VIA MEDICA

ORIGINAL ARTICLE

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Impact of angiogenesis inhibitors on inflammatory activation in human vascular endothelial cells

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Medical Research Journal 2024;
DOI: 10.5603/mrj.101643

ISSN 2451-2591
e-ISSN 2451-4101

ABSTRACT

Background: The initiation and progression of inflammation can elevate the secretion of angiogenesis activators, which bind to receptors on endothelial surfaces, thereby stimulating cell proliferation and enhancing migration which has emerged as a significant risk factor for atherosclerosis.

Material and methods: To investigate the effectiveness of angiogenesis inhibitors on changes in blood vessels, the study utilized the anti-angiogenic drugs bevacizumab, pazopanib, and KRN-633. In the study, the vascular model comprised primary human coronary artery endothelial cells (pHCAECs). Moreover, the inflammatory response was induced by the pro-inflammatory cytokine tumour necrosis factor-α (TNF-α).

Results: The compounds' effect on pHCAECs induced structural changes within the actin cytoskeleton, demonstrating the presence of entosis and apoptotic vesicle-like structures. Additionally, inflammation in the pHCAEC line exacerbated the effects of the compounds used in the study, leading to heightened disintegration of cellular cytoskeletons. Conversely, pazopanib in combination with TNF-α induced the formation of vesicular structures along the course of F-actin retraction fibres in migrating pHCAECs. Furthermore, KRN-633 combined with TNF-α resulted in the translocation of VE-cadherin to the cell nucleus in these cells.

Conclusions: It is noteworthy that current treatments for cardiovascular diseases are not entirely effective. The utilization of functional pharmacological compounds such as angiogenesis inhibitors may provide an effective approach to treating disorders and regulating cardiovascular function.

Keywords: endothelium, inflammation, angiogenesis, TNF-α, bevacizumab, pazopanib, KRN-633

Med Res J 2024

Introduction

Chronic inflammation is a significant element in the pathogenesis of cardiovascular diseases, which can be caused by endothelial dysfunction. During inflammation, the endothelium experiences dysfunction due to an elevated production of reactive oxygen species (ROS), pro-inflammatory cytokines, matrix metalloproteinases (MMPs), adhesion molecules, and disturbances in vascular tone [1–3].

Because of the local production of chemokine, leukocyte recruitment is one of the most characteristic hallmarks of inflammation [4]. Leukocyte recruitment involves a complex cascade of sequential signalling and adhesion steps, which lead to leukocyte migration through endothelial cells (ECs) [5]. This process necessitates swift and irreversible modifications in the functional expression of adhesion molecules, reorganization of cytoskeletal elements, and coordinated movements of cell and vesicular membranes [6, 7]. Leukocyte migration from blood vessels occurs via one of two routes. The first one is the intercellular (paracellular) route which takes place between ECs. It involves adhesion molecules, which facilitate connections between ECs [8]. The second type is transcellular, occurring through the endothelial cell body while maintaining connections intact [9, 10]. Notably, conditions that activate endothelial actin stress fibres reduce transcellular migration, thereby promoting increased intercellular migration. However, inhibiting the formation of stress fibres induces the creation of transitional pores in the endothelium, facilitating the transcellular extravasation

of leukocytes [11, 12]. Inflammation has the potential to stimulate angiogenesis, and the development of novel vessels may enhance tissue inflammation [13]. In response to angiogenic stimuli, ECs transform the quiescent phenotype into an active one, characterized by an enhanced capacity to migrate, matrix proteolysis, and a high mitotic index [14]. Moreover, activated ECs change the dynamics of closing and short-circuiting connections that exist between perivascular cells and neighbouring ECs and involve proteins such as claudins, vascular endothelial cadherin (VE-cadherin), neural cadherin (N-cadherin), occludin, and junctional adhesion molecule (JAMs) [15, 16].

Angiogenesis is the formation and maintenance of blood vessel structures conducted by vascular endothelial growth factor (VEGF), which is an additive responsible for vascular permeability and cell migration [17]. The mechanism based on the difference in VEGF gradient is termed sprouting angiogenesis, whereas, in its absence, it is known as invagination or division angiogenesis. In the intussusceptive angiogenesis model, intraluminal tissue pillars form through contact between ECs from opposing capillary walls or via fusion of interstitial protrusions [18].

