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Hsp90 inhibition results in glucocorticoid receptor degradation in association with increased sensitivity to paclitaxel in triple-negative breast cancer

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Abstract

Targetable molecular drivers for triple-negative breast cancer (TNBC) have been difficult to identify; therefore standard treatment remains limited to conventional chemotherapy. Recently, new-generation small molecule Hsp90 inhibitors (e.g. ganetespib and NVP-AUY922) have demonstrated improved safety and activity profiles over the first generation ansamycin class. In breast cancer, clinical responses have been observed in a subset of TNBC patients following ganetespib monotherapy, however the underlying biology of Hsp90 inhibitor treatment and tumor response is not well understood. Glucocorticoid receptor (GR) activity in TNBC is associated with chemotherapy resistance. Here we find that treatment of TNBC cell lines with ganetespib resulted in GR degradation and decreased GR-mediated gene expression. Ganetespib-associated GR degradation also sensitized TNBC cells to paclitaxel-induced cell death both *in vitro* and *in vivo*. The beneficial effect of the Hsp90 inhibitor on paclitaxel-induced cytotoxicity was reduced when GR was depleted in TNBC cells, but could be recovered with GR overexpression. These findings suggest that GR-regulated anti-apoptotic and pro-proliferative signaling networks in TNBC are disrupted by Hsp90 inhibitors, thereby sensitizing TNBC to paclitaxel-induced cell death. Thus, GR+ TNBC patients may be a subgroup of breast cancer patients who are most likely to benefit from adding an Hsp90 inhibitor to taxane therapy.

Keywords

Hsp90 inhibitor; glucocorticoid receptor; triple-negative breast cancer; apoptosis; paclitaxelresistance

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Introduction

The glucocorticoid receptor (GR) is a nuclear receptor and ligand-activated transcription factor that controls signal transduction through the modulation of genes following glucocorticoid (GC) exposure [1]. GCs regulate diverse functions in the human body including immune, metabolic, cardiovascular and behavioral physiology [2]. This diversity reflects the varying gene expression profiles elicited upon activation in different cell types of various tissues. For example, GC-initiated signaling causes apoptosis in lymphocytes [3] but appears to be anti-apoptotic in estrogen receptor- negative breast cancer cells under cytotoxic conditions such as chemotherapy exposure. Previous studies by our group showed that treatment with the synthetic GC dexamethasone diminished chemotherapy effectiveness in a mouse xenograft model of triple-negative breast cancer (TNBC) [4]. This requires regulation of genes encoding anti-apoptotic proteins such as serum and glucocorticoid-inducible protein kinase-1 (*SGK1*) [5, 6] and mitogen-activated protein kinase phosphatase 1/dual specificity phosphatase 1 (*MKP1/DUSP1*) [7]. Overexpression and increased activity of GR [8] and its downstream target *SGK1* [9] have been associated with chemotherapy-resistance and high recurrence rates in early-stage TNBC. SGK1 is a phosphatidylinositol 3-kinase-dependent serine/threonine kinase, closely related to AKT and is rapidly induced in response to cellular stressors. Activated SGK1 is an important cell survival signal in mammary epithelial cells [10]. In addition to chemotherapy resistance, increased SGK1 levels are associated with resistance to AKT-inhibitors [11]. Blocking GR activity and its downstream targets including SGK1 is therefore an attractive approach for treating chemotherapy-resistant TNBC [12].

It is well-established that the activity of the chaperone protein Hsp90 is required for proper GR structure and function [13-15]. Hsp90 activity is crucial for folding of the GR ligand binding domain (LBD) into a conformation that allows GC binding and subsequent GR transcriptional activity [16-18]. A recent study detailing the interaction of GR with Hsp70 and Hsp90 showed that Hsp70 binds and unfolds the GR LBD leading to GR inactivation [18]. Hsp90 binding to this complex promotes an ATP- dependent transfer of GR from Hsp70 to Hsp90, release of Hsp70, and recovery of ligand binding. This process is blocked by the Hsp90 inhibitor 17-AAG [18]. In addition, Hsp90 inhibition results in GR misfolding and subsequent proteasome-dependent degradation with additional loss of GR activity [19]. We have previously demonstrated that breast cancer cells treated with the Hsp90 inhibitor geldanamycin also exhibited decreased SGK1 activity [20]. Interestingly, unlike GR, steady-state SGK1 protein levels were not immediately decreased by Hsp90 inhibitor treatment [20, 21].

