

Multi-omics profiling reveals ortho-topolin riboside and protocatechualdehyde combination exhibits anti-proliferative activity by modulating metabolic pathways in *in vitro* and *in vivo* radio-resistant MDA-MB-231 cell models

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ABSTRACT

Triple-negative breast cancer (TNBC) poses a significant therapeutic challenge because of its intrinsic resistance to traditional hormonal and targeted therapies. Radio-resistant TNBC is one of the most serious problems in improving treatment outcomes. Furthermore, anticancer activity induced by co-treatment with ortho-topolin riboside (oTR) and protocatechualdehyde (PA) in radio-resistant TNBC cells has not yet been investigated. This study aimed to investigate the potential anticancer effects of oTR and PA in *in vitro* and *in vivo* radio-resistant MDA-MB-231 (MDA-MB-231/RR) cells. Transcriptomic, proteomic, and metabolomic analyses were performed to identify the major altered molecular pathways involved in tumor growth inhibition. Co-treatment with oTR and PA synergistically reduced the viability of MDA-MB-231/RR cells compared to treatment with oTR or PA alone. We identified β -alanine metabolism, hypoxia-inducible factor 1 (HIF-1) signaling, FoxO signaling, and the mitogen-activated protein kinase (MAPK) signaling pathways as altered major metabolic pathways. In xenograft mouse models, a significant reduction in tumor volume was observed in both the single treatment (oTR or PA alone) and co-treatment (low- and high-dose) groups, with no significant alterations in body weight. Our findings demonstrate the potential of co-treatment with oTR and PA as a promising therapeutic strategy for the treatment of radio-resistant TNBC.

1. Introduction

Breast cancer is one of the most common malignancies in women worldwide and remains a leading cause of cancer-related mortality [1],

[2]. It is characterized into distinct subtypes according to the expression status of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) [3]. Triple-negative breast cancer (TNBC) is characterized by the absence of expression of these

Abbreviations: ATP, Adenosine triphosphate; CI, Combination index; DPYD, Dihydropyrimidine dehydrogenase; ER, Estrogen receptor; FC, Fold change; FCCP, Carbonyl cyanide 4-trifluoromethoxy phenylhydrazone; FOXO6, Forkhead Box O6; GC, Gas chromatography; HER2, Human epidermal growth factor receptor 2; HPLC, High-performance liquid chromatography; HIF-1, Hypoxia-inducible factor 1; KEGG, Kyoto encyclopedia of genes and genomes; MAPK, Mitogen-Activated Protein Kinase; MDA-MB-231/RR, Radio-resistant MDA-MB-231; MS, Mass spectrometry; NECAB3, N-terminal EF-hand calcium binding protein 3; NSCLC, Non-small cell lung cancer; OCR, Oxygen consumption rate; oTR, Ortho-topolin riboside; PA, Protocatechualdehyde; PCA, Principal component analysis; PRKCA, Protein kinase C alpha; Rot/AA, Rotenone and Antimycin A; TNBC, Triple negative breast cancer; TNFRSF10C, TNF receptor superfamily member 10c.

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receptors. Despite advances in breast cancer treatment, TNBC remains a major therapeutic challenge due to its intrinsic resistance to hormonal and targeted therapies [4], [5].

Radiotherapy remains a critical therapeutic approach that offers substantial clinical benefits to patients with TNBC [6]. However, a significant limitation of this approach is the emergence of resistance in cancer cells that survive radiotherapy, leading to disease recurrence or the development of more aggressive phenotypes. Therefore, it is imperative to develop novel therapeutic agents to overcome this resistance and enhance treatment outcomes.

Protocatechualdehyde (PA) is a natural phenolic aldehyde found in *Salvia miltiorrhiza*, *Phellinus linteus*, and *Prunella vulgaris* [7], [8]. PA exhibits broad-spectrum anticancer activities across colon, breast, hepatocellular carcinoma, gastric, and leukaemia cancer cell lines. [9], [10], [11]. Specifically, PA degrades β -catenin in colon cancer cells, thereby inhibiting Cyclin D1 and inducing cell cycle arrest. In MDA-MB-231 and MCF-7 cells, it directly binds to CtBP1, enhancing the expression of E-cadherin and suppressing tumour metastasis [12], [13].

ortho-Topolin riboside (oTR), a naturally occurring cytokinin identified in poplar leaves, has shown potent anticancer activity across the NCI-60 panel, encompassing breast, lung, colon, central nervous system, ovarian, renal, melanoma, leukemia, and prostate cancer cell lines [14], [15]. oTR has also demonstrated efficacy in non-small cell lung cancer (NSCLC) and radio-resistant triple-negative breast cancer (MDA-MB-231/RR) models [16], [17]. Additionally, oTR administration reduced glucose and lactate levels within breast cancer cells, which is interpreted as a decrease in ATP production due to impaired glycolysis [17]. In NSCLC cells, oTR 10 μ M yielded approximately 80 % survival, whereas a low-dose co-treatment of melatonin 1 mM + oTR 1 μ M achieved a comparable effect, indicating combination synergy [16], [18]. It suggests that co-treatment strategies may achieve superior efficacy at reduced single-agent doses, thereby mitigating adverse effects. PA inhibits tumour metastasis mainly by arresting the cell cycle and increasing cell adhesion protein expression, whereas oTR impairs energy metabolism [12], [13], [17]. Based on these distinct mechanisms, we hypothesized that co-treatment with oTR and PA would demonstrate enhanced anticancer effects compared to either agent alone. Accordingly, we investigated the antiproliferation activity and molecular mechanisms of co-treatment with oTR and PA in MDA-MB-231/RR cells and xenograft mouse model. Multi-omics analyses were employed to reveal key metabolic pathways altered by the co-treatment.

2. Materials and methods

2.1. Chemicals and reagents

High-performance liquid chromatography (HPLC)-grade methanol, chloroform, ethanol, and water were purchased from Thermo Fisher Scientific (Waltham, MA). HPLC-grade hexane was purchased from Honeywell, Burdick & Jackson (Muskegon, MI). The following reagents were purchased from Sigma-Aldrich (St. Louis, MO): butylated hydroxytoluene (BHT), methoxylamine hydrochloride, pyridine, myristic- d_{27} acid, and N, O-bis (trimethylsilyl)trifluoroacetamide (BSTFA) containing 1 % trimethylchlorosilane and dimethyl sulfoxide (DMSO). We also purchased 3-(4, 4-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) from Alfa Aesar (Ward Hill, MA). Seahorse XF glycolytic rate assay and XF cell-mimetic stress test kits were procured from Agilent Technologies (Santa Clara, CA). oTR was purchased from Olchemim Ltd. (Olomouc, Czech Republic). PA was purchased from MedChem Express (USA). Pierce™ bicinchoninic acid (BCA) Protein Assay kit was purchased from Thermo Fisher Scientific. TRIzol® Reagent was procured from Ambion (Carlsbad, CA).

2.2. Cell culture

MDA-MB-231 cells were procured from the American Type Culture

Collection (ATCC, Rockville, MD), and a radio-resistant MDA-MB-231 cell line was established and characterized as previously described [19]. MDA-MB-231/RR cells were cultured in Dulbecco's modified Eagle's medium/high glucose with l-glutamine, sodium pyruvate medium with 10 % fetal bovine serum (Gibco) containing 1 % penicillin-streptomycin (Hyclone Laboratories, Logan, UT), trypsin-EDTA (0.25 %), phenol red, and 1 $\times$ phosphate-buffered saline (PBS; Hyclone Laboratories, Logan, UT). MDA-MB-231/RR cells were cultured at 37°C in a 5 % CO₂ environment.

2.3. Colony formation assay

For the colony formation assay, MDA-MB-231 and MDA-MB-231/RR cells (200 cells/dish) were seeded into cell culture dishes and incubated for 24 h. Subsequently, the cells were exposed to 0-, 3-, or 6-Gy irradiation (MDS Nordion, Ottawa, ON, Canada). After 10 d, the colonies were washed twice with PBS, fixed with 4 % paraformaldehyde, and stained with 2 % crystal violet.

2.4. Cell death assay

Cell death was monitored by propidium iodide (PI) staining and flow cytometry. Identical numbers of cells (1 $\times$ 10⁴/dish) were seeded for 24 h and exposed to 0-, 3-, or 6-Gy irradiation. The cells were washed with cold PBS, resuspended in PI (1:50), and analyzed within 30 min.

2.5. Cell viability analysis

MDA-MB-231/RR cells were plated in a 96-well plates at a density of 5 $\times$ 10³ cells/well and cultured for 24 h at 37°C under a 5 % CO₂ incubator. oTR and PA were diluted in DMSO. MDA-MB-231/RR cells were treated with four concentrations of PA (5, 10, 20, and 30 μ M) in combination with oTR (1 μ M) for 48 h, to determine cell viability. MTT (10 μ L) solution was added to each well for 1 h at 37°C under a 5 % CO₂ incubator. The medium was then removed, 100 μ L of DMSO was added to each well, and the absorbance was measured at 570 nm using a microplate absorbance spectrometer (xMARK™, BIO-RAD, USA).

The combination index (CI) was quantified using the CompuSyn software (version 1.0, ComboSyn Inc., Paramus, NJ, USA). (ComboSyn Inc., Paramus, NJ, USA), which defines effects as additive effect (CI = 1), synergism (CI < 1), or antagonism (CI > 1) [20], [21].

2.6. Xenograft mouse model

Female athymic BALB/c nude mice (4-week-old, 20 g) were acquired from Saintone Co., Ltd. (Jeju, Korea). The mice were housed in a controlled environment with an alternating 12-h light/dark cycle, and temperatures were maintained between 20°C and 25°C. Mice were housed in a pathogen-free area with *ad libitum* access to food and water. For the experiment, MDA-MB-231/RR cells (3 $\times$ 10⁷ cells/tumor), suspended in 200 μ L PBS, were injected into the fat pad of each mouse. When the tumors reached approximately 100 mm³, the tumor-bearing mice were randomly divided into the following seven groups (n = 8 mice/group): Control, oTR (0.4 mg/kg, 1.2 mg/kg); PA (2.7 mg/kg, 4 mg/kg); combination low dose (oTR 0.4 mg/kg + PA 2.7 mg/kg); and combination high dose (oTR 0.4 mg/kg + PA 4 mg/kg); treatments were administered intraperitoneally every 2 days. Tumor growth was monitored every 3 days using a caliper, and tumor volumes were calculated using the following formula: length $\times$ (width)² $\times$ 0.5. After 28 days, the animals were euthanized and tumors were collected. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Jeju National University, Jeju, Korea, and conducted in accordance with its guidelines (Approval No. 2025-0028).

For immunohistochemistry (IHC) staining, tumor tissues from MDA-MB-231/RR xenografts were excised from mice and immediately fixed in 4 % buffered formalin. After fixation, samples were processed

for paraffin embedding, and tissue sections were deparaffinized through a graded xylene–ethanol series. The sections were then blocked with 1 % bovine serum albumin (BSA) and incubated with a primary antibody against Ki-67 (1:100 dilution). Immunoreactivity was visualized using a DAB Detection Kit and a streptavidin–peroxidase system (Dako, Carpinteria, CA, USA).

