



Anti-Angiogenic Activity of Flunarizine by *In Ovo*, *In Vitro*, and *In Vivo* Assays

In Ovo, *In Vitro* ve *In Vivo* Günlüklerinden Flunarizinin Anti-Anjiyojenik Aktivitesi

Chandana KAMILI^{1*}, Ravi Shankar KAKARAPARTHY², Uma Maheshwararao VATTIKUTI³

¹CMR College of Pharmacy, Hyderabad, Telangana, India

²Sri Sai Aditya Institute of Pharmaceutical Sciences and Research, Department of Pharmacology, Surampalem, Andhra Pradesh, India

³TRR College of Pharmacy, Department of Pharmacognosy, Hyderabad, Telangana, India

ABSTRACT

Objectives: The involvement of T-type calcium channels in cell proliferation and the role of sodium channels in cell migration have been extensively studied in angiogenesis. In the present study, flunarizine, a dual sodium/calcium channel blocker; was selected to evaluate its anti-angiogenic potential. This can be therapeutically beneficial in diseases caused by pathologically excessive angiogenesis.

Materials and Methods: The anti-angiogenic activity of ion channel blocker was screened by chick chorioallantoic membrane assay (*in ovo*), rat aortic ring assay, endothelial cell proliferation assay, transwell migration assay, Matrigel cord-like morphogenesis assay (*in vitro*), and sponge implantation method (*in vivo*). The anti-angiogenic activity of the test drug was compared with the standard anti-angiogenic drug bevacizumab and, in addition, the test responses were compared with the angiogenic factor vascular endothelial growth factor at a maximal concentration of 500 pM.

Results: All the groups were compared with the control group using one-way ANOVA, followed by a post hoc test, Dunnett's test, to compare the mean of all the groups with the control mean. In the chick chorioallantoic membrane assay, the number of branching points and angiogenic score were evaluated and significant results were observed at 10^{-5} M and 10^{-4} M. In the aortic ring assay a reduction in the area of sprouts was observed with 5-10 μ M and significant reductions in the weight of sponges, number of blood vessels formed, and hemoglobin content were observed at all three tested concentrations of flunarizine in the sponge implantation method. In the studies on human umbilical vein endothelial cells the test drug (1-100 nM) showed significant inhibition of proliferation and migration and a decrease in the network length of cord-like tubes in a dose-dependent manner.

Conclusion: Flunarizine has significant anti-angiogenic action by inhibiting cell proliferation, migration, and cord-like tube formation, which resulted from blocking of the T-type calcium and sodium channels. Further studies on the structural modifications of flunarizine for repurposing this ion channel modulator will lead to treatment of the diseases due to excessive angiogenesis from the root cause.

Key words: Anti-angiogenesis, chick chorioallantoic membrane assay, rat aortic ring assay, sponge implantation method, human umbilical vein endothelial cells, flunarizine

ÖZ

Amaç: Hücre proliferasyonunda T-tipi kalsiyum kanallarının tutulumu ve hücre göçü içindeki sodyum kanallarının rolü anjiyogenezde kapsamlı olarak incelenmiştir. Bu çalışmada, ikili bir sodyum/kalsiyum kanal blokörü olan flunarizin; anti-anjiyojenik potansiyelini değerlendirmek için seçildi. Bu, patolojik olarak aşırı anjiyogenezin neden olduğu hastalıklarda terapötik olarak yararlı olabilir.

Gereç ve Yöntemler: İyon kanalı blokörünün anti-anjiyojenik aktivitesi, civciv korioallantoik membran deneyi (*in ovo*), sıçan aortik halka deneyi, endotel hücre proliferasyon analizi, transwell migrasyon deneyi, Matrigel kord benzeri morfojeniz deneyi (*in vitro*) ve sünger implantasyonu ile tarandı. Yöntem (*in vivo*). Test ilacının anti-anjiyojenik aktivitesi standart anti-anjiyojenik ilaç olan bevacizumab ile karşılaştırıldı ve buna ek olarak, test yanıtları, 500 mM'lik bir maksimum konsantrasyonda anjiyojenik faktör vasküler endotel büyüme faktörü ile karşılaştırıldı.

