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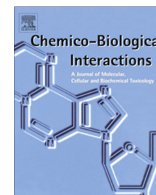

Improvement of *in vivo* anticancer and antiangiogenic potential of thalidomide derivatives

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ABSTRACT

The strategy of antiangiogenic drugs is based on inhibiting formation of new blood vessels as alternative to limit cancer progression. In this work, we investigated the antitumor and antiangiogenic potential of eight thalidomide derivatives. Most of the molecules was not cytotoxic but **2a**, **2d** and **3d** revealed weak antiproliferative activity on HL-60, Sarcoma 180 (S180) and normal peripheral blood mononuclear cells. Thalidomide, **2a** and **2b** were able to inhibit tumor growth (53.5%, 67.9% and 67.4%, respectively) in S180-bearing mice and presented moderate and reversible toxicity on liver, kidneys and spleens. Both analogs (**2a** and **2b**) inhibited cell migration of endothelial (HUVEC) and melanoma cells (MDA/MB-435) at 50 μg/mL. Immunohistochemistry labeling assays with CD-31 (PECAM-1) antibody showed microvascular density (MVD) was significantly reduced in thalidomide, **2a** and **2b** groups (30 ± 4.9, 64.6 ± 1.8 and 46.5 ± 19.5%, respectively) ($p < 0.05$). Neovascularization evaluated by Chorioallantoic Membrane Assay (CAM) with compounds **2a** and **2b** showed reduction of vessels' number (12. 9 ± 2.3 and 14.8 ± 3.3%), neovascularization area (13.1 ± 1.7 and 14.3 ± 1.7%) and total length of vessels (9.2 ± 1.5 and 9.9 ± 1.9%). On the other hand, thalidomide did not alter vascularization parameters. Consequently, addition of thiosemicarbazone pharmacophore group into the phthalimidic ring improved the *in vivo* antitumor and antiangiogenic potential of the analogs **2a** and **2b**.

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1. Introduction

Angiogenesis is a complex regulated biological process involved in the formation of new blood vessels in normal or pathological conditions [1]. Various regulatory and signaling molecules manage angiogenesis, including VEGF (vascular endothelial growth factor), PDGF (platelet-derived growth factor), FGF (fibroblast growth factor), EGF (epidermal growth factor), HGF (hepatocyte growth factor), PlGF (placenta growth factor), receptor tyrosine kinases (RTKs), transcription factors, as well as molecules involved in MAPK (Mitogen-activated protein kinases) and PI3K (Phosphatidylinositol 3-kinase) signaling [1,2]. Angiogenic events become activated in numerous illnesses, including ocular and cardiovascular diseases, inflammation and cancer [3–5].

In cancer, tumor cells stimulate angiogenesis in an effort to maintain and allow tumor progression. Thus, neovascularization improves blood supply (oxygen and nutrient) to the neoplasic cells, and consequently favors the tumor growth [1,3,4,6]. Therefore, the angiogenesis has become an attractive target in therapeutic approaches due to its key role in tumor growth, invasion and metastasis [2,3,6–8].

Thalidomide is the most known angiogenesis inhibitor. It is a synthetic derivative of glutamic acid with contains two imide rings: glutarimide and phthalimide. Due to its neurotoxicity, this drug caused devastating teratogenic effects in the 1960s [9,10]. Nowadays, thalidomide is effective to treat different diseases, including leprosy, acquired immunodeficiency syndrome (AIDS),

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multiple myeloma, cancer and other angiogenesis-dependent disorders [11,12]. Thalidomide is classified as an immunomodulatory drug (IMiD) that inhibits the production of tumor necrosis factor alpha (TNF-α) and may also affect the production of interleukin-1b (IL-1b), IL-2, IL-4, IL-5, IL-6, IL-10 and interferon-γ (IFN-γ). Moreover, secretion of both vascular endothelial growth factor (VEGF) and beta fibroblast growth factor (bFGF) from tumor and bone marrow stromal cells is suppressed upon exposure to IMiDs, resulting in reduced endothelial cell migration and adhesion [10,11]. The idea of using thalidomide in the cancer treatment occurred after the discovery of its antiangiogenic properties in the early 1990s [13]. Despite the thalidomide mechanism in cancer is not fully understood, its derivatives are known by immunomodulatory and anti-inflammatory effects that likely contribute to antiangiogenic effects [2,14]. In 2006, the Food and Drug Administration (USA) approved it against multiple myeloma [13].

To overcome risks caused by thalidomide, such as teratogenicity, many analogs have been developed with anticancer proposal. Previous reports showed that structural variations of thalidomide could change the side-effect profile, potentially eliminating them, while still maintaining the desired anticancer activity and demonstrating promising results in clinical trials [2,10]. Lenalidomide (Revlimid™) was approved for use in patients with myelodysplastic syndromes (MDS) and multiple myeloma [15]. Pomalidomide is a potent second-generation IMiD. *In vitro* studies have shown that pomalidomide is 10-fold more potent than lenalidomide and up to 15,000 times more potent than thalidomide at inhibiting TNF-α. Pomalidomide has shown significant clinical activity in phase I and II trials on patients with relapsed and/or refractory multiple myeloma [16].

In the current study, thalidomide and eight analogs were tested using *in vitro* and *in vivo* assays to assess their performance on tumor growth inhibition and tumor angiogenesis. Based on the structure–activity relationship, these novel analogs were developed with the phthalimide and thiosemicarbazone pharmacophore groups [17] (Fig. 1). Data presented here add important findings about antitumor effects of these analogs on solid tumors.

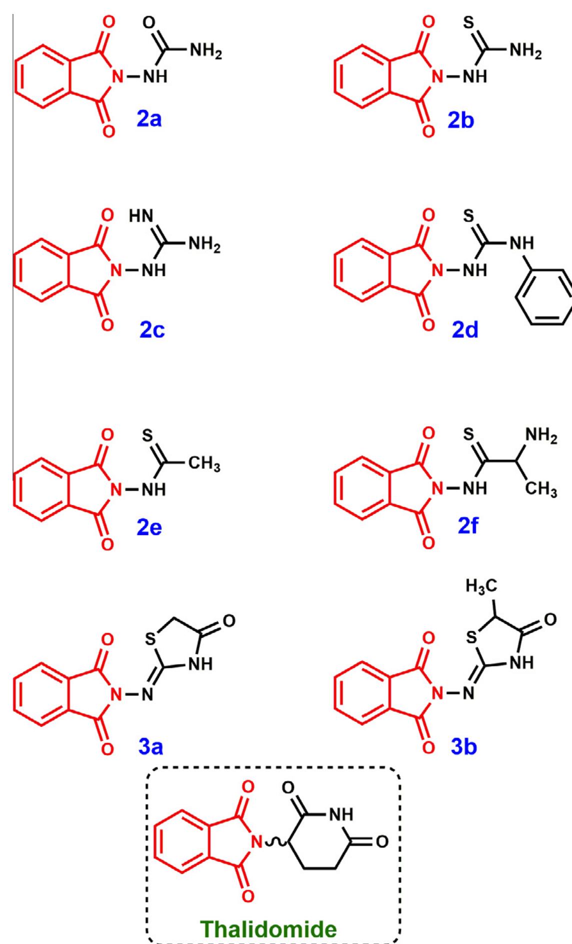

Fig. 1. Phthalimide analogs derived from thalidomide used in this study. Analogs were obtained as demonstrate by Pessoa et al. [17].

