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Carcinogenesis response modulation induced by gelonin encapsulated in liposome

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Abstract The effectiveness of gelonin to arrest protein synthesis, thereby limiting the growth of cancer cells was studied by encapsulating it into liposomes. The protein was extracted from the seeds of Indian plant *Gelonium multiflorum* by ammonium sulfate precipitation and purified using cation-exchange and gel-filtration chromatography. Biological activity of purified gelonin was determined using a rabbit reticulocyte lysate assay in the cell-free translational experiments. Gelonin was encapsulated in conventional liposomes prepared by the dry film method in order to retain biological activity of the entrapped protein. Carcinogenesis was induced in Swiss albino mice by intravenous administration of DBN (10 mg kg^{-1} body weight) at weekly intervals. Marker enzyme assays (GGT, AChE, and GST), GSH levels, cell proliferation assay, hepatocyte DNA analysis, histological examination of micro sections of liver tissues were parameters used to monitor carcinogenesis induction, and regression in mice. From the in vitro experiments conducted, it was observed that gelonin upon its encapsulation into liposome, resulted in significant destruction of the transformed liver cells by its cytotoxic effects that arrest protein synthesis. Various parameters studied to monitor regression also suggested mass cell destruction to liver upon administration of liposomal gelonin in mice exposed to DBN.

Keywords Cancer immunotherapy · Nitrosamine · Carcinogenesis · Ribosome inactivating protein · Gelonin · Luffin · Reticuloendothelial system · Liposome encapsulation · Cancer regression · Cytotoxic activity

Abbreviations

DBN	*N*,*N* nitrosodibutylamine
GGT	γ-Glutamyl transpeptidase
AchE	Acetylcholine esterase
GSH	Glutathione (reduced)
GST	Glutathione-*S*-transferase
RIP	Ribosome-inactivating protein
ED	Effective dose
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
RES	Reticulo-endothelial system
FCS	Fetal Calf Serum
DMEM	Dulbecco's Modified Eagle Medium
DPPC	Dipalmitoylphosphatidylcholine
Chol	Cholesterol
PC	Phosphatidylcholine
PE	Phosphatidyl ethanolamine
PS	Phosphatidyl serine
PG	Phosphatidyl glycerol
PEG	Polyethylene glycol
BrdU	Bromodeoxyuridine

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Introduction

Gelonin, a type-1 ribosome-inactivating protein (RIP) from the seeds of *Gelonium multiflorum* is an effective therapeutic cytotoxic agent. Gelonin and other related plant

proteins, such as abrin, luffin, trichosanthin, or pokeweed antiviral protein (PAP) are classified as ribotoxins to refer to the fact that they are cytotoxic by inactivation of ribosomes [1–4]. These proteins are RNA *N*-glycosidases that depurinate the major rRNA (28 S), thus, damaging the ribosome and eventually arrest protein synthesis [5–10]. Type-1 RIPs are innocuous to the cells in the free form but can inactivate ribosomes once they enter the cell by a receptor mediated process. This characteristic of type-1 RIPs that subject it to rapid degradation within endosomes upon cellular uptake, has generated a need for the development of a specialized cytosolic delivery strategy to enable their use as an effective anti-tumor therapeutic agent. Accordingly, numerous strategies including conjugation to folate, hormones or monoclonal antibodies, and entrapment in liposomes or polymers have been employed to effectively deliver gelonin to the cytosol of cancer cells [11–17]. Liposomes are versatile carrier systems in terms of structural characteristics and mode of drug and protein incorporation. This creates a wide range of options for the design of effective liposomal drug formulations to induce tumoricidal effector mechanisms.

In the present communication, an attempt has been made to entrap gelonin in conventional liposomes prepared by the dry film method. The dry film method was used to preserve the immunological and biological activites of entrapped gelonin. Effectiveness of such liposomal formulation on cancer regression was investigated both in vitro and in vivo. A liposome-based delivery platform has the advantages of protecting the encapsulated protein from degradation in plasma. Furthermore, the high and rapid uptake of liposomes by the reticulo-endothelial system (RES) may rule out the incorporation of specific ligands that serve as receptors for targeting. Our results show an efficient delivery of entrapped gelonin to liver tissue, which is signified by its high uptake of liposomes, and the regression effects that were observed upon intravenous administration of liposome encapsulated gelonin in DBN exposed mice. In the present investigation our approach has been to make use of a conventional liposome in order to avoid any unwanted immune response against the liposomal formulation.

Materials and methods

The seeds of *Gelonium multiflorum* were procured from United Chemicals and Allied Products, Calcutta, India, 5-Bromo-2′-deoxy-uridine Labeling and Detection Kit III and C^{14} labelled cholesterol from Roche Applied Science, Manheim and Amersham International Ltd. respectively, and *N*, *N*′-dibutyl nitrosoamine (DBN), γ-Glutamyl-*p*-nitroanilide, Glycylglycine, Acetylcholine chloride, 5, 5′ –dithiobis-(2-nitrobenzoic acid) (DTNB), Glutathione(GSH), Trypsin, Fetal Calf Serum (FCS), Dulbecco's Modified Eagle Medium (DMEM), Trypan blue, L-glutamine, L-arginine, L-asparagine, Dipalmitoylphosphatidylcholine (DPPC), Diacetyl-phosphate, Cholesterol (Chol), Phosphatidylcholine (PC), Phosphatidyl ethanolamine (PE), Phosphatidyl serine (PS), Phosphatidyl glycerol (PG) and Penicillin/Streptomycin Sigma Chemicals Co., USA. Other chemicals used were from indigenous sources.

