Cancer Therapy: Preclinical

Glucocorticoid Receptor Antagonism as a Novel Therapy for Triple-Negative Breast Cancer

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Abstract

Purpose: Triple-negative breast cancer (TNBC) accounts for 10% to 20% of newly diagnosed invasive breast cancer. Finding effective targets for chemotherapy-resistant TNBC has proven difficult in part because of TNBC’s molecular heterogeneity. We have previously reported that likely because of the antiapoptotic activity of glucocorticoid receptor (GR) in estrogen receptor (ER)-negative breast epithelial and cancer cells, high GR expression/activity in early-stage TNBC significantly correlates with chemotherapy resistance and increased recurrence. We hypothesized that pretreatment with mifepristone, a GR antagonist, would potentiate the efficacy of chemotherapy in GRþ TNBCs by inhibiting the antiapoptotic signaling pathways of GR and increasing the cytotoxic efficiency of chemotherapy.

Experimental Design: TNBC cell apoptosis was examined in the context of physiologic glucocorticoid concentrations, chemotherapy, and/or pharmacologic concentrations of mifepristone. We used high-throughput live microscopy with continuous recording to measure apoptotic cells stained with a fluorescent dye and Western blot analysis to detect caspase-3 and PARP cleavage. The effect of mifepristone on GR-mediated gene expression was also measured. TNBC xenograft studies were performed in female severe combined immunodeficient (SCID) mice and tumors were measured following treatment with vehicle, paclitaxel, or mifepristone/paclitaxel.

Results: We found that although mifepristone treatment alone had no significant effect on TNBC cell viability or clonogenicity in the absence of chemotherapy, the addition of mifepristone to dexamethasone/paclitaxel treatment significantly increased cytotoxicity and caspase-3/PARP cleavage. Mifepristone also antagonized GR-induced SGK1 and MKP1/DUSP1 gene expression while significantly augmenting paclitaxel-induced GRþ MDA-MB-231 xenograft tumor shrinkage in vivo.

Conclusions: These results suggest that mifepristone pretreatment could be a useful strategy for increasing tumor cell apoptosis in chemotherapy-resistant GRþ TNBC. Clin Cancer Res; 19(22); 6163–72. ©2013 AACR.

Introduction

Glucocorticoids (GCs) are secreted from the adrenal gland in response to exposure to emotional and physiologic stressors and are responsible for modulating essential metabolic, cardiovascular, immune, and behavioral functions (1–3). The glucocorticoid receptor (GR) belongs to a family of nuclear hormone receptors that are ligand-dependent transcription factors involved in activating and repressing gene expression, thereby changing the complement of proteins regulating key signaling pathways (4, 5). In its ligand-bound state, GR initiates or represses gene expression in a cell-type–specific manner (1). For example, GR activation can induce apoptosis in lymphocytes (6, 7), whereas its activation results in inhibition of apoptosis in breast epithelial cells (8). Furthermore, in a TNBC xenograft model, the activation of tumor GR by dexamethasone (dex), a synthetic GC, diminished chemotherapy effectiveness in vivo (9). Until now, the use of a GR antagonist in an in vivo model of GRþ triple-negative breast cancer (TNBC) has not been reported.

It was previously shown by our group and others that GR activation initiates potent antiapoptotic signaling pathways in breast epithelial cells, at least in part, via transcriptional regulation of genes encoding cell survival pathway proteins (5, 10–12). For example, genes encoding the antiapoptotic proteins serum and glucocorticoid-inducible protein kinase-1 (SGK1) and mitogen-activated protein kinase phosphatase-1 (MKP1/DUSP1) are both...
Translational Relevance

Triple-negative breast cancer (TNBC) lacks effective targeted therapies. Approximately 25% of invasive TNBCs are glucocorticoid receptor (GR)-positive (>10% of tumor cells strongly GR-positive by immunohistochemistry (IHC) and/or significantly increased tumor NR3C1 (GR) mRNA levels compared with median TNBC NR3C1 expression). High tumor GR expression significantly correlates with earlier relapse in early-stage TNBC. Mifepristone is a potent GR and progesterone receptor (PR) modulator. Here, we report that in GR+ TNBC (which lacks expression of the PR), pretreatment with mifepristone potentiates paclitaxel-induced cytotoxicity, presumably by antagonizing GR-activated TNBC cell survival pathways that otherwise contribute to chemotherapy resistance.

