The inhibitory effect of trastuzumab on BT474 triple-positive breast cancer cell viability is reversed by the combination of progesterone and estradiol

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Abstract. Breast cancer expressing the estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor-2 (HER2) is known as triple-positive (TPBC). TPBC represents 9-11% of breast cancer cases worldwide and is a heterogeneous subtype. Notably, TPBC presents a therapeutic challenge due to the crosstalk between the hormonal (ER and PR) and HER2 pathways. Patients with TPBC are treated with trastuzumab (TTZ); however, several patients treated with TTZ tend to relapse. The present study aimed to investigate the effect of the PR on inhibitory effect of TTZ on cell viability. BT474 cells (a model of TPBC) and BT474 PR-silenced cells were treated with either TTZ, progesterone (Pg), the PR antagonist mifepristone (RU486) or estradiol (E2) alone or in combination for 144 h (6 days). Cell viability assays and western blotting were subsequently performed. The results showed that Pg and E2 interfered with the inhibitory effect of TTZ on cell viability and this effect was potentiated when both hormones were combined. Pg was revealed to act through the PR, mainly activating the PR isoform B (PR-B) and inducing the protein expression levels of CDK4 and cyclin D1; however, it did not reactivate the HER2/Akt pathway. By contrast, E2 was able to increase PR isoform A (PR-A) expression, which was inhibited by Pg. Notably, in most of the experiments, RU486 did not antagonize the effects of Pg. In conclusion, Pg and E2 may interfere with the inhibitory effect of TTZ on cell viability through PR-B activation and PR-A inactivation.

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Introduction

Breast cancer that is positive for the estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor-2 (HER2) is referred to as triple-positive breast cancer (TPBC) (1). TPBC represents 9-11% of breast cancer cases worldwide and is known for its aggressive clinical behavior and poor prognosis (2,3). TPBC is a heterogeneous subtype of breast cancer in terms of gene expression profiles (4,5) and presents a therapeutic challenge, as crosstalk between hormonal and HER2 signaling pathways leads to tumor progression and treatment resistance (4,6,7).

Patients with HER2-positive breast cancer receive a treatment scheme that consists of targeted therapy, mainly trastuzumab (TTZ), plus chemotherapy, usually in the form

of anthracyclines and taxanes (8). However, 30% of patients treated with TTZ relapse after the first treatment scheme, and most patients with metastatic disease that initially respond to TTZ eventually acquire resistance (9-11). Consequently, the molecular mechanisms underlying resistance to TTZ are under investigation. Among these mechanisms, crosstalk between HER2 and hormonal signaling has been suggested in TPBC (7,12-14). In addition, previous studies have demonstrated the association between ER and resistance to TTZ (15,16). However, little is currently known about the role of progesterone (Pg) and the PR in TTZ resistance.

Pg and the PR are important regulators of cell proliferation and differentiation in the mammary gland (17). In breast cancer, they control tumorigenesis and tumor development through genes, such as CCND1, MYC, KLF4 and STAT5, which are associated with aggressive behavior and can predict poor outcomes in patients (5,17). PR isoforms are associated with the expression of EGFR family members (HER3 and HER4) and ligand activators of this family [EGF, amphiregulin, neuregulin (NRG)3 and NRG4] (12). In addition, they have been associated with cell proliferation and the expansion of cells with stem cell characteristics (18). In a previous study on TTZ-resistant TPBC cells, an increase in PR mRNA and protein expression levels has been observed in comparison to non-resistant cells (16). Therefore, the aim of the present study was to determine whether Pg, alone or in combination with estradiol (E2), interferes with the inhibitory effect of TTZ on cell viability, in a manner dependent on the main PR isoforms [PR isoform A (PR-A) and PR isoform B (PR-B)].

Materials and methods

Cell lines and cell culture. The BT474 and MDAMB361 cell lines were provided by the Mr. Salvador Jimenez-Sanchez, who oversees the panel of breast cancer cell lines purchased from the American Type Culture Collection at the breast cancer-institutional program of the Biomedical Research Institute, Universidad Nacional Autónoma de México (Mexico City, Mexico). BT474 and MDAMB361 cells were cultured in RPMI medium (cat. no. R6504; MilliporeSigma) and Leibovitz L-15 medium (cat. no. LVP01; Caisson Labs), respectively. Both media contained phenol red and were supplemented with 10% fetal bovine serum (FBS; cat. no. 26140-079; Gibco; Thermo Fisher Scientific, Inc.), penicillin (100 U/ml)/streptomycin (100 mg/ml) solution (cat. no. PS-B; Capricorn Scientific GmbH) and amphotericin B (0.25 mg/ml; cat. no. 15290026; Gibco; Thermo Fisher Scientific, Inc.). BT474 cells and MDAMB361 were incubated with 5% CO_2 and 100% air, respectively, in a humidified environment at 37˚C.

Hormonal treatment with TTZ. A total of 15,000 BT474 or MDAMB361 cells/cm^2 were seeded in 48-well plates with RPMI-1640 medium (cat. no. R8755; MilliporeSigma) without phenol red supplemented with 10% FBS stripped with activated charcoal (cat. no. C9157; MilliporeSigma) and were allowed to adhere for 24 h at 37˚C and 5% CO_2. The culture medium was then replaced, and BT474 and MDAMB361 cells were treated with an experimentally calculated IC_{50} of TTZ (Fig. S1B and C): 1.6 and 1 μg/ml TTZ (lot no. N3581B062 B20652; Roche Diagnostics), respectively, 10 nM Pg (cat. no. P0130-25G; MilliporeSigma) and/or 100 nM Mifepristone (RU486; cat. no. M8046-100MG; MilliporeSigma). The Pg and RU486 doses were previously proven to be non-toxic in BT474 cells (Fig. S1D and E). For some experiments, BT474 cells were also treated with 10 nM E2 (cat. no. E2758-1G; MilliporeSigma), a dose obtained from a previous report (19). DMSO at 0.0001% was used as a vehicle for Pg, E2 and RU486. Cells were treated for 144 h, with the culture medium and treatment changed every 48 h. Subsequently, cell viability was determined by staining cells for 20 min at room temperature with 0.1% crystal violet (dissolved in 10% formic acid) and measuring the optical density using a Multiskan GO spectrophotometer (Thermo Fisher Scientific, Inc.) at 595 nm.

Western blot analysis. BT474 cells (2x10^5 cells/cm^2) were treated for 144 h and were then lysed with lysis buffer [50 nM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM Na_3VO_4, 1 mM NaF and 1% protease inhibitor cocktail (cat. no. 11873580001; MilliporeSigma)] and sonicated on ice at 20-25 KHz for 10 sec. Protein concentration was determined using Protein Assay Dye Reagent Concentrate (cat. no. 5000006; Bio-Rad Laboratories, Inc.) and the cell lysates (30 μg) were separated by SDS-PAGE on a 9% gel. The proteins were then transferred to a PVDF membrane, blocked for 45 min at room temperature in Tris-buffered saline (TBS) with 0.1% Tween 20 (TBS-T) containing 5% nonfat milk, and washed with TBS-T. Membranes were incubated with the following primary antibodies overnight in 0.1% bovine serum albumin (cat. no. 160069; MP Biomedicals) at 4˚C: PRA/B (1:500; cat. no. sc-810; Santa Cruz Biotechnology, Inc.), ER (1:500; cat. no. sc-8005; Santa Cruz Biotechnology, Inc.), Akt (1:10,000; cat. no. sc-1618-R; Santa Cruz Biotechnology, Inc.), phosphorylated (p)Akt1/2/3 (1:250; cat. no. sc-514032; Santa Cruz Biotechnology, Inc.), CDK4 (1:500; cat. no. sc-23896; Santa Cruz Biotechnology, Inc.), cyclin D1 (1:500; cat. no. sc-8396; Santa Cruz Biotechnology, Inc.), p27^{Kip1} (1:500; cat. no. sc-1641; Santa Cruz Biotechnology, Inc.), β-actin (1:5,000; cat. no. sc-47778; Santa Cruz Biotechnology, Inc.), pPR ser345 (1:500; cat. no. 12783S; Cell Signaling Technology, Inc.), HER2 (1:10,000; cat. no. 2248; Cell Signaling Technology, Inc.) or pHER2 Y1221/1222 (1:1,000; cat. no. 2249; Cell Signaling Technology, Inc.). Subsequently, the membranes were washed with TBS-T and incubated with a secondary horseradish peroxidase-conjugated anti-mouse antibody (1:20,000; cat. no. 115-035-003; Jackson ImmunoResearch Laboratories, Inc.) or horseradish peroxidase-conjugated anti-rabbit antibody (1:20,000; cat. no. 31460; Thermo Fisher Scientific Inc.) for 45 min at room temperature. The chemiluminescent signal was visualized using Supersignal™ West Pico PLUS Chemiluminescent Substrate (cat. no. 34580; Thermo Fisher Scientific, Inc.) in the Fusion FX6 XT (serial no. 16200804; Vilber Lourmat). Densitometric analysis was performed using ImageJ software version 1.53 (National Institutes of Health).

