CHAPTER-8

Mitochondria protection with ginkgolide B loaded polymeric nanocapsules prevent diethylnitrosamine induced hepatocarcinoma in rats
INTRODUCTION

Hepatocellular carcinoma (HCC) is a deadly disease contributing one third of cancer-related death worldwide. (El-Serag et al. 2007) Past efforts have brought very limited success in treating HCC patients chemotherapeutically. Currently, liver transplantation is the most effective way to prolong the life of HCC patients. (Dutkowski et al, 2010) With the present drugs being mostly palliative in function target based new drugs are generated and investigated but with inadequate clinical efficacy. This necessitates the need for a way out to confer protection against development of a lethal disease like liver cancer. Research to find alternative “leads” for cancer prevention and therapeutics focuses on natural sources. However few of these compounds have come out of clinical trials successfully facing vital challenges of systemic toxicity and poor bioavailability.

Ginkgo biloba extract prepared from leaves of the living fossil tree Ginkgo biloba is used in traditional Chinese and in occidental medicine. It is one of the most commonly prescribed medications in Germany and France and one of the most widely used herbal dietary supplements in the United States. (Shi-hai et al, 2007; Biggs et al. 2010) Ginkgolide B (GB), a diterpene and the major unique component isolated from Ginkgo biloba extract is reported to inhibit competitively the binding of platelet-activating factor to its specific receptors in various tissues. (Lenoir et al, 2005) Recent studies revealed that GB showed antineoplastic effect in a breast cancer cell line through its antioxidant, anti-angiogenic and gene-regulatory actions. (Wen-Hsiung, 2007) Though Ginkgo biloba extract was found to significantly suppress proliferation and increase cytotoxicity in hepatoma cells in vitro, (Chao et al, 2004) beneficial effect of GB against development of HCC in vivo is quite an uninvestigated avenue.

Nanocapsules offer as interesting alternative form of drug delivery tool attributable to the feasibility of incorporating hydrophobic as well as hydrophilic molecules, long shelf life and better uptake cellular uptake (Ghosh et al. 2011; Cartiera et al, 2009) The biodegradable polymer PLGA (D. L-lactide-co-glycolide) is the most frequently used
biomaterial owing to their excellent biocompatibility and is already commercialized for a variety of drug delivery systems like blends, films, matrices, microspheres, nanoparticles, pellets, etc. (Blanco et al, 2009; Stevanoviae et al, 2007) Nanoparticles of this polymer have shown to significantly enhance efficacy of many anti cancer compounds like adriamycin, paclitaxel etc. (Davaran et al, 2006; Dong and Feng, 2005)

Since no systematic work on the effect of GB on liver carcinoma development in animals has been studied, it was thought worthwhile to investigate the efficacy of GB and its nanocapsulated forms in combating diethylnitrosamine (DEN) induced carcinogenesis in rat liver. DEN is the most intensively studied model of chemically induced liver cancer development both in rats and mice. DEN treatment has portrayed important molecular and cellular pathways involved in HCC development (eg, Stat-3P; IL6) and can be used to identify new targets in order to block chemically induced liver carcinogenesis in humans (Grivennikov et al, 2010; Naugler et al, 2007). Encouraging results of nanocapsulated GB on DEN induced HCC prompted us to study the possible anticarcinogenic mechanism of this compound. Hence we investigated its effect on some of the key players in the development of hepatocellular carcinoma like reactive oxygen species production, mitochondrial dysfunction, p53, inflammation and angiogenesis in liver.

MATERIALS AND METHODS

Chemicals

N-Nitrosodiethylamine (DEN), bovine serum albumin (BSA), Poly(lactide-co-glycolide) (PLGA) (Resomer RG 85:50H), Ginkgolide B, 2,6-dichloroindophenol (DCIP), Phenazine methosulfate (PMS), Succinic acid and Didodecyldimethylammoniumbromide (DMAB) were purchased from Sigma Aldrich (St. Louis, MO, USA) Ethyl acetate (AR Grade), HPLC grade Chloroform and Methanol were purchased from E. Merck.. All other reagents were of analytical grade.

Nanocapsulated GB preparation

A modified emulsion-diffusion-evaporation method was used to make ginkgolide B nanocapsules. GB was dissolved in ethyl acetate. The organic solution of PLGA and drug in ethyl acetate was emulsified with 5 ml of an aqueous phase containing DMAB. The resulting o/w emulsion was stirred at room temperature for 3 h before homogenizing at 15,000 rpm for 5 min. The organic solvent was removed by constant stirring on a water
bath set at 40°C. The suspension was ultracentrifuged at 105,000 g for 1 h. (Ghosh et al., 2011). The pellet of nanocapsules was washed with PBS twice and resuspended in 2 ml PBS. PEG coated nanocapsules were prepared in the same way except that PEG (4000) was taken with PLGA and drug in ethyl acetate. PEG coated nanocapsules will be referred to as N1GB and that without PEG as N2GB henceforward.

