

Effects of Methotrexate on HIF-1 α , HIF-2 α and P4H-TM Expression in Breast Cancer Cells

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ABSTRACT

Background: Methotrexate (MTX), a widely used antimetabolite, exhibits cytotoxic effects across multiple cancer types, yet its impact on hypoxia-related markers in Breast Cancer (BC) remains unclear. **Purpose:** This study investigated the effects of MTX on cell viability and on Hypoxia-Inducible Factor-1 α (HIF-1 α), Hypoxia-Inducible Factor-2 α (HIF-2 α), and Prolyl 4-Hydroxylase-Transmembrane (P4H-TM) in MCF-7 breast cancer cells and Human Telomerase Reverse Transcriptase-Immortalised Breast Epithelial Cells (HTERT-HME1). **Materials and Methods:** Cells were treated with MTX for 24, 48, and 72 hr. Viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay, and protein levels were measured by Enzyme-Linked Immunosorbent Assay (ELISA). **Results:** MTX caused a time- and dose-dependent decrease in viability, with stronger cytotoxicity in MCF-7 than in HTERT-HME1 cells. Baseline levels of HIF-1 α , HIF-2 α , and P4H-TM were higher in MCF-7 cells than in HTERT-HME1 cells. MTX treatment significantly reduced HIF-1 α levels in MCF-7 cells and P4H-TM levels in both cell lines, whereas the changes in HIF-2 α levels were not statistically significant. Moderate correlations were observed between HIF-1 α and HIF-2 α , and between HIF-1 α and P4H-TM. **Conclusion:** These findings indicate that MTX modulates selected hypoxia-related protein levels and reduces breast cancer cell viability. HIF-1 α , HIF-2 α and P4H-TM may represent potential biomarkers for distinguishing malignant from non-malignant breast epithelial cells; however, their translational utility requires further validation in preclinical and clinical studies.

Keywords: Breast cancer cells, HIF-1 α , HIF-2 α , Methotrexate, Prolyl 4-Hydroxylase-Transmembrane (P4H-TM).

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INTRODUCTION

One of the most common malignant tumours in women is Breast Cancer (BC).¹ As a typical feature of solid tumour, rapid tumour growth disrupts the organisation of the vascular network, leading to the development of a hypoxic microenvironment.² Hypoxia is therefore a frequent condition in solid tumours. When tumour cells do not receive enough oxygen, they activate adaptive genetic and metabolic responses to survive under hypoxic stress.³

Hypoxia-Inducible Factor (HIF) family members facilitate both oxygen delivery and adaptation to oxygen deprivation by regulating the expression of genes involved in glucose uptake and metabolism, erythropoiesis, angiogenesis, cell proliferation and apoptosis.^{4,5} To date, three HIF family members have been identified in human cells in response to reduced oxygen availability. Each of these heterodimers, named HIF-1, HIF-2 and HIF-3, consists of an α subunit and a β subunit that dissociate under normoxic conditions.^{6,7} Cellular and developmental responses to hypoxic conditions are mediated primarily by HIF-1 α , encoded by the HIF1A gene.⁸ Three HIF- α isoforms (HIF-1 α , HIF-2 α , HIF-3 α) produced by alternative splicing have been identified.⁹ Overexpression of HIF-1 α and HIF-2 α has been reported in various human cancers, likely due to intratumoural hypoxia or genetic alterations. As hypoxia deepens, HIF-1 α becomes the central regulator of adaptive tumour responses.¹⁰⁻¹²



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Prolyl 4-Hydroxylase-Transmembrane (P4H-TM) belongs to the family of HIF-Prolyl 4-Hydroxylases (HIF-P4Hs), which regulate the stability and activity of Hypoxia-Inducible Transcription Factors (HIFs) that play a fundamental role in oxygen homeostasis.^{13,14} P4H-TM was selected in the present study because, unlike the better-characterized PHD1-3 enzymes, it is a transmembrane prolyl 4-hydroxylase associated with the endoplasmic reticulum and has been implicated in the regulation of HIF stability and hypoxia-related signaling. Its reported involvement in tumour biology, including breast cancer, therefore provides a rationale for evaluating P4H-TM alongside HIF-1 α and HIF-2 α .¹⁵ Both the therapeutic efficacy and Toxic Effects of Methotrexate (MTX), a long-standing antimetabolite used in cancer treatment due to its inhibition of cell division, vary considerably among patients.¹⁶ Consequently, unlike many other antineoplastic agents, MTX is administered over a wide dose range.¹⁷

