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journal homepage: www.elsevier.com/locate/apjtbOriginal article <http://dx.doi.org/10.1016/j.apjtb.2017.05.005>Anti-proliferative and anti-angiogenic activities of ion-channel modulators: *In-ovo*, *in-vitro* and *in-vivo* studyChandana Kamili^{1*}, Ravi Shankar Kakataparth², Uma Maheshwararao Vattikutti³, Vijay Chidrawar⁴, Sindhuri Ammineni⁵¹Department of Pharmacology, CMR College of Pharmacy, JNTUH, 501401, India²Department of Pharmacology, Sri Sai Aditya Institute of Pharmaceutical Sciences and Research, Surampalem, India³Department of Pharmacognosy, TRR College of Pharmacy, Hyderabad, India⁴Department of Pharmacology and Toxicology, Northern Border University, Saudi Arabia⁵Department of Pharmacology, CMR College of Pharmacy, Hyderabad, 501401, India

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ABSTRACT

Objective: Angiogenesis is the development of new blood vessels. The ion channels on endothelium play a vital action in cell proliferation and so in the related angiogenesis. We aimed to investigate the anti-angiogenic effects of Mefloquine (Cl⁻ channel blocker) and 4-Aminopyridine (K⁺ channel blocker).

Methods: The anti-angiogenic activities of Mefloquine and 4-Aminopyridine (4-AP) were investigated by *in-vivo* (sponge implantation method), *in-vitro* (aortic ring assay) and *in-ovo* (CAM, Chick Chorioallantoic membrane) methods. The standard anti-angiogenic drug used was Bevacizumab.

Results: In the CAM assay, both the ion channel blockers exhibited noticeable anti-angiogenic activity at the concentrations of 10⁻⁵ M and 10⁻⁴ M where they significantly exhibited ant proliferative activity by inhibiting the new blood vessel formation. For the further confirmation anti-angiogenic activity was evaluated in vitro and in vivo. In Rat aortic ring assay reduction in the area of sprouts were observed with 40 μM of 4-AP and 7 μM of Mefloquine. A significant reduction in weight of sponges, number of blood vessels formed and hemoglobin content were observed at 4.2 mg/kg of 4-AP and 20 mg/kg and 30 mg/kg of Mefloquine.

Conclusions: These scientific findings indicate the use of Mefloquine and 4-Aminopyridine in pathological situations involving excessive angiogenesis. Negative regulation of cell volume, cell migration and proliferation of blood vessels may be the underlying molecular mechanisms.

1. Introduction

Angiogenesis is the generation of blood vessels from the existing vasculature [1]. It is also called as neovascularization. Angiogenesis has two faces, it plays vital role in maintenance of both health and diseases. Disturbance of this balance leads

to over proliferation of blood vessels that contributes to pathogenesis. Vessel growth could benefit in case of baldness, neurodegenerative ills, heart attack and could be helpful to bypass the clots in blood vessels (occlusion) as well as in tissue repair [2]. Angiogenesis is responsible for the pathological progress and metamorphosis of tumor [2]. The newly developed blood capillaries supplement cancer growth by supplying nutrients, oxygen and by taking away waste products. Angiogenesis also supports Metastasis [3]. So inhibition of angiogenesis and arresting the development of blood vessels may be a good approach for the treatment of cancer [4].

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Angiogenesis is firmly regulated through a balance of pro-angiogenic and anti-angiogenic factors. Angiogenesis is stimulated by various pro-angiogenic factors like basic Fibroblast Growth Factor (bFGF), Vascular Endothelial Growth Factor (VEGF), Platelet Derived Growth Factor (PDGF), Angiopoietins, Transforming Growth Factor (TGF), Tumor necrotic Factor- α , TNF- α , Interleukin-8, DLL4, *etc* [5]. Many natural antiangiogenic molecules including thrombospondin-1 (TSP-1) and angiostatin/endostatin act in opposition to the proangiogenic factors [6].

