

Combined effect of propranolol, vincristine and bevacizumab on HUVECs and BJ cells

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Abstract. Infantile hemangioma is one of the most common benign tumors affecting children, with ~10-15% requiring medical treatment. These tumors consist of endothelial cells and stromal components, including fibroblasts, pericytes and mast cells. Effects of propranolol treatment in combination with bevacizumab or vincristine on cell growth were compared in the current study using human umbilical vein endothelial cells (HUVECs) and BJ human normal fibroblasts (BJs) to determine potential synergic effects *in vitro*. Inhibition of cell growth was investigated using MTT assays and cytotoxicity of the drugs in various combinations was expressed as half inhibitory concentration (IC₅₀). Apoptosis was investigated using flow cytometry, with Alexa Fluor 488 and propidium iodide. Propranolol inhibited BJ and HUVEC growth in a dose-dependent manner, with increased response observed in BJ (IC₅₀, 148,32 μg/ml; standard error logIC₅₀, 0.07). Treatment with vincristine induced the strongest growth inhibition in HUVECs (IC₅₀, 17,89 μg/ml; standard error log IC₅₀, 0.07) and BJ (IC₅₀, 24,81 μg/ml; standard error log IC₅₀, 0.08) compared with propranolol (HUVEC IC₅₀, 81,94 μg/ml; standard error log IC₅₀, 0.06; BJ-IC₅₀, 148,32 μg/ml; standard error logIC₅₀, 0.07) or bevacizumab (HUVEC IC₅₀ 96,91 μg/ml; standard error log IC₅₀, 0.06; BJ IC₅₀, 182,70 μg/ml; standard error log IC₅₀, 0.09) alone.

Bevacizumab was the weakest cytotoxic agent. Combination treatment of vincristine with bevacizumab induced the highest levels of apoptosis in HUVECs compared with all other treatments and triple-drug therapy induced the levels of apoptosis in BJ. Single treatment with vincristine, propranolol or bevacizumab induced apoptosis in BJ and HUVECs. In BJ, triple treatment exhibited the greatest influence on apoptosis, compared with single and dual treatments and in HUVECs, vincristine and bevacizumab combination treatment induced apoptosis to the highest level. The present study offers novel perspectives in drug repurposing studies for the three drugs, particularly in diseases where the pathogenesis is based on healthy endothelial cell proliferation, including hemangiomas.

Introduction

Infantile hemangioma is one of the most common benign tumors affecting children, occurring in 4-10% of all infants and ~10-15% require medical treatment (1,2). The tumors consist of endothelial cells and stromal components, including fibroblasts, pericytes and mast cells (3,4). Following the proliferation phase, endothelial cells are replaced with adipocytes, which subsequently determine the involution of the tumor (5).

Before 2008, various therapies have been established to treat infantile hemangioma, including cortisone, vincristine, interferon-α and lasertherapy; however, these therapeutic treatments have been demonstrated to be associated with potentially severe adverse side effects (5). Following reports on antiproliferative effects of propranolol by Léauté-Labrèze *et al* (6) in 2008, various studies have been performed investigating the mechanisms by which propranolol induces the involution of hemangiomas (1,2). Propranolol is a non-selective β-blocker used worldwide as the first-line therapy for hemangiomas (5). Several mechanisms, including vasoconstriction, blocking of proangiogenic signals and induction of apoptosis, have been suggested as underlying mechanisms (2).

Vincristine is a chemotherapeutic agent that inhibits endothelial cell growth by blocking mitosis and the formation of

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microtubules by direct cytotoxicity (7). Furthermore, vincristine is used as a second or third-line therapeutic agent in life-threatening situations, including severe bleeding, blocked airway or Kassabach Meritt syndrome, or in steroid-resistant and steroid-dependent hemangiomas (8).

It has been established that the vascular endothelial growth factor (VEGF) and basic fibroblastic growth factor (bFGF) stimulate cell proliferation, which subsequently results in the growth of hemangiomas (5). A well-studied antiangiogenic treatment is bevacizumab, a humanized monoclonal antibody that binds to VEGF and inhibits angiogenesis (9). Bevacizumab is used in treating certain types of cancer, including colon or lung cancer, affecting adults (9). Treatment of patients with hemangiomas has not been studied extensively (10).

The aim of the present study was to assess effects of propranolol on human umbilical vein endothelial cells (HUVECs) and BJ human normal fibroblasts (BJs). Furthermore, the efficacy of vincristine and bevacizumab in combination with propranolol was assessed. To investigate the activity of the drugs and various drug combinations, the present study established an *in vitro* model consisting of normal fibroblasts and HUVECs to mimic the primary source of hemangiomas. Effects of propranolol, a clinically approved hemangioma treatment, on cell growth were compared with bevacizumab, a VEGF inhibitor, and vincristine, a mitosis inhibitor. Effects of various treatment combinations were evaluated to determine potential synergic effects.

Materials and methods

Cell cultures. BJ (CRL-2522) were purchased from the American Type Culture Collection (Manassas, VA, USA) and HUVECs were provided by Dr. Olga Soritau, from the Oncology Institute 'I. Chiricuță' (Cluj-Napoca, Romania) established from a primary source at the European Collection of Authenticated Cell Cultures (Salisbury, UK).

BJs were cultured at 37°C in a 5% CO₂ incubator in Eagle's Minimum Essential medium (MEM, Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and HUVECs were cultured in Endothelial Cell Growth medium (ECG, Cell Applications, Inc., San Diego, CA, USA); media were supplemented with 10% fetal calf serum (Sigma-Aldrich; Merck KGaA).