The cytotoxic effects of MTX have been studied in the literature and are widely known. On the other hand, it has been determined that its effects on hypoxia-related molecular pathways in breast cancer have not yet been fully elucidated. Studies in the literature that evaluate HIF-1 α , HIF-2 α , and P4H-TM markers together in both cancer and healthy breast epithelial cells are quite limited. Therefore, we hypothesized that MTX would differentially modulate hypoxia-associated proteins in malignant and non-malignant breast epithelial cells. The present study was designed to investigate the effects of MTX on cell viability and HIF-1 α , HIF-2 α , and P4H-TM protein levels in MCF-7 and HTERT-HME1 cells, thereby providing preliminary comparative evidence regarding the response of hypoxia-associated markers to MTX exposure.

MATERIALS AND METHODS

Cell Culture and Cell Viability Assay

The experiments were performed on Human Healthy Breast Epithelial Cells (HTERT-HME1) and human breast cancer cells (MCF-7). The cell lines were obtained from the American Type Culture Collection (ATCC) and cultured in high-glucose Dulbecco's Modified Eagle's Medium (DMEM; Gibco) supplemented with 10% Fetal Bovine Serum (FBS), 100 IU/mL penicillin and 100 μ g/mL streptomycin. Cells were maintained in a humidified atmosphere with 5% CO₂ at 37 °C. The cells were subcultured with 0.25% Trypsin-EDTA. The cells were treated with MTX when they had reached approximately 70-80% confluence before treatment was administered.

The cytotoxicity of Methotrexate (MTX) (Submex; Abdi Ibrahim, Istanbul, Turkey) against HTERT-HME1 and MCF-7 cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay (Merck KGaA, Darmstadt, Germany). Cells were seeded at 1×10^4 cells/well

in 96-well plates, and after overnight incubation, treated with different MTX concentrations for 24, 48 and 72 hr in a 5% CO₂ incubator at 37 °C. At the end of each incubation, 20 μ L of MTT reagent was added and cells were incubated for a further 3 hr. Formazan crystals were dissolved in 100 μ L Dimethyl Sulfoxide (DMSO) and the absorbance was measured at 570 nm with a reference wavelength of 650 nm using a microplate reader (Multiskan GO, Thermo Scientific, Waltham, MA, USA). The experiments were performed as three independent biological experiments, with three technical replicates for each experimental condition, and the half-maximal inhibitory concentration (IC₅₀) values were calculated using GraphPad Prism software (version 9, La Jolla, CA, USA). Results were expressed as the percentage of viable cells relative to untreated controls.

Experimental Groups

Groups were formed as follows: HTERT-HME1 control, MTX-treated HTERT-HME1, MCF-7 control, and MTX-treated MCF-7 cells. Cells were seeded at 3×10^5 cells/well in 6-well plates. After 24 hr, the control groups received fresh complete medium, while the treated groups received medium containing MTX at the predetermined 48-hr IC₅₀ concentration for 48 hr. The 48-hr exposure period was selected because it provided an intermediate treatment duration between the 24- and 72-hr viability assessments and allowed evaluation of biomarker responses following sustained MTX exposure while avoiding the more prolonged exposure associated with the 72-hr treatment condition. At the end of the experiments, cell lysates were prepared using 1 $\times$ Radioimmunoprecipitation Assay (RIPA) lysis buffer containing a protease inhibitor cocktail (Merck KGaA, Darmstadt, Germany).^{18,19}

Biochemical Analysis

Hypoxia-Inducible Factor-1 alpha (HIF-1 α), Hypoxia-Inducible Factor-2 alpha (HIF-2 α), and transmembrane Prolyl 4-Hydroxylase (P4H-TM) levels were measured using quantitative Enzyme-Linked Immunosorbent Assay (ELISA) kits following the manufacturer's instructions, and the measured protein concentrations were normalized to the total protein concentration of each sample before statistical analysis. HIF-1 α levels were analysed using the HIF-1 α ELISA Kit (Cat. No.: E0422Hu, BT LAB, Zhejiang, China), while HIF-2 α levels were determined using the HIF-2 α ELISA Kit (Cat. No.: E7164Hu, BT LAB, Zhejiang, China). P4H-TM concentrations were quantified using the P4H-TM ELISA Kit (Cat. No.: E7241Hu, BT LAB, Zhejiang, China). For all assays, the intra-assay coefficient of variation was <8% ($n=20$) and the inter-assay coefficient of variation was <10% ($n=20$), confirming the reliability of the measurements.

Table 1: Calculated IC₅₀ values (μM) of MTX in HTERT-HME1 and MCF-7 cells from three independent biological experiments.

