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| **Authors** | Irem Taşkın, Özlem Şen, Melis Emanet, Mustafa Culha and Bayram Yılmaz |
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| **ORCID® iDs** | Irem Taşkın - https://orcid.org/0000-0001-8388-7249; Özlem Şen - https://orcid.org/0000-0002-6237-0264; Mustafa Culha - https://orcid.org/0000-0002-3844-5190 |

# Biocompatibility evaluation of hexagonal boron nitrides on healthy mouse hippocampal cell line and their positive effect on stressed cells

Irem Culha Taskin[2], Ozlem Sen[1], Melis Emanet[1], Mustafa Culha[1], Bayram Yilmaz[2]

[1] *Department of Genetics and Bioengineering, Yeditepe University, Istanbul 34755, Turkey*

[2] *Department of Physiology, Faculty of Medicine, Yeditepe University, Istanbul 34755, Turkey*

## Abstract

In this study, hexagonal boron nitrides (hBNs) and their degradation product, boric acid (BA), are comparatively evaluated to investigate their biocompatibility and oxidative stress relieving effects on embryonic mouse hippocampal cell line (mHippoE-14). First, cell viability is assessed for a wide concentration range of 4.40-440 μg/mL of boron (B) containing hBNs and BA for 24 and 72 h exposures. Then, cell cycle, reactive oxygen species (ROS) generation, and cell death mechanisms are investigated at a concentration range of 4.4-44 μg/mL with increased incubation times. Finally, the influence of hBNs and BA on apoptotic body formation is monitored to analyze their nuclei integrity with confocal microscopy. Both hBNs and BA are found to be not cytotoxic at lower than 22 μg/mL B containing concentrations, and hBNs are much less cytotoxic compared to BA. At low concentration of hBNs, no detectable change in cell cycle, ROS production and DNA damage are observed. The study was further extended by exposing the cells to doxorubicin (DOX) to cause stress on cells before treating with hBNs and BA to investigate their positive effect on cellular metabolism. It is found that both hBNs and BA helps to increase the cell viability after the exposure to DOX. The results are envisioning the exploitation of hBNs as biocompatible agents for their possible biomedical applications including mitigating oxidative stress caused by various drugs used for neurological diseases and brain cancers. Since hBNs slowly degrade in biological media, they can be used as a controlled B releasing agent as compared to ionic BA in addition to their nanocarrier feature.

**Keywords:** Biocompatibility; boric acid; doxorubicin; hexagonal boron nitride; neuronal cells.

## INTRODUCTION

Neurons are the main cells composing the central nervous system (CNS) and carry out numerous functions. A damage to neurons caused by an infection, trauma, degenerative disorder or cancer can lead to the loss of certain functionalities [1]. In most instances, disease progression cannot be stopped or delayed with the current neurological disease treatment strategies due to the difficulties of transporting therapeutic drugs or agents across the blood-brain barrier (BBB) into the nervous system to target the proper regions of the brain [2]. Therefore, novel carriers and tools are required to overcome these difficulties for effective treatment.

Nanomedicine refers to the application of nanomaterials in medicine and a myriad of nanoparticles are evaluated for their use as nanocarrier in recent years [3]. Among many nanomaterials, boron-based ones, especially boron nitride nanotubes (BNNTs) and hexagonal boron nitrides (hBNs) are recently gaining attention as nanocarriers due to their low toxicity, high stability and flexibility, and positive effects on cell viability possibly through their degradation products [4,5]. hBNs, also called white graphene, were discovered in 1842 but their first stable forms were able to be synthesized almost 100 years later then their discovery [6]. In hBNs, boron (B) and nitrogen (N) atoms are connected through a honeycomb-shaped covalent bonding similar to graphene. Since the 2D layers of hBNs are stuck on each other through weak Van der Waals interactions, they form multilayered hBN films or spherical structures [7-9]. Similar to BNNTs, hBNs are also evaluated as nanocarrier due to large surface area, possible biocompatibility and low cytotoxicity. The hBNs are already used in 483 cosmetic formulations mainly as a slip modifier up to 25% [10,11]. Although they are used as filler in cosmetics, there are not many studies evaluating the mode of action of hBNs in biological systems. Both biocompatibility and cytotoxicity of hBNs have been gaining attention in recent years [12-14]. Their hydroxylated from was evaluated as nanocarrier [15]. It was found that hydroxylated BNs were biocompatible and it was possible to load an anticancer drug, DOX, up to contents three times exceeding their own weight. The DOX loaded BN carriers exhibited much higher potency for reducing the viability of LNCaP cancer cells compared to free drugs. In another report, hBNs were loaded with both DOX and folate to target cancer cells [16]. The cancerous HeLa cells and healthy HUVECs were used in the study to comparatively evaluate the hBNs and BNNTs as nanocarriers. In another study,

hBNs were reported to suppress the growth of prostate cancer in mice [17]. In that study, it was also predicted that hydrolyzed product of hBNs could be BA.

Boron is a common element found in Archaea, Bacteria and Eucaria [18]. Since they are involved in cellular metabolism, boron containing biomolecular structures are also vital for the human health [19-21]. Although their action of mechanisms in the biologic systems are not clear yet, the boron containing compounds including boric acid/borates are investigated for their therapeutic effects [22,23]. Among the boron containing compounds, boric acid (BA)/borate is widely investigated for its possible health and curing effects [24,25]. The preventive effect of BA on oxidative stress was also reported [26]. Moreover, BA has an important place in our study as a hydrolyzed product of hBNs. Therefore, the both hBNs and BA were evaluated for their cytotoxicity using mHippoE-14 cells with several tests including cell viability, cellular uptake, cell cycle analysis, ROS generation and cell death mechanisms. The influence of hBNs on cell nuclei integrity was monitored by confocal microscopy. Further, the study was extended by exposing the cells to doxorubicin (DOX) to cause stress before treating with hBNs and BA to investigate their positive influence on cellular metabolism. The results of the study were encouraging to extend the investigations of hBNs for their applications in medicine.

