



RESEARCH ARTICLE

## Effects of dipotassium-trioxohydroxytetrafluorotriborate, $K_2[B_3O_3F_4OH]$ , on cell viability and gene expression of common human cancer drug targets in a melanoma cell line

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### Abstract

Recently it was found that dipotassium-trioxohydroxytetrafluorotriborate,  $K_2(B_3O_3F_4OH)$ , is a potent and highly specific inhibitor of precancerous cell processes. We conducted gene expression profiling of human melanoma cells before and after treatment with two concentrations (0.1 and 1 mM) of this boron inorganic derivative in order to assess its effects on deregulation of genes associated with tumor pathways. Parallel trypan blue exclusion assay was performed to assess the cytotoxicity effects of this chemical. Treatment with  $K_2(B_3O_3F_4OH)$  induced a significant decrease of cell viability in melanoma cellline at both tested concentrations. Furthermore, these treatments caused deregulation of more than 30 genes known as common anti-tumor drug targets. IGF-1 and hTERT were found to be significantly downregulated and this result may imply potential use of  $K_2(B_3O_3F_4OH)$  as an inhibitor or human telomerase and insulin-like growth factor 1, both of which are associated with various tumor pathways.

### Introduction

Cancer research continually identifies novel deregulated carcinogenesis-related genes elucidating new mechanisms of cancer progression or treatment evasion, and potentially leading to new avenues for drug development.

$K_2(B_3O_3F_4OH)$ , is a boron inorganic derivative recently explored as useful in treatment of benign and malignant skin changes, such as nevus or skin cancer<sup>1,2</sup>. First published effects of its bioactivity, revealed a potential for inhibition of lymphocytes proliferation and cell growth of basal cell carcinoma culture as well as certain clastogenic potential<sup>3</sup>. Recently it has been confirmed that selected flavonoids may inhibit damages of genetic material in human lymphocytes induced by  $K_2(B_3O_3F_4OH)$  in concentration of 0.1 mg/ml<sup>4</sup>.  $K_2(B_3O_3F_4OH)$  has been recognized as a potent inhibitor of human carbonic anhydrases and catalase involved in the intracellular antioxidant mechanism<sup>5</sup>. Preliminary investigations imply that acute toxicity of this substance is approximately 60 mg/kg suggesting reproducible biological effects on genetic material at low treatment concentrations (B. Galic, data not published). Previous findings of antitumor activity of the halogenated boroxine *in vitro* and *in vivo*

### Keywords

Antitumor drug target, dipotassium-trioxohydroxytetrafluorotriborate, gene down-regulation, gene expression, human melanoma cell line, human telomerase, insulin-like growth factor 1

### History

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on 4T1 mammary carcinoma, B16F10 melanoma and squamous cell carcinoma SCVII revealed inhibitory effects on cell proliferation in concentrations of 1 mM while concentrations of less than 0.1 mM do not significantly affect cell growth<sup>6</sup>. *In vivo*,  $K_2(B_3O_3F_4OH)$  slows the growth of three tested tumors compared to control regardless of the route of administration (intraperitoneally, intratumor, per oral or as an ointment).

This experiment was designed to assess the effects on gene expression of selected antitumor targets under the treatment with previously established effective concentration limits of  $K_2(B_3O_3F_4OH)$  (0.1 mM and 1 mM) in comparison to untreated tumor cells (control). Differential gene expression between treated and untreated cell lines, as well as relative effects between two tested concentrations of tested substance have been evaluated. The qPCR assay that was used in this experiment precisely quantified expression of genes involved as human cancer drug targets in melanoma cell lines treated with  $K_2(B_3O_3F_4OH)$ .

### Material and methods

#### Chemistry

The boron inorganic heterocyclic compound dipotassium-trioxohydroxytetrafluorotriborate,  $K_2(B_3O_3F_4OH)$ , was prepared as reported in the literature<sup>7</sup>. All other compounds used in this study

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Table 1. Results of trypan blue exclusion assay.

Treatment	Viable cells ( $\times 10^4$ cells/ml)	Non-viable ( $\times 10^4$ cells/ml)	Total no. of cells ( $\times 10^4$ cells/ml)	No. of cells per culture ( $\times 10^6$ cells/ml)	Viability (%)
Control	74.67 $\pm$ 33.38	14.67 $\pm$ 7.64	89.33 $\pm$ 40.43	7.14 $\pm$ 3.23	83.72 $\pm$ 4.07
0.1 mM	46.33 $\pm$ 17.39	17.33 $\pm$ 10.12	64 $\pm$ 26.96	5.12 $\pm$ 2.16	73.21 $\pm$ 4.66*
1 mM	17.33 $\pm$ 7.09	10.33 $\pm$ 4.93	27.33 $\pm$ 11.15	2.18 $\pm$ 0.89	63.32 $\pm$ 5.88*

Values are presented as the mean  $\pm$  SD.

