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# Control of *vegf* Expression and Tumor Angiogenesis by Nanoparticle- Berberine- Sanazole Complexes

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**ABSTRACT**

**Background**: Growth of tumor is fully dependent on angiogenesis for the supply of nutrients which is essential for its proliferation and metastasis. Of the various pro- angiogenic factors,Vascular Endothelial Growth Factor (VEGF) plays a crucial role in the process. Tumor- induced angiogenesis was the key therapeutic target in the present study. The iron oxide nanoparticle- berberine- sanazole (NP-BBN-SAN) complexes were used to control the transcriptional level expression of *vegf* and thereby prevent neovascularization in animals bearing tumor in the peritoneal cavity. **Results**: The administration of NP-BBN-SAN complexes inhibited angiogenesis in mice-bearing tumor in the peritoneal cavity. The images of inner peritoneal membrane presented visual differences of angiogenesis in the animals treated with the complexes. At the transcription level the expression of the gene, *vegf* was found significantly down regulated in the tumor cells following the treatment with NP-BBN-SAN. The order of down regulation was NP-BBN-SAN> BBN> SAN. The complexes did not cause systemic toxicity to kidney and liver in these animals as evidenced from the results on serum biochemical parameters. **Conclusion**: The present work provide compelling evidence that nanoparticle- berberine- sanazole complexes down regulate *vegf* expression and thereby prevent neovascularization which is a must for tumor growth and metastasis. Thus the study reveals the feasibility of using the NP-BBN-SAN complexes in tumor therapy.

## INTRODUCTION

Angiogenesis is a homeostatic process of forming new blood capillaries during embryogenesis, ovarian cycle, in normal physiologic repair processes and tumor growth.

Vascular endothelial growth factor (VEGF), identified in 1989 by Leung et al., is specific for vascular endothelial cells and promote the neovascularization *in vivo*. Among the proangiogenic factors identified, VEGF is found to be the central angiogenesis initiating facor. [1,2] Malignant cells frequently express angiogenesis promoting factors.

It has been realized for long time that control of angiogenesis could inhibit tumor growth and metastasis. [3-5] A number of anti-angiogenic agents have been used to treat several cancers. The specific monoclonal antibody against VEGF was effective in reducing blood vessel density and further human tumor growth in xenograft model. [6] Bevacizumab, a monoclonal immunoglobulin G antibody, has been shown to block the expression of VEGF receptor in endothelial cells and prevented neovascularisation in tumor. [7] In metastatic colorectal cancer patients, Bevacizumab in combination with chemotherapy extended life by a few months. [8] Several natural compounds are found have anti- angiogeneic activity in cancer cells. Berberine was found to be effective in preventing tumor directed capillary formation by down regulating the expressions of hypoxia- inducible factor- 1, VEGF and pro-inflammatory mediators. [9] In the present work, iron oxide nanoparticles were complexed with Berberine (BBN), a cytotoxic plant alkaloid, and Sanazole (SAN), a hypoxic radiosensitizer. The complex, nanoparticle-berberine- sanazole (NP-BBN-SAN) has been reported to cause regression of solid tumors in mice. [10] The tumor regression could have resulted from inhibition of angiogenesis or induction of apoptosis or necrosis. Our previous work has shown that the complex could induce apoptosis in cancer cells under *in vitro* conditions [11] In the present work we examined the effect of these complexes on expression of *vegf* and formation of blood vessels in mice having tumor cells (Dalton's Lymphoma Ascites cells) growing in the peritoneal cavity. This tumor model is ideal for testing drugs for their effects on angiogenesis as formation of capillaries and blood vessels on the peritoneal membrane can be easily visualized [12].

## MATERIALS AND METHODS

### Animals

The female Swiss albino mice weighing 23- 26g were selected for the experiment obtained from Government Veterinary College, Mannuthy, Kerala and provided them with free access to food and water. All animal experiments in this study were performed with the prior approval of the Institutional Animal Ethics Committee (IAEC) and were conducted strictly adhering to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) constituted by the Animal Welfare Division of Government of India.

