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Original article

Inhibition of EGF and $CoCl_2$-Induced HIF-1α and VEGF Production in Triple Negative MDA-MB-468 Cells by Umbelliprenin: Unveiling the Role of PI3K/AKT/mTOR and MAPK Pathways

Roya Atabakhshian[1], Melina Moshirpour[2], Mohammad Hadi Farjoo[3], Shiva Ghafghazi[3], Seyed Ali Ziai[3*]

[1]Department of Clinical Biochemistry, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
[2]School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
[3]Department of Pharmacology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

Abstract

Background and objectives: Triple-negative breast cancer is a significant global health challenge, and there's growing interest in targeting multiple pathways for treatment. Umbelliprenin, derived from herbal sources, has shown anti-tumor potential. This study aimed to assess umbelliprenin's impact on key genes related to proliferation, metastasis, and angiogenesis. **Methods:** Umbelliprenin, which was synthesized by the Pharmaceutical Research Center (PRC) at Mashhad University of Medical Sciences in Iran, was utilized in this study. The study aimed to investigate the impact of umbelliprenin on EGF and $CoCl_2$-induced signaling in the PI3K/AKT/mTOR and MAPK pathways. Quantitative PCR was employed to assess the expression of EGFR, PI3K, AKT, mTOR, S6K, ERK1, ERK2, 4EBP1, HIF-1α, HIF-1β, VEGF, and VEGFR genes. Additionally, immunoblot assays were conducted to evaluate the levels of VEGF and HIF-1α in MDA-MB-468 cells. **Results:** The study found that umbelliprenin had cytotoxic effects, with an IC_{50} value of 152.5 µM. At concentrations of 10 µM and 20 µM, it upregulated genes like EGFR, VEGFR, HIF-1α, VEGF, PI3K, ERK2, and mTOR while downregulating 4EBP1. Umbelliprenin also increased VEGF protein levels. When used on EGF-stimulated cells, it enhanced VEGF and PI3K expression while inhibiting AKT, ERK2, mTOR, and antiproliferative 4EBP1 genes. Notably, VEGF and HIF-1α protein levels remained unchanged. Conversely, umbelliprenin downregulated EGFR, AKT, ERK1/2, HIF-1α, and VEGF in $CoCl_2$-stimulated cells, while elevating 4EBP1 and reducing VEGF and HIF-1α protein levels. **Conclusion:** Umbelliprenin inhibited MDA-MB-468 cell growth and impacted gene expression related to metastasis and angiogenesis, particularly under conditions of EGFR activation and hypoxia.

Keywords: angiogenesis inhibitors; breast neoplasm; medicinal plants; metastasis; umbelliprenin

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Introduction

Breast cancer is a substantial contributor to female mortality on a global scale, accounting for more than 2.3 million new cases and approximately 685,000 deaths in the year 2020 [1]. Although advancements in screening, early identification, and medical interventions have improved the chances of surviving breast cancer, raising the survival rate from 75% to 90%,

*Corresponding author: aliziai@sbmu.ac.ir

obstacles still remain. These obstacles come in the form of chemotherapy resistance and the inability to efficiently deliver drugs to the specific tumor cells, which continue to present substantial challenges in effectively treating breast cancer [2,3].

Triple-negative breast cancer (TNBC) is an aggressive form of breast cancer identified by tumor cells that lack the expression of estrogen and progesterone receptors, as well as the epidermal growth factor receptor 2. [4]. TNBC constitutes roughly 15% of all breast cancer cases [4]. Patients diagnosed with metastatic TNBC who do not respond to initial chemotherapy experience restricted progression-free survival (PFS), typically with a median PFS of 3 to 4 months. This underscores the critical requirement for the development of a medication specifically targeting TNBC [5].

The PI3K/AKT/mTOR pathway has a vital role in numerous cellular processes, encompassing cell growth, survival, migration, tumor development, and angiogenesis. [6,7]. Triple-negative breast cancers (TNBCs) are known to express other EGFR (Her1, ErbB-1) receptors [8]. Upon binding of epidermal growth factor (EGF) to its receptor (EGFR/HER1), it initiates the activation of both the mitogen-activated protein kinases (MAPK) and the phosphatidylinositol 3-kinases/protein kinase B (PI3K/AKT) pathway. [9,10]. Consequently, the serine-threonine protein kinase called the mammalian target of rapamycin (mTOR), which is located downstream of the PI3K/AKT pathway, is activated. This activation of mTOR leads to an enhanced translation of hypoxia-inducible factor-1α (HIF-1α), culminating in its accumulation within cells [11]. Enzymes downstream of the EGFR pathway, including S6K, ERK1, ERK2, and 4EBP1, are also implicated in this process. Among these enzymes, 4EBP1 has the capacity to inhibit the mTOR pathway, implying that it exerts a negative influence on angiogenesis.

