EVALUATION OF THE THERAPEUTIC EFFICACY OF NANOPARTICULATED BIO-FLAVONOIDS IN COMBATING OXIDATIVE HEPATOCELLULAR DEGENERATION BY NUCLEAR IMAGING TECHNOLOGY USING Tc-99m RADIOPHARMACEUTICALS

Synopsis submitted

by

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2023

Liver is the largest and one of the most vital organs responsible for a plethora of functions which includes metabolism, detoxification, protein synthesis and the production of bio-chemicals necessary for the sustenance of life, which also makes it one of the most vulnerable organs to toxic insults. Liver diseases, a major problem globally, incorporate several ailments, which can range from benign histological changes to serious life-threatening conditions brought on by infections, exposure to xenobiotics and unhealthy lifestyle. A report by World Health Organization (WHO) outlines that cirrhosis, hepatocellular carcinoma (HCC), chronic hepatitis B & C infections, alcoholic liver diseases and drug induced liver injury account for the majority of the deaths from all liver diseases making a major concern for the medical fraternity nowadays. Even though advanced treatment regimens have been developed over time for the treatment of various liver diseases, they are not particularly successful and satisfactory in treating liver diseases without causing side effects. Along with these, shortcomings like disease recurrence, drug resistance are also notable causes for concern. Thus, there is a need for a treatment scheme with fewer side effects and greater therapeutic efficacy. Hence comes the strategy of utilizing naturally occurring bioactive molecules found in fruits, vegetables, dry fruits, and spices as possible therapeutic agents, which have successfully demonstrated the ability to prevent or reduce the incidences of hepatic disorders in both clinical and pre-clinical models. In recent years, natural products are being increasingly used to treat liver disorders and have been accepted globally as a promising therapeutic approach for pathological conditions of the liver. Amongst these natural products, flavonoids are especially promising molecules for preventing neoplastic disorders which guards against liver carcinogens, improves the effectiveness of chemotherapeutic medications, inhibits the growth of tumor cells, reduces oxidative stress and chronic inflammation. Flavonoids are found ubiquitously in higher plants and constitute an important component of the majority of peoples' daily diet. Flavonoids like quercetin (Qr) and apigenin (API) used in this research work, are polyphenolic compounds found abundantly in nature, which exhibit antioxidant property and remarkable therapeutic potential against many pharmacological conditions including liver diseases. Even though several advancements have been made to utilize therapeutic potential of plant flavonoids, especially in cancer treatment, its clinical application is hampered by issues such as poor water solubility, limited bioavailability, poor stability, inadequate target specificity, degradation at low pH. To overcome these shortcomings and to improve clinical compliance, the application of novel drug delivery systems (NDDS) in target specific delivery of natural products is gaining importance. In formulation development, designing novel dosage forms of naturally occurring compounds ensures

various advantages including improvement of solubility and bioavailability, enhancement of pharmacological action, protection from toxicity, improvement of stability, enhancement of tissue macrophage distribution, sustained delivery, protection from physical and chemical degradation, incorporation of both hydrophilic and hydrophobic substances, and feasibility of using variable routes of administration, including oral administration and inhalation. In this context, formulation developers have focussed on various nanoparticle (NP) formulations, such as lipid NPs, polymeric NPs, nanoliposomes, nanoemulsion, etc. Biodegradable polymeric NPs formulated using poly lactic-co-glycolic acid (PLGA) are frequently being used to improve the therapeutic value of various poorly soluble bioactive molecules like flavonoids by improving bioavailability, solubility thereby improving its therapeutic index in various diseased conditions. PLGA has gained wide acceptance in global medical community due to its biocompatibility, resorbability through natural pathways and can be custom modified for drug targeting. Galactosylation of PLGA aids in liver targeting as galactose can recognize asialoglycoprotein receptors which are abundant in liver cells. Galactose-PLGA conjugate offers an amphiphilic structure and during NP formulation, the drug (flavonoids) gets encapsulated in the inner hydrophobic core and the outer hydrophilic shell with galactose moieties is available for asialoglycoprotein receptor recognition, which makes it target specific delivery system. Non-invasive monitorization of the in vivo condition of a disease is of utmost importance and nuclear scintigraphic imaging is an important tool in this regard. Various diseased states of liver like acute and chronic hepatitis, cirrhosis and cancer lead to changes in liver architecture leading to irregular hepatic blood flow which can affect biotransformation and decreased hepatic metabolism. Hence functional imaging of liver metabolism warrants particular interest. Liver scintigraphy using 99mTc-sulphur colloid and hepatobiliary scintigraphy using 99mTc-labeled mebrofenin are commonly used to evaluate liver function.

