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Original Article

# The synthetic cannabinoid 5-fluoro ABICA upregulates angiogenic markers and stimulates tube formation in human brain microvascular endothelial cells

Laith AL-Eitan, PhD * and Rawan Abusirdaneh, MSc

*Department of Biotechnology and Genetic Engineering, Jordan University of Science and Technology, Irbid, Jordan*

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**الملخص**

**أهداف البحث:** ارتبطت الكانابينويدات الاصطناعية، وهي فئة من المركبات ذات التأثير النفساني التي تحاكي تأثيرات الكانابينويدات الطبيعية، بمخاوف الإدمان والذهان. ومع ذلك، تشير الأبحاث الحديثة إلى تطبيقات دوائية محتملة، خاصة في تكوين الأوعية الدموية في الدماغ، وهي عملية فسيولوجية أساسية للنمو والإصلاح وصيانة الأنسجة من خلال تكوين أوعية دموية جديدة من الأوعية الدموية الموجودة. تستكشف هذه الدراسة القدرة المختبرية للكانابينويد الاصطناعي 5-فلورو أبيكا، لتعزيز عملية تكوين الدم الجديدة في الخلايا البطانية للأوعية الدموية الدقيقة في الدماغ البشري.

**طرق البحث:** تم إعطاء 5-فلورو أبيكا إلى الخلايا البطانية للأوعية الدموية الدقيقة في الدماغ البشري بتركيزات مختلفة (1، 0.1، 0.01، 0.001، 0.0001 مايكرو مولر). تم إجراء تحليل شامل، بما في ذلك فحص ام تي تي لبقاء الخلية، وفحص التئام الجروح لقدرة الهجرة، وفحص تكوين الأنبوب لتقييم إمكانية تكوين الأوعية الدموية للخلايا البطانية. بالإضافة إلى ذلك، تضمن التحليل قياس تعبير الرنا المرسال، ومستويات البروتين لعوامل معينة مؤيدة لتولد الأوعية، واكتشاف مستويات الفسفرة من سينثيز الجليكوجين كيناز-3بيتا في الخلايا البطانية للأوعية الدموية الدقيقة في الدماغ البشري المعالجة باستخدام تحليل الإليزا، و تحليل تفاعل البوليميراز المتسلسل في الوقت الحقيقي، وتقنيات الويسترن بلوت.

**النتائج:** 5-فلورو أبيكا يحفز بشكل فعال انتشار وهجرة وتكوين أنبوب الخلايا البطانية للأوعية الدموية الدقيقة في الدماغ البشري بطريقة تعتمد على الجرعة. كما أنه زاد بشكل ملحوظ من مستويات التعبير عن العوامل المؤيدة للتولد الوعائي، إلى جانب تنظيم مستويات الفسفرة من سينثيز الجليكوجين كيناز-3بيتا.

**الاستنتاجات:** بشكل عام، توفر النتائج التي توصلنا إليها أدلة دامغة على أن 5-فلورو أبيكا له تأثيرات تحفيزية على عملية تكوين الأوعية الدموية في الخلايا البطانية، مما يوفر خيارات علاجية في علاج الأمراض المرتبطة بتكوين الأوعية الدموية. ومع ذلك، هناك حاجة إلى مزيد من البحث لفهم الآلية الجزيئية لـ 5-فلورو أبيكا بشكل كامل في تكوين الأوعية، بما في ذلك الاعتبارات الأخلاقية لاستخدامه في البحوث الطبية.

**الكلمات المفتاحية:** الكانابينويدات الاصطناعية؛ 5-فلورو أبيكا؛ تولد الأوعية؛ الخلايا البطانية للدماغ البشري

**Abstract**

**Objective:** Synthetic cannabinoids (SCs), a class of psychoactive compounds emulating the effects of natural cannabis, have prompted addiction and psychosis concerns. However, recent research has suggested potential pharmacological applications, particularly in brain angiogenesis—an essential physiological process for growth, repair, and tissue maintenance, in which new blood vasculature is formed from existing vasculature. This study explored the in vitro ability of the SC 5-fluoro ABICA to enhance new blood formation processes in human brain microvascular endothelial cells (HBMECs).

**Methods:** HBMECs were treated with various concentrations of 5-fluoro ABICA (1 μM, 0.1 μM, 0.01 μM, 0.001 μM, and 0.0001 μM). A comprehensive analysis was conducted, including MTT assays indicating cell viability, wound healing assays indicating migration ability, and tube formation assays indicating the angiogenesis potential of endothelial cells. Additionally, mRNA expression and protein levels of specific pro-angiogenic factors were measured, and the phosphorylation levels of glycogen synthase kinase-3β were detected in treated HBMECs through ELISA, real-time PCR, and western blotting.

* Corresponding address: Department of Biotechnology and Genetic Engineering, Jordan University of Science and Technology, 22110 Irbid, Jordan.
E-mail: lneitan@just.edu.jo (L. AL-Eitan)

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**Results:** Treatment with 5-fluoro ABICA effectively stimulated proliferation, migration, and tube formation in HBMECs in a dose-dependent manner; markedly increased the expression of pro-angiogenic factors; and upregulated levels of phosphorylated-GSK-3β.

**Conclusion:** Our findings demonstrate that 5-fluoro ABICA stimulates angiogenesis in endothelial cells, thus potentially offering therapeutic options for diseases associated with angiogenesis. However, further research is needed to fully understand the molecular mechanism of 5-fluoro ABICA in angiogenesis, including ethical considerations regarding its use in medical research.

**Keywords:** 5-Fluoro ABICA; Angiogenesis; Human brain endothelial cells; Synthetic cannabinoids



## Introduction

Blood vessel formation in embryos begins with hemangioblasts, which develop into endothelial cells through vasculogenesis and angiogenesis.[1] Most embryonic blood vessels, including those in the brain, are formed by angiogenesis. Brain neovascularization is precisely regulated by the interaction of neuroectodermal elements with receptor-tyrosine kinases expressed by endothelial cells.[2] The regulatory mechanisms underlying physiological and pathological brain angiogenesis, such as hypoxia, ischemia, and brain tumor development,[3] share similarities, including key factors such as vascular endothelial growth factor (VEGF), angiopoietins, transforming growth factor-β and hypoxia-inducible factors (e.g., HIF-1), which play crucial roles in these processes.[4,5]

Recent evidence has highlighted the roles of glycogen synthase kinase-3β (GSK-3β), a serine−threonine kinase with α and β isoforms, in angiogenesis and neurogenesis. The activity of GSK-3β is finely regulated by phosphorylation at the Tyr216 and Ser9 sites, and is integral to glycogen metabolism.[6−8] Notably, GSK-3β also controls the expression of angiogenic factors including VEGF, and influences angiogenic processes associated with cell proliferation, differentiation, and apoptosis in endothelial cells.[9,10]

