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ORIGINAL ARTICLE

Investigation of cytotoxic, molecular and in silico effects of chlorambucil and tamoxifen on 2D/3D MDA-MB-231 and HeLa cancer cell models

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Abstract

Objective This study aimed to investigate the cytotoxic, morphological, and molecular effects of Tamoxifen (TMX) and Chlorambucil (CHL) on breast cancer (MDA-MB-231) and cervical cancer (HeLa) cell lines. The impact of these agents on metastatic behavior, apoptotic mechanisms, and gene expression profiles was examined in both two-dimensional (2D) and three-dimensional (3D) cell culture models.

Methods Cells were treated with varying concentrations of TMX and CHL. Cytotoxicity was assessed using the XTT assay, and morphological changes were monitored by microscopy. Migration and invasion assays assessed metastatic potential. *VEGFA* expression was quantified by qRT-PCR. In 3D cultures, treatment responses were evaluated based on size reduction and structural changes in hydrogel-based spheroids. Docking analysis was conducted to determine binding affinities of TMX and CHL.

Results TMX and CHL exhibited dose-dependent effects on breast and cervical cancer cells. Combination treatment led to significantly greater reductions in cell viability compared to controls ($p < 0.05$). Moreover, *VEGFA* expression was markedly reduced in both 2D and 3D models ($p < 0.05$). These findings support the potential therapeutic value of TMX and CHL. Docking analysis revealed highly negative binding energies, consistent with in vitro results, indicating synergistic interaction at molecular and cellular levels.

Conclusion TMX and CHL combination therapy demonstrated potent anti-cancer activity in breast and cervical cancer models, reducing cell viability, metastatic capacity, and *VEGFA* expression. These results suggest that TMX and CHL, when used together, may represent a promising strategy for developing synergistic and targeted cancer therapies. Further in vivo and clinical validation is warranted.

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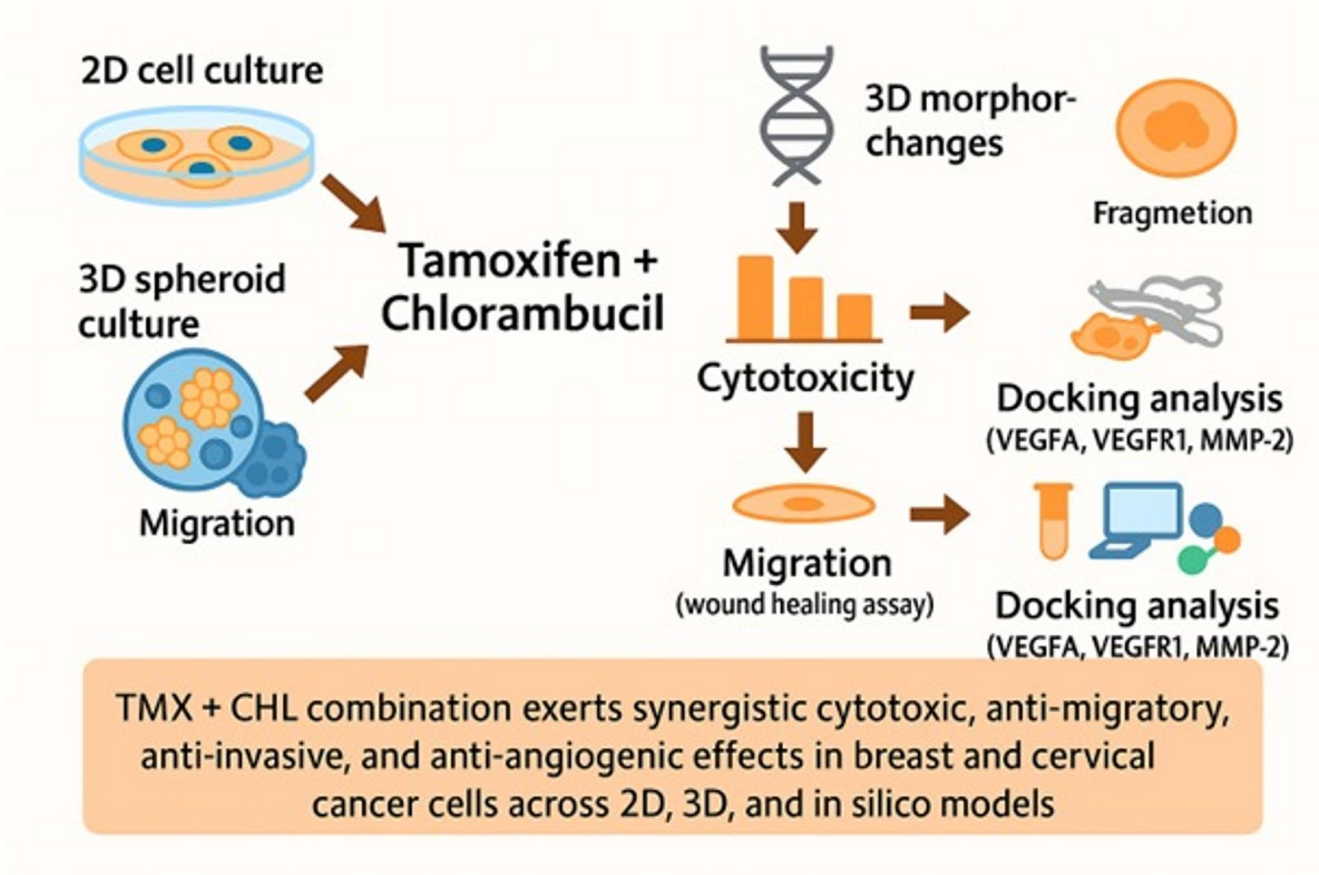
Graphical abstract



Keywords Tamoxifen · Chlorambucil · Cytotoxicity · Expression · 2D and 3D models

Introduction

Cancer remains one of the foremost causes of morbidity and mortality globally, driven by a complex interplay of environmental, genetic, and epigenetic factors. The progressive accumulation of somatic mutations in genes that regulate the cell cycle and proliferation is a central mechanism underlying tumorigenesis [1]. Among women, breast cancer is the most frequently diagnosed malignancy, with estrogen receptor–positive (ER+) subtypes exhibiting an annual incidence increase of approximately 0.87% [2]. Although the global incidence of cervical cancer has been declining, it continues to pose a significant public health challenge in low-income countries [3].

