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Radiomodulatory effect of liposome encapsulated AK-2123 on tumor in mice exposed to hepatocarcinogen

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Abstract

An attempt was made to evaluate the whole body γ-radiation effect on tumor in the presence of free and liposome encapsulated AK-2123, a hypoxic cell radiosensitizer that has widely been used in combination with a number of cancer therapies such as thermotherapy, chemotherapy and radiotherapy. Entrapment efficiency of AK-2123 into liposome was determined by LASER Raman spectroscopy. Cancer induction in mice was carried out by repeated exposure of N-nitrosodiethylamine (DEN) in combination with partial hepatectomy. Parameters such as marker enzymes activities (GGT and AChE), rates of nucleic acid synthesis, viability modification factor and the histology of liver tissues monitored, supported the induction of cancer in liver. In addition, the effect of free as well as liposome encapsulated AK-2123 on haemopoietic parameters were also studied. It was observed that AK-2123 after incorporation into liposome afforded more efficient radiomodulatory effects than that of free AK-2123 as determined by the above-mentioned parameters. Neither free AK-2123 nor liposome encapsulated AK-2123 showed any detectable toxic effects on the mice. Thus, it is seen that treatment of cancer with a combination of radiation, a radiomodifier and a drug delivery system, opens a wide scope for exploitation for the improvement of existing cancer therapies. (Mol Cell Biochem **271:** 139–150, 2005)

Key words: radiomodifier, radiosensitizer, AK-2123, radiomodulatory effect, liposome, liposome encapsulated, radioprotection, LASER Raman spectroscopy

Abbreviation: AChE: Acetylcholine esterase, BSA: Bovine serum albumin, CDNB: 1-Chloro-2,4-dinitrobenzene, DEN: N-Nitrosodiethylamine, DMEM: Dulbecco modified eagles medium, DNA: Deoxyribonucleic acid, DTNB: 5,5′-Dithiobis-(2-nitrobenzoic acid), DTT: Dithiothreitol, EDTA: Ethylene di-amine tetra-acetic acid, ELISA: Enzyme-linked immunosorbent assay, GGT: γ-Glutamyl transpeptidase, HRP: Horse reddish peroxidase, NBT: 4-Nitroblue-tetrazolium chloride, RBC: Red blood cells, RNA: Ribonucleic acid, TCA: Trichloro acetic acid, TMB: Tetramethylbenzidine, VMF: Viability modification factor, WBC: White blood cells

Introduction

Radiation causes damage to both healthy as well as transformed cells. This has been a serious limitation in enhancing clinical efficacy of radiation in cancer radiotherapy. Radiomodifiers can potentially influence the effect of radiation by either enhancing the sensitivity of the transformed cells, or shielding the normal cells from the effect of radiation.

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AK-2123, N-(2′-methoxyethyl)-2-(3″-Nitro-1″-triazolyl) acetamide is one of the promising radio-modifiers with potential applicability [1–3]. The radio-modifying properties of AK-2123 on tumor are mainly attributed to its effect on the morphology of the vascular system and on the rheology of blood of the malignant tissues [4].

AK-2123 has widely been reported as a hypoxic cell radiosensitizer [3]. It also exhibits radio-protective effects in well-oxygenated systems and in aerobic condition of irradiation [5]. It has been shown that AK-2123 markedly reduced the radiation-induced lipid peroxidation. Thus, AK-2123 acts in dual capacity as a hypoxic cell radiosensitizer and also as a radioprotector in well-oxygenated systems [5]. In addition, reports are also available about the anti-metastatic effect of AK-2123. It was suggested that this effect is related at least partially, to the inhibition of the active calcium transport [6, 7]. Therefore, AK-2123 possesses multimodal properties, which, if exploited properly, could become an effective drug in clinical radiotherapy.

Realizing the highly usefulness of a liposome mediated drug delivery system and the vast scope that AK-2123 offers for exploitation in finding out means for improving cancer therapy, an attempt was made to evaluate the whole body gamma irradiation effect in presence of liposome encapsulated AK-2123 on cancer in mice chronically exposed to a potent hepatocarcinogen DEN.

Materials and methods

Materials

N-Nitrosodiethylamine (DEN), Glycylglycine, Tris, L-γ-glutamyl-p-nitroanilide, 5,5′- dithiobis-(2-nitrobenzoic acid) (DTNB), acetylcholine chloride, Coomassie brilliant blue G-250, Trypan blue, collagenase, sodium azide, dithiothreitol (DTT) and EDTA were procured from Sigma Chemical Co., USA. 5-Bromo-2′-deoxy-uridine Labeling and Detection Kit III, penicillin and streptomycin were purchased from Boehringer Mannheim, Germany. ^{3}H-Uridine was obtained from BARC, Bombay, India. All other reagents and chemicals used were of analytical grade. AK-2123 was a generous gift from Dr. V.T. Kagiya, Japan.

Animals

Swiss Albino mice (BALB/c), bred at the animal house of the department by random inbreeding were kept on basal diet *ad libitum* and housed in plastic cages in a temperature controlled animal room (21 $\pm$ 2 °C) with a 12 h light and dark cycle. The mice weighed 20–25 g at the start of the experiment.

Cancer induction

Healthy 6–8 weeks old mice were administered an aqueous solution of DEN (150 mg/kg b. w.) intraperitoneally, and were allowed to recover for two weeks. These animals were subjected to partial hepatectomy and within 24 h the animals received another injection of DEN (100 mg/kg b. w.). After one week, animals received intravenous injection of DEN (25 mg/kg b. w.) at weekly interval for a period of 3 months. Ethyl alcohol (10%) as a promoter was given orally once in a week during the entire course of treatment. Age-matched normal mice served as controls.

Histological examination

Microtomy technique was used for histological examination of liver tissues obtained from DEN-treated and untreated normal control mice. Briefly, the tissues were fixed in Bouin's solution and then thoroughly washed under running tap water. Dehydration was carried out in increasing alcoholic grade from 30 to 100%. The tissues were further cleared using xylene and embedded in paraffin wax. Blocks prepared were trimmed and cut in ribbons. Ribbons were mounted on slides, dried and the paraffin wax was removed by xylene. Rehydration was done with alcohol by reducing its concentration from 100 to 30%. The slides were stained by haematoxylin and eosin. Again, dehydration was done and after clearing the slides, 2 drops of DPX was placed on it and a cover glass was carefully allowed to settle on it. After 2 days of drying the slides were examined under a phase contrast microscope, Leitz Dialux.

Tissue preparation for enzymatic assays

Mice were killed by cervical dislocation and the whole livers were quickly excised, rinsed in chilled normal saline (0.9% NaCl), blotted dry and weighed. A 20% homogenate was prepared in chilled 0.32 M sucrose solution in a glass Teflon tissue homogenizer. The homogenate was solubilized in 0.5% Triton X-100 (1:1 v/v) and centrifuged at 20, 000 $\times$ g for 30 min at 4 °C. The resulting supernatant containing soluble GGT and AChE was used for enzyme activities and protein estimation.