Isolation and characterization of gelonin

Gelonin extraction and Molecular weight and homogeneity determination

Gelonin was isolated from the dry seeds of *Gelonium multiflorum* by using the method originally described [18] with minor modification [14, 19]. Homogeneity and molecular weight of gelonin was determined by SDS-PAGE analysis (5–15% w/v acrylamide gradient) [20]. The gel was stained with Coomassie blue. A molecular weight reference standard from Bio-Rad provided MW marker proteins.

In vitro cell-free translation assay

The inhibitory activity of purified gelonin on cell-free protein synthesis was determined by measuring incorporation of [^{3}H]-leucine into the TCA insoluble protein fraction using a nuclease treated rabbit reticulocyte lysate as described earlier [16]. The calculated percentage inhibition of protein synthesis was expressed as: $ED_{50} = (B/B_0) \times 100$, where B is the radioactivity incorporated in the TCA precipitate in the presence of inhibitor and B_0 in absence of any inhibitor [19]. ED_{50} is the dose of inhibitor required for 50% inhibition of protein synthesis.

Gelonin entrapment into liposome

Different lipid compositions were used in the preparation of liposomes. DPPC:Cholestrol:DCP and DPPC:Cholestrol:PG were combined at a molar ratio of 1.0:0.9:0.25, whereas DPPC:Cholestrol:PE and DPPC:Cholestrol:PS were combined at a molar ratio of 0.9:0.25:0.5 respectively. Liposomes were made by the dry film method as described by Kirby and Gregoriadis [21]. The difference between free and total gelonin gave the percent entrapment efficiency into liposome. The pellet was suspended in tris-buffered saline and used for in vitro and in vivo regression studies. Some of the culture media were supplemented with PEG in order to enhance liposome fusion with liver cells.

In vivo liposome uptake by RES

Liposomes were prepared as described above using ^{3}H-cholesterol in place of cholesterol. Liposome suspension (100 µl/mouse) was administered intravenously and animals sacrificed after 30 min. Liver, kidneys and spleen were quickly excised, rinsed, weighed, homogenized (50%) in tris-buffered saline and centrifuged at 18,000*g* for 30 min. The supernatant and pellet were counted for radioactivity in scintillation cocktail and total radioactivity was calculated.

Cancer induction

Healthy mice, 5–6-weeks-old were administered an aqueous solution of DBN (10 mg kg^{-1} body weight) intravenously at weekly intervals for 12 weeks. After the exposure period, DBN-treated mice were sacrificed to monitor the induction of tumor using parameters such as marker enzymes (GGT, AChE, and GST), GSH level, histology and electrophoretic banding of genomic DNA. Sham-treated, and age-matched mice served as controls.

Sample for enzyme assays

Perfused liver (0.5 gm) obtained from mice was rinsed with chilled normal saline. The tissue was homogenized in 0.25 M sucrose and centrifuged at 20,000*g* for 30 min at 4°C. The resulting supernatant was assayed for marker enzymes.

Marker enzyme assays

γ-Glutamyl transpeptidase assay

γ-Glutamyl transpeptidase (GGT) activity was assayed according to the method described by Meister et al. (22). In a cuvette, 0.2 ml of 5 mM L-γ-glutamyl-*p*-nitroanilide (pH, 8.0), 0.2 ml of 0.1 M glycylglycine (pH, 8.0), and 0.6 ml of 0.1 M tris–HCl buffer (pH 8.0) were combined. The reaction was initiated by adding 0.2 ml of tissue homogenate prepared above and mixed well. Absorbance at 410 nm monitored release during transpeptidation for 3 min.

Acetylcholine esterase assay

Acetylcholine esterase activity was assayed according to the method described by Ellman et al. [23]. Briefly, 1.0 ml of 0.2 M phosphate buffer saline (PBS), 0.5 ml of 6.0 mM acetylcholine chloride, 0.5 ml of 0.75 mM DTNB and 0.5 ml of 0.3% triton X-100 in PBS were taken in a cuvette and mixed well. Finally, 0.5 ml of the prepared supernatant was added. Absorbance was measured at 412 nm at 10 s intervals for 3 min.

Glutathione-S-transferase assay

Glutathione-S-transferase (GST) activity in the liver was determined by the method described by Habig and Jacoby [24]. To 1 ml cuvette were added 500 µl of sodium phosphate buffer saline (0.2 M), 50 µl of GSH (20 mM), 250 µl of deionized water and 50 µl of test sample and mixed thoroughly. The reaction was initiated by addition of 50 µl of CDNB (20 mM). Absorbance at 340 nm was recorded at 10 s intervals for 3 min.

Glutathione assay

Glutathione (GSH) level was determined by the method described by Ellman [25]. Briefly, 0.1 g of perfused liver tissue was homogenized in 1.0 ml 25 mM EDTA in normal saline (pH 7.5). An equal volume of 20% TCA was added to the homogenate and vortexed. Precipitated proteins were centrifuged at 11,000*g* for 30 min at 4°C. To the supernatant, 2.0 ml Na_2HPO_4 was added and vortexed. About 1.0 ml of saline EDTA, 1.0 ml of 0.4% DTNB (dissolved in 2% trisodium citrate solution), 0.5 ml of Na_2HPO_4 solution (0.3 M) and 0.5 ml of the above supernatant mixture were mixed thoroughly and allowed to stand for 3 min. The absorbance was read at 412 nm against a reagent blank. The GSH concentration was read off from a GSH standard curve.

