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Original article

Umbelliprenin Suppresses Angiogenesis Signaling in SKBR-3 Cell Line by Downregulation of EGF/$CoCl_2$ -Mediated PI3K/AKT/MAPK

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Abstract

Background and objectives: Umbelliprenin, a prenylated coumarin from different species of *Ferula*, has demonstrated anti-cancer effects in various types of cancer cells, but the potential molecular mechanisms for the anti-angiogenic activity of umbelliprenin in breast cancer cells have not yet been studied. In this study, we investigated the possible molecular pathways involved in the anti-angiogenic effect of umbelliprenin in EGF and $CoCl_2$ stimulated SKBR-3 breast cancer cells. **Methods:** Effects of umbelliprenin on the changes in EGFR signaling genes (EGFR, PI3K, AKT, mTOR, S6K, 4EBP1, ERK1/2, HIF-1α, HIF-1β, VEGF, VEGFR) and proteins (VEGF/HIF-1α) expression were assayed in SKBR-3 via Quantitative PCR and Western blotting assays. **Results:** Umbelliprenin dramatically decreased the living cells in a concentration related manner (IC_{50}=103.9 µM) and non- toxic doses of umbelliprenin IC_5 and IC_{10} (10 and 20 µM, respectively) were used for evaluating in vitro anti-angiogenic effects. Umbelliprenin significantly reduced pro-angiogenic AKT, ERK1, ERK2, mTOR, S6K, HIF-1α, HIF-1β, VEGF and VEGFR mRNAs in EGF-treated, and AKT, ERK2, S6K, HIF-1α, HIF-1β, VEGF and VEGFR mRNAs in $CoCl_2$-treated cells. Umbelliprenin significantly increased anti-angiogenic 4EBP1 mRNA in EGF / $CoCl_2$-treated cells. It significantly decreased the levels of HIF-1α and VEGF proteins, in $CoCl_2$-treated cells. **Conclusion:** Our findings showed that umbelliprenin exhibits anti-angiogenic effects by decreasing the expression of AKT/mTOR/MAPK angiogenesis pathways in EGF or $CoCl_2$ treated SKBR-3 breast cancer cells.

Keywords: angiogenesis; breast cancer; $CoCl_2$; EGF; umbelliprenin

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Introduction

Breast cancer is the primary cause of female death worldwide. More than 1.3 million people

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are diagnosed with breast cancer, with nearly 450,000 deaths each year [1]. Nevertheless, improvements in early diagnosis and development to new treatment improved the survival rate to breast cancer patients from 75% to 90% [2]. Angiogenesis suppression is one of the most important therapeutic strategies. Angiogenesis, the cycle of new development of the blood vessel, is a critical stage of cancer progression and eventual invasion and metastases. Many factors cause angiogenesis in tumor cells including growth and environmental factors such as hypoxia [3]. Growth factors, such as the epidermal growth factor (EGF) through its receptor (EGFR), directly stimulate new vascular endothelial development and indirectly alter the expression of angiogenic regulators. [4]. EGF increases the hypoxia-inducible factor 1 alpha (HIF-1α) level in normoxic conditions through activating the mitogen-activated protein kinases (MAPK) and phosphatidylinositol 3-kinases/protein kinase B (PI3K/AkT) signaling [5,6]. Numerous studies show that PI3K/AkT/mTOR (mammalian target of rapamycin) and MAPK pathways plays an important role in the proliferation of breast cancer cells [3].

Depletion of oxygen can restrict or even impair the physiological function of the organs and cells [7]. Tumor cells also suffer hypoxic conditions due to their rapid growth [8]. HIF-1 is one of the pro-angiogenic transcription factors in the HIF family, which plays an important role in oxygen homeostasis in mammalian cells. This heterodimer, which has two separate subunits including HIF-1α and hypoxia-inducible factor 1 beta (HIF-1β) , induces transcription of more than 100 proteins [9] including vascular endothelial growth factor (VEGF) and erythropoietin, which increase the supply of oxygen to cells via erythropoiesis and angiogenesis [9,10]. In fact, HIF-1 is the main oxygen regulator, making cells adaptable to oxygen levels [9,11]. Clinical studies have shown that hypoxic tumor conditions can induce metastases in solid tumors [12] and the prognosis and pathology of breast cancer are directly linked to hypoxia [13]. Therefore, hypoxic factor evaluation may play a key role in cancer treatment [14]. Knowing the various roles of the HIF-1 protein in tumor progression and metastases has inspired the identification and development of these pathway inhibitors.

Many anti-angiogenesis therapies are focused on inhibition of VEGF and its VEGFR receptor, while tumor cells develop other angiogenic factors besides VEGF, rendering angiogenesis a complex process consisting of many signaling pathways. On the other hand, while chemotherapy is an efficient way to destroy tumor cells, it sadly has drawbacks, including problems with the distribution and penetration of drugs and, most importantly, drug resistance [15]. It is therefore important to find new compounds with more effectiveness and less side effects. In the meantime, researchers have proposed discovering herbal compounds with beneficial anti-angiogenic effects due to their abundant sources, fewer side effects and lower prices.

