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The Anti-angiogenic Potential of Thiosemicarbazide Derivative of Captopril (8) in Breast Cancer Cell Lines

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Abstract

Angiogenesis is essential for many tumours to grow and metastasise, including breast tumours. Captopril, an Angiotensin-Converting Enzyme inhibitor is known to have anti-angiogenic activity. Recently, novel derivatives of captopril that include thiosemicarbazide moiety have shown enhanced ACE inhibition activity compared to captopril. This study aimed to assess the anti-angiogenic activity of one of these derivatives designated as (8) in the Estrogen receptor-positive MCF-7, and the Estrogen-Progesterone receptor-negative AMJ13 breast cancer cells. The study included a microarray screening for 24 angiogenic factors, and genes were confirmed by RT-qPCR. Results demonstrated a stronger anti-angiogenic effect in the MCF-7 cells compared to those on AMJ13. In MCF7, the derivative caused a significant decrease in the pro-angiogenic bFGF mRNA, VEGF-A mRNA expression, thrombopoietin protein level, PECAM -1 (*CD*31) mRNA, G-CSF protein, and a significant increase in the anti-angiogenic factors MIG mRNA level, IL-13 protein level, PF-4 both protein and mRNA level. The derivative also significantly decreased TIMP-1 mRNA and IFN-γ protein levels, whereas in AMJ13, a significant increase in MIG protein expression (but not mRNA), a significant decrease in IL-β protein expression, as well as thrombopoietin and PECAM-1 mRNA were documented. This work has shown the thiosemicarbazide derivative of captopril (8) as a potential anti-angiogenic agent targeting multiple factors in the angiogenesis of breast cancer cells. This study has demonstrated derivative (8) as a very promising molecule to be further investigated in other modes of angiogenesis and types of cancer.

Keywords: Thiosemicarbazide Derivative of Captopril (8), MCF-7, AMJ13 Breast Cancer Cell Line, Angiogenesis, VEGF, bFGF, MIG, PF4.

1. Introduction

In 2020, the prevalence of breast cancer exceeded that of lung cancer among females globally (Sung et al., 2021). Even though there has been a decrease in the mortality rate (DeSantis et al., 2019)along with an increase in the relative five-year survival rates for invasive breast cancer patients(CO, 2015), challenges still exist, particularly cancer metastasis which continues to be at the lead of these challenges (Gupta and Massagué, 2006). Evidence describing the dependency of cancer growth and metastasis on angiogenesis is accumulating (Vinson et al., 2012;Madu et al., 2020).

Angiogenesis supplies oxygen and nutrients to growing cells, facilitating their metastasis (Bouquet et al., 2006; Sahib, 2022). The tumor is believed to launch this process by activating the angiogenic switch by shifting the balance between the pro-angiogenesis and anti-angiogenesis factors to the direction that serves the tumor progression (Hanahan and Folkman, 1996).

In breast cancer, this shift has been documented as overexpression or up-regulation of angiogenic inducers, particularly in vascular endothelial growth factor (VEGF) which correlated with the aggressiveness of the tumor(Relf et al., 1997). Moreover, breast cancer's increased risk of being invasive and more aggressive is associated with the increased expression of pro-angiogenic growth factors, including both the VEGF and bFGF (Hanahan and Folkman, 1996; Linderholm et al., 1999; George et al., 2001). The same is observed with the down-regulation of the angiogenic inhibitors(Volpert et al., 1997).

Hence, targeting the angiogenesis by systemic inhibitors, specifically those that inhibit VEGF in the management of metastatic cancer, is considered one of the standard modalities (Larkins et al., 2015;Tabernero et al., 2015). Few approaches have been extensively investigated, including VEGF, VEGFR monoclonal antibodies, and tyrosine kinase inhibitors; however, several challenges arose with the implication of these, most importantly the side effects and resistance(Madu et al., 2020).

Another approach that awaits to be fully elucidated is targeting factors affecting the up or downstream cellular signalling, such as the Renin-Angiotensin System (RAS)(Herr et al., 2008). RAS components are found to be involved in most carcinogenic processes, including angiogenesis, and metastasis (Ager et al., 2008;George et

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al., 2010;Pinter and Jain, 2017; Vinson et al., 2012;Wegman-Ostrosky et al., 2015).

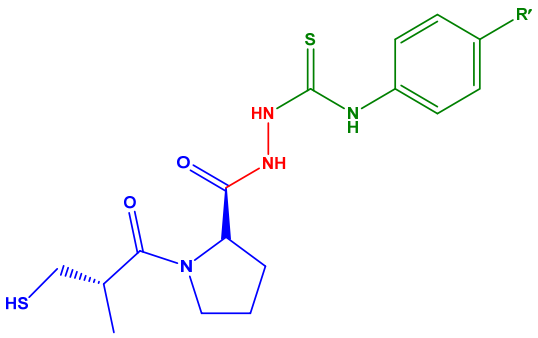
Moreover, many studies have explored the anti-angiogenic potentials of angiotensin-converting enzyme inhibitors ACEi and angiotensin II type I receptor blockers ARBS in various types of cancer (Patel and Nakka, 2017; Kosaka et al., 2007).

