PHYTOTHERAPY RESEARCH
Phytother. Res. (2017)
Published online in Wiley Online Library
(wileyonlinelibrary.com) **DOI**: 10.1002/ptr.5891

The *In Vitro* and *In Vivo* Antiangiogenic Effects of Flavokawain B

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Angiogenesis is implicated in the development of a variety of pathological processes, most commonly cancer. It is essential for tumor growth and metastasis, making it an important cancer therapeutic target. Naturally occurring substances have led to the discovery of anticancer agents. Flavokawain B (FKB), a chalcone isolated from the root extracts of kava-kava plant, inhibits proliferation and causes apoptosis in vitro and in vivo of various cancer cell lines. The antimetastatic potential of FKB has also been suggested. In our study, we confirm the antiangiogenic action of FKB in vitro and, for the first time, demonstrate its strong antiangiogenic activity in vivo, using a zebrafish model. Our data show that FKB inhibits human brain endothelial cell (HUVEC) migration and tube formation even at very low and non-toxic concentrations. Moreover, FKB blocks angiogenesis process in zebrafish, with a dramatic reduction of subintestinal vein formation in a dose-dependent manner. Flavokawain B at the concentration of 2.5 µg/mL did not exhibit any toxic effects in zebrafish larvae and caused a markedly or complete obliteration of subintestinal vein formation. Our findings along with previously published data confirm that FKB may form the basis for creating an additional tool in the treatment of cancer and other neovascularization-related diseases. **Copyright © 2017 John Wiley & Sons, Ltd.**

Keywords: angiogenesis; flavokawain B; kava-kava; zebrafish; HUVEC.

INTRODUCTION

Angiogenesis is a complex process involving the formation of new blood vessels by migration, growth, and differentiation of endothelial cells (Hanahan and Weinberg, 2011). Neovascularization is essential for tumor growth beyond 1–2 mm and crucial for tumor invasion and metastasis (Weis and Cheresh, 2011). Thus, targeting angiogenesis has been considered a promising anticancer treatment option with positive clinical outcomes both as a single agent and in combination chemotherapy (Gadducci *et al*., 2015).

Many phytochemicals have shown promising anticancer activity *in vitro*. Chalcones are precursor compounds for flavonoid synthesis in plants and have been described as potential therapeutic agents for cancer cells, as these compounds promote apoptosis, inhibition of cellular proliferation, invasion, and angiogenesis (Mahapatra *et al*., 2015). Flavokawain B (FKB) is a chalcone encountered in kava-kava root extract which is traditionally used as a drink in South Pacific islands. Epidemiological data showing less incidence of cancer in these regions (Foliaki *et al*., 2011) prompted studies that have assessed the dietary anticancer chemopreventive effects of kava consumption (Steiner, 2000). Flavokawain B is the most potent anticancer compound of kava extract and also displays activities, such as antiinflammatory, antitumorigenic, and antinociceptive properties. Flavokawain B exhibits strong cytocidal activity against prostate, uterine, lung, breast, oral, synovial, and bone cancer cells, with little toxicity against normal cells (Li *et al*., 2008; Zhou *et al*., 2010; An *et al*., 2012; Hseu *et al*., 2012; Lin *et al*., 2012; Li *et al*., 2012a,b; Ji *et al*., 2013; Kwon *et al*., 2013; Abu *et al*., 2014; Abu *et al*., 2015).

The *in vitro* antimetastatic potential of FKB was recently demonstrated by the inhibition of endothelial cell migration and tube formation. Moreover, FKB action in inhibiting metastases was further suggested by the reduction of metastasis-related proteins in breast cancer tumors and by the bone marrow smearing assay *in vivo* (Abu *et al*., 2015; Abu *et al*., 2016). However, the mechanism of metastasis inhibition was not clearly demonstrated, and the FKB antiangiogenic action using an *in vivo* model has not been described yet. Thus, the zebrafish angiogenesis models represent a promising alternative in cancer research for the development of antineoplastic and antiangiogenic therapies (Nicoli and Presta, 2007).

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Contract/grant sponsor: Fundação de Amparo à Pesquisa do Estado de Minas Gerais; contract/grant number: APQ-00220-14.
Contract/grant sponsor: Conselho Nacional de Desenvolvimento Científico e Tecnológico; contract/grant number: 405053/2013-4.