2.7. Cell lysis and protein quantification

For protein quantification, the harvested cells were sonicated for 10 min. After sonication, the samples were quenched in a liquid N₂ tank for 1 h, followed by warming in a 37°C water bath for 30 min, repeating this warming step twice. The samples were centrifuged at 1000 × g for 10 min at 4°C, and the supernatant was collected for protein quantification.

In a 96-well plate, 5 µL of the centrifuged cell supernatant and 5 µL of distilled water were added to each well. Then, 10 µL of bovine serum albumin standard was added to the respective wells of each concentration. Protein quantification was conducted using the Pierce™ BCA protein assay kit. BCA reagents A and B were mixed, and 200 µL of the mixture was added to each well. The plate was then covered with foil and incubated at 27°C for 30 min. After incubation, absorbance was measured at 570 nm using a microplate reader. The remaining cell pellet was frozen and dried using a freeze dryer (ILShinBio, Dongducheon City, Gyeonggi-do, Republic of Korea).

2.8. Metabolomic profiling analysis

We then added 500 µL of methanol containing 0.01 % BHT, 500 µL of chloroform containing 0.01 % BHT, and 10 µL of an internal standard (PE 17:0/17:0) to the lyophilized cells. The mixture was sonicated for 40 min at 4°C using a portable ultrasonic cleaner (NXP-1002; Kodo Technical Research Co. Ltd., Republic of Korea). The samples were then shaken on ice for 40 min, followed by centrifugation at 18,472 × g for 10 min at 4°C. After centrifugation, 800 µL of buffer was added, and the samples were purged with N₂ gas for 30 min [22]. Methoxylamine hydrochloride in pyridine 30 µL, BSTFA containing 1 % trimethylchlorosilane 50 µL, and myristic-d₂₇ (internal standard) 10 µL were added to a vial and the mixture was vortexed. The samples were incubated in a water bath at 65°C for 60 min to facilitate derivatization.

Gas chromatography–mass spectrometry (GC–MS) analysis was conducted using 7890 A (Agilent Technologies, Santa, Clara, CA) equipped with a 7683B autosampler (Agilent Technologies, Santa, Clara, CA) and 5975 C mass selective detector (Agilent Technologies, Santa, Clara, CA). The oven temperature program was as follows: an initial temperature of 70°C was held for 2 min, then increased to 190°C at 5 °C/min, followed by an increase to 240°C at 6 °C/min, and finally to 280°C at 5 °C/min. Metabolomic profiles were identified using multiple databases, including the NIST-Wiley Mass Spectral Library, Human Metabolome Database (HMDB, <https://hmdb.ca/>), and Golm Metabolome Database (GMD, <http://gmd.mpimp-golm.mpg.de/>). Spectral deconvolution and identification were performed using an automated mass-spectral deconvolution and identification system (AMDIS version 2.66; <https://www.amdis.net/>).

2.9. Transcriptomic profiling analysis

The MDA-MB-231/RR cells were harvested and subjected to transcriptomic analyses. The cells were seeded at a density of 1 × 10⁶ cells/well in 6-well plates. Total RNA was extracted using 700 µL of TRIzol reagent, and the samples were stored at −80°C until further analysis. RNA isolation involved phase separation and isopropanol precipitation to ensure high purity followed by DNase treatment to eliminate genomic DNA contamination. The extracted RNA was fragmented and reverse-transcribed into cDNA. Adapters were attached to both ends of the cDNA fragments, which were then amplified using polymerase chain

reaction and size-selected for library preparation. High-throughput RNA sequencing was conducted using a NovaSeq 6000 platform (Illumina, San Diego, CA) to obtain comprehensive transcriptomic data. The transcriptomic raw data format is available in the Mendeley database (doi: 10.17632/z3s6mnj76g.1).

2.10. Proteomic profiling analysis

The MDA-MB-231/RR cells were harvested for proteomic analysis. The cells were seeded at a density of 1 × 10⁶ cells/well in cell culture plates. The cell samples were lysed using a focused ultrasonicator by adding 500 µL of lysis buffer. The protein concentrations were quantified using the BCA protein assay kit. Subsequently, 500 mM tris (2-carboxyethyl) phosphine was added to reduce the proteins at 33°C for 30 min. The reduced samples were loaded onto 30 kDa molecular weight cut-off filters and centrifuged at 14,000 × g for 15 min. To alkylate proteins, 500 mM iodoacetamide was added to each filter followed by incubation at 25°C for 30 min. Samples were centrifuged again at 14,000 × g for 15 min, 50 mM ammonium bicarbonate (ABC) was added to the filters, and the proteins were digested by incubating the filters at 37°C for 12 h. After digestion, the filters were centrifuged at 14,000 × g for 10 min, and an additional 40 µL of 50 mM ABC was added to the filters, followed by a final centrifugation step. The resulting samples were dried using a Speed-Vac (Bio-Rad, Hercules, CA) and desalted using C18 microspin columns.

The proteomic analysis of MDA-MB-231/RR cells was performed using a Q-Exactive hybrid mass spectrometer (Thermo Scientific, San Jose, CA) with Ultimate 3000 system (Thermo Fisher Scientific, Waltham, MA). Peptides were separated on a PepMap™ RSLC C₁₈ column (3 µm, 100 Å, 75 µm × 2 cm, Thermo Fisher Scientific) at a flow rate of 300 nL/min. The mobile phase consisted of solvent A (0.1 % formic acid) and solvent B, with the following gradient profile: 4 % solvent B (0–1 min), 8 % solvent B (1–6 min), increasing from 8 % to 30 % (6–130 min), increasing from 30 % to 54 % (130–160 min), reaching 96 % solvent B (160–170 min), and returning to 4 % solvent B (170–185 min). Mass spectrometry parameters included a spray voltage of 2.0 kV, a capillary temperature of 320°C, and a scan range of 400–2000 *m/z*. The raw proteomic data format is available in the Mendeley database (doi: 10.17632/z3s6mnj76g.1).

2.11. Glycolytic proton efflux rate and OCR measurements

MDA-MB-231/RR cells were seeded at a density of 2 × 10⁴ cells/well in cell culture plates. After the cells reached 80–90 % confluency, they were treated with oTR 1 µM, PA 20 µM, and a co-treated group (oTR 1 µM + PA 20 µM) for 48 h. Glycolytic rate and mitochondrial function were evaluated using a Seahorse XFe 24 analyzer (Agilent Technologies, Lexington, MA). Glycolytic function was evaluated by measuring proton efflux rate (PER), which reflects the rate of proton release into the extracellular environment during glycolysis. This was determined by subtracting the protons derived from the mitochondria from the total PER, which reflects the rate of protons released into the extracellular environment during glycolysis. All analyses were conducted in accordance with the manufacturer's instructions, as detailed on the Seahorse Bioweb site (<http://www.seahorsebio.com>).

2.12. Western blot analysis

After exposure to 0-, 3-, and 6-Gy of radiation for 24 h, lysates of MDA-MB-231 and MDA-MB-231/RR cells were prepared in RIPA buffer (Thermo Fisher Scientific). The protein concentrations of the cell lysates were quantified using BCA assay kit according to the manufacturer's instructions. Primary antibodies were used at a 1:500 dilution, except for β-actin (1:10,000), and secondary antibodies (anti-rabbit and anti-mouse IgG) were used at a 1:5000 dilution. Protein bands were visualized using an enhanced chemiluminescence (ECL) substrate (Biosesang,

Yongin, Korea) and imaged with a ChemiDoc system (Bio-Rad, Hercules, CA). The developed bands were quantified using ImageJ software (version 1.48; National Institutes of Health, Bethesda, MD).

For cell transfection, FOXO6#1 (sense: 5'-AGUCCAUUUCGUC GCUGUC-3', antisense: 5'-GACAGCGACGAAUGGACU-3'), FOXO6#2 (sense: 5'-CUAACGACUUSAUGGACA-3', antisense: 5'-UGUCCAUGAA GUCGUUGAG-3'), TNFRSF10C#1 (sense: 5'-GUAGGGAUCAUAGUU CUAA-3', antisense: 5'-UUAGAACUAUGAUCCCUAC-3'), and TNFRSF10C#2 (sense: 5'-GAUACACCAACGCUUCCA-3', antisense: 5'-UGGAA GCGUUGGUGUAAUC-3') were used for gene silencing via RNA interference. MDA-MB-231/RR cells were transfected with siRNAs targeting FOXO6 or TNFRSF10C using AccuFect™ Transfection Reagent (Bioneer, Daejeon, Korea) in accordance with the manufacturer's protocol. Protein expression levels of FOXO6 and TNFRSF10C were subsequently evaluated by Western blot analysis.

2.13. Reverse transcription-quantitative PCR (RT-qPCR)

Total RNA was extracted with TRIzol reagent (Invitrogen™, Waltham, MA) from oTR (1 μM) and/or PA (20 μM) treated cells. RNA quantity and purity were determined on a NanoDrop One spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA), and samples meeting an A260/A280 of 1.8 and 2.0 were carried forward for cDNA synthesis. The resulting cDNA was subjected to quantitative PCR using TOPreal™ SYBR Green Premix (Enzynomics, Daejeon, South Korea) in 20 μL reactions on a CFX96 real-time instrument (Takara Bio Inc., Kusatsu, Shiga, Japan). Reactions were run in triplicate, and a melt-curve analysis was performed to confirm single-product amplification. Gene expression was normalized to GAPDH and reported as fold change using the $2^{-\Delta\Delta Ct}$ approach.

2.14. Annexin V-FITC/PI apoptosis assay

Apoptosis was assessed using BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit (BD Pharmingen, San Diego, CA) to assess apoptosis. Following harvest and PBS wash, cells were resuspended in 100 μL of 1 × binding buffer, incubated with Annexin V-FITC and PI for 15 min at room temperature in the dark, diluted with 400 μL buffer, and analyzed within 1 h by CytoFLEX (Beckman Coulter, Brea, CA). Compensation and quadrant gates were set using unstained and single-color controls; debris/doublets were excluded. Populations were defined as Annexin V⁻/PI⁻ (viable), Annexin V⁺/PI⁻ (early apoptosis), Annexin V⁺/PI⁺ (late apoptosis/necrosis), and Annexin V⁻/PI⁺ (necrosis).

2.15. Fluorescence staining

Hoechst staining for apoptotic bodies. For nuclear morphology, samples were incubated with Hoechst 33342 (Thermo Fisher Scientific Inc., Waltham, MA) for 10 min in the dark condition. Fluorescence images were acquired using an ECLIPSE Ts2 inverted microscope (Nikon Corporation, Tokyo, Japan).

Cells (1×10^5 per well) were seeded onto sterile coverslips in 6-well plates, allowed to adhere overnight. After treatment, cells were washed with PBS and fixed 4 % paraformaldehyde for 30 min at room temperature. Permeabilization and blocking were performed in a single buffer consisting of PBS containing 1 % BSA and 0.1 % Triton X-100. Samples were incubated with anti-HIF-1α primary antibody (NB100-448; Novus, Centennial, CO) overnight at 4 °C, washed, and then labeled with an Alexa Fluor 488-conjugated secondary antibody (Thermo Fisher Scientific Inc., Waltham, MA). Hoechst 33342 was used for nuclear counterstain; samples were mounted in antifade medium. Images were acquired on a STELLARIS 5 confocal laser scanning microscope (Leica Co., Wetzlar, Land Hessen, Germany).

