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Sunitinib malate inhibits hemangioma cell growth and migration by suppressing focal adhesion kinase signaling

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Abstract
Sunitinib malate is a small molecule that targets multiple receptor tyrosine kinases and blocks their activity. Receptors targeted by sunitinib are implicated in tumor vascularization and are overexpressed by vascular tumors encountered in infants, namely, hemangiomas. Of note is that there is still no definitive treatment for these commonly occurring tumors of infancy. The purpose of this study was to investigate the effects of sunitinib malate on hemangioma using endothelial cells isolated from a murine model of the neoplasm (sEnd.2). The effects of the drug on cell growth were evaluated using the crystal violet assay and flow cytometry, while the scratch assay was employed to measure cell migration. Proteins associated with cell migration and angiogenesis were detected using western blotting. Sunitinib was investigated further to determine its effects on the production of reactive oxygen species, a parameter associated with the promotion of neovascularization in tumors. The results showed that sunitinib significantly reduced the growth of sEnd.2 cells by causing the cells to accumulate in the sub-G1 phase of the cell cycle, and also induced a significant decrease in the migration of these hemangioma cells ($P < 0.05$). The western blot assay showed a decrease in the expression of adhesion proteins, focal adhesion kinase and paxillin at IC_{50} doses, although the expression of cadherin did not change significantly ($P < 0.05$). In addition, transforming growth factor-β1 (TGF-β1) expression was decreased in sunitinib-treated cells at the same dose. The adhesion proteins as well as TGF-β1 regulate cell movement and have been implicated in tumor progression. Thus, sunitinib malate may have potential in the treatment of hemangiomas.

Keywords: Angiogenesis; Focal adhesion kinase; Hemangioma; Migration; Sunitinib

Highlights:
- The study shows for the first time the effectiveness of VEGFR blockers, MAZ-51 and sunitinib malate in suppressing hemangioma growth.
- Data further shows the MAZ-51 and sunitinib malate are more effective in benign rather than malignant skin tumor cells.
- The expression of adhesion and angiogenesis promoting proteins that enable tumor progression (FAK, paxillin, TGF-β1) were suppressed by sunitinib malate.

Introduction

Hemangiomas are the most commonly diagnosed tumors in infants (Ionescu et al., 2008; Mabeta and Pepper, 2011). These endothelial cell neoplasms are characterized by abnormal and aggressive angiogenesis (Liu et al., 2013; Mabeta, 2018; Mabeta and Pepper, 2015). Angiogenesis, the formation of blood vessels from a pre-existing microvessels, is mediated by numerous factors which either stimulate or inhibit the process, otherwise referred to as positive and negative regulators of angiogenesis respectively (Weis and Cheresh, 2011). In hemangioma, positive regulators of angiogenesis predominate and constitutive vascular endothelial growth factor-A (VEGF-A) signalling plays a role in the development of hemangiomas (Chim et al., 2012; Mabeta, 2018; Park et al., 2020).

The main receptors for VEGF-A are vascular endothelial growth factor receptor-1 and -2 (VEGFR-1 and VEGFR-2) and both have been implicated in hemangiomagenesis (Mabeta, and Pepper, 2012; Podar and Anderson, 2011; Tugues et al., 2011). Another VEGF receptor, which binds vascular endothelial growth factor-C and -D (VEGF-C and VEGF-D), VEGFR-3, was detected in hemangioma tissue but not in adjacent normal tissue. In addition, studies have further suggested that mutations in the VEGFR-3 gene may contribute to the neoplasm (Ji et al., 2014; Walter et al., 2002). Of note is that these VEGF receptors are targeted by several small molecule inhibitors such as the VEGFR-3 inhibitor, Maz-51 and sunitinib malate (Braconi et al., 2008; Kirkin et al., 2004).

Sunitinib malate (N-(2-diethylaminoethyl)-5-[(Z)-(5-fluoro-2-oxo-1H-indol-3-ylidene)methyl]-2,4-dimethyl-1H-pyrrole-3-carboxamide) is a receptor tyrosine kinase inhibitor (RTKI)

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approved by the Food and Drug administration (FDA) for the treatment of renal cell carcinoma (RCC) and gastrointestinal stromal tumors (GIST) that are resistant to imatinib (Braconi et al., 2008; Joseph et al., 2016). It exerts its mechanism of action by blocking VEGFR-1, -2 , PDGFRs, FMS-like tyrosine kinase 3 (FLT-3) and the stem cell factor receptor c-KIT (Haas et al., 2016; Quintieri et al., 2014). Given that MAZ-51 and sunitinib block receptors implicated in hemangiomagenesis, the effects of these drugs on hemangioma cell growth were investigated. We further sought to elucidate the mechanism of action of VEGFR inhibition on hemangioma *in vitro* by studying cell migration, reactive oxygen species production as well as the expression of adhesion and angiogenic protein markers.

Materials and methods

Cell culture

The sEnd.2 endothelial cell (EC) line was isolated from mouse cutaneous hemangiomas and was obtained from Prof. M.S. Pepper (University of Pretoria, South Africa). The B16-F10 is a malignant cutaneous melanoma cell line and it was purchased from the American Type Culture Collection (ATCC, MD, USA).

Both cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Whitehead Scientific, Johannesburg, SA) supplemented with 10% fetal bovine serum (FBS) (Sigma Aldrich, St. Louis, MO, USA) and 1% penicillin-streptomycin (Sigma Aldrich, St. Louis, MO, USA) in a humidified incubator (NuAire, Plymouth, USA) at 37 °C and 5% CO_2.