2. Materials and methods

2.1. Materials

Phthalimide derivatives (**2a**–**f** and **3a**–**b**) were synthesized as described by Pessoa et al. [17]. Purity of compounds was provided by elemental analysis data.

Primary antibody CD-31 (Platelet Endothelial cell Adhesion Molecule-1, PECAM-1) and secondary antibody were purchased from Santa Cruz Biotechnology. Anti-VEGF monoclonal antibody (Bevacizumab – Avastin™) Genentech/Roche, CA, USA) was purchased from Santa Cruz Biotechnology. Endothelia basal medium (EBM), RPMI 1640 medium, fetal bovine serum (FBS), penicillin/streptomycin and glutamine were purchased from Gibco (USA). Alamar Blue™, 5-Fluorouracil (5-FU), hydrocortisone, gentamicin and mitomycin C were obtained from Sigma Aldrich (St. Louis, MO, USA). All the other chemicals were of analytical grade and used as indicated by manufacturer. Thalidomide tablets 100 mg were used (FUNED, MG, Brazil). All molecules were diluted in DMSO to a final concentration of 5 mg/mL (stock solution). Chemical names and purity data of all compounds tested are given in Supplementary material.

2.2. Animals

Adult female Swiss mice (*Mus musculus* Linnaeus, 1758) were obtained from the animal facilities at Universidade Federal do Ceará (UFC), Fortaleza, Ceará, Brazil. They were kept in well-ventilated cages under standard conditions of light (12 h with alternate day and night cycles) and temperature (22 ± 2 °C) and were housed with free access to commercial rodent stock diet (Nutrilabor, Campinas, Brazil). Ross chicken eggs of the *Gallus domesticus* species weighing 50 g, 12–24 h after laid, were purchased from CIALNE™ Company (Fortaleza, Ceará, Brazil) and stocked for 24 h at 19–20 °C before incubation. All procedures were approved by the Committee on Animal Research at UFC (Process n° 106/2007) and they are in accordance with the Brazilian (COBEA – *Colégio Brasileiro de Experimentação Animal*) and International Standards on the care and use of experimental animals (Directive 2010/63/EU of the European Parliament and of the Council).

2.3. Cytotoxicity assays

Cytotoxic potential of the compounds (thalidomide, **2a**–**2f**, **3a**, **3b**) was assessed after 72 h exposure using cancer cell lines [HL-60 (leukemia), HCT-8 (colon), MDA/MB-435 (melanoma) and SF-295 (glioblastoma)], Sarcoma 180 cells (S180) and normal peripheral blood mononuclear cells (PBMC). Human cell lines were maintained in RPMI 1640 medium supplemented with fetal bovine serum 10%, 100 U/mL penicillin/streptomycin at 37 °C with 5% CO_2 atmosphere. In similar conditions, S180 tumor cells and PBMC were cultured in RPMI 1640 medium supplemented with 20% fetal bovine serum. Quantification of cell proliferation was determined spectrophotometrically using a multiplate reader (DTX 880

Multimode Detector, Beckman Coulter). All wells were exposed to the same amount of dimethylsulfoxide (DMSO 0.1%).

2.3.1. Evaluation of the cytotoxicity on tumor cell lines

In vitro antiproliferative activity against four human tumor cell lines (HL-60, HCT-8, MDA-MB-435 and SF-295) was determined by MTT assay, a cell viability assay that detect reduction of a yellow dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to a blue formazan product [18]. Briefly, cells were plated in 96-well plates ($0.1–0.3 \times 10^6$ cells/mL) to allow adhesion. After 24 h, thalidomide and its analogs (0.78–100 μg/mL) were added to each well. Doxorubicin (0.005–5 μg/mL) was used as positive control. After 69 h of incubation, plates were centrifuged and the medium was replaced by fresh medium (150 μL) containing MTT (0.5 μg/mL). Three hours later, plates were centrifuged, and the formazan was dissolved in DMSO. The effect of the molecules was quantified at 595 nm.

2.3.2. Evaluation on PBMC proliferation

Heparinized human blood (from healthy, non-smoker donors who had not taken any drug for at least 15 days prior to sampling, aged between 18 and 35 years) was collected and PBMC were isolated by a standard method of density-gradient centrifugation over Ficoll-Hypaque. PBMC were washed and resuspended in supplemented RPMI 1640 medium and phytohemagglutinin (3%). Then, PBMC were plated in 96-well plates (5×10^5 cells/well in 100 μL of medium). After 24 h, compounds (0.78–100 μg/mL) were added to each well and cells were incubated during 72 h. Twenty-four hours before the late incubation, 10 μL of stock solution (0.312 mg/mL) of Alamar Blue™ were added to each well [19]. Doxorubicin (0.005–5 μg/mL) was used as a positive control. The effect was quantified as the percentage of control absorbance at 570 nm and 595 nm. These studies were performed in accordance to the Brazilian research guidelines (Law 466/2012, National Council of Health, Brazil) and the Declaration of Helsinki.

2.3.3. Evaluation on proliferation of Sarcoma 180 cells

Ascite-bearing mice between 7 and 9 days post inoculation were sacrificed by cervical dislocation and a suspension of S180 cells was harvested from the intraperitoneal cavity under aseptic conditions. The suspension was centrifuged at $500 \times g$ for 5 min to obtain a cell pellet and washed 3 times with RPMI medium. Cell concentration was adjusted to 0.5×10^6 cells/mL in supplemented RPMI 1640 medium, plated in a 96-well plate and incubated with increasing concentrations of the compounds (0.4–100 μg/mL). Cell proliferation was determined by the Alamar Blue assay after 72 h [20]. Eight hours before the late incubation, 10 μL of stock solution (0.312 mg/mL) of Alamar Blue™ were added to each well. The absorbance was measured at 570 nm and 595 nm and the antiproliferative effect was quantified as percentage of the control.

