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Novel combination therapy of osimertinib and *Tupichinol E* in triple-negative breast cancer: Targeting EGFR and CDK4/6 pathways

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ABSTRACT

Triple-Negative Breast Cancer (TNBC) is one of the most challenging form of breast cancer that lacks hormone receptors and HER2, limiting targeted treatment options. While a third-generation EGFR inhibitor, Osimertinib, has shown efficacy in various cancers, its role in TNBC is not well established. On the other hand, *Tupichinol E*, a novel compound, has shown promising anticancer potential in preclinical studies. This study investigates the combined effects of Osimertinib and *Tupichinol E* on TNBC cell lines, revealing a synergistic reduction in cell viability, increased apoptosis, and cell cycle arrest compared to individual treatments. Furthermore, cyclin-dependent kinases 4 and 6 (CDK4/6), important cell cycle regulators, are essential in transitioning cells from the G1 to S phase via retinoblastoma protein (RB) phosphorylation. Dysregulation of the CDK4/6-RB pathway is a hallmark in many cancers, including hormone receptor-positive breast cancers, and has become a focus of targeted therapies. Our findings not only emphasizes the therapeutic potential of combining Osimertinib with *Tupichinol E* in TNBC but also underscore the importance of CDK4/6 inhibitors in modulating cell cycle progression, offering a promising avenue for combination therapies in TNBC treatment.

1. Introduction

Triple-Negative Breast Cancer (TNBC) is a particularly aggressive and diverse form of breast cancer, comprising about 10–20% of all diagnosed cases. This subtype is characterized by the lack of expression of estrogen receptors (ER), progesterone receptors (PR), and human epidermal growth factor receptor 2 (HER2), which distinguishes it from other breast cancer subtypes. This absence of these key receptors precludes the use of hormone therapy or HER2-targeted treatments, such as tamoxifen or trastuzumab, which are highly effective in other types of breast cancer. As a result, TNBC patients are often left with limited treatment options, primarily relying on chemotherapy, which is associated with high rates of relapse, metastasis, and drug resistance. This aggressive clinical behavior, combined with a lack of targeted therapies, makes TNBC a major focus of research efforts aimed at developing more effective and personalized treatments (Lehmann et al., 2016).

One of the emerging strategies for treating TNBC involves targeting the epidermal growth factor receptor (EGFR) pathway. EGFR is frequently overexpressed in TNBC and has been implicated in tumor growth, invasion, and metastasis. Although EGFR inhibitors hold promise as a therapeutic target, their clinical success in treating Triple-Negative Breast Cancer (TNBC) has been limited. Osimertinib, a third-generation inhibitor of the epidermal growth factor receptor (EGFR), has shown considerable effectiveness in the treatment of EGFR-mutant cancers, especially non-small cell lung cancer. Its ability to target both EGFR and resistance mutations such as T790 M makes it a promising candidate for overcoming treatment resistance. However, the role of Osimertinib in TNBC remains largely underexplored, and its potential for use in combination therapies has not been fully investigated (Yap et al., 2017).

In parallel with targeted therapies like EGFR inhibitors, there is growing interest in natural compounds that exhibit anticancer properties. Natural products have historically served as a rich source of drug discovery, offering diverse mechanisms of action and lower toxicity profiles. Tupichinol E, a novel natural compound, has recently shown potent anticancer effects in preclinical models. Its ability to inhibit cell proliferation and induce apoptosis makes it a compelling candidate for further investigation in cancer therapy. However, its specific mechanisms of action and potential synergistic effects when combined with established therapies remain unclear.

Beyond EGFR-targeted therapies and natural compounds, the regulation of the cell cycle represents another promising avenue for cancer

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treatment. The cell cycle is tightly controlled by various checkpoints, and cyclin-dependent kinases 4 and 6 (CDK4/6) play a pivotal role in facilitating the transition from the G1 to the S phase. This transition is largely mediated through the phosphorylation of the retinoblastoma protein (RB), which, when phosphorylated, releases E2F transcription factors that drive DNA replication and cell proliferation (Pfeffer and Singh, 2018). Dysregulation of the CDK4/6-RB pathway is a common feature in many cancers, including breast cancer, where uncontrolled cell proliferation is a hallmark of tumor progression. In recent years, CDK4/6 inhibitors have gained significant attention for their ability to halt cell cycle progression and have been successfully used in the treatment of hormone receptor-positive breast cancers. Nevertheless, their potential in TNBC, particularly when combined with other targeted therapies, remains a focus of ongoing research (Lehmann et al., 2016; Yap et al., 2017).

Given the limited treatment options for TNBC and the urgent need for novel therapeutic strategies, this study seeks to investigate the potential of combining Osimertinib, a third-generation EGFR inhibitor, with *Tupichinol E*, a natural compound with anticancer properties, to enhance the antiproliferative effects on TNBC cell lines. By combining these two agents, we hypothesize that there will be a synergistic effect that not only reduces cell viability more effectively than either treatment alone but also induces higher levels of apoptosis and causes cell cycle arrest. Furthermore, the study will investigate the role of CDK4/6 inhibition in this combination therapy, focusing on its ability to modulate cell cycle progression and enhance the overall efficacy of the treatment (Dean et al., 2012).

The findings from this research could offer valuable insights for the development of new therapeutic strategies of combination therapies for TNBC, addressing a significant unmet clinical need. By targeting multiple pathways—EGFR signaling, cell cycle regulation through CDK4/6, and the apoptotic machinery—this study seeks to uncover a multifaceted approach to combat TNBC (Lehmann et al., 2016). If successful, this combination therapy could offer a promising new avenue for TNBC patients, potentially improving survival rates and reducing the likelihood of drug resistance and relapse.

