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A NOVEL DERIVATIVE OF BENZISOTHIAZOLE INHIBITS SOLID TUMOR GROWTH *IN VIVO* BY ANTI-ANGIOGENESIS AND APOPTOSIS

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ABSTRACT: Benzisothiazole derivative is known to have potential pharmacological activities such as anti-bacterial, anti-microbial, anti-inflammation and local anesthetic, etc. Here we have studied the anti-tumor activity of novel derivative of benzisothiazole using Ehrlich Ascites solid tumor in mice and its pro-apoptotic property *in vitro*. Our earlier study showed that 10µM of the compound-g is most effective in inhibiting cancer cells growth and proliferation, so we used the same concentration for this work. Ehrlich ascites solid tumor-bearing mice treated with compound-g showed reduced tumor size and weight by a 3 and 27 fold, respectively. The compound-g upon treatment decreases the endothelial cell proliferation marker CD31 and angiogenic molecule VEGF receptor VEGFR2 along with the proliferation biomarker Ki67. H&E staining showed a three-fold decrease in microvessel density (MVD) count. Cancer biomarker Ki67 was also found to be effectively inhibited by the compound-g in tumor sample as compared to the untreated group.

Keywords: Ehrlich ascites tumor cells (EAT), Benzisothiazole derivative, CD31, VEGFR2, and Ki67.

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1.INTRODUCTION

Mammalian cells need nutrients and oxygen to survive and therefore must be located within 100-200µm of blood vessels – the oxygen diffusion range. The growth beyond this dimension must recruit new blood capillaries by vasculogenesis and angiogenesis. During the growth and proliferation of cancer cells, without blood vessels, the size of the tumor cannot grow beyond [1]. Angiogenesis plays a critical role which is mediated by various pro-angiogenic molecules such as

VEGF, VEGFR2, CD31, etc. and found highly expressed in a malignant tumor [2], [3]. When a cytokine VEGF binds to receptor tyrosine kinases (RTKs) such as VEGFR1 (Flt-1), VEGFR2 (Flk-1/KDR), and VEGFR3, it performs respective biological functions. Among all the RTKs, VEGFR2 plays a critical role in stimulating the angiogenic signal [4]. This signal has been reported as one of the contributing factors for differentiating endothelial cells into tip cells, that migrates and forms a leading front of the growing blood vessels [5]. Aberrant in VEGFR2 activation contributes to cell proliferation, differentiation, metastasis, inflammation, apoptosis, and angiogenesis [6]. At present, several FDA approved drugs targeting VEGFR2 such as sunitinib, vandetanib, and sorafenib. Many small molecules are under pre-clinical and clinical evaluation like SKLB261 and YLT192. Similarly, CD31 also known as platelet endothelial cell adhesion molecule-1 (PECAM-1) plays an essential role in angiogenesis by stimulating endothelial cells motility, forming intercellular junctional links, and promoting extracellular matrix interactions [7]. Expression of such proteins leading to angiogenesis further promotes the proliferative activity of the tumor cells. Ki67 is one such protein associated with the progression of cell proliferation, and it is used as a marker to analyze the aggressiveness of the tumor. Malignant tissues significantly express higher Ki67 than normal tissues, and this nuclear antigen Ki67 expressed in all stages of the cell cycle except sub-Go phase (apoptotic phase) [8]. Ki67 is a strategic factor towards targeted therapy to treat the malignant tumor [9]. According to the IACR (International Agency for Research on Cancer), 13.2 million cancer-related deaths and 21.4 million new cancer cases are projected all over the world by 2030. Precise prevention measures and excellent screenings for early detection service along with improvements in cancer treatment are required to reduce the global burden. Radiotherapy and chemotherapy have been used to target both oncogenic and angiogenic pathways in cancer and tumor endothelial cells. Solid tumor represents a foremost health problem due to its existence, and the incidence is rising at an alarming rate worldwide. Majority of the solid tumors contain hypoxic regions due to oxygen-deficient caused by rapidly proliferating tumor cells. This hypoxia leads to accumulation of secreted acidic metabolite in the tumor microenvironment, making it acidosis. This later contributes to tumor growth through various means like upregulating angiogenic factors, impaired immune functions, and increased invasion [10]. In this work, we have studied the anti-tumor activity of a novel derivative of benzisothiazole i.e. 3-(4-(2-(3-(4-chlorophenyl)-4,5-dihydroisoxazol-5-yl)methyl) piperazin-1-yl) benzo[d]isothiazole (compound-g) by analyzing its inhibitory property against some of the pro-angiogenic and proliferative molecules namely CD31, VEGFR2, and Ki67 in EAT solid tumor model in mice. In our previous studies, the compound-g showed promising inhibitory activity against breast cancer cell lines [11]. Here we have studied both *in vivo* and *in vitro* anti-cancer property of the compound-g.

