ABSTRACT

Cancer, also termed as malignant tumor, is a dreadful disease of mankind and its incidence is ever increasing almost in geometric progression both in developed and developing countries. It is a group of diseases characterized by uncontrolled cell growth and proliferation. Detection of cancer at an early stage increases the chances of complete cure of the disease, but in most cases it is too late.In-spite of human attempts to eradicate this disease from ancient times and the present day's spectacular achievements in the field of medicine and science, a definite cure for cancer is yet to be found.

The major treatment modalities available as on today are surgery, chemotherapy, radiotherapy, hormone therapy, gene therapy, etc. However, most of them are associated with toxicities and concomitant side effects. Other treatments such as immune therapy, photodynamic therapy, laser therapy, stem-cell therapy, etc. are also attempted; however, all these are still in the infancy stage. Most of the chemotherapeutic agents cause systemic toxicity and side effects as they are non-specific in action. Specific targeting of therapeutic agents to tumors is the only means to avoid these problems. Nanoparticles because of their small size (1-100nm) can enter into the cells. Tumor, because of their altered vasculature and lack of lymphatic drainage system, together with changes in the extracellular matrix, can retain these nano-sized particles (Enhanced Permeability and Retention (EPR) effect). If drugs - cytotoxic/antineoplastic - are attached with nanoparticles, they can specifically kill the cells of the tumors.

The thesis comprises studies on specific targeting of cytotoxic and antineoplastic drugs, using physical and chemical targeting agents, to solid tumors in mice by complexing them with Iron-oxide nanoparticles. Iron-oxide nanoparticles have gained attention in cancer therapy because of its magnetic property and biocompatibility. An external magnetic field was used as physical agent and the nitrotriazole compound Sanazole was used as a chemical agent for tumor-directed targeting of the nanoparticle-drug complexes. Investigations were done at molecular and cellular level to understand the mechanism underlying the success of these targeted therapeutic modalities and the thesis embodies the results.

The thesis comprises ten chapters. Chapter I provides an introduction to the topic of the thesis and review of literature on the different aspects covered in the thesis. Targeted

tumor therapy using nano-drugs is a major development in recent times. Various aspects of the modality of targeted cancer therapy and their underlying mechanisms along with their advantages and deficiencies are discussed. Chapter II discusses the details of the materials and methodologies employed in the studies presented in the thesis. The chapters III to IX present the data emanating from the investigations and discuss the results of the studies in detail, and chapter X displays summary and conclusion.

Chapter III concernsstudy on magnetic nanoparticles directed delivery of Berberine - a cytotoxic phytoceutical, for tumor control.Solid tumors were developed on the hind limbs of *Swiss albino* mice by transplanting Dalton Lymphoma Ascites tumor (DLA) cells. Iron-oxide nanoparticles (NP(s)) were synthesized and complexed with a cytotoxic isoquinoline alkaloid Berberine (BBN) and these complexes (NP-BBN complexes) were targeted to the tumor site in mice - DLA solid tumor on hind limbs - by the application of an external magnetic field. The NPs were characterized by FTIR, XRD and TEM. The tumor-bearing animals were orally administered with NP-BBN complexes (100mg/kg) for seven consecutive days and targeted them to tumor specifically by the application of an external magnetic field. The tumor volume was measured and found to be reduced significantly in NP-BBN-Magnet treatment compared to control and other treatments. The transcriptional expression studies with real time PCR revealed that the underlying molecular mechanism of the tumor regression was due to altered expression of the genes *bax, bcl2, caspase 9, caspase 8* and *caspase 3*. The NP-drug complexes at the doses were found to be non-toxic as there was no change in blood and serum biochemical parameters in the treated animals. Tumor histopathology of control animals showed large number of pleomorphism as a sign of healthy cancer cells. Necrosis and condensed nuclei, as a sign of apoptosis, can be seen in tissues of NP-BBN complexes and magnetic field treated animals. The normal tissues like kidney and liver did not display any morphological alterations in these treated animals. The study suggested apoptosis through the intrinsic pathway could be the underlying mechanism of tumor regression by the magnetic NP directed delivery of BBN to tumor.

