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Unliganded progesterone receptors attenuate taxane-induced breast cancer cell death by modulating the spindle assembly checkpoint

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Abstract

Whether the presence of steroid receptors in luminal breast cancers renders them resistant to taxanes remains uncertain. Here we assess the role of progesterone receptors (PR) on taxane-

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induced cell death. We previously showed that estrogen receptor (ER)-positive human breast cancer cells that inducibly express PR-A or PR-B isoforms were protected from taxane-stimulated apoptosis when compared to the identical cells lacking PR. Surprisingly, PR-dependent protection occurred in the absence of progesterone, demonstrating that the unliganded receptors were biologically active. The present studies demonstrate that unliganded PR, focused on PR-A, protect breast cancer cells from taxane-stimulated apoptosis. The studies identify genes regulated by taxanes in isogenic ER-positive cells that either lack or express PR-A. We show that unliganded PR-A alters the gene expression pattern controlled by taxanes, especially multiple genes involved in the spindle assembly checkpoint, a group of proteins that insure proper attachment of microtubules to kinetochores during mitosis. Importantly, taxanes and unliganded PR regulate many of these genes in opposite directions. As a result, mitotic slippage is exacerbated by the presence of PR, leading to an increase in the number of multinucleated cells both in vitro and in xenograft tumors. We describe a simple new assay for assessing multinucleation in paraffin sections. We speculate that rather than inducing cell death, unliganded PR exploits multinucleation to promote cell survival from taxane therapy. This can be prevented with antiprogesterin.

Keywords

Breast cancer; Progesterone receptor; Taxane; Spindle assembly checkpoint; Cell death; Multinucleation

Introduction

Taxanes are among the most effective chemotherapeutic agents for metastatic breast cancer, producing significant benefits even in patients whose disease is resistant to other drugs [1–3]. The taxanes, docetaxel (Dx), and paclitaxel (Px) are microtubule stabilizing agents that bind polymerized β -tubulin, prevent microtubule depolymerization, induce G₂/M arrest, and promote apoptosis [4]. Although general mechanisms of taxane-induced microtubule stabilization are known, many details remain incompletely understood. For example, in addition to stabilizing microtubules and inducing apoptosis, taxanes target other pathways including transcription, the mitotic cell cycle and growth [5–7], and enrichment of drug transport proteins [4, 5]; all aspects of their mechanisms likely to be important in cancer treatments. On the other hand, neoadjuvant Dx enriches for tumor-initiating cells [8] suggesting that this important tumor-cell subpopulation is not targeted by taxanes.

The majority of breast cancers are luminal and express estrogen receptors (ER) and/or progesterone receptors (PR) [9]. Although breast cancer patients benefit from taxanes [10] it is not clear whether ER or PR-positive cancers are as responsive as receptor-negative ones. In a study of ~3000 women, a subset analysis showed a lower hazard ratio for women with steroid receptor-negative disease over women with receptor-positive disease after sequential treatment with cyclophosphamide/doxorubicin and Px [11]. In vitro, ER mediate resistance to Px by inhibiting apoptosis [12] but whether this is due to presence of the receptors or to estrogenic effects on tumor proliferation rates [13] remains unknown. Analogous to ER, PR-positive primary breast tumors appear to be less sensitive to Px than PR-negative tumors; both the presence of PR and advanced tumor stage predict resistance [14]. However, this too is controversial since a phase III trial of Px versus Px plus bevacizumab showed no difference in response based on steroid receptor status [15]. Thus, it remains unclear whether steroid receptors influence tumor responses to taxanes, and if they do, whether that influence is beneficial or harmful.

Owing to this uncertainty, we assessed the role of PR on taxane-induced human breast cancer cell death. We previously showed that ER-positive cells that inducibly express PR-A or PR-B isoforms were protected from taxane-induced apoptosis when compared to the identical cells lacking PR [16]. Surprisingly, PR-dependent protection occurred in the absence of progesterone, demonstrating that the unliganded receptors were biologically active. The present studies address mechanisms by which unliganded PR, focused on PR-A, protect breast cancer cells from taxane-induced apoptosis. We identify genes regulated by taxanes in isogenic ER-positive cells that lack or express PR-A. We show that unliganded PR-A alter the gene expression pattern controlled by taxanes, especially multiple genes involved in the spindle assembly checkpoint (SAC); a group of proteins that insure proper attachment of microtubules to kinetochores during mitosis. Importantly, taxanes and unliganded PR regulate many of these genes in opposite directions. As a result, mitotic slippage is exacerbated by presence of PR, leading to an increase in the number of multinucleated cells both in vitro and in xeno-graft tumors. Rather than inducing cell death, we speculate that unliganded PR exploit this pathway to promote cell survival from taxane therapy.

