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Abilities of β-Estradiol to interact with chemotherapeutic drugs, signal transduction inhibitors and nutraceuticals and alter the proliferation of pancreatic cancer cells

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ABSTRACT

Improving the effects of chemotherapy and reducing the side effects are important goals in cancer research. Various approaches have been examined to enhance the effectiveness of chemotherapy. For example, signal transduction inhibitors or hormonal based approaches have been included with chemo- or radio-therapy. MIA-PaCa-2 and BxPC-3 pancreatic ductal adenocarcinoma (PDAC) cells both express the estrogen receptor (ER). The effects of β-estradiol on the growth of PDAC cells has not been examined yet the ER is expressed in PDAC cells. We have examined the effects of combining β-estradiol with chemotherapeutic drugs, signal transcription inhibitors, natural products and nutraceuticals on PDAC. In most cases, inclusion of β-estradiol with chemotherapeutic drugs increased chemosensitivity. These results indicate some approaches

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involving β-estradiol which may be used to increase the effectiveness of chemotherapeutic and other drugs on the growth of PDAC.

1. Introduction

Multiple key genetic and biological factors are implicated in pancreatic and other cancers (Davis et al., 2014; Steelman et al., 2016; Chappell et al., 2018; Abrams et al., 2017, 2018, 2019; Candido et al., 2018; Chappell et al., 2018 McCubrey et al., 2017, 2018). In some cases, various mutations will confer either therapeutic sensitivity or resistance to the cancer as the proliferation may be dependent on or inhibited by the mutated gene or signaling pathway. Although various genetics and environmental factors have been identified in various cancers, additional approaches are required to improve therapy. Some treatments may involve combining commonly used chemotherapeutic drug, signal transduction inhibitors, natural products and nutraceuticals with common agents implicated in cancer growth or the induction of autophagy. An overview of where β-estradiol, chemotherapeutic drug, signal transduction inhibitors, natural products and nutraceuticals exert their effects is presented in Fig. 1.

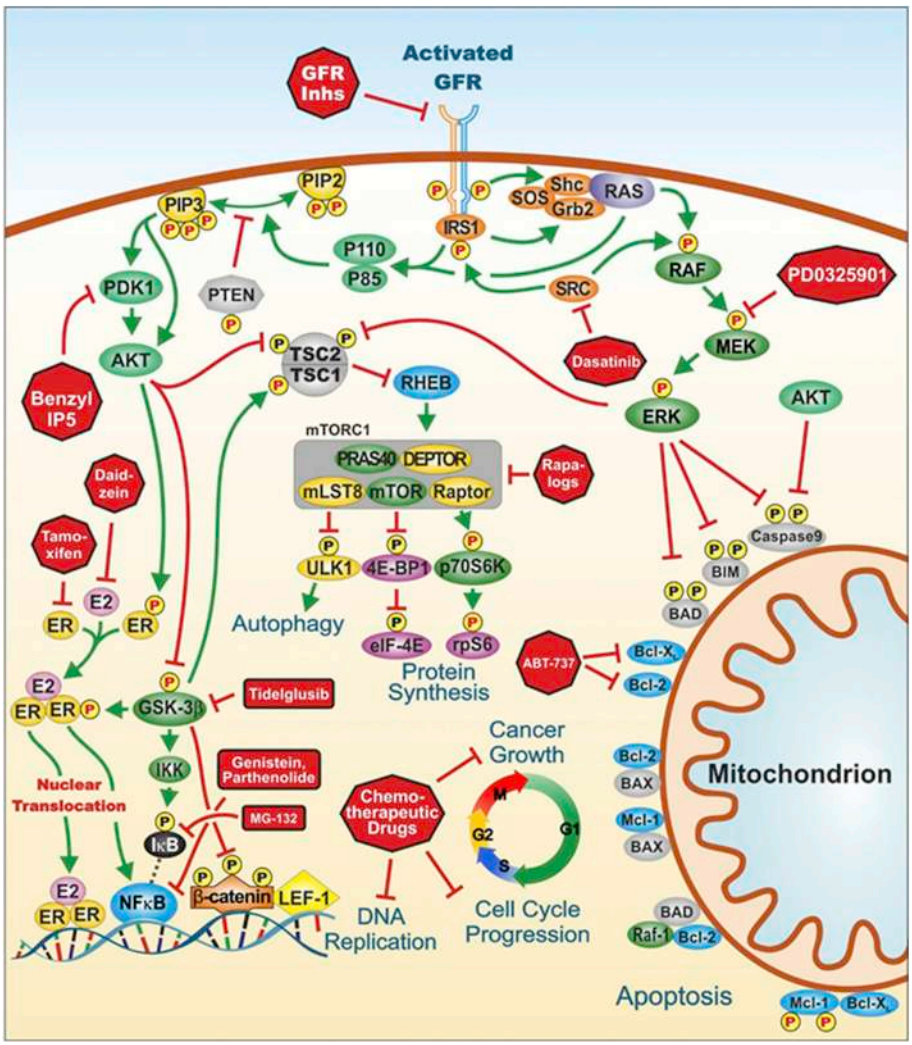

Fig. 1. Potential Interactions Between β-estradiol Signaling, Chemotherapeutic Drugs, Signal Transduction Inhibitors, Natural Products and Nutraceuticals. Sites where various inhibitors may act on signaling pathways are indicated in red octagons. This figure is meant to provide a broad overview of β-estradiol (E2) and EGFR signal pathway and indicate potential sites of cross talk and interaction by inhibitors. PIP2 = Phosphatidylinositol 4,5-bisphosphate, PIP3 = Phosphatidylinositol 3,4,5-bisphosphate. P in a red circle indicates a phosphorylation event which is activating. P in a black circle indicates a phosphorylation event which is inactivating.

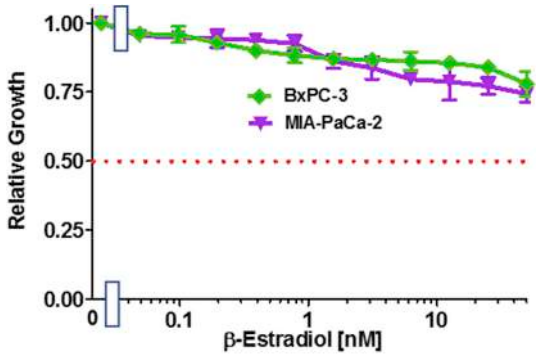

Fig. 2. Effects of the β-Estradiol on the Proliferation of BxPC-3 and MIA-PaCa-2 Cells. MTT analysis of BxPC-3 (green diamonds) and MIA-PaCa-2 cells (purple downward diamonds). All the experiments indicated in this figure were performed on the same day. These experiments were repeated 4 times and similar results were obtained.

1.1. Regulation of cancer cell proliferation by the estrogen receptor (ER)

Many different cancers are receptive to the major estrogen, 17 β-estradiol. The estrogen receptor (ER) signaling pathway is critical in breast and other cancers. ER is a key hormonal receptor which stimulates the transcription of various genes as well as other biochemical processes. ER is one of the key molecules screened for in breast cancer. Tamoxifen (Nolvadex) is an estrogen receptor antagonist used to treat ER + breast cancer (Steelman et al., 2016). BxPC-3 and MIA-PaCa-2 pancreatic cancer cells both express the

