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POLY(LACTIC-CO-GLYCOLIC ACID)-LOADED BOLDINE NANOPARTICLES ALLEVIATES CISPLATIN INDUCED HEPATOTOXICITY IN NORMAL LIVER CELLS *IN VITRO* VIA DNA TARGETING: DRUG-DNA INTERACTION

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ABSTRACT

Whether PLGA-loaded nano-Boldine (NBol) induces apoptosis and cell cycle arrest to alleviate cisplatin-induced toxicity preferentially in normal liver calls (WRL-68) primarily via DNA-targeting has been addressed in this study. Nano-encapsulation of Boldine (Bol) was accomplished by solvent displacement method. Effects of Bol and NBol were quantitatively assessed in WRL-68 and HepG2 cells in vitro in respect of cell cycle progression, reactive oxygen species (ROS) accumulation, depolarization of mitochondrial membrane potential (MMP) and apoptosis with the aid of FACS. Qualitative changes were demonstrated in respect of reactive oxygen species generation (ROS) and depolarization of MMP through fluorescence microscopy. DNA damage was assessed by DNA fragmentation assay and DAPI staining. Drug-DNA interaction was analyzed by circular dichroism (CD) spectroscopy. Both Bol and NBol reduced overall cytotoxic effects of cisplatin in normal cells, but had no or negligible effect on cancer cells. NBol protected normal liver cells from cisplatin-induced DNA damage, reduced ROS, re-polarized MMP, and reduced cisplatin's DNA binding ability leading to increased number of S-phase cell population at a greater scale than Bol. Both drugs reduced cytotoxicity of normal liver cells by targeting DNA and protecting from the adverse effect of cisplatin by interacting with DNA, presumably competing with cisplatin molecules.

Key words: PLGA- nano-encapsulation, boldine, cisplatin, DNA-targeting; cytoprotection, cell cycle, FACS.

INTRODUCTION

A low dose of Boldo (*Peumus boldus*) plant extract has recently been reported by us[1] to significantly reduce cisplatin-induced toxicity in normal liver cells, increasing their survivability after administration of cisplatin, while the co-administration did not significantly affect cisplatin-induced cytotoxicity in cancer cells. In furtherance of this work, we also recently reported[2] that Boldine (Boldine ([s]-2,9-dihydroxy- 1,10-dimethoxyaporphine) (Bol), the major bioactive alkaloid separated from Boldo crude extract having anti-oxidant and radical-scavenging properties, showed similar or stronger anticancer effect at a still lower dose and the effect could be further enhanced by nano-encapsulation with PLGA. However the question of whether Boldine and PLGA-loaded-Nano-Boldine (NBol) could act via DNA targeting and apoptosis induction, had not been addressed earlier. Therefore, our primary focus in this investigation was to ascertain if Bol and NBol acted via targeting DNA, apoptosis induction and by inhibiting cell cycle progression.

In recent years, DNA-targeted therapy has gained much importance and a drug's capacity to interact with DNA has been implicated to its capacity to hinder the process of cellular replication and protein synthesis, resulting eventually in cell growth arrest and apoptosis[3]. In this context, we tried to evaluate whether Bol/NBol has the capacity to interact with naked calf thymus DNA (ct-DNA), which had not been studied earlier.

Therefore, the hypotheses to be tested in the present study were: (i) if Bol/NBol could change ROS

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generation and depolarization of MMP, two important cellular events leading to induction of apoptosis; (ii) to examine if Bol/ NBol has DNA-binding ability by using ct-DNA as target, based on analysis of CD spectra, (d) if it can bring about any conformational change in the structure of DNA; and (e) if it does, to elucidate the possible relationship between its DNA binding behaviour and impact on cell-cycle, and (f) to correlate the findings on changes in respect of DNA-targeting, cell-cycle arrest and apoptosis.

