Anti-Cancer Study of Carvacrol in Hypoxic-Induced Colorectal Cancer Cell

Aumaima Tariq Abed [1,2*], Ahmed MH AlMudhafar [1], and Najah R Hadi [1]

Abstract

Introduction: Colorectal cancer presents a complex global health challenge, characterized by complications arising from metastasis development due to rapid proliferation, adaptation to hypoxia, and angiogenesis. Carvacrol, a natural monoterpenoid phenol derived from various plants, has interest for its diverse pharmacological properties, particularly its antitumor effects. Methods: We aimed to assess the inhibitory effect of carvacrol on cell migration and proliferation in the hypoxic colorectal cancer cell line (SW480). Chemical induction of hypoxia using cobalt chloride and serially diluted concentrations of carvacrol (400, 200, 100, 50, 25, and 12.5 µg/ml) were employed to evaluate the cytotoxic effect via the MTT assay. Smaller concentrations (low IC50) were used to examine the impact of carvacrol on SW480 cell migration through a cell migration assay (50, 25, 12.5, and 6.25 µg/ml). Results: The results demonstrated a significant, dose-dependent reduction in both cell proliferation and migration with carvacrol doses. Conclusion: The findings suggest that carvacrol could be useful as an adjuvant therapy and a promising addition for patients with highly metastatic colorectal cancer.

Significance | Effect of carvacrol on proliferation and migration of hypoxic SW480 cancer cells.

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Introduction

Colorectal (CR) cancer stands as a prominent global health concern (American Cancer Society). It ranks third in cancer-related deaths worldwide (Siegel et al., 2019), with about 50% to 60% of CR cancer patients developing distant metastasis, leading to a 50% mortality rate from CR cancer relapse (Islam et al., 2017). Prognosticating metastatic CR cancer poses a challenge, but recent years have seen significant advances in treatment, including therapies targeting angiogenesis (Hurwitz et al., 2004). Numerous studies link tumor-related factors and physiological changes to traumatic environments affecting primary tumors, such as hypoxia, oxidative stress, and acidosis (Jin et al., 2020, Taddei et al., 2013). Hypoxia, characterized by lower oxygen levels that support cell survival and malignant tumor propagation, induces changes in cancer cell metabolism. This results in therapy resistance, enhanced vascularization, and epithelial-to-mesenchymal transition (Muz et al., 2015). The presence of hypoxia activates hypoxia-inducible transcription factors HIF-1α and HIF-2α, leading to upregulation of VEGF (vascular endothelial growth factor) and other HIF targets (Shweiki et al., 1995, Maxwell et al., 1997).

The interaction between VEGF and its receptors is vital in stimulating uncontrolled angiogenesis (Ni et al., 2012). VEGF receptor activation also promotes the expression of matrix metalloproteinases (MMPs), degrading the extracellular matrix (ECM) and facilitating cell invasion into adjacent tissues. Notably, VEGF is the primary downstream mediator of HIF-1α, influencing cell migration, proliferation, and tube formation. HIF-1α

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induces epithelial-to-mesenchymal transition (EMT) in various malignant tissues, correlated with E-cadherin, N-cadherin, and vimentin expression (Lai et al., 2016). The HIF-1α/VEGF signaling pathway emerges as a potential target for treating angiogenesis-related diseases, including metastatic cancer.

Additionally, vascular endothelial growth factor (VEGF) serves as the primary downstream mediator of hypoxia-inducible factor 1-alpha (HIF-1α), it has fundamental effects in the stimulation of cell migration, proliferation, and tube formation. Although antiangiogenic drugs have been successful, they still struggle with issues like therapy resistance and side effects (e.g., high blood pressure from bevacizumab therapy). This has led researchers to focus on nutraceuticals as a significant source of compounds for treating and preventing diseases related to dysfunctional vessels (Morbidelli et al., 2018).

Carvacrol, a natural monoterpenoid phenol extracted from oregano species, exhibits diverse activities, including antimicrobial, analgesic, antioxidant, anti-inflammatory, and anti-tumor effects (Bnyan et al., 2014, Aligiannis et al., 2001, ZE Suntres et al., 2015, Can Baser et al., 2008, Abid et al., 2014). Carvacrol is recognized as Generally Recognized As Safe (GRAS) by the FDA and the Council of Europe, commonly used in foods and cosmetics (M. De Vincenzi et al., 2004). Recent interest in its antitumor effect led researchers to discover its cytotoxic and anti-cancer properties. In our study, we aimed to unveil the inhibitory effect of carvacrol on migration and proliferation in the hypoxic CR cancer cell line (SW480), addressing a gap in understanding its impact under stressful cancer mass increment conditions.