Cells were trypsin-digested at 75-85% subconfluency and seeded at 12x10³ cells/well in 96-well plates with 190 µl media (as described above). BJs were passaged 9-12 times and HUVECs were passaged 18-20 times. Passages were performed twice a week and no replacement of media was performed in between.

Drug preparations. Vincristine sulfate was purchased from Sindan Pharma SRL (Sindovin; Bucharest, Romania). A total of 1 mg vincristine (excipient, anhydrous lactose monohydrate) was dissolved in 500 µl ultrapure water (Lonza Group, Ltd., Basel, Switzerland) to obtain a 2,000 µg/ml stock and serial dilutions (1,000, 750, 500, 250, 125, 62.5, 32 and 16 µg/ml) were prepared with PBS. Concentrations in the culture medium were 100, 50, 25, 12.5, 6.25, 3.12, 1.6 and 0.8 µg/ml.

Propranolol chloral hydrate was purchased from Sintofarm SA (Propranolol; Bucharest, Romania). A total of 40 mg propranolol (excipients, lactose monohydrate, microcrystalline

cellulose and cornstarch) was dissolved in 1,000 µl of ultrapure water to obtain a 40 mg/ml stock. Serial dilutions between 2,000-15.6 µg/ml (2,000, 1,000, 500, 250, 125, 62.5, 31.25 and 15.6 µg/ml) were prepared in PBS. *In vitro* drug concentrations were extrapolated from *in vivo* doses based on previous studies (11-13).

Bevacizumab was purchased from Roche Applied Science (Avastin; Penzberg, Germany) and provided as a 25 mg/ml solution in water, containing excipients including trehalose dihydrate, sodium phosphate and polysorbate 20. Serial dilutions were prepared using the 25 mg/ml stock solution to obtain final concentrations between 19.5-2,500 µg/ml in cell culture medium.

Various propranolol concentrations were combined with a subcytotoxic concentration of vincristine and bevacizumab. All aforementioned propranolol concentrations were combined with subcytotoxic 10 µg/ml vincristine or 100 µg/ml bevacizumab.

Cytotoxicity measurements. For single drug treatments, medium was removed from the wells and replaced with 10 µl drug stock in PBS plus 190 µl of ECG for HUVECs and MEM for BJ, respectively. For combination treatments, medium was replaced with 10 µl of each drug stock solution in PBS plus 180 or 170 µl medium for dual or triple drug combinations, respectively. When cells were adherent to 96-well plates, drug treatments were administered for 24 h at 37°C as follows: in pairs; propranolol with vincristine, propranolol with bevacizumab and vincristine with bevacizumab; or with all three administered together. Cytotoxicity was then measured. Assays were performed in triplicate with untreated, viable cells used as negative controls. Blanks were based on cell culture media and were recorded in parallel.

Following incubation with the treatment, plates were subjected to MTT analyses (Sigma-Aldrich; Merck KGaA) according to a previously described protocol (14). Formazan crystals were dissolved in 150 µl dimethyl sulfoxide (Titelchimica, Pontecchio Polesine, Italy) and the intensity of coloration was measured spectrophotometrically, at 570 nm. Coloration is directly proportional to the number of living cells and resulting absorbance values were used to determine half inhibitory concentration (IC₅₀) values from dose-response curves using Graph Pad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA).

Apoptosis induction. Apoptotic and necrotic cell counts in treated BJ and HUVECs were determined by flow cytometry using the Alexa Fluor 488 Annexin V/propidium iodide (PI) Apoptosis kit (Invitrogen; Thermo Fisher Scientific, Inc.) following a previously described method (15). BJ and HUVECs were incubated in six-well plates at 2x10⁵ cells/ml in 3 ml media for 24 h and treated with the following drugs for 24 h: 50 µg/ml propranolol, 10 µg/ml vincristine, 50 µg/ml bevacizumab, propranolol (50 µg/ml) plus vincristine (10 µg/ml), propranolol (50 µg/ml) plus bevacizumab (50 µg/ml), vincristine (10 µg/ml) plus bevacizumab (50 µg/ml) and propranolol (50 µg/ml) plus vincristine (10 µg/ml) plus bevacizumab (50 µg/ml). Untreated cells were used as a negative control. Following treatment, cells were harvested, washed with Binding Buffer provided by the