Synthetic cannabinoids (SCs) mimic the effects of components of natural cannabis, such as delta-9-tetrahydrocannabinol (THC), by acting as full agonists toward two major receptors in the endocannabinoid system,[11,12] cannabinoid receptors CB1 and CB2. Thus, SCs have stronger psychoactive effects and more severe adverse effects than THC. Although they are predominantly used recreationally, SCs have multifaceted effects on the human body[13] and are sought after for their effects including relaxation, social enhancement, and an enhanced sense of well-being. However, acute intoxication results in neurological disturbances.[12,14] The widespread use of SCs has prompted concerns regarding neurodevelopment, and the disruption of processes including neurogenesis and neuroplasticity. In vitro studies have revealed diverse effects of SCs on brain cells, including apoptosis activation and neurogenesis inhibition. SCs are also involved in modulating neuroplasticity, which is crucial for neural cell survival.[15−17] Despite the rapid development of SCs, especially in relation to human brain cells, there is limited toxicological and mechanistic research examining the brain effects induced by SCs. Current data on SCs, particularly, 5-fluoro ABICA (Figure 1), also known as N-[(1S)-1-(aminocarbonyl)-2-methylpropyl]-1-(5-fluoropentyl)-1H-indole-3-carboxamide, have been derived primarily from in vitro and in vivo studies.[18,19] The compound 5-fluoro ABICA, a member of the indole family,[20] is a potent CB1 receptor agonist and a commonly used recreational drug. Despite often being unregulated and illegal, this compound's health implications are of growing interest, and are worthy of investigation in forensic and research work.[21] Because the chemical characteristics of 5-fluoro ABICA are unknown, in vitro human cell-based models expressing cannabinoid receptors are required to understand its effects.

The study focused on assessing the effects of 5-fluoro ABICA on cell survival and the angiogenic processes of human brain microvascular endothelial cells (HBMECs) in vitro. We also examined the effects of 5-fluoro ABICA on GSK-3β and angiogenesis-promoting factors, including VEGF, ANG-1, and ANG-2, to discover potential therapeutic approaches for angiogenesis-associated disorders.

## Materials and Methods

### *Drugs, solvents, cell line, and culture medium*

HBMECs (CRL-3245) were sourced from the American Type Culture Collection (Manassas, VA, USA). The cells were cultivated in a specialized growth medium comprising Dulbecco's modified Eagle's medium: F-12 nutrient mixture (DMEM/F12) provided by Euroclone S.P.A., Pero, Italy. The culture medium was supplemented with various components in the endothelial cell growth kit (PCS-110-040) from the American Type Culture Collection. The supplements included fetal bovine serum (10 %), antibiotics

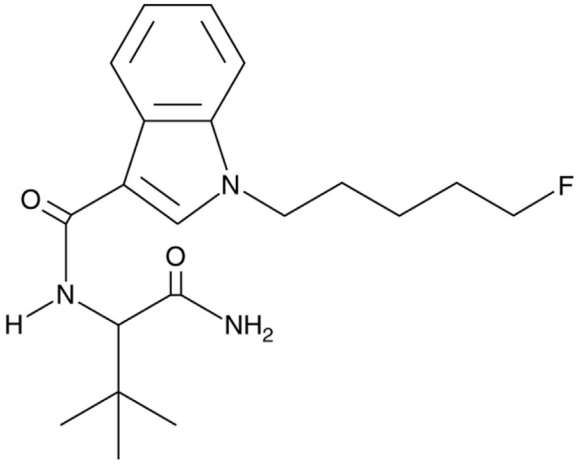


**Figure 1:** Structure of 5-fluoro ABICA.

(1 % penicillin and streptomycin), glutamine, ascorbic acid, heparan sulfate, recombinant human epidermal growth factor, hydrocortisone, and bovine brain extract. The cell culture was maintained in a controlled environment under 5 % $CO_2$ at 37 °C. Cells were passaged in a 1:4 ratio after 80 % confluence was reached. The SC 5-fluoro ABICA, sourced from Cayman Chemical in Ann Arbor, Michigan, was initially dissolved in dimethyl sulfoxide (DMSO) to create a stock solution with a concentration of 2 mg/ml. The stock solution was serially diluted to working concentrations of 0.0001 μM, 0.001 μM, 0.01 μM, 0.1 μM, and 1 μM, to explore the effects of 5-fluoro ABICA on neovascular endothelial cells. A control group was exposed to serum-free medium and 0.1 % DMSO, to serve as a reference for evaluating the influence of 5-fluoro ABICA on the cells. This experimental design allowed us to examine the effects of 5-fluoro ABICA.

*MTT assays*

The MTT assay, based on the transformation of the yellow tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H tetrazolium into purple formazan crystals, is a broadly used colorimetric technique to determine cellular metabolic function. Cells were plated on 96-well microtiter plates at a density of $5 \times 10^3$ cells/well and allowed to attach for 24 h. The cells were then exposed to five concentrations of 5-fluoro ABICA (ranging from 0.0001 μM to 1 μM) in triplicate for 24 h. The culture medium was then replaced with serum-free medium containing MTT solution (5 mg/ml) obtained from (HiMedia, Mumbai, India) and incubated for 4 h. Finally, DMSO was added to the cells, which were then agitated for 10 min to dissolve the formazan crystals. Cell viability per well was quantified with an ELISA reader, on the basis of the absorbance at 570 nm. The absorbance of the control wells (cells treated with DMEM/F-12 only) was used as the baseline value against which all other treatments were compared. Cell viability was quantified as percentage absorbance relative to that in the control wells. All experiments were performed in triplicate.

*Cell migration assessment*

Scratch wound assays was performed in vitro to examine the migration capability of HBMECs. HBMECs were cultured on a 12-well plate at a density of $5 \times 10^3$ cells/well and allowed to proliferate for 24 h until adequate confluence was reached. Uniform scratches were then created on the cell monolayer with sterile 1 ml pipette tips. After thorough washing with phosphate-buffered saline to ensure the removal of detached cells, the remaining cells were treated with 1 μM, 0.1 μM, 0.01 μM, 0.001 μM, or 0.0001 μM 5-fluoro ABICA for 24 h. The control cells were treated with an equal volume of DMSO, and each concentration was applied to triplicate wells. Four microscopic images were captured for the scratch area at (baseline), time zero, and 24 h post-wounding. The injury size was measured at baseline and 24 h after scratch wounding, with ImageJ software from the National Institutes of Health (LOCI, University of Wisconsin). The migration rate was calculated as percentage wound recovery with the following equation: (total distance of wound—mean uncovered distance)/(total wound distance) × 100 %. The experimental protocol was independently repeated three times.

In vitro *tube formation assays*

To assess the angiogenic potential of HBMECs, the formation of tube-like structures was evaluated. Initially, 96-well plates were thawed overnight at 4 °C and pre-cooled for this purpose. Each well was covered with a 50 μl matrix of the basement membrane extract, which was allowed to polymerize at 37 °C for 30 min. BME was purchased from Trevigen (Gaithersburg, MD, USA). HBMECs were seeded at a density of $2 \times 10^4$ per well in a BME-coated plate and cultured with growth-supplement-complete medium containing 5-fluoro ABICA at concentrations of 1 μM, 0.01 μM, or 0.0001 μM, or control treatment, for 24 h. Formation of tube-like structures was evaluated from photomicrographs of the tubes originating from the cells, through direct assessments, such as the quantification of the number of tube-like structures, counting and determination of the loop structures, total tube length, and branching points. In addition, ImageJ software was used to analyze the data. The tube formation assays were iteratively performed three times in triplicate.