## RESULTS AND DISCUSSION

### hBNs synthesis

The synthesized hBNs were characterized with spectroscopic and imaging techniques. Figure 1A shows the schematic representation of two layers of hBNs. As seen, B and N atoms are connected through a hexagonal network. As mentioned earlier, layers of hBNs are held together through weak Van der Waals interactions and naturally several layers are stuck together to form thicker layers with round and flat shape as seen in the TEM image in Figure 1B. The average lateral size dimension of hBNs is about 50 nm with an almost round shape. The FT-IR spectrum of the hBNs shows broad peaks at around 1364 and 820 $cm^{-1}$ that is attributed to the B-N and B-N-B vibrations, respectively (Figure 1C). hBNs are quite hydrophobic, and their dispersion in aqueous media requires long sonication times. Figure 1D shows DLS spectra of hBNs demonstrating size distribution in water and cell culture medium. The average size of hBNs in water and cell culture media is found to be 80 and 190 nm, respectively. Note that the size of hBNs is larger in aqueous medium due to solvation by

water molecules compared to the size observed on TEM images. In addition, when hBNs are added into cell culture medium, the size of hBNs gets larger since proteins and other small molecules are adsorbed onto the hBNs surfaces. It is also possible that hBNs can adhere together forming larger aggregates in cell culture medium.

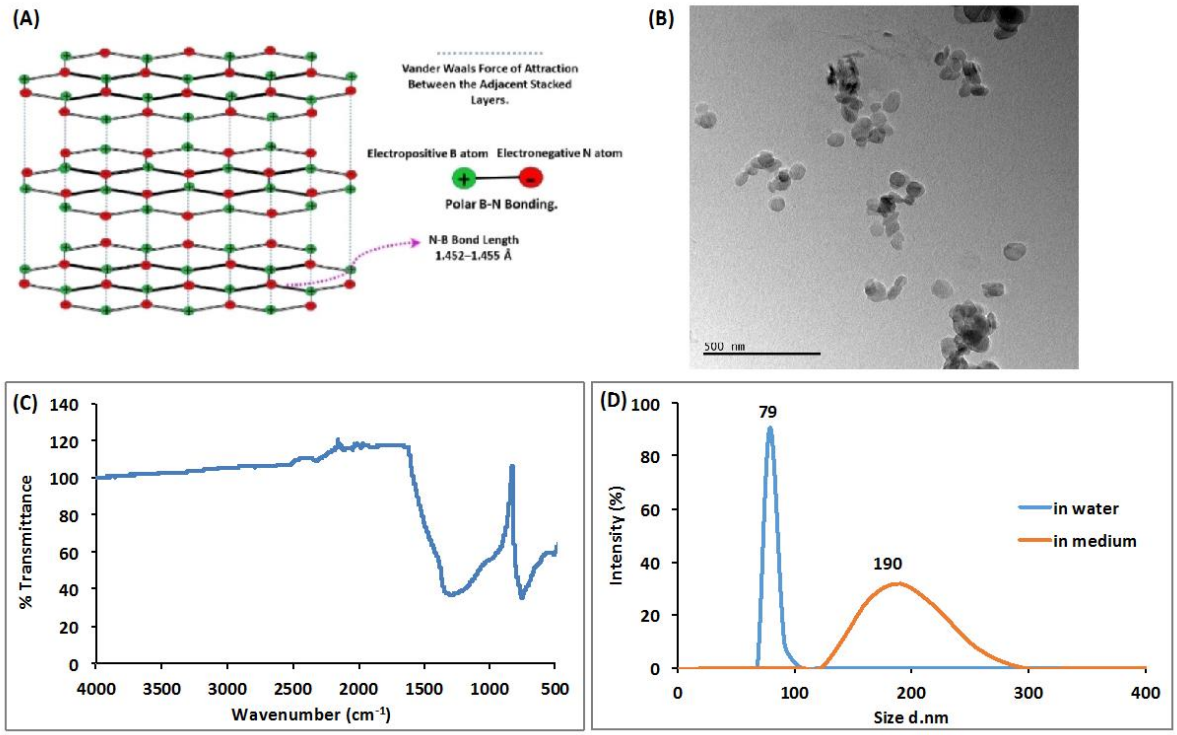


**Figure 1.** (A) Schematic representation, (B) TEM image and (C) FT-IR spectrum of hBNs. (D) DLS spectra of hBNs in water and cell culture media. Scheme in (A) reproduced with Permission from [Nirmalendu, et al. 2018]; Copyright [John Wiley and Sons] 2018.

## Cell Viability

The actual boron concentrations in hBNs and BA samples were used since boron is considered to play a role in the modulation of cell viability [28]. Thus, hBNs and BA concentrations containing from 4.4 to 440 µg/mL actual B were used instead of reagent concentrations. Figure 2 shows the cell viability assessment with WST-8 assay for 24 and 72 h of hBNs and BA exposures. As seen, hBNs are less cytotoxic compared to the same concentration range of BA for 24 and 72 h of exposure times. Figure 2A shows the viability of cells to hBNs and BA for 24 h. As seen, both hBNs and BA causes a concentration dependent decrease in cell viability while hBNs show a less cytotoxic effect. Figure 2B shows

the cells exposed to the hBN and BA for 72 h. It is clear that the cell viability increases in the cells exposed to hBNs compared to the cells exposed to BA. The difference in the cell viability increase of hBNs and BA exposed cells has become more significant as the concentration is increased. It is also clear that hBNs show much lesser cytotoxic effect than BA. The reason for the increased cell viability after 72 h of hBNs exposure can be attributed to the increased release of degradation products from hBNs with the increased incubation time. Please note that the standard deviation of hBN concentrations including 220, 330 and 440 µg/mL of B are rather higher due to the variation in the aggregation of hBNs in culture media as their concentration increases.