Standard cancer therapies—including surgery, radiotherapy, chemotherapy, targeted therapy, and immunotherapy—remain limited by suboptimal efficacy, systemic toxicity, and the development of resistance, underscoring the urgent need for innovative combination approaches [4]. In both breast and cervical cancers, the integration of endocrine therapy with chemotherapy has shown promising therapeutic potential [5].

Tamoxifen (TMX), a selective estrogen receptor modulator (SERM), is widely used in breast cancer treatment due to its ability to inhibit tumor proliferation by antagonizing estrogen signaling [6]. This triphenylethylene derivative binds to estrogen receptors through its phenyl rings, modulating estrogenic activity; it effectively suppresses proliferation in breast cancer cells while exerting partial estrogenic effects in bone and endometrial tissues [7].

Additionally, tamoxifen interferes with growth factor–mediated proliferation by modulating key signaling cascades such as PI3K/AKT/mTOR and MAPK pathways [8]. However, prolonged exposure may result in resistance, often driven by alterations in estrogen receptor (ER) signaling or the activation of alternative survival pathways [9].

Clinical studies have consistently shown that tamoxifen reduces the risk of recurrence and improves overall survival in patients with ER-positive early-stage breast cancer [10].

Although tamoxifen is primarily indicated for the treatment of ER-positive breast cancer, accumulating evidence suggests that it also exerts biological activity through estrogen receptor–independent mechanisms. Multiple studies have shown that TMX can modulate cell proliferation, apoptosis, migration, and invasion via alternative pathways, including protein kinase C (PKC), transforming growth factor-β (TGF-β), and mitochondrial signaling. These non-classical mechanisms have been observed in both ER-negative and triple-negative breast cancer (TNBC) cells. Accordingly, the use of TNBC cell lines in our study was intended to investigate the potential ER-independent anticancer effects of TMX, thereby expanding its therapeutic relevance beyond ER-positive malignancies [11].

Chlorambucil (CHL), a nitrogen mustard–based alkylating agent primarily used in the treatment of hematologic malignancies, exerts its cytotoxic effects by forming covalent bonds with DNA through its bis-β-chloroethylamine moiety, thereby inhibiting both DNA and protein synthesis. This mechanism induces cell cycle arrest at the G1 phase and suppresses cellular proliferation via modulation of β1-integrin and IGF-1 receptor signaling pathways [12]. In breast cancer therapy, chlorambucil and its derivatives have been investigated in combination with targeting moieties, particularly for estrogen receptor–positive (ER^+) cells. Evidence suggests that conjugation with amino acids bearing estrogen-like structures can enhance therapeutic efficacy [13]. More recently, chlorambucil-loaded nanolipid conjugates have been developed to improve tumor-specific delivery while minimizing systemic toxicity—an approach that has demonstrated enhanced antitumor activity in preclinical models [14]. Nevertheless, further studies are needed to elucidate the impact of such formulations on the tumor microenvironment and metastatic progression, with the aim of optimizing therapeutic strategies [15, 16].

The development of breast and cervical cancers is influenced by hormonal factors such as estrogen, progesterone, and insulin-like growth factors (IGFs), with metastatic progression shaped by the complex interplay among these signaling pathways [17, 18]. Metastatic cells enhance their invasive potential through extracellular matrix (ECM) remodeling and the induction of angiogenesis—processes mediated by dynamic crosstalk between tumor cells and the surrounding stromal components [19]. Among the key molecular drivers, vascular endothelial growth factor A (VEGFA) and matrix metalloproteinase-2 (MMP2) play pivotal roles in reconfiguring the tumor microenvironment. *VEGFA* facilitates neovascularization to support tumor growth by improving nutrient and oxygen delivery, while *MMP2* promotes invasion by degrading ECM components. Acting synergistically, these factors accelerate tumor progression and metastatic dissemination [20–22]. As such, the expression levels of *VEGFA* and *MMP2* serve as valuable biomarkers for elucidating the antitumor mechanisms of agents like tamoxifen and chlorambucil.

We hypothesized that the combination of tamoxifen and chlorambucil would elicit more potent antiproliferative, anti-invasive, and anti-angiogenic effects than either agent alone. This study aimed to evaluate the individual and combined cytotoxic, morphological, and molecular effects of these drugs in breast (MDA-MB-231) and cervical (HeLa) cancer cell lines. Experiments were conducted in both 2D and 3D culture models to assess metastatic behaviors, apoptotic responses, and *VEGFA* gene expression. Additionally, molecular docking analyses were performed to predict binding affinities to *MMP2* and other relevant target proteins.

Structural alterations within hydrogel-based 3D cultures were also examined, offering a comprehensive perspective on the therapeutic mechanisms underlying the observed anticancer effects.

Materials and methods

Cell culture

Triple-negative breast cancer (MDA-MB-231; ATCC® HTB-26™, USA) and cervical adenocarcinoma (HeLa; ATCC® CCL-2™, USA) cell lines were obtained from the Medical Pharmacology Laboratory at X University, originally sourced from the American Type Culture Collection (ATCC). Cells were maintained in RPMI-1640 medium (Capricorn Scientific, Germany; Cat. RPMI-A) supplemented with 10% (v/v) fetal bovine serum (FBS; Cat. 10-FBS-16 F) and 1% (v/v) penicillin–streptomycin (100 U/mL penicillin, 100 µg/mL streptomycin; Cat. PS-B), and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Cultures were maintained at 60–80% confluence and passaged using 0.05% trypsin–EDTA (Capricorn Scientific; Cat. TRY-2B). Prior to passaging, cells were washed once with phosphate-buffered saline (PBS), incubated with trypsin–EDTA for 3–5 min at 37 °C until detachment, neutralized with complete medium, and reseeded at a density of $3–4 \times 10^4$ cells/cm^2 in T-75 culture flasks.

