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New *in vitro* findings about halogenated boroxine cytotoxicity and deregulation of cell death-related genes in GR-M melanoma cells

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Anti-proliferative effects of halogenated boroxine – $K_2(B_3O_3F_4OH)$ (HB) – have been confirmed in multiple cancer cell lines, including melanoma, but the exact mechanism of action is still unknown. This study aimed to determine its cytotoxic effects on human Caucasian melanoma (GR-M) cell growth *in vitro* as well as on the expression of cell death-related genes *BCL-2, BECN1, DRAM1,* and *SQSTM1*. GR-M and peripheral blood mononuclear (PBM) cells were treated with different HB concentrations and their growth inhibition and relative gene expression profiles were determined using the Alamar blue assay and real-time PCR. HB significantly inhibited cell growth of both GR-M and PBM cells but was even more effective in GR-M melanoma cells, as significant inhibition occurred at a lower HB concentration of 0.2 mg/mL. GR-M *BCL-2* expression was significantly downregulated (P=0.001) at HB concentration of 0.4 mg/mL, which suggests that HB is a potent tumour growth inhibitor. At the same time, it upregulated *BCL-2* expression in normal (PBM) cells, probably by activating protective mechanisms against induced cytotoxicity. In addition, all but the lowest HB concentrations significantly upregulated *SQSTM1* (P=0.001) in GR-M cells. Upregulated *BECN1* expression suggests early activation of autophagy at the lowest HB concentration in *SQSTM1* cells and at all HB concentrations in PBM cells. Our findings clearly show HB-associated cell death and, along with previous cytotoxicity studies, reveal its promising anti-tumour potential.

KEY WORDS: anti-proliferative effect; *BCL-2*; *BECN1*, *DRAM1*; human Caucasian melanoma; peripheral blood mononuclear cell; *SQSTM1*

Malignant melanoma is a very aggressive tumour with poor prognosis and increasing incidence (1), which calls for an urgent development of new therapeutic strategies (2). Drugs that target immune checkpoint inhibitors have largely improved the response rates and survival of patients with advanced melanoma (3), yet there are still many who do not respond to or are ineligible for immune checkpoint and targeted therapies (*BRAF* mutations) (4, 5).

Because of direct exposure to a great stress, such as UVB radiation, melanocytes are programmed to survive by increasing the expression of the anti-apoptotic BCL-2 protein, which is also retained in malignant transformation and photocarcinogenesis (6). Considering that apoptosis is a key process suppressing tumour proliferation, targeting BCL-2 proteins in melanoma, regardless of the *BRAF* mutation, has a potential to overcome the issue of melanoma relapse in current treatments (6). Anti-apoptotic BCL-2 family proteins bind to pro-apoptotic ones and prevent their activation. The balance between these two groups determines cell survival or death (6).

Similarly, BCL-2 can bind to Beclin-1, an early regulator of cell autophagy, and inhibit its function, which then disrupts cross-regulation between apoptosis and autophagy and cell homeostasis (7). Autophagic cell death is yet another promising target of melanoma treatment (8), which involves the SQSTM1 and DRAM1 proteins.

Previous studies of halogenated boroxine (HB) [$K_2(B_3O_3F_4OH)$] have shown promising therapeutic effects (9–19), especially in view of the fact that cancer cells are more sensitive to HB than normal cells (11, 12, 19). Several studies reported that HB significantly affected the regulation of apoptosis-related genes in UT-7 human leukaemia (12, 19). However, the relative expression of cell death-related genes has not been studied yet in human melanoma.

Therefore, the aim of our study was to evaluate the effects of HB on GR-M and normal peripheral blood mononuclear (PBM) cell growth under the hypothesis that HB would increase melanoma susceptibility to cell death by deregulating the transcriptional activity of cell death-related genes.

MATERIALS AND METHODS

Halogenated boroxine synthesis and solution preparation

We synthesised HB as a water-soluble white powder (99.99 % purity) at the University of Sarajevo, Faculty of Science, Laboratory

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for Physical Chemistry as described elsewhere (9–18). HB stock solution was prepared by dissolving 20 mg of HB in 1 mL of the RPMI-1640 culture medium (Sigma-Aldrich, St. Louis, MO, USA).

