Chapter I

Introduction and Review of Literature

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1.1 *Tumor - major treatment modalities*

Tumors are unusual mass of tissues characterized by undisciplined growth and proliferation of cells. These can be benign (non-cancerous) and malignant (cancerous) [American Brain Tumor Association]. Cancer or neoplasm is the leading cause of human mortality, next to cardiovascular diseases. Apart from uncontrolled proliferation, the other characteristics of cancer are evasion of apoptosis, angiogenesis, invasion of tissues, and metastasis to different locations in the body [Hanahan and Weinberg, 2011]. The proliferation and metastasis are the main causes of cancer mortality [Chaffer and Weinberg, 2011].There is a steady yearly increase in the number of new cases of cancer in both developed and developing countries, in spite of the spectacular developments in medical sciences. The present treatment possibilities of cancer include – surgery, radiation therapy, chemotherapy, immune therapy, hormone therapy, gene-therapy, stem cell therapy etc. Among these, surgery, radiation therapy, and chemotherapy are most widely used.

1.1.1 Surgery: It is one of the major treatment modalities of cancer for the past few decades. It presents greatest chance to alleviate many types of cancer especially those are not spread to other parts of the body. Surgery can also be used to take samples to diagnose and predict the stage of cancer. Combination of surgery either with chemotherapy or radiotherapy is also found to be effective in curing cancer [American Cancer Society].

1.1.2 Radiation therapy:

Radiation therapy or radiotherapy is the most important modality of cancer treatment. It is highly cost effective and approximately 80% of all cancer patients require radiation therapy either for curative or palliative purpose [Delaney et al., 2005; Bernier et al., 2004]. The high energy radiations deposit energy while passing through tissues causing ionizations to produce free radicals and damaging the vital biological targets- cellular DNA and membrane - resulting in mortality of cancer cells [Jackson and Bartek, 2009; Lomax et al., 2013]. Advances in imaging techniques, computerized treatment planning systems, radiation treatment machines (with improved X-ray production and treatment delivery), use of high energy particles, etc have contributed a great deal to the success of radiation therapy [Bernier et al., 2004].Due to rapid proliferation, the tumor cells over grow their vascular supply, resulting in centrally necrotic and hypoxic regions where the

cells are refractory to radiation. To overcome this problem, either cells of the tumor have to be sensitized to radiation by using hypoxic cell sensitizers or higher doses of radiation have to be used. Clinically use of higher doses of radiation is not possible as the normal cells, surrounding the tumor, are well perfused, vascularized and remain oxygenated, and are therefore suffer more radiation damage. This necessitates the protection of the normal cells from radiation injury. Amifostin or ethyol is the only clinically approved compound available to protect the normal cells [Nair et al., 2001].

Number of compounds (radiosensitizers) have been synthesized and reported to enhance the efficacy of radiation therapy [Sheehan et al., 2010]. Most of them are completed preclinical studies successfully and failed in clinical trials due to toxicity to mammalian organisms.Also several hypoxic cell sensitizers useful in radiotherapy of cancer are at different stages of clinical trials [Coleman et al., 1988].

The nitrotriazole compound, **Sanazole or AK-2123**(figure 1.1) is an effective hypoxic cell radiosensitizer. The hypoxic cell radiosensitizing property of this compound and its lack of toxicity made it attractive as an adjuvant in radiation therapy of cancer. It has successfully completed phase III clinical trials and used in clinic along with radiation to treat different types of cancers such as head and neck, cervical cancer etc. as hypoxic radiosensitizer [Huilgol et al., 2000].

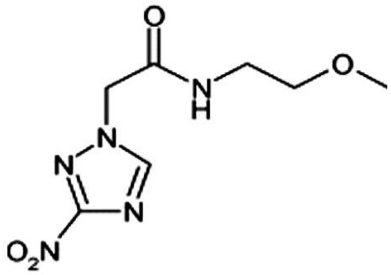

Figure 1.1: Chemical structure of Sanazole (Molecular Formula: $C_{21}H_{32}N_2O$)

The treatment with Sanazole (SAN) enhanced radiation sensitivity in post-irradiated aerobic and anaerobic cells [Imamura et al., 1995]. In patients with advanced head and neck cancer, the administration of SAN increased the sensitivity to hyper fractionated radiation treatment [Huilgol et al., 1996]. SAN has, also, the capability to enhance the therapeutic potential and reduce the effective dose of antineoplastic agents in comparison with the drug alone [Konovalova et al., 1995; Rao and Devi, 1996; Goncharova et al.,

2000].Konovalova et al (1997) revealed that the therapeutic concentration of Sanazole demonstrated anti-metastatic activity in tumor-bearing mice. AK2123 has been found to augment the antineoplastic activity of the chemotherapeutic agent Mitomycin C in tumors of multidrug resistant [Goncharova et al., 2000]. According to Schepetkin et al (2001), the bio-activation as well as the radiation and chemotherapy - sensitizing property of SAN could be due to the involvement of enzymes such as xanthine oxidase and microsomal NADPH/cytochrome p450 reductase.

The ability of SAN to accumulate in tumors was first demonstrated by Murugesan et al., in 2001 via administering Technetium-99m labelled cyclam sanazole to solid tumor-bearing animals. This study also revealed its potential in tumor imaging. Further, Das et al in 1994 explored the hypoxic tumor targeting capability of SAN. The mechanism of sensitization of hypoxic tumor by SAN is partially credited to its ability to induce increased DNA damage [Pasupathy et al., 2001]. In human lymphoma cells (U937), SAN found to cause Fas ligand-induced Caspase 8 dependent apoptosis with the down regulation of hsp70 protein [Yu et al., 2009].

1.1.3 Chemotherapy: Chemotherapy is a major therapeutic strategy in medical oncology using chemical agents or drugs to destroy cancer cells. Based on the mechanism of action, these agents are categorized mainly into alkylating agents, anti-metabolites, anti-microtubule agents, inhibitors of topoisomerase, and cytotoxic antibiotics.

- ***Alkylating agents***

These agents can alkylate macromolecules such as proteins and nucleic acids, derived from Mustard gas used in World War I [Siddik, 2005]. They can damage genomic DNA, generate inter- and intra-strand cross links in DNA resulting in inhibition of cell division (S-phase) and induce apoptosis. Other alkylating agents used in chemotherapy are cisplatin (figure 1.2.1) and its derivatives (carboplatin and oxaliplatin), nitrosoureas, cyclopho-sphamide(figure 1.2.2), mitomycin, etc. [Damia and D'Incalci, 1998; Lind, 2008].

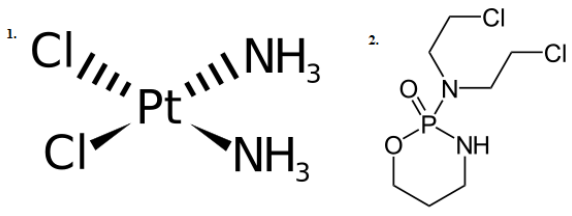

Figure 1.2: Alkylating agents. 1. Cisplatin and 2. Cyclophosphamide

- ***Anti-metabolites***

They are usually the analogues of building blocks of DNA and RNA; hence interfere with the synthesis of nucleic acids. The cell cycle dependent activity of these agents induces the programmed cell death, apoptosis. Methotrexate (figure 1.3.1) is an inhibitor of the enzyme, dihydrofolate reductase which decreases the synthesis of pyrimidine bases via inhibiting the production of folate coenzymes. Fluorouracil (figure 1.3.2) is a nucleoside analogue induces programmed cell death [Lind, 2008; Parker, 2009].

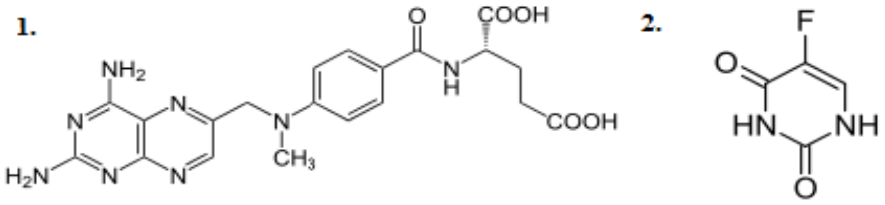

Figure 1.3: Anti-metabolites. 1. Methotrexate and 2. 5-fluorouracil

- ***Cytotoxic antiobiotics***

Anthracyclines and bleomycins are under this group. Anthracyclines (eg. Doxorubicin; figure 1.4.1) are isolated from bacterium *Streptomyces peucetius.*

Doxorubicin (DOX) is a well-known potent chemotherapeutic agent (figure 1.4.1) approved by Food and Drug Administration, widely used against a range of cancers such as leukaemia, sarcoma etc. [Carvalho et al., 2009]. DOX has the ability to fight with rapidly dividing cells, thereby decreases the tumor progression. The major mechanisms of action include DNA intercalation which prevents DNA replication and transcription, free radical generation, topoisomerase inhibition and apoptosis [Minotti et al., 2004]. However, its therapeutic usage is limited only by its toxicity especially cardiotoxicity. DOX causes toxicity to cardiac tissues through the induction of oxidative stress [Doroshow, 1983], reduced antioxidants [Doroshow et al., 1979; Olson et al., 1980], altered heart-related expression of genes [Kim et al., 2003; Aries et al., 2004; Takemura et al., 2007] and prevention of biomolecule synthesis [Odom et al., 1992].