Drug preparation and dosage

Tamoxifen (TMX; Sigma-Aldrich, USA; Cat. T5648) was dissolved in DMSO under light protection to prepare a [10–50 mM] stock, sterile-filtered through a 0.22 µm membrane, and stored at −20 °C. Chlorambucil (CHL; Sigma-Aldrich, USA; Cat. C105) was prepared as a [10–50 mM] stock in distilled water. Cells were treated in RPMI-1640 with final concentrations of 5, 10, 25, 50, 75, or 100 µM TMX or CHL.

In all TMX-treated groups, the final DMSO content did not exceed 0.01% (v/v); a vehicle control containing 0.01% DMSO (v/v) was included. A water control was used for CHL. Treatments were applied for [24/48/72 h]; [medium was refreshed with freshly prepared drug every 24 h when indicated]. The concentrations used in the combination assays (75 µM TMX + 10 µM CHL for MDA-MB-231 and 75 µM TMX + 5 µM CHL for HeLa) were selected based on preliminary IC_{50} determinations to ensure sufficient cell viability for subsequent functional assays.

Two- and three-dimensional cell culture

HeLa and MDA-MB-231 cells were cultured under standard conditions (37 °C, 5% CO_2) in RPMI-1640 medium. Three-dimensional spheroids were generated using the hanging drop method. Briefly, 10 µL drops of cell suspension (10,000 cells/mL) were placed on the inner surface of a sterile Petri dish lid, which was then inverted over a PBS-filled base to maintain humidity. Spheroid formation was observed within 24 h for HeLa cells and 48 h for MDA-MB-231 cells. Preliminary optimization studies confirmed that HeLa cells formed compact and uniform spheroids within 24 h, whereas MDA-MB-231 cells required approximately 48 h due to their mesenchymal phenotype and lower cell–cell adhesion properties.

Formed spheroids were transferred into 96-well ultra-low attachment plates and treated with tamoxifen and/or chlorambucil at predetermined concentrations. Compounds were added directly to each well without disturbing the spheroid structure. For combination treatments, both agents were administered simultaneously. No medium change was performed during the incubation period to preserve spheroid integrity. At the end of treatment, cell viability and morphological changes were assessed. These 3D models were used to evaluate drug responses under physiologically relevant conditions.

Cytotoxicity assay

Cell viability was assessed using the XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)−2 H-tetrazolium-5-carboxanilide) assay kit (Biological Industries, Cat. No: 20–300–1000), which measures mitochondrial metabolic activity. MDA-MB-231 and HeLa cells were seeded into 12-well plates and incubated until ~80% confluence. Cells were then treated with predetermined concentrations of TMX, CHL, or their combination. After 24, 48, or 72 h of exposure, the medium was replaced with fresh XTT-containing medium; cells were incubated at 37 °C in the dark for 2–4 h, and absorbance was measured at 450 nm using a Biotek microplate reader.

Cell motility and invasion analysis

For the wound-healing assay, 2×10^5 cells were seeded into each well of a 12-well plate, and a linear scratch was created upon reaching ~80% confluence. Cell migration was monitored microscopically at 24, 48, and 72 h. Wound closure rates were quantified using ImageJ™ software by measuring the area covered by migrating cells. Invasion capacity was assessed by Giemsa staining. At each time point (24, 48, and 72 h) post-treatment, cells were fixed with 3–4% formaldehyde, permeabilized with 70% ethanol, stained with Giemsa, and examined under a microscope.

Gene expression analysis

Total RNA was extracted using the GeneAll Hybrid-R™ kit (GeneAll, Korea), and its purity and concentration were assessed using a NanoDrop spectrophotometer. Complementary DNA (cDNA) was synthesized using a reverse transcription kit according to the manufacturer's instructions. The expression levels of *VEGFA* and *MMP2* were quantitatively determined using SYBR Green–based qRT-PCR, with *GAPDH* as the reference gene. All reactions were performed in technical triplicates.

Molecular Docking analysis

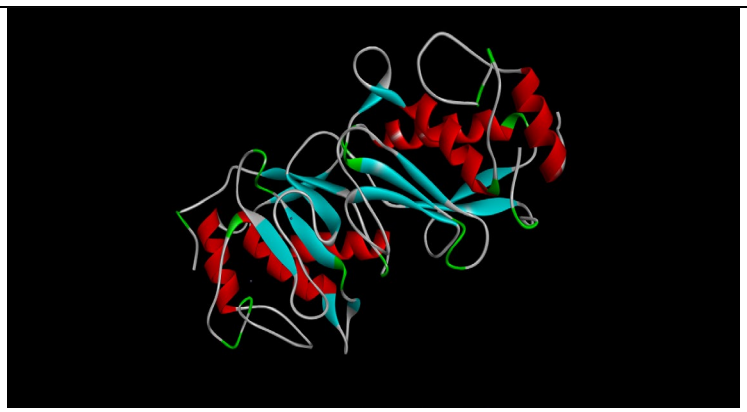
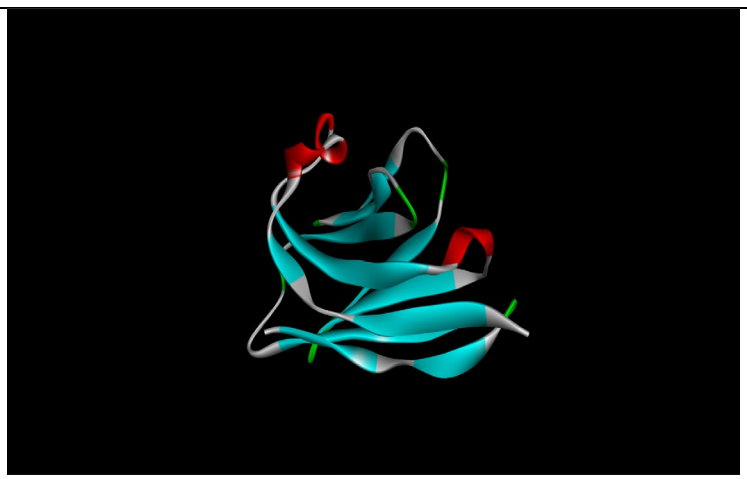
The 3D structures of VEGFR1, MMP2, and VEGFA (Protein Data Bank identifiers, PDB IDs; Table 1.) were obtained from the RCSB PDB database (https://www.rcsb.org/). The 3D structures of Chlorambucil and Tamoxifen were found in the PubChem database (https://pubchem.NCBI.nlm.nih.gov/). Molecular docking calculations were validated using both Seamdock and Autodock [20,23]. VEGFA and VEGFR1 were included in the docking analysis to determine whether the drugs directly interact with the ligand VEGFA or VEGFR1 and to assess whether these interactions are competitive or allosteric in nature.