Various species of the genus *Ferula* (Apiaceae) native to Central Asia are used as food or traditional medicines in various countries, including Iran. Several species of *Ferula* which have anti-cancer effects, synthesize terpenyloxy coumarins, including umbelliprenin (Figure 1) [16,17].

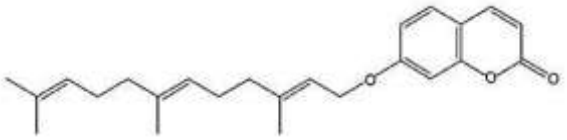

Figure 1. Structures of umbelliprenin

Previous studies have shown that umbelliprenin has anticancer properties [14,16,18-20] inducing apoptosis [21], selective cell toxicity and cell cycle arrest in the G1 phase [14]. It inhibits tumor development, angiogenesis, metastasis, inflammation and potentiates the antitumor immune response in both in vivo and in vitro studies [20,22]. The molecular mechanism of anti-angiogenic effects of umbelliprenin in breast cancer has not yet been well understood, and the purpose of this study was to simulate two models of stimulation in SKBR-3 cancer cells to investigate non-toxic doses of umbelliprenin in the EGFR signaling pathway. Early studies have shown that EGFR and Her-2 / neu expression is high in SKBR-3 cells [23].

Material and Methods

Ethical considerations

The ethical code of this research was issued by the Shahid Beheshti University of Medical Sciences Ethics Committee IR.SBMU.RETEC.REC.1395.848 and the approval date was 21.12.1395 (11 Mar 2017).

Plant material

Umbelliprenin is terpinyloxy coumarin that is synthesized by a large number of *Ferula* species including *F. asa-foetida* and can be extracted from the roots of these plants [14]. In this research umbelliprenin with the molecular formula $C_{24}H_{30}O_3$ and molecular weight 366 g/mol was bought from Department of Pharmacognosy, Mashhad University of Medical Sciences (Mashhad, Iran) Pharmaceutical Research Center (PRC) and used without further purification.

Cells and chemicals

Breast cancer cell line SKBR-3 was purchased from the Iranian Biological Resource Center (IBRC C10147). RPMI1640 medium, FBS (fetal bovine serum), penicillin-streptomycin, and trypsin-EDTA were obtained from Gibco BRL (Gibco®, Life Technologies USA). Recombinant hEGF, $CoCl_2$, DMSO (Dimethyl sulfoxide) and Trypan Blue were purchased from Sigma-Aldrich Merck KGaA (Darmstadt, Germany). Antibodies for HIF-1 alpha (MAB1536; 1:500), VEGF (MAB293R, 1:250) and beta -Actin (MAB8929; 1:50000) were procured from R&D Systems (Minneapolis, USA) and HRP conjugated goat anti-mouse IgG- (sc-2005) and RIPA buffer were obtained from Santa Cruz Biotechnology, USA. The 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) powder and RNase kit were purchased from BioBasic Co. Canada. PVDF membrane was obtained from BioRad Laboratories, Hercules, CA, USA. ECL was provided from Thermo-scientific Co. PrimeScript ® RT reagent kit was purchased from Takara Bio, Inc., Otsu, Japan. RealQ Plus 2x Master Mix Green kit was provided from Amplicon, Denmark.

Cell culture

SKBR-3 cells were cultured in RPMI1640 medium with 10% FBS and 1% penicillin-streptomycin. Culture vessels were maintained at 37 °C in a humidified 95% air and 5% CO_2 atmosphere. Cells were examined for any fungal, bacterial or mycoplasma infections using inverted microscopy.

MTT assay

To investigate the cytotoxicity of umbelliprenin, 5×10^3 cells of SKBR-3 / well were seeded in 96-well plates in a total volume of 200 µL. After 24 h, the medium was replaced with fresh one. Umbelliprenin was dissolved in DMSO immediately before use and was added to the cells, with the final concentrations of 4, 8, 17, 33, 67, 133, 267 and 533 µM in each well. It should be noted that these concentrations have been determined based on previous studies [16]. The DMSO concentration in the cell culture medium was % 0.25 and it is known as non-toxic [15,16]. Each umbelliprenin concentration was applied in triplicate and incubated for 24 h. The control group was DMSO %0.25. Twenty µL of MTT solution (5 mg/mL) was added to each well and incubated for 4 h (final MTT concentration was 0.5 mg/mL). Then the medium was replaced by 100 µL DMSO to dissolve the formazan crystals formed by alive cells. The well absorbance was determined at 550 nm with a reference wavelength at 630 nm. IC_{50} was defined as umbelliprenin concentration that decreased the alive cells to 50 percentage. IC_{10} and IC_5 of the UMB were calculated and used for further investigations.

$CoCl_2$ stock solution (24 µM) was prepared in sterile deionized water. The stock solution was sterilized by 0.22 µm filter and kept at 4 °C. Then 10^4 SKBR-3 cells / well were seeded in triplicate and were treated with serial dilutions of $CoCl_2$ (50, 100, 150, 200, 250 and 600 µM) for 24 h.