Cell culture treatment

The GR-M cells (Culture Collections No. 95032301, Public Health England, London, UK) were grown in RPMI-1640 with 10 % of foetal bovine serum (FBS) supplemented with L-glutamine, penicillin, and streptomycin in an incubator (EC160, Nuve, Akyurt/Ankara, Turkey) maintaining a humidified atmosphere 37 °C and 5 % CO_2 (20). All reagents for cell cultivation were purchased from Sigma-Aldrich (St. Louis, MO, USA).

The PBM cells were obtained by separation from 3 mL of whole blood using density gradient centrifugation with the Histopaque-1077 separation medium (Merck KGaA, Darmstadt, Germany). Blood was collected by venepuncture into a heparinised vacutainer from a healthy volunteer who previously signed informed consent. Isolated cells were re-suspended in the PBMax Karyotyping Medium (Life Technologies, London, UK) and cultivated in a 96-well plate at 37 °C for 24 h (21).

Both 24-hour cultures were then treated with 0.05, 0.1, 0.2, 0.4, and 0.8 mg/mL of HB and incubated for another 24 h. For negative control we used untreated GR-M and PBM cells.

To analyse gene expression in PBM cells we added 400 μL of peripheral blood to 5 mL of PBMax Karyotyping Medium in 15 mL sterile plastic tubes with a conical bottom (NEST, Wuxi, Jiangsu, China), incubated them at 37 °C for 24 h, and treated as described above.

Alamar blue assay

Alamar blue assay was used to determine the toxicity of HB in the GR-M and PBM cells (22, 23). Each culture containing 10.000 cells/mL was incubated with HB in a 96-well plate for 24 h. Then we added Alamar blue in the amount equal to 10 % of the culture volume, and 2 h later, measured the absorbance at 570 and 620 nm, using a Multiscan FC plate reader (Thermo Fisher Scientific, Vantaa, Finland). Each treatment was done in triplicate. For positive growth control we used cultures without the test substance, while negative control did not contain cells. For blank samples we used the medium alone. Cell growth inhibition was calculated according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA) using correction factor (22, 24), and presented as percentage of positive growth control.

RNA isolation and gene expression analysis

Total RNA was extracted from harvested melanoma cells and peripheral whole blood with a Nucleo Spin RNA isolation kit according to the manufacturer's instructions (Macherey-Nagel, Düren, Germany) and quantified with a Qubit 2.0 fluorimeter (Life Technologies, London, UK). Total RNA at concentration of 40 ng/μL was used to synthesise copy DNA (cDNA) using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Waltham, MA, USA) according to the manufacturer's instructions. Primer sequences of target cell death-related genes are presented in Table 1.

Target genes and the housekeeping *GAPDH* gene were amplified in replicates using an ABI 7300 real-time PCR instrument (Applied Biosystems, Foster City, CA, USA) (25). The reaction contained a cDNA template, Power SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA), forward and reverse primers, and nuclear-free water in a total volume of 10 μL. The obtained relative expression of the *BECN1, SQSTM1*, *BCL-2,* and *DRAM1* genes was then normalised against *GAPDH* with the Relative Expression Software Tool 384 v. 2 (REST-384) (26). REST-384 was also used to compare gene expressions at transcription level in HB treatments and controls (27).

Statistical analysis

Expression ratios (of four transcripts) were tested for significances ($p<0.05$) with a pairwise fixed reallocation randomisation test (26, 27). The test compares the cycle threshold (Ct) values of treated and control samples (target genes).

To determine the significance of cell growth inhibition between HB concentrations in GR-M and PBM cells we relied on one-way analysis of variance (ANOVA), followed by Neuman-Keuls post-hoc analysis (MedCalc 18.9. software, Ostend, Belgium). Correlations between growth inhibition in GR-M and PBM cells were determined with the Pearson correlation coefficient. The level of significance was set to $p<0.05$.

