Summary

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.

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.

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

Firstly temozolomide loaded nanoparticles (TNPs) were developed and optimized. TNPs were prepared by modified desolvation method. TNPs were preliminary optimized by OVAT design to identify the critical parameters and their working range for further optimization. During preliminary optimization, various parameters like polymer to drug ratio, polymer concentration, organic phase volume, crosslinker (gluteraldehyde) concentration, rate of addition of organic phase, effect of stirring speed, effect of pH, etc were screened. The results indicated that polymer to drug ratio ratio, organic phase volume and crosslinker concentration had significant effect on quality attributes (particle size, PDI, zeta potential and entrapment efficiency) of nanoparticles. So these parameters were further optimized using Box-Behnken response surface methodology and results indicated that all three parameters had significant effect on particle size and entrapment efficiency of TNPs while non significant effect on PDI and zeta potential. In case of particle size, GA concentration showed most significant effect as compared to polymer: drug and organic phase volume on particle size. In case of entrapment efficiency, polymer: drug ratio and interaction of polymer: drug ratio and organic phase volume was having most significant effect as compared to GA concentration and organic phase volume.

After optimization of TNPs, surface modification was done with hyaluronic acid (HA) and chondroitin sulphate (CS) using carbodiimide chemistry to achieve CD44 mediated targeted delivery of TNPs (HA-TNPs and CS-TNPs). Surface modification with HA and CS was optimized on the basis of particle size, PDI, zeta potential and conjugation efficiency. Different parameters like molecular weight of HA, ligand to NPs ratio and stirring time was selected as parameter of optimization. Conjugation of HA and CS with TNPs was also confirmed by FTIR studies. For HA-TNPs development, low molecular weight HA, HA: NPs (1:1) and 30 min of stirring was found to be optimum while in case of CS-TNPs, CS: NPs (1:1) and 12 h stirring was found to be optimum to get the highest conjugation efficiency of 78.34 % ± 1.91 % and 74.34 % ± 1.87 % respectively.

In the next step, surface modified HA-TNPs and CS-TNPs along with TNPs were characterized and evaluated on the basis of various in vitro studies. The dynamic light scattering results indicated that hydrodynamic diameter of TNPs was within the range of 150-160 nm while in case of HA-TNPs and CS-TNPs it was increased and was in range of 200-350 nm. The

increase in the hydrodynamic diameter of surface modified nanoparticles may be attributed to targeting moiety. The particle size distribution pattern indicated uniformity of nanoparticles dispersion which made them suitable for intravenous administration. Zeta potential results indicated the stability of the developed nanoparticles.

DSC study indicated that in both the cases (TNPs and surface modified TNPs) drug was molecularly dispersed in the carrier and totally encapsulated within the nanoparticles as the exothermic peak of drug was not present in the final formulations.

FTIR data also confirmed the presence of drug in the nanoparticles as all the characteristic peaks of drug was present in the developed nanoparticles with lesser intensity. FTIR data also confirmed the successful conjugation of HA and CS with TNPs as the new amide bond peak was observed in both HA-TNPs and CS-TNPs.

XRD spectrum revealed conversion of crystalline nature of TMZ in amorphous nature after encapsulation in nanoparticles as the highly intense characteristic peaks of drug was converted in the lesser intensity peaks.

TEM image of HA-TNPs and CS-TNPs showed roughly spherical shape, exhibiting a dark core surrounded by a lighter gray rim likely corresponding to the HA and CS conjugation respectively. Size of HA-TNPs and CS-TNPs obtain by TEM was lesser than obtained by zetasizer (DLS measurement). This may be due to presence of water surrounding HA-TNPs during DLS in comparison with size measured in dried state by TEM.

In vitro release data revealed biphasic release of TMZ from TNPs, HA-TNPs and CS-TNPs. All the developed nanoparticles showed initial burst release than slower and sustained release. The surface modified HA-TNPs and CS-TNPs showed more sustained release pattern of drug as compare to TNPs this may be due to slower diffusion of drug from additional HA and CS layer over the TNPs.