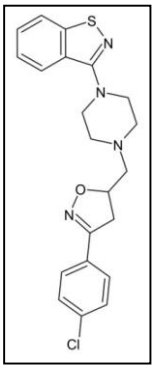

Figure 1: 3-(4-(2-(3-(4-chlorophenyl)-4,5-dihydroisoxazol-5-yl)methyl)piperazin-1-yl)benzo[d]isothiazole (compound-g)

2. MATERIALS AND METHODS

Ehrlich Solid Tumor Model In Mice

Animals experiments were performed as per the regulations of institutional animal care and use committee, University of Mysore, approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. Ehrlich ascites tumor (EAT) cells were regularly maintained and transplanted in the peritoneal cavity of mice once in two weeks ($1x10^6$ EAT cells). Experiments were performed according to the protocol, as mentioned elsewhere [12]. Swiss albino mice left thigh was injected subcutaneously with $1x10^6$ EAT cells and allowed to grow for 15 days to develop into a solid tumor. After the 15th day, mice were treated with or without compound-g (10µM/mice) on site of tumor growth every alternate day till 45th day. On the 45th day of tumor development, animals were sacrificed to collect the solid tumor from untreated and compound-g treated animals. The sizes and weight of the solid tumors were measured, and further histological studies were performed.

Immunohistochemical Analysis

The solid tumors removed were embedded in paraffin and cut into 5µm sections for immunohistochemical staining as described previously [13]. Briefly, tumor sections were deparaffinized with xylene and hydrated using a decreasing concentration of ethanol (100% and 95%) for 15 minutes. Antigen retrieval was carried with a heat treatment method with 10mM of sodium citrate. Three drops of peroxidase were incubated on the slide to quench endogenous peroxidase activity. The slides were blocked with 3% BSA for 1 hour and washed thrice with phosphate-buffered saline tween-20 (PBST). Then the individual slides were incubated with anti-pecam-1 antibody, vegfr2 antibody, and Ki67 antibody for 2 hours and secondary antibodies for 1hour. The antigen-antibody complex formed was detected by using DAB substrate and counterstained with hematoxylin. The slides were mounted with a coverslip and imaged under bright field microscope.

H & E Staining

H and E staining were performed on solid tumor sections for both the groups to count the microvessel density (MVD). The experiment was carried out, as reported earlier [14]. Briefly, solid tumor with or without compound-g treatment was formalin-fixed and later embedded in paraffin blocks. Tumors were sectioned into the 5µm thickness and stained with H&E. MVD was estimated by mean of the stained vessels from five different fields.

Calcein-AM/Propidium Iodide (PI) Dual Staining

To study the cytotoxicity of compound-g, we used two fluorescent dyes Calcein-AM and PI to distinguish between live/dead cells. Calcein stains viable and apoptotic cells and imparts green color while PI stains only those cells with a compromised cell membrane, a characteristics feature of apoptotic cells and stains red color. EAT cells were grown in 6 well-plate (2 x 10^5 cell/well) containing DMEM growth medium. Cells were then treated with/without 10µM of the compound-g for 48 hours. After treatment, cells were washed twice with PBS to remove any traces of media and then cell pellet was incubated with 10µl each of calcein-AM (10µM) and propidium iodide (1µg/ml) for 30 minutes at room temperature in dark place. The cell suspension was dropped onto glass slides, and a coverslip was placed. We observed the slides under a fluorescent microscope attached with a camera and stained cells images were captured to identify live/dead cells.