MATERIALS AND METHODS

Chemicals and Reagents

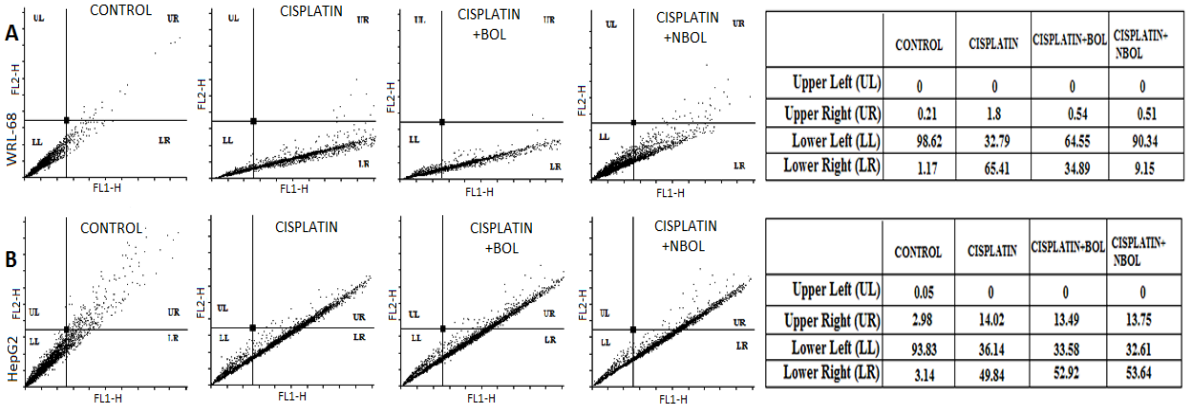
Pure form of Cisplatin [*cis*-Diamineplatinum(II) dichloride] and Boldine [$C_{19}H_{21}NO_4$[p-code 1001359485] were purchased from Sigma (USA). Dulbecco's modified Eagle medium (DMEM) and the antibiotics, namely, penicillin, streptomycin, and neomycin (PNS) were purchased from HiMedia, India. Fetal bovine serum (FBS), trypsin and ethylene di-amine tetra-acetic acid (EDTA) were procured from Gibco BRL (Grand Island, NY, USA). Tissue culture plastic wares were bought locally from Tarson, India. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) and rhodamine 123 were obtained from Sigma, USA. Acridine orange (AO) and ethidium bromide (EB) were purchased from SRL, India. Annexin V-fluorescein isothiocyanate (FITC) and primary antibodies were obtained from Santacruz Biotechnology Inc, USA. Secondary antibodies were purchased from Sigma, USA. All organic solvents used were of HPLC/analytical grade.

Preparation of blank nanoparticles and Boldine loaded nanoparticles

Boldine loaded nanoparticles were prepared by following the solvent displacement method[4]. At first, 10 mg Boldine was dissolved in 3 ml acetone and then 50 mg PLGA was further added to dissolve in the mixture. This organic phase mixture was added in a drop-wise manner (0.5 ml/min) to 20 ml of aqueous solution containing the stabilizer, F68 (1% polyoxyethylene–polyoxypropylene; w/v). The mixture was then stirred at 400 rpm by a laboratory magnetic stirrer at room temperature till the organic solvent was completely evaporated. The stabilizer was then removed from the nanoparticles by centrifugation at 25,000×g at 4 ◦C for 30 min. After that the pellet was re-suspended in Mili-Q water and washed thrice. Blank nanoparticles were also prepared in the same manner, but without adding Boldine.

Selection of doses

We used the same concentration/dose as standardized in our previous study (Mondal et al., 2015). Experimental sets were divided into 4 different groups: control (PBS treated), cisplatin treated (20µmol/L), cisplatin (20µmol/L) plus Bol treated (27µg/mL) and cisplatin (20µmol/L) plus NBol treated (27µg/mL).



	CONTROL	CISPLATIN	CISPLATIN+BOL	CISPLATIN+ NBOL
Upper Left (UL)	0	0	0	0
Upper Right (UR)	0.21	1.8	0.54	0.51
Lower Left (LL)	98.62	32.79	64.55	90.34
Lower Right (LR)	1.17	65.41	34.89	9.15

	CONTROL	CISPLATIN	CISPLATIN+BOL	CISPLATIN+ NBOL
Upper Left (UL)	0.05	0	0	0
Upper Right (UR)	2.98	14.02	13.49	13.75
Lower Left (LL)	93.83	36.14	33.58	32.61
Lower Right (LR)	3.14	49.84	52.92	53.64

Figure 1. Annexin V/PI assay indicating remarkable apoptosis induction when cisplatin was administrated on both WRL-68 cells (A) and HepG2 cells (B). Co-administration with Bol and NBol reduced apoptosis induced by cisplatin in normal cells (WRL-68) but there was no visible effect in cancer cells. In comparison with Bol, NBol result was more prominent. X-axis denotes annexin V-fluorescein isothiocyanate and Y- axis denotes propidium iodide (PI). The quadrant of lower left (LL), lower right (LR), upper right (UR) and upper left (UL) show the percentage of live (annexin-ve; PI-ve), early apoptotic (annexin+ve; PI-ve), late apoptotic (annexin+ve; PI+ve) and necrotic cells (PI +ve), respectively.

