

IN VITRO STUDY OF THE ANTI-PROLIFERATIVE EFFECTS OF DIPOTASSIUM-TRIOXOHYDROXYTETRAFLUOROTRIBORATE ON THE H520 NON-SMALL CELL LUNG CANCER CELL LINE

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Abstract

Non-small cell lung cancer has been shown to be resistant to many forms of chemotherapy and is amongst the deadliest cancers worldwide. The anti-proliferative effects of halogenated boroxine - $K_2(B_3O_3F_4OH)$, have been confirmed in multiple cancer cell lines including melanoma and breast cancer. The potential for this chemical treatment on non-small cell lung cancer cells was studied and the lower threshold concentration with the clear biological effect of halogenated boroxine, was determined. Applying MTT assays and the relative gene expression analysis of two genes of interest, *RRBP1* and *PER1*, novel knowledge on the biological potential of halogenated boroxine (HB) was gained, but did not lead to biological explanations of the mechanisms of halogenated boroxine activity. The results of MTT assay showed a significant HB effect on non-small cell lung cancer in concentration of 0.5 mg/mL, while relative expression levels of *RRBP1* and *PER1* did not significantly differ regardless the concentration applied.

Key words: $K_2(B_3O_3F_4OH)$, MTT assay, relative gene expression

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Introduction

Lung cancer has caused the majority of cancer-related deaths worldwide and with new discoveries of how lung cancer originates and proliferates, progress to find a successful treatment for the disease remains a challenge. Lung cancer is correlated with the capacity of a patient's immune system. Smoking remarkably increases the risk for lung cancer development, partially by suppressing the immune system (Domagala-Kulawik, 2015). Non-small cell lung cancer, diagnosed in more than 40% of patients over the age of 70 years (Maione et al., 2010), makes up to 85% of all lung cancers and

includes adenocarcinoma and squamous cell carcinoma. The 5-year survival rate is 17.4% (Boolell et al., 2015). Many of the current approaches to treat lung cancer include chemotherapy, radiotherapy, biological treatment, and targeted therapies (Domagala-Kulawik, 2015). However, these approaches may be detrimental to the health of the patient due to the radiation, pain, and scarring.

Dipotassium-trioxohydroxytetrafluorotriborate - $K_2(B_3O_3F_4OH)$ (Figure 1), more commonly known as halogenated boroxine (HB), is a water-soluble white powder that is synthesized at the Laboratory for Physical Chemistry, Department of Chemistry,

Faculty of Science, at the University of Sarajevo. It is currently suggested to be used as an ointment for the prevention and treatment of benign and malignant growth of epidermal skin cells (Galic, 2012; 2013). Conducted genotoxicity analysis identified HB as an agent with no observable genotoxic effects in concentrations lower than 0.1 mg/ml in human peripheral blood lymphocytes *in vitro* (Haveric et al., 2011) and in concentrations lower than 55 mg/kg *in vivo* (Haveric et al., 2016). However, bioflavonoids delphinidin and luteolin when administered in human lymphocytes may reduce genotoxic effects of HB (Hadzic et al., 2015). Additionally, HB's anti-proliferative effects in basal cell carcinoma culture, measured by alamar blue assay and in human lymphocytes, determined by calculation of the nuclear division index (NDI) and nuclear division cytotoxicity index (NDCI) have been revealed (Haveric et al., 2011).