### Chemicals

The drug Berberine was purchased from Sigma Aldrich, India. All chemicals and reagents were obtained from reputed national and international distributors. The iron oxide nanoparticles were prepared by co-precipitation method. Polyvinyl pyrrolidone (PVP) was added to protect thus prepared nanoparticles from agglomeration. The surface of nanoparticles was modified byPolyoxyethylene 25-propylene glycol stearate (POES). The drug- NP complexes were prepared by conjugating the surface with BBN and Sanazole by ultra-sonication. The preparation and characterization of NP and NP- drug complexes were explained in our previous paper.[13]

### Experiment design

Mouse Dalton's Lymphoma Ascites (DLA) cells were maintained by weekly transplantation of $3\times 10^7$ cells in the peritoneal cavity of *Swiss albino* mice. The tumor- bearing animals were divided into five groups of five each. The animals were administered orally for consecutive days with drug and drug- nanoparticle complexes after seven days of tumor transplantation. The treatment procedure was briefed as follows. Group 1(Control): *p.o.* administration of water; Group 2 (NP): administered *p.o.* with 2mg/ kg of NP; Group 3 (BBN): administered *p.o.* with 2mg/ kg of BBN; Group 4 (SAN): administered *p.o.* with 2mg/ kg of SAN; Group 5 (NP-BBN-SAN): administered *p.o.* with 2mg/ kg of NP-BBN-SAN complexes in the ratio of 1:1:1.

**Effect of NP-drug complexes on mouse peritoneum**

The animals were sacrificed on 9$^{th}$ day following various treatments. The peritoneal cavity was cut open and photographed. [12]

**Gene expression study**

The expression of the gene *vegf* was studied by both conventional reverse transcription (RT) and quantitative real time (qRT) PCR. The cells from the peritoneal cavity were collected and isolated RNA by acid guanidiumthiocyanate method. [14] cDNA of these RNAs was synthesized using random hexamer as primer by reverse transcription reactions. The primers used and the cycling conditions are given as supporting data. The amplicon obtained from RT-PCR was visualized by agarose gel electrophoresis. The result of qRT- PCR was expressed as relative fold change compared to respective control. [15]

**Serum biochemical parameters**

The quantitative determination of serum Urea and SGOT (serum glutamate oxaloacetate transaminase) were analysed using Agappe diagnostic kits. [16, 17]

**Statistical analysis**

The results were presented as Mean± SD (standard deviation) and were analyzed by GraphPad PRISM software version 5. Statistical analyses of the results were performed using One- way analysis of Variance (ANOVA) with Tukey-Kramer multiple comparisons test and the values of $p>0.05$ were considered as non- significant.

**RESULTS AND DISCUSSION**

**Serum biochemical parameters**

The levels of serum urea and SGOT in the animals treated with the drug and nano- drug complexes were in the range of normal reference values (SGOT: 54- 298 U/L and Urea: 17.12-70.62mg/dl) indicate that the administration of NP, BBN, SAN and the complexes of NP-BBN-SAN (at a dose of 2mg/ kg body weight) did not elicit systemic toxicity in liver and kidney as can be evidenced from the data presented in table. 1.

**Table 1: Serum biochemical parameters of Kidney and Liver**

| Treatments | Serum Urea (mg/dl) | Serum SGOT (U/L) |
|---|---|---|
| **Control** | 30.16± 2.9 | 56.42± 15.0 |
| **NP** | 39.68± 1.0 $^{ns}$ | 60.67± 8.6$^{ns}$ |
| **BBN** | 29.19± 5.4$^{ns}$ | 71.95± 6.4$^{*}$ |
| **SAN** | 26.07± 5.5$^{ns}$ | 67.67± 4.7$^{ns}$ |
| **NP-BBN-SAN** | 32.98± 3.3$^{ns}$ | 58.46± 6.1$^{ns}$ |

Note:all values are expressed as mean± standard deviation. * indicates the significance with $p<0.05$ and $^{ns}$ indicates non-significance with $p>0.05$ compared to control.

**Effect of NP-drug complexes on mouse peritoneum**

The photographs of peritoneum of control and treated animals were presented to visualize the difference in the angiogenesis in figure 1. The increase in branching of blood vessels, especially large blood vessels, can be seen in control while this was almost absent in the animals treated with NP-BBN-SAN.

