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A N I N T E R N A T I O N A L J O U R N A L

Genotoxicity Evaluation of Dipotassium - Trioxohydroxytetrafluorotriborate, $K_2(B_3O_3F_4OH)$, in Human Lymphocyte Cultures and Mice Reticulocytes

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ABSTRACT

Genotoxic effects of inorganic molecule dipotassium-trioxohydroxytetrafluorotriborate, K2(B3O3F4OH), a promising new therapeutic for the epidermal changes treatment, have been evaluated. In vitro analysis included evaluation of genotoxic and cytotoxic potential of K2(B3O3F4OH) in concentrations of 0.01, 0.02, 0.05 and 0.06 mg/mL applying cytokinesis-block micronucleus cytome assay in human lymphocyte culture. With the increase of concentration the frequency of micronuclei elevated but the differences were not significant. Also, there were no significant differences among the frequencies of nuclear buds and nucleoplasmic bridges between controls and treated cultures. Nuclear division index and nuclear division cytotoxycity index values did not reveal significant cytotoxic effect of K2(B3O3F4OH). In vivo genotoxic effects were analyzed on BALB/c mice applying reticulocytes micronucleus assay. K2(B3O3F4OH) was administrated intraperitoneally in final concentrations of 10, 20, 50 and 55 mg/kg. Significant decrease of reticulocytes ratio and increase of micronuclei frequencies against pre-treatments were found for both sampling periods of 48 and 72 hours of the highest applied concentration. This study confirmed that K2(B3O3F4OH) is not genotoxic in tested concentrations in vitro as well as in concentrations lower than 55 mg/kg in vivo. This study presents a reliable basis for further pre-clinical and potential clinical investigations.

Key words: genotoxicity, micronuclei, halogenated boroxine, human lymphocyte culture, BALB/c mice

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INTRODUCTION

Dipotassium-trioxohydroxytetrafluorotriborate $K_2[B_3O_3F_4OH]$ has recently attracted attention as a promising new therapeutic for prevention and/or treatment of benign or malignant changes of the epidermis[1,2]. It belongs to halogenated boroxines, derivatives of cyclic anhydride of boronic acid[3]. It is known that the cyclic anhydride form of modified dipeptidyl boronic acid is present as a trimeric boroxine in the antineoplastic agent, bortezomib (Velcade®).

A few recent studies investigated the impact of K2(B3O3F4OH) on inhibition of enzymes associated with hypothesized antitumor properties. It is reported that halogenated boroxine K2(B3O3F4OH) inhibits catalase activity[4] and human carbonic anhydrases[5]. The in vitro and in vivo antitumor activity of K2(B3O3F4OH) has also been confirmed[6]. It significantly decreases melanoma cells viability in concentrations of 1 and 0.1 mM in vitro and deregulates expression of certain genes qualified as common anti-cancer drug targets[7].

Genotoxic, cytotoxic and cytostatic effects in human lymphocyte cultures and antiproliferative effect on basal cell carcinoma culture are confirmed[8]. It has been also reported that certain genotoxic effects of K2(B3O3F4OH) in concentration of 0.1 mg/mL are inhibited in human lymphocyte culture in the presence of luteolin and delfinidin in appropriate dosage[9].

This study aimed to evaluate in vitro genotoxicity of K2(B3O3F4OH) in concentrations lower than previously reported as genotoxic as well as to determine if there are notable in vivo genotoxic effects of K2(B3O3F4OH) applied concentrations in order to determine relevant doses for the potential use.

MATERIALS AND METHODS

Tested substance

Dipotassium trioxohydroxytetrafluorotriborate, K2(B3O3F4OH), halogenated cyclic anhydride of boronic acid, was synthesized as reported in the literature[10]. For testing of genotoxic potential in human lymphocytes cultures, K2(B3O3F4OH) was directly dissolved in the culture medium to the final concentrations of 0.01, 0.02, 0.05 and 0.06 mg/mL. These concentrations were selected according to earlier evaluation of K2(B3O3F4OH) genotoxicity[8] in order to specify in vitro genotoxicity threshold.

For the in vivo testing, a physiological solution containing the K2(B3O3F4OH) was intraperitoneally administrated in final concentrations of 10, 20, 50 and 55 mg/kg, as preliminary investigation showed that the NOAEL (no observed adverse effect level) is considered to be between 50 and 60 mg/kg in rats (performed at the Centre de Recherches Biologiques – CERB, Baugy, France).

Ethical approval for the in vitro and in vivo testing was obtained from the Ethics Committee of the Institute for Genetic Engineering and Biotechnology, University of Sarajevo (Approval No. 181-1/14 dated on April 1st 2014).