Annexin V assay

After treatment, HepG2 and WRL-68 cells were washed with PBS and fixed in chilled 70% ethanol. Cells were then treated with RNase (5 mmol/L) and incubated for 10–15 min in the dark, at 37 °C. Subsequently, cells were stained with annexin V and propidium iodide (PI) as described by Matassov et al., 2004[5]. The fluorescence intensities were determined by FACS (Fluorescence-activated cell sorting) using FL-1H filter for annexin V and FL-2H for PI (BD FACSCalibur, USA) to analyze apoptotic cell percentage. Data were analyzed with Cyflogic (v.1.2.1) software.

Intra-cellular ROS production and mitochondrial membrane depolarization analysis

Changes in ROS generation and mitochondrial membrane potential (MMP) were analyzed with fluorescence microscopic and flow-cytometric methods. After 24-hour incubation with the drugs under study, cells (WRL-68 and HepG2) were collected and fixed in 4% paraformaldehyde. Fixed cells were incubated for 20 min in the dark with one of two dyes: 10 µmol/L 2',7'-di-chloro-di-hydro-fluorescein diacetate (H2DCFDA) or 10 µmol/L rhodamine 123 for ROS production and MMP evaluation, respectively. After incubation, cells were examined under a fluorescence microscope and representative photographs were taken for qualitative analyses.

Drug-DNA interaction study

To pinpoint our focus on relative ability of Bol and NBol in reducing cisplatin-induced hepatotoxicity presumably through their differential DNA-binding ability, circular dichroism (CD) spectroscopy was performed. Cisplatin (20 µmol/L), cisplatin plus Bol (20 µmol/L + 27µg/mL), cisplatin plus NBol (20 µmol/L + 27µg/mL) were mixed separately with the ct-DNA and incubated for overnight. CD spectral analysis was made using Origin pro 8 software after the 24 hour incubation[6].

4',6-Diamidino-2-phenylindole (DAPI) staining for nucleosomal fragmentation assay

Cells were washed twice with PBS and fixed in 2% paraformaldehyde solution. Cells were incubated for 30 minutes in 5 µmol/L 4', 6-diamidino-2-phenylindole (DAPI) solution, prior to analysis under a fluorescence microscope, and representative photographs were taken for qualitative analyses.

DNA gel assay

DNA gel assay was performed in both HepG2 and WRL-68 cell lines for analysis of inter-nucleosomal DNA fragmentation. Cellular DNA was isolated using the conventional phenol-chloroform DNA extraction method, DNA was precipitated from the aqueous