Chapter IVis oneffect of hypoxic cell sensitizer, Sanazole on transcription of *hif-1α* and its target genes in tumor cells. Hypoxic sensitizers have the ability to sensitize hypoxic cells in tumor. In this part of study, investigations were done on the effect of nitroimidazole hypoxic cell sensitizer, Sanazole (SAN), on the transcription of genes involved in tumor

progression under hypoxia - *hif-1α, vegf* and *egfr* -in DLA cells under *in vitro* and *in vivo* conditions. The gene *hif-1α* was up-regulated by SAN under normoxic condition while it was down-regulated under hypoxic condition, *in vitro*. The results indicate that the influence of SAN on the expression of *hif-1α* is totally dependent on the availability of oxygen in the cellular microenvironment. The same pattern of expression was observed in the case of *vegf* (angiogeneic factor) and *egfr* (cell proliferation). Under *in vivo* condition, the transcription of the gene *hif-1α* was up regulated in hypoxic tumor while it was down regulated significantly after the treatment with SAN compared to the untreated control cells. Thus, the study revealed the role of SAN in hypoxia-induced tumor growth and this compound could be useful for targeting cytotoxic drugs to hypoxic solid tumor.

Chapter Vdescribes *in vitro* study on anticancer property of complexes of iron-oxide nanoparticle with Berberine and Sanazole.NP-BBN complexes were conjugated with hypoxic cell sensitizer SAN (NP-BBN-SAN). The cytotoxic potential of NP-BBN-SAN complexes and the molecular mechanism behind it were explored in murine tumor cells (DLA) prior to *in vivo* experiments. The DLA cells were incubated with NP-drug complexes (200µg/ml) at 37^0C. The cells treated with these complexes showed significant increase in cytotoxicity, apoptosis and extensive damages in cellular DNA compared to the untreated control cells. The cells treated with NP-BBN-SAN complexes had greater DNA damage compared to other treatments. The increase in the expression of pro-apoptotic gene suggested the role of apoptosis as the underlying mechanism of cytotoxicity elicited by these nano-complexes. Complexing with SAN increased the cytotoxic potential of NP-BBN complexes.

Chapter VI describes the tissue distribution of NP-drug complexes and effect of these complexes on tumor angiogenesis. Based on the ferrozine assay (for iron concentration) and spectrophotometry analysis (for BBN concentration), faster accumulation of drug was observed in tumor tissues of animals administered orally with NP-BBN-SAN (20mg/kg), suggesting SAN assisted accumulation of the complexes in the tumor. NP-BBN-SAN complexes were administered to tumor-bearing animals and the animals were scanned in an optical imaging system, two hours after the oral administration. It can be seen that the nano-complexes got accumulated more in tumor than in other tissues, indicating the use of these complexes in tumor imaging as apart of diagnosis.

The complexes were evaluated to identify their effect on angiogenesis - an essential event for tumor progression. Of the various pro-angiogenic factors, Vascular Endothelial Growth Factor (VEGF) plays a crucial role in the process. The complexes (20mg/kg) were administered to animals inoculated with tumor cells in the peritoneal cavity. NP-BBN-SAN complexes inhibited angiogenesis in mice were visualized from the images of inner peritoneal membrane. 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 study thus provided compelling evidence that NP-BBN-SAN complexes down regulated *vegf* expression and thereby prevented neovascularization which is a must for tumor growth and metastasis.

Chapter VII describes thestudies on tumor control by targeting Nanoparticle- Berberine complexes with Sanazole**.** Hypoxia-targeted drug delivery has attained great importance in tumor therapy. Specific targeting of cytotoxic drugs to the hypoxic region in a tumor can prevent hypoxia-associated expression of genes and enhance tumor regression. The animals-bearing solid tumor on the hind limbs were administered with the drugs and nano-drug complexes (BBN, SAN, NP-BBN, NP-SAN and NP-BBN-SAN; 20mg/kg). The tumor volume was increased in the control group compared to the other treatment groups. The group treated with NP-BBN-SAN had significantly higher regression of tumor volume compared to the other groups. There was also down regulation in the expression of *hif-1α* and its target genes in the NP-BBN-SAN treated group. Thus, the results suggest that SAN enhances the therapeutic efficacy of the NP-BBN complex. The animals were observed for one month and tumor volume was calculated.

The animals treated with NP-BBN-SAN complexes were shown 100% survival up to one month. The untreated control group at the end of one month had less than 20% survival while the NP-BBN group had a survival of less than 50%. In NP-BBN-SAN group, there was no detectable tumor (complete regression of tumor) at the end of one month while in other groups the regression was only a partial and in the control there was an increase in the tumor volume. The treatment could completely eradicate the tumor, while the tumor volume was significantly increased in control (tumor-bearing untreated) animals. The mechanism of tumor regression by NP-BBN-SAN complexes could be through induction

of apoptosis in tumor tissue. There was an elevated transcription of apoptotic genes and altered morphology of the cellular architecture in the tumor of NP-BBN-SAN treated group. These results suggest the potential therapeutic utility of NP-BBN-SAN in cancer treatment.