Bulgular: Tüm gruplar kontrol grubu ile tek yönlü ANOVA kullanılarak ve post hoc testi ile karşılaştırıldı, Dunnett testi ile tüm grupların ortalamaları kontrol ortalaması ile karşılaştırıldı. Chick chorioallantoic membran testinde dallanma noktaları ve anjiyojenik skorlar değerlendirildi ve 10^{-5} M ve 10^{-4} M'de anlamlı sonuçlar gözlemlendi. Aort halkası analizinde, filiz alanında 5-10 μ M azalma gözlemlendi ve sünger implantasyon yönteminde test edilen

*Correspondence: E-mail: chandanakamili@gmail.com, Phone: +9581140044 ORCID-ID: orcid.org/0000-0001-7437-5137

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üç flunarizin konsantrasyonunda sünger ağırlığında anlamlı bir azalma, kan damarlarının sayısı ve hemoglobin içeriği gözlemlendi. İnsan umbilikal ven endotel hücreleri üzerindeki çalışmalarda, test ilacı (1-100 nM), proliferasyonun önemli ölçüde engellenmesini, doza ve kordon benzeri tüplerin ağırlığının doza bağımlı bir şekilde azaldığını gösterdi.

Sonuç: Flunarizin, T-tipi kalsiyum ve sodyum kanallarını bloke ederek hücre proliferasyonu, migrasyon ve kord benzeri tüp oluşumunu inhibe ederek önemli anti-angiogenik etkiye sahiptir. Flunarizinin bu iyon kanal modülatörünü yeniden üretmek için yapısal modifikasyonları ile ilgili daha ileri çalışmalar, kök nedeninden aşırı angiogenezden kaynaklanan hastalıkları tedavi edebilecektir.

Anahtar kelimeler: Anti-angiogenez, civciv korioallantoik membran deneyi, sıçan aortik halka deneyi, sünger implantasyon yöntemi, insan umbilikal ven endotel hücreleri, flunarizin

INTRODUCTION

The term angiogenesis or neovascularization means the formation of new blood vessels from existing vasculature. Blood capillaries supply oxygen: more capillaries can increase tissue oxygen conduction and hence improve energy production; fewer capillaries results in ischemia, hypoxia, and even anoxia in the tissues.¹ Thus, angiogenesis is important for both normal physiology and in pathological conditions.²⁻⁴

Endothelial cell (EC) structure and functional integrity are important in the maintenance of the vessel wall and circulatory functions, and most of these endothelial functions are regulated by ion channels.^{5,6} The role of ion channels in the pathophysiology of diseases has been extensively discussed.⁷⁻⁹ Despite their prime role in several diseases, there are very few drugs targeting specifically the ion channels as therapeutic inhibitors for the treatment of diseases caused by excessive angiogenesis. Such clinically approved ion channel modulators with well-known safety profiles may be reframed in the treatment of many diseases, saving significant time and money.

In the present study, flunarizine (FLN), a dual Na⁺/Ca²⁺ channel blocker, was selected in order to screen its anti-angiogenic potential. FLN, diphenylpiperazine analogue, acts on both Na⁺ and Ca²⁺ channels. The test drug is a T-type calcium channel blocker that has been studied as extensively unregulated in most tumor types. The anti-angiogenic potential of the test drug FLN was tested at three different doses in different methods by an *in ovo* method, the chorioallantoic membrane (CAM) assay; an *in vitro* method, the rat aortic ring assay, EC proliferation assay, transwell migration assay, and Matrigel cord-like morphogenesis assay; and an *in vivo* method, the sponge implantation assay.

MATERIALS AND METHODS

Chemicals

FLN, ketamine, xylazine, and tramadol were purchased from N.R. CHEM, India. Matrigel was purchased from Becton Dickinson India Pvt. Ltd, Gurgaon, India. Gel foam and Dulbecco's modified Eagle's medium were supplied by Life Technologies (India) Pvt. Ltd. Well plates were purchased from Hi Media Laboratories Pvt. Ltd, India. Bevacizumab, vascular endothelial growth factor (VEGF), penicillin, streptomycin, amphotericin, gentamycin, heparin, bovine serum albumin, gelatin, and M199 were obtained from Sigma-Aldrich (India). All the chemical and reagents used in the study were of AR grade.

Equipment

All the equipment of CMR College of Pharmacy was used. The BOD incubator, Dona analytical balance, digital pH meter, Evershine 697 homogenizer, laminar airflow unit, and Labomed trinocular microscope were purchased from MH Enterprises, Hyderabad, India.

Experimental animals

Forty-two healthy male Wistar Albino rats weighing 150-200 g were selected for the *in vivo* methods and for the *in vitro* assay. The animals were obtained from Teena Labs Pvt Ltd, Hyderabad, Telangana state, India. Fertilized leghorn chicken eggs were selected for the *in ovo* assay. All the procedures were performed according to the CPCSEA under a protocol approved by the Institutional Animal Ethics Committee (IAEC) (project license numbers CPCSEA/1657/IAEC/CMRCP/PhD-15/42).

