Chapter VI

Tissue distribution of Nanoparticle-drug complexes and effect of these complexes on tumor angiogenesis

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*****Most of the results in this chapter merited publication in International Journal of Pharmacy and Pharmaceutical Research as *S Sreeja, CKK Nair. Control of vegf Expression and Tumor Angiogenesis by Nanoparticle- Berberine- Sanazole Complexes. Int J Pharm Pharm Res.2015; 4(4):161-170.*

The iron oxide nanoparticle-berberine-sanazole (NP-BBN-SAN) complexes were administered to animals bearing solid tumor on the hind limbs and the tumor specific accumulation of these complexes was studied by ferrozine assay, spectrophotometry analysis and tumor imaging. The 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.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. 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 theNP-BBN-SAN complexes in tumor therapy.

6.1 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 [Leung et al., 1989; Olofsson et al., 1996].

Malignant cells frequently express angiogenesis promoting factors. It has been realized for long time that control of angiogenesis could inhibit tumor growth and metastasis [Folkman, 2007, 1971, 1972] 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 [Kim et al., 1993]. Bevacizumab, a monoclonal immunoglobulin G antibody, has been shown to block the expression of VEGF receptor in endothelial cells and prevented neovascularisation in tumor [Ferrara et al., 2004]. In metastatic colorectal cancer patients, Bevacizumab in combination with chemotherapy extended life by a few months [Kabbinavar et al., 2005]. 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 [Hamsa and Kuttan, 2012].

In the present work, iron oxide nanoparticles were complexed with Berberine, a cytotoxic plant alkaloid, and Sanazole, a hypoxic radiosensitizer. The complex, nanoparticle-berberine- sanazole (NP-BBN-SAN) has been reported to cause regression of solid tumors in mice [Sreeja and Nair, 2015c]. 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 [Sreeja S and Nair, 2015b]. 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 [Chandru et al., 2008].

6.2 MATERIALS AND METHODS

6.2.1***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 CPCSEA, Government of India.

6.2.2***Chemicals***

The drug BBN was purchased from Sigma Aldrich, India. All chemicals and reagents were obtained from reputed national and international distributors.

The preparation and characterization of NPs and NP-drug complexes were detailed in previous chapters.

6.2.3 ***Drug release study***

The pH (3.5, 6.6 and 7.4) triggered drug (BBN) release from NP-BBN-SAN complexes under *in vitro* condition was studied by dialysis method [Kapoor et al., 2014].

6.2.4 D*etermination of concentration of iron and berberine in tissues*

DLA tumor was transplanted on the hind limbs of animals and administered with nano-drug complexes. The iron content in tumor and liver 4hrs following the administration of NP-drug complexes was examined by ferrozine assay. BBN was extracted from the tissue homogenate by solvent extraction using isoamyl alcohol and absorbance was measured at 480nm. The concentration of BBN in tumor was calculated based on a standard curve plotted using various concentrations of BBN. The thin sections of tumor were also observed under fluorescent microscopeone hour after the administration of complexes.

6.2.5 *Drug excretion study*

The major route of excretion of the orally administered BBN is through gut as faeces. The animals bearing tumor on the hind limbs were administered with NP-BBN and NP-BBN-SAN complexes (20mg/kg) and all faecal samples were collected at time intervals. The drug BBN was extracted from the faecal homogenate by solvent extraction method. The amount of BBN in the extracts was determned spectrophotometrically.

6.2.6 *Tumor imaging*

The animals bearing tumor in the hind limb were anesthetized [Ketamine-xylazine; 100µl (1mg/ml ketamine + 0.1mg/ml xylazine) per animal] following oral administration of NP-BBN-SAN complexes and imaging was taken using XENGEN IVIS imaging system.

6.2.7*Experiment design (for analysing anti-angiogeneic potential of the complexes)*

Mouse Dalton's Lymphoma Ascites (DLA) cells were maintained by weekly transplantation of 3×10^7 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 20mg/kg of NP; Group 3 (BBN): administered *p.o.* with 20mg/kg of BBN; Group 4 (SAN): administered *p.o.* with 20mg/kg of SAN; Group 5 (NP-BBN-SAN): administered *p.o.* with 20mg/kg of NP-BBN-SAN complexes in the ratio of 1:1:1.

6.2.8*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 [Sreeja S and Nair, 2015b].