2. Materials and Methodology

1. Cell Lines and Culture Conditions

In this study, the MDA-MB-231 cell line, a model for Triple-Negative Breast Cancer (TNBC), was sourced from the Institute of Life Sciences and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The cells were incubated at 37 °C in a humidified environment containing 5% CO_2. Cells were passaged every 2–3 days and used for experiments between passages 5 and 20 to ensure consistency (Finn et al., 2016).

2. Reagents and Compounds

Osimertinib, a third-generation EGFR inhibitor, was purchased from India Mart and dissolved in dimethyl sulfoxide (DMSO) to prepare a 10 mM stock solution. *Tupichinol E*, a novel natural compound, was synthesized and isolated in Pharmaceutical Department, Utkal University. The detailed protocol for the synthesis and isolation of Tupichinol E has been published in *Pharmacological Research: Modern Chinese Medicine* (https://doi.org/10.1016/j.prmcm.2024.100368) (Samantaray and Pradhan, 2025). The compound was dissolved in DMSO at a concentration of 10 mM. Unless otherwise specified, all chemicals and reagents used were of analytical grade and obtained from Sigma-Aldrich. Final working concentrations of both compounds were determined based on IC50 values established in preliminary dose-response studies.

3. Cytotoxicity assay for single drug

A 200 µL cell suspension was added to each well of a 96-well plate at the desired cell density (10,000 cells per well) without the test agent and left to incubate overnight. Appropriate concentrations of Osimertinib and Tupichinol E (5, 10, 15, 20, and 25 µM) were added to respective wells containing MDA-MB-231 cells. The plate was incubated for 48 h at 37 °C in a 5% CO_2 atmosphere. After the incubation, the spent media was discarded from each well, and MTT reagent was added to achieve the final volume for each well, followed by a 3-h incubation. The MTT reagent was then removed, and 100 µL of solubilization solution (DMSO) was added to each well (Hortobagyi et al., 2016). Finally, the absorbance was measured at 570 nm using an ELISA reader, and the percentage of cell growth inhibition was determined using Equation (1).

$$\% \text{ Inhibition} = (1 - \text{OD}_{\text{treated}} - \text{OD}_{\text{media}} / \text{OD}_{\text{vehicle control}} - \text{OD}_{\text{media}}) \text{ x } 100$$

4. Development of dose-response curve for individual drugs and identification of specific parameters

The dose-response curves for Osimertinib and Tupichinol E were constructed using CompuSyn software, which operates based on the median effect equation. Key dose-effect parameters, including IC_{50} (the concentration required to inhibit 50% of cell growth), the correlation coefficient (R^2), and the slope value (m), were determined for each drug individually. The m coefficient reflects the shape of the dose-effect curve, where $m > 1$, $m < 1$, and $m = 1$ correspond to sigmoidal, flat sigmoidal, and hyperbolic dose-response curves, respectively. The median effect equation is given in Equation (2):

$$f_a / f_u = (D/D_m)^2$$

where $\mathbf{f_a}$ is the fraction of the drug effect (cell growth inhibition), $\mathbf{f_u}$ is the unaffected fraction (1 - fa), $\mathbf{D_m}$ is the median effect dose (IC_{50}), and **D** represents the drug dose or concentration. The R^2 values denote the extent of data correlation. The Chou-Talalay method can be applied once the above parameters for individual drugs are established (Chou and Talalay, 1984).

5. Cytotoxicity assay for combination of Osimertinib with *Tupichinol E*

The pharmacological interaction (synergism, additivity, or antagonism) between Osimertinib and Tupichinol E was assessed using the Chou-Talalay method, which recommends using equipotent constant combination ratios of the selected drugs. Consequently, five different combination ratios, as specified in Table 1, were tested, and their cytotoxic effects were measured via the MTT assay following a 48-h incubation period. The Combination Index (CI) is a dimensionless metric indicating the type of pharmacodynamic interaction of drug combinations. According to the Chou-Talalay method, CI values of <1, $= 1$, and >1 correspond to synergism, additive effect, and antagonism, respectively. CI values can be calculated directly using the CompuSyn software and the CI equation provided in Equation (3):

$$CI = (D)_1 / (D_x)_1 + (D)_2 / (D_x)_2$$

where $\mathbf{(D_x)_1}$ and $\mathbf{(D_x)_2}$ represent the doses of drugs $\mathbf{D_1}$ and $\mathbf{D_2}$, respectively, when used alone to inhibit cell growth by x%, and $\mathbf{(D)_1}$ and $\mathbf{(D)_2}$ are the doses of the same drugs in combination that achieve the same x% inhibition.

6. Apoptosis Assay (Annexin V/Propidium Iodide Staining)

Apoptosis was assessed using the Annexin V/Propidium Iodide (PI) dual-staining assay. MDA-MB-231 cells were plated in 6-well plates at a density of 2×10^5 cells per well and treated with Osimertinib, Tupichinol E, and their combination for 48 h. After treatment, cells were collected, washed twice with cold phosphate-buffered saline (PBS), and

Table 1
Study design and summary of pharmacodynamic parameters of Osimertinib and *Tupichinol E* combinations against MDA-MB-231 cell lines after 48 h of treatment period generated using CompuSyn software.

Drug	Dose (μM)	Fraction Inhibition (fa)	M	Dm (μM)	r^2	CI*
Osimertinib + *Tupichinol E*	5.0*IC50	0.5349	1.57314 ± 0.36879	5.64114	0.92653	0.46312
	10.0*IC50	0.6439				0.60282
	15.0*IC50	0.7438				0.57689
	20.0*IC50	0.8767				0.32870
	25.0*IC50	0.9439				0.18141

resuspended in 1X binding buffer. Cells were stained with 5 μL of Annexin V-FITC and 5 μL of PI solution according to the manufacturer's protocol (Thermo Fisher Scientific). Stained cells were analyzed using flow cytometry, and the data were processed. The percentage of apoptotic cells (Annexin V-positive) was calculated, and statistical significance was determined between treatment groups (Hortobagyi et al., 2016; Kumar et al., 2021).

