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In vitro anti-proliferative and anti-angiogenic activities of thalidomide dithiocarbamate analogs

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ABSTRACT

Inhibition of angiogenesis is currently perceived as a promising strategy in the treatment of cancer. The anti-angiogenicity of thalidomide has inspired a second wave of research on this teratogenic drug. The present study aimed to investigate the anti-proliferative and anti-angiogenic activities of two thalidomide dithiocarbamate analogs by studying their anti-proliferative effects on human umbilical vein endothelial cells (HUVECs) and MDA-MB-231 human breast cancer cell lines. Their action on the expression levels of IL-6, IL-8, TNF-α, $VEGF_{165}$, and MMP-2 was also assessed. Furthermore, their effect on angiogenesis was evaluated through wound healing, migration, tube formation, and nitric oxide (NO) assays. Results illustrated that the proliferation of HUVECs and MDA-MB-231 cells was not significantly affected by thalidomide at 6.25–100 μM. Thalidomide failed to block angiogenesis at similar concentrations. By contrast, thalidomide dithiocarbamate analogs exhibited significant anti-proliferative action on HUVECs and MDA-MB-231 cells without causing cytotoxicity and also showed powerful anti-angiogenicity in wound healing, migration, tube formation, and NO assays. Thalidomide analogs 1 and 2 demonstrated more potent activity to suppress expression levels of IL-6, IL-8, TNF-α, $VEGF_{165}$, and MMP-2 than thalidomide. Analog 1 consistently, showed the highest potency and efficacy in all the assays. Taken together, our results support further development and evaluation of novel thalidomide analogs as anti-tumor and anti-angiogenic agents.

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1. Introduction

Thalidomide was first synthesized in the 1950s by a German pharmaceutical company and marketed as a sedative. However, pregnant women began using thalidomide as a morning sickness drug because it helped to relieve their nausea. It was soon discovered that thalidomide is highly teratogenic, causing severe malformations in the children of women who took the drug during pregnancy [1,2]. Consequently, the drug never gained approval in the United States and was taken off the world market in 1961. In recent years, interest in thalidomide has been renewed because it appears promising for the treatment of leprosy. In 1998, the FDA approved thalidomide in the United States for treating erythema nodosum leprosum (ENL). The clinical efficacy of thalidomide in inflammatory and autoimmune diseases has been partly attributed to its ability to inhibit tumor necrosis factor-alpha (TNF-α) production [3–5].

Over three decades ago, Folkman [6] introduced the notion that solid tumors cannot grow beyond 2–3 mm^3 without the formation of new blood vessels. Inhibition of angiogenesis has thereafter been perceived as a promising strategy for treating cancer. D'Amato et al. [7] demonstrated that thalidomide inhibits basic fibroblast growth factor-induced angiogenesis in the rabbit cornea and tumor growth in rabbits. Subsequently, the anti-tumor activity of thalidomide has been evaluated in numerous clinical trials. Partial response, stable disease, or tumor regression has been reported in patients with multiple myeloma [8–10], AIDS-related Kaposi's sarcoma [11], high-grade gliomas [12], hepatocellular carcinoma [13,14], renal cell carcinoma [15,16] and advanced melanoma [16]. In androgen-independent prostate cancer, thalidomide has been found to cause a reduction in serum prostate-specific antigen and an improvement in clinical symptoms [17].

Angiogenesis is a crucial regulator of tumor growth and metastasis [18]. Tumor angiogenesis, the development of new blood vessels by the tumor, is regulated by the production of angiogenic stimulators including vascular endothelial growth factor (VEGF). Because VEGF is a key regulatory factor in the prognosis of various cancers, inhibition of VEGF production is a promising therapeutic approach for treating cancer [19].

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TNF-α, a core cytokine produced by immune cells in the bloodstream, acts as pro-angiogenic factor [20]. Thalidomide inhibits TNF-α by stimulating the degradation of messenger RNAs (mRNAs) that involved in immune responses, the stimulation of inflammation, and the suppression of certain cytokines [21–23].

Nitric oxide (NO), an important second messenger in many signaling pathways, is a potent vasodilator [24]. NO is produced by the action of nitric oxide synthase (NOS) in the transformation of arginine to citrulline and activates soluble guanylate cyclase to produce cGMP [25]. NO is involved in angiogenesis and endothelial cell (EC) migration [26]. As thalidomide interferes with angiogenesis, a process in which NO also plays a crucial role, we speculate a cross talk between thalidomide and NO signaling pathway.

Dithiocarbamates have also been attracting considerable interest because of their diverse activities. Dithiocarbamate derivatives have been described as anti-fungals [27], anti-bacterials [28], and carbonic anhydrase inhibitors [29,30]. In particular, their applications in the treatment of cancer have been explored [31,32]. Our group recently reported the synthesis of a novel thalidomide-containing dithiocarbamate moiety; several derivatives exhibited potent anti-cancer activity [33,34].

The present study aimed to investigate the anti-proliferative and anti-angiogenic activities of two thalidomide dithiocarbamate analogs by studying their ability to reduce the proliferation of human umbilical vein endothelial cells (HUVECs) and cells from the MDA-MB-231 human breast cancer cell line. Their effect on the expression of mRNAs encoding interleukin-6 (IL-6), interleukin-8 (IL-8), and TNF-α, $VEGF_{165}$, and matrix metalloproteinase-2 (MMP-2) was also determined. Moreover, we evaluated their effect on angiogenesis through wound healing, migration, tube formation, and NO assays.

2. Materials and methods

2.1. Thalidomide and its dithiocarbamate analogs

Thalidomide and its dithiocarbamate analogs 1 and 2 were synthesized by Dr. Zahran and his group at Menofia University, Egypt. Worldwide patents covering these analogs have been filed. Their chemical structures are shown in Fig. 1. The thalidomide analogs were synthesized according to a previously described procedure [33]. Stock solutions (30 mM) of thalidomide and its analogs were prepared in dimethylsulfoxide (DMSO), stored at 4 °C, and then diluted as required.

2.2. Cell lines and cell culture

HUVECs (CC2517, Lonza) were maintained in cell culture flasks coated with 0.1% gelatin (Wako Pure Chemical Industries, Ltd) and cultured in endothelial cell basal medium (EBM™, Cat. No. CC-3121, Lonza) supplemented with endothelial cell growth medium (EGM™ SingleQuots™, Cat. No. CC-4133, Lonza). Confluent cultures of HUVECs were serially passaged and used between passages 3 and 6.

MDA-MB-231 cells were obtained from RIKEN BRC, Japan. The cells were grown and subcultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (PAA Laboratories, Pasching, Austria) and 100 μg/mL kanamycin (Nacalai Tesque, Kyoto, Japan). The cells were maintained at 37 °C in a 5% CO_2-humidified atmosphere. The culture medium was replaced every other day.