Optimal antiangiogenic therapy strategies are becoming extremely important in effectively and successfully introducing them into the treatment of cardiovascular diseases. These are therapies based on substances that recognize and inhibit the activity of VEGF factors, such as bevacizumab, pazopanib, and KRN-633 [19, 20]. Bevacizumab is a chimeric monoclonal antibody containing the muMAb A4.6.1 monoclonal antibody with IgG1 immunoglobulin. It has been observed that inhibiting the activity of VEGF receptors (VEGFR-1 and VEGFR-2) in ECs, significantly enhances ECs proliferation and angiogenesis ultimately inhibiting the proliferation of ECs and angiogenesis [21]. Pazopanib is a small-molecule tyrosine kinase inhibitor (TKI) focused on platelet-derived growth factor receptors α and β (PDGFR-α, -β), VEGFR-1, -2 and -3, fibroblast growth factor receptor-1, -2 (FGFR-1 and -2) and the c-Kit matricellular factor receptor [22]. Therefore, it effectively inhibits numerous pathways affecting cell proliferation and angiogenesis [23]. KRN633, a quinazoline-urea derivative, effectively and selectively inhibits intracellular VEGF signalling, VEGFR-1, -2, and -3 tyrosine kinases, as well as PDGFR and c-Kit. It has been demonstrated that the primary mechanism of action of KRN633 involves inhibiting VEGFR-2 phosphorylation, thereby blocking the response of ECs and angiogenesis *in vitro* [24, 25].

Materials and Methods

Cell culture

To assess the correlation between the effects of anti-angiogenic substances and inflammatory activation of human vascular endothelium, primary and immortalized endothelial model cells obtained from the American Type Culture Collection (ATCC) cell bank were used in this study. In the study, the Human Coronary Artery Endothelial Cells (PHCAEC) cell line was used. The cells used in the study were obtained from a young Caucasian individual whose death was not due to a cardiovascular event. The cells that comprise the research material in this study were cultivated in sterile culture vessels designed for adherent cells with a surface area of 25 cm^3 (Eppendorf, Falcon), by the recommendations of the ATCC cell bank. The basic medium for PHCAEC cell line was the liquid growth medium supplemented with a growth kit, which, due to the specification of the research, consisted of recombinant human vascular endothelial growth factor (rhVEGF), recombinant human epidermal growth factor (rhEGF), recombinant human fibroblast growth factor (rhFGF), recombinant human insulin-like growth factor 1 (rhIGF-1), L-glutamine, heparan sulphate, hydrocortisone, ascorbic acid and 2% foetal bovine serum (FBS) (ATCC). In order to prevent bacterial contamination, the culture medium was enriched with a mixture of penicillin, streptomycin, and amphotericin B. Cells were cultivated under constant humidity, at a temperature of 37°C and in an atmosphere of 5% CO_2.

Cell treatment

To initiate the inflammatory reaction, pHCAECs were treated with recombinant human tumour necrosis factor α (TNF- α) (Sigma-Aldrich). Based on literature data, the concentration of rhTNF-α that induces an inflammatory reaction in endothelial cell lines is 100 ng/ml. Therefore, this concentration was adopted as the working concentration for the compound. Cell populations treated with rhTNF-α were incubated for 24 hours under conditions of constant humidity, 37°C temperature, and 5% CO_2. To observe the inhibition of the angiogenesis mechanism by biologically and chemically active compounds, pHCAEC were experimentally treated with selected concentrations of bevacizumab, pazopanib, and KRN-633 (TargetMol). These concentrations were based on literature data describing clinically used doses and the

IC50 values of each compound. All mentioned compounds, at the same concentrations, were also used in a 1:1 combination with rhTNF-α. Cells were incubated with these compounds for 24 hours. Simultaneously, an untreated population of cells was cultured and supplemented with a complete nutrient medium to serve as the control sample for the study. After the designated incubation period, both control and treated cells were analysed using qualitative methods to assess changes in the expression of selected proteins. This assessment was conducted to observe the effects of anti-angiogenic substances under physiological conditions and the influence of an inflammation activator.