In this study, attempts have been made to enhance the target specificity of the various polyphenolic bioflavonoids by incorporating them in PLGA based nanoparticle formulations. Qr-PLGA nanoparticles and galactose tagged API-PLGA nanoparticles have been formulated using dialysis and nanoprecipitation technique respectively, followed by their detailed characterization and monitorization of their efficacy, employing both in vitro (cytotoxicity, cellular uptake using flow cytometry and confocal microscopy and apoptosis studies on HepG2 cell line) and in vivo (estimation of different biochemical parameters, histopathological investigations, western blot analysis of proteins of interest, gelatin zymography and pharmacokinetic analysis in carbon tetrachloride induced acute and diethylnitrosamine induced chronic animal model of liver

damage) experiments. Non-invasive evaluation of the formulations through scintigraphic analysis, using 99mTc labelled sulphur colloid and mebrofenin were also done to corroborate the research findings obtained from the invasive experimentations. The thesis has been divided into 4 chapters.

Chapter 1 is the introduction section, which deals with detailed discussion on hepatic disorders with special emphasis on chronic liver diseases including HCC. Treatment of liver diseases is quite a challenge globally but natural products, specially flavonoids, are being increasingly used as a promising therapeutic approach for pathological conditions of the liver. Though phytoconstituents are potent, they have many drawbacks and to address those concerns, novel drug delivery systems have been widely utilized. These topics have been discussed in details, which provides insight into the successful implementation of novel drug delivery systems in combating liver diseases. Non-invasive imaging modality, using nuclear scintigraphy, is a useful tool to assess the in vivo condition of diseases, as well as to perceive the drug action. Thus, this technique can be exploited to safely monitor the outcome of the developed drug formulations, delivering optimal dose responsible for maximum therapeutic effect against different disease models in animals. Role of nuclear scintigraphy, along with liver imaging using 99mTc-sulphur colloid and 99mTc-mebrofenin have been discussed in this chapter as well. 99mTc-sulphur colloid localises within the liver reticuloendothelial system (RES) where damaged areas will appear as cold spots, thereby visualizing reticuloendothelial activities and 99mTc-mebrofenin, after accumulation in liver, excretes into the biliary tract where various physiological parameters of hepatic extraction, hepatic excretion, biliary tract patency and hepatobiliary clearance can be measured.

Chapter 2 is an extensive literature survey on the main phytoingredients, Qr and API used in this research, and their physico-chemical properties, biological activities and nanoformulation approaches used in tackling various diseased states have been touched and elaborated. In this chapter we have also discussed about USFDA approved polymer, PLGA and its drug delivery and biomedical applications along with its physico-chemical properties as well.

Chapter 3 deals with the development of surfactant-free quercetin loaded PLGA nanoparticles (Qr-NPs) to investigate the hepatoprotective efficacy of the product non-invasively by nuclear scintigraphy. The nanoparticles were prepared using PLGA by dialysis method and ranged in size between 50-250 nm with a narrow range of distribution. They were found to arrive at the fenestra of liver sinusoidal epithelium for accumulation. The sizes of nanoparticles (batch S1) were optimum to reach the target and offer enough protection of the hepatocytes degenerated by CCl_4 intoxication as

determined by various biochemical and histopathological tests. In vitro studies exhibited the cytotoxic effect of the formulation against HepG2 cell line. The hepatoprotective efficacy of Qr-NPs evaluated non-invasively by nuclear scintigraphic technique using 99mTc-labeled sulphur colloid revealed abnormality in liver at the area of decreased uptake in rats of CCl_4 treated group, which disappeared in Qr-NPs treated group. In dynamic studies with 99mTc-mebrofenin, excretion was severely impaired in CCl_4 treated group but was moderate in drug treated group, proving the recovery of animals from damage. The schematic outline of the work is represented in Figure 1. The findings of this study have been published in the **Journal of Nanoparticle Research. 2016; 18(7):196. doi: 10.1007/s11051-016-3504-0.**