Materials and methods:

Chemicals

Natural carvacrol (99%) and cobalt (II) chloride hexahydrate were bought from Sigma-Aldrich. MTT was bought from Solarbio (Beijing, China). Other compounds were credited from local markets and tested before use.

Cell culture

Human cancer cell lines SW-480 (colon) cells were collected from ATCC USA. The cell culture media was prepared with RPMI 1640 (Sigma, India) supplemented with DMEM, 5% serum of bovine, 1 mg/ml penicillin and 200 µg/mL streptomycin. The cells were grown upto 80% for seeding in 96-well plates (100 µL cells/well). The plates were incubated (0.25–250 µg/mL) for 48 h at 37 ºC and 5% CO2. The old media was discarded before seeding from the cell culture flask and the freshly prepared media after washing by sterile phosphate-buffered saline (PBS) (pH 7.4), 2-3 times. Trypsin was added, followed by discarding PBS and being distributed evenly onto cell surfaces. Cells were incubated at 37 ºC in 5% CO2 for 2-5 min. The flasks were gently tapped and observed under an inverted microscope. 5mL of fresh media (10% FBS) was added to neutralize trypsin activity to detach the cells properly,. Cells were counted and seeded 10,000 cells per well. The plate was incubated for 24 hr to be ready to treat cells. Six low to high doses were used in the treatment. After 48 hr of treatment, MTT reagents were added and incubated for 4 hr. 20 µl MTT lysis solution was added to each well and read the plate at 570 and 620 nm wavelengths using a high-end TecanM200 Pro multimode microplate reader. The data were collected and analyzed to determine the compound concentration that inhibited cell growth by 50% (IC50) from the optical density. 5-FU and Tamoxifen were used as the standard drug. The results are calculated from two independent experiments, each done in triplicate.

Hypoxia induction using cobalt chloride

A stock solution of carvacrol was prepared and kept in the refrigerator until usage. At the same time, cobalt chloride stock solution was prepared immediately before use. In vitro, hypoxia was induced by chemicals. Cobalt chloride ($CoCl_2$) is shown as a hypoxia inducer in vitro, as it induces the expression of HIF-1α in cancer cell lines (Dai et al., 2012, Piret et al., 2002). Different concentrations of $CoCl_2$ were tested for the cell line we had worked to create a dose-dependent examination at different incubation times to minimize $CoCl_2$ toxicity and determine the suitable concentration used in the assay (Danli and Yotnda, 2011, Li et al., 2019). The optimal concentration of $CoCl_2$ for hypoxia induction in our assay was 50µg per ml of RPMI1640 culture media. $CoCl_2$-containing media was used for preparing carvacrol dilutions (400, 200, 100, 50, 25, 12.5 ml).

Cytotoxicity assay

Cytotoxic or stimulatory effect of chemicals on cell viability in vitro was conducted by MTT. 200 µl of 1×10^5 cells/ml were implanted in flat bottom plate (96 well), incubated at 37ºC. When the cells adhered to the walls, confluence reached 70%-80%, wells were ready for treatment. $CoCl_2$-containing media was used for the chemical hypoxia induction. A serial dilution of carvacrol was prepared with 50 µg/ml $CoCl_2$ as previously described (Di Mattia et al., 2022), and the media was not changed in the exposure time. Set of eight quadruplicates were used for the assays, the first quadruplicates were treated with RPMI-1640 media only used as normoxic group, while the second quadruplicates treated 50 µg/ml $CoCl_2$ in RPMI-1640 media (hypoxic control group), the rest A quadruplicates were treated with the previously specified concentrations of carvacrol (400, 200, 100, 50, 25, 12.5 µg/ml) to be incubated for 48 hours at 37ºC. Medium was removed after the specified time (48 hr.), and wells were washed with 100 µL PBS, 100 µL of MTT (1 mg/mL) was added per well. Then the plate was incubated for 4 hr., the solution was removed, and 100µL of Formazan Dissolving Solution was added for each well. Slowly stir in a gyratory shaker for 10 min, making the crystal is completely

solubilized., Using a microplate reader (at 490 nm). The % inhibition was plotted against the concentration tested using Microsoft Excel, and the IC50 was calculated using the non-linear regression equation. According to the earlier study of Khan et al., 2016, percentage proliferation was determined.