Cell viability assay

Crystal violet, a stain that binds to deoxyribonucleic acid (DNA) in live cells was employed to study cell viability using a previously described protocol with slight modification (Feoktistova et al, 2016). The sEnd.2 and B16-F10 cells were seeded in 96-well plates at a density of 5×10^3 per well and allowed to attach overnight. The cells were treated with either sunitinib (0.1–10 μg/ml), MAZ-51 (0.1–10 μg/ml) or 0.05% dimethyl sulfoxide (DMSO) for 24 hours at 37 °C and 5% CO_2 in a humidified incubator. MAZ-51 is an indolinone that inhibits the function of VEGFR-3 by blocking the ligand induced autophosphorylation of this receptor tyrosine kinase (Kirkin et al., 2004) while sunitinib is a multi-targeting tyrosine kinase inhibitor which blocks multiple VEGFRs. The drug concentrations and exposure time of sunitinib and MAZ-51 were chosen following initial screening.

At termination, the cells were fixed in 1% glutaraldehyde (GA) for 15 min at room temperature and then stained with 0.1% crystal violet (Sigma Aldrich, St. Louis, MO, USA) solution for 30 min. The plates were washed under running water for 20 min and dried. Thereafter, 100 μl of a 0.1% Triton x-100 solution was added to each well and the cells were incubated at room temperature for 60 min. The absorbance was measured at 570 nm using a BioTek ELx800 microplate reader (BioTek instruments Inc., VT, USA). Three wells were analyzed per treatment and experiments were conducted in triplicate. The half maximal inhibitory concentration (IC_{50}) of cell growth was calculated from data obtained from crystal violet assay results using GraphPad Prism 5 Software. The calculated IC_{50} for sunitinib malate was 2.600 μg/ml (4.881 μM) for endothelial cells, and this concentration was employed for further investigation on the drug's mechanism of action.

Cell cycle analysis

For cell cycle analysis, the fluorescent nucleic acid dye propidium iodide (PI) was employed to stain cells. Endothelial (sEnd.2) cells were seeded in T25 flasks at 1×10^6 cells/ml, and therefore 5 million cells were transferred to each flask. The cells were incubated for 24 h at 37 °C and 5% CO_2 in a humidified incubator. The cells were then treated with 2.6 μg/ml (IC_{50} dose) sunitinib and a positive control, nocodazole at 0.5 μg/ml for 24 h at 37 °C and 5% CO_2 in a humidified incubator. The cells were harvested using trypsin, centrifuged at 400 rpm (580 × g) for 10 minutes and resuspended in DMEM. The cells were then counted and 1×10^6 cells were washed with PBS and centrifuged at 400 rpm (580 × g). The supernatant was removed, and the cells were fixed in 1 ml cold 70% ethanol in ddH_2O. The ethanol was added dropwise to the pellet while vortexing to minimize aggregation and to ensure fixation of all cells. The samples were stored at –20 °C overnight. The samples were then centrifuged at 2000 rpm (5800 × g) and washed twice with PBS. The supernatant was removed and 50 μl of 100 μg/ml RNase A in PBS were added directly to the pellet, followed by 400 μl of 50 μg/ml PI in PBS and then vortexed. The cells were incubated for at least 10 min to stain in the dark before being analyzed using a Beckman FC 500 Series Flow Cytometer (Beckman Coulter Life Sciences, CA, USA).

Propidium iodide fluorescence was detected on the FL3 channel (excitation/emission 496/633 nm). A minimum of 10 000 events were analyzed for each treatment.

Scratch migration assay

To measure cell migration, the scratch assay was employed as previously described (Mabeta and Pepper, 2009). Briefly, cell culture dishes were precoated with 0.2% gelatin (Sigma Aldrich, St. Louis, MO, USA) and cells were seeded at 8×10^5 cells per culture dish overnight. Thereafter, a scratch was created in the center of the cell monolayer using a pipette tip followed by rinsing of cells with PBS. The cells were then treated with sunitinib (2.6 μg/ml) or 0.05% DMSO over 24 h, and a series of images were captured at 0, 6, 20 and 24 h using a Zeiss Axiocam MRM camera mounted on a Zeiss Axiovert 40 CFL (Carl Zeiss, Oberkochen, Germany).

Western blot

The sEnd.2 cells were seeded at 200 000 cells/well in 6-well plates and incubated for 24 h at 37 °C and 5% CO_2 in a humidified incubator. The cells were treated with sunitinib at IC_{50} and double IC_{50} doses (2.6 μg/ml and 5.2 μg/ml respectively) or 0.05% DMSO for 24 h. At termination the cells were rinsed with cold 0.1 M PBS and lysed in 200 μl of RIPA (150 mM NaCl, 0.1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 8.0) lysis buffer. Protein determination was performed using a bicinchoninic acid (BCA) assay kit (Thermo Fischer Scientific, Johannesburg, RSA) to standardize all samples to the same concentration. For analysis, 4% lithium dodecyl sulphate (LDS) buffer and 2.5% Mercaptoethanol were added to the lysates.