Study design

The study groups included SKBR-3 cells as control group; IC_5 and IC_{10} (10 and 20 µM, respectively) umbelliprenin treated cells; EGF and $CoCl_2$-stimulated cells; and EGF and $CoCl_2$-stimulated cells treated with IC_5 and IC_{10} umbelliprenin for 24 h.

For activation of the EGFR signaling pathways, the cells were serum-starved overnight, then treated with EGF (20 ng / mL) for 30 min. Hypoxic conditions were induced by exposure of the normoxic cultures of the cells to $CoCl_2$, which inhibits the enzyme prolyl-4-hydroxylase (PHD) and causes stabilization of the protein HIF-1α [24]. Based on the MTT assay, 50 µM of $CoCl_2$ was selected for hypoxia induction.

Quantitative PCR analysis

The expression of HIF-1α, HIF-1β, EGFR, VEGF, VEGFR, PI3K, AKT, mTOR, S6K (Phospho-p70 S6 Kinase), ERK1/2 (extracellular signal-regulated kinases 1/2), 4EBP1 (Eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1) genes were assessed in the treated and

control cells using quantitative PCR. Total RNA was harvested using an RNase kit. The cDNA was constructed from this RNA using the PrimeScript ® RT reagent kit. Equal amounts of RNA were used for cDNA synthesis. qPCR was performed by using the RealQ Plus 2x Master Mix Green kit. Primer sequences for β-actin and other genes are presented in Table 1. The PCR conditions were as follows: 95°C for 10 min then 40 cycles of 95°C for 15sec and 60°C for 60 s by Applied Biosystem /step one plus instrument. The experiment was performed in duplicate on independent samples. Negative control was used to rule out any contamination. Gene expression results were obtained using the $2^{-\Delta\Delta Ct}$ method, and using β-actin as the housekeeping gene. REST and Linreg freeware were used for assessment of expression and the primer efficiencies, respectively.

SDS-PAGE and Western blot analysis

HIF-1α (120 KDa) and VEGF (25 KDa) protein expression were assessed by Western blotting. SKBR-3 cells were harvested with cold PBS and centrifuged at 1000 rpm at 4 °C for 5 min with 200 µL RIPA buffer, 1% v / v protease inhibitor cocktails and 1% v / v phosphatase inhibitor cocktails, the cell pellet was incubated on ice for 30 min. The lysate was sonicated and centrifuged at 13000 rpm for 15 min at 4 °C. The total concentration of proteins was quantified using Bradford method. Proteins (30 µg) were separated on SDS polyacrylamide gel (10% Tris-base) by the BioRad Criterion System and then transferred to a PVDF membrane. At room temperature, the membrane was blocked for 75 min with 2% skim milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) and then incubated overnight at 4 °C with the primary antibody after TBST wash. After 1 h, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies that were detected by chemiluminescent reaction with ECL and visualized on x-ray film. Each sample was triplicate-assayed and the relative expressions of HIF-1α and VEGF proteins were quantified using ImageJ software (NIH) relative to β-actin using a densitometry test.

Molecular docking

In order to investigate possible molecular mechanisms of umbelliprenin antiangiogenic activity, docking studies were performed on PI3K (PDB code: 5QO4), AKT (PDB code: 3QKL), mTOR (PDB code: 4JT5), S6K (PDB code: 4RLO) ERK1/2 (PDB code: 5nhj), and 4EBP1 (PDB code: 1EJ4) using Autodock vina software. In all cases, the centers of the grid boxes were the same as the center of the external ligand, and the sizes of grid boxes were set to 20*20*20. The poses with the highest affinity to the receptor were selected as the result of the docking calculations for each receptor. All images were provided using Discovery Studio Visualizer, BIOVIA, 2016.

Statistical analysis

MTT assay presentation and their IC_{50}, IC_{10}, and IC_5 calculations were rendered by Graph Pad Prism version 6.0.1, and experiments were performed in triplicates. Changes in mRNA expression and their statistical comparisons were conducted by the Relative Expression Software Tool V2.0.13 (REST 2009).

Table 1. Forward and reverse sequences of primers used in this study

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

All qPCR experiments were performed in duplicate and the results were expressed as mean ± SEM from two independent experiments. Comparisons between protein expression groups were analyzed by T-test or Mann-Whitney U test. In this study, $p \leq 0.05$ was considered statistically significant.

Results and Discussion

Mammalian cancer SKBR-3 cells were treated for 24 h with rising concentrations of UMB (4-533 µM). UMB significantly reduced cell viability in a concentration -dependent manner (Figure 2A) with the IC_{50} value of 103.9 ± 27.43 µM. Experiments were carried out using non-toxic umbelliprenin concentrations of 10 and 20 µM as IC_5 and IC_{10}, respectively. In fact, these low concentrations allow us to study the effects of the umbelliprenin on changes in the expression of the desired genes and proteins in different treatments without reducing the number of living cells.

The viability of SKBR-3 cells treated with different concentrations of $CoCl_2$ ranging from 50 to 600 µM for 24 h was assessed using the MTT assay. Results showed that IC_{50} for $CoCl_2$ was 117.5 ± 47 µM (Figure 2B) and the non-toxic $CoCl_2$ concentration of 50 µM was used for the rest of experiments in this investigation.

