

In vitro cytotoxic and genotoxic effects of newly synthesised boron ionic liquids

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ABSTRACT

In this study, new molecules containing boron were added to the family of ionic organic compounds, in particular, ionic liquids with a cationic center. This study aimed to determine the cytotoxic and genetic effects of a total of four newly synthesised borenium and borinium compounds on human peripheral lymphocytes *in vitro*. Human peripheral lymphocytes were obtained from heparinised blood samples collected from healthy females aged between 29 and 32 years, with no history of exposure to toxic agents. The cytotoxicity was assayed via the MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium-bromide) and LDH (lactate dehydrogenase) tests; the genotoxicity and cytotoxicity, via the micronucleus (MN) and sister chromatid exchange (SCE) tests. The results showed that the borenium compounds did not exhibit cytotoxic activity in the MTT and LDH tests even in high concentrations, the borinium compound did not exhibit cytotoxic effects in lower concentrations *in vitro*. The borenium compounds did not show genotoxic effects in SCE and MN tests. However, at a high concentration, borinium increased the micronucleus rate in comparison to the negative control group. The obtained results suggest that these newly synthesised cationic boron compounds can be used reliably in health, cosmetics or other industries, taking into consideration their types and concentrations.

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Borenium; borinium; MTT; LDH; micronuclei; sister chromatid exchange

Introduction

Almost all ionic compounds containing boron mentioned in the literature are ionic compounds with the BF_4^- anion. These compounds have been commonly used for many years in many reactions in both the industry and synthetic organic chemistry. In 1985, Noeth and Koelle [1] analysed the cationic boron types and systematically classified them into three groups. There are three cationic forms of boron according to the coordination numbers of 2, 3 and 4 and these are borinium, borenium and boronium (Figure 1).

Although the existence of boronium cations has been known for a very long time, the first borenium and borinium cations were only synthesised in the recent past. The borinium cations are synthesised as a result of the reaction of alkyl amino boron halides with Lewis acids (BX_3 , AlX_3 and GaX_3), which results in the

transfer of the halogen to the Lewis acid [2]. This shows that bisamido borinium ions bonded by various amido groups can easily be synthesised in the presence of appropriate conjugate anions [3,4]. The most common borenium cation synthesis is the reaction of four-coordinate neutral boron complexes with Lewis acids (a halogen or hydride split occurs). In a study by Schneider et al. [5] pyridine was used instead of acridine and the borenium cation compound was obtained as the sole product. The borenium cations were obtained by the fluorine reacting with the silyl compounds due to the effect of the silicon on the fluorine. In another study, the borenium cation was obtained through the use of these reactions, these compounds being important as they are the borenium cation derivatives of the compounds that are BODIPY derivatives. These compounds have a significant place in its photochemistry as fluorescent salts [6,7]. Tsurumaki et al. [8] obtained borenium in a planar

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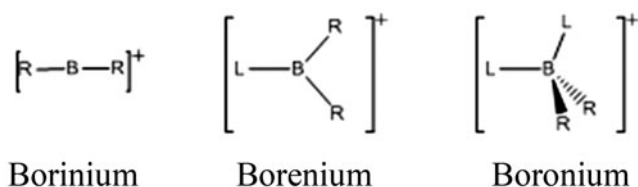


Figure 1. Cationic structures of boron.

