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DNA binding and apoptotic induction ability of harmalol in $HepG_2$: Biophysical and biochemical approaches

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ABSTRACT

Harmalol administration caused remarkable reduction in proliferation of $HepG_2$ cells with GI_{50} of 14.2 μM, without showing much cytotoxicity in embryonic liver cell line, WRL-68. Data from circular dichroism (CD) and differential scanning calorimetric (DSC) analysis of harmalol-CT DNA complex shows conformational changes with prominent CD perturbation and stabilization of CT DNA by 8 °C. Binding constant and stoichiometry was calculated using the above biophysical techniques. The Scatchard plot constructed from CD data showed cooperative binding, from which the cooperative binding affinity ($K'\omega$) of $4.65 \pm 0.7 \times 10^5$ M^{-1}, and n value of 4.16 were deduced. The binding parameter obtained from DSC melting data was in good agreement with the above CD data. Furthermore, dose dependent apoptotic induction ability of harmalol was studied in $HepG_2$ cells using different biochemical assays. Generation of ROS, DNA damage, changes in cellular external and ultramorphology, alteration of membrane, formation of comet tail, decreased mitochondrial membrane potential and a significant increase in Sub G_0/G_1 population made the cancer cell, $HepG_2$, prone to apoptosis. Up regulation of p53 and caspase 3 further indicated the apoptotic role of harmalol.

1. Introduction

Deoxyribonucleic acid that serves as the repository of genetic information of the cell is the potential therapeutic targets in cancer. Again, plant alkaloids, a natural product as chemotherapeutic agents isolated so far, have been reported to have remarkable anti-cancer applications [1–5] and consequently there is growing interest in the search for better anti-cancer drugs with high efficacy, low toxicity and minimum side effects. But most of the chemotherapeutic agents due to their rather non-selective nature and dose limiting toxicity, use is often restricted, necessitating search for newer drugs having greater potential and suitability for use. β carbonil is one such large group of natural and synthetic alkaloids (ligand) of indole derivatives. These alkaloids were originally isolated from plants like *Peganum harmala* L and *Banisteriopsis caapi*. Our previous investigation showed that beta carboline plant alkaloid, harmalol, is an excellent intercalator preferring hetero G/C sequence [6,7]. Recently, we further showed its RNA binding ability [8]. Harmalol, has been reported to have several pharmacological, neurophysiological and biochemical activities [9–15] as well as *in vivo* and *in vitro* chemopreventive action against different cancer cell line [16–18], but its detail apoptotic effect on human cancer cell line has not been studied yet.

Inspired by these results, in the present investigation we elucidate, harmalol inducing apoptotic effects in $HepG_2$ cells and consequently study its stabilization, binding and conformational changes with CT DNA using different biochemical and biophysical techniques. Further, event like ROS generation and cell cycle arrests were also accompanied in the harmalol induced apoptosis. A complete understanding of these therapeutic aspects of beta carboline alkaloid will enable the researchers for the future drug design for the betterment of mankind.

2. Materials and methods

2.1. Biochemicals

Harmalol was obtained from Sigma-Aldrich (St. Louis, MO, USA). The purity of the sample was confirmed as done earlier [6,7]. Its concentration was determined using molar extinction coefficient value of 19,000 M^{-1} cm^{-1} at 371 nm [7]. Double stranded calf thymus DNA (CT DNA) was also obtained from Sigma—Aldrich.

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Concentration was determined using the molar extinction coefficient (ε) of 6600 M^{-1} cm^{-1} at 260 nm. Harmalol and CT DNA was dissolved in 15 mM Citrate–Phosphate (CP) buffer of pH 6.8.

2.2. Cell lines and culture conditions

Five different human cell lines *viz.* HeLa (cervical carcinoma), MDA-MB-231 (breast carcinoma), A549 (lung carcinoma), $HepG_2$ (liver epitheloid carcinoma) and WRL-68 (normal hepatic cells) were chosen, obtained from National Centre for Cell Science, Pune. Cells were grown in DMEM supplemented with 10% heat inactivated fetal bovine serum (FBS) and 1% antibiotic–antimycotic, in a humidified atmosphere at 37 °C with 5% CO_2.

2.3. Cell viability test: MTT assay

We tested the percentage of cell viability and calculated the GI_{50} (50% growth inhibition) values for the above mentioned cell lines by MTT (1 mg/ml of the tetrazolium dye and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dissolved in phosphate buffer saline, pH 7.4) assay as reported earlier [19]. GI_{50} was calculated by using the equation

$$GI_{50} = (T\text{-}T_O)\times 100/(C - T_O) \quad (1)$$

where, T is the optical density of the test well after 48 h drug exposure, T_o is the optical density at time zero & C is optical density of control. The experiment was repeated three times and average was taken.