PR gene silencing. According to the manufacturer's protocols of small interfering RNA (siRNA), 15,000 BT474 cells/cm^2 were transfected for 19 h at 37˚C with 1.6 μg of a pool of three target-specific 19-25 nucleotide siRNAs targeting human PR (cat. no. sc-270221; Santa Cruz Biotechnology, Inc.) or with 1 μg

control siRNA-A (cat. no. sc-37007; Santa Cruz Biotechnology, Inc.) using 6 μl of a commercially available siRNA transfection reagent (cat. no. sc-29528; Santa Cruz Biotechnology, Inc.). Immediately after the transfection period, the subsequent experiments were performed. The sequences of the siRNAs targeting PR are shown in Table SI. siRNA-A is a scrambled sequence that is considered proprietary information.

Data mining on clinical and expression data. Normalized data from eight different datasets (Table SII) were downloaded from the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/) (20) and were annotated with Biomart (21) in the R software environment version 4.3.1 (22). The duplicate probes per gene were processed as follows: For Affymetrix microarray profiles, the probe with the highest interquartile range was selected; for the Illumina platform, the probe with the highest value was selected; for Agilent data, the mean of all probes was calculated. Clinical data for each gene set were retrieved from the GEO (20). Statistical analyses were applied to define significant differences between biological groups using a Student's unpaired t-test in R software version 4.3.1 (22). The survival plot was generated using the Kaplan-Meier plotter (https://kmplot.com/analysis/index.php?p=background) which automatically performed a log-rank test on the results produced (23).

Statistical analysis. Experimental results are presented as the mean ± SD. Data were analyzed by one-way ANOVA with a Tukey's pairwise post hoc test using Past4 software version 4.11 downloaded from https://www.nhm.uio.no/english/research/resources/past/index.html. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Pg interferes with the inhibitory effect of TTZ on viability. To determine if Pg interferes with the inhibitory effect of TTZ on cell viability, BT474 cells (Fig. 1) and MDAMB361 cells (Fig. S2) were exposed to 144 h of continuous treatment. In comparison with control cells that were left untreated for the 144 h experiment (T144), TTZ decreased cell viability by 61.7 and 47.7% in BT474 and MDAMB361 cells, respectively (Figs. 1 and S2). Compared with the TTZ group, in the TTZ/Pg-treated group, Pg reduced the inhibitory effect of TTZ on viability by 22.5 and 26.5% in BT474 and MDAMB361 cells, respectively (Figs. 1 and S2). Notably, when the PR antagonist RU486 was used, a Pg-like effect was observed. In comparison to TTZ alone, the TTZ/RU486 combination reduced the inhibitory effect of TTZ on viability by 14.9 and 65.2% in BT474 and MDAMB361 cells, respectively (Figs. 1 and S2). Similarly, compared with TTZ alone, treatment with TTZ/Pg/RU486 reduced the inhibitory effect of TTZ on viability by 20.1 and 48.9% in BT474 and MDAMB361 cells, respectively (Figs. 1 and S2). The fact that there were no statistically significant differences in viability between the TTZ/Pg, TTZ/RU486 and TTZ/Pg/RU486 groups suggests that RU486 did not antagonize Pg in these experiments. Taken together these results indicated that Pg and RU486 interfere with the inhibitory effect of TTZ on viability. Despite similar effects being observed on MDAMB361 cells, due to their lower expression of HER2 and PR-B (Fig. S1A) and their low proliferation rate, the present study focused on BT474 cells.

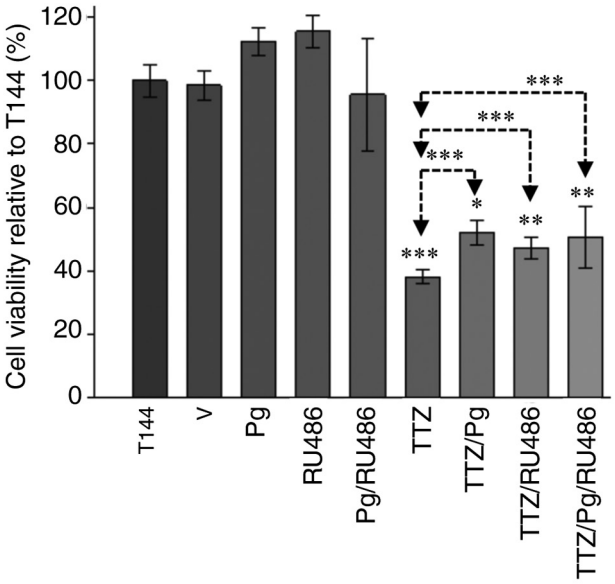

Figure 1. Inhibitory effect of TTZ on the viability of BT474 cells is interfered with by Pg, RU486 and their combination. Data are presented as the mean ± SD of three independent experiments performed in triplicate (n=9). $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ vs. T144 or as indicated. T144, control cells; V, vehicle (DMSO); Pg, progesterone; TTZ, trastuzumab; RU486, mifepristone.

Pg interference in TTZ activity is related to PR-B phosphorylation. The present study aimed to determine how Pg interferes in the inhibitory effect of TTZ on viability. Therefore, PR pathway activation was analyzed by western blotting after 144 h of treatment (Fig. 2). The results revealed that in BT474 cells, the basal levels of PR-B were higher than those of PR-A (Fig. 2A). However, neither isoform exhibited significant changes in their relative expression levels with different treatments (Fig. 2B and C). When the phosphorylation levels of PR isoforms were analyzed, no changes in PR-A were observed (data not shown). On the other hand, treatment with TTZ/Pg and TTZ/RU486 combinations induced a significant upregulation of PR-B phosphorylation compared with that in cells treated with TTZ alone (Fig. 2A and D).

Pg interference in TTZ activity is related to cell cycle-inducing proteins. After analyzing the effect of Pg on its receptor, downstream events, which could explain the interfering effects of Pg on TTZ activity, were assessed. Specifically, the proteins associated with induction (CDK4 and cyclin D1) and arrest ($p27^{Kip1}$) of the cell cycle were evaluated (Fig. 3). The results showed that Pg significantly interfered with the downregulation induced by TTZ alone of the relative expression levels of CDK4 and cyclin D1 (CDK4 activator) (Fig. 3A-C). On the other hand, RU486 interfered only with the downregulation of CDK4 induced by TTZ (Fig. 3A-C). Notably, the TTZ/Pg/RU486-treated group exhibited significantly lower expression levels of cyclin D1 in comparison with the TTZ/Pg- and TTZ/RU486-treated groups (Fig. 3A-C). For $p27^{Kip1}$, the combination of TTZ/Pg/Ru486 induced a