3. RESULTS AND DISCUSSION

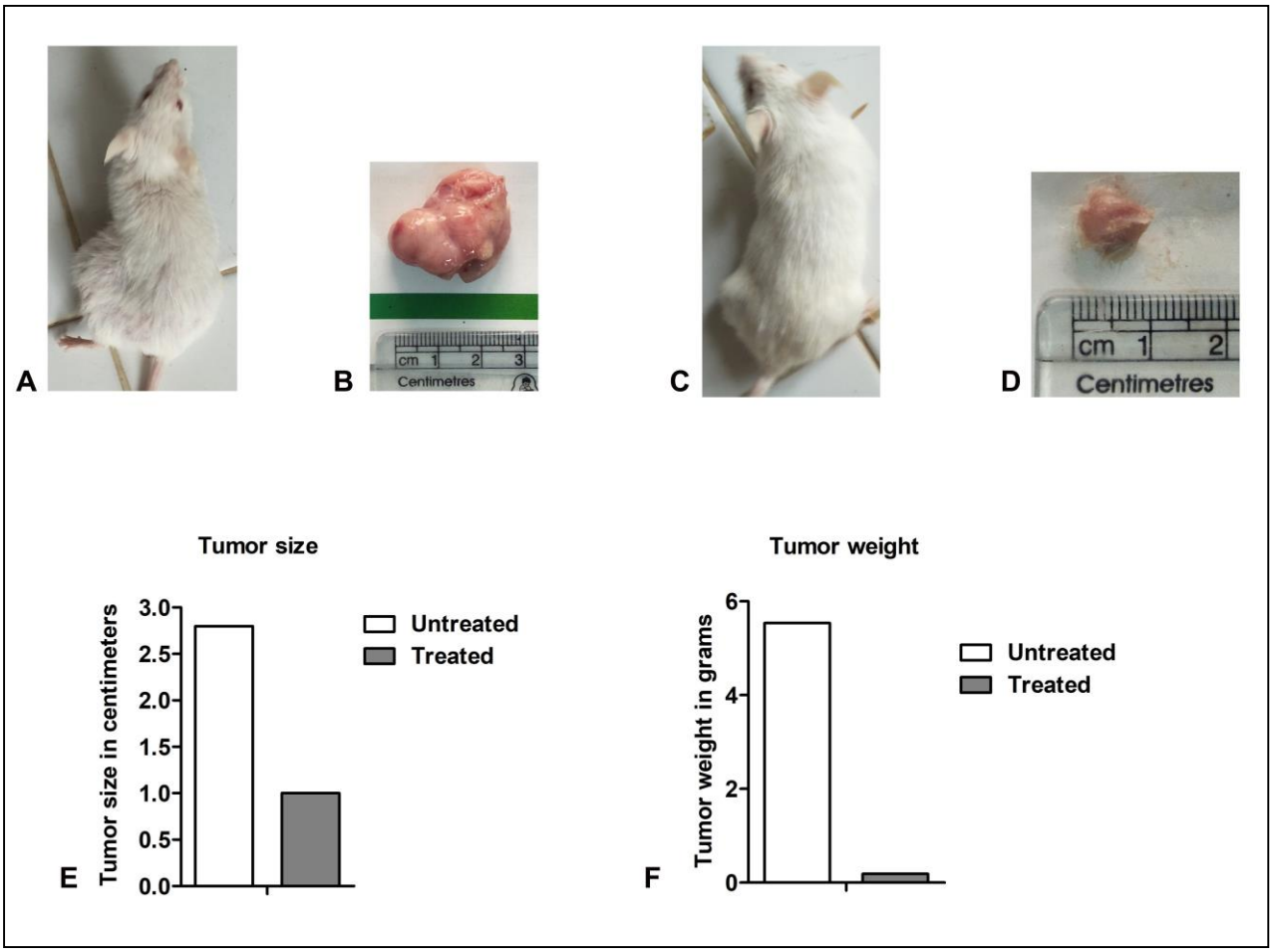

Figure 2: The antitumor activity of compound-g in solid EAT tumor model: Swiss albino mice was transplanted with $1x10^6$ EAT cells on left thigh and allowed to develop solid tumor for 15 days. On the 16th day, mice were treated with or without compound-g (10µM) on the site of tumor growth every alternate day till the 45th day of tumor transplantation. On the 45th day mice were sacrificed and tumors were removed. Untreated mice (A) with tumor (B) and treated mice (C) with tumor (D) on the thigh were removed and the tumor size was measured by both geometrical scale and vernier caliper (E) and tumor was weighed (F).

