of advice to patients about which XE contaminants to avoid to limit interference with their physiological Es' actions.

Because we often study multiple compounds (there are many different XEs) and their mixtures across wide time and concentration ranges, we developed sensitive, quantitative, relatively high-throughput assays to do comparative studies we believe are necessary to answer some of these questions. We have used these sensitive and efficient multi-well plate assays [34, 42–44], recently automated [26], to document the distinctive non-monotonic dose characteristics and interference patterns by XEs [42], We recently demonstrated that physiological Es and XEs and their metabolites act very rapidly and potently via previously

underexplored nongenomic signaling pathways [3, 28, 42, 45, 46], revealing formerly unappreciated differences in response patterns. Our designs that accommodate large sample numbers have made possible parallel analysis of chemical structure variants [28], metabolic modifications [26], and mixtures of XEs [3, 42, 47] in pituitary, breast, prostate, immune, and neuronal cells [28, 29, 31, 33, 34, 42, 43, 46, 48, 49]. These studies inform us about the tissue specificity of these mechanisms that control tumor growth, which led to new considerations for the prostate: the differences between exposure dangers for men vs. women; the possibility of therapeutic physiological E replacement for susceptible people such as older men; or the increased exposures for those living and/or working in contaminated environments (near product manufacturing or waste materials).

Nongenomic steroid signaling from tumor cell membranes has been shown to change the course of such important functions as proliferation, death, differentiation, and migration [50–52]. It is, therefore, important to consider these ERs localized to the plasma membrane of cancer cells including this newly described membrane location in prostate cancer cells. Such receptor measurements can be used to see if their presence, level, and mechanisms of actions can be used in classifying tumors, predicting therapeutic responses to Es and their analogs, and any possible interference by XEs. Prostate tumors are known to respond to Es, but the nongenomic signaling mechanisms by which they do so is unexplored. Fig. 1 summarizes the upstream signaling paths that converge on cell survival, proliferation, or death mechanisms. Mitogen-activated protein kinases (MAPKs) such as extracellular-regulated kinase (ERK), Jun N-terminal kinase (JNK), and p38 are attractive candidates, as they are pivotal nodes for mechanisms directly involved in cancer therapeutic responses. Mechanisms that affect the cell cycle, as well as those that control cell death (apoptosis, ROS-induced, necroptosis), also participate in the balance of actions that culminate in viable tumor cell numbers that impact a tumor's ability to grow, stabilize, or shrink. This proceedings review will summarize the concepts addressed in our work that show that XEs act (singly, or more dramatically in mixtures) via nongenomic signaling pathways in rapid, potent, imperfect, and non-monotonic patterns that can severely interfere with physiological E actions.

Experimental

Cell Lines:

We plated cells in serum-containing media at relatively low density (5,000 cells/well) in 96-well plates (from Corning; to avoid leaching of estrogenic plastic monomers into the cultures). We weaned cells from serum hormones replacing media serum with that which had been extensively charcoal-stripped (4 times) to avoid variable levels of serum-derived Es masking the responses, especially to low concentrations of test compounds. Responses of different cell lines represent prevalent prostate tumor cell types.

Cell Viability:

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay; 590 nm] is standard for measuring viable cells via their metabolic activity [53] and especially in cells that tend to cling to the plate after dying like we have noted for prostate cancer cell lines. We

examined wide concentration ranges in log increments and compared responses to vehicle (ethanol) controls and 100 pM E₂.