Ganetespib is a second generation Hsp90 inhibitor currently being evaluated in several large clinical trials including a Phase 2 randomized neo-adjuvant trial for women with newly diagnosed, locally advanced breast cancer (I-SPY 2). *In vitro*, treatment of cultured TNBC cells with ganetespib resulted in reduced levels of several oncogenic proteins including HIF-1 α , EGFR, JAK2, AKT, and members the mTOR signaling axis. Also depleted were p-EGFR, p-AKT and p-STAT3 [22-24]. Ganetespib monotherapy decreased TNBC xenograft growth and metastases to the lung and ganetespib enhanced sensitivity of TNBC cells to taxane chemotherapy *in vitro* and *in vivo* [23]. Here we hypothesized that GR degradation

and decreased GR transcriptional activity following treatment with Hsp90 inhibitors would allow enhanced TNBC sensitivity to chemotherapy-induced cytotoxicity. We found that Hsp90 inhibitor-mediated depletion of GR expression was indeed associated with increased tumor sensitivity to paclitaxel; moreover, GR-depleted cells no longer demonstrated a significant Hsp90 inhibitor benefit. These data suggest that GR-regulated gene expression is centrally important to the effectiveness of combining Hsp90 inhibitor activity with paclitaxel in TNBC.

Methods

Cell Culture and Treatments

MDA-MB-231 cells were purchased from the American Type Culture collection and SUM159PT cells were from Asterand. The identity of all cell lines was authenticated by analysis of short-tandem repeats by DDC Medical. MDA-MB-231 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Lonza) supplemented with 10% heat inactivated fetal calf serum (FCS, Gemini BioProducts) and 1% penicillin-streptomycin (Lonza). SUM159PT cells were cultured in Hams F12 medium (Cellgro), supplemented with 5% FCS, hydrocortisone (500 ng/ml, Sigma) and insulin (10 µg/ml, Sigma). For dexamethasone (dex, Sigma) treatments cells were cultured in 2.5% stripped FCS instead of full serum for 48 hours and treated with dex+/- ganetespib under these conditions. MG132 and dex were purchased from Sigma. Pharmaceutical grade paclitaxel was purchased from APP Pharmaceuticals and NVP-AUY922 was purchased from Sellekchem. Pharmaceutical grade ganetespib was a kind gift from Synta Pharmaceuticals.

Western blotting

MDA-MB-231 and SUM159PT Western blotting was performed as previously described [12]. Rabbit polyclonal GR phospho-Ser211 antibody (Cell Signaling Technology, 1:500 dilution, cat no: 4161) was raised against a synthetic phosphopeptide corresponding to residues surrounding serine 211 of the human GR and recognizes the GRα-A (94 kDa), GRα-B (91k Da), and GRα-C (82 kDa) translational isoforms. Mouse monoclonal GR antibody (BD Biosciences, 1:500, cat no: 611226) is raised against a peptide corresponding to amino acids 176-289 of the human GR protein and recognizes the GRα-A (94 kDa), GRα-B (91k Da), and GRα-C (82 kDa) translational isoforms.

Rabbit monoclonal GR XP (DH82) antibody (Cell Signaling Technology, 1:1000, cat no: 3660) was raised against a peptide corresponding to residues surrounding Leu378 of the human GR protein and recognizes the GRα-A (94 kDa), GRα-B (91k Da), GRα-C (82 kDa) and GRα-D (54 kDa) translational isoforms. SGK1 DB29 (Enzo cat no: ADI-KAP-PK015-D, 1:500 is a rabbit polyclonal antibody produced by immunization with a COOH-terminal SGK-1 peptide, Leu-Gly-Phe-Ser-Tyr-Ala-Pro-Pro- Thr-Asp-Ser-Phe-Leu-Cy). ACTB; 1:10,000, Santa Cruz, cat no: sc-47778), GAPDH (1:5000, Santa Cruz, cat no: sc-365062), TUBULIN (1:5000, Santa Cruz, cat no: sc-53646) were used as loading controls. Since SGK1 has a short half-life we treated cells at each timepoint with a proteasome inhibitor [MG132 (10 µM)] for 2 hours before collection to detect differences in accumulation over time. For densitometry statistics log(GR/β-Actin) was analyzed using a mixed effects

analysis of variance model (ANOVA) with treatment as the fixed effect, and biological replicate as the random effect. Differences between treatment groups were assessed by constructing appropriate contrasts based on the fitted model. Time course densitometry data were analyzed using a mixed effects model with time, treatment and time x treatment interaction. Due to the nonlinear pattern of inhibition over time, time was modelled as a categorical variable. A generalized F-test was used to test the composite hypothesis of no difference between treatment groups across all times points, effectively comparing the entire curves over time. Contrasts were constructed to estimate differences between treatments at a given time point. Analyses were performed in SAS 9.4