Cell Line	24 hr	48 hr	72 hr
MCF-7	15.01±0.8	10.07±1.3	8.01±0.9
HTERT-HME1	24.12±0.7	16.09±1.2	12.08±1.1

Statistical Analysis

The Statistical Package for the Social Sciences (SPSS) version 21.0 (IBM Corp., Armonk, NY, USA) and Microsoft Office 365 were used for data evaluation, analysis and figure generation. The Shapiro-Wilk test was used to assess normality, and homogeneity of variances was assessed using Levene's test before one-way Analysis of Variance (ANOVA). Numerical variables were presented as mean±standard deviation. One-Way Analysis of Variance (ANOVA) was used to compare continuous variables between more than two groups, and Tukey's HSD test was performed for *post hoc* analysis. Pearson correlation analysis was used to evaluate relationships between numerical variables. A *p*-value<0.05 was considered statistically significant.

RESULTS AND DISCUSSION

The anti-tumour potential of Methotrexate (MTX) at different concentrations was evaluated in HTERT-HME1 and MCF-7 cells using the MTT assay to determine its dose-dependent effect on cell viability. MTX was administered at increasing concentrations (2.5-100 μM) for 24, 48 and 72 hr, and Half-Maximal Inhibitory Concentration (IC₅₀) values were calculated (Table 1).

The IC₅₀ values of MTX were consistently higher in HTERT-HME1 cells than in MCF-7 cells at all time points, indicating lower MTX sensitivity in healthy breast epithelial cells. In contrast, MCF-7 cells showed markedly lower IC₅₀ values,

demonstrating higher cytotoxic susceptibility to MTX. MTX significantly reduced cell viability in both cell lines in a clear dose- and time-dependent manner, as shown in Figures 1 and 2. These findings support that MTX exerts potent cytotoxic effects in a time- and dose-dependent manner, as reflected by the progressive decline in cell viability across increasing MTX concentrations and longer incubation periods (Figures 1 and 2). MCF-7 cells exhibited a more pronounced reduction in viability compared with HTERT-HME1 cells, indicating greater MTX sensitivity in breast cancer cells, which is consistent with their lower IC₅₀ values. In chemotherapy, the contribution of dose and duration to therapeutic response and toxicity is well recognized.²⁰ However, the present *in vitro* findings specifically demonstrate that prolonged MTX exposure (48-72 hr) intensifies cytotoxicity in both healthy and cancerous cells. This suggests that shorter exposure durations may help reduce adverse effects on normal cells while maintaining anticancer efficacy. Although escalating MTX doses increases cancer cell death, optimising treatment regimens to balance effectiveness and safety remains crucial.²¹ Methotrexate (MTX) has been documented to exert dose- and duration-dependent cytotoxic effects not only on tumor cells but also on healthy tissues. In *in vitro* studies, although cytotoxicity and decreased cell viability observed in healthy breast epithelial cells (hTERT-HME1) were at lower levels compared to breast cancer cells (MCF-7), MTX was found to reduce Superoxide Dismutase (SOD) activity and total thiol levels, while increasing Malondialdehyde (MDA) levels, leading to significant oxidative damage in these normal cells.²² In clinical use, these pro-oxidant effects of the drug can cause serious systemic side effects such as liver damage (hepatotoxicity) and kidney failure (nephrotoxicity), which are directly related to cellular damage and increased Reactive Oxygen Species (ROS), and limit the therapeutic use of the drug.^{23,24} Statistically significant overall differences were

