Chemico-Biological Interactions 195 (2012) 206–214

Contents lists available at SciVerse ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

Nanocapsulated curcumin: Oral chemopreventive formulation against diethylnitrosamine induced hepatocellular carcinoma in rat

Debasree Ghosh [a], Somsubhra Thakur Choudhury [a], Swarupa Ghosh [a], Ardhendu K. Mandal [a], Sibani Sarkar [a], Aparajita Ghosh [a], Krishna Das Saha [b], Nirmalendu Das [a,*]

[a] Drug Development/Diagnostics & Biotechnology Department, Indian Institute of Chemical Biology, 4, Raja S.C. Mullick Road, Kolkata 700032, India
[b] Cancer & Cell Biology Department, Indian Institute of Chemical Biology, 4, Raja S.C. Mullick Road, Kolkata 700032, India

ARTICLE INFO

Article history:
Received 19 August 2011
Received in revised form 16 November 2011
Accepted 8 December 2011
Available online 16 December 2011

Keywords:
Oxidative stress
Reactive oxygen species
Diethylnitrosamine
Hepatocellular carcinoma
Drug delivery
Nanocapsules

ABSTRACT

Toxic outcome of chemical therapeutics as well as multidrug resistance are two serious phenomena for their inacceptance in cancer chemotherapy. Antioxidants like curcumin (Cur) have gained immense importance for their excellent anticarcinogenic activities and minimum toxic manifestations in biological system. However, Cur is lipophilic and thus following oral administration hardly appears in blood indicating its potential therapeutic challenge in cancer therapy. Nanocapsulated Cur has been used as a drug delivery vector to focus the effectiveness of these vesicles against hepatocellular carcinoma. The theme of work was to evaluate effectiveness in oral route of polylactide co-glycolide (PLGA) Nanocapsulated curcumin (Nano Cur) against diethylnitrosamine (DEN) induced hepatocellular carcinoma (HCC) in rat.

Nano Cur of average diameter 14 nm and encapsulation efficiency of 78% were prepared. Fourier Transform Infra Red (FTIR) analysis revealed that there is no chemical interaction between drug and the polymer. Three i.p. injections of the chemical hepatocarcinogen DEN at 15 days interval causes hepatotoxicity, the generation of reactive oxygen species (ROS), lipid peroxidation, decrease in plasma membrane microviscosity and depletion of antioxidant enzyme levels in liver. Nano Cur (weekly oral treatment for 16 weeks at 20 mg/kg b.wt) in DEN induced HCC rats exerted significant protection against HCC and restored redox homeostasis in liver cells. Nanocapsulated Cur caused cancer cell apoptosis as visualized by ApoBrdU analysis. Histopathological analysis confirmed the pathological improvement in the liver. Nano Cur was found to be a potential formulation in oral route in combating the oxidative damage of hepatic cells and eliminating DEN induced hepatocellular cancer cells in rat whereas identical amount of free Cur treatment was found almost ineffective.

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide, with a particularly high prevelance in Asian countries. Angiogenesis plays a significant role in the aggressiveness of HCC. The only potential curative modality for HCC is surgery, including transplantation, yet the recurrence rate for this particular cancer is high and long-term survival rate is rather poor [1]. Both conventional chemotherapy and radiotherapy have been found to be ineffective or only minimally effective in patients with unresectable HCC. Chemoprevention has been considered to be the best strategy in lowering the present prevalence of the disease in view of the limited treatment and negative prognosis of liver cancer [2]. The major risk factors of liver cancer are hepatitis viral infection, alcohol, fungal toxins (aflatoxins), toxic industrial chemicals, air and water pollutants [3].

ROS play a major role in the induction of carcinogenesis [4]. They are derived from the metabolism of molecular oxygen. ROS include superoxide anion radical (O_2^{-}.), singlet oxygen (O_2), hydrogen peroxide (H_2O_2), and the highly reactive hydroxyl radical (·OH). ROS normally exists in all aerobic cells in balance with biochemical antioxidants. Oxidative stress occurs when this critical balance is disrupted because of excess ROS, antioxidant depletion,

Abbreviations: Cur, curcumin; PLGA, polylactide co-glycolide; Nano Cur, Nanocapsulated curcumin; HCC, hepatocellular carcinoma; DEN, diethylnitrosamine; FTIR, Fourier Transform Infra Red; ROS, reactive oxygen species; EPR, enhanced permeability and retention effect; BSA, bovine serum albumin; DMAB, Didodecyldimethylammonium bromide; CM-H_2DCFDA, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydro-fluorescein diacetate acetyl ester; AFM, atomic force microscopy; AST, serum aspartate transaminase; AP, alkaline phosphatase; ALT, serum alanine transaminase; RLW, relative liver weight; DPH, diphenyl hexatriene; SOD, superoxide dismutase; H_2O_2, hydrogen peroxide; GSH, reduced glutathione; PVDF, polyvinylidene fluoride; BCIP/NBT, 5-bromo-4-chloro-3′-indolylphoshate p-toluidine/nitro-blue tetrazolium chloride; NOS 2, nitric oxide synthase; Cyt C, cytochrome C.

* Corresponding author. Address: Drug Development/Diagnostics & Biotechnology Department, Indian Institute of Chemical Biology, 4, Raja S.C. Mullick Road, Jadavpur, Kolkata 700032, India. Tel.: +91 33 2499 5715; fax: +91 33 2473 5197/2472 3967.
E-mail addresses: dasnirmalendu@hotmail.com, dasnirmalendu@iicb.res.in (N. Das).

0009-2797/$ - see front matter © 2011 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.cbi.2011.12.004

or both. Cellular targets attacked and damaged by ROS are lipids, proteins, sugars and nucleic acids. Various carcinogens exert their effects by generating excessive ROS during their metabolism which cause oxidative damage to cellular DNA which modulates cell signals and activate gene expression [5] that result in mutations leading to sustained proliferation of cancer cells and inhibiting proapoptotic signals responsible for apoptosis and therefore, play an important role in the initiation, promotion and progression of multistage carcinogenesis. Free radicals accelerate the rate of progression of benign tumors to malignant neoplasm.

Chemical carcinogen diethylnitrosamine (DEN) is a potent hepatocarcinogenic nitrosamine present in tobacco smoke, ground water having high level of nitrates, cheddar cheese, cured and fried meals, alcoholic beverages, occupational settings, cosmetics, agricultural chemicals and pharmaceutical agents [3,6]. The rat model of DEN-induced HCC is considered as one of the most accepted and widely used experimental models to study hepatocarcinogenesis [7]. Human livers metabolize nitrosamines similar to that of rat liver and also exhibit considerable similarities with regard to morphology, genomic alterations and gene expression, despite their different disease etiologies [8]. DEN metabolism in the liver by cytochrome isoform 2E1 (CYP 2E1) generates reactive oxygen species (ROS) causing oxidative stress [9]. DEN, being a genotoxic carcinogen, forms alkyl DNA adducts, induces chromosomal aberrations, micronuclei and sister chromatid exchanges in the rat liver [10]. These mutations induced by DEN are responsible for the development of hepatocarcinogenesis. Administration of herbal antioxidants has been shown to be preventive agents against DEN induced hepatocarcinoma [9].

Growing evidence suggests that populations with greater reliance on fruits, vegetables and spices in the diet experience a reduced risk for the major cancers. The nutraceuticals derived from nutritional sources are naturally multitargeting, and are less expensive, safer and immediately available [11]. The antitumor effects of plant polyphenols and quinone derivatives have been characterized in several cell culture and animal cancer models [12]. Curcumin (Cur) [diferuloylmethane] is a polyphenol derived from the plant *Curcuma longa*, commonly called turmeric. It has excellent antioxidant, anti-inflammatory and anti-tumorigenic properties [13]. Last 50 years extensive research has indicated that this polyphenol can both prevent and treat cancer. Cur can suppress tumor initiation, promotion and metastasis [14]. It acts on a variety of signal transduction pathways and molecular targets involved in the development of cancer [11,15]. The anti-initiation effect may result from its ability to inhibit the formation of DNA damage, whereas the antipromotion effect may be mediated through antiproliferation or apoptosis promotion of the initiated cells [16]. It is also known to reverse multidrug resistance of cancer cells. Oral Cur administration has shown a promising outcome in preventing the development of cancers of the skin, soft palate, stomach, duodenum, colon, liver, lung and breasts of rodents [17].