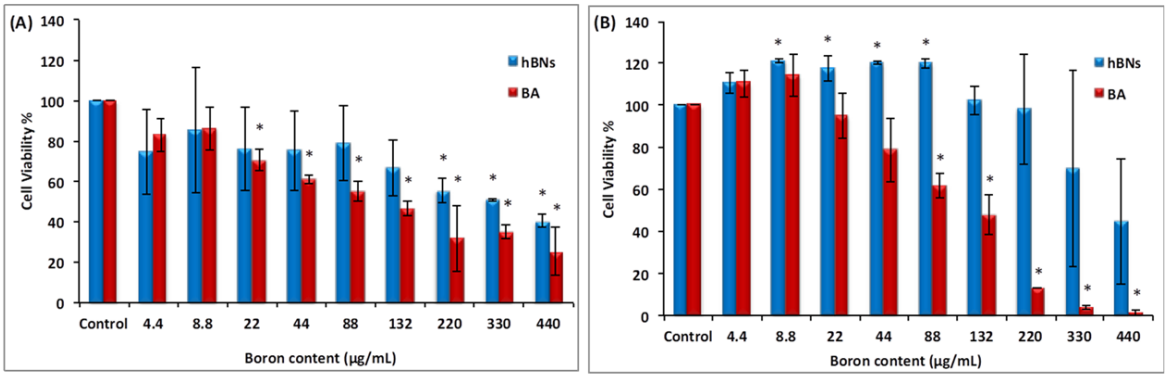


**Figure 2.** Cell viability after (A) 24 h and (B) 72 h of hBNs and BA treatment. The results are the average of two repeats. *$p<0.05$.

Although an increase in the cell viability with the treatment of both hBNs and BA is not observed with 24 h of treatment, a clear increase, especially with 44 µg/mL of B including hBNs treatment is observed. This suggests that the tested concentrations should have been lower than 44 µg/mL. Thus, three concentrations, 4.4, 22 and 44 µg/mL, were decided for the rest of the studies.

## Cellular Uptake

The cellular uptake of hBNs was investigated for 24 h (Figure 3A) and 72 h (Figure 3B) incubation times. The increase in SSC signal was used as a guide to analyze the cellular uptake of the hBNs. 24 h exposure of hBNs containing 4.4, 22 and especially 44 µg/mL B content showed a significant increase in cellular uptake with about 26% at the highest concentration. Moreover, following the 72-h incubation, 22 and 44 µg/mL B containing hBNs

caused a significant increase in cellular uptake with about 18%. The decrease in the hBNs uptake at the increased incubation times can be attributed to the death of the cells due to the high doses of hBNs.

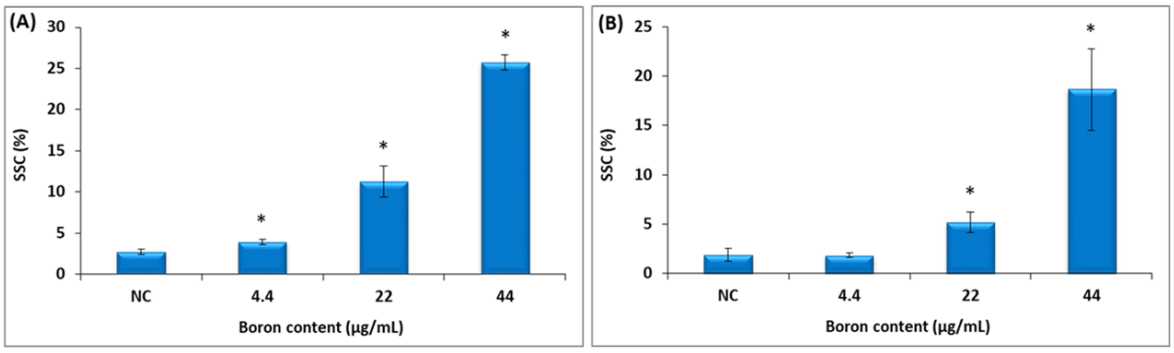


**Figure 3.** SSC intensity of cells after being exposed to increasing concentrations of hBNs after (A) 24 h and (B) 72 h. *p<0.05.

**Cell Cycle Analysis**

In this part of the study, the influence of hBNs on mHippo E-14 cell cycle stages was investigated for 24 and 72 h of incubation times. Figure 4 shows the cell cycle analysis results. The results indicate that there is no significant difference ($p<0.05$) in the cell cycle stages for all tested concentrations when the cells are exposed to hBNs and BA for 24 h exposure time as shown in Figure 4A and B. The S phase is the time when the cells undergo DNA replication. With the 24 h-hBNs exposure, as seen, no irregularity in DNA replication is observed. Furthermore, the hBNs do not cause significant changes at G0/G1 and G2/M phases either. However, as shown in Figure 4C and D, the hBNs cause a significant decrease (with about 12%) in G0/G1 phase and a significant increase (with about 25%) in G2/M phase at 22 µg/mL hBNs after 72 h of incubation. BA also causes a significant decrease (with about 50%) in G0/G1 at all concentrations following 72 h of incubation. The S phase significantly increased (25%) at 4.4 µg/mL BA, which shows increased cellular proliferation. A significant increase (with about 58%) in G2/M phase at 4.4 µg/mL is observed. As the concentration of BA increases, an increasing arrest rate in G2/M phase is observed; 70% at 22 µg/mL and 83% at 44 µg/mL.