Experimental Procedures

Materials

Paclitaxel (Sigma Cat. No.T7402) and dexamethasone (Sigma Cat. No. D4902) were purchased from Sigma-Aldrich. Initially, mifepristone was purchased from Enzo Life Sciences (Cat. No. BML-S510-0025) and later experiments were repeated with pharmaceutical-grade mifepristone provided by Corcept Therapeutics. For in vitro experiments, pharmaceutical-grade paclitaxel liquid suspension was purchased from Bedford Laboratories.

Cell culture

MDA-MB-231, BT-20, and MDA-MB-468 cell lines were purchased from American Type Culture Collection. MDA-MB-231 and BT-20 cells were cultured in Dulbecco’s modified Eagle Medium (DMEM; Lonza) and MDA-MB-468 cells in RPMI-1640 (Thermo Fisher Scientific), both supplemented with 10% fetal calf serum (FCS; Gemini Bio-Products) and antibiotics (1% penicillin-streptomycin, Lonza). All cell lines were cultured at 37°C in a humidified atmosphere with 5% CO2. Before treatment with glucocorticoids, mifepristone, and/or chemotherapy, cells were grown in DMEM or RPMI-1640 supplemented with 2.5% charcoal-stripped FCS and 1% penicillin-streptomycin.

Clonogenic assay

MDA-MB-231 cells (10,000 cells per 10 cm dish) were allowed to adhere overnight in DMEM supplemented with 10% FCS. Media was then changed to 2.5% CS-FCS for 48 hours. Cells were treated with vehicle (EtOH 0.1% v/v), dexamethasone (100 nmol/L) or mifepristone (100 nmol/L) alone or dexamethasone/mifepristone (100 nmol/L) 1 hour before paclitaxel (100 nmol/L) treatment for 72 hours. A cyanine dimer nucleic acid dye, YOYO-1 (Life Technologies, Y3601) that causes green fluorescence if the cellular membrane is compromised was used to detect dead cells. Two images (1.90 × 1.52 mm) in separate regions of each well were captured with a 10× objective at 4-hour intervals using the IncuCyte FLR HD real-time in vitro micro-imaging system (Essen Instruments). Cell death (detected as YOYO-1-positive) and total cell counts (phase contrast) were measured computationally by ImageJ Version 1.46r (16) using investigator-coded software for analysis (Supplementary Method S1). The “cytotoxic index” represents the number of dead cells/total cells for each image.

Images collected between 12 and 72 hours were used in the analysis. The cytotoxic index was log-transformed to satisfy the normality assumption. Data were analyzed using repeated measures analysis of variance models. A separate model was fitted for each cell line. The fixed effects included treatment, time, time2, time3, and all corresponding interactions between treatment and time terms. Random effects included random intercept terms for biologic and technical replicates and a random slope for the biologic replicate. Correlation between serial measurements was modeled using AR (1) covariance structure. A generalized F-test was used to test the composite hypothesis of no difference between treatment, trt × time, trt × time2, and trt × time3, effectively comparing the entire curves over time. Analyses were performed in SAS 9.2.

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A mixed-effects analysis of variance model was fitted, with colony count as the responsible variable, treatment group as the fixed effect, and biologic replicate and technical replicate nested within the biologic replicates as the random effects. Dexamethasone/paclitaxel versus dexamethasone/mifepristone/paclitaxel groups were compared on the basis of this fitted model, and the reported P values are unadjusted for multiple comparisons.

