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IN-VIVO EXPERIMENTATION OF *TUPICHINOL* E, OSIMERTINIB, AND THEIR COMBINATION USING A XENOGRAFT MODEL

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ABSTRACT: This comprehensive study investigates the *in vivo* effects of *Tupichinol E*, osimertinib, and their combination in a xenograft model of triple-negative breast cancer (TNBC), an aggressive and therapeutically challenging breast cancer subtype. By employing a multi-faceted approach, the study evaluates tumor growth inhibition, apoptosis, and proliferation through histopathological and immunohistochemical techniques, including hematoxylin and eosin (H&E) staining, TUNEL staining, and Ki67 immunohistochemistry. The results reveal significant therapeutic potential. Tumor growth was markedly suppressed in all treated groups, with monotherapy using *Tupichinol E* or Osimertinib reduces tumor volume by approximately 35% and 40%, respectively. Notably, combination therapy achieved a synergistic effect, maintaining tumor volumes at baseline levels and preventing progression over the two-week treatment period ($p<0.001$). Histological analyses demonstrated extensive necrosis and disruption of tumor architecture in the combination group, correlating with a 150% increase in apoptotic activity as indicated by TUNEL staining ($p<0.001$). Furthermore, Ki67 immunohistochemistry showed a substantial 70% reduction in proliferative activity in the combination group compared to controls ($p< 0.001$). These findings underscore the enhanced efficacy of combining *Tupichinol E* and Osimertinib, offering a novel and promising therapeutic strategy for TNBC. The synergistic effects observed in tumor suppression, apoptosis induction, and proliferation inhibition highlight the potential for this combination to address unmet clinical needs in TNBC treatment. This study provides a foundation for future research to explore molecular mechanisms and clinical applicability of this combination therapy.

INTRODUCTION: Triple-negative breast cancer (TNBC) is one of the most aggressive and clinically demanding types of breast cancer. TNBC, which accounts for 15-20% of all breast cancer cases, is distinguished by a lack of expression of the estrogen receptor (ER), progesterone receptor (PR), and HER2 amplification.

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This unique molecular profile renders TNBC unresponsive to hormone therapies and HER2-targeted treatments, such as tamoxifen and trastuzumab that have significantly improved outcomes in other breast cancer subtypes. Consequently, chemotherapy remains the primary systemic treatment for TNBC, yet it often yields suboptimal results due to the high rates of recurrence, metastasis, and resistance [1].

The clinical behavior of TNBC is marked by its aggressive nature, with higher rates of distant metastases to the lungs, brain, and liver. TNBC disproportionately affects younger women, and its

prognosis remains poor, with a 5-year survival rate significantly lower than hormone receptor-positive breast cancers. The heterogeneous nature of TNBC at the molecular and cellular levels further complicates its management, as this diversity impacts treatment response and disease progression. Emerging molecular profiling techniques have begun to reveal actionable targets within subsets of TNBC, providing hope for more tailored and effective therapies [3].

One promising avenue for targeted therapy in TNBC is the epidermal growth factor receptor (EGFR) pathway. EGFR is overexpressed in approximately 50% of TNBC cases, making it a potential therapeutic target. Osimertinib, a third-generation EGFR inhibitor, has transformed the treatment of NSCLC with EGFR mutations. Osimertinib suppresses tumor cell proliferation, survival, and migration by selectively targeting the EGFR. Despite its success in NSCLC, the role of Osimertinib in TNBC remains underexplored, warranting investigation into its efficacy and mechanisms of action in this context [2].

In parallel, there is growing interest in the use of natural compounds in cancer therapy. *Tupichinol E*, a novel bioactive compound derived from traditional medicinal plants, has shown potent anticancer activity in preclinical studies. Its actions include inducing apoptosis, inhibiting cell growth, and disrupting crucial signaling pathways involved in tumor formation. Unlike many synthetic chemotherapeutic agents, natural compounds like *Tupichinol E* often exhibits lower toxicity, making them attractive candidates for combination therapies [4].