All experiments were performed in triplicate ($n = 3$), unless otherwise stated, with consistent technical replicates for each condition to ensure reproducibility and statistical reliability.

Statistical analyses

Data were analyzed using IBM SPSS Statistics 29. One-way ANOVA followed by Tukey's post hoc test was applied for cytotoxicity, migration, and invasion assays. For gene expression analyses, the ΔΔCt method was used, and comparisons were performed using ANOVA. A p-value < 0.05 was considered statistically significant.

Table 1 3D structural representations of VEGFR1, MMP2, and VEGFA proteins used in molecular docking analyses

Proteins	PDB ID	3D Structure
VEGFR1	3hng	
MMP2	7xjo	
VEGFA	8ijz	

Results

The cytotoxic, migratory, and gene expression effects of tamoxifen (TMX) and chlorambucil (CHL) were examined in MDA-MB-231 and HeLa cell lines, both as individual agents and in combination. The treatments were associated with measurable changes in cell morphology, viability, and gene expression parameters.

To determine the antiproliferative effects of TMX and CHL, both cell lines were treated with increasing concentrations of each drug. Cell viability was assessed 48 h post-treatment using the XTT assay. Results were normalized to

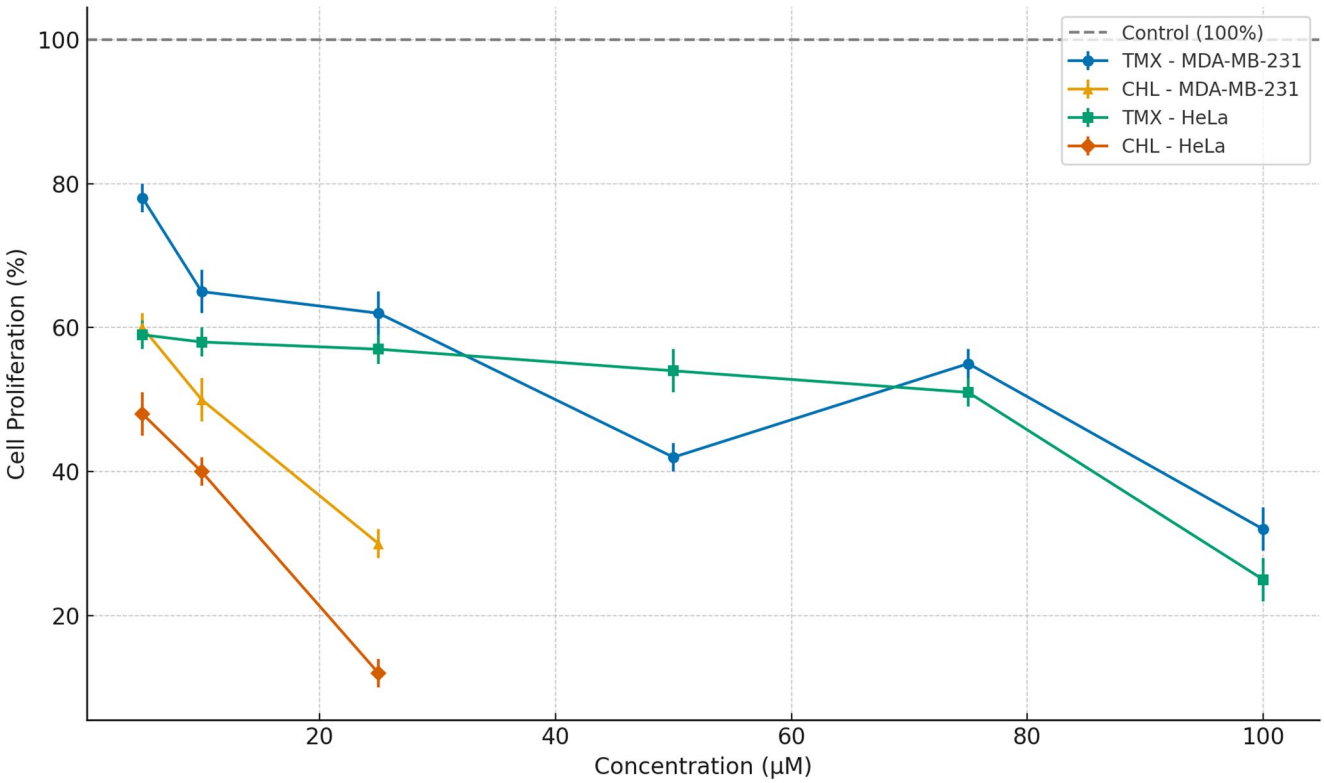


Fig. 1 Cell proliferation after treatment with Tamoxifen (TMX) or Chlorambucil (CHL) in MDA-MB-231 and HeLa cells for 48 h. Cells were exposed to increasing concentrations (5–100 μM) of TMX or CHL, and viability was measured using the XTT assay. Data are expressed as mean ± SD from three independent experiments ($n = 3$). Error bars represent standard deviation. Statistical significance was evaluated using one-way ANOVA followed by Tukey's post hoc test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