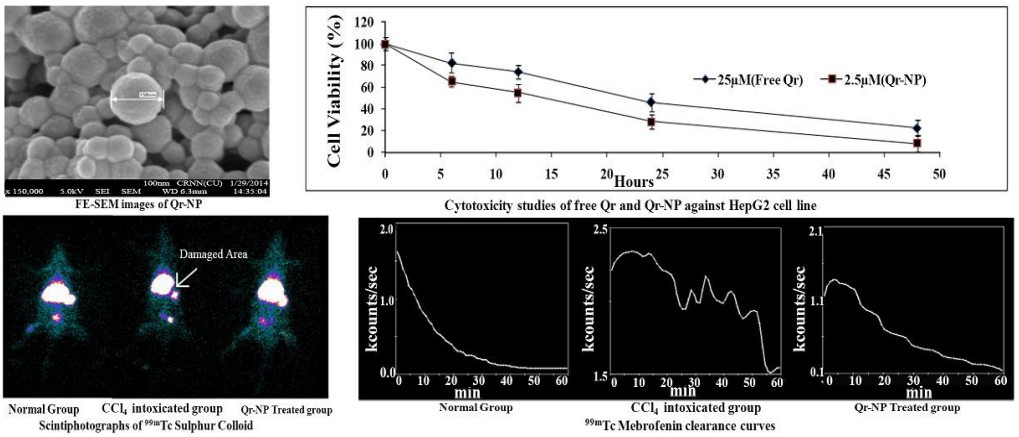

Figure 1: The hepatoprotective efficacy of Qr-NPs evaluated non-invasively by nuclear scintigraphic technique using 99mTc-labeled sulfur colloid and 99mTc-labeled mebrofenin

Chapter 4 deals with the development of galactose-tailored PLGA NPs loaded with apigenin (API-GAL-NPs) for active liver targeting to treat HCC (Hepatocellular carcinoma) in animal model. Two kinds of apigenin NPs (nanoparticles), such as apigenin-PLGA NPs (API-NPs) and API-GAL-NPs were fabricated and characterized by size, surface morphology, encapsulation efficacy, and in vitro drug release kinetics. In vitro assays were performed on HepG2 cells to check the cellular internalization, cytotoxic potential, and apoptotic potential of free apigenin (API), API-NPs, and API-GAL-NPs. In this study, API-GAL-NPs exhibited improved cellular internalization of API resulting in significantly high cytotoxic and apoptotic potentials to HepG2 cells over API and API-NPs. In in vivo studies, API-GAL-NPs exhibited a better protective effect against DEN-induced HCC in rats evidenced by the significant reduction of nodule formation, downregulation of matrix metalloproteinases (MMP-2 and MMP-9), and induction of apoptosis in the liver than API and API-NPs. Histopathological studies and scintigraphic imaging also confirmed that API-GAL-NPs treatment achieved better

therapeutic efficacy against DEN-induced HCC in rats over API-NPs. In conclusion, API-GAL-NPs may serve as a potential therapeutic agent against HCC in the future by achieving improved liver targeting. The conceptual framework of the research work is shown in Figure 2. The findings of this study have been published in the **Colloids and Surfaces B: Biointerfaces. 2021; 204: 111778. doi: 10.1016/j.colsurfb.2021.111778.**

