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Research Article

The impact of endothelin-1 on the efficacy of anti-VEGF therapy: A rationale for dual antagonism

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Abstract

Objectives: Angiogenesis-associated disease conditions are often treated with anti-angiogenic therapy. Many of the anti-angiogenic agents approved as adjuvant cancer therapy target the vascular endothelial growth factor (VEGF) axis, as VEGF signaling is regarded as the primary angiogenesis promoter. These drugs are expected to enhance immunity, antagonizing the immunosuppressive functions of VEGF, and to control angiogenesis. Despite a mechanistic rationale that strongly supports their benefits, anti-VEGF agents have shown limited success rates in most cases, along with an association with hypertensive side effects. This article briefly reviews the approved anti-VEGF agents and offers a possible explanation for their limitations.

Methods: PubMed and Scopus databases were searched with the corresponding keywords (such as anti-VEGF), and the relevant knowledge was collected. The included studies were limited to these, which report indications, responses, and side effects. In addition to the review, HuH7 and HEK293T cells were subjected to chemical induction of hypoxia by means of treatment with cobalt chloride ($CoCl_2$). This treatment induced hypoxia inducible factor 1 alpha (HIF-1) under normoxic conditions. Target protein levels were then assessed with immunoblotting to confirm the review results.

Results: The results support the fact that both VEGF and endothelin-1 (ET-1) levels are elevated in response to hypoxia. Consequently, the modulation of the proangiogenic and vasodilatory effects of the VEGF axis by anti-VEGF agents is anticipated to have an incomplete impact on angiogenesis, while resulting in hypertensive complications due to the ongoing proangiogenic activity and unopposed vasoconstrictive effects of endothelin-1.

Conclusion: Given the uncertainty regarding the capacity of anti-VEGF therapy to concurrently inhibit ET-1, the dual antagonism of VEGF and ET-1 appears to be the preferred approach for effective management of angiogenesis-related pathologies. Additional studies are necessary to validate this conclusion.

Keywords: Adjuvant therapy, angiogenesis, anti-VEGF therapy, endothelin-1, vascular endothelial growth factor

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Angiogenesis refers to the formation of new blood vessels through the migration, growth, and differentiation of endothelial cells. This process is regulated by various chemical signals within the human body, with some, such as vascular endothelial growth factor (VEGF) signaling, acting as promoters, while others function as inhibitors [1]. Under normal physiological conditions, there is a balance between angiogenesis-stimulating and inhibiting signals, ensuring the formation of new blood vessels only when and where necessary, such as during growth or tissue repair. However, disruptions in this balance can lead to pathological conditions or diseases, such as angiogenesis in cancer and metastasis or in age-related wet macular degeneration [2].

Vascular endothelial growth factor A (VEGF-A) is one of the most important and extensively studied stimulators of angiogenesis [3]. It has become a target for numerous angiogenesis inhibitors, many of which have been approved or are in advanced clinical trials for adjuvant cancer treatment. Examples of these inhibitors

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Table 1. Examples of anti-VEGF agents considered for clinical application

Drug	Mechanism of action
Axitinib (Inlyta®)	A tyrosine kinase inhibitor capable of inhibiting the angiogenic effects mediated by VEGF receptors 1–3, c-KIT, and PDGFR.
Bevacizumab (Avastin®)	Monoclonal antibody against VEGF-A.
Cabozantinib (Cometriq®)	Impedes MET (hepatocyte growth factor receptor protein), VEGFR, RET (receptor tyrosine kinase), GAS6 receptor (AXL), KIT), and Fms-like tyrosine kinase-3 (FLT-3).
Lenvatinib mesylate (Lenvima®)	A multi-kinase inhibitor targeting VEGFR1, VEGFR2, and VEGFR3 kinases.
Pazopanib (Votrient®)	A multi-kinase inhibitor that targets and inhibits the vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR), c-KIT, and fibroblast growth factor receptor (FGFR).
Ramucirumab (Cyramza®)	A direct competitive inhibitor of VEGFR2, exhibiting a high affinity for binding to the extracellular domain of VEGFR2, thereby preventing its interaction with the natural ligands (VEGF-A, VEGF-C, and VEGF-D).
Regorafenib (Stivarga®)	Exhibits binary-targeted inhibitory activity against the tyrosine kinases VEGFR2 and TIE2.
Sorafenib (Nexavar®)	A protein kinase inhibitor that demonstrates activity against VEGFR, PDGFR, and RAF kinases. Among the RAF kinases, sorafenib exhibits greater selectivity for C-Raf compared to B-Raf.
Sunitinib (Sutent®)	A multitargeted tyrosine kinase inhibitor capable of binding to PDGF receptors (PDGF-Rs), VEGF receptors (VEGFRs), CD117 (c-KIT), RET, CD114, and CD135.
Vandetanib (Caprelsa®)	Inhibits the tyrosine kinase activity of two concurrent pathways by targeting VEGFR-2 and the epidermal growth factor receptor (EGFR).
Aflibercept (EYLEA™)	A soluble fusion protein that binds all isoforms of VEGF-A, as well as VEGF-B and placental growth factor, thereby inhibiting their receptor activation.
Zivaflibercept (Zaltrap®)	A soluble decoy protein for the VEGF receptors, VEGFR-1 and VEGFR-2.
Brolucizumab (Beovu®)	A 26 kDa single-chain monoclonal antibody fragment capable of inhibiting the activation of VEGF receptors.
Ranibizumab (Lucentis®)	A recombinant humanized monoclonal antibody fragment targeting VEGF-A.

are presented in Table 1. The success rates of these agents vary due to several factors. Table 2 and Table 3 summarize some reported clinical outcomes, revealing two key limitations of these agents: Limited success rates and hypertensive side effects.