Quantitative real-time PCR
MDA-MB-231, MDA-MB-468, and BT-20 cells were seeded at approximately 50% confluence and allowed to adhere overnight in DMEM with 10% FCS, then cultured in 2.5% CS-FCS for an additional 48 hours. Media were removed and equal volumes of either vehicle (ethanol), dexamethasone (100 nmol/L) or dexamethasone/mifepristone (100 nmol/L FC) diluted in DMEM or RPMI supplemented with 2.5% CS-FCS was then added. After 4 hours of treatment, 100 μL of RNA-Solv Reagent (EaZy Nucleic Acid Isolation Kit) supplemented with 2% 2-mercaptoethanol was added to each well to harvest RNA. Total RNA was extracted using the Qiagen All-Prep DNA/RNA Mini Kit. cDNA was then reverse transcribed from 0.5 μg of total RNA with Quanta reverse transcription reagents (Quanta Biosciences) using the GeneAmp PCR System 9700 (Applied Biosystems) per manufacturer’s instruction. The cDNA was diluted in PerfeCTa SYBR Green FastMix (Quanta Biosciences), and quantitative real-time PCR (qRT-PCR) was carried out in a BioRad PCR System MyiQ (BioRad Life Sciences). The following primers were used: SGK1, 5'-AGGCCCCATCTCTTCTGCTT-3' (forward) and 5'-TTCACTGCTCCC-3' (reverse); MKP1/DUSP1, 5'-CCTGACAGTGTTGTTGCTGTT-3' (forward) and 5'-GATTTCCACCGGGC-3' (reverse); NRI3/GR, 5'-TCTGAACTCCCTGTTGC-3' (forward) and 5'-GTGGTCTCCTGTTGCTGTT-3' (reverse); Actin-B, 5'-CAGCGGAACCGCTCATTGCCAATGG-3' (forward) and 5'-GTCACCTCTGCCATCTACGA-3' (reverse). The samples were loaded in duplicate. Relative quantification of gene expression was calculated according to the standard curve method, as described by Applied Biosystems User Bulletin 2, October 2001, based on the ΔACi approach (15). A ratio of GR target gene expression to Actin-B expression was calculated.

For SGK1 and MKP1/DUSP1 qRT-PCR analysis, a mixed-effects ANOVA model was fitted with Ct as the response variable: treatment, gene type (target or reference), and treatment × gene interaction as the fixed effects and replicate as the random effect. A linear contrast was then constructed to estimate ΔACi and its confidence interval, and the results were exponentiated to obtain the estimate of 2−ΔACi and its confidence intervals; 68% confidence intervals, corresponding to ± SEM under the normality assumption. Analyses were performed in SAS 9.2.

Antibodies and Western blotting
Cells were allowed to adhere overnight in media containing 10% FCS. The following day, media were changed to 2.5% CS-FCS and cultured for 48 hours, then lysed in buffer containing phosphatase and protease inhibitor cocktails (Roche). Protein concentrations were measured using the BCA Assay Kit (Thermo Scientific) and 2× Laemmli buffer supplemented with 5% 2-mercaptoethanol was added to an equivalent volume of protein lysate. Total proteins (60 μg per lane) were resolved by SDS-PAGE and then transferred to polyvinylidene difluoride membranes (Bio-Rad). After the membranes were washed 3 times, they were incubated with 5% bovine serum albumin (BSA; Fisher Scientific) in 0.1% Tween20 in TBS (TBS/T) for 1 hour at room temperature, followed by overnight incubation at 4°C with primary antibody against human GR-alpha (GR-XP Cell Signaling), caspase-3 (Cell Signaling Technology), PARP (Cell Signaling Technology), or human β-Actin (Sigma-Aldrich). After additional washing, membranes were incubated for 1 hour at room temperature with either Alexa Fluor 680 goat anti-rabbit (Invitrogen) or 800 goat anti-mouse (LI-COR) secondary antibody, rinsed and scanned using the Odyssey infrared imaging system (LI-COR) at a wavelength of 700 or 800 nm.

Mammary fat pad xenograft studies in female SCID mice
All experiments were carried out in accordance with the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals and approved by the University of Chicago Institutional Animals Care and Use Committee. Suspensions of MDA-MB-231 cells (1 × 107) in 50 μL of PBS were injected subcutaneously into the right pectoral mammary fat pad of a 5- to 6-week-old female SCID mouse (Taconic). Tumors were allowed to reach ~200 mm3 and then mice were treated via intraperitoneal (i.p.) injection with paclitaxel (10 mg/kg) suspended in castor oil (1:10 v/v); vehicle-treated animals received two injections and paclitaxel-treated animals received an additional injection of mifepristone (15 mg/kg/d) or vehicle (ethanol and sesame seed oil 1:10 v/v) 1 hour before the paclitaxel (17). The longest (L) and shortest (S) diameters of the tumors were measured 3 times a week with electronic calipers, and tumor volume was calculated using the formula for an ellipsoid sphere: S2 × L × (0.52; ref. 9). Tumors were dissected and cut lengthwise into mirror image sections. One section was minced in lysis buffer and frozen (for protein analysis), and the other was fixed in 10% neutral-buffered formalin [for IHC and immunofluorescence (IF)]. Tumor growth data were analyzed using repeated measures analysis of variance models as previously described (9).