*Western blotting*

Protein expression of VEGF, ANG-1, ANG-2, and the phosphorylation of GSK-3β in HBMECs were assessed with western blotting. Cultured cells were initially subjected to a thorough cold phosphate buffered saline wash, then lysis with radioimmunoprecipitation assay buffer plus protease and phosphatase inhibitors. Protein quantification was performed according to the instructions of a protein assay kit from Bio-Rad, Hercules, CA, USA. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used to separate approximately 20 μg of each protein sample. The proteins were then transferred to polyvinylidene fluoride membranes, which were blocked with 2 % bovine serum albumin. Subsequently, primary antibodies, comprising anti-VEGF (ab46154; 1:500, Abcam), anti-ANG-1 (ab94684; 1:500, Abcam), anti-ANG-2 (153934; 1:500, Abcam), anti-phospho-Ser9-GSK-3β (9336S; 1:500; Cell Signaling Technology), anti-total-GSK-3β (PA5-95845; 1:1000, Thermo Fisher), and anti-β-actin (4967S; 1:1000; Cell Signaling Technology), were applied to the membrane and incubated overnight at 4 °C. The membrane was then treated with secondary antibodies for 2 h. Protein bands were detected with a ChemiDoc XRS+ system (Bio-Rad Laboratories, Hercules, CA, USA). The density of bands was measured ImageJ software, and the data were normalized to β-actin as the loading control. The western blot procedure was replicated twice.

*Enzyme-linked immunosorbent assays*

To measure the protein levels of angiogenic factors in the HBMEC lysates, we used a commercially available ELISA kit (Abcam, Cambridge, MA, USA), following the manufacturer's instructions. After a 24-h incubation with 5-fluoro

ABICA, the conditioned medium was collected and centrifuged at 10,000 RPM for 10 min. The resulting supernatant was stored at −80 °C until analysis. Each experimental condition was replicated three times, and each concentration was applied to triplicate wells. The concentrations of VEGF (ab100662), ANG-1 (ab99972), and ANG-2 (ab99971) were determined with commercially available kits from Abcam (Cambridge, MA, USA). All methods were conducted according to the manufacturer's guidelines.

*RT-qPCR analysis of gene expression*

The differential expression of genes was validated with real-time quantitative PCR. Total RNA was isolated from cultured cells with a Total RNA Purification Kit (PP-219, Jena Bioscience, Germany). Equal amounts of RNA were treated with a DNA removal kit (PP-219, Jena Bioscience, Germany) to remove any genomic DNA contamination. The total RNA concentration was determined with an ND-1000 (Bio Drop-UK) spectrophotometer. After isolation, reverse-transcription was performed on cDNA with a SOLIscript 1-Step SolisGreen kit (08-63-00250, Solis BioDyne, Tartu, Estonia), according to the manufacturer's instructions. Real-time PCR was performed with QuantStudio 1 (Applied Biosystems, Foster City, CA) and SYBR Green PCR master mix (Applied Biosystems, Foster City, CA). The reaction conditions were as follows: initial denaturation at 95 °C for 10 min; 40 cycles of denaturation at 95 °C for 30 s; and extension for 60 s at 60 °C. This entire process was repeated three times, and each run was performed in triplicate. The primer sequences used for three target genes (VEGF, ANG-1, and ANG-2), as well as the reference control gene (β-actin), can be found in Table 1. The primer selection process was based on previously published research.[22,23]

*Statistical analysis*

We conducted an initial assessment to confirm the normal distribution of our data. Subsequent data analysis was performed with one-way ANOVA and the Tukey post-hoc test in GraphPad Prism software (version 9.0, GraphPad Software, La Jolla, CA). All data are expressed as standard deviation (SD) ± mean. We considered results statistically significant when the p-value was below 0.05.

## Results

*Treatment with 5-fluoro ABICA enhances the metabolic activity of human brain endothelial cells*

After performing MTT assays on HBMECs exposed to various concentrations of 5-fluoro ABICA ranging from 0.0001 μM to 1 μM, we observed significant enhancement of cell metabolism in cells treated with 0.01 μM−1 μM concentrations compared with the control ($p = 0.0036$ for 0.01 μM, $p = 0.0046$ for 0.1 μM, $p = 0.0180$ for 1 μM). The observed enhancement of cell metabolism was positively correlated with increasing 5-fluoro ABICA concentration (Figure 2).

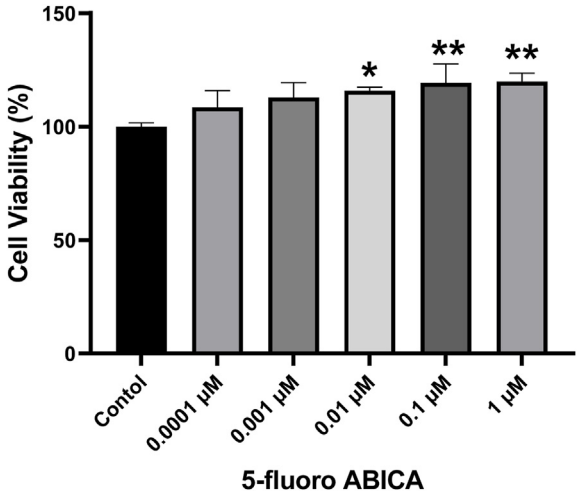


**Figure 2:** Effects of 5-fluoro ABICA on cell metabolic activity in HBMECs. For assessment of cell viability, HBMECs ($5 \times 10^3$) were initially seeded in a 96-well plate and incubated 24 h. Subsequently, the cells were exposed to five concentrations of 5-fluoro ABICA (ranging from 0.0001 μM to 1 μM) for 24 h. After treatment, the culture medium containing 5-fluoro ABICA was removed and replaced with MTT (5 mg/ml), and the cells were incubated 4 h at 37 °C under 5 % $CO_2$. The cells were then treated with DMSO and agitated for 10 min, and the resulting absorbance was quantified at 570 nm with an ELISA reader. The data are presented as SD ± mean (n = 3). Significantly greater cell viability was observed with concentrations ranging from 0.01 μM to 1 μM than the control. (**) indicates $p < 0.01$ (*) indicates $p < 0.05$.

*Treatment with 5-fluoro ABICA increases the rate of cell migration of human brain endothelial cells*

We conducted in vitro scratch wound healing assays in cultured HBMECs to evaluate the rate of cell migration—a fundamental aspect of angiogenesis. We tested the effects of five concentrations of 5-fluoro ABICA on cell migration. HBMECs showed significantly higher migration rates after treatment with 0.1 μM−1 μM 5-fluoro ABICA than control treatment (Figure 3A; $p < 0.0001$ for 0.1 μM and 1 μM). The most notable increase in cell migration was observed in the group treated with 1 μM 5-fluoro ABICA (Figure 3B).