Captopril, the first member of the ACEis was among those extensively investigated for its antiangiogenic effect. Studies attributed the antiangiogenic effects of captopril mainly to the inhibition of matrix metalloproteinase MMP-2 and MMP-9(Prontera et al., 1999;Williams et al.,2005;Volpert et al., 1996). As well as, to the reduction of VEGF (Napoleone et al., 2012;Cho et al., 2016).

Recently, Al-Saad*et al.* (2019) have synthesized novel derivatives of captopril, which integrate thiosemicarbazide moiety in an attempt to improve the ACE inhibition activity rationalizing their synthesis to the fact that these structures are versatile with a broad spectrum of activity including anti-inflammatory, antioxidants, antifungal and antibacterial (Al-Saad et al., 2019; Al-Saad et al., 2020). Higher anti-platelet (Al-Saad et al., 2019) and ACE inhibition activity (Al-Saad et al., 2020) over captopril were demonstrated by most of the derivatives, and the improvement was accredited to the thiosemicarbazide moiety that replaced the carboxylic acid group of captopril rather than thiol group (Al-Saad et al., 2019).

Recently thiosemicarbazide/ thiosemicarbazone and their derivatives attracted great interest in their biological activity against various types of cancers(Küçükgüzel and CoŦ, 2016;Dincel and Guzeldemirci, 2020). Concerning breast cancer, many derivatives have been synthesized and investigated displaying remarkable antitumor activity both on hormonal receptors positive (Malki et al., 2015) and hormonal receptors negative breast cancer cells(El Majzoub et al., 2019;Siwek et al., 2013;Yee et al., 2017;Sólimo et al., 2020, Afrasiabi et al., 2013;Bai et al., 2021).

Based on these studies and having in mind the implication of RAS in breast cancer and the fact that captopril possesses an antiangiogenic effect, it was postulated that thiosemicarbazide derivatives of captopril(Al-Saad et al., 2019;Al-Saad et al., 2020)might embrace the anti-angiogenesis activity of captopril and the anticancer effect of thiosemicarbazide creating potentially improved anti-proliferative, anti-angiogenic agents than their parent captopril and targeting multiple factors in carcinogenesis. This work sought to investigate one derivative in particular designated as derivative (8)(Al-Saad et al., 2019) (Figure 1.1) in the estrogen receptor-positive MCF-7 and the estrogen/ progesterone receptor negative AMJ13.The selection of this derivative is based on our previous work (unpublished data) that documented the highest anti-proliferative effect on both types of breast cancer cells.



Thiosemicarbazide derivatives (5,7, &8)

Compound	R'
(5)	H
(7)	Cl
(8)	OCH_3

Figure (1.1): Chemical structure of Thiosemicarbazide Derivatives of Captopril (compounds 5, 7 and 8) (Al-Saad et al., 2019).

2. Materials and Methods

2.1. Chemicals and Cell Lines

The derivative was routinely synthesized and supplied by Dr. Hiba. Najeh. AL-Saad,Department of Pharmaceutical Chemistry, College of Pharmacy, Basrah University, Basrah, Iraq. The molecular weight of derivative (8) is 380.46 and is characterized as a highly pure compound with ^{1}HNMR analysis validation(supplementary materials)(Al-Saad et al., 2019). The compound demonstrated promising results concerning the safety profile with *in vivo*histological evidence (unpublished data)

Cell Bank Unit of Experimental Therapy Department, ICCMGR, AL- Mustansiriyah University,Baghdad, Iraq supplied the cells (MCF-7 and AMJ13). AMJ13 cells were grown in RPMI-1640 medium (Gibco/USA), whereas MCF-7 were grown in MEM (U.S Biological, USA). Both were supplied with (10%) fetal bovine serum (Bio West/ USA) and 1% of (Penicillin–Streptomycin) (Capricorn-Scientific, Germany) and Incubated at 37 C^0 and (5%) CO_2.

The concentration chosen for the following experiments is based on the IC50 of derivative (8) (from our unpublished data). MCF-7 cells were treated with (IC_{50}=88.06 µM), whereas AMJ13 was treated with (IC_{50}=66.82µM) of derivative (8) for 36 hours.

2.2. Microarray

A mouse angiogenesis G1 microarray glass chip (Ray Biotech.inc USA) was used for this analysis. All steps were done according to the manufacturer's instructions. In a simple illustration, the plate was incubated with Biotin-conjugated Anti-Cytokines that were preceded and followed by several washing cycles with washing buffers. Then in a dark room, incubation with Streptavidin-Fluor was commenced and was succeeded by another washing cycle until eventually washed with deionized water and left to dry. Data were analyzed by Labworks software (UVP, USA)(Al-Shammari et al., 2015).