2.3. MDA-MB-231 proliferation assay

The inhibition of the growth of MDA-MB-231 cells by thalidomide and its dithiocarbamate analogs 1 and 2 was evaluated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, MO, USA) cleavage assay. The cells were seeded in 96-well plates at 5×10^3 cells/well in DMEM supplemented with 10% FBS. After 24 h of culture, thalidomide and its analogs were individually added in triplicate in a range of 6.25–100 μM, and the cells were further cultured for 72 h. The cells were then exposed to MTT (5 mg/mL in PBS) at a final concentration of 1 mg/mL in culture for 4 h. Formazan crystals formed during the incubation period were dissolved overnight at 37 °C by adding 10% SDS containing 0.02 N HCl. The absorbance was then measured at 570 nm. To evaluate the viability of cells treated with thalidomide analogs for 72 h, the cells were washed twice with DMEM supplemented with 10% FBS and then further incubated for 48 h in DMEM supplemented with 10% FBS. The number of the viable cells was evaluated using the MTT cleavage assay, as described above.

2.4. Quantitative real-time RT-PCR (qRT-PCR)

Total RNA was isolated using an RNA purification kit (Qiagen RNeasy®) according to the supplier's protocol. First-strand complementary DNA (cDNA) synthesis was performed using an oligo-dT primer and Superscript II reverse transcriptase (Invitrogen). qRT-PCR was performed for IL-6, IL-8, TNF-α, $VEGF_{165}$, MMP-2, and GAPDH using the SYBR green method with a real-time PCR detection system (Roche). Cycling conditions for amplification of all genes, except $VEGF_{165}$ and MMP-2, were as follows: 95 °C for 5 min, followed by 45 cycles of denaturation at 94 °C for 10 s, annealing at 60 °C for 20 s, and extension at 72 °C for 40 s. The amplification conditions for $VEGF_{165}$ and MMP-2 included annealing at 64 °C for 20 s and 66 °C for 10 s and extension at 72 °C for 20 s and 72 °C for 12 s, respectively. The transcritical cycle (Ct) values were recorded and the specificity for amplification was evaluated using melting curve analysis software (LightCycler® 480). Primer sequences for qRT-PCR are listed in Table 1. The relative gene expression levels were calculated using the comparative Ct(ΔΔCt) method, where the relative expression was calculated as $2^{-\Delta\Delta Ct}$, and Ct represents the threshold cycle [35], using *GAPDH* as the reference gene.

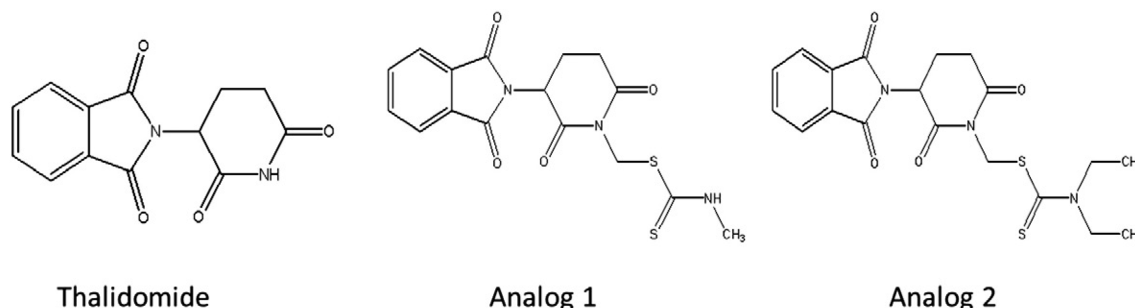

Fig. 1. Chemical structures of thalidomide and its dithiocarbamate analogs 1 and 2.

Table 1
Primer sequences for qRT-PCR.

Name	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)
IL-6	AAATTCGGTACATCCTCGAC	CCTCTTTGCTGCTTTCACAC
IL-8	TACTCCAAACCTTTCCACCC	AAAACTTCTCCACAACCCTC
TNF-α	GCCTGCTGCACTTTGGAGTG	TCGGGGTTCGAGAAGATGAT
$VEGF_{165}$	CCCTGATGAGATCGAGTACATCTT	AGCAAGGCCCACAGGGATTT
MMP-2	ATGACAGCTGCACCACTGAG	ATTTGTTGCCCAGGAAAGTG
GAPDH	CAACGACCACTTTGTC	GGTCTACATGGCAACTGTGAGG

2.5. HUVEC viability assay

HUVECs were seeded in 96-well plates at 10×10^3 cells/well in endothelial cell basal medium-2 (EBM™-2, Cat. No. CC-3156, Lonza) supplemented with endothelial cell growth medium (EGM™-2 SingleQuots™, Cat. No. CC-4176, Lonza) and allowed to attach overnight at 37 °C and 5% CO_2. The culture medium was then aspirated and fresh culture medium containing vehicle (0.3% DMSO), thalidomide, or thalidomide analogs (6.25–100 μM) was added to each well. After 48 h, the number of viable cells was evaluated using the MTT cleavage assay.

2.6. HUVEC growth curve

HUVECs, 1×10^5, were seeded overnight in 24-well culture plate, and then the medium was changed with fresh EBM-2 medium containing either DMSO (0.3%), thalidomide analogs 1 or 2 (100 μM). After 24 and 48 h of treatments, the cells were washed with EBM-2 medium and trypsinized with trypsin-EDTA (Lonza). The cell suspensions were further added with trypan blue dye (Sigma) and then counted with TC10 automated cell counter (Bio-Rad, Hercules, CA, USA). The cell growth curves were established based on the number of viable cells exhibiting trypan blue exclusion.

2.7. Wound healing assay

HUVECs were seeded into a 24-well plate at 1×10^5 cells/well in EBM™-2 supplemented with EGM™-2. After 24 h of incubation, each confluent monolayer was scratched using a 200-μL plastic pipette tip to create a wounded cell-free area and then washed twice with EBM™-2. The cells were incubated at 37 °C with vehicle (0.3% DMSO), thalidomide, or thalidomide analogs in a concentration range of 12.5–100 μM in EBM™-2 supplemented with EGM™-2. The cells were photographed at 0 and 24 h using an inverted microscope, CKX41 (Olympus, Tokyo, Japan), equipped with a digital camera, U-CMDA3 (Olympus). The distance between the edges of the cell-free areas was measured using the imaging program DP2-BSW (Olympus). Cell migration was calculated using the following equation: $\%R = [1 - (\text{wound length at } T_{24} / \text{wound length at } T_0)] \times 100$, where (%$R$) is the percent recovery, T_0 is the wound length at 0 h, and T_{24} is the wound length at 24 after injury [36]. Experiments were independently repeated thrice.