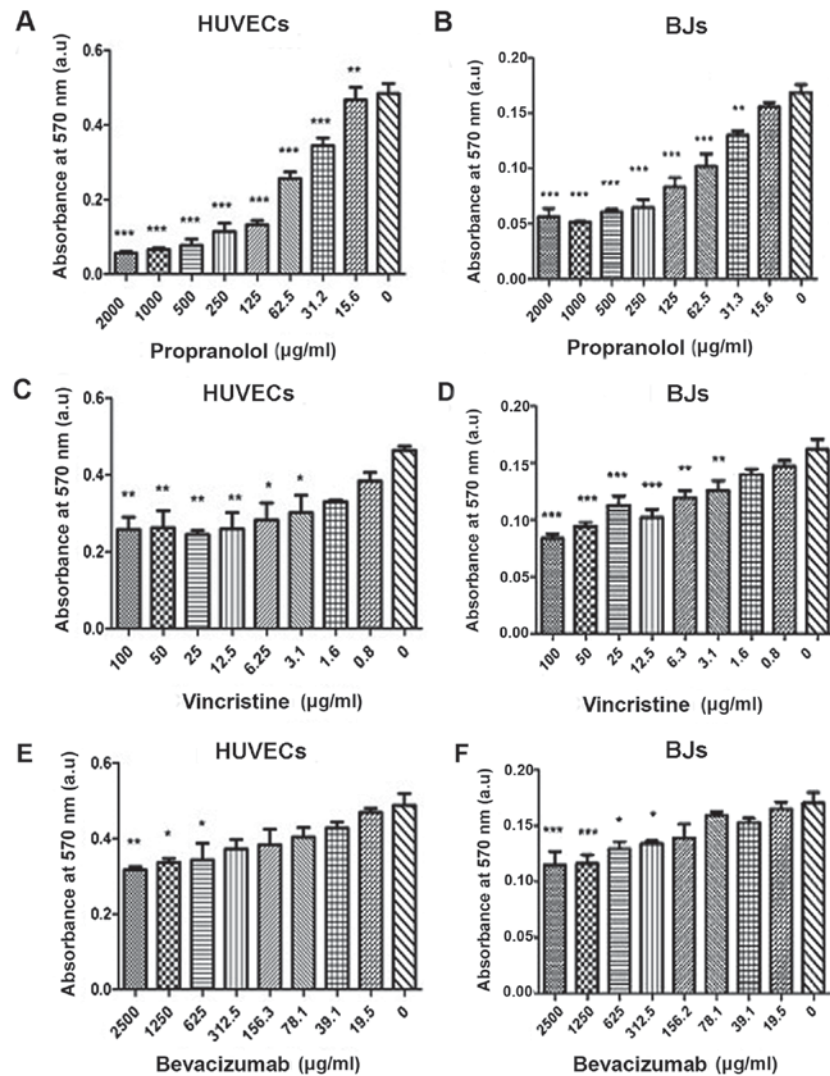


Figure 1. Inhibitory effect of propranolol, vincristine and bevacizumab in HUVECs and BJ. Cell viability determined by MTT assay is presented as a function of concentration of propranolol in (A) HUVECs and (B) BJ, vincristine in (C) HUVECs and (D) BJ, and bevacizumab in (E) HUVECs and (F) BJ. * $P < 0.05$; ** $P < 0.01$; and *** $P < 0.001$ vs. untreated (0 $\mu\text{g/ml}$). HUVEC, human umbilical vein endothelial cell; BJ, CRL-2522 human normal fibroblast.

kit, incubated for 15 min at room temperature with Alexa Fluor 488-labeled Annexin V and PI. Samples were subjected to analysis using the FACS Canto II flow-cytometer (Becton Dickinson; BD Biosciences; Franklin Lakes, NJ, USA). Two experiments were performed per treatment and mean values were determined using FACS Canto software (BD FACS Diva version 6.1; Becton Dickinson; BD Biosciences). AlexaFluor 488-labeled apoptotic cells were identified and analyzed using a 530/30 nm filter and PI-labeled necrotic cells were detected using a 575/26 nm filter.

Statistical analysis. Statistical analyses were performed using GraphPad Prism5 (GraphPad Software, Inc.). IC_{50} values were determined as the mean \pm standard deviation, by performing a non-linear regression analysis on the logarithm of the concentration vs. the normalized absorbance. Each value was determined in triplicate and three independent measurements were performed. Cell growth rates were compared using one-way analysis of variance and Dunnett's multiple comparison test, with untreated cells as reference and using a 95% confidence interval (CI). For apoptosis

evaluation, 10,000 individual single-cell measurements were evaluated and comparisons are based on one-way analysis of variances followed by Bonferroni post-hoc tests using a 95% CI. Correlation between the number of early apoptotic cells, late apoptotic cells and dead cells was assessed using a nonparametric Spearman correlation with a 95% CI. $P < 0.05$ was considered to indicate a statistically significant result.

Results

Cell growth inhibition. *In vitro* cytotoxicity was determined for propranolol, vincristine and bevacizumab using HUVECs and BJ to determine novel therapeutic strategies. Following treatment with various propranolol concentrations (15.6–2,000 $\mu\text{g/ml}$), as aforementioned, results revealed that the β -blocker inhibited growth of BJ and HUVECs in a dose-dependent manner (Fig. 1A and B, respectively). A more marked effect was observed in BJ compared with HUVECs. Vincristine inhibited growth in >50% of cell populations for BJ and HUVECS at 100 $\mu\text{g/ml}$ (Fig. 1C and D). Bevacizumab was revealed to be the weakest cytotoxic agent in the