Chick CAM assay

This is an *in ovo* angiogenesis assay for identification and quantification of anti-angiogenic agents. Eggs were collected from the hatchery on day 0 and checked for any damage. They were randomly grouped into control, VEGF, bevacizumab, and three test concentrations groups, each containing six eggs. The eggs were disinfected using ethanol and then incubated in constant humidity at a constant temperature of 37°C. On day 3, a hole was drilled at the narrow end and 2-3 mL of albumin was withdrawn using an 18-gauge hypodermic needle. The hole was sealed with sterile tape and the egg returned to incubation. On day 7, a window was opened in the shell and a sterile gel foam or sponge (3 mm×3 mm×1 mm) piece was placed on top of the membrane. The control group was given saline; the test and standard groups were impregnated with their respective doses. The eggs were incubated until day 14. On day 14, CAM tissues directly beneath the sponge were removed from control and treated CAM samples. The tissues were placed in 10% formalin, stained with hematoxylin-eosin, and then examined under a trinocular microscope. The vessel branching points in the square area were counted and analyzed for each treatment group. The resulting angiogenesis index is the mean ± standard error of mean (SEM) of the new branching points in each set of samples. An angiogenesis score of 1-4 was given to each egg based on the number of branching points. If the number of branching points is ≥35, the angiogenesis score is 4. If branches are between 25 and 34, the score is 3 and for 15-24, the score is 2. If the points are <15, the score is 1. The concentrations (10⁻⁶ M, 10⁻⁵ M, and 10⁻⁴ M) were selected based on the results

of previous studies. Previously, the concentration of 10^{-5} M resulted in submaximal efficacy of the drug. The classical molarity formula $M=m/V$ was used to find the required drug amount to provide 10^{-4} M concentration. First the concentration of 10^{-4} M was prepared, and then the other concentrations were prepared from the earlier one by serial dilutions.¹⁰⁻¹³

Rat aortic ring assay

This method is a widely used *in vitro* assay for the evaluation of both angiogenic and anti-angiogenic compounds. One healthy male Wistar albino rat from each group was selected. It was sacrificed by cervical dislocation, the thoracic cavity was cut open, and the visceral organs were separated. The thoracic aorta was identified and isolated by cutting both ends. Immediately it was transferred to cold phosphate buffer solution (PBS) supplied with aeration. The fibro-adipose tissue was isolated, and the proximal and distal 2 mm segments of the aorta were cut away. The aorta was cut into 1 mm ring sections and washed with PBS. These rings were placed in 24-well plates with 150 μ L of Matrigel. The rings were overloaded with Matrigel and were left to polymerize for 1-2 h at 37°C. Then they were exposed to hypoxia for 2 h. This hypoxic condition stimulates formation of sprouts. The rings were reoxygenated and then incubated for 7 days. The area of sprouts was quantified by the measurement of length and abundance of microvessel-like extensions from the explants.¹⁴⁻¹⁶

Sponge implantation method

In the sponge implantation method, the surgical procedure was done by a single investigator to increase the reproducibility of the process. The sponges were implanted subcutaneously (s.c.). All the surgical instruments used in the study were sterilized by autoclaving at 121°C for 25 min. Sponges of 2 cm diameter and 8 mm thickness were prepared and sterilized by soaking in 70% ethanol for 3 h and then boiling at 70°C for 30 min. This *in vivo* method was carried out by anesthetizing the rats using a cocktail of ketamine (80 mg/kg) and xylazine (5 mg/kg). Then the skin was cut open with a surgical blade. A sterile sponge was implanted s.c. by creating an air pocket, which was sutured back by 5/0 silk sutures. Two such sterile sponges were implanted on the mid-dorsal line of the body. When the animals recovered from anesthesia, they were allowed to have normal diet and water. The animals after the surgery were caged individually. Tramadol at a dose of 0.9 mg/kg was injected intramuscularly (i.m.) twice a day in the morning and evening; gentamycin at a dose of 2 mg/kg was injected i.m. in the morning. The analgesic and antibiotic drugs were given for the 3 days postoperatively. Standard and test drugs were applied to the sponges of their respective groups for 13 days after the implantation. On day 14 the animals were sacrificed and the sponges were dissected out. The sponges were weighed and the amount of hemoglobin and the number of vessels per sponge were quantified. The drug concentrations were expressed as mg/kg. The therapeutic