Umbelliprenin cytotoxicity has been evaluated and reported in different cancer cell lines. We have shown that treatment with umbelliprenin in SKBR-3 cells has led to a significant reduction in cell viability in a concentration-dependent manner. Previous umbelliprenin studies in breast cancer cells showed moderate cytotoxic activity [14,25] while Rashidi et al showed that umbelliprenin was toxic to HT29, MCF-7, CT26, A172, 4T1, and GL26 cancer cells, and their findings demonstrated inhibitory effects on cell growth in a concentration-dependent manner [16]. The most potent inhibitory activity of umbelliprenin with an IC_{50} value of 9.1 nM was documented in Raji cells [26,27]. Umbelliprenin reported moderate cytotoxicity to QU-DB (large cell lung cancer cell line) and anti-proliferative activity to A549 (adenocarcinoma cells) with 47 and 52 µM IC_{50} values, respectively [15]. The highest inhibitory effect of umbelliprenin by cell cycle arrest in the G1 process against the M4Beu melanoma cell line (IC_{50} = 12.4 ± 0.5 µM) compared to cisplatin (23.1 ± 0.8 µM) was demonstrated. Umbelliprenin has cytotoxic effects by mitochondrial-dependent pathways in the human lung cancer cell line (A549) [14,27]. Due to the hydrophobicity of UMB, delivery systems such as nanoparticles may increase their cytotoxicity [28,29].

It is estimated that more than 90% of cancer deaths are due to angiogenesis, invasion and metastases of cancer cells in vital organs [5,30]. Angiogenesis is the major cause of metastases through the HIF-1α/VEGF signaling [5]. Unlike non-genetically stable tumor cells that are rapidly resistant to drug components, the vascular endothelium is not mutated and therefore not resistant to treatment. Therefore anti-angiogenesis therapy with relatively low drug resistance and side effects is targeted-selective strategy [13]. Overexpression of HIF-1α is seen in more than 70 percent of human solid tumors, such as breast cancer [31]. HIF-1α is necessary for cell migration, invasion and metastasis in breast cancer [32].

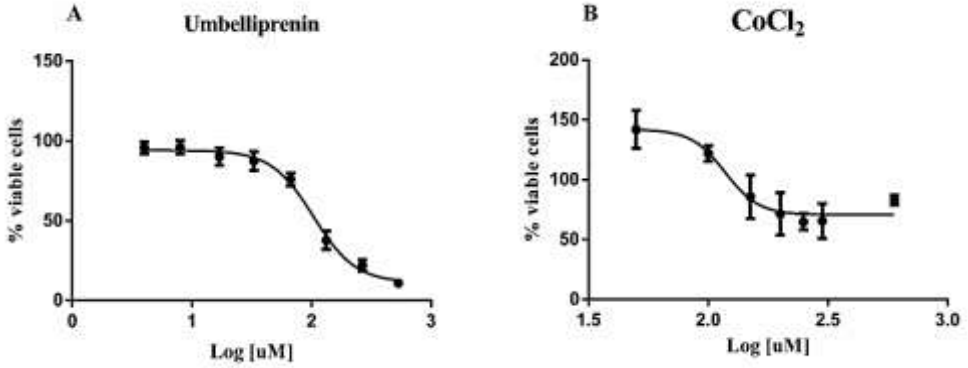

Figure 2. Viability results of SKBR-3 cells incubated with different concentrations of umbelliprenin (A) and $CoCl_2$ (B). Each point represents mean ± SD of triplicate assay.

High levels of HIF-1α in breast cancer can predict early recurrence and disease metastases and are also associated with lower clinical improvements [33]. HIF-1α and VEGF are the cardinal objectives for the prevention and treatment of breast cancer. Several types of angiogenesis inhibitors are currently used to treat cancer, and these drugs increase the efficacy of chemotherapy by targeting angiogenesis [34]. Due to significant drug resistance to chemotherapy as well as the high toxicity of chemical compounds to non-cancer cells, it is important to identify novel inhibitors with lower toxicity and fewer side effects from natural and renewable sources such as plants [20]. A large number of studies have shown the anti-cancer characteristics of herbal compounds in various cancers, among them umbelliprenin is a plant derivative that is synthesized by different plant species such as *Angelica archangelica, Coriandrum sativum* and *Citrus limon* [14,17]. It has been shown that umbelliprenin has various pharmacological [20] and anti-cancer properties [15,16]. The purpose of this study was therefore to assess the anti-angiogenic effects of non-toxic doses of umbelliprenin in SKBR-3 breast cancer cells.