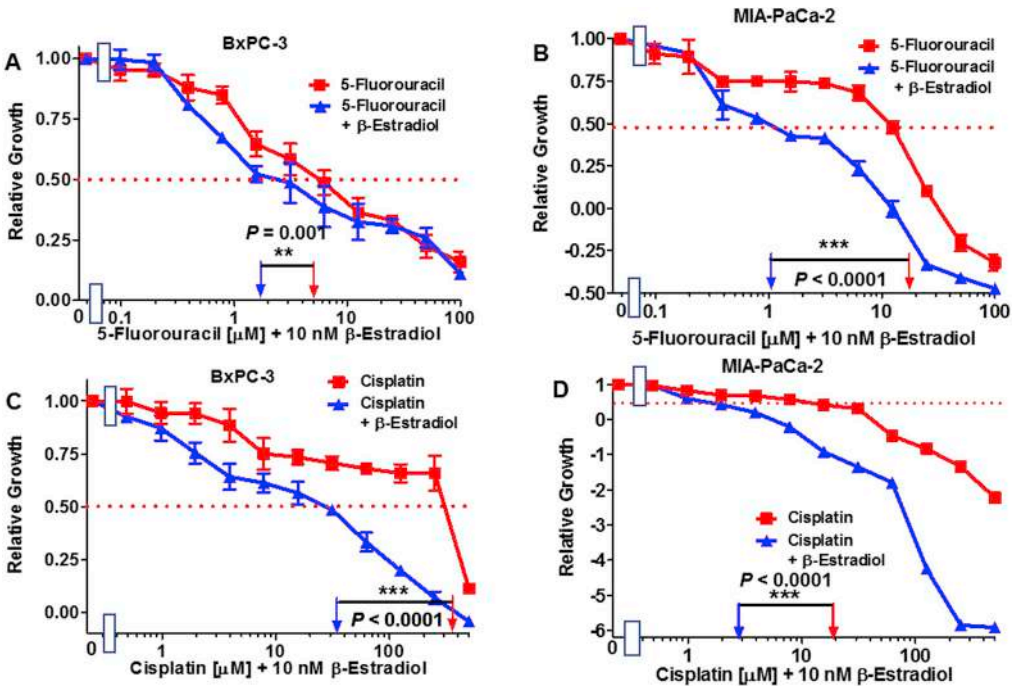

Fig. 3. Effects of Constant 10 nM β-Estradiol Dose on the Proliferation of BxPC-3 and MIA-PaCa-2 Cells Cultured with Chemotherapeutic Drugs 5-Fluorouracil or Cisplatin. Panel A) MTT analysis of BxPC-3 cells cultured with 5-fluorouracil in the absence of β-estradiol (red squares) or in the presence of 10 nM β-estradiol (blue triangles). Panel B) MTT analysis of MIA-PaCa-2 cells cultured with 5-fluorouracil in the absence of β-estradiol (red squares) or in the presence of 10 nM β-estradiol (blue triangles). Panel C) MTT analysis of BxPC-3 cells cultured with cisplatin in the absence of β-estradiol (red squares) or in the presence of 10 nM β-estradiol (blue triangles). Panel D) MTT analysis of MIA-PaCa-2 cells cultured with cisplatin in the absence of β-estradiol (red squares) or in the presence of 10 nM β-estradiol (blue triangles). Arrows pointing to the X-axis indicate where the IC_{50}s can be estimated. These experiments were repeated 3 times and similar results were obtained. Statistical analyses were performed in relationship to cells not treated with β-estradiol or with β-estradiol where appropriate by the Student T test on the means and standard deviations of various treatment groups. *** = $P < 0.0001$, ** $P < 0.005$.

Table 1
Effects of β-Estradiol on Sensitivity of BxPC-3 and MIA-PaCa-2 Cells to Chemotherapeutic Drugs, Signal Transduction Inhibitors, Natural Products and Nutraceuticals.[a]

Cells→	Target↓	BxPC-3 Cells			MIA-PaCa-2 Cells		
Compound↓		IC_{50}↓	IC_{50} with 10 nM β-Estradiol↓	Fold Difference of IC_{50} with 10 nM β-Estradiol↓	IC_{50}↓	IC_{50} with 10 nM β-Estradiol↓	Fold Difference of IC_{50} with 10 nM β-Estradiol↓
5-Fluorouracil	Thymidylate syntase	5 μM	1.8 μM	2.8 X ↓	20 μM	1 μM	20 X ↓
Cisplatin	DNA synthesis	350 μM	35 μM	10 X ↓	20 μM	2.8 μM	7.1 X ↓
Paclitaxel	Microtubules	25 nM	25 nM	–	1 nM	0.18 nM	5.6 X ↓
Docetaxel	Microtubules	1 nM	0.3 nM	66.7 X ↓	3 nM	0.05 nM	60 X ↓
Daunorubicin	Topoisomerase	1000 nM	150 nM	2 X ↓	17 nM	17 nM	–
Mitoxantrone	Topoisomerase	600 nM	300 nM	2.7 X ↓	300 nM	17 nM	17.6 X ↓
Doxorubicin	Topoisomerase	500 nM	150 nM	3.3 X ↓	400 nM	1.5 nM	267 X ↓
Dasatinib	SRC	2000 nM	> 2000 nM	–	500 nM	18 nM	28 X ↓
MG132	Proteasome	2.5 nM	1.5 nM	1.7 X ↓	4 nM	0.8 nM	5 X↓
PD153035	EGFR	180 nM	3 nM	60 X ↓	40 nM	1 ± nM	40 X↓
AG1478	EGFR	30 nM	4 nM	7.5 X↓	130 nM	20 nM	6.5 X↓
ARRY-543	EGFR. HER2, EGFR4	> 2000 nM	> 2000 nM	–	30 nM	10 nM	3 X↓
Benzyl-IP5	PDK	200 nM	6 nM	33.3 X↓	400 nM	15 nM	2.7 X↓
PD0325901	MEK1	500 nM	110 nM	4.5 X↓	2 nM	0.7 nM	2.9 X↓
Tidelglusib	GSK-3	1000 nM	250 nM	4 X↓	2100 nM	1700 nM	1.2 X ↓
Parthenolide	NF-κB, HDAC	> 100 nM	> 100 nM	–	100 nM	1.5 nM	67 X↓
ABT-737	BCL2/BCLXL	600 nM	600 nM	–	500 nM	250 nM	2 X↓
Vismodegib	Hh	> 5000 nM	> 5000 nM	–	5000 nM	3000 nM	1.7 X↓
Genistein	NF-κB	5000 nM	800 nM	6.3 X↓	800 nM	10 nM	80 X↓
Daidzein	G protein-coupled estrogen receptor 1 (binds estrogen)	2000 nM	500 nM	4 X↓	80 nM	2 nM	40 X↓

[a] Determined by MTT analysis as described in (Abrams, S.L., Lertpiriyapong, K., Yang, L.V., Martelli, A.M., Cocco, L., Ratti, S., Falasca, M., Murata, R.M., Rosalen, P.L., Lombardi, P., Libra, M., Candido, S., Montalto, G., Cervello, M., Steelman, L.S., McCubrey et al., 2018. Introduction of WT-TP53 into pancreatic cancer cells alters sensitivity to chemotherapeutic drugs, targeted therapeutics and nutraceuticals. Adv. Biol. Regul. 69, 16–34).

ER (Guo et al., 2004; Xie et al., 2014). Estrogen signals through both ERα and ERβ (Zhao et al., 2010).

Depending on the cell type and tissue culture conditions, β-estradiol has been observed to either stimulate or inhibit the proliferation of cancer cells (Joly-Pharaboz et al., 1990; Detti et al., 2008). In some studies, the effects of physiological concentrations of β-estradiol ranging from 0.1 to 10 nM have been investigated on human lens epithelial cells (Celojevic et al., 2011).

The concentration of β-estradiol in humans and other species can vary depending upon various factors, including menstruation and aging. During pregnancy, the serum concentration of 17 β-Estradiol (E2), increases from baseline 10 pM to 40 nM (Williams et al., 2002). β-Estradiol can influence the expression of many growth regulatory genes such as NF-κB (Hirano et al., 2007).

Although the role of estrogen in PDAC remains controversial, in some studies, the ratio of ERβ to ERα receptors has been shown to be important in responses of PDAC cells to various therapeutic approaches (Konduri and Schwarz, 2007). In the following studies, we demonstrate that 10 nM β-estradiol will increase the effectiveness of certain chemotherapeutic drugs, signal transduction inhibitors, natural products and nutraceuticals. Upon β-estradiol treatment, two different PDAC lines often became more sensitive to various agents which can suppress growth.

2. Materials and methods

2.1. Cell lines and tissue culture

The BxPC3 cell line (ATCC® CRL-1687™) was isolated from a 61 year old woman with pancreatic cancer (Tan et al., 1986). The MIA-PaCa-2 PDAC (ATCC® CRM-CRL-1420™) was recovered from a 65-year old Caucasian male PDAC patient (Yunis et al., 1997; Deer et al., 2010). These cell lines were obtained from the ATCC (Rockville, MD, USA).