Received 05 April 2017
Revised 27 June 2017
Accepted 21 July 2017

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Taken together, the clinical relevance of the antiangiogenic therapy in oncology and the reports of the antimetastatic action of FKB led us to investigate the *in vivo* antiangiogenic effects of FKB using a zebrafish model.

MATERIALS AND METHODS

Drug. Flavokawain B powder was purchased from the LKT Laboratories, Inc. (Product ID: F4503) and diluted in DMSO at 5 mg/mL stock solution.

Cell culture conditions and compounds. Human umbilical vein endothelial cells (HUVECs) were purchased from Life Technologies (Gibco, USA). Human brain endothelial cells were cultured in M200 basal media supplemented with large vessel endothelial supplement. Cells were grown at 37°C in 5% CO_2 incubator using T75 flasks and 100-mm dishes (Sarstedt AG & Co., Nümbrecht, Germany). All cell culture procedures were carried out under aseptic conditions in laminar flow hood. The cells were monitored with an inverted microscope Axiovert 25 (Carl Zeiss, Germany). All cell culture reagents listed were purchased from Life Technologies (Carlsbad, USA) or Sigma-Aldrich Co. (St. Louis, CA, USA).

Cell viability MTT assay. Human brain endothelial cells were plated at a density of 7.5×10^3 cells per well in 96-well culture plates (Sarstedt AG & Co., Nümbrecht Germany) in their respective cell medium containing 10% fetal bovine serum. After 24 h, the medium was treated with 0.1% DMSO vehicle control or FKB at different concentrations for 24 h. After treatment, MTT reagent was added to the wells at a final concentration of 1 mg/mL and incubated at 37°C for 3 h. After the incubation period, cell media was carefully taken to preserve the formazan crystals, and DMSO was added to dissolve them into a purple solution. The absorbance was determined at 595 nm using Victor™ X4 (PerkinElmer Inc., Whaltam, MA, USA) microplate reader. The number of viable cells was determined by uptake and reduction of MTT comparing the treated cells with the control group. All experiments were performed at least in triplicate on three separate occasions. Data are presented as mean ± SD.

Tube formation assay. Tube formation assay was performed using the Angiogenesis Starter Kit® (Life Technologies, Gibco, USA) according to the manufacturer's protocol. A 24-well plate (Sarstedt AG & Co., Nümbrecht Germany) was coated with 200 µL of Geltrex® Matrix solution and incubated at 37°C for 30 min to allow the matrix to solidify. Gently, 5×10^4 HUVEC cells were seeded into each well with 0.1% DMSO or 1.0, 2.5, and 5.0 µg/mL of FKB and incubated for 18 h at 37°C in a humidified atmosphere of 5% CO_2. At the end of the incubation period, cells were photographed with a digital camera attached to a stereomicroscope (SMZ 1500 Nikon) with 10× magnification. Human brain endothelial cells in control group formed tube-like structure which was defined as endothelial cord formations that connected at both ends. The antiangiogenic activities were assessed by manual counting branch points in which at least three tubes joined and total number of tubes using ImageJ software (National Institutes of Health, USA). The average number of branches and tubes was calculated from at least six randomly photographed fields. Three independent experiments were performed in duplicates for each condition.

Wound healing assay. The *in vitro* wound healing assay was performed to assess cell migration. Confluent HUVEC monolayers were grown on 6-well plates (Sarstedt AG & Co., Nümbrecht, Germany). The monolayer cells were wounded by scratching with 200-µL pipette tip and then washed with warm PBS to remove the non-adherent cells. M200 complete media together with 0.1% DMSO as a control or FKB 1.0, 2.5, and 5.0 µg/mL were added to the wells. The experiment was performed in duplicate for each condition. After 24-h treatment, at least three images of the wound closure were captured using a stereomicroscope (SMZ 1500 Nikon) at 5× magnification. The width of the wound from each photograph was measured using ImageJ software (National Institutes of Health, USA). The initial wound width was considered the same for all conditions, and the degree of wound regeneration was calculated as the percentage of the remaining cell free area compared with the vehicle control. Two independently experiments were performed in duplicates for each condition tested.