The effects of EGF (in order to activate the angiogenesis signaling pathway) and $CoCl_2$ (in order to establish hypoxic conditions) on the expression levels of EGFR, PI3K, AKT, mTOR, S6K, 4EBP1, ERK1/2, HIF-1α, HIF-1β, VEGF, VEGFR mRNAs (Figure 3) and HIF-1α and VEGF proteins (Figure 4) were evaluated. Our findings showed that EGFR, mTOR, S6K, HIF-1α, ERK1, and VEGF mRNA expression increased significantly in the EGF-treated SKBR-3 cells compared to the control group. So EGF stimulation increased the expression of the HIF-1α and VEGF genes while having no effect on their proteins (Figures 3, &4). EGF stimulates HIF-1α transcription through EGFR pathway stimulation in normoxia [31]. EGFR was detected in 15-45% of breast cancers [35] and as mentioned is highly expresses in SKBR-3 cells [23]. An increase in the HIF-1α protein and its transcriptional activity following EGFR stimulation in different cell lines depends on the function of the receptor tyrosine kinases and the downstream activation of the PI3K/AKT/ mTOR pathway [4]. Signaling pathways including PI3K/AKT and MAPK regulate the synthesis of HIF-1α [31]. Based on this hypothesis, we investigated the stimulation effect of the EGF on all the genes studied in the PI3K/AKT/mTOR/ERK pathway.

The EGF-treated cells were then treated with IC_5 (10 μM) and IC_{10} (20 μM) concentrations of umbelliprenin. With the exception of EGFR, and PI3K, the expression of all mentioned mRNAs decreased significantly in both IC_5 and IC_{10} groups treated with EGF, and increased significantly in 4EBP1. Significant inhibitory effects of umbelliprenin on the expression of HIF-1α and VEGF genes in EGF-stimulated SKBR-3 cells have been achieved and the inhibitory effects of umbelliprenin on angiogenesis in EGF-stimulated SKBR-3 cells have been confirmed (Figure 3). In line with our results, it has been shown that umbelliprenin prevented angiogenesis by decreasing VEGF expression in 4T1 cells [20] To the best of our knowledge, this is the first research of the effect of umbelliprenin on these pathways.

After treatment of SKBR-3 cells with $CoCl_2$, the expression of HIF-1α, VEGF, and VEGFR mRNAs increased significantly, while the expression of other mRNAs decreased compared to the control group. The $CoCl_2$-treated cells were then treated with IC_5 and IC_{10} concentrations of umbelliprenin. In the presence of both umbelliprenin concentrations, the expression of AKT, S6K, HIF-1α, HIF-1β, VEGF and VEGFR mRNAs decreased significantly, whereas the expression of 4EBP1 and ERK2 mRNAs significantly increased compared to $CoCl_2$-treated cells. In the $CoCl_2$-stimulated cells, umbelliprenin significantly reduced the HIF-1α and VEGF proteins concentration-dependently (Figure 4). Actually in this study, umbelliprenin in the $CoCl_2$-treated cells decreased pro-angiogenic HIF-1α, HIF-1β, S6K, VEGF and VEGFR mRNAs, increased the anti-angiogenesis 4EBP1 mRNAs, and inhibited protein expression of HIF-1α and VEGF significantly. About 25-40% of invasive breast cancers show hypoxic microenvironments [36]. In mammalian cells, $CoCl_2$ is successfully used as an inducer of biochemical and molecular responses similar to hypoxic conditions; hence, it is commonly used in in vitro studies as a hypoxia mimicking agent [37,38]. Hypoxia induces wide range of responses in cells and tissues. One of the important reactions to hypoxia is the expression of the transcription factor HIF-1α and its target genes, such as VEGF which are major factors in cell tolerance to hypoxic conditions [39].