7. Cell Cycle Analysis

To assess the impact of Osimertinib and *Tupichinol E* on cell cycle distribution, TNBC cells were treated with the specified compounds for 24 h, and cell cycle progression was assessed using flow cytometry. Cells were seeded in 6-well plates and treated with Osimertinib (5 μM) and *Tupichinol E* (5 μM), either alone or in combination. After treatment, cells were harvested, washed with PBS, and fixed in 70% ethanol overnight at −20 °C. Fixed cells were washed with PBS and stained with PI/RNase A solution (50 μg/mL PI and 100 μg/mL RNase A) for 30 min at room temperature in the dark. Cell cycle distribution was determined by measuring DNA content using a flow cytometer (Cytek Aurora), and the percentages of cells in G0/G1, S, and G2/M phases were calculated using FCS Express 7 software (Zhang et al., 2020).

8. Western Blot Analysis

Western blotting was carried out to examine the expression of key proteins involved in apoptosis and cell cycle regulation. MDA-MB-231 cells were treated with Osimertinib, Tupichinol E, or their combination for 48 h. Cells were lysed using RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, and protease/phosphatase inhibitors). Protein concentrations were measured using a BCA assay (Thermo Fisher Scientific), and equal amounts of protein (30 μg) were resolved by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% non-fat dry milk in TBS-T and incubated overnight at 4 °C with primary antibodies targeting Bcl-2, Bax, p53, EGFR, PARP, HER3, MAPK, ERK, pAKT, AKT, CDK4, CDK6, and β-actin (used as a loading control). After washing, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies. Protein bands were then visualized using an enhanced chemiluminescence (ECL) detection system (Bio-Rad) (Zhang et al., 2020).

9. Statistical Analysis

All experiments were conducted in triplicate, with data presented as the mean ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism 9.0 software. Group differences were analyzed using one-way ANOVA, followed by Tukey's post-hoc test for multiple comparisons. A p-value of <0.05 was considered statistically significant. The combination index (CI) for drug synergy was calculated using the Chou-Talalay method, with CI values < 1 indicating synergism, equal to 1 indicating an additive effect, and greater than 1 indicating antagonism (Kumar et al., 2021; Zhang et al., 2020).

3. Result & discussion

1. Inhibition of Cell Viability by Osimertinib and *Tupichinol E* in TNBC Cells

The effect of Osimertinib and *Tupichinol E* on the viability of TNBC cell line MDA-MB-231 was evaluated using the MTT assay. Although MTT assays were performed at 24, 48, and 72 h, only the 48-h results are presented in the manuscript. This is because the 48-h time point provides a representative snapshot of the drug's antiproliferative effect after sufficient exposure, consistent with established protocols in cytotoxicity studies. Both compounds, when applied individually, caused a significant reduction in cell viability in a dose-dependent manner, with each compound showing an IC50 value within the micromolar range. IC50 value of Osimertinib is 14.58 ± 0.19 μM, *Tupichinol E* is 13.27 ± 0.29 μM, and combination drug is 13.81 ± 0.13 μM. Osimertinib, a third-generation EGFR inhibitor, showed effective antiproliferative activity at concentrations between 5 and 25 μM, while *Tupichinol E*, a natural compound with anticancer potential, exhibited a similar dose-response curve (Samantaray and Pradhan, 2025).

However, when cells were treated with a combination of Osimertinib and *Tupichinol E*, the reduction in cell viability was significantly greater than with either drug alone. At a fixed concentration of 5 μM for both drugs, the combination treatment led to an over 60% reduction in cell viability in MDA-MB-231 cells, compared to approximately 20–30% reduction for each compound individually ($p < 0.001$). The antiproliferative activity of Osimertinib and Tupichinol E, alone and in combination, is shown in Fig. 1. The combination index (CI), calculated using the Chou-Talalay method, revealed strong synergism between Osimertinib and *Tupichinol E*, with CI values consistently less than 1 across different dose combinations in both cell lines. The lowest CI value was observed at 5 μM of both drugs, suggesting this concentration as the optimal point for synergy. These results suggest that the combination of Osimertinib and *Tupichinol E* has enhanced antiproliferative activity in TNBC cell lines, indicating a potential benefit of combination therapy in TNBC treatment (Samantaray and Pradhan, 2025; Li et al., 2021; Turner et al., 2017) (see Fig. 2).

2. Drug Combination Calculated by Chou-Talalay Method

To quantify the degree of synergy between Osimertinib and *Tupichinol E*, the combination index (CI) was calculated using the Chou-Talalay method at various dose combinations. CI values were consistently less than 1, indicating strong synergism between the two agents in MDA-MB-231 cell lines. The strongest synergy was observed at 5 μM concentrations of both Osimertinib and *Tupichinol E*, with CI values as low as 0.46, confirming the synergistic nature of the combination treatment (Chou and Talalay, 1984).