The **antibiotic actinomycin** can also intercalate DNAs and prevents the expression of genes [Sobell, 1985]. Bleomycins (figure 1.4.2) are obtained from *Streptomyces verticillus,* known to cause DNA intercalation; DNA strand breaks and free radical damage [Dorr, 1992].

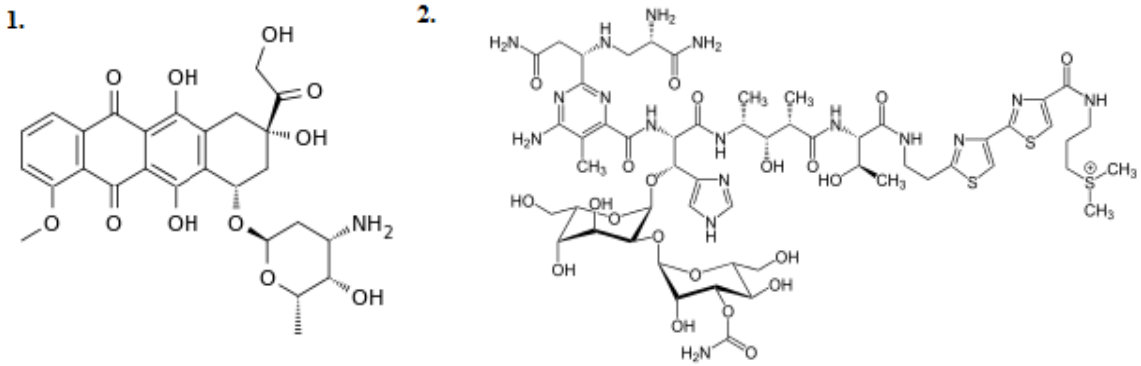

Figure 1.4: Cytotoxic antibiotics. 1. Doxorubicin and 2. Bleomycin

- ***Anti-microtubule agents and topoisomerase inhibitors***

Most of these agents are plant-derived compounds. Several natural products have been tested as anticancer agents and some of them are in clinical trials. These products are usually the secondary metabolites of microorganisms and/or plants shows distinctive structural diversity which helps the organism to adapt to the various biological situations [Bindseil et al., 2001; Firn and Jones, 2003]. The major plants with clinically verified antineoplastic activity are *Catharanthus roseus*, *Camptothecin acuminata*, *Cephalotaxus harringtonia*, *Podophyllum peltatum*, *Taxus brevifolia*, *Viscum album*, *Annona bullata*, *Onchrosia elliptica*, *Rhizoma zedoariae* and *Asmina triloba* [Ram and Kumari, 2001].

The plant-derived anticancer agents in clinical use are categorized mainly into a) the vinca alkaloids, b) the epipodophyllotoxin lignans, c) the taxane diterpenoids, and d) the camptothecin quinolone alkaloid derivatives. Apart from these, there are several plant derived molecules which have anticancer activities and are useful in chemoprevention as well as treatment of cancer.

a) Vinca alkaloids

Vincaleukoblastine (Vincristine; figure 1.5.1), an alkaloid isolated from *Vinca rosea* Linn shows anti-tumor activity in mice-bearing transplantable tumor [Cutts et al., 1960]. Johnson et al (1960) reported the potential of anticancer agent vincaleukoblastine in tumor control via obstructing the essential cellular metabolic pathways. According to Svoboda (1961), leurocristine (Vinblastine; figure 1.5.2), another Vinca alkaloid, has a wide anticancer activity in human tumors [Neuss et al., 1962]. These alkaloids are known as 'spindle poisons' as they can interact with the cellular receptor, tubulin and prevents its assembly [Guéritte-Voegelein et al., 1992; Jordan et al., 1998].

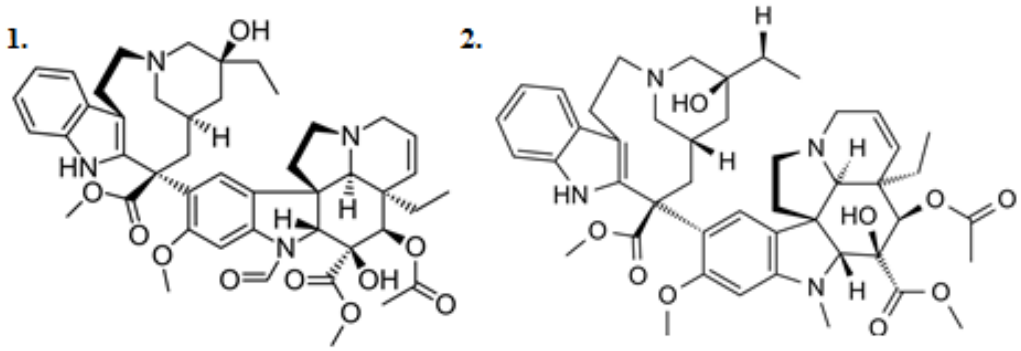

Figure 1.5: Vinca alkaloids. 1. Vincristine and 2. Vinblastie

b) Epipodophyllotoxin lignans

The podophyllotoxin (figure 1.6.1), a non-alkaloid lignan found to have antineoplatic activity, is isolated from *Phodophyllum* species [Xu et al., 2009]. It interacts with the enzyme topoisomerase II and prevents DNA unwinding as well as replication [Canel et al., 2000].

c) Taxane diterpenoids

Taxol (Paclitaxel; figure 1.6.2), isolated from *Taxus brevifolia*, is the first compound having taxane ring showed anti-leukemic and anti-tumor activities [Wani et al., 1971]. The mechanism of action of Paclitaxel is mainly through the interaction with 'tubulin', which prevents the disassembly of mitotic spindle and hence cell division [Brito et al., 2008].

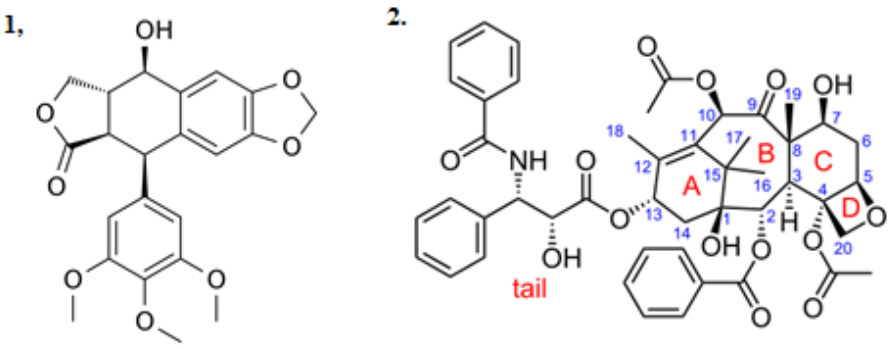

Figure 1.6: Structure of 1. Podophyllotoxin and 2. Paclitaxel

d) Camptothecin quinolone alkaloid derivatives

Camptothecin (figure 1.7), a quinolone alkaloid toxic to tumor cells, is extracted from *Camptotheca acuminata*. Topotecan and irinotecan, two analogues of Camptothecin clinically accepted as cancer chemotherapeutic agents, found to have anti-leukemic and inhibitory effect on tumors [Wall et al., 1966]. The mechanism of action is mainly through

its interaction with the enzyme topoisomerase I [Liu et al., 2000; Ulukan and Swaan, 2002].

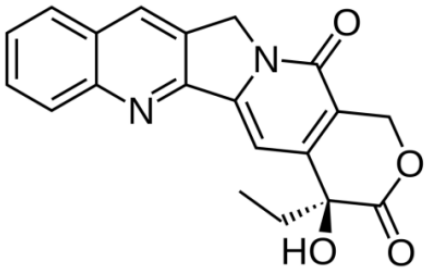

Figure 1.7: Structure of Camptothecin

Curcumin*:* It is the most studied phenolic compound with anticancer property derived from roots of *Curcuma longa* L. Phase I and phase II clinical trials revealed the therapeutic potential of curcumin against various tumors [Bar-Sela et al., 2010; Ji et al., 2012].

