# Ligand based nanocarrier therapy for Hepatocellular carcinoma (HCC)

SUMMARY OF THE THESIS

SUBMITTED IN FULFILMENT FOR THE AWARD OF THE

DEGREE OF

**Doctor of Philosophy**

IN

PHARMACEUTICAL SCIENCES


Submitted by

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Enrollment No. 1093/18

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**2024**

**Summary**

Considering the death rate associated with liver cancer, which affects one million people annually, liver cancer is crucially responsible. Because there is insufficient appropriate pharmacotherapy, it is a major cause of concern. Treatment options for HCC that have been widely accepted thus far include radiofrequency ablation, liver transplantation, transarterial chemoembolization, surgical resection, and systemic medication therapy. Nonetheless, these treatments are limited by factors like a dismal prognosis, challenges in administering a sufficient dosage to damaged liver, severe side effects, and the immunosuppressive therapy's lifetime irritation after transplantation.

Existing HCC therapeutic approaches are not desired, necessitating the development of new and alternative strategies to treat HCC, since most patients with HCC are diagnosed in advanced stages at which existing standard chemotherapies are insufficient. Consequently, the best way to manage the disease's progression and prevent the chemotherapeutics' harmful effects on healthy cells is to provide the drugs to the neoplastic hepatocytes in a site-specific manner. To significantly enhance the effectiveness of therapy for HCC, researchers worldwide have thus tried ligand-based active targeting techniques for the selective delivery of therapeutic cargo into cancerous hepatocytes.

Active targeting through receptor-mediated endocytosis is one of the most widely used tactics by HCC to enhance its targeting capabilities. The nanocarrier system (NCS) was decorated with a variety of ligands, including peptides, proteins, aptamers, polysaccharides, antibodies, vitamins, transferrin, and small molecules, to improve the affinity between NCS and tumour cells.

The presented research work in this thesis focused on the fabrication, optimization and characterization of ligand based nanocarriers i.e., liposomes and nanostructured lipid carriers (NLC) planned to target hepatocellular carcinoma. Gefitinib, an anticancer agent that selectively inhibits EGFR tyrosine kinase and encourages the development of HCC, was chosen for the current investigation. c(RGD)fK [cyclic arginine-glycine-aspartic acid-phenylalanine-lysine], a pentapeptide was further used to decorate the surface of nanocarriers to improve the target ability of nanocarrier to potentiate the liver anti-cancer activity. The chosen drug was characterized for purity and identification by FTIR, UV and HPLC spectroscopy and the selected pentapeptide for the both the nanocarriers was identified by the NMR spectroscopy.

On the other hand, solvent-emulsification and evaporation technique was used for the development of NLC because of avoidance of heat during the preparation which is the most important advantage of this method.

**c(RGDfK)-surface anchored Gefitinib loaded liposome [Gefi-c(RGDfK)-L]**

There are so many methods to prepare the liposomes but ethanol injection method was preferred here because of simplicity, rapidity, high level of reproducibility, as well as the easy scale-up of the method. For the optimization process of liposome, a three-factor, three-level box Behnken design (BBD) with three central points was chosen here which suggested 15 experimental trials with different combination of factors. The design was selected as it reduces the number of runs of a full factorial without compromising on the effectiveness of the factors. An independent variable was chosen to study the relative effects of phospholipid DSPC, solvent/non-solvent (S/NS) volume ratio, and ultrasonication time (UST) on particle size (PS), percent entrapment efficiency (EE), and percent drug loading (DL).

After preparing these 15 formulations, the acquired responses were filled in the BBD design, and the data were explored by the design of experiment software (Design Expert, Trial version 13.0). A one-way analysis of variance (ANOVA) was used to optimize the outcomes of selected independent variables. In order to get the best-fitted model, the obtained dependent variables value of the different trial formulations was fitted into the model milieu, and the best-fitted model was found to be quadratic. The numerical standards for optimized gefitinib loaded liposomes i.e., Gefi-L were PS 128.83 to 149.17 nm, EE 62.53 to 83.33%, and DL 10.32 to 12.32 %. Based on these standards, 240 mg DSPC, 0.5 solvent/non-solvent volume ratio and 10 min of UST were chosen. An optimized batch of Gefi-L was prepared based on overlay plot data. The predicted values for all the responses, such as particle size, entrapment efficiency, and drug loading, were found to be 142.547 nm, 81.5767 % and 11.8067 %, respectively. A good agreement was witnessed between predicted values and observed values of response variables for all the systems.