Zebrafish strain and drug treatment. Zebrafish (*Danio rerio*) embryos were generated by natural pairwise mating of wild-type zebrafish as described by Kimmel *et al.* (1995). For each mating, four to five pairs were set up, and on average, 100 embryos per pair were generated. Embryos were collected after natural spawning and maintained in E2 medium (EM) (15 mM NaCl, 0.5 mM KCl, 0.49 mM $MgSO_4 \cdot 7H_2O$, 0.15 mM KH_2PO4, 0.042 mM Na_2PO_4, 0.1 mM $CaCl_2$, and 0.07 mM $NaHCO_3$, pH 7.2) at 28°C on a 14-h light/10-h dark incubator during the whole experiment. The experimental procedures were approved by the Ethics Committee of the Universidade Federal de Minas Gerais (CEUA/UFMG #9/2012).

At 48-h post fertilization (hpf), healthy embryos were manually dechorionated by forceps and placed in 24-well plates (Sarstedt AG & Co., Nümbrecht, Germany) with the drug dissolved in EM at working concentrations. At this stage, zebrafish embryos do not present subintestinal vessels. In order to prevent toxicity caused by high concentrations of excreted ammonia, only five embryos were kept in each well, filled with 1.0 mL of either vehicle or drug solution. Fresh medium containing the drug of interest or vehicle was replaced every 24 h, until 72 hpf, when the fish were fixed to further analysis. Flavokawain

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B (LKT Laboratories, Inc.), dissolved in DMSO, was kept in −80°C and only thawed and diluted in EM immediately prior to administration in order to prevent degradation. *Zebrafish* larvae are tolerant to DMSO in low concentrations.

The zebrafish angiogenesis assay was performed as previously described by Serbedzija *et al*. (1999). Embryos were visually inspected for viability, morphological defects, and altered behavior. On day 3 of development, embryos were fixed in 4% paraformaldehyde for 2 h at room temperature and stained for endogenous alkaline phosphatase activity. Embryos were then washed twice in phosphate-buffered saline containing 0.1% Tween 20 (PBT) and dehydrated by immersing in 25, 50, 75, and 100% methanol in PBT. Afterwards, embryos were rehydrated stepwise to 100% PBT. Before staining, embryos were equilibrated in NTMT buffer (0.1 M Tris–HCl pH 9.5; 50 mM $MgCl_2$; 0.1 M NaCl; 0.1% Tween 20) at room temperature. After being equilibrated in NTMT for 30 min, embryos were stained with 0.34 mg/mL nitroblue tetrazolium and 0.15 mg/mL 5-bromo-4-chloro-3-indolyl phosphate disodium salt for 10–20 min at room temperature. Staining reaction was stopped by adding PBT. To remove endogenous melanin in the pigment cells and allow better visualization of the stained vessels, larvae were immersed in a 5% formamide and 10% hydrogen peroxide in PBT for 20 min. The embryos were kept in glycerol 80% for about 1 week, and then, images were taken at 10× magnification using a stereomicroscope (SMZ 1500 Nikon).

Statistical analysis. All data are shown as mean ± SD. Statistical analysis and graphical representation of the data were performed using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA). Differences between groups were examined using Student's *t*-test or one-way analysis of variance (ANOVA). Two-tailed tests were used for all the hypothesis tests in the present study. Differences were considered statistically significant when $p < 0.05$.

RESULTS

Flavokawain B inhibits endothelial cell proliferation, migration, and tube formation

Because endothelial cell proliferation is necessary for angiogenesis, we investigated the inhibitory effect of FKB on HUVEC endothelial cell growth. A survival curve was obtained with the MTT assay. Flavokawain B concentration of 1.0 and 2.5 μg/mL did not significantly change HUVEC cell viability; FKB 5 μg/mL caused around 20% of cell death, and there was a markedly reduction in cell viability at the concentrations of 7.5 and 10 μg/mL of about 80 and 90%, respectively (data no shown). The other experiments were performed with the less toxic FKB concentrations that did not significantly impair endothelial cell viability.