Hypothesis (Aim of work)

The limitations reported in the experimental and clinical studies can be attributed to several potential factors, as illustrated in Figure 1:

Table 2. Reported clinical success rates of some anti-angiogenic agents

Drug	Reported findings
Axitinib	In patients with cytokine-refractory metastatic renal cell carcinoma, Axitinib has the potential to yield an estimated 5-year survival rate of 20.6% [4].
Bevacizumab	A total of 167 patients with recurrent glioblastoma were enrolled in a multicenter, phase II, randomized, noncomparative trial. Patients who experienced a first or second relapse with progression while on temozolomide were randomized to receive either bevacizumab (10 mg/kg) alone or in combination with irinotecan, administered in 2-week cycles. The objective response rates observed were 28% in the single-agent group and 38% in the combination group. Six-month progression-free survival rates were 43% for the bevacizumab monotherapy group and 50% for the combination group. The median overall survival was 9.2 months for the bevacizumab-only arm and 8.7 months for the combination arm. The most common side effects included hypertension, seizures, neutropenia, and fatigue [5].
Cabozantinib	The phase 3 CheckMate 9ER trial randomly assigned patients with renal cell carcinoma to receive either cabozantinib in combination with nivolumab or sunitinib. The study reported an objective response rate (ORR) of 55.7% for the cabozantinib/nivolumab combination, with a complete response (CR) rate of 12.4%. In contrast, sunitinib demonstrated an ORR of 28.4% and a CR rate of 5.2%. The median duration of response (DOR) was 23.1 months for the cabozantinib nivolumab regimen, compared to 15.1 months for sunitinib [6].
Lenvatinib	According to GlobalData, the success rate of the transition phase in the phase III trial evaluating lenvatinib mesylate in patients with colorectal cancer was 43% [7, 8].
Pazopanib	In the SPIRE study, 211 patients with advanced soft tissue sarcomas were treated with pazopanib as a second-line or subsequent therapy. The median treatment duration was 3.1 months. The median progression-free survival was 3 months, while the median overall survival was 11.1 months. The overall clinical benefit rate across most histological subtypes was 46% [9, 10].
Ramucirumab	The administration of Ramucirumab in 355 patients with gastro-esophageal cancer demonstrated a response rate of 4%. However, it also showed a disease stability rate of 45%, compared to 21% in the placebo group, yielding an overall disease control rate of 45% versus 23% in the placebo group [11].

Table 2. Cont.

Drug	Reported findings
Regorafenib	Patients with metastatic colorectal cancer treated with Regorafenib had a progression-free survival of 2.9 months (interquartile range: 2.2 to 4.4 months), an overall response rate of 4% (n=2), and a disease control rate of 40% (n=19) [12].
Sorafenib	The use of Sorafenib in advanced-stage hepatocellular carcinoma demonstrated the following outcomes: a median overall survival of 26.1 months, 6- and 12-month survival rates of 92.1% and 85%, respectively, a median time to radiological progression of 8 months, and a progression-free survival rate of 64.3% [13].
Sunitinib	Objective response rates of 47% for Sunitinib and 12% for IFN-α ($p<0.001$) were observed in patients with metastatic renal cell carcinoma. The primary Sunitinib-related adverse effects included hypertension (12%), fatigue (11%), diarrhea (9%), and hand-foot syndrome (9%) [14].
Vandetanib	The use of Vandetanib in patients with locally advanced or metastatic medullary thyroid carcinoma yielded a pooled complete response rate of 0.7% and a disease stabilization rate of 47%, as determined by the RECIST criteria [15].
Zivaflibercept	Patients with colorectal cancer treated with Zivaflibercept demonstrated a median overall survival of 13.5 months and a median progression-free survival of 6.9 months, in contrast to 12.06 months and 4.67 months, respectively, for those receiving a placebo. Similarly, the response rate for the Zivaflibercept plus FOLFIRI combination was 19.8%, compared to 11.1% for the FOLFIRI-only group [16].
Dovitinib	In a mutation-specific, single-arm, phase 2 study involving 80 cancer patients with colorectal, gastrointestinal stromal, or ovarian cancers, Dovitinib demonstrated a clinical benefit rate of 13.8% [17].
	In an open-label, randomized phase 3 clinical trial evaluating dovitinib as a third-line targeted treatment for patients with metastatic renal cell carcinoma, the drug resulted in an increase of 3.7 months in progression-free survival and 11.1 months in overall survival [18].