The samples were incubated for 5 minutes at 80 °C and centrifuged for 1 minute at 12000 RPM and 21 °C. Samples were then loaded on the gel together with a protein ladder at 5 μl. Thereafter, MOPS (50 mM MOPS, 50 mM Tris Base, 1 mM EDTA, 0.1% SDS, pH 7.7) running buffer was used to run the gels at 120 V for 90 min. Proteins were transferred overnight to a methanol-activated Polyvinylidene fluoride (PVDF) membrane at 90 V at 2 °C. The membrane was blocked using 3% non-fat milk in 0.01 M PBS for 1 h, followed by incubation with 5 μl primary antibody in 5 ml blocking buffer (TGF-β1 : 1000; FAK 1 : 1000; Cadherin 1 : 1000) for 24 h at 2 °C on a roller. The membrane was washed three times with PBS-Tween for 5 min and incubated with the HRP-conjugated secondary antibody, goat IgG for 3 h and then washed with PBS-Tween. Detection

was performed using Chemiluminesence Biorad Chemidoc MP System (Bio-Rad Laboratories Inc, California, USA), and the electro chemiluminescence (ECL) kit. Quantitative results were presented as the fold change (FC), which is the change in protein abundance relative to the control. Protein expression was normalized to actin.

Oxidative stress assay

Superoxide anion levels were measured using the Muse® Oxidative Stress Kit (Merck Millipore, MA, USA) according to the manufacturer's instructions. Briefly, sEnd.2 cells were seeded in T25 flasks at 1 × 10^6 cells/ml and incubated overnight at 37 °C and 5% CO_2 in a humidified incubator. Cells were treated with sunitinib (2.6 µg/ml) or 0.05% DMSO for 24 h, harvested using trypsin, centrifuged at 400 rpm (580 × g) and resuspended in 1× assay buffer at 5 × 10^6 cells/ml.

The Muse® Oxidative Stress Reagent working solution was then added to samples and following a 30-min incubation at 37 °C samples were analyzed using the Muse® Cell Analyzer (Merck Millipore, MA, USA).

Statistical analysis

By convention sample size is chosen so that the residual degrees of freedom are at least 30. To comply with the latter, six experiments were conducted i.e. six observations (mean of three replicates per experiment) per drug-concentration combination. In each of the experiments a negative control was also included. Data analysis employed an appropriate analysis of variance (ANOVA) with fixed effects, drugs and concentrations. Data summary was by drug and concentration mean and standard deviation (SD). Data analysis was conducted with the Stata Release 14.0 statistical software. The level of significance was designated at 0.05.

Results

Cell growth

To determine the effects of sunitinib malate on cell growth, cell viability and cell cycle analysis were undertaken. Both cell viability and the cell life cycle are tightly linked to growth (Ignarro et al., 1987).

Cell viability assay

The drugs MAZ-51 and sunitinib were evaluated to determine possible effects on the viability of ECs derived from murine skin hemangiomas, the sEnd.2 cells. Another cell line which was derived from a malignant skin tumor, the melanoma B16-F10 cells, was also employed to study the effects of these drugs on cell viability. In sEnd.2 cells, MAZ-51 had no significant effect at a dose of 0.1 and 1 µg/ml, but decreased the percentage of viable cells to 48.902% only at 10 µg/ml (Fig. 1A). Sunitinib induced significant inhibition of sEnd.2 cells to 54.63% and 38.2% at doses of 1 and 10 µg/ml, with an IC_{50} value of 2.600 µg/ml. In melanoma cells, 0.1 µg/ml of MAZ-51 had no significant effect on cell survival. Nonetheless, MAZ-51 reduced cell viability at concentrations of 1 and 10 µg/ml to 68.751% and 48.914% (Fig. 1B). Sunitinib decreased the percentage of viable melanoma cells to 77.628 and 45.628 at 1 and 10 µg/ml respectively (Fig. 1B). The effects of both drugs were more pronounced on sEnd.2 cells. Sunitinib was more potent than MAZ-51 in inhibiting both endothelial and melanoma cell growth ($P < 0.05$) and thus, further studies were undertaken using sunitinib.

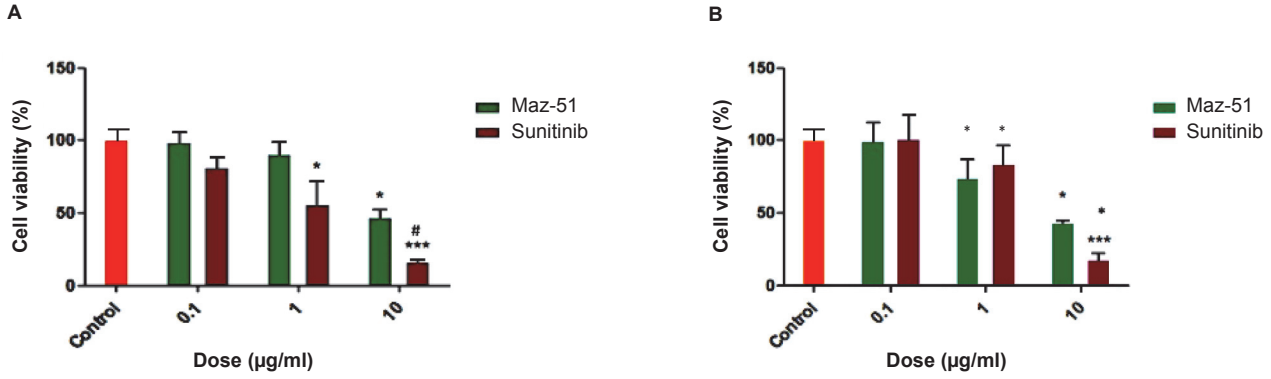

Fig. 1. The inhibition of vascular endothelial growth factor receptors with MAZ-51 and sunitinib decreases the viability of skin tumor cells, (**A**) the benign sEnd.2 and (**B**) malignant B16-F10 cells. * $P < 0.05$ compared to the negative control; *** $P < 0.001$ compared to the negative control; # $P < 0.05$ compared to MAZ-51, values represent the mean ± SD.