Cells were cultured in Dulbecco's modified Eagles medium, (DMEM), (Invitrogen, Carlsbad, CA), containing L-glutamine and antibiotics, supplemented with 5% fetal bovine serum (FBS) purchased from Atlanta Biologicals, Atlanta, GA, USA) as described in (Abrams et al., 2018; Abrams et al., 2019). Tissue culture grade 17 β-estradiol was purchased from Sigma/Aldrich and is referred to as β-estradiol. In these studies, the tissue culture medium was not charcoal-stripped as we have observed that charcoal stripping of

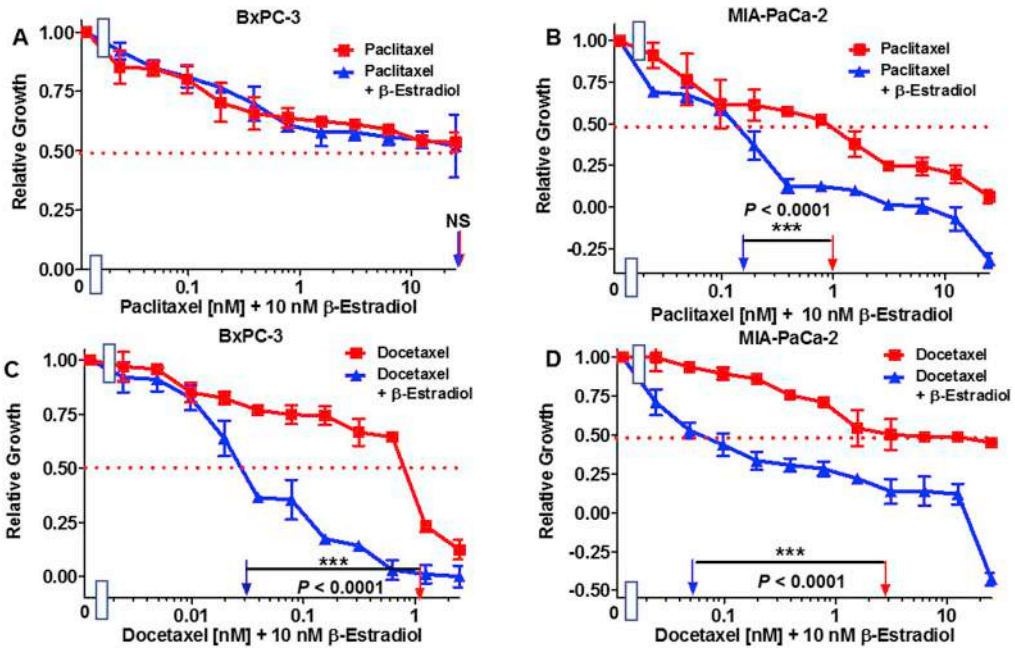

Fig. 4. Effects of a Constant 10 nM Dose of β-Estradiol on the Proliferation of BxPC-3 and MIA-PaCa-2 Cells Cultured with Chemotherapeutic Drugs Paclitaxel or Docetaxel. Panel A) MTT analysis of BxPC-3 cells cultured with paclitaxel in the absence of β-estradiol (red squares) or in the presence of 10 nM β-estradiol (blue triangles). Panel B) MTT analysis of MIA-PaCa-2 cells cultured with paclitaxel in the absence of β-estradiol (red squares) or in the presence of 10 nM β-estradiol (blue triangles). Panel C) MTT analysis of BxPC-3 cells cultured with docetaxel in the absence of β-estradiol (red squares) or in the presence of 10 nM β-estradiol (blue triangles). Panel D) MTT analysis of MIA-PaCa-2 cells cultured with docetaxel in the absence of β-estradiol (red squares) or in the presence of 10 nM β-estradiol (blue triangles). Arrows pointing to the X-axis indicate where the IC_{50}s can be estimated. These experiments were repeated 3 times and similar results were obtained. Statistical analyses were performed in relationship to cells not treated with β-estradiol or with β-estradiol where appropriate by the Student T test on the means and standard deviations of various treatment groups. *** = $P < 0.0001$, NS = not statistically significant.

medium removes other factors (lipids, metabolites, steroid hormones and growth factors) besides endogenous estrogens that are important for growth.

2.2. Methylthiazole tetrazolium assays

Methylthiazole tetrazolium (MTT) assays were performed to determine the sensitivity of the pancreatic cancer cells to the chemotherapeutic drugs, signal transduction inhibitors, natural products, nutraceutical and 17 β-estradiol as described (Abrams et al., 2018, 2019). Phenol-red free medium containing 0.5% FBS was used in MTT assays to eliminate the potential of phenol red to affect proliferation.

To test the hypothesis that the various IC_{50}s in the cells treated with the various chemotherapeutic drugs, signal transduction inhibitors, natural products, and nutraceuticals were statistically different than in the same cells treated the same day with the same drug and 10 nM β-estradiol, student's T tests were performed using Graph Pad Prism (QuickCals) statistical analysis.

3. Results

3.1. Effects of β-estradiol on proliferation of two PDAC cell lines

The effects of different doses of estrogen on the proliferation of BxPC-3 and MIA-PaCa-2 cells were determined (Fig. 2). The addition of doses of β-estradiol up to 50 nM did not suppress the proliferation of either BxPC-3 or MIA-PaCa-2 cells by more than 25%. Depending on the cell type and tissue culture conditions, β-estradiol has been observed to either stimulate or inhibit the proliferation of cancer cells (Joly-Pharaboz et al., 1990; Detti et al., 2008). In other studies, the effects of physiological concentration

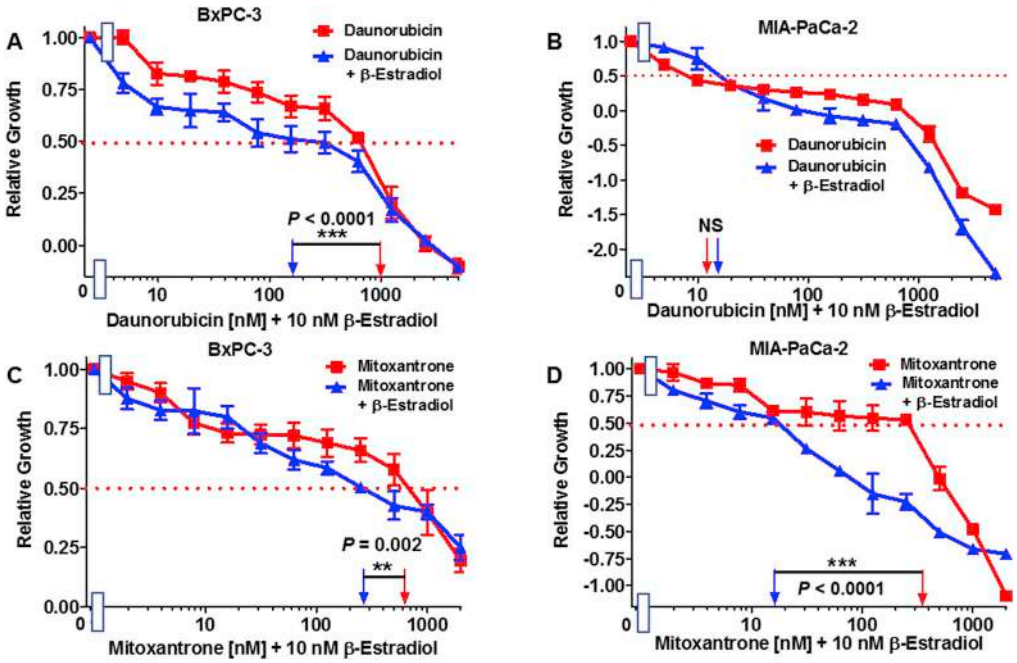

Fig. 5. Effects of a Constant 10 nM Dose of β-Estradiol on the Proliferation of BxPC-3 and MIA-PaCa-2 Cells Cultured with Chemotherapeutic Drugs Daunorubicin or Mitoxantrone. Panel A) MTT analysis of BxPC-3 cells cultured with daunorubicin in the absence of β-estradiol (red squares) or in the presence of 10 nM β-estradiol (blue triangles). Panel B) MTT analysis of MIA-PaCa-2 cells cultured with daunorubicin in the absence of β-estradiol (red squares) or in the presence of 10 nM β-estradiol (blue triangles). Panel C) MTT analysis of BxPC-3 cells cultured with mitoxantrone in the absence of β-estradiol (red squares) or in the presence of 10 nM β-estradiol (blue triangles). Panel D) MTT analysis of MIA-PaCa-2 cells cultured with mitoxantrone in the absence of β-estradiol (red squares) or in the presence of 10 nM β-estradiol (blue triangles). Arrows pointing to the X-axis indicate where the IC_{50}s can be estimated. These experiments were repeated 3 times and similar results were obtained. Statistical analyses were performed in relationship to cells not treated with β-estradiol or with β-estradiol where appropriate by the Student T test on the means and standard deviations of various treatment groups. *** = $P < 0.0001$, **$P < 0.005$, NS = not statistically significant.

of β-estradiol ranging from 0.1 to 10 nM have been investigated on human lens epithelial cells (Celojevic et al., 2011). We chose to perform studies on BxPC-3 and MIA-PaCa-2 PDAC cells with 10 nM β-estradiol.