- Hypoxia and relative ischemia are commonly observed alongside the rapid growth of solid tumors [19].
- Hypoxic conditions result in a significant decrease in NOSTRIN (Nitric-Oxide Synthase Trafficking Inducer) levels [20].
- Under hypoxic conditions, hypoxia-inducible factor 1-alpha (HIF-1α) forms a dimeric complex with HIF-1β through nuclear translocation. This complex binds to the hypoxia response element (HRE), interacting with the coactivator p300, which subsequently enhances the expression of VEGF-A, matrix metalloproteinases (MMPs), angiopoietin, and platelet-derived growth factor (PDGF) [21].
- Low NOSTRIN levels are associated with increased activity of endothelial nitric oxide synthase III (eNOS) [22], which, in turn, elevates VEGF-A [23], thereby inducing the release of soluble VEGF receptor 1 (sVEGFR-1 or sflt-1) [24].
- The presence of soluble VEGF receptor 1 (sVEGFR-1) has been reported to enhance the vasoconstrictive activity of endothelin-1 (ET-1) [25].

Clinically, low NOSTRIN has been linked to tumor progression, prognosis, and metastasis [26, 27]. Elevated levels of eNOS and VEGFs have been similarly associated with tumor progression, prognosis, recurrence, and metastasis [28]. Furthermore, high endothelin-1 (ET-1) levels have been clinically correlated with tumor progression and metastasis [29].

Therefore, the inhibition of the proangiogenic and vasodilatory effects of the eNOS/VEGF axis by anti-VEGF agents may lead to inadequate control of angiogenesis, potentially resulting in hypertension. This could be attributed to the continued proangiogenic activity and the unopposed vasoconstrictive effects of the endothelin-1 axis. The objective of this work is to validate the aforementioned principles.

Role of eNOS/VEGF axis in angiogenesis

During the conversion of l-arginine to l-citrulline, endothelial nitric oxide synthase (eNOS) acts as a catalyst, leading to the production of nitric oxide (NO). NO plays a critical role in mediating the angiogenic activity of various factors, including vascular endothelial growth factor (VEGF). The activation of eNOS is partially regulated by the upstream Akt/protein kinase B signaling pathway [29]. The VEGF family consists of seven known members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, placental growth factor (PlGF), non-human genome encoded VEGF-E, and snake venom VEGF (svVEGF) [15]. VEGF-A is vital for supporting the vascular endothelium and serves as a key regulator of angiogenesis, contributing to tumor growth, proliferation, invasion, metastasis, angiogenesis, and drug resistance [30]. VEGF-B is involved in promoting neuronal survival and cardiovascular development through angiogenesis in specific organs. The roles of VEGF-C and VEGF-D are particularly significant in tumor growth and metastasis, as they are implicated in VEGFR-3-mediated lymphangiogenesis and lymphatic metastasis [30].

Role of endothelin -1 axis in angiogenesis

Endothelin-1 (ET-1) exerts a direct angiogenic effect on endothelial and peri-vascular cells [31]. It plays a crucial role in cell growth and proliferation, and its effects are mediated through the activation of the MAPK pathway [32]. Consequently, ET-1 is actively involved in tumor angiogenesis. Furthermore, ET-1 can enhance VEGF expression and promote angiogenesis via its endothelin A receptor (ETAR), integrin-linked kinase (ILK), Akt, and hypoxia-inducible factor-1α (HIF-1α) signaling pathways [33].

Materials and Methods

A systematic review of the literature was conducted in accordance with the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines. The search

Table 3. Summary of clinical outcomes for anti-VEGF agents in solid tumors

Drug	Phase (trial)	Study population	Sample size (N)	ORR (%)	PFS (months)	OS (months)	Adverse events
Axitinib	Phase III	Metastatic RCC	Not specified	20.6 (5-yr survival)	Not specified	20.6 (5-yr)	Hypertension
Bevacizumab	Phase II	Recurrent glioblastoma	167	28–38	6-month PFS: 43–50%	9.2	Hypertension, seizures, fatigue
Cabozantinib	Phase III	Metastatic RCC	651	55.7	23.1	Not specified	Not specified
Lenvatinib	Phase III	Colorectal cancer	Not specified	43	Not specified	Not specified	Not specified
Pazopanib	Phase II	Soft tissue sarcoma	211	Not specified	3.0	11.1	Not specified
Ramucirumab	Phase III	Gastro-esophageal cancer	355	4	Not specified	Not specified	Hypertension
Regorafenib	Phase III	Metastatic colorectal cancer	Unclear	4	2.9	Not specified	Hypertension
Sorafenib	Phase III	Advanced HCC	Not specified	Not specified	8.0	26.1	Hypertension
Sunitinib	Phase III	Metastatic RCC	Unclear	47	Not specified	Not specified	Hypertension
Vandetanib	Phase III	Medullary thyroid carcinoma	Unclear	0.7	Not specified	Not specified	Not specified
Ziv-aflibercept	Phase III	Colorectal cancer	Not specified	19.8	6.9	13.5	Hypertension
Dovitinib	Phase II	Various solid tumors	80	13.8	3.7	11.1	Not specified

VEGFR: Vascular endothelial growth factor receptor; ORR: Objective response rate; PFS: Progression-free survival; OS: Overall survival; RCC: Renal cell carcinoma; HCC: Hepatocellular carcinoma.

strategy included queries in PubMed and Scopus using the following keywords and Boolean combinations: "Anti-VEGF therapy," "angiogenesis inhibitors," "VEGF antagonists," "endothelin-1," "cancer angiogenesis," and "clinical trials." The review was limited to English-language articles published between 2005 and 2024.