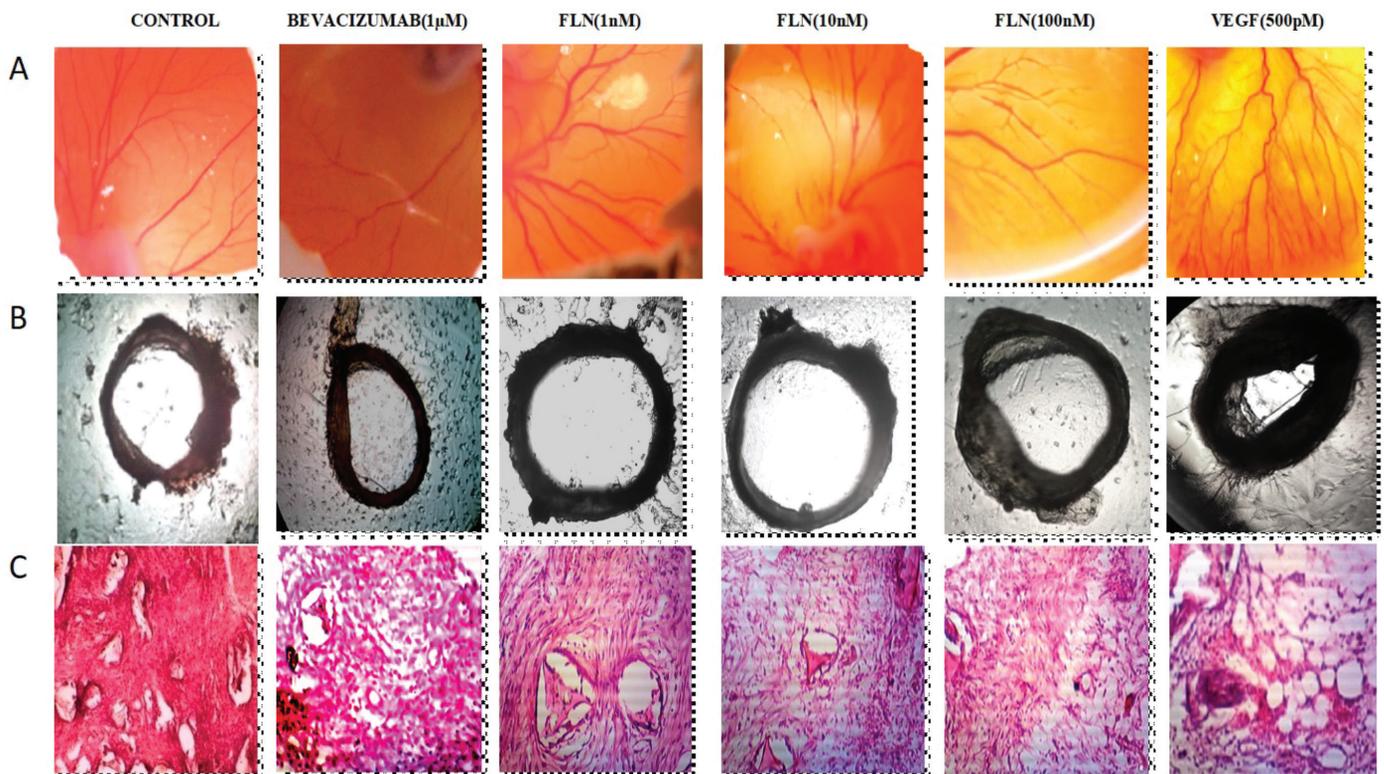


Figure 1. FLN inhibited angiogenesis *in ovo*, *in vitro* and *in vivo*. (a) In the CAM assay numbers of branching points from each major vessel were counted. (b) Photographs of explants in the aortic ring assay show the micro vessel like extensions. (c) The histological sections of the sponges show circular spaces amidst the fibroblast region representing the newly formed blood vessels

FLN: Flunarizine, CAM: Chorioallantoic membrane, VEGF: Vascular endothelial growth factor

human range of each drug in the subcutaneous route was obtained from the literature and three animal doses were calculated by the formula;

$$\text{Dose of the animal} = \frac{\text{Surface area of animal}}{\text{Surface area of human}} \times \text{Human dose}$$

Here,

Rat surface area=0.025 m²

Human surface area=1.6 m²

First the highest concentration of each drug was prepared and then the other concentrations were prepared from the earlier one by serial dilutions.¹⁷⁻¹⁹

Procedure for determining hemoglobin content: The sponges after removal from the rats were soaked in double distilled water and homogenized completely over an ice platform for 5 min. The homogenate was centrifuged at 10,000 rpm in a cooling centrifuge for 5 min and the supernatant liquid obtained was used to estimate hemoglobin content (g/dL).

Procedure for determining number of blood vessels formed per sponge: The sponges were bisected and fixed in saline at 4°C for 1 h. The sponges were immersed in 75% ethanol for 30 min and finally kept in 10% formalin. Then paraffin sections (10 µm) were prepared and stained with hematoxylin-eosin. The prepared slides were then observed under a trinocular microscope. The circular spaces amidst the fibroblast regions present were counted as they represent vessels formed in the sponges.