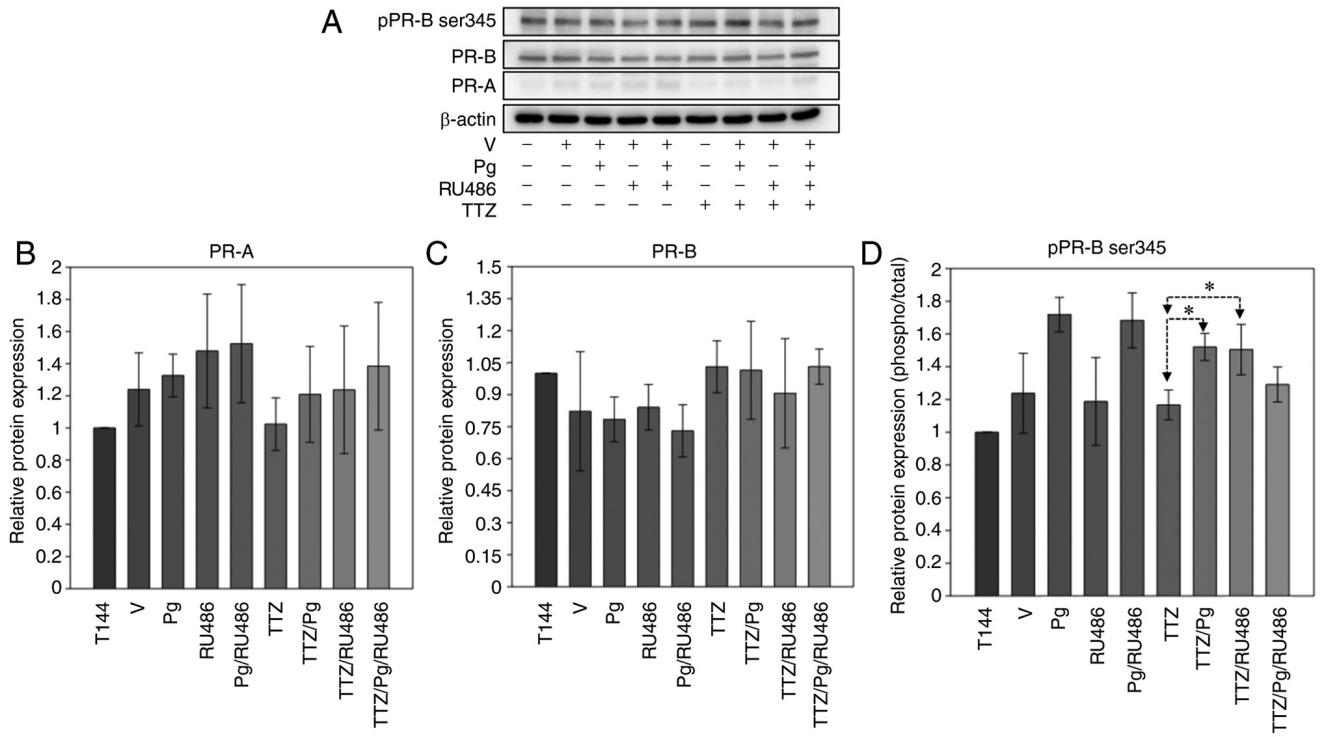

Figure 2. Effect of Pg, RU486, TTZ and their combinations on the expression and phosphorylation levels of PR isoforms. (A) Representative image of the western blot analysis of pPR-B ser345, PR-B and PR-A expression in BT474 cells treated with Pg, RU486, TTZ alone or in combination. Densitometric analysis of (B) PR-A, (C) PR-B and (D) pPR-B ser345. Data are presented as the mean ± SD of three independent experiments (n=3). *P<0.05. T144, control cells; V, vehicle (DMSO); Pg, progesterone; PR, progesterone receptor; PR-A, PR isoform A; PR-B, PR isoform B; pPR-B ser345, phosphorylated-PR in serine residue 345; TTZ, trastuzumab; RU486, mifepristone.

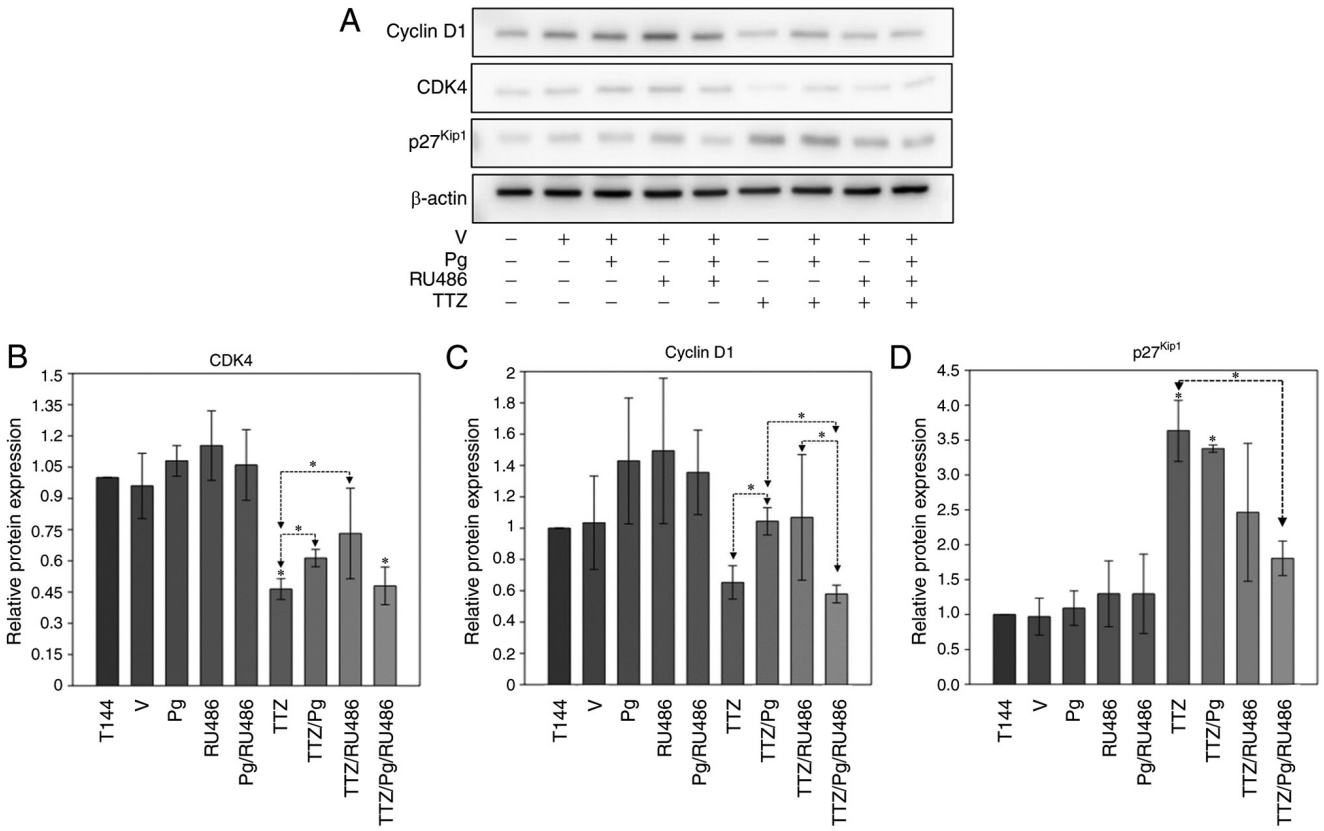

Figure 3. Effect of Pg, RU486, TTZ and their combinations on the expression of cyclin D1, CDK4 and $p27^{Kip1}$. (A) Representative image of the western blot analysis of cyclin D1, CDK4 and $p27^{Kip1}$ expression in BT474 cells treated with Pg, RU486, TTZ alone or in combination. Densitometric analysis of (B) CDK4, (C) cyclin D1 and (D) $p27^{Kip1}$. Data are presented as the mean ± SD of three independent experiments (n=3). *P<0.05 vs. T144 or as indicated. T144, control cells; V, vehicle (DMSO); Pg, progesterone; TTZ, trastuzumab; RU486, mifepristone.