Ion-channels are pore forming transmembrane proteins which plays an important role in many physiological process, Like Cell volume regulation, muscle contraction, neural depolarization, hormonal release *etc*. Recently the occurrence of ion channels on the endothelial cell surface has questioned their functional role in angiogenesis. Various ion channels like Na⁺, Ca²⁺, K⁺, volume regulated anion channels (VRAC) are identified on the endothelial cell surface [7]. Surprisingly very little research has been conducted to pin point the exact function of these specific ion channels in neovascularization. The role of Volume regulated anion channels in cell proliferation and migration was also recently found [8]. Based upon these observations we have selected Mefloquine-Hcl, a volume regulated Chloride channel blocker and 4-Aminopyridine (4-AP), voltage gated potassium channel blocker to evaluate the anti-angiogenic effect at three different doses against three different models *i.e.* Sponge implantation method (in vivo), Rat aortic ring assay (*in vitro*) and Chick Chorioallantoic membrane (CAM) assay (*in ovo*).

2. Materials and methods

2.1. Materials

2.1.1. Chemical and lab wares

Pure Mefloquine-Hcl was purchased from Sigma Aldrich Pvt. Ltd., 4-AP was purchased from NR chemical India, Ketamine, Xylazine, Tramadol and Gentamycin injections were purchased from the local market, Matrigel was purchased from Becton Dickinson India Pvt. Ltd. (Gurgaon, India). Dulbecco's modified Eagle's medium (DMEM) was supplied by Life Technologies (India) Pvt. Ltd. 24 well plates were purchased from Hi Media Laboratories Pvt. Ltd, India. All the chemicals used in the research are of AR grade.

2.1.2. Equipments

Incubator, high resolution digital camera (Cyber-Shot 6.0, Sony, Tokyo, Japan), trinocular microscope, surgical catguts (5/0) suture and needles micropipettes, oven *etc.* were used.

2.2. Methods

2.2.1. Sponge implantation method

Experimental Animals and maintenance: A total of 42 Wistar albino rats weighing in between 150 and 200 g were purchased from Teena labs Pvt. Ltd, Hyderabad, India. The animals were maintained at a controlled temperature (22–25 °C, 45% humidity) on a 12:12-h dark–light cycle. CPCSEA guidelines were strictly followed and the studies were approved by the Institutional animal ethical committee (IAEC), (Ref: CPCSEA/

1657/IAEC/CMRCP/PhD-14/30) CMR College of Pharmacy, Hyderabad, India.

Experimental Procedure: Rats were anaesthetized by a cocktail of Ketamine (80 mg/kg) and Xylazine (5 mg/kg) and sponges were implanted s.c. Sponges of 2 cm diameter and 8 mm thickness were sterilized in 70% ethanol for 3 h and then boiling them at 70° C for 30 min. All the surgical instruments were sterilized by autoclaving at 1210 °C for 25 min. Then the skin was cut open by surgical blade and sterilized sponge was inserted subcutaneously by forming an air pocket and sutured back by 5/0 silk sutures. Two such sponges were implanted on the mid-dorsal line of the body. After the animal recovered from anesthesia, they were kept separately one in a cage and were given regular diet and water. To reduce the surgical pain, Tramadol at a dose of 0.9 mg/kg was injected i.m twice a day. Gentamicin at a dose of 2 mg/kg was injected i.m for three days after surgery. In order to avoid further pain tramadol at the dose of 50 mg/kg p.o. was administered for next 1 week [9].

Animals were divided into 8 groups by keeping 6 animals in each. Standard and test drugs were given to the respective groups for successive 13 days.

Dose of drug to be given per day and per sponge were calculated and administered. Doses were selected by taking 1/10th of LD₅₀ value and the sub max and super max were selected. On 14th day the animals were sacrificed by cervical dislocation and the sponges were dissected out carefully without giving much traction on the sponges. All the dissections were done by the same person to avoid individual variations. All the sponges were stained with hematoxylin and eosin, then observed under trinocular hi-definition microscope and photographs were captured. Wet weights of sponges were calculated on digital balance of 0.01 mg sensitivity. Immediately after weighing hemoglobin content was calculated in all the sponges as per the method prescribed by Tahergorabi and Khazaei, 2012 [2].

Determination of Hemoglobin content: The sponges were soaked in double distilled water and homogenized 5 min in a cooling centrifuge and the liquid was separated, and then centrifuged at 10000 rpm for 5 min. The supernatant liquid was placed in the cell count machine and the hemoglobin content was estimated as g/dl.

