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RESEARCH ARTICLE

A Comparative Study on *In vitro* Anti-cancer and *In vivo* Anti-angiogenic Effects of TRPC Blockers Pyr-3 and SKF-96365

Hülya Tuba Kıyan[1,*], Ayca Üvez[2], Merve Erkisa[3,4], Elif İlkay Ikitimur-Armutak[2], Nadim Yılmazer[5], Osman Behzat Burak Esener[2], Deniz Erol Kutucu[6], Savaş Üstünova[7], Engin Ulukaya[8], A. Alper Öztürk[9] and Ebru Gürel-Gürevin[6]

[1]Department of Pharmacognosy, Faculty of Pharmacy, Anadolu University, Eskisehir 26470, Turkey; [2]Department of Histology and Embryology, Faculty of Veterinary Medicine, Istanbul University-Cerrahpasa, Istanbul 34500, Turkey; [3]Molecular Cancer Research Center, Faculty of Medicine, Istinye University, Istanbul, Turkey; [4]Department of Molecular Medicine, The Institute of Experimental Medicine, Istanbul University, Istanbul, Turkey; [5]Department of Biology, Faculty of Arts and Sciences, Tekirdag Namik Kemal University, Tekirdag, Turkey; [6]Department of Biology, Faculty of Science, Istanbul University, Istanbul 34134, Turkey; [7]Department of Physiology, School of Medicine, Bezmialem Vakif University, Istanbul, Turkey; [8]Department of Clinical Biochemistry, Faculty of Medicine, Istinye University, Istanbul, Turkey; [9]Department of Pharmaceutical Technology, Faculty of Pharmacy, Anadolu University, Eskisehir 26470, Turkey

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Abstract: ***Introduction*:** Angiogenesis is involved in many physiological and pathological conditions including cancer. A number of TRP channels induce angiogenesis, promote cell proliferation or induce apoptosis in several types of human cancers. Therefore, TRP channels may be considered potential pharmacological targets for therapeutic options of disorders caused by insufficient angiogenesis or aberrant vascularization.

***Aims*:** This study aimed to comparatively investigate *in vitro* anti-cancer and *in vivo* anti-angiogenic effects of TRPC blockers Pyr-3 and SKF-96365.

***Methods*:** For anti-cancer effects, four cancer cell lines (MDA-MB-231, A549, PC-3, and HCT-116) were used. *In vivo* anti-angiogenic effects were investigated by employing *in vivo* CAM assay of fertilized hen eggs.

***Results*:** Pyr-3 affected cell viability in a dose-dependent manner, all concentrations of SKF-96365 significantly reduced cell viability in all cell lines. Pyr-3 and SKF-96365 at concentrations of 2.5 µg/pellet and 50 µg/pellet, respectively inhibited *in vivo* angiogenesis significantly.

***Conclusion*:** The concentration of 2.5 µg/pellet caused no irritation, whereas 50 µg/pellet produced some slight irritation. Apart from their anti-cancer effects, our findings indicate that Pyr-3 and SKF-96365 may be promising anti-angiogenic agents for the treatment of angiogenesis-related disorders.

Keywords: Pyr-3, SKF-96365, cancer, angiogenesis, *in vivo* CAM assay, corpus luteum formation.

1. INTRODUCTION

Blood nourishes the cells of the body with oxygen and nutrients they need and removes waste and carbon dioxide away from them during both embryonic development and postnatal life. The blood vessels transport blood throughout the body, therefore they are needed to sustain life, because all of our cells rely on their functionality [1, 2]. In case of the increased requirements of oxygen and nutrients, a new vascular network is formed (neovessel formation). For example, neovessel formation occurs in embryo growth, corpus luteum formation, endometrial thickening during the menstrual cycle, embryo implantation and placentation, skeletal muscle growth and remodeling, bone morphogenesis, and wound healing. This process is represented by vasculogenesis, angiogenesis, and arteriogenesis [3]. Neovessel formation is activated by multiple pro-angiogenic signals, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), platelet derived growth factor (PDGF), epi-