6.2.9***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 guanidium thiocyanate method [Chomczynski and Sacchi, 1987]. cDNA of these RNAs was synthesized using random hexamer as primer by reverse transcription reactions. The primers used and the cycling conditions are detailed in chapter II. 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 the respective control [Thomas and Kenneth, 2008].

6.2.10 ***Serum biochemical parameters***

The quantitative determination of serum Urea and SGOT (serum glutamate oxaloacetate transaminase) were analysed using Agappe diagnostic kits [Kassirer, 1971; Winkelman et al., 1974].

6.2.11 ***Statistical analysis***

The results were presented as Mean±SDand 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.

6.3 RESULTS

6.3.1 ***Drug release study - In vitro***

As it was found (figure 6.1a) that the release of the drug, in 6hrs, was higher at pH 6.6. Further studies on longer duration of release were done only at this pH. There was a release of 68% of drug at 52hrs (figure 6.1b), suggested sustained and continuous release of BBN from the nanoparticles.

6.3.2 ***Analysis on levels of Iron and BBN in tissues following the administration NP-BBN-SAN complexes***

The tumor specific distribution of NP-BBN-SAN complexes following per oral administration of these complexes (20mg/kg) in tumor-bearing animals were analysed in liver and tumor by ferrozine assay (for iron concentration) and fluorescent assay (for BBN concentration). The ferrozine assay revealed that the NP-BBN-SAN complexes get

distributed in tumor more rapidly as can be evidenced from the data presented in figure 6.2a. However, there were no such variations observed in liver tissues (figure 6.2b). The ferrozine assay did not give satisfactory results as there was enormous variation in the iron concentration in tissues.

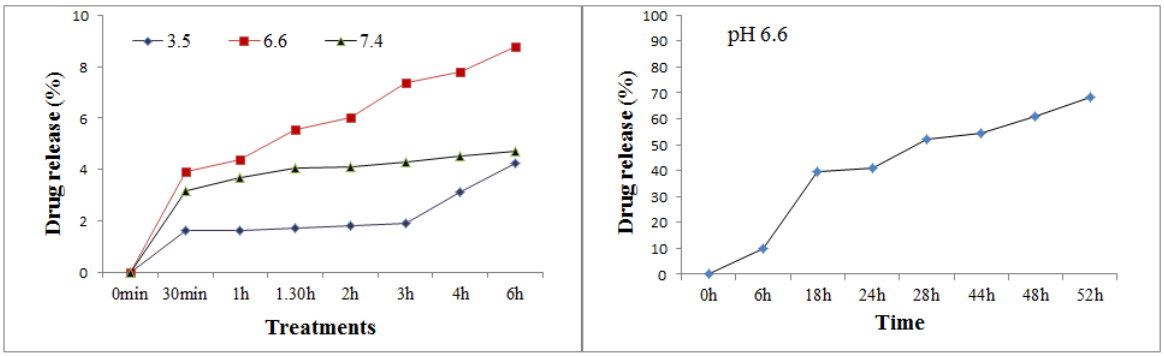

Figure 6.1: The concentration of releasedBBN from the nanocomplexes in time - and pH - dependent manner.

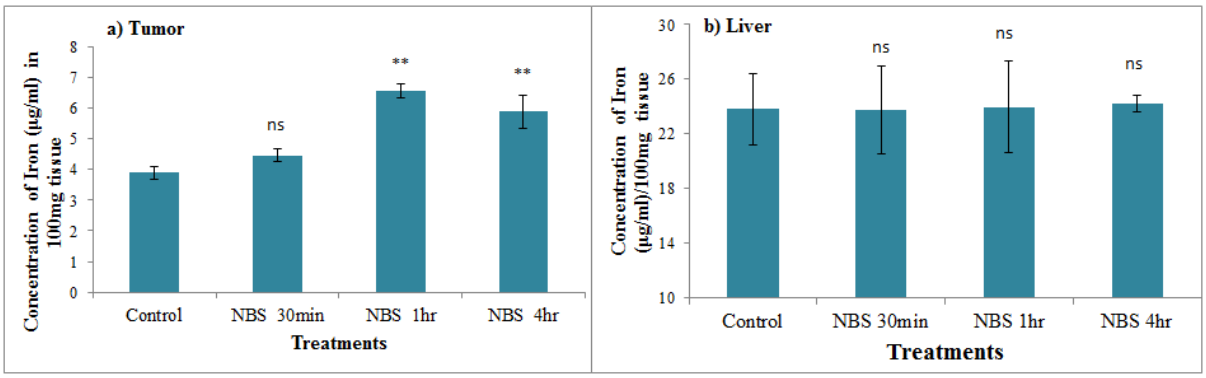

Figure 6.2: Concentration of iron in tumor (a) and liver (b) tissues. Note: all values are expressed as mean ± SD. * indicates the significance with $p<0.05$ and ^{ns} indicates non-significance with $p>0.05$ compared to control.