γ-Glutamyl transpeptidase (GGT) assay

The GGT assay was carried out by following the method described in our recent communication [8, 9]. In brief, to 0.2 ml of γ-glutamyl-p-nitroanilide (0.005 M, pH 8.0) in a cuvette, 0.2 ml of glycylglycine (0.1 M, pH 8.0) and 0.6 ml of Tris-HCl buffer (0.1 M, pH 8.0) were added. The mixture was placed

in thermo stated cuvette holder of spectrophotometer Hitachi Model U 2001 maintained at 37 °C. The reaction was initiated by adding 0.2 ml of the enzyme preparation (supernatant) and the rate of release of p-nitroaniline was recorded at 410 nm ($\varepsilon = 8.80 \times 10^4$ M^{-1} cm^{-1}). The specific activity was expressed as units per mg protein. Protein concentration was determined by Bradford's method using BSA as standard.

Acetylcholine esterase (AChE) assay

Assay was based on a method described earlier [10]. In brief, the mixture containing 1.0 ml of PBS (0.2 M, pH 7.9), 0.5 ml of acetylcholine chloride (0.006 M), 0.5 ml of DTNB (0.00075 mM), 0.5 ml of 0.3% triton X-100 and 0.5 ml of the enzyme preparation (as described above) was taken in a cuvette. The rate of release of 5-thio-2-nitrobenzoate was recorded at 412 nm ($\varepsilon = 1.36 \times 10^4$ M^{-1} cm^{-1}). The specific activity was expressed as unit per mg protein. Protein concentration was determined by Bradford's method using BSA as standard.

Assay for DNA synthesis

It is a cellular immunoassay initially described by Gratzner [11]. This immunoassay is based on the cell ELISA principle. When BrdU was added to the viable liver cells in culture medium it incorporated into DNA of proliferating cells. After fixation of cells, cellular DNA was partially digested by nuclease treatment. A peroxidase labeled antibody to BrdU was added that binds to BrdU. Absorbance was measured at 405 nm on a multiscan MS India ELISA reader after adding the substrate. The measured absorbance was directly correlated to the level of BrdU incorporated into cellular DNA.

Assay for RNA synthesis

Viable liver cells (3×10^6) were incubated for 5 min at 37 °C in 2.0 ml DMEM. Cell culture was re-incubated for 30 min at 37 °C after adding 5 μCi of [^{3}H]-U (diluted with 50% ethanol). The reaction was arrested by the addition of 2.0 ml of chilled 15% TCA, followed by thorough mixing and incubation on ice for 15 min. Cells were harvested by vacuum aspiration onto (15% TCA) pre-wetted glass microfibre filters (GF/C; 25 mm). The incorporated [^{3}H]-U in the RNA was retained over filter strip while free [^{3}H]-U was washed through the filters. Harvested cells on the filter discs were washed with 5% TCA (5 ml × 3) and air-dried. Amount of radioactivity retained on the filters was quantitated by liquid scintillation counter, following addition of 10 ml of scintillation fluid (Cocktail 'T').

Cell viability and viability modification factor (VMF)

The cell viability test was performed by Trypan Blue dye exclusion method, using a Neubauer hemocytometer as described in Sigma bulletin. This is based on principle that cells when exposed to Typan Blue dye, viable cells do not take up the dye, whereas non-viable cells take up the dye and stain blue. A 0.4% Typan Blue dye solution was prepared in 0.81% sodium chloride and 0.06% dibasic potassium phosphate. The solution was sterile and filtered before use.

The calculations were carried out using the following formulae:

Cells per ml = the average count per square
$\times$dilution factor $\times$ 10^4(count 10 squares).

Total cells = cells per ml $\times$ the original volume of fluid from which cell sample was removed.

Cell viability (%) = total viable cells (unstained)
$\div$ total cells (stained and unstained) $\times$ 100

VMF was calculated by the formulae given below:

VMF = %Viability of cells after X Gy of γ-radiation/
% Viability of cells in presence of free AK-2123
or liposomal AK-2123 after X Gy of γ-radiation

Entrapment of AK-2123 into liposome

Liposome for entrapment of AK-2123 was prepared by the method described earlier [13]. In brief, lipid film was solubilized in chloroform (250 μl) by vortexing. While vortexing AK-2123 (20 mg/ml in normal saline) was added drop-wise to the lipid dispersion under N_2 stream. Care was taken to minimize the formation of chloroform-saline emulsion. The mixture was centrifuged at 20, 000 × g for 30 min at 4 °C in a refrigerated cold centrifuge (Sorval RC 5C). The supernatant was carefully aspirated and the liposome pellet was resuspnded in 1.0 ml of normal saline, then centrifuged again at 4 °C. This step was repeated 3–4 times to ensure that unentrapped AK-2123 is completely washed away. The supernatant was pooled together and the concentration of AK2123 was estimated by using LASER Raman spectroscopy as describe below.

The intensity (I band) of $-NO_2$ group present in AK-2123 was measured by Raman spectroscopy at a known wave number of 1313 cm^{-1}. To avoid any error due to sample variation or instrumental errors, an internal standard of $(NH_4)_2$ SO_4 was used because it also exhibits symmetric mode on $-SO_4^{2-}$ group with 986 cm^{-1} wave number. AK-2123 (20 mg/ml)

prepared in normal saline was used for making standards of known concentrations. The supernatant containing free AK-2123, obtained after centrifugation was made absolutely clear by filtering through a 0.2 μ pore size glass microfibre filter. Different standard concentrations of the stock AK-2123 solution, i.e., 2.5, 5.0, 10, 15 and 20 mg/ml, and 1 ml of the supernatant containing free AK-2123, were taken in different test tubes. The volume was made up to 1 ml with normal saline followed by the addition of 100 μl of $(NH_4)_2SO_4$ as an internal standard, and the spectrum was taken in 1403 Double Monochromator, Spex.

Irradiation

Animals were whole body Gamma (γ) irradiated in a Gamma Chamber 900 (BARC, India) with ^{60}Co as the source of radiation, at a dose rate of 8.4 Gy/min.

AK-2123 Administration

Free AK-2123 and liposome encapsulated AK-2123 was administered intraperitoneally at a dose of 200 mg/kg body weight 30 min prior to whole body γ-irradiation. Within an hour following irradiation, the animals were killed by cervical dislocation and the liver was used for different assays as described above.

Hematological analysis

Analysis of the hematological parameters such as haemoglobin, WBC and RBC were carried out as described by Miale [14]. Protein was estimated by Bradford method.