Immunofluorescence

For the immunofluorescence assessment of protein localization, pHCAEC cells were seeded on sterile coverslips (18 mm) and placed in a 12-well plate (Corning) under standard conditions (37C, 5%CO_2) until the cells reached a state close to the confluence. Human endothelial cells underwent treatment in the subsequent arrangement: rhTNF-α, bevacizumab, pazopanib, KRN-633, and the combination: rhTNF-α + bevacizumab, rhTNF-α + pazopanib, rhTNF-α + KRN-633 and incubated for 24 h. At the same time, each set of treated cells had control-containing cells cultured without the presence of stress factors. After completing the 24-hour incubation, the cells were fixed with a 1% paraformaldehyde PFA solution (Sigma-Aldrich) for 20 minutes at room temperature (RT). To permeabilize the membrane, 1 mL of 0.25% Triton X-100 solution was added to the cells and left for 10 minutes. The next step was to block the non-specific background reaction using 1% (pHCAEC cell line) BSA (20 min, RT). Immediately after the background blocking procedure, double labelling for junctional proteins (ThermoFisher Scientific) was performed using a primary mouse anti-VE-cadherin antibody (1:100, Invitrogen) and a secondary antibody conjugated to Alexa Fluor 594 nm (1:200, Invitrogen). Both mentioned antibodies were incubated for 1 h (RT, dark), respectively. After the designated time, F-actin filaments were labelled using fluorescent phalloidin conjugated with FITC (1:40, Invitrogen) for 20 minutes (RT, dark). Cell nuclei were stained with DAPI counterstain (1:20,000) for 10 min. (RT, dark). All mentioned procedures above were preceded by rinsing with phosphate-buffered saline (PBS) solution for ongoing removal of residues after subsequent stages of the immunofluorescence reaction.

The stained slides were mounted with Aqua Poly/Mount medium and evaluated using a C1 laser scanning confocal microscope and an oil objective VC plan Apo 60×/1.4 and Nikon EZ-C1 software 3.80 (Nikon).

The lasers used for DAPI excitation, Alexa Fluor 488 and Alexa Fluor 594, are a 408 nm diode with a 450/35 emission filter, a 488 nm diode with a 515/30 emission filter, and a 543 nm He-Ne with a 650LP emission filter, respectively. Triple-labelling images were generated using the same parameters, including laser power, pixel exposure time, channel gain, and resolution.

Results

Fluorescence techniques and a confocal microscope were utilized to evaluate the organization and localization of F-actin and VE-cadherin in primary pHCAEC cells. The visualization of fluorescently stained structures allowed for the assessment of organizational changes in F-actin and localization of VE-cadherin in examined cells cultured under the influence of individual drugs and TNF-α. Control cells were characterized by an organized F-actin network, which encouraged mutual interactions between neighbouring cells and cooperation in mechanical signal transduction. Nonetheless, VE-cadherin was predominantly located within the sites of intercellular interaction in pHCAECs (Fig. 1a).

After 24 hours of incubation of pHCAECs with TNF-α, the organization of F-actin changed. Under the influence of TNF-α, F-actin stress fibres were rebuilt into parallel bundles of stress fibres, which resulted in the manifestation of a spindle-shaped or swollen cell shape. Due to the change in the organization of F-actin, intercellular spaces were highlighted, which indicates a limited barrier function of the examined cells. However, the junctional contact between cells assessed using fluorescently labelled VE-cadherin was limited to point interactions (Fig. 1b).

Treatment of pHCAEC cells with bevacizumab for 24 hours led to a significant expansion of the actin cytoskeleton, which resulted in cell swelling and the appearance of a continuous-discontinuous nature of intercellular interactions (Fig. 2a). Additionally, in places with continuous intercellular connections, a tendency to the phenomenon of entosis was observed. Cells were also observed about the typical stress organization of F-actin and the complete lack of interaction with neighbouring cells. The occurrence of cells with actin