Cetuximab, which is an anti-EGFR monoclonal antibody, has demonstrated limited efficacy in combination with chemotherapy for treating advanced TNBC. Its effectiveness has been assessed in various phase II clinical trials [12,13]. Angiogenesis, an essential process that plays a significant role in the invasion and spread of tumor cells, offers a promising focus for cancer treatment. In this context, the targeting of vascular endothelial growth factor (VEGF) and its receptor (VEGFR) is considered a significant therapeutic approach. However, prospective studies have not yet conclusively shown the impact of angiogenesis inhibitors on the overall survival of female TNBC patients. Bevacizumab, an antibody targeting VEGF, has been the subject of extensive research, but its incorporation into treatments has yielded minimal to no effect on overall survival [14-17].

Angiogenesis can be initiated by various factors, among them, growth factors that are released in response to hypoxia [11]. Hypoxia results in the accumulation of hypoxia-inducible transcription factor (HIF), which subsequently upregulates the expression of vascular endothelial growth factor (VEGF) [18,19]. VEGF stimulates the formation of microvessels, facilitating tumor cells in adapting to hypoxic conditions and proliferating [20,21]. The capacity of cells to adapt to hypoxia plays a pivotal role in both physiological and pathological processes, especially in the proliferation of solid tumor cells [18,22]. In normoxic (normal oxygen) conditions, HIF-1α is consistently produced but undergoes rapid degradation via the proteasomal pathway owing to its brief half-life [23]. In hypoxic environments, the degradation of HIF-1α is impeded, causing its accumulation within the cell and subsequent translocation into the nucleus. Inside the nucleus, HIF-1α forms a dimer with HIF-1β and binds to DNA, which leads to the transcription of hypoxia-responsive genes, including VEGF [24,25]. VEGF functions via its receptor, VEGFR, and assists tumor cells in adapting to low oxygen levels by stimulating angiogenesis and erythropoiesis under the regulation of HIF-1α [20,21]. Multiple pathways contribute to the accumulation of HIF-1α, and inhibiting ubiquitination prevents its degradation [26]. Furthermore, the synthesis of new proteins contributes to the elevation of HIF-1α levels in hypoxic cells. [26]. The overexpression of HIF-1α has been noted in various tumors, including breast cancer, and is linked to disease progression and treatment resistance [27]. Studies investigating alterations in gene and protein expression have revealed that hypoxic responses are more significant in TNBC [28]. Hence, the imperative lies in the development of new therapeutic compounds, particularly those derived from renewable and herbal sources, which possess selective toxicity and are more cost-effective [29].

A promising herbal therapeutic compound is Umbelliprenin, classified as a 7-prenyloxycoumarin. It is a prenylated coumarin compound synthesized by various *Ferula* species and can be discovered in different plants commonly employed in Iranian cuisine and traditional medicine, including celery and *Citrus limon*. [30]. Umbelliprenin has been the subject of extensive research and has shown a range of beneficial effects, including antioxidant and anticancer properties [2,31], anti-inflammatory and immunomodulatory activities [32], and the ability to reduce the activity of matrix metalloproteinases (MMP), which are essential enzymes involved in cancer cell migration, angiogenesis, and invasion [33,34]. Umbelliprenin has also demonstrated its capacity to induce apoptosis in human M4Beu-pigmented metastatic melanoma and Jurkat T-CLL cells [30,35]. Additionally, it has exhibited antileishmanial activity against promastigotes [36] and antibacterial properties [37]. Furthermore, umbelliprenin has inhibited the proliferation of solid tumor cells, including breast cancer [35]. In vivo studies have explored the anti-tumor effects of umbelliprenin using various cell lines [31,38].
The objective of this study was to assess how non-toxic levels of umbelliprenin affect the EGFR signaling pathway in the MDA-MB-468 cell line. This analysis was conducted under two conditions: with and without the presence of epidermal growth factor (EGF) and cobalt chloride ($CoCl_2$), which acts as an inducer for HIF-1α.

Material and Methods

Ethical considerations

The ethical approval for this research was granted by the Ethics Committee of Shahid Beheshti University of Medical Sciences under the code IR.SBMU.RETEC.REC.1395.848.

Chemicals

We purchased RPMI1640 medium, FBS (fetal bovine serum), penicillin-streptomycin, and trypsin-EDTA from Gibco BRL (USA). Recombinant human EGF (epidermal growth factor), $CoCl_2$ (cobalt chloride), DMSO (dimethyl sulfoxide), and trypan blue were sourced from Sigma-Aldrich Merck KGaA (Germany). Phosphatase inhibitor cocktails was acuared from Santa Cruz Biotechnology (USA). Antibodies for human/mouse/rat HIF-1 alpha/HIF1A (MAB1536; 1:500), human/primate VEGF (MAB293R, 1:250), and human/mouse/rat beta-actin (MAB8929; 1:50000) were obtained from R&D Systems (USA). HRP (horseradish peroxidase)-conjugated goat anti-mouse IgG- (sc-2005) was purchased from Santa Cruz (USA). Umbelliprenin was purchased from pharmaceutical research center (PRC), t Mashhad University of Medical Sciences (Mashhad, Iran). The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) powder was obtained from BioBasic Co. (Canada). RNeasy kit was bought from BioBasic (Canada). PrimeScript RT reagent kit was acquared from Takara Bio, Inc. (Otsu, Japan). RealQ Plus 2x Master Mix Green kit was purchased from Amplicon (Denmark). PVDF membrane was bought from BioRad Laboratories, Hercules, (USA).