Endothelial cell culture

Human umbilical vein EC (HUVECs) were grown on gelatinized dishes in M199 supplemented with 15% fetal calf serum, 50 U/mL penicillin, 50 mg/mL streptomycin, 50 mg/mL gentamycin, 2.5 mg/mL amphotericin B, 5 U/mL heparin, and 150-200 mg/mL EC growth supplement. Cells were used between passages 1 and 3. Each experiment shown is derived from three independent repeats, each time using different pools (isolates) and/or passages of cells.²⁰

Endothelial cell proliferation assay

The HUVECs were seeded in 24-well plates at a density of 6000 cells/cm² and incubated overnight in Dulbecco's modified Eagle's medium. The cells were exposed to different concentrations of FLN, bevacizumab, VEGF, or vehicle and allowed to proliferate for 48 h. At the end of this incubation time, the cells were trypsinized, and their number was determined using a Neubauer hemocytometer.²¹

Transwell migration assay

The capacity of EC to migrate through a pore-bearing membrane was assessed using 6.5-mm diameter transwell chambers with polycarbonate membrane inserts (8 mm pore size). Control or ECs were serum starved overnight. The cells were trypsinized and 1×10⁵ cells were added to each transwell in 100 µL of serum-free medium containing 0.2% bovine serum albumin in the control and in the presence of different concentrations of FLN

(1 nM, 10 nM, and 100 nM), bevacizumab, and VEGF. The cells were allowed to migrate for 4 h, after which the nonmigrated cells at the top of the transwell filter were removed with a cotton swab. The migrated cells on the bottom side of the filter were fixed in Carson's solution for 30 min at room temperature and then were stained with toluidine blue. The migrated cells were scored and averaged from eight random fields per transwell as previously described elsewhere.²²

Matrigel cord-like morphogenesis assay

The formation of cord-like structures by ECs (HUVECs) was assessed in growth factor-reduced Matrigel. The cell groups were plated in 96-well plates precoated with 45 µL of Matrigel per well. After 8 h of incubation, cord-like structure formation was quantified. One image per well was analyzed and used for the statistical analysis.^{21,23}

Statistical analysis

The statistical analysis was carried out using GraphPad Prism 5. The results were presented as mean ± SEM. The differences between the groups were compared by one-way ANOVA followed by post hoc Dunnett's test. In the statistical analysis all the groups were compared with the control group. The results were considered statistically significant at p values <0.05. In all the groups of the CAM assay, rat aortic ring assay, and sponge implantation method, n=6 (Figures 2, 3).

RESULTS

In the chick CAM assay (*in ovo*), the dual ion channel blocker exhibited marked anti-angiogenic activity at all the tested concentrations. In the rat aortic ring assay (*in vitro*), a reduction in the area of sprouts was observed. A noticeable reduction in the weight of sponges and inhibition in the growth of new blood vessels, and a very sharp reduction in hemoglobin content were observed, which was better than the standard drug response (*in vivo*).

Results of the chick CAM assay

In the assay, on day 14 the CAM tissues directly beneath the sponge were removed from control and treated CAM samples. The vessel branching points in the square area equal to the region of each sponge were counted (Figure 1). An angiogenesis score of 1-4 was given to each egg based on the number of branching points. Effects of the drug treatment on the two evaluation parameters, that is the number of branching points and angiogenic score, are presented in Figures 2a and 2b. The results of three doses of FLN, the standard anti-angiogenic drug bevacizumab, and VEGF were statistically compared with the control results. Significant results were observed with all three test doses selected: 10⁻⁶ M, 10⁻⁵ M, and 10⁻⁴ M.

Results of the rat aortic ring assay

Photographs showing the abundance of microvessel-like extensions from the explants are given in Figure 1. A significant reduction in the area of sprouts was observed with 5 µM and 10 µM of the drug (Figure 2c).

Results of the sponge implantation method

In the sponge implantation method, the evaluation parameters are weight of the sponge, number of vessels per sponge, hemoglobin content, and the histopathology of the sponge. A moderate reduction in weight of sponges and a prompt inhibition in the growth of new blood vessels and hemoglobin content were observed at 1.0 mg/kg and 10 mg/kg of the drug (Figures 2d-2f). Sections of the sponges were observed under a trinocular microscope. The circular spaces amidst the fibroblast regions were counted as they represent new vessels formed in the sponges. In the VEGF group large numbers of vessels were identified, in the standard very few microvessels were formed due to the strong anti-angiogenic action, and the test drug caused a dose-dependent decrease in the number of blood vessels per sponge (Figure 1).