Experimental methods

Cell culture

ER-positive T47D breast cancer cell lines that lack PR (T47D-Y) or stably express PR-A (T47D-YA) were described [17]. T47D-Y that inducibly express PR-A (YiA) under control of ponasterone A (ponA) (Invitrogen) were also described [16, 17]. All cell lines have been authenticated by short tandem repeat analysis. Cells were tagged with fluorescent ZsGreen as described [9]. YiA were plated at 10.5×10^3 cells/cm² in phenol red-free (PRF) MEM supplemented with 5% twice dextran-coated charcoal (DCC)-stripped fetal bovine serum (FBS) [16], treated 48 h with ponA or dimethyl sulfoxide (DMSO) vehicle to induce PR-A, then with 100 nM docetaxel (Dx), paclitaxel (Px) (LKT Laboratories, Inc.) or ethanol. Taxanes were given in phenol red and serum free RPMI for 24 h for total RNA and Caspase 3/7 Glo assays, and 48 h for cell lysates, multinucleation assays, and immunocytochemistry. The antiprogesterin ZK98299, 100 nM (Schering AG) or ethanol were added 48 h prior to, and during taxane treatments.

Gene expression profiling and data analysis

Experiments were performed in triplicate using time-separated samples. The six experimental groups were YiA with and without PR-A induction, and with and without vehicle, Dx or Px. RNA was extracted (RNAeasy; Qiagen), cRNA was labeled and hybridized to U133 Plus 2 micro-arrays (Affymetrix) as described [18]. Partek Genomics Suite (Partek Inc.) was used to normalize raw data by Robust Multichip Average followed by ANOVA to identify genes differentially expressed among groups in a significant manner ($P < 0.05$). For differentially regulated genes, a fold change cutoff of 1.1-fold was used. Gene Ontology (GO) and Venn diagrams were generated using Genespring GX 7.3.1 (Agilent Technologies).

Real-time polymerase chain reaction

Regulation of selected genes determined significant by microarray analysis were analyzed using real-time PCR. RNA was harvested using an RNAeasy kit according to manufacturer's directions (Qiagen). Amplification reactions were performed in MicroAmp optical tubes (PE ABI) on an ABI Prism 7700 sequence detector (Perkin Elmer Corp./Applied Biosystems) in a 50 µl mix containing 8% glycerol, 1X TaqMan buffer A (500 mM KCl, 100 mM Tris-HCl, 0.1 M EDTA, 600 nM passive reference dye ROX, pH 8.3 at room temperature), 300 µM each of dATP, dGTP, dCTP and 600 µM dUTP, 5.5 mM MgCl₂, 900 nM forward

primer, 300 nM reverse primer, 200 nM probe, 1.25 U AmpliTaq Gold DNA Polymerase (Perkin Elmer), 12.5 U Moloney Murine leukemia virus reverse transcriptase (Life Technologies, Inc.), 20 U RNasin ribonuclease inhibitor (Promega corp.) and the template RNA. Thermal cycling conditions were as follows: RT was performed at 48°C for 30 min followed by activation of TaqGold at 95°C for 10 min. Subsequently 40 cycles of amplification were performed at 95°C for 15 s and 60°C for 1 min. Following amplification, real-time data acquisition and analysis were performed. The primers and probes used were as follows: BUB1 Forward (fwd): 5'-CAAACACAT CACTGGAATGGT-3', Reverse (rev): 5'-TGCACGGTG GGTGATGG-3', BUB1 TaqMan Probe (TMP) 5'-CAGGC AACGCCATCCAAAGTGCA-3'; CDC20 fwd: 5'-AGTA CCCAACCATGGCCAAG-3', rev: 5'-GGCTCATGGTCA GACTCAGGA-3', CDC20 TMP: 5'-TGGCTGAACTC AAAGGTCACACATCCC-3'; CCNB1 fwd: 5'-CTCAA TTGCAGCAGGAGCTT-3', rev: 5'-GGTAATGTTGTAG AGTTGGTGTCCA-3', CCNB1 TMP: 5'-TTGCTTAGCA CTGAAAATTCTGGATAATGGTGA-3'; CDKN1A fwd: 5'-TGGAGACTCTCAGGGTCGAAA-3', rev: 5'-CGGCG TTTGGAGTGGTAGAA-3', CDKN1A TMP: 5'-CGGCG GCAGACCAGCATGAC-3'; KLF6 fwd: 5'-CACTGGCTT GTCTCACTTACGAA-3', rev: 5'-CAGGTACGGTACCC AGCCC-3', KLF6 TMP: 5'-CATGTCGGAGCTGTTTG CCTGGGT-3'; PLAU fwd: 5'-GGCTCTGAAGTCACC ACCAAA-3', rev: 5'-CCCTGGCAGGAATCTGTTTTC -3', PLAU TMP: 5'-TGCTGTGTGCTGCTGACCCACA GT-3'; MAD2L1 fwd: 5'-CGGGAGCGCCGAAATC-3', rev: 5'-TGCCACGCTGATATAAAATGCT-3', MAD2L1 TMP: 5'-TGGCCGAGTTCTTCTCATTCGGCAT-3'; TNFA fwd: 5'-GCTTTGATCCCTGACATCTGG-3', rev: 5'-CAA GTCCTGCAGCATTCTGG-3', TNFA TMP: 5'-TCTGGA GACCAGGGAGCCTTTGGTTCT-3'. Real-time PCR was performed at least twice on time-separated independently derived samples. Statistics were performed using an unpaired *t*-test or a non-parametric Mann-Whitney test.

