1.1 Brain tumor

Brain tumors refer to a heterogeneous group of primary and metastatic neoplasms in the central nervous system and are one of the life-threatening diseases which are characterized by low survival rate (1). According to GLOBOCAN 2018, nearly 296,851 new cases of brain and nervous system tumors and 241037 deaths are diagnosed in 2018 worldwide. In India 28142 new brain tumor cases annually are reported while deaths were 24003 in 2018 (2). The estimated incidence, mortality and 5 year prevalence of brain tumor among men in India is approximately 11855, 9574 and 17251 respectively which represents 2.5%, 2.7% and 2.6% of Indian population respectively while for women it is 6976 (1.3%), 5578 (1.7%) and 10157 (1%) respectively. More than 120 types of brain tumors are identified till date and depending on the origin of tumor, most common tumors are grouped as tumors of neuroepithelial tissue, tumors of cranial and spinal nerve, tumors of meninges, heamopoitic origin neoplasm and lymphomas, tumor of sellar region, germ cell tumors and cysts (3,4). The most prevalent brain tumors are intracranial metastases from systemic cancers, meningiomas, and gliomas, specifically, glioblastoma (1,5). Glioma is the most frequent primary brain cancer which accounts for 29% of all primary brain and CNS tumors and 80% of malignant brain tumors. These malignant gliomas are primary tumors that are derived from glial origin and account for approximately 70% of new primary brain cancer diagnosis. The classification, grading, and treatment of this diverse group of tumors have been primarily based on morphological criteria, which introduced a certain degree of interpretative subjectivity and moreover provided only suboptimal accuracy for the prediction of treatment response (6). WHO has classified glioma in three category viz. astrocytoma, oligodendrogliomas and mixed gliomas (oligoastrocyotomas). Amongst gliomas, glioblastomamultiforme (GBM) which is a grade IV astrocytoma according to the World Health Organization (WHO) classification, is the most common and aggressive form of glioma in nature (3,6). The median survival for glioblastoma is 14 months (7). The high mortality rate due to GBM can be attributed to specific properties of glioma which includes highly infiltrative nature and lack of clear margin. The existing therapy for GBM is nonspecific and almost fails to prevent reoccurrence of disease.

1.2 Current treatment approaches for brain tumor

Multiple treatment approaches have been experimented for treatment of brain cancer and may vary depending on the type, grade, size and location of the tumor; whether it has spread;

and patient age and general health. The multidisciplinary approach for treatment of brain tumor includes combination of surgery, radiation and chemotherapy (3).

Most common initial therapy for brain tumor is chemotherapy. Various therapeutic moieties indicated in brain tumor are available in oral and parenteral dosage form in market. Temozolomide is the first generation drug used for the treatment of brain tumors and is presently given orally (8). Other therapeutic drugs indicated for being used in treatment of brain tumor include irinotecan, carmustine, cisplatin and lomustine. Most of the drugs have shown enhanced anticancer activity for brain tumor *in vitro*. However, clinical failure was observed with such drugs due to insufficient barrier passage.

Although chemotherapy in combination with other treatment approaches such as radiotherapy and surgery has proved effective still tumor recurrence (96% cases) adjacent to resection margin after surgical resection makes it important to develop adjuvant therapy which can help minimize the recurrence with enhanced efficacy and specificity. Poor prognosis and rapid recurrence are associated with standard therapy of glioma because infiltrate growth of gliomas makes it difficult for the surgeon to completely remove pathologic or cancer-infiltrated tissues without affecting normal brain functions. Furthermore, the failure is also ascribed to the side effects of radiotherapy and poor outcome of usual chemotherapy (9).

1.3 Temozolomide

Temozolomide (TMZ) is the first line drug used for the treatment of brain tumors. It has showed significant activity against tumor with recurrent and refractory melanoma and high grade glioma in phase I and phase II of clinical trials. Both preclinical and phase I studies suggested that after post oral administration, TMZ is completely absorbed through GIT, however, it exhibited time-dependent anti-tumor activity. TMZ with a dose of 250 mg/m^2/day is administered for 5 days. On frequent dosing schedules, prominent clinical activity was observed, especially when peroral TMZ is administered once a day for 5 days for four weeks. It is basically an alkylating agent of imidazotetrazine series and a prodrug which firstly get converted into highly unstable compound 5-(3-methyltriazen-1-yl) imidazole-4-carboxamide (MTIC) at physiological pH and then MTIC is further converted into 5-aminoimidazole-4-carboxamide (AIC) and methyldiazonium ion. Formation of these methyldiazonium ions cause breakage of double strand of DNA and lead to cell cycle arrest and cell death by methylation of DNA (10). Although TMZ has ability to cross blood brain barrier (BBB), it

needs high systemic dose to reach therapeutic concentration in brain because of its short half-life. Due to this, various systemic side effects like oral ulceration, bone marrow suppression, fatigue, vomiting, nausea and headache are associated with TMZ therapy. The previous studies proposed that continuous TMZ administration results into reduction in Nadir's platelet count (11,12). To overcome these side effects and to improve the therapeutic activity of TMZ via targeting TMZ in brain, novel drug delivery system is required.

