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Azithromycin as Potent Inhibitor of Cell Migration in Tumor Cell Line

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Abstract: Continuous obstacles behind the discovery of novel drugs for cancer therapy have necessitated the development of alternative strategy of drug repurposing—the development of old drugs for new therapeutic purposes. With an improved understanding of the hallmarks of cancer, this strategy offers a cost-effective process for the treatment of human neoplastic disease, thereby facilitating rapid clinical translation. In this regard, macrolide antibiotics (MAs), which include a wide spectrum of activities against Gram-positive bacteria, have also been proposed as anticancer drugs for multiple tumor types. Over the past few years, significant progress has been achieved in anticancer therapy, but development of resistance and unavoidable side effects have weakened these attainments. Considering this severe condition, a number of drugs with novel antitumor mechanisms are under investigations including antimicrobials that have been shown to possess anti-inflammatory, immunomodulatory, and cytotoxic effects. In this regard, both conventional and novel antimicrobials are being studied to explore their anticancer potential along with underlying mechanisms that may render them as effective anticancer drugs in the future. Hence, in the latest study, we tested the role of a macrolide antibiotic drug, Azithromycin (AZM) alone, in combination with standard chemotherapeutic agent Sorafenib (Sorafenib/AZM) and its gold conjugated nanoparticles (AuAZM) as an anti angiogenic agent in hepatoma cell line hepG2 through wound healing assay. The migratory potential of HepG2 cells after being exposed to different treatments (AZM, Sorafenib, Sorafenib/AZM, and Au-AZM at IC50 concentrations) was observed at 0, 6, 24, 48, and 72 hours. The results of our study showed that AZM exhibited highly significant reduction in wound healing with p-value (< 0.001) up till 72 hours, while Sorafenib, Sorafenib/AZM, and Au-AZM inhibited wound healing up to 48 hours (p-value < 0.001). The current study revealed a comparatively higher antiangiogenic potential of AZM in cancer cells, thereby suggesting its clinical application for cancer treatment.

Keywords: azithromycin, wound healing potential, tumor cell line, cell migration.

阿奇霉素作为肿瘤细胞系细胞迁移的有效抑制剂

摘要：发现用于癌症治疗的新药背后的持续障碍使得有必要开发药物再利用的替代策略——开发用于新治疗目的的旧药物。随着对癌症特征的深入了解，该策略为治疗人类肿瘤疾病提供了一种具有成本效益的过程，从而促进了快速的临床转化。在这方面，大环内酯类抗生素（硕士），包括对革兰氏阳性菌的广谱活性，也被提议作为多种肿瘤类型的抗癌药物。

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在过去的几年中，抗癌治疗取得了重大进展，但耐药性的发展和不可避免的副作用削弱了这些成就。考虑到这种严重的情况，许多具有新型抗肿瘤机制的药物正在研究中，包括已被证明具有抗炎、免疫调节和细胞毒性作用的抗菌剂。在这方面，正在研究传统和新型抗菌剂，以探索它们的抗癌潜力以及可能使它们在未来成为有效抗癌药物的潜在机制。因此，在最新研究中，我们测试了大环内酯类抗生素药物阿奇霉素(AZM)单独与标准化疗药物索拉非尼(索拉非尼/AZM) 及其金缀合纳米颗粒(金氮芥)在肝癌中作为抗血管生成剂的作用细胞系肝细胞 G2 通过伤口愈合测定。在 0、6、24、48 和 72 小时观察到乙肝 G2 细胞在暴露于不同处理（AZM、索拉非尼、索拉非尼/AZM 和金-AZM 的我知道了 50 浓度）后的迁移潜力。我们的研究结果表明，AZM 在 72 小时内以 p 值 (< 0.001) 显着降低伤口愈合，而索拉非尼、索拉非尼/AZM 和金-AZM 抑制伤口愈合长达 48 小时（p 值< 0.001)。目前的研究揭示了 AZM 在癌细胞中具有相对较高的抗血管生成潜力，从而表明其在癌症治疗中的临床应用。

关键词：阿奇霉素，伤口愈合潜力，肿瘤细胞系，细胞迁移。

1. Introduction

Due to the high costs, lengthy development period, and potential for drug resistance, the drug development process is associated with a heavy financial burden [1-5]. As a consequence, the new utilization of older medicinal agents, which is commonly referred to as therapeutic switching or drug re-profiling, is the subject of significant interest among researchers seeking to assess the effectiveness of these older agents against other diseases than the known clinical indications [6, 7]. It is also evident from the literature that around 90% of drugs approved by the United States Food and Drug Administration (FDA) hold off-set targets that could be used in relation to further illnesses [8, 9]. Moreover, secondary to the enhanced understanding of the molecular and genetic basis of disease, it is now accepted that several diseases share a molecular mechanism of pathogenesis, which means that the idea of using the same drug for the treatment of more than one disease is now believable.