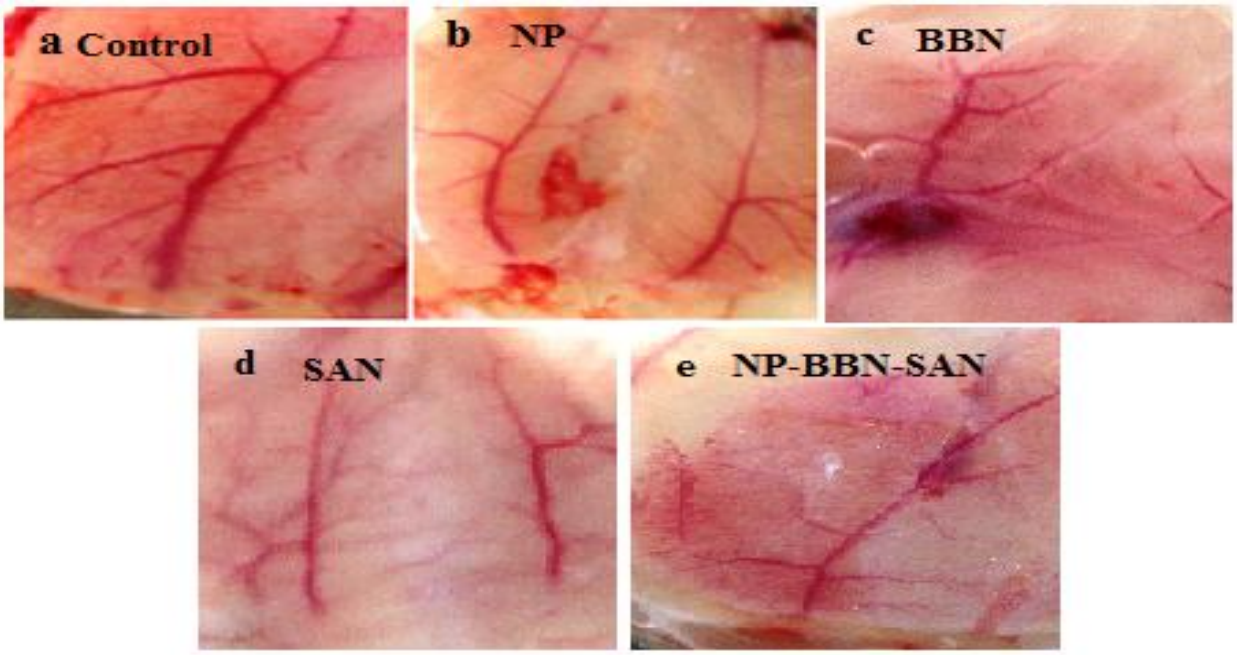


**Figure 1: Peritoneal lining of tumor-bearing mice following various treatments.**

**Expression of *vegf* in tumor cells**

The gene *vegf* is a vital pro-angiogenic factor. The gel images in figure 2 presents the results on transcription analysis of this gene by RT- PCR. The transcription level of *vegf* gene in terms of band intensity can be compared to the house-keeping gene, beta-actin. The qRT-PCR results were used to find out the fold change in *vegf* expression in comparison with control and figure 3 presents the data. In the figure, the expression of *vegf* in all treatment groups was calculated by keeping the expression in control group as baseline.