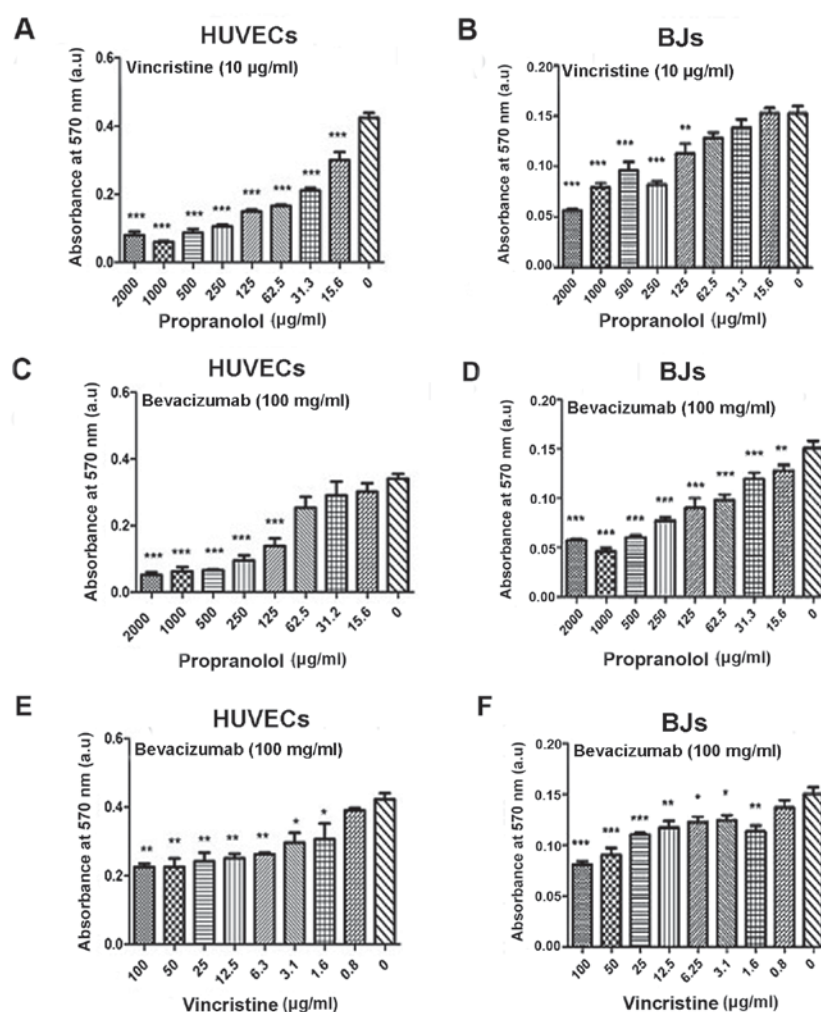


Figure 2. Inhibitory effect of propranolol plus vincristine, propranolol plus bevacizumab and vincristine plus bevacizumab in HUVECs and BJs. Cell viability determined by MTT assay is presented as a function of drug concentration, with varying propranolol plus 10 µg/ml vincristine in (A) HUVECs and (B) BJs, with varying propranolol plus 100 mg/ml bevacizumab in (C) HUVECs and (D) BJs, and with varying vincristine plus 100 mg/ml bevacizumab in (E) HUVECs and (F) BJs. *P<0.05; **P<0.01; and ***P<0.001 vs. untreated (0 µg/ml). HUVEC, human umbilical vein endothelial cell; BJ, CRL-2522 human normal fibroblast.

tested cell lines compared with propranolol and vincristine (Fig. 1E and F).

In order to investigate effects of combined treatments, various concentrations of propranolol, as aforementioned, were administered in combination with sublethal concentrations of vincristine (10 µg/ml) or bevacizumab (100 µg/ml) to BJs and HUVECs (Fig. 2).

Nonlinear dose-response curves were generated to determine IC₅₀ values for the various drug treatments using BJs and HUVECs (Table I). Decreased IC₅₀ values indicate higher levels of cytotoxicity. In every IC₅₀ value evaluation, the absorbance measurement of the untreated cells served as the reference value for 100% viability. Therefore on every sigmoidal curve, which generates the IC₅₀ value, measurements correspond to the untreated cells. The results confirmed a significantly increased treatment effect of propranolol when combined with vincristine (10 µg/ml) or bevacizumab (100 µg/ml) compared with the propranolol alone (Table I). IC₅₀ values were lower in HUVECs compared with BJs, following treatment with propranolol. Following combination treatment with bevacizumab or vincristine, IC₅₀ values in BJs decreased, increasing the cytotoxic treatment effect by 72.35 and 63.74%, respectively.

In comparison, the percentages in HUVECs were lower in combination treatment, the cytotoxic effect was increased only by 19.52% with bevacizumab and 33.49% with vincristine. In HUVECs and BJs, triple treatment with propranolol, bevacizumab and vincristine resulted in a decreased IC₅₀ compared with the single propranolol treatment and cytotoxicity increased by 94.78 and 89.55%, respectively (data not shown).

Apoptosis induction. Following 24 h treatment, apoptosis levels were determined in HUVECs and BJs, treated with one therapeutic agent or a combination of them. In the present study, the number of cells in early apoptotic stages following treatment was markedly increased compared with the untreated control samples (Figs. 3 and 4). Vincristine was the most potent inducer of programmed cell death in HUVECs and BJs *in vitro* compared with the propranolol and bevacizumab treated group (P<0.001; Table II). When using combination treatment, vincristine plus bevacizumab induced the highest levels of apoptosis in HUVECs (Figs. 3G, 4G and 5), in comparison with the BJs where triple therapy was most effective. In addition, the results revealed that among all single drug treatments, with either propranolol, vincristine or bevacizumab, the greatest

Table I. IC₅₀ values determined in HUVECs and BJ's treated with propranolol, vincristine and bevacizumab and combinations of the drugs.