Along with other diseases, the use of drug re-profiling in the field of oncology has attracted the interest of researchers over the past three decades. Unfortunately, the recurrence of resistance against anticancer agents and the limited number of targeted therapies available have shortened the interval of clinical response [10-12]. As cancer is the second most common cause of mortality worldwide, there has been a rapid increase in attempts to apply drug re-profiling based on knowledge of the multiple potential pathways involved in cancer growth and metastasis [13]. Interestingly, more than 2000 drugs have now been approved globally, each of which has, on average, more than six relevant targets that could represent beneficial off-targets leading to quick, novel, safe, and cheap therapy, which would ultimately have a substantial positive impact in the field of oncology [14, 15].

To date, there have been numerous fruitful drug re-profiling attempts in relation to many diseases, including cancers [16]. For instance, the use of thalidomide in relation to multiple myeloma, retinoic acid in relation to acute promyelocytic leukemia, zoledronic acid in relation to solid cancer with bone metastases, and non-steroidal anti-inflammatory drugs (NSAIDs) in relation to desmoid tumors have already been incorporated into the guidelines of the European Society for Medical Oncology (ESMO). In addition, when given certain co-treatments such as chemotherapeutics along with antimicrobials to eradicate infections, which usually occur in the case of chemotherapy-treated cancer secondary to the development of immunosuppression, cancer patients have shown a higher survival rate when compared with patients receiving chemotherapeutic drugs alone [17, 18].

Extensive studies have been conducted in recent years to identify the effects of certain non-chemotherapeutic drugs on increasing the efficacy of existing chemotherapeutic agents or decreasing resistance to them. In this regard, macrolides (i.e., clarithromycin, azithromycin, and erythromycin) have shown promising anticancer effects in a significant number of in vitro and in vivo studies and clinical trials. For instance, in 1997, the anticancer effect of clarithromycin was demonstrated in lung cancer patients. It has also been tested in patients with Hodgkin's lymphoma. Nevertheless, although clarithromycin is most commonly used as a mono-treatment to reduce tumor cell survival in cases of lung cancer, it is more effective when used in combination with chemotherapeutic agents [19, 20].

Similarly, in addition to their antimicrobial effects, other macrolides are known to exert antiapoptotic and antiproliferative effects through different mechanisms, such as inducing apoptosis, targeting mitochondria in cancer stem cells, and inhibiting P-glycoprotein in order to counter resistance against anticancer agents. Recently, due to its good safety profile and distinct pharmacokinetic properties when compared with the other macrolides, azithromycin has been investigated in terms of its potential against tumorigenesis after exhibiting anticancer effects in relation to cervical, hematological, gastric, and hepatocellular carcinoma. A number of studies have examined its antitumor effects, for example, in combination with imatinib in chronic myeloid leukemia (CML) cells [21], with vincristine in cervical and gastric cancer cells [22], and with tumor necrosis factor-associated apoptosis-inducing ligand (TRAIL) in colon cancer cells [23].

Another recent development pertaining to cancer therapy that has gained the attention of researchers is the use of nanotechnology-based therapeutic approaches that focus on increasing the therapeutic efficiency of drugs. Among the various nanomaterials that have been used in biomedical applications, metal and metal oxide-based nanoparticles offer unique physicochemical properties that allow for their use in combination with conventional antimicrobials, thereby increasing their potential as antimicrobial, anticancer, and immunomodulatory agents [24].

In light of the above, the present study investigated the role of azithromycin alone, azithromycin in combination with the standard chemotherapeutic agent sorafenib, and azithromycin's gold-conjugated nanoparticles as antiangiogenic agents in relation to hepatoma cell line HepG2.