Results

General Observations

Animals, on chronic exposure to DEN showed signs of lethargy and sluggishness. A decrease in the body weight was also observed. However, majority of the animals survived the complete carcinogenic induction treatment course. The photographs of liver of age-matched normal and DEN treated mice are shown in Fig. 1. A Significant morphological change was observed in the liver upon DEN treatment (Fig. 1). The liver was enlarged and its texture was rough and coarse. A similar change was also seen of the spleen in the treated animals.

Histological studies

Liver tissue of DEN-treated and that of age-matched untreated mice were examined microscopically. When compared with the control, DEN treated liver micro section showed many differences (Fig. 2). In the control animal the liver cells have well defined symmetrical, mono- and bi-nucleated cells with a regular outline and are in close contact with their neighbors. However, in the treated animal the liver cells have lost their regular morphology and possess an irregular outline with some containing several smaller nuclei that have also lost their spherical appearance as observed in the normal cells. The cells also seem to have lost their contact with some of the neighboring cells. Besides these, nuclei are more densely stained.

Marker enzymes activities

Animals after having received complete DEN treatment showed marked alterations in their marker enzyme activities in comparison to control. Control group of animals showed an average GGT activity of 0.003069 U/mg protein) while the DEN treated group showed an activity of 0.035551 U/mg, thereby exhibiting an elevation of 11.58 folds (Figs. 3A and 3B). On the other hand, AChE activity was elevated by 5.48 folds against the control group of animals showing AChE activity of 0.007584 U/mg protein while the treated group gave an activity of 0.046031 U/mg (Figs. 4A and 4B). The differences in the activities of both GGT and AChE were statistically highly significant ($p < 0.0001$) against normal.

Nucleic acids syntheses indices

Results obtained from the DNA and RNA syntheses measurement in DEN treated mice by BrdU labeling and by [^{3}H]-U incorporation experiments respectively, showed an elevation of 7.12 in the rate of DNA synthesis (Fig. 5B) and an elevation of 5.07 folds in the RNA rate of synthesis (Fig. 6B) when compared with the control group of animals (Figs. 5A and 6A). The differences in the synthetic indices of both DNA and RNA when analyzed statistically were found to be highly significant ($P < 0.0001$) in comparison to control.

Entrapment efficiency of AK-2123 into Liposome

Each Laser Raman spectrum of AK-2123 (Fig. 7) depicts a sharp peak at 1313 cm^{-1} for -NO_2 group present in AK-2123. The peak area increases proportionately with the increase in the concentration. The standard calibration curve (Fig. 8) was used to determine the concentration of AK-2123

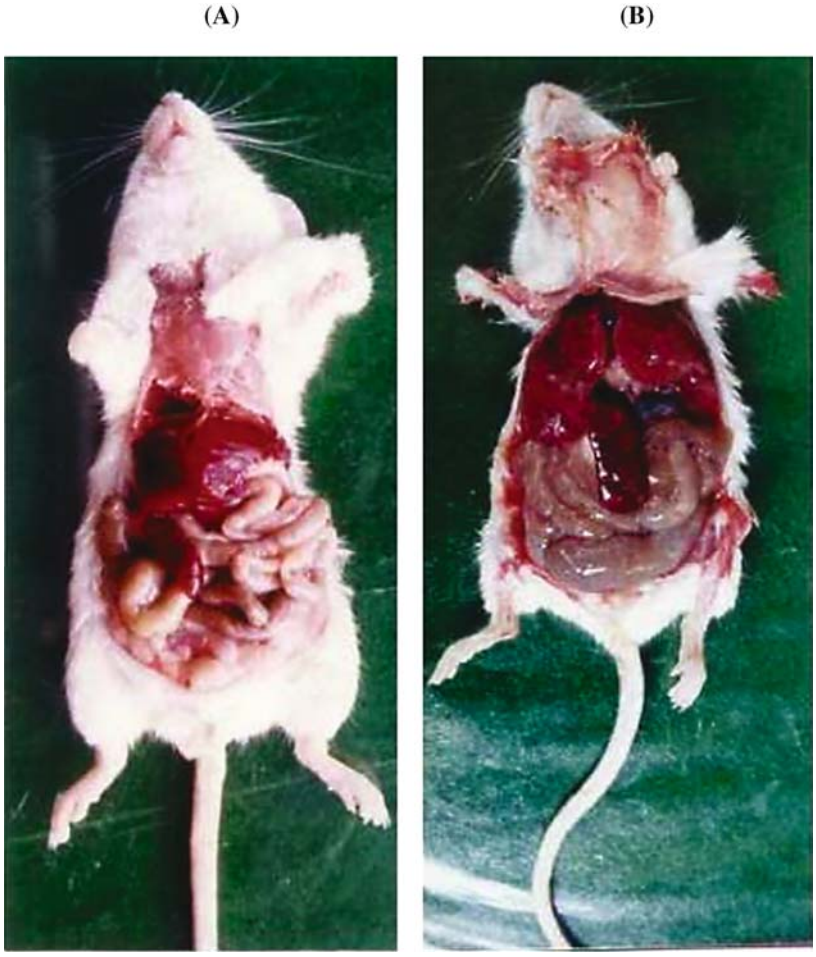

Fig. 1. The photographs of liver of age-matched DEN untreated (normal) (A) and DEN treated (B) mice. A significant morphological change was observed in the liver upon DEN treatment. The liver was enlarged and its texture was rough and coarse. A similar change was also seen of the spleen in the treated animals.

in the unknown sample. AK-2123 entrapped into liposome was thus calculated by subtracting free AK-2123 from the total concentration of AK-2123.

The concentration of AK-2123 present in each solution was read off from the spectra as follows:

Peak area = Peak intensity
× Full width at half maximum (FWHM)

Depolarization (ρ) = Peak intensity for perpendicular polarization/Peak intensity for parallel polarization, Or,

(ρ) = Peak area for perpendicular polarization/
Peak area for parallel polarization

Peak position = Position of ref. line(cm^{-1})
+ Peak position(in arbitrary unit)/
Spectrum width (in arbitrary unit)
× Spectrum width (in cm^{-1})

Based on these formulae, the following results were obtained and listed in Table 1.