2.8. Preparation of MDA-MB-231 conditioned medium (CM)

MDA-MB-231 cells were seeded at a density of 1×10^6 cells per 60 mm dish in DMEM medium supplemented with 10% FBS. After 24 h of culture, the cells were washed with DMEM medium supplemented with 2% FBS and incubated for an additional 48 h in the same medium with vehicle (0.3% DMSO), thalidomide, or thalidomide analogs (25 and 100 μM). The conditioned media (CM) were collected, centrifuged to remove insoluble materials and then stored at −20 °C until used in HUVEC migration assay.

2.9. Cell migration assay

The migration of HUVECs was assayed in 24-well plates with 8 μm pore cell culture inserts (BD, Franklin Lakes, NJ, USA). HUVECs (1×10^5), suspended in 200 μL of serum-free medium, were seeded onto the upper compartment of the insert chamber. The lower chamber was filled with 500 μL of MDA-MB-231 CM pretreated with vehicle (0.3% DMSO), thalidomide, or thalidomide analogs (25 and 100 μM). After 48 h incubation at 37 °C under 5% CO_2, the insert chambers were removed, and adherent cells on the bottom of each well were counted under microscope, CKX41 (Olympus, Tokyo, Japan). The number of migrated cells was normalized by the number of adherent cells in vehicle control.

2.10. Tube formation assay

First, 96-well plates were coated (50 μL/well) with matrigel basement membrane matrix (BD Biosciences). Following this, the plates were allowed to polymerize at 37 °C under 5% CO_2 for 30 min. HUVECs were then seeded at 2×10^4 cells/well in EBM™-2 supplemented with EGM™-2 containing vehicle (0.3% DMSO), thalidomide, or thalidomide analogs (12.5–100 μM) and incubated at 37 °C under 5% CO_2. After 8 h of incubation, the tube structures were photographed using an inverted microscope, CKX41 (Olympus, Tokyo, Japan), equipped with a digital camera, U-CMDA3 (Olympus). The digital images were analyzed using DP2-BSW imaging software. The circular images of the wells were divided into segments and the stimulation was then quantified by counting the number of branching points formed within each segment. Experiments were independently repeated thrice.

2.11. Nitric oxide assay

HUVECs were seeded at a density of 1×10^6 into 60 mm culture dishes for 24 h and then the medium was changed with fresh medium containing either vehicle control (0.3% DMSO), thalidomide, or thalidomide analogs at concentrations of 25 and 100 μM for 48 h. Production of NO by HUVECs was measured using Griess method [37] and according to the indication on the non-enzymatic colorimetric NO assay kit (Oxford Biomedical Research, USA). This step was performed by measuring the accumulation of nitrites, a stable end product of NO metabolism, in the supernatant of HUVECs. Because the NO present in biological fluids is recovered in nitrate form, the supernatant was overnight treated with desired amount of washed and dried cadmium beads with agitation at room temperature in order to reduce nitrate to nitrite. Finally, the samples were mixed with an equal volume of freshly prepared Griess reagent for 20 min. Absorbance of each sample was measured at 540 nm using a microplate reader SH-9000 Lab (CORONA ELECTRIC Co. Ltd., Hitachinaka, Japan). Concentrations of NO in the samples were determined using a calibration curve generated with standard sodium nitrite ($NaNO_2$) solutions (0.5–100 μM).

2.12. Statistical analysis

The results are expressed as the mean ± standard deviation from at least three independent experiments. The data were analyzed using Student's t test. Differences yielding p values < 0.05 were considered statistically significant.

3. Results

3.1. Effect of thalidomide and its dithiocarbamate analogs on MDA-MB-231 proliferation

As shown in Fig. 2A, compared with vehicle control (0.3% DMSO), thalidomide dithiocarbamate analogs reduced MDA-MB-231 cell proliferation in a concentration-dependent manner. However, only at higher

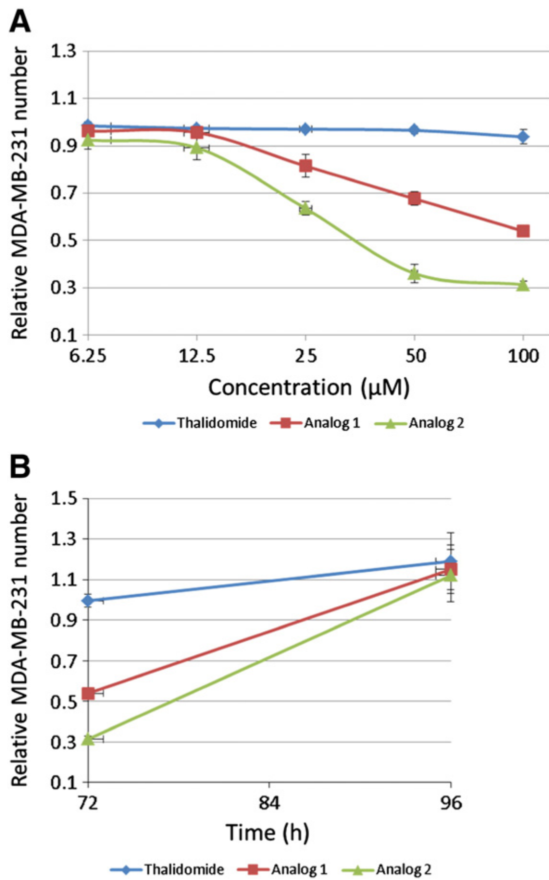

Fig. 2. Effect of thalidomide and its dithiocarbamate analogs on MDA-MB-231 cell viability. (A) The inhibition of cell growth in the presence of thalidomide and its analogs 1 and 2 for 72 h. (B) The viable cells at 72 h were kept cultured without thalidomide and its analogs up to 96 h. Cell numbers in each well were assessed using the MTT assay. The absorbance at 570 nm corresponding to the initial number of the cells was defined as 1.

concentrations (≥25 μM), the inhibition reached statistical significance. MDA-MB-231 cell proliferation was not significantly decreased by any concentration of thalidomide (6.25–100 μM). However, analogs 1 and 2 potently reduced MDA-MB-231 cell proliferation at 25 μM, by 18.6% and 36.5%, respectively. To determine whether the reduction of MDA-MB-231 cell viability by thalidomide analogs was the result of cytotoxicity or a growth-inhibitory effect, we analyzed the ability of MDA-MB-231 cells treated with 100 μM of thalidomide or its analogs for 72 h to resume their growth in the next 24 h in the absence of thalidomide or its analogs. Results illustrated that compared with vehicle control (0.3% DMSO), MDA-MB-231 cells can resume their growth in the next 48 h (Fig. 2B). Therefore, we suggest that these thalidomide analogs inhibit the growth of MDA-MB-231 through a cytostatic effect and not a cytotoxic effect.