Cell cycle analysis

There was a significant increase in the percentage of sEnd.2 cells in the Sub-G1 phase following sunitinib treatment when compared to the negative control (Fig. 2A). Also, there were significantly fewer cells in the G1 phase (Fig. 2B) following treatment with sunitinib when compared to the negative control ($P < 0.05$). The effects of sunitinib were comparable to those of the positive control (Fig. 2B), which resulted in significantly fewer cells in the G1 phase ($P < 0.001$).

Cells treated with sunitinib were slightly increased in the G2/M phase when compared to those of the negative control (Fig. 2A and B). Sunitinib treatment further induced a slight but insignificant decrease in the percentage of sEnd.2 cells that were present in the S-phase.

Scratch migration assay

At 6 h, a slight closure of the artificial gap was visible, with no clear difference between the control and the sunitinib-treated cells (Fig. 3A). At 20 h, the migration of sEnd.2 cells was evident in the control, while few migrated cells could be observed in the drug-treated cultures. After 24 h of treatment with sunitinib, there were still few migrated cells, while the control cells had migrated towards the 'wound' edge and the closure of the artificial gap was almost complete (Fig. 3A). Quantitative

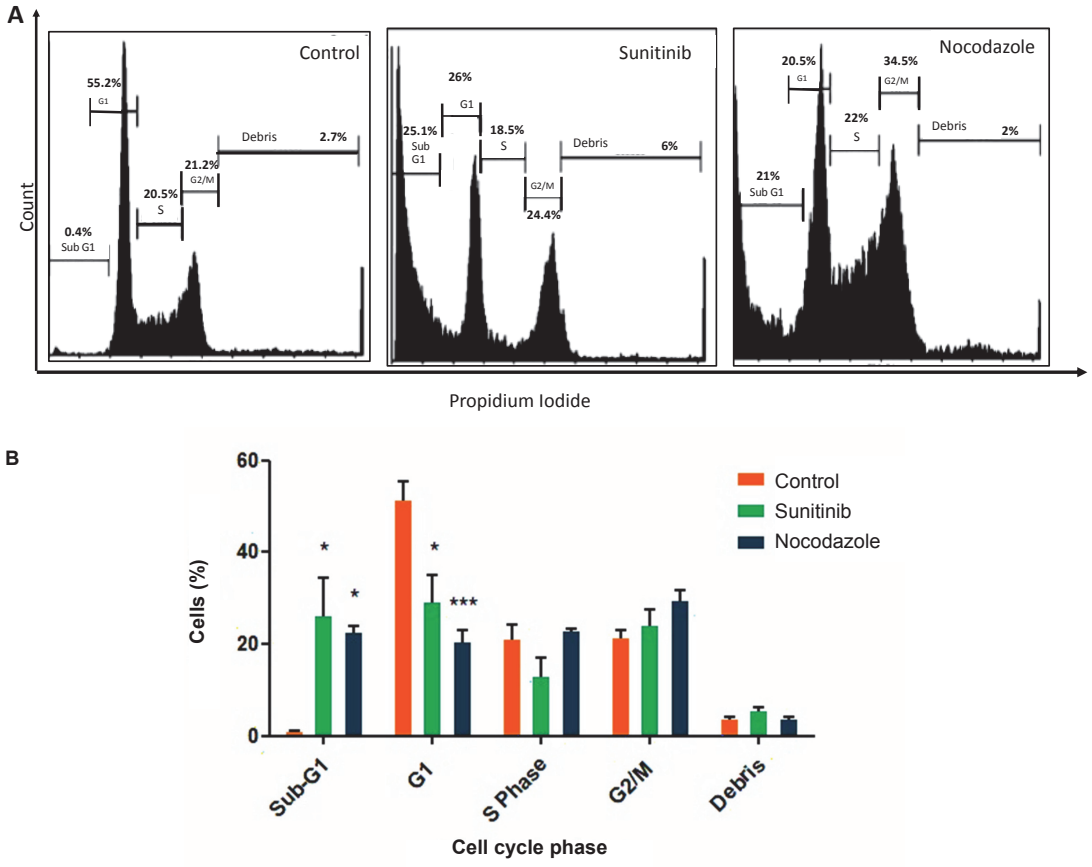

Fig. 2. Effects of sunitinib on cell cycle progression in sEnd.2 cells after 24-hour treatment. (**A**) Histograms indicating the distribution of cells in the various phases of the cell cycle (**B**) Percentage cell cycle distribution of sEnd.2 cells. Nocodazole was used as a positive control.
* $P < 0.05$ compared to the control; *** $P < 0.001$ compared to the control and values represent mean ± SD.

analysis showed that sunitinib had an inhibitory effect on the migration of sEnd.2 cells (Fig. 3B) ($P < 0.05$).