3.2. Effects of β-estradiol on sensitivity of PDAC cells to chemotherapeutic drugs

5-fluorouracil and cisplatin are two chemotherapeutic drugs used to treat PDAC patients. The effects of inclusion of a constant dose of 10 nM β-estradiol on the drug sensitivity of BxPC-3 (Fig. 3, Panels A & C) and MIA-PaCa-2 (Fig. 3, Panels B & D) cells were determined. Addition of 10 nM β-estradiol reduced the IC_{50} concentration of 5-fluorouracil 2.8-fold from 5 to 1.8 μM in BxPC-3 cells (Fig. 3, Panel A). Addition of 10 nM β-estradiol reduced the IC_{50} concentration of 5-fluorouracil 20-fold from 20 to 1 μM in MIA-PaCa-2 cells (Fig. 3, Panel B). These results are summarized in Table 1. Addition of 10 nM β-estradiol reduced the concentration of cisplatin required to reach the IC_{50} 10-fold from 350 to 35 μM in BxPC-3 cells (Fig. 3, Panel C). Addition of 10 nM β-estradiol reduced the IC_{50} concentration of cisplatin required to reach the IC_{50} 7.1-fold from 20 to 2.8 μM in MIA-PaCa-2 cells (Fig. 3, Panel D).

Paclitaxel and docetaxel are two chemotherapeutic drugs used in some situations to treat PDAC patients. The effects of inclusion of a constant dose of 10 nM β-estradiol on the drug sensitivity of BxPC-3 (Fig. 4, Panels A & C) and MIA-PaCa-2 (Fig. 4, Panels B & D) cells were determined. Addition of 10 nM β-estradiol did not reduce the IC_{50} concentration of paclitaxel required to reach the IC_{50} in BxPC-3 cells (Fig. 4, Panel A). Addition of 10 nM β-estradiol reduced the IC_{50} concentration of paclitaxel required to reach the IC_{50} 5.6-fold from 1 to 0.18 nM in MIA-PaCa-2 cells (Fig. 4, Panel B). Addition of 10 nM β-estradiol reduced the concentration of docetaxel required to reach the IC_{50} 66.7-fold from 1 to 0.03 nM in BxPC-3 cells (Fig. 4, Panel C). Addition of 10 nM β-estradiol reduced the concentration of docetaxel required to reach the IC_{50} 60-fold from 3 to 0.05 nM in MIA-PaCa-2 cells (Fig. 4, Panel D).

Daunorubicin and mitoxantrone are two chemotherapeutic drugs used to treat various cancer patients. The effects of inclusion of a constant dose of 10 nM β-estradiol on the drug sensitivity of BxPC-3 (Fig. 5, Panels A & C) and MIA-PaCa-2 (Fig. 5, Panels B & D)

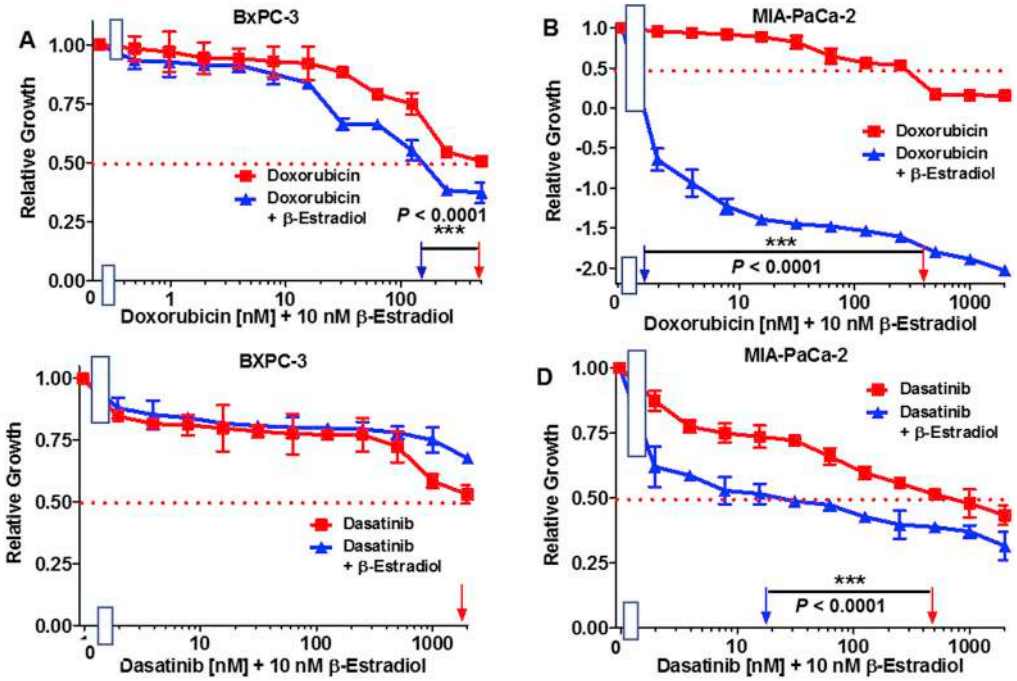

Fig. 6. Effects of a Constant 10 nM Dose of β-Estradiol on the Proliferation of BxPC-3 and MIA-PaCa-2 Cells Cultured with the Chemotherapeutic Drug Doxorubicin or the SRC Inhibitor Dasatinib. Panel A) MTT analysis of BxPC-3 cells cultured with doxorubicin in the absence of β-estradiol (red squares) or in the presence of 10 nM β-estradiol (blue triangles). Panel B) MTT analysis of MIA-PaCa-2 cells cultured with doxorubicin in the absence of β-estradiol (red squares) or in the presence of 10 nM β-estradiol (blue triangles). Panel C) MTT analysis of BxPC-3 cells cultured with dasatinib in the absence of β-estradiol (red squares) or in the presence of 10 nM β-estradiol (blue triangles). Panel D) MTT analysis of MIA-PaCa-2 cells cultured with dasatinib in the absence of β-estradiol (red squares) or in the presence of 10 nM β-estradiol (blue triangles). Arrows pointing to the X-axis indicate where the IC_{50}s can be estimated. These experiments were repeated 3 times and similar results were obtained. Statistical analyses were performed in relationship to cells not treated with β-estradiol or with β-estradiol where appropriate by the Student T test on the means and standard deviations of various treatment groups. *** = $P < 0.0001$.

cells were determined. Addition of 10 nM β-estradiol reduced the concentration of daunorubicin required to reach the IC_{50} 6.6-fold from 1000–150 nM in BxPC-3 cells (Fig. 5, Panel A). Addition of 10 nM β-estradiol did not reduce the concentration of daunorubicin required to reach the IC_{50} in MIA-PaCa-2 cells (Fig. 5, Panel B). Addition of 10 nM β-estradiol reduced the concentration of mitoxantrone required to reach the IC_{50} 2-fold from 600 to 300 nM in BxPC-3 cells (Fig. 5, Panel C). Addition of 10 nM β-estradiol reduced the concentration of mitoxantrone required to reach the IC_{50} 17.6-fold from 300 to 17 nM in MIA-PaCa-2 cells (Fig. 5, Panel D).

Doxorubicin is a chemotherapeutic drug used to treat various cancer patients. The effects of inclusion of a constant dose of 10 nM β-estradiol on the drug sensitivity of BxPC-3 (Fig. 6, Panel A) and MIA-PaCa-2 (Fig. 6, Panel B) cells were determined. Addition of 10 nM β-estradiol reduced the concentration of doxorubicin required to reach the IC_{50} 3.3-fold from 500 to 150 nM in BxPC-3 cells (Fig. 6, Panel A). Addition of 10 nM β-estradiol reduced the concentration of doxorubicin required to reach the IC_{50} 267-fold from 400 to 1.5 μM in MIA-PaCa-2 cells (Fig. 6, Panel B).