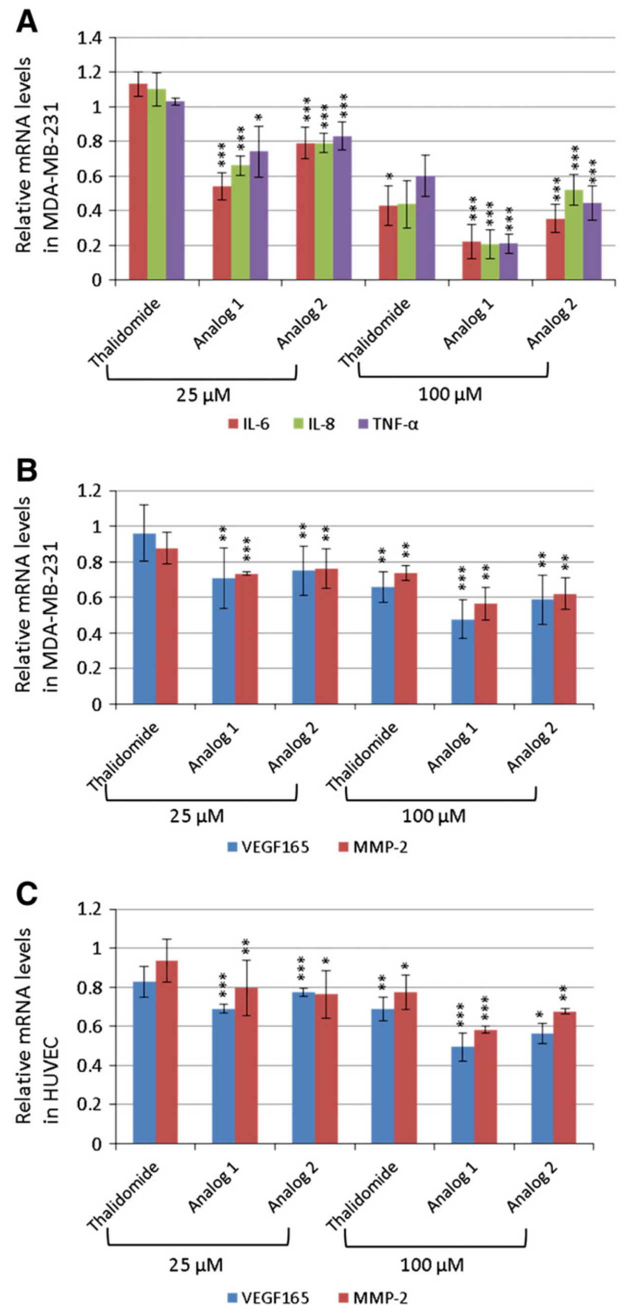

Fig. 3. Effect of thalidomide and its dithiocarbamate analogs on gene expression. (A) Effect of thalidomide and its analogs on expression of IL-6, IL-8, and TNF-α mRNA in MDA-MB-231 cells. (B) Effect of thalidomide and its analogs on expression of $VEGF_{165}$ and MMP-2 mRNA in MDA-MB-231 cells. (C) Effect of thalidomide and its analogs on expression of $VEGF_{165}$ and MMP-2 mRNA in HUVEC cells. Data are presented as mean ± standard deviation. Significantly (*$p < 0.1$, **$p < 0.05$, ***$p < 0.01$) different from the vehicle control.

3.2. Effect of thalidomide and its dithiocarbamate analogs on the expression of mRNAs encoding IL-6, IL-8, TNF-α, $VEGF_{165}$, and MMP-2

During tumor angiogenesis, VEGF is primarily produced by tumor cells. It induces the formation of new vessels in the direction of the tumor [38]. VEGF production by tumor cells is thought to be one of the most specific and critical regulators of the angiogenic signaling cascade [39]. VEGF production can be augmented by many factors, including IL-6, IL-8, and TNF-α [40].

Therefore, the effect of thalidomide and its dithiocarbamate analogs on the expression levels of IL-6, IL-8, TNF-α, $VEGF_{165}$, and MMP-2 mRNA in MDA-MB-231 was investigated. As shown in Fig. 3A and B, thalidomide at 25 μM had no effect on the expression levels of IL-6, IL-8, TNF-α, $VEGF_{165}$, and MMP-2, while, it diminished the same expression levels at 100 μM by 57.3%, 56.3%, 40%, 34.3% and 26.3%, respectively, compared with the vehicle control. Conversely, thalidomide analogs showed more potency than thalidomide toward the expression levels of IL-6, IL-8, TNF-α, $VEGF_{165}$, and MMP-2. Because, analog 1 at 100 μM showed more potent inhibition of IL-6, IL-8, TNF-α, $VEGF_{165}$, and MMP-2 (by 78%, 79.7%, 79%, 52.3% and 43.7%, respectively) than analog 2 (which reduced the same expression levels by 64.7%, 48%, 56%, 41.3% and 38%, respectively), relative to the vehicle control.

In HUVEC cells, thalidomide at 25 μM had no significant effect on the expression levels of $VEGF_{165}$ and MMP-2 compared with the vehicle control, whereas, it diminished the same expression levels at 100 μM by 31% and 22.7%, respectively. By contrast, analog 1 at 100 μM showed

more potent inhibition of $VEGF_{165}$ and MMP-2 (by 50.71% and 41.3%, respectively) than analog 2 (which reduced the same expression levels by 43.7% and 32.3%, respectively), relative to the vehicle control (Fig. 3C).

3.3. Effect of thalidomide and its dithiocarbamate analogs on HUVEC proliferation

Endothelial cell proliferation is one of the complex multistep processes involved in angiogenesis [41]. Therefore, the ability of thalidomide and its dithiocarbamate analogs 1 and 2 to inhibit the proliferation of endothelial cells was examined. As shown in Fig. 4, HUVEC proliferation was not significantly decreased by any concentration of thalidomide (6.25–100 μM). By contrast, analog 1 significantly reduced HUVEC proliferation at 50 μM by 43.3%. Compared with the vehicle control (0.3% DMSO), analog 2 reduced HUVEC proliferation by 51.6%, but only at the highest concentration. To determine whether the reduction in HUVEC viability caused by thalidomide analogs was due to cytotoxicity or a growth-inhibitory effect, we analyzed the growth curves of HUVECs treated with vehicle control (0.3% DMSO), analogs 1 or 2 (100 μM) for 48 h. As shown in Fig. 4B, HUVEC number of vehicle control increased from 1×10^5 cells at day 0 to 1.4×10^5 cells at day 1. While in the presence of 100 μM of analogs 1 or 2 the numbers of HUVECs after 24 h were 1.02×10^5 and 1.06×10^5, respectively. The growth rate revealed that HUVEC numbers treated with analogs 1 or 2 started to increase to be 1.78×10^5 and 2.17×10^5, respectively but cell numbers were still significantly lower than the vehicle control at 48 h. Consequently, we can propose that thalidomide analogs exerted growth-inhibitory effect on HUVECs but without causing significant cytotoxicity.