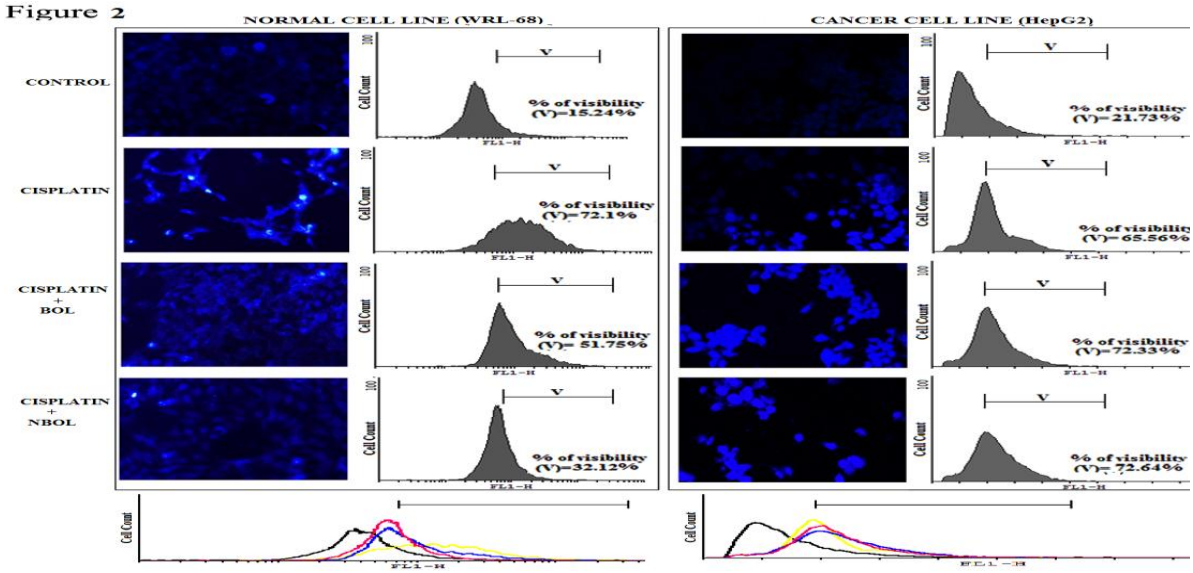


Figure 2. ROS generation was checked microscopically and flow-cytometrically for both WRL-68 (A) and HepG2 (B) cells. Cisplatin treatment increased ROS generation compared to control in both normal cells (WRL-68) and cancer cells (HepG2), but co-administration of NBol and Bol with cisplatin, respectively, reduced ROS generation only in normal cells. Here effect of NBol was more notable as compared to Bol.

layer with 100% ethanol and the precipitate was dissolved in 20 µL of Tris-EDTA buffer (10 mmol/L Tris-HCl at pH 8.0 and 1 mmol/L EDTA). This DNA solution was loaded into 1.5% agarose gel and separated by electrophoresis. Bands were visualized under a UV trans-illuminator and digitally photographed.

Cell cycle analysis

Cells of WRL-68 were fixed in 70% chilled ethanol. Fixed cells were then made RNA free by incubating in 10 mmol/L RNase for 10 min in the dark at 37 ºC. RNase-treated cells were then stained with PI (10 µmol/L; Sigma, USA) for 20 min. Fluorescence intensities were determined by FACS using a FL-2A filter (BD FACSCalibur, USA)[7]. Data were analysed with Cyflogic (v.1.2.1) software.

Statistical analysis

All data are expressed as mean±standard errors of three sets of data. Statistical significance was evaluated with analysis of variance (ANOVA) using SPSS Version 20. Comparisons among individual treatment groups were made by comparing data by the least square division (LSD) method. For all statistical tests, the threshold of $P<0.05$ was used to determine significance.

RESULTS

Annexin V assay

Cisplatin-treatment induced considerable number of apoptotic cell deaths in WRL-68 (Fig. 1A) although it caused more severe effects on cancer cells (Fig. 1B). Co-administration of NBol with