2.3. Reverse Transcription – quantitative Polymerase Chain Reaction (RT-qPCR)

The procedures were conducted according to the manufacturer's instructions.GENEzol™ Reagent (Geneaid Biotech Ltd) was used to extract total RNA and was reverse transcribed into cDNA by using AccuPower® RocketScript™ Cycle RT PreMix (Bioneer/USA). The specificprimers (Angiogenic oligo ^{TM} primers / Korea)design was based on a published sequence bythe National Center for Biotechnology Information (NCBI).

Table (2.1): Angiogenicoligo ^{TM} primers / Korea

Primer	Forward	Reverse
VEGF-A	5'-CTTCAAGCCATCCTGTGTGC-3'	5'-TCTCTCCTATGTGCTGGCCT-3'
bFGF	5'-CTTCCCCAAGGATTTCAAGATGA-3'	5'-ATGTCTTCAAACCTATAAAACAGCA-'3
TIMP-1	5'-TTGTGGGACCTGTGGAAGTA-3'	5'-CTGTTGTTGCTGTGGCTGAT-3'
MIG	5'-CTGTTCCTGCATCAGCACCAAC-3'	5'-TGAACTCCATTCTTCAGTGTAGCA-3'
THPO	5'-CCAGAGGTTCACCCTTTGCCTA-3'	5'-CCAGAATGTCCTGTGCCTTGGT-3'
PF4	5'-TCCTGCCACTTGTGGTCGCCT-3'	5'-CCTTGATCACCTCCAGGCTGG-3'
CD31	5'-AAGTGGAGTCCAGCCGCATATC-3'	5'-ATGGAGCAGGACAGGTTCAGTC-3'
Hs-GAPDH	5-'GAGTCAACGGATTTGGTCGT-3'	5'- GACAAGCTTCCCGTTCTCAG-3'

Mx Pro Mx 3500 CYBER Green software/ Agilent/USA was used for qPCR by applying the following reaction: first cycle with 25 - 95° for two minutes, followed by 45 cycles with 95° for 15 seconds, 60° for 30 seconds and 72° for 30 seconds(Ridnour et al., 2012). Data were analyzed by relative quantitation delta delta Ct method ($2^{-\Delta\Delta Cq}$) where they were normalized to GAPDH as the housekeeping gene. The method is calculated based on relative quantitation approach which is the ratio between the RG (reference gene which in this case is GAPDH) and GOI (gene of interest) from the following formulas: ΔCt= average of GOI Ct – Average RG Ct

ΔΔCt= treated ΔCt – average control group ΔCt

2.4. Statistical analysis

GraphPad Prism®, version 9 was used to analyze the data. Unpaired t-test was used to compare between two groups with a significance of < 0.05, One – way ANOVA with Tukey's multiple comparison test was used to compare the means of more than two groups. All results in this work were presented as mean ± SD.

3. Results

3.1. Non-treated cells Microarray analysis

The normal protein signal intensity in each cell line was first established. Results demonstrated different protein expressions in each cell line. In the Estrogen-positive MCF-7 cells, the high signal intensity of bFGF and TIMP2 with low if no signal at all for the other factors was reported (Figure 3.1, A). On the other hand, the highly metastatic ER/PR receptor-negative breast cancer cell line AMJ13 has demonstrated strong expression of many angiogenic factors in particular VEGF and TIMP 1 and 2(Figure 3.1, B).

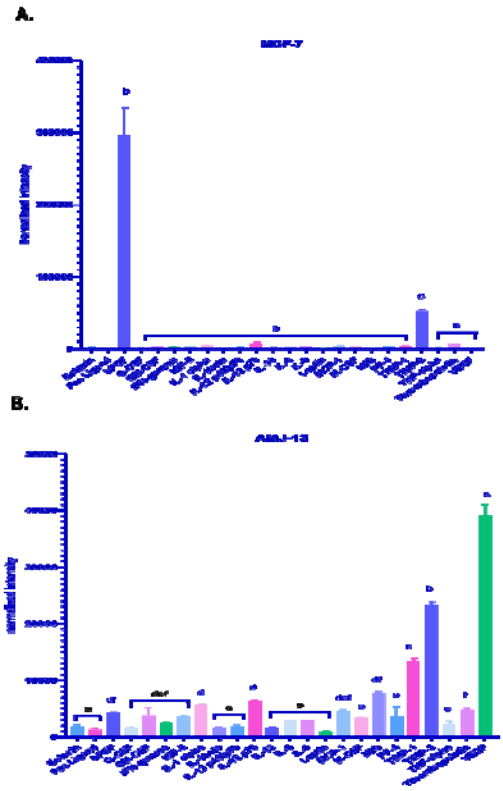


Figure (3.1): Normal signal intensity of 24 angiogenic factors in (A) Control (untreated) MCF-7 cell line. B) Control (untreated) AMJ13 cell line. The experiment was done once in two replicates.