Treatment	IC ₅₀ (μg/ml)	
	BJs	HUVECs
Propranolol	148.32±0.07 ^{aaa}	81.94±0.06 ^{aaa}
Vincristine	24.81±0.08 ^{aaa}	17.89±0.07 ^{aa}
Bevacizumab	182.70±0.09 ^b	96.91±0.06 ^b
Propranolol plus vincristine (10 μg/ml)	53.78±0.06 ^b	54.50±0.06 ^{aaa}
Propranolol plus bevacizumab (100 μg/ml)	41.01±0.05 ^{aaa}	65.95±0.03 ^b
Vincristine plus bevacizumab (100 μg/ml)	17.73±0.10 ^{aaa}	9.77±0.07 ^{aa}
Propranolol plus vincristine (10 μg/ml) plus bevacizumab (100 μg/ml)	7.73±0.11 ^{aaa}	8.56±0.04 ^{aaa}

Data are presented as mean ± standard error. ^{aa}P<0.01; ^{aaa}P<0.001 and ^bP>0.05 HUVEC, human umbilical vein endothelial cell; BJ, CRL-2522 human normal fibroblast; IC₅₀, half inhibitory concentration.

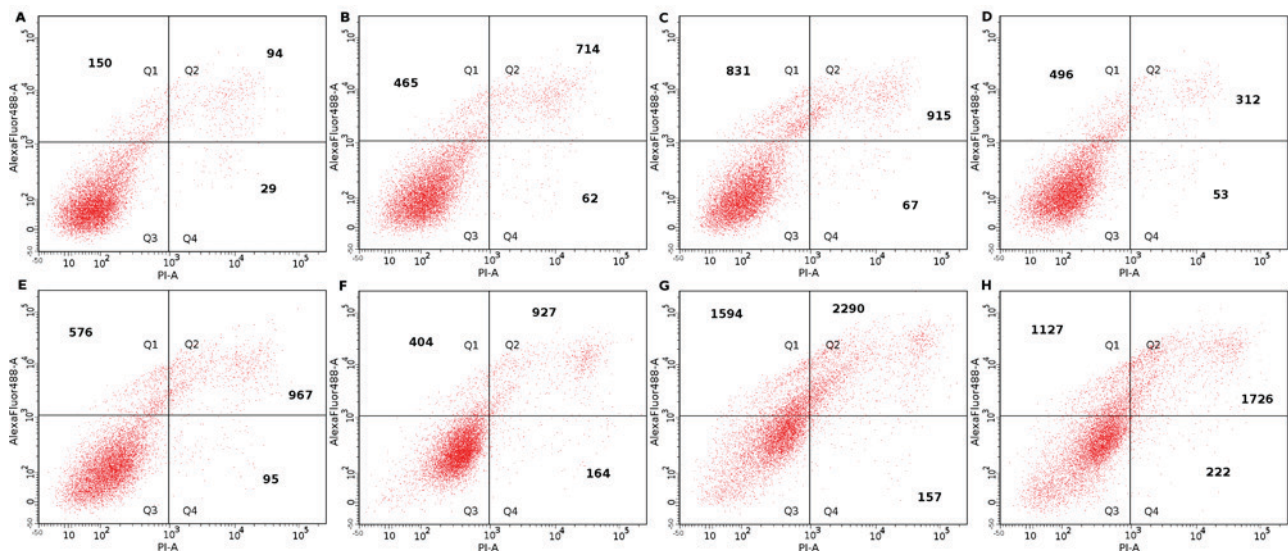


Figure 3. Apoptosis evaluation of HUVECs treated with propranolol, vincristine and bevacizumab and combinations of the drugs. Representative images of flow cytometry-based apoptosis evaluation of HUVECs (A) untreated or treated with (B) propranolol (50 μg/ml), (C) vincristine (10 μg/ml), (D) bevacizumab (50 μg/ml), (E) propranolol (50 μg/ml) plus vincristine (10 μg/ml), (F) propranolol (50 μg/ml) plus bevacizumab (50 μg/ml), (G) vincristine (10 μg/ml) plus bevacizumab (50 μg/ml), and (H) propranolol (50 μg/ml) plus vincristine (10 μg/ml) plus bevacizumab (50 μg/ml). HUVECs, human umbilical vein endothelial cell; Q1, quadrant representative of early apoptosis; Q2, quadrant representative of late apoptosis; Q3, quadrant representative of viable cells; Q4, quadrant representative of dead cells.

number of HUVECs found in the late apoptotic stage was when using vincristine ($P<0.001$; Table II). This observation was similar to the number of HUVECs found in the late apoptotic stage following combination treatment with propranolol plus vincristine. Treatment with vincristine plus bevacizumab was revealed to significantly increase cell numbers in late stage apoptosis compared with mono-treatments, combination treatment of propranolol plus vincristine and triple treatment ($P<0.05$; Table II and Fig. 5).

In BJ's, treatment with vincristine, vincristine plus propranolol and triple treatment increased the number of cells in late stage apoptosis to the greatest extent, when compared with the other cycle cell stages ($P<0.05$; Table II; Fig. 5).

Propranolol in combination treatments resulted in increased cell death, compared with combination treatments

without Propranolol; however, as subcytotoxic concentrations of propranolol were used, the number of dead cells were lower, indicating increased apoptosis (Figs. 3-5).

Furthermore, the results revealed that there was a correlation between HUVECs and BJ's regarding the number of cells in early stage apoptosis at varying treatment options (Spearman r -factor, 0.833; $P=0.077$; Table II). Correlation was further observed between HUVECs and BJ's at late stage apoptosis (r -factor, 0.743; $P=0.0288$) and with respect to the number of dead cells (r -factor, 0.895; $P=0.0109$; Table II).

Discussion

Infantile hemangiomas are benign vascular tumors that are characterized by a proliferative, involution and involuted phase (3).