Reference peak intensity = 0.13138(no. of counts)

Reference peak area = 0.7909

The value of the reference depolarization ratio lies between 0 and 3/4

Calculation for AK-2123 at 20 mg/ml:

Peak intensity for parallel polarization (in counts)
= 80/220 = 0.3636, where 80 = Peak height

Peak intensity for perpendicular polarization (in counts)
= 26/220 = 0.1181, where26 = Peak height

Relative peak intensity = I^{V1NO2}peak/I^{V1SO4}peak
= 2.76 for parallel polarization and 0.90
for perpendicular polarization

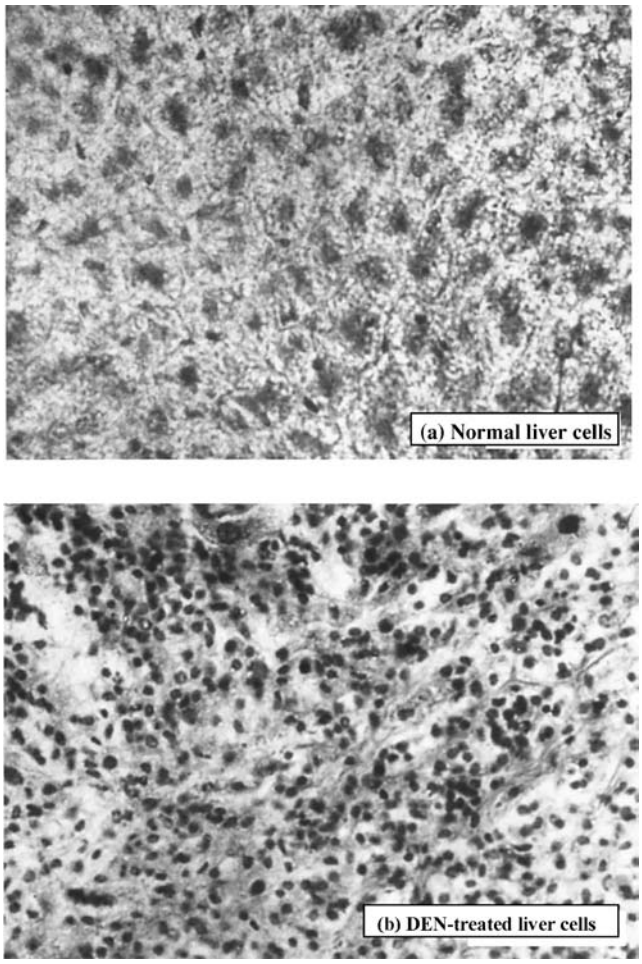

Fig. 2. Liver microphotographs of (a) normal and (b) DEN-treated mice. Microtomy technique was used for histological examination of liver tissues obtained from DEN-treated and untreated mice. The tissues were fixed in Bouin's solution and increasing the ethanol from 30 to 100% grade carried out the dehydration. 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 by xylene. The slides were stained by haematoxylin and eosin. Again, dehydration was done and the slides were examined microscopically after 2 days of drying. Magnification × 40.

FWHM = 22.9 for parallel and
21.7 for perpendicular polarization
Peak area = 8.3264 for parallel polarization and
2.5645 for perpendicular polarization.
Relative peak area = $\text{Area}^{\text{V1NO2}}/\text{Area}^{\text{V1SO4}}$
= 10.52 for parallel polarization and 3.24
for perpendicular polarization
Peak intensity depolarization ratio (ρ) = 0.3250
Peak area depolarization ratio (ρ) = 0.3076

Peak position = $1275 + 65/170 \times 100$
= 1313.2cm^{-1} for parallel polarization
Peak position = $1275 + 65/170 \times 100$
= 1313.2cm^{-1}
for perpendicular polarization

The peak area for each peak was calculated and plotted (Fig. 7) against their respective known concentrations (Table 1, Fig. 7).

Similarly the peak area of the supernatant sample (unknown) was calculated and the value was obtained from the standard curve, it was found to be 11.88 mg/ml.

Free AK-2123 in supernatant = 59.4%
Liposome entrapped AK-2123 = 100 − 59.4 = 40.6%

Therefore, the % entrapment efficiency of the prepared liposome = 40.6%

Viability modification factor (VMF)

This factor was calculated to quantify the radio-protective effects of free and liposome encapsulated AK-2123. DEN treated mice showed a decline in the cell viability at 2 Gy onward radiation, whereas in presence of AK-2123 either in free or liposome encapsulated forms the percent cell viability increased significantly (Fig. 9). VMF in presence of AK-2123 alone was found to be 1.3 at 10 Gy and 1.54 at 20 Gy. However, the same in the presence of liposome encapsulated AK-2123 was found 1.53 at 10 Gy and 1.7 at 20 Gy (Fig. 10), indicating that AK-2123 after encapsulating into liposome had a better effect.

Radiation effects on enzyme activities in presence of AK-2123

Administration of free and liposome encapsulated AK-2123 to DEN treated mice did not make any significant alteration in the GGT activity (Figs. 3C and 3D). However, upon exposure to radiation alone (10 Gy and 20 Gy) these mice showed a decrease in the enzyme activity that was below the normal enzyme level in control animals (Figs. 3E and 3F). When the treated mice were exposed to same doses of radiation in the presence of AK-2123 in its free (Figs. 3G and 3I) as well as liposome encapsulated forms (Figs. 3H and 3J) a significant decrease in the GGT activity was observed in comparison to that of DEN treated mice (Fig. 3B). A similar trend was observed with the activity of AChE upon radiation in the

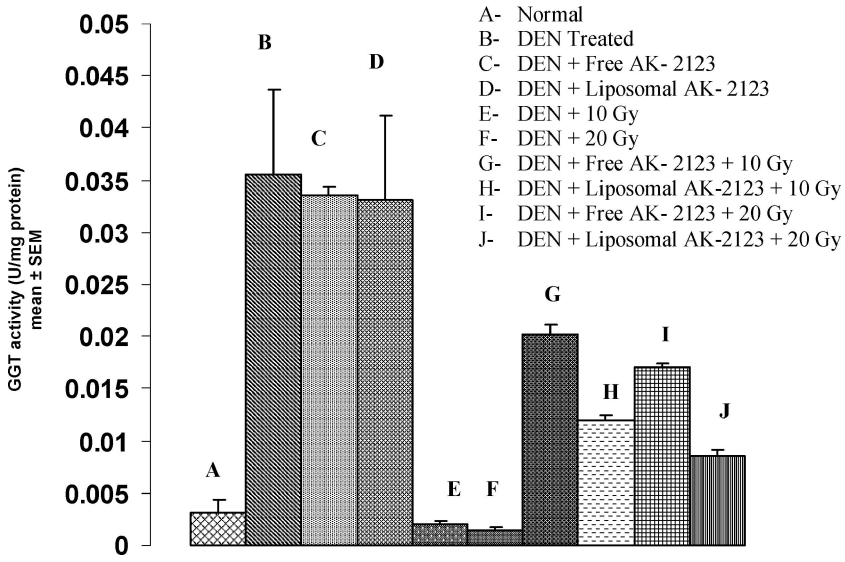

Fig. 3. γ-Glutamyl transpeptidase (GGT) activity in the liver of DBN-exposed and unexposed (normal) mice. The γ-Glutamyl transpeptidase activity was determined by the method described in the materials and method section. Briefly, 0.2 ml of γ-glutamyl-p-nitroanilide (0.005 M, pH 8.0) was mixed in a cuvette with 0.2 ml of glycylglycine (0.1 M, pH 8.0) and 0.6 ml of Tris-HCl buffer (0.1 M, pH 8.0). The reaction was initiated by adding 0.2 ml of the enzyme preparation (supernatant) and the absorbance was measured at 410 nm. The GGT activity shown in A–J corresponds to the normal and different treatments to the animals. Figures 3A and 3B show the GGT activity of DEN untreated (control) and DEN treated animals, respectively. Figures 3C–3J show the GGT activity of DEN exposed animals when further treated with, free AK-2123 (C); liposomal AK-2123 (D); 10 Gy (E); 20 Gy (F); Free AK-2123 + 10 Gy (G); Liposomal AK-2123 + 10 Gy (H); Free AK-2123 + 20 Gy (I) and Liposomal AK-2123 + 20 Gy (J).