Results of cell viability assay of different developed nanoparticles showed higher suppression of cells as compared to pure TMZ. Surface modified TNPs (HA-TNPs and CS-TNPs both) showed higher cell suppression than TNPs. This may be due to higher uptake of the

nanoparticles because of presence of targeting moiety over the surface which enhanced the cellular uptake of nanoparticles by binding with the CD44 receptor present in the surface of tumor cells.

In vitro BBB passage study also confirmed the higher permeation of nanoparticles through BBB than pure drug. This may be due to smaller size of nanoparticles than the pure TMZ. As compared to TNPs, HA-TNPs and CS-TNPs showed higher permeation this was due to the fact that CS and HA also plays role in crossing BBB. The sequence of BBB passage was as follows: HA-TNPs > CS-TNPs > TNPs > TMZ

Effect of developed nanoparticles on cell cycle arrest was also carried out and results demonstrated that as compare to pure TMZ, CS-TNPs showed higher G2/M phase cell arrest. That suggested higher inhibition of growth of cancerous cells. In case of HA-TNPs no significant changes as compare to non treated cells were observed.

Surface modification plays important role in cellular uptake of nanoparticles. HA and CS both are CD44 receptor targeting moieties. CD44 receptors are over expressed in brain tumors. To achieve the brain tumor specific targeting these CD44 receptors were targeted by HA and CS. The cellular uptake results indicated that as compared to pure drug and TNPs, cellular uptake of surface modified nanoparticles were higher in both HA-TNPs and CS-TNPs. All the developed nanoparticles were taken up by caveolae mediated pathway. CD44 receptors were also involved in the uptake mechanism and this was confirmed by the CD44 receptor blocking assay.

ROS generation in U-87 MG cells was estimated and the results demonstrated concentration dependent ROS generation. As the concentration of TNPs, HA-TNPs and CS-TNPs increased, an increase in ROS generation was also observed. This may be correlated with the fact that increase in concentration of TNPs, HA-TNPs and CS-TNPs led to increased concentration of released TMZ leading to increase in ROS generation. HA-TNPs and CS-TNPs showed higher ROS generation as compared to pure TMZ and TNPs. This may be due to higher uptake of HA-TNPs and CS-TNPs via CD44 receptor which led to increased TMZ concentration in the cells that ultimately caused increased ROS generation.

The results of scratch assay indicated slower cell migration as compared to control group after treated with TMZ and developed nanoparticles. As compared to TMZ, TNPs and surface modified TNPs (HA-TNPs and CS-TNPs) indicated significant restriction of cell migration.

In vivo pharmacokinetic and biodistribution studies revealed improvement in pharmacokinetic profile and therapeutic concentration of TMZ in brain after encapsulating it in nanoparticles.

The quantitative biodistribution study of TMZ, TNPs, HA-TNPs and CS-TNPs was performed to assess the passage of TMZ through the BBB and its distribution in various vital organs viz. brain, liver, heart, lungs, kidney and spleen. The distribution of TMZ in brain tissues was approximately six folds, eight folds and nine folds higher with TNPs, HA-TNPs and CS-TNPs respectively as compared to free TMZ. This may be due to smaller size of nanoparticles as compare to pure drug and enhanced BBB permeability. As mentioned earlier HA and CS also plays role to enhance BBB permeation, presence of HA and CS over the nanoparticles facilitate the BBB crossing which led to enhance concentration of drug in the brain. The obtained results showed correlation with in vitro BBB permeation study.

Further, the results revealed significant reduction in the distribution of drug to highly perfused organs when given as nanoparticles. This may be attributed to surface modification of nanoparticles that led to prevented opsonization. After 24 h, distribution of TMZ released from HA-TNPs and CS-TNPs were more or less similar in spleen and heart whereas in liver and lungs, distribution was significantly decreased as compared to pure TMZ. Distribution of CS-TNPs was high in kidney as compared to free TMZ may be due to presence of hydrophilic polymer over the surface of TNPs

Furthermore the biodistribution and toxicity data suggested safety of developed nanoparticles as accumulation of TMZ in other vital organs decreased and no significant changes were seen in biochemical parameters of rats treated with formulations.

The interaction of synthesized nanoparticles was also assessed with respect to blood and culture media. No significant interactions were observed.

