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The impacts of Benzisoxazole Derivative NS5 compound on Pro-apoptotic and Anti-angiogenic activities

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Abstract:

The aims of this study were to investigate the effectiveness of NS5 on the process of pro-opoptotic and anti-angiogesnesis activities which was experimented on Swiss albino mice (6-8 weeks old). The researcher applied different types of methods such as: Trypan Blue Dye Exclusion Method, Acridine orange-EtBr staining assay, Protein estimation by Lowry's method, SDS–PAGE, Western blot Analysis for Flt-1 and Flk-1 Expression, and Histological analysis (H & E staining) for Micro vessel density (MVD) scoring. These methods were used to analyze NS5 on pro-opoptotic and anti-angiogenesis works. The results indicated that employing NS5 on 6-8 weeks old mice resulted in reducing in tumor growth and decreasing in body weight compared to the control mice. Therefore, NS5 could induce down regulation of Vascular Endothelial Growth Factor receptors (VEGFR). In sum, NS5 compound had noticeable antiongiogenic and pro-opoptotic impacts on Ehrlich ascites tumor (EAT) cells in vivo and it could be operated as an angiogenesis inhibitor proapoptic in nature.

Key words: Cancer, Angiogenesis, Benzisoxazole, Angiogenesis therapy

1. INTRODUCTION

1.1 Cancer

Cancer known medically as malignant neoplastic, is an extensive group of diseases including unregulated cell growth. In cancer, cells divide and grow uncontrollably, forming malignant tumors, which may attack different parts of the body that is near. The cancer may also spread to more distant parts of the body through the lymphatic system or bloodstream. Nearly 5–10% of cancers can be traced directly to inherited genetic defects (1, 2).

1.2 Angiogenesis

Angiogenesis is a fundamental process in growth, development, wound healing and formation of granulation tissue. Therefore, it is also an essential step in the transfer of tumors from a benign state to a malignant one, resulting in the use of angiogenesis inhibitors in the treatment of cancer. Judah Folkman was the first person who founded the essential role of angiogenesis in tumor growth in 1971, who described tumors as "hot and bloody". Angiogenesis, the growth of new capillary blood vessels, is critical for development, reproduction and repair and dominates many pathological conditions (3).

1.3 Molecular mechanism

Angiogenesis can be included in various cells and stimulating factors. Some of the cells involved are the endothelial cells, lymphocytes, macrophages, and mast cells. There are two significant factors among other factors involved in this process such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) (4).

1.4 Vascular endothelial growth factor (VEGF)

Vascular endothelial growth factor (VEGF) is an important growth factor found in almost all cells of the body. When VEGF

binds to a vascular endothelial growth factor receptor (VEGFR), the resulting signaling cascade induces angiogenesis and vasculogenesis as well as promoting the proliferation, migration, and survival of endothelial cells. All of these roles are individually important during embryogenesis, skeletal growth, and reproduction (5).

1.5 Ligands and Receptors

Vascular Endothelial Growth Factor Receptors (VEGFR) is a tyrosine kinase receptor found along the cell membrane. It has seven immunoglobin-like structure found on its extracellular domain and a split tyrosine kinase found in the intracellular domain. There are three different VEGFR namely VEGFR-1, VEGFR-2, and VEGFR-3.

VEGF ligands cause varying effects by binding to different VEGFR resulting into different physiological changes. VEGFR-1 and VEGFR-2 are linked to angiogenesis whilst VEGFR-3 is related to lymph angiogenesis (6, 7).

1.6 The fibroblast growth factor (FGF)

The fibroblast growth factor (FGF) family with its prototype members FGF-1 (acidic FGF) and FGF-2 (basic FGF) consists to date of at least 22 known members (8). Most are single-chain peptides of 16-18 kDa and display high affinity to heparin and heparan sulfate. Generally, FGFs induce a variety of cellular functions by binding to cell surface FGF-receptors in the presence of heparin proteoglycans. The FGF-receptor family is included of seven members, and all the receptor proteins are single-chain receptor tyrosine kinases that become activated through autophosphorylation induced by a mechanism of FGF-mediated receptor dimerization. . Receptor activation gives rise to a signal transduction cascade that leads to gene activation and diverse biological responses, including cell differentiation, proliferation, and matrix dissolution, thus initiating a process

of mitogenic activity critical for the growth of endothelial cells, fibroblasts, and smooth muscle cell (9).