Inclusion criteria

- Peer-reviewed clinical trials or meta-analyses evaluating anti-angiogenic therapies.
- Studies reporting specific efficacy outcomes (e.g., ORR, PFS, OS) or adverse events, such as hypertension).
- Trials involving FDA- or EMA-approved anti-VEGF agents.

Exclusion criteria

- Non-clinical studies unless providing essential mechanistic insights.
- Conference abstracts without full datasets.
- Duplicate publications or interim analyses of the same trial.

After screening by title/abstract and applying inclusion/exclusion criteria, the selected studies were considered as sources for the informations included in this article.

In addition, HuH7 and HEK293T cells were sourced from affiliated research groups. The cells were harvested and washed with phosphate-buffered saline (PBS). Three independent biological replicates were performed, with 200,000 cells seeded into culture wells (6-well plates) and incubated overnight in 2 mL of supplemented medium at 37 °C in a humidified atmosphere with 5% CO_2 and ≥95% relative humidity. The medium used was DMEM, supplemented with 10% FBS (Gibco), 1X sodium pyruvate, 1X penicillin-streptomycin (Gibco), 1X Glutamax (Gibco), and 25 mM HEPES. The cells were subsequently treated with $CoCl_2$ (Sigma-Aldrich) according to the experimental protocol outlined below:

- Control non treated cells
- Cells treated with 200µM $CoCl_2$
- Cells treated with 300µM $CoCl_2$
- Cells treated with 400µM $CoCl_2$

Cells were incubated under the same conditions for an additional 72 hours before harvesting and subsequent processing. The impact of the treatments on HIF-1α and its target proteins was evaluated through immunoblotting, which was performed according to standard laboratory protocols. Equal amounts of total protein (50 µg per lane) were resolved on a 10% SDS-PAGE gel (Bio-Rad) and transferred to a nitrocellulose membrane via wet transfer. Membranes were blocked with 5% non-fat dry milk in TBST (Tris-buffered saline with 0.1% Tween-20) prior to antibody incubation. Primary antibodies specific to the target proteins were obtained from Proteintech, Germany. Band densities were analyzed using ImageJ software. An unpaired t-test was applied to compare the values of the experimental conditions to the control, with P-values less than 0.05 considered statistically significant.

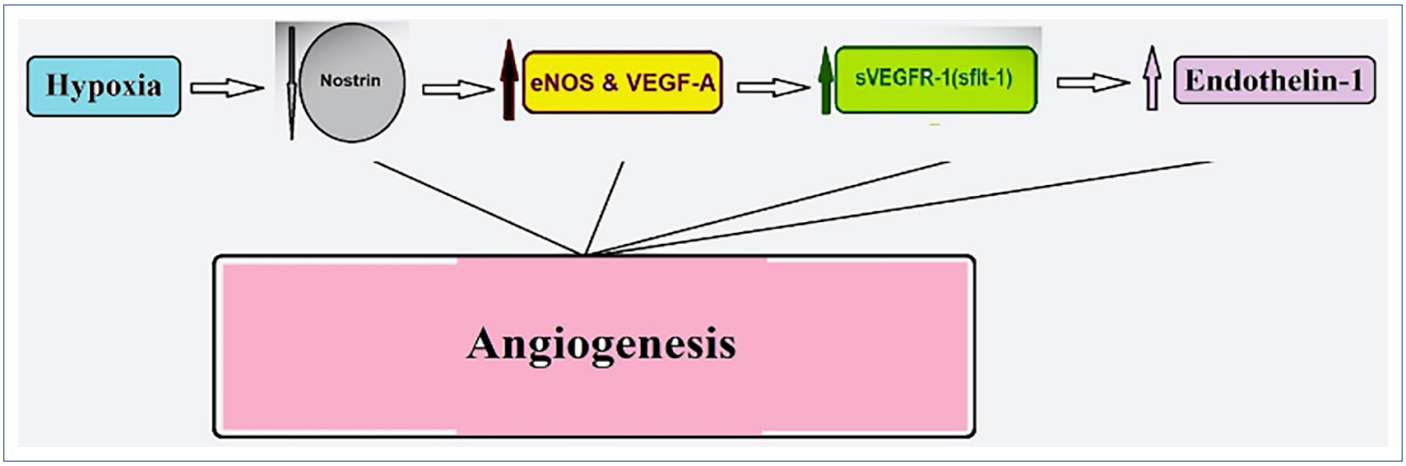

Figure 1. Diagrammatic representation of the article's hypothesis. Hypoxia is associated with decreased NOSTRIN and leads to increased eNOS, VEGF-A, sVEGFR-1 and ET-1.

eNOS: Nitric oxide synthase III; VEGF: Vascular endothelial growth factor; sVEGFR-1: Soluble VEGF receptor 1; ET-1: Endothelin-1; NOSTRIN: Nitric-Oxide Synthase Trafficking Inducer.