However, simple application of antioxidants is not effective to counter the hepatic damage, for poor oral availability of antioxidants to interact with hepatocellular membranes [18]. Despite the multiple medicinal benefits of the antioxidant Cur, it is lipophilic and has a poor systemic bioavailability following oral dosing due to its poor gastrointestinal absorption due to which very high doses (>1.5 g/day in humans) [19] are required to produce any medicinal effect and thus comprises its potential therapeutic uses which is a major challenge in cancer related therapy till date [20]. However its bioavailability increases when mixed in oils and hence various formulations are being made to capture the benefit of this compound. To maximize the therapeutic efficacy, the sufficient dose of drug should be specifically delivered to a target with minimal exposure to non-target cells.

Hence, there is a need to develop a delivery system for vectoring curcumin to hepatic cells. In this context, nanocapsules have been used here as potent drug carriers because of their several important technological advantages, e.g. long shelf life, high carrier capacity, feasibility of incorporation of both hydrophobic and hydrophilic substances and feasibility of variable routes of administration including oral route [18]. Also, nanocapsules are known to accumulate mostly at tumour sites because of poor lymphatic drainage of macromolecules in solid tumors and the enhanced permeability and retention effect (EPR) of tumor cells due to which the nanocapsules can extravasate through enlarged pores in the capillary endothelium of tumor cells [21].

Since poor systemic bioavailability following oral dosing of Cur limits its potential therapeutic uses, many groups have focussed on ways to improve its bioavailability. Co-administration of oral Cur with piperine appeared to increase serum concentrations of Cur [22]. Till date effectiveness of liposomal and nanoparticulated Cur has been seen against pancreatic cell lines [23,24] but not in an in vivo model of HCC. Recently nanoparticulated Cur has been tested against a number of cancer cell lines [20]. Oral anticancer formulations are preferred by the patients. However poor gastrointestinal absorption of Cur following oral dosing made it indispensable to formulate Cur nanoparticles for the treatment of hepatocellular carcinoma in vivo. In this context, present in vivo study has been designed to optimise the dose of the antioxidant Cur in polylactide-co-glycolide (PLGA) nanocapsules to judge the efficacy of oral nanocapsulated Cur over free Cur feeding against DEN induced hepatocellular carcinoma in rat model. According to the best of our knowledge and belief, this is the first in vivo study demonstrating the chemopreventive activity of curcumin encapsulated in biodegradable nanoparticles against chemically induced hepatocarcinoma.

2. Materials and methods

2.1. Materials

N-Nitrosodiethylamine (DEN), bovine serum albumin (BSA), Polylactide-co-glycolide (PLGA) (Resomer RG 85:15H), Didodecyldimethylammonium bromide (DMAB), and curcumin were purchased from Sigma–Aldrich (St. Louis, MO, USA). CM-H_2DCFDA was purchased from Invitrogen. Ethyl acetate (AR Grade) was purchased from Rankem Fine Chemicals (New Delhi, India). Chloroform and Methanol were purchased from E. Merck. All other reagents were of analytical grade.

2.2. Preparation of Nano Cur

A modified emulsion–diffusion–evaporation method [25] was used to make the Cur nanocapsule. In brief, 100 mg of PLGA was dissolved in 5 ml of ethyl acetate at room temperature. Cur (10 mg) was dissolved in 1 ml of ethyl acetate. The organic solution of PLGA and drug in ethyl acetate was then emulsified with 10 ml of an aqueous phase containing Didodecyldimethylammonium bromide (DMAB). The resulting o/w emulsion was stirred at room temperature for 3 h before homogenizing at 15,000 rpm for 5 min with a high-speed homogenizer (Polytron PT4000; Polytron Kinematica, Lucerne, Switzerland). The organic solvent was removed by constant stirring on a water bath set at 40 °C. The suspension was ultracentrifuged at 105,000*g* in Sorval RC 5B Plus using the rotor Sorval T-865 for 1 h. The pellet of nanocapsules was washed with PBS twice and re-suspended in 2 ml PBS [26].

To estimate the intercalated drugs in nanocapsules, the pellets were then dissolved in 2 ml of ethyl acetate and kept for 3 days at 4 °C. The O.D. was measured at λ_{max} (Cur) 422 nm and percent

of incorporation was calculated. The percent encapsulation of drugs in nanocapsule was found to 78% of the added amount.

2.3. Characterization of Nano Cur

2.3.1. Atomic force microscopy (AFM)

The AFM observations were performed with an Agilent Technologies, 5500 Pico Plus AFM system. All the images were obtained with the Aquastic mode using cantilevers having resonance frequency150–300 kHz, Tip height 10–15 μm and Tip length 225 μm. Mica was chosen as a solid substrate and used immediately after cleavage in a clean atmosphere. During this characterization experiment, the probe and cantilever were immersed completely in the water solution. The nanocapsule suspension on mica was dried in air (65% humidity) for 30 min. Images were captured and analysed using Picoscan 5.33 software of Molecular Imaging Corporation.

2.3.2. Fourier transform infrared spectroscopy (FTIR)

Potassium bromide (KBr) technique was used for FTIR analysis. First KBr was dried at 105 °C and grounded finely. Drug sample was added to it (Sample: KBr = 1:3) and was finely grounded again. It was compressed under high pressure to prepare pellets of 10.0 mm and 1–2 mm thick. The pellets were scanned over a range of 4000 cm^{-1} to 400 cm^{-1} and spectra was recorded using FTIR (Jasco FTIR 460 Plus, Japan).

2.4. Animals and treatment

Adult male Swiss Albino rats, each weighing approximately 100–120 g, were acclimatized to conditions in the laboratory (26–28 °C, 60–80% relative humidity, 12-hr. light/dark cycle) for 7 days prior to the commencement of the treatment during which they received food and drinking water. All the rats used in this study received proper care and handling in compliance with Animal Ethics Committee. India, Registration No. 147/99/CPC SEA, India. All animal experiments were conducted following the guideline of the "Principles of laboratory animal care" (NIH publication No. 85-23, 1985) and only after receiving the approval of the institutional animal ethics committee.

Animals were randomly selected for groups and carcinogen and drug were administered as per individual body weight of rat. Rats were divided into five groups with five animals in each group. Rats in the normal group (Group A) were injected (i.p.) with three doses of olive oil (0.5 ml) at an interval of 15 days. All rats in experimental groups were injected with three doses of DEN (i.p.) (200 mg/kg b. wt. in 0.5 ml olive oil) at 15 days interval. Group B animals were kept as DEN administered control. Animals in Group C were treated with empty nanocapsule (0.5 ml) orally once in a week from the day of 1st DEN administration upto 16 weeks, Group D animals were orally treated with free Cur (0.5 ml suspension of 0.2% Tween 80 aqueous solution containing 20 mg Cur/kg b.wt) once in a week from the day of 1st DEN administration upto 16 weeks. Group E animals were treated orally with nanocapsulated Cur (0.5 ml containing 20 mg Cur/kg b.wt) as a weekly dose from the day of 1st DEN administration upto 16 weeks. All animals were kept with normal diet and drinking water without any treatment for the next 2 weeks and sacrificed thereafter [9].