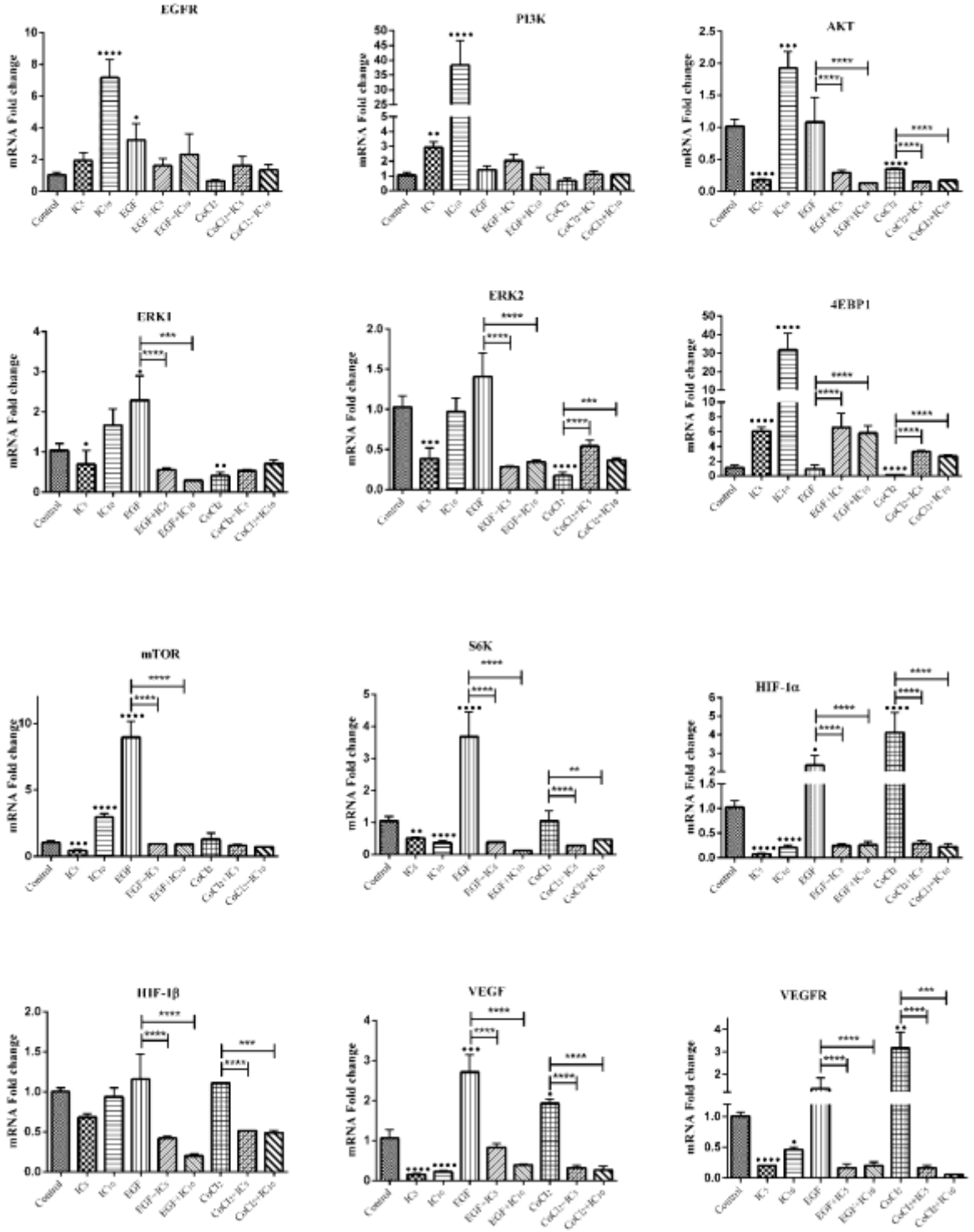

Figure 3. EGF (20 ng) and $CoCl_2$ (100 µM)-stimulated SKBR-3 cells treated with IC_5 (10 µM) and IC_{10} (20 µM) umbelliprenin concentrations. The mRNA levels were determined by real-time PCR. Each bar represents the mean ± SEM of the three independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ compared to EGF or $CoCl_2$ groups. ● $p < 0.05$; ●● $p < 0.01$; ●●● $p < 0.001$; ●●●● $p < 0.0001$ compared to control group