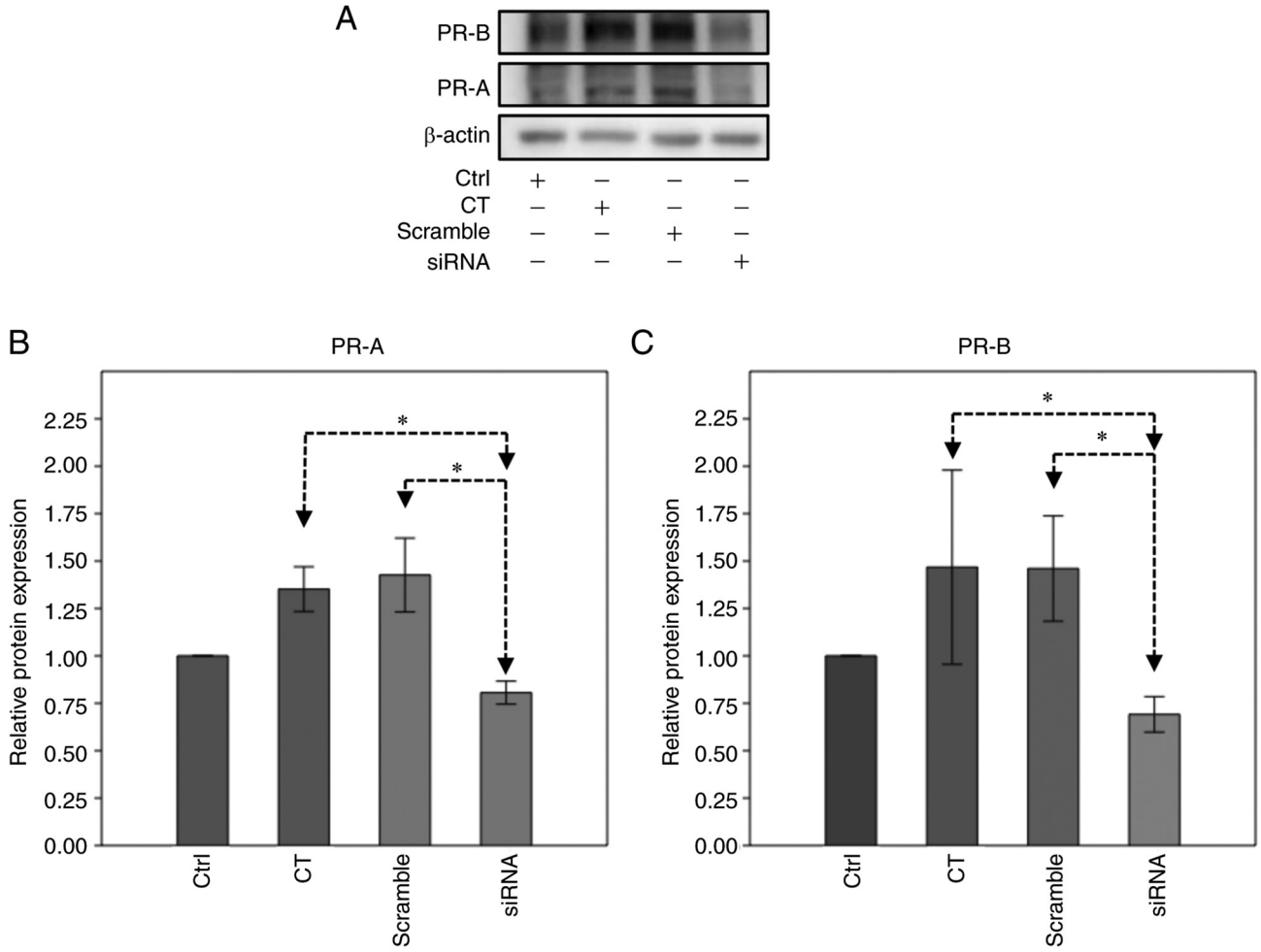

Figure 4. Effect of a PR-siRNA on the protein expression levels of PR isoforms. (A) Representative image of the western blot analysis of PR-B and PR-A protein expression levels in BT474 cells post-transfection. Densitometric analysis of (B) PR-A and (C) PR-B. Data are presented as the mean ± SD of three independent experiments (n=3). *P<0.05. PR, progesterone receptor; PR-A, PR isoform A; PR-B, PR isoform B; siRNA, small interfering RNA; CT, transfection control; Ctrl, untransfected cells.

significant downregulation in its expression, in comparison with treatment with TTZ alone (Fig. 3A and D). Taken together, these results suggested that Pg may interfere with the effect of TTZ on the expression of CDK4 and cyclin D1, but not on the expression of $p27^{Kip1}$. On the other hand, it is inconclusive if RU486 modulates the effect of TTZ on the expression levels of CDK4, cyclin D1 and $p27^{Kip1}$, as TTZ/RU486 and TTZ/Pg/RU486 had contrasting results, and the TTZ/RU486-treated group had an unusually high SD.

PR silencing suppresses Pg interference in TTZ activity. To confirm that the interfering effects of Pg on the inhibitory effect of TTZ on viability were PR-dependent, PR gene silencing was performed (Fig. 4). Using this strategy, PR-A and PR-B expression levels were silenced by 20 and 31%, respectively, compared with in untransfected cells (Fig. 4B and C).

Pg treatment of PR-silenced cells did not interfere with the inhibitory effect of TTZ on viability (Fig. 5). Notably, RU486 significantly increased cell viability by 12.7% only in the TTZ/Pg/RU486 combination treatment group compared with TTZ alone (Fig. 5). Taken together, these findings indicated that Pg and RU486-induced interference with TTZ activity is PR pathway-dependent.

Pg-induced interference with TTZ activity does not reactivate the HER2/Akt signaling pathway. To determine whether the effects of Pg on the inhibitory effect of TTZ on viability were due to HER2/Akt reactivation, the changes in the expression and phosphorylation state of these proteins were analyzed after 144 h of treatment (Fig. 6). The combination of TTZ with Pg or RU486 did not alter HER2 or Akt protein expression levels or phosphorylation status, compared with treatment with TTZ alone (Fig. 6A-E). However, when both Pg and RU486 were combined with TTZ, an increase in pHER2 and in the expression levels of Akt occurred in comparison with treatment with TTZ alone (Fig. 6A, C and D); despite this, Akt phosphorylation remained unchanged (Fig. 6A and E). These results suggested that the interfering effects of Pg on the inhibitory effect of TTZ on viability were not due to reactivation of the HER2/Akt pathway.

E2 potentiates the interfering effects of Pg on the inhibitory effect of TTZ on viability. In hormone-dependent breast cancer, a close relationship between Pg and E2 has been observed (1,24). For this reason, an assay to investigate whether the combination of both hormones interfered in the inhibitory effect of TTZ on viability was performed (Fig. 7). Compared with TTZ alone, when E2 was combined with TTZ,

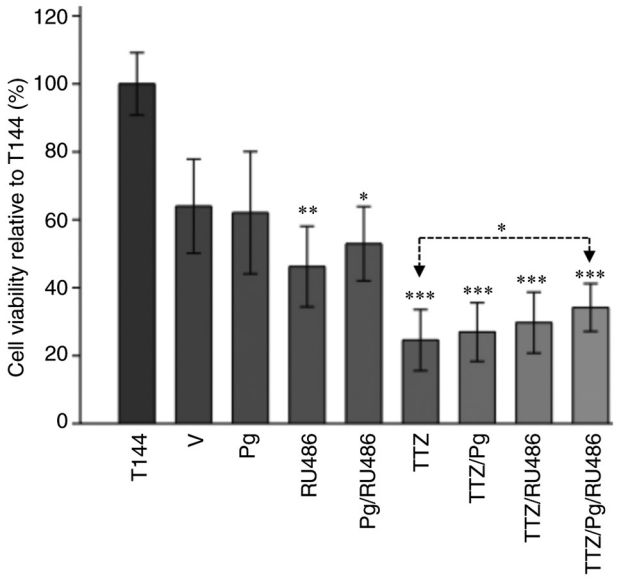

Figure 5. Effect of Pg, RU486, TTZ and their combinations on the viability of PR-silenced BT474 cells. Data are presented as the mean ± SD of three independent experiments performed in triplicate (n=9). *P<0.05, **P<0.01 and ***P<0.001 vs. T144 or as indicated. T144, control cells; V, vehicle (DMSO); Pg, progesterone; TTZ, trastuzumab; RU486, mifepristone.

a significant interference in the inhibitory effects of TTZ on viability was observed (20.8%), although the interference was like that achieved with TTZ/Pg treatment (14%). Notably, the combination of TTZ/Pg/E2 had a greater effect on cell viability, which was statistically different to the effects of TTZ/Pg and TTZ/E2. Taken together, these results indicated that an interaction between Pg and E2 potentiates their interference in the inhibitory effect of TTZ on viability.