Cytokinesis-block micronucleus cytome assay

In vitro analysis of cytotoxic and genotoxic potential of $K_2(B_3O_3F_4OH)$ was performed applying cytokinesis-block micronucleus cytome assay (CBMN-Cyt assay) in human lymphocyte cultures. Four volunteers signed informed consent forms and donated 4 mL of peripheral blood samples for this study. Cultures were set up by adding 400 µl of peripheral blood in 5 mL of PB-MAX Karyotyping Medium (GIBCO-Life Technologies, Grand Island, NY, USA), in 15-mL sterile, plastic tubes with a conical bottom (Isolab GmbH, Wertheim Germany). Cultivation lasted 72 hours at 37ºC. K2(B3O3F4OH) was added to the cultures in the 25th hour of cultivation to the final concentrations of 0.01, 0.02, 0.05 and 0.06 mg/mL. Untreated cultures were set up as negative controls. Cytochalasin B (Sigma-Aldrich Co., St Louis, MO, USA) was added to the final concentration of 4.5 µg/ mL, in order to block cytokinesis.

After the cultivation period, cultures were centrifuged for 10 minutes at 1.000 rpm and subjected to hypotonic treatment with 0.56% KCl and centrifuged immediately after the hypotonic addition. Hypotonic treatment was followed by three fixations in ice-cold glacial acetic acid + ethanol (1+3) fresh fixative. Fixed lymphocytes solution was dropped on coded microscope slides. Air-dried slides were stained in 5% Giemsa for 7 minutes.

Slides were analyzed at 400x magnification on Olympus BX51 microscope (Tokyo, Japan). At least 2.000 binuclear (BN) cells[11] for each blood

sample and tested concentration were scored in order to determine genotoxic potential through the frequencies of micronuclei, nucleoplasmic bridges, and nuclear buds. Frequencies of mononuclear, binuclear, trinuclear, and quadrinuclear cells, as well as apoptotic and necrotic cells, were scored in the total number of at least 500 counted cells. All genotoxicity and cytotoxicity parameters were recognized according to the criteria given by Fenech[12-14]. Cytostatic and cytotoxic effects of K2(B3O3F4OH) were examined by the calculation of the nuclear division index (NDI)[12,15] and nuclear division cytoxicity index (NDCI)[12].

Mice reticulocytes micronucleus assay

In vivo experiment was performed on adult BALB/c mice, obtained from the breeding colony from the Institute for Pharmacology, Clinical Pharmacology and Toxicology, Faculty of Medicine, University of Sarajevo. The animals were kept in conventional conditions and treated according to the Animal Welfare Regulations. Each of the four experimental groups consisted of 4 animals that were used for testing of selected dose (10, 20, 50 and 55 mg/kg). Animals were treated with K2(B3O3F4OH) intraperitoneally. Apical tail cutting was used to collect peripheral blood before treatment (pre-treatment, control - 0 h) as well as 48 and 72 h upon administration. Peripheral blood smears were prepared on cleaned slides and fixed in absolute methanol. Slides were directly stained in acridine orange solution and rinsed in phosphate buffer. Air-dried slides were immediately analyzed on the epi-fluorescent microscope Olympus BX51, using U-MWIB2 filter, at 1000x magnification. Reticulocytes are identified by the red-orange and micronuclei by the yellowish-green fluorescence. *In vivo* genotoxicity evaluation of $K_2(B_3O_3F_4OH)$ were conducted by the analysis of the frequencies of normochromatic erythrocytes (NCE), polychromatic erythrocytes (PCE) and micronuclei in polychromatic erythrocytes (MNPCEs), according to the previously described criteria[16-18] and the Guideline for the testing of chemicals[19].

Statistical analysis

The significance of differences between tested concentrations of $K_2(B_3O_3F_4OH)$ was tested by one-way analysis of variance (ANOVA), using MedCalc software Version 10.4.0.0 (Mariakerke, Belgium). Significant level was set at $p < 0.05$.

RESULTS AND DISCUSSION

Results of the analysis of micronuclei, nuclear buds and nucleoplasmic bridges frequencies as well as nuclear division index and nuclear division cytotoxicity index in human lymphocyte culture are presented in the table 1 as the means ± standard deviation. The frequency of micronuclei rose with the concentration but the differences in comparison to controls are not significant. ANOVA showed no significant differences among the frequencies of nuclear buds and nucleoplasmic bridges in controls and treated cultures. NDI and NDCI values did not significantly differ, although the means of both values were slightly decreased in treated cultures in comparison to controls.

Table 1. CBMN-cyt assay in human lymphocytes treated with $K_2(B_3O_3F_4OH)$

Treatment	MN	NB	NPB	NDI	NDCI
control	27 ± 8.869	6 ± 4	2 ± 0	1.565 ± 0.1	1.56 ± 0.099
0.01 mg/mL	33.5 ± 13.026	4.25 ± 2.363	1.5 ± 1.732	1.454 ± 0.028	1.448 ± 0.025
0.02 mg/mL	33.5 ± 13.916	6 ± 4.397	1.5 ± 1.732	1.512 ± 0.025	1.502 ± 0.029
0.05 mg/mL	40 ± 20.445	5.5 ± 3.109	1.75 ± 1.258	1.456 ± 0.047	1.45 ± 0.043
0.06 mg/mL	49 ± 30.31 [a]	4 ± 3.559	2.75 ± 0.5	1.446 ± 0.093	1.441 ± 0.092

Values are presented as the mean ± SD.
[a] Significantly different compared to controls $p< 0.05$.
MN – micronuclei; NB – nuclear buds; NPB – nucleoplasmic bridges;
NDI – nuclear division index; NDCI – nuclear division cytotoxicity index.