Plant material

Umbelliprenin is a compound derived from plants and was synthesized and purified by the Pharmaceutical Research Center (PRC) at the Department of Pharmacognosy, Mashhad University of Medical Sciences, Iran. The substance used in this study had a molecular weight of 366 g and a molecular formula of $C_{24}H_{30}O_3$, with a purity exceeding 95% (Figure 1).

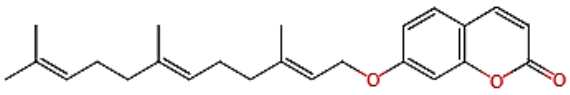

Figure 1. structure of Umbelliprenin

Cell line

The MDA-MB-468 cell line was acquired from the Iranian Biological Resource Center, Tehran, Iran.

Cell culture

MDA-MB-468 cells were cultured in RPMI1640 medium containing 10% fetal bovine serum and 1% penicillin-streptomycin. The cultures were maintained in a humidified environment with 5% CO_2 at a temperature of 37 °C.

MTT assay

To assess the toxicity of umbelliprenin, 5×10^3 cells/well were seeded in 96-well plates with 200 µL of complete culture medium. After 24 hours, the medium in each well was replaced with fresh

medium, and various concentrations of umbelliprenin dissolved in DMSO were added to achieve final concentrations of 4, 8, 17, 33, 67, 133, 267, and 533 µM in each well. Each concentration of umbelliprenin was tested in triplicate and incubated for 24, 48, and 72 h with 5% CO_2 at 37°C. The control group contained only 0.025% DMSO. After the specified incubation period, 20 µL of MTT solution (5 mg/mL) was added to each well and incubated for 4 h. Subsequently, the culture medium containing the MTT solution was removed, and 100 µL of DMSO was added to dissolve the formazan crystals generated by viable cells. The absorbance of the wells was assessed at a wavelength of 550 nm, using 630 nm as the reference wavelength. The concentration of umbelliprenin required to inhibit the growth of 50% of the cells was determined as the IC_{50}. Additionally, IC_{10} and IC_5 values of umbelliprenin were computed and subsequently employed in further investigations.

Study design

To activate the EGFR, the cells were deprived of serum overnight, followed by pretreatment with EGF (20 ng/mL) for 30 min. Subsequently, IC_5 and IC_{10} concentrations of umbelliprenin (10 and 20 µM, respectively) were introduced and incubated for 24 h.

To induce hypoxic conditions, cells were exposed to cobalt chloride ($CoCl_2$). Approximately 10^4 cells/well were seeded in triplicate and subjected to increasing dilutions of $CoCl_2$ (50, 100, 150, 200, 250, 300, and 600 µM) for 24 h. Based on the MTT results, a concentration of 100 µM $CoCl_2$ was selected to induce hypoxia. Cells were then treated with either 10 µM or 20 µM of umbelliprenin in the presence or absence of 100 µM $CoCl_2$.

Quantitative PCR analysis

The expression levels of EGFR, PI3K, AKT, mTOR, S6K, ERK1, ERK2, 4EBP1, HIF-1α, HIF-1β, VEGF, and VEGFR genes were evaluated in both treated and control cells through real-time PCR. Total RNA extraction was carried out using a RNeasy kit. The concentration and purity of the extracted RNA (with a 260/280 ratio) were determined in duplicate using NanoDrop (NanoDrop Technologies). Subsequently, the total RNA was reverse-transcribed into cDNA using the PrimeScript RT reagent kit, with equal RNA amounts for each synthesis.

Real-time PCR was performed utilizing the RealQ Plus 2x Master Mix Green kit, with primer sequences provided in Table 1. Beta-actin served as the housekeeping gene in this analysis. The PCR conditions consisted of an initial step at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 60 s, employing the Applied Biosystem/StepOne Plus instrument (USA). The experiment was conducted in duplicate with independent samples, and a negative control was included to rule out any contamination.

Gene expression results were analyzed using the $2^{-\Delta\Delta Ct}$ method, and REST and Linreg freeware were employed to assess expression levels and efficiencies, respectively.

SDS–PAGE and Western blot analysis

The expression of HIF-1α and VEGF proteins was assessed using the western blotting technique.