Determination of Number of blood vessels formed per sponge: The sponges were cut into two halves, soaked in normal saline at 4 °C for 1 h and then put in 75% ethanol for 30 min and finally fixed in 90% ethanol. Paraffin sections (10 pm) were prepared and stained with Hematoxylin and Eosin. The slides were observed under trinocular microscope and the circular spaces amidst the fibroblast growth regions represent the blood vessels formed in the sponges.

2.2.2. Rat aortic ring assay method

Rat Aortic Ring Assay method is widely used *in vitro* method for the evaluation of angiogenic and antiangiogenic drugs [10]. A healthy male Wistar rat weighing in between 180 and 200 g sacrificed by cervical dislocation, thoracic cavity was opened and the visceral organs were separated. Thoracic aorta was identified and isolated by cutting both the ends. Immediately it was transferred to cold PBS supplied with aeration. Fibro adipose tissue was removed. Aorta was cut into 1 mm ring sections and washed with DMEM (Dulbecco's modified Eagle's medium). These rings were put into the 24

well plates with 150 μ l of matrigel. Rings were overloaded with matrigel and were allowed to polymerize for 1–2 h at 37 °C and then exposed to hypoxic conditions for 2 h. This hypoxic condition stimulates the formation of sprouts from the rings. They were reoxygenated for 7 days and the abundance of blood vessels was quantified. Doses were selected based on the IC₅₀ values [11,12]. Length of the branches formed was measured under microscope at 400 \times magnification using stage micrometer.

2.2.3. Chick Chorioallantoic membrane assay

Forty two fertilized chicken eggs were collected from a local hatchery on the day '0' and were checked for any damage. They were grouped randomly into 7 groups each containing 6 eggs. The eggs were superficially sterilized using 70% ethanol and then incubated under constant humidity at 37°C by placing them in a horizontal position. On the 3rd day a hole was made at the narrow end to withdraw 3 ml of albumin. The hole was sealed with surgical tape and the eggs were put back for incubation. On the 7th day of incubation a small window was cut opened on the shell and sterile gel foam (3 mm \times 3 mm \times 1 mm) piece was placed on the membrane. The standard and test doses were placed on the sterile gel foam. Then eggs were incubated undisturbed till day 14. On the 14th day the CAM tissues were removed out [13]. Tissues were placed in 10% formalin and stained with hematoxylin and eosin then examined under trinocular microscope. The number of vessel branch points in a unit square region were counted and analyzed. Angiogenesis score 1–4 was given to each egg based on the number of branching points. If the no. of branching points is \geq 35, the angiogenesis score is 4. If the branching points are 25–34, score is 3 and for 15–24, the score is 2. If the branching points are < 15, the score is 1.

2.3. Statistical analysis

The statistical analysis was carried out by using graph pad Prism 5. The differences between the groups were compared by one way ANOVA followed by post hoc Dunnett's test. All of the data obtained from the experimental groups have been compared to the control group. Values are significant at * $p < 0.05$. Comparison of the test groups were done with the control group. Values are expressed as mean \pm SEM; $n = 6$. *** ($p < 0.001$) compared to disease control group, ** ($p < 0.01$) compared to disease control group, * ($p < 0.05$) compared to disease control group.

3. Results

3.1. Sponge implantation method

In sponge implantation method, weight of the sponges, number of blood vessels formed and hemoglobin content were estimated. At 20, 30 mg/kg of Mefloquine and 2.1, 4.2 mg/kg 4-AP significantly less sponge weights, less number of blood vessels per sponge and hemoglobin (Hb) content per sponge were observed when compared with the control. Results are quite comparable with the Standard antiangiogenic drug, a VEGF inhibitor, Bevacizumab (0.25 mg) (Figures 1–4).