$$\% \text{ inhibition} = 1 - \frac{\text{absorbance of treated}}{\text{absorbance of untreated}} \times 100$$

Cell migration assay

The cell migration assay was conducted with the SW-480 colon cancer cell line. 20,000 cell were seeded per well in 24 well plates with the confluence cells. The cells were allowed to be 95% confluent monolayer, so the wound was created at the middle of the well from corner to corner by sketching a straight line using 200 µl micropipette tips with a sterile ruler. PBS was used to wash the cells as 2 times. The inhibitory effect of carvacrol on hypoxic CR cancer cells migration can be evaluated in vitro by wound-healing assay (Si L Yan et al., 2018). Cells in 24 wells were treated with $CoCl_2$-containing media or as a combination carvacrol + CoCl2-containing media (50, 25, 12.5, 6.25 µg/ml $CoCl_2$-containing media). The cells tended to migrate after that. The width of wounds was measured each 12 hr., starting from zero time to 48 hr., and images were captured for the migrated cells from the scratch edges by an inverted microscope camera. The experiment was worked in duplicate and images for each well were captured from three different scratch zones. The concentrations used for carvacrol were (50, 25, 12.5, 6.25 µg/ml $CoCl_2$-containing media). Five to six pictures were captured by inverted microscope for the wound of each well at 0 hr, 6 hr, 12 hr, 18 hr and 24 hr. Image-J software version-1 was used to measure the width of the wound. Three readings were recorded for each well. The data were collected, and the following equation was used to determine the percentage of migration inhibition:

$$\% \textit{ wound closure} = 1 - \frac{\text{width at the indicated times (h)}}{\text{width at zero time}} \times 100$$

Statistical analyses

GraphPad Prism 7.0. was used for statistical analysis. Data for the same group were denoted as the mean ± standard deviation. Unpaired Students' t-tests evaluated tests for two different groups. While one-way analysis of variance was used for more than two groups. Images were analyzed by Image J software analysis. A value of $P < 0.05$ was considered as significant statistically.

Results

Carvacrol's Inhibitory Effects on Viability in Hypoxic SW480 Colorectal Cancer Cells

In our research on colorectal cancer, we focus on the SW480 cell line to understand how carvacrol, a natural compound with various medicinal properties, affects the growth and movement of cancer cells under stressful conditions. The inhibitory effects of carvacrol on the viability of hypoxic SW480 cells ($CoCl_2$ used for induction HIF1α in SW480 CR cancer cell line as 50 µg/ml RPMI1640 media) assessed by MTT assay as shown in Figure 1 (a), higher doses of carvacrol significantly decreased cells viability in a concentration-dependent mode after 48 hr incubation. IC_{50} values of carvacrol after 48 hr of incubation were ≈324 µg/ml (figure 1 (b)).

Hypoxic CR cancer cells possess higher metastatic capacity than normoxic CR cancer cells

Our study looks at the impact of carvacrol on the proliferation and migration of hypoxic SW480 cancer cells, a common occurrence in colorectal cancer. Hypoxia, characterized by low oxygen levels, adds complexity to cancer progression, contributing to therapy resistance and other challenges. We induce hypoxia in SW480 cells to simulate rapid cancer growth using cobalt chloride. We then introduce carvacrol in varying concentrations to assess its cytotoxic effects and influence on cell migration through specific assays. Cancer cells cannot metastasize unless they have the ability of migrate. Figure (2) represented that the hypoxic control group showed high migration rate, the scratched wound was entirely healed within 36 hr., while approximately same results were seen after 48 hr. for normoxic cells (untreated neither with $CoCl_2$ nor carvacrol). Hypoxic cells were more aggressive than normoxic cells. The migration ability of SW 480 cells was decreased by carvacrol depending on the dose. A significant effect was seen for cells treated with 50 µg/ml carvacrol compared with hypoxic control group, as shown in figure (3). Our results show a promising trend, with carvacrol reducing both the proliferation and migration of hypoxic SW480 cancer cells in a dose-dependent manner. These findings suggest that carvacrol could be a potential adjuvant therapy for colorectal cancer, especially in cases with high metastatic tendencies.