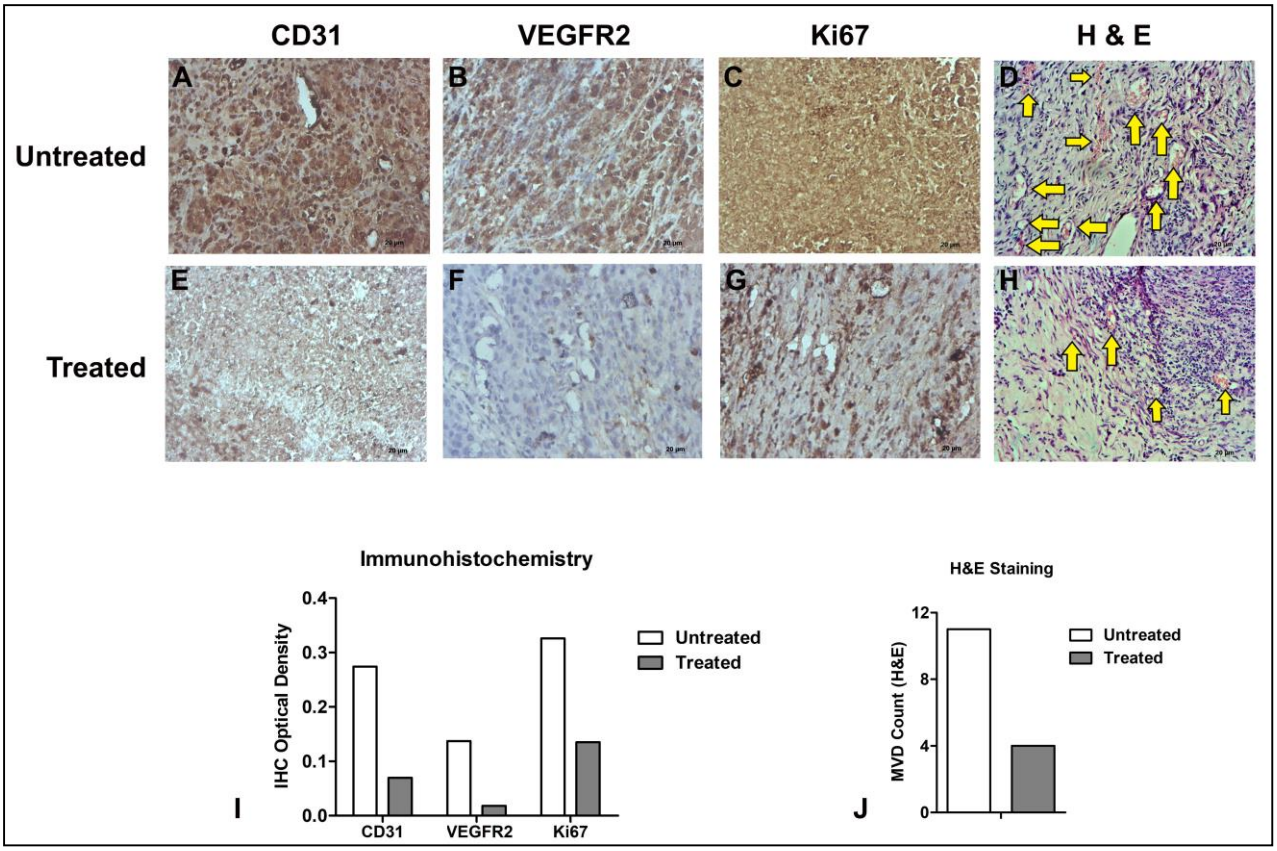

Figure 3: Immunohistochemical detection of CD31, VEGFR2 and Ki67 and H&E staining of EAT solid tumor sections: the expression study of various proteins involved in the progression of cancer was studied using IHC method and the microvessel density count by H&E staining. IHC optical density was measured using Fiji ImageJ (I) and MVD was counted by three different investigators.