Following *p.o.* administration of NP-BBN and NP-BBN-SAN complexes to tumor-bearing animals, the highest concentration of BBN in tumor reached at around 4hrs in case of NP-BBN while animals treated with NP-BBN-SAN showed highest concentration at 1hr and much lower level at 4hrs (figure 6.3). This would suggest that presence of SAN facilitates fast accumulation of the complexes in the tumor compared to the complexes without SAN. This assumption is further supported by the studies on the fluorescent microscopic examinations of the tumor tissue sections as can be evidenced from figure 6.4.

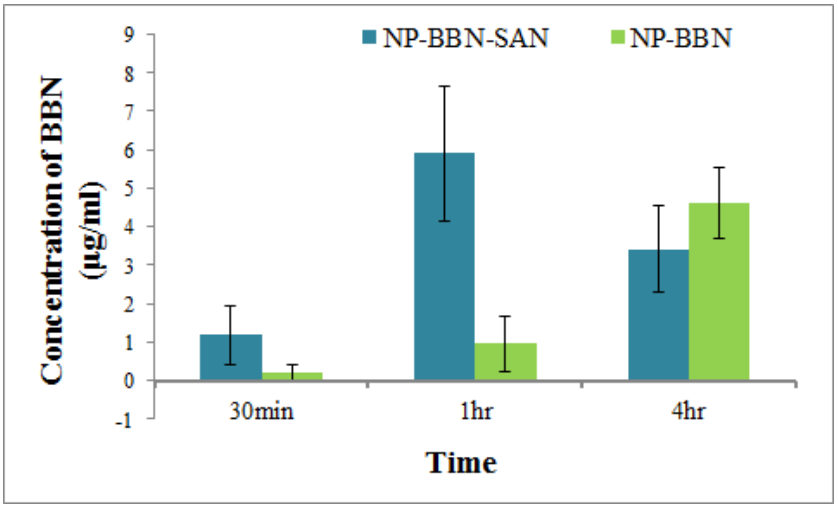

Figure 6.3: Concentration of BBN in tumor tissue following the administration of NP-BBN and NP-BBN-SAN complexes.

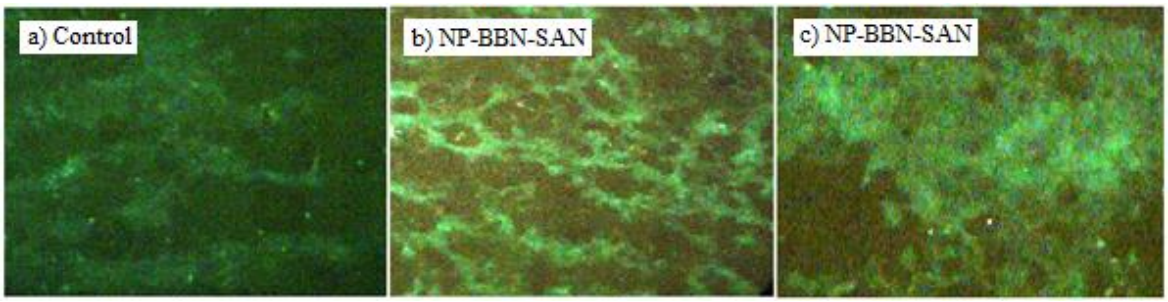

Figure 6.4: Fluorescent microscopic images of tumor tissue following the treatment with NP-BBN-SAN. Note: a: Control (untreated, tumor-bearing); b and c: NP-BBN-SAN