3.2. Effect of Derivative (8) on MCF7 angiogenic factors (Microarray and RT-qPCR analysis)

Derivative (8) has imposed several alterations in the expression of many angiogenic factors. The derivative significantly decreased the protein levels of G-CSF (p-value 0.0472), IFN-gamma (p-value 0.0394), and Thrombopoietin (p-value 0.0213). In addition to that, the derivative significantly increased the protein level of the anti-angiogenic factor PF4 (p-value 0.0236) along with a significant increase in the pro and anti-inflammatory cytokines (IL-1 α and IL-13) (0.0394, 0.036 respectively) (Figure 3.2).

Reverse transcriptase–quantitative polymerase chain reaction (RT-qPCR) results showed a significant increase in PF4 (p-value 0.0169) and MIG mRNA (0.0118) (Figure 3.3 C, D). In addition to that, the derivative significantly and highly significantly decreased the most important contributors in the angiogenic process (VEGF-A, bFGF) mRNA (p-value 0.0119, 0.0008 respectively) (Figure 3.3. A, B) along with a significant decrease in TIMP-1 (p-value = 0.0283) and CD31mRNA level (p-value = 0.0109) (Figure 3.3.E, F). Derivative (8), however, insignificantly decreased Thrombopoietin (Figure 3.3. *G).*

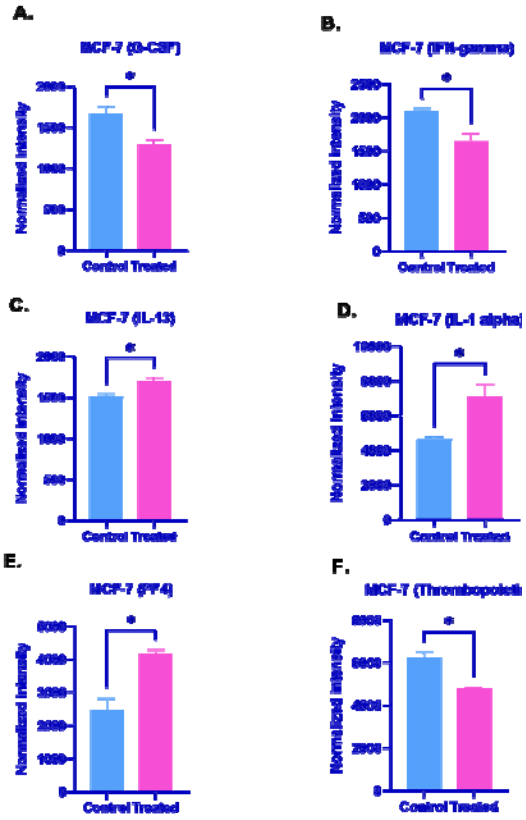


Figure (3.2): Microarray analysis of MCF-7 cell line demonstrating the significant effect of derivative (8) on various angiogenic factors. The cells were treated with (IC_{50}=88.06 µM) of captopril derivative (8) for 36 hours. The experiment was done once with two replicates. An unpaired t – testwas conducted on each angiogenic factor. All results are represented as mean ± SD.

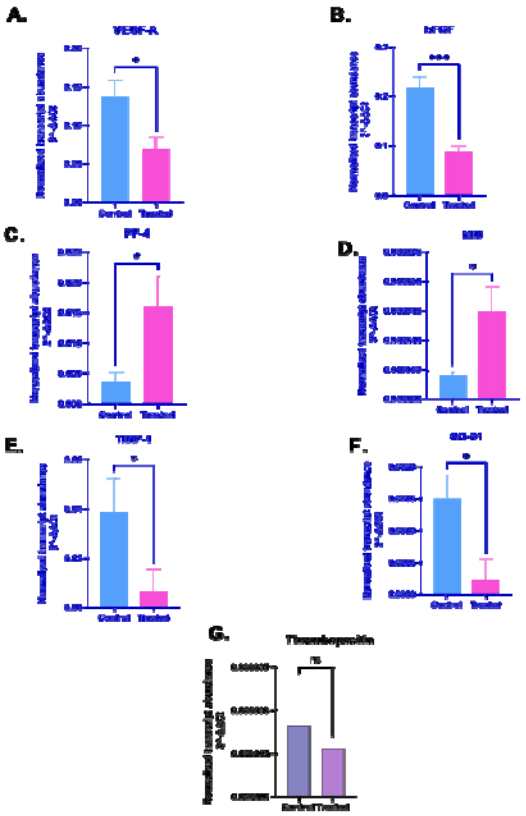


Figure (3.3): RT-qPCR analysis for seven-angiogenic gene expression in MCF-7 cell line after being treated for 36 hours with (IC_{50}= 88.06µM) of derivative (8). The experiment was conducted with three biological replicates for each GOI (control and treated) with duplicate Ct values and was normalized to GAPDH. All results represented here are mean ± SD.

3.3. 3.3 Effect of derivative (8) on AMJ13 angiogenic factors (Microarray and RT-qPCR analysis

The derivative did not cause significant changes in all of the angiogenic factors except a significant increase in MIG (p-value 0.0412), and a significant decrease in IL-1 β (p-value 0.0222) (Figure 3.4 a).