Table 1. Primers sequences of studied genes

Gene	Forward	Reverse
HIF-1α	AGATTTTGGCAGCAACGACAC	GAAGTGGCTTTGGCGTTTCA
VEGF	ACAAATGTGAATGCAGACCAAA	CACCAACGTACACGCTCCA
VEGFR	GGTTGTGTATGTCCCACCCC	TACCAGTGGATGTGATGCGG
HIF-1β	AGCAAGCCCCTTGAGAAGTC	TGCCTTTACTCTGATCCGCA
EGFR	GTGAAAACACCGCAGCATGT	AAACAGTCACCCCGTAGCTC
PI3K	AAGAGCCCCGAGCGTTTCT	TGATGGTCGTGGAGGCATTG
AKT	GCAAAGGATGAAGTGGCACA	AAAACAGCTCGCCCCCATTA
mTOR	TGGGGACTGCTTTGAGGTTG	ACACTGTCCTTGTGCTCTCG
S6K	TTATTTCGGGAGCAAGGGGG	CCATGCCAAGTTCATATGGTCC
ERK1	TCAGACTCCAAAGCCCTTGAC	TCAGCCGCTCCTTAGGTAGG
ERK2	AATTTGTCAGGACAAGGGCTCA	CCAAACGGCTCAAAGGAGTC
4EBP1	GGAGTGTCGGAACTCACCTG	ACTGTGACTCTTCACCGCC
β-Actin	CACACAGGAGAGGTGATAGCAAGT	GACCAAAAGCCTTCATACATCTCA

MDA-MB-468 cells were collected using cold PBS and centrifuged at 1000 rpm at 4 °C for 5 min. PBS was then discarded, and the cell pellet was subjected to lysis with a buffer comprising 200 µL of RIPA buffer, 1% v/v protease inhibitor cocktail, and 1% v/v phosphatase inhibitor cocktails. The cell pellet was incubated on ice for 30 min in this lysate. Subsequently, the lysate was sonicated and centrifuged at 13000 rpm for 15 min at 4 °C. The total protein concentration was determined using the Bradford method.

Equal amounts of protein (30 µg) from each sample were separated on an SDS polyacrylamide gel (10% Tris-base) employing the BioRad Criterion System. Subsequently, the separated proteins were transferred onto a PVDF membrane. The membrane was blocked for 75 min at room temperature using 2% skim milk in Tris-buffered saline containing 1% Tween-20. Following the blocking step, the membrane was incubated overnight at 4 °C with the primary antibody after being washed with TBST.

Next, the membranes were incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies and then detected through a chemiluminescent reaction using ECL (Thermo Scientific, USA). The results were visualized using X-ray film.

Statistical analysis

Gene expression changes were analyzed using REST software. Statistical analysis of group means was conducted using the GraphPad Prism version 9.0 program; p-values less than 0.05 were regarded as statistically significant.

Results and Discussion

MDA-MB-468 cells were exposed to increasing concentrations of umbelliprenin (4, 8, 17, 33, 67, 133, 267, and 533 µM) for 24, 48, and 72 hours. The cell viability exhibited a significant decrease in both a concentration-dependent and time-dependent manner, as illustrated in Figure 2. The IC_{50} values for umbelliprenin at 24, 48, and 72 hours were determined to be 152.5 µM (95% CI: 114.4 to 203.3), 77.22 µM (95% CI: 58.97 to 101.1), and 71.77 µM (95% CI: 55.76 to 92.37), respectively. For subsequent experiments, non-toxic concentrations of umbelliprenin were selected as IC_5 (10 µM) and IC_{10} (20 µM) at the 24-hour time point.

The cytotoxicity of $CoCl_2$ on MDA-MB-468 cells was assessed using the MTT assay. The outcomes indicated that treatment with 100 µM $CoCl_2$ for 24 h did not induce cytotoxicity in these cells, as depicted in Figure 3. Consequently, a dosage of 100 µM $CoCl_2$ was selected for inclusion in this study.

We assessed the impact of umbelliprenin on the PI3K/AKT/mTOR and MAPK pathways both in isolation and in the context of EGF or $CoCl_2$ stimulation in MDA-MB-468 cells.

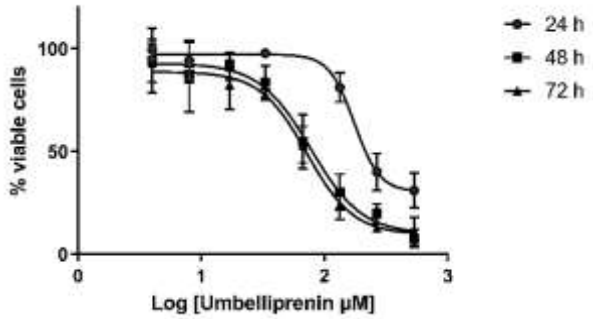

Figure 2. Umbelliprenin inhibited cellular viability in a concentration- and time-dependent manner at 24, 48, and 72 hours. Each data point represents the mean±SD of triplicate assays. *p<0.05 compared to the control

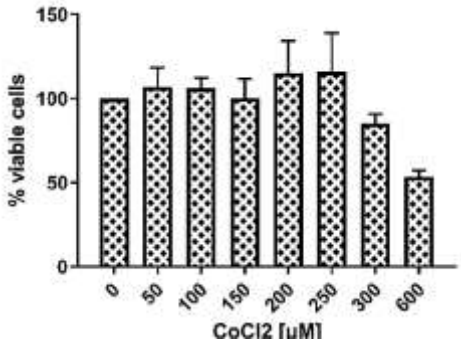

Figure 3. The graph illustrates the viability results of MDA-MB-468 cells when exposed to varying concentrations of $CoCl_2$. Each bar on the graph represents the mean value along with the standard deviation (mean±SD) obtained from a minimum of three repeated assays.