2. Materials and Methods

2.1. Cell Line Revival and Sub-Culturing of HepG2

The cryovial containing the cell line was stored in liquid nitrogen at -196°C. It was sterilized with 70% ethanol, thawed in warm water at 37°C, and then transferred to a falcon tube containing complete Dulbecco's modified Eagle's medium (DMEM). Following centrifugation, it was seeded in a tissue culture-treated T75 flask containing 9 ml of high glucose DMEM and then incubated at 37°C in a 5% CO_2 incubator for the purposes of cell proliferation.

The sub-culturing of the cells was performed once the cells had become 80% confluent when observed under an inverted microscope. Following medium aspiration, the cells were washed with 2 ml of 1X phosphate buffer saline (PBS). After the removal of the PBS, 4 ml of trypsin was added to detach the cells from the surface for 4–5 minutes. The trypsin reaction was terminated with the addition of complete DMEM and the suspended cells were transferred to a 15 ml falcon tube, which was then centrifuged at 1000 rpm for 5 min. Once the cell pellet was formed, the supernatant was discarded. Next, the pellet was dissolved in 1 ml of fresh media by means of reverse pipetting. It was then aspirated and seeded in two separate T75 tissue culture flasks each containing 9 ml of fresh complete DMEM.

2.2. Procedure for the Optimization and Preparation of the Serially Diluted Drugs

The drugs used in this study (i.e., sorafenib and azithromycin [AZM]) were dissolved in 100% dimethyl sulfoxide (DMSO) in order to prepare the stock solution from which the serial dilutions required for the different concentrations were formulated, depending on the optimization of drugs. The gold-conjugated form of AZM was kindly provided by the Hussain Ebrahim Jamal (HEJ) Research Institute of Chemistry at Karachi University.

2.3. Grouping of the Treated Cells

The treated cells were grouped as follows:

- *Group 1:* Untreated HepG2 cells (control group);
- *Group 2:* HepG2 cells treated with AZM (AZM group);
- *Group 3:* HepG2 cells treated with sorafenib (sorafenib group);
- *Group 4:* HepG2 cells treated with a combination of AZM and sorafenib (sorafenib/AZM group);
- *Group 5:* HepG2 cells treated with gold-conjugated AZM (Au-AZM group).

2.4. Cell Counting

Cell counting using Neubauer counting was done by observing the cells under an inverted microscope. The number of cells counted is the sum of all cells counted across squares in one chamber, and the final count is derived with the help of the following formula:

$= 2 * \text{no of cells in a chamber} * 10^4$

2.5. Scratch Assay/Cell Migration Assay

The scratch assay was performed to assess the anti-migratory potential of control and treated groups. For this experiment, cells were seeded along with media in a 6-well plate. After 24 hours, when cells became confluent on the surface of the plate, vertical scratches were made in each well through a sterile 10µL tip. After inducing scratch in the wells, media was discarded and replaced with fresh media along with the drugs (at IC50 concentrations). Subsequently, post-incubation imaging was done at 0, 6, 24, 48, and 72 hours. Cellular migration and closure of the wounded area were analyzed by Image J software (Version: 1.50 b). Following is the formula for wound closure:

$\%\ \text{of wound closure} = [(At = 0h - At = \Delta h) / At = 0h] \times 100\%$

where:

$A_{t=0h}$ is the area of the wound measured immediately after scratching;

$A_{t=\Delta h}$ is the area of wound measured 0, 6, 12, 24, or 48

h after scratching.

2.6. Statistical Analysis

For the data analysis, the SPSS program (Version 20) was used. All the numerical data has been presented as mean ± SE of mean (SEM), which was generated by applying ANOVA (analysis of variance) followed by Tukey's post hoc tests to observe the intergroup comparison. The significant difference between and within the treatment groups was considered significant at a set P-value < 0.05.

3. Results

3.1. The Wound Healing Potential of Untreated and Treated Groups at Different Time Intervals on HepG2 Cell Line

In vitro scratch assay was used to analyze the wound healing potential of untreated (control) and treated (AZM, Sorafenib, Sorafenib/AZM, and AuAZM) groups. Then it was observed under the inverted microscope at different time intervals such as 0, 6, 24, 48, and 72 hours. Fig. 1 depicts the migratory potential of control/untreated cells at different time intervals, which completed wound healing after 24 hrs. Fig. 2 showed the migratory potential of azithromycin-treated cells (at IC50 concentration) at different time intervals, which halted the wound healing for up to 72 hrs, whereas Fig. 3-5 indicate the migratory potential of Sorafenib, combination (AZM/Sorafenib) and AuAZM treated cells (at IC50 concentrations) which exhibited complete wound healing after 48 hours.