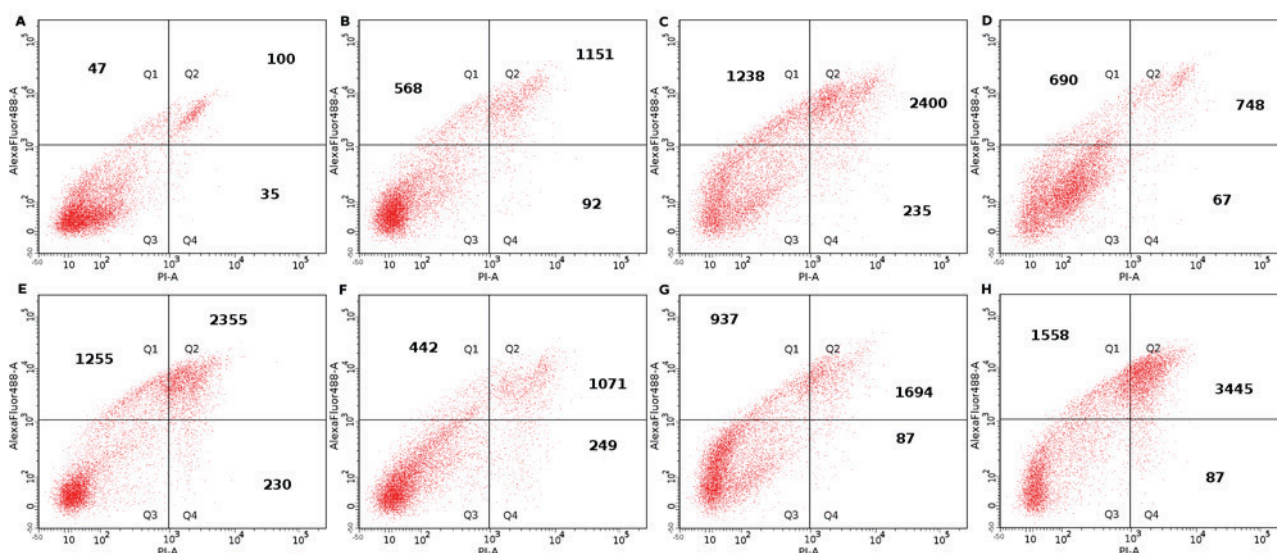


Figure 4. Apoptosis evaluation of BJ cells treated with propranolol, vincristine and bevacizumab and combinations of the drugs. Representative images of flow cytometry-based apoptosis evaluation of BJ cells (A) untreated or treated with (B) propranolol (50 μ g/ml), (C) vincristine (10 μ g/ml), (D) bevacizumab (50 μ g/ml), (E) propranolol (50 μ g/ml) plus vincristine (10 μ g/ml), (F) propranolol (50 μ g/ml) plus bevacizumab (50 μ g/ml), (G) vincristine (10 μ g/ml) plus bevacizumab (50 μ g/ml), and (H) propranolol (50 μ g/ml) plus vincristine (10 μ g/ml) plus bevacizumab (50 μ g/ml). BJ, CRL-2522 human normal fibroblast; Q1, quadrant representative of early apoptosis; Q2, quadrant representative of late apoptosis; Q3, quadrant representative of viable cells; Q4, quadrant representative of dead cells.

They appear during the first few months of life and proliferate following stimulation by several growth factors, including VEGF and bFGF (5). Following the proliferative phase, hemangiomas rapidly enter the involution phase, where levels of secreted VEGF and bFGF fall and cells undergo apoptosis (16). To date, several studies performed on hemangioma-derived cells (16) and HUVECs (17) have demonstrated an arrest in cell proliferation and subsequent involution of the tumor.

HUVECs represent a primary endothelial cell model (18). Furthermore, HUVECs are similar to hemangioma cells, as they react similarly to endothelial cells present in infantile hemangiomas (19). Several studies were performed using HUVECs to analyze the effects exhibited by Propranolol that result in the involution of hemangiomas (16,17). Further studies used HUVECs as control cell cultures to compare hemangioma-derived stem cells (4) or hemangioma-derived endothelial cells (20). In addition, BJ cells were used in the present study, to mimic the connective tissue of infantile hemangiomas, composed of fibroblasts, pericytes and mast cells (3,4,21). The present study investigated therapeutic effects of vincristine, propranolol and bevacizumab on HUVEC and BJ cells.

Ji *et al* (16) demonstrated that propranolol induced apoptosis in hemangioma-derived cells via activation of caspase-3 and -9, following the intrinsic pathway. Pan *et al* (22) revealed that propranolol *in vitro* induces the inhibition of cell cycle progression, concomitantly with decreased nitric oxide and VEGF levels through the downregulation of the phosphoinositide 3-kinase/protein kinase B/endothelial nitric oxide synthase/VEGF signaling pathway. In addition, Tu *et al* (23) revealed that apoptosis is induced both via the intrinsic and the extrinsic pathway. Ou *et al* (20) performed a study on hemangioma-model cells (hemEC) that demonstrated that the inhibition of proliferation and induction of apoptosis is associated with downregulation of VEGF receptor 2. Lamy *et al* (17) investigated effects of propranolol

on HUVECs and demonstrated inhibition of cell growth in a dose-dependent manner, which was in line with the results of the present study. This effect was further observed in BJ cells in the present study, which suggested that the therapeutic effect of propranolol on hemangiomas may be due to the inhibition of growth in the sustaining tissue. However, this observation requires further experimental evidence based specific hemangioma cells for validation. Cell growth inhibition exerted by propranolol was significantly increased in BJ cells and HUVECs, when treatment was combined with subcytotoxic concentrations of vincristine and bevacizumab, as presented in Figs. 1 and 2. In addition, IC_{50} values decreased when propranolol was administered together with either the mitosis or the VEGF inhibitors. The cytotoxicity of each drug was stronger in HUVECs compared with BJ cells; however, the synergistic effect following treatment with propranolol plus the two inhibitors was enhanced in BJ cells compared with HUVECs.