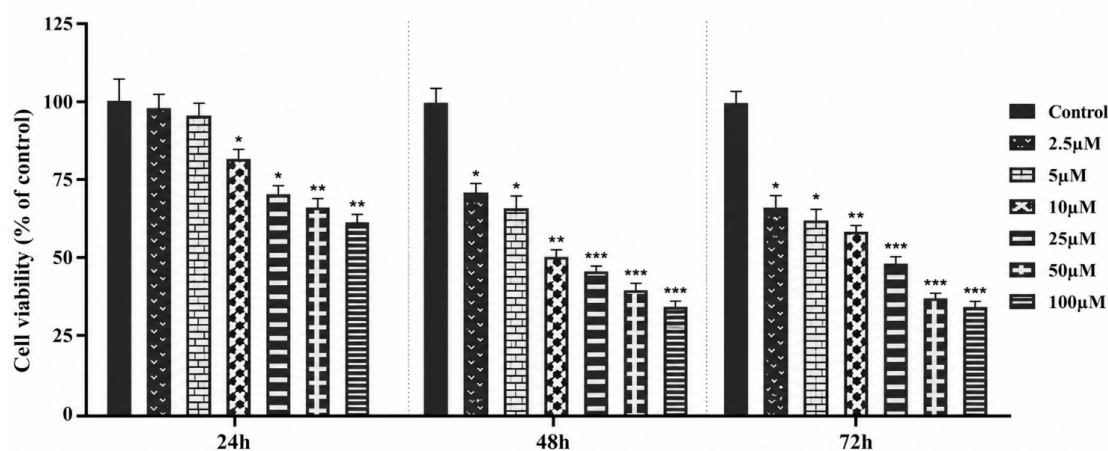


Figure 1: Cell viability of HTERT-HME1 cells treated with Methotrexate (MTX) at concentrations of 2.5-100 μM for 24, 48, and 72 hr. Viability was determined using the MTT assay and normalized to untreated controls. Data are expressed as mean±SD. Statistical significance relative to the control group is indicated by **p*<0.05, ***p*<0.01, and ****p*<0.001.

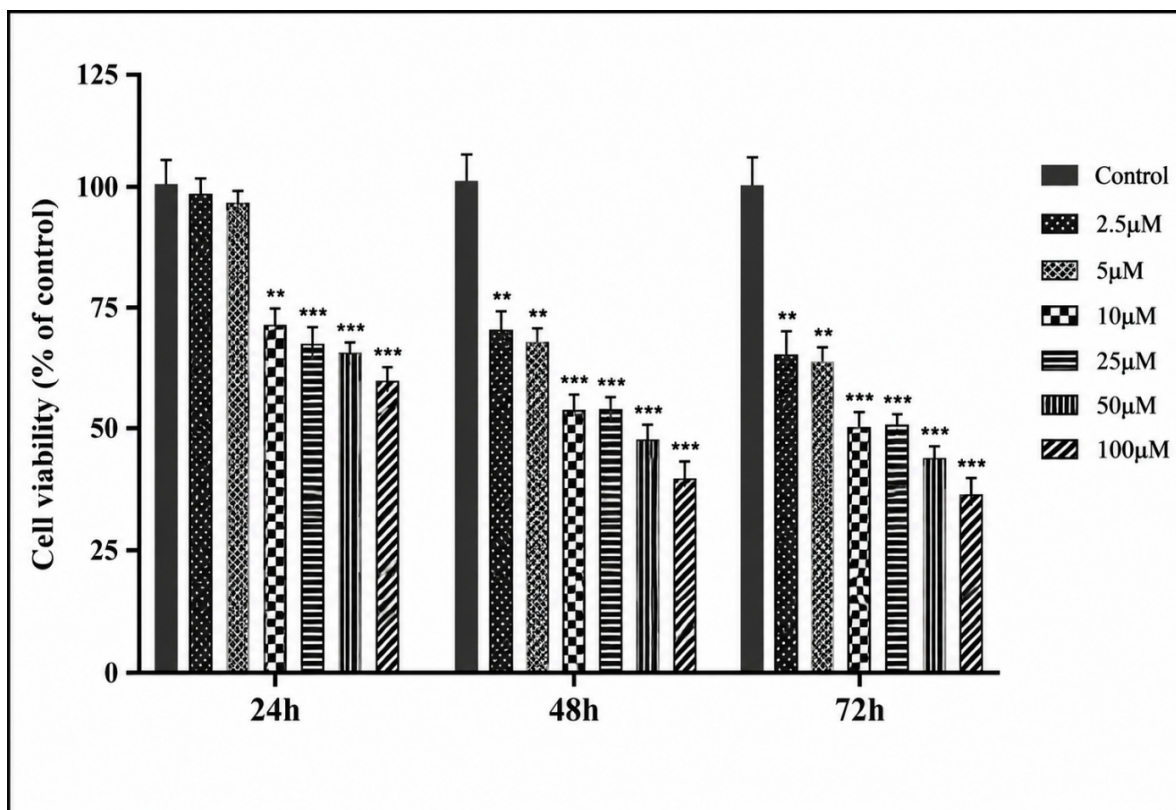


Figure 2: Cell viability of MCF-7 breast cancer cells treated with Methotrexate (MTX) at concentrations of 2.5-100 µM for 24, 48, and 72 hr. Cell viability was quantified using the MTT assay and normalized to untreated control cells. Data are expressed as mean±SD. Statistical significance relative to the control group is indicated by * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