Histopathological examination
For anti-GR IF analysis of tumor xenografts, samples were formalin-fixed for 24 hours and embedded in paraffin immediately after necropsy. Sections (5 μm thick) were adhered to positively charged slides, dewaxed in xylene, and hydrated using graded ethanol washes. Heat-induced antigen retrieval was performed using Tris-EDTA Buffer (10 mmol/L Tris base, 1 mmol/L EDTA solution, pH 9.0) incubation in a pressure cooker for 3 minutes. After 30 minutes of blocking in 10% normal goat serum in PBS,
slides were incubated with either a 1:100 dilution of anti-GR rabbit polyclonal antibody (Santa Cruz Biototechnology, H-300 sc-8992) followed by a secondary Alexa Fluor goat anti-rabbit IgG (Cell Signaling Technology).

Entire scans of each tumor section were captured using the CRi Panoramic Whole Slide Scanner (PerkinElmer Life Sciences). Twenty random individual images of each scan were then analyzed from different locations of the slide. The proportion of GR+ staining cells over the total cell count for each image was calculated (details in Supplementary Method S2) and log-transformed to satisfy the normality assumption. A mixed-effects model was fitted, with treatment as the fixed effect, and tumor as the random effect, to account for the correlation between multiple images of the same tumor section.

Results

Mifepristone enhances paclitaxel-induced TNBC cell death

We have previously showed that high GR-expressing ER-negative breast cancer cells exposed to physiologic stress dose concentrations of glucocorticoids (1 μmol/L) are relatively resistant to chemotherapy-induced cell death (13). Here, we examined whether or not pharmacologically relevant concentrations of mifepristone (100 nM) could reverse the cytotoxic effects of physiological glucocorticoid concentrations (100 μmol/L) in MDA-MB-231, MDA-MB-468, and BT-20 TNBC cell lines (18). We used the IncuCyte system, a real-time microscopic-imaging system (16), to determine the percentage of cell death continuously over several days using software outlined in Supplementary Method S1. Cells were treated with vehicle (EtOH 0.1% V/V), dexamethasone (100 nM), mifepristone (100 nM), or dexamethasone/mifepristone (100 nM) 1 hour before paclitaxel (100 nM) before being placed in the IncuCyte assay system. Figure 1A shows the percentage of cell death measured over several days. The addition of mifepristone to MDA-MB-231 (Fig. 1A, top) and BT-20 (Fig. 1A, middle) cells significantly reversed the protective effect of physiologic glucocorticoid concentrations (dexamethasone, 100 nM). The addition of mifepristone to MDA-MB-468 cells also increased cell death from dexamethasone/paclitaxel, although not significantly (P = 0.68; Fig. 1A, bottom). Interestingly, treating MDA-MB-231, BT-20, and MDA-MB-468 cells with mifepristone alone (no chemotherapy) had no significant effect on either apoptosis (Fig. 1) or cell proliferation (Supplementary Fig. S1). A representative image of cells before counting is shown in Supplementary Fig. S2. Figure 1B shows images from the IncuCyte detection system where green cells reflect apoptotic cells reported in Fig. 1A (19). Trypan blue exclusion assays (Supplementary Fig. S3) further supported the conclusion that mifepristone treatment partially reversed GR-mediated protection from chemotherapy-induced apoptosis in TNBC.

We next measured GR (NR3C1) mRNA transcript expression in MDA-MB-231, MDA-MB-468, and BT-20 cells by qRT-PCR (Fig. 1C) and total GR protein expression by Western blot analysis (Fig. 1D). Q-RT-PCR confirmed expression of GR (NR3C1) mRNA levels in all 3 cell lines. The highest total GR mRNA and protein levels were found in MDA-MB-231 cells; interestingly, several translational isoforms (GR-A, GR-B, GR-C, GR-D) were also observed in all three cell lines (20, 21).