It has been established that VEGF is one of the most potent growth factors inducing proliferation in hemangiomas (14). However, single treatment with bevacizumab, a VEGF inhibitor, exhibited the weakest effect on viability in the tested cell lines of the current study. This may be associated with the mechanism of action of VEGF inhibitors, which do not directly affect apoptosis levels, but indirectly affect apoptosis by decreasing levels of growth factor, resulting in decreased levels of proliferation and subsequent cell cycle arrest (24,25). However, combination treatment of bevacizumab plus vincristine resulted in a significant increase in apoptosis levels compared with bevacizumab and vincristine single treatments and the determined IC_{50} significantly decreased in HUVECs for vincristine plus bevacizumab compared with vincristine single treatment, as shown in Table II. This synergy between a direct cytotoxic and a VEGF inhibitor bevacizumab has been established in malignant solid tumors, including lung or

Table II. Number of apoptotic and dead HUVECs and BJ's treated with propranolol, vincristine and bevacizumab and combinations of the drugs, determined by flow cytometry.

A, Early apoptotic cells within a population of 10,000 cells

Treatment	HUVECs	BJs	R	P-value
Untreated	150±3	47±4		
Propranolol	465±14 ^c	568±2 ^c		
Vincristine	831±6 ^c	1238±34 ^c		
Bevacizumab	496±27 ^c	690±31 ^c		
Propranolol plus vincristine (10 µg/ml)	576±31 ^c	1255±58 ^c		
Propranolol plus bevacizumab (50 µg/ml)	404±12 ^c	442±13 ^c		
Vincristine plus bevacizumab (50 µg/ml)	1,594±53 ^c	937±8 ^c		
Propranolol plus vincristine (10 µg/ml) plus bevacizumab (50 µg/ml)	1,127±41 ^c	1558±4 ^c	0.833	0.077

B, Late apoptotic cells within a population of 10,000 cells

Treatment	HUVECs	BJs	R	P-value
Untreated	94±2	100±11		
Propranolol	714±8 ^c	1151±98 ^c		
Vincristine	915±58 ^c	2400±69 ^c		
Bevacizumab	312±10 ^b	748±4 ^c		
Propranolol plus vincristine (10 µg/ml)	967±7 ^c	2355±57 ^c		
Propranolol plus bevacizumab (50 µg/ml)	927±44 ^c	1071±14 ^c		
Vincristine plus bevacizumab (50 µg/ml)	2290±86 ^c	1694±50 ^c		
Propranolol plus vincristine (10 µg/ml) plus bevacizumab (50 µg/ml)	1726±22 ^c	3445±27 ^c	0.743	0.0288

C, Dead cells within a population of 10,000 cells

Treatment	HUVECs	BJs	R	P-value
Untreated	29±3	35±2		
Propranolol	62±6 ^d	92±16 ^a		
Vincristine	67±9 ^d	235±23 ^c		
Bevacizumab	53±1 ^d	67±9 ^d		
Propranolol plus vincristine (10 µg/ml)	95±4 ^b	230±1 ^c		
Propranolol plus bevacizumab (50 µg/ml)	164±10 ^c	249±27 ^c		
Vincristine plus bevacizumab (50 µg/ml)	157±12 ^c	87±7 ^a		
Propranolol plus vincristine (10 µg/ml) plus bevacizumab (50 µg/ml)	222±32 ^c	267±22 ^c	0.895	0.0109

Data are presented as mean ± standard deviation. ^aP<0.05; ^bP<0.01; ^cP<0.001 and ^dP>0.05 vs. untreated. R, Spearman correlation coefficient with 95% confidence interval; HUVEC, human umbilical vein endothelial cell; BJ, CRL-2522 human normal fibroblast.

colon cancer and has been revealed to reduce VEGF levels and enhance cytotoxic effects of the chemotherapeutical agents, which subsequently results in improved therapeutic outcomes (26,27). As a result, cell growth may not only be dependent on growth factors, but may be associated with numerous other mechanisms, including mitosis inhibition. A similar effect was observed in BJ's. Liu *et al* (28) demonstrated that combination treatment using bevacizumab and TRC105, two angiogenesis inhibitors with varying mechanisms of action, suppresses HUVEC growth to a greater extent when compared with single treatments. The results of the present

study demonstrated that using a therapeutic approach based on different mechanism of action increased the efficacy in tumor arrest and involution, when compared with treatment with two agents that have a similar mechanism. Effects of bevacizumab may be increased in *in vivo* studies, particularly when combined with other treatments, compared with the *in vitro* model due to the increased secretion of growth factor observed in viable cells; however, further experimental proof is required. Hayot *et al* (29) investigated several agents, including vincristine, vinblastine, vindesine and vinorelbine, to determine their effects on HUVECs and demonstrated that