**Characterization of nanocapsules**

To estimate the intercalated drug in nanocapsules, the pellet was then dissolved in 2 ml of ethyl acetate and kept for 3 days at 4°C. The O.D. was measured at λ max (GB) 220 nm and % of incorporation was calculated against a standard plot of GB. Percent encapsulation was calculated. The size and shape were measured by atomic force microscopy (AFM) (Ghosh et al., 2010). Zeta potential and polydispersity index (PDI) were measured using a zetasizer (Nano ZS, Malvern Instruments). The *in-vitro* release study was done following the method of Win et al., 2005.

**Animals and experimental design**

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 and 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 the Animal Ethics Committee, India and experimental procedures were carried out 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 six groups with five animals in each group. Rats in the normal group (Group A) were kept as untreated control and injected (i.p.) with three doses of olive oil (0.5 ml) at an interval of 15 days. All other rats 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, D, E and F were treated orally with GB (100 mg/Kg bd. wt of ginkgolide B suspended in neutral oil), GB (5 mg/Kg bd. wt of ginkgolide B suspended in neutral oil), N1GB containing 5mg / Kg b.wt of ginkgolide B and N2GB containing 5mg / Kg b.wt of ginkgolide B respectively once in a week 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. At the end of 18 weeks, final body weight
was measured and blood was collected from heart from each group animals. Serum aspartate transaminase (AST), serum alkaline phosphatase (AP), serum alanine transaminases (ALT) were determined using a standard kit manufactured by Coral Clinical Systems; India. 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. 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. (Mandal et al, 2008).

**Histological examination of liver of experimental animals**

Postfixed liver portions of experimental animals were embedded in paraffin, and 5 μm sections were cut on a microtome. The sections were stained with Hematoxylin-Eosin (HE) using standard staining protocol and examined under a light microscope.

**Liver mitochondria and submitochondrial particles preparations**

Liver mitochondria were isolated from the rat tissue by differential centrifugation following the method of Navarro and Boveris. (Navarro and Boveris, 2004)

**Mitochondrial ROS measurement**

Intracellular ROS level was measured in liver mitochondria (Ghosh et al, 2011). 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%.

**Lipid peroxidation, mitochondrial membrane fluidity, reduced glutathione (GSH) succinate dehydrogenase, NADH oxidase and mitochondrial membrane potential assays**

Lipid peroxidation in the mitochondrial membrane was determined by measuring the amount of conjugated diene. 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. GSH level of a part of tissue homogenate was determined by the method mentioned in Ghosh et al, 2011 with the help of a spectrophotometer and using tetrachloroacetic acid with EDTA as protein precipitating reagent. Succinate dehydrogenase and NADH oxidase activity in liver mitochondria were assayed using the

**In situ DNA fragmentation assay**

Formalin fixed, paraffin embedded glass slide mounted liver and brain sections of experimental rats were stained with Anti-BrdU; FITC antibody using the Apo-BrdU in situ DNA fragmentation assay kit (Biovision K401-60) as per the manufacturer’s protocol.

**Immunofluorescence**

Deparaffinized, rehydrated liver sections were subjected to standard immunostaining techniques followed by incubation with primary antibody (NOS 2 and VEGF, Santa Cruz Biotechnology, USA) solution and then incubation with texas red and fluorescein isothiocynate-conjugated secondary antibody (Santa Cruz Biotechnology, USA) solutions separately. The images were observed under fluorescence microscope. (Chakraborty et al, 2012)

**p53, p21, NFκβ and cox2 immunoblotting**

Liver tissues were subjected to standard SDS/PAGE procedures, followed by electrophoretic transfer to Polyvinylidene Fluoride (PVDF) membrane (Millipore) at 15 V for 20 min by using Semi dry (Bio-Rad) transblot apparatus, blocked with BSA in PBS followed by incubation with the primary p53, p21, cox2, NFκβ antibodies separately for 3 h and then in secondary alkaline phosphatase conjugated anti rabbit goat IgG antibody (1:1000) for 1 h 30 min. Bands were visualized by the development of colour using Sigma premixed BCIP/NBT substrate solution. Their pixel density was analysed with Image J software system.

**Quantitation of ginkgolide B in liver**

Liver tissues were homogenized in 5 volumes of 25mM HEPES. Homogenates from experimental rats were deproteinized with 1% phosphoric acid, vortexed and centrifuged. The supernatant was mixed with 3 volumes of ethyl acetate, vortexed and centrifuged at 1000 rpm for 5 min. The ethyl acetate layer was removed and dried under nitrogen atmosphere. The residue was dissolved in 120 μl of methanol, 50 μl was injected onto a HPLC C18 Symmetry column. (Waters Corporation, USA). The mobile phase consisted of methanol and water (60:40, v/v) at a flow rate of 0.3ml/ min. (Tang et al, 2006)
Statistical analysis
The mean and standard deviation were calculated for all data. Significant differences between means were evaluated by an analysis of variances. A difference was considered significant when \( p<0.05 \).