Effect of E2, Pg, TTZ and their combinations on the expression and phosphorylation status of PR isoforms. To investigate how E2 and Pg interact to interfere with TTZ activity, whether E2 modifies the expression and phosphorylation status of PR isoforms in a similar manner to Pg was evaluated (Fig. 8). In comparison to TTZ and TTZ/Pg treatment, E2 in combination with TTZ significantly upregulated the protein expression levels of PR-A (Fig. 8A and B). Notably, E2 induced the presence of an additional band that had a lower molecular weight than PR-B (Fig. 8A). The additional PR-B band was upregulated when E2 was combined with TTZ but was downregulated when Pg was added to the TTZ/E2 combination. The significance of this additional band is currently unknown and could be explored in future studies.

Regarding the phosphorylated status of the PR isoforms, PR-A phosphorylation at ser345 was significantly downregulated by the combination of TTZ/Pg/E2 compared with treatments with TTZ and TTZ/Pg (Fig. 8A and C). No statistically significant changes were observed in the expression or phosphorylation levels of PR-B in response to any of the treatments (Fig. 8D and E).

Association between TTZ treatment response and the expression profile of hormone receptors, and their downstream genes in patients with TPBC. To define the possible coordinated and unique activity of PR, ER, cyclin D1, CDK4 and $p27^{Kip1}$, clinical data from patients (ER^+ and PR^+, $HER2^+$) treated with neoadjuvant or adjuvant TTZ, as a monotherapy or in conjunction with chemotherapy or hormone therapy, were analyzed. The expression profiles of these genes in different clinical groups according to the response to treatment or presence of any clinical event were observed (Figs. 9 and S3). In one of the datasets (Fig. 9A), in patients with a partial response to therapy, a significant enrichment of PGR (gene that codes for PR) expression and joint ESR1 (gene that codes for ER)/PGR expression, but a non-significant upward trend in the expression of ESR1 and CCND1 (gene that codes for cyclin D1), was determined. Additionally, in these patients, the expression of CDK4 was not significantly enriched and CDKN1B (gene that codes for $p27^{Kip1}$) was not significantly decreased (Fig. 9A). Nevertheless, in the rest of the datasets (Fig. S3), like the one depicted in Fig. 9B, the enrichment of PR expression in partial responders to treatment was not significant, and the significance of the changes in expression of the rest of the genes of interest differed between each database. Notably, throughout the clinical cohorts in which there was no optimal therapeutic response, i.e., in patients with partial response or residual disease after TTZ treatment, there was a higher frequency of TPBC tumors compared with other immunochemical subtypes (Fig. 9C). Moreover, when looking at overall survival in patients with $HER2^+$ and ER^+ tumors, PR expression may have an impact on clinical outcome as depicted in the Kaplan-Meier plot, where tumors overexpressing the PR transcript exhibited a trend towards a lower overall survival compared with tumors with low PR expression (Fig. 9D).

Discussion

TTZ is the primary treatment for HER2-positive breast cancer (1); however, this therapy does not differentiate between breast cancer subtypes with HER2 positivity, which may explain the benefits and/or failures of HER2 therapies. In TPBC, it has been suggested that the interaction between hormonal receptors and HER2 may explain the decrease in efficacy of anti-HER2 therapy (25,26). It has been demonstrated that Pg and its receptor are responsible for tumor heterogeneity, conferring plasticity, proliferative ability, and progression (27,28). In TPBC cell lines, treatment with anti-HER2 therapies, such as TTZ, has been found to increase PR expression (16,29). Notably, a report on patients treated with lapatinib (another anti-HER2 therapy) confirmed that an increase in PR expression is associated with HER2 inhibition (29).

The aim of the present study was to provide further information on how Pg, the PR, RU486 and E2 interfere with the inhibitory effect of TTZ on viability. The findings of the present study support those of a previous report where Pg was shown to interfere with TTZ activity in a 3D system (30). However, the present data also showed that the inhibitory effect of TTZ on viability was poorly interfered with by Pg. The main limitation of the present study is that a single cell line was used for most of the experiments. During the preliminary phase of the project, two human TPBC cell lines were used, BT474 and MDAMB361. The effect of TTZ, Pg and their combination on the viability of MDAMB361 cells was like that observed in BT474 cells. Notably, MDAMB361 cells had an increased response to RU486 when compared with BT474 cells. However,