2.16. Data processing and statistical analysis

Raw GC-MS data were converted to an analysis base file (ABF) format using an ABF converter (version 11.0; www.moleculardevices.com). Data processing was performed using MS-DIAL (version 4.9; <https://systemsomicslab.github.io/comprms/msdial/main.html>). The GC-MS data were normalized to internal standards and their respective protein quantification values. The data were subjected to fold-change analysis using MetaboAnalyst (version 6.0; <https://www.metaboanalyst.ca/home.xhtml>). Significant differences were determined by Student's *t*-test and Mann-Whitney *U* test using SPSS software (version 26, IBM, Somers, NY). All data were subjected to principal component analysis (PCA) using the Pareto scale for clustering and categorization among the sample groups. GraphPad Prism (version 10.0; GraphPad Software, San Diego, CA) was used to generate volcano plots. Proteomic data were analyzed using Proteome Discover™ (version 3.1, Thermo Fisher Scientific) and the UniProt database (<https://www.uniprot.org/>). We performed multi-omics integration of transcriptomics, proteomics, and metabolomics data using the MetScape plugin in Cytoscape (version 3, <https://cytoscape.org>).

3. Results

3.1. Characteristics of radiation-resistant breast cancer MDA-MB-231/RR cells

MDA-MB-231/RR cells were established by irradiating MDA-MB-231 cells with 2 Gy of ionizing radiation for 25 cycles [23]. Colony formation analysis showed that irradiation reduced the viability of MDA-MB-231 cells compared to that of MDA-MB-231/RR cells (Fig. S1A). Furthermore, the radiation-induced cell death in MDA-MB-231 cells was higher than that in MDA-MB-231/RR cells at 3 Gy and 6 Gy (Fig. S1B). Moreover, MDA-MB-231 cells exhibited markedly higher levels of γ-H2AX expression, a DNA damage marker, compared to MDA-MB-231/RR cells upon exposure to 3 Gy and 6 Gy of irradiation (Fig. S1C). These results suggest that MDA-MB-231/RR cells have stronger radioresistance characteristics than the parental MDA-MB-231 cells.

3.2. Effect of co-treatment with oTR and PA on cell viability

The anti-proliferative effects of combined oTR and PA treatment on MDA-MB-231/RR cells were evaluated. MDA-MB-231/RR cells were exposed to increasing concentrations of PA (1, 5, 10, 20, and 30 μM) and oTR (1 μM) for 48 h. As shown in Fig. 1B, the viability of MDA-MB-231/RR cells was significantly reduced upon treatment with a combination of oTR and PA compared to that after treatment with oTR alone. The CI values, a measure of drug synergy, were 0.77, 0.65, 0.37, and 0.38 for concentrations of 5, 10, 20, and 30 μM PA with oTR 1 μM, respectively (Fig. 1C). As all CI values were < 1, the interaction between oTR and PA indicated a synergistic effect.

The IC₅₀ for oTR was 7.78 μM, whereas that of PA was significantly higher at 142.59 μM. Co-treatments with 5, 10, 20, and 30 μM PA each with oTR 1 μM demonstrated synergy with the most potent effect observed at PA 20 μM (CI = 0.37). Based on the data in Fig. 1A and B, a significant enhancement in reducing cell viability was observed on co-treatment compared to those using the control and oTR, PA single treatment; PA 20 μM concentration was selected as the optimal combination dose with oTR 1 μM.

3.3. Inhibition of tumor growth by co-treatment of oTR and PA in xenograft model

Compared with the control group, the administration of oTR or PA alone moderately suppressed the growth of subcutaneous xenograft tumors. Representative images of excised tumors showed that co-treatment with oTR and PA markedly reduced tumor size compared to

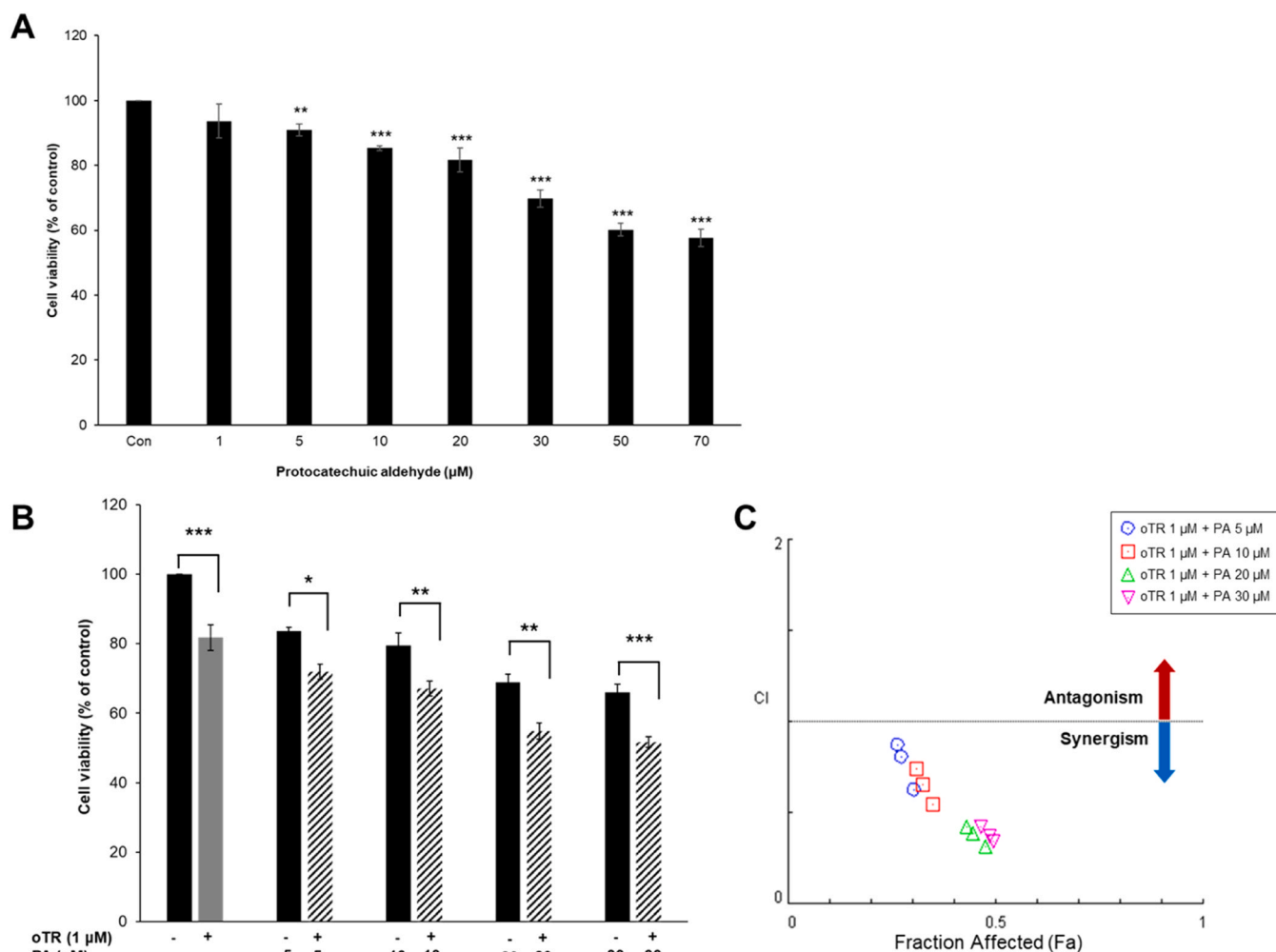


Fig. 1. Effects of co-treatment with ortho-topolin riboside (oTR) and protocatechualdehyde (PA) on cell viability and combination index (CI) values in MDA-MB-231/RR cells. (A) Cell viability following treatment with PA (1–70 μM) for 48 h in MDA-MB-231/RR cells. (B) MDA-MB-231/RR cells viability on co-treatment with oTR and PA. (C) CI plot showing synergistic interaction (CI < 1) between oTR and PA at various concentrations, calculated using the Chou–Talalay method. The bars and error bars represent the mean value and standard deviation (SD; n = 3, three replicates). * $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$ (Student's *t*-test).

single-agent treatment or the control (Fig. 2A). Co-treatment with oTR and PA significantly reduced the tumor volume and weight, particularly in the high-dose combination group (Figs. 2B, 2C). None of the treatment groups showed significant changes in body weight during the treatment period, indicating that the treatments were well tolerated and did not cause systemic toxicity (Fig. 2D). Further immunohistochemical staining was conducted to assess the expression of Ki-67, a proliferation marker indicative of cancer cell division, in tumor tissues. The results revealed that Ki-67 expression was downregulated in both the oTR and PA groups, with the lowest expression observed in the combination group (Fig. 2E).

3.4. Alteration of metabolic profiles in MDA-MB-231/RR cells by co-treatment with oTR and PA

A total of 45 significantly altered metabolites were identified in MDA-MB-231/RR cells, compared to those in the co-treatment group (oTR 1 μM + PA 20 μM). Increased levels of amino acids (alanine, glycine, isoleucine, leucine, lysine, pyroglutamate, serine, threonine, tyrosine, valine), fatty acids (palmitic acid), organic acids (aminomalonic acid, 3-phosphoglycerate, succinate), pyrimidine (uridine), sugars (sucrose), ethanolamine, and glycerol-3-phosphate were observed in co-treated (oTR 1 μM + PA 20 μM) MDA-MB-231/RR cells compared to those in untreated cells. Decreased levels of alcohols (*myo*-inositol),

amino acids (β-alanine, asparagine, aspartate, γ-aminobutyric acid, glutamate, ornithine, proline), organic acids (erythronic acid, glycerate, lactate, malate), purines (guanine, hypoxanthine, inosine), pyrimidine (uracil), sugars (galactose, glucose, glucose-6-phosphate), and creatinine were observed in co-treated (oTR 1 μM + PA 20 μM) MDA-MB-231/RR cells compared to those in untreated cells (Table 1).

As listed in Table S1, starch and sucrose metabolism involved three metabolite hits (sucrose, glucose-6-phosphate, glucose); six metabolite hits (aspartate, asparagine, alanine, glutamate, γ-aminobutyric acid, succinate) in alanine, aspartate, and glutamate metabolism; one metabolite hit (tyrosine) in phenylalanine, tyrosine, and tryptophan biosynthesis; four metabolite hits (serine, glycine, threonine, glycerate) in glycine, serine, and threonine metabolism; four metabolite hits (sucrose, galactose, glucose, *myo*-inositol) in galactose metabolism; and three metabolite hits (β-alanine, aspartate, uracil) in β-alanine metabolism were observed. The PCA-derived score plot showed clustering of the quality control samples, indicating the robustness and reproducibility of the analytical methods in the cell systems (Fig. S2).