structure, with boron bonded to the porphyrin structure. These structures are interesting because of their optical properties. While studies on boron's derivatives in cationic forms continue at a fast pace, research into the derivatives of these structures in ionic liquid form is unfortunately very scarce. Boron cationic liquids containing optically active carbons have not been found in the literature. In this study, we synthesised the first boron cationic liquids where ionic liquids and amino acids with chiral structures were used as anions in all four types of boron cations. We performed cytotoxic and genotoxic analyses of these newly synthesised ionic boron cations in order to determine their potential usability both in basic science and different industries. Studies in the literature indicate that boron compounds have no carcinogenic or mutagenic effects on cells and living organisms [9]. These studies were carried out with well-known boron compounds, such as boric acid, borax and sodium tetraborate. However, a recent study reported that boric acid had some cytotoxic and apoptotic effect in the HL60 cell line, and its effect was higher compared to sodium tetraborate [10]. In another study, the application of boric acid, borax pentahydrate and disodium pentaboratedecahydrate to CCL-62 cells affected the proliferation of these cell lines while having no additive or reducing effect on the cytogenetic degeneration present in these cell lines [11]. In experimental cytotoxicity studies on cancer cells, it was revealed that boron could have antiproliferative effect on cells depending on the dosage and the duration of exposure [12]. In another study by Turkez et al. [13], boric acid, borax, colemanite and ulexite compounds applied on peripheral blood cultures within certain ranges of concentration did not have any genotoxic effects, even at high concentrations. The studies performed evaluated the well-known boron compounds, while the cytotoxic and genotoxic properties of newly synthesised borenium and borinium compounds were evaluated for the first time in this study.

Materials and methods

Synthesis of the borenium ionic liquids

Dialkylarylboron and anion compound chlorobenzene were dissolved. After homogenising, an aromatic

amine compound (dimethyl amino pyridine) was added to this reaction solution and it was boiled at a temperature of $>120^{\circ}\text{C}$. The reaction mixture was centrifuged at 2000 rpm (Nuve NF800R) and the solvent was removed.

Centrifugation was performed and the dissolvent was separated. The synthesis reaction of the three synthesised borenium compounds, and their code numbers are shown in [Figures 2, 3 and 4](#).

We used ^1H NMR, ^{13}C NMR (nuclear magnetic resonance) and IR (infrared absorption) to characterise the synthesised borenium compounds structurally. The ^1H NMR, ^{13}C NMR and IR spectrum peaks of the synthesised borenium compounds with code numbers 1, 2 and 3 and the borinium compound with code number 4, are provided as [Supplemental material](#).

Synthesis of the borinium ionic liquids

Dialkylaryl boron and the anion compound chlorobenzene were dissolved. After homogenisation, this reaction solution was boiled at a temperature of $>130^{\circ}\text{C}$. The reaction mixture was cooled down to room temperature overnight and was centrifuged at 2000 rpm (Nuve NF800R), after which the solvent was removed. The synthesis reaction of the synthesised borinium ion is shown in [Figure 5](#).

Experimental design

We used heparinised blood samples from four healthy non-smoking female donors between the ages of 29 and 32 with no history of occupational exposure to any genotoxic agent. For all volunteers involved in our investigation, hematological and biochemical analyses were performed and no pathology was detected. The culture conditions were previously described [14]. For *in vitro* cytotoxicity and genotoxicity testing, 15 mL of peripheral whole blood was collected from each donor. Then, 6.5 mL of PB-MAX Karyotyping Medium (Gibco, Carlsbad, CA, USA) was added along with 0.5 mL heparinised blood to each tube. Different concentrations (0, 1.56, 3.12, 6.25, 12.5, 25, 50, 100, 200 and 400 mg/L) of the compounds borenium (codes 1, 2 and 3) and borinium (code 4) were tested *in vitro*. Cultures without boronated compounds were used as a control (–) group. Triton-X (1%, Sigma, St. Louis, MO, USA) and mitomycin C (MMC, $8.97\ \mu\text{mol/L}$, Sigma-Aldrich, Steinheim, Germany) were used as controls (+) in cytotoxicity and genotoxicity testing, respectively [15].