Flavopiridol is a cytotoxic flavone in clinical trials found to induce cell death in human lung carcinoma cells [Bible and Kaufmann, 1996] and also found to induce p53-independent programmed cell death in small cell lung carcinoma cells [Shapiro et al., 1999]. **Lycopene** is a tomato carotenoid shows a variety of biological functions, especially anticancer activities in various cancers, both *in vitro* and *in vivo* conditions [Bhuvaneswari and Nagini, 2005]. ***Resveratrol***, a phenolic compound, found to interfere with tumor initiation and the various steps of tumor progression [Aggarwal et al., 2004]. The prevention of growth of hepato-cellular cancer cells by resveratrol revealed its anticancer potential [Bishayee et al., 2010].

Berberine**(BBN)** is an isoquinoline alkaloid derived from plants in Berberidaceae family such as *Berberis vulgaris* (barberry), *B. aristata* (tree turmeric), *B. aquifolium* (Oregon grape), *Hydrastis canadensis*, *Coptis chinensis* (golden thread) and *Arcangelisia flava* (Menispermaceae) [Imanshahidi and Hosseinzadeh, 2008]. BBN (figure 1.8) is known to have anti-microbial, anti-helmintic, anti-viral and anti-inflammatory activities [Franzblau and Cross, 1986; Singhal, 1976; Romero et al., 2005; Kuo et al., 2004]. Recently, research has been focussing mainly on the antineoplastic activities of BBN.

BBN might contribute anticancer activity through inhibiting the growth of *Helicobacter pylori*, an organism known to cause peptic ulcer and gastric cancer [Ferreira et al., 2008; Farinati et al., 2008]. BBN also found to influence the activation of proto-oncogene to

oncogene. The activation of proto-oncogene, c-Ki-ras2 contributes to oncogenic processes in human embryonic carcinoma cells- Tera 1 and Tera 2. BBN is found to influence the morphological differentiations of teratocarcinoma cells into neuronal cells through the negative regulation of c-Ki-ras2 [Chang, 1991]. Since it was found that Activator Protein (AP-1) plays pivotal role in tumor progression [Shen et al., 2005], BBN has an inhibitory effect on AP-1 activity as evidenced from a reporter gene assay done in human hepatoma cells [Fukuda et al., 1999].

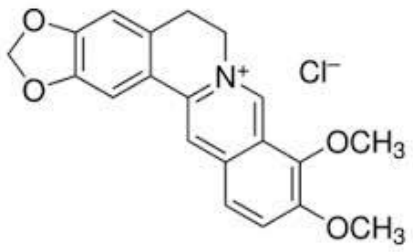

Figure 1.8: Structure of BBN

Mantena et al in 2006(a and b) reported that BBN inhibits the growth of human epidermoid carcinoma A431 cells through cell cycle arrest at G1 phase and apoptosis via regulating Cdki-Cdk-cyclin cascade, activation of caspase 3 and poly (ADP-ribose) polymerase (PARP). BBN was also reported to induce apoptosis in U937 cells while it enhances cell death in melanoma B16 cell line through the induction of apoptosis [Letasiová et al., 2006].

Grisendi et al in 2006 reviewed the importance of Nucleophosmin/B23 in tumor progression as it was recognized as a potent tumor marker for several human tumors. The importance of the enzyme telomerase in cancer opens new avenues for developing strategies for cancer therapy [Shay and Keith, 2008; Xu and Goldkorn, 2016]. BBN was shown to reduce the activity of telomerase and nucleophosmin/B23, and induce programmed cell death (apoptosis) in human leukemia HL-60 cells [Wu et al., 1999]. In 1969, Krey and Hahn reported that BBN can interact with DNA molecules. Later in 1981 Rungsitiyakorn et al showed the influence pH in the binding of BBN to DNA. According to Liu et al (2008), the p53-associated cell cycle arrest, apoptosis and DNA double strand breaks were induced in human osteosarcoma cells by the treatment with BBN. This BBN is found to induce cell death through apoptosis in human colon cancer cells by various biochemical reactions [Chidambara Murthy, 2012].

1.1.4 Immunotherapy: Cancer cells express different molecules on its surface in order to enhance its proliferation. These molecules may be cancer antigens or carbohydrates. Immunotherapy is basically used to enhance the immune system by targeting these molecules on the surface, to kill cancer cells. The cell death mechanisms include antibody - dependent cell-mediated cytotoxicity (use antibodies to attack specific surface molecules), complement system (use blood proteins after antigen-antibody interaction), cell signalling (binding of antibodies initiates several signalling pathways to activate cell death mechanisms) and payload (antibody is conjugated with drug, toxin, small interfering RNA or radioisotope against antigen on the cell surface [Weiner et al., 2010; Wang et al., 2015; Scott et al., 2012; Gelderman et al., 2004]. A combination of antibodies with radionuclides, radio-immunotherapy, has also been reported to be effective in cancer therapy [Sharkey and Goldenberg, 2011].

1.1.5 Photodynamic therapy: In this mode of treatment light-sensitive compounds, which are non-toxic to cells, have been used. When exposed to light these compounds become toxic. In the presence of light, photo sensitizers get excited and produce highly reactive free radicals, destroy the target cells. Some examples of photosensitizers areAminolevulinic acid (natural), Allumera, Photofrin, Visudyne, Levulan etc. (commercially available). The application of photosensitizing agent to the body is purely depends on the part of the body being treated [Chen et al., 2002].

1.1.6 Hyperthermia: The controlled use of high body temperature than normal is often capable to wipe out cancer cells. Exposing the cells to higher temperature than normal temperature, cause alterations and make the cells more likely to be affected by the treatments such as radiotherapy and chemotherapy. Hyperthermia can be used in two different ways.

- Local hyperthermia or thermal ablation: Very high temperatures are used to kill a small area of cells.
- Regional hyperthermia or whole-body hyperthermia: A part of the body or whole body is exposed to slightly higher body temperature to destroy the cells and helps other cancer treatments work better. In whole body hyperthermia, body heated to 39^{o}C to 43^{o}C to treat cells [Wust et al., 2002].

1.1.7 Laser therapy: Laser, a narrow beam of light, has a single wavelength and can be used instead of scalpel in surgery. The commonly used types of lasers in these treatments are Carbon dioxide (CO_2), Argon, Nd:YAG (Neodymium: Yttrium-Aluminum-Garnet), Er:YAG (erbium: yttrium aluminum garnet); Ho:YAG (holium: yttrium aluminum garnet), copper vapor, and diode lasers [Lasers in Cancer Treatment. American Cancer Society].

✸ However, most of these treatment strategies are coupled with several harmful side effects and hence the therapeutic efficacy in terms of specificity gets compromised, causing damage to biological systems [Prise and O'Sullivan, 2009].The treatments such as immunotherapy, laser therapy, hormone therapy and photodynamic therapy are still in the developmental stage. Most of the chemotherapeutic agents are known to cause systemic toxicities due to the lack of its therapeutic specificity. For example, the antineoplastic agentsDoxorubicin cause severe toxicity to heart tissues [Chatterjee et al., 2010] and Cisplatin has been reported to generate renal injury in association with damages in tumor cells [Yao et al., 2007].

✸ Unique feature of all tumors is the rapid proliferation of the cells. The therapeutic strategy for tumor is particularly directed towards the rapidly proliferating cells. Chemotherapeutics, predominantly, act on rapidly proliferating tumor cells through interfering cell division, metabolic processes in the cells or cause damage to vital cellular targets such as DNA and membrane. However, rapidly dividing normal cells, such as cells of the bone marrow, intestine and hair follicles are also affected. The therapeutic effectiveness of drug increases with increasing doses of administration. However, with increase in doses of administration there is an increase in systemic toxicities and side effects which compromises the therapeutic dose.

✸ By targeted delivery of drugs specifically to the tumor, one can achieve maximum therapeutic efficacy without side effects. Drugs can be delivered directly intumors by intratumoral injection at the site [Voulgaris et al., 2002; Duvillard et al., 2004]. This is possible only in case of peripheral tumors. For most other tumors other means of delivering drugs have to be adopted. Intratumoral injection of carrier-based chemo-therapeutics has also been tried [Lammers et al., 2006]. Delivery vehicles such as liposomes [Medina et al., 2004] and membrane sacs of red blood cell [Muzykantov et al., 2010] have been tried and the success was limited. Thermo labile liposomes carrying the

therapeutics are of great advantage [Zellmer and Cevcas, 1996], following administration, increasing the temperature at the tumor site can specifically release the contents in the tumor, while the liposomes remaining intact in other tissues. This would specifically destroy the tumor without affecting normal tissues. The recent upsurge in nanotechnology and nano-medicine has contributed to the development of elegant novel strategies for delivering drugs to the tumor [Landesman-Milo et al., 2015; Peer et al., 2007].

