2.1. Epidemiology of CNS and brain tumours.

The tumours originating from the brain and spinal cord are known as CNS tumours. The brain tumour consists of 2-3% of all cancers which are found more in males as compared to females globally. There is a wide variation of occurrence of brain tumours in various regions of the world. In the past 20 years there has been a dramatic increase in the occurrence of brain among patients of all age groups. The incidences of brain tumours are higher in developed western countries and Europe as compared to Asian countries. Lowest incidences are reported in African countries. Among 100,000 people the prevalence rate was 2.5 for females and 3.6 for males globally. The difference of prevalent rates in different regions of the world can be due to the availability of diagnostic capabilities, socio economic status, genetic differences and risk factors. The mortality rate of neuronal cancers is 3.4 per 100000 worldwide which is due to the advances in field of medicine and early diagnosis (1).The one third of all brain tumours are represented by gliomas. They are 80% of primary malignant brain and spinal cord tumours. The survival after diagnosis varies with age and is highest in young age groups. Various environmental, non-environmental and genetic risk factors play a major role in the epidemiology of gliomas (2).

2.2. Role of molecular markers in glioma.

2.2.1. Isocitrate dehydrogenase (IDH).

It is an enzyme which is involved in krebs cycle and is associated with the conversion of isocitrate to alpha-ketoglutarate. Isocitrate dehydrogenase has two sub groups IDH 1 and IDH 2. The mutation in IDH 1 results in abnormal synthesis of 2 hydroxy glutarate which in turn causes methylation of histones and DNA promoting tumour growth by upregulation of vascular endothelial growth factor (VEGF). Mutation in IDH 2 causes 2-hydroxyglutaric aciduria which causes variety of symptoms associated with seizures and cerebrum damage. If the tumours have variation in IDH enzyme then they are known as IDH mutant and if it is absent then it is known as wild type. The IDH marker is used in diagnostic purposes for differentiation of tumours. Drugs such as temozolamide are under clinical trials for inhibition of IDH pathway (3).

2.2.2. O6-methylguanine-DNA methyltransferase methylation (MGMT).

It is an expressed nuclear protein which is associated with the dealkylation of O6-methyl guanine residues in the DNA. The elevation of MGMT can result in reduction in the activity of alkylating chemotherapeutic agents used for treatment of glioma while on the opposite the reduction in the MGMT levels can enhance the effect of the alkylating agents. The MGMT levels can be determined by immunochemistry which can be used to assess the activity of alkylating agents (4).

2.2.3. 1p and 19 q deletion.

This consists of co deletion of short arm of chromosome 1 and log arm of chromosome 19 which is observed in oligodendrogliomas of Grade II and III. It is also associated with IDH mutation and is useful for deciding the treatment for glioma. The deletion can be detected by using techniques such as florescence in situ hybridization(FISH), next generation sequencing(NGS) and chromosomal microarray(CMA) (5).

2.2.4. p53 tumour protein.

It is a well-known tumour suppression gene positioned at short arm of chromosome 17. Due to its mutation it can cause damage to DNA, activate oncogenes and promote proliferation of cancerous cells including glioma. p53 protein mutation was found in 50% of the samples taken from glioma patients. Thus it can be concluded that this mutation plays a vital role in oncogenesis and can serve as a target for many drugs (6).

2.2.5. Telomerase reverse transcriptase (TERT).

The ribonuclear proteins responsible for maintenance of length of telomeres in chromosomes are known as telomerase. In case of normal cells the length on telomeres become shorter with each division while in case of cancerous cells they are elongated. The telomere lengths are important to assess the survival rate of the patients and resistance to therapies like radiotherapy. The telomerase comprise of a telomerase reverse transcriptase (TERT) with RNA sub unit. The mutations at C250 and C228T of the promoter region have been identified for their role in glioma (7).

2.2.6. Alpha-thalassemia/mental retardation syndrome X-linked (ATRX).

It is a gene located on the X chromosome at q arm and plays a major role in transcription of genes, DNA replication and repair, maintenance of telomere length. It has been found that in 75% of cases of WHO grade II and III gliomas the mutation of ATRX is present. The ATRX expression is lacking in glioma cells which can be used for the diagnostic purposes. In most of the cases the ATRX mutation is seen along with other mutations like IDH, p53 etc. (8).

2.2.7. H3K27 mutation.

The maintenance of the structure of chromatin and regulation of gene transfer is done by histones. The class of histones like H4,H3,H2A and H2B bind with H1 after formation of an octamer giving a secondary unit which controls the gene transcription. The methylation of H3 class of histone at lysine 27 is having a major role in carcinogenesis. The variants of H3 histone include H3A and H3B which are found in specifically in pediatric patients of glioma which are also age specific with median age of 11 years. Drugs having histone deacetylation activity can be exploited for treatment of the glioma occurring by this mutation (9).

2.2.8. RELA fusion and BRAF.

RELA is an effector protein in NF-κB signalling which are mediators of central inflammatory responses and are responsible in conversion of neural stem cells to tumorous cells. The fusion of RELA with C11 or f95, an uncharacterised gene due to chromothripsis which is a rare rearrangement phenomena involving chromosomes is responsible for certain types of gliomas (10).The development of tumours and their propagation is regulated by mitogen activated protein kinase pathway(MAPK).This pathway is regulated by RAS and RAF proteins. The RAF kinases include BRAF gene which is responsible for cell proliferation, signalling and survival .The mutation in BRAF known as V600E is responsible for many types of cancers. BRAF inhibitors such as vemurafenib and many others can be used to target cancerous cells (11).