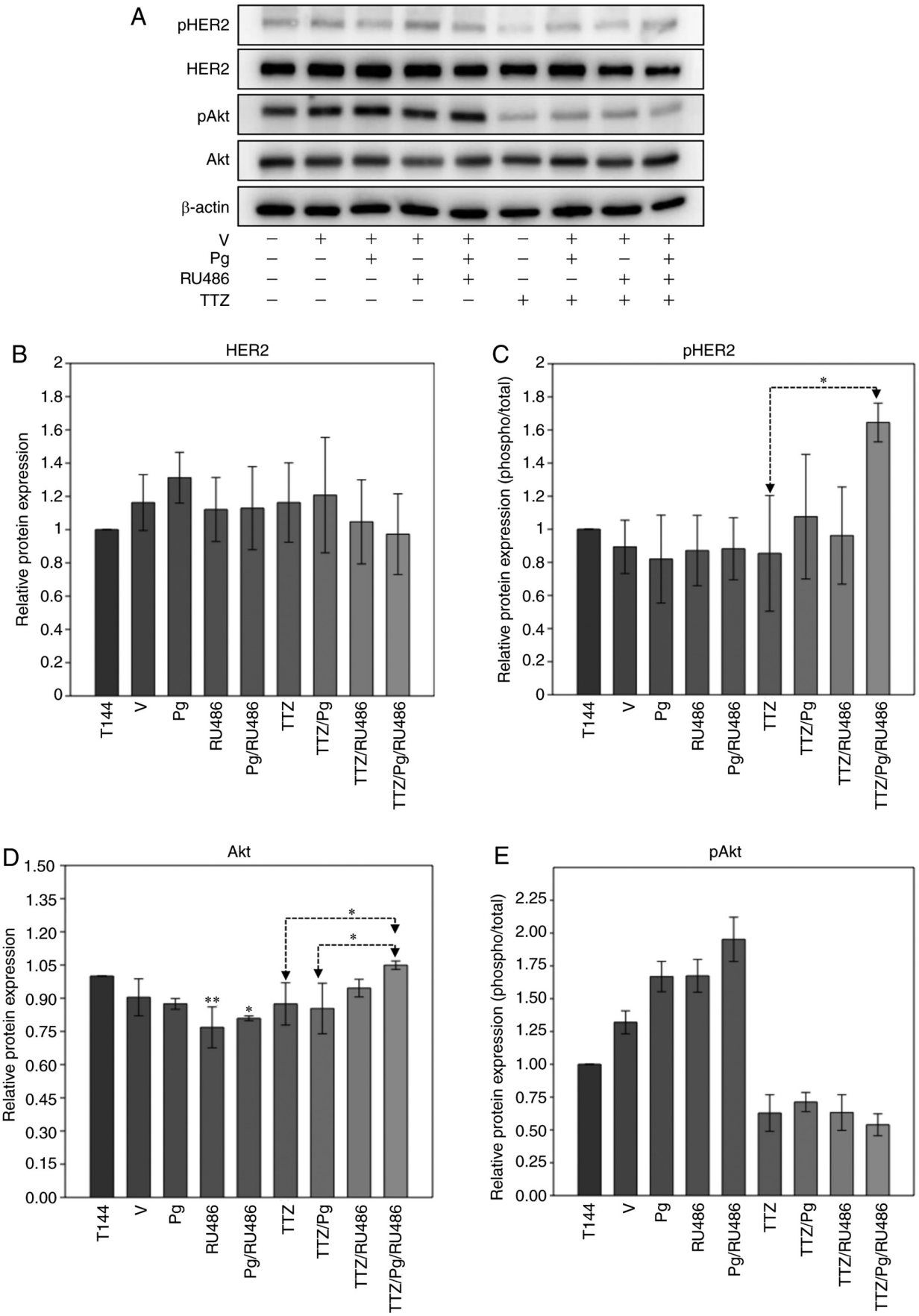

Figure 6. Effect of Pg, RU486, TTZ and their combinations on the expression and phosphorylation levels of HER2 and Akt in BT474 cells. (A) Representative western blot analysis of the protein expression levels of pHER2, HER2, pAkt and Akt in BT474 cells treated with Pg, RU486, TTZ alone or in combination. Densitometric analysis of (B) HER2, (C) pHER2, (D) Akt and (E) pAkt. Data are presented as the mean ± SD of three independent experiments (n=3). *P<0.05 and **P<0.01 vs. T144 or as indicated. T144, control cells; V, vehicle (DMSO); Pg, progesterone; TTZ, trastuzumab; HER2, human epidermal growth factor receptor-2; pHER2, phosphorylated HER2; pAkt, phosphorylated Akt; RU486, mifepristone.

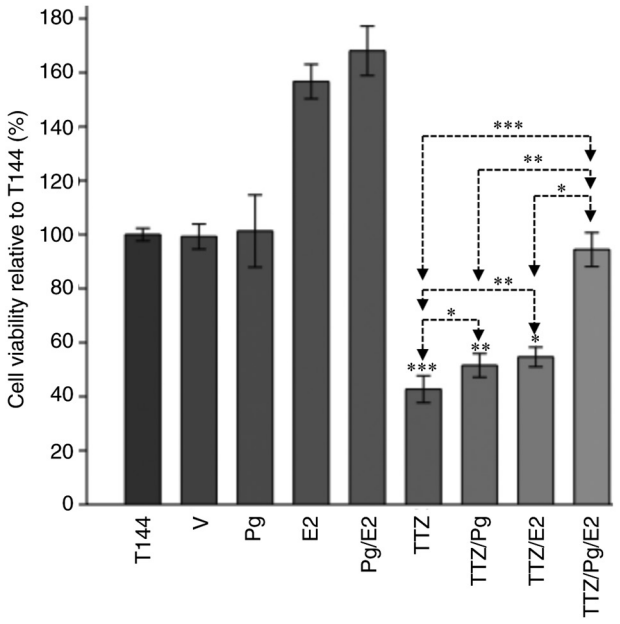

Figure 7. Inhibitory effect of TTZ on the viability of BT474 cells is interfered with by Pg, E2 and by their combination to a greater extent. Data are presented as the mean ± SD of three independent experiments performed in triplicate (n=9). *P<0.05, **P<0.01 and ***P<0.001 vs. T144 or as indicated. T144, control cells; V, vehicle (DMSO); Pg, progesterone; TTZ, trastuzumab; E2, estradiol.

MDAMB361 cells had a low proliferation rate and expression of HER2 and PR-B (Fig. S1A) compared to BT474 cells. Therefore, the subsequent experiments used BT474 cells.

In the present study, a known antagonist of the PR, RU486, induced interference with the inhibitory effect of TTZ on viability. Previous reports on BT474 cells used RU486 at a concentration of 1 μM to block the action of Pg (31,32) and treated cells 1 h before the addition of Pg (33). In the present study, 100 nM RU486 was used to treat cells 1 h before Pg. The dose of RU486 was chosen because it was not toxic to BT474 cells. However, it is unclear if, in the present study, RU486 stabilized the agonist or antagonist structures of the PR, coupled to coactivator or corepressor proteins, respectively. To corroborate this, future studies would need to show, for example, that RU486 in the conditions of the present study could inhibit the expression of a reporter gene under the control of Pg response elements, as we did in a previous publication (34). In other words, RU486 in the present study may be at a sub-optimal dose to be able to antagonize the effects of Pg. Moreover, even though the RU486 used is of the same brand as previous reports (32-34), the present lot number, and thus the biological activity, may be different. Based on the present counterintuitive results with RU486, for future experiments other PR antagonists could be used, such as Proellex, which inhibits PR-B biosynthesis (35).

Pg binding to the PR is associated with transcriptional activation. Individual overexpression of PR isoforms regulates the differential expression of genes, as follows: PR-A regulates the expression of genes related to cell stemness, whereas PR-B is related to the regulation of genes associated with cell proliferation (CCND1, MYC) (5,18). In the present study, in the absence of E2, Pg treatment induced an increase in PR-B phosphorylation and in the expression of cell cycle-inducing proteins, CDK4 and cyclin D1. This effect occurred even if Pg was combined with TTZ, but not in the TTZ/Pg/RU486 combination. Nevertheless, the latter triple combination downregulated $p27^{Kip1}$ protein levels, which is possibly linked to RU486. $p27^{Kip1}$ downregulation has previously been associated with TTZ resistance (36). Therefore, $p27^{Kip1}$ may be involved in the mechanism by which RU486 interferes in the inhibitory effect of TTZ on viability.

It has been noted that Pg promotes the expression of growth factors that induce reactivation of the HER2/HER3-dependent signaling pathway (30). However, these data were obtained with short-term Pg exposures and without TTZ. The present results, at an extended exposure time (144 h), excluded the possibility that Pg interference in TTZ's inhibitory effect on viability was caused by reactivation of the HER2/Akt signaling pathway or alternative growth factors pathways where Akt is involved.

Under standard cell culture conditions, it has been reported that nuclear receptors are activated; therefore, to analyze PR activation, elimination of all possible unknown ligands (using charcoal-stripped FBS) or the agonistic effect of phenol red is required. Only under these conditions can it be certain that activation of PR was due to the Pg added. By contrast to Pg, RU486 binds to several different nuclear receptors, such as the glucocorticoid receptor (32). The present study also attempted to describe the relevance of the observed phenomena when estrogens are present. The results confirmed those shown in previous reports (16,32) that, in TPBC, E2 functions as an escape and/or survival mechanism against TTZ. However, the present results also demonstrated that the interference effect of E2 is like that of Pg. Notably, the effect of E2 was enhanced when it was combined with Pg. Additionally, E2 can induce an increase in PR-A and a low-molecular weight variant of PR-B, as PR is a gene regulated by E2 and its receptor (1,24). These findings corroborated previously reported data where TTZ treatment increased PR levels (16). Despite these increases in the protein levels of the PR isoforms, the Pg/E2 combination reduced the phosphorylation of PR-A, but not of PR-B. This suggests that PR-A phosphorylation may act as a negative regulator in the interference of TTZ activity.