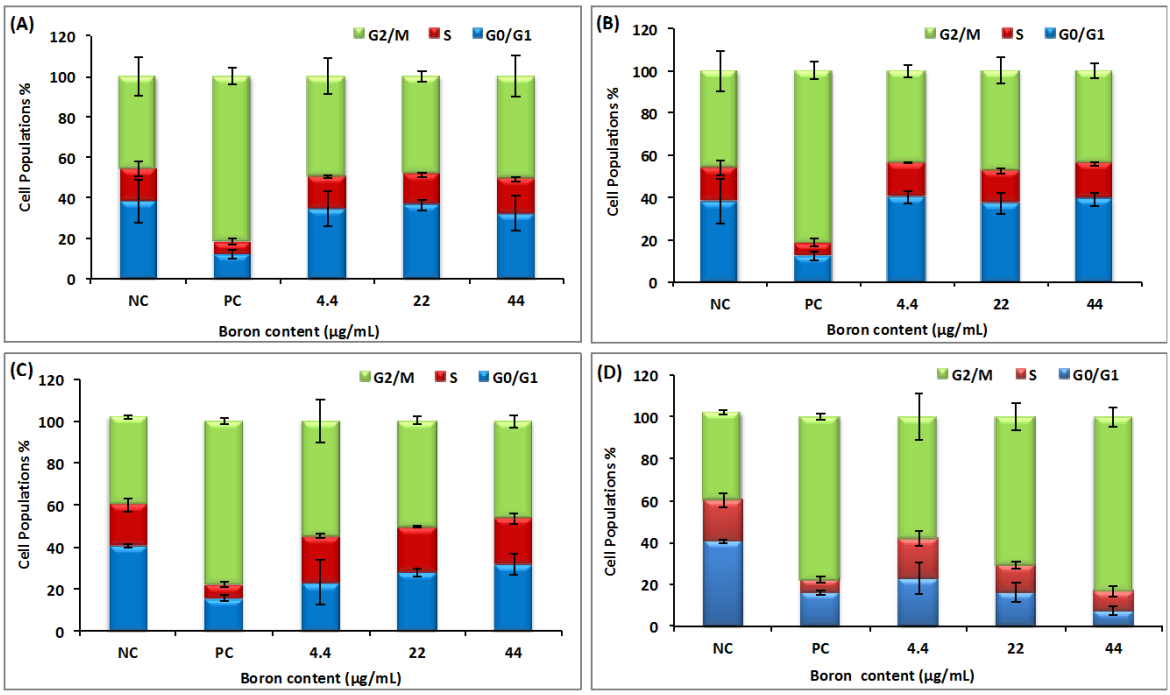


**Figure 4.** Cell cycle analysis of (A) hBNs and (B) BA for 24 h, (C) hBNs and (D) BA for 72 h treated mHippoE-14 cells.

## ROS Generation

The cancer cells are metabolically more active and are under an increased oxidative stress due to dysfunctional metabolic regulation with uncontrolled cell proliferation and increased ROS generation [29]. The ROS production in mHippo E-14 cells exposed to hBNs and BA for 24 and 72 h were monitored. The concentrations; 4.4, 22 and 44 µg/mL, were used in the experiments and the results are provided in Figure 5. As seen, with the 24 h exposure time, only 44 µg/mL B containing hBNs had a significant increase in ROS production with 2.8% of DCF intensity while there was no significant change in ROS production for all other concentrations. Meanwhile, the ROS production is not significantly increased in BA exposed cells at the 24 h of incubation as shown in Figure 5A.

When the cells were exposed to hBNs for 72 h, a significant increase in ROS production at 44 µg/mL B containing hBNs were observed as given in Figure 5B. For BA, a significant increase in ROS production was observed at each concentration levels. The results indicate that the high concentrations of hBNs at each incubation times stimulate the ROS production in the cells while BA significantly stimulated ROS production for each concentration at increased incubation times.

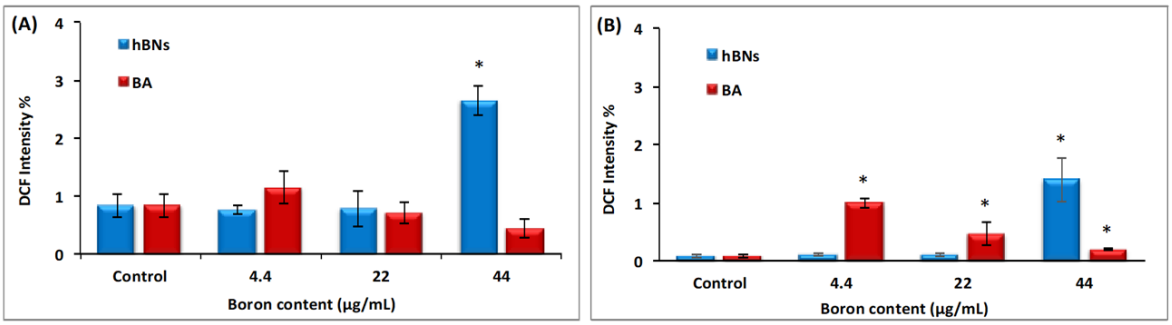


**Figure 5.** ROS analysis of (A) 24 h and (B) 72 h of hBNs and BA treated mHippoE-14 cells. *p<0.05.

## Cell Death Mechanism

The death mechanism of mHippo E-14 cells was also investigated for 24 and 72 h exposure times. As seen on Figure 6, with the 24 h treatment, all concentrations of hBNs had no significant effect on viability, necrosis and apoptosis excluding 44 µg/mL. At this highest tested concentration, the cell necrosis was increased from 10% to 15% and the cell viability decreased from 84% to 73% as shown in Figure 6A. All BA concentrations at 24 h incubation showed no significant effect on cell viability and necrosis except 22 µg/mL B containing BA, which caused a significant increase of early apoptosis from 3% to 6% and a 15% significant increase in late apoptosis (Figure 6B).

The hBNs showed no significant effect on necrosis at all tested concentrations while a significant increase from 1% to 10% in late apoptotic cells was observed when incubation was increased to 72 h as shown in Figure 6C. In addition, a significant increase in early apoptosis at 44 µg/mL was observed. However, a significant decrease in cell viability and a significant increase in necrotic cells for all tested concentrations of BA were observed.

The reason behind the decrease in cell viability and the increase in late apoptosis exposed to hBNs could be due to the stress caused increased incubation time of 72 h when compared to the cell line, which is needed a passage almost every 48 h. As the incubation time increases, the media volume decreases, and more cells come into contact with each other and cause more stress.