**Table 1: RT-PCR primer sequences.**

| Primer | Forward sequence | Reverse sequence |
|---|---|---|
| Beta-actin | 5′-GGAGATTACTGCCCTGGCTCCTA-3′ | 5′-GACTCATCGTACTCCTGCTTGCTG-3′ |
| VEGF | 5′-GCACGTTGGCTCACTTCCAG-3′ | 5′-TGGTCGGAACCAGAATCTTTATCTC-3′ |
| ANG-1 | 5′-ACCGTGAGGATGGAAGCCTAGA-3′ | 5′-AATGAACTCGTTCCCAAGCCAATA-3′ |
| ANG-2 | 5′-CTTCAAGTCAGGACTCACCACCA-3′ | 5′-CCACCCATGTCCATGTCACAG-3′ |

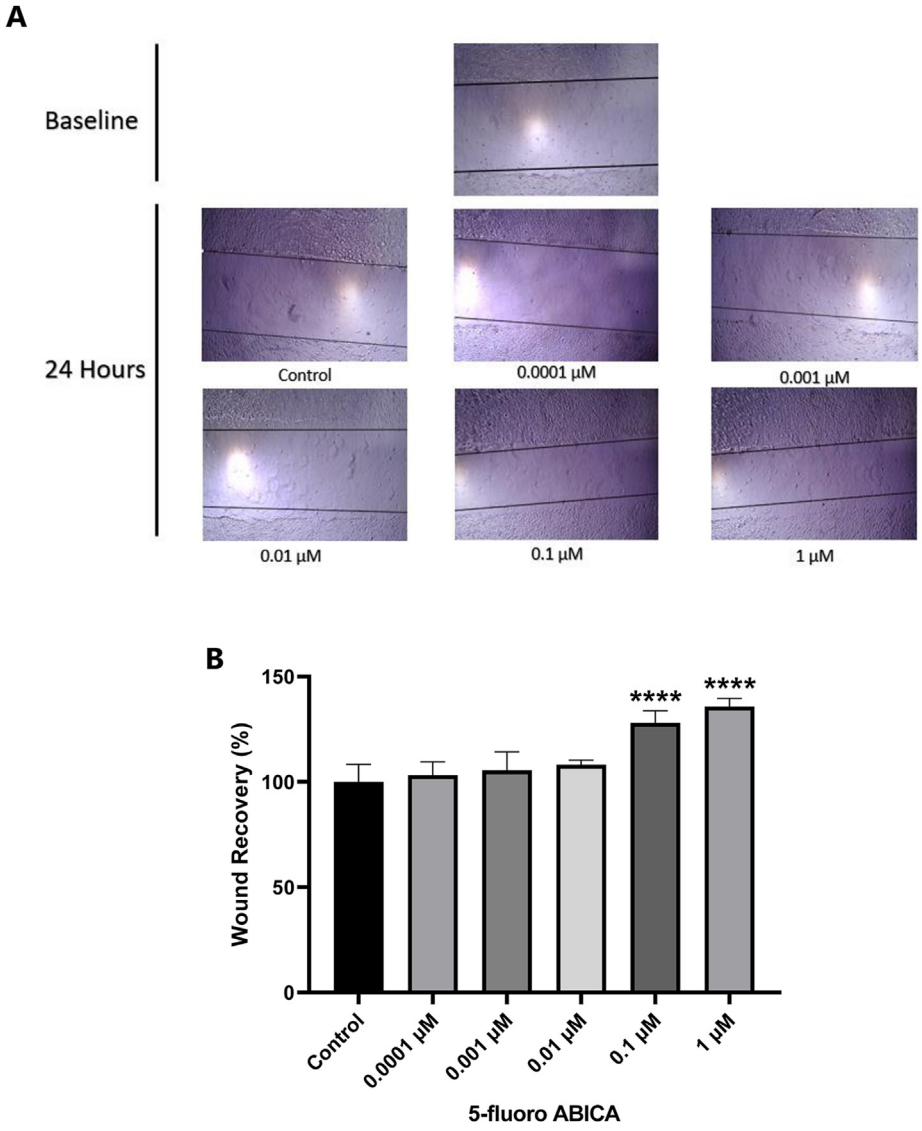


**Figure 3:** Treatment with 5-fluoro ABICA enhances t HBMEC migration rate. The effects of 5-fluoro ABICA on cell migration in HBMECs were evaluated with the following protocol. HBMECs were cultured in a 12-well plate for 24 h. After the desired confluence was reached, a cell monolayer was gently scratched with a 1000 µl pipette tip to induce a wound. (A) Microscopic images were captured to document migration levels at the starting point (time zero) and 24 h after treatment with various concentrations of 5-fluoro ABICA at 0.0001 µM−0.1 µM. (B) Quantitative analysis of the migration data shown in A, revealing that doses of 5-fluoro ABICA ranging from 1 µM, and 0.1 µM elicited greater rates of migration than the control. The data were measured and assessed in three separate experiments, each conducted in duplicate, and are depicted as SD ± mean (n = 3). (****) indicates $p < 0.0001$.

*Treatment with 5-fluoro ABICA promotes tube-forming activity in HBMECs*

We next assessed angiogenesis with in vitro tube formation assays to assess the effects of 5-fluoro ABICA treatment on HBMECs. Notably, HBMECs exposed to 5-fluoro ABICA at concentrations 1 µM and 0.01 µM showed significant elevation in several angiogenic parameters, including the number of tubes, total tube length, number of loops, and number of branch points (Figure 4A−E; $p < 0.0001$ for 1 µM and 0.01 µM).

*Treatment with 5-fluoro ABICA increases VEGF, ANG-1, and ANG-2 mRNA expression in HBMECs*

To investigate the effects of 5-fluoro ABICA on the mRNA expression levels of VEGF, ANG-1, and ANG-2 in HBMECs, we used quantitative real-time PCR. The absolute quantification of VEGF copy numbers with RT-PCR indicated a significant correlation between VEGF mRNA expression and different concentrations of 5-fluoro ABICA. VEGF expression increased after treatment with 1 µM, 0.1 µM, or 0.01 µM 5-fluoro ABICA (Figure 5A; $p < 0.0001$