Dasatinb (SPRYCEL®) is a small molecule inhibitor which targets SRC and other kinases (Araujo and Logothetis, 2010). It is used in cancer therapy including treatment of imatinib-resistant chronic myeloid leukemia. SRC can phosphorylate and activate the EGFR (Sato et al., 2003). In addition, EGFR can have effects on SRC activity [Taniguchi et al., 2013] Dasatinib can inhibit the effect of SRC and EGFR activity [Nautiyal et al., 2009] The effects of inclusion of a constant dose of 10 nM β-estradiol on the sensitivity of BxPC-3 to dasatinib (Fig. 6, Panel C) and MIA-PaCa-2 (Fig. 6, Panel D) cells were determined. Addition of 10 nM β-estradiol did not reduce the concentration of dasatinib required to reach the IC_{50} in BxPC-3 cells (Fig. 6, Panel C). Addition of 10 nM β-estradiol reduced the concentration of dasatinib required to reach the IC_{50} 28-fold from 500 to 18 nM in MIA-PaCa-2 cells (Fig. 6, Panel D).

MG-132 is a small molecule inhibitor which targets the proteasome. MG-132 suppresses degradation of ubiquitin-conjugated proteins. The effects of inclusion of a constant dose of 10 nM β-estradiol on the sensitivity of BxPC-3 (Fig. 7, Panel A) and MIA-PaCa-2 (Fig. 7, Panel B) cells to MG-132 were determined. Addition of 10 nM β-estradiol reduced the concentration of MG-132 required to

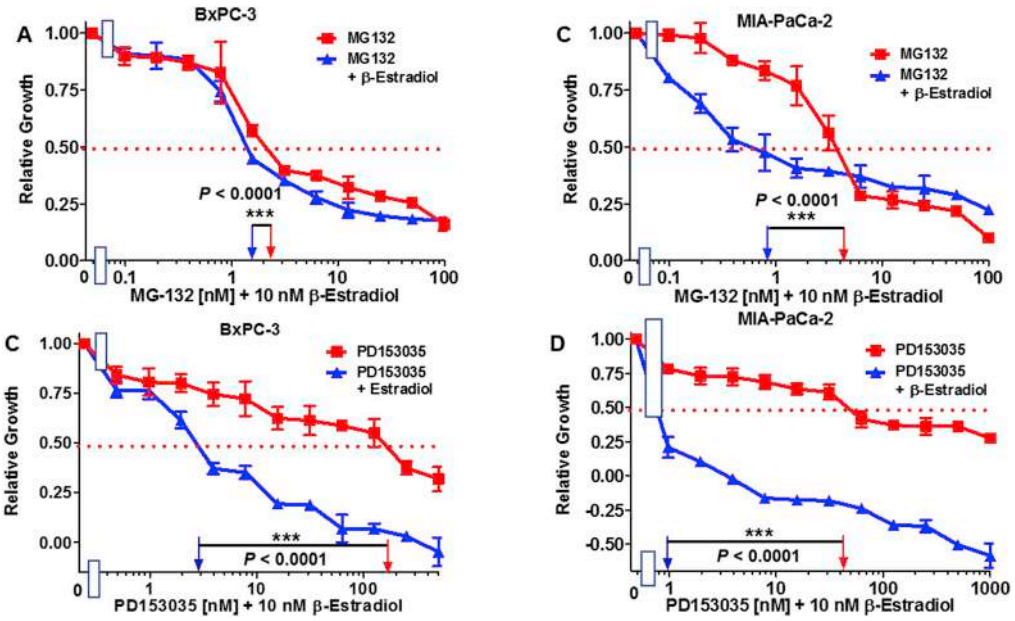

Fig. 7. Effects of a Constant 10 nM Dose of β-Estradiol on the Proliferation of BxPC-3 and MIA-PaCa-2 Cells Cultured with the Proteasomal Inhibitor MG-132 or the EGFR Inhibitor PD153035. Panel A) MTT analysis of BxPC-3 cells cultured with MG-132 in the absence of β-estradiol (red squares) or in the presence of 10 nM β-estradiol (blue triangles). Panel B) MTT analysis of MIA-PaCa-2 cells cultured with MG-132 in the absence of β-estradiol (red squares) or in the presence of 10 nM β-estradiol (blue triangles). Panel C) MTT analysis of BxPC-3 cells cultured with PD153035 in the absence of β-estradiol (red squares) or in the presence of 10 nM β-estradiol (blue triangles). Panel D) MTT analysis of MIA-PaCa-2 cells cultured with PD153035 in the absence of β-estradiol (red squares) or in the presence of 10 nM β-estradiol (blue triangles). Arrows pointing to the X-axis indicate where the IC_{50}s can be estimated. These experiments were repeated 3 times and similar results were obtained. Statistical analyses were performed in relationship to cells not treated with β-estradiol or with β-estradiol where appropriate by the Student T test on the means and standard deviations of various treatment groups. *** = $P < 0.0001$.

reach the IC_{50} 1.7-fold in BxPC-3 cells from 2.5 nM to 1.5 nM (Fig. 7, Panel A). Addition of 10 nM β-estradiol reduced the concentration of MG-132 required to reach the IC_{50} 5-fold from 4 to 0.8 nM in MIA-PaCa-2 cells (Fig. 7, Panel B).

PD153035 is a small molecule inhibitor which targets the EGFR much more selectively than HER2 (Bos et al., 1997). The effects of inclusion of a constant dose of 10 nM β-estradiol on the sensitivity of BxPC-3 (Fig. 7, Panel C) and MIA-PaCa-2 (Fig. 7, Panel D) cells to MG132 were determined. Addition of 10 nM β-estradiol reduced the concentration of PD153035 required to reach the IC_{50} 60-fold in BxPC-3 cells from 180 nM to 3 nM (Fig. 7, Panel C). Addition of 10 nM β-estradiol reduced the concentration of PD153035 required to reach the IC_{50} 40-fold from 40 to 1 nM in MIA-PaCa-2 cells (Fig. 7, Panel D).

AG1478 is a small molecule inhibitor which targets the EGFR much more selectively than HER2 (Egeblad et al., 2001). The effects of inclusion of a constant dose of 10 nM β-estradiol on the sensitivity of BxPC-3 to AG1478 (Fig. 8, Panel A) and MIA-PaCa-2 (Fig. 8, Panel B) cells to AG1478 were determined. Addition of 10 nM β-estradiol reduced the concentration of AG1478 required to reach the IC_{50} approximately 7.5-fold from 30 to 4 nM in BxPC-3 cells from 180 nM to 3 nM (Fig. 8, Panel A). Addition of 10 nM β-estradiol reduced the concentration of AG1478 required to reach the IC_{50} 6.5-fold from 130 to 20 nM in MIA-PaCa-2 cells (Fig. 8, Panel B).

ARRY-543 (Varlitinib), is a small molecule inhibitor which targets EGFR, HER2 and HER4 (Liu et al., 2019). It is referred to as a PAN EGFR inhibitor. Certain pancreatic cancer cell lines such as MIA-PaCa-2 express high levels of HER2 and are sensitive to MoAb antibodies such as Herceptin (Büchler et al., 2001). BxPC-3 cells also express HER2, however, they express more EGFR than HER2 (Larbouret et al., 2012). ERB4 has been shown to have some important growth regulatory roles in PDAC cells, including MIA-PaCa-2 cells (Mill et al., 2010).

The effects of inclusion of a constant dose of 10 nM β-estradiol on the sensitivity of BxPC-3 (Fig. 8, Panel C) and MIA-PaCa-2 (Fig. 8, Panel D) cells to ARRY-543 were determined. Addition of 10 nM β-estradiol did not reduce the concentration of ARRY-543 required to reach the IC_{50} in BxPC-3 cells. Addition of 10 nM β-estradiol reduced the concentration of ARRY-543 required to reach the IC_{50} 3-fold from 30 to 10 nM in MIA-PaCa-2 cells (Fig. 8, Panel D).