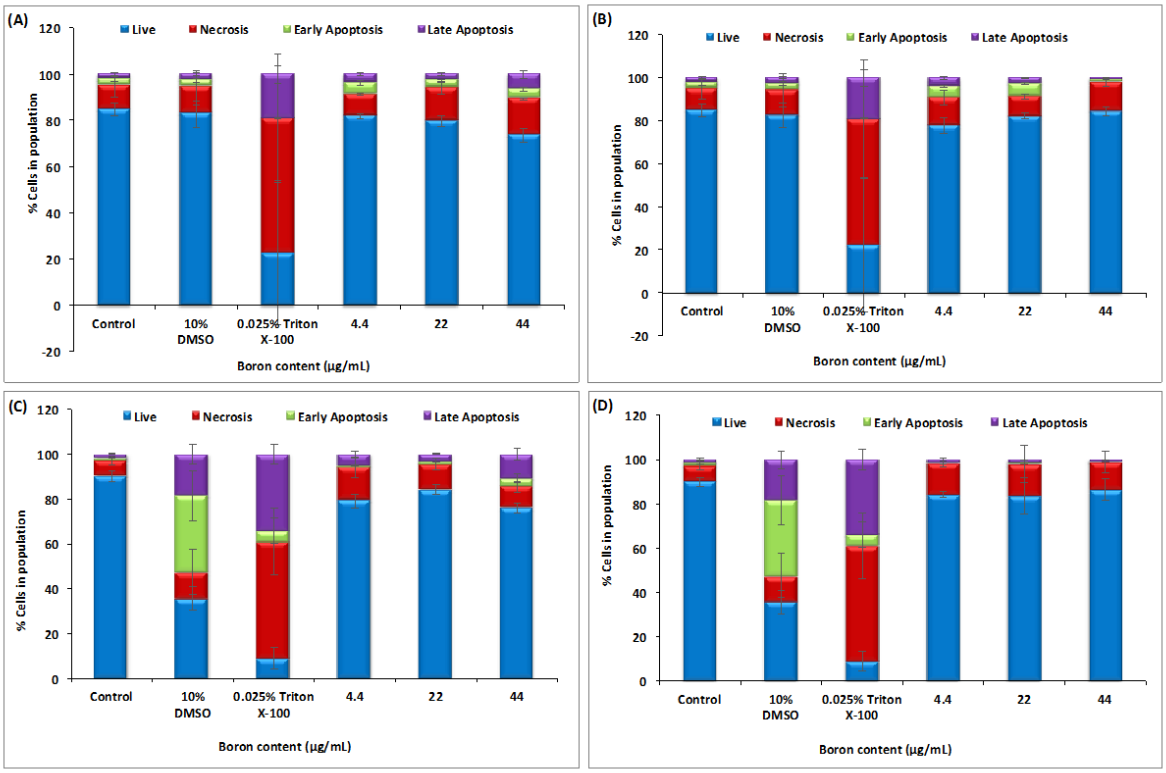


**Figure 6.** Cell death mechanism detection of (A) hBNs and (B) BA for 24 h, (C) hBNs and (D) BA for 72 h treated mHippoE-14 cells.

## Nuclei Imaging

The nuclei of the mHippo E-14 cells were monitored using confocal microscopy imaging at 4.4, 22 and 44 µg/mL of B containing hBNs and BA treatment for 24 and 72 h. The images are provided in Figure 7 and 8. The results indicate that no detectable changes in the cell nuclei integrity including apoptotic formations were observed at all tested concentrations and incubation times.

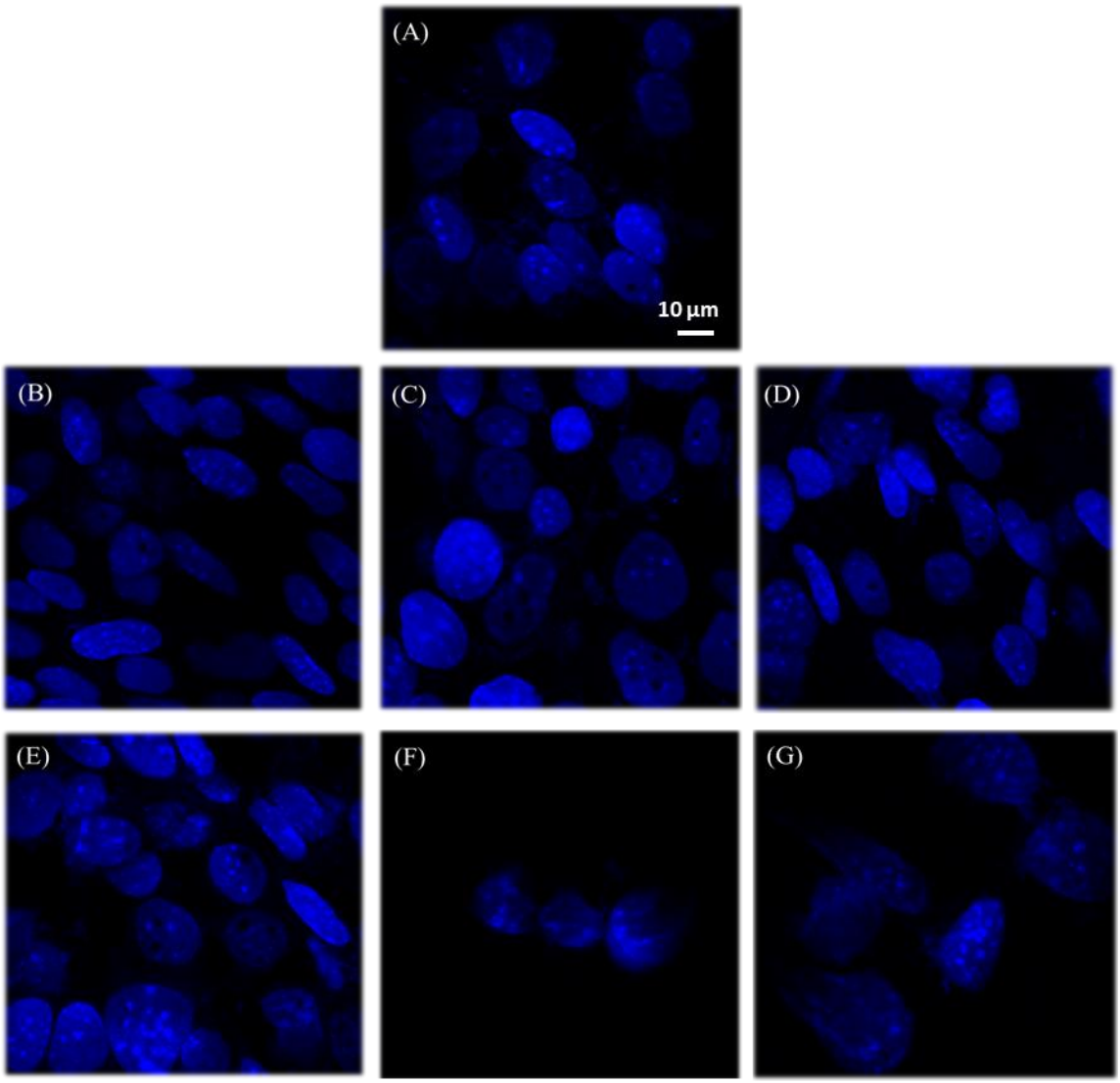


**Figure 7.** Detection of apoptotic bodies in cells treated with hBNs and BA exposed for 24 h. (A) Control cells, (B) 4.4, (C) 22 and (D) 44 µg/mL B containing hBNs with 24 h exposure, and (E) 4.4, (F) 22 and (G) 44 µg/mL B containing BA with 24 h exposure.