The RT-qPCR analysis for AMJ13 showed that the compound as in the microarray screening insignificantly decreased VEGF-A (Figure 3.4b/A), with an insignificant increase on bFGF, MIG, TIMP-1, (Figure 3.4b/B, D, E) and no significant effect on PF-4 (figure 3.4 b/C). The compound, however, significantly decreased thrombopoietin (p-value 0.0222) and PECAM-1 (p-value 0.0138) (Figure 3.4 b/ G, F)

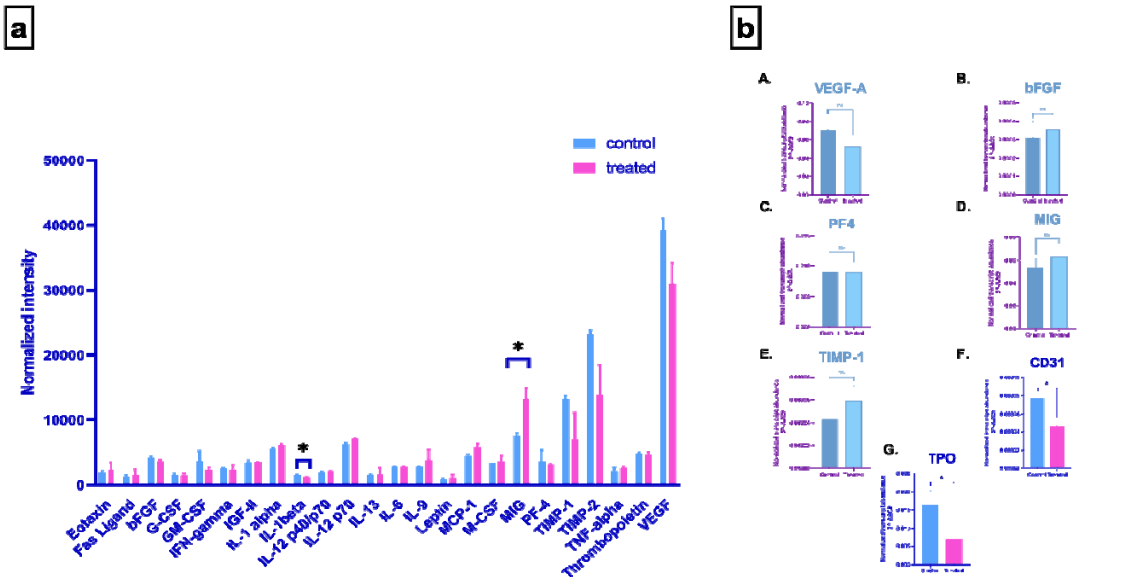


Figure (3.4): Assessment of the anti-angiogenic potential of derivative (8) in AMJ13 breast cancer cells. a) Microarray analysis of AMJ13 demonstrating the effect of compound (8) on various angiogenic factors after being treated with (IC_{50}=66.82µM) of derivative (8) for 36 hours. A significant effect was documented on IL-β and MIG. The experiment was done once with two replicates. An unpairedt–test was conducted on each angiogenic factor. b) RT-qPCR analysis for seven-angiogenic gene expression in AMJ13 cell line after being treated for 36 hours with (IC_{50}=66.82µM) of derivative (8). The experiment was conducted with three biological replicates for each GOI (control and treated) with duplicate Ct values. All results represented here are mean ± SD.

4. Discussion

Studies have proven angiogenesis as a crucial step for breast tumors to metastasize and acquire a more aggressive form (Relf et al., 1997). Thus, several approaches have been developed to target breast cancer angiogenesis. One of these approaches that awaits full elucidation is targeting the Renin-Angiotensin System (RAS) by the use of RAS blockers such as the ACE inhibitor captopril(Napoleone et al., 2012;Cho et al., 2016).

This study investigated the antiangiogenic activity of a thiosemicarbazide derivative of captopril termed derivative (8) in the estrogen receptor-positive MCF-7 and the estrogen/progesterone receptor-negative AMJ13 breast cancer cell lines.

The anti-angiogenesis activity of captopril derivative (8) was investigated based on two main reasons. First, the compound is a derivative of captopril that is known for its anti-angiogenic action in various types of cancer beyond the ACE activity(Hii et al., 1998;Napoleone et al., 2012;Nakagawa et al., 1995;Prontera et al., 1999;Volpert et al., 1996;Williams et al., 2005;Wang et al., 2008;Yoshiji et al., 2001).

Second,Tumour angiogenesis modulation is one of the major mechanisms proposed for RAS to induce the pro-tumour effect and most of its components have been implicated in angiogenesis*(Ager et al., 2008;George et al., 2010;Pinter and Jain, 2017;Vinson et al., 2012;Wegman-Ostrosky et al., 2015)*. Diverse human tumours demonstrated an association between VEGF, VEGFR expression as well as microvessel density with the expression of AT1R (Arrieta et al., 2015;Ino et al., 2006;Shirotake et al., 2011).