Wound healing assay was also performed to evaluate endothelial cell migration, an essential step in angiogenesis. The percentage of cell migration was determined compared with the control group. In control group, the wound was completely closed by migrated cells after 24 h. In contrast, FKB caused a clear and significant inhibition of HUVEC migration. At the non-toxic concentration of 2.5 μg/mL, the percentage of migrated cells was just 29.12 ± 13.74% compared with control (Fig. 1).

Tube formation assay was conducted to analyze the effects of FKB on HUVEC capacity to form capillary tube-like structures. Flavokawain B dramatically inhibited HUVEC tube formation in a dose-dependent manner, suggesting that it indeed regulated angiogenesis *in vitro*. The percentage of tube formation in FKB 2.5 and 5.0 μg/mL treated groups were 38.44 ± 3.95 and 25.44 ± 5.69 relative to control, respectively, considering branch point number (Fig. 2).

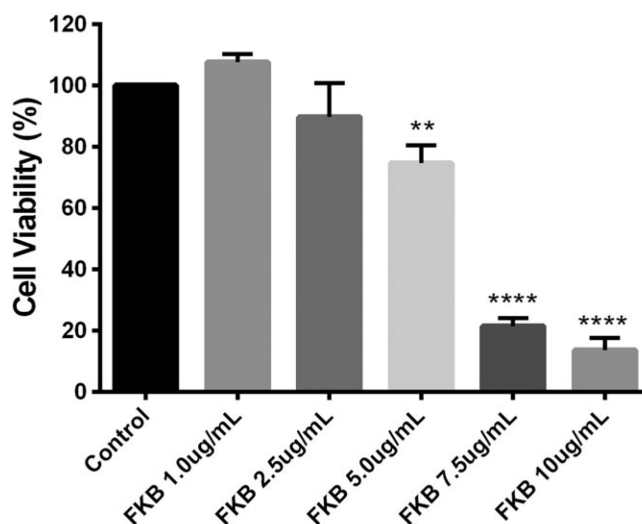

Figure 1. Cell viability of human brain endothelial cells treated with flavokawain B for 24 h. Human brain endothelial cells were plated in 96-well culture plates. After 24 h, the medium was changed to fresh medium and treated with 0.1% DMSO alone or flavokawain B at the indicated doses. After 24 h of treatment, cell viability was measured by MTT assay. Columns are representative of three independent experiments; each condition was performed in triplicates. Data are represented as mean ± SD, $^{**}p < 0.01$; $^{***}p < 0.001$; significance was tested by one-way ANOVA test.

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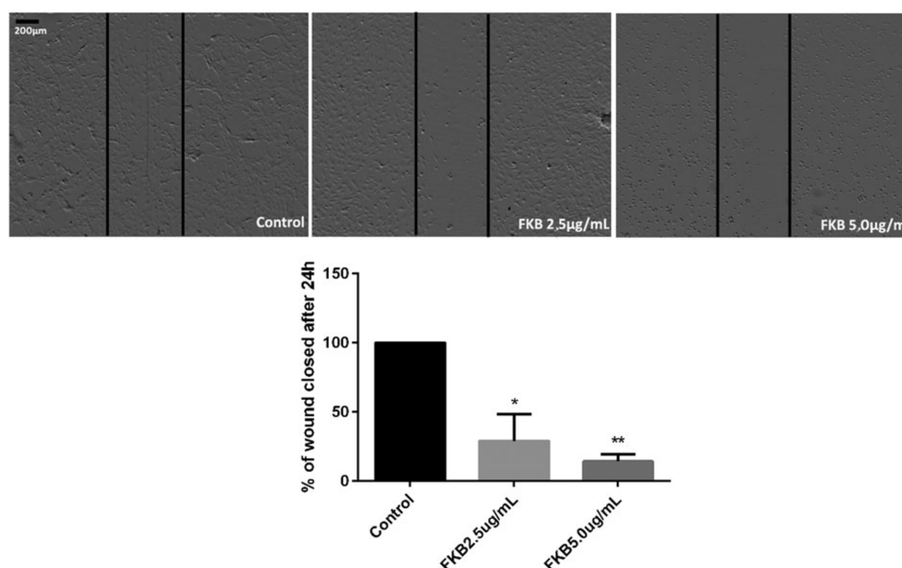