Histological study

Microtomy technique as described by Ratcliffe, N. A. [26] was employed for histological examination of liver tissues. In brief, the excised liver was cut into pieces and fixed in the freshly prepared Bouin's fluid for 24 h. Excess fixative was washed overnight under running tap water. Fixed tissue was then dehydrated an increasing alcoholic grade of 30%, 50%, 70%, 90%, and 100% successively for 2 h each. Alcohol was then cleared by placing the tissue in xylene for about 5 h till it become translucent. Wax infiltration steps were carried out at 55°C initially in a 1:1 mixture of xylene and wax for 1 h followed by 100% molten wax for 1–2 h with two changes in 100% wax. This was allowed to cool. The embedded tissue was cut in ribbons 7 µm sections using a microtome and mounted on slides smeared with Haupt's adhesive, which was disinfected with 4% formalin. The sections were warmed. Slides were then left overnight for drying. Mounted slides were immersed in xylene to de-wax. Rehydration was done in decreasing grades of alcohol: 100%, 90%, and 70%.

The sections were then stained with haematoxylin for 10–15 min and the excess washed under running tap water. Slides were then dipped in 70% alcohol for few seconds and counterstained with eosin for 5–10 min. Excess stain was again washed off in running tap water. The stained sections were now successively treated with 70%, 90%, and 100% alcohol, and the alcohol was cleared in xylene. The sections were mounted in DPX and left to dry for two days after which they were examined.

Cell proliferation assays

Hepatocyte preparation

The method described by Fry [27] and Seglen [28] was used to isolate intact hepatocytes from collagenase-treated perfused liver. This method involved a two-step procedure, in which the mouse liver was perfused first with a Ca^{2+}-free buffer, then with collagenase and Ca^{2+}. Perfusion was done with the perfusion buffer (207.5 g NaCl, 12.5 g KCl, 60 g Hepes, and 6 g solid NaOH in 1,000 ml water, pH 7.4). This was replaced by collagenase buffer containing 1.25 g collagenase and 1.75 g $CaCl_2 \cdot 2H_2O$ in 200 ml water to which 250 ml solution containing 10 g NaCl, 1.25 g KCl, 60 g Hepes, and 6.6 g solid NaOH were added and the final volume made to 500 ml. The solution was filtered through a Millipore serum filter and pH adjusted to 7.6.

After perfusion the liver was cut into pieces and transferred to a wide petridish containing 80–100 ml of ice-cold suspension buffer (40 g NaCl, 4.0 g KCl, 1.5 g KH_2PO_4, 1 g Na_2SO_4, 72 g Hepes, 65 g tricine, and 21 g solid NaOH dissolved in 1,000 ml of water, pH 7.6). The liver was held by the portal connective tissue with forceps and the cells were carefully raked out. The suspension was incubated at 37°C for 30 min and centrifuged at 4°C for 3 min. in flat bottomed centrifugation tubes. The cells were gently suspended in ice-cold perfusion buffer. The yield of viable cells was checked by trypan blue exclusion method and was used for BrdU incorporation assay.

BrdU incorporation assay

A cellular immunoassay initially described by Gratzner was used to monitor cell proliferation and DNA synthesis with slight modification as has been reported elsewhere (29, 30). About 1×10^6 viable cells were used for the assay.

Cellular DNA analysis

DNA was extracted using a method originally described by Blin and Stafford [31] with slight modification. Briefly, 100 mg of liver tissue was chopped and suspended in ice-cold PBS. Tissue debris was removed by spinning at low speed (600 rpm) and cells were collected by further spinning at 1000*g* for 5 min. Digestion buffer (1.2 ml/0.1g tissue) was added to and incubated for 12–18 h at 50°C. DNA was then extracted by adding an equal volume of a solution consisting of Phenol/Chloroform/isoamyl-alcohol in the ratio 25:24:1 (v/v). The mixture was vortexed vigorously for 10 s and kept to settle for 2–3 h. The top aqueous layer was carefully collected in a test tube. To 1.0 ml of the extract, 0.5 ml of ammonium acetate and 2.0 ml of absolute ethanol were added and centrifuged at 1000*g* for 2 min. The pellet was rinsed thrice with 70% alcohol followed by centrifugation at 6000*g* for 2 min each and dried in an incubator at 37°C. DNA thus isolated was dissolved in TE buffer (10 mM Tris–HCl and 1 mM EDTA, pH 7.6). The sample was electrophoresed on 1% agarose gel. The gel was stained with ethidium bromide solution (0.3 g/ml H_2O). DNA intercalated ethidium bromide fluorescence was visualized on a UV transilluminator and photographed. Densitometric readings were calculated in triplicate and mean values were taken in the graph.

In vitro inhibition effect of free- and liposome-encapsulated gelonin

Hepatocytes ($\sim 1 \times 10^6$) each were incubated in the presence of free gelonin and liposome encapsulated gelonin in a culture medium (DMEM supplement with 10% FCS, L-arginine, L-glutamine, and L-aspartate) for 16 h at 37°C in a humidified atmosphere of 5% CO_2. The inhibition effect was monitored by determination of the number of viable cells as described above.

In vivo inhibition effect of free- and liposome-encapsulated gelonin

DBN-exposed mice were divided in two groups comprising 8–10 animals each. To the first group, liposome encapsulated gelonin (~ 100 μg equivalent) was administered thrice by intravenous route at weekly intervals. The second group did not receive liposome encapsulated gelonin. A third group of mice were sham treated and served as control. Samples were prepared for the enzyme assays (GGT, AChE, GST), GSH level, histology, cell viability, DNA synthetic index and electrophoretic study of cellular DNA using the same methods as described above.

Results

Gelonin purification and characterization

Molecular weight of gelonin obtained was shown by SDS-PAGE to be ~ 30 kDa (Fig. 1).