2-O-benzyl-myo-inositol 1,3,4,5,6-pentakisphosphate (2-O-Bn-InsP5, benzyl-IP5 is a PDK inhibitor (Falasca et al., 2010). The effects of inclusion of a constant dose of 10 nM β-estradiol on the sensitivity of BxPC-3 (Fig. 9, Panel A) and MIA-PaCa-2 (Fig. 9, Panel

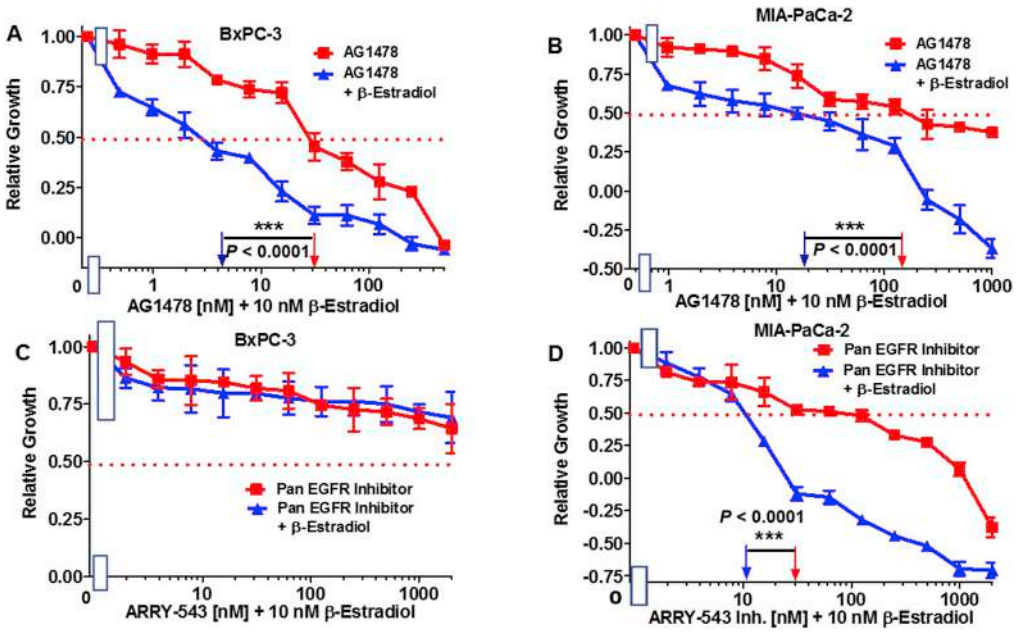

Fig. 8. Effects of a Constant 10 nM Dose of β-Estradiol on the Proliferation of BxPC-3 and MIA-PaCa-2 Cells Cultured with the EGFR Inhibitor AG1478 or the Pan EGFR Inhibitor ARRY-543. Panel A) MTT analysis of BxPC-3 cells cultured with AG1478 in the absence of β-estradiol (red squares) or in the presence of 10 nM β-estradiol (blue triangles). Panel B) MTT analysis of MIA-PaCa-2 cells cultured with AG1478 in the absence of β-estradiol (red squares) or in the presence of 10 nM β-estradiol (blue triangles). Panel C) MTT analysis of BxPC-3 cells cultured with ARRY-543 in the absence of β-estradiol (red squares) or in the presence of 10 nM β-estradiol (blue triangles). Panel D) MTT analysis of MIA-PaCa-2 cells cultured with ARRY-543 in the absence of β-estradiol (red squares) or in the presence of 10 nM β-estradiol (blue triangles). Arrows pointing to the X-axis indicate where the IC_{50}s can be estimated. These experiments were repeated 3 times and similar results were obtained. Statistical analyses were performed in relationship to cells not treated with β-estradiol or with β-estradiol where appropriate by the Student T test on the means and standard deviations of various treatment groups. *** = $P < 0.0001$.

B) cells to benzyl-IP5 were determined. Addition of 10 nM β-estradiol reduced the concentration of benzyl-IP5 required to reach the IC_{50} 33.5-fold from 200 nM to 6 nM in BxPC-3 cells (Fig. 9, Panel A). Addition of 10 nM β-estradiol reduced the IC_{50} concentration of benzyl-IP5 required to reach the IC_{50} 2.7-fold from 400 to 150 nM in MIA-PaCa-2 cells (Fig. 9, Panel B).

PD0325901 is a potent MEK1 inhibitor (Ricciardi et al., 2012). MEK is frequently activated in PDAC cells due to the high frequency of upstream *KRAS* mutations (Fitzgerald et al., 2015). BxPC-3 cells do not have a *KRAS* mutation (Waters and Der, 2018). The effects of inclusion of a constant dose of 10 nM β-estradiol on the sensitivity of BxPC-3 (Fig. 9, Panel C) and MIA-PaCa-2 (Fig. 8, Panel D) cells to PD0325901 were determined. Addition of 10 nM β-estradiol reduced the concentration of PD0325901 required to reach the IC_{50} 4.5-fold from 500 nM to 110 nM in BxPC-3 cells (Fig. 9, Panel C). MIA-PaCa-2 cells were more sensitive to the PD0325901 inhibitor than BxPC-3 cells (compare Panels C & D). This may have resulted from MIA-PaCa-2 cells possessing a mutant *KRAS* gene while BxPC3 cells have a WT-*KRAS* gene. Addition of 10 nM β-estradiol reduced the concentration of PD0325901 required to reach the IC_{50} 2.9-fold from 2 to 0.7 nM in MIA-PaCa-2 cells (Fig. 9, Panel D).

Tidelglusib is a GSK-3 inhibitor that has been evaluated in clinical trials (Lovestone et al., 2015). GSK-3 activity may be dysregulated in PDAC cells due to the high frequency of upstream *KRAS* mutations which can have effects on AKT activity and downstream GSK-3 which is a target of AKT (Marchand et al., 2015; Fitzgerald et al., 2015; Mancinelli et al., 2017). The effects of inclusion of a constant dose of 10 nM β-estradiol on the sensitivity of BxPC-3 (Fig. 10, Panel A) and MIA-PaCa-2 (Fig. 9, Panel B) cells to tidelglusib were determined. Addition of 10 nM β-estradiol reduced the concentration of tidelglusib required to reach the IC_{50} 4-fold from 1000 nM–250 nM in BxPC-3 cells (Fig. 10, Panel A). Addition of 10 nM β-estradiol reduced the concentration of tideglusib required to reach the IC_{50} 1.2-fold from 2100 to 1700 nM in MIA-PaCa-2 cells (Fig. 10, Panel B).

Parthenolide is a natural product isolated from the fruits of the plant feverfew. It is a sesquiterpene lactone. Some of the targets which it suppresses the activity of are NF-κB and histone deacetylase I (HDACI) and not to affect other HDACs (Yip et al., 2004; Rajendran et al., 2011). It has been examined in the treatment of certain leukemias including acute myeloid leukemia (AML) as it may have more effects on the AML stem cells (Guzman et al., 2005).

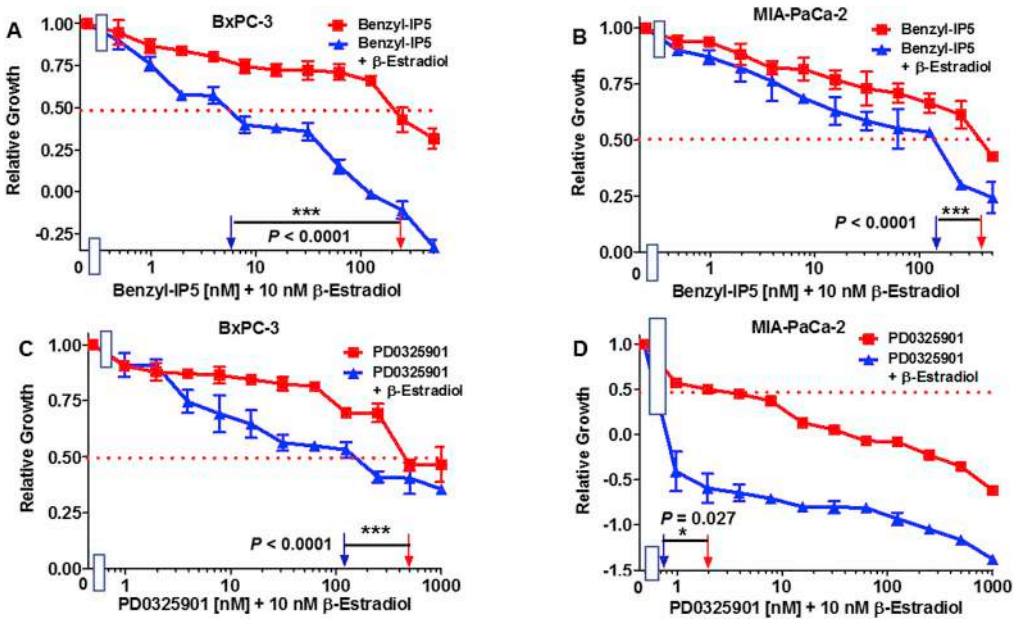

Fig. 9. Effects of a Constant 10 nM Dose of β-Estradiol on the Proliferation of BxPC-3 and MIA-PaCa-2 Cells Cultured with the PDK1 Inhibitor Benzyl-IP5 or the MEK1 Inhibitor PD0325901. Panel A) MTT analysis of BxPC-3 cells cultured with Benzyl-IP5 in the absence of β-estradiol (red squares) or in the presence of 10 nM β-estradiol (blue triangles). Panel B) MTT analysis of MIA-PaCa-2 cells cultured with Benzyl-IP5 in the absence of β-estradiol (red squares) or in the presence of 10 nM β-estradiol (blue triangles). Panel C) MTT analysis of BxPC-3 cells cultured with PD0325901 in the absence of β-estradiol (red squares) or in the presence of 10 nM β-estradiol (blue triangles). Panel D) MTT analysis of MIA-PaCa-2 cells cultured with PD0325901 in the absence of β-estradiol (red squares) or in the presence of 10 nM β-estradiol (blue triangles). Arrows pointing to the X-axis indicate where the IC_{50}s can be estimated. These experiments were repeated 3 times and similar results were obtained. Statistical analyses were performed in relationship to cells not treated with β-estradiol or with β-estradiol where appropriate by the Student T test on the means and standard deviations of various treatment groups. *** = $P < 0.0001$.