Figure 2. Flavokawain B inhibits endothelial cell migration. Confluent monolayers of human brain endothelial cell on 6-well plates were wounded with a 200-µL pipette and treated with flavokawain B or vehicle for 24 h. (A) Images of the wound closure were captured using a stereomicroscope (SMZ 1500 Nikon) at 5× magnification. The width of the wounds from each photograph was measured using ImageJ software. The initial wound width was considered the same for all conditions and is represented by the black vertical lines. (B) Total monolayer regeneration was expressed as a percentage of mean wound width of each treatment compared with the vehicle control. Two independent experiments were performed in duplicates for each condition. Data are represented as mean ± SD, $*p < 0.05$; $**p < 0.01$. Significance was tested by one-way ANOVA test.

Flavokawain B suppresses angiogenesis *in vivo* in a zebrafish model

Endogenous alkaline phosphatase (AP) staining of zebrafish embryos allowed adequate visualization of subintestinal veins (SIVs) which are normally completely formed at 72 hpf. Prior to the experiments, 48 and 72 hpf untreated larvae were AP stained to assess SIV formation. At 48 hpf, SIVs were absent, and at 72 hpf, SIVs were completely formed with normal characteristic patterns.

A dose–response toxicity curve was also performed, based on the concentration of FKB utilized for cells in the previous experiments (0.5, 1.0, 1.5, 2.5, 5.0, and 10 µg/mL). Noteworthy, zebrafish embryos did not show signs of toxicity up to the concentration of 5.0 µg/mL. At that concentration, embryos died within 24 h of treatment. At the concentration of 10 µg/mL, the formation of SIVs was absent; however, FKB was toxic, and pigment alterations were observed in the embryos (Fig. 3). Treatment with FKB for 24 h in concentrations between 0.5 and 2.5 µg/mL did not alter morphological or behavior pattern, screened visually (data not shown). Therefore, the non-toxic concentrations of 0.5, 1.0, 1.5, and 2.5 µg/mL of FKB were used in further experiments.

The angiogenesis rate was quantified by manual counting of the SIV intersegmental vessels observed on the left and right sides of the embryo. Figure 4 shows the representative results of two replicated experiments.

The exposure of the embryos to FKB for 24 h showed a dramatic reduction of SIV in a dose-dependent manner. At concentrations of 1.0, 1.5, and 2.5 µg/mL of FKB, SIV formation relative to control was, respectively, 74.36 ± 4.47, 57.84 ± 2.90, and 23.96 ± 6.62% on the left side and 72.59 ± 4.92, 55.55 ± 5.73, and 22.96 ± 6.89% on the right side. A complete inhibition of SIV formation was observed in 2.5 µg/mL FKB-treated embryos.

To determine if FKB had any effect on zebrafish embryo development, the embryos' body length was measured according to Parichy *et al.* (2009) before treatment at 48 hpf and after treatment at 72 hpf (data not shown). When we compared the 72 hpf larvae exposed to FKB with 48 hpf larvae, we observed that 72 hpf embryos had a significant greater length (Fig. 5). These findings suggest that the decrease on larva growth seen after FKB treatment at 1.5 and 2.5 µg/mL might be due to vascular impairment and not because of postponed maturation of the embryos, because they continue to grow even in the presence of FKB.

DISCUSSION

Angiogenesis is a complex multistep process, involving cell proliferation, migration, and tube formation. It is a critical step for tumor growth and metastasis. When a tumor lesion grows, hypoxia triggers neovascularization signals to allow cancer cells to spread (Weis and Cheresh, 2011). Numerous growth factors and cytokines are involved in this process, but VEGF is its major key mediator (Hanahan and Weinberg, 2011). The identification of antiangiogenic drugs is a promising new target for antineoplastic therapy, as exemplified by the positive results in the treatment of cancer patients with bevacizumab, a monoclonal anti-VEGF antibody (Gadducci *et al.*, 2015).