*Address correspondence to this author at the Department of Pharmacognosy, Faculty of Pharmacy, Anadolu University, Eskisehir 26470, Turkey; E-mail: htkiyan@anadolu.edu.tr

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dermal growth factor (EGF), stromal derived factor-1a (SDF-1a), placental growth factor (PGF), cytokines as interleukins (IL-1, IL-8), prostaglandin E2 (PGE2), angiopoietin-2, angiostatin, angiogenin, proliferin, endostatin, prolactin, interferon-α and β, tumor necrosis factor-α (TNF-α), vasostatin, and regulatory proteins and cell adhesion molecules which are released from the extracellular matrix (ECM) and the cells surrounding ECM [4, 5]. Apart from these, endothelial Ca^{2+} signals play a crucial role in angiogenesis and arteriogenesis [3].

The transient receptor potential (TRP) channels are cation-specific ion channels, most of which are permeable for Ca^{2+}, and some also for Mg^{2+} and Na^{+} [6, 7]. In vertebrates, they are divided into seven subfamilies by amino acid sequence homology: TRPC ("C" for canonical), TRPV ("V" for vanilloid), TRPM ("M" for melastatin), TRPN ("N" for NOMPC-like), TRPA ("A" for ankyrin), TRPP ("P" for polycystic), and TRPML ("ML" for mucolipin). Each subfamily contains at least one vertebrate member; mammals bear 28 members from all subfamilies except TRPN as follows: TRPC1-7 (seven members), TRPV1-6 (six members), TRPM1-8 (eight members), TRPA1 (one member), TRPP2, TRPP3, and TRPP5 (three members), and TRPML1-3 (three members) [8-10]. It has long been known that a number of TRP channels, *e.g*., TRPV4, TRPC1, and TRPC6, promote vasculogenesis, angiogenesis, and arteriogenesis by mediating extracellular Ca^{2+} entry into cells [11]. Insufficient angiogenesis may cause ischemic disorders, including myocardial infarction, stroke, peripheral artery disease, ischemic retinopathies, and pre-eclampsia, and other diseases such as hypertension, neurodegenerative disorders and obesity-associated diseases, whereas aberrant vascularization promotes various diseases such as cancer, atherosclerosis, inflammatory (*e.g*., arthritis, psoriasis) and eye diseases, and pulmonary arterial hypertension [3, 5]. Therefore, TRP channels may be considered potential pharmacological targets for future therapeutic options of above-mentioned pathologies. Additionally, several TRP channels are overexpressed in several types of human cancers to promote cell proliferation or to induce apoptosis or inhibit metastasis, and this makes them new prognostic parameters [11-16].

In the current study, we aimed to comparatively investigate *in vitro* anti-cancer and *in vivo* anti-angiogenic effects of two TRPC blockers, Pyr-3 and SKF-96365, by employing cultured human cancer cell lines and chick CAM assay.

2. MATERIALS AND METHODS

2.1. *In vitro* Anti-cancer Effects

2.1.1. Chemicals

The following chemicals were used as received in this study: RPMI 1640 medium (Gibco, Grand Island, NY, USA), penicillin G (Gibco, Grand Island, NY, USA), streptomycin (Gibco, Grand Island, NY, USA), L-glutamine (Gibco, Grand Island, NY, USA), 10% fetal bovine serum (Gibco, Grand Island, NY, USA), Sulforhodamine B (Sigma,USA), trichloroacetic acid (Sigma,USA), acetic acid (Sigma,USA), and Tris base (Sigma,USA). Pyr-3 and SKF-96365 were obtained from Abcam, UK.

2.1.2. Cell Culture

The cell lines being human breast cancer (MDA-MB-231), lung cancer (A549), prostate cancer (PC-3), and colon cancer (HCT-116) were purchased from American Type Culture Collection (Manassas, Virginia, United States). Cells were grown in RPMI 1640 medium supplemented with penicillin G (100U/ml), streptomycin (100 µg/ml), L-glutamine, and 10% fetal bovine serum in a humidified incubator at 37°C and 5% CO_2.