4.1 Effect of derivative (8) on angiogenic inducers (VEGF, bFGF, thrombopoietin and, PECAM-1, G-CSF, and IL-β)

Derivative (8) significantly decreased VEGFA mRNA expression in the MCF-7 cell line; if this result would be rationalized based on the fact that the compound is a derivative of captopril and might target RAS components, then it is consistent with many studies that documented the decrease in VEGFA expression in response to captopril. This decrease was observed in many types of cancer, such as triple-negative breast cancer(Napoleone et al., 2012), gastric cancer (Wang et al., 2008), and hepatocellular carcinoma (Yoshiji et al., 2002).

In addition to VEGFA, the compound exerted a stronger effect on the pro-angiogenic factor basic fibroblast growth factor. Various tumors have documented deregulated bFGFsignalling, including breast, lung, prostate, and colon cancer(Lieu et al., 2011).

This growth factor is a very powerful inducer of angiogenesis, where it induces the endothelial cells to invade and form capillary-like tubules with a potency twice that of VEGF(Pepper et al., 1992). Unlike VEGF, the factor was correlated with the angiogenesis maintenance rather than in its initiation (Compagni et al., 2000).

In addition to that, FGF induces angiogenesis synergistically with VEGF, an action that was believed to be achieved by up-regulating VEGF and VEGFR in the endothelial cells. However, each factor imposes a distinct effect on the vessel function and tumor survival(Giavazzi et al., 2003). Furthermore, FGF and FGFR are upregulated in response to anti-VEGF therapies as a resistance mechanism to these therapies(Casanovas et al., 2005); hence, targeting both growth factors and their kinases is a very tempting goal(Lieu et al., 2011).

In this study, Derivative (8) successfully and significantly decreased both growth factors, with a more potent effect on the bFGF, such an effect might decrease the likelihood of resistance rising as a potential future issue.

In addition to that, Captopril derivative (8) also significantly decreased the hematopoietic cytokine thrombopoietin. Hematopoietic cells are known to differentiate and mature to be megakaryocytes by the action of this cytokine, these cells will eventually become platelets(Lin et al., 2014).

It is thought that the platelets potentiate the tumor growth; meanwhile, the tumor hijacks some of the most important activities of the platelet, creating a feedback loop. One of the most important activities is potentiating angiogenesis through the release of their α – granules which are enriched with pro- and anti-angiogenic factors(Battinelli et al., 2014).

Perhaps the greatest proof of this relationship is the correlation between paraneoplastic thrombocytosis and the significant reduction in survival/ and or response that has been recorded in many types of solid tumours(Buergy et al., 2012). Among the many studies that displayed this correlation was that of Stone and colleagues (2012), where not only did they describe this correlation in recently diagnosed ovarian cancer women, but they also demonstrated that both IL-6 and thrombopoietin levels were higher in patients displaying thrombocytosis compared to those without. Moreover, they revealed a reduction in these two factors and restoration of normalized platelet count upon treatment with small interfering RNA(Stone et al., 2012).

In another study, silencing of the hepatic TPO gene in MMTV-PyMT mice significantly reduced TPO, platelet, PF4, and VEGF levels, as well as tumor growth and pulmonary metastasis(Shirai et al., 2019).

Interestingly, in this study, TPO level was decreased in both the estrogen receptor-positive MCF-7 cells, as well as the ER/PR receptor-negative AMJ13 cell lines. In addition to that, a closer look at the microarray results (supplementary materials) would demonstrate a decrease in IL-6 particularly in the MCF-7 cell line even though it is not a significant result; it does warrant further investigation.

Noteworthy, both ACE and RAS components were found to be implicated in the hematopoietic system evolution at the earliest stages, where hematopoietic potential was observed in ACE (+) cells of cultured chicken yolk embryo, while such observation was not detected in ACE(-) cells(Okwan-Duodu *et al.*, 2013).

Another platelet-related factor that has been affected by captopril derivative (8) is the platelet endothelial cell adhesion molecule – 1 (PECAM -1) or (CD31). The compound significantly decreased the mRNA level of this glycoprotein in both cell lines the MCF-7 and the AMJ13. It is worth mentioning that PECAM-1 is considered a reflection of the level of tumor angiogenesis, as well as the presence of tumor endothelial cells since both young and mature endothelial cells tend to express it on their surfaces(Feng et al., 2016).

Several studies have described a reduction in PECAM-1 level upon blocking AT1R, among which one displayed a reduction in tumor growth of the murine melanoma model by losartan. This was justified by the reduction in the mRNA level of both CD31 and VEGFR1 (Flt-1) rather than of VEGF itself(Otake et al., 2010). In another study, angiogenesis and proliferation of nasopharyngeal xenograft tumors were inhibited in response to apoptosis induced by valsartan and losartan; moreover, the AT1R blockers reduced the level of both VEGF and CD31(Lin et al., 2021).