2.5. General procedures

At the end of 18 week starting from the 1st day of DEN administration, the final body weight was measured and blood was collected from heart in the rats of each group. Serum aspartate transaminase (AST), alkaline phosphatase (AP), serum alanine transaminases (ALT) were determined using a standard kit manufactured by Coral Clinical Systems; India [9]. After collection of blood, all rats were dissected and their livers were isolated promptly and washed with cold physiological saline. Final liver weights of all animals were recorded and relative liver weights (RLW) were calculated. A part of the organ was immediately used for mitochondria preparation and another part was fixed in 10% formaldehyde and processed for histological examination. The rest part was kept at −80 °C for further experiments.

2.6. Liver mitochondria preparation

Liver mitochondria were isolated from the rat tissue by conventional differential centrifugation [27,28]. The tissue was homogenized in 250 mM sucrose, 1 mM EDTA and 5 mM HCl–Tris buffer (pH 7.4). The 600 g pellet was discarded and mitochondria were pelleted at 6800 g, washed twice with the same solution, and the final pellet suspended in 240 mM sucrose, 34 mM KCl, 5 mM $mgCl_2$, 9 mM HCl–Tris, 1 mM EDTA, 6 mM Na_2HPO_4–KH_2PO_4 (pH 7.4) (1 ml buffer/g liver). All experiments were performed within 3 h following mitochondrial isolation.

2.7. Liver cytosolic fraction preparation

A portion of the liver was homogenized in 0.25 M sucrose solution. The homogenate was centrifuged at 8,200*g* for 10 min. using a Sorvall SS34 rotor. The supernatant obtained was again spun at 105,000*g* for 1 h in an OTD-50B Sorvall ultracentrifuge (4 °C) [18]. The supernatant from the second centrifugation was collected as the cytosolic fraction of liver cells.

2.8. Biochemical analysis and enzyme assays

2.8.1. Mitochondrial ROS level measurement

Intracellular ROS level was measured in liver mitochondria [29]. Briefly, isolated mitochondria (0.4 mg protein/ml) were loaded with the cell permeant probe CM-H_2DCFDA (5-(and-6)-chloromethyl-2′,7′-dichlorodihydro-fluorescein diacetate acetyl ester) (2 μM) for 15 min at 30 °C in dark, and fluorescence was measured through a spectrofluorometer (LS 3B, Perkin Elmer, USA) by using 499 nm as excitation and 520 nm as emission wavelengths. The data were normalized to normal values, and the normal was expressed as a value of 100%.

2.8.2. Mitochondrial membrane fluidity measurement

The fluorescence depolarisation, associated with the hydrophobic fluorescence probe diphenyl hexatriene (DPH), was used to monitor the changes in the fluidity of the lipid matrix accompanying the gel to liquid crystalline phase transition. Mitochondrial membrane fractions of hepatic cells were incubated at 37 °C by the addition of DPH dissolved in tetrahydrofuran (DPH/lipid molar ratio 1:500). The excitation and the emission maxima were kept at 365 and 430 nm, respectively. The fluorescence anisotropy was calculated by using the equation,

$$r = (I_{II} - I_{\perp})/(I_{II} + 2I_{\perp})$$

where I_{II} and $I_{\perp}$ are the fluorescence intensities in parallel and perpendicular to the direction of polarization of the excited light. The micro viscosity parameters $[(r_o/r) - 1]^{-1}$ were calculated in each case, knowing the maximal limiting fluorescence anisotropy r_o which for DPH is 0.362 [30].

2.8.3. Lipid peroxidation assay

Lipid peroxidation in the whole liver membrane was determined by measuring the amount of conjugated diene by the method of Mandal et al. using a spectrophotometer [31]. Lipids in cyclohexane solvent were assayed at 234 nm and the results were

expressed as μmol of Lipohydroperoxide/mg protein by using ∈m of 2.52×10^4 l/mol/cm. Total protein was measured by the method of Lowry et al. [32].

2.8.4. Estimation of SOD and catalase

The assay of Superoxide Dismutase (SOD) (EC 1.15.1.1) in cytosolic fractions of liver homogenate was estimated by following the method of Markland and Markland [33]. The activity of SOD was expressed as percentage inhibition of pyrogallol autooxidation detected in Reyleigh UV 2601 double beam spectrophotometer. One unit of SOD is described as the amount of enzyme required to bring 50% inhibition of pyrogallol autooxidation per 2 ml of assay mix.

A part of the cytosolic fraction was used for the assay of catalase activity spectrophotometrically by the method of Moragon et al. [34] with slight modifications. The activity was assayed by monitoring the decrease in absorbance at 240 nm as a consequence of hydrogen peroxide (H_2O_2) consumption and enzyme activity is expressed as μmol of H_2O_2 decomposed per minute per milligram of protein.

2.8.5. Reduced glutathione (GSH) assay

Glutathione level of a part of tissue homogenate was determined by the method of Davila et al. [35] with the help of a spectrophotometer by using tetrachloroacetic acid with EDTA as protein precipitating reagent. After completion of the total reaction, solutions were read at 412 nm. Absorbance values were compared with a standard curve generated from known GSH concentration to evaluate liver homogenate GSH levels.

2.9. Immunoblotting

Liver tissues were homogenized (1:10, W/V) in 10 mM Tris–HCl, pH 7.4, 150 mM NaCl, containing freshly added protease and phosphatise inhibitors and cytosols were prepared by centrifugation at 15,000*g* for 10 min at 4 °C. Protein concentrations were determined in cytosolic fractions [32]. SDS/PAGE was performed by subjecting 30 μg total protein under reducing conditions on 10% polyacrylamide gels, followed by electrophoretic transfer to polyvinylidene fluoride (PVDF) membrane (Sigma) at 15 V for 20 min by using Semi dry (Bio-Rad) transblot apparatus. Membrane was blocked in 4% BSA in PBS (overnight) at 4 °C, followed by incubation with the primary rabbit anti-cytochrome C (Cyt C) antibody (1:100) in blocking solution (0.2% Tween 20 in PBS and 2% BSA) and NOS 2 (1:1000) for 3 h at room temperature. After five washes with 0.2% Tween20 in PBS, the membrane was incubated in secondary alkaline phosphatase conjugated anti rabbit goat IgG antibody (1:1000) for 1 h 30 min. After five washes with 0.2% T-20 in PBS and 2% BSA, proteins were visualized by the development of colour using Sigma premixed 5-bromo-4-chloro-3′-indolylphosphate p-toluidine/nitro-blue tetrazolium chloride (BCIP/NBT) substrate solution. Colour intensity of bands were analysed with Image J software system.

2.10. Histological studies

Liver sections were fixed in 10% formalin overnight, and paraffin wax embedded. Sections were stained with hematoxylin and eosin (H&E) [36] for histopathological examination.

2.11. In situ DNA fragmentation assay

Apoptosis was determined by Apo-BrdU *in situ* DNA Fragmentation Assay Kit [Biovision K401-60].

2.12. Statistical analysis

The mean and standard error were calculated for all data. Statistical analysis was performed with Student's *t*-test. In all instances, $P < 0.05$ was considered as the minimum level of significance.

3. Results

3.1. Characterization of Nano Cur and their encapsulation efficiency

AFM analysis of the Cur nanocapsules revealed spherical shaped nanocapsules with mean average diameter of 15 nm (Fig. 1a). The size of 30 nm diameter is the result of aggregation of two nanocapsules of 15 nm diameter. The height of the nanocapsules above the substratum was found to be 3 Å.

The FTIR analysis of PLGA, Nano Cur and free Cur (Fig. 1b–d) indicates no drug-polymer interactions in the nanocapsulated formulation Nano Cur.

The encapsulation efficiency of the Cur nanoparticles was found to be 78%.

3.2. Effect of Nano Cur on RLW and marker enzymes of liver

DEN (3 doses of i.p. 200 mg/kg b.wt at 15 days interval) treatment causes an increase in relative liver weight (RLW) and marker enzymes of liver in rats. Nano Cur prevented reduction in RLW and marker enzymes of liver in rats significantly in comparison to DEN administered control group. Free Cur treatment or empty nanocapsules were not effective in giving any significant prevention from DEN administered control group of rats (Table 1).