Results of the endothelial cell proliferation assay and transwell Matrigel and cord-like morphogenesis assay

Na⁺ and Ca²⁺ channels are important for cell proliferation, migration, and cord-like network formation. To further test the link between channel inhibition and anti-angiogenesis, EC-based assays triggering proliferation and mobilization were performed. In the cell proliferation assay VEGF resulted in elevated proliferation (increase of 49%), whereas bevacizumab and the three doses of FLN showed significant inhibition of

proliferation (inhibition by 50%, 79.3%, 69.7%, and 58.3%, respectively). In addition, test doses of FLN inhibited cell motility through transwell compartments comparable to the vehicle control, respectively. To further assess the anti-angiogenic property of the test drug, a cord-like tube formation assay was performed. Significant inhibition was observed with the test doses (69.3%, 59.7%, and 48.3%, respectively) (Figures 3 and 4).

DISCUSSION

FLN is a dual sodium/calcium blocker.²⁴ It acts on sodium and Ca²⁺ channels, blocking influx of Ca²⁺ ions. Ca²⁺ ions have long been known to be secondary messengers in various cellular signaling resulting in angiogenesis. The fact that deprivation of extracellular Ca²⁺ leads to cell growth arrest in G1/S indicates that Ca²⁺ is required for cell cycle progression.²⁵⁻²⁷

One of the Ca²⁺ regulation mechanisms is binding of calcium to calmodulin protein. Intracellular Ca²⁺ binds with calmodulin II, in turn activates calcium-calmodulin-dependent protein kinases, and regulates pro-survival transcriptional proteins.

In the chick CAM assay, the ion channel blocker exhibited potent anti-angiogenic activity at all three test concentrations of 10⁻⁶ M, 10⁻⁵ M, and 10⁻⁴ M. A reduction in the area of sprouts in the