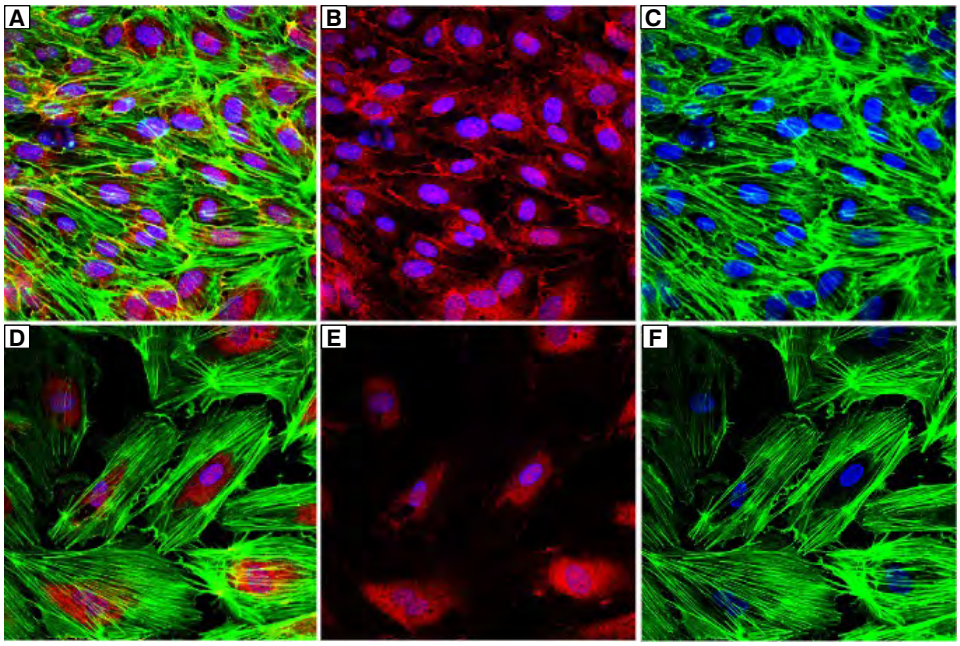

Figure 1A. Representative confocal image showing F-actin (green) and VE-cadherin (red) fluorescence in control pHCAECs. Cell nuclei were labelled with DAPI (blue). A1. Overlay; B1. DAPI + VE-cadherin; C1. DAPI + F-actin. (Magnification ×100) Bar = 100μm.

Figure 1B. Representative confocal image showing F-actin (green) and VE-cadherin (red) fluorescence in pHCAECs treated with 100 ng/ml TNF-α. Cell nuclei were labelled with DAPI (blue). A2. Overlay; B2. DAPI + VE-cadherin; C2. DAPI + F-actin. (Magnification ×100) Bar = 100μm

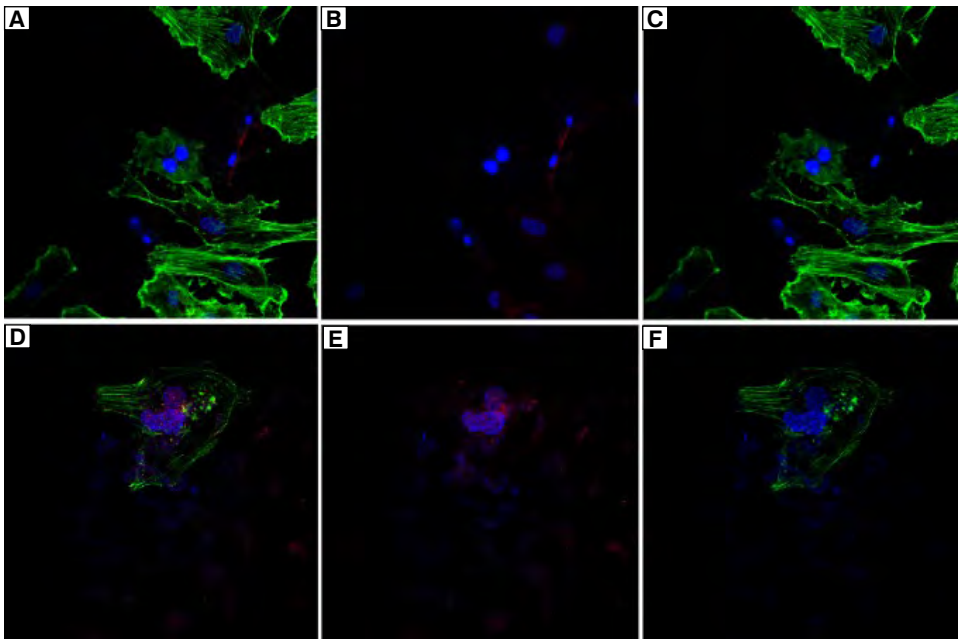

Figure 2A. Representative confocal image showing fluorescence of F-actin (green) and VE-cadherin (red) in pHCAECs treated with bevacizumab 6.7 × 10–6 nmol. Cell nuclei were labelled with DAPI (blue). A1. Overlay; B1. DAPI + VE-cadherin; C1. DAPI + F-actin. (Magnification ×100) Bar = 100μm

