Formulation and Development of Nanotherapeutics System Utilizing Surface-engineered Approach for Cancer Cell Specificity

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Summary

The present study is focused on the development and assessment of lyotropic liquid crystalline nanoparticles (cubosomes) designed to target hepatocellular carcinoma. The targeted delivery system is believed to deliver drugs specifically to the cancer cells, resulting in a high payload and thus enhanced therapeutic efficacy. Such systems are capable of easily discriminating between cancer and normal cells, thereby reducing dose-related toxicity.

The anticancer drug selected for the current study was imatinib mesylate and bosutinib. Imatinib mesylate (IMS) is an inhibitor of tyrosine protein kinase approved by the FDA for treating chronic myeloid leukaemia (CML) and gastrointestinal stromal tumour. IMS is also used as an important targeted therapy candidate for the inhibition of tumour growth in numerous malignancies, including thyroid, ovarian, pancreatic, osteosarcoma, prostate, and other solid tumours. Numerous investigations proposed that the anticancer property of IMS is predominantly because of mitochondrial apoptotic pathway activation, with the involvement of various antiapoptotic proteins (Bcl-2, Bcl-xl), caspases (caspase 3 and 9), and proapoptotic proteins (BAX, BAD). Bosutinib (BST) is a second-generation tyrosine kinase inhibitor and acts as a dual inhibitor of SRC and ABL kinases. BST inhibits the BCR/ABL fusion gene product and its anti-cancer effects are reported in imatinib-resistant chronic myeloid leukaemia (CML). It has been approved for the treatment of CML by the U.S. Food and Drug Administration (FDA) in 2012 (8). Studies on BST reported that SRC plays an essential role in the pathways that mediate HCC development, invasion, and metastasis as well as cellular proliferation. Along with this ABL1 also plays a significant role in HCC development by mediating the c-MYC/NOTCH1 axis. BST also downregulates inflammatory cytokines and produces apoptosis through a caspase-mediated apoptotic pathway.

This exciting pharmacological potential and multi-targeting ability make it the perfect drug candidate for targeting HCC. However, its low water solubility, low bioavailability, instability, low bioavailability and high dose were major barriers in the designing of the formulation. The selected drug candidates for the present study were characterized for identification and purity by melting point, FTIR and UV spectroscopy.

Cubosomes are found to be the most suitable nanoformulation as they can load poor water-soluble drugs in three-dimensional cubic phases, leading to pronounced increases in solubility,

stability and bioavailability. Cubosomes have attained special interest in enhancing the oral delivery of various compounds comprising poorly soluble drugs and drugs with large molecular sizes. Cubosomes can facilitate the absorption of drugs administered by the oral routes owing to their bioadhesive properties, secretion of physiological surfactants in the gastrointestinal tract (GIT) or interaction with the cell membrane. They also have excellent loading capacity and can protect the drug from degradation. In the GIT, cubosomes keep the encapsulated drug in a solubilized state by its entrapping into the mixed micelles that are formed by the digestion of cubosomes. Thus, they improve drug absorption resulting in enhanced oral bioavailability.

The Box-Behnken design was chosen to develop the process parameters for the estimation of IMS and BST cubosomes. The design was selected as it reduces the number of runs of a full factorial without compromising on the effectiveness of the factors. 3 factors at 3 levels with 3 center points were selected for the study. The impact of independent variables like glyceryl monooleate (GMO) (X1), poloxamer (X2), and sonication time (X3) was assessed on the response variables such as particle size (PS) and % entrapment efficiency (% EE). The experiential data for both (IMS and BST) cubosomes systems were analysed mathematically, using quadratic equations and the best-fit model that generated best-fitted equations was obtained. A Response Surface Model (RSM) was implemented which best validated the relationships among selected factors and responses accompanied by interactions among factors. Further, the design space was generated using an overlay plot and the optimized formulation was recognised by putting definite criteria for different response factors. The criteria applied for the cubosomes optimizations were fixed at the values *viz.* particle size range of 100−150 nm and maximum % EE. A good agreement was witnessed between predicted values and observed values of response variables for both cubosomes.

The optimized imatinib mesylate loaded cubosomes (IMS-LCNPs) surface was modified with PEG and lactoferrin. IMS-LCNPs were also surface modified with chitosan and hyaluronic acid. The optimized bosutinib-loaded cubosomes (BSTF) surface was modified with folic acid.