Previous reports suggested that FKB could have an antimetastatic action. Lin *et al.* (2012) demonstrated that FKB treatment reduced the expression of metastasis-related proteins such as matrix metalloproteinase-9 and urokinase plasminogen activator on human squamous carcinoma cells. Moreover, Abu *et al.* (2015) using the clonogenic assay and bone

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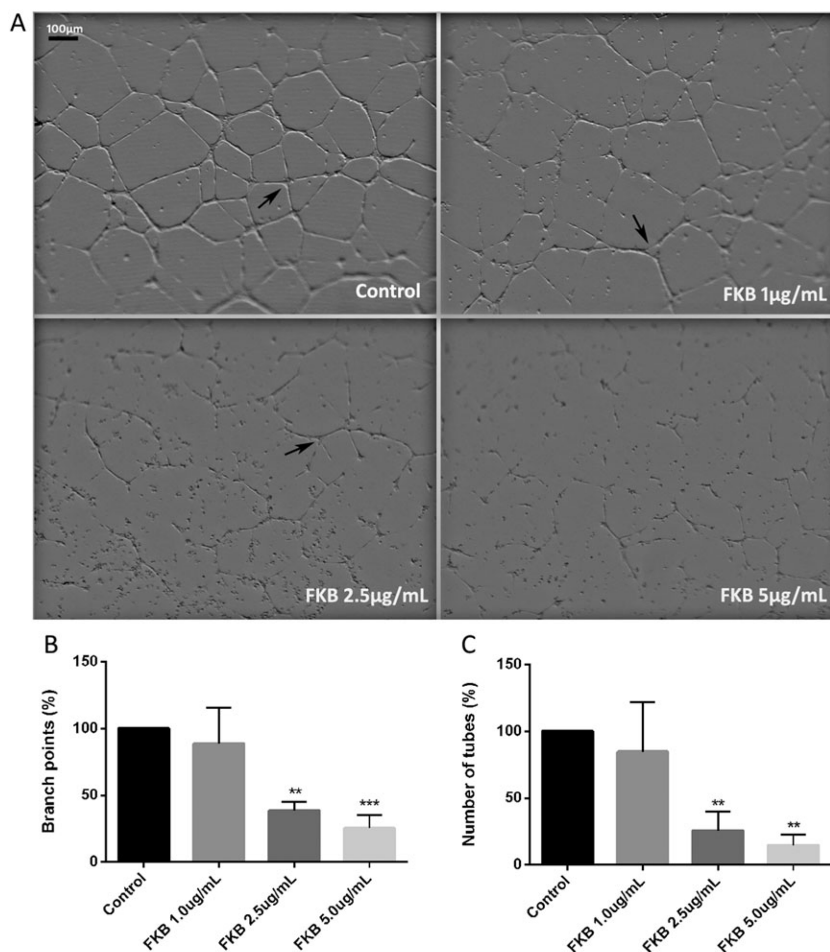

Figure 3. Flavokawain B inhibits capillary tube formation in cultured endothelial cells. (A) Human brain endothelial cells were seeded on Geltrex®-coated 24-well plates and incubated with flavokawain B and vehicle for 18 h. At least six images were randomly taken from each well. This figure is representative of three experiments performed in duplicates. (B, C) Branch points (indicated by the arrows) and tube structures were counted to quantify endothelial tube formation. Tube formation was calculated as the percentage of branch points or tubes compared with vehicle control. Data are represented as mean ± SD, $^{**}p < 0.01$. Significance was tested by one-way ANOVA test.

marrow smearing assay showed that FKB reduced metastatic process in a breast cancer mice xenograft model. Based on tumor proteome profiles, Abu and co-workers (2015) showed that FKB reduced the expression of many pro-angiogenic-related proteins including angiogenin, coagulation factor 3, SDF-1, serpin F1, TSP-2, pentraxin 3, and VEGF. Furthermore, these same researchers (Abu *et al.*, 2016) recently demonstrated that FKB inhibited cell migration and tube formation *in vitro*, reduced sprouting vessels from an *ex vivo* rat aortic ring assay, and regulated many tyrosine kinase angiogenesis-related proteins in breast cancer cells, such as VEGF. In the present study, we confirmed the *in vitro* antiangiogenic effects of FKB and demonstrated for the first time the *in vivo* antiangiogenic actions of FKB. The data reported herein suggest that the antimetastatic action of FKB demonstrated *in vivo* by Abu *et al.* (2015) might possibly be related to the inhibition of angiogenesis.