2.4. In vivo antitumor assay

Female Swiss mice (20–25 g) obtained from the facilities of the Federal University of Ceará were used. Animals were kept in cages with free access to food and water under a 12/12 h light–dark cycle (lights on at 6:00 a.m.). Sarcoma 180 cells were maintained in the peritoneal cavities of the mice. Ten-day-old sarcoma 180 ascite tumor cells were removed from the peritoneal cavity, counted and subcutaneously implanted into the right hind axillary of the animals (4×10^6 cells/mL). In the following day, thalidomide and its analogs (50 mg/kg/day) dissolved in DMSO 10% were intraperitoneally administered for 7 days. Negative and positive controls received DMSO 4% and 5-FU (25 mg/kg/day), respectively. On the 8th day, mice were sacrificed and tumors, livers, spleens and kidneys were dissected out, weighed and fixed in 10% formaldehyde. The inhibition ratio of tumor growth (%) was calculated as follows: inhibition ratio (%) = $[(A - B)/A] \times 100$, where A is the average tumor weight in the negative control, and B is the average in each separately treated group.

2.4.1. Histopathology and morphological observations

Tumors, livers, spleens and kidneys fixed with formaldehyde were grossly examined for size or color changes and hemorrhage. Subsequently, these structures were cut into small pieces to prepare histological sections (4–7 μm) and stained with hematoxylin and eosin (H&E). Histological analyses were performed under light microscopy.

2.5. Antiangiogenic assays

2.5.1. Migration assay

The migration assay, also called Wound Healing Assay, was performed as previously described by Bürk [21] with some modifications. HUVEC and MDA-MB-435 cells were used in this test. The human umbilical vascular endothelia cells (HUVEC-CC2517) was cultured in endothelia basal medium (EBM) supplemented with 20% FBS, human epidermal grow factor (hEGF) 10 μg/mL, hydrocortisone 1 mg/mL, gentamicin 50 mg/mL, bovine brain extract 3 mg/mL (BBE) and used at early passages. These cells were incubated at 37 °C with 5% CO_2 atmosphere. For this, HUVEC-CC2517 cells were cultured until a confluent monolayer formed (>90%) and MDA/MB-435 cells (2×10^5 cells/mL) were both cultured in 12 well plates. After confluence, each well was treated with mitomycin C (5 μg/mL) for 15 min. Wells were washed with PBS and appropriated medium was added. Using a sterile plastic pipette tip, a wound was made in the center of the plate by the removal of the cell monolayer [22]. After washing, cells were incubated with a nontoxic dose of thalidomide and analogs (**2a** and **2b**, 50 μg/mL). Negative control of both cells was treated only with culture medium and vehicle (DSMO). After 24 h of incubation, the wells were visualized and photographed using inverted microscope (Nikon, UK, magnification of 200×) to observe cellular migration through the wound.

2.5.2. Assessment of angiogenesis by immunohistochemistry

In order to verify pathways involved on inhibiting neovascularization, the same *in vivo* model of the antitumor activity evaluation was used to quantify intratumoral vessels staining with CD-31 (PECAM-1) antibody. To assess the antiangiogenic potential, the treatment with thalidomide and its analogs (**2a** and **2b**) were started after 5 days of inoculation of Sarcoma 180 cells and extended for 10 consecutive days at 50 mg/kg/day. Negative and positive controls received 10% DMSO and 5-FU (25 mg/kg/day), respectively. Additionally, the monoclonal antibody bevacizumab (5 mg/kg/day) was used as standard drug. For each group of animals, chosen tumors were those that apparently had a larger number of peri- and intra-tumoral vessels.

Histological image samples were acquired under light microscope (BX4, Olympus Optical Co. Ltd., Japan) using a digital camera (C7070 Wide Zoom, Olympus Imaging America Inc., USA). The procedure included an initial scan of the tumor at 40× magnification in order to identify areas with increased vascular density called 'hot spots'. Three hot spots selected for each tumor were color-scanned at magnification of 200×. Images were saved as Windows® bitmaps (512 × 384 pixels since 1 pixel = 24 bits, RGB color scheme). Morphometric analyses of slides were processed by the System Quantification of Angiogenesis (SQAN), software specifically developed for this purpose [23]. The system was previously adjusted to the color spectrum produced by the staining techniques employed. This step enabled the software to automatically identify and segment (single out) blood microvessels in both

Table 1
In vitro cytotoxic activity of thalidomide and its synthetic analogs on tumor cell lines evaluated by the MTT and on primary culture of Sarcoma 180 and peripheral blood mononuclear cells (PBMC) quantified by Alamar Blue assay after 72 h of incubation.

Compound	CI_{50} (μg/mL)					
	MDA-MB-435	HCT-8	SF-295	HL-60	Sarcoma 180	PBMC
2a	>100	>100	74.3 (49.0–75.0)	72.0 (39.6–96.6)	>100	>100
2b	>100	>100	>100	>100	>100	>100
2c	>100	>100	>100	>100	>100	>100
2d	>100	>100	>100	58.6 (52–65.3)	11.3 (7.8–16.5)	>100
2e	>100	>100	>100	>100	>100	>100
3a	>100	>100	>100	>100	>100	>100
2f	>100	>100	>100	>100	>100	>100
3b	>100	>100	>100	>100	>100	26.6 (19.6–30.9)
Thalidomide	>100	>100	>100	40.3 (30.3–54)	>100	90.7 (83.6–98.6)
Doxorubicin	0.8 (0.8–1.6)	0.8 (0.8–1.6)	1.9 (1.5–2.0)	1.6 (0.8–1.6)	0.3 (0.2–0.5)	0.9 (0.5–1.8)

Data are presented as IC_{50} values and 95% confidence intervals for leukemia (HL-60), melanoma (MDA/MB-435), colon (HCT-8) and glioblastoma (SF-295) human tumor lines and for primary culture of Sarcoma 180 and peripheral blood mononuclear cells. Doxorubicin was used as positive control. All *in vitro* studies were carried out in triplicate represented by three independent biological evaluations.

full images and user-defined regions. However, interactive segmentation (making changes in segmentation parameters) remained an option whenever the automatic mode was deemed inadequate. After the segmentation, the software provided the microvascular density of areas studied. The area density was obtained by dividing the area of microvasculature by the total area analyzed [24].

2.5.3. In vivo chorioallantoic membrane (CAM) assay

To evaluate the activity of thalidomide and its synthetic analogs (**2a** and **2b**) on the blood vessels' formation, the chorioallantoic membrane (CAM) assay was used [25,26]. Briefly, eggs were incubated in a hatcher at 38.5 °C under a humidified atmosphere (33% relative humidity) and with rotary movement every 2 h. After 72 h of incubation (embryonic day – E3 stage 20-HH; Hamburger and Hamilton [27]), a hole in the eggshell (approximately 100 mm^2) was done until reach the air camera, causing a displacement between the inner shell membrane and vitelline membrane. The albumin was withdrawn using a sterile syringe through a hole made on the opposite side. Subsequently, eggs were sealed and re-incubated. At day 10 of incubation, eggs ($n = 8$) were treated with filter paper disk (2 mm) impregnated with 20 μL of thalidomide and analogs (1 e 5 mg/mL) and these disks were implanted over the chorioallantoic membrane. Negative control was treated with 10% DMSO. After treatment, the eggshell window was sealed and eggs were re-incubated at 38.5 °C. At 12th day of incubation, eggs were removed from the incubator. A solution of white ink in distilled water (2:1) was injected within the allantoic sac and a white background was obtained underneath the treatment disks. Antiangiogenic response was determined by the number of blood vessels around the disk, and expressed as percent of vessels in the limit of the disk. For this, digital images of the CAM were standardized in four quadrants (top, bottom, right and left) corresponding to the region around the filter paper disk containing the tested compounds. Images acquired were analyzed by the System Quantification of Angiogenesis (SQAN) as described above [24]. The parameters analyzed for angiogenesis quantification included area of neovascularization, vascular length and total number of blood vessels.