The effects of inclusion of a constant dose of 10 nM β-estradiol on the sensitivity of BxPC-3 (Fig. 10, Panel C) and MIA-PaCa-2 (Fig. 10, Panel D) cells to parthenolide were determined. Addition of 10 nM β-estradiol did not reduce the concentration of parthenolide required to reach the IC_{50} in BxPC-3 cells (Fig. 10, Panel C). Addition of 10 nM β-estradiol reduced the concentration of parthenolide required to reach the IC_{50} 67-fold from 100 to 1.5 nM in MIA-PaCa-2 cells (Fig. 10, Panel D).

The expression of the anti-apoptotic BCL2/BCLXL proteins are dysregulated in many cancers including PDAC (Miyamoto et al., 1999). The effects of inclusion of a constant dose of 10 nM β-estradiol on the sensitivity of BxPC-3 (Fig. 11, Panel A) and MIA-PaCa-2 (Fig. 11, Panel B) cells to the BCL2/BCLXL inhibitor ABT-737 were determined. Addition of 10 nM β-estradiol did not reduce the concentration of ABT-737 required to reach the IC_{50} in BxPC-3 (Fig. 11, Panel A). Addition of 10 nM β-estradiol reduced the concentration of ABT-737 required to reach the IC_{50} 2-fold from 500 to 250 nM in MIA-PaCa-2 cells (Fig. 11, Panel B).

The hedgehog (Hh) signaling pathway is an important signaling pathway involved in the metastasis of many cancers including PDAC (Thayer et al., 2003). Vismodegib is a small molecule inhibitor which targets Hh and has been examined in some clinical trials with PDAC patients (Catenacci et al., 2015). The effects of inclusion of a constant dose of 10 nM β-estradiol on the sensitivity of BxPC-3 (Fig. 11, Panel C) and MIA-PaCa-2 (Fig. 11, Panel D) cells to the Hh pathway inhibitor vismodegib were determined. Addition of 10 nM β-estradiol did not reduce the IC_{50} concentration of vismodegib in BxPC-3 (Fig. 11, Panel C). Addition of 10 nM β-estradiol reduced the IC_{50} concentration of vismodegib 1.7-fold from 5000 to 3000 nM in MIA-PaCa-2 cells (Fig. 11, Panel D).

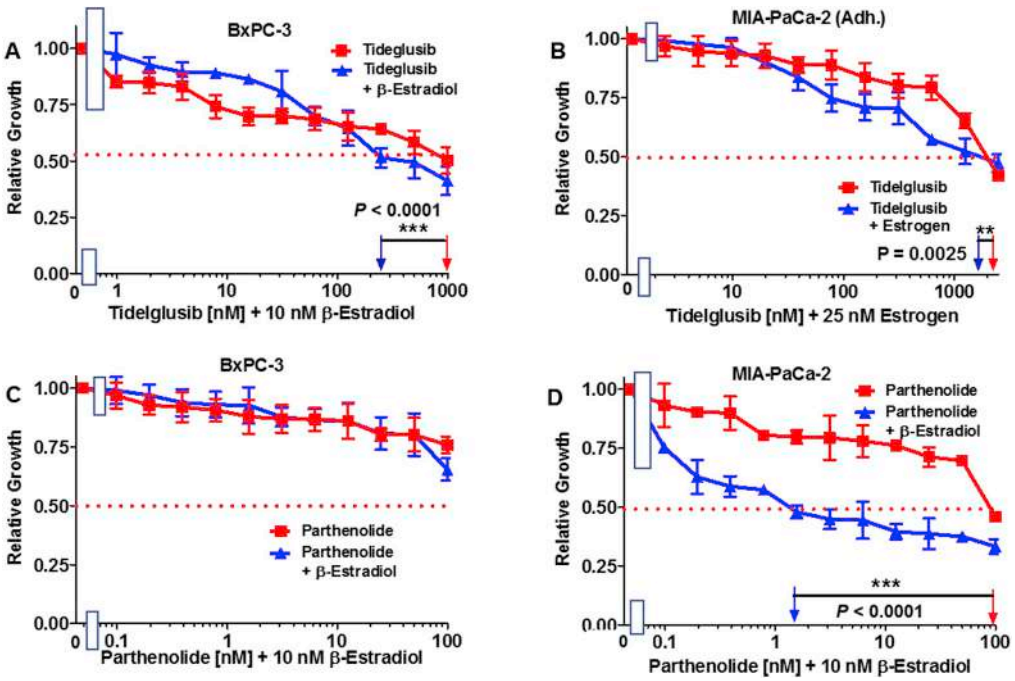

Fig. 10. Effects of a Constant 10 nM Dose of β-Estradiol on the Proliferation of BxPC-3 and MIA-PaCa-2 Cells Cultured with the GSK-3 Inhibitor Tidelglusib or the Natural Product Parthenolide. Panel A) MTT analysis of BxPC-3 cells cultured with tidelglusib in the absence of β-estradiol (red squares) or in the presence of 10 nM β-estradiol (blue triangles). Panel B) MTT analysis of MIA-PaCa-2 cells cultured with tidelglusib in the absence of β-estradiol (red squares) or in the presence of 10 nM β-estradiol (blue triangles). Panel C) MTT analysis of BxPC-3 cells cultured with parthenolide in the absence of β-estradiol (red squares) or in the presence of 10 nM β-estradiol (blue triangles). Panel D) MTT analysis of MIA-PaCa-2 cells cultured with parthenolide in the absence of β-estradiol (red squares) or in the presence of 10 nM β-estradiol (blue triangles). Arrows pointing to the X-axis indicate where the IC_{50}s can be estimated. These experiments were repeated 3 times and similar results were obtained. Statistical analyses were performed in relationship to cells not treated with β-estradiol or with β-estradiol where appropriate by the Student T test on the means and standard deviations of various treatment groups. *** $= P < 0.0001$, ** $P < 0.005$.

Genistein is a soy isoflavone. It is natural product commonly found in soy beans and other plants. It has apoptosis-inducing effects on PDAC cells. It induces some of its anti-proliferative effects by inhibition of NF-κB (Li et al., 2004). The effects of inclusion of a constant dose of 10 nM β-estradiol on the sensitivity of BxPC-3 (Fig. 12, Panel A) and MIA-PaCa-2 (Fig. 12, Panel B) cells to genistein were determined. Addition of 10 nM β-estradiol reduced the concentration of genistein required to reach the IC_{50} in BxPC-3 6.3-fold from 5000 nM–800 nM (Fig. 12, Panel A). Addition of 10 nM β-estradiol reduced the concentration of genistein required to reach the IC_{50} approximately 80-fold from 800 to 10 nM in MIA-PaCa-2 cells (Fig. 12, Panel B).

Daidzein is an isoflavone also found in soy and other legumes Gabriely et al., 2004. Daidzein has been shown to inhibit the growth of ER+ and ER-pancreatic cancer cells. The estrogen receptor may be a target of daidzein (Guo et al., 2004). The effects of inclusion of a constant dose of 10 nM β-estradiol on the sensitivity of BxPC-3 (Fig. 12, Panel C) and MIA-PaCa-2 (Fig. 12, Panel D) cells to daidzein were determined. Addition of 10 nM β-estradiol reduced the concentration of genistein in BxPC-3 required to reach the IC_{50} 4-fold from 2000 nM–500 nM (Fig. 12, Panel C). Addition of 10 nM β-estradiol reduced the concentration of daidzein required to reach the IC_{50} approximately 40-fold from 80 to 2 nM in MIA-PaCa-2 cells (Fig. 12, Panel D).