3.5. Transcriptomic profiles in MDA-MB-231/RR cells by co-treatment with oTR and PA

The alterations in gene expression profiles between the control and co-treatment groups (oTR 1 μM + PA 20 μM) were analyzed, revealing

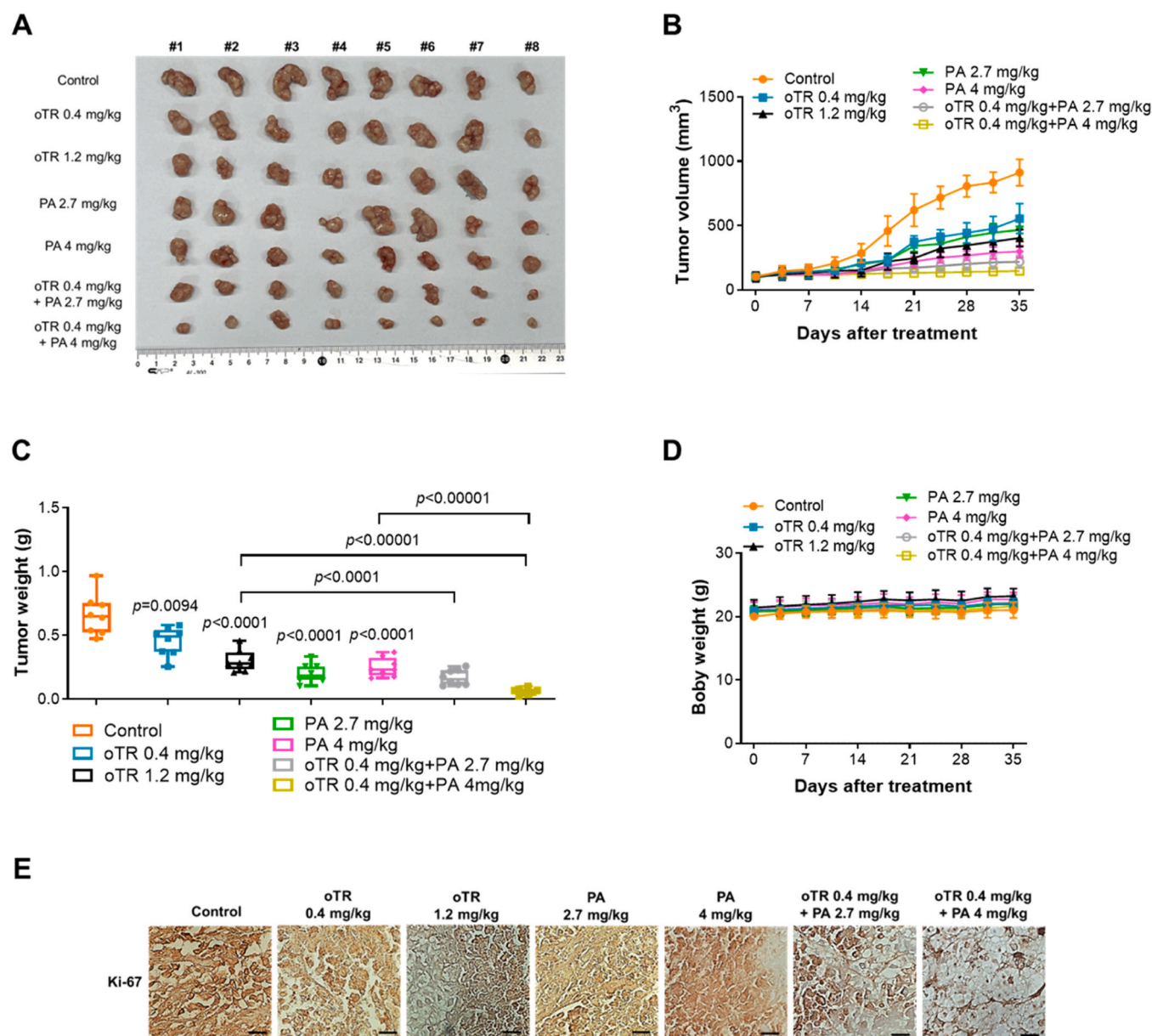


Fig. 2. Co-treatment with oTR and PA suppresses tumor growth in the MDA-MB-231/RR xenograft model. (A) Representative images of subcutaneous tumor tissues derived from mice at the endpoint. Mice were treated with oTR (0.4 mg/kg and 1.2 mg/kg), PA (2.7 mg/kg and 4 mg/kg), a low-dose combination (oTR 0.4 mg/kg + PA 2.7 mg/kg), and a high-dose combination (oTR 0.4 mg/kg + PA 4 mg/kg), respectively. (B) Tumor volume, (C) tumor weight, and (D) body weight changes were observed following treatment with oTR and PA in the xenograft model. (E) Immunohistochemical analysis of Ki-67 in tumor tissue samples from each experimental group. Scale bars: 100 μ m. Data are expressed as mean $\pm$ SD ($n = 8$ per group). Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparisons test or multiple t -tests with Holm-Sidak correction.

916 significantly identified genes, of which 616 transcripts were upregulated, and 300 were downregulated. Furthermore, notable differences in expression were observed among the control, single oTR treatment, and co-treatment with oTR and PA (Fig. S3). Although there were alterations in gene expression in the single oTR treatment compared to the control group, more substantial alterations were observed in the co-treatment group. In particular, the additional expression changes induced by co-treatment seemed to be attributable to PA. Pathway enrichment analysis indicated that several pathways were significantly altered at the transcriptomic level in MDA-MB-231/RR cells after the co-treatment. As listed in Table S2, the major altered pathways in the transcriptomic profiles were one carbon pool by folate pathway, mitogen-activated protein kinase (MAPK) pathway, and cell cycle.

3.6. Proteomic profiles in MDA-MB-231/RR cells by co-treatment with oTR and PA

Proteomic profiling alterations between the control and co-treatment groups were investigated, resulting in the identification of 2385 protein expression profiles. Among these, 147 proteins exhibited significant alterations in expression following co-treatment, with 69 proteins upregulated and 78 proteins downregulated. Alterations in protein expression profiles were observed in the control, single oTR treatment, and co-treatment groups. In particular, there was a distinct alteration in the proteomic profiles of single oTR treatment and co-treated groups (Fig. S4). Pathway enrichment analysis revealed that several pathways were significantly altered at the proteomic level in MDA-MB-231/RR and co-treated MDA-MB-231/RR cells. Proteomic analysis revealed

Table 1
Identification of metabolites and fold-change in the relative levels of identified metabolites in MDA-MB-231/RR cells and the single oTR, PA-treated MDA-MB-231/RR cells and co-treatment of oTR and PA as determined using gas chromatography–mass spectrometry.

No.	Compound	m/z	RT	Fragmentation ion (m/z)	TMS	log ₂ (FC)		
						PA+oTR/Con	oTR/Con	PA/Con
Alcohols								
1	Glycerol	205	11.531	103, 117, 133, 205	3	0.70	−0.77**	−0.39
2	myo-Inositol	305	29.272	191, 217, 305, 318	6	−0.26***	−0.41**	0.21
Amino acids								
3	β-Alanine	102	9.431	75, 102, 176, 218	2	−0.19*	−0.12	0.06
		174	15.427	86, 174, 248, 290	3			
4	Alanine	116	7.244	59, 75, 116, 190	2	0.44**	0.79***	−0.38*
5	Asparagine	116	21.159	75, 116, 132, 231	3	−0.4***	0.22	−0.99***
6	Aspartate	232	17.729	75, 100, 218, 232	3	−1.0***	−0.57**	−0.43***
7	Cysteine	220	18.523	59, 75, 100, 220	3	−0.16	0.30	−1.44***
8	γ-Aminobutyric acid (GABA)	174	17.911	59, 75, 174, 304	3	−0.57*	0.44	−0.59*
9	Glutamate	246	20.091	75, 84, 128, 246	3	−0.21***	−0.27***	−0.26***
10	Glutamine	156	23.437	75, 131, 156, 245	3	−0.16	0.73**	−1.88***
11	Glycine	102	7.713	59, 102, 176, 204	2	1.22***	0.93***	0.05
		174	12.312	86, 133, 174, 248	3			
12	Isoleucine	158	12.004	86, 158, 218, 232	2	0.77***	0.74***	−0.29**
13	leucine	158	11.474	86, 100, 158, 232	2	0.67***	0.70***	−0.20**
14	Lysine	174	26.394	128, 156, 174, 317	4	0.69***	1.13***	−0.12
15	Ornithine	142	24.321	75, 142, 175, 193	4	−0.72**	−0.1	−0.68
16	Proline	142	12.093	59, 70, 142, 216	2	−1.2***	−0.52**	−1.26***
17	Pyroglutamate	156	17.666	75, 156, 230, 258	2	0.47***	−0.01	−0.56***
18	Serine	204	13.744	75, 100, 204, 218	3	0.23*	0.54***	−0.63***
19	Threonine	218	14.377	75, 117, 218, 291	3	0.27***	0.53***	−0.61***
20	Tryptophan	202	31.238	202, 218, 281, 291	3	−0.34	0.62**	−1.01**
21	Tyrosine	179	25.681	75, 179, 208, 219	2	0.43***	0.75***	−0.54***
22	Valine	218	26.689	75, 179, 218, 280	3			
	Valine	144	10.007	59, 100, 144, 218	2	0.65***	0.78***	−0.28**
Fatty acids								
23	Palmitic acid	117	28.665	75, 117, 129, 313	1	2.97***	0.62	1.51***
Organic acids								
24	Aminomalonate	218	16.468	133, 174, 218, 320	3	1.54***	1.38***	0.49*
25	Fumarate	245	13.545	75, 83, 143, 245	2	0.14	−0.17**	0.28**
26	Erythronic acid	292	18.255	75, 205, 220, 292	4	−2.1***	−1.06**	−0.63***
27	Glycerate	189	12.964	75, 103, 189, 292	3	−0.93**	−0.50**	−0.30*
28	Lactate	117	6.268	66, 75, 117, 191	2	−1.0***	−1.00**	−0.69***
29	Malate	233	16.966	75, 133, 233, 245	3	−0.16**	−0.33**	0.18
30	3-Phosphoglycerate	357	24.137	227, 299, 357, 387	4	0.31***	0.27***	0.37***
31	Succinate	75	12.591	56, 75, 129, 247	2	1.29***	0.68*	0.56*
Purines and pyrimidines								
32	Cytidine	223	38.028	75, 217, 223, 245	4	0.13	0.21	0.88***
33	Guanine	352	29.889	264, 280, 352, 367	3	−1.0***	−0.98**	−0.81***
34	Hypoxanthine	265	24.055	84, 193, 265, 280	2	−1.0***	−0.56**	−0.65***
35	Inosine	217	36.463	75, 103, 217, 230	4	−0.76***	−0.41**	−0.83***
36	Uracil	241	13.137	99, 113, 241, 255	2	−0.67***	0.29**	−1.26***
37	Uridine	217	34.654	75, 103, 169, 217	3	0.43**	−1.07**	0.50***
Sugars								
38	Erythritol	217	17.423	83, 103, 204, 217	4	−0.13	−0.2	−0.34**
39	Galactose	204	26.007	129, 191, 204, 217	5	−1.4***	−0.79**	−1.28***
40	Glucose	204	25.812	129, 191, 204, 217	5	−2.2***	−1.12**	−1.87***
		204	27.463	129, 191, 204, 217	5			
41	Glucose−6-phosphate	387	33.66	129, 204, 299, 387	6	−0.22**	−0.36**	−0.49***
		387	33.824	129, 204, 299, 387	6			
42	Sucrose	361	37.333	103, 129, 217, 361	8	1.66	0.27	0.80***
Others								
43	Creatinine	115	18.412	75, 100, 115, 329	3	−2.1***	−1.18**	−1.17***
44	Ethanolamine	174	11.304	59, 75, 100, 174	3	0.98***	0.68	0.86**
45	Glycerol−3-phosphate	357	23.194	75, 101, 299, 357	4	1.00**	−1.00**	0.72

log₂(FC) indicates the log₂ ratio for the peak intensity of metabolites in the treated groups (single oTR, single PA, and co-treatment with oTR and PA) versus the untreated group. The *p* values were evaluated using the Mann–Whitney *U* test. *n* = 9, three biological and two experimental replicates; RT, retention time; TMS, trimethylsilylation; significantly altered levels compared to those in MDA-MB-231/RR cells (**p* < 0.05; ***p* < 0.01; ****p* < 0.001).