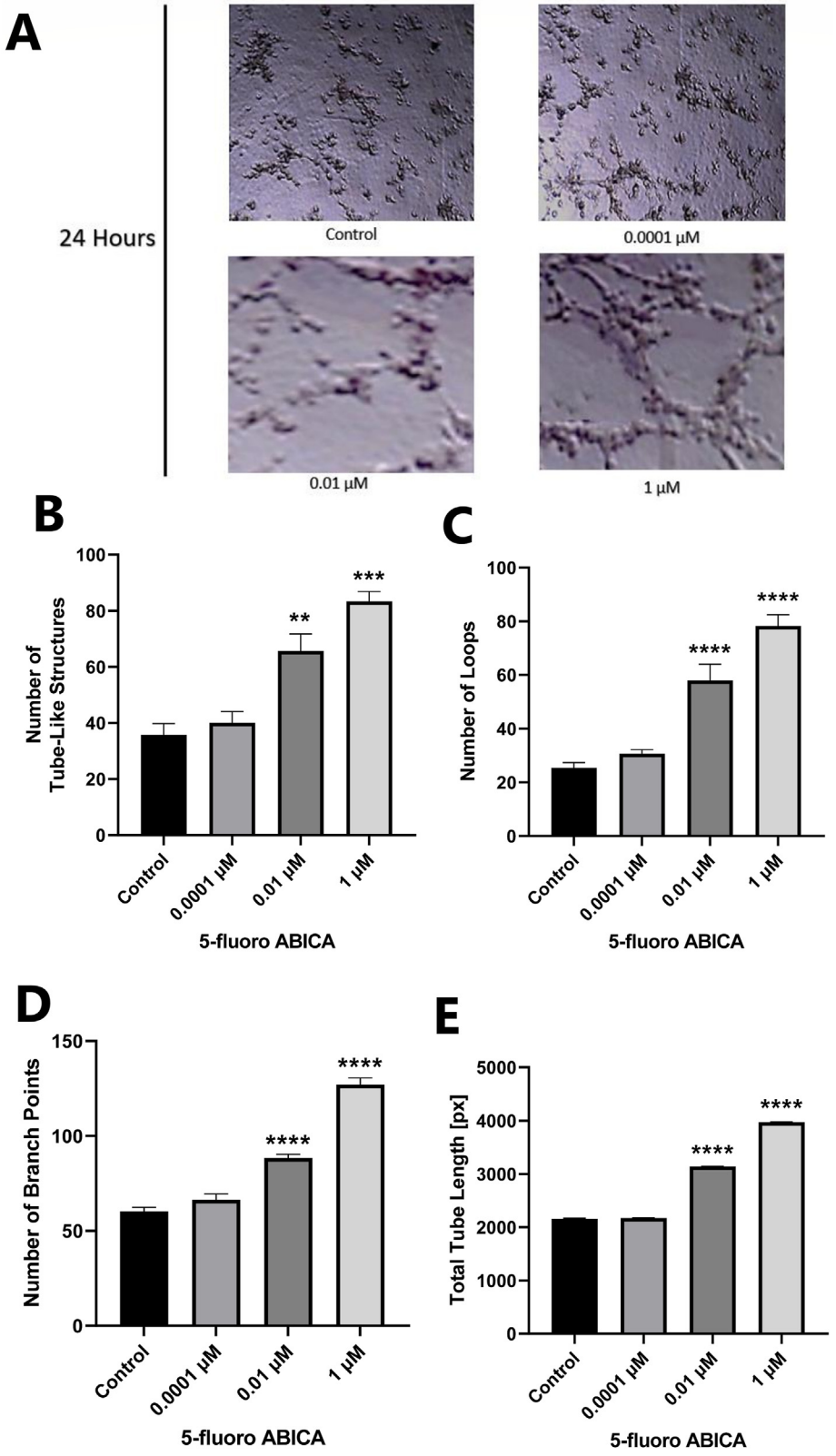


**Figure 4:** Treatment with 5-fluoro ABICA enhances HBMEC functionality. Angiogenic potential was assessed with tube formation assays. BME-coated plates were initially seeded with $2 \times 10^4$ HBMECs maintained in serum-free medium and treated with varying concentrations of 5-fluoro ABICA (0.001 µM, 0.1 µM, and 1 µM) or control for 24 h. (A) After 24 h of treatment, microscopic visual images of tubular structures produced by HBMECs were captured. (B) Quantity of tube-like structures, (C) number of loops, (D) number of branch points, and (E) total tube length. The data are presented as SD ± mean (n = 3). (****) indicates a significance level of $p < 0.0001$.

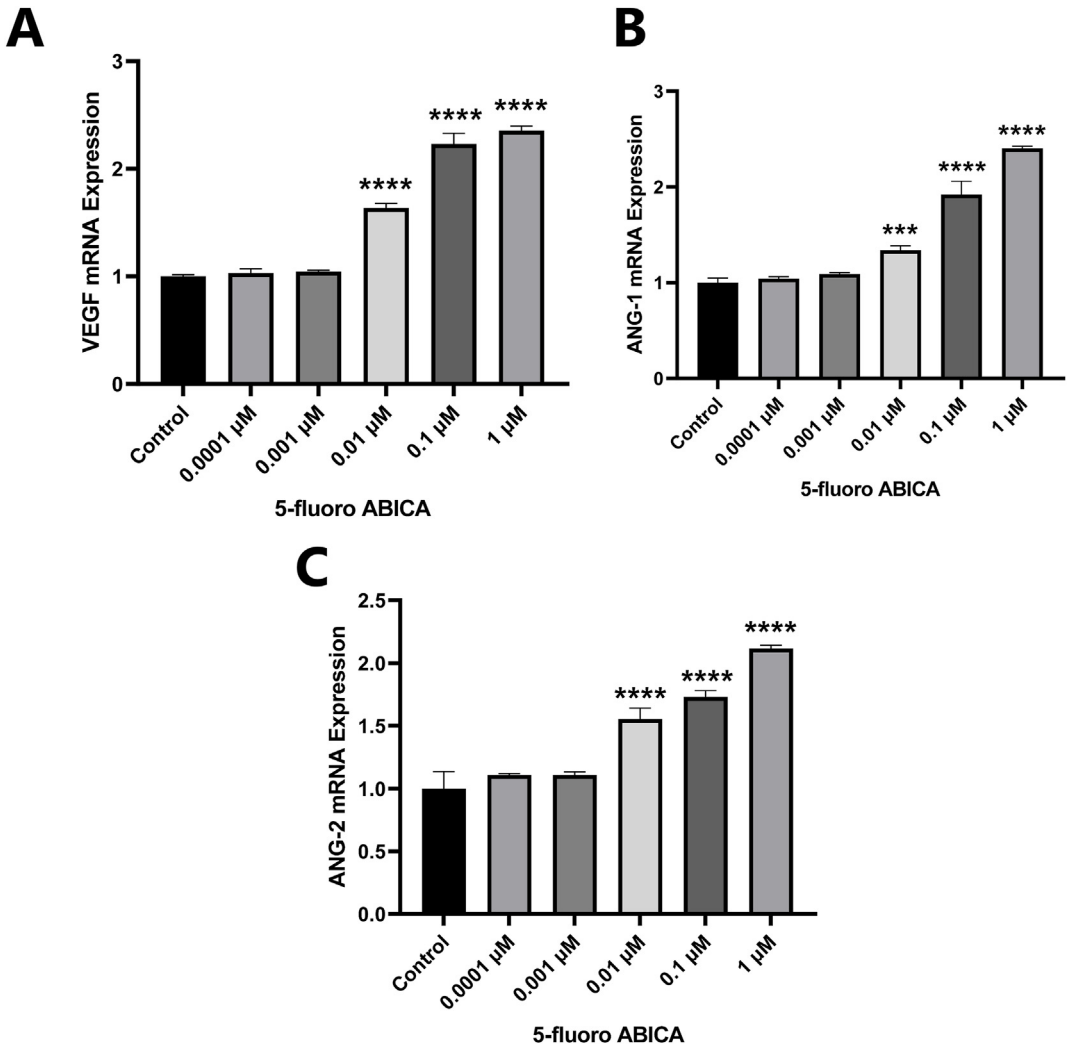


**Figure 5:** Treatment with 5-fluoro ABICA enhances angiogenesis-associated gene expression in HBMECs: RT-PCR analysis. (A) RT-PCR quantification of VEGF mRNA expression in HBMECs treated with different concentrations of 5-fluoro ABICA. (B) Effects of 5-fluoro ABICA on the mRNA expression levels of ANG-1 and (C) ANG-2 in comparison to the control. Gene expression levels were calculated for each sample and are presented in the graph as units. The data are presented as SD ± mean (n = 3). (****) indicates $p < 0.0001$, (***) indicates $p < 0.001$.

for 1 µM, 0.1 µM, and 0.01 µM). Additionally, ANG-1 exhibited significantly greater upregulation in the 0.01 µM ($p = 0.0001$) and 1 µM, 0.1 µM ($p < 0.0001$ for 0.1 µM and 1 µM) groups than the control group (Figure 5B). ANG-2 mRNA expression levels were also significantly higher in the groups treated with the same concentrations of 5-fluoro ABICA ($p < 0.0001$ for 1 µM, 0.1 µM, and 0.01 µM) than the control group (Figure 5C).