Immunoblotting

Cell lysates [17] were normalized to protein concentration (Bio-Rad Protein Assay Reagent), loaded on 7.5 or 15% SDS PAGE gels, separated, transferred to nitrocellulose, blocked in 5% non-fat dry milk in TBST (Tris-buffered saline, 0.3% Triton-X), and incubated with primary antibody and secondary antibodies. Blots were visualized (Western Lightning Chemiluminescence Plus, Perkin Elmer) and developed on x-ray film. Densitometry used Fluor Chem Software (Alpha Innotech). Primary antibodies: β -Actin, 1:10,000 (Sigma-Aldrich); Bub1, 1:200 (Abcam); cdc20, 1:2,000 (Novus Biologicals); Cyclophilin B1, 1:1,000 (Abcam); Mad2, 1:1,000 (MBL International Corp.); PARP, 1:1000 (Cell Signaling Technology). Secondary Antibodies: Rabbit Anti-Mouse-HRP, 1:10,000 (Sigma-Aldrich); Goat Anti-Rabbit-HRP, 1:10,000 (Sigma-Aldrich).

Immunocytochemistry and confocal microscopy

Cells were plated at 10^5 /3.5 cm dish on sterile glass coverslips and grown in PRF MEM with DCC-stripped FBS with or without ponA for 48 h, then with phenol red- and serum-free RPMI containing vehicle or 100 nM Dx for 24 or 48 h. Coverslips were fixed and processed for immunocytochemistry [16] using: PR Clone 1294, 1:100 (Dako); DAPI, 1 μ g/ml (Sigma-Aldrich); RNA Pol II (Phospho S5), 1 μ g/ml (Abcam Inc.); anti-mouse Alexa Flour 488, 1:500, or goat anti-rabbit Alexa Flour 488, 1:500 (Invitrogen). Mitotracker Red 580 (500 nM) was from Invitrogen. Confocal microscopy was previously described [16]. Multinucleation was quantified in ten 400 $\times$ fields/treatment group for three time-separated experiments, imaged on an Eclipse E600 microscope equipped with a Photo-metrics Cool Snap fx camera (Nikon).

Caspase 3/7 assay

YiA cells were plated in 96-well dishes at 5000 cells/well, with or without ponA 48 h, then with or without 100 nM Dx 24 h. Caspase 3/7 Glo (Promega) was assayed on a Microtiter Plate Luminometer (Dynex Technologies). Each experiment was performed three times in triplicate.

Cell cycle analysis

YiA cells, treated as mentioned, were suspended at 10^6 cells/ml in Krishan Stain (3.8 mM Sodium Citrate, 68.97 μ M propidium iodide, 0.01% NP40, 0.01 mg/ml RNase A), and incubated overnight at 4°C. Cell cycle analysis used a Coulter FC500 flow cytometer (Beckman) with ModFit LT software (Verity Software House).

Mitotic arrest

Cells were treated 16 h with 100 ng/ml Nocodazole (Sigma-Aldrich) or DMSO. Ten 100 $\times$ fields/treatment group were imaged (Eclipse Ti with Elements Software; Nikon Instruments Inc.). Total and mitotically arrested (rounded) cells/field were counted.

Time-lapse microscopy

PR-negative or PR-positive cells in 6 well plates were treated with or without 100 nM docetaxel for 48 h during which they were continuously imaged using a motorized stage (ASI Imaging, MS-2000) incubator (Pathology Devices, Inc) on a Nikon Eclipse Ti microscope mounted with a Nikon DS-Qi1Mc digital camera and Nikon's Perfect Focus System. Nikon Instruments Software (NIS) Elements was used to program the time-lapse movie. The incubator was controlled for humidity (95%), CO₂ (5%) and temperature (37°C). Stage coordinates were set using NIS Elements, 5 per well. Images were taken with a 10 $\times$ Phase objective of each position every 15 min for the duration of docetaxel treatment. Following imaging, 120 cells per condition were analyzed through the 48 h movie to quantify length of time in mitosis.

Xenografts

Animal procedures were performed under a protocol approved by the University of Colorado Institutional Animal Care and Use Committee. Ovariectomized athymic mice (Taconic) 4–6 weeks of age, were implanted subcutaneously with silastic pellets containing 2 mg 17 β -estradiol [9], and injected into contralateral 4th mammary glands with 10^6 ZsGreen-tagged T47D-Y or T47D-YA cells in 100 μ l Matrigel (Becton-Dickinson Biosciences). Tumor size was measured weekly and fluorescence was visualized (Illumatool 9900; Lighttools Research). Mice were randomized at 6 weeks, and injected by tail-vein on days 0, 4, and 8 with 150 μ l 5 mg/kg Dx [19] or vehicle (250 mg/ml polysorbate 80, 9.75% ethanol in PBS) and tumors were measured daily. At necropsy, tumors were removed for histology and immunohistochemistry [9].

Multinucleation in xenografts

Paraffin-embedded tumors were sectioned at 2 μ m, stained for pan-cadherin (1:50; Cell Signaling) and counterstained with hematoxylin. De-identified sections were imaged and counted for multinucleated versus total cells in ten high power fields/treatment/3 tumors.