2.6. Statistical analysis

For cytotoxicity studies, IC_{50} values and their 95% confidence intervals were obtained by nonlinear regression. All *in vitro* studies were carried out in triplicate represented by independent biological evaluations. In order to determine differences among treatments, data expressed as mean ± standard error of the mean (S.E.M.) were compared by one-way analysis of variance (ANOVA) followed by the Newman–Keuls test ($p < 0.05$) using the Graphpad program (Intuitive Software for Science, San Diego, CA).

3. Results

3.1. In vitro antiproliferative action

Outcomes revealed that none of the studied molecules showed potent cytotoxic action on tumor lines (HL-60, HCT-8, MDA-MB-435 and SF 295). *In vitro* weak antiproliferative activity was found in HL-60-treated cells with **2a** [72.0 (39.6–96.6) μg/mL], **2d** [58.6 (52–65.3) μg/mL] and thalidomide [40.3 (30.3–54) μg/mL] (Table 1).

To evaluate *in vitro* cytotoxic activity on primary cell cultures, the Alamar Blue™ assay was used. Once again, majority of the molecules showed weak action on sarcoma 180 and polymorphic blood mononuclear cells, and compounds **2d** and **3b** exhibited IC_{50} values of 11.3 (7.8–16.5) μg/mL and 26.6 (19.6–30.9) μg/mL on sarcoma and PBMC, respectively.

3.2. Evaluation of the antitumor activity of thalidomide and its analogs in mice

The evaluation of the antitumor activity of thalidomide and its analogs was performed in Sarcoma 180-bearing mice. Thalidomide (0.98 ± 0.15 g), 5-FU (0.34 ± 0.04 g), compound **2a** (0.67 ± 0.12 g) and compound **2b** (0.69 ± 0.09 g) reduced tumor growth (53.5 ± 7.2, 83.9 ± 2.3, 67.9 ± 6.0 and 67.4 ± 4.6%, respectively) when compared to negative control (2.11 ± 0.18 g) ($p < 0.05$) (Table 2).

3.2.1. Tumor morphological alterations

Analysis of the tumor tissue showed polygonal and mitotic cells, cellular pleomorphism and presence of anisokaryosis in all groups (Fig. 2). Areas of coagulative necrosis and hemorrhage were especially seen in 5-FU, **2a** and **2b**-treated groups (Fig. 2B–D). Changes about relative weight of the livers and kidney were not noticed (Table 2). On the other hand, **2b** and **3b**-treated groups showed a significantly increasing in their spleen relative weights in comparison with the control group (0.73 ± 0.05, 0.82 ± 0.08 and 0.55 ± 0.05 g, respectively) ($p < 0.05$) while positive control treated with 5-FU suffered from a reduction in the relative weight of this organ (0.22 ± 0.01 g). Kidneys showed glomerular and interstitial hemorrhage and presence of hyaline cylinders in all groups, especially in **2e**-treated mice, which also showed intense cellular

Table 2
Effect of thalidomide and its synthetic analogs (50 mg/kg/day) on tumor growth in Swiss mice bearing Sarcoma 180 after 7 days of treatment.

Treatment	Dose (mg/kg/day)	Mice weight (g)	Liver	Kidney	Spleen	Tumor mass (g)	Tumor inhibition (%)
			g/100 g body weight				
Negative control	–	31.38 ± 1.25	5.26 ± 0.14	1.38 ± 0.03	0.55 ± 0.05	2.11 ± 0.18	–
5-FU	25	24.60 ± 0.40*	4.86 ± 0.27	1.28 ± 0.03	0.22 ± 0.01*	0.34 ± 0.04*	83.9 ± 2.3*
Thalidomide	50	29.00 ± 0.94	4.93 ± 0.09	1.38 ± 0.02	0.46 ± 0.03	0.98 ± 0.15*	53.5 ± 7.2*
2a	50	29.38 ± 0.82	5.20 ± 0.16	1.50 ± 0.06	0.49 ± 0.03	0.67 ± 0.12*	67.9 ± 6.0*
2b	50	30.33 ± 0.76	5.13 ± 0.16	1.42 ± 0.04	0.73 ± 0.05*	0.69 ± 0.09*	67.4 ± 4.6*
2c	50	29.77 ± 1.83	5.47 ± 0.12	1.32 ± 0.04	0.54 ± 0.05	1.66 ± 0.35	28.9 ± 16.6
2d	50	29.67 ± 1.40	5.30 ± 0.16	1.40 ± 0.08	0.51 ± 0.02	1.74 ± 0.28	17.9 ± 12.5
2e	50	30.50 ± 0.89	5.67 ± 0.24	1.39 ± 0.06	0.69 ± 0.05	1.69 ± 0.22	20.1 ± 10.6
2f	50	30.00 ± 0.00	5.94 ± 0.21	1.43 ± 0.07	0.69 ± 0.05	1.90 ± 0.16	10.4 ± 7.6
3a	50	30.50 ± 1.16	6.03 ± 0.33	1.42 ± 0.09	0.71 ± 0.06	1.68 ± 0.19	20.6 ± 8.9
3b	50	31.43 ± 0.92	5.94 ± 0.26	1.39 ± 0.05	0.82 ± 0.08*	1.49 ± 0.28	29.6 ± 13.1

Values are means ± S.E.M., $n = 10$ animals/group. Negative control was treated with the vehicle used to dilute the drug (10% DMSO). 5-Fluorouracil (5-FU) was used as positive control.
* $p < 0.05$ compared with the negative control by ANOVA followed by Newman–Keuls test.