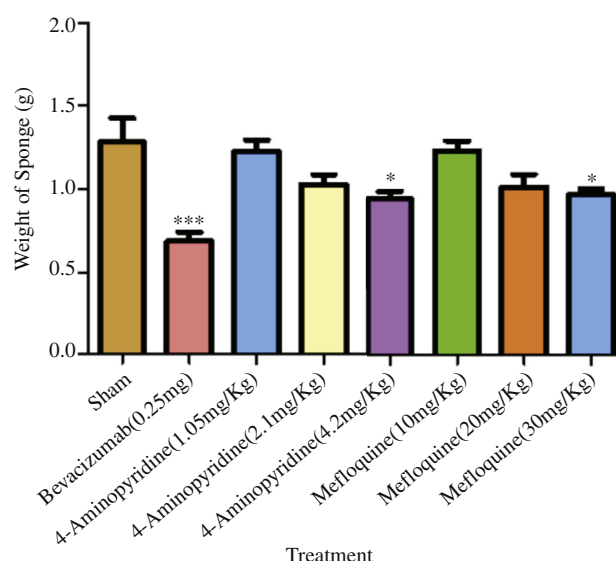


Figure 1. Graph showing the effect of treatment groups on wet weight of Sponge.

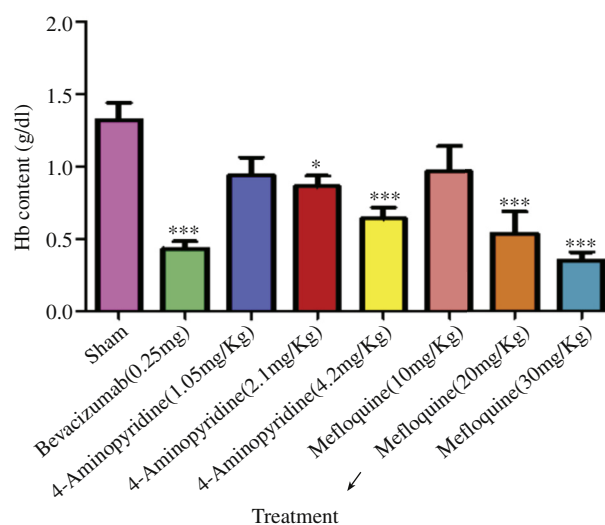


Figure 2. Graph showing the effect of treatment groups on hemoglobin content per sponge.

3.2. Aortic ring assay

A well noticeable reduction in the area of sprouts were observed by the treatment with 5 μ M/ml and 7 μ M/ml of Mefloquine, 4-AP at the doses of 20, 40 μ M/ml in aortic rings. Our results suggest that Mefloquine and 4-AP have good inhibitory effects on angiogenesis, with the most effective inhibition being obtained with the above mentioned doses (Figures 5 and 6).

3.3. Chick Chorioallantoic membrane (CAM) assay

The numbers of branching points were counted by observing under the microscope. Our results show significant reduction in the branching points at 10^{-5} M, 10^{-4} M of Mefloquine and 4-AP treated groups (Figures 7–9).