that the ‘lysine degradation’ pathway had the lowest *p* value and the highest impact value in the co-treated MDA-MB-231/RR cells. Significant alterations in apoptotic pathways were also observed. In addition, the citrate cycle (TCA cycle) and the valine, leucine, and isoleucine degradation pathways were identified as the main pathways (Table S3).

3.7. Glycolytic rate and mitochondrial respiration inhibition

Fig. 3A shows the real-time glycolytic rate measured based on the

glycolytic PER in the control, oTR 1 μM, PA 20 μM, and co-treatment with oTR 1 μM and PA 20 μM. As shown in Fig. 3B, basal glycolysis was highest in the control group, indicating normal glycolytic activity. Both oTR 1 μM and PA 20 μM treatments significantly decreased basal glycolysis relative to that in the control group (*p* < 0.05). Co-treatment with oTR 1 μM and PA 20 μM further decreased basal glycolysis, with significant decreases in the three experimental groups (oTR 1 μM, PA 20 μM, oTR 1 μM + PA 20 μM) (*p* < 0.05).

Compensatory glycolysis was evaluated after the addition of

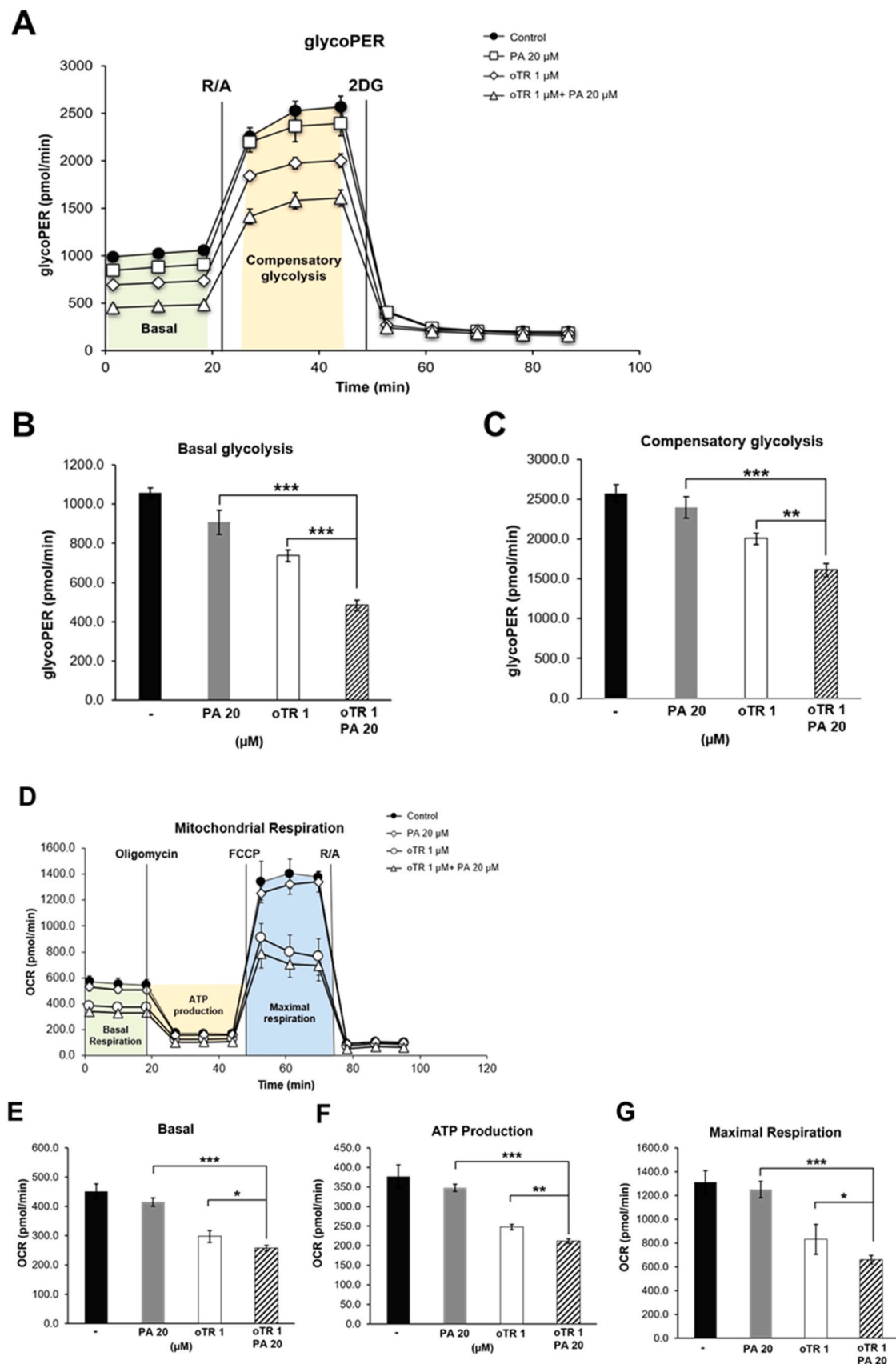


Fig. 3. Comparison of glycolytic rate and mitochondrial respiration following co-treatment with oTR and PA in MDA-MB-231/RR cells. (A) Glycolytic proton efflux rate (glycoPER), (B) Basal respiration, (C) Compensatory glycolysis, (D) Mitochondrial respiration, (E) Basal respiration, (F) ATP production, and (G) Maximal respiration in MDA-MB-231/RR cells after co-treatment with oTR and PA. The cells were treated with oTR 1 μ M, PA 20 μ M, and co-treatment (oTR 1 μ M + PA 20 μ M) for 48 h. Each graph indicates mean values with error bars indicating SD ($n = 3$, three biological replicates). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Student's t -test).

Rotenone and Antimycin A (Rot/AA), which inhibit mitochondrial respiration and made the cells more dependent on glycolysis. As shown in Fig. 3C, glycolysis rates in the control group increased significantly with the addition of Rot/AA, indicating compensatory glycolysis in the control group. However, compared to the control, co-treatment with oTR 1 μ M and PA 20 μ M significantly reduced compensatory correspondence.

As shown in Figs. 3D and 3E, the control group exhibited the highest basal oxygen consumption rate (OCR), confirming normal mitochondrial function. Compared to the control, both oTR 1 μ M and PA 20 μ M treatments significantly decreased basal respiration. Furthermore, the combination treatment (oTR 1 μ M + PA 20 μ M) showed the greatest decrease in basal respiration, indicating a synergistic effect on mitochondrial dysfunction ($p < 0.05$).

Fig. 3F shows ATP production after the addition of oligomycin. Here, the control group exhibited the highest OCR associated with ATP production, which decreased significantly on treatments with both oTR 1 μ M and PA 20 μ M ($p < 0.05$). The co-treatment (oTR 1 μ M + PA 20 μ M) resulted in the most pronounced reduction in ATP production, emphasizing the synergistic inhibitory effect on ATP synthesis ($p < 0.05$).

Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) is an uncoupling agent that collapses proton gradients and disrupts mitochondrial membrane potential. After the addition of FCCP, electron flow through the electron transport chain was not inhibited, and oxygen consumption by complex IV was maximized. The control group reached the highest level of maximal respiration, which significantly decreased on treatment with oTR 1 μ M and PA 20 μ M ($p < 0.05$). In addition, maximal respiration was significantly decreased with combination treatment (oTR 1 μ M + PA 20 μ M), which showed the greatest decrease compared to that on single treatment, thus confirming its strong inhibitory effect on mitochondrial respiratory capacity ($p < 0.05$).

The maximal respiratory capacity, defined as the difference between maximal and basal respiration, was then calculated. Correspondingly, the reserve respiratory capacity represents the mitochondrial ability to respond to increased energy demand; the control group exhibited the highest reserve respiratory capacity (Fig. 3G). However, the oTR 1 μ M and PA 20 μ M treatments significantly decreased the reserve respiratory capacity relative to that of the control ($p < 0.05$).