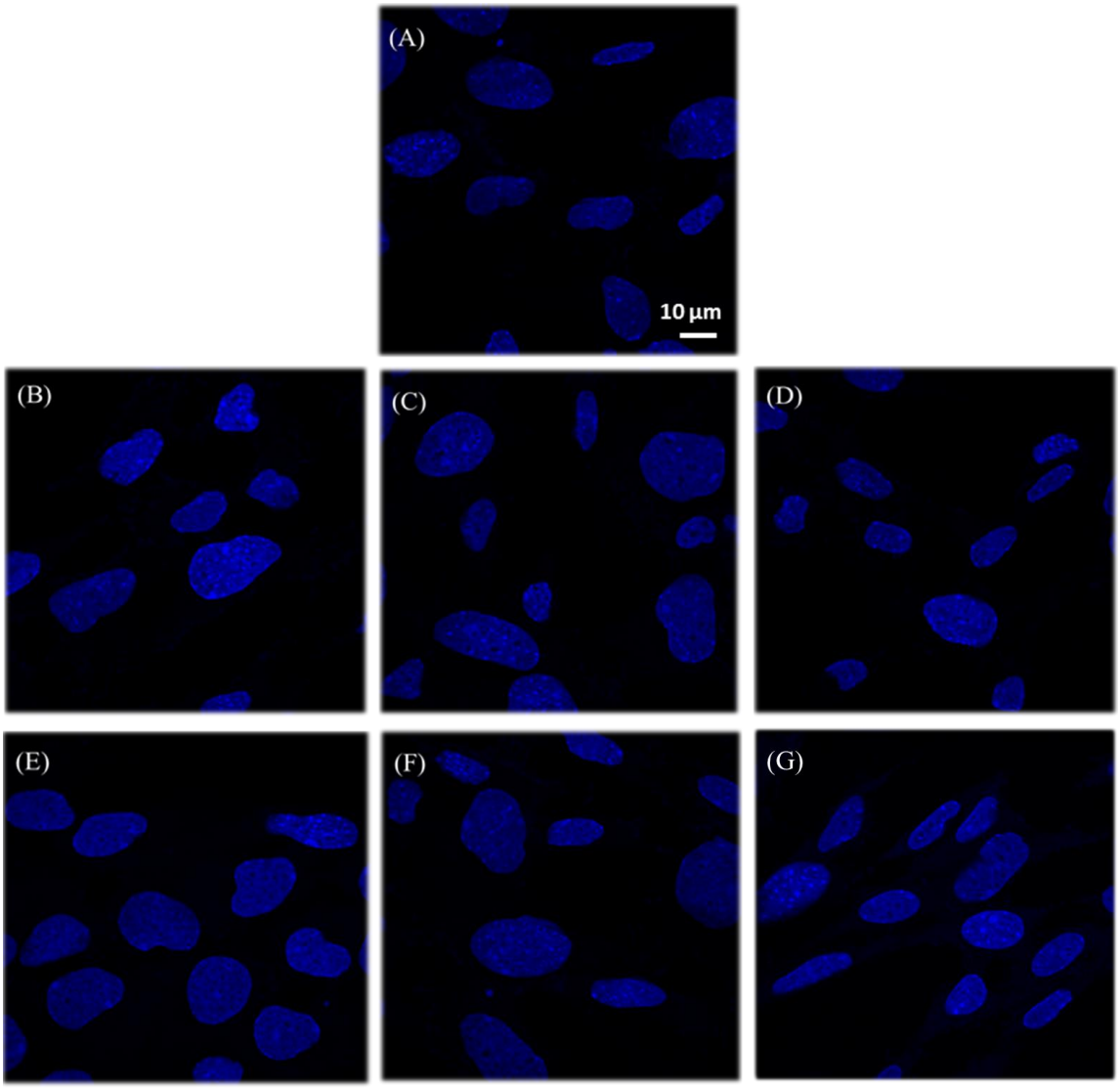


**Figure 8.** Detection of apoptotic bodies in cells treated with hBNs and BA exposed for 72 h. (A) Control cells, (B) 4.4, (C) 22 and (D) 44 µg/mL B containing hBNs with 72 h exposure, and (E) 4.4, (F) 22 and (G) 44 µg/mL B containing BA with 72 h exposure.

**Cell Viability Analysis of hBN treated cells after DOX exposure**

The aim of the study was to understand whether hBNs and BA could help to decrease the stress caused by therapeutic drugs such as DOX. DOX, is a chemotherapeutic agent that produces ROS reactive superoxide anions by redox cycling of the quinone moiety in the presence of molecular oxygen [30]. DOX is al1so a fluorescent neurotoxin, which can be transported in the reverse direction in the brain and can kill neurons in the brain [31]. As DOX is used for cancer treatment, killing neurons results in unbearable side effects [32]. In this study, hippocampal cells were used to determine whether hBNs and BA could possibly

decrease the deleterious effects of drugs on healthy cells or tissues such as DOX. Thus, the cells exposed to 4 µM DOX for 5 h, just enough concentration and time, to cause stress on the cells. After the exposure, the hBNs and BA were added into the cell culture medium at increasing concentrations and the cells were incubated for 24 and 72 h. As it is seen on Figure 9A, the group only treated with 4 µM DOX has significantly decreased the cell viability to compared to the control group, but when exposed to hBNs or BA containing equal concentrations of boron, the cell viability significantly increased ($p<0.05$) compared to the group only exposed to 4 µM DOX. As it did not reach the control groups cell viability level, it still showed a significant increase in cell viability at 24 h exposure. 72 h exposure of hBNs or BA showed a significantly higher increase in the cell viability compared to 24 h exposure. The cell viability increase seen in 72 h exposure is similar to the cell viability of the non-treated group.