RESULTS

Physicochemical characterization of nanocapsules
The physicochemical characteristics like particle size, zeta potential, PDI and drug encapsulation efficiency of N1GB and N2GB are summarized in Fig 1. The images revealed that formulated nanocapsules have regular spherical shape with narrow size distribution and smooth surface. No apparent aggregation was detected, PEG coating increased particle size. Zeta potential values indicated stable suspensions of both N1GB and N2GB. In vitro drug release exhibited a biphasic pattern characterized by a fast initial burst during the first 24 h (Fig 1), followed by a slow, sustained release for both type of particles. N1GB showed a faster release than N2GB. After 5 days, the cumulative drug release was 38% and 31% respectively.

Ginkgolide B level in liver following N1GB and N2GB feeding to rats exposed to DEN
Table 1 indicates the amount of GB level in rat liver at the end of the experimental regimen i.e. after 18 weeks from the commencement of the study. GB was detected by HPLC only in nanocapsules treated groups and not in the “free” drug treated groups. A significantly higher amount of GB was found to be present in DEN + N2GB treated rat liver than in DEN + N1GB treated rat liver.

Table 1: Amount of ginkgolide B present in liver homogenates of experimental rats subjected to DEN induced hepatocellular carcinoma

<table>
<thead>
<tr>
<th>GB in liver homogenate (µg/gm tissue)</th>
<th>DEN + N1GB treated</th>
<th>DEN + N2GB treated</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>34.32 ± 3.16</td>
<td>83.4 ± 4.52*</td>
</tr>
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</table>

Results are expressed as mean ± S.E; \( n=5 \) animals. DEN + N1GB treated group was compared with DEN + N2GB treated group * indicate \( p<0.001 \).
Fig. 1. (A) Atomic Force Microscopic images of ginkgolide B nanocapsules, (B) Physicochemical characterization and (C) in vitro release profiles of the two nanocapsules prepared. Each point represents the mean obtained from triplicates of samples.

Body growth, liver weight, liver morphology and marker enzymes of liver

DEN (3 doses of i.p. 200 mg/kg at 15 days interval) treatment caused an increase in liver weight with a decrease in body growth. (Fig 2) The liver exhibited small grayish white nodules on the surface of cirrhotic liver. The marker enzymes of liver function were found significantly altered from their normal counterparts. Alpha-fetoprotein expression, a marker of HCC
development was found high in DEN exposed rats. (Table 2) Oral treatment of GB (5mg) was found completely ineffective; GB (100mg) could significantly inhibit DEN induced micronodule formation and hepatic function alteration. But maximum protection was achieved through N2GB oral treatment (p<0.001) against DEN induced HCC incidence.

**Table 2: Effect of nanocapsulated ginkgolide B on hepatic toxicity in DEN induced hepatocellular carcinoma**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal treated</th>
<th>DEN treated</th>
<th>DEN + GB (5 mg/kg) treated</th>
<th>DEN + GB (100 mg/kg) treated</th>
<th>DEN + N1GB treated</th>
<th>DEN + N2GB treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline Phosphatase (KA Units)</td>
<td>37.82 ± 4.15</td>
<td>79.52 ± 7.06b</td>
<td>73.11 ± 6.92NS</td>
<td>65.74 ± 5.34c</td>
<td>56.59 ± 5.12b</td>
<td>47.38 ± 4.63a</td>
</tr>
<tr>
<td>Serum Aspartate Transaminase (IU/L)</td>
<td>121.36 ± 9.02</td>
<td>238.59 ± 14.68a</td>
<td>215.42 ± 11.63NS</td>
<td>182.38 ± 9.55c</td>
<td>157.18 ± 7.13b</td>
<td>139.73 ± 6.85b</td>
</tr>
<tr>
<td>Serum Alanine Transaminase (IU/L)</td>
<td>32.54 ± 3.06</td>
<td>114.23 ± 5.19a</td>
<td>107.06 ± 4.58NS</td>
<td>95.82 ± 3.92c</td>
<td>54.93 ± 3.56b</td>
<td>41.76 ± 3.28b</td>
</tr>
<tr>
<td>a-Fetoprotein (ng/ml)</td>
<td>2.42 ± 0.25</td>
<td>8.67 ± 0.71a</td>
<td>8.43 ± 0.81NS</td>
<td>5.37 ± 0.78c</td>
<td>5.25 ± 0.46b</td>
<td>3.98 ± 0.27a</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± S.E; n=5 animals. DEN treated group was compared with normal and experimental groups were compared with DEN treated group. NS indicates non significant, * indicate P<0.001, b indicate P<0.01 and c indicate P<0.05