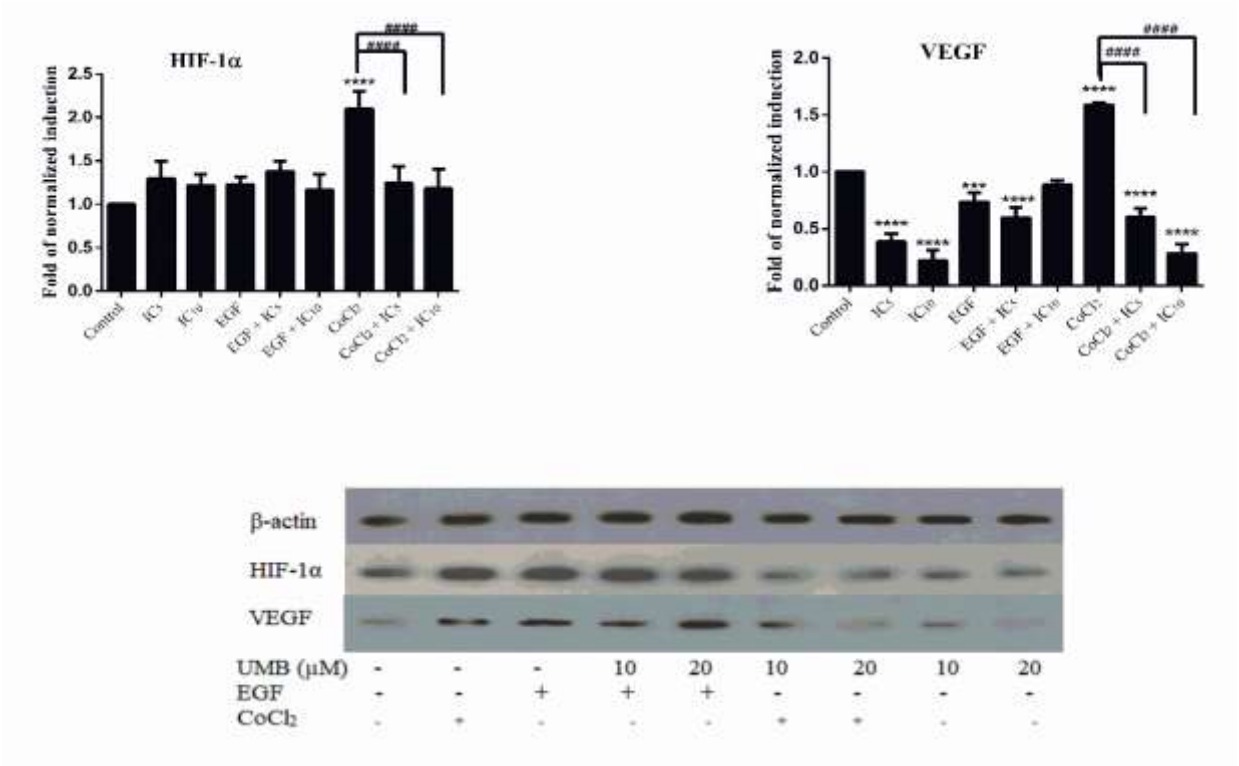

Figure 4. The levels of HIF-1α and VEGF proteins were determined by western blot analysis. Each bar represents the mean ± SD of three separate experiments quantified by the ImageJ software. There's a photograph of the blots in the bottom. *** $p < 0.001$; **** $p < 0.0001$ compared to the control, #### $p < 0.0001$ compared to $CoCl_2$ group.

Increasing the levels of HIF-1α as a response to hypoxia or growth factor is different in two critical aspects; First, hypoxia increases the expression of HIF-1α in all cell types, whereas growth factors increase cell-specific HIF-1α expression; Second, hypoxia is associated with decreased denaturation of HIF-1α while growth factors promote synthesis of HIF-1α through activation of the PI3K and MAPK pathways [40]. HIF-1α has a short half-life and its lifetime depends on elimination more than synthesis. This may explain why EGF treatment did not increase the amount of HIF-1α in our study. In normoxic status, poly-ubiquitinylation of HIF-1α is catalyzed by oxygen-dependent prolyl hydroxylases through the E3 ligase complex of the von Hippel-Lindau protein (VHL) and consequent degradation of HIF-1α. Under hypoxia condition, however, HIF-1α is not degraded owing to a block in prolyl hydroxylation. Stabilized HIF-1α forms an active complex in the nucleus with another subunit of HIF called HIF-1β and stimulates the transcription of target genes such as VEGF after binding to hypoxia-response elements (HREs) in the promoter area [31,41] As predicted in this study, $CoCl_2$-stimulated hypoxia increased both transcriptional and translational levels of the HIF-1α and VEGF significantly. These results are similar to another study suggesting that hypoxia in addition to stabilizing HIF-1α can increase the expression of its mRNA as well [30].

VEGF transcription in hypoxic conditions and in the presence of growth factors is primarily controlled by HIF-1α [31,32]. VEGF, a ligand for VEGFR1 and R2, is the most active angiogenic factor and plays a major role in angiogenesis caused by hypoxia [30]. VEGF binding to VEGFR activates various signaling networks that lead to survival, vascular permeability, migration, proliferation, and mobilization in endothelial cells [30]. For further research, it is proposed that EGFR pathway kinase inhibitors should be used and that the phosphorylate form of the proteins studied should also be determined to establish the exact umbelliprenin target in this pathway.

In cells treated with concentrations of 10 and 20 umbelliprenin, the expressions of PI3K and 4EBP1 mRNA increased significantly, while the expressions of S6K, HIF-1α, VEGF and VEGFR

mRNA decreased significantly compared to the control group. Although IC_5 significantly decreased AKT, ERK1, ERK2, mTOR, IC_{10} significantly increased AKT, ERK1, ERK2, mTOR, plus EGFR. Also UMB reduced VEGF protein expression concentration-dependently. We observed that umbelliprenin inhibited all key factors involved in angiogenesis in the AKT/mTOR /MAPK signaling pathways except for PI3K. Expression of 4EBP1, a negative controller for HIF-1α formation, increased four times significantly.

Table 2 indicates the docking score of the pose with the highest affinity.

Table 2. Scores of the umbelliprenin docking poses in the proposed target proteins

Target receptor	PDB code	Affinity (kcal/mol)
PI3K	5QO4	-8.7
AKT	3QKL	-9.0
mTOR	4JT5	-8.7
S6K	4RLO	-7.7
4EBP1	1EJ4	-8.0
ERK1/2	5nhj	-9.5

To inspect the alignment of docked molecules with crystallographic external ligands, for each PDB file, the pose with the highest affinity, the structures of protein and the external ligand were combined and demonstrated in Figure 5A. Also, 2D interaction maps of docked umbelliprenin are shown in Figure 5B. The results from docking studies show that umbelliprenin might have affinity to PI3K, AKT, mTOR, S6K, 4EBP1, and ERK1/2 and interact with these receptors which can be due to presence of various rotatable bonds and the similarities between the inhibitors or modulators of these receptors. This also makes umbelliprenin a lead compound for further research. Rigidifying rotatable bonds in different stereochemistry considering the poses resulted from docking and also addition of required functional groups may result in new drugs with higher potency and selectivity. Umbelliprenin significantly inhibited VEGF protein levels in non-stimulated cells concentration-dependently, while the same impact was not observed in EGF-stimulated cells.

Conclusion

The effects of umbelliprenin tend to strengthen this compound as an anti-cancer agent with mechanisms, such as inhibition of angiogenesis, by decreasing the yield of HIF-1α and VEGF and thus angiogenesis through AKT/mTO /MAPK pathways in SKBR-3 cells.