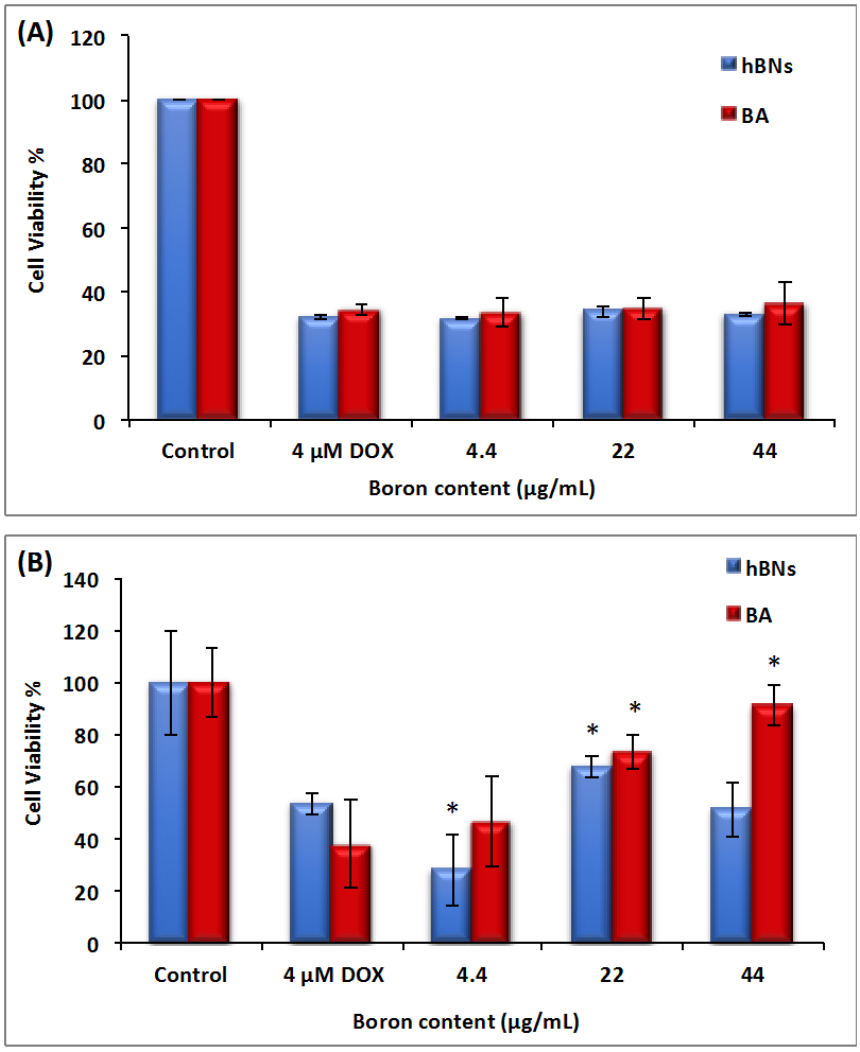


**Figure 9.** Viability of DOX exposed mHippo cells 24 h (A) and 72 h (B) exposure of hBNs and BA. *$p<0.05$.

The results show that as incubation time increases, hBNs and BA concentrations cause cell viability to significantly increase, almost as high as in the control group. The results suggest that both hBNs and BA can be used as therapeutic agents to relieve oxidative stress caused by an anticancer-drugs such as DOX, which have prolonged toxic effects after the treatment.

## CONCLUSION

In conclusion, this study reveals that both hBNs and BA are not cytotoxic at lower than 22 µg/mL boron containing concentrations on brain neuronal mHippo E-14 cells. However the BA cause significant cell viability decrease at high concentrations while hBNs are much less cytotoxic than BA. When a low concentration of hBNs is used, there is not almost a detectable change in cell cycle, ROS production and DNA damage. Although both hBNs and BA helped to increase the cell viability after exposure to DOX used to cause a reasonable stress, the slow degradation of hBNs offers a controlled release feature for possible use not only in the treatment of neurological disorders, but also to relieve the stress caused by the treatment. Besides, the serious cell viability decrease at the high concentration of BA emphasize the importance of treating the cells with low concentration of BA released form even at the high concentrations of hBNs. The results of this study suggest that hBNs should be further investigated for their use as possible therapeutics to relieve stress caused by drugs used to treat several diseases.

## EXPERIMENTAL

### Synthesis and Characterization of hBNs

The synthesis of hBNs was performed by firstly suspending 2 g of boric acid in 3 mL of 13.38 M ammonia solution. This mixture was transferred onto a silicon carbide boat and dried on a hot plate adjusted to 100°C for approximately 20 min. Then, this silicon carbide boat was placed in a Protherm Furnace PTF 14/50/450 and heated until 1300°C with a heating rate of 10°C/min under ammonia gas flow for 2 h. Following the heating, the silicon carbide boat was removed from furnace at around 550°C, and the hBNs were scratched from the surface of the silicon carbide boat with the help of spatula and stored at room conditions. The synthesized hBNs were characterized with imaging and spectroscopic techniques such as

TEM and IR. TEM images were obtained using a JEOL-2100 HRTEM microscopy system at 200 kV (equipped with LaB6 filament and an Oxford Instruments 6498 EDS system). A Thermo NICOLET IS50 Spectrometer was used in order to acquire IR spectrum [33].

**Cell culture**

Embryonic mouse hippocampal cell line (mHippoE-14) was utilized to assess the cytotoxicity of hBNs and BA, which is used as control boron including agent. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin/ampicillin (PSA) antibiotic solution. The cells were incubated in water-jacketed incubator in a 5% $CO_2$ and 95% air atmosphere at 37°C.