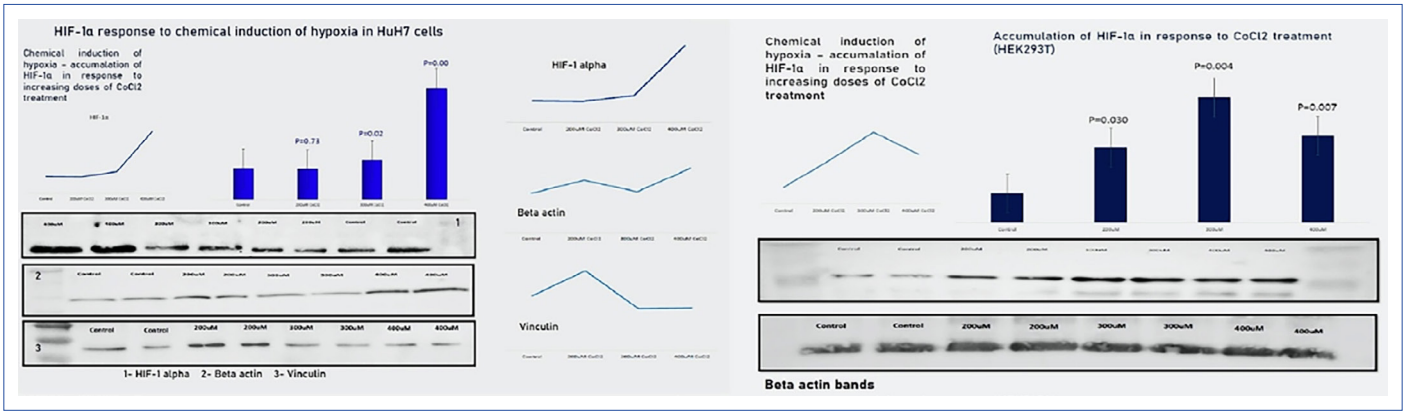

Figure 2. HIF-1α induction in HuH7 and HEK293T cells following treatment with various concentrations of $CoCl_2$. HIF-1α band intensities were normalized to their respective loading controls. Protein concentrations were estimated using the Bradford assay prior to gel loading. Equal amounts of total protein were loaded for each sample, and vinculin and β-actin were used as loading controls. Vinculin was favored over β-actin as a reference, given reports of β-actin's reactivity to hypoxia—an effect observed at the 400 μM treatment in HuH7 cells. $CoCl_2$ treatment induced dose-dependent changes in HIF-1α expression in HuH7 cells (p=0.73, 0.02, and 0.0001 for 200 μM, 300 μM, and 400 μM, respectively) and in HEK293T cells (p=0.03, 0.004, and 0.007 for 200 μM, 300 μM, and 400 μM, respectively).

HIF-1α: Hypoxia-inducible factor 1-alpha.

Results

The chemical induction of hypoxia was successfully achieved, as evidenced by the upregulation of HIF-1α (Fig. 2). In response to HIF-1α activation, the secretion of eNOS, VEGF-A, sVEGFR1, and Endothelin-1 (ET-1) was elevated in a dose-dependent manner (Fig. 3). Similar experiments conducted on human umbilical vein endothelial cells (HUVECs) also demonstrated an increase in ET-1 secretion (Fig. 4).

Discussion

The selected target proteins are well-established effectors that play crucial roles in endothelial physiology and vascular pathology. These proteins serve as indicators of tissue hypoxia and are involved in the process of angiogenesis [31]. To mitigate potential variations that may arise in studies utilizing physical hypoxia, such as differences in the type (e.g., sustained or intermittent), duration (e.g., short-term or long-term), or extent of hypoxia, this study employed the previously validated chemical induction of hypoxia through the use of $CoCl_2$, which promotes the accumulation of HIF-1α and HIF-2α under normoxic conditions [34].

This study demonstrates that the secretion of ET-1 and VEGF-A increases concurrently in response to hypoxia, prior to the statistical significance peak of HIF-1α. This observation suggests that both effectors may exhibit heightened sensitivity to hypoxia and/or play simultaneous leading roles in the tissue's response to hypoxia, particularly at the paracrine and/or remote levels.

The roles of eNOS, NO, and VEGF-A in angiogenesis have been extensively investigated and thoroughly documented. The majority of clinically approved anti-angiogenic therapies target this specific pathway (Table 1). VEGF-A stim-