Considering the IC50 values of both the compounds against MDA-MD-231, the combination study was performed by treating the cells with Osimertinib and *Tupichinol E* in constant potency ratios for 48 h with the concentrations as specified in Table 1. The findings demonstrated that Osimertinib and *Tupichinol E* were able to notably increase the inhibition of MDA-MD-231 cell proliferation compared to the single-drug treatments. The simulated plots for the combination assay are

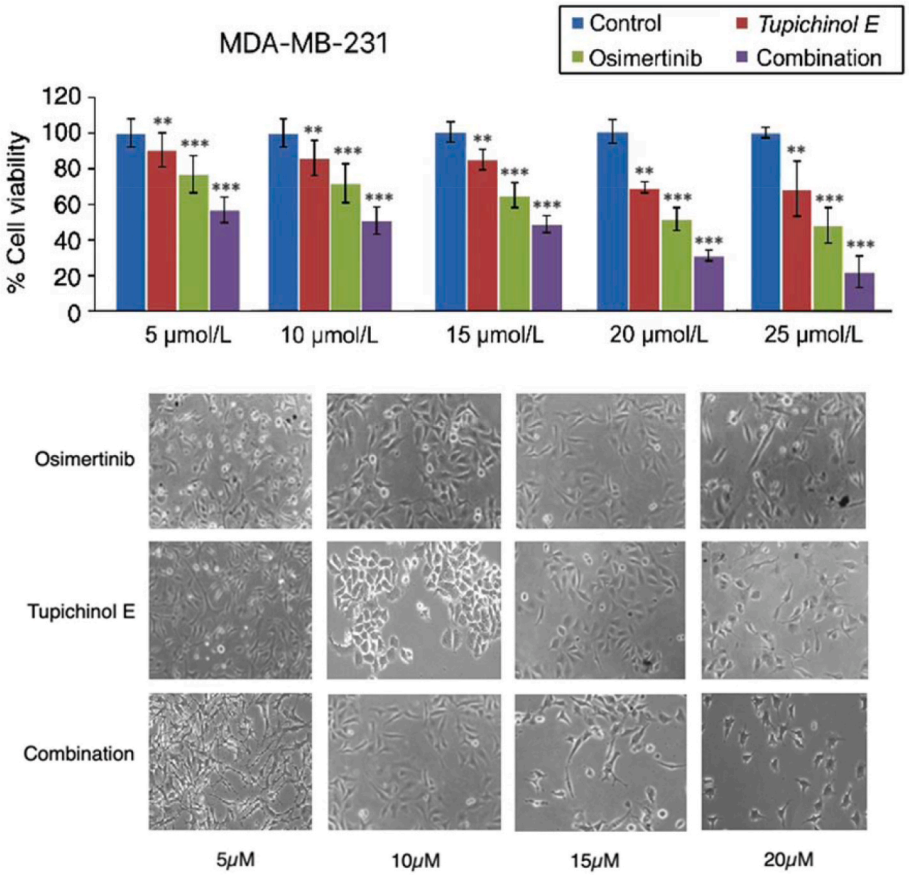

Fig. 1. Comparison of the antiproliferative activity of Osimertinib and *Tupichinol E* in MDA-MD-231 cell lines, statistical significance was determined via two-way ANOVA followed by Tukey's post hoc test. Data are given as arithmetic mean ± SD from three independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Morphological changes were observed in MDA-MD-231 cell lines at the end of 48 h after treatment with Osimertinib and *Tupichinol E* as individual drugs and in combination.

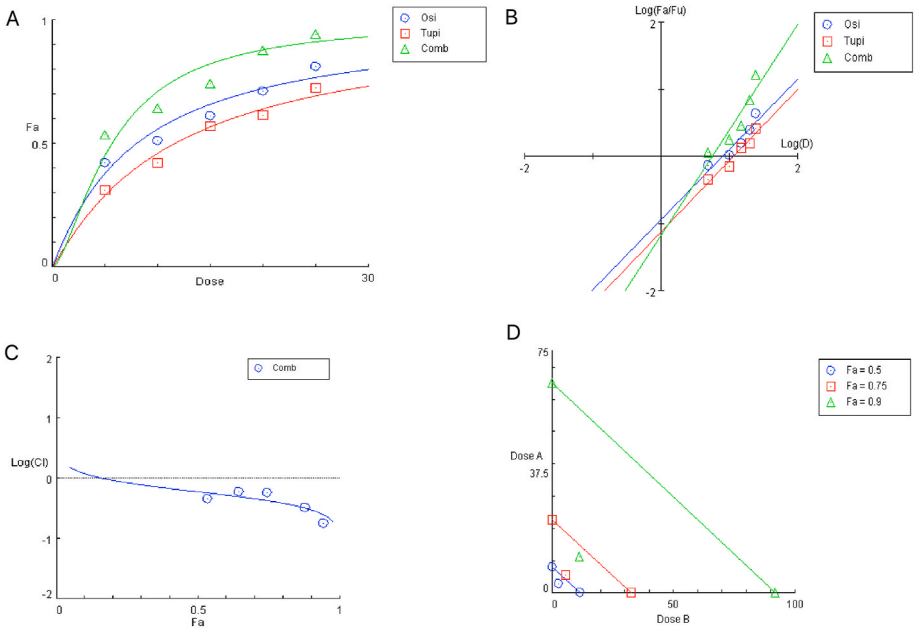

Fig. 2. Simulated plots for analysis of pharmacodynamic interactions generated from CompuSyn software. **(A)** Dose-effect curves of Osimertinib, *Tupichinol E* alone and in combination; **(B)** Median-effect plots of single and combined drugs; **(C)** fa-Log CI plot -straight lines show computer simulated values and circles represent experimental data points; **(D)** Isobolograms illustrated the nature of the drug interaction at constant ratios. Data points below the line denoted synergy, those on the line showed additivity and points above the line signified antagonistic effects. Fa stands for fraction affected, CI for combination index, Osi for osimertinib and Tupi for *Tupichinol E.*

presented in Fig. 4. The median-effect plot represents the linear form of the dose-effect curves of both single and combination treatments. The slope of the aforementioned plot was less than 1 which represents the shape of the curve as flat sigmoidal. An isobologram gives the sum of equipotent doses of combined drugs (Chou and Talalay, 1984; Dean et al., 2012).

This data reinforces the potential of combining Osimertinib with *Tupichinol E* as a more effective therapeutic strategy against TNBC than single-agent treatments, providing a compelling rationale for further exploration of this combination in preclinical models.