Figure 3

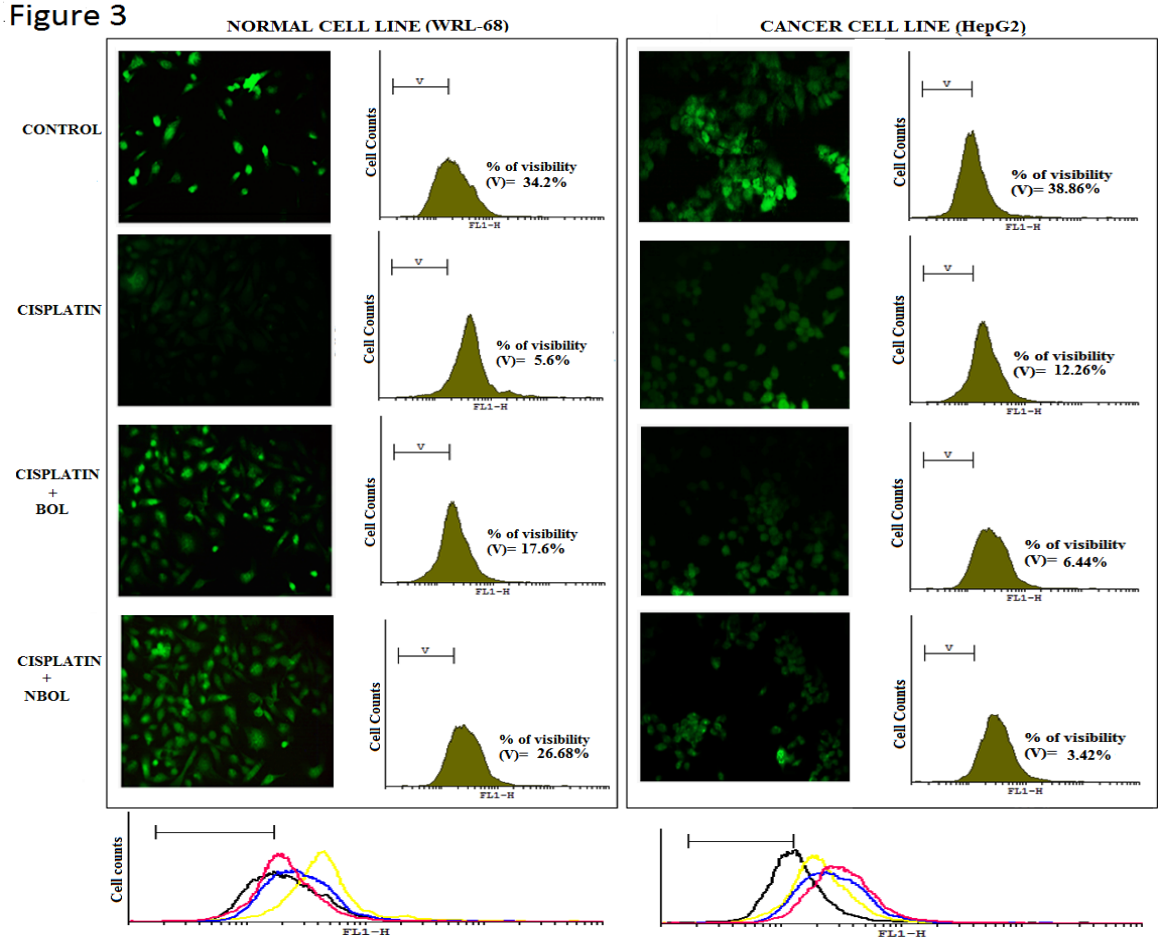


Figure 3. Mitochondrial membrane potential (MMP) depolarization was checked microscopically and flow-cytometrically in both HepG2 and WRL-68 cells with cisplatin, cisplatin plus Bol and cisplatin plus NBol treatment, respectively. Co-administration of cisplatin with Bol and NBol, respectively, decreased MMP towards normality in normal WRL cells, NBol to a greater degree, but not observed significantly in cancer cells (HepG2).

cisplatin reduced cell death more significantly than Bol in normal cells, but neither had any significant protective effect on the cisplatin-induced cytotoxicity in cancer cells.

ROS generation

Cisplatin treatment caused increase in ROS generation compared to normal control set but co-administration of cisplatin with either Bol or NBol reduced ROS generation in normal cells, more - administration of cisplatin with either Bol or NBol reduced ROS generation in normal cells, more prominently in the NBol conjoint treatment group (Fig. 2A). In HepG2 cells, both cisplatin treated and cisplatin plus Bol or NBol treated groups generated production of reactive oxygen species (ROS). This result is significant in that it clearly indicates the capability of both NBol and Bol to reduce cisplatin induced ROS generation (Fig. 2B).

Determination of mitochondrial membrane de-polarization

Only cisplatin treatment dramatically depolarized mitochondrial membrane potential (MMP) in both normal and cancer cell lines. Co-administration of cisplatin with either Bol or NBol reduced depolarization of mitochondria in the WRL-68 cells

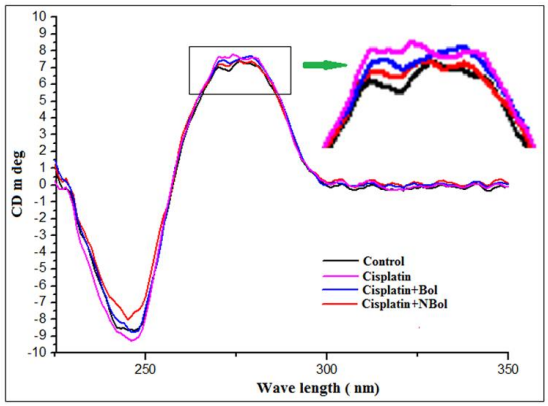


Figure 4. Drug-DNA interaction study; CD spectral analysis of calf thymus DNA-binding ability of cisplatin, co-administration of cisplatin with Bol and NBol, respectively. (B) GSH depletion in WRL-68 and HepG2 cells [LN1=control. LN2=cisplatin, LN3=cisplatin+Bol, LN4=cisplatin+NBol]. Data are represented as percentage of control and are presented as mean ± standard error of mean. Statistical significance was considered as *P<0.01 (for WRL-68) or #P<0.01 (for HepG2) versus untreated control.

