Enhancement of Oral Bioavailability and Antiproliferative Potential of Betulinic Acid by PLGA Loaded Nanoparticle Approach

A SUMMARY SUBMITTED TO
BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY
(A CENTRAL UNIVERSITY), LUCKNOW

FOR THE DEGREE OF

Doctor of Philosophy

IN

PHARMACEUTICAL SCIENCES

Submitted by

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(2020)

1. Introduction

Hepatocellular carcinoma or hepatoma is considered the chief widespread malignant tumor of liver. Liver comprises of diverse cellular components like hepatocytes (80%, major cell form), blood cells, billiary cells, ito cells, Kupffer cells, perisinusoidal cells, etc. The mainstream of principal liver cancer (approximately >90%) occurs from the hepatocytes and is referred as hepatocellular carcinoma. During the process of hepatocarcinogenesis, preliminary carcinogen offences result in instigated cells from the normal liver parenchyma cells or hepatocytes by alteration in the genetic levels which is followed by an interaction generally with DNA. The chronic exposure of hepatocytes to tumor promoter or carcinogens like Phenobarbital leads to subsequent promotion of tumors and produces hepatic altered foci (clonally selected expansions of initiated cell populations). These carcinogenic contents cause further alterations at the genetic levels where under extreme accumulated levels produce hyperplastic nodules that eventually changes into hepatocellular carcinoma.

Triterpenoids, originally a terpenoid (additionally known as isoprenoids), the biggest gathering of characteristic items to which the betulinic acid analogs additionally have a place. These chemical compounds comprise of six isoprene units and can be segregated from various plant sources. Virtually all terpenes have same pharmacological activities in animals, including man and withal play a paramount role in human medicine. From this perspective, the most consequential group of terpenes are triterpenes, triterpene glycosides, (withal known as saponines), and other triterpenoids, representing one of the numerous classes of natural compounds. Betulinic acid, a product found in nature, was obtained from the *Dillenia indica* stem bark (family: Dilleniaceae), having a pentacyclic triterpene nucleus with the broad spectrum of biological and pharmacological activities like anti-cancer, anti-malarial, anti-HIV, anti-bacterial, anti-inflammatory, anthelmintic, anti-nociceptive activities, etc.

Betulinic acid (B) is a pentacyclic triterpenoid of natural origin with remarkable anticancer activity; however, its less oral bioavailability makes itself towards poor therapeutic efficacy. Recent investigations suggested that the bioavailability may be increased through the preparation of nanoparticles using PLGA, poly(lactic co-glycolic acid). Therefore, we aimed to prepare PLGA, loaded B nanoparticle (BNP) and characterization would be done with various analytical parameters. Later, pharmacokinetic studies of B and BNP would be find out using the Albino

Wistar rats and data will be compared with standard B. Finally, antiproliferative potential against the hepatocellular carcinoma would be tested by *in vitro* human hepatoma cell lines (Hep-G2 cells).

Further, *in vivo* antiproliferative effects would be assessed using N-nitrosodiethylamine (NDEA)-induced hepatocellular carcinogenic rats. Various physiological, biochemical, morphological parameters in plasma and hepatic tissues would be performed to find out and compare the effects of B and BNP after oral administration.

Again, various enzyme linked immunosorbent assay (ELISA), (Caspases, i-NOS, e-NOS and interleukins: IL-1ß, IL-2, IL-6, IL-10), qRT-PCR and western blot based gene expression study would be performed to determine the mechanism of action of B and BNP. Further qRT-PCR data based mathematical modelling would be performed to perform to determine the prospective of B and BNP to induce apoptosis to the cancer cells. Finally, metabolic perturbations in plasma would be carried out through NMR to evaluate metabolic changes during cancerous condition and after treatment. In conclusion, BNP may be a better alternative for hepatic carcinoma therapy for future drug design perspectives.

2. Objectives

2.1 Isolation of betulinic acid (B)from *Dillenia indica* and its characterization

2.2 Preparation of betulinic acid nanoparticle (BNP) and its characterization

2.3 *In vitro* ameliorative effect using human hepatoma cells (Hep-G2)

2.4 *In vivo* pharmacokinetic study using albino Wistar rats

2.5 *In vivo* antiproliferative action using N-nitrosodiethylanine (NEDA)-induced albino Wistar rats

2.6 NMR based metabolomic studies

Summary

Hepatocellular carcinoma, especially in the later stages, is a major problem in the clinic and serious complication of cirrhosis or other chronic liver disease. At present, the treatment strategies are limited and there is a clear need for new therapies. Only a few medications are available in the market for the treatment of HCC, possession of contraindication and side effects are the biggest unacceptability for the patient. Sorafenib is only FDA approved drug for the treatment of HCC with limited response rate due to local metastasis and chemotherapeutic resistance. Betulinic acid (B) is a naturally occurring product acquired from *Dillenia indica* stem bark, belonging to the family Dilleniaceae, having a pentacyclic triterpene nucleus with the broad range of pharmacological activities and biological activities like anticancer, antimalarial, anti-HIV, antibacterial, anti-inflammatory, anthelmintic, antinociceptive activities. The chief disadvantage associated with natural products is poor oral absorption, which may lead to lower effectiveness. Therefore, the foremost purpose of my research work is to isolate B, formulate nanoparticle of B (BNP) for treating HCC at molecular and cellular level.