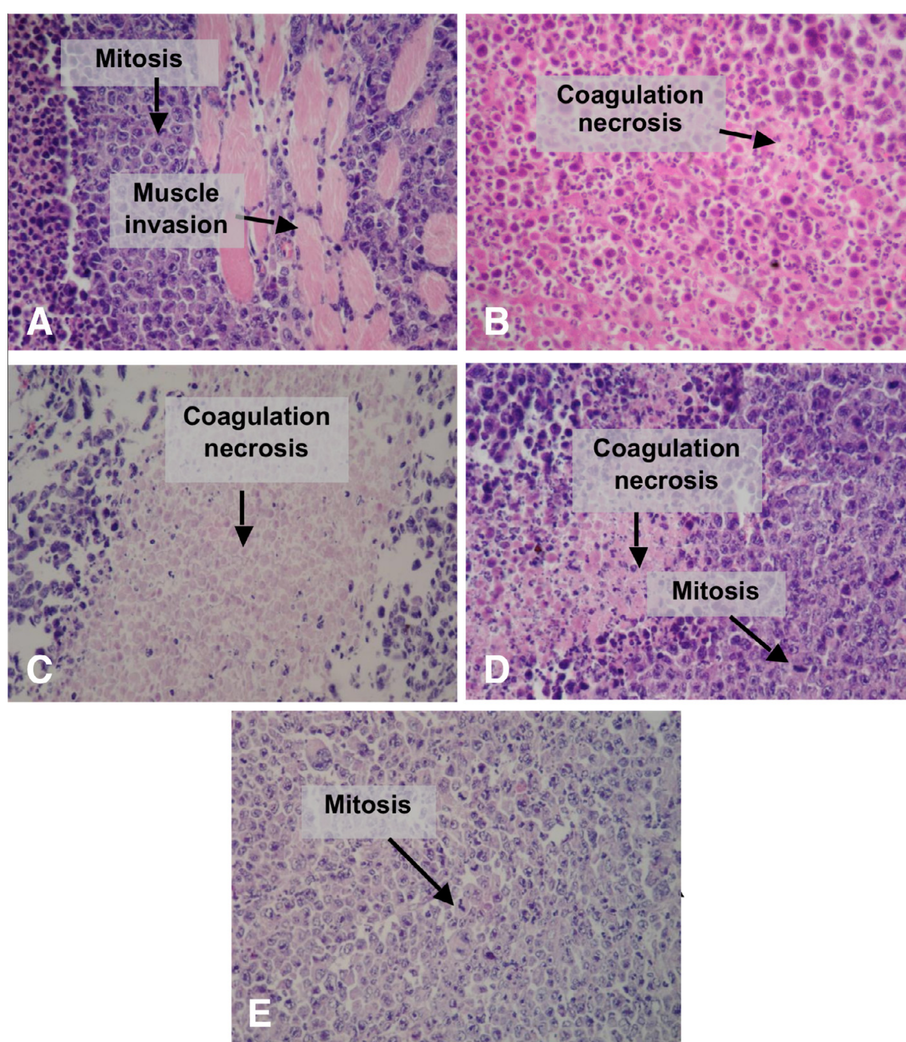

Fig. 2. Histotological analyses by light microscopy of tumors in sarcoma-180 bearing mice intraperitoneally treated with thalidomide and its analogs during 7 days. Negative control was treated with the vehicle used for diluting the tested substances (DMSO 10%). (A) Control (DMSO 10%); (B) 5-Fluouracil (5-FU) (25 mg/kg/day); (C) **2a** (50 mg/kg/day); (D) **2b** (50 mg/kg/day); (E) Thalidomide (50 mg/kg/day). Hematoxylin–eosin staining. Magnification, 400×.

swelling in the proximal tubules epithelium and hydropic degeneration. Histological structure of the spleen did not reveal microscopic changes. In general, many megakaryocytes and preserved architecture of the white and red pulp were observed, even in the **2b** and **3b** groups. In **2e** and **2f** groups, light congestion and disorganization in the red pulp were noted. Hemosiderin pigments were also found. Hyperplasia of the hepatic Kupffer cells, inflammatory foci and focal microvesicular steatosis were exhibited in all treated and untreated animals. Intense cellular swelling and necrosis areas were observed in the **2e** group. However, all samples

analyzed suggested preservation of the hepatic parenchyma and glomerular architecture.

3.3. Antiangiogenic potential

The most active analogs against *in vivo* tumor (**2a** and **2b**) were selected to execute detailed studies about the mechanism of action.

3.3.1. Cell migration inhibition

In order to assess the inhibitory effect of analogs, *in vitro* assays were carried out to verify the ability to inhibit HUVEC and MDA-MB-435 migration (Fig. 3). Both analogs (**2a** and **2b**) inhibited cell migration at a concentration of 50 μg/mL, leading to the migration inhibition of 65.3 ± 1.6 and 65.2 ± 2.4% on endothelial cells and 23.8 ± 0.8 and 98.5 ± 0.3% of inhibition on melanoma cells (Fig. 4). Thalidomide was not able to reduce cell migration.

3.3.2. Tumor angiogenesis inhibition analyzed by immunohistochemistry

Antitumor activity of thalidomide, **2a** and **2b** (50 mg/kg/day) was also performed to determine tumor vasculature related parameters. The treatment started at 5th day after the Sarcoma 180 tumor inoculation and was performed for 10 consecutive days. Subsequently, the animals were sacrificed on the 15th day and tumors were dissected out. Thalidomide (2.29 ± 0.13 g) and analogs (**2a**: 2.73 ± 0.59 g; **2b**: 3.09 ± 0.49 g) reduced tumor growth (56.6 ± 6.5, 48.2 ± 11.2 and 41.4 ± 9.4%, respectively) in comparison with negative control (5.28 ± 0.71 g) ($p < 0.05$) (Table 3). Only thalidomide-treated animals presented weight loss in the posterior treatment (29.14 ± 1.16 g) compared to control (34.08 ± 1.25 g) ($p < 0.05$). Fig. 5A shows a tumor section, indicating vessels and cells immunostained with CD-31 antibody. Immunohistochemistry assays showed that microvascular density (MVD) was significantly reduced in thalidomide, **2a** and **2b** groups (30 ± 4.9, 64.6 ± 1.8 and 46.5 ± 19.5%, respectively) in comparison with negative control group (Fig. 5B) ($p < 0.05$).

3.3.3. Angiogenesis assay in the chorioallantoic membrane (CAM) of chicken embryo

To evaluate the *in vivo* antitumor effect of thalidomide and analogs, the chorioallantoic membrane assay, also known as *in ovo* chicken embryo method, was performed in attempt to correlate the inhibition of vessel formation with antitumor potential. Compounds **2a** and **2b** (5 mg/mL) were able to reduce number of vessels (12. 9 ± 2.3 and 14.8 ± 3.3%), neovascularization area (13.1 ± 1.7 and 14.3 ± 1.7%) and the total length of vessels in mm^2 (9.2 ± 1.5 and 9.9 ± 1.9%) as shown in Fig. 6. Thalidomide, on the other hand, did not alter these vascularization parameters. Concentration dependent analyzes are in progress in attempt to observe pharmacological outcomes and possible toxic effects.