Table 1 Primer sequences used for selected cell death-related genes

Gene	Reference sequence No. (FASTA format)	Primers
BECN1	NM_003766.5	Forward: CTCCCGAGGTGAAGAGCATC Reverse: GGGGGATGAATCTGCGAGAG
SQSTM1	NM_003900.5	Forward: CCGTGAAGGCCTACCTTCTG Reverse: TCCTCGTCACTGGAAAAGGC
BCL-2	NM_000633.3	Forward: GGGGTCATGTGTGTGGAGAG Reverse: GAAATCAAACAGAGGCCGCA
DRAM1	NM_018370.3	Forward: TTGGTGCAGCCACGATGTAT Reverse: ACACCACAGACAAAGGCCAA

RESULTS

Cytotoxic effects of halogenated boroxine

HB exhibited cytotoxic activity on both GR-M and PBM cells (Figure 1). Higher concentrations (0.2, 0.4, and 0.8 mg/mL) resulted in a significantly higher cell growth inhibition than 0.05 and 0.1 mg/mL (F=12.965; p=0.001) in GR-M cells. In PBM cells growth inhibition was significantly higher with HB concentrations of 0.4 and 0.8 mg/mL compared to 0.05 and 0.1 mg/mL (F=54.861; p<0.001). Growth inhibition correlated significantly between GR-M and PBM cells (Pearson correlation coefficient r=0.633; p=0.01; 95 % CI 0.1789–0.8649).

Changes in cell death-related genes

Tables 2–5 show changes in the relative expression of cell death-related genes in both cultures exposed to different HB concentrations. In GR-M cells, HB significantly downregulated the relative expression of *BCL-2* only at the concentration of 0.4 mg/mL and upregulated it at 0.1 and 0.2 mg/mL compared to negative control. In PBM cells, significant was only the upregulation at the concentrations of 0.05, 0.2, and 0.8 mg/mL (Table 2).

BECN1 was significantly downregulated only at 0.8 mg/mL and upregulated at 0.05 mg/mL in GR-M cells, whereas upregulation was the only significant effect in PBM cells with all HB concentrations (Table 3).

DRAM1 in GR-M cells was significantly downregulated at the highest HB concentrations of 0.4 and 0.8 mg/mL, whereas in PBM cells significant were its upregulations at 0.1, 0.2, and 0.4 mg/mL. We could not detect the Ct values for the highest HB concentration in PBM cells (Table 4).

The relative expression of *SQSTM1* in the GR-M cells was also significantly upregulated with all but the lowest HB concentration. In PBM cells, it was significantly upregulated at 0.2 and 0.4 mg/mL (Table 5).

DISCUSSION

Changes in cell death-related gene expression in GR-M cells confirm our hypothesis that HB would increase melanoma susceptibility to cell death. Our findings corroborate recent *in vitro* studies reporting evidence of anti-tumour effects of HB in different cancer cell types (9, 11–13, 19), including GR-M (9). Cytotoxic effects were more pronounced against tumour GR-M cells which additionally supports reported selective toxicity of HB (11, 12, 19). However, downregulation of *BCL-2* was significant only at 0.4 mg/mL, which suggests that HB concentrations above 0.2 mg/mL induce apoptosis. This HB effect coincides with the highest growth inhibition rate of 70.09 % at the concentration of 0.4 mg/mL (Figure 1). The observed upregulation of *BCL-2* expression in PBM cells suggests that healthy cells activate protective mechanisms against HB-induced cytotoxicity (12). This kind of rather selective downregulation of *BCL-2* expression in GR-M cells is a valuable finding, as the evasion of apoptosis and impaired apoptotic signalling have been crucial for cancer proliferation and resistance to treatment. Our findings support recent reports (12, 19) of HB-downregulated BCL-2 protein expression and induction of apoptosis in human leukaemia UT-7 but not normal cells.