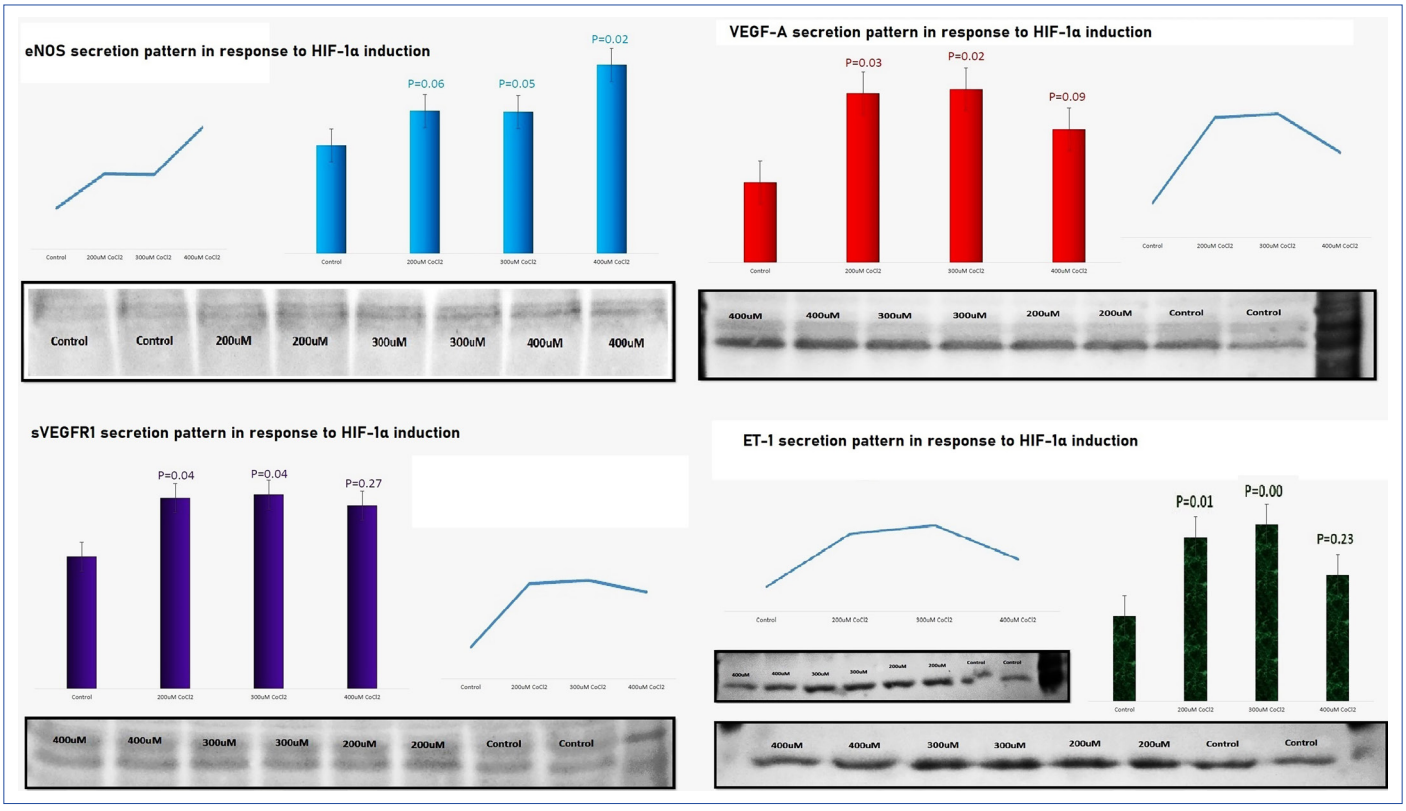

Figure 3. Secretion patterns of eNOS, VEGF-A, sVEGFR1, and ET-1 in response to chemically induced hypoxia. Band densities were quantified using ImageJ software. Each treatment group was compared independently to the control using an unpaired t-test. A P-value of less than 0.05 was considered statistically significant. No corrections for multiple comparisons were applied, as the values were analyzed independently.

eNOS: Nitric oxide synthase III; VEGF: Vascular endothelial growth factor; sVEGFR-1: Soluble VEGF receptor 1; ET-1: Endothelin-1; NOSTRIN: Nitric-Oxide Synthase Trafficking Inducer.

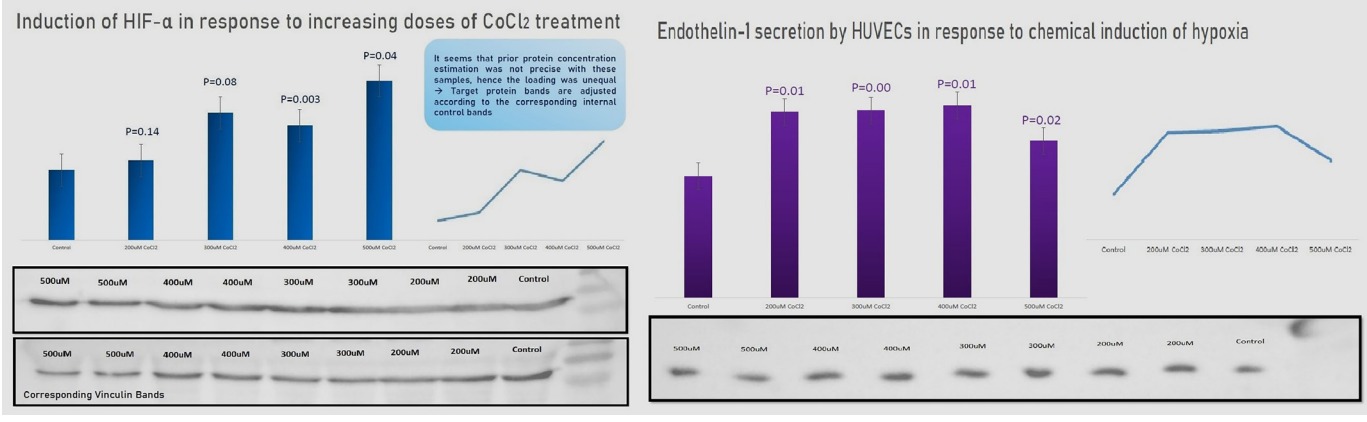

Figure 4. Effect of various doses of $CoCl_2$ treatment on HIF-1α in HUVECs and the corresponding changes in ET-1 secretion. The induction of HIF-1α was associated with corresponding increase in ET-1 secretion ($p<0.05$).