Western blot

Western blot analysis was conducted to determine the expression levels of proteins which regulate cell migration and angiogenesis (FAK, cadherin and paxillin). Results showed that sunitinib induced a decrease in the expression of paxillin and FAK (Fig. 4A, B, D), and that the decrease in the expression of these proteins was more pronounced at double the IC_{50} dose (5.2 µg/ml). The pan-cadherin antibody, which cross--reacts with various members of the cadherin family, namely, P-cadherin, N-cadherin, E-cadherin and R-cadherin, was employed to study the expression of cadherin. Sunitinib had no significant effect on the expression of cadherin at IC_{50} doses, 2.6 µg/ml (Fig. 4A, C). However, higher doses of sunitinib resulted in a decrease in the expression of cadherin. Furthermore, the expression of a proangiogenic cytokine which promotes tumour invasion and stimulates tumor angiogenesis, TGF-β1, was investigated. Results also showed that TGF-β1 expression was decreased in sunitinib treated cells compared to DMSO-treated cells (Fig. 5A, B).

Reactive oxygen species assay

The amount of ROS positive cells in the control was 4.37%, while it was 7.55% following treatment with sunitinib (Fig. 6A, B). Although there was a slight increase in ROS+ cells in sunitinib-treated cultures compared to the control, the drug did not induce any significant change ($P < 0.05$) in the ROS profile of the sEnd.2 cells (Fig. 6A, B).

Discussion

The effects of VEGFR signaling blockade were studied on different facets pertaining to tumor progression such as growth, migration and adhesion/angiogenic protein levels. From the data, sunitinib proved to have the most potent effect on the viability of the two skin tumor cells, although its effects were more pronounced in hemangioma cells. At the doses used (0.1–10 µg/ml or 3–30 µM), MAZ-51 has been shown to inhibit the VEGF-C and VEGF-D autophosphorylation of VEGFR-3, with no effect on VEGFR-2 (Kirkin et al., 2001; 2004). The potency of sunitinib in inhibiting hemangioma cell growth may be due to the fact that at tested doses MAZ-51 inhibits VEGFR-3 while sunitinib blocks several tyrosine kinase receptors, namely VEGFRs, platelet derived growth factor receptors (PDGFRs) and c-KIT. Importantly, the overexpression of PDGFRs and c-KIT mutation have been associated with the development of haemangiomas (Ji et al., 2014). Furthermore, receptors targeted by both MAZ-51 and sunitinib are mainly expressed in endothelial cells (ECs), thus explaining the more pronounced effect of these drugs on these benign endothelial tumor cells than on melanoma cells.