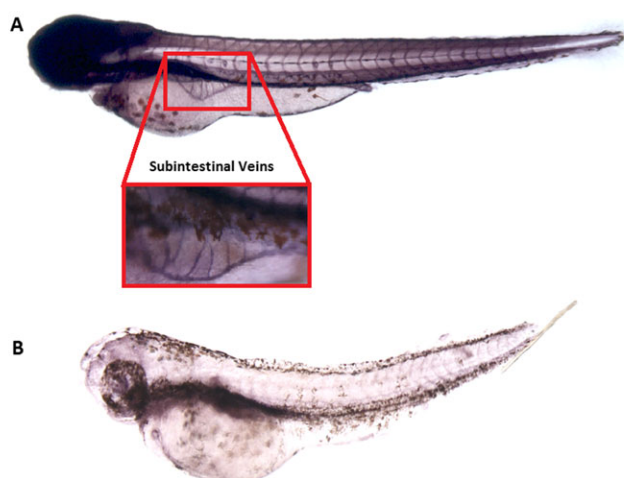

Figure 4. Subintestinal vein (SIV) formation and toxic effect of flavokawain B toward zebrafish. (A) Lateral view scheme at 3× and 10× magnification of normal SIV development in a zebrafish 72 hpf control embryo after alkaline phosphatase staining. The black arrow indicates the intersegmental vessels. (B) Lateral view at 3× magnification of flavokawain B 10 µg/mL 72 hpf treated embryo with completely inhibited SIV formation and significantly altered pigmentation. [Colour figure can be viewed at wileyonlinelibrary.com]

Angiogenesis requires endothelial cells to degrade the basement membrane in order to migrate into the perivascular stroma. Hence, to investigate cell migration ability, the wound healing assay was performed on the premise that endothelial cells in monolayer need to migrate for covering the denude area after scratching (Liang *et al.*, 2007). Our results utilizing HUVEC cells showed a significant inhibition on cell migration at low FKB concentrations, and this result was found by Abu *et al.* (2015) who also performed wound healing assay, but with human breast cancer cells, and described a

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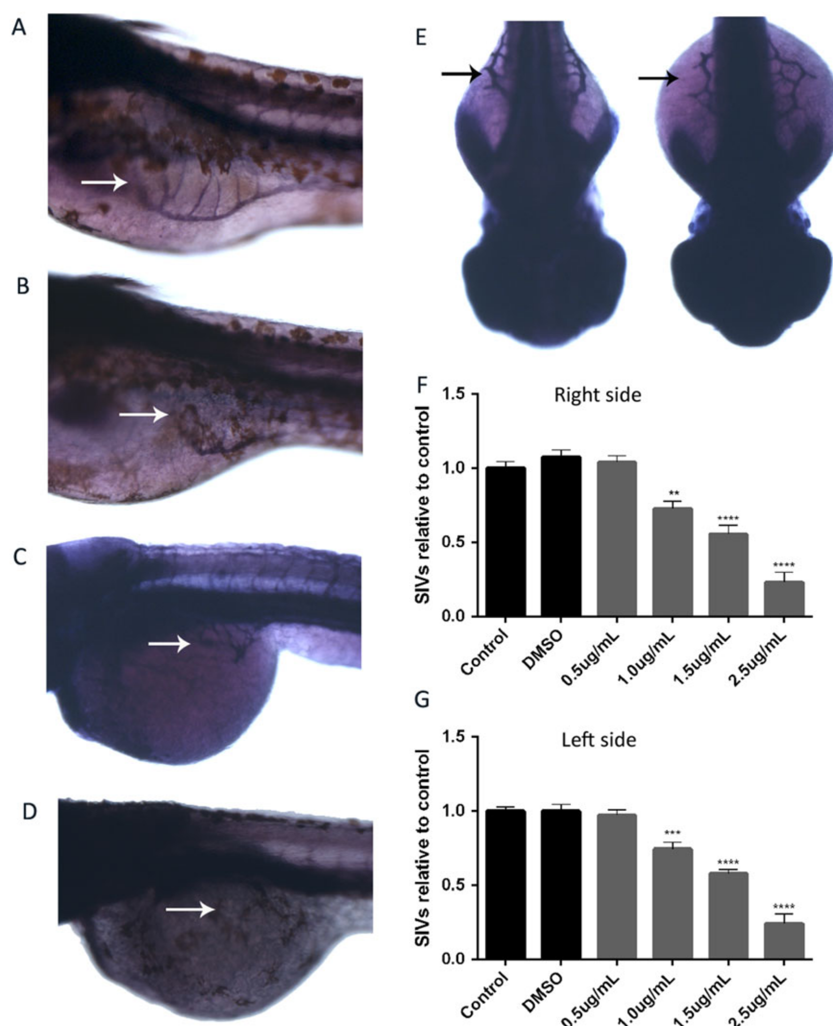