**Pathomorphology of liver sections**

HE stained liver sections of normal rat exhibited (Fig 3a) cords of normal hepatocytes, central veins, normal looking sinusoids lined by Kupffer cells. were normal and the histology was within normal limit. DEN exposed rats showed hyperplastic nodule (Fig 3b inset), cells having higher nuclear to cytoplasmic ratio with atypical nuclei, loss of architecture and large fluid filled spaces. cells not arranged in a single plane, (Fig 3 c) and formation of septa and large accumulation inflammatory infiltrates (Fig 3d) GB(100 mg) + DEN exposed rat liver sections showed no signs of HCC but prominent cirrhotic damage characterized by accumulation of fibrous tissue and septa formation (Fig 3e) N1GB + DEN treated rat liver sections showed interportal bridging fibrosis (Fig 3f) whereas N2GB + DEN treated rat liver sections show near normal portal tract with regular hepatic cellular arrangement. (Fig 3g)
Fig. 3. Liver sections of experimental animals (under 10X) after 18 weeks of 1st DEN administration. Rats received 3 i.p injections of DEN (200mg/kg) at 15 days interval. Experimental rats except normal and DEN treated control groups were orally administered with different doses and formulations of GB on a weekly basis from the day of 1st DEN administration upto 16 weeks. (a) showing liver section of normal rats having proper arrangement of hepatic cords, (b) showing liver sections of DEN exposed rats having hyperplastic nodule (inset) with cells having higher nuclear to cytoplasmic ratio and atypical nuclei, (c) DEN treated rat liver showing loss of architecture and large fluid filled spaces, cells not arranged in a single plane, (d) liver sections of DEN treated rats showing formation of septa and large accumulation inflammatory infiltrates indicated by (→). (e) GB (100 mg) + DEN exposed rat liver sections show accumulation of fibrous tissue and septa formation indicated by (→→). (f) N1GB + DEN treated rat liver sections show interportal bridging fibrosis (g) N2GB + DEN treated rat liver sections show near normal portal tract with regular hepatic cellular arrangement.

**Lipid peroxidation and antioxidant status in liver**

Conjugated diene generated as a result of lipid peroxidation caused by DEN induced oxidative stress was studied. DEN exposed control animals showed a significant increase (p<0.001) in diene level in liver than normal animals. GB (100mg) could decrease diene level to 3.18μmol/mg protein (p<0.05) and N1GB could arrest the level to 3.53μmol/mg protein (p<0.01) in liver. However maximal protection was achieved through N2GB oral treatment post DEN exposure depicting antioxidative behavior (p<0.001) of the formulation. (Table 3) GSH the most important intracellular endogenous
antioxidant and GST the free radical detoxifying enzymes were studied. Rats exposed to DEN showed a marked increase in liver GSH level from 18.32 to 39.57 µg/mg tissue (Table 3). No significant protection was observed in the case of rats treated with free GB (5mg). However GB (100 mg) and N1GB treatment could retain the GSH levels as compared to DEN treated animals but maximal retention towards normal values at the higher level of significance (p<0.001) were showed in case of rats treated with N2GB. Similar pattern was seen in GST activities where N2GB most proficiently showed less GST activity than DEN induced control animals. (Table 3)

Table 3: Effect of nanocapsulated ginkgolide B on mitochondrial ROS, lipoxypheroxide content, reduced glutathione and GST in DEN induced hepatocellular carcinoma in rats

<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>Lipophyroxide content (mmol/mg protein)</th>
<th>Reduced glutathione (µg of GSH/mg tissue)</th>
<th>GST (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0.49 ± 0.03</td>
<td>18.32 ± 1.14</td>
<td>0.92 ± 0.07</td>
</tr>
<tr>
<td>DEN treated (A)</td>
<td>4.54 ± 0.48⁸</td>
<td>39.57 ± 1.32⁴</td>
<td>3.17 ± 0.27⁸</td>
</tr>
<tr>
<td>A + GB (5 mg/kg) treated</td>
<td>4.27 ± 0.53⁸</td>
<td>36.12 ± 1.27³</td>
<td>2.93 ± 0.21⁵</td>
</tr>
<tr>
<td>A + GB (100 mg/kg) treated</td>
<td>3.18 ± 0.37⁷</td>
<td>33.57 ± 1.48â</td>
<td>2.31 ± 0.17⁶</td>
</tr>
<tr>
<td>A + N1GB treated</td>
<td>2.53 ± 0.06⁵</td>
<td>31.23 ± 1.57³</td>
<td>1.78 ± 0.14⁵</td>
</tr>
<tr>
<td>A + N2GB treated</td>
<td>0.95 ± 0.03¹</td>
<td>25.41 ± 1.08³</td>
<td>1.18 ± 0.08⁴</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± S.E; n=5 animals. DEN treated group was compared with normal and experimental groups were compared with DEN treated group. NS indicates non significant. ⁸ indicate P<0.001, ⁷ indicate P<0.01 and ⁶ indicate P<0.05.