Quantitative reverse-transcriptase PCR

Cells were seeded and RNA extracted for quantitative reverse transcriptase PCR (qRT-PCR) as previously published [12] and the reactions were run on a BioRad PCR System MyIQ (BioRad Life Sciences). The following primers were used: SGK1, 5'-AGGCCCATCTTCTCTGTTT-3' (forward) and 5'-TTCAGTCTCCCTCAGTCT-3' (reverse); MKP1/DUSP1, 5'-CCTGACAGCGCGGAATCT-3' (forward) and 5'-GATTTCCACCGGGCCAC-3' (reverse); RPLP0, 5'-GGAGAACTGCTGCCTCATATC-3' (forward), 5'-CAGCAGCTGGCACCTTATT-3' (reverse). The samples were loaded in triplicate. 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 C_t approach [25]. For *SGK1* and *MKP1/DUSP1* qRT-PCR analysis, a mixed effects ANOVA model was fitted with C_t as the response variable: treatment, time, gene type (target or reference), and all of their interactions as fixed effects, and replicate and gene within replicate as the random effect. A linear contrast was then constructed to estimate C_t and its confidence interval, and the results were exponentiated to obtain the estimate of 2^{-C_t} and its confidence intervals.

Quantitative reverse-transcriptase PCR profile multiplex assay

A human glucocorticoid signaling qRT-PCR multiplex assay (RT² Profiler PCR Array, Qiagen) was used. This is a multiplex assay for 84 known GR target genes from lung and adipose tissue. MDA-MB-231 cells were pretreated with 100 nM dex for one hour then ganetespib (100 nM) was added for an additional 4 hours. Samples were prepared according to manufacturer's instructions. Data analysis was carried out using the C_t described above where the fold changes for Dex/Veh and Dex/Gan samples compared to vehicle were calculated. Genes were considered significantly regulated between the Dex/Veh and Dex/Gan treatment groups if they changed by at least 25% (Supplementary Table 1).

GR depletion and re-expression in TNBC cells

A stable MDA-MB-231 GR-depleted cell line was created as previously described [26]. Briefly, MDA-MB-231 and SUM159PT cells were transduced with a GR specific (antisense sequence: TTAATAATCAGATCAGGAG) doxycycline-inducible shRNA-mir- expressing lentivirus using the TRIPZ lentiviral shRNA-mir system per manufacturer's instructions (Dharmacon). A negative control (GR-intact) cell line was also produced using an integrated provirus with a controllable shRNA-mir that does not target any human gene (non-silencing

control antisense sequence CTTACTCTCGCCCAAGCGAGAG). Cells containing the shRNA constructs were selected for with puromycin (400 ng/ml) and stable clones and pools were propagated and tested for GR knockdown by Western blot. For GR-depletion experiments, cells were treated once with doxycycline (dox, 1 μ M) 24 hours after plating to induce depletion. To re-express GR, a GFP-GR construct was transiently transfected into GR-depleted TNBC cells 48 hours prior to inducing GR depletion by doxycycline. This GFP-GR construct has been previously shown to be capable of translocating to the nucleus and transactivating gene expression [27-29]. A GFP construct was also transiently transfected into GR-depleted TNBC cells as a control. Incucyte analysis was then performed as described below.

Cell death assay

MDA-MB-231 and SUM159PT cells (1×10^3) were plated in DMEM (10% FCS) or HamsF12 (5% FCS) media respectively and allowed to adhere for 24 hours. Cells were treated with ganetespib (50 nM), paclitaxel (10 nM) or the combination. Each experiment was performed at least twice and each treatment had three replicate wells per experiment for each treatment condition. To detect dead cells YOYO-1 Iodide (ThermoFisher Scientific, Y3601) or YOYO-3 Iodide (Y3606) was added. YOYO is a cyanine dimer nucleic acid dye that fluoresces if the cellular membrane is compromised. Two images (1.90×1.52 mm) in separate regions of each well were captured with a 10x objective at 4-hour intervals using the IncuCyte FLR HD or Incucyte ZOOM 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 using investigator-coded software for analysis [Supplementary Method S1[12]]. The “cytotoxic index” represents the number of dead cells/total cells for each image. Images collected between 0 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 were treatment, time, time², time³, and all corresponding interactions between treatment and time terms. Random effects included random intercept terms for biological and technical replicates and a random slope for the biological 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), trt $\times$ time, trt $\times$ time², and trt $\times$ time³, effectively comparing the entire curves over time.

TNBC mouse xenografts

All xenograft 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 either Synta Pharmaceutical Corp. or the University of Chicago Institutional Animals Care and Use Committee. Suspensions of SUM159PT cells (1×10^7) in 50 μ L of PBS were injected subcutaneously into the right pectoral mammary fat pad of 6- to 7-week-old female SCID mice (Taconic). MDA-MB-231 (5×10^6) cells were subcutaneously implanted into female SCID mice (Charles River Laboratories, 7 to 12 weeks of age) at Synta as described above. For both xenograft experiments mice bearing tumors (150-200 mm³) randomized into groups of eight and treated with ganetespib (100 mg/kg for SUM159PT xenografts for 150