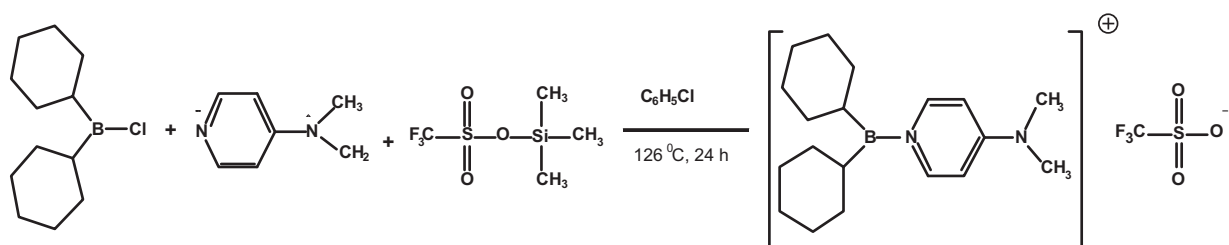


Figure 2. Synthesis reaction of dicyclohexylboronium dimethyl amino pyridine trifluoro methane sulphonate (Compound 1).

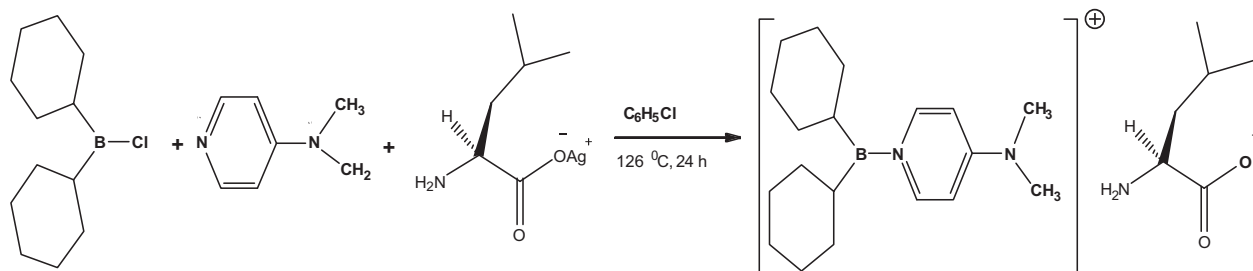


Figure 3. Synthesis reaction of dicyclohexyl boronium dimethyl amino pyridine 2-amino-4-methylpentanoate (Compound 2).

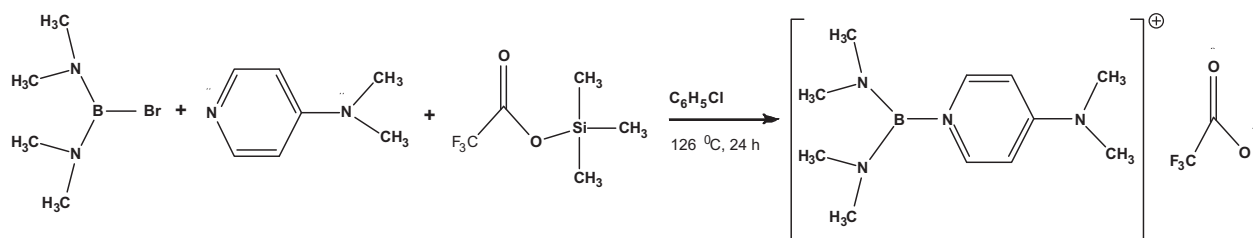


Figure 4. Synthesis reaction of bisdimethyl amino boronium dimethyl amino pyridine trifluoroacetate (Compound 3).