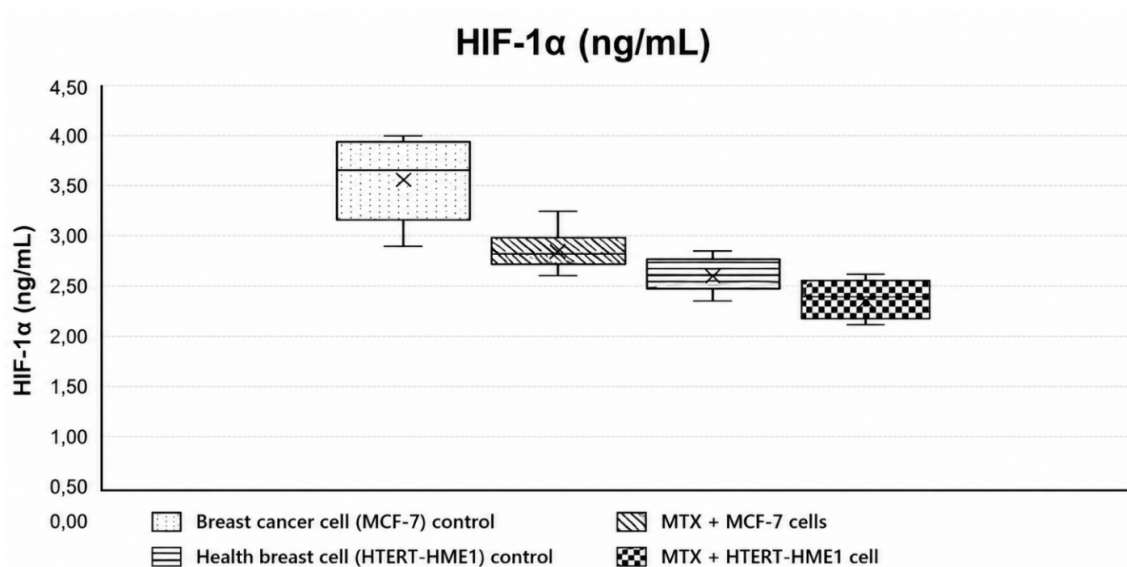


Figure 3: Box plot showing HIF-1α levels in control and MTX-treated MCF-7 and HTERT-HME1 cells. HIF-1α, hypoxia-inducible factor-1 alpha; MTX, methotrexate; SD, standard deviation. Data are expressed as mean±SD.

Table 2: Comparison of HIF-1 α , HIF-2 α , and P4H-TM levels between treatment and control groups.

Parameter	Breast cancer cell (MCF-7) control	MTX + MCF-7 cells	Healthy breast cell (HTERT-HME1) control	MTX + HTERT-HME1 cells	p value
HIF-1 α (ng/mL)	3.56 $\pm$ 0.4	2.88 $\pm$ 0.18	2.63 $\pm$ 0.17	2.40 $\pm$ 0.19	<0.001
HIF-2 α (ng/mL)	10.04 $\pm$ 0.72	9.43 $\pm$ 0.43	7.32 $\pm$ 0.77	8.45 $\pm$ 0.92	<0.001
P4H-TM (ng/mL)	2.35 $\pm$ 0.48	1.79 $\pm$ 0.20	1.99 $\pm$ 0.08	1.52 $\pm$ 0.22	0.001

Note: One-way ANOVA test was applied. As shown in Table 2, untreated MCF-7 cells exhibited the highest levels of HIF-1 α , HIF-2 α , and P4H-TM; MTX treatment significantly reduced HIF-1 α and P4H-TM levels in the evaluated groups, whereas the reduction in HIF-2 α observed in MCF-7 cells was not statistically significant.

Table 3: Post hoc Tukey HSD comparison of HIF-1 α , HIF-2 α , and P4H-TM levels.

Parameter	MCF-7 control vs MTX + MCF-7	MCF-7 control vs HTERT-HME1 control	MCF-7 control vs MTX + HTERT-HME1	MTX + MCF-7 vs HTERT-HME1 control	MTX + MCF-7 vs MTX + HTERT-HME1	HTERT-HME1 control vs MTX + HTERT-HME1
HIF-1 α (ng/mL)	0.001	<0.001	<0.001	0.343	0.018	0.416
HIF-2 α (ng/mL)	0.482	<0.001	0.006	<0.001	0.131	0.062
P4H-TM (ng/mL)	0.015	0.163	<0.001	0.638	0.372	0.045