mg/kg for MDA-MB-231 xenografts) or vehicle (DRD= 10 % DMSO, 18 % Cremaphor RH 40, 3.6 % dextrose) intravenously via the lateral tail vein. Intraperitoneal (SUM159PT) or intravenous (MDA-MB-231) injection of paclitaxel (10 mg/kg) or vehicle [castor oil (1:10 v/v for SUM159PT and DRD for MDA-MB-231)] was administered an hour later. 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: $S^2 \times L \times 0.52$ [4]. Animals received four consecutive once weekly treatments of ganetespib, paclitaxel or the combination of both drugs, and were sacrificed 24 hours after the last treatment. At time of sacrifice tumors were minced in lysis buffer and frozen for protein analysis. One ganetespib and paclitaxel treated SUM159PT xenograft mouse died during the course of the experiment, and was removed from the data analysis. Tumor growth data were analyzed using repeated measures analysis of variance models similar to [4], with one exception that the model included treatment day and their interaction as fixed effects.

Results

Hsp90 inhibition results in rapid loss of activated (pSer211) and total GR

Because GR activation is associated with chemotherapy-resistance in TNBC and GR antagonist treatment increases chemotherapy-induced cell death in GR+ TNBC [12], we hypothesized that GR degradation following Hsp90 inhibitor treatment would similarly increase taxane chemotherapy-induced cytotoxicity. To test this hypothesis, we first assessed the stability of total and phosphorylated GR following ganetespib treatment on TNBC cell lines. As predicted, Western analysis showed that total GR levels declined rapidly in MDA-MB-231 (Fig 1) and SUM159PT (Fig S1) TNBC cells treated with ganetespib. Because Hsp90 activity is required for the GR LBD to fold correctly and bind glucocorticoid [18], which in turn is required for GR Ser211 phosphorylation (GR pSer211) and transcriptional activity [30, 31], we also determined GR pSer211 levels following Hsp90 inhibition. Decreased dex-mediated GR Ser211 phosphorylation was detected starting one hour after ganetespib exposure and continued for the duration of the experiment (Fig 1). The proteasome-dependent (Fig S2) decrease in steady-state GR protein also began early on (Fig 1C). A time course of ganetespib-treated MDA-MB-231 cells showed that Hsp70 levels increased 4 hours following ganetespib while Hsp90 levels increased after 24 hours (Fig S3). Increased Hsp70 is a well-established response to Hsp90 inhibition [23]. Since Hsp70 can bind to and inhibit GR LBD activity [18], the increase in Hsp70 levels may allow sustained GR inactivity.

Hsp90 inhibition significantly disrupts GR transcriptional activity

Based on the rapid ganetespib-induced loss of pSer211 GR and the subsequent decrease in total GR steady-state protein, we hypothesized that GR-mediated transcription would be reduced by ganetespib treatment. Indeed, we found that dex-induced *SGK1* and *MKP1/DUSP1* steady-state mRNA transcript levels were both significantly decreased following ganetespib treatment of MDA-MB-231 (Fig 2 A, B) and SUM159PT cells (Fig S4 A, B). This relative decrease in GR target gene expression was maintained throughout the experiment. We also used a multiplex qPCR assay to determine the effect of ganetespib on a larger transcriptional network of known GR target genes. Table 1 shows breast cancer-

related GR target genes that were decreased (by at least 25%) following the addition of ganetespib to dex in MDA-MB-231 cells. These included genes encoding proteins involved in breast cancer growth and proliferation (*C/EBP β* , *BCL6*, *ANGPLT4*, *END1*, *MT1E*, *MT2A* and *SLC19A2*), apoptosis inhibition (*BCL6*, *ANGPLT4*, *EDN1*) and metastasis (*ANGPLT4*, *BCL6*, *LOX*, *MT1E*, *MT2A*, *USP2*).

Western analysis of SGK1 protein in MDA-MB-231 (Fig 2C) and SUM159PT (Fig S4C) cells confirmed decreased SGK1 accumulation as early as 1 hour post-gan treatment. This depletion of SGK1 protein was presumably due to decreased GR activity and transcriptional induction of *SGK1* expression (Fig 2A and S4A) as well as possible direct effects of ganetespib on SGK1 protein stability. Because SGK1 has such a short half-life, its decreased induction was most clearly demonstrated by the addition of the proteasome inhibitor MG132 for two hours prior to cell lysis. This enhanced accumulation and detection of steady-state SGK1 protein levels over time (Fig S5 [20]). Interestingly, treatment of the SUM159PT cell line with ganetespib showed more dramatic depletion of SGK1 than MDA-MB-231 cells, also seen best in a time course with MG132 added prior to each cell lysis (Fig S4C). Taken together, these results suggest that Hsp90 inhibition both reduces GR target gene expression and SGK1 protein expression, both of which are known effectors of GR-mediated cell survival.