**ROS level in rat liver mitochondria, mitochondrial respiratory complex enzyme activities and mitochondrial membrane potential dissipation**

The fluorescence intensity produced by H₂DCFDA on oxidation to H₂DCF is proportional to the amount of ROS produced by the mitochondrion. DEN exposure of experimental animals caused a 2.5 fold increase in mitochondrial ROS generation with concomitant increased NADH oxidase activity and reduced SDH activity in rat liver. Rats treated with GB (100mg) and N1GB showed a significant decrease in mitochondrial ROS level and NADH oxidase activity and elevated the activity of SDH (p< 0.05 and p< 0.01)
N2GB offered maximum benefit (p<0.001) against DEN induced ROS generation in mitochondria and respiratory complex enzyme dysfunction. (Table 4)

Table 4: Effect of nanocapsulated ginkgolide B on ROS generation, SDH and NADH oxidase activity in rat liver mitochondria by the induction of DEN induced hepatocellular carcinoma

<table>
<thead>
<tr>
<th>Groups</th>
<th>DCF fluorescence % of normal</th>
<th>NADH Oxidase level (nmole of oxidised NADH/min/mg protein)</th>
<th>SDH activity (μl DCIP reduced/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>100 ± 6.5</td>
<td>5.89 ± 0.18</td>
<td>1.52 ± 0.09</td>
</tr>
<tr>
<td>DEN Treated (A)</td>
<td>247 ± 15.8(^a)</td>
<td>10.77 ± 0.38(^a)</td>
<td>0.42 ± 0.04(^a)</td>
</tr>
<tr>
<td>A + GB (5 mg/kg) treated</td>
<td>238 ± 6.2(^\text{NS})</td>
<td>10.69 ± 0.41(^\text{NS})</td>
<td>0.40 ± 0.02(^\text{NS})</td>
</tr>
<tr>
<td>A + GB (100 mg/kg) treated</td>
<td>192 ± 11.5(^c)</td>
<td>8.83 ± 0.54(^c)</td>
<td>0.68 ± 0.08(^c)</td>
</tr>
<tr>
<td>A + N1GB treated</td>
<td>164 ± 12.7(^b)</td>
<td>7.98 ± 0.48(^b)</td>
<td>0.73 ± 0.07(^b)</td>
</tr>
<tr>
<td>A + N2GB treated</td>
<td>127 ± 10.6(^a)</td>
<td>6.27 ± 0.39(^a)</td>
<td>0.93 ± 0.06(^a)</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± S.E; n=5 animals. DEN treated group was compared with normal and experimental groups were compared with DEN treated group. NS indicates non significant, \(^a\) indicate P<0.001, \(^b\) indicate P<0.01 and \(^c\) indicate P<0.05.

DEN exposure showed a decrease in mitochondrial membrane potential from 1.93 to 0.67 in using succinate as substrate at the end of 18 weeks from the commencement of study. (Fig 4) Considerable repression in membrane dissipation (p < 0.05) was achieved through oral treatment with GB (100mg) and N1GB in liver mitochondria. On the other hand animals treated orally with N2GB along with DEN administration showed a significant increase in mitochondrial membrane potential (p < 0.001).

Fig.4: Liver cell membrane microviscosity and membrane potential of mitochondria isolated from experimental rat liver after 18 weeks of 1st DEN administration. DEN treated control groups were compared with normal animals and drug treated groups were compared with DEN control group. Values are mean ± SEM for 5 rats. \(^a\) indicates p<0.001, \(^b\) indicates p<0.01 and \(^c\) indicates p<0.05.
Fig. 5: (A) Western Blot analysis of p53 and p21 protein expression in cytosolic fraction of experimental rat liver. Histogram showing representative pixel intensities (arbitrary units of densitometric analysis using Image J software) of the immunoblot performed with different individual rats. (B) Representative photomicrographs of BrdU positive cells observed by double staining with BrdU (under FITC filter) and PI staining of liver sections. ai, a2, a3 are liver sections from normal rats (10X), b1, b2, b3 are liver sections from DEN treated control rats (10X), c1, c2, c3 are sections from DEN + GB(100) treated rats (10X), d1, d2, d3 are sections from DEN + N1GB treated animals (10X) and e1, e2, e3 are liver sections from DEN + N2GB treated rats (20X).