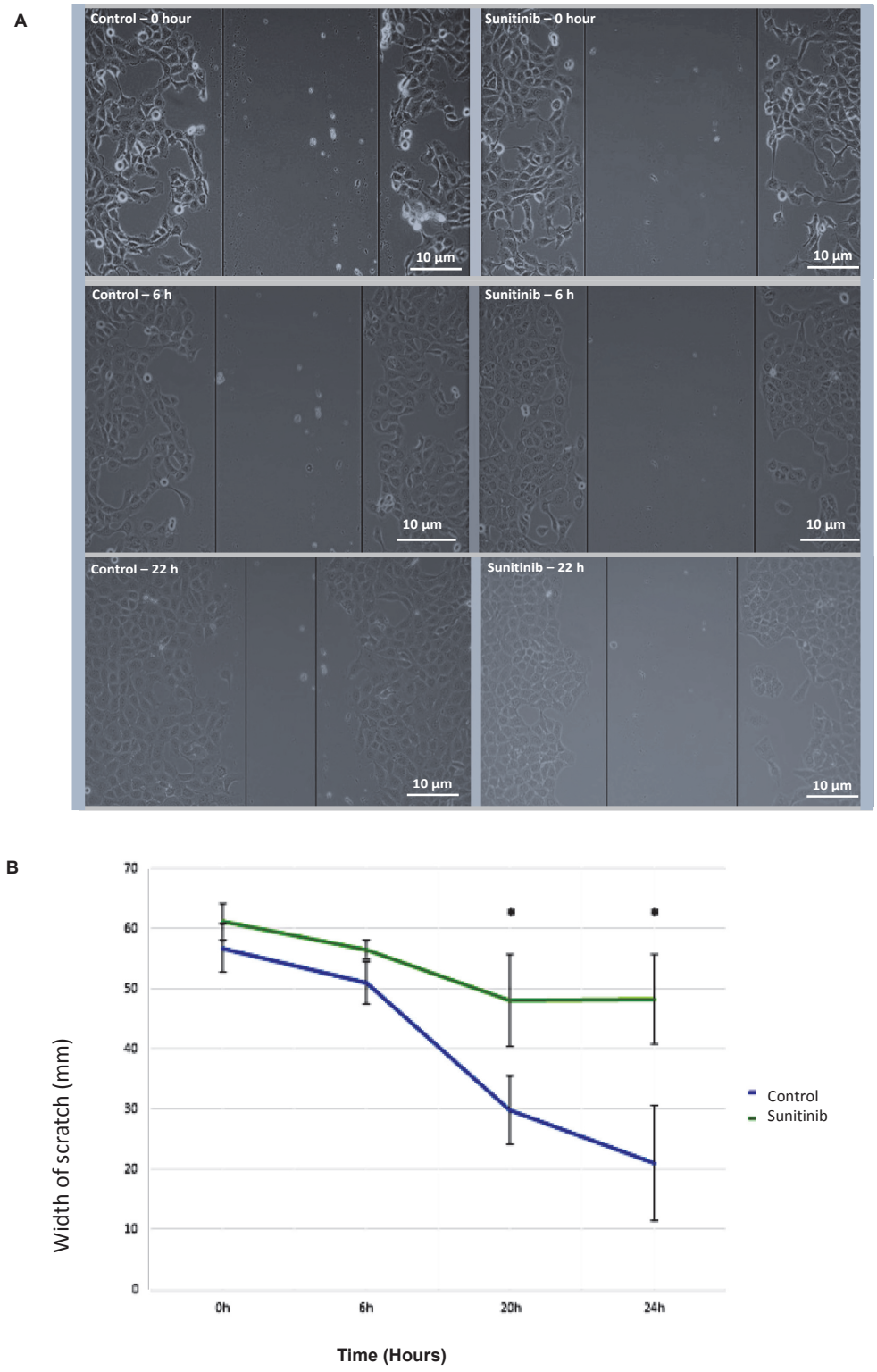

Fig. 3. Sunitinib inhibits cell motility. (**A**) Control and sunitinib (2.6 µg/ml) treated sEnd.2 cells at different time-points. Lines indicate the edge of the monolayer. Bar = 10 µm. (**B**) Quantitative analysis of sEnd.2 cell migration. Sunitinib showed a potent inhibition of endothelial cell migration. * $p < 0.05$ compared to the control, and values represent mean ± SD of three separate experiments.

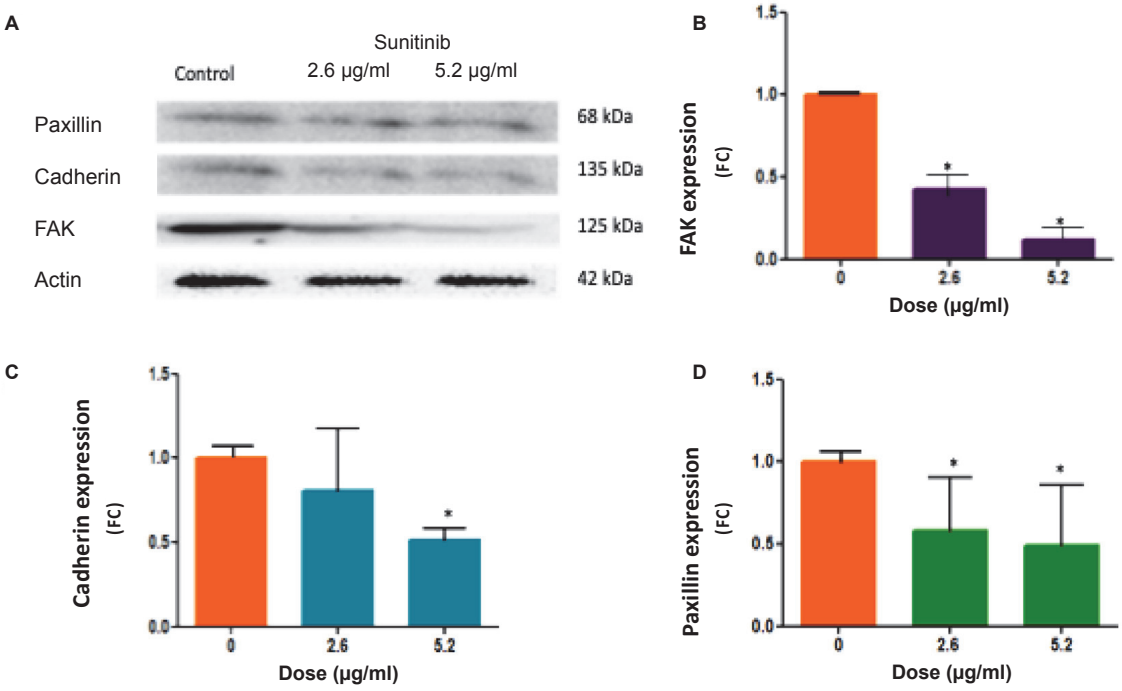

Fig. 4. Effects of sunitinib on the expression of adhesion proteins in sEnd.2 cells. Actin was employed as a housekeeping protein. (**A**) Protein bands visualized in control and drug-treated cells, (**B, C, D**) Qualitative expression of FAK, Cadherin and Paxillin in control and Sunitinib-treated sEnd.2 cells. Values represent the mean fold change (FC) protein expression normalized to actin ± SD. * indicates $P < 0.05$ compared to the DMSO control.

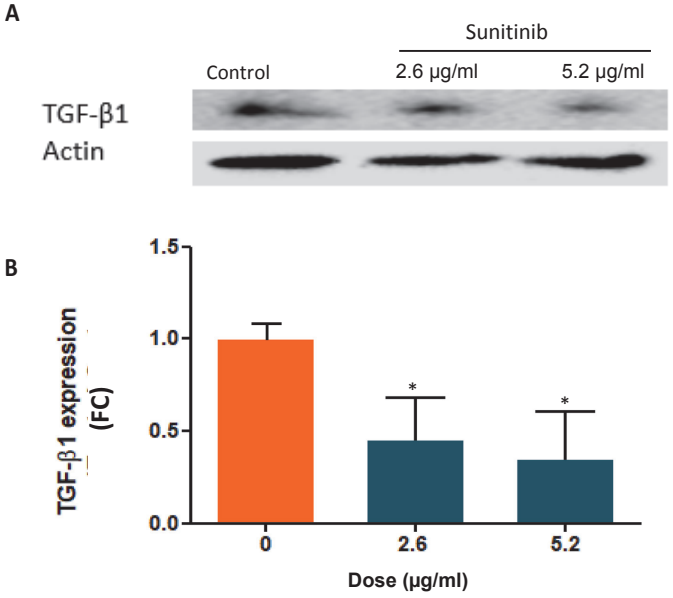

Fig. 5. Sunitinib treatment induces a decrease in the expression of transforming growth factor-β1 (TGF-β1) in sEnd.2 cells. Actin was employed as a housekeeping protein. (**A**) Protein bands visualized in control and drug-treated cells, (**B**) Qualitative expression of TGF-β1 in control and sunitinib-treated sEnd.2 cells. Values represent the mean fold change (FC) protein expression normalized to actin ± SD. * $P < 0.05$ compared to the DMSO control.