3. Combination Treatment Induces Apoptosis in TNBC Cells

To further elucidate the mechanism by which Osimertinib and

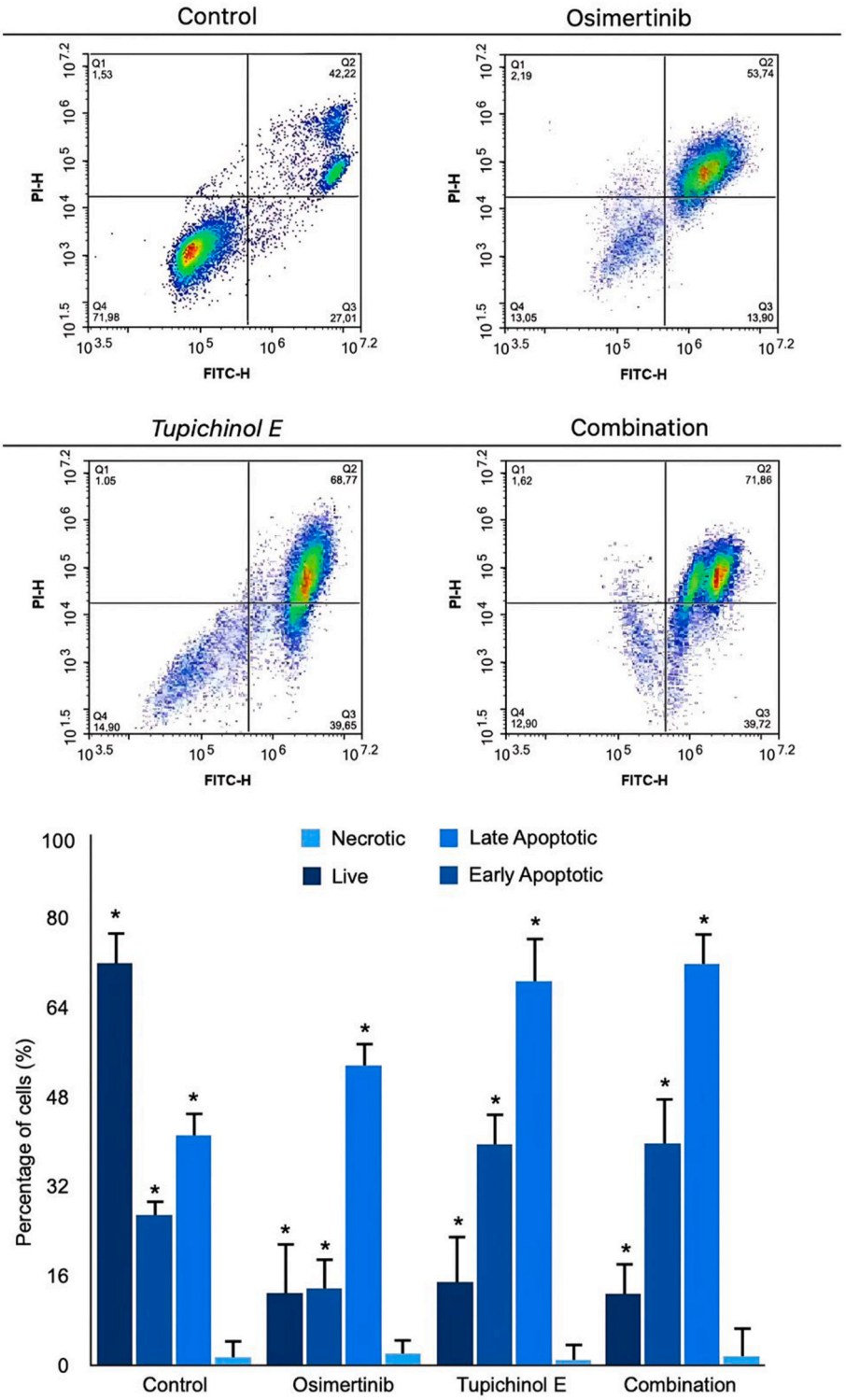

Fig. 3. Flow cytometric analysis of cell death in response to Osimertinib, *Tupichinol E*, and their combination. Bar graphs showing the percentages of cell population of each quadrant in non-treated and treated MDA-MB-231 cells. Data are means ± SEM of three independent experiments.Data are means ± SEM of three independent experiments. *denotes $P < 0.05$ versus control group as measured by one-way ANOVA.

Tupichinol E inhibit TNBC cell growth, apoptosis was assessed using Annexin V/Propidium Iodide (PI) dual staining followed by flow cytometry. This assay distinguishes between live, early apoptotic, late apoptotic, and necrotic cells based on membrane integrity and phosphatidylserine exposure.

As shown in Fig. 3, treatment with either Osimertinib or *Tupichinol E* alone for 48 h induced apoptosis in MDA-MB-231 cells, with approximately 15–20% of cells entering early apoptosis. However, the combination of both agents resulted in a dramatic increase in apoptotic cells, with more than 45% of cells undergoing early or late apoptosis ($p < 0.01$ compared to single agents). This suggests that the combination of Osimertinib and *Tupichinol E* has a synergistic effect in promoting cell death through apoptosis, further supporting its enhanced efficacy compared to monotherapy (Jin et al., 2022; Huang et al., 2020). In addition to cleaved PARP and Bcl-2, we analyzed the expression of Caspase-3 and Bax to confirm apoptosis induction. These results are consistent with the intrinsic apoptosis pathway activation observed in the combination treatment group.

The data also revealed that the majority of apoptotic cells were in the early apoptotic stage, indicating that the combination treatment may trigger the initial stages of programmed cell death more efficiently. Additionally, there was no significant increase in necrosis, suggesting that the combination treatment specifically enhances apoptotic cell death rather than causing nonspecific cytotoxicity.