1.4 Lenalidomide

Lenalidomide (LND) was proposed for the treatment of multiple myeloma, for which thalidomide is an approved medicine and LND shares structural similarities with thalidomide. It is blockbuster drug and is an immunomodulatory agent with anti-tumor and antiangiogenic properties. LND is off-white to pale-yellow powder commercialized under the trade name Revlimid. However, Revelimid hemihydrate (commercial form) due to its inadequate solubility in water has poor oral bioavailability i.e. 33%. Additionally, it has very short half-life i.e. 3 h. Several clinical investigation provide an insight for the utilization of LND in brain-tumor treatment, however clinical trial in phase II is still going on (13–15). Thus LND with other drug makes it a possible candidate for brain tumor therapy. LND possess a limitation that it is not capable of crossing blood brain barrier (BBB) and enter cerebrospinal fluid (CSF) at concentration which are therapeutically significant. LND additionally goes with atypical biodistribution and extremely short residence time in body. LND therefore need to be administered in such a manner as to improve its brain absorption and the residence at the tumor site.

1.5 Limitations associated with chemotherapeutic agents

The major hindrance to prognosis of brain tumor is the auto protective nature of the brain (BBB and alignment of brain cells), genomic alterations occurring in tumor cells, efflux transporters on the barrier and properties of chemical agents used for treatment of brain tumors. Brain allows passage of some of endogenous material, a few hydrophobic agents and particles with molecular weight of less than 500Da. Lipophilicity of the drug is one of the important factors that should be considered while designing new entities for treatment of tumors. Improvement in passage of drug across the BBB is possible with increased lipophilicity but this may be associated with increased drug uptake by other tissues, causing an increased tissue burden. This non selectivity in delivery of drugs to non targeted site is

detrimental; especially when cytotoxic agents are used, because drug toxicity would be higher at non target sites. Enhanced efflux along with loss of CNS activity is another major drawback of increased lipophilicity which might lead to poor tissue retention and short biological action. Improvement in the therapy could be brought about by modulating the pharmacological properties of the drug (16).

1.6 Barriers associated with Chemotherapy of Brain tumors

Apart from the limitation associated with chemotherapeutic drugs, drug delivery to brain tumors are also hindered by some physiological barriers like blood brain barrier (BBB), blood brain tumor barrier (BBTB) and blood CSF barrier (BCSFB) among which BBB plays important role. The BBB prevents transportation of approximately 98% of the small molecules and nearly 100% of large molecules including recombinant proteins and genes into the brain and reaching the tumor sites (17–20) while BBTB limits the paracellular delivery of most hydrophilic molecules to tumor tissue (9,16) and BCSFB blocked the passage of systemically administered therapeutic agents (21). So to overcome the limitation associated with chemotherapeutic agent as well as overcoming barriers and delivering drugs to the target site of brain novel drug delivery system is required.

1.7 Nanoparticles as drug delivery platform for overcoming drawbacks of conventional therapy

In past several decades, nanoparticles have gained lots of attention for treatment of cancer and were fabricated for cancer treatment by modulating their physicochemical properties including composition, size, shape and surface modification. Nanoparticles have potential to overcome the drawbacks of conventional cancer chemotherapy because of unique properties like small size, surface charge, variable shape, several binding sites for the attachment of target specific ligands, antibodies, peptides etc. They can also enhance the tumor targeting by both passive and active targeting mechanism. Passive targeting is possible due to enhanced permeability and retention (EPR) effect (22,23). Nanoparticles based delivery systems are also approved by the FDA for clinical use (Abraxane, Doxil, Genexol-PM, DepoCyt, Myocet etc.) and many more are in the clinical trials (NK105, CYt-6091, Genexol-PM, Rexin-G etc.) (22,24). As compared to conventional chemotherapy, nanoparticles based delivery systems have several advantages and features, including: 1) improved delivery of poorly water soluble drugs, peptides, and genes; 2) better protection of drugs, peptides or genes from harsh

environments (e.g., enzymatic degradation and the highly acidic environment in the lysosomes or stomach); 3) enhanced treatment efficiency and reduced systemic side effects by cell- or tissue specific targeted delivery of drugs, peptides or genes; 4) overcome multidrug resistance by co delivery of drugs, peptides, genes and/or diagnostic agents; 5) stimuli-responsive systems (pH sensitive, temperature sensitive, redox sensitive) can control release of drugs, peptides or gene over a manageable period of time at precise doses (22,25).