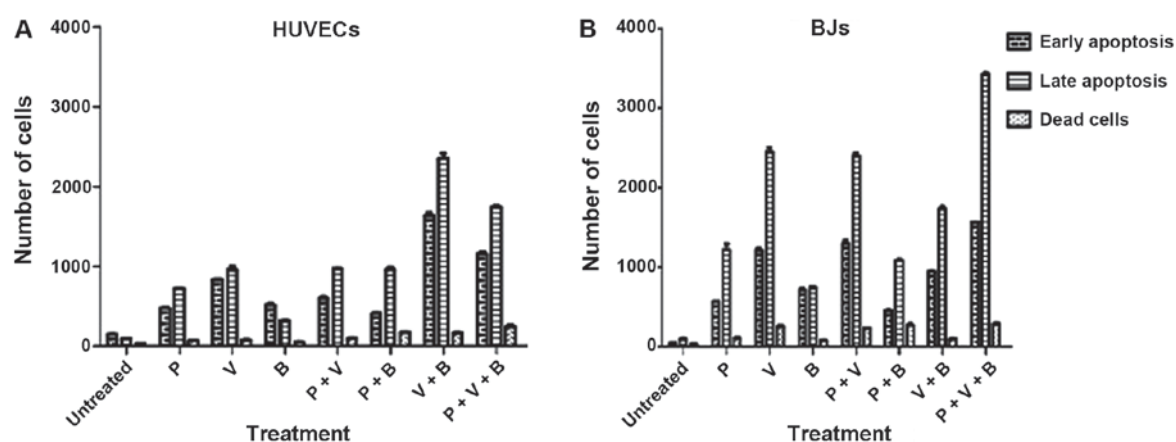


Figure 5. Quantitative evaluation of apoptosis in HUVECs and BJs treated with propranolol, vincristine and bevacizumab and combinations of the drugs. Number early and late apoptotic and dead cells determined by flow cytometry using (A) HUVECs and (B) BJs treated with propranolol (50 $\mu\text{g/ml}$), vincristine (10 $\mu\text{g/ml}$) and bevacizumab (50 $\mu\text{g/ml}$) and combinations of the drugs. Values are per population of 10,000 cells. HUVEC, human umbilical vein endothelial cell; BJ, CRL-2522 human normal fibroblast; P, propranolol; V, vincristine; B, bevacizumab.

vincristine has an antiangiogenic effect, even when administered at non-cytotoxic concentrations (10 $\mu\text{g/ml}$). The present study demonstrated marked cytotoxic effects of vincristine administered at 100 $\mu\text{g/ml}$ in single or at 10 $\mu\text{g/ml}$ in combination treatment with bevacizumab and/or propranolol. The results suggested that the cytotoxic effects of vincristine, via antiangiogenesis or direct cytotoxicity, were dependent on the concentration. Consequently the question remains, whether the concentration of vincristine administered *in vivo* has antiangiogenic or directly cytotoxic effects, suggesting that the range of vincristine administrable to patients is large and adaptable to patient needs. However, further investigations in this direction are required.

Following treatment, early apoptosis can occur within 30 min or thereafter, depending on cell and exposure type (30). In the current study, cells in early and late apoptosis were differentiated. In HUVECs, combination of vincristine and bevacizumab increased cells numbers in early and late stage apoptosis compared with triple treatment with propranolol, vincristine and bevacizumab. In BJs, triple treatment with propranolol, vincristine and bevacizumab exhibited the most pronounced apoptosis induction compared with all other treatment options. This difference between the cell lines may be due to varying proliferation rates and longer doubling times of BJs.

Regarding monotherapy, vincristine was revealed to be the most potent therapeutic agent in BJs and HUVECs inducing early and late stage apoptosis compared with the other single treatment regimens. The next best results were obtained with propranolol, which induced higher levels on late apoptosis compared with cells in early apoptosis. Treatment with bevacizumab plus vincristine exhibited a greater combinatory therapeutic effect compared with propranolol plus vincristine. This may be due to the similar mechanism of action exhibited by propranolol and vincristine, while bevacizumab and vincristine exhibit varying mechanisms of action. Future studies are required to elucidate the antiangiogenic mechanism of the combination of these drugs.

Patients with infantile hemangioma require prolonged treatment with propranolol to achieve tumor regression, which

may be due to the fact that propranolol predominantly induces late apoptosis, as was shown in our study. In hemangiomas that are difficult to treat or life threatening and where rapid therapeutic effects are required, vincristine is used (5). The various combinations of the drugs used in the current study require further investigation on specific hemangioma cell cultures or tissues, to evaluate novel therapeutic strategies for patients with infantile hemangiomas.

In conclusion, a marked synergistic effect on HUVECs and BJs was observed following administration of propranolol with bevacizumab and vincristine. Apoptosis was induced by the various treatments trialed in the present study; however, triple treatment exhibited the most marked increase in apoptosis in BJs and vincristine plus bevacizumab exhibited the strongest effect in HUVECs. The results of the present study may constitute a basis for novel drug testing and drug repurposing regarding the treatment of infantile hemangiomas.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

MB generated and analyzed data and contributed to the writing of the article. EFF designed the cytotoxicity studies, performed the experiments and revised the manuscript. OVB

documented research and conceived and designed the current study. MC designed and performed the flow cytometry analysis, and elaborated and revised the manuscript. GP and CLB were involved in the drafting of the manuscript and its critical revision for important intellectual content. AT coordinated the study, supervised its progress and approved the final version of the article for publication. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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