Captopril derivative (8) also significantly reduced the protein level of Granulocyte- Colony Stimulating Factor (G-CSF). Even though this factor is a known mobilizer of hematopoietic progenitor stem cells, yet recently it was found to be implicated in tumour growth, angiogenesis, metastasis and resistance to chemotherapypromotion (Liu et al., 2020). In breast cancer, G-CSF expression was found to be upregulated, specifically in triple-negative breast cancer cells, where higher levels were detected than those in T47D and MCF-7 cells(Lee et al., 2013). It is worth mentioning that AngII has a Stimulatory effect on G-CSF(Jiang et al., 2013).

IL-1β is a pro-angiogenic cytokine that can stimulate angiogenesis(Fahey and Doyle, 2019). The cytokine was also found to be associated with chemoresistance to tamoxifen in TNBC(Jiménez-Garduño et al., 2017). In colorectal cancer, IL-1β release was stimulated by Ang II and its level was successfully decreased by ARBs [reviewed in(Asgharzadeh et al., 2020). Captopril and lisinopril also decreased the expression of IL-β as demonstrated by several studies in several cell types(Nemati et al., 2011;Miguel-Carrasco et al., 2010).

Microarray results displayed a significant increase in IL-1α protein level. Studies have demonstrated a promoted release of this cytokine by AngII(Zaidi and Research, Smith and Missailidis, 2004;George et al., 2010). It is difficult to explain this increment without further investigations, yet two hypotheses can be suggested. First, the derivative did display time-dependent ROS generation (unpublished data). Second, this change might be induced by the derivative independent action from RAS as a system or from the Ang II abrogated effect specifically.

4.2 Effect of captopril derivative (8) on angiogenic inhibitors (PF-4, MIG, TIMP-1, IL-13, and IFN-γ)

Platelet factor 4 is a very potent anti-angiogenic chemokine that was found to bind with VEGF and bFGF in a heparin and heparin – and heparin-independent manner(Teleanu et al., 2019;Belperio et al., 2000). It is suggested that PF4 mainly target microvasculature during angiogenesis owing to its selective binding to the endothelium of the active angiogenesis area only (Hansell et al., 1995). Moreover, high levels of the factor were detected in pre- metastatic stage of lung cancer that later decreased as the tumor progressed to metastasis. In addition to that, the factor inversely correlated with tumour grade and positively with survival(Jian et al., 2017).

In MCF-7 cells, derivative (8) significantly increased both the protein level, as well as the mRNA expression of PF4, with a concurrent decrease in both VEGFA and bFGF.

Another potent anti-angiogenic factor affected by compound (8) is the monokine induced by interferon-gamma (MIG) or (CXCL9). As the term designated, the soluble protein is released from the dendritic cells and macrophage located in the tumour microenvironment in response to interferon–gamma (IFN-γ) (Belperio et al., 2000;Rossi and Zlotnik, 2000).

The anti-angiogenic activity of this protein has been described by several studies; for instance, tumour growth and metastasis inhibition of NSCLC have been correlated with the overexpression of CXCL9 and justified by the decrease of vessel density (Addison et al., 2000). In addition to that, Th1–dependent immunity is promoted by CXCL9 through recruitment of T cells and NK that express CXCL9 receptor (CXCR3) (Groom et al., 2011).

Interestingly, derivative (8) exerted a significant increase in the mRNA level of MIG, but not the protein in MCF-7. This can be correlated with decreased IFN-gamma expression observed in the microarray results (supplementary materials). This might be attributed to the observation that ACE increase the release of the pro-inflammatory mediators independently from Ang II in ACE 10/10 (overexpress ACE) mice compared to ACE KO mice (ACE knock out)(Okwan-Duodu et al., 2013;Bernstein et al., 2018). Whatever the cause might be, it does warrant further investigation.

On the other hand, in AMJ13 cells the derivative increased the MIG protein level, but not the mRNA with no apparent effect on IFN-gamma.

IFN-γ anti- tumour immune response is well documented; however, recent studies have displayed paradoxical effects of this factor promoting tumour genesis. This effect was specifically recognized in the equilibrium and escape phases of the tumor immunoediting paradigm [reviewed in (Zaidi and Research, 2019).The cytokine can be either a pro-apoptotic or proliferative inducer in human lymphocytes depending on the expression of IFN-γR2, where IFN-γ stimulated proliferation and inhibited apoptosis in cells expressing low levels of IFN-γR2. Contrary to that, the pro-apoptotic effect of IFN-γ was seen in cells expressing high levels of IFN-γR2(Bernabei et al., 2001). Studies displayed the involvement of this pro-inflammatory mediator in the suppression of T cells and NK cell activity. This suppression is exerted through the activation and induction of immune checkpoint genes particularly PD-L1 and 2 expressed on the tumour cells as well as due to stimulation of binding to their immune inhibitory receptors PD-1(Abiko et al., 2015;Bellucci et al., 2015;Sharma et al., 2017).