Results

PR-A attenuate taxane-induced apoptosis

YiA cells were generated from ER-positive, PR-negative T47D-Y cells engineered to inducibly express PR-A under control of ponasterone A (ponA) [17]. Throughout these experiments the levels of induced PR-A were monitored by immunoblotting and/or immunocytochemistry both without and with taxane treatment (Supplemental Figure S1) and found to be unchanged. Uninduced YiA cells undergo apoptosis in response to Px, but when PR-A are induced, apoptosis is significantly decreased [16]. To study taxanes in more detail, apoptosis was assayed by poly-ADP-ribose polymerase (PARP) cleavage (Fig. 1a) and caspase activation (Fig. 1b). Induction of PR-A in the absence of taxanes had no effect on PARP cleavage, while both Dx and Px increased PARP cleavage. However, taxane effects were markedly reduced by PR-A induction. Staurosporine-treated HeLa cells served as a control. Since Dx was more effective than Px it was used for subsequent experiments. PARP is cleaved by caspases 3 and 7 in an apoptotic signaling cascade. Activated caspase 3/7 activity (Fig. 1b) was low in control cells, and significantly ($P = 0.023$) reduced by induction of PR-A. Dx strongly increased caspase 3/7 activity, which was significantly ($P = 0.002$) decreased by presence of PR-A. Effects of PR-A did not require progesterone and confirm that the unliganded receptors can protect against taxane-induced apoptosis. The power of our PR-inducible breast cancer model is that it allows study of the identical cells in either the absence or presence of PR-A.

PR-A do not alter proliferation

The protective effects of unliganded PR-A could be explained if the receptors elicit taxane resistance by reducing proliferation [13]. To address this, PR-negative or PR-A induced YiA cells were treated with vehicle or Dx and cell cycle phases were quantified by flow cytometry (Fig. 1c). Induction of PR-A for 48 h had no effect on cell cycle. In contrast, Dx treatment significantly altered cell cycle distribution [7], characterized by a 16% decrease in G1 ($P < 0.05$) (red bars), and a 14% increase in G2 M ($P < 0.05$) (green bars) (Fig. 1c). Importantly, expression of PR-A did not alter the cell cycle distribution of Dx-treated cells. In separate studies, both a non-radioactive MTS cell proliferation assay and live cell quantification assays also showed that PR-A do not influence proliferation (not shown). We conclude that the protective effects of unliganded PR-A against Dx-induced apoptosis cannot be explained by suppressed proliferation.

Gene expression profiling

Since both taxanes [5–7] and unliganded PR-A [6, 16, 17] modify transcription, uninduced or PR-A-induced YiA cells were treated with vehicle, Dx or Px, gene expression was profiled and duplicate gene symbols were removed. Venn diagrams (Fig. 2a) show (left) that Dx regulated 1476 genes and Px regulated 1276 genes. While some genes were uniquely regulated by Dx (487) or Px (287) the majority (989) were regulated by both taxanes. On the right are genes regulated by PR-A in the presence of taxanes: 2690 were regulated by PR-A in the presence of Dx, of which 614 were unique; 2494 were regulated by PR-A in the presence of Px, of which 418 were unique. 2076 genes were regulated by PR-A in the presence of either taxane. Further analyses focused on defining the overlap between the 989 genes regulated by both taxanes, and the 2076 genes regulated by PR-A in the presence of taxanes. This yielded 423 genes regulated by taxanes and by unliganded PR-A in the presence of taxanes (bottom Fig. 2a and Supplemental Table 1).

The 423 statistically significantly regulated probesets were further narrowed down by imposing a filter of 1.1-fold change [20], resulting in 358 regulated genes. These genes were further subcategorized into four classes based on their patterns of regulation by taxanes

and by PR in the presence of taxanes (Fig. 2b and Supplemental Table 2): Class I (146 genes) were increased by taxanes and decreased by PR-A; Class II (60 genes) were increased by taxanes and increased by PR-A; Class III (94 genes) were decreased by taxanes and increased by PR-A; and Class IV (58 genes) were decreased by taxanes and decreased by PR-A. The fold changes in gene expression were modest; the highest fold change observed with Dx treatment was threefold; however, the fold changes were consistent and repeatable.

Some genes of each class were confirmed by real-time PCR (Fig. 2c and data not shown), including tumor necrosis factor (TNFA), kruppel-like factor 6 (KLF6), plasminogen activator urokinase (PLAU), and cyclin-dependent kinase inhibitor 1A (CDKN1A). Both the fold changes and the pattern of regulation confirmed the expression profiling data. For example, TNFA was upregulated twofold by Dx and downregulated 1.6-fold by PR in the presence of Dx on the array; real-time PCR data showed 2.7-fold upregulation by Dx and 1.4-fold down-regulation by PR in the presence of Dx. In most cases realtime PCR showed a greater fold regulation of gene expression than the array. To determine functional pathways represented by each class a Gene Ontology Analysis was performed (Supplemental Table 3). Remarkably, the functional categories in Class I were overwhelmingly focused on mitosis. Class II contained genes involved in cell adhesion and JAK/STAT signaling. Class III genes included ones involved in neural development, hormone biosynthesis, and metabolism. Class IV genes were also involved in metabolism.