2.1.3. Sulforhodamine B (SRB) Assay for cell viability

Cells were seeded at a density of 5×10^3 cells/well in 96-well plates and treated with different concentrations of TRPC blockers (0.62, 1.25, 2.5, 5, 10, 20, and 40 µg/ml of Pyr-3, 3.2, 6.25, 12.5, 25, 50, 100, and 200 µg/ml of SKF-96365) for 48 h. Then, cells were *in situ* fixed with 50 µL of 50% (w/v) trichloroacetic acid at 4°C for 1 h. At the end of fixation, wells were washed 5 times with deionized water and allowed air dry at room temperature for 50 µL of 0.4% SRB solution was added into each well and incubated at room temperature for 30 min. Then, plates were washed with 1% acetic acid to eliminate non-specific bindings and unbounded dye. The bounded SRB dye was dissolved by adding 150 µL 10 mM Tris base per each well. Measurements were carried out at 564 nm using Lumistar Omega microplate reader. Cell viability of treated cells was calculated in reference to the untreated control cells using the formula as viability (%) = 100 × (Sample Absorbance)/(Control Absorbance). The experiments were performed in triplicate.

2.2. *In vivo* Anti-angiogenic Effects

2.2.1. Fertilized Eggs and Chemicals

The fertilized hen eggs were provided by a hatchery (Has Tavuk Company, Bursa and Sivrihisar, Turkey). Immediately after collection, the eggs were transported to our laboratory in a cooler. Standards have been provided from commercial sources: agarose (Sigma-Aldrich, Germany), SDS (Fluka-Biochemika, Germany), and (±)-Thalidomide (Sigma-Aldrich, Germany).

2.2.2. Chick Chorioallantoic Membrane (CAM) Assay

The *in vivo* CAM assay was performed as described in our previous articles [17, 18]. As soon as the eggs arrived in our lab (day 0), they were examined by means of visual inspection for any damage. The study eggs were disinfected with ethanol and then placed in a humidified incubator that can maintain 36.5°C and 80% relative humidity (RH). During the first 72 h of incubation, the horizontally positioned eggs are rotated several times to prevent attachment of the embryo to the eggshell. On day 3, 10-15 mL of albumen was aspirated from a hole on the pointed end, and then the eggs were opened on the snub end; they were traced with a scalpel at the two third of the height from the pointed end, and the shells were removed with sterile forceps. These holes are sealed with stretch film to prevent dehydration and contamination of the embryo before incubation at 36.5°C/80% RH for another 72 h. The pellets of the experimental, blank, and control compounds were placed on the CAM (1 pellet/egg), and the holes were sealed again (day 6). After a 24-hours of

incubation, the eggs were scored for angiogenesis under a stereo-microscope. 10-15 eggs were evaluated for each compound. Three independent experiments were performed for each compound.

2.2.2.1. Preparation of Pellets

Pyr-3 (2.5 and 5 µg/pellet) and SKF-96365 (50 and 75 µg/pellet) and all standards (50 µg/pellet) were suspended in a 2.5% (w/v) agarose solution. For ease of application, the pellets of these solutions (10 µL) were prepared, and applied dropwise on circular stainless-steel supports of 5 mm diameter, cooled down to room temperature for solidification, and subsequently applied to the CAM on the 6th day of the incubation.

2.2.3. Scoring the Anti-angiogenic Effects on the CAM

In vivo anti-angiogenic potentials were evaluated using a semi-quantitative microscopic angiogenesis score system (Kıyan *et al*., 2014) [17]. The anti-angiogenic scores were calculated using the formula [1]. (±)-thalidomide and sodium dodecyl sulphate (SDS) at the concentration of 50 µg/pellet were also tested as positive and negative controls, respectively. CAMs treated with solidified agarose solution in pellet form (2.5%, w/v) were also used as blank. Each compound was tested in triplicate [17, 18].