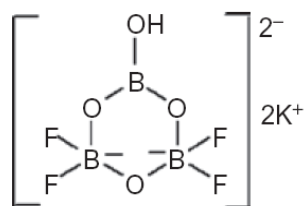


Figure 1. Structural formula of halogenated boroxine

$\text{K}_2(\text{B}_3\text{O}_3\text{F}_4\text{OH})$ was first described more than 50 years ago (Ryss & Slutskaya 1951) but its anti-tumor activity has been recently accessed *in vitro* and *in vivo* in 4T1 mammary carcinoma, B16F10 melanoma and squamous cell carcinoma SCVII. Higher tested concentrations of $\text{K}_2(\text{B}_3\text{O}_3\text{F}_4\text{OH})$ (0.5 mg/ml and 1 mg/ml) were highly cytotoxic in all tested tumor lines. Effects of $\text{K}_2(\text{B}_3\text{O}_3\text{F}_4\text{OH})$ on the growth inhibition of tumor cells are significant in the growing medium containing low levels of Ca^{2+} ions (0.42 mM) (Ivankovic et al., 2016). *In vivo*, regardless of the administration mode used (intraperitoneally, intratumor, *per os* or topical) $\text{K}_2(\text{B}_3\text{O}_3\text{F}_4\text{OH})$ slowed the growth of all three tested tumor types compared to the controls (Ivankovic et al., 2015). Significant decrease of cell viability for concentrations of 0.025 and 0.25 mg/ml of $\text{K}_2(\text{B}_3\text{O}_3\text{F}_4\text{OH})$ was also shown in human Caucasian melanoma cell line (GR-M). The same $\text{K}_2(\text{B}_3\text{O}_3\text{F}_4\text{OH})$ concentrations deregulated

expression of more than 30 anti-tumor targeted genes in GR-M cell line (Pojskic et al., 2015). $\text{K}_2(\text{B}_3\text{O}_3\text{F}_4\text{OH})$ also reduces the activity of catalase (Islamovic et al., 2014) and human carbonic anhydrases (hCA), especially hCA IX isoform (Vullo et al., 2015).

Ribosome binding protein 1 (*RRBP1*) and period circadian clock 1 (*PER1*) genes are differentially expressed in lung cancer tissue. The *RRBP1* gene functions by alleviating intracellular stress-induced apoptosis through the activation of 78 kDa glucose-regulated protein (GRP78), that is frequently over-expressed in lung cancer but not in normal lung tissue (Wang et al., 2005; Uramoto et al., 2005). GRP78 also plays a critical role in chemotherapy resistance in some cancers (Wang et al., 2008). *RRBP1*, originally identified to be located on the rough endoplasmic reticulum (ER) and associated with unfolding protein response (UPR), is frequently over-expressed in breast cancer (Telikicherla et al., 2012). Expression levels of *RRBP1* have varied between non-small cell lung cancer (NSCLC) samples showing a trend of higher expression in the early tumor stages, without a significant correlation between *RRBP1* expression and tumor stages (Tsai et al., 2013).

The *PER1* gene is among significant molecular clock mechanisms involved in regulating a cell's circadian clock (Zheng et al., 2001). *PER1* has been shown to be under-expressed in lung cancer tissue compared to healthy lung tissue.

Down-regulation of *PER1* gene, associated with DNA hypermethylation and histone H3 acetylation in NSCLC (Gery et al., 2007), leads to uncontrolled cell growth and division due to limitations of the cell's circadian rhythm. It has been shown that down-regulation of *PER1* also accelerates breast cancer cells growth (Gery et al., 2006; Yang et al., 2009). In contrary, its up-regulation results in cell growth inhibition and apoptosis promotion in colon (Gery et al., 2006), prostate (Cao et al., 2009) and lung cancer (Gery et al., 2007).

Hypothesizing under-expression of *RRBP1* and over-expression of *PER1* due to the influence of halogenated boroxine and decrease in cell proliferation of H520 non-small lung cancer cells,

we aimed to determine the effects of halogenated boroxine using MTT assay and relative gene expression analysis of *RRBP1* and *PER1* genes.

Materials and methods

Dipotassium trioxohydroxytetrafluorotriborate, ($K_2[B_3O_3F_4OH]$)

Dipotassium trioxohydroxytetrafluorotriborate, halogenated boroxine (HB), a water-soluble white powder (7.1% at 20°C) with 99.99% purity, was synthesized in the Laboratory for Physical Chemistry, Department of Chemistry, Faculty of Science - University of Sarajevo. For this study, HB was dissolved in RPMI-1640 culture media. After the non-small lung cancer cells had reached 70% of confluency, medium containing HB was added in final concentrations of 0.125 mg/mL, 0.25 mg/mL and 0.5 mg/mL.