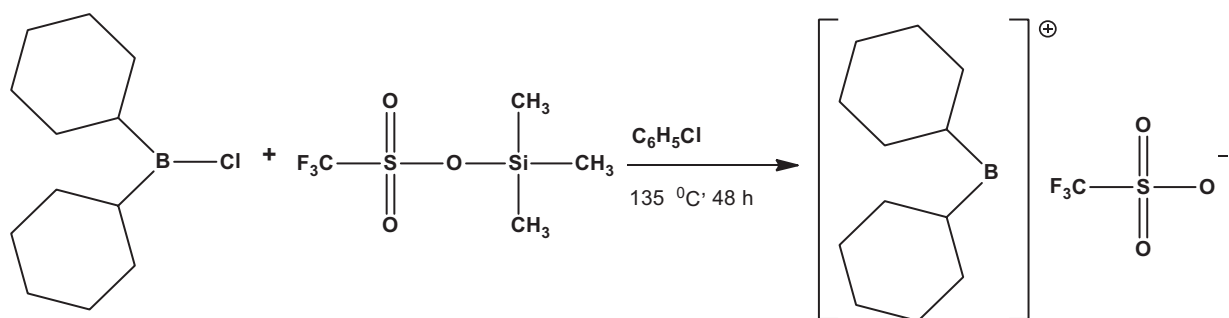


Figure 5. Synthesis reaction of dicyclohexyl boronium trifluoro methane sulphonate (Compound 4).

Ethics of experimentation

All experiments were conducted according to the guidelines of the World Medical Assembly (Declaration of Helsinki).

Cytotoxicity testing

The collected heparinised human blood cells were seeded in 48-well plates. Cells were incubated in a humidified 5% CO₂/95% air mixture at 37 °C and exposed to boronated compounds for 72 h. MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium-

bromide) substrate solution was used according to the provider's manual (Cayman Chemical Company, Ann Arbor, MI, USA). MTT solution was added to the plates for 4 h. Formed formazan crystals were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich), then the plates were analysed using a microplate reader at 570 nm. Lactate dehydrogenase (LDH) released from damaged cells in the culture medium was measured via LDH assay kits (Cayman Chemical Company). After incubation, supernatants were obtained and LDH levels were determined following the provider's guide. A total of 150 µL of supernatant was used for LDH release

measurement. Released LDH catalyses the oxidation of lactate to pyruvate and the reduction of NAD^+ to NADH at the same time. The rate of NAD^+ reduction was determined as an elevation in absorbance at 490 nm. The rate of NAD^+ reduction was considered directly proportional to the LDH amount in the medium [16].

Genotoxicity testing

Sister chromatid exchanges (SCEs) were visualised using 5-bromo-2'-deoxyuridine (Sigma) added at the culture initiation step. The treated and non-treated cultures were incubated in complete darkness for 72 h at 37 °C. Exactly 70 h and 15 min after setting the cultures, demecolcine (*N*-diacetyl-*N*-methylcolchicine, Sigma) was added into the culture tubes. Following the hypotonic treatment (as 0.075 mol/L KCl), three repetitive cycles of fixation in methanol/acetic acid solution (3:1, v/v), centrifugation (2000 rpm, 10 min, Hettich Zentrifugen Universal 320R) and re-suspension steps, the cell suspension was dropped on cold microscopic slides. After these steps, the fluorescence plus Giemsa (FPG) technique was applied [17]. For each treatment condition, 32 well-spread dividing metaphases containing 42–46 chromosomes in each cell were analysed and the values obtained were presented as SCEs/cell.

The micronucleus (MN) test was performed via adding cytochalasin B (Sigma) after 44 h at the of culture initiation. At the end of the 72 h incubation period, the lymphocytes were fixed with ice-cold methanol/acetic acid (1:1, v/v). The fixed cells were placed on slides using a cytospin, then stained with Giemsa solution. The formation of MN was scored in 2.000 bi-nucleated cells [18].

Statistical analyses

All tests were repeated at least four different times. The results are presented as mean values with standard deviation (\pm SD). The statistical analyses were carried out using Statistical Package for Social Sciences (SPSS, version 20.0, NY, USA). Differences in the cytotoxic and genotoxic potential of the borenium and borinium compounds were assessed using analysis of variance (ANOVA) followed by Duncan's test and values of $p < 0.05$ were considered to indicate statistically significant differences.

Results and discussion

Synthesis

In this study, we synthesised four new molecules containing cationic boron. The cationic forms of boron

we synthesised were classified as borinium and borenium compounds. The spectrum peaks of ^1H NMR, ^{13}C NMR and IR of the synthesised borenium compounds with code numbers 1, 2 and 3 and the borinium compound with code number 4, are provided as [Supplemental material](#).