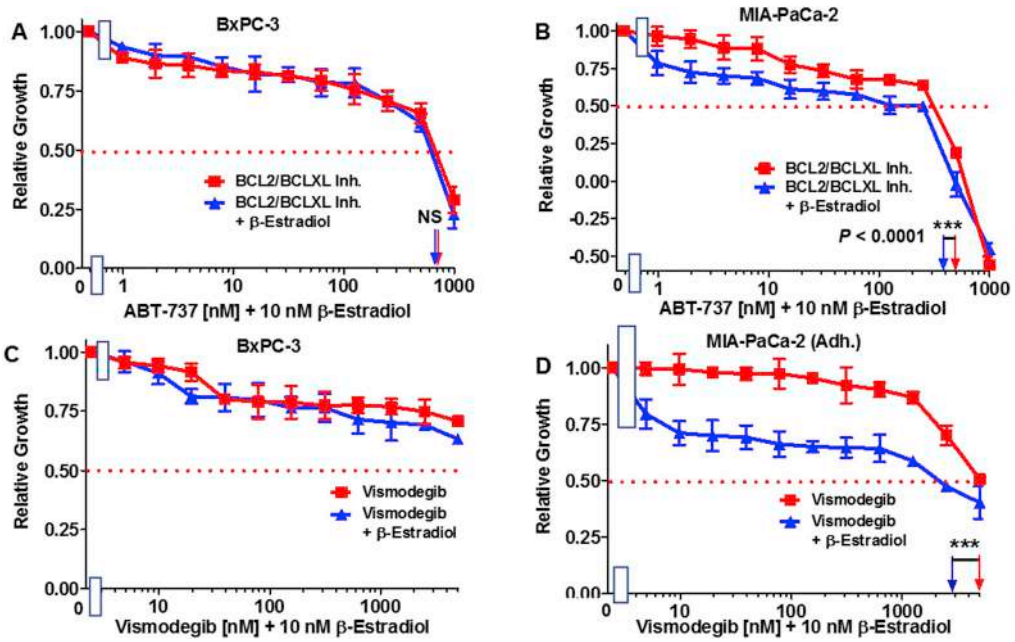

Fig. 11. Effects of a Constant 10 nM Dose of β-Estradiol on the Proliferation of BxPC-3 and MIA-PaCa-2 Cells Cultured with the BCL2/BCLXL Inhibitor ABT-737 or the Hedgehog Inhibitor Vismodegib. Panel A) MTT analysis of BxPC-3 cells cultured with ABT-737 in the absence of β-estradiol (red squares) or in the presence of 10 nM β-estradiol (blue triangles). Panel B) MTT analysis of MIA-PaCa-2 cells cultured with ABT-737 in the absence of β-estradiol (red squares) or in the presence of 10 nM β-estradiol (blue triangles). Panel C) MTT analysis of BxPC-3 cells cultured with vismodegib in the absence of β-estradiol (red squares) or in the presence of 10 nM β-estradiol (blue triangles). Panel D) MTT analysis of MIA-PaCa-2 cells cultured with vismodegib in the absence of β-estradiol (red squares) or in the presence of 10 nM β-estradiol (blue triangles). Arrows pointing to the X-axis indicate where the IC_{50}s can be estimated. These experiments were repeated 3 times and similar results were obtained. Statistical analyses were performed in relationship to cells not treated with β-estradiol or with β-estradiol where appropriate by the Student T test on the means and standard deviations of various treatment groups. *** = $P < 0.0001$, NS = not statistically significant.

4. Discussion

Our studies have revealed that a sub-IC_{50} dose of 10 nM β-estradiol could lower the concentrations of many chemotherapeutic drugs, signal transduction inhibitors and natural products that were required to reach their IC_{50} in two different human PDAC cells. BxPC-3 and MIA-PaCa-2 cells express both ERα and ERβ (Konduri and Schwarz, 2007; Xie et al., 2015). These results demonstrate that in vitro, β-estradiol can increase efficacy and reduce the side effects of chemotherapy.

Estrogens elicit multiple effects on cell cycle progression in various cell types (Prall et al., 1998). Estrogen has been shown to stimulate G_1 to S phase transition in ER + breast cancer cells (Foster et al., 2001a, 2001b).

Estrogen has effects on PDAC proliferation and tumor formation (Seeliger et al., 2018). Pharmacological activation of the ER has been shown to influence the extent of metastasis of certain cancers such as melanoma. Activation of the ER in this system improved the sensitivity of the melanomas to immune checkpoint blockade inhibitors (Natale et al., 2018). Thus β-estradiol treatment could improve the responses of certain PDAC and other cancer to chemotherapeutic drugs, signal transduction inhibitors and nutraceuticals. We propose that the low dose β-estradiol treatment stimulates cell cycle progression which makes more of the cycling cancer cells sensitive to the various drugs. It should also be remembered that estrogens are a key target in certain cancers (e.g. breast and ovarian) cancers. Some of these cancers receive treatment with tamoxifen which is an ER antagonist.

Potential interactions between β-estradiol signaling, chemotherapeutic drugs, signal transduction inhibitors, natural products and nutraceuticals is summarized in Fig. 1.

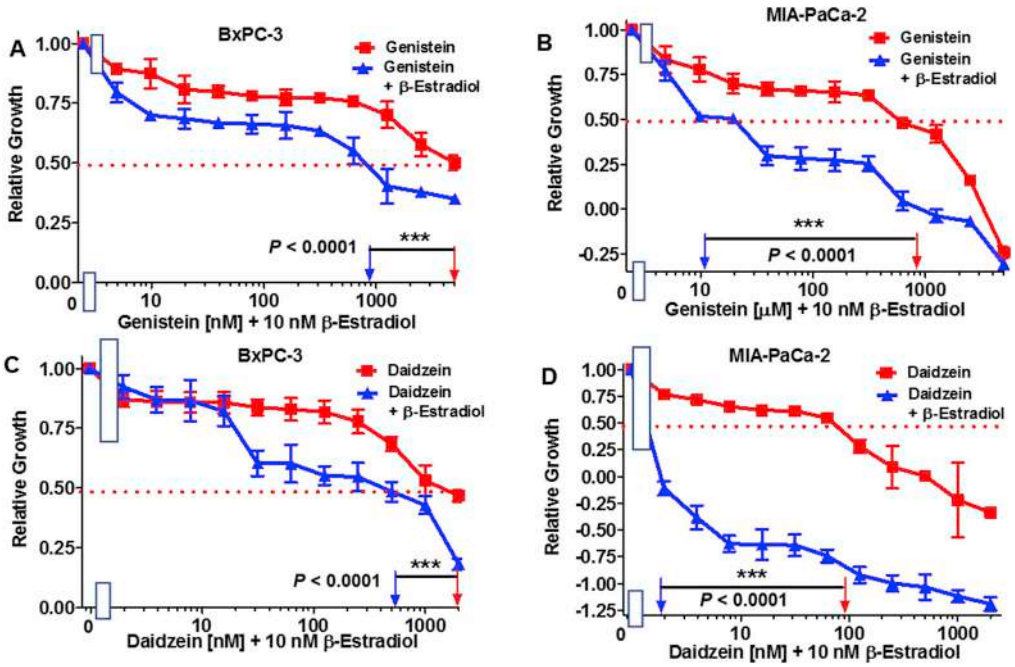

Fig. 12. Effects of a Constant 10 nM Dose of β-Estradiol on the Proliferation of BxPC-3 and MIA-PaCa-2 Cells Cultured with the Nutraceuticals Genistein or Daidzein. Panel A) MTT analysis of BxPC-3 cells cultured with genistein in the absence of β-estradiol (red squares) or in the presence of 10 nM β-estradiol (blue triangles). Panel B) MTT analysis of MIA-PaCa-2 cells cultured with genistein in the absence of β-estradiol (red squares) or in the presence of 10 nM β-estradiol (blue triangles). Panel C) MTT analysis of BxPC-3 cells cultured with daidzein in the absence of β-estradiol (red squares) or in the presence of 10 nM β-estradiol (blue triangles). Panel D) MTT analysis of MIA-PaCa-2 cells cultured with daidzein in the absence of β-estradiol (red squares) or in the presence of 10 nM β-estradiol (blue triangles). Arrows pointing to the X-axis indicate where the IC_{50}s can be estimated. These experiments were repeated 3 times and similar results were obtained. Statistical analyses were performed in relationship to cells not treated with β-estradiol or with β-estradiol where appropriate by the Student T test on the means and standard deviations of various treatment groups. *** = $P < 0.0001$.

Declaration of competing interest

The authors declare that they have no conflicts of interest with publication of this manuscript.

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