Treatment with umbelliprenin at a concentration of 10 µM resulted in significant upregulation of EGFR, VEGFR, HIF-1α, VEGF, and PI3K, along with a downregulation of 4EBP1, as illustrated in Figure 4. Meanwhile, at the concentration of 20 µM, umbelliprenin significantly increased the expression of VEGFR, HIF-1α, VEGF, PI3K, ERK2, and mTOR, while decreased 4EBP1 (Figure 4).

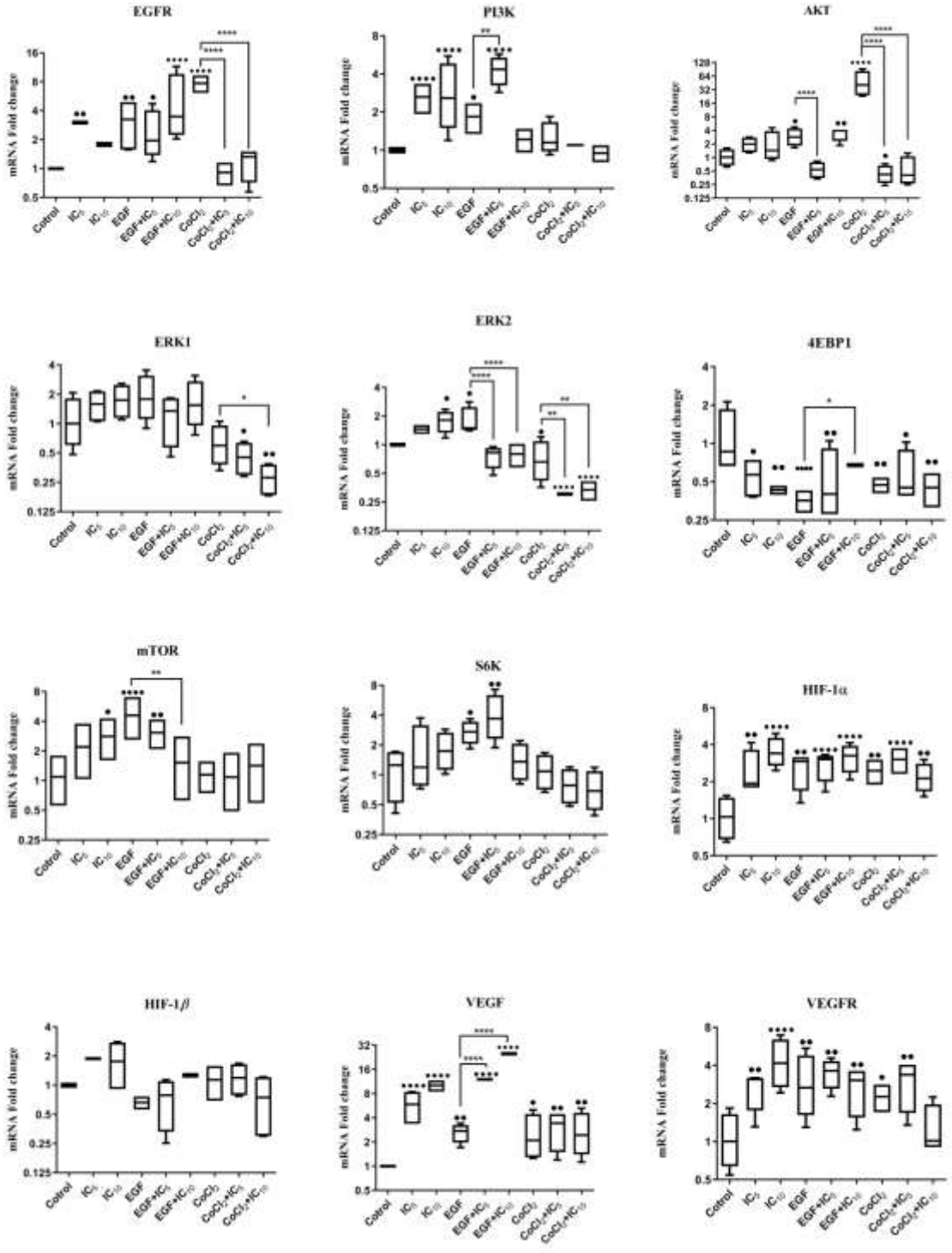

Figure 4. The figure presents min-max boxplots showing the mRNA expression fold change of the studied genes compared to β-actin as the housekeeping gene in various conditions, including: control (MDA-MB-468 cells); the cell line treated with umbelliprenin 10 μM (IC_5); the cell line treated with umbelliprenin 20 μM (IC_{10}); the cell line stimulated by EGF; EGF-stimulated cells treated with umbelliprenin (EGF + IC_5 and EGF + IC_{10}); hypoxia induced by $CoCl_2$ in the cell line ($CoCl_2$); $CoCl_2$-induced hypoxia cells treated with umbelliprenin ($CoCl_2$ + IC_5, $CoCl_2$ + IC_{10}). Each bar in the graph represents the results of three independent experiments. Dots (●) on the boxplots indicate the significance compared to the control group (●$p<0.05$, ●●$p<0.01$, ●●●$p< 0.001$, ●●●● $p< 0.0001$). Stars (*) on the boxplots represent the significance compared to the control EGF or $CoCl_2$ (*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p< 0.0001$).

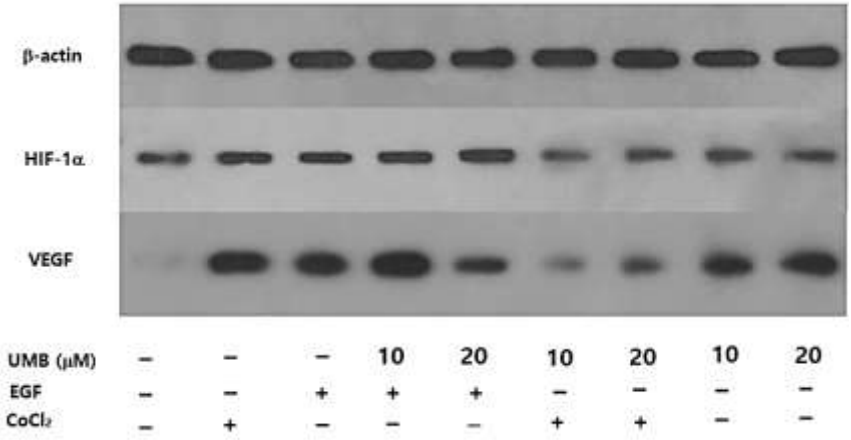

Figure 5. The figure demonstrates the effects of umbelliprenin on the translated proteins HIF-1α and VEGF in both control and stimulated cells

These effects were predominantly concentration-dependent. Umbelliprenin did not influence the protein levels of HIF-1α; however, it notably elevated the expression of VEGF protein, as demonstrated in Figure 5.

These findings imply that umbelliprenin, when administered alone, may stimulate proliferation, metastasis, and angiogenesis in MDA-MB-468 cells.Stimulation of MDA-MB-468 cells with EGF led to a significant increase in the expression of EGFR, VEGFR, PI3K, AKT, ERK2, mTOR, S6K, HIF-1α, and VEGF genes, while decreasing the expression of 4EBP1 genes.