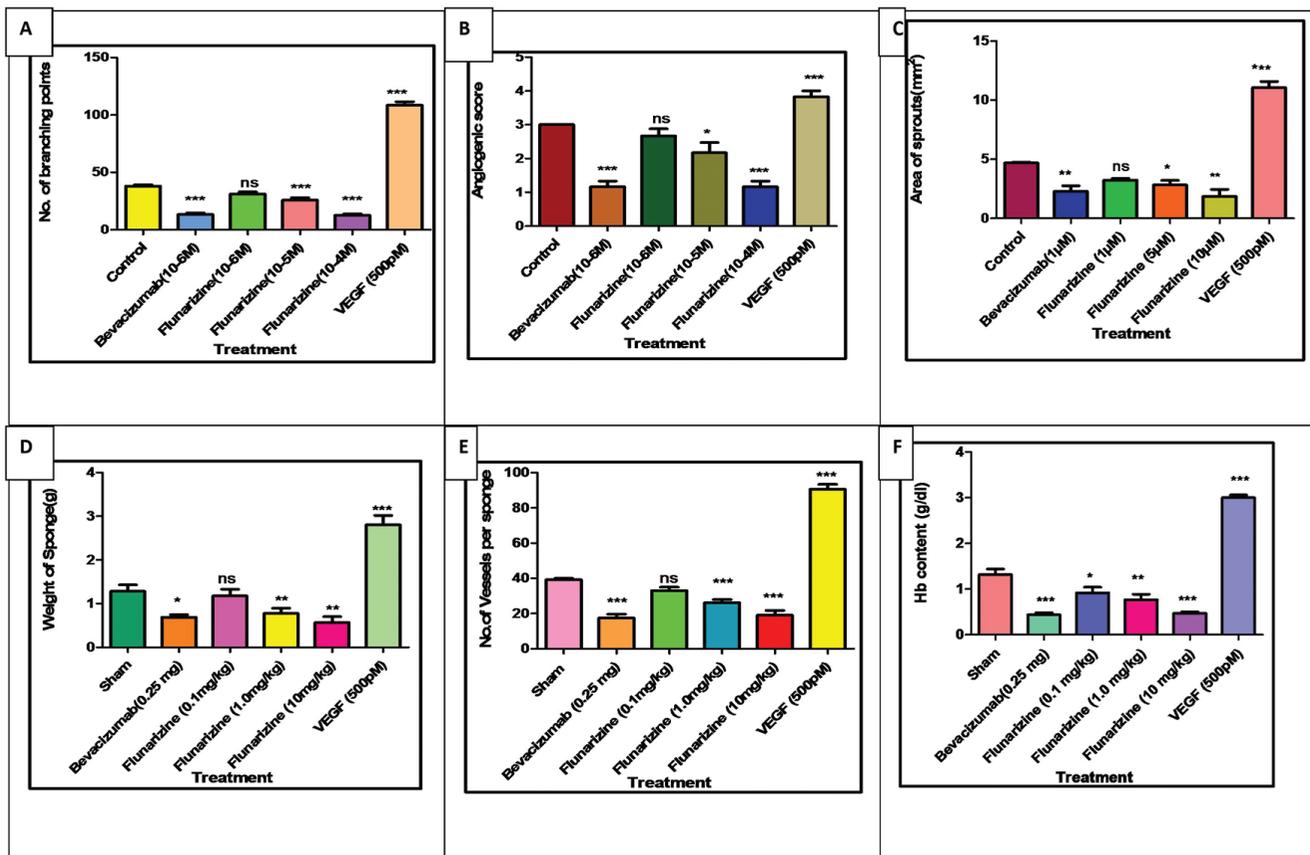


Figure 2. Graphs showing the effect of FLN on (A) number of branching points in CAM assay (B) angiogenic score in CAM assay (C) area of sprouts in aortic ring assay (D) weight of sponge in sponge implantation method (E) number of vessels per sponge (F) Hemoglobin content per sponge in sponge implantation method. All the results were expressed as mean \pm standard error of mean; n=6. ***p<0.001, **p<0.01, *p<0.05 vs control

ns: Non-significance, FLN: Flunarizine, CAM: Chorioallantoic membrane, VEGF: Vascular endothelial growth factor