The murine mammary carcinoma EAT cells were subcutaneously injected into the thigh of experimental mice, and their growth inhibition upon treatment with or without compound-g (10µM) was studied. The results showed that compound-g relatively reduced the size and weight of EAT solid tumor growth by more than 2 and 12 fold, respectively (Figure 1.E, F). The movement of untreated mice was found to be difficult and stressful, while the compound-g treated group mice were normal. To investigate the distribution and intensity of few proteins (CD31, VEGFR2, and Ki67) involved in cancer progression between treated and untreated mice, tumors were made into sections of 5µm and processed for immunohistochemistry. Among all the proteins, Ki67 was found to be highly expressed, followed by CD31 and VEGFR2 in both the groups as measured in IHC OD using Fiji ImageJ software by three different investigators. Comparatively lower distribution of oncoproteins in treated mice was observed, the maximum difference in protein expression between the two groups was found in VEGFR2 followed by CD31 and Ki67 with IHC OD value fold difference of 7.48, 3.93 and 2.411 respectively (Figure 1.I). These results suggested that the compound-g inhibited the growth of the solid tumor by downregulating above mentioned proteins. Since there was a lower abundance of the angiogenic marker, we studied the microvessel density

count from the tumor sample by H&E staining. The MVD count from both the sample showed that compound-g effectively reduced the angiogenesis as compared to untreated with MVD count of 4 and 11, respectively (Figure 2.J).

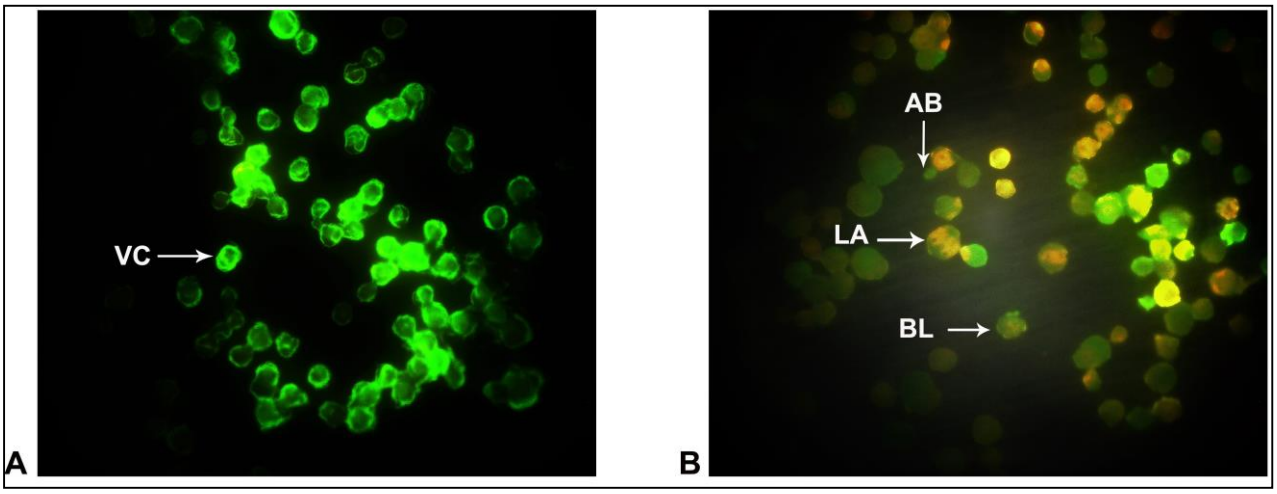

Figure 4: Cell viability study using differential staining: EAT cells without (A) or with (B) treatment of compound-g were stained with dual fluorescent dye (Calcein-AM/PI). In untreated cells no uptake of PI dye was observed while many cells are PI stained in treated cells, indicating its cytotoxicity against cancer cells. VI: viable cells; AB: apoptotic body; LA: late apoptosis; BL: membrane blebbing.