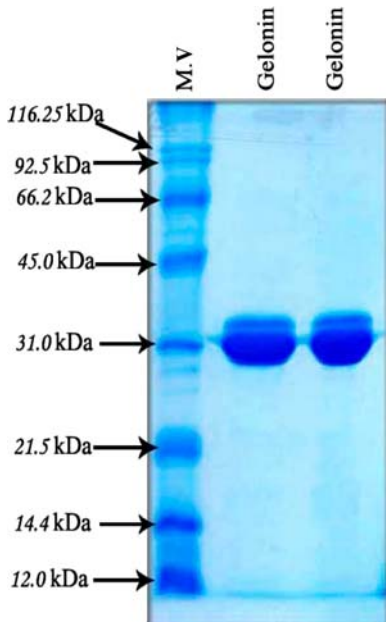

Fig. 1 Molecular weight determination on SDS-PAGE of protein after purification on Sephadex G-200 with molecular weight markers from Bio-Rad. Wells were loaded with 30 μg of protein each. The gel was fixed in methanol/acetic acid, stained with Coomassie blue and destained with methanol/acetic acid for 24 h

Cell-free translation assay

The results of cell-free translation experiments using purified gelonin and luffin (Fig. 2) show dose-dependent relationships between the percentage of protein synthesis and both toxins. The percentage ribosome-inactivating activity was determined after measuring ED_{50}. Gelonin ED_{50} was found to be twofold higher than that of luffin when calculated from the inhibition curve (Fig. 2).

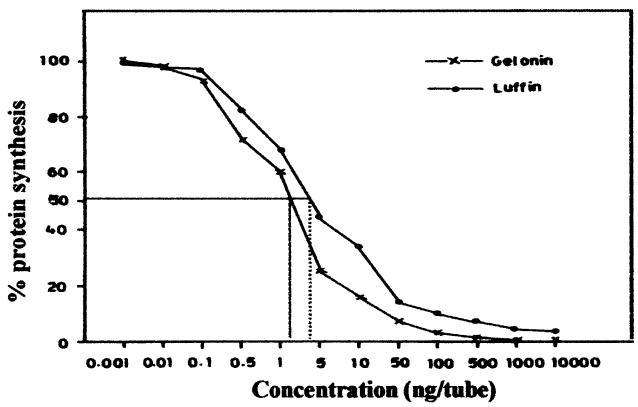

Fig. 2 Inhibition of protein synthesis in the cell-free translational system from rabbit reticulocyte lysate assay by gelonin (—x—) and luffin (—•—). Protein synthesis was measured by counting the ^{3}H-Leucine incorporated into TCA protein precipitate. ED_{50} of gelonin and luffin were calculated from the graph. ED_{50} of gelonin = 2.25 ng and ED_{50} of luffin = 4 ng

In vivo liposome uptake by RES

The percentage entrapment efficiency of gelonin into liposomes, determined by separating free gelonin from encapsulated gelonin by centrifugation, were found to be approximately 25% in all three preparations (data not shown). Liposome uptake by the RES upon intravenous administration was determined. Results shown in Fig. 3 indicate that liposome taken up by liver was very high as compared to other tissues like kidney and spleen. Liposomes made from DPPC, PG, ^{3}H Cholesterol and DPPC + PE + ^{3}H cholesterol exhibited more uptake by liver and kidney.

Liver histology

Liver tissues upon DBN exposure were examined and compared with that of age-matched normal control mice. Significant morphological changes were seen in the liver following DBN treatment. The liver was enlarged and in some mice gray-white nodules were visible (Fig. 4a, b insets). In most cases the tissue was swollen and hardened, whereas in untreated control mice no such signs were noticed. Nodules were not detected in livers of mice receiving liposome encapsulated gelonin. (Fig. 4c inset).

Liver tissue histology also showed distinct differences. Hepatocytes in normal mice had well-defined outlines with tightly packed mono- and bi-nucleated cells with a regular morphology (Fig. 4a). DBN–exposed mice lost their regular arrangement and showed variations in shape and size (Fig. 4b). No defined outlines were visible and cells seemed to have lost contact with their neighboring cells. It was also observed that most of the cells became multinucleated. DBN-exposed mice liver subsequently treated

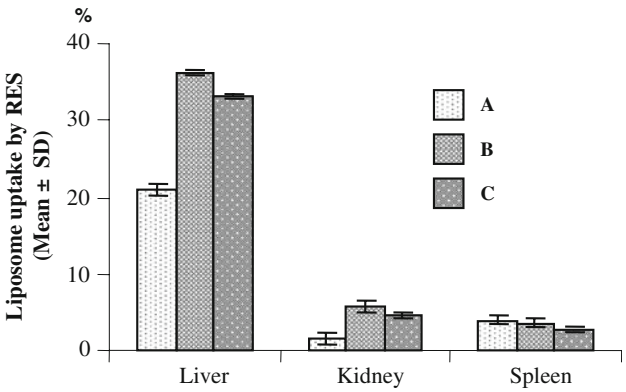

Fig. 3 Liposome uptake in vivo by the Reticulo Endothelial System (RES). Liposomes made from different lipids and labeled with ^{3}H Cholesterol were administered intravenously. Mice were sacrificed by cervical dislocation 30 min after administration. Radioactivity measured in liver, kidneys and spleen separately and the percent uptake for each tissue was determined. Liposome compositions: A = DPPC + DCP+ ^{3}H Cholesterol, B = DPPC + PG +^{3}H Cholesterol, and C = DPPC + PE + ^{3}H Cholesterol