Discussion

In the present study, CoCl2-induced hypoxia in SW-480 cancer cell lines might increase the cell line's invasive ability. We have studied the carvacrol to determine the inhibitory potential of proliferation and migration in different doses. Carvacrol weakened the cell migration abilities in a hypoxic environment. These results contrast previously reported findings of increased invasion of cancer cells(Munoz-Najar et al., 2006, Canning et al., 2001). When cancer grows rapidly, especially in solid tumors, it creates a lack of oxygen and nutrients due to the inability of local blood vessels to supply enough (Bhandari et al., 2019). Research has shown that this lack, known as hypoxia, strongly promotes tumor progression by encouraging tumor growth and spread (Casillas et al., 2018, Yang et al., 2017). Metastasis, the process of

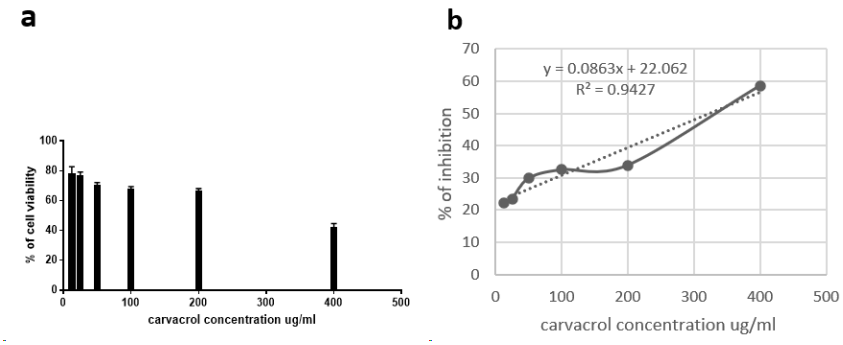

Figure 1. cytotoxic effects of carvacrol on hypoxic SW480 cells. (a) %of cell viability and (b) % of inhibition of hypoxic SW480 cells cultured with the specified concentrations of carvacrol (12.5,25,50,100,200, and 400 µg/ml) for 48 hr. Cell viability was measured by MTT assay. The represented values are the means ± SD from quadruplicates. *p < 0.05 indicates a significant variance comparing with the control group.

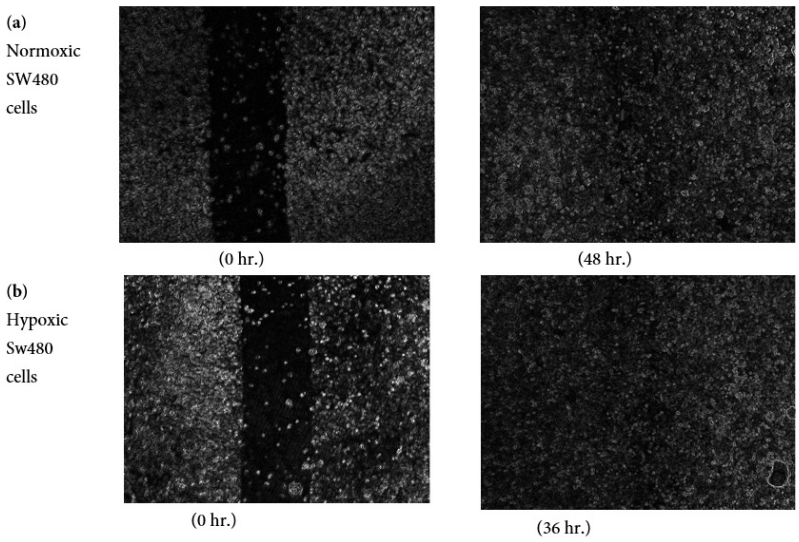

Figure 2. Migratory ability of SW480 cells. a) normoxic cells, b) hypoxic cells. Cell migration was evaluated by wound-healing assay. Images were taken at 0 and 48 hr. for normoxic cells and at 0 and 36 hr. for hypoxic cells till complete healing of scratch obtained.