Mifepristone induces caspase-3–associated cell death

The molecular mechanisms underlying GR-mediated cell survival in the context of chemotherapy-induced apoptosis are not well understood (12). We therefore characterized caspase-3 and PARP cleavage in association with paclitaxel-induced apoptosis. We found that chemotherapy-induced apoptosis and accompanying caspase-3 and PARP cleavage increased following the addition of mifepristone (Fig. 2). The increased cleavage of caspase-3 and PARP is seen after 24 hours of treatment but enhanced after 48 hours of treatment. A second biologic replicate of caspase-3 cleavage at 24 and 48 hours is reported in Supplementary Fig. S4. These data suggest that mifepristone reverses GR-mediated cell survival, at least in part, through blocking an apoptotic mechanism involving increased cleavage of caspase-3 and PARP.

Mifepristone antagonizes GR-mediated gene expression in TNBC cell lines

SCK1 and MKP1/DUSP1 genes are directly upregulated by GR transactivation in mammary epithelial cells (2). To examine whether mifepristone antagonizes this GR-mediated gene expression, we treated MDA-MB-231, BT-20, and MDA-MB-468 cells with dex (100 nM) ± mifepristone (100 nM) for 4 hours. In MDA-MB-231 cells, Q-RT-PCR showed an average dex-associated 2.13-fold increase in SCK1 and 6.62-fold increase in MKP1/DUSP1 mRNA levels over vehicle alone; the dex-mediated increase in both GR target genes was significantly reversed with the addition of mifepristone (Fig. 3A). In BT-20 cells, SCK1 (2.10-fold increase) and MKP1/DUSP1 (1.70-fold increase) mRNA levels were both inhibited by mifepristone (Fig. 3B). In MDA-MB-468 cells, the dex-associated increase in SCK1 (2.85-fold increase) was significantly inhibited by mifepristone, whereas MKP1/DUSP1 (1.52-fold increase) expression was reversed although it did not meet statistical significance (Fig. 3C). These data suggest the degree of GR inhibition with mifepristone is variable depending on cell type and the particular gene studied.

Mifepristone treatment potentiates paclitaxel effectiveness in an MDA-MB-231 TNBC xenograft model

In cell lines with robust mifepristone-mediated antagonism of GR target gene expression, we hypothesized that mifepristone administered in vivo might reverse the endogenous GR activity of TNBC and increase chemotherapy sensitivity. Therefore, SCID mice bearing MDA-MB-231 GR+ TNBC mammary fat pad tumor xenografts (~200 mm3) were treated with either vehicle, paclitaxel (10 mg/kg/d), or mifepristone (15 mg/kg/d) 1 hour before paclitaxel (10 mg/kg/d) for 5 consecutive days (17). Tumors were...
then measured 3 times per week for 35 days (Fig. 4A) except for vehicle-treated animals, which were sacrificed once the tumor had reached approximately 3,000 mm$^3$. Analysis of the mean tumor volumes ± SEMs for each treatment group are shown in Fig. 4A. Repeated measures ANOVA model found a significant treatment × day interaction ($P < 0.001$) indicating significant differences in the pattern of tumor growth over time. Differences at each time point in paclitaxel versus mifepristone/paclitaxel treatment groups were evaluated using post hoc testing. Significant differences between the paclitaxel and mifepristone/paclitaxel treatment groups appeared at day 18 ($P = 0.02$) and remained significantly different throughout the rest of the experiment. The average tumor volumes for the paclitaxel cohort were 500 mm$^3$ (SEM = 13) on day 35, whereas the average tumor volumes for the mifepristone/paclitaxel cohort were 300 mm$^3$ (SEM = 12) on day 18 and 800 mm$^3$ (SEM = 12) on day 35. Finally, the generalized F-test showed a significant difference between mifepristone/paclitaxel- and vehicle/paclitaxel-treated tumors over time ($P = 0.0417$). Thus, the addition of mifepristone treatment 1 hour before chemotherapy significantly improved the efficacy of paclitaxel in MDA-MB-231 xenografts.