1.2 *Nanoparticles - the drug delivery vehicle*

The nanoparticles or nanomaterials have distinctive optical, magnetic and electronic properties, and are capable to carry therapeutic or diagnostic agents. By utilizing these unique properties including their large surface-to-volume ratio, it is possible to develop new theranostic strategies. Since 1980s to the present, several technologies were implemented to enhance the activity and clinical success of nano-based therapeutics.

The concept of **PEGylation**(poly ethylene glycol conjugation)was found to enhance the biopharmaceutical properties of proteins and biologically active substances [Jain and Jain, 2008]. The non-toxicity, water-solubility and less immunogenicity make PEG differ from other polymers [VeroneseandPasut, 2005].

'**Active targeting**' of the drug can be achieved by conjugating the nanoparticles with specific ligand molecules for the cellular receptors, antibodies or peptides, providing specific interaction between receptors and ligands if they are in close proximity. Béduneau et al in 2007 demonstrated the effectiveness of active targeting by conjugating lipid nanocapsules of functionalized PEG with monoclonal antibodies against transferrin receptors (TFR) which are over expressed in cerebral epithelium, to facilitate specific drug delivery to the brain. Antibody-mediated cancer treatment was demonstrated by Daniels-Wells and Penichet (2016) using TFR-1 as a potent target. Several PEGylated products are under various stages of clinical trials and some are in the clinic such as Doxil® (liposome-doxorubicin product) and albumin-based nano-drug carriers (Abraxane®- nanoparticle-albumin-paclitaxel and Albuferon-α® - albumin andinterferon-α) [Hoffman, 2008].

The conjugation of a new targeting peptide SP90 with doxorubicin-encapsulated liposomes was found to enhance the therapeutic index by improving its accumulation in tumors and reducing the drug-induced systemic toxicities [Lu et al., 2013]. For tumor mitochondria

specific photodynamic therapy, Wei et al (2016) developed a surface-modified Grapheme oxide based nano-drug in conjugation with the integrin αvβ3 monoclonal antibody.

The **passive targeting** of nanoparticle-drug complexes is purely based on the phenomenon Enhanced permeability and Retention Effect. These nano-sized particles are entrapped by solid tumors because of their leaky blood vessels and inefficient lymphatic drainage system [Noguchi et al., 1998; Maeda et al., 2000 and 2001]. The size, shape, surface chemistry and stability [Burt et al., 1999] of nanoparticles have influence on its cellular uptake [Jin et al., 2009],plasma clearance and bio-distribution.

The receptor-ligand interaction and subsequent downstream signalling cascade is influenced by the size of the particles. The metal nanoparticles (gold and silver) with size less than 100nm were effective to induce cell mortality [Jiang et al., 2008]. The particles having size >150nm will be cleared through reticuloendothelial system mediated by macrophage activation, while particle having size less than 10nm gets removed through renal clearance. Nanoparticles of the size 10-100nm will have decent pharmacokinetic properties.

As the size of the particles increases above 150nm, the chance to get cleared from the circulation increases. The serum proteins get adsorbed on these particles coated with targeting molecules, prevents the accumulation of nano-complexes to solid tumors. The PEGylation of nanoparticles can improve the circulation time and passive targeting by inhibiting the interaction with serum proteins preventing macrophage - mediated plasma clearance [Moghimi et al., 2001; Walkey et al., 2012; Lazarovits et al., 2015].

The enhanced retention can further be improved by coating with cell specific targeting molecules. Several studies reported that the size and surface chemistry including surface charge of nanoparticles have a strong impact on targeted delivery and circulation time of the particles [Hirnet al., 2011; Sonavane et al., 2008]. The smaller particles, in comparison with larger particles, can penetrate deeply in to the tumor interstitium [Perrault et al., 2009].

1.3 *Magnetic nanoparticles in targeted drug delivery*

Nanoparticles can be of non-metallic or metallic origin. Non-metallic NPs constitutes natural carbohydrate polymers like chitin, chitosan, carrageenan, polylactic acid etc.

Metallic NPs comprise oxides as well as salts of several metals including silver and gold. Nanoparticles containing paramagnetic elements such as iron, manganese etc. will have magnetic property and are called as magnetic nanoparticles. Iron-oxide nanoparticles - Fe_3O_4 and γ-Fe_2O_3 - are of special relevance. These are used in large number of studies for diagnostic and therapeutic purpose. Our tissues and blood do contain iron and iron-oxide nanoparticles, are biocompatible. These NPs are cost-effective compared to several other nanoparticles of metals.

Magnetic iron-oxide nanoparticles (NP) have gained attention in cancer diagnosis (Imaging) and therapy (drug delivery). Because of the magnetic property, they can be directed to specific areas in the body by the application of an external magnetic field. Super-paramagnetic NPs can be used in magnetic resonance imaging of tumors (MRI), as contrast agents [Sun et al., 2008; Corot et al., 2006]. Several magnetic NPs are in the clinic as contrast agents such as AMI-25 for liver/spleen imaging, AMI-227 forlymph node imaging (size is 20-40nm), NC100150 (Clariscan, size is 20nm) for perfusion imaging and NC100150 for MR angiography [Wang et al., 2001].

NPs have been extensively employed as drug delivery vehicle in several studies. The importance of NPsin controlling tumor is mainly due to its capability to 1) transport and localize under the influence of an external magnetic field, 2) generate hyperthermia in the presence of alternating magnetic field and 3) carry targeting molecules to enhance active drug delivery.

1.3.1 Magnetic hyperthermia

IONP are capable of generating heat (hyperthermia) in the presence of an alternating magnetic field. The quantity of heat generated depends merely on the magnetic properties of the material and intensity of the magnetic field [Hergt et al., 2006; Glöckl et al., 2006]. Under hyperthermia (40-46^{0}C) cancer cells can not survive, while normal cells are unaffected [Wanga et al., 2005]. The altered microenvironment makes the tumor cells more sensitive to higher temperatures with the exception of central nervous system (CNS) [Raaphorst, 1990]. As CNS was found to be sensitive at temperature 40-43^{0}C for more than 6hrs, it is possible to treat tumor associated with CNS only under special conditions [Sminia et al., 1994]. During hyperthermia (41.8^{0}C), cytoplasmic heat shock protein 72 has

been reported to over express on tumor cells, which is absent in normal cells [Multhoff et al., 1995].

1.3.2 Magnetic drug targeting

The magnetic property has given more attention to magnetic NPs in solid tumor therapy as these particles can be attracted to a desired region with the application of magnetic field either external or internal. Hence, the non-specificity linked with conventional chemotherapy can be overcome by complexing them with magnetic NPs [Gupta and Gupta, 2005].

For the drug targeting, these nanocarriers should be biocompatible, hydrophilic and non-toxic. As mentioned earlier in this review; the diameter, shape, surface charge, surface modifications and composition of the magnetic nanoparticles have influence on magnetic property and drug delivery. Ma et al (2004) developed a targeting strategy to overcome the deleterious effects of conventional chemotherapeutic agent, doxorubicin (having systemic toxicities, particularly cardiotoxicity and hepatotoxicity) by conjugating it with spherical carbon magnetic nanoparticles of size 40-50nm. Doxorubicin was specifically targeted to solid tumor by conjugating with NPs and application of magnetic field externally [Jayakumar et al., 2009] in an animal model. The clinical application of magnetic targeting of 4'-epidoxorubicin, with an external magnetic field, was successfully demonstrated in patients with solid tumors [Lubbe et al., 1996], proving the advantage of this technique over conventional therapy.

The tumors which are >2cm away from the periphery of the body, cannot be targeted by the application of an external magnetic field because the strength of magnetic field decreases with increase in distance [Rudgea et al., 2000]. Takeda et al (2007) demonstrated the essential application of magnetic NP-drug targeting to tumors, which are deep in site, using super conducting magnets. Use of magnetic field internally to target NP-drug complexes to tumors was also investigated. The NP was coated with doxorubicin and this complex was targeted to tumor by implanting magnet inside the desired region using laparoscopic technique [Fernández-Pacheco et al., 2009]. However, this strategy of drug targeting was found to cause several problems while applying to human situations [Ritter et al., 2004].

1.3.3 Magnetic nanoparticles in gene delivery

The magnetic NPs can also be used as a carrier for the delivery of nucleic acids to cells as nucleic acids can bind to magnetic IONPs [Majidiet al., 2016]. This binding property has been exploited in the purification of DNA [Saiyed et al, 2008]. This gene transfer-mediated by the magnetic nanoparticle is called magnetofection. Thus, both RNA and DNA can be conveniently transport using this technique. The magnetic NP–DNA complex can be taken up by cells and the genes can be expressed in the recipient cells. This technique is extremely useful technique for controlling gene expression by introducing antisense oligonucleotides [Krötz et al., 2003] and siRNA [Mykhaylyka et al., 2007].