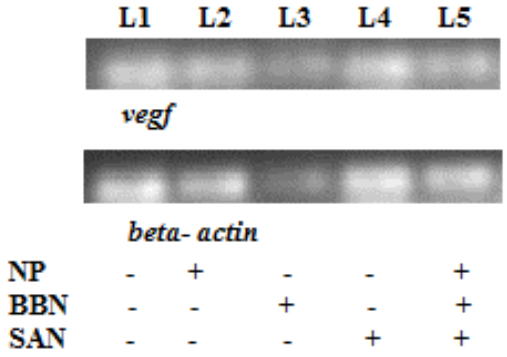


**Figure 2: Image of agarose gel electrophoresis. L1: Control, L2: NP, L3: BBN, L4: SAN and L5: NP- BBN- SAN.**

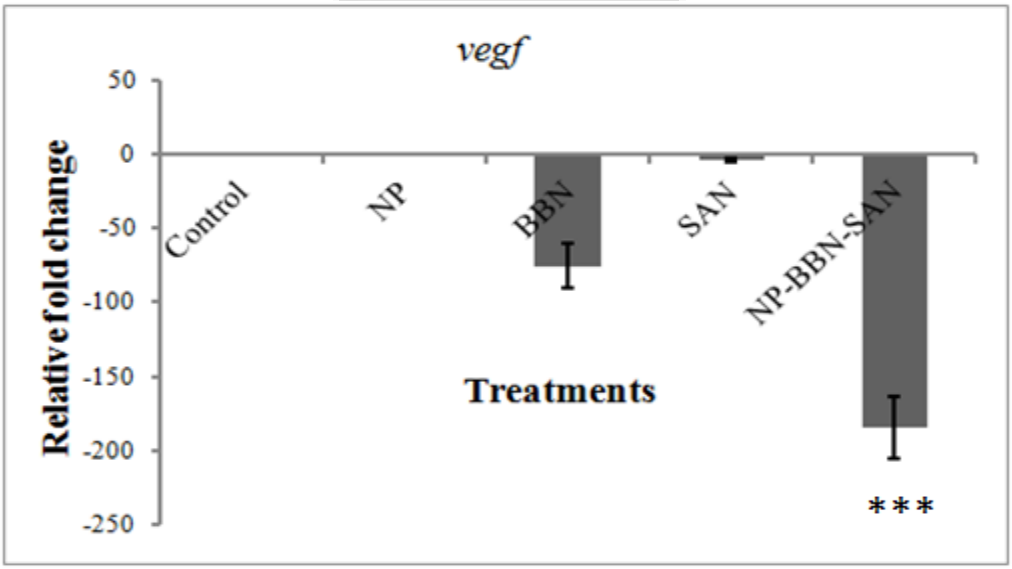


**Figure 3: Relative fold change in the expression of *vegf*after treatments. Note: all values are expressed as mean± SD. *** indicates the significance with p<0.001 compared to control.**

Higher level of angiogenesis was discernible in the control group compared to the treated ones. The expression of *vegf* in the tumor cells was down regulated in all treatment groups compared to the control group, In NP-BBN-SAN treated tumor cells the expression was significantly ($p<0.001$) down regulated. These results from qRT-PCR corroborated the results presented in figure 1.

## DISCUSSION

Inhibition of tumor- induced angiogenesis has been used as a major target to control tumor growth and invasion. Anti-angiogenesis therapy was found to be more effective in combination with chemotherapy and radiotherapy. The combination therapy of anti-angiogenic drug with cytotoxic chemotherapeutic agent would have increased the reduction in tumor growth. [18-20]Most anti- angiogenesis agents including natural products mainly act on VEGF as it can stimulate proliferation and migration of endothelial cells to support growth and metastasis of tumor. [21-23].

In our study, NP-BBN-SAN complexes were shown inhibitory effect on tumor- induced neovascularisation. The cytotoxic drug BBN decreases the expression of *vegf* however; a synergistic effect can be seen in the case of NP-BBN-SAN. The significant down regulation of *vegf* expression in this treatment could substantiate the result observed in peritoneum images and indicate that the reduction in tumor-induced vascularisation in NP-BBN-SAN treatment is due to the inhibition of *vegf* transcription. The tumor suppressor gene p53 was found to be a key regulator of angiogenesis by activating the inhibitors of vegf-A. [24]. Hypocia- inducible factor-1a (HIF-1a) binds to and stabilizes p53. The interaction of p53 with HIF-1a results in inhibition of HIF-stimulated transcription. The transcription of genes involved in tumor survival under hypoxic conditions including VEGF are stimulated by HIF-1. The mutations in p53 could enhance expression of angiogenic factors. The induction of p53 under tumor hypoxia requires associated induction of HIF-1α, which forms a heterodimer with HIF-1β to form HIF-1 which inturn positively regulate transcription of *hif-1α* and several angiogenic factors including *vegf* and subsequent VEGF expression [25,26] Several alternative VEGF- related pathways are involved in the process, angiogenesis. One possilble mechansm is through the interaction between VEGF and its receptor, results in the autophosphorylation of Tyrosine residues. Further downstream signalling activates molecules such as protein kinase B (Akt/PKB), endothelial

nitric oxide synthase and GTP-associated Rac protein. The activated Akt/PKB inhibits B-cell lymphoma 2 (Bcl2) associated cell death mechanism and niric oxide generated by endothelial nitric oxide synthase enhances vascular permeability, lead to increase in cell survival and migration. [27,28]

The analyses on the serum biochemical markers such as urea and SGOT indicate the absence of systemic toxicity of the treatments.

## CONCLUSION

The NP-BBN-SAN complex was found to down regulate *vegf* expression and thereby prevent tumor- induced angiogenesis. This finding could be a stepping stone in the future application of the complexes in tumor therapy.

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### Competing interests

The authors have no financial and nonfinancial competing interests. Also the authors have no conflict of interest.

### Author Contributions

The authors have contributed equally.

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