Culture treatment

Squamous lung cancer NCI-H520 cells were obtained from the American Type Culture Collection (ATCC). H520 cells were grown in RPMI-1640 medium with 10% FBS. Growth conditions were optimized and media was replaced every two to three days based on the manufacturer's instructions. Cells were observed by the Zoe Fluorescent Cell Imager at a magnification of 100 micrometers.

MTT Assay

The MTT (Trevigen) was conducted in quadruplicate in the 96-well plate using 103 cells per each well. Treatment periods with HB lasted for 70, 40 and 20 hours. Shorter treatments were chosen as no significant results were observed with 70 hours long treatment. MTT assay was performed prior to relative gene expression analysis. The optical density was measured using Bio Rad iMark Microplate Absorbance Reader (Bio-Rad Laboratories, Inc., USA) at a wavelength of 595 nm.

RNA Isolation and RT-qPCR

Cells for RNA isolation and following relative gene expression analysis were cultivated in 96-well plates. According to the results of MTT assay, the lowest tested concentration of HB (0.125 mg/mL)

was not used for RNA isolation and RT-qPCR. Total RNA was isolated from cells treated with HB for 70 hours using TRIzol Reagent (Thermo Fisher Scientific Inc., USA). For each treatment five identical samples were established and used for RNA isolation. For cDNA synthesis, performed by the standard methods (Applied Biosystems, Foster City, CA), 50 ng/μl of each RNA sample was used. Primers were obtained from Integrated DNA Technologies, Inc. (USA). The primer sequences were as follows:

- *RRBP1* Forward: CTC TAT CTC AGC ACA CCA GAA C and
- *RRBP1* Reverse: CCC TTA CCT GAT CTG AGC TTT C.
- *PER1* Forward: CCT AAT GCT CCT CTC TCC TTT G and
- *PER1* Reverse: CTC TAG CTT GGG CAC CTT ATT.

The expression of the *RRBP1* and *PER1* gene were monitored and normalized with 18S rRNA as a reference gene (Paule & White, 2000).

Total cDNA template was amplified using Power SYBR Green Master Mix (Applied Biosystems, USA), with the cycling conditions: 48°C for 30 min, 95 °C for 10 min, and then 40 cycles of 95 °C for 15 s and 60 °C for 1 min following standard program for dissociation curve. The $2^{-\Delta\Delta CT}$ method was used for relative quantification of gene expression in quantitative real-time polymerase chain reaction (qPCR) data analysis. The StepOne software was used for analysis of RT-PCR results.

Statistical analysis

Initially, experiments were performed in quadruplicate for each assay and duplicate for the RT-qPCR. However as standard deviation values were too high for the first two MTT assays conducted, for the following 70-hours' treatment and 40-hour's treatment, up to 10 replicates were performed. Arithmetic means and variability were calculated per treatment and applied assay.

The significance between differences in analyzed effects of tested concentrations of $K_2(B_3O_3F_4OH)$ was determined by a one-way analysis of variance (ANOVA) followed by post-hoc analysis with the significance level set at 0.05.

Results and Discussion

MTT Assays of H520 Cells Treated with Halogenated Boroxine

MTT assays were conducted in treatment periods of 20, 40, and 70 hours. The concentrations of HB were: 0.0 mg/mL (control), 0.125 mg/mL, 0.25 mg/mL, and 0.5 mg/mL. Higher absorbance indicates higher cell count and increased proliferation. Sample size for each level was three wells per 10000 cells.

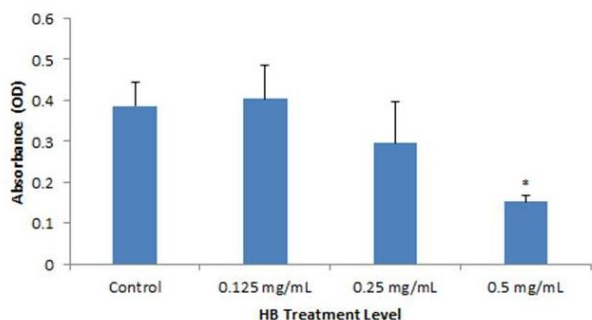


Figure 2. MTT assay - absorbance levels after 20 hours' treatment (*p = 0.033; between control and 0.5 mg/mL)