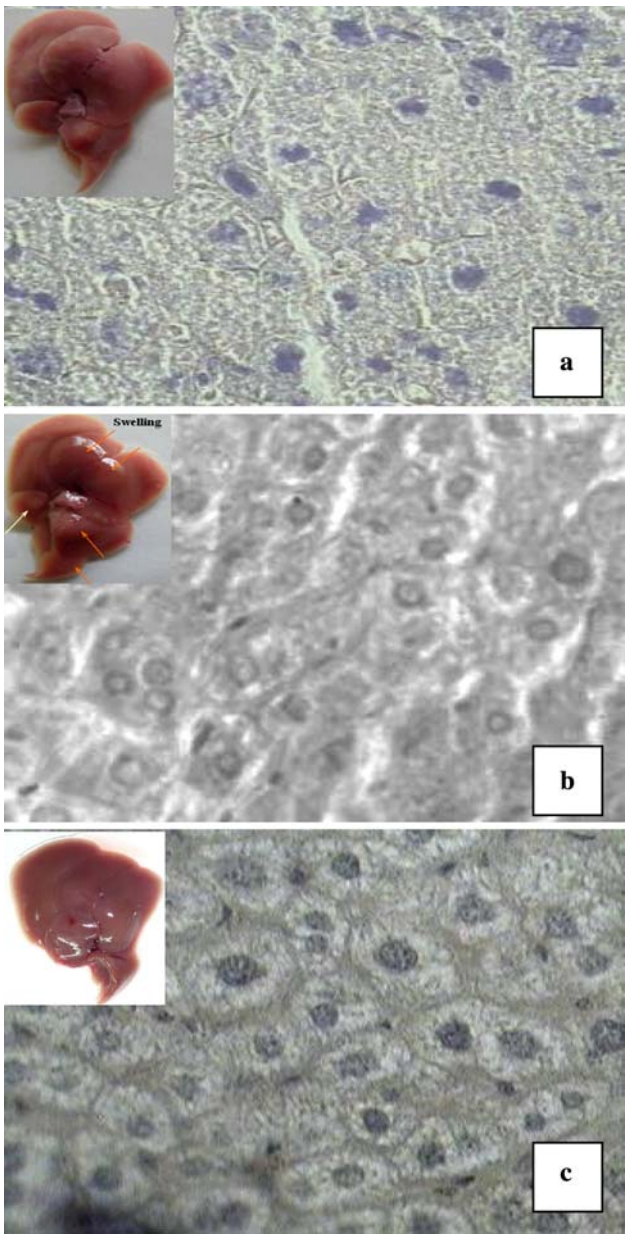

Fig. 4 Liver microphotographs of (**a**) normal control, (**b**) DBN-exposed and (**c**) liposome encapsulated gelonin treated DBN-exposed mice. The tissues were fixed in Bouin's solution by increasing the ethanol from 30 to 100% dehydration grade. The tissues were further cleared by using xylene and embedded in paraffin wax. Blocks prepared were trimmed and cut in ribbons and mounted on slides, dried and the paraffin wax was removed with xylene. The slides were stained by haematoxylin and eosin. Slides were examined microscopically after drying. Magnification × 40. Arrows indicate the nodule formation and swelling in the liver of DBN-exposed mouse

with liposome-encapsulated gelonin did not exhibit any noticeable changes in comparison to DBN-exposed animals (Fig. 4c). The only major difference possibly resulting from gelonin treatment was the disappearance of multinucleated cells and the chromatin appeared more condensed.

Marker enzymes activities

DBN-exposed mice showed marked alterations in their marker enzyme activities in comparison to normal control. GGT and AChE activities in the DBN-treated mice were found to be significantly higher as compared to that of the normal control group of animals (Fig. 5a-I and II, b-I and II). However, after one week of liposome-encapsulated gelonin treatment both enzyme activities decreased to normal levels (Fig. 6a, b-III). A marginal increase in enzyme activities was seen after two and four weeks of treatment (Fig. 5a-IV and V, b-IV and V)

Activity of GST in DBN-exposed mice was significantly reduced and was approximately 1.5-fold less than the normal control (Fig. 5c-I and II). Upon treatment with liposome-encapsulated gelonin the DBN-exposed mice showed a marked elevation (∼twofold) in GST activity, which sharply decreased after 2 and 4 weeks of treatment (Fig. 5c-III, IV and V).

DBN-exposed mice showed an elevated level of GSH in comparison to control (Fig. 5d-I and II). However, upon treatment with liposome encapsulated gelonin, the level significantly decreased and reached close to normal (Fig. 5d-III, IV and V).

In vitro inhibition effect of free- and liposome-encapsulated gelonin

DBN-exposed mouse liver cells ($\sim 1 \times 10^6$) were cultured and incubated separately with 100 mg free gelonin and liposome-encapsulated gelonin (equivalent to 100 mg). Liposomes consisting of four different lipid compositions as given in Table 1 were made. While no detectable cell death was observed with free gelonin, following encapsulation into liposomes 13–16% of cells were killed. In order to enhance the fusion of liposomes with liver cell some of the culture media were supplemented with PEG (shown by +sign) that causes a small increase in cell death.

DNA synthetic index

BrdU incorporation in liver cells of DBN-exposed and age-matched unexposed control mice was determined by in vitro experiments. Liver cells of DBN-exposed mice exhibited a marked increase (threefold) in BrdU incorporation in comparison to the normal control. However, one week after treatment with liposome-encapsulated gelonin these cells exhibited decreased BrdU incorporation (Fig. 6a).