Nucleic acid can be conveniently attracted towards magnetic nanoparticle by coating them with polyethyleneimine having positive charge [Huth et al., 2004]. The nucleic acid complexed with NPs is taken up by the cells. Magnetofection can reduce time of transfection and the dose of the vector. The uptake of the complexes by the cells can be enhanced by the use of dynamic magnetic field through oscillating high intensity magnetic field. Recently, *in vivo* applications of the magnetofection with enhanced tissue penetration by oscillating magnetic field have been demonstrated [McBain et al., 2008]. The oscillating magnetic field imparts extra energy to the system which in turn results in particle uptake against external barriers. The underlying mechanism involves non-linear motion of the particles under the influence of the oscillating magnetic field facilitate tissue penetration, overcoming external barriers like muscle surrounding the tumor. Carbon nanotubes coated with nickel has also been found useful in transferring DNA to the cells under *in vitro* condition using magnetic field [Cai et al., 2005]

Oxidative therapy using complexes of magnetic NPs and D-aminoacid oxidase have been demonstrated in an animal model. This has proved even enzymes can be specifically targeted to tumor using magnetic NPs with the help of external magnetic field [Divakaran et al., 2011; Bava et al., 2013].

1.4 *Tumor microenvironment: Challenges and opportunities*

For many years, cancer research has been focused mainly on genetic alterations in a cell as an indicator of tumorigenesis [Hanahan and Weinberg, 2011]. Recently, this paradigm has been extended to epigenetic changes that include distraction in DNA methylation, modifications of histone and nucleosome positioning, resulting changes in gene

expression. And these genetic and epigenetic changes were found to be intertwined each other as epigenetic changes can induce alterations in the genes and vice versa [Baylin and Jones, 2011; Kasinski and Slack, 2011]. The alterations in genomics and epigenomics could be assessed from the enormous DNA sequence data generated through next-generation sequencing and microarray-based technologies [Sandoval and Esteller, 2012]. Very recently cancer researchers have recognized the potential influence of various tumor microenvironmental (TME) factors in the progression of tumor.

The growing tumor has a unique microenvironment which supports its proliferation and metastasis. The characteristics of TME such as altered extracellular matrix, undeveloped leaky vasculature, hypoxia, acidity, lack of lymphatic drainage etc. provide ample opportunities for designing strategies for diagnosis and therapy.

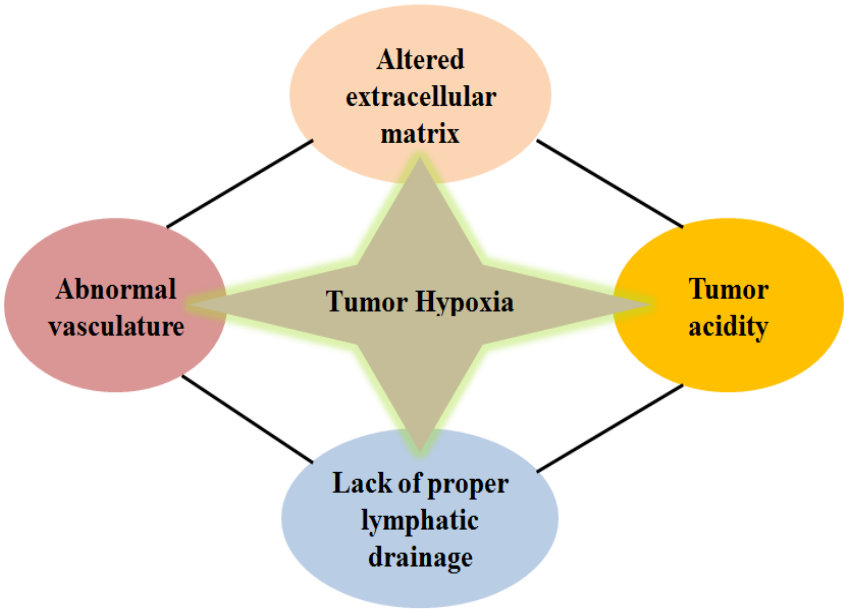

Figure 1.9: The interlinked major tumor microenvironment factors

The transformation of pre-malignant tumor into malignant cancer is associated with alterations in genetic, epigenetic and even more on the microenvironment. In recent years, more specific treatment methods have been facilitated via proper understanding of these interlinked alterations in tumor. The TMEalong with genetic and epigenetic variations contributes to drug resistance [Correia and Bissell, 2012]. Compared to normal tissues, tumor microenvironment is found to be entirely different in its composition and function. The common treatment modalities of tumor - radiation therapy and chemotherapy- are

toxic to normal tissuesdue to their lack of specificity, leading to dose limitations affecting therapeutic gain.

The proper understanding of the differencesin the microenvironment of normal and tumor tissues at cellular and molecular level is essential for successful tumor therapy. The important microenvironment factors of the tumor are presented in figure 1.9. The heterogeneity of tumor microenvironment together with genetic and epigenetic changes contributes to tumor progression and drug resistance [Hamm et al., 2010; Mumenthaler et al., 2015; Correia and Bissell, 2012].

1.4.1 The extracellular matrix (ECM): a dynamic contributor of tumor progression

The ECM,consisting of complex network of highly cross-linked proteins, provides architectural support to the cells in normal tissues. It exists as interstitial (within organs)and specialised forms (to surround certain tissues and cell types as basement membranes).It is composed of proteoglycans and several fibrous proteins, mainly collagens- most abundant, elastins, fibronectins and laminins [Jarvelainen et al., 2009]. The alterations in the organization of ECM are the hallmark of cancer progression. These include up regulation in integrin signalling through higher collagen deposition and/or ECM stiffness[Wozniak et al., 2003]. The higher collagen accumulation and ECM stiffness because of lysyl oxidase over production enhance facilitated oncogene Neu-mediated malignant transformation [Levental et al., 2009]. Abnormal ECM can support cancer cells to survive, grow and invade nearby tissues, thereby promoting loss of tissue integrity which results in cancer cell migration. ECM remodelling also facilitates apoptotic evasion in mutant tumor cells [Mottand Werb, 2004].

1.4.2 Heterogeneity in tumor vasculature: basis for unique physiology

In normal tissue systems, the blood vessels are properly oriented and regulated by the balanced expression of pro-angiogenic and anti-angiogenic factors based on the metabolic demand. The vascular network is hierarchically organized and distributed evenly in the tissue system. The systematic lymphatic systems drain fluids and waste products of metabolism from the tissue interstitium. However, in tumors the aggressive growth and proliferation of cells promote the hyper-activation of pro-angiogenic factors leading to the improper development of vascular networks. The vasculature in tumor tissues is heterogeneous since it contains normal blood vessels from which tumor cells invade and

tumor-induced microvasculature.The tumor blood vessels are often dilated, convoluted and the branching pattern is entirely different in comparison with normal vasculature [Correia and Bissell, 2012].

In some cases the vasculature is not properly organized into capillaries, arterioles and venules end up in sudden terminations. The tumor vasculature is leaky as their walls comprisealtered endothelial linings, defective basement membranes and less pericytes compared to normal vessels. These features support permeability of the vessels to small molecules and drugs [Carmeliet and Jain, 2000; Hashizume et al., 2000; Yonenaga et al., 2005; Konerding et al., 2001]. Functionally, the immature blood vessels diminish the proper supply of oxygen and nutrients to tumor cells. The absence of functional lymphatic drainage hindersthe waste material clearance from tumor mass [Leu et al., 2000; Carmeliet and Jain, 2000].

1.4.3 Tumor hypoxia and acidity

Tumor hypoxia is a pathophysiological condition manifesting a much lower tissue oxygen level than the physiological level. The availability of oxygen is a major factorin the therapeutic efficacy of radiation in cancer treatment as the hypoxic tumor cells are refractory requiring higher doses of radiation for mortality [Gray et al., 1953]. The inventive work of Gray et al (1953) proved that the damages induced by radiationdepend on the availability of oxygen at the time of irradiation. The histological analysis of human lung adenocarcinoma by Thomlinson and Gray (1955) revealed the presence of hypoxia in solid tumors. The uncontrolled growth and proliferation of tumor cells restricted the entry of blood vessels inside the tumor so that the tumor cells are farther away from the nearest blood vessels than in normal cells results in a reduction in oxygen concentration.