3.3. Effect of Nano Cur on the generation of mitochondrial ROS and microviscosity of liver

DEN administration induced a significant increase of ROS with a significant decrease in the membrane microviscosity of liver tissue in the control as compared with rats of the normal group. Free Cur or empty nanocapsules treatment were unable to prevent mitochondrial ROS generation and microviscosity parameters as compared to DEN administered control group. However, Nano Cur showed a significant prevention in mitochondrial ROS generation with an elevation of microviscosity as compared to the control group (Table 2).

3.4. Effect of Nano Cur on lipid peroxidation and antioxidant status in liver

Conjugated diene is an important index of lipid peroxidation. DEN administered control rats showed a significant elevation in conjugated diene formation. Free Cur or empty nanocapsules did not show any significant prevention. However Nano Cur showed a significant prevention from increase in the levels of conjugated diene in the liver cell membrane (Table 3).

Antioxidant enzyme assay showed a significant reduction in the levels of the enzymatic antioxidants, SOD and CAT as well as reduced GSH in the DEN administered control group of rats. Free Cur and empty nanocapsules did not show any major deviation in the expression levels of these enzymes from those of the control group. However Nano Cur treatment prevented reduction of these antioxidant enzyme levels (Table 3).

3.5. Effect of Nano Cur on the expression of inducible nitric oxide synthase (NOS 2) expression in cytosol and release of cytochrome C (Cyt C) from the liver mitochondrial membrane

Western Blot analysis showed a significant increase in NOS 2 expression in the liver cell in DEN administered control group of

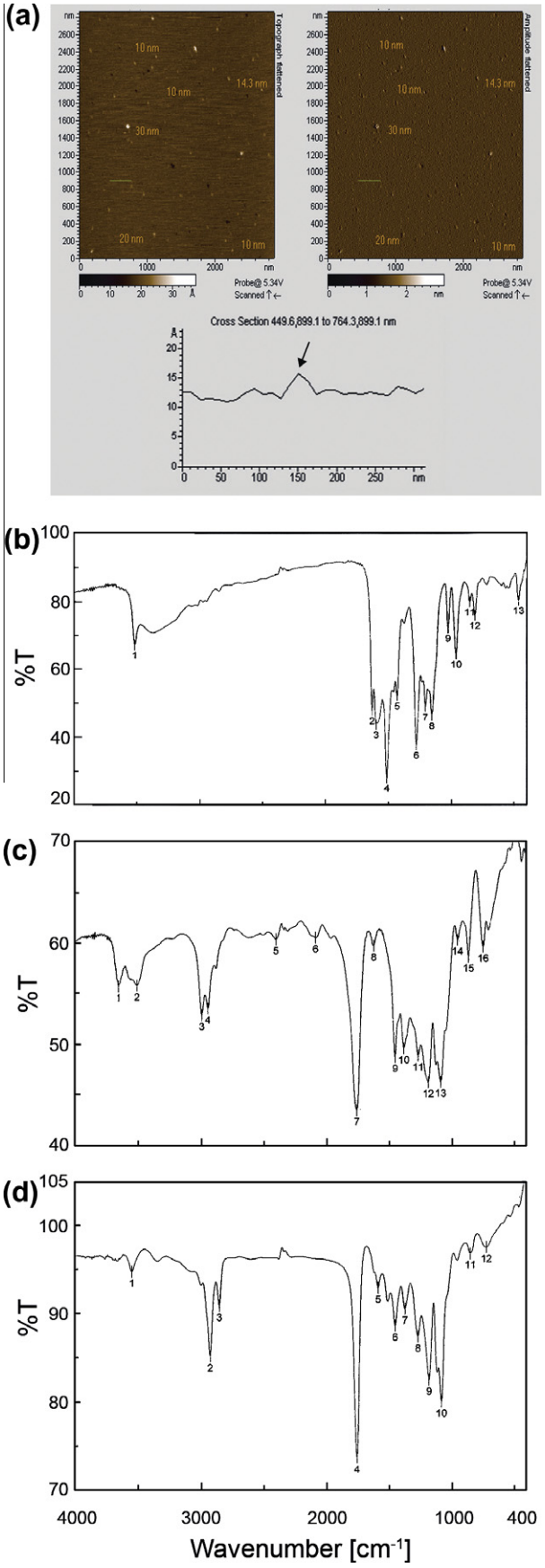

Fig. 1. AFM images of nanoparticles (a) under water obtained 30 min after deposition on mica sheet (Topography and amplitude flattened). Graph indicates height of nanoparticles from substratum i.e. mica sheet. Arrow indicates the height measured. FTIR images of PLGA (b), NanoCur (c) and Free Cur (d).

rats. Free Cur did not show any significant decrease in the expression levels of NOS 2 in the liver. However nanocapsulated Cur showed a significant decrease in the NOS 2 levels in the liver as compared to DEN administered control group of rats (Fig. 2a).

Release of Cyt C, from the mitochondrial membrane to the cytosol is a key event in cell apoptotic signalling following cell death. It was observed that normal cells released Cyt C in the cytosol as a consequence of the normal process of Programmed Cell Death (PCD). DEN administered rat showed an increase in the Cyt C levels as compared to normal rats. However Nano Cur treatment led to a significant higher release in Cyt C as compared to DEN induced control group of rats (Fig. 2b).

3.6. Effect of Nano Cur on histopathological and histochemical analysis of liver sections

Haematoxylin–Eosin-stained liver sections of normal rat (Fig. 3a1) showed hepatocytes are arranged in cords around hepatic vein forming hepatic lobules. Portal tracts are normal. DEN injected animals shows dialated hepatic veins (Fig. 3a2) and hepatic micronodules separated by thin fibrous septum (Fig. 3a3). In higher magnification fatty changes in hepatocytes with irregular nuclear membrane, with some eosinophilic cytoplasm are evident (Fig. 3a4). Free drug treatment to DEN injected animals was unable to produce any significant change to DEN induced hepatocyte pathomorphological structures. Hepatic micronodules separated by thin fibrous septa, with dialated hepatic veins and some amount of periportal fibrosis are also evident (Fig. 3a5). In higher magnification fatty changes are also evident. However nuclear membrane has not lost its structural integrity (Fig. 3a6). However Nano Cur have prevented the liver from developing hepatocarcinoma. Normal looking hepatic vein, portal tracts and hepatocytes are present here (Fig. 3a7).

ApoBrdU staining has shown some amount of apoptosis in normal cells as a consequence of normal Programmed Cell Death (Fig. 3b1). We observed increased BrdU positive cells indicating apoptosis in liver of Nano Cur treated group in comparison to DEN administered control group (Fig. 3b2 and b4). Free Cur treatment showed visibly less apoptotic cells in liver as compared to Nano Cur (Fig. 3b3).

4. Discussion

The limited progress achieved by cancer therapy in the last three decades has increased the interest of researchers in cancer chemoprevention. The process of carcinogenesis can take several decades to complete; hence it makes more sense to prevent cancer at its earliest stages by using low-toxic chemicals than to wait until the disease has reached its final stages, where it becomes necessary to use more toxic chemicals.

A dose escalation study of Cur has been done at three different doses of curcumin encapsulated in nanocapsules (5, 10 and 20 mg/kg b.wt). The effective dose of Cur has been found to be 20 mg/kg b.wt in combating DEN induced hepatocellular carcinoma in rat model. The data's for the effective dose have only been shown in the article. Pharmacologically, Cur has been found to be safe even at very high doses according to the USA-FDA in both animals as well as humans [17].

The small particle size (average diameter 15 nm) of the Cur nanocapsules formulated by us might be responsible for its longer circulation time and also for targeting the liver tumor by seepage through the leaky vasculature characteristic of the tumor tissues [37]. Also the uniformity in the dimensions of the different nanocapsules indicates the uniformity of the nanocapsules formed. FTIR analysis revealed no interaction between the polymer and the drug

Table 1
Effect of Cur (Free and Nanoencapsulated forms) on% of increase in RLW and marker enzymes of liver in DEN induced hepatocellular carcinoma.