Regulation of spindle assembly checkpoint (SAC) genes by taxanes and PR

Detailed pathway analyses of the Class I genes counter-regulated by taxanes and PR-A showed that many were components of the SAC, which arrests mitosis until all chromosomal kinetochores are properly captured by spindle microtubules [21]. These SAC genes included: budding uninhibited by benzimidazoles 1 homolog (yeast) (*BUB1*); Cyclin B1 (*CCNB1*); cell division cycle protein 20 (*CDC20*); centromere Protein A (*CENPA*); centromere Protein E (*CENPE*); polo-like kinase 1 (*PLK1*); and survivin (*BIRC5*). PR-A also decreased expression of other SAC genes in the presence of Tx including: Aurora Kinase B (*AURKB*); budding uninhibited by benzimidazoles 1 homolog beta (yeast) (*BUB1B*); Ndc80 homolog *S. cerevisiae*(*KNTC2*); MAD2 mitotic arrest deficient-like 1 (*MAD2L1*); TTK Protein Kinase (*TTK*); and ZW10 inter-actor (*ZWINT*). Regulation of key SAC genes was confirmed by real-time PCR (Fig. 3a), showing Dx mediated upregulation of *CDC20*, *CCNB1* and *BUB1* and PR mediated downregulation of these genes. Real-time PCR also confirmed PR mediated downregulation of *MAD2L1*. Again the fold change of these genes is low; however, they are consistent between the micro-array and real-time PCR. To determine whether changes in gene expression reflect changed protein levels of Bub1, Cdc20, and Mad2, immunoblotting was performed (Fig. 3b). Bub1 was not regulated by PR-A in the absence of taxanes, but it was upregulated by Dx, then attenuated by PR-A. Cdc20 expression was slightly decreased by PR-A alone, but increased by Dx, and decreased by PR-A. Similarly, Mad2 was slightly decreased by PR-A, increased by Dx, then decreased by PR-A (Fig. 3b). While subtle, these patterns of regulation were reproducible.

SAC regulation by PR-A was tested using nocodazole, a direct activator of the SAC [22, 23]. The number of mitotically arrested cells was low (<3%) in the absence of nocodazole regardless of PR-A expression (Fig. 3c). No-codazole significantly ($P < 0.001$) increased the number of arrested cells to 46% and this was significantly ($P < 0.001$) decreased to 19% by presence of PR-A, indicating that PR mediated regulation of SAC proteins allows cells to partially bypass the SAC. Furthermore, as measured by time-lapse video analysis, PR-A significantly decreased (~1 h) the time that cells stayed in mitosis in response to Dx treatment (Fig. 3d). We postulated that the PR-mediated decrease of SAC proteins allows

cells to slip through the checkpoint. Together these data suggest that unliganded PR-A have the remarkable ability to alter the SAC both genetically and functionally.

PR-A increase multinucleation following taxane treatment in vitro

Cell cycle and cell division defects associated with dys-regulation of the SAC can result in formation of multinucleated, polyploid cells [4, 21]. To analyze this, YiA cells were rendered PR-A positive, treated with Dx, then imaged by phase contrast microscopy (Fig. 4a), and high resolution confocal microscopy (Fig. 4b). Phase contrast reveals many multinucleated cells (Fig. 4a). Shown in Fig. 4b is a cell triple-stained for mitochondria (red), nuclei (blue), and PR (green). This representative cell contained six well-structured nuclei, all of which expressed PR-A, with no sign of DNA fragmentation or condensation associated with apoptosis. Indeed, multinucleated cells (Fig. 4c, d; arrows) were transcriptionally active as shown by the punctate pattern of nuclear staining for activated RNA-Polymerase II (green). The percent of multinucleated cells in response to Dx treatment of PR-A negative and PR-A positive cells was quantified in >650 total cells per condition (Fig. 4e). In control PR-negative, Dx untreated cells, <1% were multinucleated and PR-A induction alone had no effect. Dx significantly ($P < 0.05$) increased the number of multinucleated cells to 6.5% of total (red bar). This was significantly increased further ($P = 0.002$) to 17% by unliganded PR-A (blue bar). To show that this was a PR-specific effect, the actions of the receptors were blocked with the pure antiprogesterin, ZK98299. The antagonist completely prevented the PR-A induced component of multinucleation without affecting the Dx-induced component (Fig. 4e). Thus, in the presence of Dx, a multinucleation pathway is activated that is exacerbated by unliganded PR.

PR attenuate solid tumor response to Dx

We next tested whether the protective effects of PR-A could be recapitulated in vivo. These studies used isogenic ZsGreen-tagged PR-negative T47D-Y cells or stable PR-A-positive T47D-YA cells [9]. One million cells in Matrigel were injected into bilateral 4th mammary glands of ovariectomized immunocompromised mice supplemented with 17β -estradiol-releasing pellets. After 6 weeks, tumor-bearing mice were randomized and intravenously injected with vehicle or 5 mg/kg Dx, every 4 days for 3 cycles. Tumor response to therapy in breast cancer patients is calculated by measuring tumor volume pre- and post-treatment. Similarly, tumor size was measured prior to and following Dx treatments and the percent change in tumor volume was calculated (Fig. 5a). In the absence of Dx, tumors grew well whether or not they contained PR-A. In the absence of PR-A, Dx treatment strongly suppressed tumor volume ($P = 0.001$), but Dx was much less effective ($P = 0.033$) in PR-A-positive tumors. This confirmed the in vitro data and demonstrated in solid tumors that unliganded PR promote taxane resistance.