The cells that are far away from blood vessels are presumably necrotic, while the cells in the hypoxic region are found to be viable, but not rapidly proliferating. Further nutrient deprivation in hypoxic cells can potentiate the migration of hypoxic tumor cells into necrotic zone [Tannock, 1968; Vaupel et al., 1989]. Since most of the anticancer drugs are primarily effective against rapidly proliferating cells, the hypoxic cells can escape from the treatment. The cells in normoxic region that are dividing rapidly are highly exposed to the treatments undergo cell death, leading to improved nutrient supply to the previously hypoxic cells allowing them to divide rapidly to regenerate the tumor.

The tumor cells follow anaerobic glycolytic pathway- generation of ATP by the conversion of glucose into lactate- for energy generation which necessitate greater demand for nutrientsfor their rapid growth and proliferation [Vaupel et al., 1989; Warburg, 1956]. Due to the absence of a proper lymphatic drainage system, the major by-products of this metabolic pathway –CO_2 and carbonic acid- were accumulating in hypoxic tumor cells. The reduced clearance of these acidic metabolic products results in tumor acidity [Tannock and Rotin, 1989]. Further, lactate to pyruvate ratio is predictive of cancer.

1.4.4 Influence of tumor microenvironment on drug resistance

The proliferation rate decreases with increasing diffusion distance from the blood vessel and the slow proliferating hypoxic cells escape from chemotherapeutic drugs aimed to rapidly dividing cells [Tannock, 1968].Thus, the rate of proliferation of tumor cells playsvital role in drug sensitivity [Tannock, 1978].

Cell-adhesion mediated drug resistance has been observed in a variety of cancer types, suggesting the role of interaction between tumor cells and their microenvironment in sensitivity to chemotherapy [Shain and Dalton, 2001]. The importance of these interactions in drug resistance has been implied from the observationon potentiation of resistance of mouse colon cancer cell line against cytotoxic agents by Insulin like growth factor-I. The inhibition of beta4-integrin protein prevents DNA damages and apoptosis induced by chemotherapeutics in small cell lung cancer,also deliberate the importance of cell interactions and their microenvironment in drug resistance [Guo et al., 1998; Sethi et al., 1999; Weaver et al., 2002]. Transient hypoxia and associated nutrient deprivation have also been reported to cause increased expression of genes encoding P-glycoprotein confering multidrug resistance [Ledoux et al., 2003].

The pH of tumor microenvironment influences the cellular uptake of drugs and thereby reduces its cytotoxicity. In uncharged form, the molecules can easily diffuse into the cells. Low pH in tumor prevents uptake of weakly basic cytotoxic drugs with an acid dissociation constant of 7.5 to 9.5 such as Vinca alkaloids and doxorubicin. Alkalinization of the TME can enhance the cellular uptake of these drugs. The uptake of weakly acidic drugs is also found to be inhibited by the slightly acidic pH of the tumor extracellular microenvironment [Gerweck et al., 2006; Raghunand et al., 1999].

1.5 *Hypoxic environment in tumor: a feasible target for tumor therapy*

The altered diffusion geometry in solid tumors reduces the supply of oxygen and nutrients to the tumor. These hypoxic cells are known to be alive, nevertheless not able to divide rapidly. These cells have been known to be a cause of malignancy and resistant to chemo- and radiation therapy [Rankin and Giaccia, 2016].In order to adjust with the situation, hypoxic cells induce change in the expression of genes known to be involved in tumor cell proliferation and metastasis. Among them, hypoxia-inducible factor-1 (HIF-1) plays a crucial role in hypoxia-induced tumor progression. HIF-1 was identified by the recognition of hypoxia response element in the 3' enhancer region of the gene erythropoietin - a hormone involved in erythrocyte proliferation and undergoes hypoxia-induced transcription [Goldberg et al., 1988; Semenza et al.,1991]. The heterodimer HIF-1 comprises a cytoplasmic subunit HIF-1α and a nuclear subunit HIF-1β. The oxygen dependent regulation of HIF-1α was schematically represented in figure 1.10.

The best mechanism behind the regulation of HIF-1α protein is mediated by von Hippel-Lindau (vHL) protein [Maxwell et al., 2001]. vHL protein in the presence of oxygen recruits HIF-1α for ubiquitination via 26S proteasome degradation pathway. The hydroxylation of prolyl residues in HIF-1α by prolyl-4-hydroxylase (PHD) promotes the binding of HIF-1α with vHL. However, in hypoxic condition tricarboxylic acid cycle intermediates such as succinate and fumarate, or mitochondrial reactive oxygenspecies can stabilize HIF-1α by inhibiting the activity of PHD. The accumulated HIF-1α in association with HIF-1β binds to the hypoxia response elements in target genes.

The promoter region of the key angiogeneic factor - vascular endothelial growth factor (VEGF) - consists of hypoxia response element which is a binding site for HIF-1 [O'Rourke et al., 1997]. This binding is essential for the expression of VEGF which in turn promotes angiogenesis and subsequent tumor progression [Jain, 2002]. The reduction in oxygen in hypoxic milieu in tumors leads to increased expression of epidermal growth factor receptors (EGFR) [Wing et al., 1988]. EGFR signalling has been attributed to increase in the proliferation of tumor cells, decreased apoptosis in these cells, tumor angiogenesis, and metastasis [Baselga, 2002].Hypoxia has been shown to increase the expression of EGFR in several human cell lines [Franovic et al., 2007]. Hypoxia has been

shown to facilitate tumor metastasis through regulation of factors involved in matrix degradation, cell-cell adhesion and autocrine mobility [Pugh and Ratcliffe, 2003].

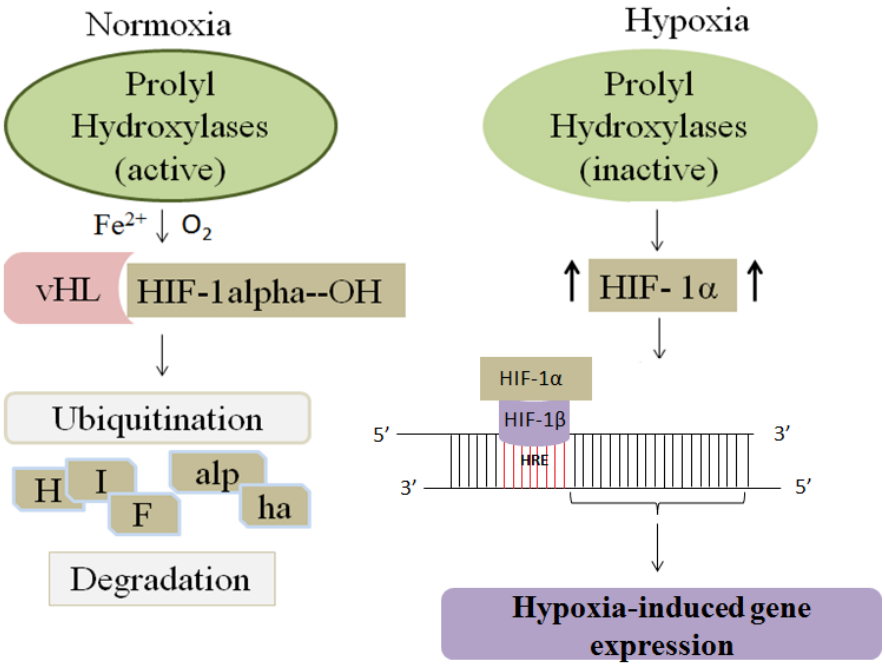

Figure 1.10: Schematic representation of the regulation of HIF-1α.

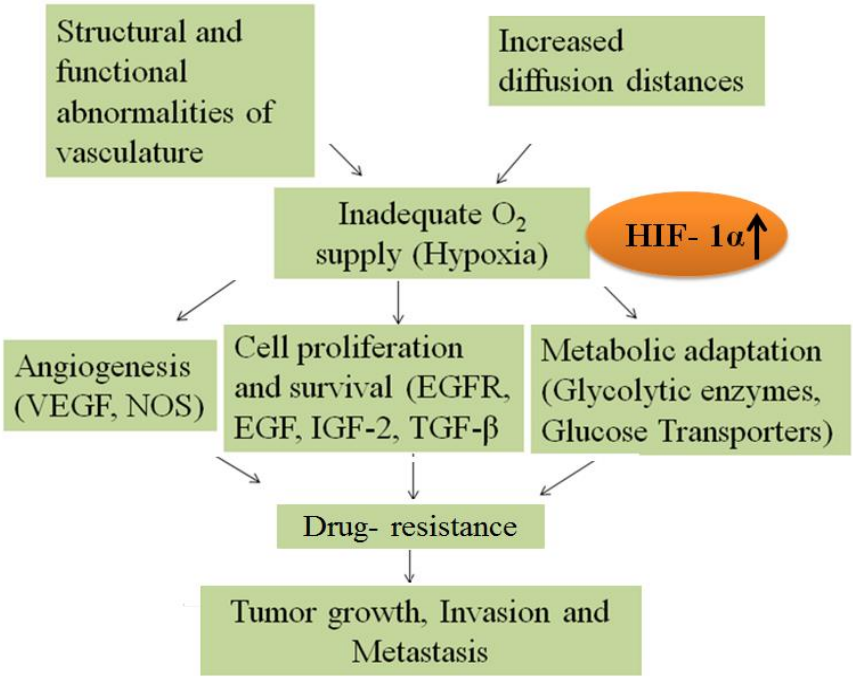

Figure 1.11: Tumor microenvironment-induced drug resistance and tumor progression.