The targeted synthesis of the borenium ionic liquids was performed by the interaction of the dialkyl halogen boron compound with the various silver salt compounds of trimethyl silyl trifluoro methyl sulphonate and trifluoroacetic acid and leucine amino acid in the presence of 4-dimethyl amino pyridine. The borinium compound was synthesised through the reaction of the dicyclo hexyl chloro boron compound with the trimethyl silyl trifluoro methyl sulphonate without the presence of 4-dimethyl amino pyridine. The presence of the peaks in the ^1H -NMR and ^{13}C -NMR spectra of the synthesised compounds indicates that the synthesis of the targeted compounds was performed successfully.

Cytotoxic potential

Cytotoxicity tests provide basic information on the behaviour of a substance which may possibly become a drug or whose toxic effects are unknown and provide a basis for subsequent animal experiments or clinical trials. That is why these tests are commonly used in *in vitro* toxicology studies [19]. In our study, to determine the viable cell rates after treatment with the newly synthesised boron compounds, we used MTT analysis, which is a colorimetric method, and LDH analysis, which is an enzymatic method.

The results obtained from MTT and LDH release analysis are shown in [Table 1](#). The Triton-X solution as control (+) reduced the cell viability percentages to 23.4% and 27.3%, in MTT and LDH assays, respectively. On the contrary, both the MTT and the LDH release assay results revealed that compounds **2** and **3** did not show statistically significant difference from the negative control, suggesting that they did not exhibit cytotoxic action on cultured human blood cells even at their highest concentrations (400 mg/L). Likewise, the lower concentrations of the borenium compounds (code **1**) and borinium (code **4**) were non-cytotoxic *in vitro*. However, the relatively higher concentrations of compounds **1** (200 and 400 mg/L) and **4** (50, 100, 200 and 400 mg/L) led to significant reductions of cell viability as compared to the control (–) group.

Genotoxic potential

To evaluate the potential genotoxic effects of the newly synthesised cationic boron compounds, we used

Table 1. Cell viability rates of cultured human blood cells after exposure to boron compounds for 72 h.

Groups	MTT assay (% cell viability)	LDH release assay (% cell viability)
Control (–)	100	100
Control (+)	23.4 ± 2.8*	27.3 ± 3.4*
1		
1.56 mg/L	97.9 ± 8.1	99.8 ± 11.5
3.12 mg/L	98.6 ± 7.2	99.6 ± 9.9
6.25 mg/L	97.4 ± 6.5	98.9 ± 10.8
12.5 mg/L	99.3 ± 7.1	99.3 ± 9.6
25 mg/L	96.2 ± 7.9	97.3 ± 10.2
50 mg/L	94.8 ± 6.4	98.2 ± 8.7
100 mg/L	95.3 ± 5.9	96.1 ± 9.3
200 mg/L	87.1 ± 7.7*	86.7 ± 8.5*
400 mg/L	75.3 ± 7.0*	82.5 ± 8.7*
2		
1.56 mg/L	98.3 ± 7.4	97.4 ± 10.3
3.12 mg/L	97.7 ± 7.7	98.8 ± 9.5
6.25 mg/L	94.9 ± 8.1	98.4 ± 8.9
12.5 mg/L	98.3 ± 6.5	98.6 ± 9.3
25 mg/L	96.3 ± 6.9	97.4 ± 10.4
50 mg/L	95.7 ± 8.0	96.5 ± 9.3
100 mg/L	96.8 ± 7.8	96.3 ± 8.9
200 mg/L	95.3 ± 7.5	95.3 ± 9.2
400 mg/L	94.6 ± 6.3	94.8 ± 9.7
3		
1.56 mg/L	98.3 ± 6.6	98.0 ± 8.9
3.12 mg/L	97.7 ± 7.2	99.3 ± 8.6
6.25 mg/L	98.0 ± 9.3	97.3 ± 10.3
12.5 mg/L	95.8 ± 8.7	96.6 ± 9.4
25 mg/L	96.2 ± 10.3	98.8 ± 8.5
50 mg/L	96.0 ± 8.5	97.4 ± 8.7
100 mg/L	97.1 ± 7.0	96.5 ± 9.0
200 mg/L	96.4 ± 9.3	97.2 ± 10.5
400 mg/L	94.2 ± 8.1	95.8 ± 9.6
4		
1.56 mg/L	99.7 ± 8.9	98.3 ± 10.0
3.12 mg/L	98.6 ± 7.9	98.6 ± 9.4
6.25 mg/L	97.3 ± 8.0	98.4 ± 10.6
12.5 mg/L	97.5 ± 9.6	97.3 ± 9.5
25 mg/L	96.2 ± 9.1	95.5 ± 8.3
50 mg/L	91.0 ± 8.3*	94.2 ± 8.6
100 mg/L	85.6 ± 6.9*	87.8 ± 9.1*
200 mg/L	63.6 ± 6.5*	72.9 ± 7.9*
400 mg/L	57.2 ± 5.1*	62.1 ± 7.3*