Table 2 Cell proliferation (%) after combined treatment with TMX and CHL in MDA-MB-231 and HeLa cells

Cell line	Drug	Concentration (μM)	Cell proliferation (%)	*P* value
MDA-MB-231	TMX + CHL	75 + 10	22 ± 1	$P < 0.05$*
	Control	No treatment	100	
HeLa	TMX + CHL	75 + 5	18 ± 1	$P < 0.05$*
	Control	No treatment	100	

*$P < 0.05$: Statistically significant compared to control (one-way ANOVA followed by Tukey's post hoc test)

untreated control cells and expressed as percentages, illustrating the concentration-dependent cytotoxic profiles of TMX and CHL (Fig. 1).

In MDA-MB-231 cells, treatment with increasing concentrations of TMX (5–100 μM) resulted in a dose-dependent decrease in cell viability, from 78% at 5 μM to 33% at 100 μM. CHL produced a greater reduction in viability at lower concentrations, with values of 60% at 5 μM and 30% at 25 μM. In HeLa cells, TMX reduced viability from 60% at 5 μM to 51% at 75 μM, and to 25% at 100 μM. CHL exhibited more pronounced cytotoxic effects in HeLa cells than in MDA-MB-231 cells, with viability decreasing to 49% at 5 μM and 12% at 25 μM (Fig. 1).

Combined treatment (75 μM TMX + 10 μM CHL for MDA-MB-231; 75 μM TMX + 5 μM CHL for HeLa) resulted in further reductions in viability compared to either drug alone, reaching 22% in MDA-MB-231 and 18% in HeLa cells. These decreases were statistically significant relative to untreated controls ($p < 0.05$, Table 2).

In 3D spheroid cultures, the same concentrations of TMX and CHL used in 2D experiments were applied to evaluate morphological changes at 24 and 72 h using phase-contrast microscopy. TMX and CHL monotherapies led to a partial loss of spheroid compactness and moderate volume reduction (~15–25%) relative to untreated controls. In contrast, combination treatment was associated with pronounced structural disruption, reduced spheroid integrity, and greater shrinkage in volume (~45–60%) in both MDA-MB-231 and HeLa cell lines (Fig. 2).

Wound healing assays were conducted to evaluate the effects of TMX and CHL on cell migration in MDA-MB-231 and HeLa cells. Cells were treated with IC_{50} concentrations of each drug, either alone or in combination, and the assay was performed 48 h post-treatment. Untreated cells served as the control group. Wound closure percentages

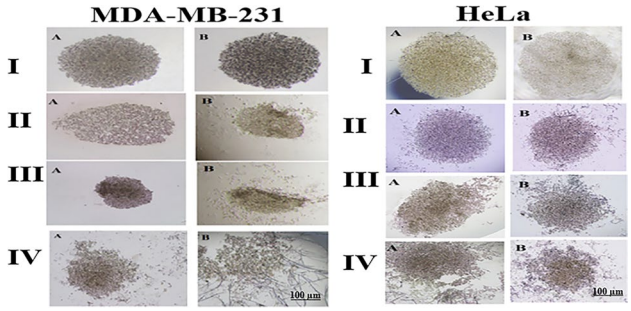


Fig. 2 Representative spheroid morphologies of MDA-MB-231 and HeLa cells after treatment with Tamoxifen (TMX), Chlorambucil (CHL), and their combination (TMX + CHL) for 24 h (**A**) and 72 h (**B**). Rows I–IV correspond to the following treatment conditions: **I**—Control (untreated), **II**—TMX (75 µM for both cell lines), **III**—CHL (10 µM for MDA-MB-231 and 5 µM for HeLa), **IV**—TMX + CHL combination (75 µM TMX + 10 µM CHL for MDA-MB-231, 75 µM TMX + 5 µM CHL for HeLa). The treatments caused a time-dependent reduction in spheroid compactness and structural integrity, which was most pronounced in the TMX + CHL combination group. Scale bar = 100 µm

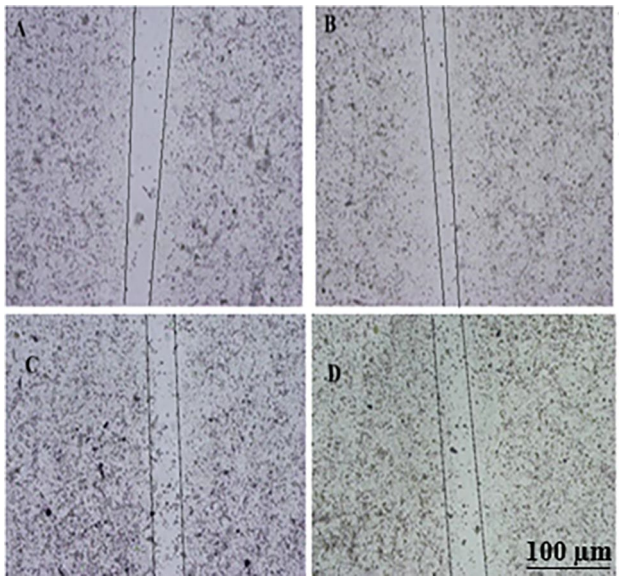


Fig. 3 Representative images of wound-healing assays showing the effects of Tamoxifen (TMX), Chlorambucil (CHL), and their combination (TMX+CHL) on cell migration in MDA-MB-231 cells after 24 h. Panels **A**–**D** correspond to the following treatment conditions: (**A**) Control (untreated), (**B**) TMX (75 µM), (**C**) CHL (10 µM), and (**D**) TMX+CHL (75+10 µM). The combination treatment (TMX+CHL) markedly reduced wound closure compared to single treatments, indicating a synergistic inhibition of cell migration. Scale bar = 100 µm

were calculated to assess the impact on migratory capacity (Fig. 3). In MDA-MB-231 cells, TMX (75 µM) and CHL (10 µM) resulted in wound closure rates of 65% and 60%, respectively, compared with 25% for the combination group and 72% in controls (Table 3). In HeLa cells, wound closure was 59% with TMX (75 µM), 53% with CHL (5 µM), 32% for the combination, and 81% in controls. Migration appeared reduced in the combination group, aligning with the quantitative data in Table 3 (Fig. 3).