To explore how the addition of mifepristone treatment increases chemotherapy-induced TNBC tumor shrinkage, paraffin-embedded tumor xenograft sections were examined with a green fluorescence–labeled anti-GR antibody and stained with DAPI (representative image Supplementary Fig. S5). As shown in Fig. 4B, tumors from

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**Figure 1.** In vitro analysis of paclitaxel-induced cell death in mifepristone in MBA-MB-231, BT-20, and MDA-MB-468 cells. A, TNBC cell lines were treated with vehicle (EtOH 0.01%), mifepristone (mif; 100 nmol/L), paclitaxel (pac; 100 nmol/L), dexamethasone (dex; 100 nmol/L)/paclitaxel (100 nmol/L), or dexamethasone (100 nmol/L)/mifepristone (100 nmol/L)/paclitaxel (100 nmol/L). Error bars represent ±SEM. **B,** representative phase-contrast and cytotoxic fluorescent dye images of 3 TNBC cell lines using automated IncuCyte imaging. C, total GR (NR3C1) mRNA expression was first normalized to β-actin mRNA expression and then compared (fold difference) to one technical replicate associated with MDA-MB-231 NR3C1 levels (error bars reflect ±SEM of 3 independent experiments). D, Western blot analysis of GR and β-actin. GRs translational isoforms are labeled.
mice (n = 6) treated with paclitaxel alone had a trend toward a higher percentage of strongly GRþ residual tumor cells compared to tumors from mice (n = 5) treated with mifepristone and paclitaxel (19% vs. 8%, P = 0.11). Software code is described in Supplementary Method S2. This difference in GR expression was further supported by Western blot analysis of total GR protein expression in the residual tumors (Supplementary Fig. S6). These data suggest that the addition of mifepristone treatment results in improved cytotoxicity of highly GR-expressing MDA-MB-231 cells, ultimately resulting in a smaller population of high GRþ tumor cells and greater tumor shrinkage.

The effect of mifepristone in combination with glucocorticoids and paclitaxel on colony formation in vitro was further evaluated with a clonogenic assay (Fig. 4C). MDA-MB-231 cells were treated with vehicle (EtOH 0.1% V/V), dex (100 nmol/L), mifepristone (100 nmol/L), or dexamethasone/mifepristone (100 nmol/L) 1 hour before paclitaxel (100 nmol/L) in 2.5% CS-FCS. Colonies were counted 96 hours after treatment. The addition of mifepristone to dex/pac significantly decreased the number of colonies formed in comparison to dex/pac alone (P = 0.0047). A representative image of each treatment at 3 different magnifications (15×, 24×, and 38×) is reported in Supplementary Fig. S7. These data suggest that mifepristone significantly reduces tumor viability in the setting of chemotherapy independently of an effect on cell proliferation.

Discussion

The limited success of targeted treatments for patients with TNBC highlights the complex heterogeneity of ER−/PR−/HER2− breast cancer (22, 23). Next-generation sequencing of TNBC samples also strongly suggests that this type of breast cancer can be further classified into several molecular subtypes (24). Defining the major driver pathways dividing TNBC into various phenotypic subtypes is a critical question to be answered to improve TNBC outcome. Over a decade ago, we discovered that GR activation by physiologic concentrations of glucocorticoids mediates potent antiapoptotic signaling in the context of either growth factor deprivation (8, 14) or chemotherapy-induced apoptosis (13) in GRþ, ER-negative premalignant breast epithelial and TNBC cell lines. Furthermore, mifepristone, a GR antagonist, was observed to reverse the cell survival effects of GR-expressing MDA-MB-231 cells, ultimately resulting in a smaller population of high GRþ tumor cells and greater tumor shrinkage.

Here we hypothesized that increased sensitivity to chemotherapy-induced apoptosis would result from concomitant GR antagonism with mifepristone of TNBCs. Indeed, we found that cotreatment with the GR-antagonist mifepristone both reverses GR-mediated gene expression in 3 TNBC cell lines and augments chemotherapy-induced apoptosis. The mechanism by which GR activation inhibits apoptosis appears to require GR-mediated transcriptional induction of both SGK1 and MKP1/DUSP1 (13). SGK1 overexpression was previously found to inhibit PARP-dependent apoptosis in a variety of cell lines (8), in part, through phosphorylation and inactivation of the Forkhead transcription factors (8). Here, we find that GR activation blunts paclitaxel (and growth factor deprivation)-induced cleavage of caspase-3 and PARP. The addition of mifepristone to dexamethasone reversed the reduced caspase-3 and PARP cleavage resulting from growth factor deprivation as well as paclitaxel therapy, suggesting that GR activity is an important mediator of caspase-3 and PARP-dependent apoptosis,
regardless of initiating cellular insult (8). We are currently characterizing additional antiapoptotic GR target genes and networks modified by GR activity to better understand the transcriptional regulatory mechanisms through which glucocorticoids and mifepristone modify chemotherapy-induced cytotoxicity.