Cellular DNA

Cellular DNA samples extracted from liver tissues of normal control, DBN-exposed, and liposome-encapsulated

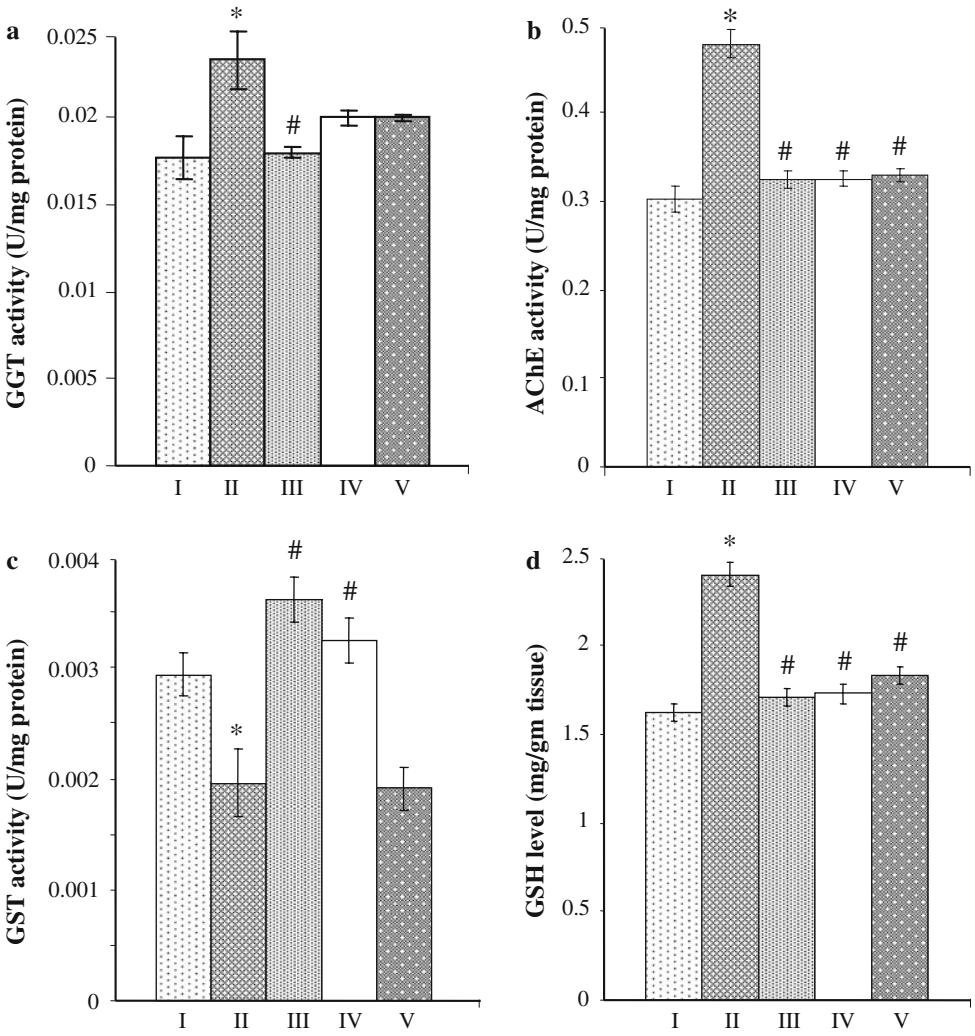

Fig. 5 Marker enzymes activities in mouse liver tissue. The marker enzyme activities were determined as described in Materials and Methods. (**a**) GGT activity, (**b**) AChE activity, (**c**) GST activity and (**d**) GSH level. In each diagram the bar-I corresponds to enzyme activity in normal control mice, bar-II corresponds to enzyme activity in DBN-exposed mice and bars- III to V correspond to enzyme activity in DBN-exposed mice upon treatment (1, 2 and 4 weeks respectively) with liposome encapsulated gelonin. The values in the bars are expressed as mean ± SEM. $n = 8$. *Statistically significant ($P < 0.05$) in comparison to control, #statistically significant ($P < 0.05$) in comparison to DBN-exposed.

gelonin treated DBN-exposed mice were run on a 1% agarose gel. DBN-exposed liver cell extract showed broad DNA band of high intensity in comparison to normal control (Fig. 6b-L1 and L2). However, upon treatment of these mice with liposome encapsulated gelonin the cellular DNA appeared to be sheared and more intensely stained as supported by densitometric analysis of the bands (Fig. 6c).

Discussion

Gelonin, a type-1 RIP was isolated and purified from the seeds of an Indian plant *Gelonium multiflorum.* Homogeneity and molecular weight of gelonin was determined by SDS-PAGE under reducing conditions along with the appropriate molecular weight markers (Fig. 1). The purified preparation of protein was homogeneous and possessed a molecular weight of ~30 kDa which was in agreement with the molecular weight of gelonin reported earlier [32].

Biological activity of purified gelonin was determined in a cell-free translational in vitro system of nuclease-treated rabbit reticulocyte lysate. In order to compare its relative biological activity another type-1 RIP i.e. luffin (obtained from the seeds of *Luffa cylindrica*) was run in parallel. The relationship between the percentage protein synthesis inhibition at different doses of gelonin and luffin was determined (Fig. 2). Gelonin ED_{50} was found to be 2.5 ng which was lower than luffin (ED_{50} = 4.0 ng). A twofold difference in ED_{50} of gelonin and luffin has earlier been reported where the former showed lower ED_{50} than the latter [32].

Before performing regression studies gelonin was encapsulated into liposomes and percentage entrapment efficiency was determined. The entrapment efficiency of gelonin into liposomes produced by the dry film method was found to be ~25% in all preparations. Low entrapment efficiency for proteins by dry film method is reported [33].

Tissue distribution of liposome was monitored by measuring its uptake in vivo by liver, spleen and kidney.