Conjugation of pentapeptide to optimized liposome was accomplished via carbodiimide reaction between an amine group of peptides and a carboxylic group of DSPE–$PEG_{2000}$–COOH by using activation of EDC/NHS chemistry. The targeting c(RGD)fK pentapeptide was successfully conjugated on the surface of gefitinib loaded liposome was evidenced by means of FTIR and NMR spectroscopy.

Size, shape, surface morphology, zeta potential, percentage of drug loading, and entrapment efficiency were the parameters tested for in the optimised batches of c(RGD)fK-surface modified gefitinib loaded liposomes and gefitinib loaded liposomes.

The physicochemical characterization of Gefi-L and Gefi-c(RGDfK)-L displayed the particle size of 143.5 ± 2.14 nm and 152.6 ± 1.86, zetapotential –19.39 ± 3.57 mV and – 21.45 ± 4.92. Furthermore, The %EE of Gefi in Gefi-L & Gefi-c(RGDfK)-L was found to be 79.75 ± 4.01 & 75.16 ± 3.03 %, respectively and %DL of Gefi-L was 11.46 ± 1.26 whereas Gefi-c(RGDfK)-L exhibit the %DL 11.21 ± 1.17. According to the obtained data, surface alteration resulted in a modest decrease in both %EE and % DL. SEM and TEM micrograph showed that liposome vesicles were smooth outer surface and spherical in shape. Additionally, The TEM and SEM micrographs revealed a noticeable separate surface modified layer in Gefi-c(RGDfK)-L.

Based on the obtained data from FTIR, XRD and DSC it was concluded that weakened and/or disappeared peak proved an effective entrapment of the Gefi inside the liposome lipidic matrix. The *in-vitro* dissolution behaviour of Gefi-L and Gefi-c(RGDfK)-L point towards a biphasic release pattern of gefitinib for 48 h. In the initial 8 h, approximately 50% Gefi was released from Gefi-L and Gefi-c(RGDfK)-L. Due to the adsorption of some amount of drug on the surface of liposomes, indicating a burst release pattern in the initial time point. Various kinetic models were implemented to the percent Gefi release to categorize the release phenomenon. The R square value (R2) was calculated from diverse kinetic models by using DD solver software. The highest $R^2$ value indicated the best-fit model, so the Korsmeyer-Peppas model was considered the best-fit model for Gefi-L and Gefi-c(RGDfK)-L both.

The Gefi-c(RGDfK)-L were found to be stable during the studies performed as per ICH guidelines over a period of three months.

On a HepG2 cell line the cytotoxicity tests of free Gefi, blank liposomes, blank modified liposomes, Gefi-L, and Gefi-c(RGDfK)-L were conducted using an MTT assay at varying drug concentrations over the course of 72 h. The findings showed $IC_{50}$ values of the free Gefi, Gefi-L, and Gefi-c(RGDfK)-L formulations were 41.05 ± 1.16, 30.50 ± 1.94, and 18.32 ± 1.84 µg/ml, respectively. It indicated that the surface-modified nanocarrier significantly impacted cytotoxicity, as the $IC_{50}$ values of surface-modified formulations were around 2.3-fold lower than that of free Gefi. it is evident that the formulations are non-toxic to the HepG2 cells because cell viability was greater

than 85% under all conditions tested. Since the liposomal system did not affect cell viability, supporting the improved delivery method's safety profile, it is a potential choice for delivering Gefi.

FITC-labelled c(RGDfK)-surface modified liposomes produced stronger green fluorescence signals in cells than FITC-labelled non-modified liposomes, implying that the c(RGDfK)-surface modified liposomes could boost cellular uptake via αvβ3 integrins receptor-mediated endocytosis. DAPI staining revealed that apoptosis and nonspecific necrosis were increased in the treatment cells in modified liposomes at a concentration of 18.32 μg/ml compared to normal cells.

The pharmacokinetic data revealed that compared to free Gefi suspension, Gefi-c(RGDfK)-L had a nine-fold higher AUC and a two-fold higher half-life. A longer duration in circulation in the blood may be responsible for the increased half-life and AUC, which in turn may have resulted from increased Gefi-c(RGDfK)-L accumulation in the cancer tissue via EPR effect and, thus, a higher therapeutic index. The longer residence time of the drug in the body and higher values for Gefi-L and Gefi-c(RGDfK)-L indicates that the formulation is a controlled release formulation that improves the bioavailability of Gefi and allows for further dosage reduction.

Organ biodistribution study showed that the surface-modified liposome demonstrated greater activity at the periods considered, possibly because of increased Gefi accumulation when compared with the free Gefi and Gefi-L treated group. Increased Gefi accumulation in liver tissue, attributed to improved targeting capability of cancer tissues thanks to c(RGDfK) pentapeptide surface modification, likely contributed to the prolonged Gefi-c(RGDfK)-L's retention at tumorous locales for 48 h.