Following the migration analysis, a dye-based invasion assay was conducted to evaluate the effects of TMX and CHL on the invasive capacity of MDA-MB-231 and HeLa cells. Cells were treated with the respective drug concentrations, and invasion was assessed after 48 h. In MDA-MB-231 cells, TMX (75 µM) and CHL (10 µM) reduced invasion rates to 36% and 20%, respectively, compared to 95% in the control group. Combination treatment (75 µM TMX+10 µM CHL) further decreased invasion to 8% ($p<0.01$). In HeLa cells, invasion rates were reduced to 29% with TMX (75 µM) and 23% with CHL (5 µM), while the combination (75 µM TMX+5 µM CHL) lowered invasion to 5%, compared to 99% in controls ($p<0.01$). All treatment conditions demonstrated statistically significant reductions in invasion relative to the control group (Table 3; Fig. 4).

Molecular findings

VEGFA gene expression

In MDA-MB-231 cells, both TMX and CHL treatments significantly reduced *VEGFA* expression levels compared to the control group. In 2D cultures, the fold change in *VEGFA* expression decreased from ~1.1 in controls to ~0.1 with CHL, ~0.15 with TMX, and as low as ~ –1.2 with the TMX+CHL combination. This indicates that the combination treatment induced more than a twofold downregulation relative to control, demonstrating a stronger inhibitory effect than either monotherapy ($p<0.05$). A similar trend was observed in 3D cultures, where *VEGFA* expression decreased from ~1.0 in the control group to ~0.05–0.1 with single-agent treatments and dropped further to ~ –0.3 following the combination therapy. Given that 2D and 3D culture systems exhibit distinct microenvironmental features—such as cell–cell interactions, oxygen gradients, and extracellular matrix composition—that can affect gene expression, VEGFA expression was assessed in both models to capture more physiologically relevant drug responses. These results suggest that the synergistic effect of the drug combination persists in the three-dimensional tumor model. In HeLa cells, *VEGFA* expression was also downregulated, although the reduction was less pronounced compared to MDA-MB-231 cells. In 2D cultures, fold change values declined from ~1.0 in controls to ~ –0.8 with TMX, ~ –0.6 with CHL, and ~ –1.6 with the combination therapy. In 3D HeLa models, *VEGFA* levels decreased from ~1.0 to ~0.1 following combination treatment, while monotherapies produced a more modest reduction (~0.05–0.1).

Table 3 Effects of Tamoxifen (TMX), Chlorambucil (CHL), and their combination on migration and invasion in MDA-MB-231 and HeLa cells

Cell Line	Treatment	Time (h)	Concentration (μM)	Wound Closure (%)	*P* value	Invasion Rate (%)	*P* value
MDA-MB-231	TMX	48	75	65 ± 2	$P<0.05$*	36 ± 1	$P<0.01$**
	CHL	48	10	60 ± 2	$P<0.05$*	20 ± 2	$P<0.01$**
	TMX + CHL	48	75 + 10	25 ± 1	$P<0.01$**	8 ± 1	$P<0.01$**
	Control	48	–	72 ± 2	–	95 ± 2	–
HeLa	TMX	48	75	59 ± 1	$P<0.05$*	29 ± 1	$P<0.01$**
	CHL	48	5	53 ± 2	$P<0.05$*	23 ± 1	$P<0.01$**
	TMX + CHL	48	75 + 5	32 ± 1	$P<0.01$**	5 ± 1	$P<0.01$**
	Control	48	–	81 ± 1	–	99 ± 2	–

*$P<0.05$, **$P<0.01$, ***$P<0.001$: Statistically significant compared to control (one-way ANOVA followed by Tukey's post hoc test)

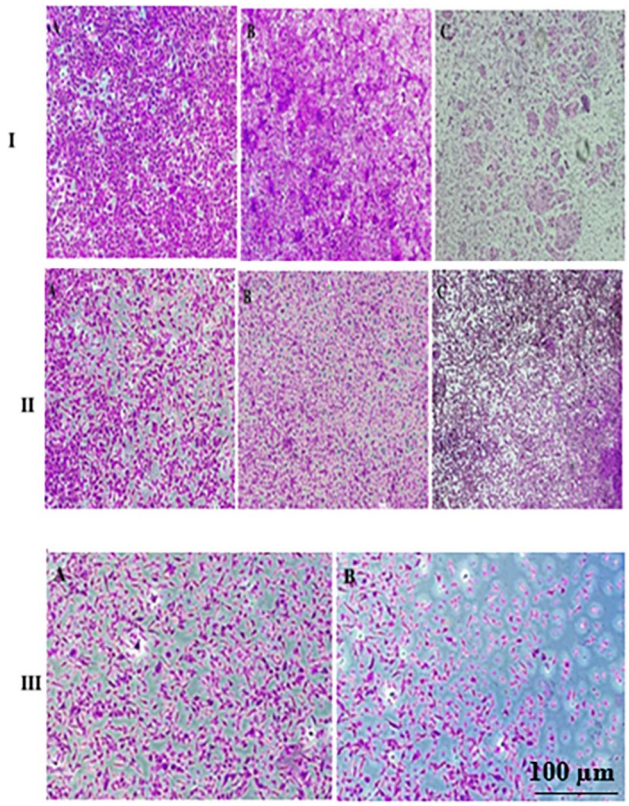


Fig. 4 Representative images from the invasion assay showing the effects of Tamoxifen (TMX), Chlorambucil (CHL), and their combination (TMX + CHL) on MDA-MB-231 cells at 24, 48, and 72 h. Rows **I**–**III** correspond to the following treatment conditions: **I**—TMX (75 μM); **II**—CHL (10 μM); **III**—TMX + CHL (75 + 10 μM). Panels **A**–**C** represent images taken at 24, 48, and 72 h, respectively (for the combination group, 24 h and 48 h images are shown). The treatments caused a time-dependent decrease in the number of invasive cells, with the TMX + CHL combination showing the most pronounced inhibitory effect on cell invasion. Scale bar = 100 μm