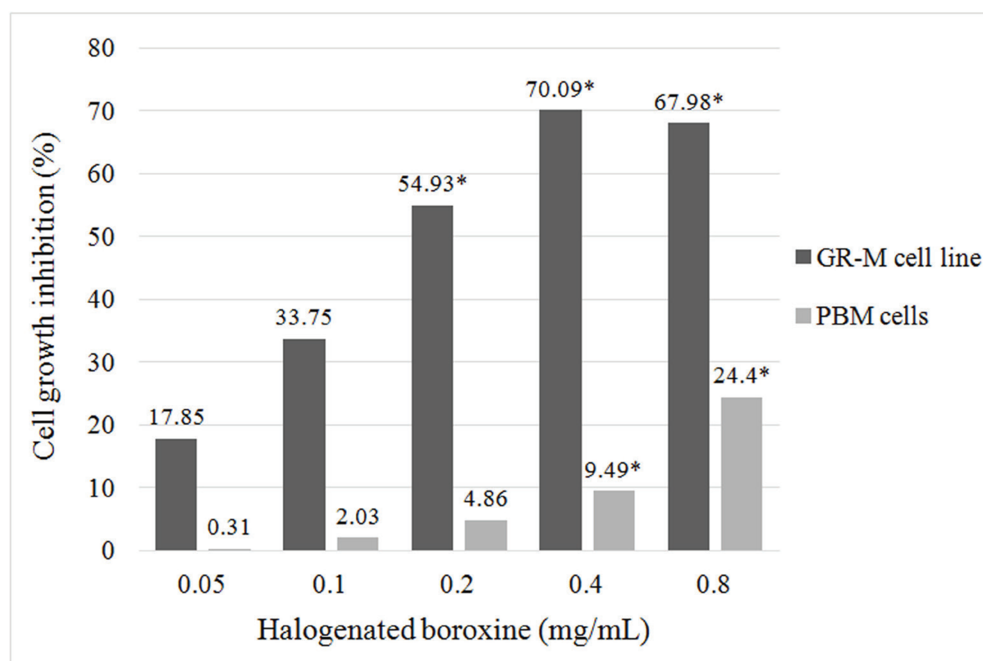

Figure 1 Growth inhibition of GR-M and PBM cells treated with different concentrations of halogenated boroxine; *p<0.05

Table 2 Changes in relative *BCL-2* gene expression after GR-M and PBM cell treatment with halogenated boroxine compared to negative control

HB concentrations (mg/mL)	GR-M cells		PBM cells	
	↑↓	Fold change/ P value	↑↓	Fold change/ P value
0.05	down	-3.805/0.672	up	2.04/**0.001**
0.1	up	11.769/**0.001**	down	-1.158/0.677
0.2	up	1.568/**0.001**	up	2.14/**0.001**
0.4	down	-10.933/**0.001**	down	-1.262/0.169
0.8	down	-1.041/0.848	up	6.365/**0.001**

HB – halogenated boroxine; GR-M – human Caucasian melanoma; PBM cells – peripheral blood mononuclear cells

Table 3 Changes in relative *BECN1* gene expression after GR-M and PBM cell treatment with halogenated boroxine compared to negative control

HB concentrations (mg/mL)	GR-M cells		PBM cells	
	↑↓	Fold change/ P value	↑↓	Fold change/ P value
0.05	up	1.788/**0.001**	up	3.884/**0.001**
0.1	down	2.143/0.0815	up	1.848/**0.001**
0.2	up	2.202/0.322	up	15.735/**0.001**
0.4	up	1.04/0.83	up	62.542/**0.001**
0.8	down	3.188/**0.001**	up	72636.933/**0.001**

HB – halogenated boroxine; GR-M – human Caucasian melanoma; PBM cells – peripheral blood mononuclear cells

Table 4 Changes in relative *DRAM1* gene expression after GR-M and PBM cell treatment with halogenated boroxine compared to negative control

HB concentrations (mg/mL)	GR-M cells		PBM cells	
	↑↓	Fold change/ P value	↑↓	Fold change/ P value
0.05	down	1.661/0.3405	up	15.778/0.667
0.1	down	1.098/0.671	up	459.792/**0.001**
0.2	down	1.209/0.828	up	7968.193/**0.001**
0.4	down	4.195/**0.001**	up	6014.928/**0.001**
0.8	down	2.344/**0.001**	/	/

HB – halogenated boroxine; GR-M – human Caucasian melanoma; PBM cells – peripheral blood mononuclear cells

Table 5 Changes in relative *SQSTM1* gene expression after GR-M and PBM cell treatment with halogenated boroxine compared to negative control

HB concentrations (mg/mL)	GR-M cells		PBM cells	
	↑↓	Fold change/ P value	↑↓	Fold change/ P value
0.05	up	1.387/0.1695	down	1.078/0.6465
0.1	up	3.643/**0.001**	down	1.951/0.153
0.2	up	11.612/**0.001**	up	5.764/**0.001**
0.4	up	6.92/**0.001**	up	7.424/**0.001**
0.8	up	3.295/**0.001**	up	1.004/0.6465