2.3. Glioblastoma multiforme: Most aggressive form of glioma.

The term glioblastoma multiforme (GBM) was devised by Cushing and bailey in the year 1926.The term multiforme indicates differentiation into multiple types of cells based on the histology. The glioblastoma multiforme is the most aggressive, proliferative and lethal form of the brain tumour which is characterised by the world health organisation (WHO) as class IV astrocytoma in the classification of 2016 which was an update to the older system of classification (12). The GBM accounts for 60% of brain tumours in adults and its global occurance rate is less than 10 per 100,000 people which has increased dramatically as compared to last 15 years. The patients having GBM have a median survival rate of 10 months. Early diagnosis and initiation of therapy may improve the survival of patients. GBM can further be classified as IDH1 or IDH 2 mutant, IDH wild type or NOS (not otherwise specified) based upon the presence of molecular markers (13).

2.3.1. Pathophysiology and Risk factors associated with Glioblastoma multiforme.

The incidences of brain tumours are more in case of Caucasian people as compared to Asian or Hispanics. Age is also an important factor which is higher in developed countries. As compared to females, males are more affected but post-menopausal women are at higher risk of development of glioblastoma due to the low levels of sex hormones. It was found from animal studies that estrogen plays a major role in survival of rats implanted with glioma cell lines. Thus it can be concluded that sex hormones play a major role in survival of patients. There were mixed results with women undergoing hormonal replacement therapy or using contraceptives. Genetic factors such as mutation of tumour protein 53, neurofibromatosis (NF1 and NF2), tuberous sclerosis (TSC1 and TSC2), tucrot syndrome and retinoblastoma(RB1). Mutation of genes involved in DNA repair can also cause gliomas. Some viruses like human cytomegalovirus can also induce glioma development. These viruses encode genes which disrupt the signalling mechanisms involved with apoptosis, mutagenesis, cell division and inflammatory pathways. Granulocyte colony stimulating factor is also involved with the malignancy and proliferation of glioblastoma. Exposure to ionizing radiation such as X-rays and γ-rays increases risk of glioma. Radiation workers, radiologist and lab technicians are at higher risk of development of glioma.

Glioblastoma multiforme is also seen in workers at the pesticide, chemical and petrochemical factories who are exposed to carcinogenic chemicals. It is still unclear whether the electromagnetic radiation from telecom equipment has any role in carcinogenesis.

Lifestyle factors should also be considered while assessment of the risk of glioma. Dietary habits have not been a proven risk factor although consumption of fresh vegetables and fruits have been inversely associated with risk of glioma as compared to consumption of meat and processed foods. Body mass Index (BMI) is also a risk factor. Higher BMI indicates obesity which increases risk of glioma along with type 2 diabetes. Alcohol and smoking have been associated with carcinogenesis since a long time although the relation with glioma is still not clearly understood. High carbohydrate intake is directly linked to increased risk of cancer including glioma as cancerous cells depend on glycolysis for their survival and glucose uptake of cancerous cells is higher as compared to normal cells. Consumption of vitamins such as A, D and E and minerals like zinc and calcium show reduced risk for glioma. Intake of caffeine from tea and coffee consumption(>100ml/day) has also shown reduction in risk associated with glioma (14, 15).

2.3.2. Magnetic resonance imaging (MRI).

The main principle of MRI is based on the excitation of hydrogen nuclei in the applied magnetic field using pulses of radiofrequency which excites the nuclei and transition from lower energy level to higher energy level takes place by absorption of energy. After absorption of energy and transition to higher energy state there is emission phase in which energy emitted in the form of radiofrequency induces voltage in the measurement coils which is processed to get images. The images produces by MRI can be T1 or T2 weighed which is based on the relaxation as shown in figure 2.1. T1 relaxation is spin lattice relaxation where the relaxation energy is transferred to the surrounding nuclei and is also known as longitudinal “relaxation”. In case to T2 relaxation the energy is distributed in the spin of the nuclei and is also known as transverse relaxation (16).

MRI imaging requires contrast agents which can be classified as paramagnetic or superparamagnetic based on their magnetic properties. The paramagnetic agents consist of lanthanide complexes of gadolinium and transition series metal manganese complexes which are water soluble. Gadolinum based imaging agents reduce the T1 and T2 relaxation times of neighbouring protons of water which increases T1 intensity and reduces T2 intensity of

images. The superparamagnetic imaging agent consists of iron oxides which are available in the form of dispersed colloids or nanoparticles. They reduce the T2 signals in the tissues where they are localised which produces T2 weighed images. Iron oxide was the first contrast enhancing agent used in MRI for imaging of liver (17).

Many novel techniques using MRI have been developed and are currently in medical use. Diffusion weighed imaging is based upon the Brownian motion of water molecules inside the tissues. The diffusion of water molecules creates a contrast by alteration of signals, which are quantified by calculating the apparent diffusion coefficient map. This technique is useful in imaging brain tumours. Diffusion tensor imaging consists of imaging of white matter by the help of mean diffusivity and fractional anisotropy of water molecules. This technique is useful for tumour imaging and grading of brain tumours. Perfusion weighed imaging utilizes the tumour vasculature and a paramagnetic contrast agent which accumulated in highly vascularized tumour where the blood perfusion is high. Magnetic resonance spectroscopy is useful tool to detect biochemical changes in brain tissues non invasively. This technique can detect compounds which contain choline, creatinine, N-acetylaspartate and lactate which have altered levels in case of gliomas. Functional magnetic resonance imaging consists of T2 weighed images due to deoxygenation of haemoglobin due to brain activity and perfusion changes in the brain (18).