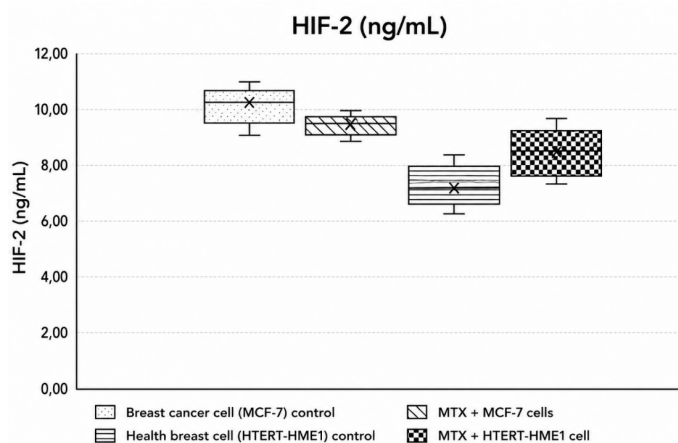


Figure 4: Box plot showing HIF-2 α levels in control and MTX-treated MCF-7 and HTERT-HME1 cells. HIF-2 α , hypoxia-inducible factor-2 alpha; MTX, methotrexate; SD, standard deviation. Data are expressed as mean $\pm$ SD.

observed among the four experimental groups for HIF-1 α , HIF-2 α , and P4H-TM (Table 2; Figures 3 and 5). *Post hoc* Tukey HSD analysis was subsequently performed to identify the specific group comparisons responsible for these differences (Table 3).

As shown in Table 2, untreated MCF-7 cells exhibited the highest levels of HIF-1 α , HIF-2 α , and P4H-TM. MTX treatment significantly reduced HIF-1 α levels in MCF-7 cells and P4H-TM levels in both cell lines, whereas the changes in HIF-2 α levels were not statistically significant in either cell line. Table 3 confirms that the most significant differences originated from comparisons involving MCF-7 controls, particularly versus MTX-treated groups, indicating strong MTX-induced suppression of hypoxia markers.

Figure 3 illustrates that HIF-1 α levels were markedly elevated in MCF-7 control cells and decreased significantly following MTX treatment, while HTERT-HME1 cells showed overall lower levels with a modest MTX-induced reduction.

As shown in Figure 4, HIF-2 α levels were highest in MCF-7 control cells; although HIF-2 α levels decreased numerically following MTX treatment in MCF-7 cells, this difference was not statistically significant ($p=0.482$), and the numerical increase observed in MTX-treated HTERT-HME1 cells compared with untreated HTERT-HME1 cells was also not statistically significant ($p=0.062$).

Figure 5 demonstrates that P4H-TM expression was highest in untreated MCF-7 cells and significantly reduced after MTX exposure, with the lowest levels observed in MTX-treated HTERT-HME1 cells.

Overall, HIF-1 α levels were significantly lower in MTX-treated MCF-7 cells than in untreated MCF-7 cells, whereas the corresponding reduction in HTERT-HME1 cells was not statistically significant. P4H-TM levels were significantly reduced following MTX treatment in both cell lines. Although HIF-2 α levels decreased numerically in MTX-treated MCF-7 cells, the difference was not statistically significant ($p=0.482$), and the numerical increase in HTERT-HME1 cells following MTX treatment was also not statistically significant ($p=0.062$). Correlation analysis demonstrated moderate positive associations between HIF-1 α and HIF-2 α ($r=0.595$, $p=0.002$) and between HIF-1 α and P4H-TM ($r=0.441$, $p=0.031$), whereas no significant correlation was observed between HIF-2 α and P4H-TM ($r=0.169$, $p=0.429$) (Table 4).