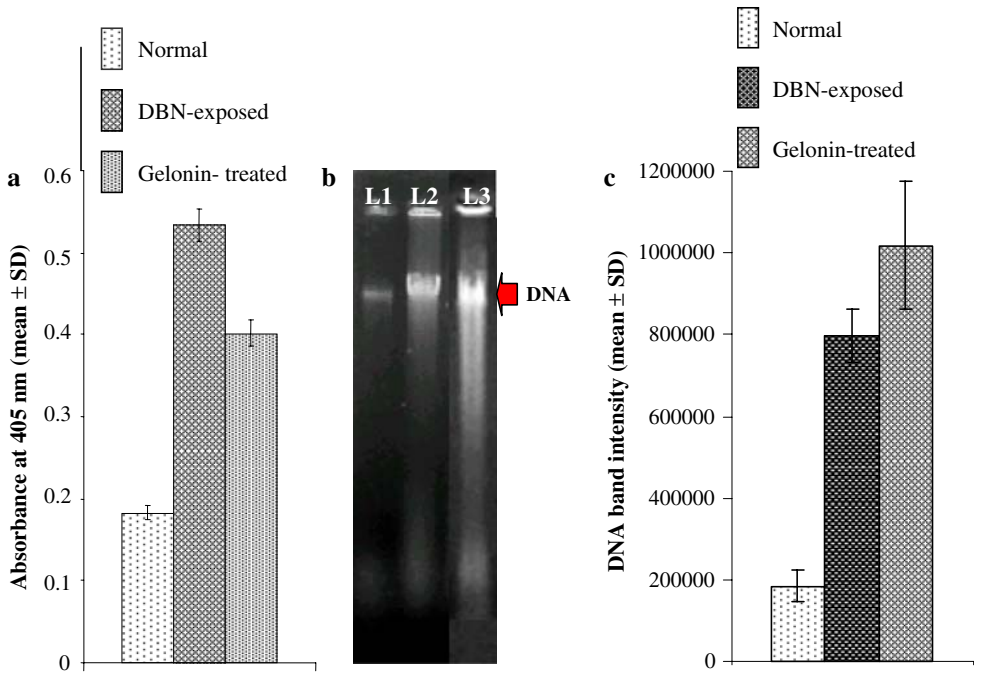

Fig. 6 a-*DNA synthesis*. BrdU incorporation into DNA of proliferating liver cells was measured by taking absorbance at 405 nm on a multiscan MS India ELISA reader. The measured absorbance was directly correlated to the level of BrdU incorporation into cellular DNA in normal control mice, DBN-exposed mice and DBN-exposed mice upon treatment with liposome-encapsulated gelonin for three weeks at weekly intervals. The values in the bar diagrams are expressed as mean ± SD. $n = 10$. b-*Liver cell DNA in mice*. L1 = normal control, L2 = DBN-exposed and L3 = DBN- exposed upon treatment with liposome-encapsulated gelonin. Crude extract was run on 1% agarose gel. The gel was stained with ethidium bromide. c-*Densitometric analysis*. Densitometric analysis of DNA extracted from liver of control, DBN-exposed and DBN-exposed gelonin-treated mice. Mean of triplicate values were plotted

The majority of the liposomes (∼25–40%) was captured from circulation by liver. However, liposome uptake by other tissues such as kidney and spleen was found very low (Fig. 3). High uptake of liposomes from circulation by liver is known [34]. It was further observed that liposomes made from DPPC, PG and H^3 cholesterol and DPPC, PE, H^3 cholesterol showed significantly higher uptake by liver in comparison to the liposome made from DPPC, DCP and H^3 cholesterol. This increase in uptake may be due to increase in stability of liposome in blood circulation.

Tumor induction in Swiss albino mice was achieved with weekly intravenous administration of DBN without promoter. Mice were sacrificed after DBN treatment and the liver was examined for any visible changes. Nodule formation, swelling, and enlargement of liver in DBN-exposed mice (Fig. 4) support induction of carcinogenesis. However, the available reports state that carcinogenesis in liver is induced only when the carcinogens are coupled with a promoter [35].

The abnormal production of enzymes, proteins and hormones is generally associated with the initiation of carcinogenesis. Thus, upon DBN exposure marker enzymes activities, and GSH level were monitored in liver tissue of mice. Treated mice exhibited significant elevation in GGT and AChE activities (Fig. 5a, b). However, GST activity decreased in mice upon DBN exposure (Fig. 5c). Such alterations have been shown earlier in mice exposed to DBN [29]. Further, marked elevation in GGT activity provides strong evidence of neoplastic transformation in hepatocytes [36–37]. The liver GSH level in mice upon DBN treatment was found significantly high in comparison to normal control (Fig. 5d). An increase in GSH immediately prior to nuclear division has also been reported [38].

Cancer is a state where the cells do not respond to normal growth controls and hence uncontrolled proliferation of cells occur in such condition. This was monitored by quantitating BrdU incorporation into the DNA of replicating hepatocytes. The assay results showed threefold higher BrdU incorporation in liver cells of DBN-exposed mice when compared with normal control (Fig. 6a). It clearly indicates an increase in DNA synthesis upon DBN treatment. This observation was further confirmed by analysis of cellular DNA. The DNA band corresponding to DBN-treated mice was much more intense in comparison to control. High DNA content thus signifies increased replication of DNA in the proliferating cell upon DBN treatment. Although, the mechanism by which cell proliferation exerts its unique effect in the initiation phase of carcinogenesis is not clearly understood, replication of DNA with carcinogen-induced lesion prior to repair offers

an alternative mechanism by which carcinogen-induced critical damage may be fixed into the newly made DNA and thus result in an initiated cell [39].