The targeting module lactoferrin was successfully conjugated on the surface of IMS-LCNPs via a reaction of the carboxyl groups of PEG-lipids and free amino groups of lactoferrin with the help of EDC and NHS, as evidenced by employing FTIR. The characteristic peaks for various groups and structural configurations were observed and confirmed through the FTIR spectra. The physicochemical characterization of IMS-modified cubosomes (IMS-LF-LCNPs) revealed a

particle size of 120.40 ± 2.75 nm, a zeta potential of 12.5 ± 0.23 mV, entrapment efficiency of 85.11 ± 1.82 percent and drug loading of 13.58 ± 0.41% respectively.

The hyaluronic acid (HA) was successfully anchored on the surface of IMS-loaded cubosomes (IM-CBs) employing layer by layer technique (LBL). Chitosan and HA were used to surface-modify IM-CBs. Since both IM-CBs and HA have a negative surface charge, they repel each other. Thus, to coat the IM-CBs with HA, cationic chitosan was used to form LBL assembly. The physicochemical characterization of HA-modified cubosomes (HA-IM-CBs) displayed a particle size of 130.7 ± 2.92 nm, zeta potential -31.40 ± 2.76 mV, entrapment efficiency of 87.76 ± 0.79 % and drug loading of 14.01 ± 0.69% respectively.

The folic acid was successfully conjugated on the surface of BSTF with help of NHS and EDC as denoted by characteristic peaks of FTIR. Estimation of folate content further witnessed the folate conjugation to the BSTF surface. The amount of folate attached was found to be 6.32 μM. The physicochemical characterization of folate conjugated cubosomes (BSTMF) displayed a particle size of 188.5±2.25 nm, zeta potential -20.19±2.01 mV, entrapment efficiency of 90.31±3.15 % and drug loading of 14.91±2.03%, respectively.

The *in-vitro* drug release of all optimized and surface-modified formulations was found to be sustained throughout 48 h. An insignificant change in the release profiles was observed before and after surface modification of cubosomes in all three formulations, The release kinetics of formulation was best explained by Higuchi's equation, and the results revealed that the release is controlled by diffusion.

The surface morphology of the cubosomes was confirmed by using the HR-TEM and AFM. The result revealed that surface modification does not alter the cubic phase internal structure of the cubosomes (IMS-LF-LCNPs, HA-IM-CBs and BSTMF).

The lactoferrin-modified, HA-modified and folate-modified cubosomes were found to be stable during the studies performed as per ICH guidelines over three months.

The *in vitro* cytotoxicity of plain IMS, unmodified cubosomes, blank-modified cubosomes and surface-modified cubosomes and plain BST, unmodified cubosomes, blank-modified cubosomes and surface-modified cubosomes were evaluated against the Hep G2 cell line at a concentration of 10−80 μg/mL and 0.25-10 μg/mL by MTT assay. The results showed dose-dependent or concentration-dependent cytotoxicity for plain IMS, BST, and modified cubosomes, whereas the blank-modified cubosomes exhibited low cytotoxic potential activity. It was observed that

modified cubosomes exhibited a superior cytotoxic potential compared to unmodified and plain drugs. The results of *in vitro* cytotoxicity were further confirmed by using cellular uptake studies. DAPI staining assay was utilized for visualizing the nuclear changes in the Hep G2 cells. The supremacy of modified formulations over unmodified formulations as concluded from *in vitro* cell lines data specified that surface modifications facilitated these formulations in improved cellular internalization. through overexpressed receptors. Also, formulation being nanosized, possess passive targeting resulting in enhanced permeation and retention.

The *in-vivo* pharmacokinetic profile of plain drug (IMS, BST), unmodified formulation (IMS-LCNPs, IM-CBs and BSTF) and modified formulation (IMS-LF-LCNPs, HA-IM-CBs and BSTMF) revealed the controlled release pattern of the formulation. The result revealed that the modified formulation showed higher plasma concentration as compared to the pure drug and unmodified formulation. This is due to the surface decoration of cubosomes through targeting moiety, which enhances the blood residence time, escapes from RES uptake in the body and maintains a higher concentration, which results in improved bioavailability.

Thus, it was considered worthwhile to evaluate the *in-vivo* efficacy of prepared formulations against NDEA-induced hepatic cancer.