Molecular docking analyses

Molecular docking was performed to evaluate the binding affinities of TMX, CHL, and their combination to key targets involved in angiogenesis and invasion—namely *MMP2*, *VEGFA*, and *VEGFR1* (Table 4). Binding energies were expressed in kcal/mol, with more negative values indicating stronger predicted interactions. For MMP2, TMX and CHL exhibited binding energies of −7.3 and −5.6 kcal/mol, respectively, whereas the combination treatment yielded a more favorable binding energy of −8.3 kcal/mol, enhanced affinity. Similarly, strong interactions were observed with *VEGFA*: TMX and CHL demonstrated binding energies of −13.4 and −11.6 kcal/mol, respectively, while the combination showed the most negative value at −14.8 kcal/mol. Against *VEGFR1*, TMX and CHL recorded binding energies of −7.9 and −6.0 kcal/mol, with the combination achieving −8.2 kcal/mol. In all cases, the TMX + CHL combination produced more negative binding energies than either monotherapy, indicating a potential synergistic interaction at the molecular level and supporting the enhanced inhibitory effects observed experimentally.

Table 4 Binding energies (kcal/mol) of Tamoxifen, Chlorambucil, and their combination with MMP2, VEGFA, and VEGFR1

Target protein	Drug	Binding energy (kcal/mol)
MMP2	Tamoxifen	−7.3
	Chlorambucil	−5.6
	Tamoxifen + Chlorambucil	−8.3
VEGFA	Tamoxifen	−13.4
	Chlorambucil	−11.6
	Tamoxifen + Chlorambucil	−14.8
VEGFR1	Tamoxifen	−7.9
	Chlorambucil	−6.0
	Tamoxifen + Chlorambucil	−8.2

Discussion

Breast and cervical cancers are among the most prevalent malignancies affecting women globally and are associated with significant mortality. A major challenge in managing these cancers is the emergence of resistance to conventional chemotherapeutic agents, which compromises treatment efficacy and worsens patient outcomes [24]. To address this issue, combining chemotherapeutic agents at lower doses to achieve synergistic effects has emerged as a promising

strategy to enhance therapeutic efficacy while reducing systemic toxicity.

In the present study, we systematically investigated the in vitro effects of combining tamoxifen, a selective estrogen receptor modulator, with chlorambucil, a DNA-alkylating agent, in MDA-MB-231 and HeLa cell lines. Cytotoxicity, migration, invasion capacity, and *VEGFA* gene expression were evaluated in both 2D and 3D culture models. In addition, molecular docking analyses were conducted to explore the potential molecular interactions underlying the observed biological effects.

Cytotoxicity analyses (Tables 2 and 3; Figs. 1 and 2) revealed that the combination of TMX and CHL elicited significantly greater antiproliferative effects than either agent alone in both MDA-MB-231 and HeLa cell lines ($p < 0.05$). This synergistic activity likely arises from the dual mechanisms of action: CHL induces genotoxic stress through DNA alkylation, while TMX inhibits proliferation by modulating estrogen receptor signaling [13]. The convergence of these pathways provides a mechanistic rationale for the enhanced cytotoxicity observed with the combination treatment. Comparable synergistic effects have been reported when TMX is paired with other compounds, such as polyphenols [25, 26].

However, not all combination or hybrid drug strategies yield additive or synergistic effects. In some instances, the combined activity may be diminished compared to monotherapies. Such discrepancies can stem from various factors, including the structural linkage of chlorambucil to its partner pharmacophore, altered pharmacokinetics, off-target effects, the molecular profile of the cancer cells, or the activation of resistance pathways. The heterogeneity of tumor biology and the complexity of drug resistance mechanisms often pose challenges to combination therapy efficacy [13]. Furthermore, while TMX is classically associated with estrogen receptor blockade, it also modulates alternative signaling cascades—such as PI3K/Akt/mTOR and NF-κB—which may influence therapeutic outcomes and contribute to resistance in a context-dependent manner [27].

Supporting these observations, scratch assay results (Table 3; Fig. 3) demonstrated a pronounced inhibition of cell migration following combination treatment, with wound closure reduced to 25% in MDA-MB-231 cells and 32% in HeLa cells ($p < 0.01$). These findings are consistent with previous studies indicating that TMX can attenuate metastatic potential under specific conditions [28]. Similarly, chlorambucil has been shown to inhibit matrix metalloproteinase activity, thereby limiting extracellular matrix degradation and reducing cellular motility [13]. Extensive evidence in the literature highlights the pivotal roles of matrix metalloproteinases—particularly *MMP-2* and *MMP-9*—in promoting tumor progression and metastasis in both breast and cervical cancers. In breast cancer models, especially in triple-negative subtypes such as MDA-MB-231 cells, *MMP-2* is known to exhibit high basal expression and serves as a primary mediator of invasive behavior [29]. Similarly, in cervical cancer, *MMP-2* has been identified as a key contributor to tumor aggressiveness and metastatic potential. In contrast, MMP-9 is typically expressed at low basal levels in HeLa cells and is only significantly upregulated in response to strong external stimuli [30, 31]. Accordingly, the present study focused on *MMP-2* due to its well-established functional significance and constitutive expression patterns in both cancer types, aligning with prior findings in the literature.