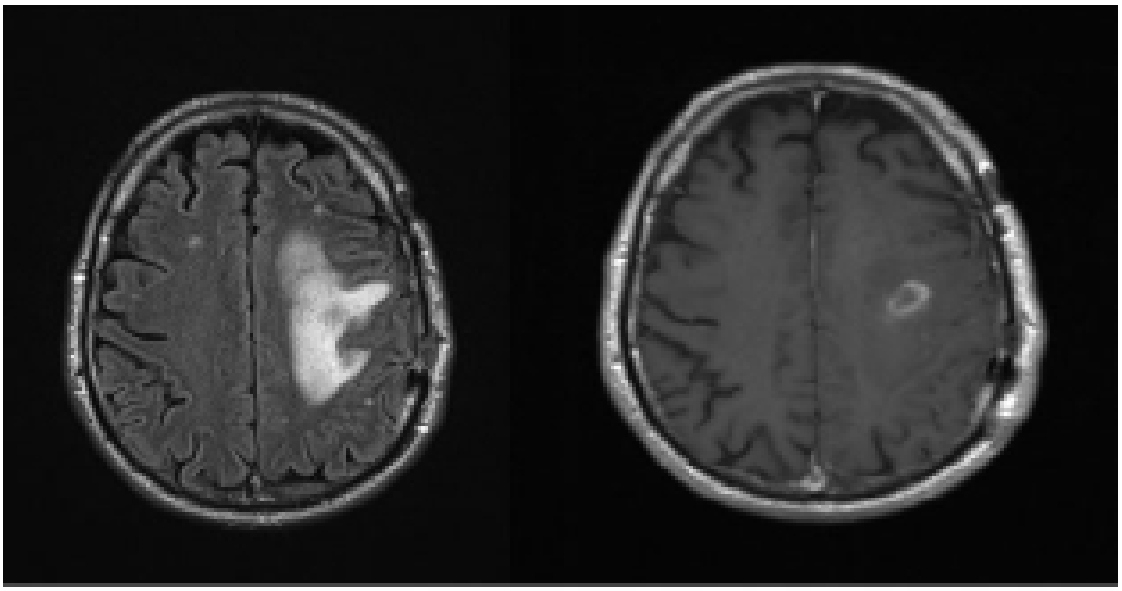

Figure 2.1 :MRI image of grade III astrocytoma(19).

Table 2.1: Commercially FDA approved MRI contrast agents(20, 21).

Commercial name	**Generic name**
Gadolinium based complexes	
OptiMark™ (US)	Gadoversetamide
Gadavist™ (US) Gadovist™ (Britain)	Gadobutrol
ProHance™ (US)	Gadoteridol
Dotarem™ (US)	Gadoterate meglumine
Omniscan™ (US)	Gadodiamide
Magnevist™ (US)	Gadopentetate dimeglumine
Primovist™ (US) Eovist™ (US)	Gadoxetate disodium
Ablavar/Vasovist™ (EU)	Gadofosveset trisodium
MultiHance™ (US)	Gadobenate dimeglumine
Iron oxide nanoparticle based imaging agents	
Feridex™ (US) Endorem™ (Britain)	Dextran coated iron oxide (ferumoxide)
Faraheme™ (US)	Polyglucose sorbitol carboxymethylether-coated iron oxide (ferumoxytol)
Resovist™ (EU,US) Cliavist™ (France)	Carboxydextran-coated iron oxide (ferucarbotran)
Sinerem™ (EU), Combidex™ (US)	Dextran-coated iron oxide (ferumoxtran)

.

2.4. Chemotherapy for glioma.

The current standard treatment of glioblastoma multiforme includes use of temozolamide which methylates DNA at specific sites. The drug is taken by oral route and has near complete bioavailability and can cross blood brain barrier. The cytotoxic effect of the temozolamide is correlated with the intracellular levels of O6-methylguanine-DNA-methyltransferase (MGMT) which is associated with DNA repair. High levels of MGMT is associated with resistance to temozolamide. The drug combined with radiotherapy shows high effectiveness as compared to drug therapy.

The other targeted therapies exploit the growth receptors which are expressed on the gliomas such as epidermal growth factor receptor (EGFR), platelet derived growth factor receptor(PDGFR), vascular endothelial growth factor receptor(VEGFR) etc.. Many molecules such as tyrosine kinase receptors (TKIs), mammalian target of Rapamycin (mTOR) and integrin inhibitors are currently evaluated for their anticancer efficacy. Some of the agents used in targeted therapy for glioblastoma are shown in table 2.2. (22, 23).

Table 2.2 : Examples of targeted molecules for treatment of glioblastoma(22).

Class	Examples
PDGFR inhibitor	Imatinib mesylate Tandutinib
Inhibitors of EGFR	Cetuximab Gefitinib Erlotinib Lapatinib Canertinib Pelitinib BIBW-2992
VEGFR inhibitors	Pazopanib Sunitinib Cediranib Vandetanib Vatalinib Bevacizumab
Integrins	Cilengitide
mTOR inhibitors	Everolimus Sirolimus Temsirolimus

2.5. Drawbacks of conventional therapy for glioblastoma.

Although many approaches are available for the treatment of glioblastoma the key factor lies in its early diagnosis which requires use of sophisticated imaging techniques as the symptoms are generally not precisely experienced in the patients which delays the diagnosis. The Surgical procedures are complex and tumour regrowth may be observed if after surgery diagnostic monitoring is not done and acute morbidity is present in patients. Radiotherapy is usually done in combination with other approaches but the damage induces by radiation to the normal tissues and radioresistance are hurdles in the approach.

Selection of the treatment approach can be difficult due to the mutation of the cancerous cells. Chemotherapy which is the first line approach can also lead to failure as the glioma cells also show resistance to cytotoxic drugs due to upregulation of resistant proteins. For most of the molecules used in chemotherapy the blood brain barrier acts as a physical barrier and inhibits movement of molecules across itself resulting in accumulation in non target organs and toxicity. Apart from these drawbacks the patient compliance is also very low due to adverse effects arising from all the treatment approaches (24).