*Treatment with 5-fluoro ABICA increases levels of secreted vascular endothelial growth factor, angiopoietin 1, and angiopoietin 2*

We next sought to investigate whether 5-fluoro ABICA might affect angiogenic factor release. Using ELISA, we determined the levels of VEGF, ANG-1, and ANG-2 angiogenic factors released by cells subjected to 5-fluoro ABICA treatment. VEGF levels were higher after treatment with 1 µM, 0.1 µM, 0.01 µM, 0.001 µM, and 0.0001 µM 5-fluoro ABICA than control treatment ($p < 0.0001$ for 1 µM, 0.1 µM, 0.01 µM, $p = 0.0009$ for 0.001 µM, and $p = 0.0460$ for 0.0001 µM; Figure 6A). Furthermore, ANG-1 levels were significantly higher after treatment with 1 µM, 0.1 µM, 0.01 µM, and 0.001 µM ($p < 0.0001$ for 1 µM, 0.1 µM, 0.01 µM, and $p = 0.0008$ for 0.001 µM) than control treatment (Figure 6B). ANG-2 levels were also significantly higher after treatment with 1 µM, 0.1 µM, 0.01 µM, and 0.001 µM 5-fluoro ABICA ($p < 0.0001$ for 1 µM, 0.1 µM, 0.01 µM, and $p = 0.0270$ for 0.001 µM) than control treatment (Figure 6C).

*Treatment with 5-fluoro ABICA increases angiogenic factor protein levels and enhances levels of phosphorylated GSK-3β*

We investigated the profiles of intracellular protein levels of VEGF, ANG-1, ANG-2, and phosphorylated GSK-3β by

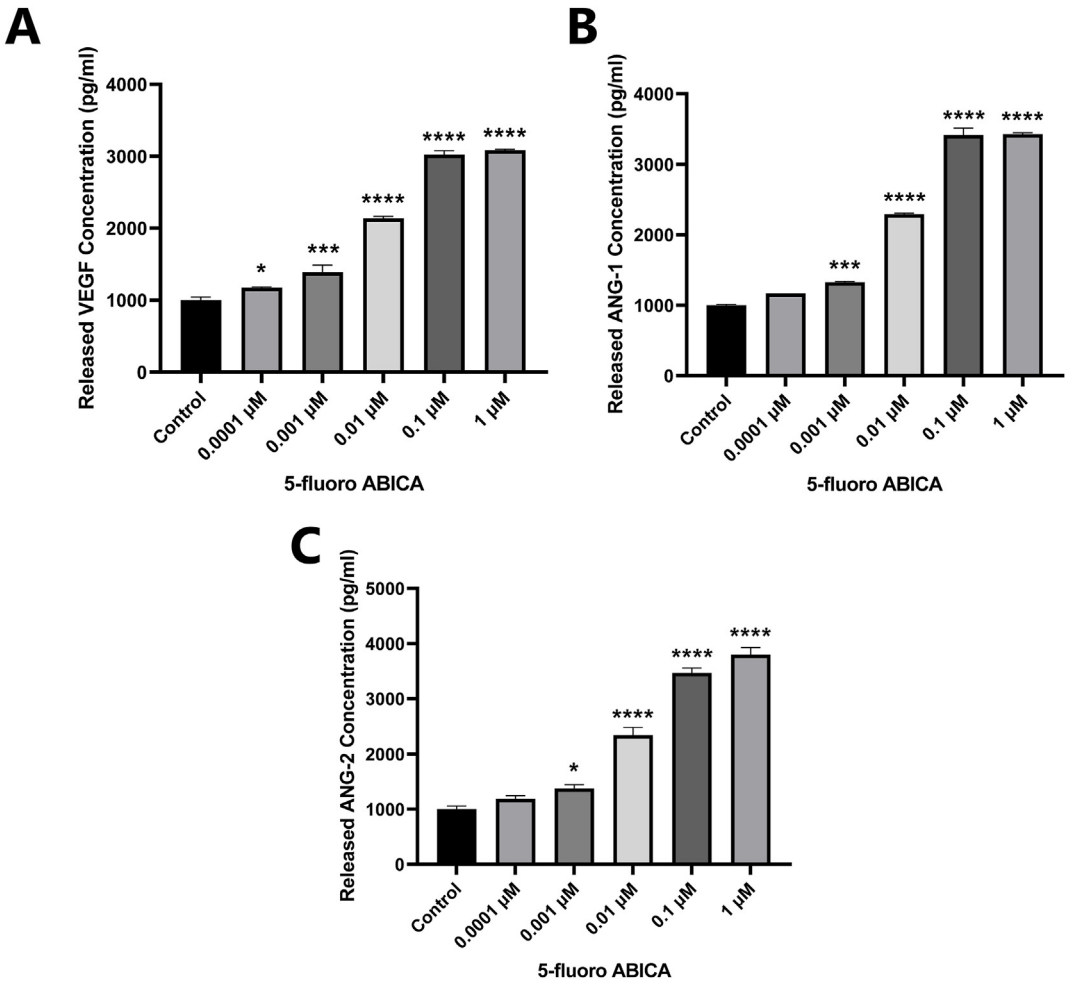


**Figure 6:** ELISA quantification of gene expression in HBMECs treated with different concentrations of 5-fluoro ABICA. ELISA was conducted on HBMECs treated with 5-fluoro ABICA at different concentrations, to quantify the levels of secreted proangiogenic factors. (A–C) Significant increase in the release of VEGF, ANG-1, and ANG-2 concentrations in a dose-dependent manner after treatment with 5-fluoro ABICA compared with the control. Gene expression was calculated for each sample and is presented in the graph as units. The data are presented as SD ± mean (n = 3). (****) indicates $p < 0.0001$, (***) indicates $p < 0.001$, (*) indicates $p < 0.05$.

western blotting in HBMECs exposed to 5-fluoro ABICA. VEGF bands were observed at approximately 27 kDa in both 5-fluoro ABICA and control treated HBMECs. The expression of VEGF was significantly upregulated after treatment with 1 μM and 0.01 μM 5-fluoro ABICA ($p = 0.0002$ for 1 μM, and $p = 0.0013$ for 0.01 μM; Figure 7A and B). Similarly, ANG-1 and ANG-2 protein bands, observed at approximately 57 kDa, were significantly upregulated in HBMECs treated with 5-fluoro ABICA at various concentrations. ANG-1 demonstrated a notable increase after 1 μM ($p = 0.0002$) and 0.01 μM ($p = 0.0007$) 5-fluoro ABICA treatment, and ANG-2 also exhibited a significant elevation after 1 μM ($p = 0.0001$) and 0.01 μM ($p = 0.0006$) 5-fluoro ABICA treatment (Figure 7C and D). Furthermore, bands corresponding to phosphorylated GSK-3β, at approximately 46 kDa, were significantly upregulated after 1 μM ($p = 0.0002$) and 0.01 μM ($p = 0.0039$) 5-fluoro ABICA treatment, compared with the control treatment (Figure 7E).