The rationale for combining Osimertinib with *Tupichinol E* lies in their complementary mechanisms of action. While Osimertinib specifically targets EGFR-driven pathways, *Tupichinol E* exerts broader anticancer effects, including modulation of apoptosis and cell cycle regulators. Together, these agents have the potential to overcome the limitations of monotherapies, enhance therapeutic efficacy, and mitigate resistance mechanisms [5]. This study aims to evaluate the therapeutic potential of *Tupichinol E*, Osimertinib, and their combination in a xenograft model of TNBC. Using advanced histological techniques, including hematoxylin and eosin (H&E) staining, TUNEL staining, and Ki67 immunohistochemistry, this research seeks to elucidate the effects of these treatments on tumor growth, apoptosis, and proliferation. By providing a deeper understanding of their mechanisms, the findings from this study could pave the way for the development of innovative therapeutic strategies for TNBC, addressing critical gaps in its treatment landscape. Furthermore, this research underscores the importance of integrating molecularly targeted therapies and natural compounds in the quest for effective and personalized cancer care.

MATERIALS & METHODOLOGY:

Reagents and Compounds: The *in-vivo* experimentation utilized a range of specialized reagents and components to ensure the precision and reliability of model [6]. MDA-MB-231 cells, a human triple-negative breast cancer cell line, were selected to establish the xenograft tumors. These cells were suspended in phosphate-buffered saline (PBS) and mixed in a 1:1 ratio with Matrigel to enhance tumor engraftment and stability post-injection. *Tupichinol E*, a naturally derived bioactive compound, was prepared by dissolving it in saline containing 5% dimethyl sulfoxide (DMSO) for intraperitoneal administration [7, 9]. Osimertinib, a marketed third-generation EGFR inhibitor, was prepared and administered at a dose of 15 mg/kg intraperitoneally, once weekly, tailored for the murine model [26].

***In-vivo* Experiment:** Female BALB/c nude mice were procured from the National Institute of Animal Biotechnology (NIAB) in Hyderabad. The experimental protocol was reviewed and approved by the Institutional Animal Ethics Committee at The Pinnacle Biomedical Research Institute (PBRI) in Bhopal, under the protocol approval number PBRI/IAEC/21-03-2024/035. Upon arrival, the mice were individually housed in ventilated disposable cages, provided with food and water, and maintained under controlled environmental conditions (20 ℃, 50% ± 20% relative humidity, and a 12-hour light/dark cycle) [8, 10]. After a 7-day acclimatization period, each mouse was subcutaneously injected at the right flank with a suspension of 4×10^6 MDA-MB-231 human breast cancer cells.

The cells were resuspended in phosphate-buffered saline (PBS), mixed in a 1:1 ratio with serum-free medium containing Matrigel. Tumor growth was monitored, and once the average tumor volume reached approximately 100 mm^3 (calculated using the formula: Volume = ½ × width² × length), the mice were randomly assigned into four treatment groups (n=5 per group) as follows:

- **Group 1 (Control):** Saline water (intraperitoneal injection, 6 days per week).
- **Group 2:** *Tupichinol E* (5 mg/kg, intraperitoneal injection, 6 days per week).
- **Group 3:** Osimertinib (15 mg/kg, intraperitoneal injection, once per week).
- **Group 4:** Combination treatment with *Tupichinol E* (5 mg/kg, intraperitoneal injection, 6 days per week) and Osimertinib (15 mg/kg, intraperitoneal injection, once per week).

Both *Tupichinol E* and Osimertinib were dissolved in saline water containing 5% dimethyl sulfoxide (DMSO). Tumor volumes and body weights were measured twice weekly. After two weeks of treatment, the mice were euthanized. Tumor tissues were excised, weighed, and collected for histological and immunohistochemical analyses [11].

Hematoxylin and Eosin (H&E) Staining: Tumor tissues were fixed in a 10% neutral buffered formalin solution (Sigma-Aldrich) before undergoing paraffin embedding. Thin sections of the tissues were prepared and subsequently stained using the H&E solution (Merck). The stained sections were then analyzed and imaged using a fluorescence microscope (Olympus BX51, Japan) [15].