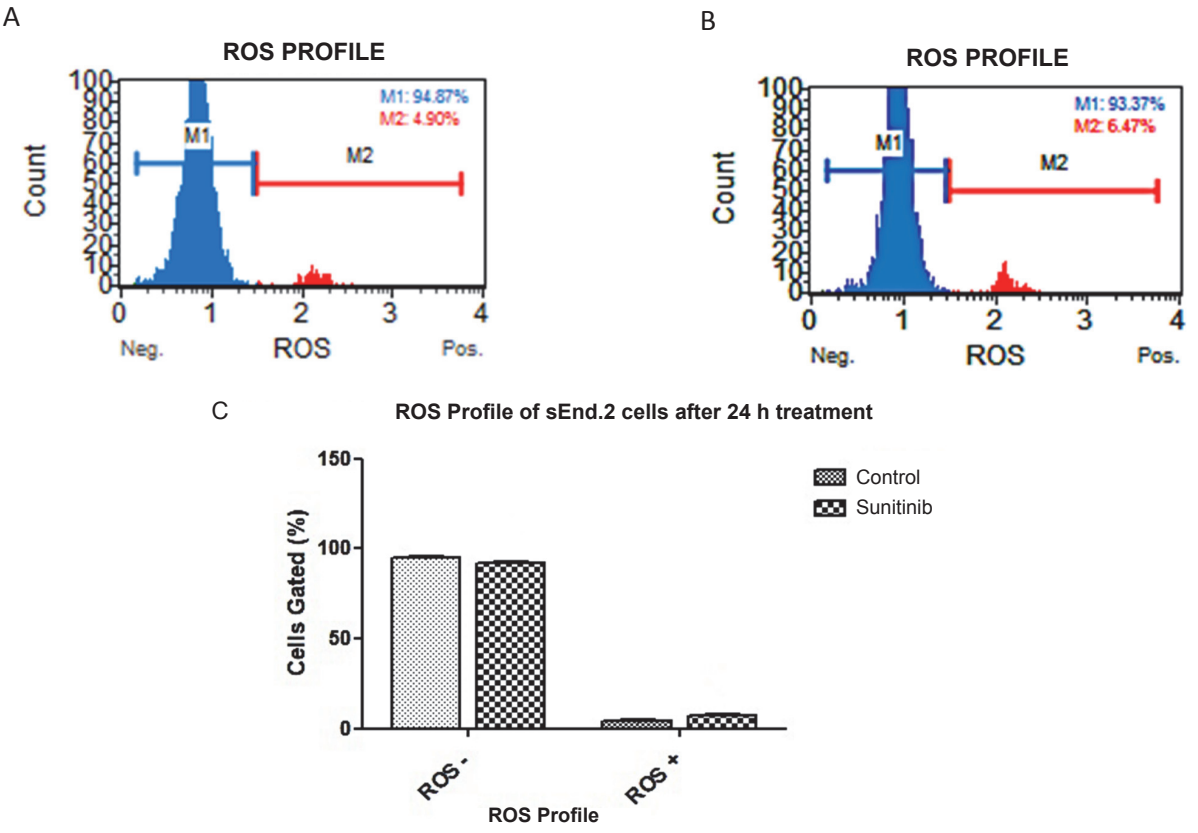

Fig. 6. Effects of sunitinib on reactive oxygen species production by sEnd.2 cells. Representative graphs showing ROS profiles of (**A**) Control and (**B**) Sunitinib (2.6 μg/ml)-treated sEnd.2 cells. (**C**) ROS profile of sEnd.2 cells after 24-hour treatment with Sunitinib 2.6 μg/ml. The mean percentage of ROS positive cells in the control was 4.37, while it was 7.55 following treatment with sunitinib. Values represent the mean of three separate experiments ± SD; there was no significant difference at $P < 0.05$ between control and sunitinib-treated cells.

The decreased sEnd.2 cell viability observed following sunitinib treatment correlated with an increase in the percentage of these cells in the Sub-G1 phase ($p < 0.05$). Noteworthy is that the Sub-G1 phase is associated with cell death (Kang et al., 2020). In a study by Di Desidero et al. (2013), sunitinib inhibited the proliferation of human dermal microvascular endothelial cells (HMVEC-d) and induced an increase in the Sub-G1 cell population. Similarly, in another study sunitinib induced a significant increase in the percentage of human umbilical vein endothelial cells (HUVECs) in the Sub-G1 phase (Brossa et al., 2015). These studies also showed that sunitinib caused apoptosis in ECs (Brossa et al., 2015). It is thus possible that sunitinib may inhibit the growth of sEnd.2 cells partly by inducing cell death.