Histological examination of liver section from DBN-treated mouse exhibited several morphological changes (Fig. 4b). Among other changes discussed earlier, some cells were found to be multinucleated and the nuclei appeared more densely stained, which was likely due to more condensed chromatin. Our earlier study supports these observations [29]. The loss of regular cell shape upon DBN-treatment indicates changes in membrane structure and composition, which may be related to a change in function. These changes may also be related to the perturbation in enzyme activities, especially those of GGT and AChE and the level of GSH as these being cellular components and associated with membrane function directly or indirectly. The densely stained nuclei showed condensed chromatin normally observed in dividing cells. Thus, the morphological changes observed in the liver cells of DBN-exposed mice indicate that the cells were in a state of rapid division. The parameters, thus monitored to follow carcinogenesis induction revealed induction of hepatocellular carcinogenesis in mice when exposed to DBN.

After having established cellular transformation in mice upon chronic exposure of DBN, regression studies were carried out by in vitro and in vivo experiments. Through in vitro experiments we made an attempt to assess the relative efficacy of gelonin-containing liposome formulation against tumor growth. We treated liver cells of DBN-exposed mice with both the free- and liposome-encapsulated gelonin. The data indicated that liposome-encapsulated gelonin made access to its target subcellular compartment, the cytosol and showed its potential to inactivate ribosomes and arrest protein synthesis leading to cell death as observed in the cell viability assay (Table 1). By contrast, cells treated with equivalent concentration of free gelonin exhibited no detectable cytotoxicity. PEG addition to the culture medium did not cause any significant improvement in cytotoxic effect of liposome encapsulated gelonin (Table 1). PEG was used to enhance the membrane fusion. Significant cell killing by listeriolysin O-liposome-mediated delivery of gelonin has recently been shown through in vitro experiment [17]. Our approach has been to achieve the maximum cytotoxic effect of the protein toxin gelonin with conventional type of liposomal formulation permissible for human use. Thus a further detailed investigation with other potent membrane fusion agent(s) may be of significant importance.

The relative in vivo efficacy of liposome-encapsulated gelonin against hepatocarcinogenesis was determined in mice chronically exposed to DBN by direct intravenous administration. Parameters monitored to study the regression effect were same as those followed for cancer induction. The post gelonin treatment data of marker enzyme activities are shown in Fig. 5. Upon comparison with control as well as DBN-exposed animals, GGT and AChE activities significantly decreased and reached close to normal (Fig. 5a, b). A small increase in GGT activity was however noticed after 2 and 4 weeks of gelonin treatment. These levels are again, lower than that in untreated DBN-exposed mice. Initial lowering in activity may be attributed to the cytotoxic effect of gelonin on tumor cells. The subsequent increase in GGT activity could be due to the presence of proliferating hepatocytes that are not necessarily transformed.

Activity of GST of transformed cells followed a different trend upon gelonin treatment (Fig. 5c). The activity increased initially after one week and progressively decreased after 2 and 4 weeks of DBN-treatment. A similar observation has been reported earlier [29], in which a carcinogenic condition showed an increase in GGT activity accompanied by a corresponding decrease in GST activity. In this report, with increase in time of exposure to DBN however, the level of GST activity showed an increase. The GSH level decreased significantly upon treatment with gelonin in the first week, which maintained after 2 and 4 weeks of treatment (Fig. 5d).

Electrophoretic profiles of hepatocyte DNA extracted from DBN-exposed, gelonin-treated DBN-exposed and control mice are shown in Fig. 6. The broad band observed in the extract from DBN-treated mice indicates a high DNA content in comparison to normal. The band from gelonin-treated mice is broad and highly diffused. This could be due to increase in DNA content of liver tissue accompanying regenerative cell division in response to cytotoxic tumor cell destruction by gelonin. Diffusion of DNA band may be a result of DNA fragmentation which may in turn, be due to uncondensed DNA in these normal proliferating cells. The DNA from gelonin-treated mice was found to be of highest intensity. This could be due to the fact that uncondensed and fragmented DNA may bind more ethidium bromide than the condensed form.

Two observations were made from the synthetic index of DNA in hepatocytes from gelonin-treated tumor-induced mice (Fig. 6). The index showed a decrease as against that of DBN-exposed mice. However, the level was not lowered to that of the normal control mice. Cytotoxic effect of gelonin may be responsible for the lower level of BrdU incorporation in the first observation while a higher level than normal tissue in subsequent weeks is indicative of normal proliferation due to regeneration. This data further supports the DNA electrophoretic test discussed above.

After gelonin treatment, a restoration of several features comparable to normal liver was seen (Fig. 4c). The cells attained its regular shape and were bi-nucleated and mono-nucleated as well. Condensed chromatin material which

Table 1 Liver cell death (percentage) of DBN-exposed mice with free and liposome encapsulated gelonin

Liposomal compositions	Free gelonin (μg) $n = 8$	Liposome encapsulated gelonin (μg) $n = 8$	PEG (1mg/ml) $n = 8$	% cell death (Mean ± SEM)
–	100	–	–	N D
DPPC : Chol : DCP	–	100	–	16.00 ± 0.46
DPPC : Chol : DCP	–	100	+	19.00 ± 0.45
DPPC : Chol : PG	–	100	–	16.00 ± 0.59
DPPC : Chol : PG	–	100	+	18.50 ± 0.30
PC : Chol : PE	–	100	–	13.46 ± 0.64
PC : Chol : PS	–	100	–	13.60 ± 0.46

was observed in liver cells during DBN-exposure was also seen in those that received gelonin treatment. These small morphological changes in liver cells of the treated animals may be considered a positive indication of cancer regression caused by liposome encapsulated gelonin upon its administration. The long term toxic effect of gelonin treatment on tumor-bearing mice and on normal mice is currently being carried out.

RIPs have proven to be a potent anticancer agent for therapy. Our preliminary observations, therefore, indicate that the effectiveness of type-1 RIPs can further be improved by their encapsulation into liposomes, especially when liver is the target tissue for cancer therapy.

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