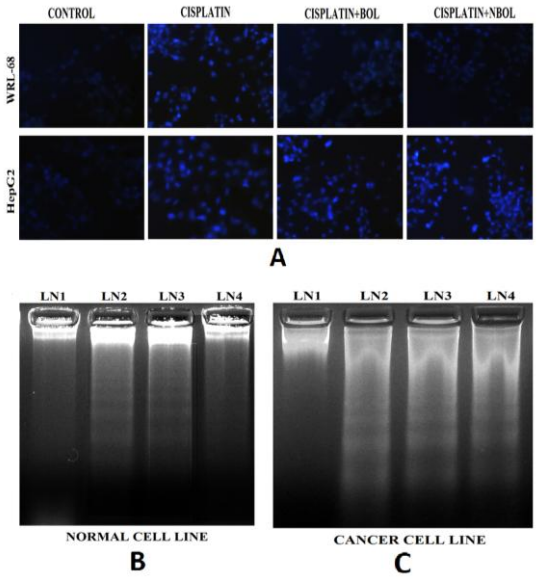


Figure 5. (A) DAPI staining; indicates that there was damage in DNA when cisplatin was administrated on both HepG2 and WRL-68 cells. Co-administration of NBol reduced the damage induced by cisplatin in WRL-68 cells but not in HepG2 cells more significantly than Bol. (B) and (C) DNA-gel assay; normal cells (WRL-68) (B) and cancer cells (HepG2) (C): reveals that cisplatin treatment in both normal and cancer cells induced DNA damage. Co-administration of NBol reduced damage in DNA of normal cells but not in cancer cells more significantly as compared to that by Bol.

but not significantly in HepG2 cells. The result further indicates that NBol yielded better effect to reduce cisplatin-induced depolarization of MMP in normal cell lines (Fig. 3).

Drug-DNA interaction study

Analysis of CD spectra revealed that cisplatin interacted quite strongly with ct-DNA and caused extensive damage. However, when NBol was co-administered with cisplatin, there appeared to be some distinct changes in the spectral pattern which could be attributed to the effect of NBol treatment. Further, co-administration Bol produced spectra seemingly located at somewhat intermediate position between that of only cisplatin and cisplatin plus NBol treatment (Fig. 4).

Qualitative estimation of DNA damage: DAPI staining

Wide-ranging damage of cellular DNA was observed in both WRL-68 and HepG2 cells treated

with cisplatin alone. Co-administration of cisplatin with either Bol or NBol resulted in reduction of DNA damage in normal cell line (WRL-68) as revealed from the decreased fluorescence intensity, and the decrease was more markedly demonstrable in the NBol treatment group. However, DNA damage of HepG2 cancer cells was apparently not reduced by either Bol or NBol co-treatment group along with cisplatin. Thus, when NBol and cisplatin were combinedly treated, they preferentially protected normal cells by reducing DNA damage, while no significant preventive change occurred in DNA damage induced by cisplatin the cancer cells (Fig. 5A).

Confirmatory test on DNA damage: Fragmentation assay

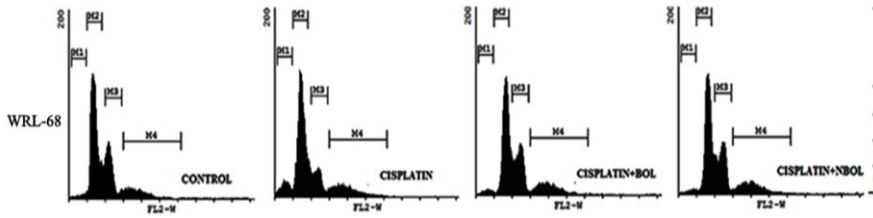
Cisplatin-induced DNA damage was further confirmed in both WRL-68 (Fig. 5B) and HepG2 (Fig. 5C) cells by DNA fragmentation assay which showed positive results. Co-administration of both Bol with cisplatin and NBol with cisplatin reduced the DNA damage in the normal liver cells, NBol showing more pronounced effect than that of Bol. However, no significant change in cisplatin-induced DNA damage could be observed with co-administration of either Bol or NBol in the cancer cells, showing the ability of the drugs to protect the normal cells rather than the cancer cells.