Results of the analysis of normochromatic erythrocytes, polychromatic erythrocytes and micronuclei in polychromatic erythrocytes frequencies are presented in figure 1. Significant differences between controls and treatments are indicated in table. Significant differences were registered for the relative frequency of PCE, 72 hours upon treatment in concentration of 20 mg/kg when compared to the pre-treatment. However, significant differences against pre-treatments for PCE (%) and MNPCEs were found for both sampling periods upon treatment with the highest tested concentration (55 mg/kg).

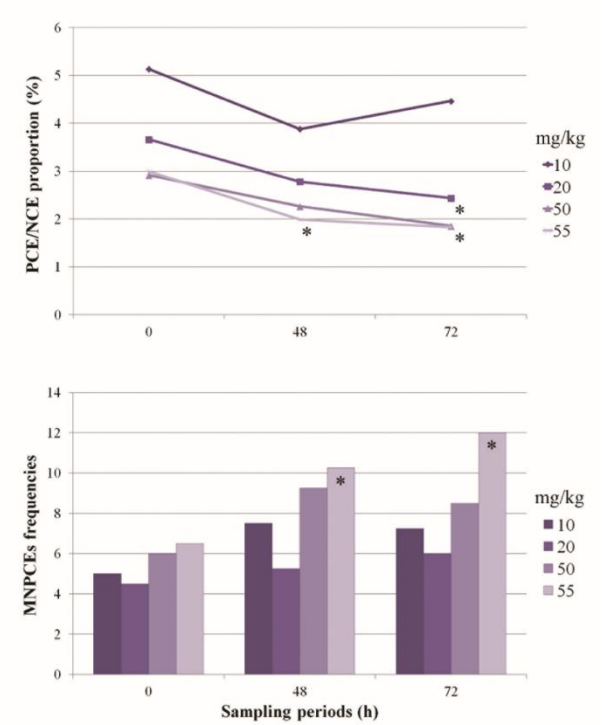

Figure 1: Results of reticulocytes micronucleus assay
Legend: * Significantly different against the pre-treatment, p< 0.05. PCE (%) – proportion of polychromatic among total erythrocytes; MNPCEs – micronuclei in polychromatic erythrocytes.

Importance of genotoxicity evaluation of K2(B3O3F4OH) is emphasized by the generally accepted aim of genotoxicity testing which is to identify potentially hazardous drug candidates.
The first published bioactivity analysis of K2(B3O3F4OH) confirmed that concentrations of 0.1 and 0.2 mg/mL in human lymphocyte cultures cause significant increase in frequencies of structural chromosome aberrations, micronuclei, nuclear buds and nucleoplasmic bridges8.

Additionally, results of genotoxicity analysis in combination with acute and sub chronic toxicity present the basis to approve clinical trials of drug candidates[20].
As notable antitumor potential of K2(B3O3F4OH) has been already reported[6-8] emphasizing its clinical potential, genotoxicity observation of K2(B3O3F4OH) in vitro and in vivo were performed in concentrations different of those already reported as genotoxic in vitro[8] as well as lower and the same, to the estimated NOAEL values in vivo. Applying CBMN-cyt assay in human peripheral blood lymphocytes in concentrations of 0.01, 0.02, 0.05 and 0.06 mg/mL and mice reticulocytes micronucleus assay in concentrations of 10, 20, 50 and 55 mg/kg the following findings are recognized:

I. $K_2(B_3O_3F_4OH)$ in final concentrations of 0.01, 0.02, 0.05 and 0.06 mg/ml *in vitro* is not genotoxic and cytotoxic to human lymphocytes.
II. Results of the mice reticulocytes micronucleus assay are the contribution to the understanding of the $K_2(B_3O_3F_4OH)$ *in vivo* genotoxicity, preliminary already reported[21]. Genotoxic effects, expressed as the significant increase of the micronuclei frequencies and decrease of the reticulocytes ratio in mice blood, are detected for the highest tested concentration of 55 mg/kg.
III. $K_2(B_3O_3F_4OH)$ did not induce genotoxic effects against controls regarding micronuclei frequency (MNPCEs) *in vivo* in concentrations lower than 55 mg/kg that is in accordance to the considered NOAEL concentrations.

CONCLUSION

This study confirmed that $K_2(B_3O_3F_4OH)$ is not genotoxic in tested concentrations *in vitro* as well as in the concentrations lower than 55 mg/kg *in vivo*, that were not previously tested but may have pharmaceutical potential. These findings present the bases for the determination of applicable $K_2(B_3O_3F_4OH)$ doses in future promising clinical applications.

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