An *in-vivo* animal study was done in male Wistar rats as per procedure consented by the Institutional Animal Ethics Committee (IAEC). Body weight variation, liver weight and percent survival of animals were measured at regular time interval. The rat liver weight and body weight variation were significantly higher in the carcinogen control (CC) group compared to the normal control group. According to the obtained result, Gefi-c(RGDfK)-L treated group exhibited a substantial reduction in the liver weight, whereas the Gefi, Gefi-L-treated group exhibited a minor reduction. Compared to the CC group, the treatment effectiveness was considerably enhanced.

A noticeable visual transformation in the number of carcinogenic nodules from the normal control to Gefi-c(RGDfK)-L treated group were observed through histopathological analysis by hematoxylin & eosin (H & E) staining. The H & E

staining of normal control group confirmed the normal architecture of hepatic cells, healthy copious nucleus and normal Kupffer cells. In contrast, the carcinogen control group exhibited the distinctive architecture of hepatic cells, degenerated nucleus, ruptured hepatic cells and occurrence of binucleate. Treatment with Gefi-c(RGDfK)-L displayed a noteworthy improvement in the gross microscopic manifestation of hepatic tissues, followed by Gefi-L and plain Gefi.

Assessment of liver enzymes and catabolic byproducts were done successfully. Result indicated that the ALP, ALT, and AST enzyme levels were elevated in the CC groups' serum as compared to the normal control group. Consequently, treatment of the injured liver with Gefi-c(RGDfK)-L normalized the liver enzyme levels, indicating the protective effect of Gefi-c(RGDfK)-L. In addition, the effects of Gefi-L and Gefi-c(RGDfK)-L on catabolic pigments bilirubin in blood serum were evaluated, and the results demonstrated that therapy with modified liposomes could considerably restore these levels.

For the *in vivo* biosafety assessment of Gefi-L and Gefi-c(RGDfK)-L histopathological analysis was done. The images indicated that the major organs were unaffected and showed no signs of inflammation or injury in either formulation group. Fortunately, the therapeutic doses of Gefi-L and Gefi-c(RGDfK)-L are well tolerated and have great biocompatibility.

The above summarised result concluded that the Gefi-L was successfully prepared and optimized Gefi-L to potentiate liver anticancer activity, and then its surface was modified with c(RGDfK) pentapeptide for tumour targeting. The receptor-mediated targeted delivery and small particle size of the produced Gefi-c(RGDfK)-L make the formulation attractive for use in cancer medication delivery. In HepG2 cells and solid tumours, c(RGDfK) surface-modified Gefi-loaded liposomes exhibited better cytotoxic/ apoptotic activities, superior biodistribution, tumour selectivity features and *in vivo* pharmacokinetic profiles in an animal model, when compared with Gefi-L and free Gefi. Our *in vitro* and *in vivo* findings conclude that the Gefi-c(RGDfK)-L formulation is an efficient feasible approach for targeting hepatocellular cancer.

**Pentapeptide cRGDfK-Surface Engineered Nanostructured Lipid Carriers (NLCs)**

In the present research work, the NLCs contained cholesterol, oleic acid, Pluronic F-68, and Phospholipon 90G. The NLC surface was functionalized to enhance targeting with

the cRGDfK-pentapeptide, which binds to the αvβ3 integrin receptor overexpressed on hepatocarcinoma cells.

Solvent-emulsification and evaporation technique followed by ultrasonication method was used for the development of NLC because of avoidance of heat during the preparation which is the most important advantage of this method.

To optimize the formulation of gefitinib loaded NLC i.e., GF-NLCs, a Box-Behnken design (BBD) was employed, utilizing a three-factor, three-level design. Design-Expert® software (Trial version 13.0) was utilized for this purpose. oleic acid, surfactant PF-68, ultrasonication time, were the independent variables, chosen for optimization of formulation. A total of 15 experimental trials were generated based on this design and evaluated for particle size, percentage entrapment efficiency, and percentage drug loading as the dependent variables. By employing a quadratic polynomial model, the cause-and-effect relationship between the independent and dependent variables was determined through mathematical modelling. The measured value of dependent variables of the various trial formulations was fitted into the model milieu, and a quadratic polynomial model was found to be the best-fitting model.