Ganetespib treatment potentiates chemotherapy-induced TNBC cell death

Because ganetespib treatment inhibited GR-mediated gene expression, we next tested whether ganetespib could potentiate paclitaxel cytotoxicity in GR+ TNBC cell lines. We co-treated GR+ MDA-MB-231 and SUM 159PT cells with pharmacologically-relevant ganetespib (50 nM) and paclitaxel (10nM) concentrations and measured cellular cytotoxicity using microscopy-based live cell imaging [12, 32]. Fig 3A and C show that co-treatment caused significantly more cell death compared to paclitaxel treatment alone in both MDA-MB-231 ($p=0.005$) and SUM159PT ($p=0.0007$) cells. Western analysis (Fig 3B, D) showed a concomitant loss of total GR following either single agent ganetespib or ganetespib/paclitaxel treatment, but not paclitaxel alone. MDA-MB-231 and SUM159PT live cell counting over time revealed that there was concomitant decreased cell accumulation following treatment with ganetespib and paclitaxel (Fig S6), with the biggest effect seen in SUM159PT cells, where there were no live cells after 28 hours of ganetespib treatment. These data demonstrate that SUM159PT cells were generally more sensitive to either single agent ganetespib or paclitaxel cytotoxicity compared to MDA-MB-231 cells. SUM159PT cells highly express GR and AR while MDA-MB-231 express GR and low levels of AR [33]. High AR-expressing TNBC (e.g. SUM159PT cells) may be more chemotherapy- and Hsp90 inhibitor-sensitive. Additionally, SUM159PTs rapidly lost SGK1 protein following ganetespib treatment (Fig S4C), whereas for MDA-MB-231 SGK1 protein levels were sustained for longer (Fig 2C).

***In vivo* ganetespib treatment leads to tumor GR depletion and a significant increase in paclitaxel effectiveness**

Based on our *in vitro* results, we hypothesized that addition of ganetespib to paclitaxel treatment would decrease GR activity, thereby leading to increased paclitaxel-induced

cytotoxicity and TNBC tumor shrinkage compared to paclitaxel alone. We found significantly less MDA-MB-231 (Fig 4A, B) and SUM159PT xenograft (Fig 4C, D) tumor growth following dual treatment compared to paclitaxel treatment alone ($p < 0.0001$ for both xenografts). To determine whether ganetespib treatment had in fact reduced GR expression in tumor xenografts, we examined xenografted tumors 24 hours following the final paclitaxel +/- ganetespib treatment. Western analysis of GR from MDA-MB-231 tumor lysates (Fig 4B) showed that paclitaxel alone (right panel) did not alter steady-state GR protein levels compared to vehicle treated tumors (left panel). However, ganetespib treatment, either alone or administered immediately prior to paclitaxel, resulted in significantly reduced tumor GR expression. For SUM159PT tumors although there was more variation in GR levels in the paclitaxel treated mice, overall there was decreased tumor GR expression with ganetespib or with ganetespib/paclitaxel treatment (Fig 4D).

GR expression contributes to ganetespib-mediated enhancement of paclitaxel-induced cell death

Because GR expression and activity are associated with resistance to paclitaxel-induced cytotoxicity [6] we next asked whether GR depletion would increase sensitivity to paclitaxel and reduce the cytotoxic effect of adding ganetespib to paclitaxel. We used a doxycycline-inducible GR shRNA to deplete GR expression (Fig 5 A-C) and then measured cytotoxicity of tumor cells treated with ganetespib, paclitaxel or the combination using automated microscopy (Fig 5D, E, S7, S8). As expected, MDA-MB-231 and SUM159PT cells expressing a control shRNA exhibited significantly greater cell death with the addition of ganetespib to paclitaxel compared to paclitaxel alone ($p < 0.01$ for both cell lines). However, the combination of ganetespib and paclitaxel in GR-depleted cells no longer caused a significant increase in paclitaxel-induced cytotoxicity compared to single agent paclitaxel (Fig 5D, E). GR depletion in MDA-MB-231 cells resulted in more single agent paclitaxel-induced cytotoxicity (Fig 5D, S7), consistent with the well-established role of GR in mediating chemoresistance. However co-treatment of GR-depleted MDA-MB-231 cells no longer demonstrated a significant increase in cytotoxicity compared to paclitaxel alone. As above, AR+/GR+ SUM159PT cells were more sensitive to either single agent ganetespib or paclitaxel compared to MDA-MB-231 cells (Fig 3, 5E, S8); however, there was still a significant increase ($p < 0.01$) in cytotoxicity with drug co-treatment. As with GR-depleted MDA-MB-231, cell death from ganetespib/paclitaxel was blunted in GR-depleted SUM159PT cells compared to treatment with paclitaxel alone. These data suggest that GR expression and activity contribute to the ability of ganetespib to enhance paclitaxel cytotoxicity.