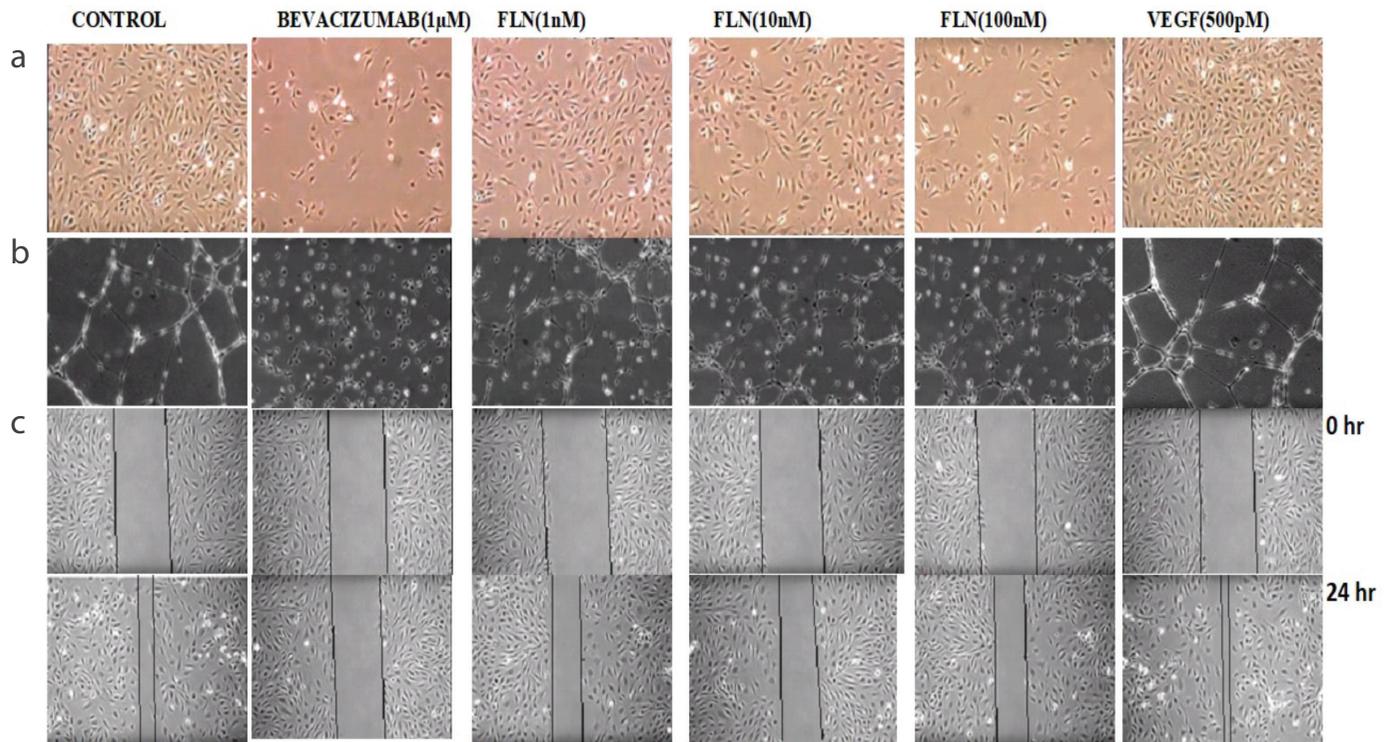


Figure 3. Modulation of endothelial cell responses to FLN, Bevacizumab and VEGF. (a) Cell proliferation was determined by cell counting with a hemocytometer. (b) Representative images of tube formation after being treated with FLN for 2 h following VEGF stimulation. (c) Quantitative data of scratch wound-healing inhibition in HUVECs treated with FLN for 24 h under VEGF stimulation. Cord-like network morphogenesis in vitro is affected by K_{ATP} modulation
FLN: Flunarizine, VEGF: Vascular endothelial growth factor, HUVECs: Human umbilical vein endothelial cells

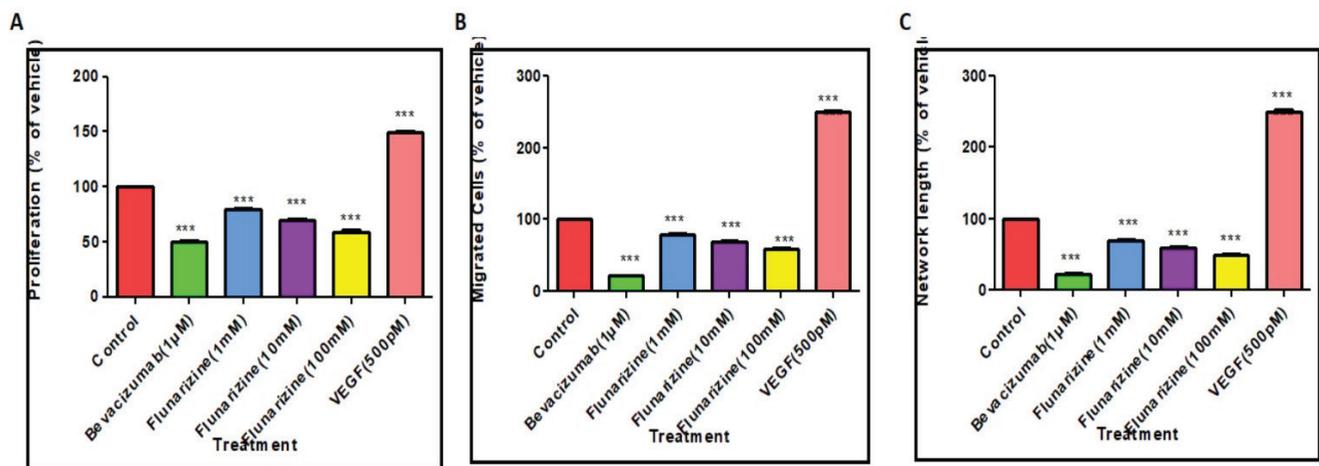


Figure 4. Graphs showing the effect of FLN on (A) Proliferation assay (B) Migrated cells (C) Network length. All the results were expressed as Mean \pm SEM; n=6, ***p<0.001, **p<0.05 vs control, ns-Non significance

rat aortic ring assay was observed with 5 μ M and 10 μ M of FLN. A significant reduction in the weight of sponges, number of blood vessels formed, and hemoglobin content were observed at 1 mg/kg and 10 mg/kg. The results revealed that FLN has significant inhibition of sprout formation and branching in a dose-dependent manner. Modulation of EC response to FLN was significant at all the test doses of 1 nM, 10 nM, and 100 nM on the EC proliferation, migration, and tube formation assays. FLN, being a strong blocker of Ca^{2+} ion influx, gave significant anti-angiogenic results. This drug serves as good chemical template

that can be structurally modified for more site-specific actions for anti-angiogenic therapy.

CONCLUSIONS

The anti-angiogenic property of an ion channel modulator, FLN, was thoroughly evaluated by *in ovo*, *in vitro*, and *in vivo* studies. The test drug showed very potent anti-angiogenic activity, even better than that of the standard drug bevacizumab at a concentration range of 5-10 μ M. The very strong anti-

angiogenic potential is due to effective blockage of Ca^{2+} influx. $\text{Na}^+/\text{Ca}^{2+}$ dual blocker inhibits the Ca^{2+} influx with double the strength. Calcium dynamics play a crucial role in the critical steps of angiogenesis like cell migration, proliferation, and even cell death. Molecular modifications of the ion channel modulator used in the present study will evolve EC targeted chemical moieties. Furthermore, such endothelial targeted chemical moieties can be formulated suitably to achieve a site specific action that minimizes side effects.

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Conflict of Interest: No conflict of interest was declared by the authors.

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