Although most cell lines used in TNBC research express GR-alpha, levels vary (14). In a cohort of more than 300 patients with early-stage ER-negative cancer, we reported that the highest quartile of GR-expressing primary tumors (by Affymetrix gene arrays) had a significantly worse long-term prognosis compared to patients with tumors expressing the bottom quartile of GR expression (2). In these studies, increased SGK1 and MKP1/DUSP1 tumour expression also correlated with high GR expression, suggesting that these GR target genes as well as others are likely reflecting increased GR activity (2). On the basis of the results reported here, we anticipate that GR antagonism will be a particularly useful treatment for patients with GRþ tumors that are likely chemotherapy-resistant TNBC.

Recently, next-generation sequencing of more than 100 primary TNBCs identified the NR3C1 (GR-encoding) gene as among the top genes undergoing somatic mutation in association with significant changes in expression of shared GR network genes (24, 25). These findings support the hypothesis that GR activity is a significant driver in the biology of a subset of TNBC.

A previous study by Pietenpol and colleagues (26) characterized somatic mutations in a panel of TNBC cell lines including the MDA-MB-231, BT-20, and MDA-MB-468 lines studied here. MDA-MB-468 cells, the least sensitive to the addition of mifepristone in our assay, were classified as "heavily enriched for mutations relating to cell cycle and cell division pathways" including mutations in both p53 and RB (ref. 26; see Table 1). Interestingly, this cell line was most sensitive to paclitaxel monotherapy (approximately twice the percentage cell death was observed in MDA-MB-468 cells compared to either MDA-MB-231 or BT-20 cells). In contrast, MDA-MB-231 and BT-20 cells predominately harbor somatic mutations in genes encoding proteins related to cell survival and growth factor signaling (respectively PDGFRA and PIK3CA) and were the least sensitive to paclitaxel monotherapy (ref. 26; Table 1). This suggests that TNBCs with mutations in survival signaling pathways (rather than cell-cycle pathways) could benefit the most from GR antagonism because GR antagonism blocks cell survival but has little effect on proliferation in ER– cells (Supplementary Fig. S1). Further clinical/translational studies could test the hypothesis that the "basal" type TNBC harboring p53 and RB mutations (both affecting cell cycle) will benefit less from GR antagonism; these tumors also

and MKP1/DUSP1 (P = 0.005) mRNA expression with the addition of mifepristone. C, in MDA-MB-468 cells, dexamethasone-induced SGK1 (P = 0.001) steady-state mRNA levels were significantly higher compared with dexamethasone/mifepristone-treated cells, although the difference in MKP1/DUSP1 mRNA levels was not significant (NS), * P < 0.05, ** P < 0.01, *** P < 0.001.
tend to be more sensitive to initial chemotherapy treatment compared to the "mesenchymal" TNBC subtypes that include MDA-MB-231 (26).

The residual MDA-MB-231 xenograft tumors we excised and examined approximately one month following treatment with paclitaxel/C6 mifepristone suggest that residual tumor GR expression is relatively low following dual paclitaxel and mifepristone treatment compared to tumor remaining after paclitaxel monotherapy. Interestingly, not all individual MDA-MB-231 cells had the same intensity of immunofluorescent GR expression (Fig. 4B). This implies that mifepristone may preferentially target high GR-expressing MDA-MB-231 cells for chemotherapy-induced cell death. Another possibility is that mifepristone results in increased non-metabolized paclitaxel concentrations based on the ability of GR ligands (such as mifepristone and dexamethasone) to inhibit the CYP3A4 liver enzyme, thereby increasing active paclitaxel levels (27). However, we have previously measured plasma paclitaxel levels from SCID mice treated with either vehicle/paclitaxel or dex/paclitaxel and found no significant differences in active paclitaxel concentrations (9). Therefore, the lower percentage of GR-expressing cells following mifepristone treatment is more likely due to greater sensitivity of high GR-expressing cells to chemotherapy-induced apoptosis with the addition of mifepristone.