For the 20-hours' experiment a statistically significant difference ($p=0.033$) between the optical density of the control ($OD=0.385$) and 0.5 mg/mL HB treatment ($OD=0.152$) was observed. In addition, the 70-hours' treatment showed significant differences between the optical density of the control ($OD=0.798$) and 0.25 mg/mL HB treatment ($OD=0.691$) ($p=0.017$) as well as between the control and the 0.5 mg/mL HB treatment ($OD=0.634$) ($p=0.0017$). The percent decrease in the absorbance values were 13.4% and 20.6% respectively for 0.25 mg/mL and 0.5 mg/mL treatment groups. Results are presented in Figures 2 and 3 with the standard error bars included. With the increase of HB concentration, proliferation of H520 non-small lung cancer cells decreases, most likely due to the specific binding of the anion, $[B_3O_3F_4OH]^{2-}$, to the active site on the tumor cell lines.

RT-PCR Relative Expression of Apoptosis and Circadian Rhythm Genes

Relative expression of *RRBP1* and *PER1* genes was only analyzed for 70 hours' treatment and two HB

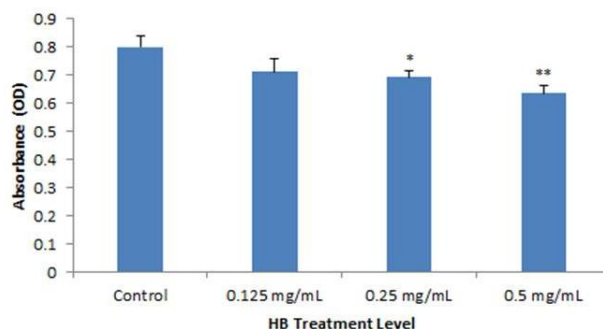


Figure 3. MTT assay - absorbance levels after 70 hours' treatment (*p value = 0.017; between control and 0.25 mg/mL **p = 0.0017 between control and 0.5 mg/mL)

concentrations: 0.25 mg/mL and 0.5 mg/mL since the lowest treatment group (0.125 mg/mL) did not show significant differences in the MTT assay. As treatment concentration of HB increased, the relative expression of *RRBP1* decreased. Levels of relative expression of *RRBP1* gene in different concentrations were compared by t-test. Significant differences were not found as p values were 0.389 between the control and 0.25 mg/mL treatment and 0.202 between the control and 0.5 mg/mL treatment.

Registered underexpression of *PER1* gene as HB concentration increased was also insignificant. The p values were 0.684 between the control and 0.25 mg/mL and 0.211 between the control and 0.5 mg/mL.

The apparent percent decreases between the control and 0.5 mg/mL treatments for both the *RRBP1* and *PER1* genes were 81.6% and 83.0% respectively. Even though the apparent percent decreases were large they were insignificant due to the wide variations in data and the small sample size of 5 samples per treatment. However, alternative primers and/or normalization genes selected may provide more reliable data of HB effects on relative gene expression of *RRBP1* and *PER1* genes.

Conclusions

Halogenated boroxine (HB) has shown anti-proliferative effects on different cancer cell lines but had never been tested against lung cancer cells. This study aimed at determining whether HB could be a potential treatment for lung cancer and at which lowest threshold concentration it would have anti-

proliferative effects. Additionally, we evaluated relative expression of *RRBP1* and *PER1* gene hypothesizing that increased HB concentration would lead to increased *PER1* and decreased *RRBP1* expression.

The results of MTT assay generally showed a significant effect of HB in the highest applied concentration (0.5 mg/mL).

Our predictions that increased HB concentration would lead to increased *PER1* expression and decreased *RRBP1* expression as HB treatment concentration increased were not supported. Although the expression of both genes decreased, statistical analysis showed that the decrease is insignificant for both *RRBP1* and *PER1* but future analysis with the better designed primers should be performed. Furthermore, relative gene expression analysis should be followed by protein expression analysis.

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