Formula (1) used in angiogenesis scoring:

$$Average\ score = \frac{Number\ of\ eggs\ (score\ 2)\ x\ 2 + number\ of\ eggs\ (score\ 1)\ x\ 1}{Total\ number\ of\ eggs\ (score\ 0,\ 1,\ 2)}$$

2.3. Statistical Analysis

One-way ANOVA followed by Tukey's Multiple Comparison Test in Graphpad Prism 6.0 statistical software for Windows was used to determine statistical significance. A p-value ≤0.05 was considered statistically significant. Activity results were expressed as mean ± standard deviation (SD).

3. RESULTS

3.1. *In vitro* Anti-cancer Effects of Pyr-3 and SKF-96365

Upon treatment with different concentrations of Pyr-3 (0.62, 1.25, 2.5, 5, 10, 20, and 40 µg/ml) for 48 h the results of the cell viability assay for the cell lines of this study are presented in Fig. (**1**). As seen from the results, Pyr-3 decreased cell viability in a dose-dependent manner, and MDA-MB-231 cells could not survive nearly at all concentrations whereas PC-3, HCT-116 and A549 cells were affected only at higher concentrations. For 48-h treatment, the calculated IC_{50} values of Pyr-3 were 15.4 µg/ml, 7.9 µg/ml, 1.1 µg/ml, and 2.3 µg/ml for A549, HCT-116, MDA-MB-231, and PC-3 cells, respectively (Table **1**).

Unlike Pyr-3, all concentrations of SKF-96365 (3.2, 6.25, 12.5, 25, 50, 100, and 200 µg/ml) significantly reduced cell viability in all cell lines in a dose-dependent manner. Even the lowest concentration (3.2 µg/ml) resulted in a significant decrease in the percentage of viable HCT-116 cells (Fig. **2**). The calculated IC_{50} values of SKF-96365 for A549, HCT-116, MDA-MB-231 and PC-3 cells were 11.3 µg/ml, 3.2 µg/ml, 5.3 µg/ml and 7.2 µg/ml at 48 h, respectively (Table **1**).

3.2. In vivo Anti-angiogenic Effects of Pyr-3 and SKF-96365

The results of *in vivo* CAM assay are summarized in Table **2**. Furthermore, anti-angiogenic effects were scaled, scored and described based on the observations on CAM

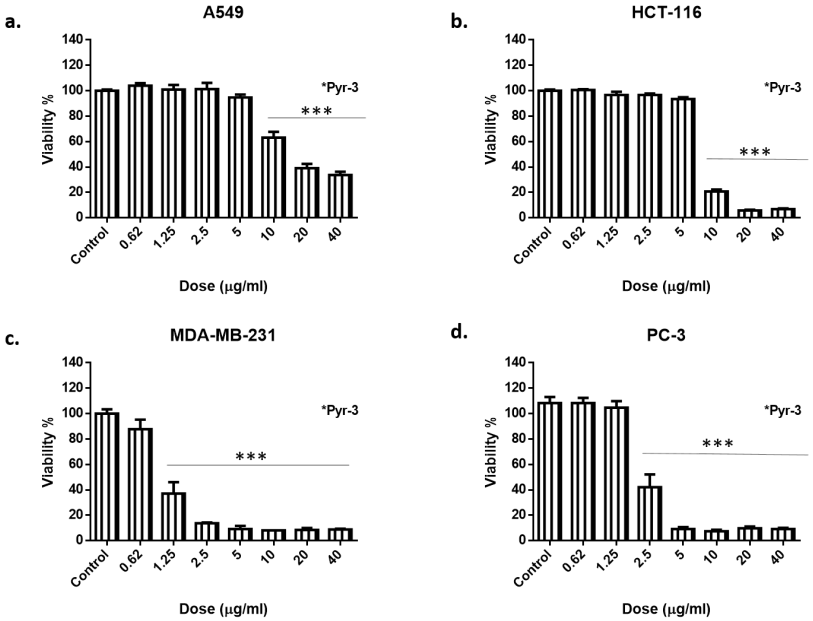

Fig. (1). Cell viability of A549 (**a**), HCT-116 (**b**), MDA-MB-231 (**c**), and PC-3 (**d**) cell lines treated with Pyr-3 for 48 h. Pyr-3 suppresses cell viability in a dose dependent manner. **Note:** * denotes statistically significant differences in comparison with the control: ***($p < 0.001$). Data are presented as mean ± SD (n=3). (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