In order to achieve the desired outcome ie, minimum PS, maximum % EEand % DL, the formulation was optimized using both desirability and overlay plot criteria. Desirability function and the obtained observed values of optimized GF- NLC via overlay plot are displayed in Figure 3J and K), respectively. The optimized GF-NLC specifications were PS 114.22 nm to 141.17 nm, EE 68.94% to 89.33%, and DL 9.63% to 15.87%. These standards led to the selection of 25% w/w oleic acid, 1% PF-68 and 9 min ultrasonication time. Based on these standards, the optimized GF-NLC formulation was developed.

The cRGDfK@GF-NLC-designated cRGDfK undergoes a carboimide reaction with DSPE-PEG2000-COOH. It was confirmed by the FTIR and 1H-NMR results that the NLC surface satisfactorily conjugated cRGDfK to DSPE- PEG2000-COOH.

According to the DLS results, the PS values of the GF-NLC and cRGDfK@GF-NLC were 137.83 ± 5.55 nm, 140.66 ± 2.17 nm, and their respective PDIs were 0.243 ± 0.09 and 0.166 ± 0.07. Both GF-NLC and cRGDfK@GF-NLC had negative ZP of −26.22 ± 4.01 mV and −31.76 ± 5.21, respectively. The SEM and TEM micrograph showed that the NLCs were round and had a smooth exterior. cRGDfK@GF-NLC, had a distinct, surface-functionalized layer. These findings proved that the developed NLCs were

effective at delivering drug by exploiting their increased permeability and retention effect.

Based on the obtained data from FTIR, XRD and DSC it was concluded that weakened and/or disappeared peak proved an effective entrapment of the GF inside the lipid matrix. XRD pattern of pure GF indicated crystalline nature of drug and exhibits a remarkably high intense peak at 15.95, 19.45, 22.6, 24.37, 26.38 and 26.49. However, the characteristic GF peak was either significantly attenuated or completely absent in the GF-NLC and cRGDfK@GF-NLC, demonstrating substantial entrapment of GF inside the lipid.

The observed and predicted values of % EE and % DL of GF-NLC and cRGDfK@GF-NLC were obtained by the BBD and result showed a slight variation from the observed value. It follows that these parameters were unaffected by the addition of cRGDfK to non-functionalized NLC.

The *in vitro* GF release profile exhibited an initial burst of drug followed by a sustained release up to 48 h. According to the findings, approximately 80% of GF was released from the GF-suspension, whereas approximately 40% of GF was released from the GF-NLC and cRGDfK@GF-NLC within 4 hours, followed by the slow and prolong release pattern up to 48 hours. The observed burst effect in release studies can typically be attributed to the presence of unencapsulated drug on the surface of NLC. Additionally, the sustained release patterns of NLC can be attributed to the release of drug particles from the NLC core, as they partition between the aqueous phase and the lipid matrix, while also considering the barrier function of the interfacial membrane.

The Korsmeyer-Peppas model provided the best fit to the release data, as measured by the strength of their respective correlation coefficients ($R^2$). This finding is evident that Fickian diffusion was the key driving force behind the transport of GF from the GF-NLC and cRGDfK@GF-NLC.

Cytotoxicity studies were conducted by MTT assay with both blank NLC and GF-loaded NLC (both non-functionalized and functionalized) at 5–25 µg/mL concentration to ascertain the impact of target ligand cRGDfK on HepG2 cell lines. With no change to the percent cell growth inhibition, the NLC system is a promising choice for GF delivery, validating the optimised delivery system's favourable safety profile. In contrast, GF-loaded non- functionalized and cRGDfK-surface functionalized NLC had a substantial effect on the percentage of growth inhibition. A linear relationship

between GF, GF-NLC, and cRGDfK@GF-NLC concentrations and growth inhibition was observed.

Evaluation of the targeting effect of cRGDfK-surface functionalized NLC can be accomplished by monitoring their internalisation into cells via Confocal laser scanning microscopy (CLSM). Compared to FITC-NLC, cRGDfK@FITC- NLC exhibited greater cellular internalisation after 8 hours of incubation. Endocytosis mediated by αvβ3-integrins receptors was responsible for the increased internalisation of cRGDfK-surface functionalized NLC, suggesting the involvement of interactions between the cRGDfK group and its receptor.

An *in-vivo* animal study was done in male Wistar rats as per procedure consented by the Institutional Animal Ethics Committee (IAEC). Several physiological parameters such as animal body weight, weight variation, rat liver weight, liver index, tumour burden, and tumour incidence number were assessed to evaluate the therapeutic efficacy of GF, GF-NLC, and cRGDfK@GF-NLC. Results demonstrates that in the DEN control group, both liver weight and body weight variation in rats were significantly higher compared to the normal control group. However, treatment with GF, GF-NLC, and cRGDfK@GF-NLC restored normal body weight fluctuations. Moreover, compared to the GF, GF-NLC treated group, the cRGDfK@GF-NLC treated group showed a significant decrease in liver weight. These findings suggested that cRGDfK@GF-NLC curtailed DEN-induced liver enlargement significantly.