Groups	Liver weight (g)	RLW	% of increase in RLW	Alkaline phosphatase (KA Units)	Serum aspartate transaminase (IU/L)	Serum alanine transaminase (IU/L)
Normal	8.2 ± 1.1	4.2 ± 0.2	–	30.2 ± 1.6	115.4 ± 5.3	36.4 ± 2.5
DEN (A)	10.5 ± 1.9	6.9 ± 0.7**	64.2	82.4 ± 2.3*	256.6 ± 6.2*	130.1 ± 3.2*
A + empty nanocapsule	10.4 ± 2.0	6.8 ± 0.3	61.9	76.5 ± 1.5	248.3 ± 7.4	125.2 ± 3.4
A + Free Cur	10.1 ± 2.4	6.5 ± 0.4	54.7	71.3 ± 2.1	225.4 ± 3.5	113.3 ± 2.2
A + Nano Cur	8.8 ± 2.2	4.7 ± 0.1##	11.9	37.1 ± 3.2#	122.2 ± 5.5#	32.4 ± 1.5#

Relative liver weight (RLW) = liver weight/final body weight × 100.
Results are expressed as mean ± S.E, of five animals.
* $P < 0.0001$ is significantly different from normal.
** $P < 0.01$ is significantly different from normal.
# $P < 0.0001$ significantly different from the DEN treated control group (A).
$P < 0.05$ significantly different from the DEN treated control group (A).

Table 2
Effect of Cur in free and nanoencapsulated forms on changes in the generation of ROS and mitochondrial membrane microviscosity ($[r_0/r-1]^{-1}$) in rat hepatocarcinoma.

Groups	DCF fluorescence (% of normal)	Membrane microviscosity ($[r_0/r-1]^{-1}$)
Normal	100 ± 7.43	0.656 ± 0.032
DEN treated(A)	250 ± 12.30*	0.244 ± 0.014*
(A) + empty nanocapsule treated	248 ± 11.72	0.256 ± 0.023
(A) + free Cur treated	230 ± 7.56	0.306 ± 0.011
(A) + Nano Cur treated.	120 ± 5.34#	0.590 ± 0.010#

Results are expressed as mean ± S.E, of five animals.
* $P < 0.0001$ is significantly different from normal.
# $P < 0.0001$ significantly different from the DEN treated control group (A).

Table 3
Effect of Cur (free and nanoencapsulated forms) on lipid peroxidation and antioxidant enzymes in liver of DEN induced rats.

Groups	Conjugated diene (μg/mg protein)	SOD level (% inhibition of pyrogallol autooxidation)	Catalase activity (μmol of H_2O_2 reduced/min/mg protein)	GSH level (mg/mg protein) [10^{-4}]
Normal	1.6 ± 0.11	74.3 ± 1.47	8.5 ± 0.18	0.0016 ± 0.0002
DEN treated (A)	3.6 ± 0.22*	33.3 ± 2.40*	2.6 ± 0.35*	0.0003 ± 0.0001**
A + Empty Nanocapsule treated	3.4 ± 0.23	33.6 ± 1.85	2.7 ± 0.31‘	0.0003 ± 0.0001
A + Free Cur	3.2 ± 0.12	39.6 ± 2.72	3.3 ± 0.17	0.0006 ± 0.0001
A + Nano Cur	2.1 ± 0.17#	71.1 ± 1.73##	8.2 ± 0.35##	0.0014 ± 0.0002###

Results are expressed as mean ± S.E, of five animals.
* $P < 0.0001$.
** $P < 0.001$ is significantly different from normal.
# $P < 0.001$ significantly different.
$P < 0.0001$.
$P < 0.01$significantly different from the DEN treated control group (A).

in Nano Cur, which indicates that Cur can be incorporated in the nanocapsules without altering its individual structural identity (Fig. 1).

Relative liver weight is an important parameter in judging the pathological condition of the liver. Lowering in the relative liver weight of rats by Nano Cur treatment is an important indication of the pathological improvement of the liver. Reduction of SGPT, SGOT and AP levels, important markers of liver function tests, by Nano Cur indicate its preventive effect in hepatocarcinoma (Table 1).

Oxidative stress is an obvious outcome of human cancer. It is produced either through an increased ROS generation with or without an alteration of antioxidant defence of the target cells or tissues [38,39]. Mitochondria are the major sites of cellular ROS production. ROS binds with a number of cellular components of cell including lipid, protein, DNA, carbohydrate, thiols, and other low-molecular-weight antioxidants causing oxidation of macromolecules and ultimately leading to pathogenesis. DEN administration generates a high amount of ROS in the hepatocarcinoma cells and the high amount of ROS generation is also responsible for a decrease in membrane fluidity due to rigidity of the mitochondrial membrane. Nano Cur treatment prevented the oxidative stress to a major extent by inhibiting ROS production, which in turn prevented decrease in membrane fluidity as compared to DEN treated control group of animals (Table 2).

Conjugated diene is one of the major end products of peroxidative degradation of the polyunsaturated fatty acid constituents of biological membranes and has mutagenic and carcinogenic properties as evident from in vitro systems and in experimental animals [40]. The present study shows a significant prevention of conjugated diene formation on Nano Cur treatment which is believed to be due to ROS generation in the liver (Tables 2 and 3). The detoxification ability of the liver resides in its rich metabolizing enzyme profile and intracellular antioxidant reserve. The antioxidant

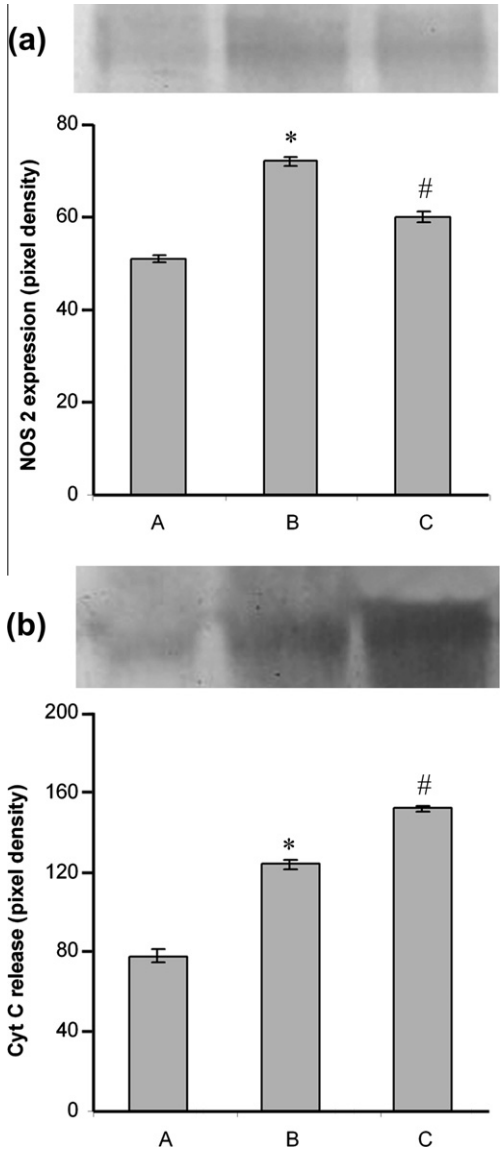

Fig. 2. Western blot analysis showing expression of Nos 2 (a) and Cyt C (b) protein in cytosolic fraction of liver tissue. Lane 1: Olive oil treated normal (A), Lane 2: DEN treated (B), Lane 3: DEN + Nano Cur treated (C). Histogram showing representative pixel intensities (arbitrary units of densitometric analysis using Image J software) of the immunoblot performed with different individual rats. Values are mean ± S.E. of 5 rats. $^{*}P < 0.0001$ significantly different from DEN treated control and $^{\#}P < 0.0001$ significantly different from Nano Cur.

reserve of liver includes two important enzymatic antioxidants, SOD, Catalase and an important non-enzymatic antioxidant reduced glutathione (GSH). SOD and Cat are the major free radical scavenging enzymes present in the biological systems. GSH plays a crucial role in the detoxification process of majority of alkylating agents including DEN. It neutralizes the electrophilic site by providing a –SH group and renders the metabolite more water soluble [6]. Our results agree with the hypothesis that DEN treatment in rat causes depletion of enzymatic and non-enzymatic antioxidant defence in hepatic tissue and induces hepatocarcinoma in rat (Table 3) with a substantial increase of mitochondrial ROS as indicated by (Table 2). Numerous reports have demonstrated that Cur may act as an antioxidant or as a pro-oxidant. In our experiments Nano Cur is acting as an antioxidant by preventing generation of high cellular levels of ROS and hence prevents the process of carcinogenesis, therefore acting as a cancer chemopreventive agent (Tables 2 and 3).