PR-A increase multinucleation following taxanes in vivo

Tumor sections from vehicle or Dx-treated PR-negative and PR-positive xenografts were examined histologically and quantified for multinucleation by an assay we developed for this purpose. The tumors were Bloom-Richardson Grade III carcinomas with cellular morphology quite similar among the four groups: tumors were composed of markedly enlarged pleomorphic cells growing predominantly in cords and nests, with areas of solid growth. There were abundant mitoses present, as well as areas of necrosis and apoptosis. Tumors were thinly (2 μ m) sectioned to generate single-cell layers, then stained with pan-cadherin to mark plasma membranes and counterstained with hematoxylin to visualize nuclei. Examples of multinucleated (black arrows) and mononucleated (yellow arrows) cells in two images from PR-positive Dx-treated tumors are shown in Fig. 5b. Multinucleation was quantified as a percent of total cells (Fig. 5c). Vehicle-treated PR-

negative and PR-positive tumors lacked multinucleated cells and were not statistically different from one another. Dx treatment significantly ($P < 0.001$) increased the percent of multinucleated cells to $>3.5\%$, which were further increased ($P = 0.023$) by PR-A expression, confirming the in vitro results (Fig. 4e).

Discussion

Taxanes and gene regulation

There are several predictors of taxane resistance including mutations and altered expression of tubulin isotypes, overexpression of the drug efflux pumps MDR-1 and MRP1, and overexpression of microtubule-associated proteins including Tau, Stathmin, and MAP4 [4]. In an attempt to identify other pathways that interfere with taxanes, investigators have analyzed gene expression profiles of human breast cancer cell lines and clinical samples [5, 7, 24–27]. Two studies used ER+ PR+, MCF7 cells and ER-PR-, MDA-MB-231 cells. Thirty-two genes, many involving the SAC, were regulated by Dx [7] or Px [5] in MCF7 cells and also regulated in our ER+ PR+ T47D models, including: cell division cycle 2 (*CDC2*); cell division cycle 6 (*CDC6*); cyclin-dependent kinase 2 (*CDK2*); *BUB1B*; *MAD2L1*; cyclin A2 (*CCNA2*); centromere protein A (*CENPA*), and the taxane resistance genes, caspase-7 (*CASP7*) and tubulin beta 2 (*TUBB2*). Six of our genes overlapped with taxane-regulated genes in MDA-MB-231 cells.

Three studies profiled genes following taxane mono-therapy of breast cancer patients. A 92 gene signature predicts response to Dx in a group of pre- and post-menopausal women [24]. However, potentially important predictor genes expressed at low levels, such as aurora kinase A (*AURKA*) were excluded from the study [24]. A second study identified overexpression of cellular redox pathways in a group of pre- and post-menopausal breast cancers as markers of Dx resistance [25]. A third found 51 genes differentially expressed between Dx-responding and non-responding breast cancers, with 9 genes identified as predictive of response [26]. 21 genes in our studies overlapped with genes reported in these studies, including interleukin-6 (*IL6*); survivin (*BIRC5*); catenin beta 1 (*CTNNB1*); and serpin peptidase inhibitor clade A member 3 (*SERPINA3*). Most clinical studies, however, combine taxanes with other chemotherapeutic agents making it difficult to ascribe their regulation to taxanes specifically [27].

Chemotherapy resistance and the SAC

Our study is the first to assess the role of PR in transcriptional and cellular responses to taxanes and focuses on luminal breast cancers. We find that a large number of SAC genes, which have become important targets of therapy [28–31], were regulated by taxanes and counter-regulated by PR-A. Examples include: *BIRC5*, *CENPE*, and *PLK1*. Aurora kinases also play a key role by mediating micro-tubule attachment to centromeres and specific aurora kinase inhibitors are now in clinical trials [31]. SAC genes are also important in development, and homozygous null knockouts for several such genes, including *AURKA* [32], *BUB1* [33], *BUB3* [21], *BUBR1* [21], *CENPE* [34], and *MAD2L1* [21] are embryonic lethal. Key evidence that SAC proteins play a role in tumorigenesis comes from mice that are heterozygous or hypomorphic for SAC genes. Such mice survived embryonic development but were prone to tumor formation later in life [33, 35]. However, the direction of SAC protein expression and the consequences of this remain controversial. Studies implicate decreased expression of SAC proteins in carcinogenesis, increased chromosomal instability, decreased senescence, and increased anchorage-independent growth [36]. Along those lines, colorectal cancers with increased chromosomal instability have decreased levels of non-mutated BUB1 [37]. Knockdown of Mad2, a SAC protein, increased multinucleation [38]. For antimicrotubule drugs like taxanes, a weakened SAC can lead to apoptotic

resistance [21]; a major hurdle in successful treatment outcomes. On the other hand, some studies find that an increase in SAC proteins in breast cancer cell lines correlates with chromosomal instability [39]. For instance, overexpression of Mad2 in an inducible mouse model increased chromosomal instability and promoted tumorigenesis [40]. Amplification of Aurora A in HeLa cells decreased Px-induced apoptosis by circumventing the SAC [41]. Therefore, appropriate expression of SAC proteins, which balance is modified by taxanes and PR, significantly affects cell survival.