2.6. Lenalidomide-A novel anticancer drug.

The thalidomide analogue, lenalidomide an immumodulatory was approved by the USFDA in the year 2005 with restricted usage in treatment of complications associated with myelodysplactic syndromes but in the year 2006 it was approved to be used in patients with multiple myeloma. It is available in the market as capsules with brand name Revlimid®, Celegene Corporation, USA with strengths 2.5mg-25mg. The drug also exhibits anti angiogenic and antitumour activity (25).

2.6.1. Chemistry .

The chemical name of lenalidomide is 3-(4-amino-1-oxo 1,3-dihydro-2H-isoindol-2-yl) piperidine-2,6-dione as shown in figure 2.2. It has molecular weight of 259.3gm/mol with empirical formula $C_{13}H_{13}N_3O_3$. The drug physically is white to pale yellow powder with solubility in water/organic phase mixtures with low solubility in less acidic buffers ranging from 0.4-0.5 mg/ml. Due to one asymmetric carbon it exists as racemic mixtures with optically actives forms of S(-) and R(+) forms with a net zero optical rotation (26).

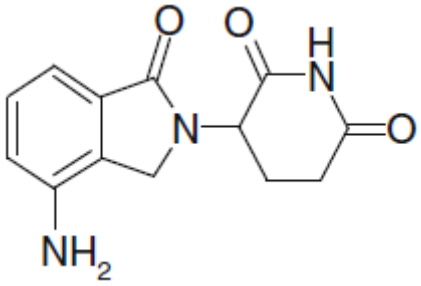

Figure 2.2: Chemical structure of lenalidomide(26).

2.6.2. Pharmacokinetics.

The drug followed by oral administration shows maximum plasma concentration after 0.625 to 1.5 hr post dosing and food co-administration does not alter the bioavailability, which is less than 33% orally but reduces the Cmax by 36%. The plasma binding of the drug is 30% and its elimination occurs unchanged through urinary tract in healthy individuals.

The half-life of the drug is 3 hours. Lenalidomide is stable at room temperature away from sunlight and excessive heat and cold (26, 27).

2.6.3. Mechanism of action.

Lenalidomide has multiple modes of action. It has immunamodulatory activity on different components of the immune system. It inhibits the production of inflammatory cytokines TNF-α and IL-1,6 and 12 with elevation of IL-10 which is an anti-inflammatory cytokine. It also stimulates T cell proliferation with augmentation of natural killer cells. Apart from these mechanisms the key mechanism of action is inhibition of angiogenesis. The tumour cells overexpress vascular endothelial growth factor (VEGF) which signals cell proliferation and neovascularization which are essential for survival of tumours. Lenalidomide inhibits expression of VEGF which inhibits angiogenesis and promoted apoptosis in multiple myeloma and other cancers (28).

2.7. Use of Vascular endothelial growth factor inhibitors in glioma.

Various researchers have found out that VEGF inhibitors including lenalidomide are effective in treatment of glioma. Hanashima et. al. have investigated that glioma cells lines when treated with 10µM of lenalidomide showed increased expression of p21 and p53 proteins which are involved in regulation of apoptosis which resulted in arrest of cell cycle(29). In a Phase I clinical trial involving lenalidomide in pediatric recurrent CNS tumours the doses of 40mg/m^2 were found to be safe and the immunomodulatory response was observed with minimal toxicity and further evaluation was required in Phase II clinical trials (30). Currently many Phase II trials are going in pediatric and younger age groups for treatment of glioma as per the details shown in U.S national library of medicine.

2.8. Work done on lenalidomide based nanoformulations.

The work done on delivery of lenalidomide for treatment of cancer using nanotechnology is described in table 2.3.

Table 2.3: Nanoformulations based delivery of lenalidomide.

Nanoplatform	Application	Research findings	Year	Reference
Hybrid gold nanoparticles	Pancreatic cancer	Pegylated gold nanoparticles were synthesized and lenalidomide was conjugated on the nanoparticles using carbodiimide chemistry. Particle size was 42 ± 2 0.434 and zeta potential was found to be −29 ± 4. Drug loading was 88% for 9µg drug. Folic acid was also conjugated into the nanoparticles which demonstrated anticancer activity against PANC-1 cell lines	2020	**(31)**
Hyaluronic acid functionalised Iron-Platinum nanoparticles	Glioblastoma	Iron-platinum nanoparticles were synthesized by solvothermal process and lenalidomide was conjugated using cis-aconitic anhydride as spacer using carbodiimide chemistry. Hyaluronic acid was conjugated as a targeting moiety. Particle size and zeta potential were found to be 21.3 ± 2.9 nm and 25.8 ± 2.1 respectively. The nanoparticles were found to have anticancer activity against U87MG cell lines and permeation across nasal mucosa.	2019	**(32)**

PLGA nanoparticles	Multiple myeloma	Lenalidomide loaded PLGA nanoaparticles were prepared by nanoprecipitation method and had particle size and zeta potential of 179.4±0.9nm and -24.4±0.2mV respectively. The entrapment efficiency was found to be 78±0.92% The cytotoxic potential of the nanoparticles were evaluated in U2688 cell lines.	2017	**(33)**
Chitosan nanoparticles	Breast cancer Multiple myeloma	Chitosan nanoparticles were prepared by ionic cross linking using Sodium hexamataphosphate. The nanoparticles had size range of 220–295 nm and entrapment efficiency was 99.35%. Cytotoxicity was evaluated for MCF-7 and U266 cell lines.	**2014**	**(34)**

2.9. Role of nanotechnology in treatment of glioma.

After decades of advancement in the field of nanotechnology various forms of nanoparticles have been developed with loading of variety of chemotherapeutic moieties. These nanoparticles have unique characteristics which make them suitable candidates for drug delivery to the brain. The small size range of 10-200nm aids in permeation of nanoparticles across tight junctions of blood brain barrier. The small size also assists in accumulation inside the leaky tumour vasculature by the phenomenon known as enhanced permeation retention (EPR). This type of drug delivery is known as passive targeting of tumours while the active targeting involves use of targeting moieties.