We aimed to study cytotoxicity and the potentiality of the compound-g to induced apoptosis in tumor cells. We selected Calcein-AM/PI staining to assess the difference in cell staining upon treatment with compound-g. Microscopic observation revealed the uptake of PI dye in EAT cells treated with 10µg of compound-g. After 48 h treatment, some of the cells exhibited features of apoptotic cells such as blebbing of the cell membrane, reddish-orange color by binding of PI to denatured DNA, and released of the apoptotic body (Figure 4.B), while none of the cells in untreated showed sign of apoptosis (Figure 4.A). This result signifies that the compound-g induces apoptosis in EAT treated cells. Calcein-AM and PI are used for cell staining, which emits green fluorescence in viable cells and reddish-orange fluorescence in cells undergoing apoptosis or in apoptotic cells, respectively. Benzisothiazole derivative reported to have many potent pharmacological effects such as antimicrobial, antibacterial, anti-inflammatory, local anesthetic and psychotropic activity [15], [16], [17], [18]. In our previous study, the drug-like property of the compound-g using bioinformatics tool has been showed [19]. Here, we studied the antitumor property of novel benzisothiazole derivative (compound-g) (Figure 1.) with respect to anti-angiogenesis, tumor size growth, and pro-apoptotic activity. The size and weight of the solid tumor in treated mice were much lesser than that of untreated animal (Figure 2.A-E). The angiogenic molecules such as CD31, VEGFR2 were under-expressed in mice upon treatment with compound-g (Figure 3.E, F) which might have caused the

reduction of tumor growth as a similar result was also reported [20]. It was also found that VEGFR is profusely expressed by a majority of the solid tumors [21]. Nuclear transcription factor NFκB was reported to be one of the factors which abundantly expresses VEGFR2 [22]. The down-regulation of VEGFR2 upon treatment could be due to the binding affinity of compound-g on transcription factor NFκB as reported in our earlier study [19] and making them unavailable for downstream signaling pathway and inhibiting expression of proteins. Similarly, angiogenic marker CD31 expression was considerably linked with larger tumor size, advanced stage, and higher grade of the tumor [23]. Our results showed that there was a correlation between the size of the tumor and CD31 expression as similar to reported elsewhere [24]. After treatment with compound-g, the size of the tumor and CD31 expression was lesser compared to untreated EAT cells solid tumor-bearing mice. It was reported that CD31 expression is directly involved in angiogenesis [2]. It was reported earlier that CD31 and CA9 showed early response upon treatment with antiangiogenic therapy such as sunitinib [25]. Down-regulation of angiogenic markers in the compound-g treated group was reflected on the microvessel density (MVD) count using H&E staining. There was an almost three-fold decrease in MVD count between the treated and untreated group (Figure 3.J). Similarly, cancer proliferation biomarker Ki67 is known to express highly in the malignant tumor [26], and IHC staining of the antigen is used as a clinical indicator for breast cancer [27]. Targetting this biomarker have potential implications for the treatment of malignant tumor [28]. The compound-g treated group showed more than a twofold lesser expression of Ki67 as compared to the untreated sample (Figure 3.I). This reduction in Ki67 expression could be due to the inhibition of angiogenesis in a treated solid tumor as analyzed by MVD. Apart from anti-angiogenesis for cancer therapy [29], apoptosis also plays an important for the inhibition of tumor growth [30]. Cancer cells undergo apoptosis by DNA fragmentation which leads to its death [31], [32]. For that, invitro pro-apoptotic property of the compound-g was studied *in vitro* on EAT cells using calcein/PI fluorescent dye where the compound inhibited around 40% of the total cell population when observed under the microscope from 10 different fields while no apoptotic cells were observed in untreated (Figure 4.A, B). From this study, the compound-g showed antiangiogenic and pro-apoptotic activities in mouse mammary carcinoma. Further, the signaling pathway of those activity has to be studied to get insights into its mechanism of action.

4. CONCLUSION

The work presented herein shows that compound-g has several modes of action involving (i) downregulating of angiogenic molecules such as VEGFR2 and CD31 leading to reduced blood vessels formation and subsequently preventing further tumor growth (ii) inhibition of Ki67 leading to reduced tumor cell proliferation (iii) killing of tumor cells by promoting apoptosis. All the substituent molecule of compound-g is known to be clinically safe to use, and further validation tests are required to define the precise mode of action to use it as a targetted therapy.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The authors confirm that the data supporting the findings of this research are available within the article.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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