In EGF-stimulated cells, treatment with 10 μM umbelliprenin resulted in a significant increase in the expression of VEGF and PI3K (exhibiting a synergistic effect) and a decrease in AKT and ERK2 (demonstrating an antagonistic effect), as shown in Figure 4. Conversely, treatment with 20 μM umbelliprenin significantly increased the expression of VEGF and 4EBP1, while reducing ERK2 and mTOR in EGF-stimulated cells (Figure 4). Immunoblotting revealed that umbelliprenin either had no effect or increased the protein levels of VEGF and HIF-1α (Figure 5).

Interestingly, the effects of umbelliprenin on EGF-stimulated MDA-MB-468 cells did not appear to exhibit a concentration-dependent pattern (Figure 4).

Stimulation of MDA-MB-468 cells with $CoCl_2$ resulted in a significant increase in the expression of EGFR, AKT, ERK2, HIF-1α, VEGF, and VEGFR genes, while reducing the expression of 4EBP1 genes (Figure 5).

Treatment with 10 and 20 μM umbelliprenin significantly decreased the expression of EGFR, AKT, ERK1 (only at 20 μM), and ERK2 in $CoCl_2$-stimulated cells. Notably, these effects were not influenced by the concentration of umbelliprenin (Figure 4). Umbelliprenin also led to a reduction in the protein levels of HIF-1α and VEGF in $CoCl_2$-stimulated cells (Figure 5).

The current study revealed that umbelliprenin, significantly reduced the viability of MDA-MB-468 cancer cells in a manner dependent on both its concentration and the duration of exposure. The application of umbelliprenin at concentrations corresponding to IC_5 and IC_{10} levels activated a range of genes associated with processes such as cell proliferation, angiogenesis, and metastasis. This activation included the upregulation of EGFR, VEGFR, HIF-1α, VEGF, PI3K, ERK2, and mTOR genes, while downregulating the 4EBP1 gene. These genetic changes were further supported by an increase in the levels of HIF-1α and VEGF proteins.