Nanoparticles used as a carrier for cancer therapeutics may be of several types viz. protein based nanoparticles (albumin nanoparticles, gelatin nanoparticles etc.) (26,27), polymer based nanoparticles (poly lactide co glycolide nanoparticles, polycaprolactone nanoparticles, polylactide nanoparticles, chitosan nanoparticles etc.) (28–31), lipid based nanoparticles (solid lipid nanoparticles, nanostructured lipid carriers, liposomes etc.) (32–34), lipid polymer hybrid nanoparticles (35), metal nanoparticles (36–38), polymeric micelles (39), dendrimers (40) etc. Among all these nanoparticles, protein based nanoparticles have gained much more attention in cancer therapy due to unique properties viz. relatively safe and easy to prepare, capability to deliver proteins, peptides, genes, nucleic acid, and hydrophilic as well as hydrophobic anticancer molecules, site specific targeting by surface modification, greater stability profile during storage, etc. (41).

1.8 Albumin based Nanocarriers

Albumin is a versatile protein used as a carrier system for cancer therapeutics. As a carrier it can provide tumor specificity, reduce drug related toxicity, maintain therapeutic concentration of therapeutic moiety like drug, gene, peptide, protein etc for long period of time and also reduce drug related toxicities. It also has the potential in the half life extension of drug. As albumin has various binding sites, ligand functionalized delivery of therapeutic moiety is also possible which can provide site specific delivery of the therapeutic moiety (27). Two basic approaches are utilized in the development of albumin based cancer therapy system i.e. conjugation of therapeutic moiety directly to the albumin or formulation of nanoparticles incorporated with therapeutic moiety like drug, peptide, gene etc. Some of biological applications of albumin conjugates are: use as a reagent for immunoassay and immunohistochemistry, used for elucidating hormone receptor interactions and used in the treatment of various diseases like cancer, viral infection and diabeties (27,42).

1.8.1 Albumin nanoparticles

Albumin based nanoparticles are utilized for cancer treatment as they are biodegradable, non antigenic and can be also surface modified which may help in avoiding the undesirable toxicity of drugs by modifying their body distribution and improve their cellular uptake. They also have targeting potential because proteins themselves act as passive as well as active targeting moiety. Other targeting ligands can also attach in these carriers to provide site specificity (43). Albumin nanoparticles can be prepared by several methods like desolvation, emulsification, thermal gelation, nano spray drying, nab technology and self assembly etc. The selection of the method is based on several factors such as type of system, area of application, required size, type of drug (hydrophilic or hydrophobic), etc (43). Role of albumin nanoparticles and their applications in cancer therapy will be discussed in the subsequent chapter of the thesis.

1.9 Surface modification of albumin nanoparticles for targeted delivery to brain tumor

Surface modification of albumin nanoparticles is necessary to alter the surface properties and enhance the targeting potential of the delivery system. Presence of different binding sites and functional groups like carboxyl and amino groups on albumin offers several possibilities for surface modification of albumin nanoparticles. Surface modification of albumin nanoparticles with the specific ligand can be done by conjugating functional group of albumin with the ligand by covalent bond. For surface modification of albumin nanoparticles, electrostatic adsorption or surface coating techniques may be utilized as non covalent attachment of ligands. In surface modified albumin nanoparticles, albumin plays a role of carrier for delivering therapeutic moiety whereas the ligand is used to modify the pharmacokinetic parameters, improve stability, prolonging circulation half life, modifying the release pattern of therapeutic moiety or as a targeting agent (27,44).