Re-expression of GR reinstates ganetespib-mediated enhancement of chemotherapy sensitivity

To determine whether ganetespib-mediated chemotherapy sensitization was in fact mediated, at least in part, by GR expression, we ectopically expressed a GFP-GR construct in GR-depleted SUM159PT cells (Fig 6A). GR-depleted MDA-MB-231 cells proved extremely resistant to efficient transient transfection and therefore GR re-expression for MDA-MB-231 could not be achieved. Western analysis of SUM159PT GR-depleted cells showed expression of GFP-GR (Fig 6C, band at approximately 130 kDa) despite co-

treatment of doxycycline (to induce GR knockdown) and ganetespib which lead to further GR degradation (Fig 6C). The end result was increased expression of GR compared to SUM159PT GR- depleted cells transfected with a GFP control vector (Fig 6B). Cytotoxicity of cells treated with ganetespib/paclitaxel compared to paclitaxel was measured (Fig 6D, S9) and demonstrated that with GR re-expression there was again a significant increase in cytotoxicity following ganetespib/paclitaxel treatment compared to paclitaxel-treated cells ($p<0.05$).

Discussion

GR expression is associated with chemotherapy resistance in TNBC [4, 6]. Here we show that destabilization and transcriptional inactivation of GR following Hsp90 inhibitor treatment (Fig 1 and 2) increased sensitivity of GR+ TNBC cells to paclitaxel (Fig 3 and 4). As shown previously with the GR antagonist mifepristone, we demonstrate that inhibition of GR activity in GR+TNBC (here with the Hsp90 inhibitor ganetespib) decreased the expression GR target genes associated with paclitaxel resistance (Table 1, Fig 2). Mifepristone inhibited GR-mediated *SGK1* and *MKPI/DUSP1* expression and increased paclitaxel- induced caspase 3 and PARP associated cell death [12]. Loss of GR activity following Hsp90 inhibition “primed” GR+ TNBC cells for more efficient cell death by paclitaxel (Fig 3 and 6). The kinetics of GR loss after ganetespib treatment (4 hours) occurred prior to the onset of paclitaxel-induced cell death (Fig 3); therefore, we hypothesize that by the time paclitaxel-initiated cell death, GR and its downstream oncogenic signaling was diminished by Hsp90 inhibition thereby increasing paclitaxel sensitivity. Thus, GR appears to be required, at least in part, for ganetespib- mediated sensitization to paclitaxel (Fig 5) and re-expression of GR restored sensitivity of TNBC to the combination of ganetespib and paclitaxel compared to paclitaxel alone (Fig 6). These studies highlight the importance of disrupting the GR-mediated transcriptional network in GR+TNBC to increase sensitivity to chemotherapy-induced cell death.

As expected, following GR depletion by ganetespib, GR transcription was effectively disrupted (Fig 2, Table 1 and S1). Previously, the GR transcriptional network has been implicated in TNBC oncogenesis by Shah and colleagues who demonstrated that somatic mutations in *NR3C1* result in significant changes in GR network gene expression [34]. Here we show that a number of GR target genes involved in breast cancer growth and metastasis were downregulated by addition of ganetespib. Subsequent ganetespib-induced decreased live cell count of the TNBC cell lines (Fig S6) could be the result of increased apoptosis or decreased cell proliferation which is related to the decreased expression of anti-apoptotic and pro-proliferative genes after ganetespib treatment (Table 1). For example in addition to known GR target genes *SGK1*, *MKPI/DUSP1* and *GILZ*, *BCL6* and *ANGPTL4* were all downregulated following Hsp90 inhibitor treatment. *BCL6* promotes proliferation and inhibits apoptosis in cytokeratin 5 positive/ER-negative [35] and TNBC cells [36]. *ANGPTL4* is involved in proliferation and metastasis [37, 38] and is upregulated in TNBC cell lines [39]. A number of other proteins closely associated with GR function are key Hsp90 inhibitor drug targets. For example, HIF-1 α and GR interact to modulate gene transcription during the stress response [40]. Interestingly *ANGPTL4* expression has been shown to also be regulated by HIF1- α in breast cancer [39]. Ganetespib blocks HIF1- α

activation and inhibits TNBC tumor growth, vascularization, stem cell maintenance, invasion, and metastasis in mice [24]. The PI3K and mTOR pathways are both upstream of SGK1 phosphorylation and activation [20, 41] and mTOR signaling pathways are both disrupted by Hsp90 inhibition in TNBC [22, 23].