P53, p21 and apoptosis in liver

P53 expression in liver cytosol of DEN exposed rats was significantly lower than normal. (Fig 5 A) Very strong p53 expression was observed in DEN exposed rats treated with GB (100mg). Basal level of p53 expression was observed in N2GB treated animals. P53 induced p21 expression was observed in DEN + GB (100mg) treated rats. Liver sections when studied for apoptosis and DNA fragmentation showed no more than normal fluorescence in DEN exposed control rats. BrdU fluorescence indicates DNA fragmentation and apoptosis. Maximum number of fluorescent cells was observed in DEN + GB (100mg) treated rats indicating induction of apoptosis in the initiated cancer cells. However an improved histology along with lesser number of fluorescent cells was observed in N2GB treated rats. (Fig 5 B)
Chapter-8  Ginkgolide B nanocapsules against hepatocarcinoma

Inflammation and iNOS, COX2 and NFκβ expression in liver

iNOS, COX2 and NFκβ are some of the key players involved in inflammation. Since hepatocarcinogenesis is inherently associated with inflamed liver, we studied the expression of these proteins. Immunofluorescent staining of liver sections with anti iNOS and Texas red conjugated anti rabbit IgG antibodies showed normal rat liver with minimal red fluorescence (Fig 6a) while strong intense fluorescent signal was recorded in DEN treated control rats (Fig6b). Considerable fluorescence was observed in DEN + GB (100 mg) treated rats (Fig 6c) with a gradual decrease in fluorescent signal in DEN + N1GB treated rats (Fig 6d) while a near normal level of fluorescence was observed in DEN + N2GB treated rats (Fig 6e). Western Blot analysis of COX2 expression in cytosolic fraction of experimental rat liver showed a significant increased expression in DEN exposed rats. A gradual decrease in expression was observed with different formulations of GB with N2GB offering maximum reduction in expression. NF-κβ (p65) protein expression was studied in the cytosolic fraction of liver. A sharp disappearance of NF-κβ protein from cytosol was observed in hepatocarcinogenic condition induced by DEN exposure. DEN + N2GB treated animals show almost normal NF-κβ expression.

VEGF expression and neangiogenesis in rat liver

VEGF expression is a marker of neangiogenic signal. Effect of GB nanocapsules on hepatic VEGF expression due to DEN induced hepatocarcinogenesis in rats was studied. VEGF immunofluorescent localization was visualized in liver sections from animals sacrificed after 18 weeks of the commencement of the study. Green fluorescence indicates VEGF expression. Normal rats showed no fluorescence (Fig 7a) while strong intense fluorescent signal was recorded in DEN treated control rats (Fig 7b). A significantly lower level of fluorescence was observed in DEN + N1GB treated rats (Fig 7c) and a near normal minimal level of fluorescence in DEN + N2GB treated rats (Fig 7d).
Fig. 6: (A) Effect of GB nanocapsules on DEN induced expression of inflammation modulators in hepatic cells of rats sacrificed. (A) Representative photomicrographs of rat liver sections showing iNOS localization visualized by immunofluorescent staining of sections with anti iNOS and Texas red conjugated anti-rabbit IgG antibodies using standard procedures. Red fluorescence indicates iNOS activity. Normal rats show minimal red fluorescence (a) while dark intense fluorescent signal recorded in DEN treated control rats (b), considerable fluorescence observed in DEN + GB(100) treated rats (c) with a gradual decrease in fluorescent signal in DEN + N1GB treated rats (d) and a near normal level of fluorescence seen in DEN + N2GB treated rats (e). Lower panel micrographs are phase contrast images of their corresponding fluorescence images. (B): Western Blot analysis of COX2 and NF-κβ (p65) protein expression in cytosolic fraction of experimental rat liver. Histogram showing representative pixel intensities (arbitrary units of densitometric analysis using Image J software) of the immunoblot performed with different individual rats.

Fig. 7: Effect of GB nanocapsules on hepatic VEGF expression due to DEN induced hepatocarcinogenesis in rats. Representative photomicrographs of rat liver sections following sacrifice after 18 weeks of the commencement of the study. VEGF immunofluorescent localization visualized by tagging sections with anti VEGF and FITC conjugated IgG antibodies using standard procedures. Green fluorescence indicates VEGF expression. Normal rats show no fluorescence (a) strong intense fluorescent signal recorded in DEN treated control rats (b), significantly lower level of fluorescence in DEN + N1GB treated rats (c) and a near normal minimal level of fluorescence seen in DEN + N2GB treated rats (d). a, b, c, and d micrographs are phase contrast images of their corresponding fluorescence images.
DISCUSSION

In this study we formulate GB in two different nanocapsules and demonstrate that a concurrent administration of hepatocarcinogen DEN along with oral feeding of GB loaded polymeric nanocapsules can prevent development of hepatocarcinogenesis in rats. N2GB offers a significantly higher level of GB payload to liver and is most effective in preventing DEN induced carcinogenesis. In relation to cancer development, GB poses to be a useful chemoprevention tool but the challenge remains to fix the effective dose and proper time of administration.

Polymeric nanocapsules equipped with sustained drug releasing ability offer a unique nontoxic, biodegradable, non-immunogenic solution of delivering drugs, peptide, aptamers and other bioactive molecules in biological systems. PLGA, a widely preferred polymer was used to nanoencapsulate GB and was further modified with PEG coating.