The observed anti-migratory effect of the TMX + CHL combination is likely attributable to the synergistic interaction between TMX's suppression of proliferation and epithelial–mesenchymal transition (EMT) via estrogen receptor modulation, and CHL's dual role as a DNA-alkylating agent and MMP inhibitor. However, the cellular response to tamoxifen can vary substantially depending on the molecular characteristics of the cancer type; divergent outcomes have been reported, particularly in tamoxifen-resistant or estrogen receptor–negative cell lines [7, 10, 28]. These findings emphasize the need to interpret the observed effects within the specific biological context of each cell model.

Invasion assays (Table 3; Fig. 4) demonstrated that the combination treatment reduced invasion rates to 8% in MDA-MB-231 cells and 5% in HeLa cells ($p < 0.01$). These results align with previous studies showing that tamoxifen can inhibit invasion in triple-negative breast cancer models [27, 28]. Chlorambucil has also been reported to limit extracellular matrix degradation [32]; however, some studies have observed a neutral impact on the invasive phenotype [13], suggesting that its effect may depend on tumor microenvironmental conditions.

Analysis of *VEGFA* expression revealed a significant reduction in both cell lines following combination treatment ($p < 0.05$). Given the central role of *VEGFA* in tumor angiogenesis, this downregulation is particularly relevant for impairing tumor vascularization. While tamoxifen has been shown to suppress *VEGF* release in both in vivo and in vitro models, intracellular *VEGF* levels may paradoxically increase, implying that its antiangiogenic effect could also arise from reduced vessel density rather than *VEGF* suppression alone [33]. In tamoxifen-resistant cells, *VEGFA* expression can be upregulated via *MAPK* and Pin1 signaling pathways [27]. Additionally, redox-sensitive heparin–chlorambucil conjugates have been shown to reduce *VEGF* production and inhibit endothelial cell proliferation [34]. The present findings are consistent with these reports, suggesting that the TMX + CHL combination may exert potent antiangiogenic effects through *VEGFA* downregulation. The

observed reduction in VEGFA expression following TMX–CHL combination treatment may underlie the reduced migratory and invasive capacities observed in wound healing and invasion assays. VEGFA-driven angiogenesis and extracellular matrix remodeling are closely linked to tumor cell motility. Therefore, the downregulation of VEGFA, together with the observed inhibition of MMP-2 activity, suggests a coordinated anti-angiogenic and anti-metastatic mechanism. Moreover, molecular docking results indicating strong binding affinities of TMX and CHL to both VEGFA and VEGFR1 support this mechanistic relationship.

Molecular docking results (Table 4) demonstrated that the TMX + CHL combination yielded lower—thus stronger—binding energies for MMP2, VEGFA, and VEGFR1 compared to either agent alone. Notably, the highest binding affinity was observed for VEGFA, supporting the proposed molecular mechanism underlying the combination's anti-angiogenic activity observed in vitro. Similar correlations between docking affinity and biological inhibition have been reported for other compound classes reinforcing the predictive value of in silico modeling [35, 36].

Furthermore, docking simulations revealed that TMX and CHL interact with amino acid residues located within or near the VEGFR binding pocket, with partial overlap at the VEGFA interface. This suggests that both compounds may disrupt VEGFA–VEGFR interactions by sterically hindering or competitively occupying key residues critical for ligand binding. These insights provide mechanistic support for the observed suppression of angiogenic signaling in vitro and highlight the potential of the TMX + CHL combination as a dual-targeted therapeutic strategy.

Conclusion

This study demonstrates that the combination of tamoxifen and chlorambucil exerts multifaceted anticancer effects in both MDA-MB-231 and HeLa cell lines. Compared to single-agent treatments, the combination significantly decreased cell viability, robustly inhibited metastatic behaviors such as migration and invasion, and markedly downregulated *VEGFA* expression—a key mediator of tumor angiogenesis. Importantly, results from 3D spheroid models indicate that these effects are maintained under physiologically relevant conditions that better mimic the hypoxic and heterogeneous tumor microenvironment, underscoring the therapeutic potential of this drug combination in targeting aggressive and treatment-resistant cancers.

Molecular docking analyses demonstrated strong binding affinities of the TMX–CHL combination to MMP2, VEGFA, and VEGFR1, reinforcing the molecular rationale for the observed biological effects. To our knowledge, this is the first study to comprehensively demonstrate the broad-spectrum anticancer activity of TMX–CHL combination therapy across 2D, 3D, and in silico models. By targeting complementary pathways, the combination effectively suppresses both invasive and angiogenic phenotypes. These findings highlight the potential translational value of this combination as a synergistic, multi-targeted therapeutic approach—particularly at lower, less toxic doses.

We acknowledge that the migration and invasion assays in this study were conducted at concentrations that led to more than a 50% reduction in cell proliferation. While this approach revealed robust antimigratory effects, it may also reflect compromised cell viability. Ideally, such functional assays should be performed at concentrations that reduce proliferation by 25–50% to more accurately isolate migration-specific effects. Additionally, we recognize that cell proliferation may have partially contributed to wound closure in the migration assay—a known limitation that will be addressed in future studies through the use of proliferation inhibitors or serum starvation protocols.

Furthermore, this study was limited to in vitro and in silico models; protein expression and functional activity were not validated, and the models used do not fully recapitulate tumor heterogeneity or the complexity of the in vivo microenvironment. Future research should include in vivo studies to evaluate therapeutic efficacy, pharmacokinetics, and potential toxicity.

In conclusion, the TMX–CHL combination presents a promising, repurposing-based therapeutic strategy with significant anti-angiogenic and anti-metastatic potential. These findings support its development as a novel, multi-targeted anticancer approach with high translational relevance.

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Data availability Raw data are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors declare that the research was conducted in an environment in which there was no commercial or financial relationship with any institution or person that could be construed as a

potential conflict of interest.

Ethics, Consent to participate, and Consent to publish Not applicable

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