**Cell Viability**

Cells were seeded into 96-well plates at a density of $5\times10^3$ cells/well and incubated for 24 h. Then, the culture media were replaced with hBNs or BA containing media at concentrations suitable to achieve 4.4, 8.8, 22, 44, 88, 132, 220, 330 and 440 µg/mL of B. After 24 and 72 h of incubation, the media were removed, and the cells were washed with PBS. Then, they were replaced with fresh culture media containing WST-8 reagent with 1:10 ratio and incubated for a further 1 h. The cell viability upon exposure to varying concentrations of hBNs and BA was calculated by measuring the absorbance of formed formazan salts at 450 nm with a microplate reader.

**Cellular Uptake**

Cellular uptake of the hBNs was investigated by using a flow cytometer (Guava® easyCyte Flow Cytometry System) based on side scattering (SSC) values. The mHippo E-14 cells were seeded in 24-well plates at a density of $25\times10^3$ cells/well and incubated for 24 h at 37°C. Then, the cells were treated with 4.4, 22 and 44 µg/mL B including hBNs and further incubated for 24 or 72 h. Following the incubation, the cells were collected in PBS. The intensity of cell side scattering signal was analyzed using the flow cytometer (FACS Comp software).

**Cell Cycle**

Cell cycle investigation was performed to deeply analyze the proliferation performance of mHippo E-14 cells exposed hBNs and BA. A density of $25\times10^3$ cells/well were seeded in 24-well plates and allowed to adhere overnight. The cells were then treated with 4.4, 22 and 44 µg/mL B including hBNs and BA dissolved in media for 24 and 72 h. For positive control, the cells were exposed to 0.1 µM colchicine. After the treatment, the cells were collected and fixed overnight using 70% ethanol at -20°C. The following day, the cells were centrifuged at 2500 rpm at 5 min and then re-suspended in 0.5% BSA (in PBS). Then, they were centrifuged at 2500 rpm for 10 min and then re-suspended in 50 µg/mL RNAse (in PBS). Finally the cells were centrifuged at 2500 rpm for 5 min. After that, the cells were re-suspended in 0.1 µg/mL propidium iodide (PI) for each sample. The samples were analyzed using a flow cytometry.

**Reactive Oxygen Species (ROS) Generation**

ROS detection test was performed on mHippo E-14 cells exposed to hBNs and BA in order to assess whether intracellular stress was increased. The cells were seeded in 24-well plates at a density of $25\times10^3$ cells/well and incubated for 24 h at 37°C. In the following day, the cells were exposed to 4.4, 22 and 44 µg/mL B including hBNs and BA containing media and incubated for 24 and 72 h at 37°C. Following the incubation, the cells were treated with buffer containing 20 µM ROS detection reagent (2' ,7' dichlorofluorescindiacetate, DCFDA) and incubated for 45 min at 37°C in the dark. Following the transformation of DCFDA to a non-fluorescent compound by deacetilation reaction, they were oxidized into the fluorescent 2,7-dichlorofluorescein (DCF) by the presence of ROS in the cells that were analyzed with a flow cytometer.

**Cell Death Mechanism**

In order to determine the death mechanisms of mHippo E-14 cells treated with hBNs and BA, the Annexin-V and PI staining was carried out using an Annexin-V FITC apoptosis detection kit (Sigma, Germany). The cells were seeded in 24-well plates at a density of $25\times10^3$ cells/well and incubated for 24 h. As a positive control for apoptosis, 10% dimethyl sulfoxide (DMSO) was used, and 0.025% Triton X-100 was used as a positive control for necrosis. Following the treatment of the cells with 4.4, 22 and 44 µg/mL B including hBNs and BA for 24 and 72 h, the cells were collected and washed with PBS. Subsequently, the cells were re-

suspended in 200 µL of 1×binding buffer solution containing 0.5 µl of Annexin-V FITC and incubated for 10 min at 37°C in the dark. Then, the cells were centrifuged at 2500 rpm for 5 min. After centrifugation, a 200 µL of PI (0.1 µg/mL) solution was added and incubated for 15 min at 37°C in the dark. After incubation, the flow cytometry was used to quantify the number of viable, apoptotic and necrotic cells.

**Nuclei Imaging**

The genotoxic effect of the hBNs and BA were preliminarily analyzed by monitoring the nucleus of cells using a confocal microscopy. In the experiment, $15\times10^4$ and $5\times10^4$ cells/well were respectively seeded in coverslip inserted 6-well plates for 24 and 72 h to analyze the cell nuclei following the hBN and BA treatment. The cells were incubated for 24 h at 37°C. The following day, the cells were treated with 4.4, 22 and 44 µg/mL B including hBNs and BA for 24 and 72 h. Then, the cells were fixed with 4% paraformaldehyde dissolved in PBS for 20 min at 4°C. Next, the cells were washed with PBS for three times. Then, they were treated with 1 mg/mL of sodium borohydride ($NaBH_4$) to reduce background fluorescence and incubated for 5 min at room temperature. After washing the cells with PBS for three times, they were stained with 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI, 5 µg/mL per well) and incubated at room temperature in the dark. Then, they were rinsed again with PBS for three times. Finally, each coverslip was inverted onto a drop of mounting media placed on a glass slide and analyzed with a confocal microscope (Zeiss LSM 700 confocal laser-scanning microscope).

**Cell Viability Analysis of hBN treated cells after DOX exposure**

The possible effects of the hBNs and BA against DOX side effects were studied with WST-8 assay on mHippo E-14 cells. The cells were seeded into 96-well plates at a density of $5\times10^3$ cells per well and after 24 h of incubation, 4 µM DOX was added to stimulate intracellular stress and was incubated for 5 h, then they were treated with 4.4, 22 and 44 µg/mL boron including hBNs and BA for 24 h and 72 h. The hBNs and BA containing media in 96-well plates was replaced with fresh culture media containing WST-8 reagent with 1:10 ratio and incubated for a further 1 h. The cell viability upon exposure to varying concentrations of hBNs and BA was calculated by measuring the absorbance of formed formazan salts at

450 nm with a microplate reader. Besides, DOX non-treated cells and hBN and BA non-treated cells following the incubation with DOX were measured as control samples.

### Statistical Analysis

All data were expressed as mean ± standard deviation (SD) and to determine weather each two-sample datasets were different significantly when compared by Student's *t*-test. A value of $p<0.05$ was considered statistically significant.

## ACKNOWLEDGEMENT

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