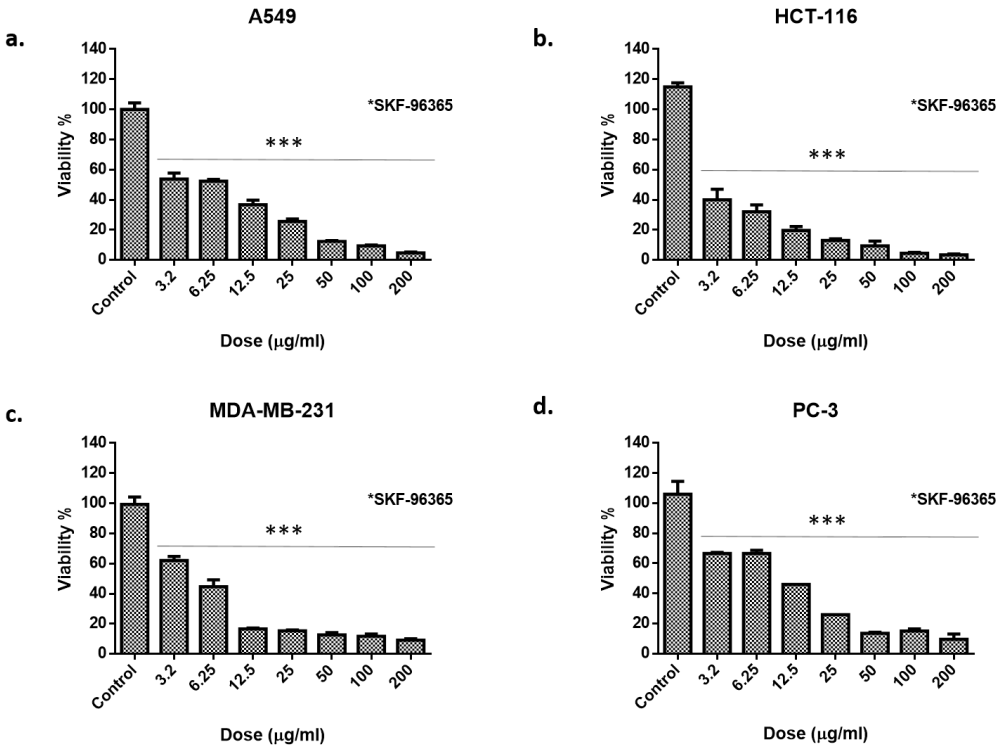

Fig. (2). Cell viability of A549 (**a**), HCT-116 (**b**), MDA-MB-231 (**c**), and PC-3 (**d**) cell lines treated with SKF96365 for 48 h. SKF96365 suppresses cell viability in a dose dependent manner. **Note:** * denotes statistically significant differences in comparison with the control: ***($p < 0.001$). Data are presented as mean ± SD (n=3). (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Table 1. IC_{50} values of Pyr-3 and SKF-96365 at 48 h in the studied cancer cell lines.

Cell Lines	IC_{50} Values	
	Pyr-3	SKF-96365
A549	15.4 ± 1.6	11.3 ± 0.6
HCT-116	7.9 ± 0.8	<3.2
MDA-MB-231	1.1 ± 1.8	5.3 ± 1.2
PC3	2.3 ± 2.2	7.2 ± 0.2

Table 2. *In vivo* anti-angiogenic effects of Pyr-3 and SKF-96365.

Compounds	Concentration (µg/pellet)	Average Anti-angiogenic Score[a]	Anti-angiogenic Potential	Irritation/Embryotoxicity (%)
Pyr-3	2.5	1.00 ± 0.27	Strong	-
Pyr-3	5	1.25 ± 0.35	Very strong	41.66 ± 35.3
SKF-96365	50	1.0 ± 0.12	Strong	57.14 ± 10.0
SKF-96365	75	0.88 ± 0.22	Strong	85.71 ± 14.02
Agarose (Blank)	%2.5, w/v	0.5 ± 0.15	Inactive	-
(±)-thalidomide (Positive control)	50	0.8 ± 0.2	Strong	-
SDS (Negative control)	50	0.3 ± 0.2	Inactive	85 ± 7

Note: Values are mean of average score ± SD (n = 45 for the compounds, and n = 30 for the standards), $p < 0.05$ compared with (±)-thalidomide.