The main advantage of using nanoparticles is delivery of the drug to the desired site and protection of the encapsulated drug with sustained activity at the site of action thereby prolonging the half-life of the drug. Biodegradable nanocarriers are more preferred due to their minimal toxicity (35).

Polymeric nanoparticles of natural origin such as chitosan, albumin, gelatin etc.. and synthetic polymers such as polycaprolactone, polylactide co glycolic acid (PLGA), polyacrylate have been widely researched for delivery of cytotoxic agents out of which PCL and PLGA are approved by the USFDA for use in biomedical applications considering their biodegradable and low toxicity profile. Lipid carriers consisting of liposomes which are phospholipid vesicles are used for both hydrophilic as well hydrophobic drug delivery while solid lipid nanoparticles are used for hydrophobic drugs only. Dendrimers which are composed of central core unit and repeating mono or oligomeric branches with reactive groups are used for conjugation of small molecules. Polymaidoamine (PAMAM) dendrimers have been investigated for delivery of small molecules. Although the toxicity profile of the dendrimers is high as compared to the polymeric and lipid nanoparticles (36, 37).

Inorganic nanoparticles are a separate class of nanoparticles which consist of metallic nanoparticles such as iron oxide, gold etc.., quantum dots, silica nanoparticles and carbon nanotubes. The inorganic nanoparticles offer many advantages over their counterparts such as high tunability, stability and diagnostic applications. The fabrication of the inorganic nanoparticles should be done carefully to avoid toxicity (38).

2.10. Role of mitochondria in cancer.

The mitochondrion is a double-membrane-bound organelle found in most eukaryotic organisms as shown in figure 2.3. Mitochondria provide approximately 95% energy for cellular activities, so as called the “power house of the cell”. Mitochondrion also regulates cell apoptosis by release of cytochrome C into mitochondrial intermembrane space. In presence of deoxy adenosine triphosphate cytochrome C combines with apoptotic protease activation factor and caspase precursor protein forming apoptotic bodies thereby causing cell apoptosis. Mitochondria also regulate ROS homeostasis. Imbalance in metabolic activities and improved resistance to mitochondrial cell death are the causes of cancer (39, 40).

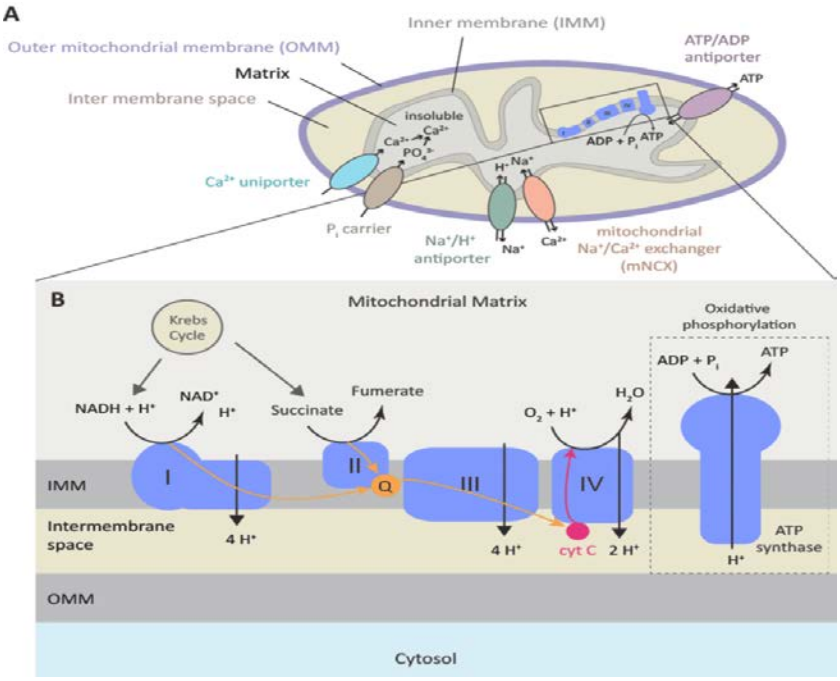

Figure 2.3: Structure of mitochondria.

2.10.1. Warburg effect.

In 1920s, Otto Warburg discovered that tumours were consuming excessive amounts of glucose as compare to surrounding tissue. Glucose was fermented to lactate even in presence of oxygen which is different from normal cells as shown in figure 2.4. This phenomenon is referred as “Warburg effect”. In 1929 Herbert Crabtree confirmed the findings of Warburg and studied heterogeneity in respiration in different types of tumours. Recent studies suggest that the mutation in the mitochondrial DNA or enzymes of TCA cycle may be responsible for the phemomenon. Many oncogenes such as ATK which encodes a protein associated with enhanced glucose uptake may be contributors to oncogenesis and cancer (41).