The size and size distribution of nanoparticles are important in determining their stability for drug release and cellular uptake efficiency. (Storm et al, 1995) Small size of the particles have the property of getting opsonized and cleared out of systemic circulation by the reticulo-endothelial system. PEG functionalization has been a major approach to modify nanocarriers, reduces the chances of getting opsonized and subsequent macrophage uptake thereby reducing phagocytosis (Storm et al, 1995). PEG coating imparted a negative zeta potential to the particles. (Fig 1) The positive potential residing on N2GB surface was a result of the positive cationic surfactant DMAB used in the formulation. The in vitro drug release patterns indicate PEG coating obviously enhancing drug release than the uncoated PLGA nanoparticle. (Fig. 1) HPLC data confirmed that the nanocapsulated formulations were able to maintain the drug in its active form for a quite extended period as compared to the free doses. (Table 1) However higher hepatic GB level in N2GB fed animals indicate a slower and extended release of the drug at the organ. Bigger size of the particles along with initial burst of drug as observed in in vitro release study caused a faster release of drug from N1GB as compared to only DMAB stabilized uncoated particles i.e. N2GB. A faster release from N1GB might have been responsible for a rapid clearance of the drug from the organ.

Carcinogenesis is a long-term multi-step process involving a spectrum of changes to occur in the biological system. We have found DEN administration leading to the
development of hyperplastic nodules in rat liver with a concomitant increase in serum ALT, AST and AFP level. (Table 2) N2GB was able to arrest hepatocellular carcinoma incidence reflected from body growth, liver weight and micronodule formation. (Fig. 2) There might be several mechanisms by which GB could exert its anticarcinogenic potential. Since DEN metabolism produce toxic free radicals, a major factor involved in all steps of carcinogenesis, ie, initiation, promotion and progression (Karbownik et al, 2001) the first step would have been to quench ROS. High DCH$_2$FDA fluorescence generated from mitochondria of DEN administered rats were put out by N1GB and N2GB. DEN induced elevated ROS production disrupts cellular redox balance and mitochondrial output. (Table 4) Our findings have shown a significantly higher (p<0.001) conjugated diene level in liver mitochondria of DEN exposed rats. (Table 3) DEN induced decrease in membrane microviscosity of hepatic cells (Fig 4) might also be attributed to the accumulation of lipids and protein oxidative damage due to cellular ROS buildup as well as ROS generated from defective mitochondria created during the carcinogenetic process. Lipid peroxidation products may play an important role in mediating the decreased membrane microviscosity causing hyperpermeable membrane thus allowing serum ALT and AST that originally resides within the cytoplasm to be released in to the blood stream. (Table 2) Lower ROS level in N2GB treated animals explain the lesser amount of conjugated diene in cell membrane as well as higher membrane microviscosity. GST as well as GSH content and other glutathione metabolizing enzymes is overexpressed in actively proliferating cells. (Huang et al, 2001) Therefore the elevated level of GSH and GST might be due to an overexpression of these antioxidants as a consequence of enhanced proliferation and rapid sequestration of antioxidants by tumor cells. (Table 3) Thus lowering of GSH and GST in N1GB and N2GB treated animals reflect a state of lower ROS and lesser dividing cells in the liver giving a positive indication in the reduction of oxidative burden generated in liver due to DEN administration. Warburg, 1956 in his pioneering work mentioned about the “injury” created in mitochondrial respiratory machinery as a result of carcinogenesis with a subsequent “compensatory” glycolytic ATP production. An increased activity of NADH oxidase suggests a higher ROS production. The impaired electron transport system and oxidative phosphorylation in mitochondria is reflected from the decreased SDH activity (Table 4) causing a lower proton accumulation and a dissipation of mitochondrial membrane potential. (Fig 4) The administration of N1GB and N2GB could inhibit DEN induced mitochondrial dysfunction.
that drives hepatic cells towards carcinogenic damage with N2GB offering highest protection.