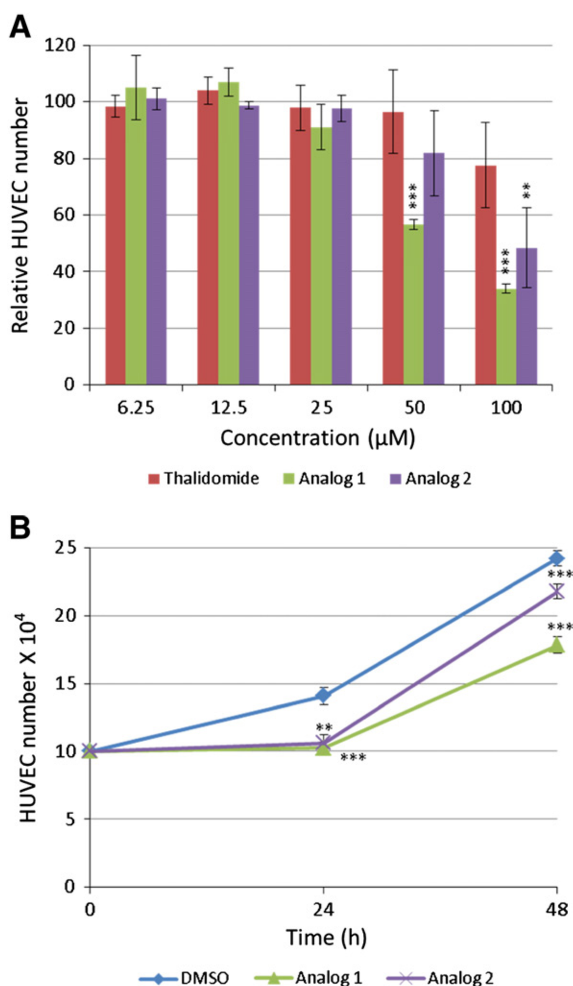

Fig. 4. Effect of thalidomide and its dithiocarbamate analogs on HUVEC viability. (A) The inhibition of cell growth in the presence of thalidomide and its analogs 1 and 2 for 48 h. Cell numbers in each well were assessed using the MTT assay. The absorbance at 570 nm corresponding to the initial number of the cells was defined as 100%. (B) The growth curves of HUVEC treated with control (0.3% DMSO) or thalidomide analogs (100 μM) for 24 and 48 h. Data are presented as mean ± standard deviation. Significantly (**$p < 0.05$, ***$p < 0.01$) different from vehicle control at the same time point.

3.4. Effect of thalidomide and its dithiocarbamate analogs on HUVEC migration through wound healing assay

The wound-healing model is used to estimate the migration potential of endothelial cells in monolayer culture [42]. Therefore, the wound healing migration assay was performed to determine the effect of thalidomide and its dithiocarbamate analogs 1 and 2 on HUVEC migration. As shown in Fig. 5A, HUVEC migration was not significantly reduced by any concentration of thalidomide, in a range of 12.5–100 μM, within 24 h. On the other hand, analog 1 was found to be more potent and efficacious (inhibiting migration at 25 μM by 32.2%) than analog 2 (which significantly reduced migration only at the highest concentration by 62.2%). Representative migration images are shown in Fig. 5B. To prove that the change in wound distance after 24 h was due to the effect of thalidomide and its analogs on the migration of endothelial cells, and not due to their effect on endothelial cell proliferation, we measured endothelial cell proliferation using identical concentrations of thalidomide and its analogs during the same time of exposure. Proliferation was measured using the trypan blue exclusion method. The results (data not shown) demonstrated that there was no loss of endothelial cell viability.

3.5. Effect of thalidomide and its dithiocarbamate analogs on migration ability of HUVEC co-cultured with MDA-MB-231

To check the effect of thalidomide and its dithiocarbamate analogs 1 and 2 on the migration ability of HUVEC co-cultured with MDA-MB-231 CM, we firstly treated MDA-MB-231 cells with vehicle (0.3% DMSO), thalidomide, or thalidomide analogs (25 and 100 μM) for 48 h, then CM was harvested and its angiogenic activity was checked by examining the level of HUVEC migration. Compared with the vehicle control, HUVEC migration was not significantly reduced by CM pre-treated with thalidomide (25 or 100 μM) within 48 h (Fig. 6). Conversely, MDA-MB-231 CM pre-treated with thalidomide analog 1 was found to be more effective (inhibiting HUVEC migration at 25 μM by 35.58%) than CM pre-treated with thalidomide analog 2 (which significantly reduced HUVEC migration at 25 μM by 23.68%), relative to vehicle control.

3.6. Effect of thalidomide and its dithiocarbamate analogs on HUVEC tube formation

To investigate the morphogenic potential of thalidomide and its dithiocarbamate analogs 1 and 2, a tube formation assay was performed. This assay is one of the most popular in vitro angiogenesis tests [43]. As demonstrated in Fig. 7A, thalidomide in the concentration range of 12.5–100 μM failed to significantly block tube formation. Tube formation appeared to be more sensitive to the effect of thalidomide analogs. Compared with the vehicle control (0.3% DMSO), significant inhibitions of 86.4% and 64.3% were observed at 12.5 μM with analogs 1 and 2, respectively. Representative tube images are shown in Fig. 7B.