The results of stability studies indicated no significant change in particle size, % assay and zeta potential of prepared nanoparticles at both refrigerated condition and room temperature for three months which indicated their stability.

Lenalidomide loaded albumin nanoparticles (LNPs) were also prepared by desolvation method and optimized by OVAT design. Then according to optimized formula scale up batch (4X) was prepared and subjected to lyophilization.

After optimization of LNPs, surface modification was done using lactoferrin (Lf) to achieve lactoferrin receptor targeting. As LNPs has negative surface charge and Lf has positive surface charge at neutral to slight alkaline medium, Lf is physically coated over the LNPs. For the optimization of Lf coating two different parameters like Lf concentration and time of stirring was considered and optimization was done with placebo nanoparticles on the basis of particle size and zeta potential measurement. The results indicated that 1% w/v Lf and 1 hr stirring was sufficient to coated LNPs for achieving desired particle size and zeta potential. Confirmation of the coating was done on the basis of zeta potential. Initially zeta potential of LNPs was negative (-16.7 ± 2.9 mV) which was shifted to positive side (0.9 ± 0.5 mV) after Lf coating. Percentage coating and presence of Lf was also estimated by Bradford protein estimation assay which indicated approximate 31.78 % ± 2.3 % coating of Lf with the LNPs. To increase the percentage of Lf coating higher concentration of Lf also tried but particle size was significantly enhanced so for preparing final optimized batch of Lf coated LNPs (LF-LNPs) 1% w/v Lf and 1hr stirring was used. After that scale up batch was prepared and lyophilized.

Surface modified Lf-LNPs along with LNPs were further characterized and evaluated on the basis of various in vitro studies. The dynamic light scattering results indicated that hydrodynamic diameter of LNPs was within the range of 120-130 nm while in case of Lf-LNPs it was increased and was in range of 145-150 nm. The increase in the hydrodynamic diameter of surface modified nanoparticles may be attributed to Lf coating. The particle size distribution pattern indicated uniformity of nanoparticles dispersion which made them suitable for intravenous administration. Zeta potential results indicated the successful coating and stability of the developed nanoparticles.

Entrapment efficiency of LND in LNPs and Lf-LNPs was measured by free drug estimation and entrapment was found to be 92.96 % ± 2.37 % and 90.05 % ± 2.87 % respectively while drug loading was found to be 12.13 % ± 1.77 % and 11.15 % ± 1.17 % respectively.

FTIR data also confirmed the presence of drug in the nanoparticles as all the characteristic peaks of LND was present in the developed nanoparticles with lesser intensity. FTIR data also confirmed the successful coating of Lf over LNPs.

XRD spectrum revealed conversion of crystalline nature of LND in slightly amorphous nature after encapsulation in nanoparticles as the highly intense characteristic peaks of drug was converted in the lesser intensity peaks and also confirmed presence of LND in the developed formulation.

TEM image of Lf-LNPs showed spherical shape, exhibiting a dark core surrounded by a lighter gray rim likely corresponding to the Lf coating. Size of Lf-LNPs obtain by TEM was lesser than obtained by zetasizer (DLS measurement). As mentioned earlier, this may be due to presence of water surrounding Lf-LNPs during DLS in comparison with size measured in dried state by TEM as mentioned earlier.

In vitro release data revealed biphasic release of LND from LNPs and Lf-LNPs in which initial burst release observed and after that slower and sustained release was observed. The initial fast release may be due to release of surface associated drug while sustained release may be due to slower diffusion of drug from nanoparticles.

Results of cell viability assay of developed nanoparticles showed higher suppression of cells as compare to pure LND. Surface modified Lf-LNPs showed higher cell suppression than LNPs. This may be due to higher uptake of the nanoparticles because of presence of targeting moiety over the surface which enhanced the cellular uptake of nanoparticles.

In vitro BBB passage study also confirmed the higher permeation of nanoparticles through BBB than pure drug. This may be due to smaller size of nanoparticles than the pure LND. As compared to LNPs, Lf-LNPs showed higher permeation this was due to Lf receptor

mediated targeting as Lf receptors are over expressed in BBB. The sequence of BBB passage was as follows: Lf-LNPs > Lf-PNPs > LNPs > PNPs > LND.