3.2. Comparative Effect of Different Treatments on the Migratory Potential of HepG2 Cells by Scratch/Wound Healing Assay

Fig. 6 shows a bar graph representing the anti-migratory potential of cancer cells after exposure to different treatments (AZM, Sorafenib, Combination of Sorafenib and AZM, and Au-AZM at IC50 concentrations) for 0, 6, 24, 48, and 72 hrs. AZM showed a highly significant reduction in wound healing with a p-value < 0.001 for up to 72 hrs, while Sorafenib, the combination of Sorafenib and AZM, and Au-AZM inhibited wound healing for up to 24 hrs (p-value < 0.001). The healing area of the scratch was then calculated at different hours (6, 2, 48, and 72 hours) in all the groups. The healing potential of cells after AZM treatment was 1.93% at 6 hrs, 38.6% at 24 hrs, 60.9% at 48 hrs, and 66.2% at 72 hrs; after Sorafenib treatment, it was 5.2% at 6 hrs, 49.1% at 24 hrs, and 100% at 48 and 72 hrs; after combination treatment (AZM and Sorafenib), it was 6.3% at 6 hrs, 69.7% at 24 hrs, and 100% at 48 and 72 hrs; and finally, after Au-AZM treatment, it was 9% at 6 hrs, 81% at 24 hrs, and 100% at 48 and 72 hrs.

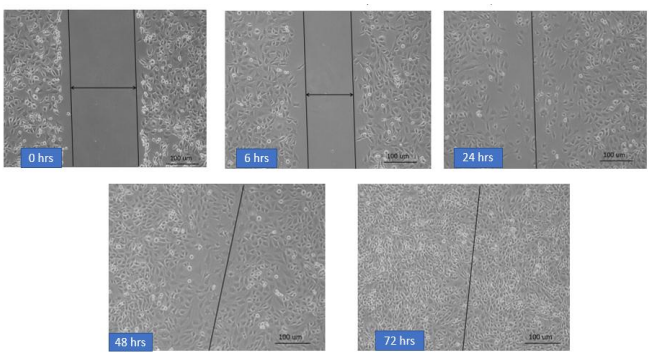

Fig. 1 Healing/migration potential of control/untreated cells of HepG2 cell line (Images of HepG2 cells taken at 0, 6, 24, 48, and 72 hours after induction of scratch)

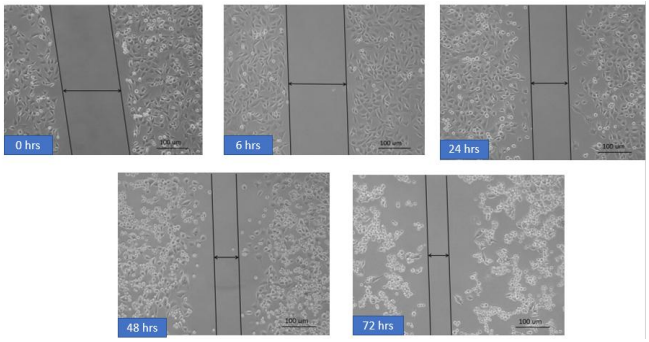

Fig. 2 Effects of treatment on healing/migration potential of AZM-treated HepG2 cell line (Images of HepG2 cells taken at 0, 6, 24, 48, and 72 hours after induction of scratch)

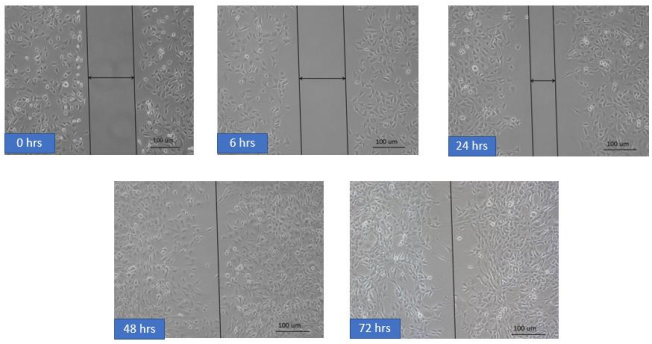

Fig. 3 Effects of treatment on healing/migration potential of Sorafenib treated HepG2 cell line (Images of HepG2 cells taken at 0, 6, 24, 48, and 72 hours after induction of scratch)