In vivo studies were performed on male albino Wistar rats. The protocol for animal testing was approved through an institutional ethical committee.

Animals were randomized and divided into five groups of 6 animals each for lactoferrin-modified, HA-modified and folate-modified cubosomes. Hepatic cancer was induced by administering N-DEA (100 mg/kg) i.p. 6 weeks (once a week). During the treatment period, various formulations were administered orally. The treatment was continued for the next 20 days after 6 weeks of toxicant injection. On the twenty-first day, animals were sacrificed an approved procedure was followed to investigate various parameters.

The carcinogen control exhibited the highest tumour incidence number, increased hepatic weight, and lower body weight, representing severe cancer. Orally administered surface-modified formulations put back the level of markers to a more considerable extent than pure drug and unmodified formulation thus demonstrating the targeted delivery on the hepatic site.

The NDEA-induced hepatic carcinoma brings about a significant imbalance in biochemical mechanisms of cells. It was indicated by noticeable alteration in the levels of oxidative stress markers like TBARS, protein carbonyl, GSH, SOD and catalase. Treatment with different

formulations restored the levels of these imbalanced markers towards normal but surface-modified cubosomes revealed the highest efficiency in the restoration of altered levels of oxidative stress markers towards normal, which can be ascribed to the property of targeting moiety to reduce oxidative stress.

Tumour-induced hyperlipidemia promotes liver cirrhosis, which results in tissue damage. In our study, we discovered that the CC group had elevated levels of VLDL TG, LDL, and TC, as well as low levels of HDL, indicating hepatic injury. All three modified formulations restored (IMS-LF-LCNPs, HA-IM-CBs and BSTMF) all of these enzyme levels compared to pure drug and unmodified formulation. Furthermore, the blood enzyme levels (ALP, ALT, and AST) increase due to numerous enzymes leaking at the hepatic site. A similar finding was noticed in the N-DEA treated group, where elevated expressions of ALT, ALP, and AST enzymes were noted. Therefore, all three modified formulation treated groups restored these damaged hepatic enzyme levels to normal, demonstrating the protective efficacy of the targeting molecule. Similarly, increased LDH serum level indicates a generalised fluctuation in cell membrane integrity and cirrhosis. As a result, higher LDH levels in the CC group suggested NDEA-induced HCC, which can be reversed by orally administrating surface-modified cubosomes.

Moreover, HCC is also associated with elevated bilirubin and biliverdin levels. These markers increased during NDEA exposure and then returned to normal after treatment with modified formulations indicating that hepatic damage had been controlled. In comparison to all other treated groups, the modified formulations treated group protects against HCC by normalising these enzyme levels, demonstrating its superior anticancer action.

The CC groups' histological investigations showed abnormally shaped nuclei, RC, TA, and vacuole formation, most probably caused by the free radical generation following N-DEA exposure. An improvement in cell structure and the re-establishment of hepatic parenchymal cells was noticed in the IMS-LF-LCNPs, HA-IM-CBs and BSTMF groups, which are almost similar to NC liver parenchymal cells. Since LF, HA and FA specifically bind to the ASGPR, CD44 and folate receptor, the active liver targeting of IMS-LF-LCNPs, HA-IM-CBs and BSTMF was increased.

In disease states such as cancer, serum cytokines can increase or decrease inflammation (Lv et al., 2016). Nitrative stress activates the inducible isoform of NOS (i-NOS) and oxidative Nitric oxide (NO) metabolism induces liver damage (Yu et al., 2018). NO plays an important role in

the liver, as it has both cytoprotective and cytotoxic properties. According to reports, altered expressions of i-NOS and e-NOS are analogous to caspase-mediated cell apoptosis. When ASGPR and CD44 interact with lactoferrin and HA, it regulates some inflammatory events. During HCC, CD44 regulates apoptosis by interacting with HA and apoptotic indicators such as Bcl-2 family proteins and caspases (Xie et al., 2008). The abrogation of cytokines, reactive nitrogen species, and apoptosis induced by mitochondrial pathway activation or the initiation of caspases are mainly responsible for HA and IM's anti-inflammatory and anticancer action. CC group demonstrates elevated cytokine levels, while the treatment groups had lower levels. The total nitrite or nitrate ratios and NOS action demonstrated that the NO levels in HCC rats were reduced after N-DEA therapy, representing hepatic damage. The NO level was raised after being treated with all IMS groups indicating a protective effect. Apoptosis is induced by increased NO levels, The findings showed that downregulated and upregulated i-NOS and e-NOS expression, respectively, in the CC group contributed to the development of Hepatic cancer. On the other hand, all treated groups normalised the NOS isoform expression levels and nitrative stress indicators. The protective effect of modified cubosomes was superior to that of plain IM and unmodified cubosomes, demonstrating receptor-mediated endocytosis uptake by the tumour cells, possibly as a result of drug accumulation in the hepatic tissues.