NOS 2 is an inflammation responsive enzyme, involved in wound healing, angiogenesis and carcinogenesis [41]. The inducible isoform (NOS 2) is expressed de novo by cell types, including macrophages and hepatocytes, in pathological stressed conditions [41] and is responsible for enhanced tumor growth in hepatocellular carcinoma [42]. NOS 2 upregulation and increased nitric oxide (NO) production affect the redox balance of cells, NOS being a downstream target of ROS, can induce protein, lipid, and DNA modifications [43]. Estimating the levels of NOS 2 expression is therefore an important parameter for anticancer formulations. DEN administration increase the NOS 2 levels indicating the generation of ROS promoting carcinogenesis and possibly leading towards angiogenesis. A low level of NOS 2 in the Nano Cur treated group is indicative of the low levels of ROS generation which might have helped prevent tumor growth and inhibit angiogenesis (Fig. 2a).

Cancer therapy is modulated either by a suppression of proliferation of cancer cells or by apoptosis of the cancer cells. The anti-tumor effect of curcumin has also been attributed in part to the suppression of cell proliferation, reduction of tumor load and induction of apoptosis in various cancer models both in vitro and in vivo [44]. The mechanism of cancer cell apoptosis is mitochondria-dependent or mitochondria independent [14]. Release of cytochrome C, from the mitochondrial membrane to the cytosol is a key event in cell death, which can lead the cell towards apoptosis or necrosis. In our experiments, increased cytochrome C levels above the normal cells in DEN administered control group of rats indicates the death of some cells on exposure to the genotoxic carcinogen. Increased cytosolic cytochrome C levels above the DEN administered control group of rats on Nano Cur treatment indicates an increased amount of death of the liver cells as compared to DEN administered rats. ApoBrdU positive cells indicates apoptosis. We observed increased BrdU positive cells in Nano Cur treated group as compared to DEN administered control group indicating DNA fragmentation leading to apoptosis. Since Cur preferably induces apoptosis in highly proliferating cells, [44,45] death is much more pronounced in tumor cells than normal ones. (Fig. 3b, b1 and b2) Hence it can be attributed that this higher amount of Brdu positive cells indicates apoptosis of the initiated cancer cells on Nano Cur treatment. Nano Cur follows the identical mechanism that cur follows for cancer cell killing. Since cytochrome C release in the cytosol in earlier studies has been proved to be associated with the intrinsic pathway [14], possibly in our experiment the intrinsic pathway is involved in the induction of apoptosis by Nano Cur. Cytochrome C release in the cytosol from the mitochondrial inner membrane on curcumin treatment converts procaspase 9 to caspase-9, with activation of caspase-3, ultimately leading the liver cells towards apoptosis [14]. In our studies release of cytochrome C in the cytosol from the mitochondrial inner membrane indicates that Nano Cur might have followed the same mechanism of apoptosis as reported in earlier studies with curcumin alone.

Curcumin at low doses has been shown to possess antioxidant property with chemopreventive effect in the development of cancers of the skin, soft palate, stomach, duodenum, colon, liver, lung and breast of rodents [17]. Apoptosis inducing property was shown by curcumin in HL-60 (human acute myelogenous leukemia) cell lines at doses of 25 μM of curcumin in a time dependent manner by Anto et al. [46]. In our study we found the antioxidant effect of curcumin in Nano Cur treated animals (Table 2 and 3; Fig. 2a). Besides acting as an antioxidant it might have exerted its antiapoptotic effect on the transformed malignant cells that proliferate at high rates [44,45]. The apoptosis inducing property of Nano Cur (Figs. 2b, 3b) might have been due to the inhibition of NF kappa β, a feature commonly seen in the antiproliferative and apoptosis-inducing effects of curcumin. This NF kappa β inhibition

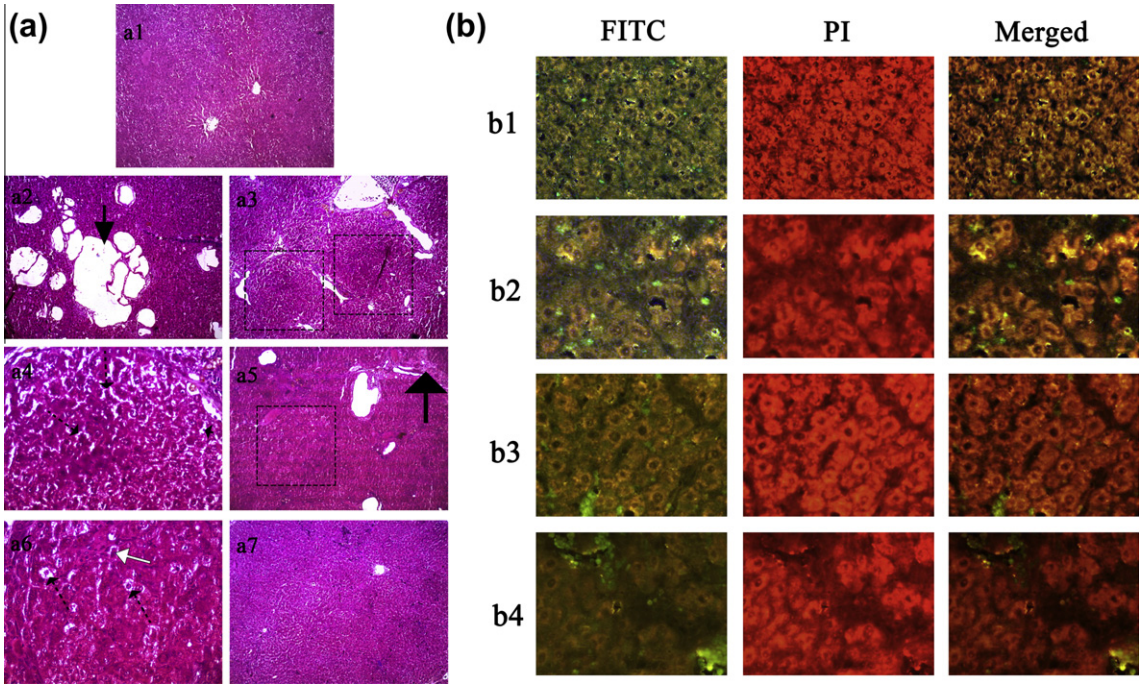

Fig. 3. Histological and histochemical examination of liver sections of experimental rats. (a) Eosin–haematoxylin stained liver sections [a1] Olive oil treated Control (10×) showing normal liver architecture, [a2] DEN treated control (10×) showing (↓) dialated hepatic veins [a3] DEN treated control (10×), Inset showing hyperplastic nodules, [a4] DEN treated control (40×), showing (⇣) fatty changes, [a5] DEN + Free Cur treated (10×) showing hyperplastic nodules (inset) with marked periportal fibrosis (↓), [a6] DEN + Free Cur treated (40×) showing (⇩) atypical nuclei and fatty changes (⇣), [a7] DEN + Nano Cur treated (10×), showing architecture similar to normal liver. (b) Representative photomicrographs (20×) of BrdU positive cells observed by double staining with BrdU (under FITC filter) and PI staining of liver of normal (b1), DEN treated (b2), DEN + Free Cur treated (b3) and DEN + Nano Cur treated (b4) rats.

might have led the cancerous cells towards apoptosis and cytochrome C release into the cytosol.