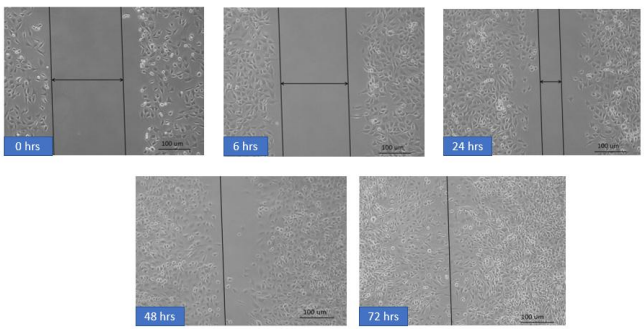

Fig. 4 Effects of treatment on healing/migration potential of the combination (AZM and Sorafenib) treated HepG2 cell line (Images of HepG2 cells taken at 0, 6, 24, 48, and 72 hours after induction of scratch)

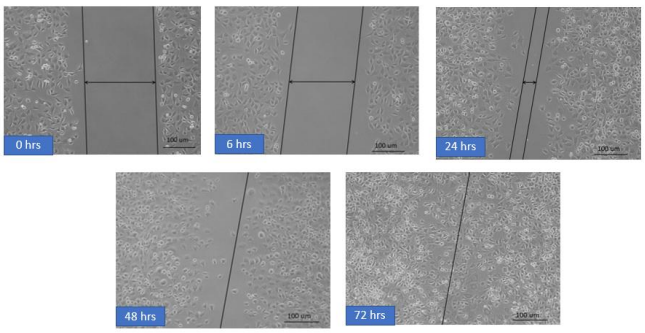

Fig. 5 Effects of treatment on healing/migration potential of Au-AZM treated cells HepG2 cell line (Images of HepG2 cells taken at 0, 6, 24, 48, and 72 hours after induction of scratch)

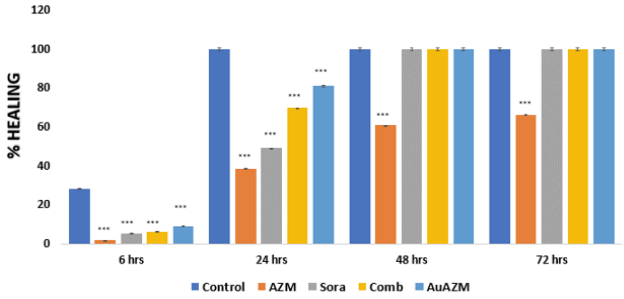

Fig. 6 Comparison of the effect of different treatments on the migratory potential of HepG2 cells by Scratch / Wound healing assay (Experiments were run in triplicates, data represented as mean ± SEM)

4. Discussion

The antimigratory potential of AZM, Sorafenib, the combination treatment, and Au-AZM was assessed (at the IC50 concentrations mentioned earlier) via performing a scratch/wound healing assay. The results of the assay were observed at 0, 6, 24, 48, and 72 hrs, and it was noted that Au-AZM, Sorafenib, and the combination treatment showed a highly significant P-value (< 0.001) at 6 and 24 hrs only; however, AZM was associated with the continuous inhibition of wound healing until 72 hrs with a P-value of <0.001. These results indicate that AZM revealed a superior antimigratory capacity to stop the proliferation or migration of cancer cells compared to Sorafenib alone, a combination of Sorafenib and AZM, and Au-AZM, signifying its potential role against metastasis.