The pharmacokinetic study exposed that the plasma concentrations of GF in rats treated with GF-NLC and cRGDfK@GF-NLC formulation were consistently higher than in rats treated with GF suspension across all time points. Plasma distribution of cRGDfK@GF-NLC was roughly four times higher than that of GF-NLC, as measured by the area under the concentration-time curve (AUC). It is possible that the nanoformulation' enhanced permeation and retention (EPR) in the tumour microenvironment is the reason of the longer half-life and higher AUC. Furthermore, the administration of GF-NLC and cRGDfK@GF-NLC resulted in a noteworthy increase in both the Area under the mean curve (AUMC) and the mean residence time (MRT) after 48 hours, when compared to the use of free GF suspension. Based on these findings, GF-NLC and cRGDfK-functionalized GF-NLC were significant better compared to free GF suspension after 48 hours. The increased duration of drug presence in the body and higher values of $t_{1/2}$ observed for GF-NLC and cRGDfK@GF-

NLC indicate that these formulations exhibit controlled release properties. This leads to enhanced bioavailability of GF and allows for potential dosage reduction.

Compared to the GF-treated group, the surface-functionalized NLC exhibited greater activity throughout the time periods observed in the *in-vivo* organ biodistribution study.This was probably because the former accumulated GF more rapidly. Growing evidence suggests a correlation between improved cancer tissue targeting and elevated GF accumulation in the liver. The interaction between cRGDfK@-GF-NLC and the αvβ3-integrin receptor overexpressed on HCC cells allows GF to maintain cRGDfK-surface functionalized NLC in tumour sites for an additional 48 hours. This interaction is likely facilitated by the surface functionalization of cRGDfK-pentapeptide.

Histopathological examination showed that the number of cancerous nodules dramatically decreased in the cRGDfK@GF-NLC treated group compared to the normal control group. The microscopic examination of liver tissues revealed a notable improvement in their overall microscopic appearance with the administration of cRGDfK@GF-NLC, followed by GF-NLC and frees GF.

The DEN control group exhibited increased levels of triglycerides, cholesterol, low-density lipoprotein, and very low-density lipoprotein in the serum lipid profiles compared to the NC group. Conversely, HDL levels were significantly lower in the DEN control group compared to the NC group. When cRGDfK@GF-NLC was administered intravenously, it normalised TC and TG levels while reducing LDL and VLDL.Prioritizing treatment with cRGDfK@GF-NLC, serum HDL levels returned to normal in the GF-NLC-treated group, followed by the free GF-treated group.

The levels of GSH and SOD showed a significant decrease ($p < 0.001$) following DEN administration, whereas the levels of MDA and CAT (measured as the disappearance of H2O2/min/μg of protein) were elevated in the DEN control group compared to the NC group. Treatment with either developed GF-NLC or cRGDfK@GF-NLC significantly decreased these parameters, with hepatic SOD and GSH activities peaking in the latter and being significantly higher ($p < 0.001$) than in the DEN control group.

Enzyme markers ALP, ALT, and AST were significantly regained to normal levels in the cRGDfK@GF-NLC and GF-NLC treatment groups compared to the free GF treatment groups. The effect of cRGDfK@GF-NLC was more noticeable than that of GF alone and GF-NLC. Therefore, the protective activity of cRGDfK@GF-NLC was demonstrated by the return of liver enzyme levels to near normal following treatment of the damaged liver with cRGDfK@GF-NLC.

ELISA was employed to determine the levels of proinflammatory cytokines (IL-2, IL-6, and IL-1β) and the apoptotic signal initiator (caspase-9) in the blood serum. Results showed a notable increase in the levels of IL-2, IL-6, and IL-1β, while the level of caspase-9 was significantly reduced (approximately 4-fold) in the DEN control group. After being treated with free GF, GF-NLC and cRGDfK@GF-NLC these inflammatory cytokines were reduced to a certain level whereas caspase 9 levels were increased significantly ($p < 0.001$) restored to normal levels. This finding, though, was most pronounced in the cRGDfK@GF-NLC treatment group as compared to the GF-NLC and free GF drug. We found that cRGDfK@GF-NLC surpassed both non-functionalized NLC and free GF in terms of its restorative potential.

The results of *in vitro* and *in vivo* experiments confirming their primary effects of enhancing drug efficacy provided strong support for their use. This leads us to believe that cRGDfK-surface functionalized NLC for the GF delivery is a desirable and potent vehicle for the treatment of HCC.