HIF-1α: Hypoxia-inducible factor 1-alpha.; ET-1: Endothelin-1; HUVECs: Human umbilical vein endothelial cells.

ulates eNOS expression and enhances NO production by vascular endothelial cells. A reduction in NO production impairs angiogenesis and decreases the vascular permeability typically induced by VEGF-A [35].

Similarly, the elevated secretion of ET-1 by cultured cells in response to hypoxia has been previously reported, [36] along with other contradictory findings. *In-vivo* preclinical and clinical studies have also reported similar outcomes, with intermittent hypoxia linked to ET-1 overexpression in animal models [37–39], and chronic intermittent hypoxia, as observed in patients with obstructive sleep apnea, associated with the accumulation of HIF-1α and elevated circulating ET-1 levels [40–42]. Increased circulating ET-1 levels have been associated with vascular complications and endothelial dysfunction in humans [43].

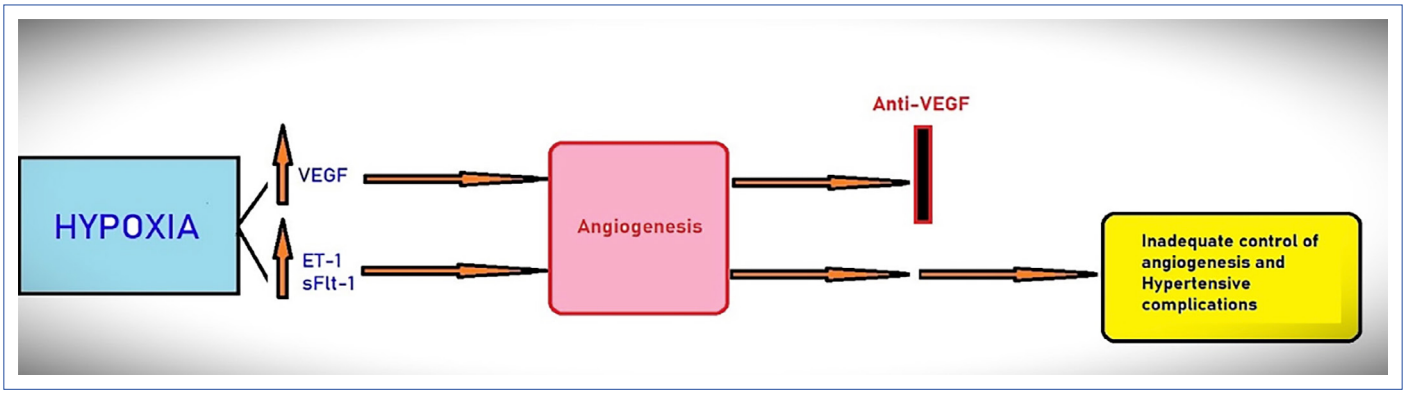

Figure 5. Diagrammatic representation of the main conclusion of the work: Anti-VEGF therapy may antagonize the effects of VEGF during hypoxia-induced angiogenesis; however, sVEGFR1 (sFlt-1) and ET-1 would remain elevated, which may explain the reported low success rates and hypertensive side effects.

VEGF: Vascular endothelial growth factor; sVEGFR-1: Soluble VEGF receptor 1; ET-1: Endothelin-1.

A recent study examined the impact of sustained and intermittent hypoxia (SH and IH, respectively) on HIF-1α, VEGF, and ET-1 in HepG2 cells (hepatocellular carcinoma cell line). The study found an overexpression of HIF-1α and VEGF in response to IH, but not to SH, whereas no such effect was observed for ET-1 [44]. While these findings may seem contradictory to those of the present study, several key considerations should be taken into account when interpreting these results; the hypoxia induction in the study was achieved physically through exposure to a low oxygen gas mixture. The cells employed were of cancerous origin, which may be associated with specific proangiogenic alterations that could make it challenging to detect additional induction of ET-1. In other words, cancerous cells may undergo a degree of hypoxia in culture, as indicated by their accelerated growth rates. As demonstrated in my experiments, ET-1 secretion increased with a 200 μM $CoCl_2$ treatment but tended to decrease at the 400 μM treatment, where HIF-1α exhibited its peak expression (Fig. 3). In addition, the primary findings of this study focus on ET-1 secretion, which may not directly correspond to changes in mRNA or protein expression levels. In the context of cancer-related angiogenesis, where localized relative hypoxia is a constant feature of the tumor microenvironment, multiple studies have documented elevated circulating levels of ET-1 [45]. Therefore, the results of the present study appear to reflect a more realistic scenario.

Hypoxia-induced VEGF also stimulates the production of its truncated soluble form, VEGFR1, via the VEGFR-2-MEK-PKC signaling pathway, [46] which functions as a regulatory mechanism to prevent excessive activity. The ultimate consequence of hypoxia is the activation of angiogenesis, [47] a process characterized by a balance between proangiogenic and anti-angiogenic factors. Soluble VEGFR1 is part of the endogenous anti-angiogenic factors that help protect against uncontrolled angiogenesis, although it may also be actively involved in angiogenesis [48, 49]. These findings align with the results of the current study, which demonstrated an increase in sVEGFR1 following chemical induction of hypoxia (Fig. 3). However, a study reporting contradictory findings indicated that hypoxia led to a reduction in sVEGFR1 expression. This discrepancy may be attributed to the fact that their experiments were conducted on human microvascular endothelial cells isolated from neonatal dermis [50].