The cellular antioxidant parameters - GSH, GPx and SOD - in tumor of the animals were found depleted upon administration of NP-BBN-SAN complexes. Also, there was an increase in the levels of peroxidation of lipid in the cells of tumor tissue. The levels of β-D glucuronidase and myeloperoxidase were found to be significantly reduced in tumor of these animals. Myeloperoxidase not significantly affected kidney and liver in these animals.Thus, the study revealed the potential application of SAN and NP in targeted delivery of the cytotoxic alkaloid BBN to solid tumor for higher therapeutic efficacy.

Chapter VIII displaysa comparative study on tumor targeting of nanoparticle-drug complex by external magnetic field and hypoxic cell sensitizer.This chapter focuses on the comparative study between tumor specific delivery of NP-BBN complexes by a physical method using an external magnetic field and a chemical method using hypoxic cell sensitizer, SAN which gets concentrated in solid tumor (Das et al., 2004). The tumor-bearing animals were administered with the complexes and tumor volume was measured to monitor tumor-regression. The regression of the tumor was observed in animals treated with NP-BBN-SAN complexes and NP-BBN complexes along with the application of external magnetic field, compared to the untreated control.

The results obtained from the histopathology of tissues (tumor and liver) and serum biochemical parameters of the treated animals, suggesting the absence of systemic toxicity particularly to liver. Molecular studies and comet assay substantiated that the mechanism underlying the tumor regression was due to induction of apoptosis by both intrinsic and extrinsic pathways. The data provide that in the case NP-BBN-Magnet treatment intrinsic pathway of apoptosis was more predominant. The comparative study revealedthat both methods are useful, however; the physical method can be operative only with magnetic nanoparticles and also, may be in peripheral tumors, not in deep-seated ones.

Chapter IX describesstudies onchemo-directed specific targeting of nanoparticle-doxorubicin complexes to tumor in animal model.The therapeutic potential of antineoplastic drugs is often limited by systemic toxicities. Targeted drug delivery totumor

sites can eliminate the toxicities and side effects. In the present study the chemotherapeutic doxorubicin (DOX), which causes cardiotoxicity, was complexed with iron-oxide nanoparticles together with a hypoxic cell radiosensitizer SAN for specific targeting to tumor sites. The complexes (NP-DOX-SAN) were characterized by FTIR, TEM and Size analysis. The IR spectrum of the complexes showed the characteristic peaks of DOX and SAN indicated that the drugs were adsorbed on NPs. The shape of the complexes was somewhat spherical with the size of less than 50nm as evidenced from TEM analysis. The hydrodynamic size of the particle in aqueous medium determined by nanosize analyser was 188.07±32.3nm. NP-DOX-SAN complexes (50µg/ml) increased apoptosis in tumor cells under *in vitro* condition as characterized by apoptotic morphology, typical comet tail formation and the expression of genes - *bax* and *bcl2*.

The tumor volume was reduced significantly in mice administered with NP-DOX-SAN complexes (8mg/kg) compared to untreated control. Morphological studies of tumor and normal tissues(heart, liver and kidney), and also the data on serum biochemical parameters revealed the specific action of the complexes on tumor without any systemic toxicity to the liver, heart and kidney. In tumor, studies by qRT-PCR revealed significant down regulation in the transcription of the gene *hif-1α* and up regulation of *bax* and *caspases*, further confirm the apoptosis induction by the complexes. Thus, the study reveals the potential application of SAN to direct and specifically target nano-complexes of antineoplastic drugs to the cells of solid tumors, to cause tumor regression without unwanted systemic toxicities and side effects.

Conclusions: Tumor specific targeting of cytotoxic drugs using SAN and NPs enhanced the therapeutic efficacy without systemic toxicities. A decline in the transcription of hypoxia associated genes, tumor markers and antioxidant levels were observed in NP-BBN-SAN treated animals which would indicate the antitumor effect of the complexes. The comparison between the two methods of specific targeting - physical (applying an external magnetic field) and chemical (complexing with SAN) - revealed that the chemical directed targeting has more potential benefits and convenience than the physical method. This chemical targeting strategy was effective in the specific delivery of currently used chemotherapeutic agent, DOX, to solid tumors in mice. However, more studies are needed for clinical trials of this strategy, of tumor targeting nano-drug complexes with SAN, for therapeutic application.