Hepatocarcinoma in rat was also examined by histopathological examination. A single dose of DEN administration (200 mg/kg b.wt, i.p. injection) initiates hepatocarcinogenesis, promoted by phenobarbital in drinking water (orally in drinking water for 14 weeks and animals were sacrificed at the end of 15/16 weeks starting from the day of DEN administration) has been reported earlier by Sreepriya et al., Bishayee et al. [47,2]. DEN administration (3 doses i.p) at 15 days interval has also been reported earlier by Mandal et al. [9]. Development of hyperplastic nodules is a characteristic feature of DEN induced hepatocellular carcinoma shown earlier by Bishayee et al., Das et al., Mandal et al. [2,40,7]. Also the presence of atypical nuclei is another marker of hepatocellular carcinoma shown by Sreepriya et al. [47]. Our studies have shown distorted histopathological changes in the liver with formation of hyperplastic nodules (Fig. 3a3) and atypical nuclei (Fig. 3a6) on DEN administration which are indications of DEN induced hepatocarcinogenesis. Remarkable pathological improvement was noticed in rats treated with Nano Cur whereas the same dose of free Cur did not show any encouraging results (Fig. 3a).

It appears that when any essential component of a signal transduction pathway of a cell is rendered hyperactive or autonomous, it may acquire the ability to drive the cell into unchecked proliferation leading to tumor promotion. Cur attenuates or suppresses the hyperactivity of these components of signal transduction and maintains simultaneously the normal cell function [15,11]. Our results show that in animals these biodegradable Cur nanocapsules are more bioavailable than free curcumin without any toxic effects at the dose employed herein. Due to their greater bioavailability these Cur nanocapsules have proved to be much more effective than free Cur at much lower dose than the effective dose of free Cur in combating DEN induced hepatocarcinoma in rats.

5. Conclusions

Curcumin in nanocapsulated drug delivery system has proved to be an effective free radical quencher, could protect the rat liver from DEN induced altered hepatic functioning, prevented DEN induced hyperplastic nodule formation, checked any upregulation of iNOS expression which in a way prevented angiogenesis in tumor sites, as well as promoted apoptosis of the initiated cancer cells as evidenced by DNA fragmentation, Cyt C release. Nano Cur has shown its effectivity against DEN induced hepatocellular carcinoma in rat. Though further studies on quantitating bioavailability remains to be done Nano Cur in oral route might be a promising anticancer alternative to prevent HCC.

Conflict of interest statement

The authors declare that they have no conflict of interest.

Acknowledgements

This work has been supported financially by the Council of Scientific and Industrial Research (CSIR), Government of India, CSIR-SRF Project and Network project (IAP 0001), IICB Kolkata. The authors acknowledge Dr. Anjan Kumar Das, Associate Professor, Department of Pathology, Calcutta National Medical College and Hospital, Kolkata for histopathological interpretation, Mr. T.Muruganandan, Technical Officer for AFM operation and Mr. Satyabrata Samaddar for FTIR operation.

References

[1] P. Chintana, Role of Curcumin on tumor angiogenesis in hepatocellular carcinoma, Naresuan. Univ J. 16 (3) (2008) 239–254.