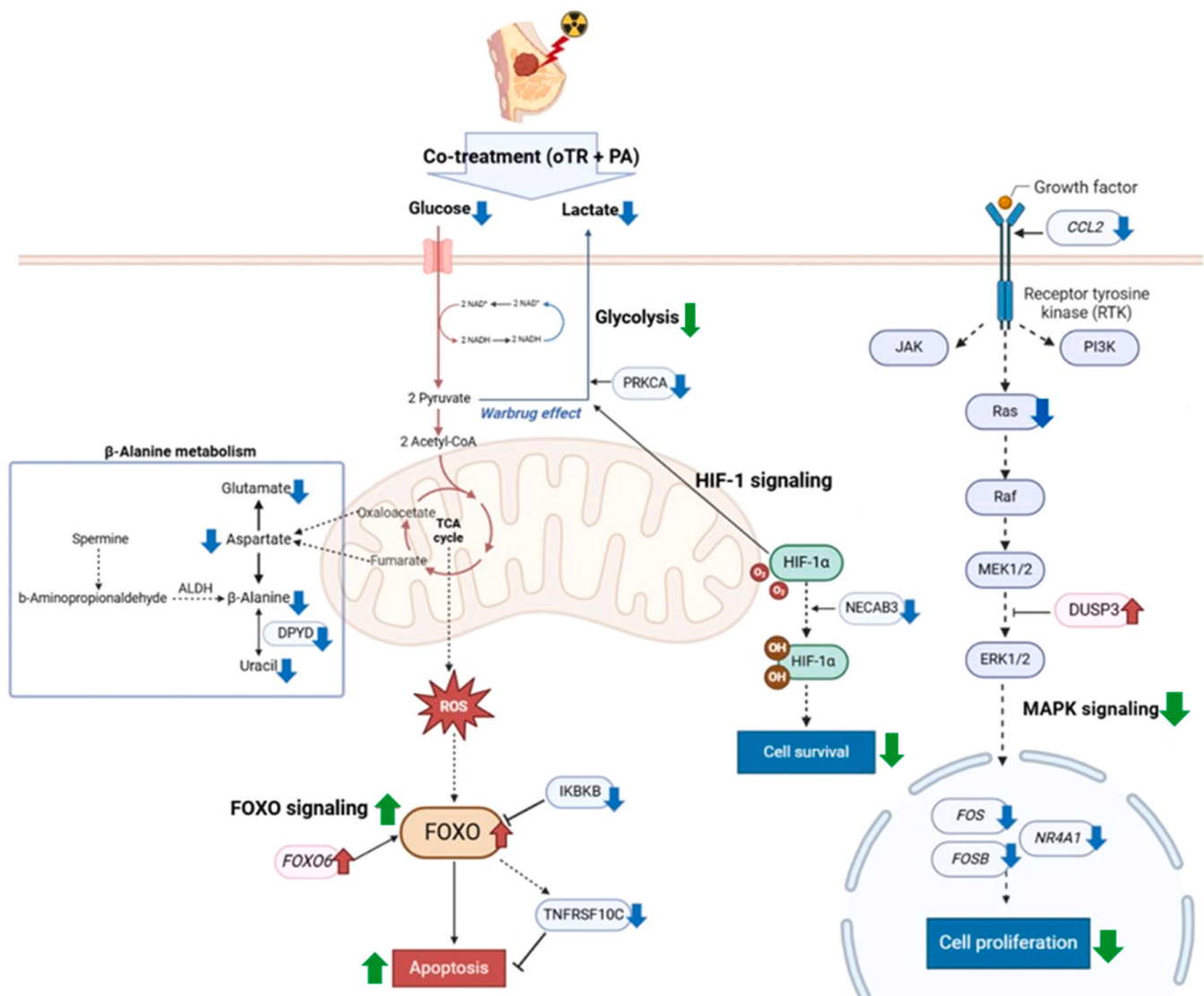


Fig. 4. Integrated pathway analysis by which the transcriptome, proteome and metabolome are simultaneously altered by co-treatment with oTR and PA in MDA-MB-231/RR cells. Arrows indicate the direction of change in metabolites, transcripts, and proteins where measured relative to the control: red ↑, significant increase; blue ↓, significant decrease ($p < 0.05$). Green arrows denote alterations in cellular signaling and functional state inferred from the integrated data.

3.8. Multi-omics profiling

Transcriptomic, proteomic, and metabolomic profiling data were integrated for multi-omics analyses. The analysis involved a one-to-one matching of expression alterations between the transcriptomic and proteomic datasets. The correlation between the transcriptomic and proteomic data was assessed using Pearson's correlation coefficients. The correlation coefficient was 0.62, indicating a significant correlation (Fig. S5). This suggests a link between gene expression and the subsequent alterations in protein expression.

Integrative analysis of transcriptomic, proteomic, and metabolomic data identified the major metabolic pathways affected by co-treatment with oTR and PA. In the pathway enrichment analysis, pathways such as galactose metabolism and glycine, serine, and threonine metabolism showed statistically significant differences with low *p* values. However, these pathways were only prominent in metabolomic data.

Consequently, the MetScape plugin of Cytoscape was employed to collectively analyze the transcriptomic, proteomic, and metabolomic data (Fig. 4). MetScape analysis revealed interactions between the three data types, facilitating the identification of pathway interactions and regulatory networks that were not discernible in the individual data. This analysis identified hypoxia-inducible factor 1 (HIF-1) signaling pathway, β -alanine metabolism, FoxO signaling, and the MAPK pathway as notably affected with the effects of co-treatment of oTR and PA (Table 2).

In the HIF-1 signaling pathway, glucose ($\log_2\text{FC} = -2.2$), lactate ($\log_2\text{FC} = -1.0$), and protein kinase C alpha (PRKCA) ($\log_2\text{FC} = -1.2$), and N-terminal EF-hand calcium binding protein 3 (NECAB3) ($\log_2\text{FC} = -1.9$) expression were significantly reduced in the co-treatment group compared to those in oTR treatment alone. In β -alanine metabolism, alterations were observed in the transcripts and proteins, including dihydropyrimidine dehydrogenase (DPYD) ($\log_2\text{FC} = -0.4$), and aldehyde dehydrogenase 6 family member A1 (ALDH6A1) ($\log_2\text{FC} = 0.48$). Additionally, metabolomic profiling revealed decreases in β -alanine ($\log_2\text{FC} = -0.19$), aspartate ($\log_2\text{FC} = -1.0$), uracil ($\log_2\text{FC} = -0.67$), and GABA ($\log_2\text{FC} = -0.57$) levels. In the FoxO signaling pathway, alterations were identified in glucose ($\log_2\text{FC} = -2.2$), glutamate ($\log_2\text{FC} = -0.21$), TNF receptor superfamily member 10 C (*TNFRSF10C*) ($\log_2\text{FC} = -2.11$), Forkhead box O6 (*FOXO6*) ($\log_2\text{FC} = 4.61$), and inhibitor of nuclear factor kappa B Kinase Subunit Beta (IKBKB) ($\log_2\text{FC} = -1.33$) expression. In the MAPK pathway, transcriptomic changes included chemokine (C-C motif) ligand 2 (*CCL2*) ($\log_2\text{FC} = -2.02$), Nuclear receptor 4A1 (*NR4A1*) ($\log_2\text{FC} = -3.18$), *FOS* ($\log_2\text{FC} = -2.17$), and *FOSB* ($\log_2\text{FC} = -2.91$). Proteomic alterations included changes in the

expression of dual specificity phosphatase 3 (*DUSP3*) ($\log_2\text{FC} = 1.48$).

3.9. Co-treatment with oTR and PA modulates the expression of key factors FOXO6, PRKCA, and DUSP3 in MDA-MB-231/RR cells

To validate the main results from the multi-omics profiling, we conducted RT-qPCR and Western blot analyses to evaluate the expression levels of the key regulatory molecules of FOXO6, PRKCA, and DUSP3 in MDA-MB-231/RR cells. RT-qPCR analysis revealed a significant downregulation of *PRKCA* in the co-treatment group compared with both the control and single treatment groups, whereas *FOXO6* and *DUSP3* transcript levels were upregulated (Fig. 5A). Consistent with these results, Western blotting showed that co-treatment with oTR and PA significantly decreased PKC α and increased FOXO6 and DUSP3 compared with the control and single treatment groups (Fig. 5B). Collectively, these findings indicate that co-treatment oTR and PA may exert anticancer effects in MDA-MB-231/RR cells by modulating key regulators of apoptosis and signaling pathways.

3.10. Co-treatment with oTR and PA induces apoptosis via the FoxO signaling pathway in MDA-MB-231/RR cells

To assess the apoptotic effects of oTR and PA treatment, Annexin V-FITC/PI staining followed by flow cytometry was performed in MDA-MB-231/RR cells. The proportion of apoptotic cells was markedly higher in the co-treatment group than in either the single treatment or control groups (Fig. 6A). Western blot analysis confirmed a significant elevation of cleaved caspase-3, a definitive marker of apoptosis, in the co-treatment group (Fig. 6B). Consistently, confocal microscopy revealed the cleaved, active form of caspase-3, which was substantially increased under co-treatment (Fig. 6C). Hoechst 33342 staining further revealed pronounced chromatin condensation and nuclear fragmentation in co-treated cells relative to control groups (Fig. 6D). To delineate the roles of *FOXO6* and *TNFRSF10C* in apoptosis, knockdown of each gene was performed in MDA-MB-231/RR cells. Western blot analysis revealed that *FOXO6* knockdown reduced cleaved caspase-3 levels, whereas *TNFRSF10C* knockdown increased them, indicating opposing effects on apoptosis (Fig. 6E). Concordantly, Annexin V-FITC/PI analysis showed a reduced proportion of apoptotic cells following *FOXO6* knockdown, whereas an increased proportion was observed after *TNFRSF10C* knockdown (Fig. 6F). Furthermore, under *TNFRSF10C* knockdown, co-treatment with oTR and PA further decreased residual *TNFRSF10C* protein and increased cleaved caspase-3 levels compared with knockdown alone (Fig. 6G), and this was accompanied by a greater

Table 2
List of selected pathways exhibiting significant alteration in transcript, protein, and metabolite profiles with the co-treatment of oTR and PA in MDA-MB-231/RR cells.

Related pathway	Transcript	$\log_2(\text{FC})$	Protein	$\log_2(\text{FC})$	Metabolite	$\log_2(\text{FC})$
		$\frac{\text{oTR+PA}}{\text{Con}}$		$\frac{\text{oTR+PA}}{\text{Con}}$		$\frac{\text{oTR+PA}}{\text{Con}}$
HIF-1 signaling	nd		PRKCA*	-1.2	Glucose*	-2.2
			NECAB3*	-1.9	Lactate*	-1.0
			DPYD*	-0.4	β -Alanine*	-0.19
β -Alanine metabolism	nd		ALDH6A1	0.48	Aspartate*	-1.0
					Uracil*	-0.67
					GABA*	-0.57
					Glucose*	-2.2
FoxO signaling	<i>TNFRSF10C</i> *	-2.11	IKBKB*	-1.33	Glutamate*	-0.21
	<i>FOXO6</i> *	4.61				
	<i>CCL2</i> *	-2.02				
	<i>NR4A1</i> *	-3.18				
MAPK pathway	<i>FOS</i> *	-2.17	<i>CCL2</i> **	-2.63	nd	
	<i>FOSB</i> *	-2.91	<i>RAS</i> **	-0.95		
			<i>MEK</i> **	-2.91		
			<i>ERK</i> **	-3.98		

The transcriptomic, proteomic, and metabolomic data were analyzed collectively using the MetScape plugin of Cytoscape; nd, no detection; *, Significantly altered transcript, protein, and metabolite by co-treatment of oTR and PA (*p* < 0.05). **, Levels of the proteins were obtained from an additional western blot validation experiment (*p* < 0.05).