## Discussion

SCs are a novel class of drugs with a broad spectrum of effects, acting through CB1 and CB2 receptors. Several previous in vitro and in vivo studies have presented compelling evidence that SCs exert pharmacological effects 2 to 100 times more potent than those of THC.[24–26] These effects are relevant to conditions as diverse as cancer growth, inflammatory responses, and neurodegenerative diseases. Notably, SCs, like 5-fluoro ABICA, lack medical approval and are illegal in many countries, because of their potential for addiction. Although 5-fluoro ABICA is known to have strong affinity toward CB1R, this study provides the first report of the effects of 5-fluoro ABICA on brain endothelial cells.

Recent studies have explored how SCs affect various human cell types and may potentially influence cell metabolic activity by modulating intracellular processes. However, these effects vary, depending on factors such as CB receptor

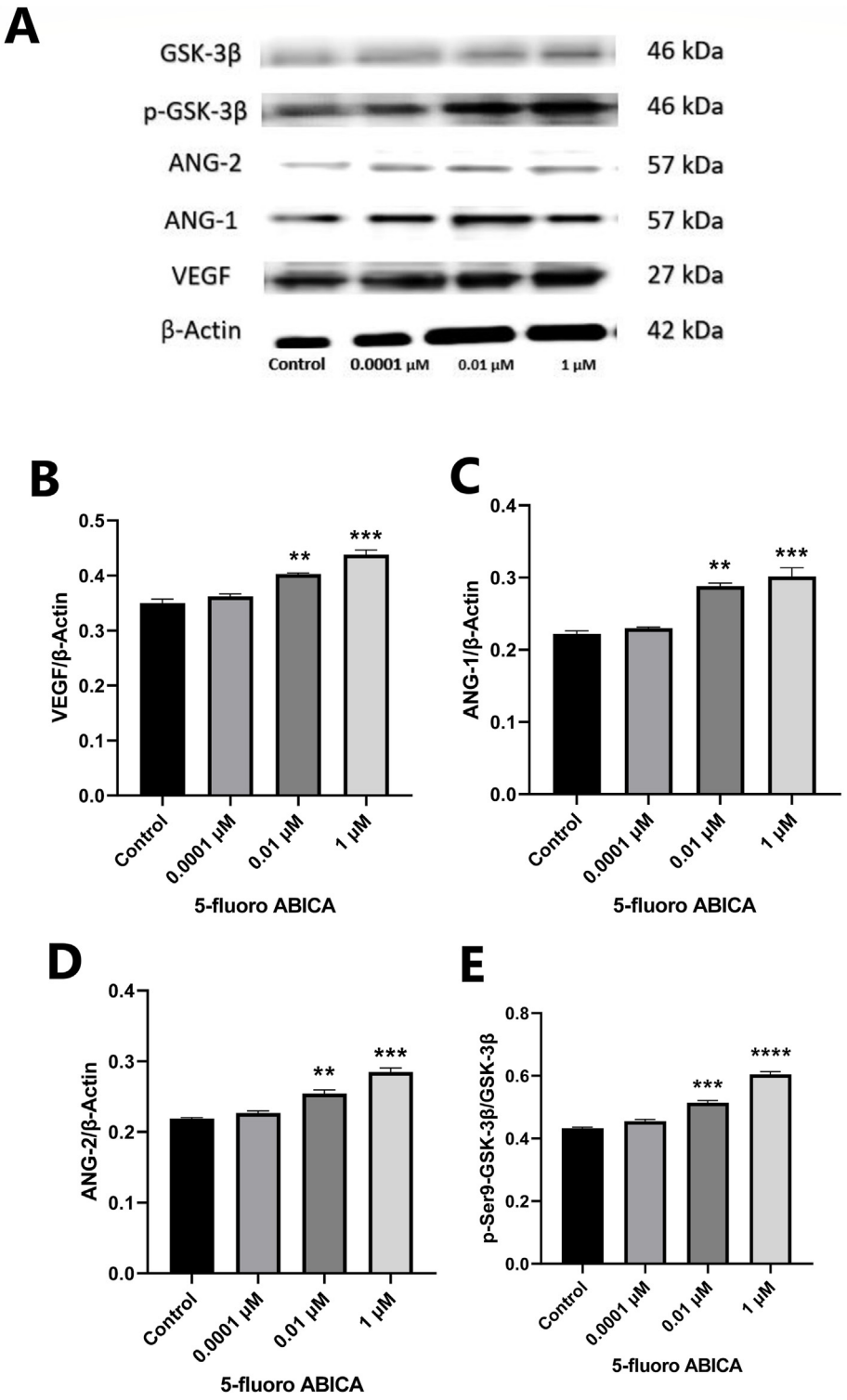


**Figure 7:** Effects of 5-fluoro ABICA treatment on the protein expression of VEGF, ANG-1, ANG-2, GSK-3β, and p-GSK-3β in HBMECs, assessed with western blotting. (A) Protein extracts were obtained from HBMECs treated with 5-fluoro ABICA at varying concentrations (0.1 μM, 0.01 μM, or 0.0001 μM). These extracts were then used to quantify the protein levels of VEGF, ANG-1, ANG-2, GSK-3β, and p-GSK-3β within the cells; β-actin served as the reference protein. Proteins were isolated with radioimmunoprecipitation assay lysis buffer containing phosphatase-protease inhibitors, and 20 μg of each protein sample was separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The separated proteins were transferred to a polyvinylidene fluoride membrane, which was blocked with 2 % bovine serum albumin and probed with primary antibodies during an overnight incubation. HRP-conjugated secondary antibodies were used to detect chemiluminescence signals. (B) The VEGF expression levels, normalized to those of β-actin, were significantly higher in cells treated with 5-fluoro ABICA than the control. (C) Significantly elevated ANG-1 in treated HBMECs. (D) Increase in ANG-2 expression after treatment with 5-fluoro ABICA compared with the control. (E) Substantial increase in the expression of p-GSK-3β with respect to GSK-3β. The data are presented as SD ± mean (n = 3). (****) indicates $p < 0.0001$, (***) indicates $p < 0.001$, (**) indicates $p < 0.01$.

distribution, cell-specific responses, culture conditions, and the complex interactions among these compounds. Although several studies have suggested that SCs promote cell survival, contrasting findings have suggested pro-apoptotic properties of SCs. For instance, the SC MAM-2201 has been shown to induce cytotoxicity and inhibit cell growth in astrocytes.[27] CP55940 and WIN552122 have been reported to inhibit proliferation in C6.9 glioma cells.[28,29] In contrast, other studies have reported that SCs such as HU-210, THJ-2201, and 5F-PB22 increase the proliferation of Neuro-2A cells and NG108-15 cells.[15,30] Our investigation of cell metabolic activity in vitro indicated that 5-fluoro ABICA significantly altered cell metabolic activity (MTT reduction) and increased the viable number of brain endothelial cells. These findings suggested that 5-fluoro ABICA has a specific concentration range at which it effectively promotes the metabolic activity of cells. This compound appears to have a narrow therapeutic window, given the narrow range of test dose effects. These observations align with findings from a prior study indicating stimulatory effects of XLR-11 on the cell metabolism rate in HBMECs.[31]

Angiogenesis is a requisite developmental process involving extracellular matrix degradation, chemotactic migration, proliferation of endothelial cells, and differentiation into a capillary-like structure. SCs have been shown to decrease proliferation and migration, and to induce proapoptotic effects. Most studies to date have demonstrated the ability of various SCs to exert anti-proliferative effects and induce apoptosis in various cell lines through targeting receptors beyond the classical CB1 and CB2.[32–35] In our study, a CB1 or CB2 ligand did not induce cell death, but markedly enhanced the cell migration and tube formation rates of the cells in the presence of 5-fluoro ABICA. These findings align with those from our previous studies on SCs, specifically XLR-11, 5F-MDMB-PICA, and AB-CHMINACA,[31,36,37] and their effects on HBMEC angiogenic ability. Although the effects of 5-fluoro ABICA on migration and the tube formation rates were concentration dependent and showed threshold effects, HBMECS showed significantly greater potential to enhance wound healing and tissue regenerative processes 24 h after initiation of the wound healing assay. These findings suggested that the drug might have potential therapeutic benefits at higher doses but might lack effects at lower doses. Therefore, further studies and clinical trials are required to determine the optimal drug concentration.