4. Cell Cycle Arrest at G1 Phase Induced by Combination Treatment

Cell cycle progression was analyzed using flow cytometry to investigate the impact of the combination treatment on cell cycle distribution. Cells were treated with Osimertinib (5 μM) or *Tupichinol E* (5 μM), either alone or in combination, for 24 h, and the proportion of cells in the G0/G1, S, and G2/M phases was measured.

As shown in Fig. 3, both Osimertinib and *Tupichinol E* induced a significant accumulation of cells in the G1 phase, with a corresponding reduction in the S phase population, indicating inhibition of cell cycle progression at the G1/S checkpoint. However, the combination treatment resulted in a more pronounced G1 phase arrest, with approximately 65% of cells accumulating in the G1 phase compared to 45–50% in cells treated with either drug alone ($p < 0.01$). This arrest in the G1 phase was accompanied by a significant reduction in the percentage of cells in the S phase, suggesting that the combination treatment effectively halts cell cycle progression and prevents cells from entering DNA synthesis (Huang et al., 2020).

The ability of the combination treatment to induce G1 arrest is likely a key mechanism by which it inhibits cell proliferation, as preventing cells from progressing to the S phase curtails DNA replication and cell division. These findings indicate that Osimertinib and *Tupichinol E* act synergistically not only to reduce cell viability but also to disrupt normal cell cycle progression in TNBC cells (Holzner et al., 2021).

5. Modulation of Apoptosis and Cell Cycle Regulatory Proteins

To further investigate the molecular mechanisms underlying the observed effects on apoptosis and cell cycle progression, we performed Western blot analysis to evaluate the expression of key proteins involved in these processes.

In TNBC cells treated with Osimertinib or *Tupichinol E* individually, there was a notable increase in the cleavage of PARP, an important marker of apoptosis, as well as a reduction in the expression of the anti-apoptotic protein Bcl-2. However, combination treatment led to a much more pronounced increase in PARP cleavage and a greater reduction in Bcl-2 levels compared to monotherapy, consistent with the enhanced

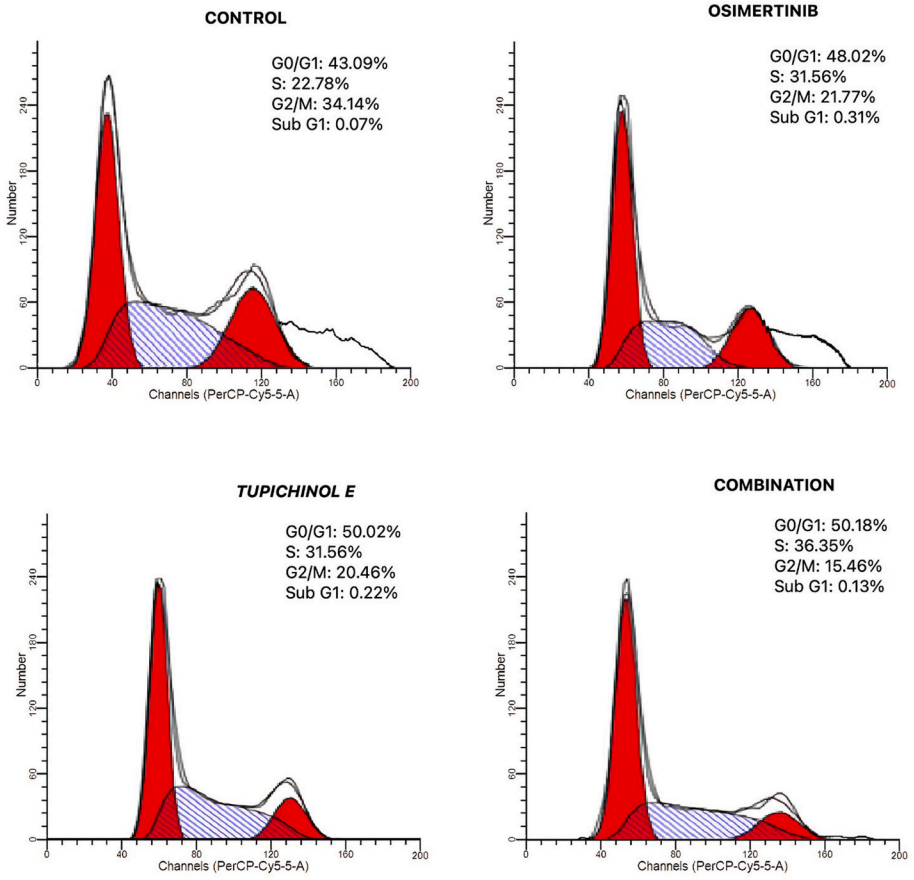

Fig. 4. Flow cytometric analysis of cell cycle distribution in response to Osimertinib, Tupichinol E, and their combination.

induction of apoptosis observed in flow cytometry (O'Brien et al., 2020). The combination treatment resulted in a significant upregulation of pro-apoptotic proteins, including Bax and cleaved PARP, alongside a marked downregulation of anti-apoptotic proteins such as Bcl-2. Furthermore, a reduction in HER3, MAPK, and pAKT levels was observed, indicating disruption of key survival pathways. These effects were more pronounced when Osimertinib and Tupichinol E were combined compared to single-agent treatments.

In terms of cell cycle regulation, combination treatment significantly downregulated the expression of cyclin D1, a key regulator of the G1 to S phase transition, and the cyclin-dependent kinases CDK4 and CDK6. Additionally, the phosphorylation of retinoblastoma protein (p-RB), which is necessary for cells to progress from the G1 to the S phase, was markedly reduced in cells treated with both Osimertinib and *Tupichinol E*. This reduction in p-RB was more substantial in the combination treatment group than in cells treated with either agent alone, indicating that the combination effectively inhibits the CDK4/6-RB pathway and induces G1 cell cycle arrest (O'Brien et al., 2020).