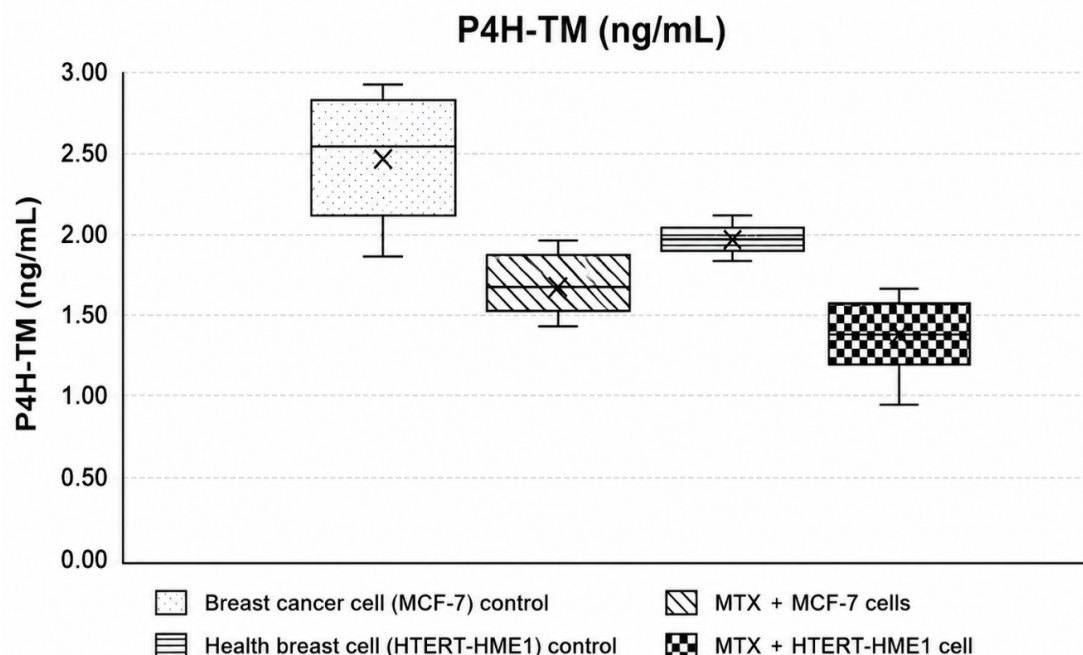


Figure 5: Box plot showing P4H-TM levels in control and MTX-treated MCF-7 and HTERT-HME1 cells. P4H-TM, prolyl 4-hydroxylase-transmembrane; MTX, methotrexate; SD, standard deviation. Data are expressed as mean $\pm$ SD.

Table 4: Pearson correlation analysis between HIF-1 α , HIF-2 α and P4H-TM levels.

	HIF-2	P4H-TM
HIF-1		
r	0.595	0.441
p value	0.002	0.031
HIF-2		
r	-	0.169
p value	-	0.429

Although the mechanisms underlying MTX-induced toxicity are not fully defined, several regulatory pathways have been implicated. In the present study, MTX was associated with differential changes in HIF-1 α , HIF-2 α , and P4H-TM levels in breast cancer and healthy breast epithelial cells, with statistically significant reductions observed for HIF-1 α in MCF-7 cells and P4H-TM in both cell lines. HIF-1 α , a central mediator of cellular adaptation to hypoxia, may act as a key regulator linking P4H-TM activity with hypoxic signalling in MTX-treated cells.²⁵ Elevated HIF activity has long been associated with tumour progression across diverse cancer types, including lung, prostate, breast, endometrial and colon tumours.²⁶ Bos *et al.*²⁷ Further reported that HIF-1 α expression increases with tumour grade and correlates with oestrogen receptor positivity. At the same time, multiple studies have linked high HIF-1 α levels with poor prognosis and aggressive disease behaviour.²⁸⁻³² In addition, a large retrospective analysis by Dales *et al.*³³ demonstrated that high

HIF-1 α expression predicts early recurrence, reduced survival and elevated metastatic risk even in node-negative breast cancer, reinforcing its prognostic value. In our study, untreated MCF-7 cells exhibited significantly higher levels of HIF-1 α and HIF-2 α than healthy breast cells, aligning with these observations. HIF-1 α appeared more responsive to MTX exposure than HIF-2 α in the present study, as the reduction in HIF-2 α levels in MCF-7 cells did not reach statistical significance. This difference may reflect differential regulation, protein degradation kinetics, or distinct functional roles of the two HIF isoforms; however, the underlying mechanism cannot be established from the present experiments.³⁴ Moreover, silencing HIF-1 α has been shown to increase MTX sensitivity in MCF-7 cells,³⁵ suggesting a possible association between HIF-1 α signaling and MTX response; however, the present study did not directly investigate whether MTX-induced changes in hypoxia-associated proteins contribute causally to altered drug sensitivity.

P4H-TM, an endoplasmic reticulum-localised enzyme known to hydroxylate and stabilise HIF-1 α ,³⁶ also demonstrated its highest expression in untreated MCF-7 cells and was markedly reduced following MTX exposure. The moderate positive correlation identified between HIF-1 α and P4H-TM levels suggests an association between these markers in the present experimental setting, while HIF-2 α showed no significant correlation with P4H-TM; however, these correlation findings do not establish a direct functional or causal relationship. Given that P4H-TM expression

is induced during hypoxia,³⁷ MTX-mediated suppression of P4H-TM may contribute to reduced hypoxic signalling in breast cancer cells.