We also found that exposure to Hsp90 inhibitors resulted in degradation of both the androgen receptor (AR) and Janus Kinase (JAK) in TNBC cells (Agyeman and Conzen, unpublished). AR activity has been implicated in TNBC proliferation and apoptosis [33] and GR and AR have been shown to have overlapping roles in activating oncogenic gene expression in prostate cancer [42]. SUM159PT cells express significantly more AR than MDA-MB-231s [33] and these cells were more sensitive to ganetespib alone. This suggests that ganetespib's ability to decrease both AR and GR expression may be associated with increased SUM159PT cytotoxic sensitivity compared to MDA-MB-231 cells. The role of JAK/STAT signaling in TNBC tumorigenesis and chemoresistance is well established [43, 44]; interestingly, GR and JAK/STAT pathways crosstalk to inhibit cytokines and promote apoptosis of immune cells, leading to immunosuppression and suppressing inflammation [45]. The interplay between GR signaling and Hsp90 client proteins suggest that proteins encoded by the GR transcriptional network can also be inactivated by Hsp90 inhibition. Thus, inhibiting GR and its transcriptional network proteins simultaneously via Hsp90 inhibition may effectively block oncogenic bypass mechanisms arising following GR depletion.

Small molecule Hsp90 inhibitors appear potent and yet relatively well-tolerated [46]. NVP-AUY922 was in phase II clinical trials for ER+ and Her2+ breast cancer ([47], NCT00526045 and NCT01271920) while PU-H71 [48] and PF-4942847 [49] showed some pre-clinical anti-tumor activity in TNBC; PU-H71 is in a phase I clinical trial (NCT01393509). Ganetespib monotherapy is currently in a phase II clinical trial for metastatic breast cancer that includes TNBC (ENCHANT Trial; NCT01677455) [23, 50]. The combination of ganetespib and paclitaxel is being analyzed as neoadjuvant treatment for breast cancer, including TNBC, in the ongoing I-SPY 2 trial (NCT01042379). In summary, our results suggest that high GR-expressing TNBCs will demonstrate increased sensitivity to paclitaxel-induced cytotoxicity following the addition of an Hsp90 inhibitor to paclitaxel therapy. Immunohistochemical studies have shown that anywhere between 30-50% of early stage TNBC patients express significant levels of GR ([51] and our unpublished observations). However, we suspect that the percentage with recurrent, chemo-resistant TNBC will be much higher due to selection of GR+ cells following adjuvant chemotherapy treatment. A neoadjuvant clinical trial examining 2 weeks of ganetespib monotherapy followed by 12 weeks of ganetespib and paclitaxel in TNBC is planned to address the hypothesis that GR depletion will occur in GR-overexpressing TNBC and that adding ganetespib to paclitaxel will therefore improve TNBC complete response rates compared to using paclitaxel alone.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Hsp90 inhibitor treatment results in rapid decrease of GR activation as measured by reduction in GR Ser211 phosphorylation and GR target gene expression. In all experiments cells were pre-treated with dexamethasone (dex, 100 nM) an hour prior to ganetespiib (gan, 100 nM). **A**, Treatment of MDA- MB-231 cells with gan resulted in decreased dex-induced GR Ser211 phosphorylation (pSer211) and total GR protein levels. This figure is representative of two independent experiments. **B**, Densitometry of GR pSer211. **C**, total GR levels from Figure 3A show a significant decrease in both proteins over time following the addition of gan to dex compared to dex alone ($p < 0.05$, mixed effects ANOVA based on this experiment and one replicate).

Figure 2.

The GR transcriptional network is disrupted by Hsp90 inhibitor treatment. **A**, Dex-induced *SGK1* and **B**, *MKP1/DUSP1* transcripts were reduced after gan treatment of MDA-MB-231 cells. Cells were pre-treated with dexamethasone (dex, 100 nM) for an hour prior to ganetesipib (gan, 100 nM) for the indicated times on the x-axis. Real-time quantitative reverse transcription PCR (qRT-PCR) was used to measure mRNA. This figure is representative of two independent experiments. The error bars are standard error of the mean (S.E.M) of the triplicate C_t values. For both genes there is a significant decrease in expression over time with the addition of gan ($p < 0.0001$, mixed effects ANOVA based on this experiment and one replicate). **C**, Western blot and densitometry analysis of dex-

induced SGK1 protein levels following gan treatment. MDA-MB-231 cells were pre-treated with 1 μ M Dex and 20% FBS to induce SGK1, 1 hour prior to treatment with veh or gan (100 nM), for the indicated times. Cells were treated with the proteasome inhibitor MG132 (10 μ M) 2 hours before lysis to increase the amount of steady-state SGK1. This experiment was repeated twice.

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Figure 3.

Hsp90 inhibition sensitizes TNBC cell lines to paclitaxel-induced cell death and results in GR depletion. **A**, Incucyte live-cell imaging of MDA-MB-231 cell death over 72 hours. YOYO-1 fluorescent staining was used to monitor cell death and Image J software was used to analyze percentage cell death [YOYO-1 positive (fluorescent)/total cell number (phase contrast)]. This figure is representative of two independent experiments. Error bars represent the S.E.M of three cell culture wells. A comparison of cell death following gan/pac vs. pac demonstrates a significant increase in cell death with the addition of gan ($p=0.005$). In both cases the statistics were done by a mixed effect ANOVA based on two independent experiments. **B**, Time course Western blot following treatment of cells with ganetespi (50 nM, gan) +/- paclitaxel (10 nM, pac) that were cultured in parallel to cells in Figure 3A. **C** and **D** show results using the same conditions as in Figures 3A and 3B respectively, except

SUM159PT cells were used instead. The comparison of percentage cell death following gan/pac treatment is again significantly higher compared to pac alone ($p=0.0007$).