Figure 2B. Representative confocal image showing fluorescence of F-actin (green) and VE-cadherin (red) in pHCAECs treated with 100 ng/ml TNF-α and bevacizumab 6.7 × 10–6 nmol. Cell nuclei were labelled with DAPI (blue). A2. Overlay; B2. DAPI + VE-cadherin; C2. DAPI + F-actin. (Magnification ×100) Bar = 100μm

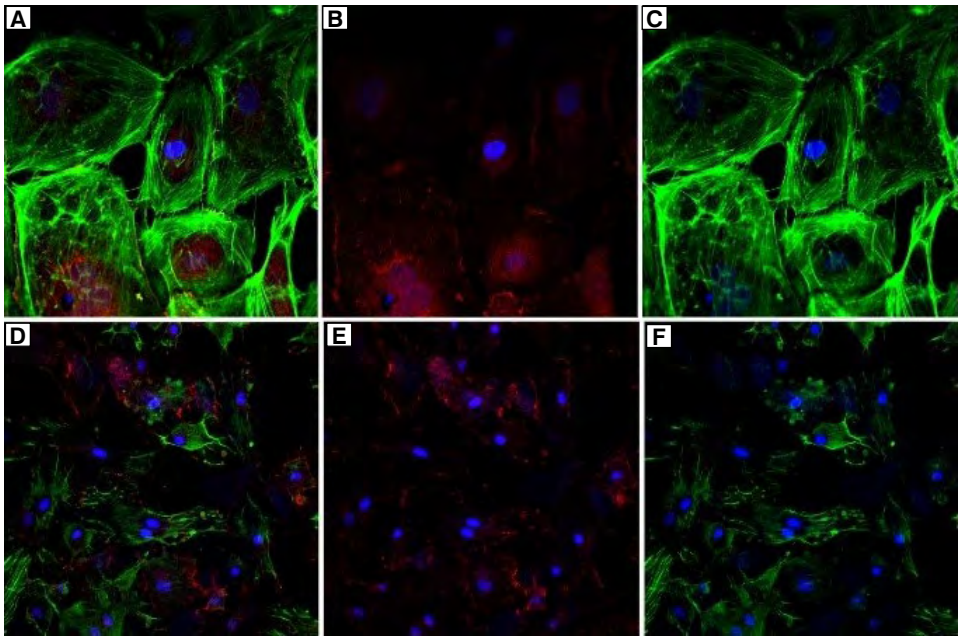

Figure 3A. Representative confocal image showing fluorescence of F-actin (green) and VE-cadherin (red) in pHCAECs treated with pazopanib 1 × 10–5 nmol. Cell nuclei were labelled with DAPI (blue). A1. Overlay; B1. DAPI + VE-cadherin; C1. DAPI + F-actin. (Magnification ×100) Bar = 100μm

Figure 3B. Representative confocal image showing fluorescence of F-actin (green) and VE-cadherin (red) in pHCAECs treated with 100 ng/ml TNF-α and pazopanib 1 × 10–5 nmol. Cell nuclei were labelled with DAPI (blue). A2. Overlay; B2. DAPI + VE-cadherin; C2. DAPI + F-actin. (Magnification ×100) Bar = 100μm