3.7. Effect of thalidomide and its dithiocarbamate analogs on NO production

NO plays a crucial role in angiogenesis process and migration of endothelial cells [26]. Therefore, the effect of thalidomide and its analogs 1 and 2 on NO production level in HUVEC was studied. As shown in Fig. 8, thalidomide failed to significantly reduce NO production level in HUVEC supernatants either at 25 or 100 μM. On the other hand,

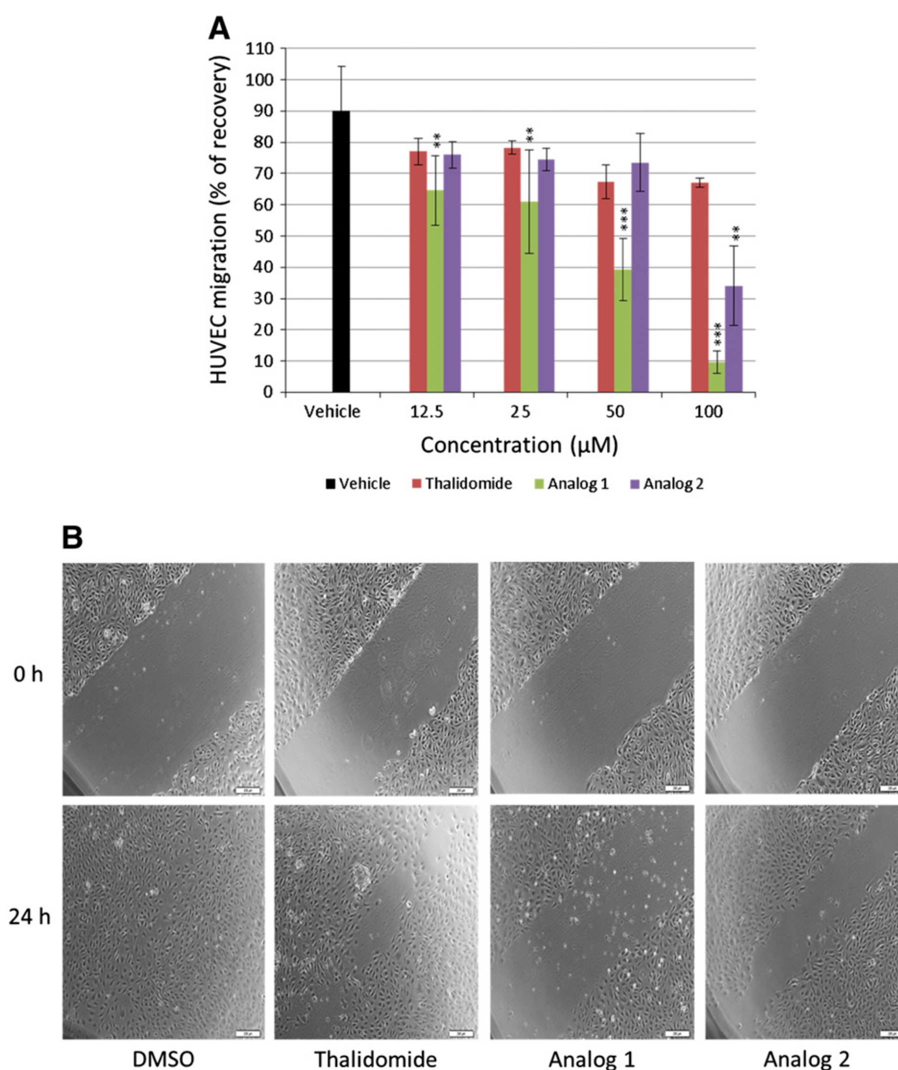

Fig. 5. Effect of thalidomide and its dithiocarbamate analogs on HUVEC migratory ability. (A) The percent recovery as determined in the scratch-wound assay. Data are presented as mean ± standard deviation. Significantly (** $p < 0.05$, *** $p < 0.01$) different from vehicle control. (B) Representative images of wounds treated with the vehicle control (0.3% DMSO), thalidomide, or thalidomide dithiocarbamate analogs at 100 μM.

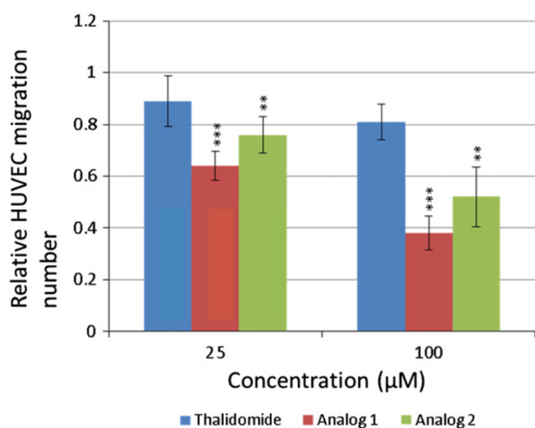

Fig. 6. Effect of thalidomide and its dithiocarbamate analogs on migration ability of HUVEC co-cultured with MDA-MB-231, as determined in the migration assay. Translocated cell numbers were normalized against those in the vehicle control. Data are presented as mean ± standard deviation. Significantly (** $p < 0.05$, *** $p < 0.01$) different from vehicle control.

analogs 1 and 2 could reduce NO production level by 8.56% and 4.49%, respectively at 25 μM in comparison with the vehicle control (0.3% DMSO). Furthermore, NO production was more sensitive to the effect of thalidomide analogs at 100 μM, as significant inhibitions of 24.51% and 18.65% were observed with analogs 1 and 2, respectively.

4. Discussion

Cancer, a leading cause of death globally, causes almost 8 million deaths each year and poses a major socioeconomic hazard to humanity at large. Among all factors resulting in the ultimate failure of cancer treatment, drug resistance is a significant player [44]. The development of new molecules to fight cancer is urgently required because most anticancer drugs are ineffective owing to drug resistance. Molecular hybridization, which covalently combines two or more drug pharmacophores into a single molecule, is an effective tool to design highly active novel entities [45]. These merged pharmacophores may act on multiple therapeutic targets and offer the possibility of circumventing drug resistance. In addition, the hybrids also minimize unwanted side effects and enable synergic action [46]. The molecular hybridization approach has already been applied to the development of novel antimalarial agents for overcoming drug resistance [47]. Due to the anti-angiogenic properties