*Statistically significant differences as compared to the control (–) group ($p < 0.05$). Values are means ± S.D.

SCE and MN analyses *in vitro*. We studied the cytogenetic alterations in human lymphocyte cultures after exposure to different boronated compounds for 72 h. The results revealed that the compounds of borenium (code 1, 2 and 3) had no genotoxic effects on the cultured lymphocytes as determined by SCE and MN analysis (Table 2). However the highest concentrations of the borenium compound **3** (400 mg/L) and borinium compound **4** (200 and 400 mg/L) led to increases in the MN rates as compared to the control (–) group without altering the SCE frequencies. Consequently, the cytogenetic analysis indicated that, in the tested concentration range, the boron compounds had no clastogenic or aneugenic (except for the compound **3** and **4**) potential in human lymphocytes.

Overall, the results indicated that the most reactive of these cationic boron types is the borinium cation. This is because it is easily surrounded by electron donating groups due to its low coordination number

Table 2. SCEs and MN frequencies in human lymphocytes after treatment with different boron compounds for 72 h.

Groups	SCEs/cell	MN/1000 cell
Control (–)	4.3 ± 0.5	1.9 ± 0.2
Control (+)	11.4 ± 1.6*	5.7 ± 0.7*
1		
1.56 mg/L	4.0 ± 0.3	1.8 ± 0.2
3.12 mg/L	4.4 ± 0.4	1.9 ± 0.3
6.25 mg/L	4.2 ± 0.4	1.6 ± 0.2
12.5 mg/L	3.5 ± 0.4	1.7 ± 0.3
25 mg/L	4.1 ± 0.5	1.9 ± 0.2
50 mg/L	4.0 ± 0.6	2.1 ± 0.3
100 mg/L	4.4 ± 0.5	2.0 ± 0.2
200 mg/L	4.5 ± 0.6	2.1 ± 0.3
400 mg/L	4.6 ± 0.4	2.1 ± 0.3
2		
1.56 mg/L	4.4 ± 0.4	1.6 ± 0.2
3.12 mg/L	4.7 ± 0.6	1.8 ± 0.3
6.25 mg/L	4.4 ± 0.5	1.8 ± 0.2
12.5 mg/L	4.5 ± 0.3	2.0 ± 0.2
25 mg/L	4.6 ± 0.5	2.1 ± 0.3
50 mg/L	4.1 ± 0.5	2.1 ± 0.4
100 mg/L	4.5 ± 0.5	2.0 ± 0.3
200 mg/L	4.4 ± 0.3	2.2 ± 0.2
400 mg/L	4.8 ± 0.5	2.1 ± 0.3
3		
1.56 mg/L	4.0 ± 0.5	2.1 ± 0.3
3.12 mg/L	3.7 ± 0.6	2.0 ± 0.4
6.25 mg/L	4.1 ± 0.4	1.9 ± 0.3
12.5 mg/L	3.8 ± 0.6	2.0 ± 0.3
25 mg/L	3.6 ± 0.4	1.9 ± 0.2
50 mg/L	4.5 ± 0.7	2.0 ± 0.3
100 mg/L	4.4 ± 0.6	2.1 ± 0.3
200 mg/L	4.6 ± 0.5	2.2 ± 0.4
400 mg/L	4.6 ± 0.7	2.7 ± 0.5*
4		
1.56 mg/L	4.4 ± 0.6	1.7 ± 0.2
3.12 mg/L	4.5 ± 0.5	1.9 ± 0.3
6.25 mg/L	4.6 ± 0.5	1.8 ± 0.2
12.5 mg/L	4.2 ± 0.6	2.0 ± 0.2
25 mg/L	4.4 ± 0.4	2.0 ± 0.3
50 mg/L	4.4 ± 0.5	1.7 ± 0.2
100 mg/L	4.6 ± 0.4	2.2 ± 0.4
200 mg/L	4.6 ± 0.5	2.9 ± 0.4*
400 mg/L	4.8 ± 0.4	3.5 ± 0.6*