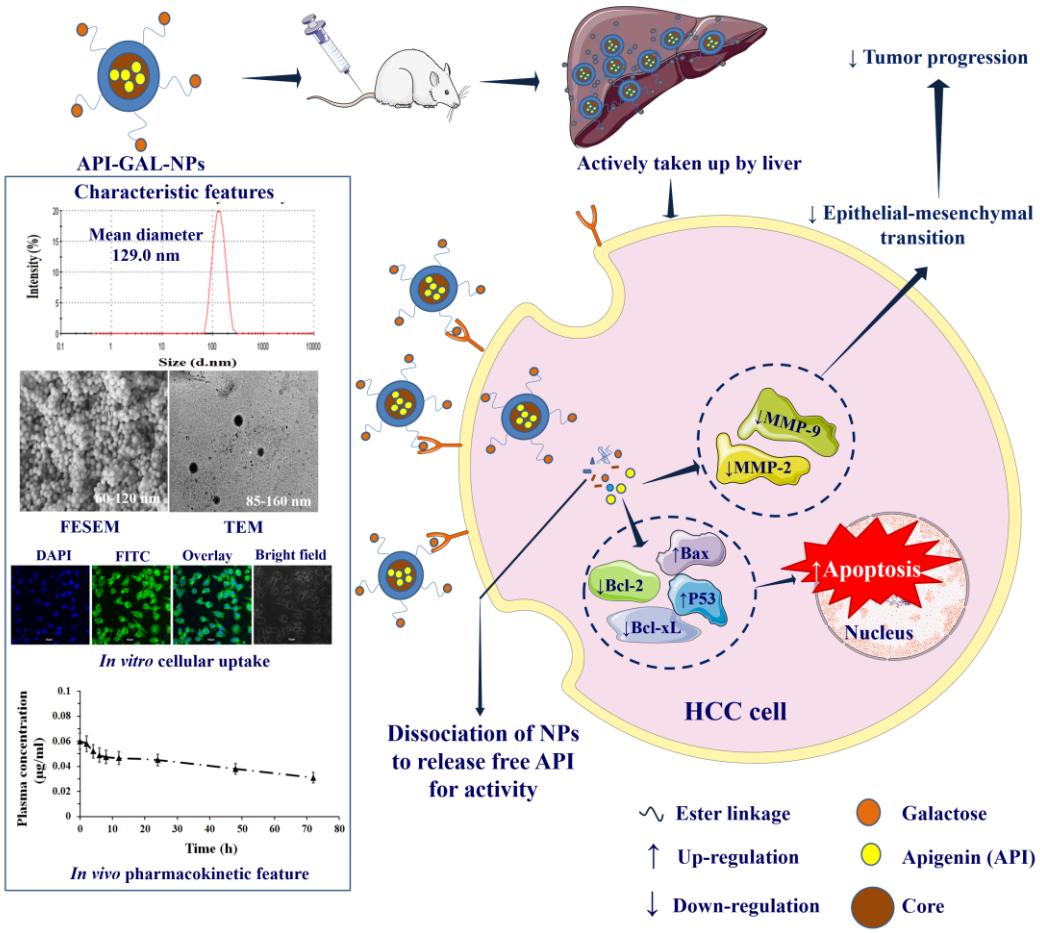

Figure 2. The graphical presentation detailing the scheme of the research work along with its results demonstrating the target specificity API-GAL-NPs in combating HCC.

List of Publications:

1. **Ganguly S**, Gaonkar RH, Sinha S, Gupta A, Chattopadhyay D, Chattopadhyay S, Sachdeva SS, Ganguly S, Debnath MC. Fabrication of surfactant-free quercetin-loaded PLGA nanoparticles: evaluation of hepatoprotective efficacy by nuclear scintigraphy. J Nanopart Res. 2016; 18(7):196. doi: 10.1007/s11051-016-3504-0.
2. **Ganguly S**, Dewanjee S, Sen R, Chattopadhyay D, Ganguly S, Gaonkar R, Debnath MC. Apigenin-loaded galactose tailored PLGA nanoparticles: A possible strategy for liver targeting to treat hepatocellular carcinoma. Colloids Surf B Biointerfaces. 2021; 204: 111778. doi: 10.1016/j.colsurfb.2021.111778.

List of Patents: Nil

List of presentations in National/International Conferences

1. **Ganguly S,** Debnath MC. Evaluation of the therapeutic efficacy of Nanoparticulated Quercetin in combating oxidative hepatocellular degeneration by nuclear imaging technology using 99mTc radiopharmaceuticals. SNMICON 2014 (46th National Annual Conference of Society of Nuclear Medicine India 2014), Kolkata.

2. **Ganguly S,** Gaonkar RH, Debnath MC. Evaluation of the therapeutic efficacy of nanoparticulated apigenin in combating oxidative hepatocellular degeneration by non-invasive nuclear imaging technology using 99mTc radiopharmaceuticals. ISCBDD 2016 (International Symposium on Chemical Biology and Drug Discovery, 2016), Kolkata.

3. **Ganguly S**, Debnath MC. Dialysis Method: A Novel Technique for Preparing Surfactant Free Nanoparticles. National Seminar on Pharmacy and Healthcare: Traditional Knowledge to Modern Techniques, Jadavpur University, Kolkata, September, 2018.

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