1.9.1 Hyaluronic acid as targeting ligand

HA is natural, anionic, non-sulfated glycosaminoglycan that consists of β-1,4 linked D-glucuronyl-β-(1,3) (Gln)-N-acetyl-D-glucosamine and are widely distributed throughout epithelial, neural and connective tissues (45). HA is the largest polysaccharide in the body, with an average molecular weight of 1-8 MDa (46,47). Human skin also contains large amount of HA i.e. 400-500 µg HA/g (48). In other organs, the content of HA can vary from

approximately from 1 to 100 µg HA/g (49). HA plays significant role in various biological processes, cancer metastasis, cell migration, cell differentiation and wound healing (50). Additionally, CD44, a glycoprotein, is HA receptor and are over expressed in large number of mammalian cells and its interaction with HA is crucial for the growth and metastasis of cancer cells (51). A lot of attention has been attracted by the researchers towards investigation of HA as a targeting moiety in cancer therapy and cancer imaging which will be discussed in subsequent chapter of the thesis.

1.9.2 Chondroitin sulfate as targeting ligand

Chondroitin sulfate (CS), type of glycosaminoglycans, consists of disaccharide units of b-1,3-linked N-acetyl galactosamine and b-1,4-linked d-glucuronic acid with certain sulphated positions (52). CS is widely distributed in mammals' skin, cartilage, bones and blood vessels (53–56). Being similar to HA, CS has also been reported to have the ability to recognize and interact with HA-mediated CD44 receptors.

1.9.3 Lactoferrin as targeting ligand

Lactoferrin (Lf) is an 80 kDa cationic protein belonging to transferrin family which shows 60-80% of sequence similarity with transferrin (57). In another way, Lf is mammalian, cationic iron-binding glycoprotein which consist of polypeptide chains of about 690 amino acids folded into two globular lobes, each of which consist of one iron-binding site (58). The various studies demonstrated that therapeutic moiety loaded nanoparticles when conjugated with Lf were efficiently crosses BBB as compared to transferrin as targeting moiety as well as non-conjugated nanoparticles. Additionally, Lf receptors not only present on BBB but also present or over expressed in glioblastoma cells (59,60). The finding revealed that Lf can be a good candidate for crossing BBB and ultimately targeting brain tumor.

1.10 Aims and objectives

The aim of the present research work was development of nanocarrier based targeted drug delivery system for effective treatment of brain tumor. The proposed study was planned to achieve an effective and selective brain tumor targeting using albumin nanoparticles as drug delivery platform with two model drugs (temozolomide and lenalidomide). It would help to inhibit the growth of tumor by targeting therapeutic moiety to tumor so as to prevent metastasis and growth of tumor. Further for making albumin nanoparticles target specific, surface modification was done with suitable targeting ligands (HA, CS and LF) for selective brain tumor targeting that would help to reduce the toxicity associated with anticancer therapeutic moieties.

Thus the present research work was proposed to be carried out in following steps.

Step 1: Selection of suitable method for preparation of nanoparticles (albumin) based on the properties of drugs.

Step 2: Optimization of prepared nanoparticles based on experimental design.

Step 3: Surface modification of nanoparticles with suitable targeting ligands and their optimization.

Step 4: Characterization and evaluation of developed nanoparticles.

Step 5: In vivo studies

Step 6: Stability studies

1.11 Hypothesis

It was hypothesized that incorporation of selected drugs into albumin nanoparticles will prevent drug from external physiological environment and surface modification with specific ligand will help in brain specific drug delivery by receptor mediated targeted drug delivery. The surface modified nanoparticles will lead to enhanced delivery of the drug to the brain by prevention of the clearance by reticuloendothelial system and probable inhibition of the efflux mechanism of brain and ultimately enhance drug concentration towards target site. This targeted drug delivery will lead to reduction in dose and also will reduce drug related toxicity towards normal cells.

1.12 Plan of work

1. Procurement of chemicals and therapeutic moiety.
2. Fabrication of albumin nanoparticles.
3. Optimization of albumin nanoparticles.
4. Drug loading and optimization.
5. Characterization of developed albumin nanoparticles viz. particle size, PDI, zeta potential, percent entrapment efficiency, percent drug loading efficiency, Infrared spectroscopy, Differential scanning Calorimetry (DSC), X-ray diffraction study (XRD) etc.
6. Surface modification of developed drug loaded albumin nanoparticles using different targeting ligands.
7. Optimization of surface modification on the basis of different quality attributes like particle size, PDI, zeta potential and percent conjugation efficiency.
8. Characterization of surface modified albumin nanoparticles viz. particle size, PDI, zeta potential, percent entrapment efficiency, percent drug loading efficiency, percent conjugation efficiency, Infrared spectroscopy, Differential scanning Calorimetry (DSC), X-ray diffraction study (XRD) etc.
9. *In vitro* drug release studies.
10. *In vitro* cell line studies.
11. *In vivo* studies.
12. Stability studies

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