Finally, the significance of PR expression in the resistance to TTZ was assessed using a data mining analysis of several cohorts of patients with TPBC that were subjected to a treatment scheme that included TTZ (Fig. 9). The plots showed that PR expression tended to be higher in patients that had reduced overall survival, partial pathological response, or a recurrence event. Nevertheless, this trend only achieved statistical significance in one of the datasets. A limitation of this data mining analysis was that each dataset had a limited number of patients with TPBC. In the future, the transcriptome of more patients with TPBC could become available and the analysis may be repeated. In addition, better results could be obtained by analyzing the phosphorylation status of PR-B in patients with TPBC. The reasoning being that *in vitro* results showed that PR-B phosphorylation was associated with Pg interference with TTZ. However, the necessary phospho-proteomic data needed to be able to perform such data mining analysis

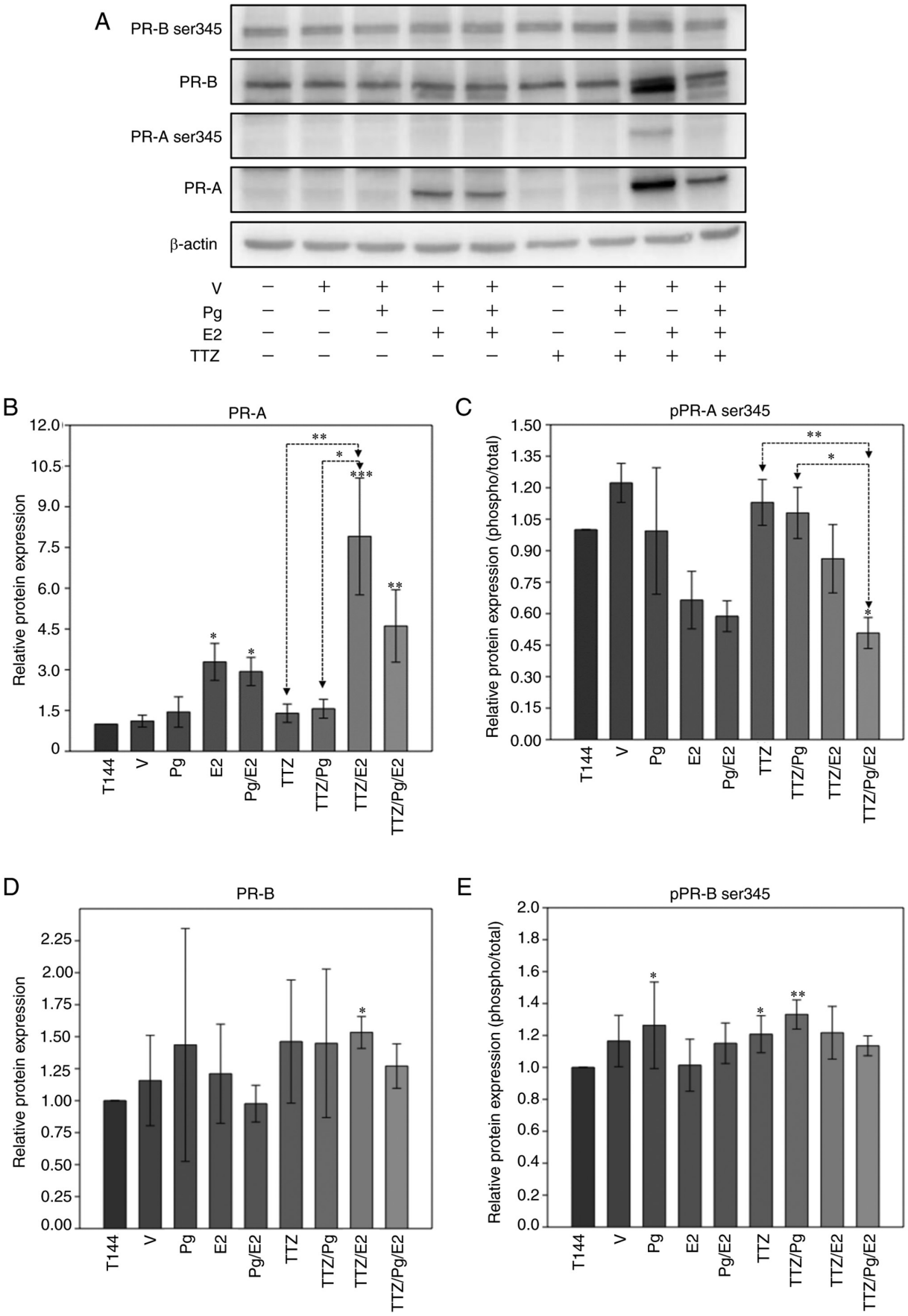

Figure 8. Effect of Pg, E2, TTZ and their combinations on the expression and phosphorylation levels of PR isoforms. (A) Representative western blot analysis of the protein expression levels of pPR-B ser345, PR-B, pPR-A ser345 and PR-A in BT474 cells treated with Pg, E2, TTZ alone or in combination. Densitometric analysis of (B) PR-A, (C) pPR-A ser345, (D) PR-B and (E) pPR-B ser345. Data are presented as the mean ± SD of three independent experiments (n=3). *P<0.05, **P<0.01 and ***P<0.001 vs. T144 or as indicated. T144, control cells; V, vehicle (DMSO); Pg, progesterone; TTZ, trastuzumab; E2, estradiol; PR, progesterone receptor; PR-A, PR isoform A; PR-B, PR isoform B; pPR-A/B ser345, phosphorylated-PRA/B in serine residue 345.