1.7 Matrix Metalloproteinase (MMP)

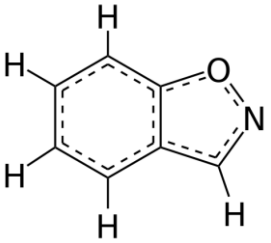
Another major contributor to angiogenesis is matrix metalloproteinase (MMP). MMPs help degrade the proteins that keep the vessel walls solid. This proteolysis permits the endothelial cells to escape into the interstitial matrix as seen in sprouting angiogenesis. Inhibition of MMPs impedes the formation of new capillaries. These enzymes are highly regulated during the vessel formation process because destruction of the extracellular matrix would decrease the integrity of the microvasculature (10).

1.8 Anti-angiogenic therapy

In recent years, anti-angiogenesis has been at the core of alternate cancer therapy research. There have been recent successes in treating mouse cancers. Evidence demonstrates that by neutralizing antibodies against VEGF-A, and antibodies that block VEGF-A receptors, tumor growth can be significantly reduced. More recently, antibodies have been designed to selectively recognize VEGF-A forms found on tumor vessels, hence avoiding side effects that might result from inactivation of free VEGF-A (11).

1.9 Benzisoxazole

Benzisoxazole is an aromatic organic compound with a molecular formula C_7H_5NO containing a benzene- fused isoxazole ring structure. It is heterocyclic compound, used in research as a starting material for the synthesis of larger, usually bioactive structures. It is found within the chemical structures of pharmaceutical drugs such as the antipsychotic risperidone and anticonvulsant zonisamide. It has reactive sites which allow for functionalization and used primarily in industry and research.



2. MATERIALS AND METHODS

NS5 was chemically synthesized and obtained from the department of chemistry, Manasagangotri, Mysore. Stock solutions (1mM) of the compound were prepared using 0.1% dimethylsulfoxide (DMSO). The working solution of the compound was 100µM and 500µM. All primary antibodies used were obtained from Santa Cruz Biotechnology Inc. USA. Secondary HRP tagged antibodies were from Merck India. Recombinant VEGF was obtained from Genentech USA. All other reagents used were fresh and of the highest analytical grade. Swiss albino mice (6-8 weeks old) were obtained from the animal house, Department of Studies in Zoology, University of Mysore, Karnataka, India. EAT cells/mouse mammary carcinoma cells were maintained in our laboratory from last few years and thus used for *in vivo* transplantation. The animal experiments were approved by the Institutional animal care and use committee, University of Mysore, Karnataka, India.

2.1 Treatment of compound on EAT cells *in vivo*

The compound (NS5) was used for *in vivo* treatment in Swiss albino mice transplanted with EAT cells. Mice were inoculated with EAT cells intra-peritoneally and weight of the animals was monitored from the day one till the 21th day in order to ensure the tumor growth. The compound was injected at a concentration of 50 µM concentration daily intra- peritoneally from the 5th day to monitor the effect of the compound inhibition on EAT cell growth *in vivo*. The animals were

sacrificed on 21st day and dissected to observe the effect of the compound (NS5) inhibition on peritoneal angiogenesis.

2.2 Cell number and ascites volume

After scarification of the animal, cells along with the ascites fluid were collected both from the control and treated animals. The harvested cells were washed with ammonium chloride solution to remove residual RBC contamination. Then cells were resuspended in PBS and counted using a haemocytometer by trypan blue dye exclusion method. The volume of ascites obtained from control and treated animals were recorded.

2.3 Trypan Blue Dye Exclusion Method

Firstly, EAT cells were collected from the sacrificed mice and were counted on a haemocytometer. Secondly, 10 µL of EAT cells was taken from the total cell volume. Thirdly, 10 µL trypan blue + 980 µL of PBS was added to it. Mixture was placed on a haemocytometer for counting. The dead cells uptake the dye turning blue in color and only the viable cells that are transparent were counted. Finally, Calculation: A x 10^4 x dilution factor, where 'A' is the average cell number. Graph was plotted for viable cells.

2.4 Acridine orange-EtBr staining assay

EAT cells from sacrificed mice (1:6 diluted), control and treated (with compound) were used for staining.

	Smeared	on	slides	and	fixed	with
	methanol- acetic acid (3:1).					

Stained with 100 µL of a mixture of 1:1 (0.1 M) Acridine orange-EtBr and washed immediately once with PBS and viewed under 40 X objective.