Table 3. Semi-quantitative microscopic angiogenesis score system.

Scale	Anti-angiogenic Effect	Effects Observed on CAM following the Treatment
<0.5	Inactive	No change (Normal embryo growth)
0.5-0.75	Weak	No capillary free area Area with reduced density of capillaries around the pellet not larger than its own area
>0.75-1	Strong	Small capillary free area or area with significantly reduced density of capillaries Effects not larger than double the size of the pellet
>1	Very strong	Capillary free area around the pellet at least double the size of the pellet

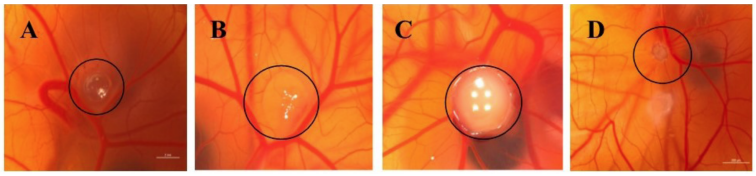

Fig. (3). *In vivo* anti-angiogenic results of CAM assay: (**A**) Strong anti-angiogenic effect of (±)-thalidomide (average score = 0.8 ± 0.2; 50 µg/pellet); (**B**) Strong anti-angiogenic effect of Pyr-3 (average score = 1.00 ± 0.27; 2.5 µg/pellet); (**C**) Strong anti-angiogenic effect of SKF-96365 (average score = 1.0 ± 0.12, and 57.14 ± 10.0% irritancy; 50 µg/pellet); (**D**) Irritant effect of SDS (85 ± 7%; 50 µg/pellet). Note: White spots may be the reflection of the pellet surface, or some fragments of the egg shell. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

(Table **3**) by using the semi-quantitative microscopic angiogenesis score system.

As photographed in Fig. (**3**), stereo-microscopic observations revealed that 5 µg/pellet Pyr-3 exhibited a very strong anti-angiogenic effect with a score of 1.25 ± 0.35, but with slight irritant potential (41.66 ± 35.3%), whereas 2.5 µg/pellet Pyr-3 showed a strong activity with a score of 1.00 ± 0.27 without any membrane irritation or embryotoxicity when compared to the positive control (±)-thalidomide (0.8 ± 0.2, 50 µg/pellet). At the concentrations of both 50 and 75 µg/pellet, however, SKF-96365 showed a strong anti-angiogenic effect with scores of 1.0 ± 0.12 and 0.88 ± 0.22, respectively; but the former caused a slight membrane irritation (57.14 ± 10.0%), while the latter produced a strong membrane irritation (85.71 ± 14.02).

4. DISCUSSION

To the best of our knowledge, the novelty of the current study includes: 1) This is the first comparative study that investigates the effects of Pyr-3 and SKF-96365 on cancer cell proliferation and angiogenesis; 2) The human colon cancer cell line HCT116 was *in vitro* used in a study of TRPC blockers for the first time; 3) The *in vivo* CAM assay was first employed to evaluate the effects of these blockers in angiogenesis.