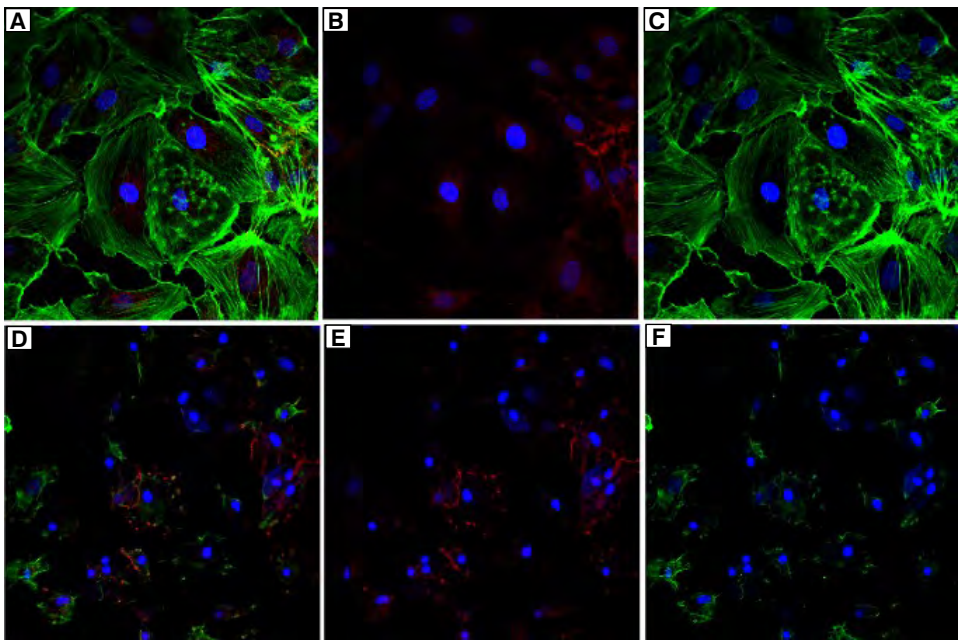

Figure 4A. Representative confocal image showing fluorescence of F-actin (green) and VE-cadherin (red) in pHCAECs treated with KRN-633 1.7 × 10–4 nmol. Cell nuclei were labelled with DAPI (blue). A1. Overlay; B1. DAPI + VE-cadherin; C1. DAPI + F-actin. (Magnification ×100) Bar = 100μm

Figure 4B. Representative confocal image showing fluorescence of F-actin (green) and VE-cadherin (red) in pHCAECs with 100 ng/ml TNF-α and KRN-633 .7 × 10–4 nmol. Cell nuclei were labelled with DAPI (blue). A2. Overlay; B2. DAPI + VE-cadherin; C2. DAPI + F-actin. (Magnification ×100) Bar = 100μm

within their membranes' vesicular structures resembling apoptotic buds has also been reported.

Fluorescence assessment of the examined structures in pHCAECs treated with both bevacizumab and TNF-α showed swelling or bundle shrinkage of the cells. However, both of these changes were accompanied by almost complete degradation of the actin cytoskeleton and loss of continuity of the endothelial layer (Fig. 2b).

In the case of the 24-hour action of pazopanib in pHCAEC cells, a significant expansion of F-actin was observed, which resulted in cell swelling and led to the continuous-discontinuous nature of intercellular interactions. Moreover, cells freshly after cytokinesis showed the characteristics of cells undergoing the phenomenon of entosis into swollen cells. Shrunken cells were also observed with vesicular structures resembling apoptotic buds (Fig. 3a). In the case of combined treatment of cells with pHCAECs pazopanib and TNF-α degradation of the cell cytoskeleton was observed. In addition, vesicle structures were observed in parts of migrating cells along the course of actin retraction fibres (Fig. 3b).

Treatment of cells with pHCAECs KRN633 for a period of 24 hours led to cell swelling and a continuously discontinuous nature of intercellular connections (Fig. 4a). pHCAEC cells treated with both KRN-633 and TNF-α led to cell swelling and the formation of a cell migration phenotype, which conditioned the point nature of intercellular connections. Moreover, translocation was observed in VE-cadherin into the cell nucleus (Fig. 4b).