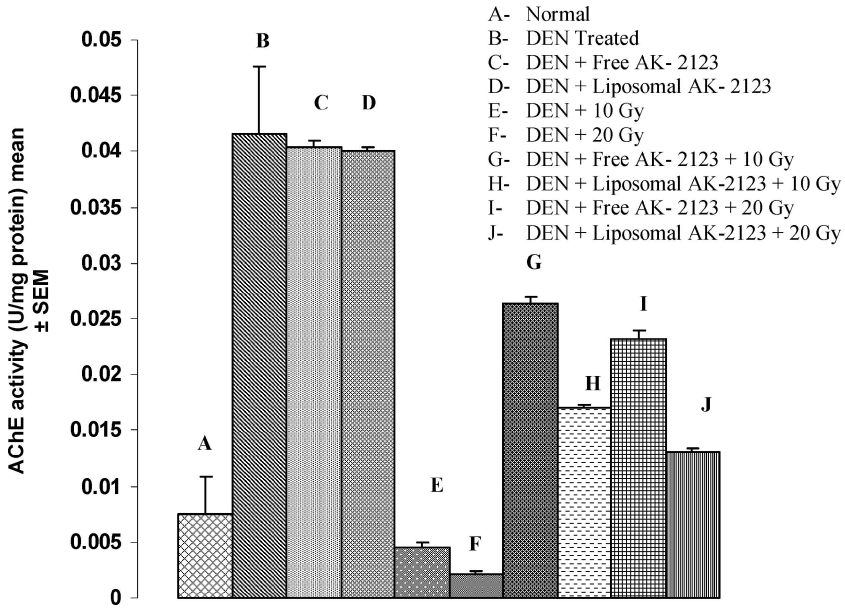

Fig. 4. Acetylcholine esterase (AChE) activity in liver of DBN-treated and unexposed (normal) mice. The acetylcholine esterase activity was determined by the method described in the materials and method section. Briefly, to a mixture of 1.0 ml of PBS (0.2 M, pH 7.9), 0.5 ml of acetylcholine chloride (0.006 M), 0.5 ml of DTNB (0.00075 mM) and 0.5 ml of 0.3% triton X-100, 0.5 ml of the enzyme preparation (as described above) was added to initiate the reaction. The absorbance at 412 nm was measured. The *AChE* activity shown in A–J corresponds to the normal and different treatments to the animals. Figures 4A and 4B show the GGT activity of DEN untreated (control) and DEN treated animals, respectively. Figures 4C–4J show the GGT activity of DEN exposed animals when further treated with, free AK-2123 (C); liposomal AK-2123 (D); 10 Gy (E); 20 Gy (F); Free AK-2123 + 10 Gy (G); Liposomal AK-2123 + 10 Gy (H); Free AK-2123 + 20 Gy (I) and Liposomal AK-2123 + 20 Gy (J).

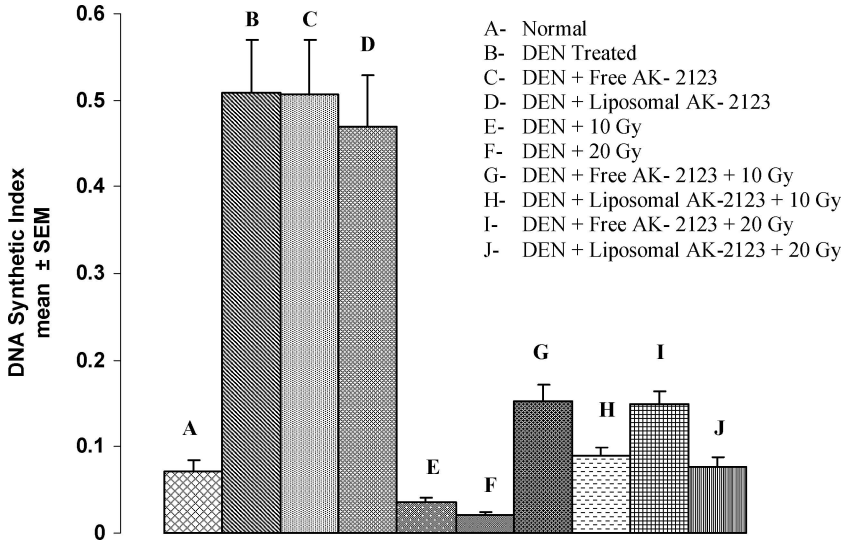

Fig. 5. DNA Synthesis. This is a cellular immunoassay initially described by Gratzner [11]. This immunoassay is based on the cell ELISA principle. When BrdU was added to the viable liver cells in culture medium it incorporated into DNA of proliferating cells. After fixation of cells, cellular DNA was partially digested by nuclease treatment. A peroxidase labeled antibody to BrdU was added that binds to BrdU. Absorbance was measured at 405 nm on a multiscan MS India ELISA reader after adding the substrate. The measured absorbance was directly correlated to the level of BrdU incorporated into cellular DNA. Figures 5A and 5B show the DNA synthetic index of DEN untreated (control) and DEN treated animals, respectively. Figures 5C–5J show the DNA synthetic index of DEN exposed animals when further treated with, free AK-2123 (C); liposomal AK-2123 (D); 10 Gy (E); 20 Gy (F); Free AK-2123 + 10 Gy (G); Liposomal AK-2123 + 10 Gy (H); Free AK-2123 + 20 Gy (I) and Liposomal AK-2123 + 20 Gy (J).