In cells that were stimulated with EGF, umbelliprenin demonstrated synergistic effects on the expression of VEGF and PI3K, while concurrently exhibiting antagonistic effects on AKT, ERK2, mTOR, and the antiproliferative 4EBP1 gene. However, the overall impact on the protein levels of HIF-1α and VEGF exhibited minimal alterations. Conversely, in cells subjected to $CoCl_2$-induced hypoxia, umbelliprenin acted to antagonize the expression of EGFR, AKT, ERK1, and ERK2 genes, as well as the levels of HIF-1α and VEGF proteins.

According to the American Cancer Society (http://www.cancer.org/Cancer/BreastCancer), breast cancer stands as the second most

widespread cancer and the second primary contributor to cancer-related fatalities among women in the United States. In our investigation, umbelliprenin showcased inhibitory effects on the MDA-MB-468 cancer cell line, and these effects were contingent on both concentration and exposure time. Previous research has reported the cytotoxic effects of umbelliprenin on the SKBR-3 breast cancer cell line [39], and it has demonstrated similar cytotoxic effects on various other cancer cell types [29,35,40].

Receptor tyrosine kinases (RTKs) have crucial roles in controlling cell proliferation and are linked to two downstream signaling pathways: the Ras/mitogen-activated protein kinase (MAPK) pathway and the phosphoinositide 3 kinase (PI3K)/AKT/mTOR (PAM) pathway. One well-known RTK is the epidermal growth factor receptor (EGFR), which is frequently dysregulated in triple-negative breast cancer (TNBC) tumors. Approximately 60-80% of TNBC tumors exhibit elevated EGFR levels [41,42]. The EGFR signaling pathway, particularly involving the PI3K and ERK enzymes, plays a critical role in promoting tumor cell proliferation and growth [43]. Activation of this pathway enhances HIF-1α stability, especially in breast cancer, through mTOR [28], ultimately leading to increased VEGF secretion via both HIF-1α-dependent and -independent mechanisms [44]. Growth factors either directly or indirectly stimulate the lipid kinase PI3K, which converts phosphatidylinositol 4, 5-diphosphate (PIP2) into phosphatidylinositol 3, 4, 5-triphosphate (PIP3). This, in turn, triggers AKT phosphorylation and its relocation to the cell membrane, where it becomes active through the action of PDK1 and the mTOR complex 2 (mTORC2) [45,46]. Activated AKT phosphorylates various factors involved in cell survival, proliferation, and motility, including mTOR, thereby promoting cell growth [46]. Since mTOR is downstream of PI3K, it can be targeted therapeutically independently of PI3K activation, with inputs from MAPK signaling and LKB1 [47]. TNBC exhibits notably higher levels of AKT phosphorylation, a hallmark of AKT activation, in comparison to luminal breast cancer [48]. The PAKT trial demonstrated the advantages of adding a PI3K inhibitor, capivasertib, to paclitaxel in TNBC patients as opposed to paclitaxel alone [49]. The PI3K pathway is also subject to regulation through cross-talk with other pathways, particularly the RAS-MAPK pathway, which can both activate and inhibit PI3K signaling [45]. Inhibition of mTOR, AKT, and PI3K can trigger feedback loops that may limit the effectiveness of these inhibitors. For instance, mTOR inhibition can upregulate upstream receptor tyrosine kinases (RTKs), leading to a rebound activation of AKT [50]. Inhibition of AKT induces FOXO-dependent transcription and activates RTKs, while PI3K inhibition enhances MAPK signaling [51,52]. Given the complexity of the PI3K/AKT/mTOR system and its interactions with other kinase pathways, combination therapies or drugs with multiple inhibitory effects on these enzymes may represent potential treatment options for TNBC [6]. In our study, umbelliprenin significantly reduced the expression of AKT and mTOR in EGF-stimulated MDA-MB-468 cells, suggesting its potential as a therapeutic candidate warranting further investigation.

The mTORC1, a protein complex, adds phosphate groups to two important proteins: 4EBP1 (eukaryotic initiation factor 4E-binding protein) and S6K (p70 ribosomal S6 kinase). These proteins have critical roles in facilitating the production of ribosomes and the translation of proteins, essential processes for cell growth and proliferation [53]. Phosphorylation of 4EBP1 prevents it from binding to the eIF4E mRNA cap-binding protein, enabling the formation of the cap-binding complex and the initiation of translation [54]. A study demonstrated that introducing mutants with altered phosphorylation sites in 4EBP1 into breast carcinoma cells reduced their tumorigenicity, and the removal of these mutants reversed the malignant characteristics [55]. Non-phosphorylated 4EBP1 can reduce resistance to apoptosis in breast cancer cells [55]. In our research, umbelliprenin increased the expression of 4EBP1 in cells treated with EGF. This elevation has the potential to decrease resistance to apoptosis, emphasizing its possible utility as a therapeutic strategy for TNBC treatment.