The influence of tumor microenvironment in the progression of tumor is presented in figure 1.11.Thus, hypoxia can mediate tumor progression by activating genes responsible

for crucial events such as angiogenesis, cell proliferation and metastasis [Denko, 2008]. Putting the above mentioned problems together, it is necessary to develop a modern therapy basically involving tumor hypoxia.

1.6 *Hypoxia targeted drug delivery*

Improper development of vasculature in solid tumors leads to the heterogeneity of the cell population. The solid tumor mass will contain areas where nutrients and oxygen do not available – necrotic area. There is also another area where the availability of nutrients and oxygen is inadequate – hypoxic area. Apart from these two, at the periphery of the tumor, the cells get adequate supply of nutrients and oxygen - normoxic area. Hypoxic area makes the tumor refractory to different modalities of the treatment. However, this hypoxia provides opportunities for selective tumor therapy and there are four different approaches developed in this direction.

***1.6.1 Selective therapy using hypoxia-activated pro-drug*:** Hypoxia selective drugs in this category can be included in two major categories - quinone based bio-reductive alkylating agents (eg. mitomycin-C) [Rauth et al., 1983] and nitroimidazole hypoxic cell radiosensitizers (eg. Misonidazole) [Hall and Roizin-Towle, 1975; Mohindra and Rauth, 1976; Moore et al., 1976; Hirst et al., 1982].Lin et al. (1972) described that the benzoquinone derivatives had the potential to inhibit the growth of adenocarcinoma 755 ascites cells, and increased the lifespan of tumor-bearing mice. These compounds undergo alkylation following bio-reduction in hypoxic condition. Zeman et al. in 1986 proposed that the compound 3-amino-1,2,4-benzotriazine-1,4 dioxide (tirapazamine) was effective in killing cancer cells under hypoxic condition as selective bio-reductive agent. The product of one electron reduction, produced under hypoxia, was found to be more toxic in hypoxic cells than two electron reduction product. Denny and Wilson in their review in 1993 pointed out the drawbacks of currently available hypoxia-selective cytotoxins as they are designed to destroy hypoxic cells which comprise small fractions. Hence, it was essential to use them in combination treatments with either radiation or chemotherapy or photodynamic therapy to completely eliminate tumor population.

To overcome these difficulties they designed diffusible cytotoxins, such as nitro-deactivated aromatic mustards and cobalt (III) complex-deactivated aliphatic mustards, having bio-reduction potential. The nitrogen mustards which cause DNA damages with

less cell cycle specificity got activated upon reaction with reductive triggers -nitro and Co (Ill) - in hypoxic cells. The most of the metabolic transformations of drugs were prevented in normoxic cells, explored the use of hypoxia in its bio-reduction [Wilson, 1992]. These agents can be activated by the reductive metabolism under low oxygen pressure to produce toxic products. The basic mechanism behind is that the oxygen-sensitive one electron reductase such as cytochrome p450 reductase generate free radicals [Ortiz de Montellano, 2013], upon reaction with these pro-drugs, which are toxic to cells capable of generating cell death [Brown 1993]. While the two-electron product of these drugs generated by NAD(P)H dependent quinone oxidoreductase, which is oxygen- independent, found to be less toxic in hypoxic cells than one-electron product. The free radicals generated through one-electron reduction get converted to superoxide radicals in reaction with oxygen under normoxia [Murphy, 2009] which is found to be less toxic than the free radicals generated in hypoxic condition.

Several hypoxia-selective bio-reductive drugs are in clinical trials including Tirapazamine (TPZ). Brown (1993), Lee and Wilson (2000) were identified the cytotoxicity of TPZ in hypoxic condition. The importance of TPZ (SR-4233) has been increased mainly due to its cytotoxic potential in hypoxic mammalian cells [Zeman et al., 1986] and its ability to enhance toxicity to radiation. The treatment along with TPZ could enhance the anti-tumor potential of a well-known chemotherapeutic agent, Cisplatin in hypoxia-dependent manner[Dorie and Brown, 1993; Kovacs et al., 1999].

The therapeutic potential of TPZ has been attributed mainly to its protonated form in low oxygen condition [Brown 1993]. However, later it appears that this protonated form is not the final toxic product, since evidences shows the involvement of new radicals either hydroxyl or benzotriazinyl [Daniel and Gates, 1996; Zagorevskii et al., 2003; Anderson et al., 2003], as presented in figure 1.12.Anthraquinone (AQ4N), another pro-drug in clinical trails. It gets activated to hypoxia-selective cytotoxin by an unusual two electron reduction mechanism achieved by the CYP3A members of the cytochrome P450 family [Patterson, 2002; Patterson and Murray, 2002].

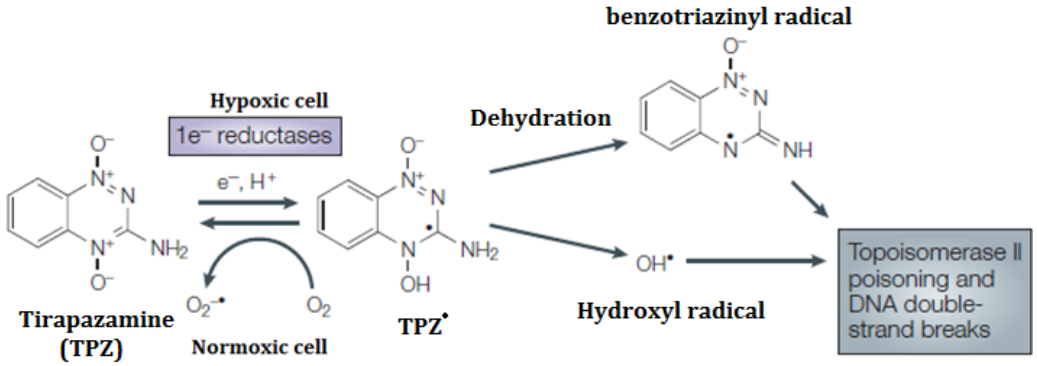

Figure 1.12: The activation of pro-drug Tirapazamine to hypoxia selective cytotoxins [Brown and Wilson, 2004].

***1.6.2 Hypoxia-selective gene therapy*:** Tumor specific proteins arising from differential expression of genes, confer characteristic properties to tumor cells, and thereby provide opportunities for delivering strategies for tumor control through gene targeting method. At the gene level, the expression of HIF-1 is higher in hypoxic tissues than in normoxic tissues [Zhong et al., 1999]. The cells in hypoxic condition acquire the potential to enhance tumor growth through the activation of HIF-1. The over expression of HIF-1α and HIF-2α in hypoxic tumor enhances tumor progression through the activation of genes responsible for angiogenesis, invasion and metastasis [Talks et al., 2000; Semenza, 2000]. The transcription of these genes is promoted by the binding of HIF-1 dimer, HIF-1α and HIF-1β, to the hypoxia-response elements in the target genes.

A new approach of gene-directed enzyme prodrug therapy has been designed to deliver the genes of specific enzymes with promoters having hypoxia-response elements. Following gene transfer, the genes are transcribed and translated to produce the required enzymes to convert a pro-drug to active cytotoxic drug [Greco and Dachs, 2001]. Elegant protocols have been developed using the strategy of introducing genes of enzymes, which can activate prodrug into cytotoxin, into tumor cells and some of these protocols are in clinical trials [Shibata et al, 2002]. Binley et al. in 2003 developed an optimized hypoxia responsive promoter to stimulate the hypoxia-targeted expression of human cytochrome P450 (CYP2B6) in the context of adenoviral vectors, which interrupts tumor growth by the activation of pro-drugs to active cytotoxic agents [Binley et al., 2003]. Some of the fascinating enzyme/pro-drug gene therapy include the combinations of herpes simplex-1

virus thymidine kinase/ganciclovir and cytosine deaminase/5-fluorocytosine [Trinh et al., 1995]

Another approach in gene-directed enzyme pro-drug therapy achieved noticeable success is the delivery of the gene encoding a one-electron reductase such as cytochrome P450 3A4 for the hypoxia-specific activation of pro-drug AQ4N [McCarthy et al., 2000]. One of the major problems in these therapeutic approaches is the delivery of the enzyme/pro-drug specifically to cells with high HIF-1 or with hypoxia. An alternative strategy is by transduce human macrophages using hypoxia-assisted adenoviral vector to deliver a reporter gene or a gene encoding the enzyme which will be effective to activate pro-drug as cytotoxin [Griffiths et al., 2000; Burke et al., 2002].