Multinucleation: a survival mechanism of taxane resistant breast cancer cells

We demonstrate that in taxane-treated PR-A positive cells, modification of SAC protein expression results in decreased cell death and increased multinucleation (Figs. 1, 3, 4, 5). Interestingly, time-lapse video of individual cells shows multinucleation is not due to cell-cell fusion but rather nuclear division without cytokinesis, further implicating the SAC in multinucleation. We speculate that this is a mechanism by which breast cancers survive taxane treatments. While multinucleation is physiologically normal for cells such as osteoclasts, hepatocytes and skeletal muscle, for most normal cells it is a consequence of aberrant mitosis and results in cell death. This may not be the case for cancer cells, however, where multinucleation is often associated with resistance to a variety of chemotherapeutic agents, including aurora kinase inhibitors [42] and taxanes [43–46]. Px-resistant MCF7 breast cancer cells exhibit a higher incidence of multinucleation than their Px-sensitive counterparts [43] and Px-resistant PC-3 prostate cancer cells are also reportedly multinucleated [44]. The mechanisms underlying multinucleation are unclear, but decreased caspase activity is one proposed cause [44]. Interestingly, we observed decreased caspase activation in PR-A positive cells (Fig. 1b). Another proposed multinucleation mechanism is faulty cytokinesis associated with inappropriate formation of a nuclear envelope around groups of chromosomes in response to degradation of the cell cycle regulator cyclin B1 [47]. Multinucleation can also supply a new diploid population of malignant cells through de-polyploidization [45]; a process that may restore normal mitosis. We speculate that in this respect, multinucleation can serve as a mechanism for cell survival following chemotherapy. There is evidence for that in p53 mutant Burkitt's lymphoma cells, where polyploidy is an alternative to mitotic catastrophe, and multinucleation associated with de-polyploidization, follows irradiation [48]. These cells repair the resultant double-strand DNA breaks [49] and Aurora B likely plays a role in their survival [48]. The p53 mutant chronic myelogenous leukemia cell line K562 also developed polyploidy in response to taxol resulting in taxol-resistance [46]. This is consistent with our results (Figs. 1, 4, 5) where PR-A positive cells exhibited increased multinucleation following Dx treatment, associated with decreased apoptosis and diminished response to Dx in vivo.

We propose (Fig. 6) that taxane-treated breast cancers, which SAC protein levels are attenuated by presence of PR-A, fail to appropriately maintain mitotic arrest and instead slip into multinucleation, which can lead to mitotic catastrophe in some cases [6, 50] or to cell survival in others [43, 45]. We speculate that our results represent an example of the latter, and that multinucleated cells give rise to cells that are capable of repopulating the tumor.

Combinatorial taxane and antiprogesterone treatment of PR-positive breast cancer

If, as we propose, multinucleation is a mechanism for cancer cell survival to chemotherapeutic drugs, and we find that multinucleation induced by taxanes is exacerbated by the presence of PR, then blocking PR function with an antiprogesterone could increase the efficacy of taxanes. Indeed, we find that the pure antiprogesterone ZK98299 suppresses the PR-dependent multinucleation component (Fig. 4e). Importantly ZK98299 does not alter ER or PR levels [51] and has decreased antiglucocorticoid activity and lacks antiandrogen activity

often ascribed to earlier antiprogestins [52]. ZK98299 therefore allows us to conclude that the tumor-cell protective effects we observe are due to the presence of unliganded PR.

Clinical studies of antiprogestins in breast cancer are few. In a preliminary treatment trial of refractory breast cancers in post-menopausal or oophorectomized women, the antiprogestin RU486 showed an 18% response rate after 3 months, where all responders with known steroid receptor status were ER+ PR+ [53]. Third generation antiprogestins like ZK230211 are in Phase II trials for metastatic breast cancers in post-menopausal women [54]. These newer antagonists might be extremely useful in combination with taxanes to improve therapeutic outcomes in luminal disease.

In summary, we show that unliganded PR alter transcriptional regulation by taxanes, modifying the SAC and increasing multinucleation. We propose that this is a tumor-cell survival mechanism. If so, it would explain the inadequate response of luminal breast cancers to taxane therapies, and suggests that taxane responsiveness could be improved by blocking the actions of unliganded PR with antiprogestins. While PR are routinely measured in breast cancers, multinucleation is rarely analyzed. We have developed a novel method (Fig. 5b) using extremely thin sections to eliminate depth-of-field issues, coupled with a plasma membrane stain, to quantify multinucleated cells in solid tumors. This assay could be easily adopted by pathology laboratories.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

PR attenuate taxane-induced apoptosis but do not affect the cell cycle. YiA cells were treated 48 h with vehicle or ponA to induce PR-A, followed by 24–48 h with vehicle, docetaxel (Dx), or paclitaxel (Px). **a** Whole-cell extracts of 48 h taxane-treated cells were resolved by SDS-PAGE and immunoblotted with an anti-PARP antibody. Staurosporin-treated HeLa cells were the positive control. Tubulin served as the loading and densitometry control. **b** Caspase 3/7 Glo assay reporting activity in relative light units (RLU) using 24 h Dx-treated cells. Data representative of 3 independent experiments are shown. **c** Cells were harvested after 48 h of Dx, stained with propidium iodide and sorted by flow cytometry. The average of 3 time-separated experiments is shown. ANOVA was performed with Tukey post-test; *and # equal $P < 0.05$

Fig. 2.