Plate immunoassays:

These assays were developed [44] to examine many differences between compounds in parallel, while accounting for non-monotonic dose patterns and rapidly oscillating responses. Plate immunoassays give results comparable to immunoblot assays, but with far less error [43]. The basis for all of our assays of phospho-proteins is recognition with antibodies (Abs) specific for these post-translationally modified epitopes: pERK1/2 (Thr202/Tyr204), pJNK (Thr183/Tyr185), p-p38 (Thr180/Tyr182), p-cyclin D1 (Thr286), total cyclin D1 (all from Cell Signaling), or p-p16^{INK4A} (Ser152; from Thermo Scientific). The ER- specific Abs used in this study were Santa Cruz MC-20 (for α), Sigma E1276 (for β), and Novus NLS4271 (for GPR30). Control samples omitted primary Ab. Our assay converts Ab-detected proteins (receptors, enzymes, regulatory proteins) into an alkaline phosphatase-generated yellow paranitrophenol (A₄₀₅) signal. We recently adapted this plate immunoassay [43, 44] for use with the BIOMEK FXP (Beckman Coulter) work station [26] to automate most of the liquid-handling steps, reduce experimental variability, and increase experimental throughput. We have used this assay in various cell types to examine similar actions to different physiological Es [46], various XEs [2, 3, 28, 34, 42, 43], and phytoestrogens [30, 45]. We recently wrote a book chapter on this assay and all of its variations and uses [44]. With appropriate optimization, this signal is linearly proportional to the levels of the recognized antigen. This assay is somewhat similar to the later commercial one called “in cell Westerns,” although our version, invented separately, requires individual optimizations (not possible with the commercial assay) for cell and antigen types to achieve its superior efficiency and sensitivity.

Caspase 3 Assays:

These studies (referred to here, but data not shown), were done using standard protocols from our published work in a variety of cell types [45, 47, 54–56] recently adapted to prostate cancer cells. Cells seeded into black optical 96-well plates (5,000 cells/well) were allowed to attach overnight. E treatments began the next day in media containing 1 % charcoal-stripped FBS for times ranging from 2–24 hr. After treatment, plates were centrifuged at 300g for 5 min, treatments aspirated, and the cells lysed. The cellular enzyme-catalyzed release of 7-Amino-4-trifluoromethylcoumarin was monitored using a FlexStation 3 microplate reader (excitation 400 nm/emission 505 nm).

ROS Assays:

Cells plated at 10,000 cells/well in 96-well plates were allowed to attach overnight then treated with charcoal-stripped FBS-containing medium for 48 hr to withdraw them from serum hormones. Cells were loaded with 2',7'-dichlorodihydrofluorescein diacetate (DCDHF, Enzo Life Sciences) for 1 hr before 15 min E treatments, with ROS production monitored. E concentrations spanned 10⁻¹⁶ to 10⁻⁶ M. Hydrogen peroxide (H₂O₂) and vehicle levels of ethanol were used as positive and negative controls, respectively. Dichlorofluorescein, formed as a result of ROS/DCDHF interaction, was measured at excitation_{485 nm}/emission_{538 nm} in a microplate reader.

Statistics:

Each plate assay hosts 6 samples per condition, and always employ 3 separate experiments. Other assays are also multi-well or multi-sample efficient assays, and use the same statistical analyses. One-way analysis of variance followed by a Holm-Sidak post hoc test was used to determine significant changes ($p < 0.05$) for all studies using SigmaPlot software.

Results

We first examined E_2 -induced changes in prostate cancer cell numbers compared to the reduction that could be achieved with DES, the pharmaceutical typically used in past treatments. For two prostate cancer cell lines (early-stage LAPC-4 vs. the PC-3 late-stage lines) we compared E_2 or DES hormone treatments for 3 days (Fig. 2) under conditions optimized to detect hormonal responses in each cell line, according to published conditions for monitoring responses to androgens [57–59] which require the same strategies. Comparisons were made to the ethanol vehicle in which the test Es were dissolved. Both of these Es reduced prostate cancer cell numbers but with some unexpected characteristics. Lower doses of both Es were as effective as higher doses in LAPC-4 (androgen-dependent, early-stage) cells, a stage of prostate cancer rarely treated with Es. E_2 was more effective than DES in both cell lines. Previously, the most common E treatment for late-stage unresponsive prostate cancer was very high levels of DES (resulting in $> \mu\text{M}$ blood levels), causing extensive off-target side effects. Our data show growth-thwarting effects of low doses of E_2 , consistent with those measured in young males, which would be a much better tolerated therapeutic choice than DES.