2.5 Protein estimation by Lowry's method

First, 0.2 ml of BSA worked standards in 5 test tubes and made up to 1ml using distilled water. The test tube with 1ml distilled

water serves as blank. Second, add 4.5 ml of Reagent 1 and incubate for 10 minutes. Third, after incubation added 0.5 ml of Reagent 2 and incubated for 30 minutes. Measured the absorbance at 660nm and plotted the standard graph. In the end, estimated the amount of protein presented in the given sample from the standard graph.

2.6 SDS – PAGE

Resolving gel was prepared first and poured into an SDS PAGE gel casting unit. A layer of ethanol was added on top of the resolving gel and it was allowed to solidify after the resolving gel solidified, ethanol was removed and stacking gel was poured on top of the solidified resolving gel. A comb was inserted on top of the stacking gel. The samples were prepared by adding sample buffer (loading buffer).The samples were then heated in boiling water for 5-10 min. The samples were then loaded into the wells. Protein marker was loaded into the first lane. Then covered the top and connected the electrodes. The unit was initially run at 50V till the dye crossed the line between the stacking and resolving gel. Then the voltage was increased to 100V. The voltage was stopped after the dye front reached the end of the gel plate.

2.7 Western blot Analysis for Flt-1 and Flk-1 Expression

Remove gel from the gel-cast and place into Transfer buffer. Cut filter paper in half and soak in transfer buffer. Cut a piece of nitro-cellulose membrane for transfer and soak in methanol for at least one minute. Place 7 pieces transfer-buffer soaked filter paper on the transfer apparatus (roll out any air bubbles every time).Take the membrane from out of the methanol and rinses it in transfer buffer. Place the membrane onto the 7 pieces of filter paper and gently roll out any air bubbles. Place the gel on top of the membrane in the correct orientation. The marker should be on the same side that it was when the gel was

loaded. Gently roll out any air bubbles. Place three more pieces of filter paper on top of the gel. Set the transfer apparatus to 15V for 45 minutes and hit start. When the transfer is complete, remove the nitro-cellulose membrane and place directly in TBST (Be careful not to let the membrane dry at this point.) This step removes any excess methanol from the blot. Place the nitro-cellulose membrane into a fresh tray with your choice of blocking buffer. We recommend 5% BSA in TBST for most applications. Incubate the membrane in blocking buffer for at least two hours with gentle agitation on a shaker. Dilute the primary antibody 1:1000 in 5% BSA in TBST. Remove the western blot membrane from the tray with blocking buffer and place into a fresh, clean tray with the primary antibody solution. Incubate 2 hours to overnight under gentle agitation on a shaker. If incubating overnight, place the membrane at 4°C. After primary antibody incubation, wash the western blot membrane 3 times for 5 minutes each with TBST. Be sure to use at least 25 ml of TBST for each wash. Incubate the washed nitro-cellulose merbrane with secondary antibody at a 1:2000 dilution in 2.5% BSA in TBST. The secondary antibody can be diluted further if the signal is too strong. However, for most applications, a dilution of at 1:1000-1:2000 will give the best signal to background ratio for your western blots.

Leave the secondary antibody on for 30 minutes to 1 hour. Wash the membrane 4 times with TBST with gentle agitation on a shaker. Use at least 25 ml for each wash. After the fourth wash, place your western blot membrane into a fresh, clean tray of TBST. The ECL should be kept at 4°C. Remove the ECL from the fridge and mix an equal amount of substrate and oxidizing agent in a fresh, clean tray. Remove the membrane from its tray of TBST and dab the excess buffer off of a corner onto a clean paper towel**.**

Do not let the membrane dry out**.** Just remove the excess buffer before placing the membrane into the ECL. Place the membrane into the ECL for 45 seconds. Remove the membrane

from the ECL tray and dab off excess ECL onto a clean paper towel. The membrane should stay wet. Place the ECL-soaked membrane into a clean cassette between transparent page protectors. Roll out any air bubbles that may obstruct the film development. Close the cassette, grab a time and film, and go to the nearest darkroom to develop. Generally, a good starting exposure time is 30 seconds. This time can be adjusted to get an exposure in the linear range.