4. Discussion

In the fight against cancer, search for new chemical entities (NCEs) with chemotherapeutic properties is really worthy and numerous methods have been utilized to acquire compounds, including isolation from plants and animals and the use of synthetic and combinatorial chemistry and molecular modeling [19,28–31]. Thalidomide, for example, was the first drug with significant clinical outcomes against multiple myeloma (MM) and can be considered as alternative treatment for patients treated with conventional therapy or after transplantation [32]. Benefits of thalidomide for MM patients may be due to its ability to inhibit

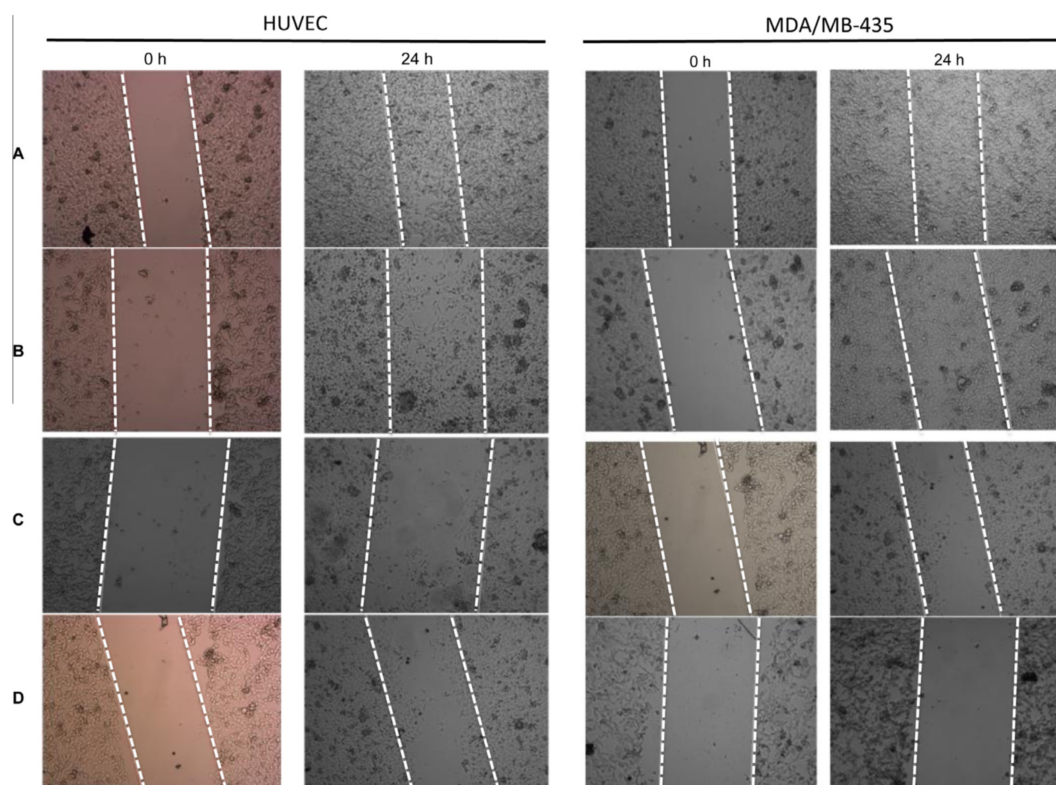

Fig. 3. Effects of thalidomide and its analogs on the migration of human umbilical vein endothelial cells (HUVEC) and melanoma cells (MDA/MB-435) before and after 24 h of incubation. Each well was pretreated with mitomycin C (5 μg/mL) for 15 min. (A) Negative control (DMSO 0.1%); (B) Thalidomide (50 μg/mL); (C) **2a** (50 μg/mL); (D) **2b** (50 μg/mL). Magnification, 100×. Studies were carried out in triplicate represented by three independent biological evaluations.

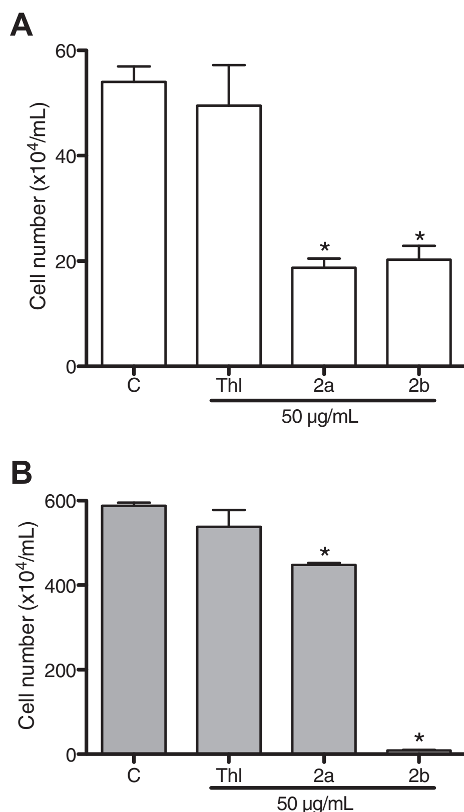

Fig. 4. Effects of thalidomide (Thl) and analogs (**2a** and **2b**) analyzed by the Wound Healing Assay measured after 24 h of incubation. (A) Human umbilical vein endothelial cells (HUVEC); (B) Melanoma cells (MDA-MB-435). Negative control (C) was treated with the vehicle used for diluting the tested substances (DMSO 0.1%). Results are mean ± S.E.M. of two independent experiments performed in triplicate obtained by cells counting that invaded the scar. $^{*}p < 0.05$ compared to negative control by ANOVA test followed by Newman–Keuls test.

the growth of new blood vessels (angiogenesis); a phenomenon observed in different tumor types. Due to this potential, chemical modeling of the thalidomide is a source of novel precursors with therapeutic properties. Taking into consideration structure–activity related studies (SAR), analogs and metabolites of thalidomide clearly showed that the presence of the pharmacophoric unit phthalimide is essential for its pharmacological activity [33]. Thiosemicarbazone was another important pharmacophoric group used in this study. The anticancer effects of thiosemicarbazones and its analogs (semicarbazones and *N*-acyl-hydrazones) have been quite promising, acting on cell proliferation and combating chemotherapy resistances [34].

Table 3
Effect of thalidomide and synthetic analogs (50 mg/kg/day) on tumor growth in Swiss mice bearing Sarcoma 180. The treatment started after 5 days of Sarcoma 180 inoculation and extended for 10 consecutive days.

Treatment	Dose (mg/kg/day)	Mice weight (g)	Tumor (g)	Tumor inhibition (%)
Negative control	–	34.08 ± 1.25	5.28 ± 0.71	–
5-FU	25	30.63 ± 1.21	3.09 ± 0.41*	41.5 ± 7.8*
Thalidomide	50	29.14 ± 1.16*	2.29 ± 0.13*	56.6 ± 2.5*
2a	50	31.43 ± 0.75	2.73 ± 0.59*	48.2 ± 11.2*
2b	50	31.86 ± 1.24	3.09 ± 0.49*	41.4 ± 9.4*

Values are means ± S.E.M., $n = 10$ animals/group. Negative control was treated with the vehicle used to dilute the drug (10% DMSO). 5-Fluorouracil (5-FU) was used as positive control.
* $p < 0.05$ compared with the negative control by ANOVA followed by Newman–Keuls test.