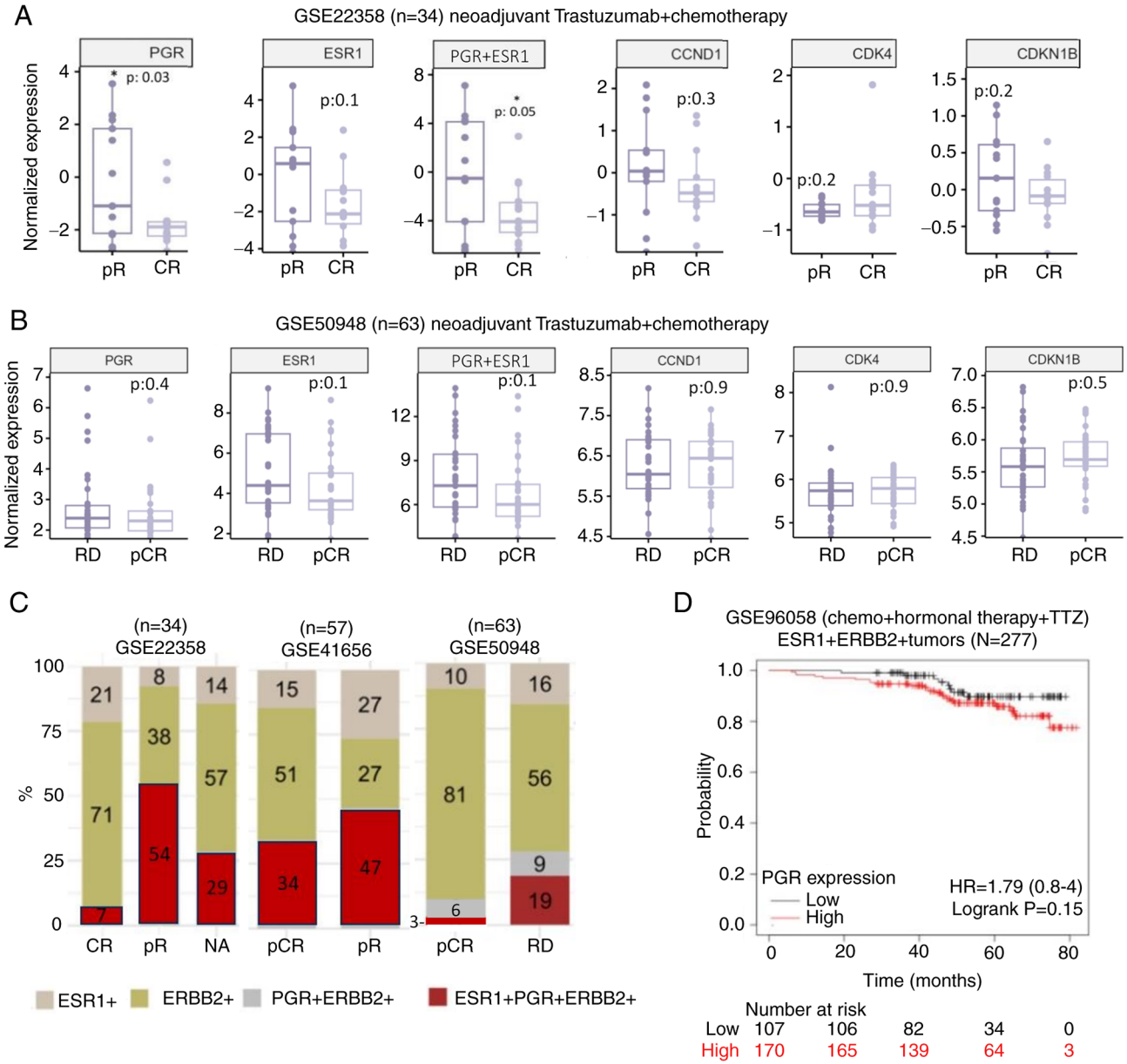

Figure 9. Association between TTZ treatment response and the expression profile of PGR, ESR1 and their downstream genes in patients with TPBC. Normalized mRNA expression levels of PGR, ESR1, CCND1, CDK4 and CDKN1B in (A) patients with TPBC and pR or CR to therapy from the GSE22358 dataset (37) or in (B) patients with TPBC and RD or pCR from the GSE50948 dataset (38). (C) Percentage of patients from the GSE22358, GSE50948 and GSE41656 (39) datasets with varied responses to therapy for which their tumors express ESR1, PGR, ERBB2 or a combination of these genes. (D) Survival curve of patients from the GSE96058 dataset (40) with $ER^+/HER2^+$ breast cancer, low or high levels of PGR and following a treatment scheme that included TTZ. *P<0.05 vs. CR. pR, partial response; CR, complete response; PGR, progesterone receptor; ESR1, estrogen receptor 1; CCND1, cyclin D1; CDKN1B, $p27^{Kip1}$; RD, residual disease; pCR, pathological complete response; ERBB2, human epidermal growth factor receptor-2; TPBC, triple-positive breast cancer; HR, hazard ratio.

could not be found. Alternatively, future studies could analyze the levels of Pg levels in the metabolome of patients with TPBC, to see if they are associated with response to TTZ treatment. Notably, the present study revealed that patients with TPBC tended to have a partial response to treatment more often than other breast cancer subtypes. Even if patients with triple-negative breast cancer do not appear in this analysis, future research could be directed at identifying the reason behind this, which could be related to the effect of Pg, but not necessarily through its canonical genomic pathway, as PR expression did not seem to be robustly enriched in non-responders to treatment.

In conclusion, the inhibitory effect of TTZ on viability in BT474 TPBC cells may be interfered with by Pg and E2, and to a greater extent by their combination (Fig. 10). Pg, in the absence of E2, may promote interference in TTZ activity by reactivating cell proliferation and promoting PR-B phosphorylation (Fig. 10B), and when E2 is present, by inducing PR-A dephosphorylation (Fig. 10C). Pg-dependent reactivation of cell proliferation did not induce the HER2/Akt signaling pathway, suggesting an alternate pathway dependent on Pg and the PR. In addition, the PR antagonist RU486 did not produce the expected effect in the present study.

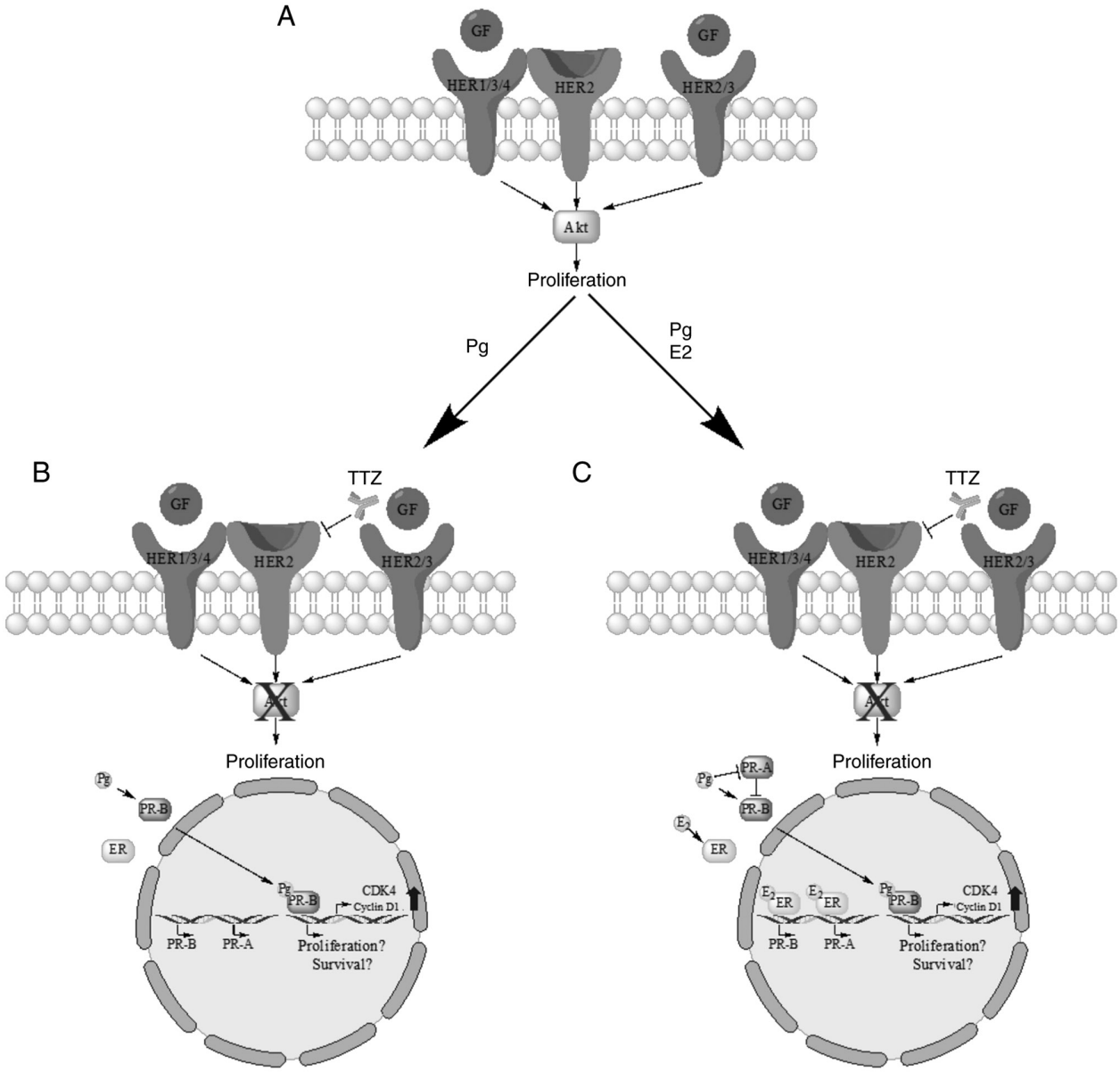

Figure 10. Schematic diagram of the findings on the effect of Pg, E2, TTZ and their combinations on BT474 cells. (A) HER2/Akt-dependent proliferation pathway in tumor cells. (B) Interference of TTZ activity by Pg is associated with PR-B phosphorylation and induction of CDK4 and cyclin D1. (C) Combinatorial effect of Pg and E2 on TTZ activity is associated with PR-A dephosphorylation. GF, growth factor; Pg, progesterone; TTZ, trastuzumab; E2, estradiol; HER2, human epidermal growth factor receptor-2; PR, progesterone receptor; PR-A, PR isoform A; PR-B, PR isoform B.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. The GEO datasets analysed in this study are available in the links provided in Table SII.

Authors' contributions

AZD conceptualized the project and procured the necessary funds and infrastructure for it. JALM and AZD designed the

project. JALM performed the *in vitro* experiments with aid from JLVG and AAPV. JALM and AZD confirm the authenticity of all the raw data. JALM performed the statistical analysis of the *in vitro* data. SLRC conceived and designed the part of the study related to clinical and transcriptomic data; in addition they curated, analysed and aided in the interpretation of such data. AZD, ICA, ML, MMV, NJJH and ALDR supervised the study, and substantially contributed to the conception and design of the experiments. JALM and AJCQ interpreted the data and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Use of artificial intelligence tools

During the preparation of this work, AI tools (ChatGPT-4 through the Microsoft Bing chat interface) were used to improve the readability and language of the manuscript, and subsequently, the authors revised and edited the content produced by the AI tools as necessary, taking full responsibility for the ultimate content of the present manuscript.

Competing interests

The authors declare that they have no competing interests.

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