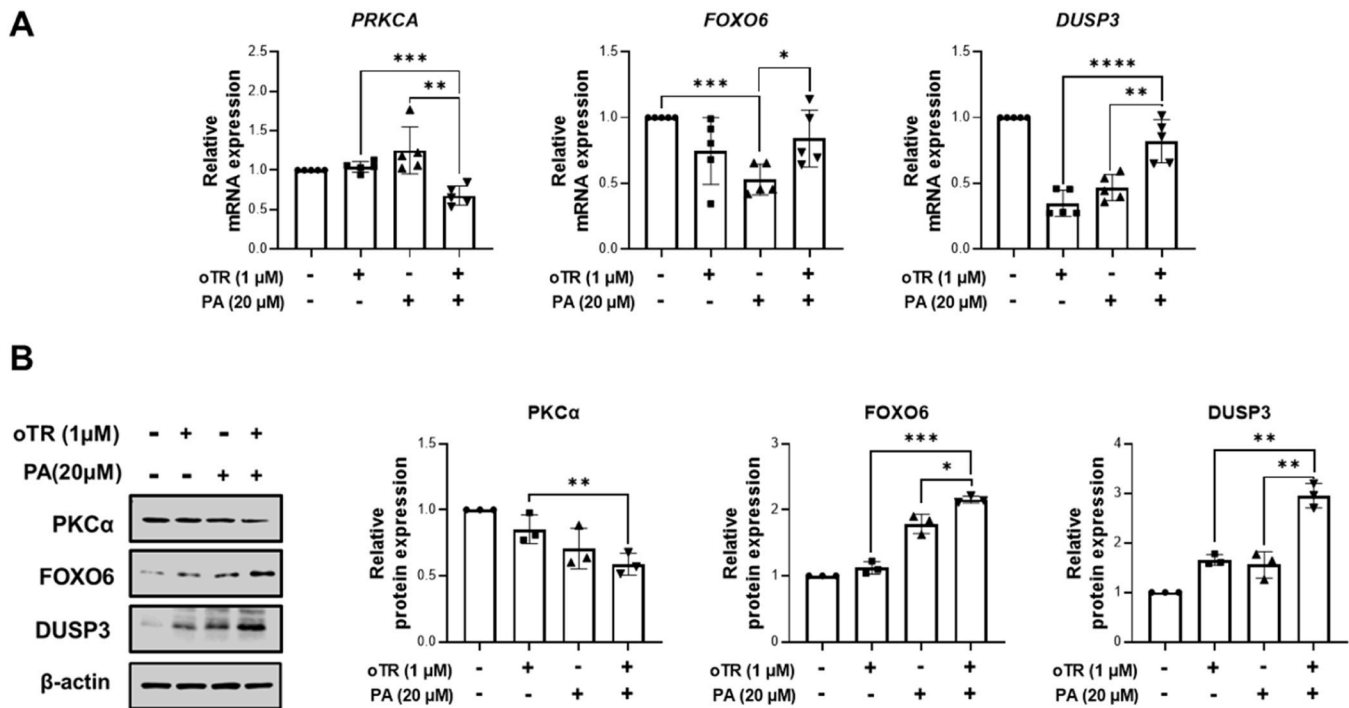


Fig. 5. Altered PRKCA, FOXO6, and DUSP3 expression following combined oTR and PA treatment in MDA-MB-231/RR cells. (A) mRNA expression levels of *PRKCA*, *FOXO6* and *DUSP3* in MDA-MB-231/RR cells following treatment with oTR (1 μM), PA (20 μM), or co-treatment (oTR 1 μM + PA 20 μM). (B) Protein expression levels of PKCα, FOXO6, and DUSP3 detected by western blot under the same treatment conditions. Each graph represents mean values with error bars indicating SD (n = 3, three biological replicates). **p* < 0.05, ***p* < 0.01, ****p* < 0.001, and *****p* < 0.0001 (two-way ANOVA).

presence of bright blue condensed chromatin, a hallmark of apoptosis, as observed by Hoechst 33342 staining, compared with the control and single treatment groups (Fig. 6H). Collectively, these findings suggest that the co-treatment with oTR and PA induces apoptosis in MDA-MB-231/RR cells through modulation of FoxO signaling.

3.11. Co-treatment with oTR and PA attenuates nuclear HIF-1α and ERK activity in MDA-MB-231/RR cells

To investigate the effects of oTR and PA on nuclear HIF-1α localization and ERK signaling in MDA-MB-231/RR cells, we first performed cell fractionation followed by Western blot analysis after treatment with oTR (1 μM), PA (20 μM), or their co-treatment. This analysis demonstrated a marked reduction in nuclear HIF-1α levels in the co-treatment group compared with the control group (Fig. S6A). Consistently, confocal microscopy confirmed a pronounced decrease in nuclear HIF-1α accumulation under co-treatment conditions (Fig. S6B). Next, western blot analysis showed that treatment with oTR, PA, or their combination significantly reduced p-ERK/ERK level (Fig. S6C). In line with these findings, RT-qPCR analysis confirmed that oTR, PA, and their co-treatment significantly downregulated the ERK downstream targets *FOS*, *FOSB*, and *NR4A1* in MDA-MB-231/RR cells, consistent with the observed decrease in p-ERK/ERK (Fig. S6D). Collectively, these results suggest that co-treatment with oTR and PA suppresses nuclear accumulation of HIF-1α and attenuates ERK activity within the MAPK signaling cascade in MDA-MB-231/RR cells, thereby leading to down-regulation of downstream transcriptional targets and potentially impairing cell proliferation.

4. Discussion

We aimed to elucidate the anticancer mechanisms of the combination of PA, a plant-derived natural product, and oTR, a plant growth regulator (a cytokinin derivative), in radio-resistant TNBC cells and xenograft mouse models using multi-omics analysis. Our analyses

identified the major altered molecular pathways involved in tumor growth inhibition. Co-treatment with oTR and PA synergistically reduced the viability of MDA-MB-231/RR cells compared to treatment with oTR or PA alone. Identified major altered pathways were β-alanine metabolism, hypoxia-inducible factor 1 (HIF-1) signaling, FoxO signaling, and mitogen-activated protein kinase (MAPK) signaling. In xenograft mouse models, a significant reduction in tumor volume was observed in both the single treatment and co-treatment groups, with no significant alterations in body weight. The findings of this study demonstrate the potential of co-treatment with oTR and PA as a promising therapeutic strategy for the treatment of radio-resistant TNBC. This is the first study to elucidate the mechanisms underlying the anti-proliferative activity of co-treatment with oTR and PA in *in vitro* and *in vivo* MDA-MB-231/RR cell models.

PA exhibits antioxidant, anti-inflammatory, and anticancer properties [24]. It also exerts anticancer effects through multiple mechanisms in different cancer types, and suppresses proliferation and migration by targeting CtBP1 and restoring tumor-suppressive gene expression in breast cancer [25]. In colorectal cancer models, PA downregulates cyclin D1 and HDAC2 [12], induces ATF3-mediated apoptosis [9], and triggers S-phase arrest via the p27^{KIP1}-cyclin A/D1-CDK2 axis [26]. In melanoma cells, PA synergizes with dacarbazine by destabilizing O⁶-methylguanine-DNA methyltransferase (MGMT) and augmenting DNA double-strand breaks, thereby enhancing apoptosis [27]. PA inhibited the proliferation of ER-positive MCF-7 breast cancer cells, whereas this effect was not observed in ER-negative MDA-MB-231 cells [28].

oTR (5 μM) influences various metabolic pathways and exhibits anticancer efficacy in MDA-MB-231/RR cells [21]. In addition, 1 μM oTR shows no significant toxicity in normal cell lines (MCF-10A and fibroblast cells) [29]. Therefore, to reduce potential toxicity in normal cell lines, we selected 1 μM oTR in combination with PA. Notably, although PA alone showed little activity in MDA-MB-231/RR cells, co-treatment with oTR and PA produced a significant synergistic reduction in cell viability, demonstrating its synergistic anticancer

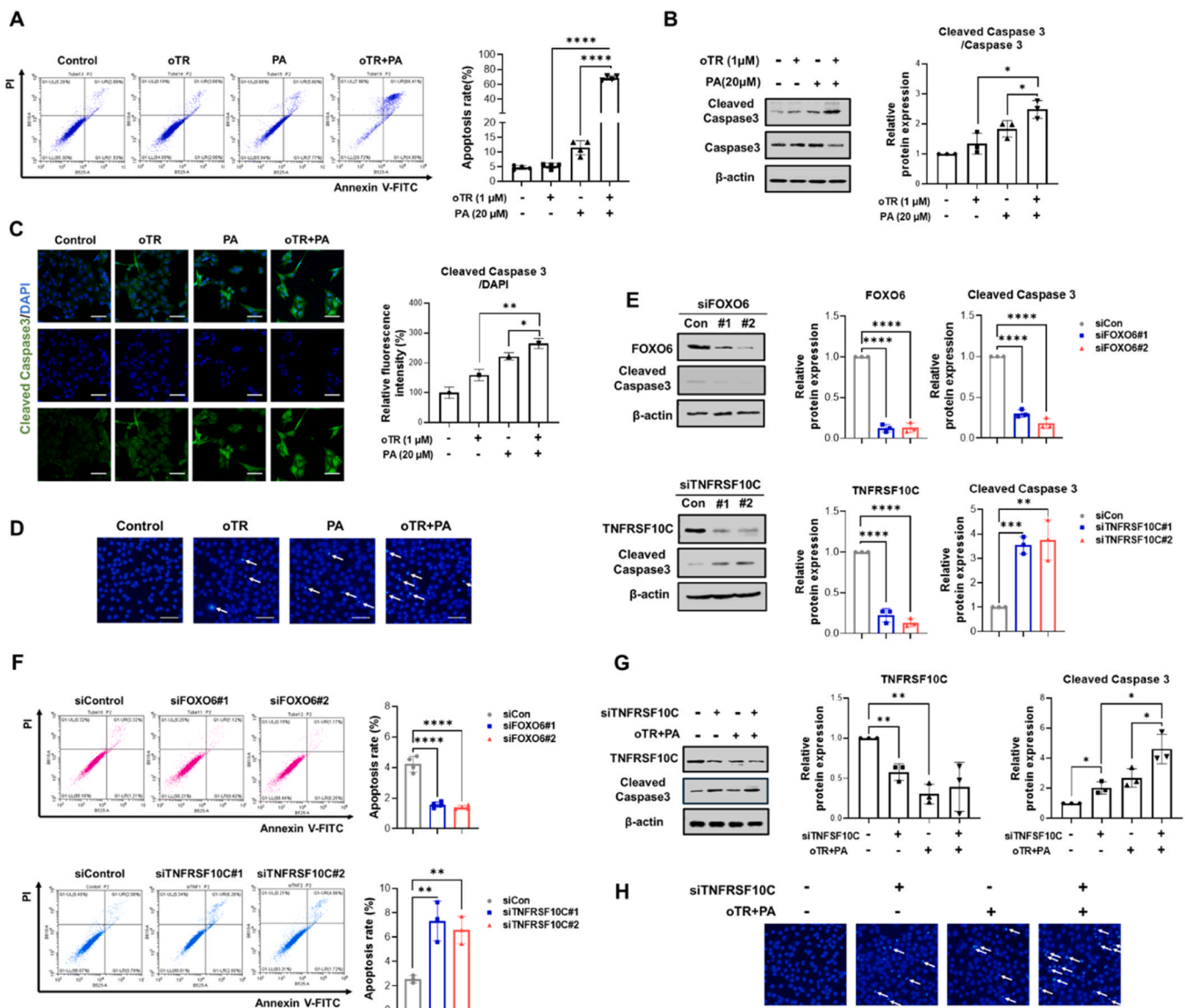


Fig. 6. Co-treatment with oTR and PA is associated with the induction of apoptosis via the FoxO signaling pathway in MDA-MB-231/RR cells. (A) Representative flow cytometry plots using Annexin V-FITC/PI staining to assess apoptosis in MDA-MB-231/RR cells following treatment with oTR (1 μ M), PA (20 μ M), or co-treatment (oTR 1 μ M + PA 20 μ M). (B) Western blot analysis of cleaved caspase-3 and total caspase-3 expression in MDA-MB-231/RR cells. (C) Confocal immunofluorescence images showing cleaved caspase-3 (green) and nuclei (blue) in MDA-MB-231/RR cells treated with oTR (1 μ M), PA (20 μ M), or co-treatment (oTR 1 μ M + PA 20 μ M). Scale bar: 50 μ m. (D) Hoechst 33342 staining of MDA-MB-231/RR cells, with arrows indicating condensed chromatin. Scale bar: 100 μ m. (E) Western blot analysis of FOXO6, TNFRSF10C, and cleaved caspase-3 expression in MDA-MB-231/RR cells transfected with FOXO6 and TNFRSF10C siRNA. (F) Representative flow cytometry plots using Annexin V-FITC/PI staining to assess apoptosis in MDA-MB-231/RR cells transfected with FOXO6 and TNFRSF10C siRNA. (G) Western blot analysis of TNFRSF10C protein levels in MDA-MB-231/RR cells transfected with TNFRSF10C siRNA, with or without co-treatment (oTR 1 μ M + PA 20 μ M). (H) Hoechst 33342 staining of MDA-MB-231/RR cells transfected with TNFRSF10C siRNA, with or without co-treatment (oTR 1 μ M + PA 20 μ M). Arrows indicate condensed chromatin. Scale bar: 100 μ m. Each graph represents mean values with error bars indicating SD ($n = 3$, three biological replicates). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ (two-way ANOVA).

effects.