Tunel Staining: Apoptotic activity in the tumor tissues was evaluated through TUNEL staining, following the manufacturer's protocol. Briefly, the tissue sections were deparaffinized and rehydrated using a graded ethanol series. After rinsing with 0.85% sodium chloride (NaCl) and phosphate-buffered saline (PBS), the sections were fixed with 4% formaldehyde for 15 minutes. Proteinase K solution was applied to the tissue sections for 8–10 minutes following a PBS wash. A second fixation with 4% formaldehyde for 5 minutes was performed before equilibration in equilibrium buffer for 5–10 minutes. The TdT reaction mixture was then applied, and the sections were incubated for 1 hour in a dark environment. After this, the sections were treated with SSC solution for 15 minutes and washed with PBS. Finally, after counterstaining with DAPI, the sections were visualized and photographed under a fluorescence microscope (Olympus BX51, Japan). The average fluorescence signal from three images per treatment group was quantified [13].

Ki67 Immunohistochemistry: For Ki67 analysis, the tissue sections were first deparaffinized and subjected to antigen retrieval using citrate buffer. After blocking with 2% fetal bovine serum for 20–30 minutes, the sections were incubated with rabbit anti-human Ki67 antibody (1:200) for 1 hour at room temperature. Post-incubation, the sections were washed with PBS and treated with chicken anti-rabbit IgG TR-conjugated antibody (1:500) for 1 hour in a dark setting. The sections were counterstained with DAPI and subsequently imaged using a fluorescence microscope (Olympus BX51, Japan). Quantification of the fluorescence signals was performed by analyzing three images per treatment group [14, 13].

Statistical Analysis: All experiments were conducted in triplicate, and the results were expressed as the mean ± standard deviation (SD). Statistical evaluations were performed using GraphPad Prism 9.0 software. Group comparisons were analyzed via one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test for multiple comparisons. A p-value of less than 0.05 was considered statistically significant [8, 9].

RESULT:

***Tupichinol E* Suppresses Breast Tumor Growth in Nude Mice:** To establish a breast tumor xenograft model, MDA-MB-231 breast cancer cells were subcutaneously injected into the right flank of female nude mice. As illustrated in **Fig. 1**, the tumor volume in the vehicle-treated group exhibited a marked increase after two weeks, growing from approximately 100 mm^3 to 330 mm^3. Treatment with 5 mg/kg *Tupichinol E* and 15 mg/kg Osimertinib significantly slowed tumor progression; however, neither treatment completely

eliminated the tumors or restored the tumor volume to baseline. Notably, the combination treatment (5 mg/kg *Tupichinol E* + 15 mg/kg Osimertinib) demonstrated a more pronounced inhibitory effect on tumor growth compared to either treatment alone. While the combination therapy did not completely eradicate the tumor, it successfully maintained tumor volume near the baseline level throughout the two-week treatment period [21]. Additionally, a slight reduction in mouse body weight was observed across treatment groups, but no visible adverse effects were detected.