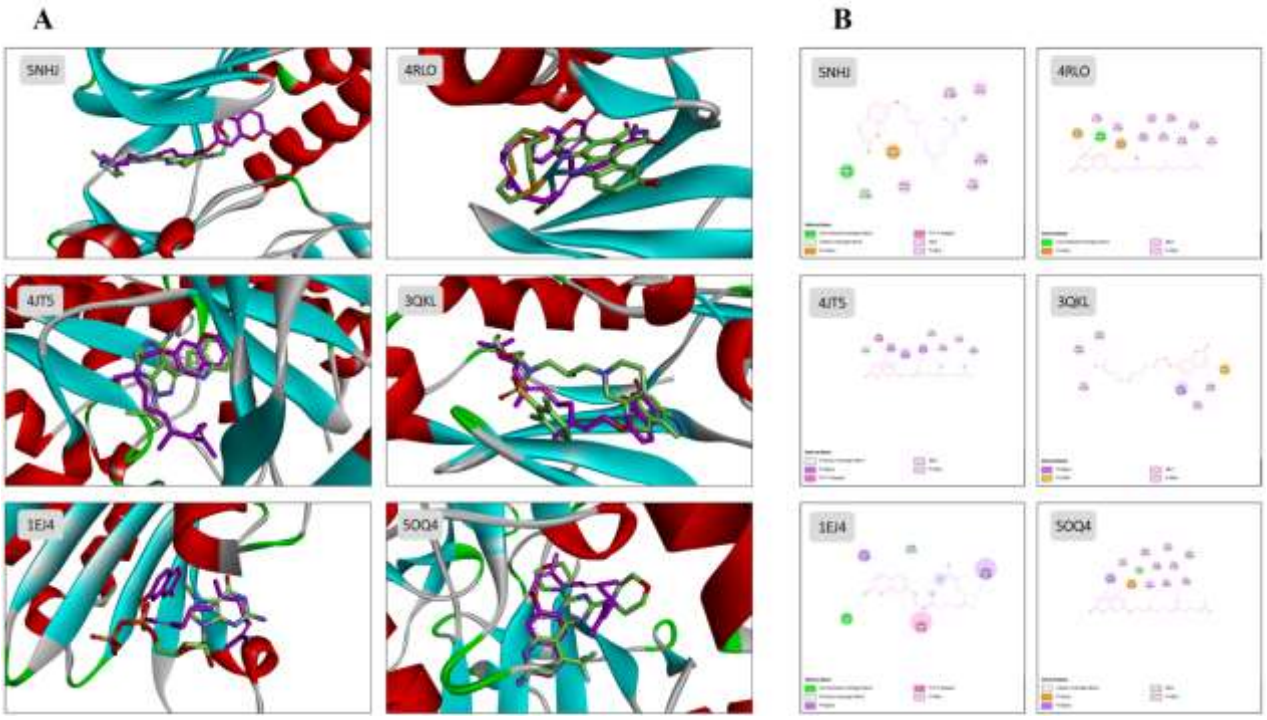

Figure 5. The crystallographic structures of protein and the external ligand, together with the pose with the highest affinity, resulted from docking. The molecules with green carbon atoms are external ligand, and the purple carbon atoms belong to umbelliprenin (A) 2D map of the interactions between umbelliprenin and receptors(B)

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Author contributions
Roya Atabakhshian and Seyed Ali Ziai were involved in the practical and technical phase of the study; Roya Atabakhshian, Somayeh Mahmoodi Khatonabadi and Shiva Ghafghazi performed the acquisition of data; Roya Atabakhshian, Siamak Salami, Reza Mirfakhraie, Majid Sirati-Sabet, Bahram Gholamali Yaghmaei, Mitrasadat Rezaei, and Seyed Ali Ziai were involved in analysis and interpretation of data; Roya Atabakhshian prepared the draft of the manuscript; Siamak Salami, Reza Mirfakhraie, Majid Sirati-Sabet, Bahram Gholamali Yaghmaei, Mitrasadat Rezaei, and Seyed Ali Ziai were involved in drafting the manuscript and revising it critically for important logical content; Amirreza Dowlati Beirami took part in in-silico analysis and interpretation of data; Seyed Ali Ziai provided the conception and design of study. The authors have agreed to be responsible for all aspects of the work and ensure that problems relating to the quality or credibility of any section of the work are adequately investigated and resolved.

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

EGF: epidermal growth factor; $CoCl_2$: cobalt chloride; HIF -1α: hypoxia-inducible factor 1 alpha; VEGF: vascular endothelial growth factor; MAPK: mitogen-activated protein kinases; PI3K: phosphatidylinositol 3-kinases; AkT: protein kinase B; mTOR: mammalian target of rapamycin; HIF-1β: hypoxia-inducible factor 1 beta; FBS: fetal bovine serum; DMSO: dimethyl sulfoxide; PHD: prolyl-4-hydroxylase; S6K: phospho-p70 S6 kinase; ERK1/2: extracellular signal-regulated kinases ½ ; 4EBP1: eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1