Despite the low mutation rate (2%) in the Ras/MAPK signaling pathway in triple-negative breast cancer (TNBC), copy number variations in specific genes within this pathway have been linked to TNBC [56]. Patients with TNBC who exhibit overexpression of ERK, for example, have a higher mortality rate [57]. The Ras and

ERK-MAPK pathway can be triggered by a range of stimuli, including growth factors, polypeptide hormones, neurotransmitters, chemokines, and phorbol esters [58,59]. In our investigation, umbelliprenin reduced the expression of ERK in both EGF- and $CoCl_2$-induced cells.

Activation of the Ras-ERK and PI3K-AKT pathways can mutually inhibit each other. Inhibiting one pathway can trigger the activation of the other pathway, illustrating their reciprocal inhibition. For instance, MEK inhibitors can enhance the activation of AKT induced by EGF [45]. Robust activation of IGF1 has a similar inhibitory effect on both AKT and Raf, and AKT prevents ERK activation by phosphorylating inhibitory sites in the N-terminus of Raf [45]. The Ras-ERK and PI3K-mTORC1 pathways are frequently activated in cancer development, as they govern essential cellular processes like cell survival, growth, metabolism, and movement. However, this activation can potentially result in resistance to treatments that target only one pathway, due to cross-activation and pathway convergence. In fact, concurrent mutations in KRAS/BRAF and PI3K/PTEN diminish the cytostatic response of cancer cell lines to AKT and mTOR inhibitors [45]. Elevated levels of active ERK, EGFR, and Ras-like transcriptional patterns in basal-like breast tumors suggest that these cancers may halt their growth in response to MEK inhibitors. Nonetheless, the use of MEK inhibitors for treating basal-like cell lines can enhance AKT activity, leading to cytostasis instead of cytotoxicity. In xenograft models, the combination of MEK inhibition with PI3K inhibition has demonstrated the ability to induce tumor regression and in vitro cell death [45]. In our study, umbelliprenin significantly reduced the expression of both AKT and ERK in cells induced by both EGF and $CoCl_2$, which suggests its potential as a therapeutic agent that could simultaneously target these two pathways.

Given the intricate nature of the PI3K/Akt/mTOR system and its intricate interactions with other kinase pathways, an effective therapy for TNBC could potentially involve a medication that exerts multiple inhibitory effects on enzymes or a combination of treatments targeting various intracellular components [6]. Umbelliprenin has shown promising effects on various downstream enzymes within these pathways. However, to comprehensively evaluate the overall effectiveness of umbelliprenin, it is advisable to assess the expression levels of proteins and mRNAs in the EGFR pathway.

In summary, hypoxia plays a pivotal role in stimulating angiogenesis in tumor cells [60]. Our study demonstrated that umbelliprenin displayed antiangiogenic properties by reducing the expression of crucial angiogenesis-related proteins, including VEGF and HIF-1α, in CoCl2-induced hypoxia in the MDA-MB-468 cell line. Therefore, umbelliprenin has the potential to act as a natural compound for inhibiting angiogenesis, proliferation, and metastasis pathways in TNBC MDA-MB-468 cells.

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Author contributions

Roya Atabakhshian contributed in investigation, writing original draft, conceptualization and methodology; Melina Moshirpour was involved in writing, review and editing; Shiva Ghafghazi helped in resources and methodology; Mohammad Hadi Farjoo contributed in writing original draft; Seyed Ali Ziai contributed in conceptualization, methodology, formal analysis, supervision and funding acquisition.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper content.

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Abbreviations

4EBP1: eukaryotic translation initiation factor 4e binding protein 1; AKT: Ak strain transforming (protein kinase B); BRAF: v-raf murine sarcoma viral oncogene homolog B1; CLL: chronic lymphocytic leukemia; EGFR: epidermal growth factor receptor; eIF4E: eukaryotic translation initiation factor 4E; ERK1/2: extracellular signal-regulated kinase ½; FBS: fetal bovine serum; FOXO: Forkhead box protein O1; HER: Human epidermal growth factor receptor; HIF-1α/β:hypoxia-inducible factor-1; HRP: horseradish peroxidase; IC_{50}: inhibitory concentration 50%; KRAS: Kirsten rat sarcoma viral oncogene homolog; LKB1: liver kinase B1; MAPK: mitogen-activated protein kinases; MEK: mitogen-activated protein kinase kinase; mTOR: mammalian target of rapamycin; mTORC: mammalian target of rapamycin complex; PI3K: phosphatidylinositol-3 kinase; PIP2: phosphatidylinositol bisphosphate; PIP3: phosphatidylinositol 3, 4, 5 trisphosphate; PTEN: phosphatase and tensin homolog; RAS: rat sarcoma virus; RTKs: receptor tyrosine kinases; S6K: Ribosomal S6 kinase; TNBC: triple-negative breast cancer; UMB: umbelliprenin; VEGF: vascular endothelial growth factor; VEGFR: vascular endothelial growth factor receptor