***1.6.3 Interfering HIF-1 activity*:** The hypoxia induced tumor growth can be prevented by interfering with HIF-1 activity. The inhibition of HIF-1 can prevent the major adaptive responses of tumor progression. Developing inhibitors for HIF-1 is of great importance in tumor control.

Several approaches have been developed to regulate the potential target, HIF-1. One of the tactics is the inhibition of HIF-1 associated transactivation of genes, such as vascular endothelial growth factors and epidermal growth factors, to cause the prevention of hypoxia-induced tumor growth. Kung et al (2000) have shown that the use of a polypeptide that interrupts the interaction of HIF-1 to its co-activators p300/CREB can hinder the transcription of its target genes involved in tumor progression. Another approach is to inhibit the HIF-1 protein via the inhibition of translation or destabilization. By enhancing the degradation of HIF-1 protein, geldanamycin could reduce its level in tumor and thereby tumor regression [Mabjeesh et al., 2002]. Intratumoral introduction of an antisense HIF-1alpha containing plasmid resulted in down regulation of VEGF and thereby reduced microvessel density. Though this treatment did not exhibit any effect on T cell-mediated immunity, it synergized B cell-, NK cell-, and CD8 T cell- dependent cure of tumors. The gene therapy with antisense HIF-1α thus enhanced efficacy of immunotherapy [Sun et al., 2001] and also improved the therapeutic efficacy of doxorubicin against [Liu et al., 2008].

Recently, remarkable advances have been made in the development and use of HIF-1α inhibitors for tumor control. Most of the HIF-1α inhibitory molecules are discovered for

other endogenous targets and later the molecules were found to inhibit HIF-1α. One such example is the cardiac glycoside digoxin, which reduces the proliferation and viability of hepatic cancer cells [Tahervand et al., 2016].The chemotherapeutic agent cisplatin has shown to enhance HIF-1α degradation in cisplatin sensitive ovarian cancers [Ai et al., 2016]. An antisense oligonucleotide, EZN-2698, successfully completed phase I clinical trial in subjects with advanced solid tumors [Masoud and Li, 2015; Jung et al., 2015]. An HIF inhibitor-topotecan- also completed clinical trials in non-small cell lung cancer [Jung et al., 2015]. The anti-tumor activity of gemcitabine was shown to induce immunogenic cancer cell death in pancreatic ductal adenocarcinoma through the inhibition of HIF-1α by PX-478 [Zhao et al., 2015].

***1.6.4 Therapy with anaerobic bacteria*:**Anaerobic bacterial therapy is of relevance because of the necrotic regions in the tumor where blood supply and oxygen are totally absent.The use of bacterial therapy has been documented for more than hundred years ago [Nowotny, 1985] and still active research is being done in this area. The recent developments in this strategy encompass targeting therapeutic agent to anaerobic necrotic areas in the tumor, using genetically engineered non-pathogenic strain of the bacterial genus *Clostridium* which can grow and localize in these regions [Lemmon et al., 1997].

The major approaches in bacterial therapy include the usage of bacteria- for enhancing the therapeutic potential of drugs, use as carrierof anti-neoplastic drugs, and vectors in gene therapy[Jain, 2001]. However, the most favourable approaches are the use of genetically modified bacterial strains for tumor destruction and for bacterial gene-directed enzyme/pro-drug therapy. The gene encoding Cytosine deaminase, anenzyme present in Escherichia coli which can convert pro-drug 5-fluorocytosine to the cytotoxic chemotherapeutic agent 5-fluorouracil, was cloned in *Clostridium* with an expression vector. This method could increase the sensitivity of murine EMT6 carcinoma cells to 5-fluorocytosine several fold [Fox et al., 1996]. Gardlik et al., (2011) reviewed the approach of Bacteria-mediated anti-angiogenesis therapy in tumor tissues.

1.7 Combination therapy

Use of more than one therapeutic agent or drug shown to possess great advantage in many instances as combination of drugs with different mechanisms of action provide synergism in cancer therapy which could also prevent the treatment associated multi-drug resistance.

Highly potent combinations of drugs are quite often associated with deleterious effects due to toxicities and side effects. Nanoparticle combinations of drugs are an alternative to overcome these deleterious effects [Hu et al., 2010]. Single nanoparticle combinations of multiple drugs have great advantage over combined administration of nanoparticle – single drug combinations, since the former offers uniformity of the vehicle size, proper loading of drugs in desired proportion and time-dependent time release[Hu et al., 2010].

Nanoparticle plat forms such as liposomes, dendrimers, polymeric nanoparticles etc. are employed in many instances for the co-delivery of chemotherapeutic drugs, siRNA, sensitizers etc. for tumor control [Kaneshiro and Lu, 2009; Bai et al., 2015; Allémann et al., 1995]. Specific targeting of neoplastic drug doxorubicin to tumors have been achieved by complexing them with nanoparticles of iron-oxide and silver-oxide together with a hypoxic sensitizer Sanazole [Nair and Nair, 2014; Sreeja and Nair, 2016].

1.8 *Future perspectives*

The tumor specific proteins arising from differential expression of genes confer characteristic properties to tumor cells and provide opportunity for developing strategies of tumor control through gene targeting methods. Tumor cells, in general, display reduced apoptosis and if one can enhance the apoptosis, either by reducing the expression of anti-apoptotic proteins or increasing the expression of pro-apoptotic proteins, will have therapeutic benefit. Tumor hypoxia and associated expression of genes enhances tumor progression. Developing siRNA or antisense RNA techniques for the inhibition of expression of these genes (*vegf*, *egfr*, etc.) can be thought of as therapeutic strategy for tumor control. Certain killer peptides/proteins are expressed in cells, which are in severe stress and trauma, trigger pathways to induce cell death [Magliani et al., 2011; Ellerby et al., 2008]. Tumor can be controlled by specific targeting of killer peptides to them using any of the nanocarriers. Thus, nanotechnology and the characteristics of tumor microenvironment can be harnessed to develop suitable strategies for tumor control.

SCOPE OF THE THESIS

The work reported in the thesis comprises development of strategies for targeted tumor therapy using magnetic iron-oxide nanoparticle-cytotoxic drugs complexes with physical and chemical agents. As a physical agent an external magnetic field and as a chemical agent Sanazole, a hypoxic-cell radiosensitizer, were used. The studies were mainly focussed on regression of DLA solid tumor, developed on hind limbs of mice, and the underlying cellular and molecular mechanisms. Investigations included histopathological examinations and biochemical alterations in the tissues of tumor-bearing animals following the treatments along with the transcriptional expression of various genes-associated with tumor hypoxia, tumor progression and apoptosis.

NPs were prepared using ferric chloride and ferrous chloride by alkaline co-precipitation method. The magnetic NPs thus obtained were surface-modified with polyoxyethyl stearate (POES). The cytotoxic isoquinoline alkaloid BBN and hypoxic cell sensitizer SAN were complexed with these nanoparticles to get NP-BBN-SAN complexes. Similarly, the NPs were also complexed with the antineoplastic agent DOX and SAN to get NP-DOX-SAN complexes. These complexes were characterized by FTIR, XRD, TEM and nano-size analysis. The complexes were orally administered to mice-bearing transplanted DLA solid tumors on hind limbs. The physical targeting was carried out using NPs with only BBN. The administration and the treatments continued for several days and the tumor volume was monitored. Also, tumor tissues were excised following the treatments for undertaking cellular, molecular and biochemical studies.

The hypoxic cell sensitizer was very effective in targeting the nano-drug complexes to tumors. It was also found that NP-SAN complexes could be effective in tumor imaging if complexed with a fluorescent compound such as rhodamine or BBN. Thus, the study provided compelling evidences for the therapeutic efficacy of hypoxia-targeted treatment of tumor using NP-drug-SAN complexes. The salient findings of the studies presented in thesis are given in the abstract. The thesis contains 10 chapters and bibliography in addition to the list of publications. The chapters are written in the format of manuscripts for journals excluding the references. Some of the chapters have already been published in different journals as mentioned in thetitle pages of the chapters. As the chapters are written in the form of independent papers, there could be some duplication of statements particularly ofthe chapters I and II.