Discussion

It is believed that cardiovascular diseases (CVDs) and other lifestyle-related diseases may be initiated by common factors, posing a challenge in designing effective therapeutic regimens [26, 27]. This need prompted the experimental approach of using bevacizumab, pazopanib, and KRN-633 in antiangiogenic tests on human endothelial cell lines suitable for arteries (pHCAECs). Due to the diverse phenotypes of ECs in the vascular tree, which vary based on their function and may have a significant impact on specific susceptibility to pathological conditions, this study placed special emphasis on the morphology of pHCAECs. This study highlighted a significant reduction in the number of live cells treated with bevacizumab, which correlated with decreased VE-cadherin expression in the pHCAEC cell line. Studies on VE-cadherin-deficient mouse models showed embryonic stage mortality due to severe vascular defects. Flemming et al. (2015) showed that TNF-α significantly inhibits the expression of VE-cadherin and time-dependently affects the formation of spaces between ECs [28, 29]. Similar results were obtained in this study. Cells incubated with TNF-α exhibited a reduction in membrane VE-cadherin expression, which correlated with the loss of continuous fluorescent signals at the cell edges. This change was associated with a shift in the nature of intercellular interactions from continuous to punctate. Similarly to the studies discussed in this work, the authors also observed changes in cell morphology and the appearance of intercellular spaces. Additionally, it has been shown that TNF-α induces the expression of MMP9, which can break the homophilic interaction of VE-cadherins located on the surface of two neighbouring cells [30]. As shown, TNF-α enhances blood vessel remodelling by influencing the activation of ECs and promoting pericyte recruitment. In turn, chronic inflammation affects, through the induction of Angiotensin 2 (Ang-2) in ECs, destabilization and increased vascular permeability [31]. The Ang-1/Tie-2 system is crucial in maintaining blood vessel stability. Ang-1 acts as an antagonist to Ang-2, which is released from Weibel-Palade bodies in response to various stimuli. As a consequence, the ratio of Ang-1 to Ang-2 is reduced, thereby contributing to the destabilization of the endothelial monolayer and the formation of new blood vessels [32]. It is worth noting that Ang-2 also affects the phosphorylation of VE-cadherin, simultaneously regulating the activity of this protein. Studies confirm that phosphorylated VE-cadherin induces blood vessel permeability and destabilization of intercellular connections [33]. Wang et al. (2019) performed a fluorescence analysis of VE-cadherin protein, showing its increased expression in SACC-LM cells stimulated with VEGF-A. However, incubation of cells with bevacizumab contributed to the reduction of VE-cadherin expression and disruption of the formation of vascular-like structures, which confirms the anti-angiogenic nature of the drug [34].

The cytoskeleton is integral to several crucial biological functions at the cellular level. Among these, it plays key roles in cell movement, intracellular organization, endocytosis, cytokinesis, and even apoptosis. The actin cytoskeleton is composed mainly of actin. This protein actively adapts to the changing intra- and extracellular environment by polymerizing actin into the form of F-actin filaments or depolymerizing them. Destabilization of F-actin induces features in cells that define the image of apoptotic cells [35]. These cells are characterized by the condensation and fragmentation of genetic material, fragmentation of the cell nucleus, swelling and shrinking of cells, and the formation of small

vesicles. Moreover, during the early phase of apoptosis, cells undergo shrinkage, and their intracellular contents become compacted. The late phase is characterized by fragmentation of the cell nucleus and the formation of vesicular structures of the plasma membrane [36]. In this study, the morphological picture was observed indicating the induction of this form of cell death. It was demonstrated that cells from both tested cell lines developed surface-like actin structures resembling apoptotic vesicles. These structures were observed in pHCAEC cells following treatment with bevacizumab and pazopanib. Interestingly, the study presented in this paper demonstrated the ability to induce entosis in pHCAEC cells incubated with bevacizumab and pazopanib. Entosis is a fascinating phenomenon where a living cell is engulfed completely by a neighbouring cell. Moreover, cells engulfed by entosis primarily die, suggesting that this process serves as a mechanism for cell survival and supports proliferation under conditions of limited nutrient availability. However, studies have also demonstrated that cells can divide inside the host cell or escape from it, subsequently continuing their cell cycle [37]. These findings suggest that cells may utilize entosis as a survival mechanism under conditions unfavourable for growth.

Fluorescence analysis of cells treated with both bevacizumab and TNF-α in pHCAECs shows a significant decrease in the intensity of F-actin and VE-cadherin, which was associated with almost complete degradation of the actin cytoskeleton and loss of continuity of the endothelial layer. Carneiro et al. (2009) demonstrated the antiangiogenic activity of bevacizumab on VEGF-treated HUVECs. This drug has been shown to dose-dependently reduce the number of proliferating cells. This study also showed that blocking VEGF induces apoptosis, limits migration to the damaged area and reduces vessel formation in vitro [38]. Zhang et al. (2020) demonstrated the inconclusive anti-angiogenic effect of bevacizumab. They found that the migration of HUVEC cells under hypoxic conditions, treated with bevacizumab, was actually enhanced. Additionally, an in vitro vessel formation assay showed that this drug accelerates cell angiogenesis [39]. Therefore, it can be suggested that bevacizumab may exhibit both pro- and anti-angiogenic characteristics depending on the microenvironmental conditions (normoxia or hypoxia) of the cells.