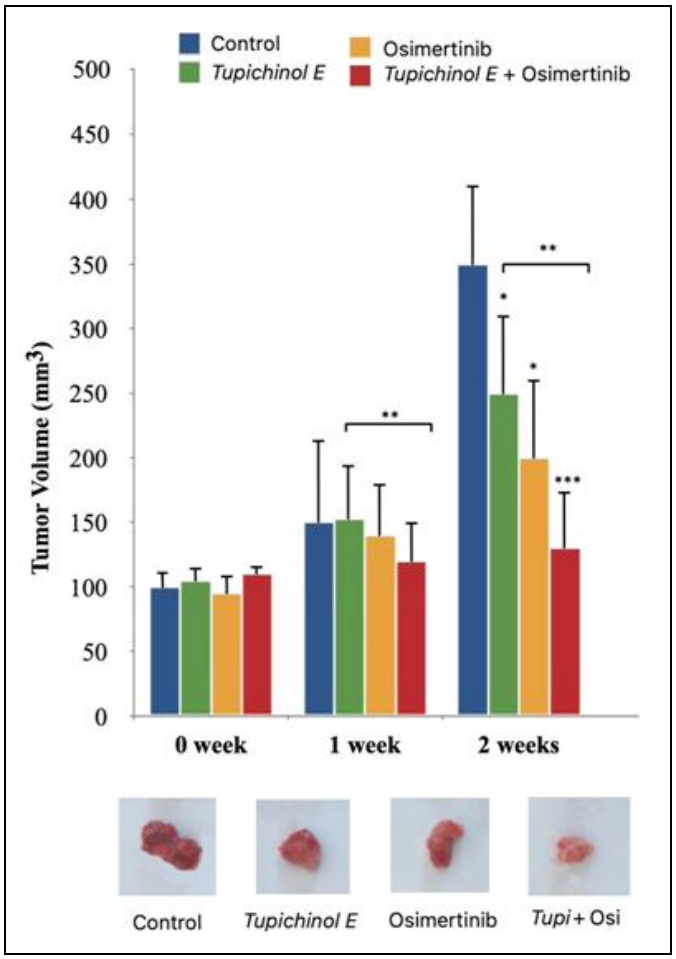

FIG. 1: *TUPICHINOL E* REDUCES BREAST TUMOR GROWTH IN NUDE MICE MDA-MB-231 CELLS WERE INJECTED SUBCUTANEOUSLY INTO THE RIGHT FLANK OF EACH NUDE MOUSE. Once tumors reached a volume of 100 mm^3, the mice were randomly assigned into four groups (n=5/group). Group I received saline water (intraperitoneally, six days per week). Group II received 5 mg/kg *Tupichinol E* (intraperitoneally, six days per week). Group III received 15 mg/kg Osimertinib (intraperitoneally, six days per week). Group IV received a combination of 5 mg/kg *Tupichinol E* (intraperitoneally, once per week) and 15 mg/kg Osimertinib (intraperitoneally, six days per week). Tumor volumes are presented as means ± SD. *p<0.05, ***p<0.001 compared to the control group. **p<0.05 denotes comparison between indicated groups.

Histological and Immunohistochemical Analysis of Tumor Tissues: Tumor tissues were subjected to H&E staining to assess structural alterations [23, 24]. In the vehicle-treated group, high-grade tumors were observed with irregular cellular arrangements **Fig. 2A**. In contrast, tumors from drug-treated groups exhibited structural changes, characterized by increased cellular debris and reduced stromal content. TUNEL staining was performed to evaluate apoptosis levels in the tumor tissues. A comparison of green fluorescence signals revealed approximately 50% higher TUNEL staining in tumors treated with 5 mg/kg *Tupichinol E* or 15 mg/kg Osimertinib. Notably, the combined treatment group displayed a 150% increase in TUNEL staining compared to the vehicle group, indicating a synergistic enhancement in apoptotic activity **Fig. 2B.** Immunohistochemical analysis targeting the Ki67 protein, a well-established marker of cellular proliferation, showed significant

reductions in Ki67 levels in all drug-treated groups relative to the vehicle-treated group. The lowest Ki67 expression was observed in the combined treatment group, highlighting the effectiveness of the combination therapy in suppressing tumor cell proliferation **Fig. 2C.**