Based upon the compatibility, desired biocompatible and biodegradable properties PLGA polymer was selected. The selected polymer was anticipated to possess capability of giving stable formulation, having high entrapment efficiency and drug loading. For polymeric nanoparticles, PLGA and PVA concentration was selected based on the particle size, %EE, drug loading and *in vitro* release.

Taking this scaffold as parent molecule B was isolated and formulated BNP by emulsifcation and the solvent evaporation technique with PLGA and different concentration of PVA. All batches of BNP subjected to zeta potential, particle size, PDI, entrapment efficiency, drug loading, *in vitro* drug release, SEM and FTIR analysis for optimization.

Once the formulation and characterization have been completed, optimized formulation BNP and B were subjected to *in vitro* screening on Hep-G2 cell line to investigate their anti-proliferative potential against HCC cells. Although the parent compound B represented the moderate cytotoxic potential (GI_{50} = 10 µg/mL) towards the Hep-G2 cell line, BNP exhibited high order of cytotoxicity (GI_{50} < 10µg/mL) against the Hep-G2 cell line. Again cellular uptake analysis was performed against Hep-G2 cells to reveal their cellular internalization potential. This action was

substantiated through confocal images where we established that BNP had good penetration power and entered inside the Hep-G2 cell to a greater extent than the parent compound B. Both these experiments suggested that BNP had more antineoplastic potential than the parent compound B.

On the basis of *in vitro* result, both BNP and B introduced for detailed *in vivo* pharmacological study. Prior to initiate this study, we performed *in vivo* pharmacokinetic studies for the estimation of drug concentration in the plasma as stated by the profile of BNP and B where experiment demonstrated that BNP had a higher volume of distribution in plasma than the parent compound B. This action may be due to the lower particle size of BNP, which was ultimately absorbed to a greater extent after the oral administration in the rats.

We, therefore, conducted *in vivo* anti-proliferative screening of both BNP and B in the NDEA-induced HCC model using male albino Wistar rats and provides few notable findings related to the mechanism underlying the BNP action. In our preliminary investigations, we assured for the induction of carcinogenic condition in NDEA-exposed rats by the reduced body weight of animals and higher incidence number of carcinogenic nodules in the liver tissue. The examined lessening in these alterations after BNP and B administration was principal proposition of its shielding effect against carcinogenic condition, which suggests the necessitation of additional biochemical and pathophysiological investigations. It has now been depicted that there is a decline in the action of anti-oxidants during the HCC conditions. The reduction in the enzymatic defense of SOD, CAT, and GSH and was profoundly observed after the NDEA treatment that unveils their increased utilization during excessive cellular proliferation. Further, the NDEA treatment increased the production of MDA and PC that validated the damage of the proteins and cellular lipids, respectively. It is notable that BNP and B administration reduced the levels of PC and MDA with restoration of enzymatic antioxidant defense of CAT, SOD and GSH, corroborated its tumor shielding capability with remarkable antioxidant effects.

Further, biliverdin and bilirubin are the catabolic by-products of RBCs, and the raised the levels of the following biomarkers which pointed toward hepatic damage. The elevation in these markers during the exposure to NDEA and their restoration after BNP and B administration also hold up the control of hepatic disease. Moreover, NDEA exposure elevated the levels of enzymes imperative for the liver function (AST, ALT and ALP) which reflect the advancement of

carcinogenesis condition. The observed efficacy of BNP to restore these enzyme levels indicated the ability of BNP to prevent hepatic damage. Similarly, the high concentration of LDH in the serum attained in the NDEA-exposed group could be accountable for NDEA-mediated damage to the liver due to incidence of pre-neoplastic lesions. Treatment with BNP led to the enhancement in LDH level which probably caused the attenuation in mitigation of pre-neoplastic lesions and hepatic damage and thereby potentiating its antitumor property.

Recent studies suggested that few pro-inflammatory cytokines and are engaged in the cancer progression inflammation pathogenesis. B usually brings to bear anticancer activity moreover by activating the apoptotic mitochondrial pathway or by inducing cellular stress, such as DNA damage and cytokine withdrawal. Therefore, we examined the modified stages of caspases and cytokines between diverse faction through the ELISA and investigated the consequences of BNP and B treatments over the carcinogen control group. In NDEA-exposed rats, the inflammatory cytokines were raised and were declined to a definite degree after BNP and B administrations. On the contrary, caspase-8 and caspase-3 were rapidly decline in the carcinogen control group and were notably raised again to the normal stages after BNP and B administrations. It was recognized that distinctive to the tested cytokines, the stages of caspase-8 and caspase-3 in the NDEA-exposed group were rapidly re-establish to the regular level after administration of BNP and B, and this effect was further distinct in the BNP-treated group as compared to the B-treated group. Further protective effect of BNP was evidenced through SEM analysis and histopathology. The microscopic image of histopathology showed irregular shaped cytoplasm and nuclei in the NDEA-treated rats that were most likely owing to unwarranted generation of free radical during the NDEA exposure. The potential reduction in denatured and ruptured cells (RC and dN) in BNP treated groups denoted the ameliorative perspectives of BNP against HCC. A similar trend was found in SEM analysis also.