Surface modification plays important role in cellular uptake of nanoparticles. Lf receptors are over expressed in BBB as well as brain tumor cells so Lf receptor mediated targeting strategy was utilized the deliver the drug (LND) to the target site by coating LNPs with Lf. For confirming the Lf receptor mediated targeting, the cellular uptake study was performed and the cellular uptake results indicated that as compared to pure drug and LNPs, cellular uptake of surface modified nanoparticles (Lf-LNPs) was higher. LNPs were taken up by caveolae mediated pathway while uptake of Lf-LNPs was clathrin mediated. Lf receptors were also involved in the uptake mechanism and this was confirmed by the Lf receptor binding assay.

ROS generation in U-87 MG cells was estimated and the results demonstrated concentration dependent ROS generation. As the concentration of LNPs and Lf-LNPs increased, an increase in ROS generation was also observed. This may be correlated with the fact that increase in concentration of LNPs and Lf-LNPs led to increased concentration of released LND leading to increase in ROS generation. Lf-LNPs showed higher ROS generation as compared to pure LND and LNPs. This may be due to higher uptake of Lf-LNPs via Lf receptor which led to increased LND concentration in the cells that ultimately caused increased ROS generation.

The results of scratch assay indicated slower cell migration as compared to control group after treated with LND and developed nanoparticles. As compared to LND and LNPs and Lf-LNPs indicated significant restriction of cell migration.

The interaction of synthesized nanoparticles was also assessed with respect to blood and culture media. No significant interactions were observed.

After getting satisfactory results from in-vitro characterization and evaluation, Lf-LNPs were subjected to in vivo tumor regression study for assess the anticancer potential of developed formulation. The results indicated that as compared to untreated model control group and standard control (LND treated), lesser tumor growth was observed in Lf-LNPs treated animals which indicated better anticancer activity of LND encapsulated in the NPs.

The results of stability studies indicated no significant change in particle size, % assay and zeta potential of prepared nanoparticles at both refrigerated condition and room temperature for three months which indicated their stability.

Conclusion

In the present work, hyaluronic acid conjugated, chondroitin sulphate conjugated and lactoferrin coated albumin nanoparticles were developed and optimized for CD44 receptor mediated and lactoferrin receptor targeted delivery of temozolomide and lenalidomide respectively. Various physicochemical properties of developed nanoparticles were investigated. The developed nanoparticles were characterized and evaluated for various physicochemical properties like particles size, PDI, zeta potential, morphology, drug release, stability etc. In vitro release profile demonstrated the sustained release behavior of both the drugs after encapsulating in the nanocarrier. In vitro cell line studies demonstrated that developed nanoparticles were able to cross blood brain barrier and reached to target tumor site by caveolae mediated endocytosis pathway (TNPs and LNPs). Hyaluronic acid and chondroitin sulphate enhanced the cellular uptake of nanoparticles (HA-TNPs and CS-TNPs) by CD44 receptor mediated delivery which was investigated by CD44 receptor blocking assay while lactoferrin enhanced the cellular uptake of Lf-LNPs by Lf receptor mediated delivery which was investigated by Lf receptor binding assay and enhanced the therapeutic activity and performance of temozolomide and lenalidomide towards cancerous cells as compared to pure drug. The results obtained by in vivo studies were also correlated with in vitro studies. Pharmacokinetic studies proved the enhanced concentration of temozolomide in brain when delivered by nanoparticles and toxicity data indicated safety of developed nanoparticles as no significant changes were seen in biochemical parameters of rat. In case of lenalidomide, results of in vivo tumor regression study suggested that Lf-LNPs showed better anticancer activity than standard control.

In conclusion, the results of this study suggested that the developed hyaluronic acid conjugated and chondroitin sulphate conjugated albumin nanoparticles loaded with temozolomide (HA-TNPs and CS-TNPs) and lactoferrin coated albumin nanoparticles (Lf-LNPs) loaded with lenalidomide have potential to target brain tumor and reduce toxicity towards normal cells. The finding suggest that the efficient brain tumor targeting with maximum safety

and efficacy and minimum toxicity can be achieved for effective treatment of brain tumors by employing surface modified albumin nanoparticles as delivery platform.