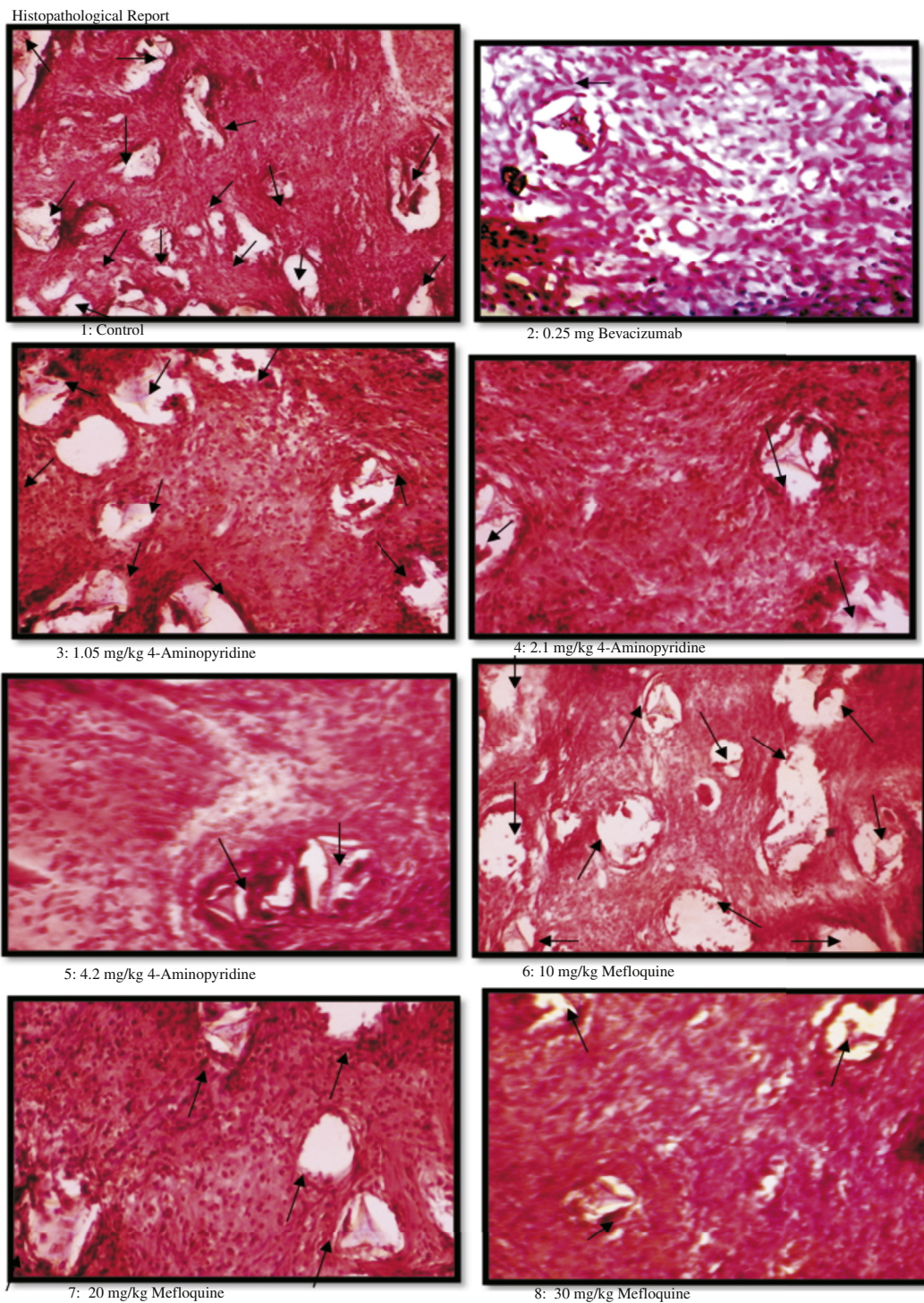


Figure 3. Representative photographic results showing new capillaries formed in the control and test groups. Image from Trinocular Microscope at magnification of 400 \times . Arrows indicate the newly formed microvessels.

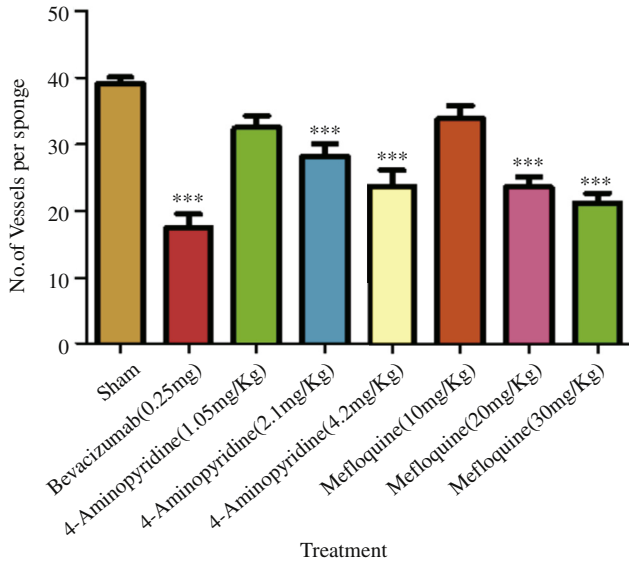


Figure 4. Graph showing the effect of treatment groups on number of blood vessels formed per sponge.