The second drug used in the research on which this work was based was pazopanib. Subsequent detailed fluorescence analysis of F-actin in pHCAEC cells incubated with pazopanib and TNF-α revealed structures resembling the originally described migrasomes. Ma et al. (2015) demonstrated that migrating cells leave behind retraction fibres where membrane-covered vesicles are located. However, subsequent studies have established that actin polymerization is essential for the formation of migrasomes. The process by which cells form migrasomes and secrete them into the extracellular space may play a crucial role in cell communication [40]. However, considering the above, it should be expected that this image is an expression of cell death during dynamic processes, such as division or migration.

In the present study on the pHCAECs cell model, it was noted that the induction of KRN-633 and KRN-633 combined with TNF α induced both cell apoptosis and cell swelling, which was characterized by a specific arrangement of F-actin filaments, but also complete disintegration of F-actin. The observed swelling correlated with the parallel arrangement of F-actin stress fibres and led to the spindle shape of the cells. This image of pHCAEC cells is characterized by their increased migration potential. It is worth noting that incubation with KRN-633 contributed to a reduction in the intensity of membrane VE-cadherin. It is intriguing that in cells treated simultaneously with KRN-633 and TNF-α, VE-cadherin was predominantly localized in the nucleus and cytoplasm around the perinuclear region. Research conducted by Liu et al. (2017) shows that thrombin induces phosphorylation of VE-cadherin while contributing to increased vascular permeability. Fluorescence analysis reveals that incubation of endothelial cells with thrombin leads to increased expression of VE-cadherin in the perinuclear area and cytosol, accompanied by decreased expression of this protein on the cell membrane. It has been suggested that the effect of such an event may be to protect the protein from degradation [41]. Similarly, in the present findings, VE-cadherin translocation may serve as a mechanism to protect the protein against factors influencing cell degradation, although current studies do not definitively confirm this hypothesis. However, one can suggest that the inflammatory induction of pHCAECs inhibits the antiangiogenic effect of KRN-633.

Conclusions

Based on the conducted research, it can be concluded that TNF-α induces an inflammatory response in pHCAEs, characterized by remodelling of the actin cytoskeleton into parallel-arranged F-actin stress fibres. This alteration changes the nature of cell-cell interactions, leading to increased permeability of the cell monolayer

through discontinuous intercellular connections and the formation of gaps between cells.

Antiangiogenic compounds induce structural changes in the actin cytoskeleton of pHCAECs, leading to cell swelling, disruption of intercellular interactions, entosis occurring within strong cell-cell interactions, and the appearance of vesicular structures resembling apoptotic vesicles.

Additionally, endothelial inflammatory activation by TNF-α treatment of cells enhances the disintegrative cytoskeletal effect of bevacizumab, pazopanib, and KRN-633 in pHCAECs. In the presence of TNF-α, pazopanib induces the formation of vesicular structures along the F-actin retraction fibres of migrating pHCAECs. This finding may contribute to reevaluating the mechanism proposed by the Chinese team from Tsinghua University led by Li Yu regarding "migrasome" formation, particularly in the context of cell death during dynamic processes such as cell migration or cytokinesis.

Article information

Data availability statement: *The data are available at the Department of Histology and Embryology CM UMK in Bydgoszcz.*
Ethics statement: *None.*
Author contributions: *Conceptualization; KP, DS, MG, AG; Data curation: KP; Formal analysis KP, Funding acquisition KP, MG; Investigation and Methodology; KP, DS, DJ, KB; Supervision: MG, AG; Visualization KP, DS; Roles/Writing — original draft KP, DS, MG; Writing — review & editing KP, DS, KB, DJ.*
Funding: *The project was financed by research grants awarded by the authorities of the Ludwik Rydygier Collegium Medicum in Bydgoszcz Nicolaus Copernicus University in Toruń.*
Acknowledgements: *None.*
Conflict of interest: *None.*
Supplementary material: *None declared.*

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