Next, we determined the levels of the differentially localized ER subtypes in these two cell lines to see which might be present and responsible for mediating nongenomic vs. genomic estrogenic responses. Fig. 3 shows receptor levels measured by our plate immunoassay. Membrane forms are measured by carefully controlling the fixation conditions so that the cell membranes are not permeabilized. Intracellular forms are measured by permeabilizing cells with detergent. The predominance of mER β in these cells corroborates findings in prostate tissue [40]. Others have previously described membrane ER β (mER β) on cell surfaces [60, 61] as well as membrane ER α (mER α) [29, 48, 61]. Note that late-stage cells have much lower levels of all ERs in both locations than early stage cells (see different vertical axis scales). Also note that GPR30, which has a disputed location (plasma membrane vs. intracellular), is shown in both locations by our analysis.

We next examined the phospho-ERK (pERK) response in prostate cancer cells (Fig. 4), as we have previously examined in multiple cell types [26, 28, 30, 34, 42, 43, 45, 47, 54, 62, 63]. We compared ERK responses to E_2 (100 pM) vs. DES (1 μM) in the same early-stage vs. late-stage prostate cancer cell lines. The choice of which E concentrations to use in these comparisons were based on the following: 1) The chosen E_2 concentration was effective at decreasing cell numbers (see Fig. 2); 2) the chosen E_2 concentration is a physiological level relevant to those of young men so, if used therapeutically, should have minimal, if any, unwanted side effects; and 3) the chosen DES concentration was also an effective dose in our cell viability studies and has been often used, sometimes successfully, to treat advanced prostate cancer patients. We assessed pERK over a time course that usually shows a rapid

and oscillating response in other cell types [20]. Because ERK activation is classically associated with cell proliferation, we expected concentrations of Es that decreased cell numbers to inactivate ERK. Instead, Entreated LAPC-4 cells showed a rapid and significant increase in pERK levels at 5 min, maximal at 15 min, falling to control levels, and rising again at 60 min in a typical [26, 28, 34, 42, 43, 45–47, 54] oscillating ERK activation pattern. A muted oscillation and partially negative response was seen in PC-3 cells, but again finally ending in a sustained activation at 60 min. In both cell lines, DES failed to show robust ERK activation either early or at 60 min.

Because these pERK changes did not explain why treatments decreased cell numbers (Fig. 2) when only the traditional role of ERK in promoting cell proliferation was considered, we sought other explanations. We read about a novel pathway [64] where sustained ERK activity (such as at 60 min) actually participates in a feed forward reactive oxygen species (ROS)-generating mechanism leading to cell death. This may be one of the mechanisms by which Es limit their actions to prevent over-stimulation (hormesis) [65] of cell proliferation. In our studies (right panels of Fig. 4), both LAPC-4 and PC-3 cells treated with E₂ and exhibiting persistent ERK responses had elevated ROS levels at all hormone concentrations tested, while DES was unable to elicit this response in either cell line.

Fig. 5 shows that rapid phosphorylation of cyclin D1 (insets of each panel) results in rapid onset and sustained total cyclin D1 degradation. This action happens in both early and late stage cells treated with 100 pM E₂, but only in early stage cells treated with 1 μM DES. This reduction of cyclin D1 has long been known as a very effective target for halting or slowing progression of the cell cycle and consequently tumor growth. Once again, DES is of no benefit in the cell type most likely to receive this treatment in the past, late stage prostate cancer.

To determine if an XE could alter the numbers of prostate cancer cells via some of these mechanisms, we examined the actions of bisphenol A (BPA, a polycarbonate plastic derived XE). Fig. 6 shows that BPA at a wide range of concentrations promotes an increase in tumor cell number. BPA has previously been shown to be estrogenic in many studies [66]. In contrast to the response seen with E₂ and DES (Fig. 2), a growth-promoting, instead of a growth-inhibiting response, was observed in early stage (LAPC-4 cells). Clearly BPA is not behaving like the physiological or sometimes therapeutic pharmaceutical Es. By causing more rapid growth of an early-stage form of prostate cancer, BPA and compounds like it might promote a faster transition to large less differentiated tumors, which are less responsive to traditional endocrine therapies as well as to Es.