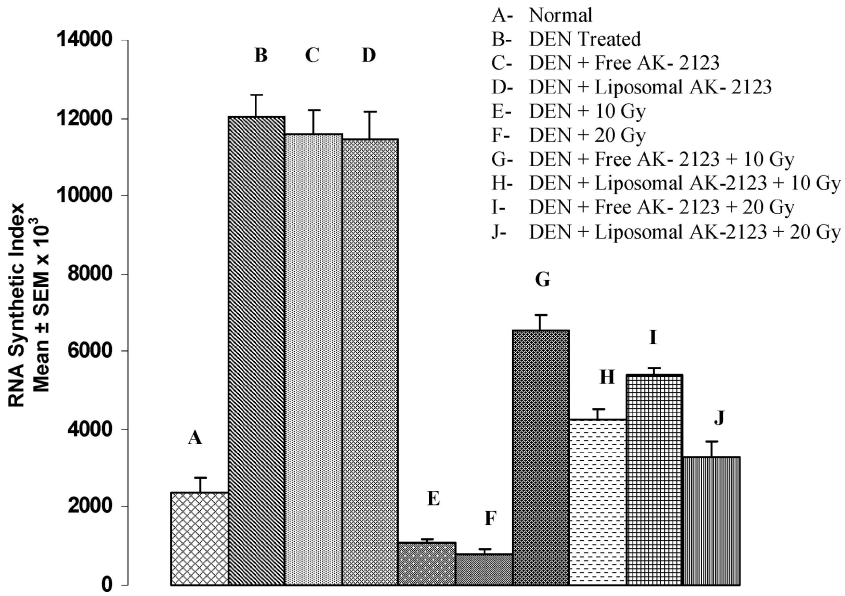

Fig. 6. RNA synthesis. Viable liver cells (3×10^6) were incubated for 5 min at 37 °C in 2.0 ml DMEM. Cell culture was re-incubated for 30 min at 37 °C after adding 5 μCi of [^{3}H]-U (diluted with 50% ethanol). The reaction was arrested by the addition of 2.0 ml of chilled 15% TCA, followed by thorough mixing and incubation on ice for 15 min. Cells were harvested by vacuum aspiration onto (15% TCA) pre-wetted glass microfibre filters (GF/C; 25 mm). The incorporated [^{3}H]-U in the RNA was retained over filter strip while free [^{3}H]-U was washed through the filters. Harvested cells on the filter discs were washed with 5% TCA (5 ml × 3) and air-dried. Amount of radioactivity retained on the filters was quantitated by liquid scintillation counter, following addition of 10 ml of scintillation fluid (Cocktail 'T'). Figures 6A and 6B show the RNA synthetic index of DEN untreated (control) and DEN treated animals, respectively. Figures 6C–6J show the RNA synthetic index of DEN exposed animals when further treated with, free AK-2123 (C); liposomal AK-2123 (D); 10 Gy (E); 20 Gy (F); Free AK-2123 + 10 Gy (G); Liposomal AK-2123 + 10 Gy (H); Free AK-2123 + 20 Gy (I) and Liposomal AK-2123 + 20 Gy (J).

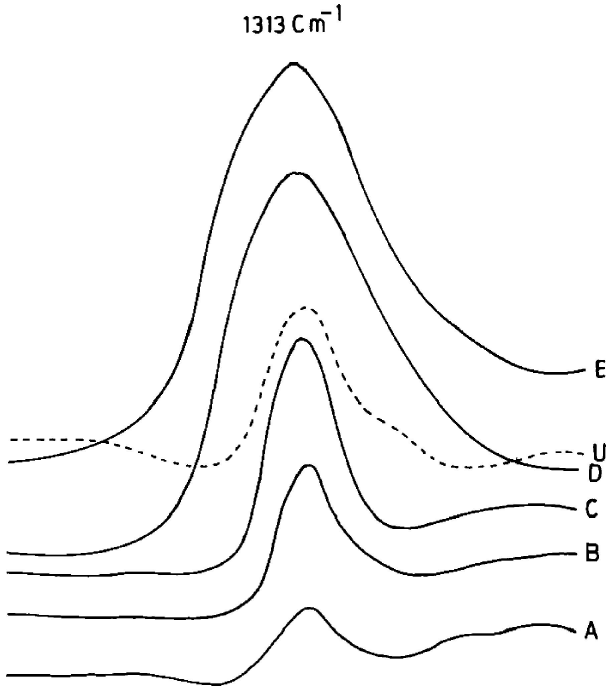

Fig. 7. LASER Raman spectral analysis. AK2123 concentration was estimated in the supernatant by using LASER Raman spectroscopy. The intensity (I band) of $-NO_2$ group present in AK-2123 was measured by Raman spectroscopy at a known wave number of 1313 cm^{-1}. To avoid any error due to sample variation or instrumental errors, an internal standard of $(NH_4)_2SO_4$ was used because it also exhibits symmetric mode on $-SO_4^{2-}$ group with 986 cm^{-1} wave number. AK-2123 (20 mg/ml) prepared in normal saline was used for making standards of known concentrations. The supernatant containing free AK-2123, obtained after centrifugation was made absolutely clear by filtering through a 0.2 μ pore size glass microfibre filter. Different standard concentrations of 2.5, 5.0, 10, 15 and 20 mg/ml, and 1 ml of the supernatant containing free AK-2123, were taken in different test tubes. The final volume of each sample was made up to 1 ml with normal saline followed by the addition of 100 μl of $(NH_4)_2SO_4$ as an internal standard, and the spectrum was recorded in 1403 Double Monochromator, Spex. The LASER Raman spectra of standard AK-2123 of 2.5, 5.0, 10, 15 and 20 mg/ml are shown as A, B, C, D and E, respectively. The spectra of the unknown sample are shown as U. The calibration curve and the quantitation of unknown sample are given in Fig. 8.

presence of free and liposome encapsulated AK-2123 (Figs. 3E–J, respectively).

Radiation effects on DNA and RNA synthesis in presence of AK-2123

The rate of DNA and RNA syntheses did not alter significantly upon administration of free and liposome encapsulated AK-2123 to DEN treated mice (Figs. 5C and 5D). Upon radiation (10 Gy and 20 Gy) of DEN treated animals the synthetic indices fall below the normal level (Figs. 5E and 5F). However, the significant decline in DNA synthetic

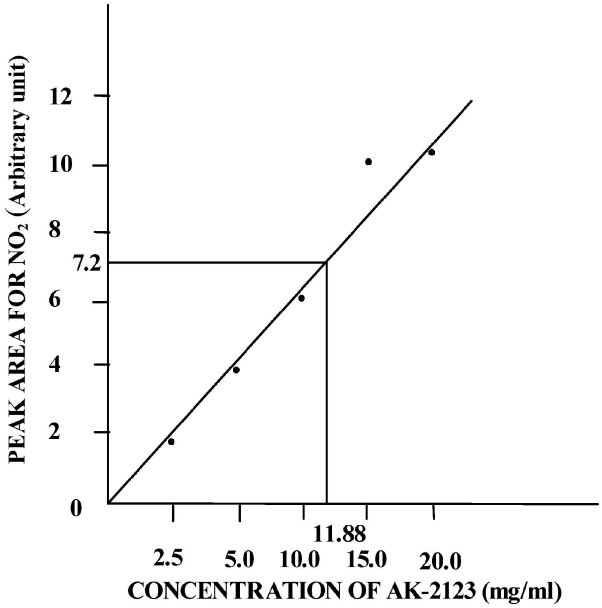

Fig. 8. Calibration curve for AK-2123 as determined by LASER Raman spectra of Fig. 7. A relationship between different concentrations of AK-2123 *versus* the peak area of NO_2 as determined by LASER Raman spectroscopy is given in Fig. 7. The concentration of the unknown AK-2123 was determined by interpolating from the same standard curve.

index observed upon radiation in the presence free AK-2123 (Figs. 5G and 5I) as well as liposome encapsulated AK-2123 (Figs. 4H and 4J). A similar trend was observed for RNA synthetic index upon radiation in the presence of free and liposome encapsulated AK-2123 (Figs. 6-E–J, respectively). Thus, the syntheses indices of DNA and RNA reduced significantly in DEN exposed animals upon irradiation at 10 Gy and 20 Gy. However, in presence of free and liposome encapsulated AK-2123 the effect was found significantly less when compared with radiation alone, the latter showing a better response than the former.