Co-treatment with oTR and melatonin inhibits glycolysis and mitochondrial respiration and modulates glycine, serine, and threonine metabolism; tyrosine metabolism; pyrimidine *de novo* synthesis and pyrimidine salvage; and Wnt/ β -catenin signaling [18], [29]. Co-treatment with oTR and PA significantly altered β -alanine metabolism and the FoxO, HIF-1, and MAPK signaling pathways, leading to pronounced apoptosis induction (FOXO6 upregulation, TNFRSF10C downregulation) and metabolic stress in radio-resistant TNBC cells. Our study shows that co-treatment with oTR and PA exerts different network-level modulation across signaling and metabolic pathways compared with previous reports on co-treatment with oTR and

melatonin.

In the xenograft mouse model, tumor size was significantly reduced by co-treatment with oTR and PA compared to that in the control. The tumor volume and weight were also significantly reduced by co-treatment compared to the administration of oTR, or PA (Figs. 2B, 2C). No significant differences in body weights were observed between the control and treatment groups. Those findings indicate the efficacy and safety of co-treatment with oTR and PA.

Cancer cells primarily rely on glycolysis for rapid proliferation because this process generates ATP at a faster rate than that achieved through oxidative phosphorylation within the mitochondria [30]. This phenomenon is known as the Warburg effect, and is a typical metabolic

feature of cancer cells [31]. We found that both basal and compensatory glycolysis were reduced in both single- and co-treatments (Fig. 3). Similar trends were observed in co-treatment of NSCLC with oTR (1 μ M) and melatonin (1 mM), and the treatment of MDA-MB-231/RR cells with oTR alone (5 μ M) or the co-treatment with oTR (1 μ M) and melatonin (1 mM) [17], [18], [29]. Glycolysis is the main energy metabolic process in cancer cells and is associated with adaptability to metabolic stress for growth and proliferation [32]. Reduced glycolysis may alter the energy metabolism of MDA-MB-231/RR cells and reduce their adaptability to metabolic stress, ultimately inhibiting cancer cell growth and proliferation. This inhibitory effect is linked to the HIF-1 signaling pathway, which regulates cellular metabolism in hypoxic conditions by promoting ATP production and reducing mitochondrial dependence upon oxygen [33], [34].

In the HIF-1 signaling pathway, PRKCA, NECAB3 proteins, glucose, and lactate were significantly reduced by the co-treatment. PRKCA is an isoform of protein kinase C (PKC), and PKC phosphorylates Ser226 of GLUT1 to increase glucose uptake [35]. NECAB3 promotes glycolysis by activation of HIF-1 α under normoxic conditions [36]. Furthermore, the inhibited HIF-1 signaling pathway reduces glucose utilization and ultimately impairs cell survival [37], [38]. We showed that co-treatment with oTR and PA potently suppresses HIF-1 α nuclear localization and thereby attenuates HIF-1 signaling (Fig. S6). Thus, the reduced expression of PRKCA and NECAB3 by the co-treatment may restrict the glucose uptake (decreased glucose) and glycolysis (decreased lactate) in MDA-MB-231/RR cells, thereby inhibiting cell survival. These observations are consistent with prior oTR-based studies, in which oTR alone reduced the HIF-1 signaling pathway and glycolysis in MDA-MB-231/RR cells [17], and co-treatment with oTR and melatonin attenuated glycolytic flux and mitochondrial respiratory capacity in NSCLC models [18].

The β -alanine metabolism was also one of the major altered metabolic pathways after co-treatment by oTR and PA compared to the control. β -Alanine, an end product of pyrimidine metabolism, declines when nucleotide metabolism is reduced [39], [40]. Co-treatment significantly reduced the levels of DPYD, ALDH6A1, β -alanine, aspartate, uracil, and GABA therein. NSCLC models have also shown that oTR (1 μ M) alone significantly decreases β -alanine within β -alanine metabolism [18]. Aspartate plays a role in the survival and proliferation of cancer cells by facilitating ATP production via its conversion to oxaloacetic acid during the tricarboxylic acid cycle [41], [42]. Co-treatment with oTR and PA decreased aspartate levels, and may result in the inhibition of energy metabolism in cancer cells. β -Alanine level was significantly reduced, which might have a detrimental impact on cancer cell survival and proliferation in our study. DPYD catalyzes the conversion of pyrimidine to β -alanine, and ALDH6A1 converts malonate semialdehyde to acetyl-CoA and reduces lactate production, inhibiting cell proliferation [43], [44]. Therefore, a decrease in DPYD, increase in ALDH6A1, and decrease in β -alanine, aspartate, uracil, and GABA by the co-treatment may result in the inhibition of tricarboxylic acid cycle and pyrimidine metabolism, thereby impairing cell survival and proliferation.

The FoxO signaling pathway is an important pathway that regulates cancer cell survival, apoptosis, and oxidative stress responses [45]. Co-treatment with oTR and PA significantly increase *FOXO6* and decrease *TNFRSF10C*, *IKBKB*, glucose, and glutamate levels, which are involved in the FoxO signaling pathway. *TNFRSF10C* decreases the sensitivity of cancer cells to apoptosis [46]. *IKBKB*, a regulator of NF- κ B signaling, could inhibit cell death by impairing survival signaling [47], [48]. Reduced glucose and glutamate levels can also impair energy metabolism in cancer cells [49]. *FOXO6* inhibits cancer cell migration and invasion by upregulating E-cadherin [50]. *FOXO6* expression decreases with a single oTR treatment, but significantly increases with co-treatment with oTR and PA, suggesting a potential role of PA in enhancing *FOXO6* expression. It was also confirmed that *FOXO6* functions as a pro-apoptotic regulator, whereas *TNFRSF10C* acts as an anti-apoptotic mediator in MDA-MB-231/RR cells (Fig. 6). These results

imply that the co-treatment with oTR and PA activated the FoxO signaling pathway, simultaneously disrupting cell survival signaling, promoting apoptosis, and impairing the energy metabolism of MDA-MB-231/RR cells.

Transcriptomic and proteomic profiling revealed that the MAPK pathway was significantly altered in the co-treatment group. The MAPK pathway was the main altered pathway by the single treatment of oTR (5 μ M) in MDA-MB-231/RR cells [17]. Furthermore, the pathway is involved in cancer cell proliferation and survival [51]. Co-treatment of oTR and PA reduced the levels of *CCL2*, *NR4A1*, *FOS*, and *FOSB*, and increased *DUSP3* in the MAPK pathway. *CCL2* is a chemokine involved in cancer progression that promotes cancer cell proliferation and survival by activating the MAPK/ERK pathway [52]. Co-treatment with oTR and PA reduced *CCL2* expression, whereas oTR treatment alone increased *CCL2* expression. The co-treatment-induced reduction in *CCL2* expression inhibited MAPK/ERK activity, thereby demonstrating the synergistic effect of co-treatment in attenuating cancer cell growth and metastasis. RAS protein is a key activator of the RAS/MAPK signaling pathway [53], and its reduction may suppress the MAPK pathway, thereby reducing cancer cell growth in our study. *NR4A1*, *FOS*, and *FOSB* contribute to proliferation of ovarian, breast, and melanoma cancer cells [54], [55]. *DUSP3* suppresses cell proliferation and induces apoptosis by inhibiting MAPK-related proteins [56]. Therefore, the co-treatment with oTR and PA may also suppress the MAPK pathway, and result in decreased MDA-MB-231/RR cell proliferation.

To validate the regulatory effects of key pathway molecules, we confirmed the expression levels of the key regulatory transcripts and proteins from the multi-omics analysis by employing RT-qPCR and Western blot analyses. Co-treatment with oTR and PA significantly downregulated PRKCA while upregulating FOXO6 and DUSP3 resulted in apoptosis induction and inhibited cell survival (Figs. 5, 6). In addition, we showed that co-treatment with oTR and PA potently suppresses HIF-1 α nuclear localization and thereby attenuates HIF-1 signaling (Fig. S6). The combination also reduced ERK phosphorylation and downregulated the expression of its downstream targets (*FOS*, *FOSB*, *NR4A1*), indicating that co-treatment with oTR and PA disrupts MAPK signaling and suppresses cell proliferation (Table 2, Fig. S6).

5. Conclusion

The co-treatment resulted in a synergistic reduction in cell viability. Tumor growth was significantly reduced by the co-treatment, with no significant changes in body weight in the xenograft mouse model. Multi-omics profiling and analyses of target transcripts and proteins revealed significant alterations in multiple metabolic pathways, including HIF-1 signaling, β -alanine metabolism, FoxO signaling, and the MAPK pathway. Co-treatment with oTR and PA could be a promising anticancer strategy for patients with radio-resistant TNBC. In future studies, oTR and PA formulations should be developed and optimized for better application in preclinical and clinical trials.

CRediT authorship contribution statement

Seung Min Nam: Writing – original draft, Visualization, Validation, Investigation, Data curation, Conceptualization. **Dae Kyeong Kim:** Visualization, Investigation, Data curation, Conceptualization. **Ji Soo Kim:** Visualization, Investigation, Data curation. **Seungcheol Lee:** Visualization, Data curation. **Junyoung Ahn:** Visualization, Data curation. **Somi Kim cho:** Writing – review & editing, Writing – original draft, Resources, Project administration, Conceptualization. **Hyung-Kyoon Choi:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Conceptualization.

Compliance with ethical standards

All animal experiments were approved by the Institutional Animal

Care and Use Committee (IACUC) of Jeju National University, Jeju, Korea, and conducted in accordance with its guidelines (Approval No. 2025–0028).

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.biopha.2025.118631](https://doi.org/10.1016/j.biopha.2025.118631).

Data availability

Data will be made available on request.

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