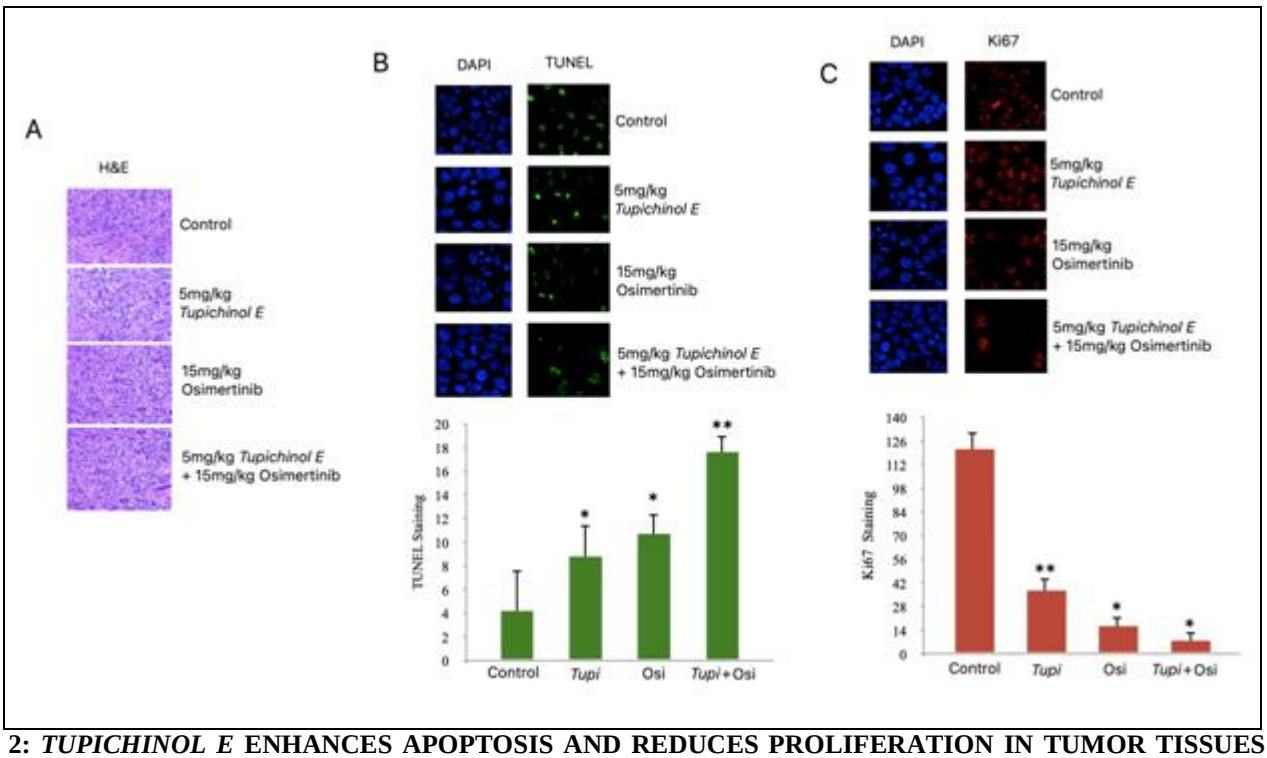

FIG. 2: *TUPICHINOL E* ENHANCES APOPTOSIS AND REDUCES PROLIFERATION IN TUMOR TISSUES. (A) H&E staining revealed structural changes in tumor tissues across treatment groups. (B) TUNEL staining demonstrated enhanced apoptosis, with quantification based on the average number of green fluorescent dots per group. Values represent means ± SD from three images. *p<0.05, **p<0.01 compared to the vehicle group. (C) Ki67 immunohistochemical staining showed reduced proliferation, with quantification based on fluorescence signals. Values are means ± SD from three images. *p<0.05, **p<0.01 compared to the vehicle group.

DISCUSSION: The findings of this study underscore the potential of combining *Tupichinol E* and Osimertinib as a novel therapeutic strategy for triple-negative breast cancer (TNBC). The results demonstrated that this combination therapy offers superior tumor growth inhibition, enhanced apoptosis induction, and significant suppression of cell proliferation compared to either agent alone. These outcomes suggest that the combination of *Tupichinol E* and Osimertinib acts synergistically to target multiple pathways involved in TNBC progression [12, 19].

One of the most striking findings was the ability of the combination therapy to maintain tumor volumes at baseline levels throughout the treatment period. This outcome highlights the potential of this approach to effectively control tumor progression in aggressive cancers like TNBC. The histological and immunohistochemical analyses further validated these results, with extensive necrosis and disruption of tumor architecture observed in the combination group. This degree of tumor cell death was corroborated by the significant increase in TUNEL staining, which indicated a 150% rise in apoptotic activity compared to the control group [15].

The reduction in Ki67 positivity in the combination group also provides critical insights into the mechanisms of action. Ki67, a marker of cellular proliferation, was significantly decreased by 70% in the combination therapy group. This reduction underscores the efficacy of this approach in curbing the rapid proliferation that is characteristic of TNBC cells. These findings align with the proposed mechanisms of *Tupichinol E* and Osimertinib, wherein the former induces apoptosis and modulates key signaling pathways, while the latter inhibits EGFR-driven proliferation and survival [16]. The observed synergy between *Tupichinol E* and Osimertinib may be attributed to their complementary mechanisms of action.