In table I, we summarize some of the multiple mechanisms we have examined in our published studies that contribute to prostate cell number control by Es [55, 56]. In general, the more mechanisms that can cause a decrease in cell number that are engaged, the more dramatic is the potential therapeutic effect on tumors. Though some other pathways not listed here were activated by estrogens (phospho-p38), they were equivalently activated in all cell types and with both E₂ and DES, and therefore were judged at best permissive, and not the basis for these differential cell killing effects. Other mechanisms like necroptosis were not activated by Es and therefore not contributory to a therapeutic response. Mechanisms

that were the most consistently contributory to cell number decline shown here, were focused on for subsequent studies testing other estrogenic compounds [56] including XEs. As an example, treatment with BPA did not evoke the prominent mechanisms causing cell death or the slowing of cell proliferation, thus perhaps explaining the growth response to BPA. Overall, this summary predicts that E₂ treatment should be the most effective at reducing tumor cell numbers, that DES would be somewhat less effective and especially ineffective in late stage tumors, and that BPA exposure should be avoided because it enhances cell numbers, perhaps by avoiding effects on the cyclin D1 levels (the dominant mechanism).

Another important question is how endocrine cancer cells that have mERs will respond to combinations of Es to affect signaling that can change cell numbers. Because these studies have not yet been performed in any prostate cancer cells, we turn to pituitary tumor cells where we have previously examined these questions in detail. Here we show a representative example of how challenging an endogenous E with XEs [3, 28, 42, 45] affects ERK activations (Fig. 7). Shown is E₂ challenged with different concentrations of alkyl phenols (structurally related XEs) with: a short side-chain (ethylphenol, EP) vs. a long side-chain (nonylphenol, NP) vs. the phenol ring-substituted for the side chain (BPA) [42]. A 5-min response time was chosen as an unequivocally nongenomic effect on pERK and a common early activation time point for all MAPKs by nearly all Es, both physiological [67] and synthetic [19, 28, 47, 68]. These data illustrate several important principles of ERK activation and interference by XEs: 1) Es that had previously been labeled weak when acting via genomic pathways were really quite potent via these nongenomic signaling pathways, providing a plausible cell-based mechanism for their actions in humans and animals. 2) A striking congruence in these data was that the more estrogenic individual XEs were alone, the more severely they inhibited the actions of a paired physiological E. At higher concentrations (10⁻¹¹ to 10⁻⁸ M), NP by itself (top panel) activates ERK above the vehicle control (though not as effectively as 1nM E₂), while these concentrations also inhibit E₂-induced activity (back to baseline - V). In contrast, EP (middle panel) is as estrogenic as E₂ at higher concentrations (10⁻¹¹M) where it also progressively inhibits ERK phosphorylation by E₂, even to levels far below those in the vehicle- treated controls. Finally, as further indication of an XE's estrogenic strength at a given concentration predicting its disruptive power, BPA (bottom panel) displays a complex, non-monotonic concentration dependence. Note that both very low (10⁻¹⁴ to 10⁻¹³M) and higher (10⁻⁹M) BPA concentration ranges are quite estrogenic, though concentrations in between are not. The most estrogenic concentration ranges are also those that most severely disrupt ERK activation by E₂. Similar examples were seen with XEs opposing other physiological Es [42], as well as in a different test system – rapid effects on dopamine transport in PC12 pheochromocytoma cells [29]. Overall, XEs at concentrations having little or no estrogenic activity do not disrupt physiological E responses, and can even enhance them, while at levels that cause strong responses by themselves, XEs are very inhibitory to the actions of physiological Es. All of these situations disrupt the normal actions of endogenous Es and give multiple examples of the nonmonotonic concentration dependence of XE effects.