2.8 Histological analysis (H & E staining) for Micro vessel density (MVD) scoring

The effect of NS5 compound on the angiogenic response induced by VEGF was further verified by analysis of micro vessel density in EAT bearing mice which were treated regularly for 7 days with the compound after the sixth day of EAT cells transplantation, the peritoneum of the mice treated with or without the compound was fixed in formalin, dehydrated with alcohol and embedded in paraffin. The 5µm sections were taken using microtome and stained with hematoxylin and eosin stain. MVD was determined by 'hotspot' method (Goddard, 2001) using Nikon binocular microscope. In brief, 10 fields with highly vascularized areas were screened at low magnification (10x), and further magnification was changed to high-power field (HPF) (40x) and the microvessels were counted.

3. Results

Days	Body weight in grams			
	Control 1	Control 2	NS5 (1)	NS5 (2)
0	38.69	38.69	38.69	38.69
1	42.07	41.20	35.20	36.34
2	43.26	40.12	34.29	35.86
3	41.65	37.32	35.16	34.89
4	42.43	39.47	33.35	33.15
5	42.02	39.31	36.58	34.95
6	43.25	40.16	35.50	35.29
7	44.19	42.41	35.05	35.83
8	45.68	43.33	36.32	37.74
9	45.89	44.46	37.54	39.09
10	47.70	47.71	38.86	40.25
11	49.99	50.44	39.09	41.93
12	48.29	47.95	40.52	44.06
13	50.35	49.19	39.66	41.92
14	48.74	50.83	43.28	44.51
15	47.58	49.77	42.83	44.40

Table.1 Body weights of control and compound (NS5) treated animals

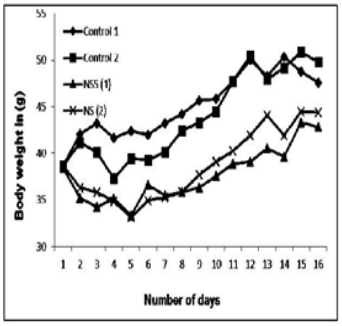


Figure 1 The effect of NS5 compound on tumor growth in vivo. EAT cells were injected to mice (i.p) and the body weight was noted from day 0 to 22 days. Compound treatment was given from 5th day to till scarification**.**

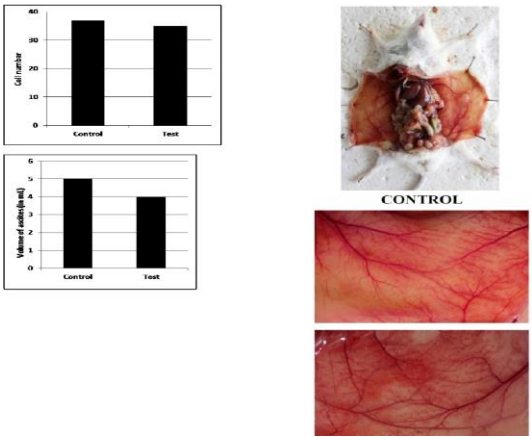


Figure 2 The effect of NS5 compound on ascites volume and cell number (A) Ascites volume, (B) cell number (determined by trypan blue dye exclusion method).

The EAT bearing mice treated with NS5compound showed reduction in tumor growth and thus decrease in body weight compared to the control (Fig. 1). The volume of ascites formed due to tumor induction increased slightly in the control, whereas in the compound (NS5) volume of ascites is slightly less to that of control (Fig. 2).

Figure 3 The Photographs of the peritoneum untreated (control) and NS5 treated EAT bearing mice.

The peritoneum of untreated EAT bearing mice showed extensive angiogenesis, while compound treated EAT bearing mice showed significantly less angiogenesis (Fig. 3)

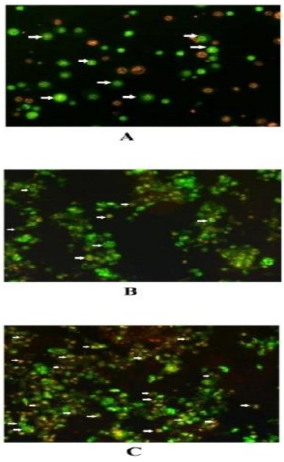


Figure 4 The identification of apoptotic cells by Acridine Orange/Ethidium bromide staining in EAT cells. EAT bearing mice were treated in vivo, i.p. Representative micrographs (40X) of treated and untreated EAT cells. (A). Control – vehicle treated EAT cells. (B) EAT cells treated with 100 μM NS5 compound and (C) EAT cells treated with500 μM NS5 compound. The morphological changes associated with apoptosis such as chromatin

condensation, nuclear fragmentation, and margination of nucleus are evident in the treated and untreated cells (shown in Fig. 4).