Further, compared to all other treatment groups, surface-modified cubosomes displayed significantly elevated expression of caspases (-3 and 9), indicating that they are effectively delivered to the hepatic site. Unlike cytokine levels and caspase levels in the NDEA-treatment group quickly normalised after IM, IM-CBs, and HA-IM-CBs.

Apoptosis in cells may occur in two ways: extrinsic and intrinsic mitochondrial pathways. Anti-apoptotic (Bcl-xl and Bcl-2) and proapoptotic (BAX and BAD) regulate the mitochondrial processes. After receiving IMS formulation(s), surface-modified cubosomes (IMS-LF-LCNPs and HA-IM-CBs), the result of qRT-PCR indicated upregulated anti-apoptotic markers and downregulated expressions of proapoptotic markers in the CC group. The modified cubosomes significantly increased these levels compared to the other treatment groups, suggesting receptor-mediated endocytosis uptake of modified cubosomes by tumour cells, perhaps attributable to drug accumulation in hepatic organs via ASGPR and CD44 targeting.

The results of biodistribution studies contribute to the notion that all three modified formulations (IMS-LF-LCNPs, HA-IM-CBs and BSTMF) can target and accumulate in the liver whilst

restricting entry into other essential organs. This could be attributed to highly expressed ASGPR, CD44 and FRs on the surface of liver cancer cells which provides a unique opportunity to specifically target liver cancer cells by the high affinity of all overexpressed receptors.

^{1}H NMR-based metabolic profiling was performed to explore the biochemical changes associated with colon cancer and to further see how these changes get modulated in the rats receiving the treatment with IMS formulation(s). The observed metabolic variations in the CC group, such as augmented levels of lipids, NAG, lipoproteins, PUFA, and lactate and decreased levels of citrate, amino acids, and creatine, clearly indicate significant perturbations in the amino acid, glycolysis, tricarboxylic acid (TCA) cycle, and energy metabolic pathways. The higher levels of lipid metabolites (including VLDL/ LDL, PUFA) in carcinogenesis are generally observed owing to altered growth patterns observed in tumour tissues. A drop in citrate and creatine levels indicates the growth in energy uptake due to rapid cell division. Hepatic carcinoma can also increase protein degradation from cell necrosis leading to altered concentrations of amino acids in the serum. The increased levels of lactate and the decreased glucose levels suggest an increased glycolytic rate and enhanced glycolysis in tumour cells. Almost all metabolic changes in NDEA-treated animals returned towards normal after IMS formulation(s) treatment, suggesting that the formulation(s) has the potential to normalise the NDEA-altered metabolic changes.

Conclusion:

A targeted drug delivery system was found therapeutically effective and safe for regulating cancer proliferation in hepatic carcinoma. The cubosomes containing IMS and BST were developed, optimized and surface modified with targeting modules successfully. The cubosomes formulation(s) were evaluated for numerous physicochemical parameters and *in-vitro* and *in-vivo* performances which exhibited the development of a consistent, stable as well as an effective delivery system for cancer therapy. Thus, these have to be taken up through receptor-mediated endocytosis, high-affinity ASGPR receptor for lactoferrin uptake and high-affinity folate receptor for folate uptake. Also, CD44 overexpressed in hepatic cancer cells that bind to its primary ligand HA and are stated to be responsible for cellular signalling, leading to the regulation of biological processes within cells. Thus a swift uptake of cubosomes carrying such molecules is witnessed via these overexpressed receptors, delivering the drug payload. The overexpression of these receptors over cancer cells and selective uptake of such targeting

modules in the cancer cells could be a probable justification for the selective uptake of molecularly designed targeted NPs for hepatic cancer. Conjugation of targeting moiety utilizing the help of a linker yielded an effective ligand-receptor molecule and supported its internalization into cancer cells.