Tumor angiogenesis plays an essential role during lung cancer progression and metastasis. As there is a scarcity of anti-angiogenic agents, the identification of more effective drugs with anti-angiogenic activity by drug repurposing is important for improving cancer therapy. In this regard, the literature reports the anti-angiogenic role of some of the macrolide antibiotics [24]. There is evidence that CAM inhibits tumor cell proliferation directly as well as causes tumor-induced angiogenesis in mice [26]. Tumor tissue from mice treated with 50–100 mg/kg CAM showed significantly lower vessel density than tumor tissue from the control group in the Lewis lung cancer model and the B16BL6 melanoma model [27]. CAM inhibited the formation of the endothelial tube in a dose-dependent manner at concentrations greater than 10 μM in vitro. Saad et al. conducted a randomized trial that indicated that the addition of CAM to a CVP (cyclophosphamide, vincristine, and prednisone) regimen was able to reduce the soluble vascular endothelial growth factor (sVEGF) level more than CVP alone. As these findings were interconnected with the clinical benefit in terms of response, they point toward the anti-angiogenic effect of CAM, which involves the VEGF pathway [28]. Another study conducted on HLT-ECs (human lung tumor associated-endothelial cells) isolated from human lung tumors to evaluate the anticancer effects of AZM revealed its role as an inhibitor of angiogenesis, which is considered crucial in tumor progression and the transition of a tumor from the dormant to the malignant stage. VEGF is a potent pro-angiogenic factor and a pivotal mediator of angiogenesis in malignant tissues. Increased VEGF and high microvascular density are strongly affiliated with poor prognosis in cancers, such as those affecting the lung and liver. In this study the anti-angiogenic effects of AZM were tested in vitro, in vivo, and using the lung tumor xenograft model. Azithromycin effects were tested on VEGF-stimulated angiogenesis as VEGF is the most important endothelial cell's (EC) specific angiogenic growth factor. It revealed that AZM effectively inhibits capillary network formation on the complete Matrigel matrix in a dose-dependent manner. AZM, in addition to suppression of multiple aspects of VEGF-induced angiogenic response, also showed inhibition of VEGFR2 (vascular endothelial growth factor receptor 2) mediated other downstream signaling pathways. The overall finding of this study was that AZM inhibited the spreading, proliferation, and survival of endothelial cells via suppression of VEGF-VEGFR2-mediated PI3k/Akt and other signaling pathways [29]. These findings are also evident in our study by significant wound healing assay results in the AZM group, which showed that AZM remained successful in inhibiting the migration of cancer cells till 72 hours.

A study was done to examine the in vivo antiangiogenic activity of the 14-membered ring macrolide antibiotic roxithromycin (RXM), chosen for its bioavailability. It was observed that RXM interferes with the migration of endothelial cells of HUVEC when tested for their ability to migrate in the presence or absence of RXM (0–50 mM). It was observed that at concentrations greater than 20 mM, RXM inhibited endothelial cell migration. Furthermore, the effects of RXM on the differentiation of endothelial cells into tube-like structures were studied by culturing endothelial cells on a Matrigel substrate. In the presence of RXM, endothelial cells formed small aggregate structures whose network of tube-like structures was less extensive than those in control cultures. Using this assay, it was inferred that the anti-angiogenic effect was dose-dependent as RXM reduced

the surface area of endothelial cells to about 20 and 40% at 20 and 50 mM doses, respectively [25].

5. Conclusion

In the current study, we found that the macrolide antibiotic azithromycin has significant anti-angiogenic effect in a HepG2 cell line. These results suggest that azithromycin may be a possible therapeutic option that can halt angiogenesis in tumor tissue. Current cancer treatments have many harmful side effects since commonly used antineoplastic drugs target all rapidly dividing cells rather than the solely cancerous ones. In contrast, certain antimicrobial proteins seem to show specificity towards malignant cells. The electrostatic attraction between the negatively charged components of bacterial and cancer cells on one hand and the positively charged antimicrobial proteins on the other is believed to play a major role in the strong binding and selective disruption of bacterial and cancer cell membranes, respectively. A growing number of studies have shown that some of the cationic antimicrobial peptides, which are toxic to bacteria but not to normal mammalian cells, exhibit a broad spectrum of cytotoxic activity against cancer cells. Such studies have considerably enhanced the significance of these synthetic and natural agents, which have been of importance both for an increased understanding of the immune system and for their potential as clinical antibiotics. Consequently, the results of our study are also comparable with previous studies suggesting the antitumor potential of antimicrobials.

6. Limitation

Due to budget constraints, we could not perform further assays to investigate the underlying molecular mechanism behind the inhibition of angiogenesis by azithromycin.

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Ethical Approval

Ethical approval was taken from the institutional ethical review committee (Reference number 0750119DZPHA).

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