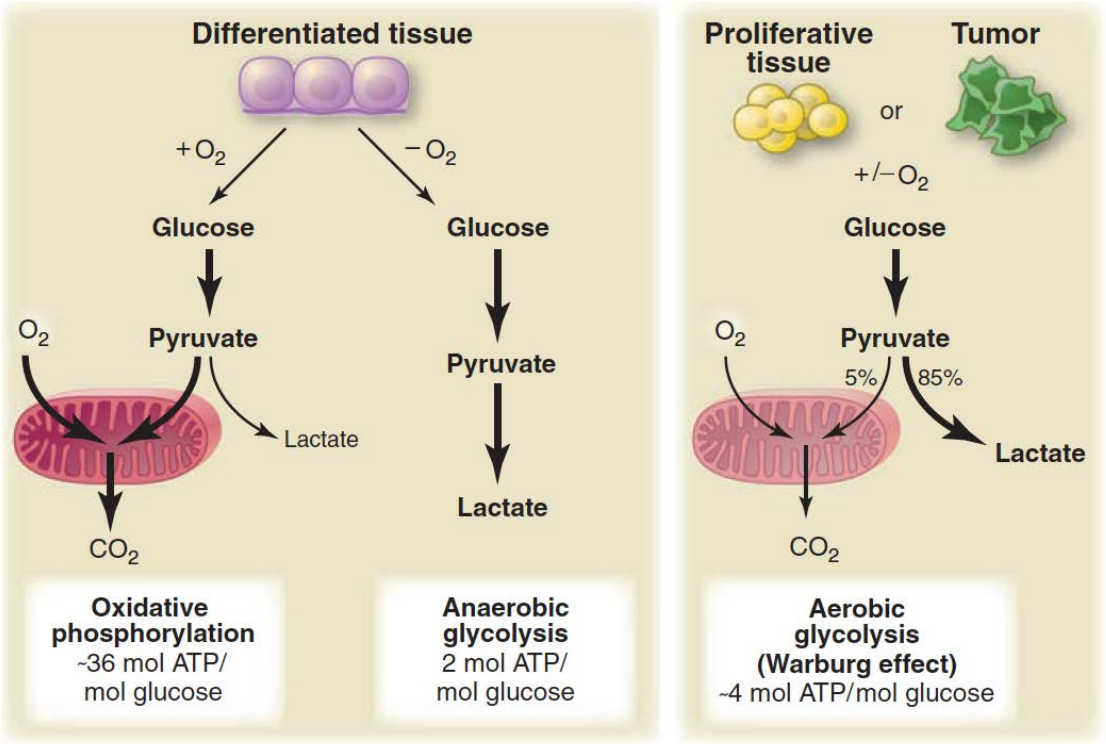

Figure 2.4: Warburg effect (42).

2.10.2. Mitochondrial dysfunction in glioma.

Glioma cells generate ATP through aerobic glycolysis exclusively and are unable to metabolise ketones and fatty acids in absence of glucose. There are abnormalities in the apoptotic pathways such as overexpression of BCL-2 proteins which are associated with resistance against chemotherapy and radiotherapy. The protein is also related to the mitochondrial membranes where its interaction facilitates release of apoptotic proteins.

Cardiolipin which is a mitochondrial membrane bound phospholipid whose dysfunction causes abnormalities in the respiratory chain and metabolic process in glioma cells. The mutations in mitochondrial DNA have role in malignancy of brain tumours although the mechanisms have not been fully understood (43).

2.10.3. Considerations for mitochondrial targeting.

The mitochondria is a complex organelle with four parts the outer mitochondrial membrane (OMM), the intermembrane space (IMS), the inner mitochondrial membrane (IMM), and the matrix. There is presence of cardiolipin and a strong negative charge of approximately ($\Delta\psi m$) of -160to-180 mV across the membranes which makes diffusion of small molecules across it

very difficult. To target mitochondria several strategies have been proposed. Dequalinium which a single chain amphiphilic molecule with two carbon cationic centres can self-assemble like liposomes and can be used to deliver agents to target mitochondria. Triphenylphosphonium (TPP) is another molecule used to target mitochondria which acts by the depolarization of the membranes. Short peptide sequences can also be used to target mitochondria (44, 45)

2.11. Polyethylenimine and its application in theranostic nanoparticles.

Polyethlyenimine (PEI) is a synthetic polymer which are available in two forms-branched PEI(bPEI) and linear PEI(lPEI) as shown in figure 2.5 .The branched PEI is synthesized by cationic ring opening of substituted or unsubstitited azaridines while linear PEI is synthesized by acid catalysed polymerization of azaridine monomers. Linear PEI has secondary amino groups in the chain with primary amines in the terminal region while branched PEI has primary, secondary and tertiary amines in the ratio of 1:2:1(approximately).Wide ranges of PEI are available commercially based on the applications. At room temperature, branched PEI exists as a viscous liquid while linear PEI as a solid powder. Apart from many applications, branched PEI has been widely used as a DNA transfection agent due to the ability of PEI to from stable complexes with DNA and prevent its degradation due to proton sponge effect and pH buffering capacity (46).

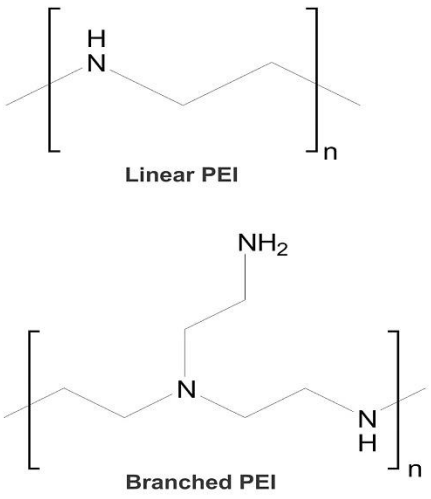

Figure 2.5 : Linear and branched forms of PEI (47).

Non modified PEI is cytotoxic which limits its applications in drug delivery. The modifications of PEI with various polymers/ligands improve its biocompatibility. PEI has been modified with polymers such as dextran, chitosan and pullulan to increase the transfection efficiency. Modification of PEI with PEG (polyethylene glycol) also increases the circulation time of the nanoparticles/nanocomplexes and provides stealth against the cells of immune system. PEI can also be modified with hydrophobic molecules to enhance its interaction with the cell membranes. Many targeting ligands such as folic acid, transferrin, galactose, peptides can be conjugated with PEI to fabricate nanoparticles for targeting the overexpressed receptors on the cancerous cells (48).