Further, we investigated the mechanism of anti-HCC perspectives of BNP at molecular level. The levels of total nitrite/nitrate ratios, NOS, i-NOS are increased and e-NOS level decreased after NDEA treatment revealed that the NO concentration was decreased in HCC rats, representing liver injury. Interestingly, the concentration of NO was increased after BNP and B treatments, demonstrating their protective action, higher for BNP than parent B.

Further, gene expression analysis revealed that the BNP and B treatment provided a rapid reduction in i-NOS, Bcl-2, Bcl-xl and induction in e-NOS, Cyt-C, BAD, caspase-3, BAX, and caspase-9 mRNA expression which followed a trend similar to those measured in ELISA. Furthermore western blot study disclosed that increased expressions of Bcl-xl, Bcl-2, and decreased expressions of BAD in CC group re-established to the normal level after BNP and BP treatments Considering altogether, both BNP and B causes induction of apoptosis through e-NOS and i-NOS induced activation of Bcl-2 family proteins → Cyt C → Caspase-3 and Caspase-9 signaling cascade. This action was more prominent for BNP than the pure B.

Again we also applied a data based mathematical modeling approach in the present work. To accomplish the following, we deciphered the quantitative assessment of qRT-PCR analysis into the formulated method through the MATLAB software. It was recognized from the mathematical modeling that the decrease in concentration of cancer cells is maximum for BNP than B. It was further observed that stimulation of e-NOS and i-NOS resulted in elevation of BAX+BAD, declination of Bcl-2+Bcl-xl in the cytoplasm and increase in the concentrations of Caspase-9 and Caspase-3. Thus, the quantitative qRT-PCR and data-based mathematical modeling strongly supported the potential of BNP to induce apoptosis to the cancer cells mediated *via* stimulation of e-NOS and i-NOS.

In addition, the tumor-induced hyperlipidemia leads to the progression of liver cirrhosis during the HCC development, which ultimately led to the liver damage. Liver damage during carcinogenic condition was observed through increased TC, TG, LDL, VLDL and decreased HDL concentrations in CC rat sera as compared to the NC. The BNP and B treatments normalized these concentrations, which further accounted protective activities of BNP were more prominent than pure B.

We further implemented ^{1}H-NMR-based metabolomics to estimate whether BNP and B have the capability to reinstate the metabolic perturbations linked to NDEA-exposed HCC condition. The box-cum-whisker plots and OPLS-DA score plots by utilizing the MetaboAnalyst were achieved from the 1D-^{1}H-CPMG-NMR spectral results of the rat serum and clearly demonstrated significant metabolic modifications in the NDEA-exposed carcinogenic condition. Interestingly, a decreased glucose level and augmented lactate level were detected in the NDEA-exposed

group, which was well supported by earlier findings that demonstrate the carcinogenic condition. These findings completely prop up the Warburg effect and may be associated with a superior quantity of glucose utilization by the proliferating tissues pursued by the formation of the by-product, lactate. The noteworthy incidence witnessed in this investigation manifested the exceptional capability of BNP and B to reinstate two foremost metabolic perturbations, *i.e.*, elevated lactate levels and reduced glucose levels, a characteristic feature for the progression of tumor. Furthermore, VLDL/LDL was observed to be augmented in CC rat as both metabolites are the precursor for the production of cholesterol and cholesterol is utilized by cell for cell membrane production. B and BNP administration again normalized these metabolites concentration near to normal, which further demonstrated their anticancer properties. The metabolic perturbations showed a reduced tricarboxylic acid (TCA) cycle (glutamine) and elevated β-oxidation (lipoproteins and lipids) glycolysis or gluconeogenesis (glucose and lactate) during HCC condition.

In conclusion, developed PLGA based nanoformulation (BNP) demonstrated enhanced oral bioavailability, pharmacotherapeutic efficiency and anticancer effect. The current study substantiates the biochemical, pathophysiological and molecular link of BNP treatment and demonstrates the mechanism of anti-tumor response. Our obtained *in vivo* data confirms those of the earlier *in vitro* investigation. Although the molecular insights discovered for BNP action provides the caspase activation mediated mitochondrial apoptotic pathway. Moreover, using a metabolomics approach in an *in vivo* model, we discovered an advanced mechanistic understanding of BNP action at cellular level. Altogether, the results indicate that BNP has outstanding potential to obliterate HCC and could serve as prospective component for the growth of anti-HCC drugs.