Soluble VEGFR-1 plays a significant role in angiogenesis, where perivascular cells interact with its isoforms via GM3 ganglioside. This interaction impacts actin cytoskeleton dynamics by destabilizing pericyte-endothelial cell interactions and altering adhesion contacts with the basement membrane, thereby contributing to vessel sprouting [51]. Moreover, the presence of sVEGFR-1 has been shown to shift α5β1 integrin signaling from a traditional adhesion pathway to a more dynamic one [52], while also enhancing its expression [53]. Consequently, the presence of sVEGFR-1 in the endothelial cell microenvironment during vessel sprouting is crucial [54]. These findings support the critical role of sVEGFR-1 in vessel sprouting and angiogenesis through mechanisms beyond VEGF binding [55], which aligns with the conclusions of the present study.

The intervention in the present study involved $CoCl_2$ treatment (chemical induction of HIF-1α), and thus, the observed changes can be attributed to the activities of HIF-1α. While the dependence of certain effectors on the upregulation or downregulation of others may be somewhat less considered based on the variations in response to different treatments, though it cannot be completely excluded yet.

The aim of this article was to confirm the dual activation of the eNOS/VEGF and ET-1 axes in response to hypoxia, which has been experimentally demonstrated, as well as to investigate the persistence of ET-1 activation despite anti-VEGF therapy (Fig. 5). As previously mentioned, the concurrent or parallel pattern of changes in VEGF-A and ET-1 secretion in response to hypoxia may suggest independent responses of both effectors. However, the appropriate approach to fully address this issue would have involved the introduction of various anti-VEGF agents followed by a reassessment of the levels of both effectors. Due to significant resource limitations, this investigation has not yet been conducted; thus, this issue will be further discussed based on existing published literature and experimental findings.

The secretion of VEGF-A in response to hypoxia persists for as long as the hypoxic stimulus is present [56]. Hypoxia induces angiogenesis, which is the process of new vessel formation [57]. Although the precise sequence of events remains unclear, this process involves both vascular endothelial and smooth muscle cells. VEGF-A acts as a specific mitogen for vascular endothelial cells, promoting their proliferation, while ET-1 stimulates the proliferation of vascular smooth muscle cells [58]. Therefore, both effectors are expected to increase concurrently, as observed in my experiments. Furthermore, it is anticipated that each effector can influence the expression of the other [58, 59].

From a biological perspective, the reduction in ET-1 observed following anti-VEGF therapy, despite the persistence of hypoxia and/or the initial stimulus, should be limited to the inhibition of the additional induction caused by VEGF overexpression, as its actions are suppressed by the therapy [60]. However, the response to the initial stimulus may remain unaffected. Consequently, reports of decreased ET-1 levels after anti-VEGF therapy may reflect scenarios where the stimulus for abnormal angiogenesis is simultaneously eliminated during the therapy [61]. In contrast, when the pathology persists, increased ET-1 levels have been observed post-therapy [61]. Therefore, in response to hypoxia and tumor-associated angiogenesis, driven by relative hypoxia within the tumor microenvironment, a reduction in ET-1 due to anti-VEGF therapy cannot be anticipated. For instance, VEGF-A, which normally reduces ET-1 production by 29%, loses this capability when its VEGFR2 receptor is blocked by SU5416, resulting in a 16% increase in ET-1 production under therapy [62].

Nevertheless, the current study demonstrated a notable increase in the levels of sVEGFR1 in the culture medium following hypoxia induction (Fig. 3). Soluble VEGFR1 is an endogenous antagonist of VEGF, and pharmacological anti-VEGF monoclonal antibodies exhibit structural and functional similarities to it [63]. Despite the elevation of sVEGFR1 in the culture medium, a significant increase in ET-1 levels was also observed (Fig. 3).

Conclusion

In summary, the limited success rates of anti-VEGF agents as adjuvant therapies in cancer treatment may be attributed to the principles discussed above. Additionally, any hypertensive side effects associated with these agents may be linked to the unopposed increase in ET-1 (Fig. 5). To achieve effective angiogenesis control without inducing hypertension, a dual antagonism of VEGF and ET-1 may be considered. Preclinical and clinical studies are necessary to evaluate the efficacy of such a dual therapy. Furthermore, since the secretion of the four effectors (eNOS, VEGF-A, sVEGFR1, and ET-1) significantly increases in response to hypoxia, which is a hallmark of angiogenesis, their levels may serve as biomarkers for monitoring the efficacy of therapy in angiogenesis-related pathologies, both before and after treatment. Thus, the SHEHATA MARKER OF ANGIOGENESIS has been introduced as a biomarker panel and is planned for further clinical validation [64].

Informed Consent: Not applicable. This study did not involve human participants, and no new patient data were collected or used. All data are derived from previously published sources or the author's own experiments on commercial cell lines.

Conflict of Interest Statement: The author has no conflicts of interest to declare.

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