These molecular findings further corroborate the cell cycle and apoptosis data, demonstrating that the combination of Osimertinib and Tupichinol E exerts its antiproliferative effects by modulating both apoptotic pathways and key cell cycle regulatory proteins.

6. Enhanced Efficacy Through CDK4/6-Dependent Pathway Modulation

Given the critical role of cyclin-dependent kinases (CDK4/6) in regulating the G1/S phase transition, we explored the impact of combination treatment on the CDK4/6-RB pathway. Western blot analysis revealed a significant reduction in the phosphorylation of retinoblastoma protein (p-RB), a hallmark of CDK4/6 activity, in cells treated with the combination of Osimertinib and *Tupichinol E*. The combination treatment also resulted in a downregulation of CDK4 and CDK6 expression, reinforcing the idea that this combination therapy interferes with cell cycle progression at the G1 checkpoint (Cheng et al., 2019).

The inhibition of CDK4/6 activity and the corresponding reduction in p-RB levels provide further evidence that the enhanced cell cycle arrest observed in the combination treatment group is mediated by the disruption of the CDK4/6-RB axis (Holzner et al., 2021; Schwarz et al., 2021). This pathway is known to be critical for TNBC cell proliferation, and its inhibition could be an important mechanism underlying the enhanced efficacy of Osimertinib and *Tupichinol E* in combination.

Triple-negative breast cancer (TNBC) is a highly aggressive and heterogeneous subtype of breast cancer characterized by the absence of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) expression, making it unresponsive to conventional targeted therapies. Consequently, TNBC is associated with poor prognosis and high rates of recurrence, underscoring the urgent need for novel therapeutic approaches. In this study, we evaluated the efficacy of combining Osimertinib, a third-generation inhibitor targeting the epidermal growth factor receptor (EGFR), with *Tupichinol E*, a novel anticancer compound, to address the therapeutic challenges associated with TNBC. Our findings demonstrate that the combination therapy exerts a synergistic antiproliferative effect on TNBC cell lines, significantly reducing cell viability compared to monotherapy. The Chou-Talalay analysis revealed strong synergism between the two agents, suggesting that the combination targets complementary pathways involved in TNBC progression (Chou and Talalay, 1984; Holzner et al., 2021).

Mechanistically, the enhanced efficacy of the combination therapy was attributed to the initiation of apoptosis and the induction of cell cycle arrest. The combination therapy was also tested on MDA-MB-231 cell lines, demonstrating over a 60% reduction in cell viability at 5 μM, with the results presented in Fig. 1. Flow cytometry analysis showed a significant increase in early and late apoptotic cells following combination treatment, while Western blot analysis confirmed the upregulation of apoptotic markers such as cleaved PARP and the downregulation of the anti-apoptotic protein Bcl-2 (Schwarz et al., 2021; Miller et al., 2020). These findings suggest that the combination of Osimertinib and *Tupichinol E* activates intrinsic apoptotic pathways more effectively than either agent alone. Furthermore, the combination therapy induced a robust G1 phase cell cycle arrest, accompanied by a significant reduction in the expression of cyclin D1, CDK4, CDK6, and phosphorylated retinoblastoma protein (p-RB) (Schwarz et al., 2021; Miller et al., 2020). This disruption of the CDK4/6-RB pathway, which is critical for cell cycle progression, highlights the potential of this combination to inhibit TNBC cell proliferation by targeting key regulatory checkpoints (Shebaby et al., 2014; Yang et al., 2022).

The dual-targeting approach of Osimertinib and *Tupichinol E* offers several advantages in the context of TNBC, a cancer subtype characterized by its resistance to conventional therapies. By simultaneously inhibiting the EGFR signaling pathway and modulating additional cancer-related pathways, the combination therapy addresses the inherent heterogeneity of TNBC and reduces the likelihood of resistance development. Moreover, the ability of the combination to induce apoptosis and cell cycle arrest in TNBC cells underscores its potential to overcome the limitations of single-agent therapies, which often fail to achieve sustained tumor control. These findings align with emerging evidence suggesting that combination therapies targeting multiple molecular pathways are more effective in managing aggressive cancers like TNBC. While this study focused on TNBC cells, future studies will aim to evaluate the selectivity and safety of the therapy in normal breast epithelial cells to better understand its targeted effects (Adyasa et al., 2021; Samantaray et al., 2023).

4. Conclusion

This study highlights the significant therapeutic potential of combining Osimertinib, a third-generation EGFR inhibitor, with *Tupichinol E*, a novel anticancer compound, in treating triple-negative breast cancer (TNBC). TNBC is one of the most aggressive breast cancer subtypes, with limited targeted treatment options due to the absence of hormone receptors and HER2 expression. As a result, current treatment options are limited, and patient outcomes remain poor. Our findings indicate that the combination of Osimertinib and *Tupichinol E* holds promise for addressing these challenges, offering a novel and more effective therapeutic strategy (Cheng et al., 2019).

For apoptosis and Western blot analysis, Osimertinib and *Tupichinol E* were used at a concentration of 5 μM, as detailed in Figs. 1 and 5. These concentrations were selected based on their observed IC50 values in preliminary experiments. The results demonstrated a synergistic antiproliferative effect when Osimertinib and *Tupichinol E* were used together, significantly reducing the viability of TNBC cell lines (MDA-MB-231) compared to monotherapies (Holzner et al., 2021). The combination index (CI) values consistently below 1 confirm that this synergism enhances the efficacy of the treatment, making it a powerful approach for TNBC. The combination therapy induced significant apoptosis, as evidenced by increased PARP cleavage and a marked reduction in the anti-apoptotic protein Bcl-2. The findings confirmed a synergistic activation of intrinsic apoptotic pathways in the combination treatment group, evidenced by Caspase-3 activation and significant PARP cleavage. This amplified apoptosis suggests that the combination treatment triggers more efficient programmed cell death, which is crucial for combating highly proliferative and resistant TNBC cells (Miller et al., 2020; Adyasa et al., 2021).