2.4. LDH assay

LDH activity was measured quantitatively by counting the number of floating dead cells and adherent viable cells for both control and drug treated cells according to the protocol of *Biswas* et al., [20]. The percent apoptotic and percent necrotic cell deaths were determined as follows:

$$\%\text{Apoptosis} = (LDH_p \times 100\%)/(LDH_p + LDH_i + LDH_e) \quad (2)$$

$$\%\text{necrosis} = (LDH_e \times 100\%)/(LDH_p + LDH_i + LDH_e) \quad (3)$$

The floating cells were collected from culture media by centrifugation (3000 r.p.m) at 4 °C for 4 min, and the LDH content from the pellets was marked as an index of apoptotic cell death (LDH_p). The released extracellular LDH in the culture supernatant was marked as an index of necrotic cell death (LDH_e), and the LDH_i present in the adherent viable count was used as intracellular LDH. $HepG_2$ cells were seeded in 6-well plates at a density of 2×10^4 cells/well and treated with harmalol of 7, 14 and 21 μM concentrations at 37 °C for 48 h.

2.5. FITC-Annexin V/PI FCM double staining

Double staining for FITC (fluorescein isothiocyanate) - Annexin V binding and for cellular DNA using PI (propidium iodide) FCM (flow cytometry) was performed as described by Mallick et al. [21].

2.6. Fluorescence microscopy

For the identification of nuclear changes such as chromatin condensation, nuclear fragmentation and MMP (mitochondrial membrane potential) alteration by JC-1, cells were visualized using a fluorescence microscope (Olympus). DAPI, a blue fluorescent stain, that preferentially stains nuclei (apoptotic or viable). Briefly, cells were placed on coverslips at 2×10^4/well in a 6 well plate for 48 h before treatment. Cells were then stained for 15 min with DAPI (5 μM) at room temperature. Cells on cover slips were mounted for fluorescence microscopic observation.

2.7. Scanning electron microscopy (SEM)

To study the external morphology of cell, they were first fixed in glutaraldehyde and dehydrated through a graded series of ethanol. Subsequently, they were cleaned in tetrachloromethane, air-dried and coated with gold in IB2 ION COATER and observed using S530 Hitachi scanning electron microscope.

2.8. Transmission electron microscopy (TEM)

Cultured materials were centrifuged at 3000 rpm for 4–5 min to discard the supernatant. The pellet was suspended in 0.1 M phosphate buffer (pH 7.4), dispersed and centrifuged again. Resuspended the pellet and fixed in a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in buffer for 3–4 h at 4 °C. Centrifuged for 10 min at 4 °C and the supernatant were discarded. The samples were finally observed using TECNAI 200 KV TEM (Fei Electron Optics), 35 mm Photography System from All India Institute of Medical Science, N. Delhi.

2.9. Estimation of DNA damage (comet assay and DNA gel electrophoresis)

Comet assay was performed on isolated $HepG_2$ cells by single cell gel electrophoresis as reported by Liao et al. [22]. It consists of mainly the following steps- Slide preparation and cell lysis to liberate the DNA, DNA unwinding, electrophoresis, neutralization of the alkali, DNA staining and scoring. Reagents required were- 1% normal agarose; 1% low melting agarose; electrophoresis buffer (pH 10); neutralization buffer (pH 7.2) and lysis solution (pH 13 to 14). Cell suspensions of control and three different concentrations (7, 14 and 21 μM) of harmalol treated sets of cells were mixed with 1% low melting agarose at 37 °C in 1:3 ratio and 100 μl of this mixture was applied for three times quickly on top of the fully frosted four different glass-slides which were coated previously with the 1% normal agarose. After solidification of the gel, these glass-slides of control and three harmalol treated samples, containing about 1000–2000 cells, were conveniently immersed in ice-cold lysis solution at 4 °C for 16 h. After washing with neutralization buffer these slides were placed on the platform of the electrophoresis apparatus and the electrophoresis was carried out at 0.8 V/cm for 15 min. Ethidium bromide solution of 0.5 μg/ml was added to the gel and the slides were covered with cover slips. The stained DNA in the cells was examined at 200× magnification with the help of OLYMPUS Fluorescence Microscope. We determined the extent of DNA damage by measuring the % DNA in comet tail by using software CASP. One hundred of each class of visually classified comets from undamaged or no discernible tail to the category having almost all DNAs in tail was selected for image analysis using CASP. We compared 100 comets of different categories using 100× magnifications as the use of 100 × magnification allows a faster analysis of gels.

For performing the DNA gel electrophoresis in 1% agarose gel, we extracted DNA of $HepG_2$ cells by standardized phenol-chloroform method.

2.10. Cell cycle inhibition analysis

$HepG_2$ cells were grown at a density of 2×10^5 cells/ml in 70 mm culture plate and treated with different concentrations of