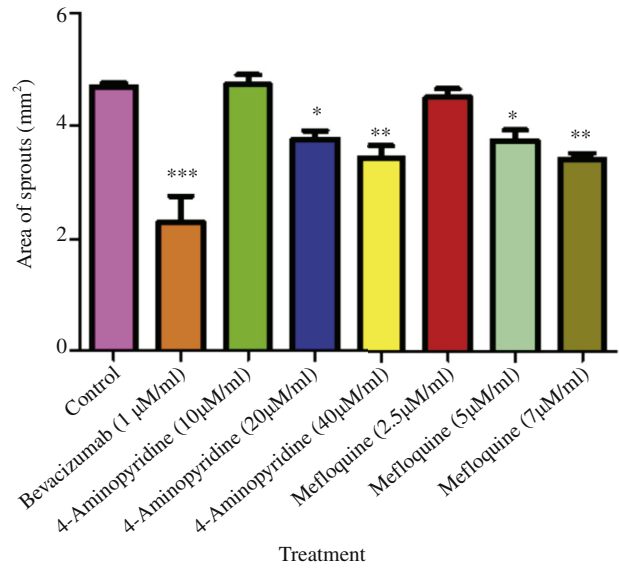


Figure 6. Graph showing the effect of treatment groups on the area of sprouts formed in aortic ring assay of angiogenesis.

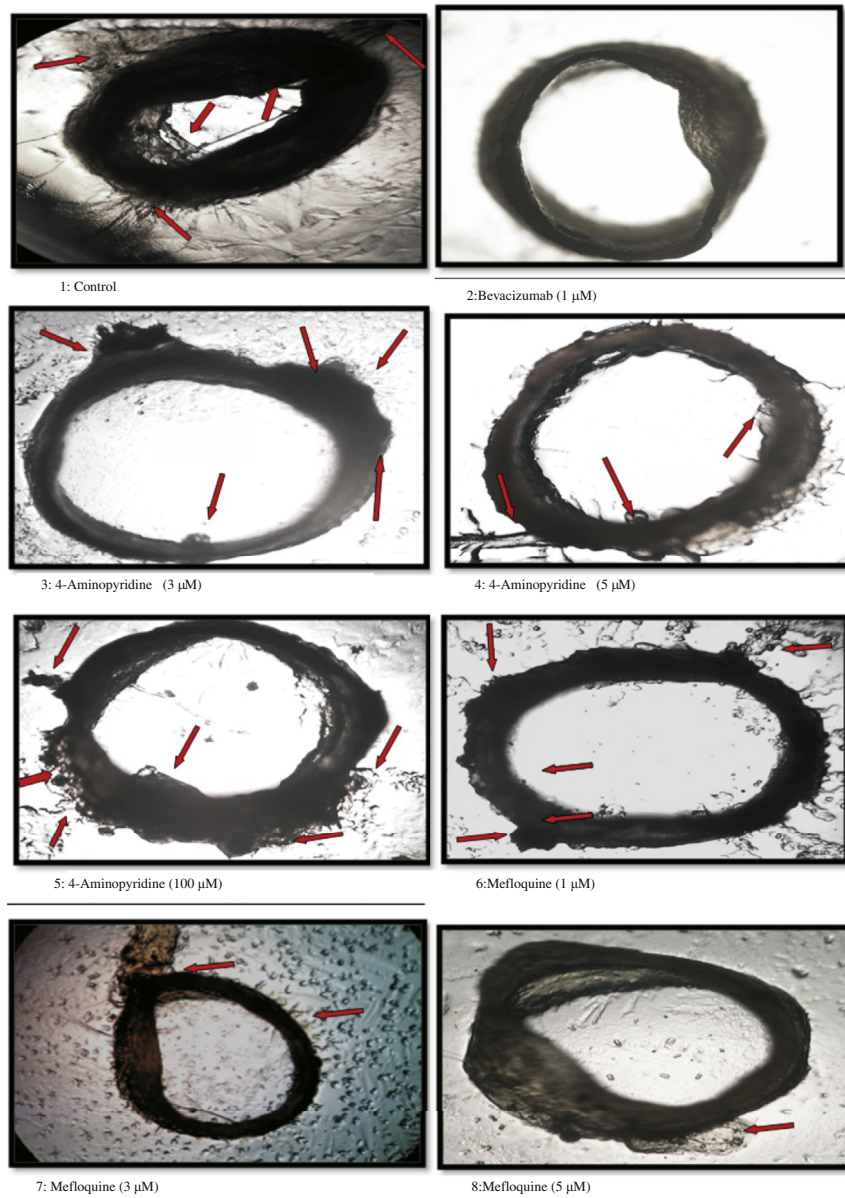


Figure 5. Effect of test drugs on sprouting of aortic rings in aortic ring assay of angiogenesis.