6.3.3 *Tumor imaging*

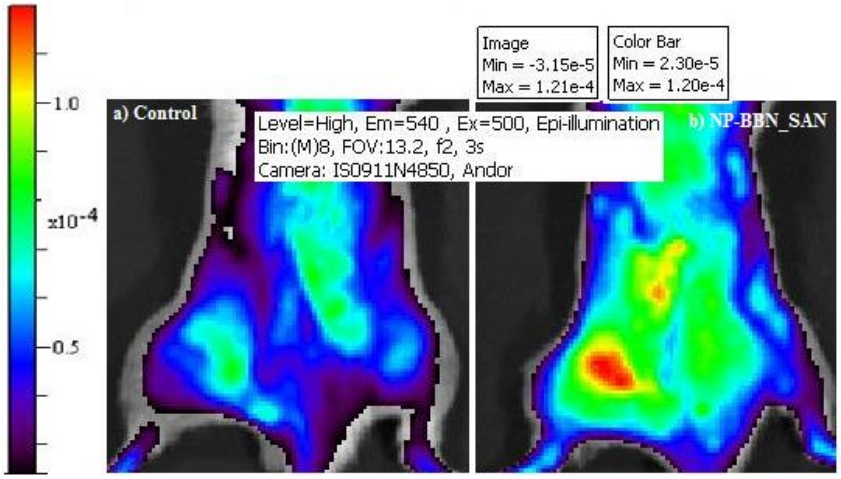

Figure 6.5: Imaging of solid tumor- bearing animals following oral administration of NP-BBN-SAN complexes [a) Control; b) NP-BBN-SAN].

We also investigated whether the complexes NP-BBN-SAN can be used for tumor imaging. The complexes were administered to tumor- bearing animals and the animals were scanned in an imaging system (XENGEN IVIS, USA). Two hours after administration, it can be seen that the nano-complexes got accumulated more in tumor than in other tissues as can be evidenced from figure 6.5. Thus, the complexes have good potential to be used for tumor imaging for diagnosis.

6.3.4 *Analysis on drug excretion - in vitro*

The faecal samples collected for 24hrs from the animals following treatment with NP-BBN-SAN (500μg/animal) complexes contained about 22% of BBN administered and about 16% in 48 hr while in case of NP-BBN (500μg/animal) treatment the BBN contents in faecal samples were ~48% and ~20% respectively at these intervals (Table 6.1). The balance amounts of the administered BBN in the complexes were most probably retained in tumor tissues or excreted through urine.

Table 6.1: Amount of BBN excreted through faeces following the administration of NP-BBN and NP-BBN-SAN complexes (The amount of BBN administered through the complexes was 500μg).

Treatments	0hr	24hrs	48hrs
	Amount of BBN released (μg)		
NP-BBN-SAN	0.0	114.23	83.32
NP-BBN	0.0	242.08	102.9

6.3.5 *Effect of NP-drug complexes on tumor-induced angiogenesis*

6.3.5.1 *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 20mg/ kg body weight) did not elicit systemic toxicity in liver and kidney as can be evidenced from the data presented in table 6.2.

6.3.5.2 ***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 6.6. 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.

Table 6.2: 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^{ns}
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.