Of TRPC channels, TRPC3 serves to control specific functions in the cardiovascular system, the brain, the immune system, cancer progression, and tissue remodeling [19, 20]. Previous studies have shown that TRPC channels, especially TRPC3 and TRPC6, govern proliferation and migration of a variety of cancer cells, including bladder [21], blood [22], brain [15, 23-26], breast [13, 27], cervix [28], colon [29], kidney [30], liver [31], lung [14, 32], oesophagus [33], ovary [34, 35], skin [36], stomach [37, 38], and prostate [39-41]. To elucidate the role of TRP channels in these cancer cells, TRP blockers have been commonly used. For example, the studies by Bomben and Sontheimer [23], Song *et al*. [26], Yang *et al*. [14], Cai *et al*. [37], Jing *et al*. [29], and Selvaraj *et al*. [41] used SKF-96365, whereas Pyr-3 was the blocker in the studies by Abdoul-Azize *et al*. [22], Chang *et al*. [15], and Lin *et al*. (2021) [38]. In these studies, both blockers inhibited the proliferation of cancer cells, being congruent with the results of our study. The imidazole derivate SKF-96365 (1-(beta-[3-(4-methoxyphenyl] propoxy]-4- methoxyphenethyl)-1H-imidazole) is a nonselective TRP blocker [19, 42], and Pyr-3 (Ethyl-1-(4-(2, 3,3-trichloroacrylamide) phenyl)-5-(trifluoromethyl)-1H-pyrazole-4-carboxylate) is a TRPC blocker with a striking preference for TRPC3 [15, 19]. In this study, all concentrations of SKF-96365 significantly reduced cell viability in all cell lines can be explained by the fact that it is a nonselective blocker. Similarly, the selectivity of Pyr-3 on TRPC3 might cause a decrease in cell viability in a dose-dependent manner.

A non-animal and well-designed test, *in vivo* CAM assay is cheap and simple to carry out, produces reproducible results, and offers great opportunity for the study of anti-angiogenic, irritant, anti-inflammatory effects and embryotoxic responses of natural products, cosmetics as well as chemicals and synthetic drugs [17, 43-45]. It is also the most preferred *in vivo* angiogenic assay in the studies of cancer biology and therapy to reveal angiogenic responses [46, 47]. As far as we know, the anti-angiogenic effects of Pyr-3 and SKF-96365 have never been tested on CAM before the current study. In animal studies, Chen *et al*. [48] found that, in addition to blocking tumor growth, SKF-96365 destroyed tumor feeding vessels. TRPC3 silencing with siRNA significantly suppressed endothelial tube formation, an indicator of

angiogenesis [49]. Similarly, silencing the expression of TRPC3, TRPC4, or TRPC5 also inhibited tube formation in human umbilical vein-derived endothelial cell line EA.hy926 and human umbilical vein derived endothelial cells [50]. Furthermore, the pharmacological blockade of TRPC3 with Pyr-3 inhibited the proliferation of endothelial precursor cells, suggesting that this channel might be relevant for angiogenesis [51]. Concerning the participation of TRP channels in angiogenesis, more studies can be found in Smani *et al.* studies [52]. Being congruent with the literature, our study revealed that Pyr-3 and SKF-96365 at concentrations of 2.5 µg/pellet and 50 µg/pellet, respectively, inhibited *in vivo* angiogenesis significantly. With no or slight irritation, these concentrations may have potential therapeutic benefits.

In conclusion, this study provides the first evidence of *in vivo* anti-angiogenic effects of Pyr-3 and SKF-96365 on CAM. It indicates that TRPC are key regulators of both cancer cell proliferation and angiogenesis.

CONCLUSION

Therapeutic targeting of angiogenesis may be an effective strategy to treat angiogenesis-related pathologies such as cancer. Due to the regulatory role of TRPC channels in angiogenesis and cancer progression, these channels may have great potential as therapeutic targets in clinical medicine. In search of new bioactive agents that are more effective with less toxicity, TRPC blockers such as Pyr-3 and SKF-96365 may offer significant hope as novel angiogenesis inhibitors for the future treatment of related diseases.

LIST OF ABBREVIATIONS

bFGF	=	Basic Fibroblast Growth Factor
EGF	=	Epidermal Growth Factor
IL-1, IL-8	=	Cytokines as Interleukins
PDGF	=	Platelet Derived Growth Factor
PGE2	=	Prostaglandin E2
PGF	=	Placental Growth Factor
SD	=	Standard Deviation
SDF-1a	=	Stromal Derived Factor-1a
TNF-α	=	Tumor Necrosis Factor-α
TRP	=	Transient Receptor Potential
VEGF	=	Vascular Endothelial Growth Factor

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data and supportive information is available within the article.

FUNDING

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Declared none.

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