Figure 7. Effect of test drugs on number of branches in CAM assay angiogenesis.

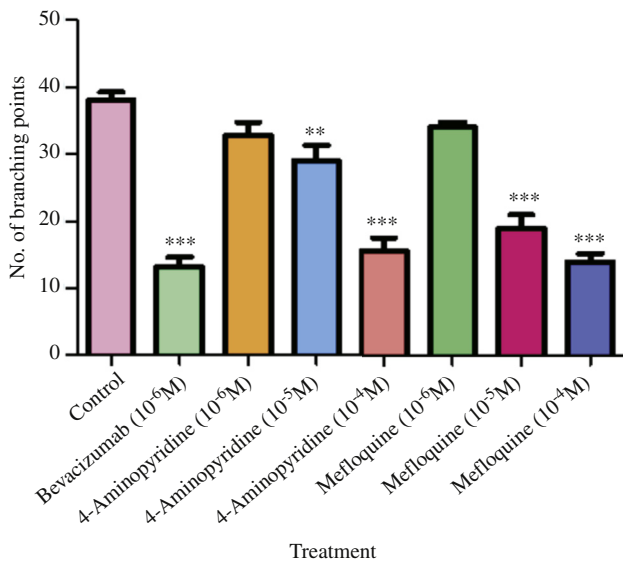


Figure 8. Graph showing the effect of treatment groups on the no. of branching points in CAM assay of angiogenesis.

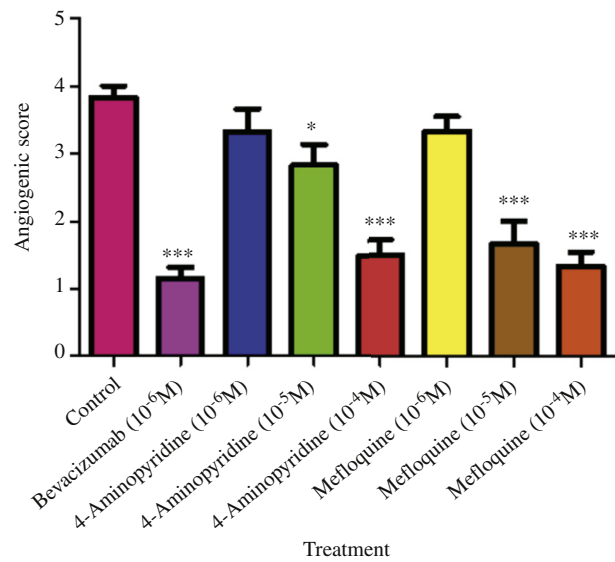


Figure 9. Graph representing the effect of treatment groups on the angiogenesis score in CAM assay.

4. Discussion

Angiogenesis has an important role in the pathological progress and metamorphosis of tumor [3]. So inhibition of angiogenesis may be a good approach for the treatment of cancer [4,10].

Ion-channels are transmembrane minute opening proteins that regulates the flow of ions such as K^+ , Na^+ , Ca^{2+} , Cl^- etc. across the cell membrane and govern many physiological functions like skeletal muscle contraction, nerve conduction, cell volume regulation etc. Defects in the ion-channels can results in many diseases like myriad disorder such as heart arrhythmia, cystic fibrosis and many [14]. Data suggest that ion-channels plays critical role in cell volume regulation and are over expressed in tumor formation, but surprisingly very few studies has been conducted to find their role in the angiogenesis and hence tumor formation.

More preciously, Volume-regulated anion channels (VRACs), Na^+ Channels, Ca^{++} Channels and K^+ channels participate in the regulation of the membrane potential and cell-volume regulation [15]. Cell proliferation needs an increase of cell volume. Change in cell volume requires the involvement of ion transporters across the membrane. Cl^- channels mediate osmolyte flux and thus influence cell volume. K^+ channels sensitize the cell for a programmed cell death mechanism [16].