*Statistically significant differences as compared to the control (–) group ($p < 0.05$). Values are means ± S.D.

and empty orbitals [20]. Meanwhile, the borenium cations have 2 sigma bonds and 3 coordination numbers and they complete their coordination by the electron transfer of the neutral ligand to the empty p orbitals of boron [21]. Due to the electron insufficiency of boron based on its empty orbitals, its reactivity increases with the electron donation from the donor ligand. SCE provides information on the mutagenic and carcinogenic effects of various agents, especially the structural changes occurring in chromosomes as a result of DNA damage and DNA repair [22–24]. As micronuclei are formations that occur during the mitotic division of the cell and originate from whole chromosome or acentric chromosome fragments that are not included in the main nucleus, an increase in the MN number is considered to be an indirect indication of the numeric and structural chromosome anomalies that various chemical agents generate in cells.

Agents stimulating aneuploidy lead to MN formation by causing centromere division errors and dysfunction in spindle fibers, while clastogens lead to MN formation by causing chromosome breakage [25]. For this reason, the MN test is the only biomarker allowing both the clastogenic and aneugenic effects to be assessed together [26]. In both human and laboratory studies, boron reportedly has no genotoxic effect and in fact appears to have antigenotoxic properties [27]. In addition, borax has a protective effect of 30–50% against SCEs and MNs induced by aflatoxin B1 [28]. These data were supported by our experimental evidence that the borenium ions had no genotoxic effects in lymphocyte cell cultures. Boric acid and borax were previously tested by the American National Toxicology Program (N.T.P. 1987) and it was concluded that they were not mutagens, either in bacteria or in cultured Chinese hamster ovary (CHO) cells [29].

Whereas we identified no cytotoxic effects of the borenium compounds numbered **2** and **3** in low and high concentrations using both MTT and LDH measurements, the borenium compound with code number **1** decreased the cell viability in high concentrations when compared to the positive control group. On the other hand, the borinium compound decreased the cell viability in different concentrations. In a study where boric acid was applied to HeLa cells in a dose of 100–500 mg/L, it was shown that boric acid raised the proliferation of the cells to a high level by demonstrating mitogenic effect, but that it suppressed the proliferation of the cells when it was applied in a dose of 1000 mg/L or higher. The suppression of cell growth and reproduction based on boron revealed that boron could have an impact on signalling pathways related to cell growth [30,31].

Conclusions

The obtained results showed that the four newly synthesised cationic boron compounds are effective in cell proliferation depending on the type of compounds and their concentrations, but that they are not likely to affect the frequency of cytogenetic anomalies existing in peripheral lymphocytes in vitro. We suggest that these new boron compounds could be used safely in many areas such as health, cosmetic or industrial areas.

Consent for publication

The authors confirm that informed consent was obtained from four volunteer participants.

Availability of data and materials

The datasets used and/or analysed in this study are available from the corresponding author on reasonable request.

Disclosure statement

The authors declare that they have no competing interests.

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