While Osimertinib specifically targets EGFR, *Tupichinol E* has broader anticancer effects, including modulation of apoptosis-related proteins and inhibition of angiogenesis. This multi-pronged approach likely disrupts the ability of TNBC cells to adapt and survive under therapeutic pressure, thereby enhancing overall efficacy [27].

In the context of existing therapies for TNBC, the results of this study are particularly significant. Current treatment options for TNBC are largely limited to chemotherapy, which is associated with high toxicity and limited efficacy in the face of drug resistance. The combination of *Tupichinol E* and Osimertinib presents a targeted, less toxic alternative that could improve patient outcomes. Moreover, the natural origin of *Tupichinol E* offers the added advantage of potentially reduced side effects, which is a critical consideration in cancer therapy [17, 21].

Future research should focus on elucidating the molecular pathways underlying the observed synergy. For instance, investigating the effects of this combination on EGFR downstream signaling pathways, such as PI3K/AKT and MAPK, could provide deeper mechanistic insights. Additionally, exploring the role of apoptosis regulators like Bcl-2 and caspases could help clarify how *Tupichinol E* enhances the apoptotic effects of Osimertinib [22].

Clinical translation of these findings will require further validation in larger preclinical models and eventually in clinical trials. Optimizing the dosing regimen and identifying biomarkers for patient stratification will be crucial steps in this process. Given the heterogeneity of TNBC, understanding which subsets of patients are most likely to benefit from this combination therapy will be essential for its successful implementation [25].

In conclusion, the combination of *Tupichinol E* and Osimertinib represents a promising therapeutic strategy for TNBC. By leveraging the complementary mechanisms of these agents, this approach addresses critical gaps in current treatment options, offering hope for improved outcomes in this challenging cancer subtype.

CONCLUSION: This study highlights the promising potential of combining *Tupichinol E* and Osimertinib as a therapeutic strategy for triple-negative breast cancer (TNBC), a cancer subtype with limited treatment options and poor prognosis. The results demonstrate that this combination therapy not only effectively inhibits tumor growth but also significantly enhances apoptosis and reduces cellular proliferation. These effects, driven by the complementary mechanisms of action of the two agents, underscore the potential of this approach to address the critical challenges posed by TNBC [18].

The ability of the combination therapy to maintain tumor volumes at baseline levels throughout the treatment period represents a significant advancement in the therapeutic management of aggressive cancers. By targeting both EGFR-driven pathways and broader tumor survival mechanisms, this approach effectively disrupts TNBC progression while potentially minimizing the toxicity associated with conventional therapies. Histological analyses revealing extensive necrosis and immunohistochemical findings of reduced Ki67 positivity provide strong evidence of the therapy's efficacy [20].

While these preclinical findings are highly encouraging, the path to clinical application involves addressing several key aspects. First, further studies should explore the molecular pathways involved in the observed synergy between *Tupichinol E* and Osimertinib. Understanding how these agents interact at a cellular and molecular level will facilitate the development of biomarkers to predict patient response. Second, larger preclinical studies should be conducted to validate these results and establish optimal dosing regimens. Third, the safety and efficacy of this combination therapy must be assessed in clinical trials, with an emphasis on identifying patient subsets most likely to benefit from this treatment [26, 28].

In conclusion, the combination of *Tupichinol E* and Osimertinib represents a highly promising therapeutic approach for TNBC. By leveraging their complementary mechanisms of action, this strategy offers the potential for improved outcomes and reduced toxicity compared to current treatment options. This study provides a strong foundation for future research and highlights the importance of integrating natural compounds and molecularly

targeted therapies in the fight against aggressive cancers like TNBC. Continued investigation and clinical validation of this approach could pave the way for a new paradigm in TNBC treatment, offering hope to patients facing this challenging disease.

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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 from the corresponding author, upon reasonable request.

Author Contribution:
Adyasa Samantaray: Article Drafting, Analysis and interpretation of the data.

Debasish Pradhan: Supervision, Analysis, Conception and design.

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