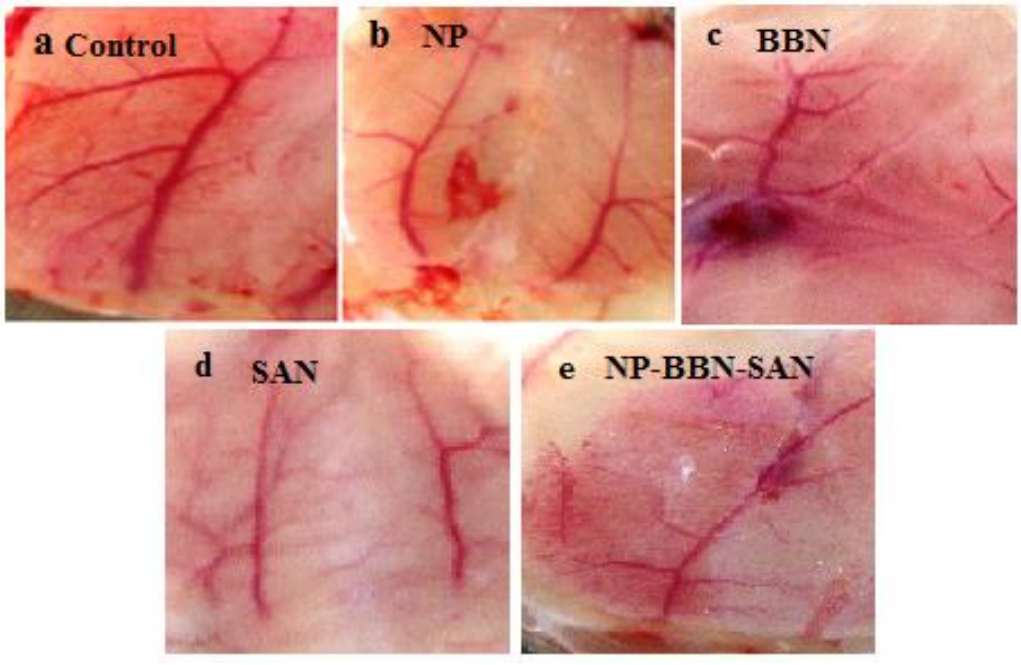

Figure 6.6: Peritoneal lining of tumor-bearing mice following various treatments.

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

The gene *vegf* is a vital pro-angiogenic factor. The gel images in figure 6.7 presents the results on transcription analysis of this gene by RT-PCR. The qRT-PCR results were used to find out the fold change in *vegf* expression in comparison with control and figure 6.8 presents the data. In this figure, the expression of *vegf* in all treatment groups was calculated by keeping the expression in control group as baseline.

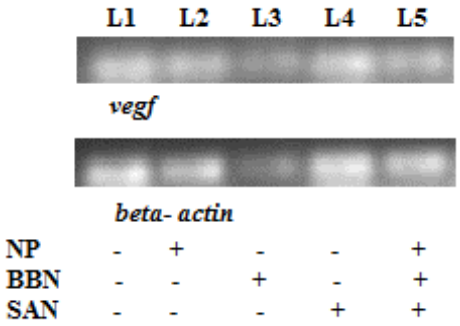

Figure 6.7: Image of agarose gel electrophoresis. L1: Control, L2: NP, L3: BBN, L4: SAN and L5: NP- BBN- SAN.

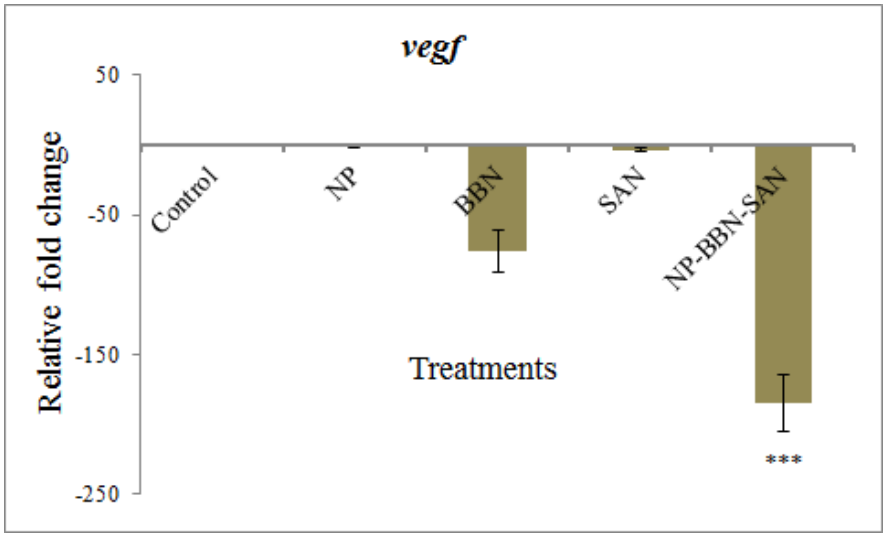

Figure 6.8: Relative fold change in the expression of *vegf*. Note: all values are expressed as mean± SD. *** indicates the significance with $p<0.001$ compared to the control.

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 6.6.

6.4 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 [Ansiaux et al., 2005; Ma and Waxman, 2008; Bonner et al., 2006]. 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 [Folks, 1995; Riasu, 1997; Folkman, 1986].

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 [Fontanini et al., 1998]. 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 is 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 [Zhong et al., 1999; Blagosklonny et al., 1998]. 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 [Gerber et al., 1998; Cross et al., 2003]. The analyses on the serum biochemical markers such as urea and SGOT indicate the absence of systemic toxicity of the treatments.

6.4 CONCLUSION

The NP-BBN-SAN complex was found to accumulate more in tumors, and 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.