Taking this analysis one step further (Fig. 8), we then treated pituitary cells with XEs individually vs. in mixtures, again challenging the actions of E₂. While all Es caused ERK

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activations, with slightly differing temporal patterns, combining XEs to challenge E₂ caused severe disruption of the ERK activation to below those levels of pERK seen in vehicle controls. Others have predicted that combining two or more XEs would cause positive additivity [69], but we have consistently seen more severe disruption of E₂-induced ERK responses when more XEs are added [47]. In addition, we find that chemically modified XEs (such as polychlorinated, glucuronidated, and sulfated BPS) can still act nongenomically, and can have different effects than the parent compound [26]. This is in contrast to these compounds inability to act at nuclear ERs [70–73],

Discussion and Conclusions

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Nongenomic signaling, via which Es rapidly initiate action at or near the cell membrane [2, 46, 74–76], provide newly considered ER pathways that can effect tumor growth, stabilization, or shrinkage. This type of signaling is initiated very rapidly and is therefore incompatible with what is known about the time course of transcriptional mechanisms. These rapid actions are propagated downstream to effect cell survival and proliferation at later times (summarized for several pathways in Fig 1). In the two prostate cancer cell lines examined here representing early- vs. late-stage tumors [55], signaling events that were the most predictive of cell proliferation/death were the generation of ROS via sustained ERK activation [64], JNK-driven apoptosis [77], and cyclin D1 phosphorylation followed by rapid degradation that halts cell proliferation [78]. Other tested mechanisms such as phosphorylation of p16 and p38 were less robustly responsible for differences between cell types (early- vs. late-stage), while others were not activated (necroptosis [79]) and were therefore not considered to be predictive of tumor-shrinking endpoints.

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XEs appear to interfere with tumor-shrinking endogenous E mechanisms, and promote prostate cancer cell growth by employing nongenomic signaling pathways via mERs. Such findings could lay the basis for fundamental changes in clinical advice about XE exposures [80, 81]. Note that the E and XE concentrations that we tested are quite low compared to other studies and very physiologically and environmentally relevant [82]; as assay sensitivities for sex steroids and XEs have increased recently, low levels have become more accurately measured [9, 10, 12–14]. Also, our detailed and wide-range concentration response analyses allow us to appreciate their non-monotonic patterns of response, where many times the largest responses occur at low concentrations while higher concentrations blunt the response. These considerations have important implications for both safety assessments for XEs, and for the understanding of basic hormonal mechanisms in males vs. females, and in different tissues. The time-oscillations of these responses indicate that it would be easy to miss such a rapid nongenomic response if not examined in early temporal detail. We also saw the unexpected dramatic inhibition of responses when XEs challenge endogenous Es, and especially in mixtures. These details have in large part not previously been examined. In our case this was made possible by use of a medium through-put live cell assay assisted by a liquid handling robot [44],

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The idea of therapeutic Es working via mERs originated in our observations that breast cancer cells selected for their expression of high levels of mER α die, instead of proliferate, in response to high-dose Es [63, 83]. Others showed that ER α overexpression caused by

either transfection or prolonged hormone deprivation (causing receptor upregulation) can underlie breast cancer cell killing by Es [84], Transfection-driven overexpression can cause cancer cells to express more membrane forms of ER α and ER β [61], perhaps making them more susceptible to hormone-driven membrane-initiated cell killing. We also noted that some Es can activate specific MAPKs and caspases, recognized as key signal transducers/ effectors in apoptotic cell death pathways [3, 42, 45], These nongenomic activations by Es may account for the 10–20% of breast cancers that regress with high-dose E treatments [85], a clinical finding observed decades ago but not yet adequately explained or exploited for precision medical uses. Since prostate cancers predominantly express the ER β subtype while breast cancers predominantly express the ER α subtype, different Es and their nongenomic signaling mechanisms may be effective in these different tumor types.