On these scientific basis we would like to suggest the hypothesis and identified structurally related drug *i.e.* Mefloquine, a selective volume regulated chloride channel blocker and 4-AP, a voltage gated potassium channel blocker and examined the effect of these test drugs against three distinct models of angiogenesis *i.e.* *in-vivo*, *in-ovo* and *in-vitro* models.

The treatment with Mefloquine and 4-AP reduced the wet weight of the sponges signifying the anti-proliferative activity of the compounds at all three doses. Estimation of hemoglobin content of the sponges is one of the sensitive markers for angiogenesis [17]. There was dose dependent decrease in the sponge Hb content that was observed and compared with the negative control group by both the test drugs. Angiogenesis after implantation of the sponge was estimated and augmented micro vascularisation in test compound treated animals was analyzed. Histopathology of the control and test compound treated implants demonstrated noticeable divergences. As depicted in Figure 5, in the control sponges, there is high degree of neovascularization compared to test groups.

The chorioallantoic membrane (CAM) of chick embryos is a suitable *in ovo* model for the study of extensive vascular network using various angiogenic and anti-angiogenic stimuli [18,19]. Factors to be tested are delicately applied on the surface of CAM on 7–10 day-old embryos. Results can be scheduled after an incubation of at least 48 h. Vascular endothelial growth factor (VEGF) receptor activation may have potential contributions to induce angiogenesis acting via its receptor tyrosine kinases (RTK). Earlier literature shows that the Mefloquine antagonizes K^+ channel through receptor tyrosine kinase and hence inhibit angiogenesis [20].

Rat Aortic assay is another major model implemented in this research. The assay is based on the generation of neovascular capillary network from the endothelial cells when the aortic ring is incubated in matrigel under hypoxic conditions. The neovascular capillary network generated from the rings is very similar to those formed during *in-vivo* angiogenesis. In this assay the major steps of angiogenesis like endothelial cell invasion, cell migration, proliferation, differentiation, and new vessel

formation can be reported [21]. Hypoxia created during the assay induces the free radical formation provoking angiogenesis. Mefloquine has antioxidant and free radical scavenging property which rescues the endothelium from oxidative stress [22]. In the blood vessels, production of free radicals is majorly responsible for the oxidative damage of endothelium resulting in its dysfunction which is the basic root cause for diseased condition [23]. As superoxide anion scavenger and antioxidant, Mefloquine would resume the normal function of the endothelium. There was a drastic change in the area of sprout formation by the Mefloquine treated group in dose dependent manner. Mefloquine is a Volume regulated Cl^- Channel blocker. During cell division there is increase in the cell cytoplasmic content, cell volume and Cl^- has important role in cell proliferation, migration and proteolysis. Cl^- ions maintains osmosis to increase in cytoplasmic cell volume. VRAC maintain membrane potential that is broadly recognized to regulate the cell cycle primarily by regulating the driving force for Ca^{++} which in turn regulates secondary messenger of cell proliferation [24]. Cl^- also has promising role in the cell migration which is a major step in angiogenesis. Chloride is needed for the expression of Ras, a small G-protein molecule that regulates a large number of intracellular signaling pathways affecting cell proliferation and motility. So these could be the probable mechanism of Mefloquine in the prevention of proliferation and angiogenesis.

Anti-angiogenesis by 4-Aminopyridine may be due to blocking the Voltage gated K^+ channel. K^+ channels have prime role in the progression of cell cycle. G1 phase progression, G1/S and G2/M transition in several cell types require Ca^{2+} signaling. The relationship K^+ channels, Ca^{2+} influx and membrane potential, are reported to be related in a way that the membrane hyperpolarization generated by the K^+ channel opening potentiates the Ca^{2+} influx, resulting in the activation of Ca^{2+} -dependent transcriptional factors leading to the expression of cell cycle regulatory proteins, such as cyclins, CDKs leading to the cell proliferation [25,26].

So, voltage-gated K^+ channel blockade could inhibit Ca^{2+} influx and thus cell proliferation.

Based on the scientific finds in the research, We report here that VRAC blocker, Mefloquine and volume regulated K^+ channel blocker, 4-AP inhibit new vessel formation in all the three models. These results suggest that Mefloquine and 4-AP may be useful in the therapy of angiogenic-dependent tumor growth and other angiogenic-dependent diseases.

Conflict of interest statement

The authors declare no conflicts of interest.

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