Role of mitochondria in tumor suppression have been recently elucidated (Gottlieb and Tomlinson, 2005; Cuezva et al, 2009). In tumors associated with mutations in complex II subunits, accumulation of succinate is suggested to be the underlying cause of carcinogenesis because it encourage hypoxic acclimatization (Zanssen and Schon, 2005; Selak et al, 2005) Mitochondrial electron transport chain complexes are encoded by two genetic systems- mitochondrial DNA (mt DNA) and the nuclear DNA (nDNA). While the complex II is entirely nDNA encoded, complex I is encoded by both types of DNA. Thus decreased SDH activity observed in DEN exposed rats give an indication of any DNA damage occurring in the nuclear genome due to the application of the carcinogen. Impaired p53 signaling is the most common genetic modification found in cancer and it plays a decisive role in apoptosis (Joerger and Fersht, 2008). p53 also engaged in controlling cellular metabolism regulates glycolysis and oxidative metabolism in divergent directions (Matoba et al, 2006; Bensaad et al, 2006; Vousden and Ryan, 2009), and is therefore considered as one of the molecular moderators steering cells from oxidative metabolism toward glycolysis. In the present study down-regulated p53 expression together with lower SDH activity in DEN exposed rats reflects an impairment of p53 expression and function due to mitochondrial dysfunction. Nanocapsulated delivery of GB could successfully safeguard hepatic cells from such a fate (Fig 5) Thus by protecting the mitochondria from dysfunctioning N2GB might have prevented p53 inactivation and inhibit the process of carcinogenesis. A quite unexpected strong p53 expression in GB (100 mg/kg) treated cells indicate P53 accumulation due to the stress generated by DEN but not its inactivation as found in DEN treated control animals. DNA damage make p53 protein levels to increase and induce the synthesis of p21, an inhibitor of cyclin-dependent kinases, leading to arrest of the cell cycle. In our study DEN exposed group show low levels of p21 and a high level in GB (100 mg/kg) treated group. P53 inactivation in DEN exposed animals might have caused p21 not to be expressed in response to stress but oral administration of GB (100 mg/kg) actually stimulated the DEN induced increase in p53 and p21 in rats. Upstream regulation of ROS and mitochondrial dysfunction might have prevented DNA damage and subsequent up regulation of p53-p21 signaling in N2GB treated animals. Liver sections of DEN exposed rats showed GB was able to induce apoptosis in hyperplastic nodules containing cancerous cells in DEN exposed animals. (Fig 5) This is consistent with our
p53 results. The lesser number of apoptotic cells in N2GB treated animals indicate lesser number of initiated cancer cells in that group, a clear reflection of the anticarcinogenic activity of GB.

Histological evidences indicate HCC usually develops in the backdrop of chronic hepatitis or cirrhosis, conditions that result in hepatocyte death and activation of resident liver macrophages and newly recruited inflammatory cells. (Demaria et al, 2010) NF-kB is the central mediator of inflammation (Aggarwal, 2004) regulating other inflammatory molecules either upstream or downstream. Increased expressions of COX-2 and iNOS have been observed in several human HCC and in chemically induced animal tumors. (Surh et al, 2001; Wu, 2006) Our study corroborates previous findings showing a strong COX-2 and iNOS expression in DEN administered animals. (Fig 6) Involvement of platelet-activating-factor (PAF) in the tumor microenvironment has been implicated in numerous pathophysiologic processes. (Han et al, 2002) PAF effects are mediated by a G-protein linked transmembrane receptor (Patel et al, 1992) and binding on its receptor leads to tumor induced angiogenesis and metastasis. In addition, PAF can induce the expression of several angiogenic factors such as TNFa, IL-1β, FGF, VEGF etc in endothelial cells through the transcription of NF-kB. (Izumi and Shimizu, 1995) N2GB was most successful in controlling DEN induced COX 2, iNOS and VEGF expression. (Fig 6, 7) GB, the PAF antagonist might have played the most crucial role in bringing down the DEN induced inflammatory and neoangiogenic signals.

Usually cancer cells via the enhanced permeability and retention effect have the ability to concentrate drug 20 times more when delivered through nanoparticulated system. (Verdun et al, 1990) Polymeric nanocapsules with a positive or neutral charge enter most of the cell lines studied, while that with a negative charge internalizes mostly in the cancer cell lines. Moreover, upon entry into the cells, nanoparticles are localized to different subcellular parts (e.g., cytoplasm and lysosomes) depending on the nanoparticle’s surface charge. (Mous and Bharali, 2011; Asati et al, 2010) The positive surface charge possessed by N2GB might have been the key factor controlling hepatic cellular internalization of nanocapsules followed by their rapid mitochondrial accumulation. A higher mitochondrial accumulation might have led to a buildup of GB concentration in mitochondria, capable of protecting the organelle from dysfunctioning that subsequently leads towards hepatocellular carcinoma development.
Nanocapsulation of GB in biodegradable polymer PLGA stabilized with cationic surfactant DMAB was found most effective in preventing DEN induced HCC in rat liver. Dysfunctioning mitochondria arising out of DEN administration appears to be the key factor in controlling redox status, inflammation, cell proliferation and neoangiogenesis in liver. Efficient “cargo unloading” at hepatic mitochondria by N2GB could prevent mitochondrial dysfunction associated inflammation and neoangiogenesis. Protecting the mitochondria thus plays the most decisive role in inhibiting the DEN induced hepatocarcinogenesis process. Human beings are constantly exposed to various carcinogens and hence are threatened with the chances of developing cancer during their lifetime. This pre-clinical study of using a nontoxic compound of natural origin in polymeric nanocapsules stabilized with cationic surfactant provides its prospect in clinical use against development of a deadly killer disease like liver cancer.