HB – halogenated boroxine; GR-M – human Caucasian melanoma; PBM cells – peripheral blood mononuclear cells

As for autophagy genes in melanoma cells, we found that significantly downregulated *DRAM1* expression at the highest HB concentrations coincided with *SQSTM1* upregulation and *BCL-2* downregulation, whereas its expression in PBM cells was significantly upregulated. DRAM1 is involved in the elimination of autophagosomes (28), and the knock-down of *DRAM1* gene affects *SQSTM1* -mediated autophagy (29). Along with downregulation of *BCL-2,* it suggests that HB promotes cell death in GR-M cells.

We also determined a significant upregulation of *BECN1* at the lowest HB concentration (0.05 mg/mL) in both cell types, and upregulation of *SQSTM1* at all except the lowest concentration in GR-M but not in PBM cells. We know that the *BECN1*-encoded beclin 1 protein is responsible for the nucleation and maturation of autophagosomes and that higher autophagosome counts indicate higher cell degradation but may point to a blocked autophagy flux as well (30). Upregulated expression of *SQSTM1* suggests higher cell content that needs to be labelled for degradation, because the sequestosome 1 protein encoded by *SQSTM1* is a selective autophagy receptor (31).

CONCLUSION

Halogenated boroxine inhibited the growth of both GR-M and PBM cells but was significantly more cytotoxic to the tumour cells. Furthermore, it promoted cell death processes in GR-M cells. Even though our study is somewhat limited as it did not include protein analysis that would confirm the gene expression profile, this remains for future *in vitro* studies to establish and expand their interest to a number of other tumour cell lines to get a better insight into the HB therapeutic potential as a cell death promotor.

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Conflicts of interest

None to declare.

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Nova *in vitro* otkrića o citotoksičnosti halogeniranoga boroksina i deregulaciji gena povezanih sa staničnom smrću u stanicama GR-M melanoma

Antiproliferativni učinci halogeniranoga boroksina – $K_2(B_3O_3F_4OH)$ (HB) – potvrđeni su u više staničnih linija raka, uključujući melanom, ali točan mehanizam djelovanja još uvijek nije poznat. Cilj ovoga istraživanja bio je utvrditi njegove citotoksične učinke na rast stanica ljudskoga melanoma (GR-M) *in vitro,* kao i na ekspresiju gena *BCL-2*, *BECN1*, *DRAM1* i *SQSTM1,* povezanih sa staničnom smrću. GR-M melanom i mononuklearne stanice periferne krvi (PBM) tretirane su različitim koncentracijama HB-a, a njihova inhibicija rasta i relativni profili ekspresije gena određeni su *Alamar blue* testom i *real-time* PCR-om. HB je značajno inhibirao rast GR-M melanoma i PBM stanica, no u GR-M melanomu učinci su registrirani pri nižim koncentracijama HB-a. Ekspresija *BCL-2* gena u GR-M melanomu bila je značajno smanjena (P=0,001) pri koncentraciji od 0,4 mg/mL, što sugerira da je HB snažan inhibitor rasta tumora. Istodobno, pojačao je ekspresiju *BCL-2* u normalnim PBM stanicama, vjerojatno aktiviranjem zaštitnih mehanizama protiv inducirane citotoksičnosti. Osim toga, sve osim najniže koncentracije HB-a značajno su povećale ekspresiju *SQSTM1* (P=0,001) u GR-M melanomu. Povećana ekspresija *BECN1* u najnižoj koncentraciji HB-a u GR-M stanicama i pri svim koncentracijama u PBM stanicama sugerira ranu aktivaciju autofagije. Naša otkrića jasno pokazuju indukciju stanične smrti povezane s HB-om i zajedno s prethodnim studijama citotoksičnosti otkrivaju njegov obećavajući antitumorski potencijal.

KLJUČNE RIJEČI: antiproliferativni učinak; *BCL-2*; *BECN1*, *DRAM1*; ljudski kavkaski melanom; mononuklearne stanice periferne krvi; *SQSTM1*