PEI has tremendous potential use in development of theranostic nanoparticles. Iron oxide nanoparticles coated with PEI have been reported for delivery of anticancer drugs. PEI conjugated with PEG for delivery of doxorubicin with pH sensitive release mechanism have been developed using iron oxide core (49). Many nanoparticles for bioimaging have been developed using PEI coated on the upconversion nanoparticles which can be excited by near infrared light and in turn emit short wavelength light for detection. Luminescent polymeric nanoparticles involving use of modified polyethyleneimine and polylactic acid (PEI-PLA) copolymer has been used to monitor tumours and their localization. Branched PEI-carbon nanodots have been designed to generated reactive oxygen species using activation of carbon nanodots by light irradiation for treatment of Alzheimer's disease. PEI complexes have also been reported to deliver drugs and therapeutics across blood brain barrier (50).

2.12. Iron oxide nanoparticles- A theranostic platform.

Superparamagnetic iron oxide nanoparticles have been researched thoroughly since their discovery. The driving force behind the intensive research is variety of applications like catalysis, drug delivery, diagnostic agents etc.. Iron oxide nanoparticles consists of nanoparticles of maghemite γ-Fe_2O_3 or magnetite Fe_3O_4 and hematite α-Fe_2O_3. Magnetite is used for anti cancer therapy as it contains $Fe^{2+}$and Fe^{3+} ions in the ratio of 1:2 in which Fe^{2+} ions cause generation of reaction oxygen species by fenton reaction. Magnetite also has the ferromagnetic properties and which it retains even when magnetic field is removed. The physical, chemical and structural properties of magnetic nanoparticles depend on their method of synthesis which can be selected based on the application and feasibility (51).

2.12.1. Methods of synthesis of iron oxide nanoparticles.

2.12.1.1. Coprecipitation method.

This method is based on the precipitation of Fe^{2+} and Fe^{3+} aqueous salt solutions which is done by addition of alkali. The type of nanoparticles synthesized by this method depend on many factors such as the stoichiometric ratio of Fe^{2+}/Fe^{3+},type of the precursor used (nitrate, chloride etc..), type of alkali used (sodium hydroxide, ammonium hydroxide etc..), temperature of the medium, pH and stirring speed. This method is most cost effective but the main drawback in this method is lack of control over particle size and polydispersity. This method produces less crystalline nanoparticles and many variations of the technique are available which carry almost the same drawbacks (52).

2.12.1.2. Thermal decomposition (solvothermal method).

This method involves use of the metal salts (actetylacetonates) which decompose at very high temperatures in high boiling point organic solvents. It also involves use of surfactants or stabilisers such as oleyl amine, oleic acid, hexadecylamine etc... However after completion of the reaction the iron oxide have to be washed with toxic organic solvents such as chloroform, hexane, toluene etc.. followed by its phase transfer into aqueous phase. The main drawback of this method is that the removal of the toxic organic impurities and solvents is difficult which be harmful for biomedical use.

The method is also tedious and time consuming and requires careful heating for the decomposition reaction under inert atmosphere. The main advantage of this method is that the iron oxide nanoparticles produced have narrow size distribution and are relatively monodisperse (53).

2.12.1.3. Sol-gel method.

It consists of hydrolysis of metal alkoxide precursors resulting in generation of metal oxide nanoparticles which are gelled by removal of solvent. This method produces large sized relatively monodispersed nanoparticles although further heat treatment is required in the next step to get desired crystalline structure and post purification is required. Modification of the iron oxide with polymers such as chitosan, gelatin, PVA etc.. can be done to prevent agglomeration. Coating with inorganic materials such as carbon silica and precious metals such as gold and platinium is also possible simultaneously (54).

2.12.1.4. Microemulsion method.

In this method, the iron oxide nanoparticles are synthesized by intermicellar nucleation by using iron chloride salts and reducing agents. Ampiphilic surfactants such as cetyltrimethylammonium bromide (CTAB), dioctyl sodium dodecyl sulfate (DSDS), sodium dodecyl sulfate (SDS) and polyethoxylates (e.g. Tween-20 and -80) are used for the preparation of microemulsion. The limitations of the method are low yield, difficulty in removal of surfactants and post purification after thermal treatment(55).

2.12.1.5. Hydrothermal method.

This method is associated with the treatment of the metal precursors at high temperature and pressure in the range of 130-250°C and 0.4-4MPa respectively in a sealed jacketed conatainer. The method produces highly crystalline and monodisperse nanoparticles but the final size of nanoparticles is difficult to control.The method requires precise control over reaction conditions(56).

2.12.1.6. Polyol method.

The polyol process consists of use of diols majorly 1,2 diols like ethyleneglycols and its analogues such as diethylene, triethylene, tetraethylene and polyethylene glycols which have high boiling points from 197°C (ethylene glycol) to 328°C (tetraethylene glycol) due to increase in chain lengths.

The use of polyols in the process have multiple advantages as they act as metal precursor solubilizers, surfactants/stabilizers and reducing agents and do not require any additional excipient. Metal precursors used in the reaction are nitrates, chlorides, sulphated, hydroxides, acetates and acetylacetonates. The metal precursors are heated with the polyols at high temperatures under inert atmosphere. By controlling the reaction temperature and time the shape and size of nanoparticles can be varied. The method offers excellent control over particle size and polydispersity with high crystalline yield and purification step is also easy. By using this method, various types of metallic nanoparticles such as monometallic, alloy and core shell type can be synthesized. This method is comparably cheaper, eco-friendly and scalable (57, 58).

2.12.1.7. Miscellaneous methods.

Sonochemical process involves use of ultrasonication which causes cavitation in aqueous medium where microbubbles occur and temperature of around 5000°C and pressure of 1800 Kpa is generated. 3nm Ytrium nanoparticles have been prepared by this method (59). Electrochemical involves use of iron electrode in aqueous solution of DMF and cationic surfactants. The particle size depends on the current density applied. Aerosol technology is also used in preparation of iron oxide nanoparticles in which the ferric salts along with a reducing agent is sprayed in reactors where the solvent evaporates leaving nanoparticles in the size range of 5-60nm. Laser pyrolysis is a technique in which lower reaction volumes can be used for synthesis of nanoparticles with particle sizes of 2-7nm (60).