Additionally, the combination treatment induced G1 phase cell cycle arrest, halting cell division by downregulating cyclin D1, CDK4, CDK6, and retinoblastoma protein (p-RB) phosphorylation. This arrest is critical for preventing the unchecked proliferation characteristic of TNBC (Schwarz et al., 2021; Patnaik et al., 2016; Samantaray et al., 2023). By targeting the CDK4/6-RB pathway, the combination therapy effectively blocks cell cycle progression, further enhancing its antiproliferative

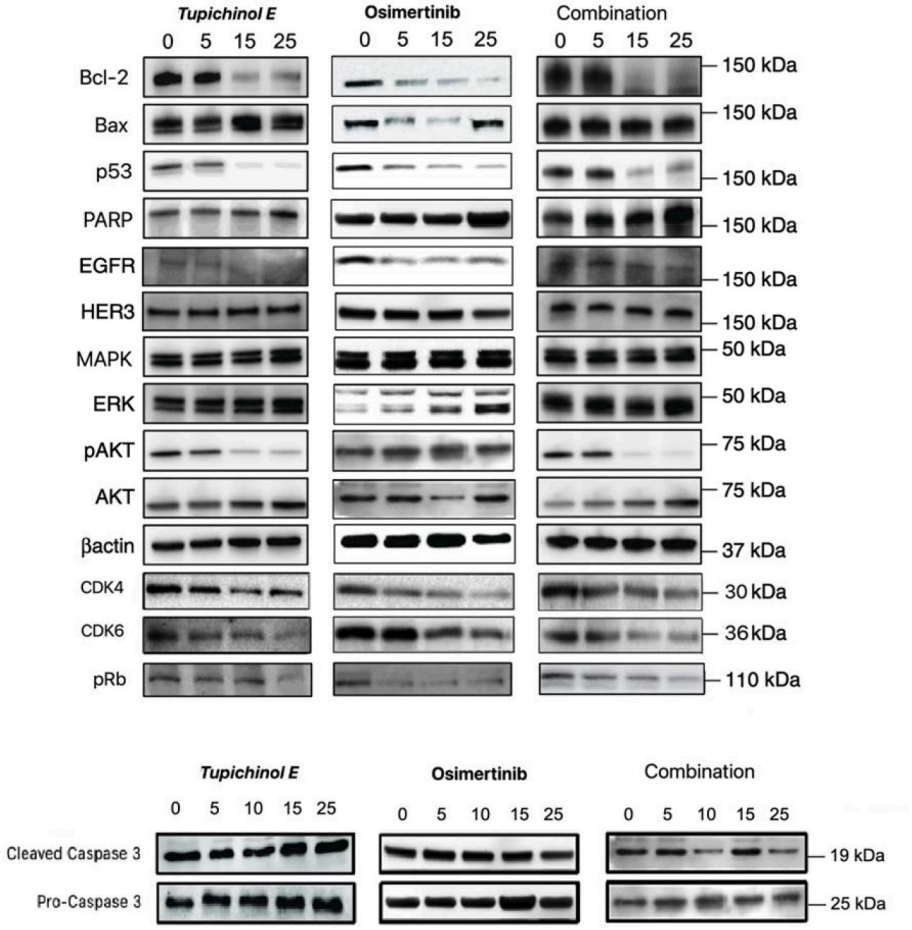

Fig. 5. The cells were lysed, and the specified proteins were detected by western blotting following immunoprecipitation of the target proteins.

properties. Importantly, while CDK4/6 inhibitors have shown efficacy in hormone receptor-positive breast cancers, the ability of this combination to inhibit TNBC, a subtype with fewer therapeutic options, is particularly noteworthy (Smith et al., 2020; Murphy and Dickler, 2015).

The dual-targeting approach of Osimertinib and *Tupichinol E* is a significant advancement because TNBC tumors often exhibit heterogeneity and prone to developing resistance to single-agent therapies. By simultaneously inhibiting the EGFR pathway (via Osimertinib) and other cancer-related pathways (potentially modulated by *Tupichinol E*), this combination therapy addresses multiple aspects of TNBC pathology, leading to better control over tumor growth and progression. This multipronged approach may overcome the resistance mechanisms that often limit the efficacy of conventional treatments (Viale et al., 2021; Klein et al., 2018).

While the in vitro findings are promising, the translation of these results into clinical settings will require further preclinical studies in animal models to evaluate the safety, pharmacokinetics, and overall therapeutic potential of the combination therapy in vivo. Additionally, further investigation into the precise molecular mechanisms of *Tupichinol E*'s anticancer activity is necessary to optimize the combination and develop targeted strategies for clinical application. The potential of Tupichinol E to target pathways beyond EGFR could open new avenues for enhancing the combination's effectiveness and broadening its applicability.

In summary, this study provides compelling evidence that the combination of Osimertinib and *Tupichinol E* offers a novel and more effective treatment option for TNBC. The synergistic inhibition of cell proliferation, disruption of cell cycle progression, and induction of apoptosis suggest that this dual therapy could fill a critical gap in the management of TNBC, where current treatment options are insufficient. With further validation, this combination could potentially improve clinical outcomes and survival rates for patients with this highly aggressive form of breast cancer, offering a new hope for individuals facing limited treatment options.

CRediT authorship contribution statement

Adyasa Samantaray: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation. **Debasish Pradhan:** Writing – review & editing, Supervision, Formal analysis, Conceptualization.

Data availability declaration

The authors confirm that the data supporting the findings of this study are available within the article. If any supporting data required, it is available form the corresponding author, upon reasonable request.

Declaration of competing interest

The authors declare that there is no financial or potential competing interest.

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