Table 1. Raman data for V_1 of NO_2 excited by 488.0 nm of Ar ion Laser at 4 cm^{-1} slit

AK-2123 (mg/ml)	Peak position (cm^{-1})	Depolarization ratio (peak intensity)	FWHM (cm^{-1})	Relative peak area
20	1313.2	0.32	22.9	10.52
15	1313	0.39	22.9	9.97
10	1313	0.35	22.46	6.24
5	1312.3	0.43	22.98	3.91
2.5	1313	0.36	22.5	1.79
Sample (Supernatant)	1313	0.42	21.73	7.2

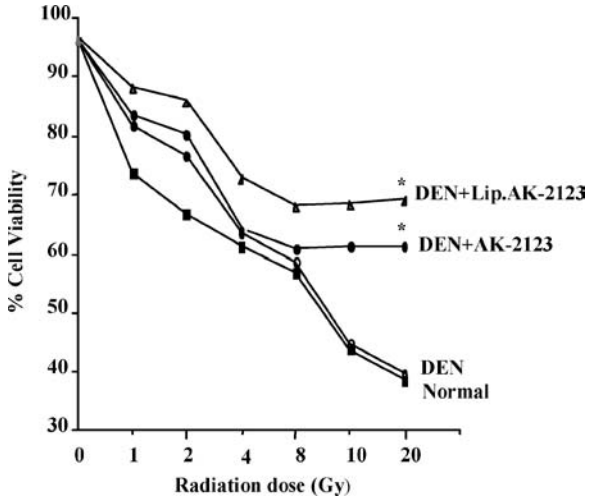

Fig. 9. Percent viability of hepatocytes. Percent viability of hepatocytes following whole body γ -irradiation at different doses in normal mice and those exposed to DEN alone was determined by the incorporation of Trypan Blue dye as described in the Materials and Method section. The figure also shows the percent viability of hepatocytes upon γ-irradiation of DEN treated mice exposed to free AK-2123 and liposome encapsulated AK-2123. No bar means that SEM was smaller than the thickness of the point. $^{*}P < 0.0001$ against DEN treated samples.

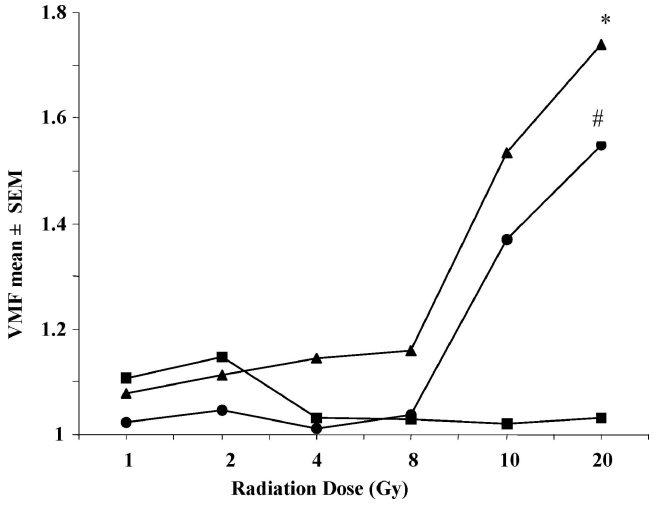

Fig. 10. Viability modification factor VMF. The cell viability test was performed by Trypan Blue dye exclusion method, using a Neubauer hemocytometer as described in Sigma bulletin. This is based on principle that cells when exposed to Typan Blue dye, viable cells do not take up the dye, whereas non-viable cells take up the dye and stain blue. The calculations were carried out using the formulae as described in the materials and method section. A relationship was determined between VMF versus the different doses of γirradiation in normal, DEN treated and DEN treated exposed to free AK-2123 and liposome encapsulated AK-2123. $^{\#}p < 0.0001$ against DEN treated samples. $^{*}p < 0.0001$ against DEN and DEN + AK2123 + irradiation.

Effects of free and liposome encapsulated AK-2123 on WBC and RBC

Total WBC counts in the blood of normal mice and those treated with DEN alone, and the DEN treated administered with free and liposome encapsulated AK-2123 are shown in Table 2. WBC counts increased two times in mice simply upon DEN exposure but it decreased when AK-2123 administered either free or liposome encapsulated, the latter showed less decrease than the former. A similar trend was observed for the total RBC counts (Table 2).

Discussion

Chronic exposure of DEN when combined with hepatectomy triggered initiation of carcinogenesis in the liver of Swiss albino mice. The induction of cancer was much more pronounced and was achieved in a short course of time in comparison to DEN treatment alone [13]. Earlier it was also observed that partial hepatectomy promotes the initiation process of carcinogenesis [15, 16]. Changes of activity (inhibition or activation) of marker enzymes, viz. GGT and AChE are common finding in hepatic tissues during cellular transformation. In liver GGT has been recognized as a positive marker for hepatocytes which has undergone malignant transformation [17]. Thus, the hyper-activation of GGT in DEN-exposed mice signifies hepatocellular transformation (Fig. 3B). On the other hand increase in AChE activity also support the development of a cancerous situation in hepatocytes as the cancer induction is accompanied by membrane changes and AChE is a membrane bound enzyme (Fig. 4B). Cancer induction in DEN-treated mice was further confirmed by several folds increase in DNA and RNA synthetic indices (Figs. 5B and 6B). Since cancer is a state where the cells are no longer responsive to the normal growth controlling factors, thereby uncontrolled proliferation of cells occurs in such a condition [15]. In order to keep up with this uncontrolled cell division, the nucleic acids also have to step up their rates of syntheses and therefore, accordingly result in an increase in their synthetic indices [18–20].