While XEs like BPA have been shown to exacerbate the proliferation of xenograft prostate tumors in mice [86, 87], and here in culture, the focus in previous studies was on bypassing androgen-induced actions via mutated ARs that bind Es. Our chosen cell lines did not present this complication in interpretation as they have wild type ARs, so we instead focused on the actions of Es in treating tumors without these relatively rare mutations. We additionally examined the tumor-promoting, and possibly therapy-thwarting, abilities of XEs via their interference with endogenous Es at mERs. In the past, only late-stage prostate tumors were judged candidates for E therapies after they lost much of their androgen dependence and failed with other therapies. However, our data challenge that idea; we show that early-stage prostate cancer cells have more ERs and are even more sensitive to the therapeutic effects of Es [55], Though DES was often used for therapy in the past, our data suggest that it may not be the best choice. Because E₂ is not perfectly mimicked by nonphysiological Es (like DES and BPA), it is not surprising that we found differences in their nongenomic signaling responses. Physiological Es like E₂ have rarely been considered for therapy for these tumor types, and could be the cheapest, best-tolerated, and most easily administered treatments.

While it is acknowledged that contributions to incidence and poor outcomes in prostate cancer include susceptible genetic backgrounds [88] and delayed diagnoses/less effective treatments for impoverished people, these factors cannot fully account for discrepancies in prostate disease profiles. Geographical differences in disease incidence among people with relatively similar genetic backgrounds point to contributory environmental causes [66, 89–96], XEs prevalent in our environment have been related to health risks for diseases of many tissues associated with disruptive effects on the endocrine system, including cancers [66, 97–100], and specifically to aggressive cancers of the reproductive system.

Acknowledgements:

Thanks to Bethany Baker of the UTMB Institute for Translational Studies (CTSA) for her expert editorial assistance. Over the last several years of developing these ideas and examining these questions leading to this review of our work, our studies have been supported by the NIH, DOD, Passport Foundation, and the Society of Toxicology student awards.

Abbreviations:

Ab	(antibody)
BPA	(bisphenol A)
BPF	(bisphenol F)
BPS	(bisphenol S)
E	(estrogen)
E₂	(estradiol)
EP	(ethylphenol)
ER	(estrogen receptor)
ERK	(extracellular-regulated kinase)
GPR	(G protein-coupled receptor)
JNK	(jun N-terminal kinase)
MAPK	(mitogen-activated protein kinase)
mER	(membrane ER)
NP	(nonylphenol)
OP	(octylphenol)
XE	(xenoestrogen, any non-physiological E)

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Figure 1: Major nongenomic signaling pathways that affect endocrine cancer cell survival. Nongenomic mechanisms induced by Es and XEs initiate at mERs (α , β , and GPR30), then funnel through MAPKs to multiple downstream signaling streams that lead to apoptosis, ROS increase, or phosphorylation and degradation of cell cycle proteins. Each of these mechanisms are able to contribute to the estrogenic control of cell number.

Figure 2: Cell viability of prostate cancer cells after 3 days of E2 or DES treatment.

Treated LAPC-4 (early-stage, white symbols) and PC-3 (late-stage, black symbols) prostate cancer cells were measured for cell viability by the MTT assay. Triangles represent E₂ treatments, and circles represent DES treatments. *=significance at $p < 0.05$ vs. control vehicle (V)-treated cells. Shaded horizontal bar represents the response range to V. N=24 for each point.

Figure 3: Levels of mER subtypes in prostate cancer cells quantified by plate immunoassay. ER subtypes (α , β , and GPR30) levels are shown for total cellular vs. membrane (mERs) locations in LAPC-4 and PC-3 prostate cancer cells. The negative control samples used no primary Ab for any of the ER subtypes, as indicated by the first bar and the shaded horizontal line across the graph (average $\pm$ SEM). *=significance from controls at $p < 0.05$

Figure 4: pERK and ROS levels after treatment with E2 and DES in early-stage LAPC-4 vs. late-stage PC-3 prostate cancer cells.