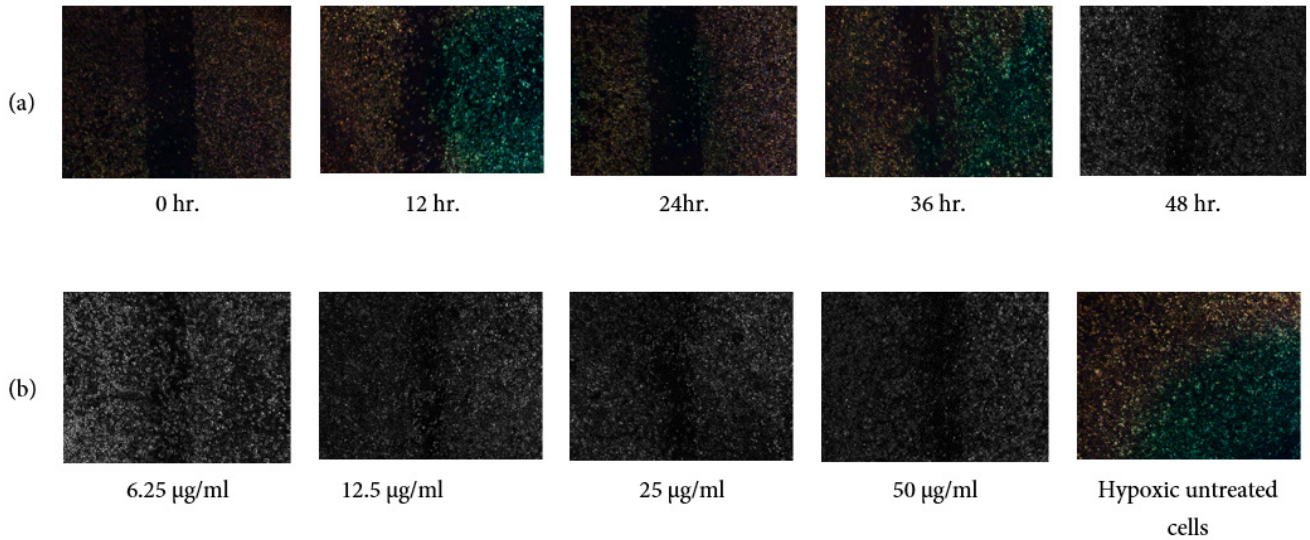

Figure 3. Effects of carvacrol on the migratory ability of hypoxic SW480 cells. (a) effect of specified concentration of carvacrol (50 µg/ml), at different times (0,12,24,36,48 hr.). (b) effect of different concentrations of carvacrol (50,25, 1205, 6.25) after 48 hr. Images were taken under the inverted microscope.

cancer spreading, involves steps like cell migration, invasion, and adhesion. Active nutraceuticals targeting these processes have been explored for managing metastatic cancers (Morbidelli et al., 2018). Carvacrol, a natural compound from aromatic plants approved by the FDA as a safe food additive, has various medicinal properties (Haidan et al., 2014, Fenaroli et al., 1975, Mezzoug et al., 2007, Nostro et al., 2012). Recent studies indicate that carvacrol can suppress cancers with minimal toxic effects, suggesting its potential as a co-therapy to hinder cancer hallmarks (Fatima et al., 2022).

Our study on SW480 cancer cells shows that carvacrol significantly inhibits proliferation, especially at higher doses. While researchers have highlighted the role of the tumor microenvironment in cancer processes, some studies, like one by Khan and colleagues, focused on carvacrol's impact on cell migration and angiogenesis without considering the influence of hypoxia and the HIF-1α/VEGF signaling pathway (Khan et al., 2019). Our observations suggest that carvacrol might disrupt this pathway in colorectal cancer cells, reducing the angiogenic response of hypoxic cells.

Conclusion

In conclusion, the results indicate that carvacrol can significantly reduce cell growth and migration dose-dependently. This study is crucial as metastatic colorectal cancer remains a challenge. Carvacrol shows promise as an additional therapy and a potential supplement for patients with highly metastatic colorectal cancer. However, the exact mechanism of carvacrol's action requires further investigation through in vivo studies and clinical trials.

Author contribution

A.M., and H.N.R. conceptualized, performed the methodology, analsyzed the data and wrote the paper.

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Competing financial interests

The authors have no conflict of interest.

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