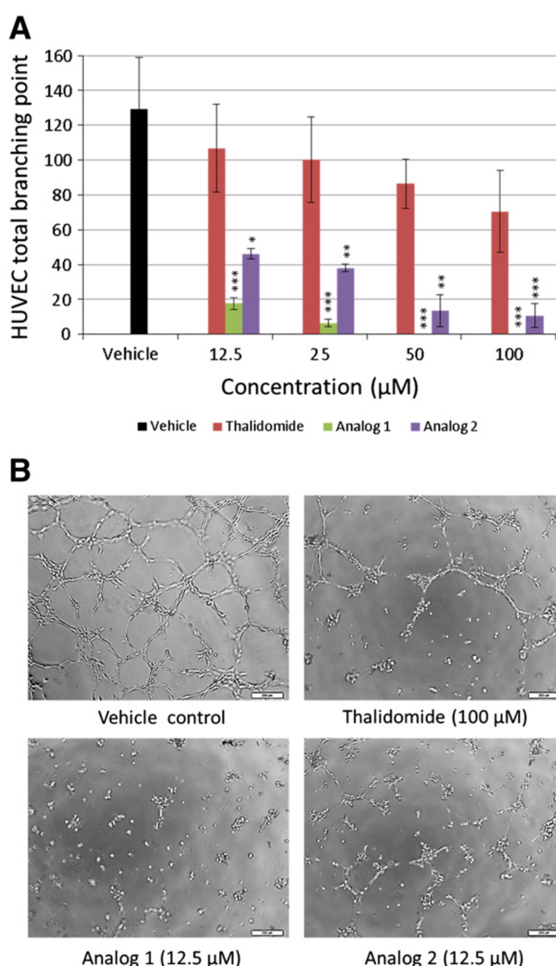

Fig. 7. Effect of thalidomide and its dithiocarbamate analogs on HUVEC capillary morphogenesis. (A) The number of branching points formed within each segment. Data are presented as mean ± standard deviation. Significantly (*$p < 0.1$, **$p < 0.05$, ***$p < 0.01$) different from the vehicle control. (B) Representative images from the tube formation assay showing a higher potency of thalidomide dithiocarbamate analogs than that of thalidomide. Vehicle control is 0.3% DMSO.

of thalidomide [7,48] as well as the numerous biological activities of dithiocarbamates and their application in cancer treatment [49,50], the idea of combining or merging thalidomide and a moiety of dithiocarbamate caught the attention of our research group. This approach was pursued to design and synthesize new bioactive molecules with higher activity than thalidomide itself. In addition, thalidomide dithiocarbamate analogs had no negative co-later effects and this was obviously noticed from our previous study which showed that they had no necrotic effect on the liver of tumored mice compared with the liver of mice treated with the parent thalidomide [33,34].

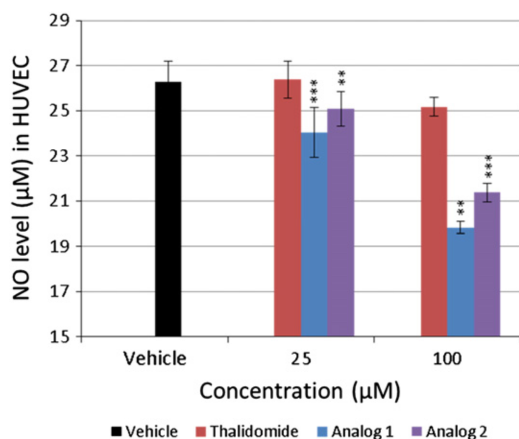

Fig. 8. Effect of thalidomide and its dithiocarbamate analogs on NO production level in HUVEC cells. Data are presented as mean ± standard deviation. Significantly (**$p < 0.05$, ***$p < 0.01$) different from the vehicle control.

The present study was designed to investigate the anti-proliferative and anti-angiogenic activities of two thalidomide dithiocarbamate analogs in comparison with the activity of parent thalidomide against HUVECs and the MDA-MB-231 breast cancer cell line.

Angiogenesis is the process of generating new capillary blood vessels. It plays an important role in the proliferation, invasion, and metastasis of malignant tumors. Blocking tumor-induced angiogenesis continues to be an attractive strategy for cancer therapy [51]. As shown in our results, MDA-MB-231 proliferation was not significantly reduced by any concentration of thalidomide in a range of 6.25–100 μM. On the other hand, compared with vehicle control (0.3% DMSO), analogs 1 and 2 potently reduced MDA-MB-231 proliferation at 25 μM by 18.6% and 36.5%, respectively (Fig. 2A). This finding was consistent with a previous report that thalidomide analogs containing sulfur atoms have greater inhibitory activity than thalidomide on Ehrlich ascites carcinoma cell lines [33]. Thalidomide analogs inhibited MDA-MB-23 cell proliferation through cytostatic effects rather than cytotoxic effects. This was demonstrated by the ability of MDA-MB-231 cells treated with 100 μM of thalidomide analogs for 72 h to resume their growth in the next 24 h in the absence of thalidomide or its analogs (Fig. 2B). Consequently, we conclude that the greater inhibitory activity of these analogs than thalidomide may be attributed to the presence of the dithiocarbamate group in their chemical structures.

Tumor angiogenesis has been recently observed to correlate with hypoxia and the inflammatory process within the tumor [38]. When a solid tumor grows beyond 2 mm in diameter, insufficiency of oxygen and nutrients becomes obvious. Hypoxia in the inner part of the tumor will attract the infiltration of many inflammatory cells. Many factors are produced from the hypoxic region of the tumor and inflammatory cells, including IL-6, IL-8, TNF-α, and VEGF. All these factors are involved in tumor angiogenesis. Therefore, the effect of thalidomide and its dithiocarbamate analogs on the expression of IL-6, IL-8, TNF-α, $VEGF_{165}$, and MMP-2 mRNA was investigated. As shown in Fig. 3A and B, compared with vehicle control, thalidomide at 100 μM diminished the expression levels of IL-6, IL-8, TNF-α, $VEGF_{165}$, and MMP-2 mRNA in MDA-MB-231 cells by 57.3%, 56.3%, 40%, 34.3%, and 26.3%, respectively. These findings are consistent with a previous report that thalidomide suppresses the induction of VEGF in co-cultures of multiple myeloma cell lines and bone marrow stromal cells [52]. In addition, thalidomide inhibited the expression of VEGF and IL-6, which is thought to be the mechanism underlying the attenuation of angiogenesis by thalidomide [52]. Moreover, the inhibition of TNF-α production was initially considered to be a key mechanism of action of thalidomide [4].

On the other hand, analog 1 at 100 μM demonstrated more potent inhibition ability to suppress the expression levels of IL-6, IL-8, TNF-α, $VEGF_{165}$, and MMP-2 in MDA-MB-231 cells (by 78%, 79.7%, 79%, 52.3%, and 43.7%, respectively) than analog 2 (which reduced the same expression levels by 64.7%, 48%, 56%, 41.3%, and 38%, respectively), relative to the vehicle control. Reportedly, thalidomide reduces the production of TNF-α by enhancing TNF-α mRNA degradation [4]. In addition, Rosinol et al. [53] stated that the decrease in circulating levels of VEGF correlates with the therapeutic efficacy of thalidomide in patients with multiple myeloma and myelodysplastic syndromes. Furthermore, IL-6 is a potent growth factor for malignant plasma cells, and its inhibition may be partly responsible for the action of thalidomide in myeloma [54]. IL-8 promotes leukin chemotaxis into tumors, leading to tumor neovascularization and acceleration of tumor growth and metastasis [55]. Therefore, the activity of thalidomide analogs could be interpreted to be resulting from the viability of these analogs, particularly analog 1, to effectively reduce the expression levels of IL-6, IL-8, TNF-α, $VEGF_{165}$, and MMP-2.