Herein, the cytotoxic analyses of eight phthalimide and thiosemicarbazone analogs were performed by the MTT and Alamar Blue™ assays, revealing weak *in vitro* antitumor activity against tumor cells. Thus, the addition of the thiocarbonyl group to the phthalimide ring did not improve the cytotoxic action. However, compound **2d** showed promising action on sarcoma 180 cells. Previously, a series of 16 novel thalidomide sulfur analogs containing one and two sulfur atoms were screened for *in vitro* antitumor activity against Ehrlich ascites carcinoma cell line and also exhibited potent cytotoxic action [35]. A relationship between the thiosemicarbazone subunit and antiproliferative effects on experimental tumors has been established, since this chemical structure inhibits ribonucleotide reductase processes, alters DNA structure, presents chelating capacity with potent and selective antineoplasic activity and generates iron complexes with redox activity [34,36,37].

In vitro effects can be not directly extrapolated for *in vivo* models. Thus, evaluating the pharmacological actions of these compounds in complete biological systems becomes an obligatory requirement. In order to assess the *in vivo* antitumoral action, the compounds were evaluated using the Sarcoma 180 experimental model, a mouse-derived tumor widely exploited in antitumor research [20,38–40]. In Sarcoma-180-bearing animal assays, only thalidomide and compounds **2a** and **2b** revealed antitumor action. Interestingly, the compound **2d** was not able to inhibit *in vivo* tumor growth, though it has displayed moderate *in vitro* cytotoxicity against Sarcoma 180 cells. Probably, *in vivo* metabolic activation is required to generate compounds with antiproliferative effect on solid tumors as seen with thalidomide, **2a** and **2b**.

It has been demonstrated that inhibition of angiogenesis and tumor growth by thalidomide or analogs requires metabolic activation [12,41–43]. In a similar way, Pessoa et al. [17] also suggest that a prior metabolic activation is necessary to produce one or more active metabolites from *N*-phthaloyl amino acids derivatives, explaining their antiproliferative activity on *in vivo* models only. Therefore, it is also possible that the metabolizing of the molecules also explains, at least in part, the *in vivo* antitumor action, since it is very known that some molecules undergo hepatic enzymatic reactions to generate active metabolites [44]. Zahran et al. [35], using an *in vivo* model of experimental carcinoma, also demonstrated that thalidomide dithiocarbamate and dithioate analogs have activity against Ehrlich carcinoma-induced solid tumor in Swiss albino mice.

Histopathological analyses of livers in treated animals showed Kupffer cell hyperplasia, microvesicular steatosis and preserved hepatic parenchyma. The liver possesses a pronounced regenerative capacity: even when necrosis is found with conjunctive tissue preservation, there is often complete tissue restoration [44]. Thus, hepatic alterations observed in **2a** and **2b** treated groups were reversible.

In spite of only compounds **2a** and **2b** have exhibited antitumor action, both **2b** and **3b** caused spleen enlargement, a finding suggestive of immunostimulant action of these molecules. Indeed, immunomodulatory drugs based on thalidomide, mainly amino-substituted thalidomide analogs, have been reported to be superior candidates as antitumor agents [45,46]. Despite thalidomide had not led to spleen morphological alterations in this work, it is known that some phthalimide analogs are co-stimulatory substances, increasing the response of T-lymphocytes to T-cell-receptor-mediated stimulation, the production of interleukin-2 and interferon-γ as well as the number of natural killer cells [17,47]. On the other hand, most clinical chemotherapy drugs are immunosuppressive and has negative side effects [48], such as leucocyte suppression, hypoplasia of the splenic white pulp and small lymphoid aggregates in 5-FU-treated mice [46]. These findings display the importance about enhancement of host

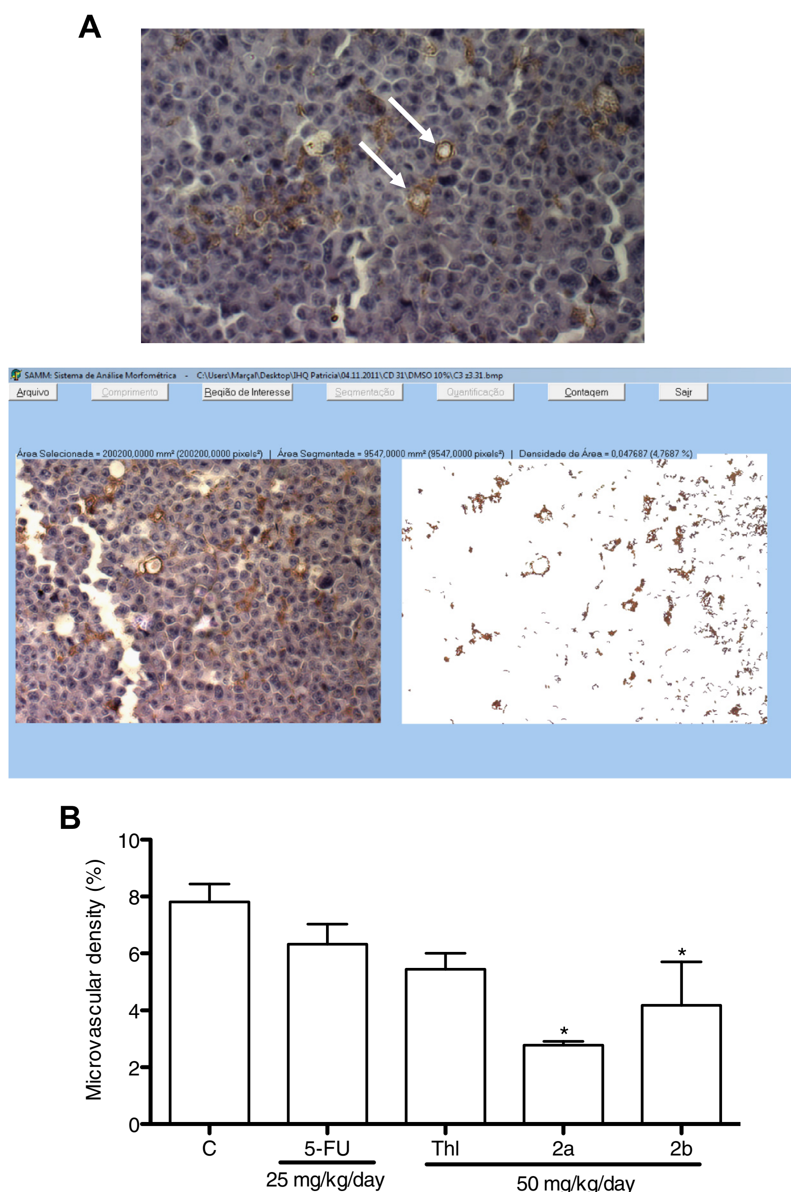

Fig. 5. Effects on tumor angiogenesis evaluated by immunohistochemistry. (A) Images of a hot spot on the tumor obtained by inverted microscope and processed by the System Quantification of Angiogenesis (SQAN). White arrows indicate vessels and cells immunostained with CD-31 (PECAM-1) antibody (Magnification, 200×); (B) Effects of thalidomide (Thl) and analogs (**2a** and **2b**) on microvascular density analyzed by immunohistochemistry in Sarcoma 180 tumors after 10 days of treatment started after on the 5th inoculation day. Negative control (C) was treated with the vehicle used to dilute the drug (DMSO10%). 5-Fluorouracil (5-FU) was used as positive control. Values are mean ± S.E.M. Microvascular density of areas was analyzed in 3 slides/group. $^{*}p < 0.05$ compared with the negative control by ANOVA followed by Newman–Keuls test.

defenses as alternative to the traditional cancer cytotoxic chemotherapy since it involves slight side effects.