Clinically, cancer is confirmed based on biopsy and histological verification of the disease. Therefore, histological study was taken up for monitoring the induction process. The microphotographs exhibited a vast difference in the morphology of the liver in normal and treated situations (Fig. 2). The treated liver seemed to have undergone a lot of alterations. The DEN treated hepatocytes distributed in somewhat disarrayed fashion. The architecture of the supporting matrix seemed coarser, disorganized and disoriented. There were marked variations in their shapes and size and increase in cell number and more intense staining of nuclei. However, in the normal condition a more uniform supporting

Table 2. Effects of free and liposome encapsulated AK-2123 on haemopoietic parameters

Group	Total WBC counts $\times 10^6$cells/ml blood Mean ± S.D. ($n = 6$)	Total RBC counts $\times 10^7$cells/ml blood Mean ± S.D. ($n = 6$)	Haemoglobin (mg/ml) Mean ± S.D. ($n = 6$)	Total protein (in blood) (mg/ml) Mean ± S.D. ($n = 6$)
Normal	13.06 ± 0.46	7.54 ± 0.23	107.69 ± 3.53	10.00 ± 0.21
DEN-treated	22.06 ± 0.46*	12.14 ± 0.23*	118.80 ± 4.24*	11.55 ± 0.16*
Normal + Free AK-2123	09.35 ± 1.13*	05.04 ± 0.23*	96.20 ± 1.20*	09.19 ± 0.29*
Normal + Lip- AK-2123	10.08 ± 0.45*	06.04 ± 0.23*	107.03 ± 3.43	09.99 ± 0.21
DEN + Free AK-2123	16.92 ± 0.60*	09.95 ± 0.37*	82.44 ± 1.64*	10.41 ± 0.21*
DEN + Lip-AK-2123	20.00 ± 0.42*	11.89 ± 0.23	94.14 ± 2.13*	10.91 ± 0.22*

n = number of observations, *statistically significant ($p < 0.0001$) in comparison to normal group, Lip- = Liposome encapsulated.

matrix and distinct and defined arrangement of the cells was observed.

The entrapment efficiency of AK-2123 into liposome was determined by LASER Raman spectroscopy. As the UV absorption spectrum of AK-2123 gives a very broad band with no specific absorption maximum and its insolubility in organic solvents restricted the use of IR spectroscopy. In contrast, the presence of a free nitro group (–NO_2) in AK-2123 is ideally suited for the use of LASER Raman spectroscopy, since — NO_2 is known to show a characteristic Raman peak at 1313 cm^{-1}. For each concentration of AK-2123 we obtained a characteristic peak for –NO_2 at 1313 cm^{-1} and the peak areas increased proportionately with the increase in the concentration (Fig. 7). The peak area for the unknown sample when interpolated in the standard plot and calculated, gave entrapment efficiency of 40.6%, which is reasonably high (Fig. 8).

Whole body γ-irradiation effects in the presence of free as well as liposome-encapsulated AK-2123 were studied in DEN-exposed and age-matched untreated mice. Cell viability, viability modification factor (VMF), marker enzyme activities, nucleic acid synthetic indices in liver tissues of treated mice were parameters to study the effects. Beside these some other parameters such as the RBC and WBC counts and the total protein were also monitored. Radiation alone inflicted normal and transformed cell destructions signified by the very low levels of marker enzyme activities (Figs. 3E, 3F and 4E, 4F) and nucleic acids synthetic indices (Figs. 5E, 5F and 6E, 6F). The presence of AK-2123 either in it's free or liposome encapsulated form, along with radiation, inhibited this effect of radiation on the cells (Figs. 3–6, G–J). However, in the absence of radiation both the free and encapsulated AK-2123 could not exhibit statistically significant protective effects (Figs. 3–6, C and D). This observation indicates that AK-2123 alone has no toxic effect on the liver as well as on the nucleic acids. However, in presence of radiation, AK-2123 sensitized the transformed cells to radiation and hence resulted in the fall of enzyme activity levels and the rates of nucleic acids syntheses. The liposome-encapsulated AK-2123 was found to be better responder to radiation indicating that AK-2123 after encapsulation into liposome exhibits better radio-sensitizing effect than free AK-2123. This observation can be explained from the fact that liposome have an inherent preferential migration towards tissues rich in reticuloendothelial cells like the liver [21]. Therefore, a larger proportion of AK-2123 reaches the target tissue, the liver, and thus, exhibited higher radio-sensitizing property than its free counterpart.

The assay of percent cell viability at different doses of radiation showed that AK-2123 shielded the cells from the harmful effects of radiation. This protective effect was relatively more pronounced in case of liposome encapsulated AK-2123 at the radiation doses of 10 Gy and 20 Gy (Fig. 9). Further, the hepatocytes following whole body irradiation in presence of free AK-2123 exhibited a VMF of 1.37 and 1.54; while it was 1.53 and 1.74 in presence of liposome encapsulated AK-2123 at 10 and 20 Gy, respectively (Fig. 10). These observations further support better protection by liposome encapsulated AK-2123 over the free AK-2123 at the same radiation doses.

Haemopoietic system is also a predominant target organ of chemical carcinogens. Effects of free and liposome encapsulated AK-2123 on certain blood parameters were studied. DEN treatment caused an increase in haemoglobin, blood cells and total protein contents (Table 2). Administration of free AK-2123 inhibited the haemoglobin, blood cells and total protein contents of the animal. No such effects were observed upon administration of liposome encapsulated AK-2123 (Table 2). It may be due to preferential migration of liposome towards the reticuloendothelial cells rich tissues which limits its free distribution elsewhere. Consequently, its free contact with blood is restricted. This may prevent the encapsulated AK-2123 from imparting its toxic and suppressive effects in the blood unlike in the case of free AK-2123. Hence, encapsulated AK-2123 exhibited lesser toxic and suppressive effects on the parameters under study.

The initial laboratory investigations on radiomodifying properties of AK-2123 were conducted *in vitro* systems.

Based on these studies, AK-2123 was categorized as a hypoxic cell radiosensitizer, which encouraged considering AK-2123 in the limited clinical trials [1–3]. The results of *in vivo* studies were not in the line with those of *in vitro* studies. A notable difference in the two conditions (*in vitro* and *in vivo*) is the level of oxygenation. Investigation that followed shows that AK-2123 behaved differently under oxic and anoxic conditions. The present *in vivo* study also finds that AK-2123 showed rather weak radio-sensitizing effects coupled with radio-protective effects on certain parameters. Since the mouse *in vivo* system is inherently oxic, but has different levels of oxygenation in different organ systems, the results are not unexpected. One interesting conclusion could be drawn from this investigation is that AK-2123 may have differential radio-modifying effects in different tissues *in vivo*, and in different conditions of irradiation. Therefore, AK-2123 may be a suitable drug for chemo-radiotherapy of only certain types of cancer. Since solid tumors in late stage develop anoxic core, AK-2123 could be a potentially useful drug for their chemo-radiotherapy.

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