VEGF and angiopoietins are critical angiogenesis regulators. VEGF, the most potent angiogenic mediator of neovascularization, and normal and abnormal angiogenesis, acts through binding two specific membrane receptors, the tyrosine kinase receptors VEGFR-1 and VEGFR-2.[38] Activation of these receptors leads to endothelial cell survival, proliferation, vessel sprouting, and increased vessel permeability. Maintaining a basal level of VEGF expression is essential for vessel stability in mature conditions. However, elevated VEGF levels in hypoxic stromal or tumor cells contribute to the stimulation of branching angiogenesis. Angiopoietin belongs to a family of angiogenic factors affecting blood vessel formation and maintenance. Angiopoietins bind endothelial cells expressing the tyrosine kinase receptor Tie-2 and have multifaceted roles in modulating the equilibrium between promoting angiogenesis and triggering angiostasis. In the angiopoietin family, ANG-1 and ANG-2 have been extensively investigated and found to have contrasting effects: ANG-1 promotes angiogenesis via Ties2 activation, whereas ANG-2 has varying effects depending on the context, either facilitating or hindering angiogenesis.[39] Several in vitro experiments have revealed that Ang-1 and ANG-2, together with VEGF, constitute a platform that controls endothelial plasticity and angiogenesis.[40,41] The SCs XLR-11, AB-CHMINACA, and (R)-5-Fluoro-ADB[31,37,42] have all been found to elevate VEGF, ANG-1, and ANG-2 mRNA expression, secretion into the medium, and intracellular presence. In the present study, we identified significant upregulation of the expression of VEGF, ANG-1, and ANG-2 in HBMECs treated with 5-fluoro ABICA. Therefore, 5-fluoro ABICA has the potential to induce brain angiogenesis. Moreover, the drug has concentration-dependent effects on proangiogenic protein/mRNA expression levels, such that higher concentrations have more pronounced effects. However, these effects reached a plateau beyond a dose threshold, thereby indicating that the effectiveness of this drug may be limited to higher doses. Consequently, we concluded that 5-fluoro ABICA is likely to have an optimal therapeutic concentration for inducing brain angiogenesis between 1 and 0.01 μM. These concentration-dependent effects may be correlated with the complex nature of the HBMEC biological systems involved. For example, this drug may target specific pathways and proteins at low concentrations, whereas at higher concentrations, broader effects may occur and exert different effects.

GSK-3 serves as a nodal point of convergence of signaling mechanisms in endothelial cells. Non-phosphorylated active GSK-3β inhibits the migration of endothelial cells to the VEGF angiogenic factor and promotes apoptosis of endothelial cells. In contrast, phosphorylation of GSK-3β increases angiogenesis and enhances capillary formation.[7] Moreover, the activation of phosphorylated GSK-3β has been suggested to have anti-apoptotic properties and to promote cell survival through increasing the expression of anti-apoptotic proteins and inhibiting the transcription of pro-apoptotic genes, such as caspase9 and p53. GSK-3β also regulates angiogenesis by upregulating hypoxia inducible factor 1α (HIF-1α) expression, thereby inducing VEGF transcriptional activation.[43] Under hypoxia or low-oxygen conditions, HIF-1α, a master transcription factor regulating VEGF expression and oxygen homeostasis, promotes the expression of angiogenic factors such as VEGF and TGF-α[44]—key players in angiogenesis under oxygen scarcity. Interestingly, GSK-3β has an indirect role in suppressing angiogenesis in hypoxic conditions, by inducing the phosphorylation and subsequent degradation of HIF-1α. Consequently, VEGFR-2 downregulation inactivates hypoxia-associated angiogenesis factors, particularly those in the VEGF signaling pathway.[45]

Cannabinoids have been found to activate the PI3K/Akt pathway by interacting with the CB1 and CB2 receptors. Activation of Akt by cannabinoids leads to inactivation of GSK-3 by phosphorylation at specific sites. THC and HU-210 have neuroprotective effects on primary cortical neurons through stimulation of the PI3K/Akt pathway, thereby increasing phosphorylation of GSK-3β.[46] Another study has noted a decrease in the phosphorylated active form of GSK-3β in PC12 cells treated with arachidonyl-2-

chloroethylamide, cannabidiol, and WIN55,212-2.[47] Furthermore, HBMECs treated with XLR-11, AB-CHMINACA, and (R)-5-fluoro-ADB have shown elevated phosphorylated GSK-3β levels.[31,37,42,48] Our study revealed significant up-regulation of the levels of phosphorylated GSK-3β compared with GSK-3β, thus suggesting that 5-fluoro ABICA activates p-GSK-3β in a dose-dependent manner, and promotes cell proliferation and survival. Despite the lack of response at lower concentrations, which may indicate limited activity of the drug in upregulating the expression of p-GSK-3β at low doses or a need for high doses to be effective, the drug may activate various signaling pathways involved in GSK-3β phosphorylation.

## Conclusion

Overall, 5-fluoro ABICA exposure in vitro alters hallmarks of angiogenesis, such as proliferation, migration, and tube formation in HBMECs, and drives activation of proangiogenic factors and GSK-3β expression. Our findings suggested that 5-fluoro ABICA has a therapeutic concentration range between 1 μM and 0.01 μM. Notably, this study reports the first examination of the physiological effects of 5-fluoro ABICA, to our knowledge. However, these effects were observed in vitro, and the effects of the drug may be more complex in vivo, depending on variables such as the disease stage, the target cell or tissue, and the type of drug used. The incorporation of CB1R and CBR2 and additional proliferation assays will be critical in elucidating the precise signaling pathway triggered by 5-fluoro ABICA, thereby advancing understanding of its role in angiogenesis. Therefore, additional investigations are required to determine the optimal therapeutic concentration of 5-fluoro ABICA, and to fully understand the mechanism underlying its effects. Moreover, the potential and safety of 5-fluoro ABICA in treating human brain disorders should be investigated.

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## Conflict of interest

The authors have no conflict of interest to declare.

## Ethical approval

There are no ethical issues.

## Authors contributions

LA conceived and designed the study. LA and RA conducted research, collected and organized data, analyzed and interpreted data, and wrote the initial and final drafts of the article. All authors contributed to the manuscript's review. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

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