Noteworthy, immune checkpoint inhibitors (ICI) have been investigated by several studies to address this type of immune evasion and were found to act synergistically with anti-VEGF in various types of tumours(Yi et al., 2019).

In this work, it is suggested that compound (8) might exert potent anti-angiogenic action by affecting multiple factors including the most important angiogenesis contributors (VEGF and bFGF). Simultaneously, the derivative might minimize immune evasion by decreasing IFN-γ.

Another factor affected by compound (8) was the Tissue inhibitor of matrix metalloproteinase – 1. TIMP-1 has been known to exert its anti-angiogenic activity by inhibiting MMPs proteolytic activities; however, recent studies have shifted the attention to the complicated roles of TIMP-1 in carcinogenesis that is imposed independently from MMPs inhibition activity. Various types of cancers, including breast have been associated with high levels of TIMP-1; the factor was also correlated with both advanced-stage and fast-relapse tumors(Jackson et al., 2017). Moreover, immunostaining free of TIMP-1 was associated with an improved prognosis of positive node high-grade breast tumours(Kuvaja et al., 2005). Contrary, pre-treatment high serum level of TIMP-1 was associated with endocrine therapy poor response in hormonal-positive breast cancer patients(Lipton et al., 2008). In addition to that, an upregulation in VEGF has been observed in the MCF-7 cell line after being transfected with TIMP-1 cDNA (Yoshiji et al., 1998;Campochiaro et al., 2001). Recently, TIMP-1 has been found to be implicated in tumour cell proliferation through PECAM-1 modulation of the tumour microenvironment, where it is suggested that TIMP-1 might be released from the endothelium in response to PECAM-1 homophilic-dependent binding(Abraham et al., 2018).

In this work, derivative (8) significantly decreased TIMP-1, VEGFA, and PECAM-1 mRNA in the MCF-7 cell line. Noteworthy, AngII has been found to be involved in the regulation of TIMP-1 expression in various cell types (Chen et al., 2003).

Lastly, captopril derivative (8) significantly increased IL-13. It is believed that this anti-inflammatory pro-fibrotic cytokine induces an anti-angiogenic effect similar to those produced by the closely related IL-4, an effect that was attributed to signaling through JAK2/ STAT6(Nishimura et al., 2008).

This study has documented different anti-angiogenic effects of derivative (8) on breast cancer cell lines. As such raised an important question of whether these differences arise because of the distinct characteristics of each cell line, or because the derivative tends to behave differently based on the assumption of its versatility of targets that may or may not involve RAS components.

Without a doubt, the cells are both distinct from each other in their histological characteristics (Al-Shammari et al., 2015;Lee et al., 2015), as well as in RAS involvement. Differences in RAS regulation to angiogenesis have been documented between the two sub-types (hormonal positive and negative receptors)(Herr *et al.*, 2019). Moreover, it was revealed that both cell lines hormonal receptor-positive and receptor-negative express components of RAS(Chen et al., 2003;Herr et al., 2008), yet only in negative cells were the VEGF, HIF-2-α, and TIMP-1 gene expression increased in response to AngII stimulation, which was abolished by candesartan (Herr *et al*., 2008).

Interestingly, in this research derivative (8) exerted an action on the MCF-7 cell line that was likely more dependent on the inhibition of the AngII/ AT1R pathway. At the same time, a different effect was seen on the AMJ13 cell line.

Perhaps, the most essential issue to be mentioned here is the actual expression of RAS components in AMJ13 since, until this date and to our knowledge, no paper has investigated the presence of RAS components in the AMJ13 cell line. This limitation is in fact a very appealing area for future investigation. Examining the expression of RAS components in AMJ13 and exploring the effect of thiosemicarbazide derivatives on these components can provide robust information both on these cells as well as on captopril derivatives' potential mechanism of action.

5. Conclusion

Breast cancer angiogenesis continues to be one of the major challenges in the treatment of this disease as it fuels

the tumor to an invasive and more aggressive form. One of the approaches that recently attracted interest to tackle this challenge is targeting the Renin-Angiotensin System by the use of ACEinhibitors. This study investigated the antiangiogenic activity of a thiosemicarbazide derivative of the ACEi Captorpril. Results of this work have demonstrated the thiosemicarbazide derivative of captopril (8) as a very promising agent with high anti-angiogenic activity, especially in the Estrogen receptor-positive breast cancer cell line (MCF-7). The derivative decreased many vital pro-angiogenic factors specifically VEGF and bFGF, and increased several anti-angiogenic factors such as MIG and PF4.The derivative is a very promising leading molecule for the development of other derivatives. Further investigations will provide robust information on the action of this derivative, revealing potential targets and pathways involved in the regulation of breast cancer angiogenesis.

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Data Availability Statement

Data is within the article and can be provided by the corresponding author upon reasonable request.

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Conflict of Interests

The authors declare no conflict of interest**.**

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