Cell cycle analysis

Treatment with cisplatin alone caused an increase in Sub-G population of WRL-68 cells and decrease of S-phase populations. Co-administration of cisplatin with either Bol or NBol caused reduction in the number of cells at sub-G stage, and a corresponding increase in number of cells at S-phase in WRL -68 cells, more obvious in the NBol plus cisplatin treated cells (Fig. 6).

DISCUSSION

Results of our present study showed that cisplatin caused severe damage to the DNA of cancer cells that might be the reason for causing death to the cells. Incidentally, one major way through which cisplatin exerts its anti-cancer effect is believed to be by binding with DNA and causing detrimental structural changes to the DNA, thereby interfering with both replication and transcription processes, and resulting in prevention of active proliferation and growth of cancer[8,9]. But cisplatin also damaged DNA of normal cells, thereby interfering with normal metabolic activities and divisional processes of normal cells as well. Results of this study further show that both Bol and NBol also had ability to bind with DNA, apparently NBol having greater ability. This was evident from our results on CD-spectrometric analysis. Therefore, it could be quite possible that NBol and Bol competed with cisplatin for binding with active sites of DNA more effectively in normal cells than in cancer cells, showing preferential protection to the normal cells. This inference could be drawn from our results in respect of the CD spectra obtained in the different series of treatment. DNA fragmentation assay results also supported this contention. Such a hypothesis gains further ground because in the FACS analysis of cell cycle, cells were found to be arrested at sub-G stages by only cisplatin treatment, while in the co-treatment group, the cell population in normal cells was not found to be blocked at Sub G stage. Thus, in other words, cell cycle analysis results revealed reduction of S-phase cell population by cisplatin with respect to the controlled cells without cisplatin treatment as compared to the co-treatment groups where more number of cell population was found in the S phase. Spectral analysis of DNA of cells which had undergone co-treatment of Bol/NBol and cisplatin indicated that there was a significant



Treatment set	% of visibility of total cell	
	M3= S phase	M1=Sub G phase
CONTROL	32.58	1.6
CISPLATIN	14.23	8.3
CISPLATIN+BOL	28.63	2.48
CISPLATIN+NBOL	28.84	2.27

Figure 6. Flow-cytometric analysis of cell cycle in cisplatin, cisplatin with Bol and cisplatin with NBol treated normal cells (WRL-68) indicated that increase in number of sub-G cells with reduction in S-phase population, while cisplatin alone was administrated. But both Bol and NBol treatments along with cisplatin reduced sub-G cells and increased DNA synthesis [M1=Sub G, M2=G0/G1, M3=S, M4=G2/M]. Y axis denotes counts of cells.

change in the binding pattern with the DNA, as compared to that of only cisplatin treatment group. Structural changes in the DNA molecule have also been claimed to generate reactive oxygen species (ROS) inside the cells by other workers[10,11,12] which was also evident in our study.

Additionally, elevated ROS level can also cause damage to DNA, inducing cellular death[13]. Our study on DNA damage by fragmentation assay revealed that cisplatin exposure generated DNA damage and co-treatment with Bol/NBol helps to protect DNA in normal cells and to reduce ROS level, but not in cancer cells.

Nanocarriers appear to be a promising system of drug delivery for several advantages, such as increased protection of encapsulated drug targeting specific sites for localized action[14]. However, among several known carriers, PLGA has gained importance for the encapsulation of a wide variety of drugs as it is biodegradable, biocompatible, capable of controlled release of the incorporated entity, and considered efficient carrier system for the delivery of drugs within the cells[15] and less toxic[16] in nature. Further, PLGA has the ability to form stable nanoparticles, and has already been approved for use in humans by the US Food and Drug Administration[17]. Therefore, overall results point out that PLGA-loaded NBol at a low dose could act as a better supportive drug than Bol in cisplatin oncotherapy, and has great potential for being used in future drug formulation for its non-toxic nature and greater efficacy.

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Conflict of interest

There is no conflict of interest to declare.

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