Further evaluation of the effects of sunitinib on the migration of hemangioma cells showed changes in the width of the wound from 6 h, although quantitative analysis showed that the inhibition of sEnd.2 cell motility was more pronounced from 20 h. In a study investigating the effect of sunitinib on the migration of non-transformed HUVECs employing the same assay over 24 h, sunitinib inhibited cell migration after 9 h (Moravcik et al., 2016). In another separate study sunitinib inhibited the migration of prostate tumor derived ECs after 4–6 h of treatment (Pla et al., 2014). Furthermore, sunitinib has been shown to inhibit the migration of human benign and malignant meningioma cells as well as ECs and to inhibit angiogenesis (Andrae et al., 2012; Brossa et al., 2015; Grunewald et al., 2019). Taken together, the reported studies as well as the current study reveal that sunitinib inhibits the migration of tumor endothelial cells. Furthermore, previous studies reveal that sunitinib's anti-migratory effects in tumor-derived ECs are evident after shorter periods of exposure than in normal ECs (Andrae et al., 2012; Brossa et al., 2015; Grunewald et al., 2019; Pla et al., 2014). This may imply that ECs derived from tumor vessels such as hemangioma cells are more susceptible to the effects of sunitinib than normal ECs.

Sunitinib induced a decrease in the expression of FAK, an adhesion protein that is actively involved in several pathways that modulate cell migration and angiogenesis. In our laboratory we have shown that the blocking of FAK with PF573,228 in sEnd.2 cells led to reduced cell migration and invasion (Mabeta, 2016). In another study, FAK inhibitors induced a decrease in EC migration, similar to the results of the present study (Cabrita et al., 2011). In a separate study, FAK inhibition in glioblastoma xenografts reduced tumor microvascular density (Roberts et al., 2008). In contrast to FAK, there was no significant change in the expression of cadherin, a protein that modulates calcium-dependent cell surface adhesion. However, a reduced expression of the protein was observed when cells were exposed to double the IC50 dose (5.2 μg/ml).

On the other hand, the expression of paxillin, an adapter adhesion protein downstream of FAK signaling, was also decreased in sunitinib-treated cells. Paxillin plays a crucial role in adhesion by recruiting structural and signaling molecules that regulate cell migration. Upon integrin engagement with the extracellular matrix (ECM), paxillin is activated through phos-

phorylation at Tyr31, Tyr118, Ser188, and Ser190, resulting in the promotion of cell movement (López-Colomé et al., 2017). This further implies that by blocking VEGFR-2 activation, sunitinib could have led to a decrease in FAK and paxillin expression and therefore, reduced the migration of sEnd.2 cells as observed in this study. Of note is that the ligand for VEGFR-2, VEGF-A, is involved in the activation of FAK and the induction of angiogenesis (Abedi and Zachary, 1997; Cabrita et al., 2011; Koch and Claesson-Welsh, 2012). Thus, our findings indicate that the drug-induced decrease in FAK expression may lead to an inhibition of hemangioma cell migration and possibly the associated angiogenesis. Indeed, our findings show that sunitinib induced a decrease in the expression of a cytokine that regulates angiogenesis, TGF-β1, which is the TGF-β isoform also commonly implicated in tumorigenesis.

The growth factor acts in the vascular microenvironment by increasing the expression of matrix metalloproteinases (MMPs), ultimately leading to the degradation of the ECM and an increase in cell motility and neovascularization (Akhurst and Derynck, 2001; Drabsch and ten Dijke, 2012; Padua and Massagué, 2009).

In a study comparing the effects of sunitinib and another RTK inhibitor, sorafenib on prostate tumor derived ECs, sunitinib proved to be superior with regards to its inhibitory effects on cell proliferation, survival and motility (Pla et al., 2014). Sunitinib has been incorporated in various drug combination studies in preclinical models and in clinical trials, with promising results (Bianchini et al., 2019; Kuang et al., 2018; Nikolaou et al., 2012; Yeramian et al., 2012). It is plausible that sunitinib could be incorporated in combination treatments targeting receptors expressed by hemangioma cells, although studies are required to investigate combination approaches.

Reactive oxygen species contribute to angiogenesis by stimulating VEGF-A production. Exploratory studies were thus conducted to evaluate the effects of sunitinib on ROS. There was no significant alteration in the number of ROS+ cells following sunitinib treatment when compared to the control at doses that inhibited both cell growth and migration. This observation is of significance since ROS, which promotes angiogenesis, is also a marker of endothelial dysfunction (ED) and is linked to cardiovascular disorders such as atherosclerosis and hypertension (Xia et al., 2007).

It is worth noting that cardiotoxicity is a major side effect of sunitinib treatment and several other drugs that target the VEGF-VEGFR pathway (Jain et al., 2006; León-Mateos et al., 2015). Recently, we observed that at the same doses, sunitinib did not increase the levels of nitric oxide (NO) and angiotensin II, both important molecules in vascular homeostasis and contributors to ED. Thus, the possibility of employing sunitinib to target multiple receptors involved in tumor growth at doses that may not induce ED provides an opportunity for hemangioma treatment.

Conclusions

This study showed that VEGFR signaling blockade with sunitinib was the most effective in inhibiting hemangioma cell growth. The drug also inhibited cell migration, an important step in the progression of the tumor. In addition, there was a decrease in the expression of FAK, paxillin and TGF-β1, important proteins in EC migration and angiogenesis. This study further revealed that sunitinib does not elicit significant changes in ROS production at IC_{50} doses (2.6 μg/ml). This is a desirable outcome as oxidative stress supports tumor angiogenesis and is also linked to endothelial dysfunction. Therefore, blocking VEGFR signaling with a multi-targeting RTKI such as sunitinib may be effective in the treatment of hemangiomas.

Conflict of interests

The authors declare no conflict of interests.

Acknowledgements

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Author contributions

WS conducted experiments; PM assisted with the analysis of results and prepared the manuscript.

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