Thus, the more potent inhibitory effects of the thalidomide analogs in comparison with the effects of the parent thalidomide may be attributed to the presence of the sulfur atoms in these novel compound structures. This change may have led to an increase in inabilities to suppress the levels of IL-6, IL-8, TNF-α, $VEGF_{165}$, and MMP-2 mRNA expression.

To gain additional insight into the anti-angiogenic activity of thalidomide dithiocarbamate analogs, their ability to inhibit HUVEC proliferation, migration, and tube formation was assessed.

The proliferation of endothelial cells is considered to be a primary event in angiogenesis and is therefore a potential target for anti-tumor therapy [41]. Therefore, the anti-proliferative activity of thalidomide and its analogs against HUVECs was investigated. The results demonstrated that HUVEC proliferation was not significantly decreased by any concentration of thalidomide in a range of 6.25–100 μM. By contrast, compared with vehicle control (0.3% DMSO), thalidomide analogs significantly reduced HUVEC proliferation, as shown in Fig. 4A. Parallel to our finding, Ng et al. [56] observed that thalidomide, at concentrations of 12.5–200 μM, failed to inhibit HUVEC growth, while other N-substituted analogs significantly inhibited cell proliferation. Thalidomide analogs inhibited HUVEC proliferation through a cytostatic effect rather than a cytotoxic effect. This was deduced from the growth curves of HUVEC treated with 100 μM of thalidomide analogs 1 and 2 for 48 h (Fig. 4B). This suggests that the replacement of the imido hydrogen in the glutarimide ring (N-substitution) with dithiocarbamate moieties generated analogs with more potent anti-proliferative activity than thalidomide.

Endothelial cells serve as a barrier between the intravascular compartment and the surrounding tissues. Thus, they are directly exposed to various stresses, including hypoxia and insufficient nutrient supply. If the stress is sufficiently severe to cause endothelial injury, wound repair processes are immediately initiated to restore this barrier function. Wound repair is a complex process that includes cell adhesion, migration, and proliferation [57].

Our data, through wound healing assay, showed that HUVEC migration was not significantly reduced by thalidomide at concentrations of 12.5–100 μM; however, thalidomide analogs, particularly analog 1, exhibited forceful activity to inhibit HUVEC migration (Fig. 5A). With regard to thalidomide, our finding was in agreement with the previous report [58], which mentioned that thalidomide at concentrations of 25–75 μM had no effect on the rate of endothelial cell migration, while thalidomide at 150 μM caused only a 20% reduction in the rate of wound healing.

In addition, Fig. 6 revealed that, compared with the vehicle control, HUVEC migration was not significantly reduced by MDA-MB-231 CM pre-treated with thalidomide, while CM pre-treated with thalidomide analogs 1 and 2 was found to be more effective to inhibit HUVEC migration. VEGF is probably the most important inducer of angiogenesis, owing to its potency and selectivity for endothelial cells and its ability to regulate the proliferation and migration of endothelial cells [57,59]. MMP-2, predominately expressed in the endothelial cells, was directly involved in endothelial cell migration and vascular remodeling during angiogenesis [60,61]. Moreover, the present study revealed that thalidomide analogs, particularly analog 1, demonstrated potent activity to reduce the expression levels of MMP-2 and $VEGF_{165}$ (Fig. 3C). In addition, they strongly suppressed HUVEC proliferation (Fig. 4). Thus, the potency of both analogs, particularly analog 1, to reduce HUVEC migration could be related to their potent inhibitory action on MMP-2 and VEGF expression levels in addition to HUVEC proliferation.

The abilities of endothelial cells to differentiate in response to specific stimuli and to migrate out of existing blood vessels to organize new capillaries are important for angiogenesis [38]. To examine the morphogenic potential of thalidomide and its analogs, we used an in vitro assay that allows the visualization of multicellular tube-like structures, which resemble microvascular networks, on matrigel.

The results proved that thalidomide in a concentration range of 12.5–100 μM had no significant effect on the formation of capillary tubes. On the other hand, compared with vehicle control (0.3% DMSO), significant inhibitions of 86.4% and 64.3% were observed at 12.5 μM concentrations of analogs 1 and 2, respectively (Fig. 7A). With regard to thalidomide, this finding was consistent with a previous report that thalidomide at concentration of 12.5–200 μM failed to block angiogenesis in vitro, while some thalidomide analogs significantly inhibited microvessel outgrowth [56].

With regard to thalidomide analogs, it has been suggested that TNF-α indirectly mediates the angiogenesis process and that its mechanism may depend on the synthesis of secondary mediators, including IL-8 and VEGF [62–65]. Furthermore, VEGF plays a pivotal role in normal and pathological angiogenesis because its activation triggers multiple signaling networks that result in endothelial cell survival, mitogenesis, migration, and differentiation [66]. Moreover, IL-8 mediates the inhibition of endothelial cell apoptosis, endothelial cell proliferation, and capillary tube formation [67]. Therefore, the maximum inhibition of tube formation was observed with thalidomide analogs, particularly analog 1. This is expected from the present study because thalidomide analogs, particularly analog 1, demonstrated more potent ability to reduce the expression levels of IL-6, IL-8, TNF-α, $VEGF_{165}$, and MMP-2. They also exhibited strong suppression of HUVEC proliferation and migration.

NO is an important physiological messenger and effecter molecule in many biological systems, including immunological, neuronal and cardiovascular tissues [68,69]. It plays an important role in various phenomenon involved in angiogenic process by modulating the activity of angiogenic factor released by tumor cells such as VEGF [70]. As shown in Fig. 8, thalidomide failed to significantly reduce NO production level in HUVEC supernatants either at 25 or 100 μM (Fig. 8). This finding is consistent with previously reported results which concluded that thalidomide has been found to exhibit weak NOS inhibitory activity [71]. On the contrary, level of NO production was reduced after the treatment of HUVECs with analogs 1 and 2. This potency of both analogs, particularly analog 1, to reduce NO production level could be related to their potent inhibitory effect on the VEGF expression. Recent studies showed that VEGF increases NO production in rabbit or human endothelial cells and NO is thought to be an important mediator in VEGF-induced endothelial cell proliferation [72,73].

In summary, our results suggest that the anti-cancer action of synthetic thalidomide dithiocarbamate analogs results from both direct action in cancer cells (inhibition of growth as well as IL-6, IL-8, TNF-α, $VEGF_{165}$, and MMP-2 expression levels) and indirect action through endothelial cells (inhibition of proliferation, migration, and tube formation as well as NO production). These findings further suggest that thalidomide dithiocarbamate analogs may be excellent leads for the development of anti-angiogenic and anti-cancer drugs.

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