The steroid binding affinity of mouse corticosterone to human GR is 1.5 to 3 times lower than cortisol (28). While we observed a statistically significant difference between the pac and mif/pac treatment in vivo, the decreased binding affinity of mouse corticosterone to the human MDA-MB-231 xenografted cells could dampen the antagonistic effect of mifepristone. However, we also conducted in vitro cell viability assays with dexamethasone at 100 nmol/L, which is similar to plasma cortisol concentrations in patients (29). In these experiments, we also observed an increase in tumor cell death with the addition of mifepristone to dex/pac. Furthermore, clonogenicity of MDA-MB-231 cells was

Table 1. Subtypes and associated somatic mutations in TNBC cell lines (26)

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<tr>
<th>TNBC subtype</th>
<th>Cell line</th>
<th>Mutated genes</th>
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<tbody>
<tr>
<td>MDA-MB-231</td>
<td>Mesenchymal-like</td>
<td>TP53, CDKN2A, PDGFRα, BRAF, KRAS, NF2</td>
</tr>
<tr>
<td>BT-20</td>
<td>Unclassified</td>
<td>TP53, CDKN2A, PIK3CA</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>Basal-like</td>
<td>TP53, RB1, PTEN, SMAD4</td>
</tr>
</tbody>
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NOTE: The underlined somatically mutated genes encode proteins having primary roles in cell cycle regulation, while those without underlining have primary roles in malignant transformation including epithelial/mesenchymal transition (EMT). BT-20 and MDA-MB-231 are predicted to have mainly cell cycle gene mutations, while MDA-MB-231 cell have many mutations involving genes regulating cell cycle and transformation.

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significantly decreased with dex/mif/pac compared to dex/pac. Taken together, these data support the hypothesis that mifepristone co-treatment targets high GR-expressing tumor cells that would otherwise be resistant to chemotherapy alone.

Mifepristone has been studied extensively since the 1980s for its ability to antagonize progesterone and glucocorticoid receptors in various human tissues (30). However, previous studies using mifepristone in breast cancer have only considered the use of mifepristone to target the PR in ER+/PR+ tumors, never to target the GR in TNBC. For example, several ER+/PR+ breast tumor xenograft studies have suggested a decrease in volume following treatment with mifepristone alone (subcutaneous administration, doses ranging from 25 to 50 mg/kg) or in combination with anti-estrogen therapy. In addition, a phase II clinical trial with 28 patients found that daily single-agent oral mifepristone treatment (200 mg) for recurrent ER+/PR+ breast cancer resulted in 3 partial responses for an overall response rate of 10.7% and only mild-to-moderate side effects (nausea, lethargy, anorexia, and hot flashes were noted; ref. 31).

In our studies, an in vitro mifepristone concentration of 100 nmol/L was used to target the GR in PR-nonexpressing breast tumors based on previous human pharmacokinetic studies where subjects received between 100 and 800 mg/d of drug. The concentration of mifepristone in the serum 24 hours after administration was found to be approximately 2.0 nmol/L irrespective of dose (32). In addition, chorionic villi tissue levels in women receiving a single dose of 200 mg of mifepristone found cytosolic concentrations on average of 238 nmol/L (18). These human data suggest that 100 nmol/L is a physiologically achievable mifepristone concentration in tumor tissue.

An ongoing phase I clinical trial is evaluating the safety of mifepristone (300 mg/d) for 2 days followed immediately on day 2 by a weekly dose of nab-paclitaxel. In this study, the safety and tolerability of mifepristone in combination with chemotherapy for advanced ER-negative, PR-negative, HER2-negative but GR-positive breast cancer will be determined.

Disclosure of Potential Conflicts of Interest
The University of Chicago, S.D. Conzen and M. Kocherginsky were assigned a patent for the use of GR antagonists in ER-negative breast cancer while this manuscript was under review. No other conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.N. Skor, M. Kocherginsky, Y. Cai, S.D. Conzen
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