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Figure 4.

Addition of ganetespi to weekly paclitaxel significantly inhibits TNBC xenograft growth.

A, For MDA-MB-231 and **C**, SUM159PT SCID mouse xenografts, the addition of ganetespi (gan) to paclitaxel (pac), resulted in significantly decreased tumor growth compared to pac monotherapy ($p < 0.0001$). There were eight mice per treatment group and error bars represent the S.E.M for each treatment group. **B** and **D**, Western blot and densitometry analysis of four tumor lysates from four mice in each treatment group show relative depletion of GR α translational isoforms following ganetespi treatment.

Figure 5.

Sensitization of TNBC cells to paclitaxel by Hsp90 inhibitor is reduced following GR depletion. **A**, Timeline for GR-depletion experiments. Western blots of GR-depleted (GR shRNA) and GR-intact (control shRNA) **B**, MDA-MB-231 or **C**, SUM159PT cells treated with dox 24 hours after plating, followed by, vehicle (veh), ganetespib (gan 50 nM), paclitaxel (pac, 10 nM) or gan/pac 24 hours later. **D**, Incucyte analysis was then performed and bar graph represents percentage cell death at 72 hours and is the average of two independent experiments. Gan/pac vs. pac cell death is significantly increased for control cells (light grey bars, $p < 0.0001$) but not significantly (NS) increased for GR- depleted cells (dark grey bars, $p = 0.6$). **E**, Incucyte analysis at 72 hours for SUM159PT cells. Gan/pac vs. pac cell death is significantly increased for control cells (light grey bars, $p < 0.01$) but not significantly (NS) increased for GR-depleted cells (dark grey bars, $p = 0.1$).

Figure 6.

Re-expression of GR recovers benefit of adding Hsp90 inhibitor to paclitaxel. **A.** Timeline for GR-depletion and re-expression experiments. Western blot timecourse analysis of GR expression following transfection of **B**, GFP or **C**, GFP-GR into GR shRNA expressing SUM159PT cells. Cells were then treated with vehicle or dox (1 μM) 48 hours later then +/- Gan (50 nM) 24 hours after dox treatment. **D.** Incucyte live cell imaging analysis of GR-depleted (GR shRNA-expressing cells transfected with GFP) or GR re-expressed (GR shRNA-expressing cells transfected with GFP-GR). Bar graph represents percentage cell death at 72 hours. Gan/pac vs. pac cell death is not significantly (NS) increased for GR-depleted cells (dark grey bars, $p=0.8$) but significantly increased for GR re-expressing cells (light grey bars, $p<0.05$). This graph represents the average of two independent experiments.

Table 1
GR target gene induction is disrupted by treatment with ganetespib in MDA-MB-231 cells.

Gene Symbol	Gene name	Fold change relative to Veh/Veh		P-value	Function in ER-negative breast cancer	Ref
		Dex/Veh	Dex/Gan			
TSC22D3(GILZ)	TSC22 domain family, member 3	22.8	3.4	0.0036	Unknown	
C/EBPβ	CCAAT/enhancer binding protein, beta	4.1	1.2	0.0003	Growth	[52]
ANGPTL4	Angiotensin-like 4	8.5	3.5	0.0203	Growth, Metastasis, Anti-Apoptosis	[37]
DUSP1 (MKP1)	Dual specificity phosphatase 1	3.7	1.6	0.0041	Anti-apoptosis	[7]
BCL6	B-cell CLL/lymphoma 6	2.6	1.1	0.0063	Growth, Metastasis, Anti-apoptosis	[36]
SGK1	Serum/glucocorticoid regulated kinase 1	2.9	1.4	0.0036	Anti-apoptosis	[6]
MTIE	Metallothionein 1E	2.7	1.3	0.0159	Growth, Metastasis	[53]
LOX	Lysyl oxidase	2.4	1.5	0.0055	Metastasis	[54]
SLC19A2	Solute carrier family 19 (thiamine transporter), member 2	2.0	1.3	0.0206	Growth	[55]
EDN1	Endothelin 1	2.3	1.6	0.0616	Growth, Anti-Apoptosis	[56]
MT2A	Metallothionein 2A	3.0	2.1	0.0141	Growth, Metastasis	[53]
USP2	Ubiquitin specific peptidase 2	3.1	2.3	0.0614	Metastasis	[57]