Figure 5. Flavokawain B (FKB) blocks angiogenesis process in zebrafish. Lateral view (A, B, C, D) and dorsal view (E, F) at 10× magnification of alkaline phosphatase-stained embryos at 72 hpf. Subintestinal vein (SIV) locations are indicated by the arrows. Embryos were treated with FKB or vehicle control for 24 h. (A) DMSO (0.1%) had no effect on vessel formation. (B) FKB 1 μg/mL caused reduction of SIV intersegmental vessels. (C) FKB 1.5 μg/mL caused reduction and distortion of SIV. (D) FKB 2.5 μg/mL completely blocked SIV formation. (E) Dorsal view of control larva. (F) Dorsal view of FKB 1.5 μg/mL treated embryo with distorted SIV formation. (G, H) Intersegmental vessels in the right and left sides of the embryos were manual counted. Images are representative of two experiments independently done ($n = 10$ per group). Data are represented as mean ± SD. $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$. Significance was tested by one-way ANOVA test. [Colour figure can be viewed at wileyonlinelibrary.com]

reduction of about 50% on wound closure after treatment with FKB.

The tube formation assay represents later steps on the angiogenic process (Arnaoutova and Kleinman, 2010), and a significant dose-dependent reduction on endothelial cell differentiation into capillary structures under FKB treatment was demonstrated. At the concentration of 2.5 μg/mL, which did not show any toxicity toward endothelial cells, we can see a decrease of around 62% on branch points and 75% on total tubule formation. Recently, similar to our results, Abu *et al.* (2016) demonstrated that FKB inhibited HUVEC tube formation in a dose-dependent manner.

Zebrafish (*D. rerio*) possesses a complex circulatory system, sharing genes and mechanisms of angiogenesis with mammals, and provides many advantages as a vertebrate angiogenesis model system such as permeability to small molecules, easy blood vessel imaging, and rapid development allowing the assays to be performed in a relatively short time (e.g. days) (Nicoli and Presta, 2007; Santoro, 2014). Thereby, to confirm our *in vitro* findings regarding to the FKB antiangiogenic action, we used the zebrafish AP staining angiogenesis model. Zebrafish exposure to increasing concentrations of FKB has demonstrated an impressive dose-dependent reduction on SIVs and intersegmental vessel formation, as well as a disruption of the vascular morphology of SIVs. The concentration of 2.5 μg/mL FKB produced maximal desired effect (complete inhibition of SIV formation) with no toxicity against zebrafish larvae, being considered the ideal concentration according to our results. The measure of larvae length 48 and 72 hpf also have proved that the embryos are growing and developing in the presence of FKB, so vascular impairment might not be due to postponed maturation. Other studies also corroborate that zebrafish model is well suited for studying the properties of potential anticancer drugs as antiangiogenic potential of compounds such as synthetic flavonoid WYCO2-9, polyphllin D, barbigerone, and marine bromophenol has been demonstrated (Chan *et al.*, 2011; Chen *et al.*, 2013; Li *et al.*, 2012b; Qi *et al.*, 2015). The antiangiogenic action

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of FKB *in vivo* utilizing a zebrafish model, however, was first demonstrated in the present study.

Taken together, the results of the current study identify FKB as a potential antimetastatic drug acting as an antiangiogenic agent at non-toxic low concentrations. However, more studies should be done in order to determine the mechanism of action involved in the antiangiogenic properties of FKB in different environments and the possible clinical utility of FKB-derived drugs.

Acknowledgements

This work was partially funded by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq # 405053/2013-4) and Fundação de Amparo à Pesquisa de Minas Gerais (FAPEMIG # APQ-00220-14).

Conflict of Interest

The authors declare no conflicts of interest.

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