[2] A. Bishayee, N. Dhir, Resveratrol-mediated chemoprevention of diethyhlnitrosamine-initiated hepatocarcinogenesis: inhibition of cell proliferation and induction of apoptosis, Chem. Biol. Interact. 179 (2009) 131–144.
[3] V. Sivaramkrishnan, P.N.M. Shilpa, V.R.P. Kumar, S.N. Devaraj, Attenuation of *N*-nitrosodiethylamine-induced hepatocellular carcinogenesis by a novel flavonol-Morin, Chem. Biol. Interact. 171 (2008) 79–88.
[4] J.E. Klaunig, L.M. Kmendulis, B.A. Hocevar, Oxidative stress and oxidative damage in carcinogenesis, Toxicol. Path. 38 (2010) 96–109.
[5] G. Waris, H. Ahsan, Reactive oxygen species: role in the development of cancer and various chronic conditions, J. Carcinogenesis 5 (14) (2006).
[6] C. Gupta, A. Vikram, D.N. Tripathi, P. Ramarao, G.B. Jena, Antioxidant and antimutagenic effect of quercetin against DEN induced hepatotoxicity in rat, Phytother. Res. 24 (1) (2010) 119–128.
[7] W. Hai, C. Kim, S. Song, C. Kang, Study on mechanism of multistep hepatotumorigenesis in rat; development of hepatotumorigenesis, J. Vet. Sci. 2 (2001) 53–58.
[8] F. Feo, P.M. Pascale, M.M. Simile, M.R. De Miglio, M.R. Muroni, D. Calvisi, Genetic alterations in liver carcinogenesis; implications for new preventive and therapeutic strategies, Crit. Rev. Oncog. 11 (2000) 19–62.
[9] A.K. Mandal, S. Das, M. Mitra, R.N. Chakrabarti, M. Chatterjee, N. Das, Vesicular flavonoid in combating diethylnitrosamine induced hepatocarcinoma in rat model, J. Exp. Ther. Oncol. 7 (2008) 123–133.
[10] M.C. Jagadeesh, M. Sreepriya, G. Bali, D. Manjulakumari, Biochemical studies on the effect of curcumin and embelin during N-nitrosodiethylamine/Phenobarbital induced-hepatocarcinogenesis in wistar rats, African J. Biotechnol. 8 (18) (2009) 4618–4622.
[11] S.C. Gupta, J.H. Kim, R. Kanappan, S. Reuter, P.M. Dougherty, B.B. Aggarwal, Role of nuclear factor- kβ-mediated inflammatory pathways in cancer-related symptoms and their regulation by nutritional agents, Exp. Biol. Med. 236 (2011) 658–671.
[12] M.A. Indap, M.S. Barkume, Efficacies of plant polyphenolics on sodium butyrate induced anti-tumoractivity, Indian J. Exp. Biol. 41 (2003) 861–864.
[13] A. Altunbas, S.J. Lee, S.A. Rajasekaran, J.P. Schneider, D.J. Pochan, Encapsulation of curcumin in self-assembling peptide hydrogels as injectable drug delivery vehicles, Biomaterials 32 (2011) 5906–5914.
[14] B.B. Aggarwal, A. Kumar, A.C. Bharti, Anticancer potential of curcumin: Preclinical and clinical studies, Anticancer Res. 23 (2003) 363–398.
[15] S.C. Gupta, J.H. Kim, S. Prasad, B.B. Aggarwal, Regulation of survival, proliferation, invasion, angiogenesis, and metastasis of tumor cells through modulation of inflammatory pathways by nutraceuticals, Cancer Metastasis Rev. 29 (2010) 405–434.
[16] M.H. Pan, W.L. Chang, S.Y. Lin Shiau, C.T. Ho, J.K. Lin, Induction of apoptosis by garcinol and curcumin through cytochrome C release and activation of caspases in human leukemia HL-60 cells, J. Agric. Food. Chem. 49 (2001) 1464–1474.
[17] G. Sa, T. Das, S. Banerjee, J. Chakraborty, Curcumin: from exotic spice to modern anticancer drug, Al. Ameen. J. Med. Sci. 3 (1) (2010) 21–37.
[18] D. Ghosh, S. Ghosh, S. Sarkar, A. Ghosh, N. Das, K.D. Saha, A.K. Mandal, Quercetin in vesicular delivery systems: Evaluation in combating arsenic-induced acute liver toxicity associated gene expression in rat model, Chem. Biol. Interact. 186 (2010) 61–71.
[19] R.A. Sharma, S.A. Euden, S.L. Platton, D.N. Cooke, A. Shafayat, H.R. Hewitt, T.H. Marczylo, B. Morgan, D. Hemingway, S.M. Plummer, M. Pirmohamed, A.J. Gescher, W.P. Steward, Phase I clinical trial of oral curcum: biomarkers of systematic activity and compliance, Clin. Cancer Res. 10 (2004) 6847–6854.
[20] P. Anand, H.B. Nair, B. Sung, A.B. Kunnumakkara, V.R. Yadav, R.R. Tekamal, B.B. Aggarwal, Design of curcumin-loaded PLGA nanopaticles formulation with enhanced cellular uptake and increased bioactivity in vitro and superior bioavailability in vivo, Biochem. Pharmacol. 79 (2010) 330–338.
[21] T. Lammers, W.E. Hennink, G. Storm, Tumor targeted nanomedicines: principles and practice, Br. J. Cancer 99 (2008) 392–397.
[22] J. Shaikh, D.D. Ankhola, V. Beniwal, D. Singh, M.N.V. Ravi Kumar, Nanoparticle encapsulation improves oral bioavailability of curcumin by at least 9-fold when compared to curcumin administered with piperine as absorption enhancer, Eur. J. Pharm. Sci. 37 (2009) 223–230.
[23] L. Li, S.F. Braiteh, R. Kurzrock, Liposome encapsulated curcumin. In vitro and in vivo effects on proliferation, apoptosis, signaling and angiogenesis, Cancer 104 (6) (2005) 1322–1331.
[24] S. Bisht, G. Feldmann, S. Soni, R. Ravi, C. Karikkar, A. Maitra, Polymeric nanoparticles-encapsulated curcumin (nanocurcumin): a novel strategy for human cancer therapy, J. Nanobiotechnol. 5 (3) (2007) 1–18.
[25] S. Hariharan, V. Bhardwaj, I. Bala, J. Sitterberg, U. Bakowsky, M.N.V. Ravi Kumar, Design of Estradiol loaded PLGA Nanoparticulate formulations: a potent oral delivery system for Hormone therapy, Pharm. Res. 23 (1) (2006) 184–195.
[26] S. Lala, S. Gupta, N.P. Sahu, D. Mandal, N.B. Mondal, S.P. Moulik, M.K. Basu, Critical evaluation of the therapeutic potential of basic acid incorporated in oil-in-water microemulsions Poly-D, L-lactide nanoparticles against experimental leishmaniasis, J. Drug Target. 14 (2006) 171–179.
[27] M. Dubin, P.H. Carrizo, A.M. Biscardi, S.H. Fernandez Villamil, A.O.M. Stopanni, Effect of 5-nitroindole on adenylate energy charge, oxidative phosphorylation and lipid peroxidaion in rat hepatocytes, Biochem. Pharmacol. 48 (1994) 1483–1492.
[28] N.V. deWitte, A.O.M. Stopanni, M. Dubin, 2-Phenyl-β-lapachone can affect mitochondrial function by redox cycling mediated oxidation, Arch. Biochem. Biophys. 432 (2004) 129–135.
[29] C. Betainder, E. Fontaine, C. Keriel, X.M. Leuerve, Determination of mitochondrial oxygen species: methodological aspects, J. Cell Mol. Med. 6 (2002) 175–187.
[30] A.K. Mandal, N. Das, Sugar coated liposomal flavonoid: a unique formulation in combating carbontetrachloride induced hepatic oxidative damage, J. Drug Target. 13 (2005) 1305–1315.
[31] A.K. Mandal, J. Sinha, S. Mandal, S. Mukhopadhyay, N. Das, Targeting of liposomal flavonoid to liver in combating hepatocellular oxidative damage, Drug Delivery 9 (2002) 181–185.
[32] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.G. Randel, Protein measurement with Folin Phenol reagent, J. Biol. Chem. 193 (1951) 265–277.
[33] S. Markland, G. Markland, Involvement of superoxide anion radical in the autooxidation of pyrogallol and a convenient assay of Superoxide Dismutase, Eur. J. Biochem. 47 (1974) 469–474.
[34] A.C. Moragon, G.N. De Lucas, F.M. Encarnicon Lopez, A.S. Rodriguez-Manzaneque, F.J.A. Jimenez, Antioxidant enzymes occupational stress and burnout in works of a prehospitalary emergency service, Eur. J. Emerg. Med. 12 (2005) 11–115.
[35] J.C. Davila, P.J. Davis, D. Acosta, Changes in glutathione and cellular energy as potential mechanisms of Papaverine-induced hepatotoxicity in vitro, Toxicol. Appl. Pharmacol. 108 (1) (1991) 28–36.
[36] S.N. Kim, K.C. Park, H.J. Kim, K.H. Cho, J.H. Chung, K.H. Kim, H.C. Eun, J.S. Lee, K.D. Park, Effects of collagen IV and laminin on reconstruction of human oral mucosa, J. Biomed. Mater. Res. 58 (2001) 108–112.
[37] N.P. Praetorius, T.K. Mandal, Engineered nanoparticles in cancer therapy, Recent Patents on Drug Delivery Formulation. 1 (2007) 37–51.
[38] M.A. Trush, T.W. Kensler, An overview of the relationship between oxidative stress and chemical carcinogenesis, Free Radic. Biol. Med. 10 (1991) 201–209.
[39] C. Rice-Evans, R. Burdon, Free radical–lipid interactions and their pathological consequences, Prog. Lipid Res. 32 (1993) 71–110.
[40] T. Das, F. Patra, B. Mukherjee, Effect of antisense oligomer in controlling c-raf.1 overexpression during diethylnitrosamine-induced hepatocarcinogenesis in rat, Cancer Chemother. Pharmacol. 65 (2010) 309–318.
[41] M.A. Rahman, D.K. Dhar, E. Yamaguchi, et al., Coexpression of Inducible Nitric Oxide Synthase and COX-2 in Hepatocellular carcinoma and surrounding liver: Possible involvement of COX-2 in the angiogenesis of Hepatitis C Virus-positive cases, Clin. Cancer Res. 7 (5) (2001) 1325–1332.
[42] M.L. Majano, C. Garcia-Monzon, M. Lopez-Cabrera, E. Lara-Pezzi, E. Fernandez-Ruiz, Inducible nitric oxide synthase expression in chronic viral hepatitis, evidence for a virus induced gene upregulation, J. Clin. Invest. 101 (1998) 1343–1352.
[43] S.A. Glynn, B.J. Boersma, T.H. Dorsey, M. Yi, H.G. Yfantis, L.A. Ridnour, D.N. Martin, C.H. Switzer, R.S. Hudson, D.A. Wink, D.H. Lee, R.M. Stephens, S. Ambs, Increased NOS 2 predicts poor survival in estrogen receptor-negative breast cancer patients, J. Clin. Invest. 120 (11) (2010) 3843–3854.
[44] G. Sa, T. Das, Anti cancer effects of curcumin: cycle of life and death, Cell Division 3 (14) (2008) 1–14.
[45] T. Choudhuri, S. Pal, T. Das, G. Sa, Curcumin selectively induces apoptosis in deregulated cyclin D1-expressed cells at G_2 phase of cell cycle in a p-53 dependent manner, J. Biol. Chem. 280 (2005) 20059–20068.
[46] R.J. Anto, A. Mukhopadhyay, K. Denning, B.B. Aggarwal, Curcumin(diferuloylmethane) induces apoptosis through activation of caspase-8, BID cleavage and cytochrome C release: its suppression by ectopic expression of Bcl-2 and Bcl-xl, Carcinogenesis 23 (1) (2002) 143–150.
[47] M. Sreepriya, G. Bali, Effects of administration of embelin and curcumin on lipid peroxidation, hepatic glutathione antioxidant defence and hematopoietic system during *N*-nitrosodiethylamine/phenobarbital-induced hepatocarcinogenesis in wistar rats, Mol. Cell. Biochem. 284 (2006) 49–55.