Gene expression profiling of taxane and PR regulated genes. YiA cells were treated 48 h with vehicle or ponA to induce PR-A, followed by 24 h 100 nM Dx, Px, or vehicle. mRNAs were extracted, cRNA was labeled and hybridized to a U133 plus 2.0 Affymetrix gene expression microarray. Data were analyzed in Partek Genomics Suite and Genespring at $P < 0.05$ using a 1.1-fold change cutoff. **a** Venn diagrams show the number of genes regulated by: (1) Dx or Px in the absence of PR; (2) PR-A in the presence of Dx or Px; (3) both taxanes (Tx) and PR-A in the presence of Tx. **b** Pie chart showing breakdown of 358 genes regulated by Tx and PR-A in the presence of Tx. Arrows indicate the direction of transcriptional regulation. **c** Real-time PCR was performed on multiple genes, at least twice. Four representative genes are shown (TNFA, KLF6, PLAU, CDKN1A) * $P < 0.05$ from control (none). + $P < 0.05$ for Px + PR versus Px. # $P < 0.05$ for Dx + PR versus Dx

Fig. 3.

Spindle Assembly Checkpoint proteins are counter-regulated by taxanes and PR. **a** Real-time PCR was performed on multiple spindle assembly checkpoint genes, at least twice. Four representative genes are shown (*CDC20*, *MAD2L1*, *CCNB1*, and *BUB1*) * $P < 0.05$ from control (none). # $P < 0.05$ for Dx + PR versus Dx. **b** PR-negative Y cells and PR-positive YA cells were treated 48 h with Dx where indicated. Whole-cell extracts were resolved by SDS-PAGE and probed with antibodies directed against Bub1, Cdc20, and Mad2. Cyclophilin B1 served as a loading control. A representative blot of three is shown. **c** Y and YA cells were treated 16 h with 100 ng/ml nocodazole where indicated. The number of mitotically arrested cells was quantified in ten 100 $\times$ fields of 3 time-separated experiments; >4500 cells were counted per condition. A representative experiment is shown. **d** PR-negative Y cells and PR-positive YA cells were treated 48 h with Dx under timelapse microscopy observation. The amount of time spent in mitosis was quantified for 120 cells per condition

Fig. 4.

PR increase viable multinucleated cells following Dx. YiA cells were treated 48 h with vehicle or ponA to induce PR, followed by 48 h with vehicle or Dx. **a** 400× Phase contrast image of PR-positive multinucleated cells overlapped with an image counterstained with DAPI. **b** Confocal image stained for mitochondria (Mitotracker Red), PR (green), and DAPI (blue). **c, d** 1000× images counterstained with DAPI (**c**; blue) and stained for Phosphorylated RNA-Polymerase II (**d**; green). Arrows show multinucleated cells. **e** Percent of multinucleated cells among total cells were quantified in ten 400× fields for each condition, each in triplicate. A subset of cells was treated as above plus vehicle or 100 nM

ZK98299 for the 96 h course of the experiment. The average of 3 independent experiments is shown

Fig. 5. PR attenuate Dx response and increase multinucleated cells in vivo. Immunocompromised, ovariectomized, and estrogen-supplemented mice were injected in contralateral #4 mammary glands with ZsGreen-tagged PR-negative or PR-A positive T47D human breast cancer cells. After 6 weeks, tumor-bearing mice were randomized and injected intravenously 3 times, 4 days apart, with vehicle or 5 mg/kg Dx. Tumor volume was measured every 7 days and daily during Dx treatments. **a** Average percent change in four experimental groups of 5 (vehicle) or 6 (Dx) mice/group. Statistical significance was determined by *t*-test. **b** 3 days after last treatment, tumors were paraffin-embedded, sectioned at 2 μ m, and stained for pan-cadherin, with hematoxylin counterstaining. Multinucleated cells were quantified in ten fields from 3 separate tumors. Two 1000 $\times$ fields of PR-positive Dx-treated tumors are shown. *Black arrows* show multinucleated cells; *yellow arrows* mononucleated cells. **c** Percent multinucleated cells in xenograft tumors, showing significance (*t*-test). *N* = 3 tumors per group

Fig. 6.

Model of PR action in response to taxanes. Taxanes stabilize microtubules, altering microtubule tension and activating the SAC. The SAC orchestrates mitotic arrest, which under normal circumstances allows the cell to correct spindle attachment problems or to accumulate death signals and undergo apoptosis [50]. We propose that unliganded PR allow cells to slip out of mitotic arrest prematurely resulting in microtubule attachment problems and failure to accumulate death signals. This results in multinucleated cells that either die by mitotic catastrophe [47, 50] or survive [43–46, 49, 55, 56] leading to drug resistance