Collectively, these findings highlight the need to balance MTX dose and exposure duration to maximise therapeutic benefit while limiting cytotoxicity to healthy cells. Furthermore, the differential levels of HIF-1 α , HIF-2 α , and P4H-TM observed between malignant and non-malignant breast epithelial cells suggest that these proteins may warrant further investigation as potential biomarkers. Further validation in additional breast cancer models, hypoxic experimental systems, animal studies, and clinical samples will be necessary to determine their potential prognostic or therapeutic relevance. Methotrexate is associated with systemic toxicities in clinical settings, including hepatotoxicity, nephrotoxicity, and myelotoxicity, particularly with high-dose or prolonged exposure.³⁸⁻⁴¹ However, these systemic toxicities were not directly evaluated in the present *in vitro* study and therefore are discussed only as clinical context rather than as findings of the current investigation.

This study has several limitations. First, only one breast cancer cell line, MCF-7, was evaluated, which limits the generalizability of the findings to other molecular subtypes of breast cancer. Second, the experiments were performed under normoxic culture conditions and therefore may not fully reproduce the hypoxic microenvironment of solid breast tumors. Third, although changes in HIF-1 α , HIF-2 α , and P4H-TM protein levels were evaluated, mechanistic confirmation using approaches such as Western blotting or RT-qPCR was not performed. In addition, apoptosis, oxidative stress, and downstream signaling pathways were not directly investigated. Finally, the findings were obtained exclusively from *in vitro* cell models and require validation in additional breast cancer models, hypoxic experimental systems, animal studies, and clinical samples before their translational significance can be established.

CONCLUSION

The findings demonstrate that methotrexate exerts dose- and time-dependent cytotoxic effects on breast cancer (MCF-7) and normal breast epithelial (HTERT-HME1) cells. MTX exposure was associated with significant reductions in HIF-1 α and P4H-TM levels, whereas the numerical reduction in HIF-2 α levels in MCF-7 cells was not statistically significant, suggesting that MTX may differentially modulate hypoxia-associated proteins. The moderate positive correlation between HIF-1 α and P4H-TM suggests an association between these proteins in the present experimental setting, although the biological and causal significance of this relationship remains to be established. These findings provide preliminary evidence supporting further investigation of HIF-1 α , HIF-2 α , and P4H-TM as potential biomarkers; however, validation in additional breast cancer

subtypes, hypoxic models, animal studies, and clinical samples is required before their prognostic or therapeutic utility can be determined.

ABBREVIATIONS

ANOVA: Analysis of Variance; **ATCC:** American Type Culture Collection; **BC:** Breast Cancer; **DHFR:** Dihydrofolate Reductase; **DMEM:** Dulbecco's Modified Eagle's Medium; **DMSO:** Dimethyl Sulfoxide; **ELISA:** Enzyme-Linked Immunosorbent Assay; **FBS:** Fetal Bovine Serum; **GSH:** Reduced Glutathione; **HD-MTX:** High-Dose Methotrexate; **HIF:** Hypoxia-Inducible Factor; **HIF-1 α :** Hypoxia-Inducible Factor-1 Alpha; **HIF-2 α :** Hypoxia-Inducible Factor-2 Alpha; **HTERT-HME1:** Human Telomerase Reverse Transcriptase-Immortalised Breast Epithelial Cells; **IC₅₀:** Half-Maximal Inhibitory Concentration; **MCF-7:** Michigan Cancer Foundation-7 Human Breast Cancer Cell Line; **MDA:** Malondialdehyde; **MTT:** 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay; **MTX:** Methotrexate; **NF- κ B:** Nuclear Factor Kappa B; **P4H-TM:** Prolyl 4-Hydroxylase-Transmembrane; **RIPA:** Radioimmunoprecipitation Assay Buffer; **ROS:** Reactive Oxygen Species; **SD:** Standard Deviation; **SPSS:** Statistical Package for the Social Sciences; **SOD:** Superoxide Dismutase.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS CONTRIBUTIONS

Orcun Can: Conceptualization, study design, manuscript preparation, and final approval. **Seyma Dumur:** Study design, data collection, data analysis, manuscript preparation, and final approval. **Dilek Duzgun Ergun:** Study design, data collection, data analysis, manuscript preparation, and final approval. **Berrin Papila:** Study design, critical review of the manuscript, and final approval. **Cigdem Usul Afsar:** Study design, critical review of the manuscript, and final approval. **Abdulhalim Senyigit:** Study design, critical review of the manuscript, and final approval. **Hafize Uzun:** Study design, supervision, critical review of the manuscript, and final approval.

ETHICAL APPROVAL

Ethical approval was not required because this study involved only commercially available established cell lines and did not involve human participants or animals. Therefore, informed consent was not applicable.

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