2.12.2. Applications of iron oxide nanoparticles in cancer treatment.

The iron oxide nanoparticles have numerous applications in various fields but the most promising application is in the biomedical use for diagnosis and treatment of cancer. The following are the applications of iron oxide nanoparticles.

2.12.2.1. Photothermal therapy.

It is a non-invasive therapy of cancer which utilizes light radiation in visible or near infrared spectrum by using laser, which is absorbed by the specialised materials such as inorganic nanoparticles including iron oxide nanoparticles which in response emit thermal radiation which is responsible for destruction of cancerous cells. High intensity laser causes necrosis of cancerous cells while low intensity causes apoptosis (61).

2.12.2.2. Magnetic hyperthermia.

When the iron oxide nanoparticles are exposed to the alternating magnetic fields of optimised amplitude and frequency, heat is generated locally which causes death of cancerous cells. However, large quantities of nanoparticles need to be injected which raises concern over their toxicity. There are three mechanisms which are responsible for this property. Neel relaxation is associated with internal friction with respect to crystal lattice. Brown relaxation is the internal friction of nanoparticles and surrounding environment while hysteresis loss which is shift of domain walls contributes to the hyperthermia effect (62).

2.12.2.3. Site specific drug delivery.

The magnetic property of iron oxide nanoparticles can be utilised for drug delivery of cytotoxic agents at specific sites which requires higher saturation magnetization of the nanoparticles to control its movement. Strong magnetic field is also required at the region of interest which needs to be focused at the specific site which requires specialised instrumentation (63).

2.12.2.4. Magnetic resonance imaging contrast agents.

The approved clinical use of iron oxide nanoparticles is contrast agent in MRI. The ultrasmall superparamagnetic iron oxide nanoparticles (USPIO) having mean diameter of less than 50 nm and superparamagnetic iron oxide nanoparticles (SPIO) which have mean diameter of more than 50 nm are approved for use in MRI imaging. The iron oxide nanoparticles are T2 contrast agents and are administered by intravenous administration. The nanoparticles can also penetrate blood brain barrier and reach the brain which is useful for imaging of glioma (64). Doping of iron oxide nanoparticles with lanthanides can also enhance the T1 contrast thereby providing dual contrast in MRI which enhances the diagnostic capabilities of the same (65).

Table 2.4 shows the list of iron oxide nanoparticles currently used or under clinical trials for imaging and therapy.

Table 2.4: Current status of formulations of iron oxide for diagnosis and therapy(66).

Iron oxide nanoparticles for diagnostic purpose				
Product name	**Hydrodynamic size (nm)**	**Coating**	**Application**	**Regulatory status**
Ferumoxytol (Feraheme®/Rienso®)	30	Polyglucose sorbitol carboxymethylether	Brain tumour imaging	Clinical trials
Feruglose (Clariscan™, MION-46, NC100150)	20	PEGylated starch	Liver lesions/perfusion imaging	Clinical trials
Ferucarbotran (Resovist® ,Cliavist™,SHU 555A)	60	Carboxydextran	Liver/spleen imaging	Approved
Iron oxide nanoparticles as therapeutic agents				
NanoTherm®	15	Aminosilane	Magnetic hyperthermia on brain tumours	Approved/in use in Europe
Feraheme®	30	Polyglucose sorbitol carboxymethyether	Treatment of IDA in Adult patients with CKD	Approved/in use
SPION-epirubicin	50–150	Anhydroglucose	Magnetic drug targeting	Clinical trials

2.12.3. In vivo fate/Pharmacokinetics of iron oxide nanoparticles.

In most of the cases, the iron oxide nanoparticles are injected intravenously as it is the most effective route for reaching the organs but other routes such as intraperitoneal, intratumoral, intrapulmonary and oral route have been reported. Nanoparticles more than 50nm show passive targeting by reticuloendothelial system while particles with diameter of less than 50 nm show less accumulation. Particles under 10 nm show faster excretion from blood through renal clearance into urine while particles > 200nm show accumulation in liver and spleen. The distribution of IONPs below 20 nm can be found in major organs like heart, lungs, brain, stomach and small intestine while the blood brain barrier was also found to be breachable by the nanoparticles. The magnetic field targeting was more promising rather than relying on the passive diffusion.

Intravenously injected nanoparticles undergo phagocytosis by macrophages and organs of the reticuloendothelial system and are metabolised in acidic lysosomes where they are integrated into the normal iron metabolism with the help of proteins like ferritn and hemosiderine for synthesis of haemoglobin and myoglobin inside the body.In case of oral administration the iron oxide nanoaprticles slowly degrade into acidic environment of stomach from which the iron ions are absorbed and enter the iron metabolic pathway of the body. The oral route shows slower metabolism as compared to intravenous route. The polymeric coatings and functionalization on iron oxide nanoparticles increase the circulation time and the half-life (67).

2.12.4. Toxicological aspects of iron oxide nanoparticles.

The iron oxide nanoparticles are considered safer as compared to other inorganic nanoparticles and are available for clinical use. The LD50 (dose required to kill half of the animals under trial) was found to be 300-600 mg Fe/kg of body weight while the value increased to 2000-6000 mg Fe/kg, when the nanoparticles were coated with biocompatible polymers such as dextran. However, the toxicological studies have not been reported with other capping agents. In case of animal models, minimal toxicity was reported for intravenous and oral routes of administration as all the biochemical parameters were normal.

For the FDA approved nanoparticles clinical trials have reported elevated haemoglobin levels with no serious adverse reactions in clinical trials although long term toxicity has not been evaluated. Thus considering all the facts it can be concluded that the iron oxide nanoparticles are the future of drug delivery and diagnostics and are backbone of theranostics (68).

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