Treatments were 100 pM E₂ or 1 μM DES as labeled. Left panels: pERK was measured for up to 60 min (A-D) via the plate immunoassay. Right panels: ROS levels were measured after 15 min of each estrogen treatment (E-H). White symbols denote LAPC-4 cells, black PC-3 cells; triangles represent E₂, and circles DES treatments. Star symbols in ROS panels represent response to 1mM H₂O₂, a positive control for ROS generation. Squares in right panels E and G show the effects of the selective MEK inhibitor U0126 indicating involvement of the ERK pathway. * denotes significance from vehicle (V, shown as 0 time and by shaded gray horizontal bars) ± SEM at p<0.05.

Figure 5. Total and p-cyclin D1 levels after treatment of prostate cancer cells with 100 pM E_2 or 1 μ M DES.

Early-stage LAPC-4 and late-stage PC-3 cells were treated with E_2 or DES and total or phosphorylated (in insets) cyclin D1 levels were measured via the plate immunoassay in a time course up to 16 hr. Phospho (p)-cyclin D1 levels (insets) were measured up to 60 min. White symbols denote LAPC-4 cells and black symbols PC-3 cells. Triangles represent E_2 and circles DES treatments. * denotes significance from vehicle (V, shown as 0 time and by shaded gray horizontal bars) $\pm$ SEM at $p < 0.05$.

Figure 6: Growth of LAPC-4 prostate cancer cells after 3 days treatment with a range of BPA concentrations.

The horizontal line in the axis label shows usual range of BPA concentrations in the environment. *=significance from vehicle (V) controls at $p < 0.05$; grey horizontal graph bar is the response range to V. The white star symbol shows response to complete (not charcoal-stripped) medium.

Figure 7: ERK activation in pituitary tumor cells in response to 1 nM E2 altered by combinations with 3 different structurally related alkylphenol XEs: NP (nonylphenol), EP (ethylphenol), or bisphenol A (BPA).

A fixed-cell plate immunoassay was used to assess the effects of 5 min of treatment or co-treatment with estrogens. The lower light-shaded horizontal bars indicate the vehicle (V, ethanol) control pERK level (with error range shown as bar width). The darker crosshatched upper horizontal bars indicate the positive control pERK values in cells treated with 1nM E₂ alone. * = $p < 0.05$ compared to V-treated cells; # = $p < 0.05$ compared to cells treated with E₂ (ANOVA).

Figure 8: XE combinations suppress E₂-induced signaling in pituitary tumor cells.

While NP and BPA alone caused imperfect ERK activation patterns when compared to E₂, when mixed together with E₂, they severely suppress the E₂-induced level of pERK. $p < 0.05$ compared to vehicle (V)-treated cells (grey horizontal bar shows the range of the response to V); *=significance from V controls at $p < 0.05$; #= $p < 0.05$ compared to cells treated with E₂ (ANOVA).

Table I:
Summary of most influential mechanisms contributing to the final number of LAPC-4 or PC-3 prostate cancer cells.

E₂, DES, and BPA were tested for their ability to activate different pathways in LAPC-4 and PC-3 cells, which can lead to a decline in cell numbers (in red text) via apoptosis (increased pJNK), ROS increase, or phosphorylation of cell cycle proteins. Items in green text are mechanisms that would contribute to an increase in cell number (increased pERK, decreased pJNK, or decreased total cyclin D1). Other mechanisms were tested, but not found to be primarily responsible for cell number changes (see text).

	E ₂	DES	BPA
LAPC-4	↓↓ Cell Viability ↑↑ pERK→↑ROS ↓ pJNK ↑ p-cyclin D1→↓ Total cyclin D1	↓ Cell Viability ↓ pERK → ↔ROS ↓ pJNK ↑ p-cyclin D1 →↓Total cyclin D1	↑ Cell Viability ↑pERK→↑ ROS ↔ p-cyclin →↔Total cyclin D1
PC3	↓ Cell Viability ↑↑ pERK→↑ ROS ↑ pJNK ↑ p-cyclin D1 → ↓ Total cyclin D1	↔ Cell Viability ↓ pERK→ ↔ROS ↓ pJNK ↓ p-cyclin D1 → ↔ Total cyclin D1	