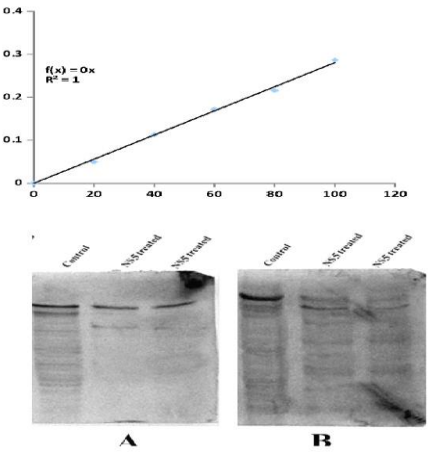


Figure 5 The Western blotting analysis of (A) flt-1 and (B) flk-1 expression in NS5 treated (100 µM & 500 µM concentration respectively) and vehicle treated control EAT whole cell lysate. It was found that the NS5 treated EAT cells (100 µM and 500 µM) could effectively induce significant down regulation of VEGF receptors Flt-1 and Flk-1, whereas there was no change in VEGFR expression in vehicle treated EAT control cells. This result indicates that subtoxic doses of NS5 can induce down regulation of VEGF receptors (Fig. 5).

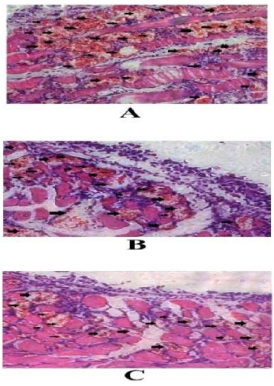


Figure 6 Micro vessel density in mouse peritoneum (A) vehicle treated control, (B) 100 µM NS5 treated, (C) 500 µM NS5 treated. The micro vessel density (MVD) was counted in the peritoneum sections of NS5 compound treated, as well as the untreated EAT bearing mice. There was a reduction of

almost 50% (MVD) in NS5 treated peritoneam section when compared to untreated (Fig. 6).

4. DISCUSSION & CONCLUSIONS

The concept of anti-angiogenic therapy was first brought forward some four decades ago by Folkman. Today it is the most promising area of oncology and therapeutics (12). Tumor growth is dependent on angiogenesis and other factors like VEGF,bFGF play a crucial role during this process. Understanding the molecular mechanism behind angiogenesis and in addition in identifying pro-and anti-angiogenenic factors can lead to strategies that prevent tumor nourishment. Anti-angiogenic therapies encounter many challenges; one of them is being the need for combination of therapies and agents. The challenge does not lie on combing them, but depends on the agent-dose and the time-window. It is important to determine the best time when these therapies, administered together, give the best results. Thus the discovery of angiogenic inhibitors with pro-apoptotic activity would provide an important therapeutic value. With the goal of finding a potent pro-apoptotic and anti-angiogenic compounds, we have initiated a screening program in our laboratory designed to test a wide variety of chemically modified compounds for anti-angiogenic and pro-apoptotic activity. I have worked on NS5, a chemically modified compound, to determine its anti-angiogenic and pro-apoptotic activity.

In the present study, we have shown that NS5 compound has considerable anti-angiogenic and pro-apoptotic effects in Ehrlich ascites tumor (EAT) cells *in vivo*. We carried out many experiments to see that the NS5 compound inhibit angiogenesis and induce apoptosis in the EAT cell lines *in vivo*. Various experiments such as body weight, ascites volume, EAT cell number, nuclear staining like Acridine orange/ ethidium bromide, and other apoptotic assays were done to observe the

effect of NS5 on EAT cells *in vivo*. It was suggested that NS5 induced high level of apoptotic activity, which may be linked to slower tumor growth. Our results show that NS5 compound inhibits angiogenesis and induce apoptosis in EAT cells *in vivo.*

In conclusion, we demonstrate that NS5 compound may act as an angiogenesis inhibitor and is pro-apoptotic in nature. Further studies can be done on this chemically synthesized compound to determine its effects on the signals that promote or regulate the cell cycle, growth factors and their receptors, signal transduction pathways and pathways affecting apoptosis and angiogenesis. Consequently, the studies of these anti-angiogenic and pro-apoptotic pathways will advance our knowledge and understanding of the efficacy of many chemo-preventive compounds some of which may become very potent therapeutic drugs of the future.

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