In general, the *in vivo* antitumor effect of thalidomide is not commonly observed in the literature. Gutman et al. [49], testing the efficacy of thalidomide daily administered by gavage (0.3–1.0 mg until 10 days), reported no growth retardation in CT-26 bearing mice nor in mice with pulmonary or peritoneal metastases of B16-F10 melanoma. On the other hand, antitumor effects of thalidomide have been associated with its antiangiogenic properties. It is well known that new analogs of thalidomide, including the second generation, have a great therapeutic potential. Therefore, these novel and active pharmacologically compounds were divided into two classes: SelCIDs (selective cytokine inhibitory drugs) and IMiDs (immunomodulatory drugs) [50].

In order to evaluate the antiangiogenic potential of the molecules **2a** and **2b**, antiangiogenic assays were performed. Cell migration technique has been widely used as a screening assay for new compounds with antiangiogenic activity [51,52]. Both molecules (**2a** and **2b**) that showed promising *in vivo* results inhibited cell migration of endothelial (HUVEC) and melanoma cells (MDA-MB-435) at 50 μg/mL. On the other hand, thalidomide was not able to reduce cell migration, whose inefficacy can be explained by the fact that thalidomide requires metabolic activation to produce one or more active metabolites after incubation with microsomal proteins, as previously described [12,53]. These data suggested that the absence of migration was not caused by cytotoxicity or inhibition of proliferation and other factors are probable involved in the antiangiogenic potential, a finding that needs to be investigated.

The continuous growth of tumors requires oxygen and nutrients and these substances are provided by the blood flow. *In vivo* antiangiogenic action of the compounds were performed in Sarcoma 180-bearing mice assay with a longer period of exposure (10 days) since effects of antiangiogenic therapy cannot be

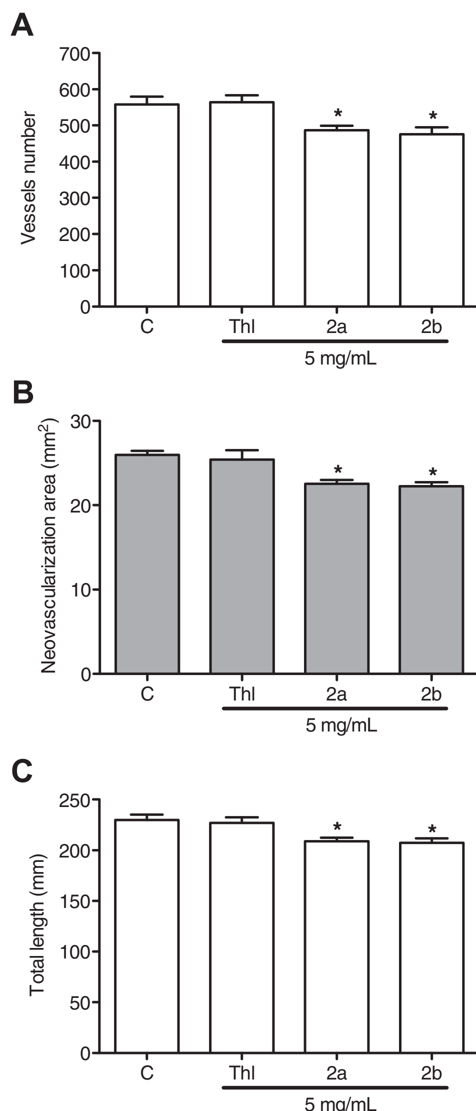

Fig. 6. Effects of thalidomide (Thl) and analogs (**2a** and **2b**) on embryonic angiogenesis analyzed by the *in ovo* Chorioallantoic Membrane Assay (CAM) after 24 h exposure. (A) Number of vessels; (B) Neovascularization area; (C) Total length of the vessels. Graphs were obtained by the software SQAN from the average of the four quadrants photographed in a stereoscopic microscope (BX4, Olympusb Optical) equipped with a digital camera (C7070 Wide Zoom, Olympus). Negative control (C) was treated with the vehicle used for diluting the tested substances (DMSO 0.1%). Studies were carried out in triplicate represented by two independent biological evaluations. Values are mean ± S.E.M. $^{*}p < 0.05$ compared with the negative control by ANOVA followed by Newman–Keuls test.

detected *in vivo* in shorter trials [54]. Thus, CD-31 or PECAM-1 labeling was determined. CD-31 or PECAM-1 is an adhesion molecule present in endothelial cells and platelets. It is a member of the immunoglobulin superfamily expressed on the surface of endothelial cells, platelets, monocytes, neutrophils and T cells [55–58]. Thalidomide and analogs (**2a** and **2b**) inhibited tumor growth but intratumor vessels stained with the CD-31 antibody revealed reduction in microvascular density (MVD) only in **2a** and **2b** groups.

In vivo chorioallantoic membrane (CAM) assay demonstrated effects of molecules on the neovascularization. The results confirmed that only compounds **2a** and **2b** were able to reduce the number of vessels, neovascularization area and the total length of vessels at 5 mg/mL after 24 h exposure. These findings indicate that addition of the thiosemicarbazone pharmacophore group to the phthalimidic ring in thalidomide increased its antitumor potential and angiogenesis activity. Thalidomide, on the other hand, did not change vascularization parameters probably because *in vivo* hepatic biotransformation is necessary [12,53]. In fact, thalidomide antiangiogenic activity is significantly increased by human microsomal activation, but different findings are observed in rats. It is known that hydroxylation of thalidomide in positions 1′ and 5′ keep its antiangiogenic potential, but the hydroxylation at 4′ position generates an inactive compound [59].

5. Conclusions

The addition of thiosemicarbazone pharmacophore group into the phthalimidic ring improved the *in vivo* antitumor and antiangiogenic potential of the analogs **2a** and **2b**. These molecules have special interest for the development of new anticancer therapies and additional studies are in progress to elucidate the mechanism of action of these bioactive phthalimides.

Conflict of Interest

The authors have declared no conflict of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cbi.2015.06.037.

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