\*Significant difference in comparison to control ( $p < 0.05$ ).

Table 2. List of genes with changed expression profile after  $K_2(B_3O_3F_4OH)$  treatment with 1 mM and 0.1 mM: negative values show gene down-regulation of  $\geq 2$  fold compared to control – untreated melanoma cells; positive values show gene up-regulation of  $\geq 2$  fold compared to control – untreated melanoma cells).

Gene symbol	Gen bank reference	Gene description	Treatment concentrations	
			1 mM	0.1 mM
AKT1	NM 005163	V-akt murine thymoma viral oncogene homolog 1	-2.57	
AKT2	NM 001626	V-akt murine thymoma viral oncogene homolog 2	-2.99	
AURKA	NM 003600	Aurora kinase A	-3.20	
BIRC5	NM 001168	Baculoviral IAP repeat containing 5	-2.93	
CDK1	NM 001786	Cyclin-dependent kinase 1	-3.81	
CDK2	NM 001798	Cyclin-dependent kinase 2	-2.01	
CDK4	NM 000075	Cyclin-dependent kinase 4	-2.04	
CDK9	NM 001261	Cyclin-dependent kinase 9	-2.48	
CTSB	NM 001908	Cathepsin B	-4.44	
CTSS	NM 004079	Cathepsin S	-7.56	
EGFR	NM 005228	Epidermal growth factor receptor	-3.01	
ERBB3	NM 001982	V-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)	6.64	2.46
ERBB4	NM 005235	V-erb-a erythroblastic leukemia viral oncogene homolog 4 (avian)		3.30
ESR1	NM 000125	Estrogen receptor 1	2.62	2.59
ESR2	NM 001437	Estrogen receptor 2 (ER beta)	3.16	4.57
GRB2	NM 002086	Growth factor receptor-bound protein 2	-6.86	
HDAC6	NM 006044	Histone deacetylase 6	-3.46	
HIF1A	NM 001530	Hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	-3.63	
HSP90B1	NM 003299	Heat shock protein 90 kDa beta (Grp94), member 1	4.11	
IGF1	NM 000618	Insulin-like growth factor 1 (somatomedin C)		-2.26
KDR	NM 002253	Kinase insert domain receptor (a type III receptor tyrosine kinase)	-4.96	
KRAS	NM 004985	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	-3.27	
NFKB1	NM 003998	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	-2.34	
PARP2	NM 005484	Poly (ADP-ribose) polymerase 2	2.35	
PARP4	NM 006437	Poly (ADP-ribose) polymerase family, member 4	-2.34	
PDGFRA	NM 006206	Platelet-derived growth factor receptor, alpha polypeptide	-8.75	
PDGFRB	NM 002609	Platelet-derived growth factor receptor, beta polypeptide	-2.48	
PGR	NM 000926	Progesterone receptor		2.81
PLK1	NM 005030	Polo-like kinase 1	-4.08	
PLK2	NM 006622	Polo-like kinase 2	-2.41	
PRKCA	NM 002737	Protein kinase C, alpha	-6.49	
PRKCB	NM 002738	Protein kinase C, beta		5.10
PRKCD	NM 006254	Protein kinase C, delta	-3.11	
PRKCE	NM 005400	Protein kinase C, epsilon	-2.91	
TERT	NM 198253	Telomerase reverse transcriptase	9.72	-2.00
TOP2A	NM 001067	Topoisomerase (DNA) II alpha 170 kDa	-7.56	
TOP2B	NM 001068	Topoisomerase (DNA) II beta 180 kDa	-2.36	
TP53	NM 000546	Tumor protein p53	-5.77	

were commercially available, highest purity reagents (Sigma-Aldrich, St. Louis, MO).

### Melanoma cell line

The Human Caucasian melanoma cell line (GR-M) was obtained from Culture Collections, Public Health England, London, UK (Cat. No. 95032301). Cell line was cultured in RPMI 1640 (Gibco-Invitrogen, Grand Island, NY) with L-glutamine

supplemented with 10% of fetal bovine serum (FBS) and 100 units/ml penicillin, 100 mg/ml streptomycin (Sigma-Aldrich, St. Louis, MO). T-25 flasks (NUNC, Rochester, NY) with vented caps were used as a culture dishes and cultures were maintained in CO<sub>2</sub> incubator (EC 160, Nuve, Ankara, Turkey) at 37 °C in a 5%CO<sub>2</sub> atmosphere with 95% humidity. Cell cultures were treated with  $K_2(B_3O_3F_4OH)$  in concentrations of 0.025 mg/ml and 0.25 mg/ml at 70% of confluence. Cells were exposed to  $K_2(B_3O_3F_4OH)$  for 48 h.

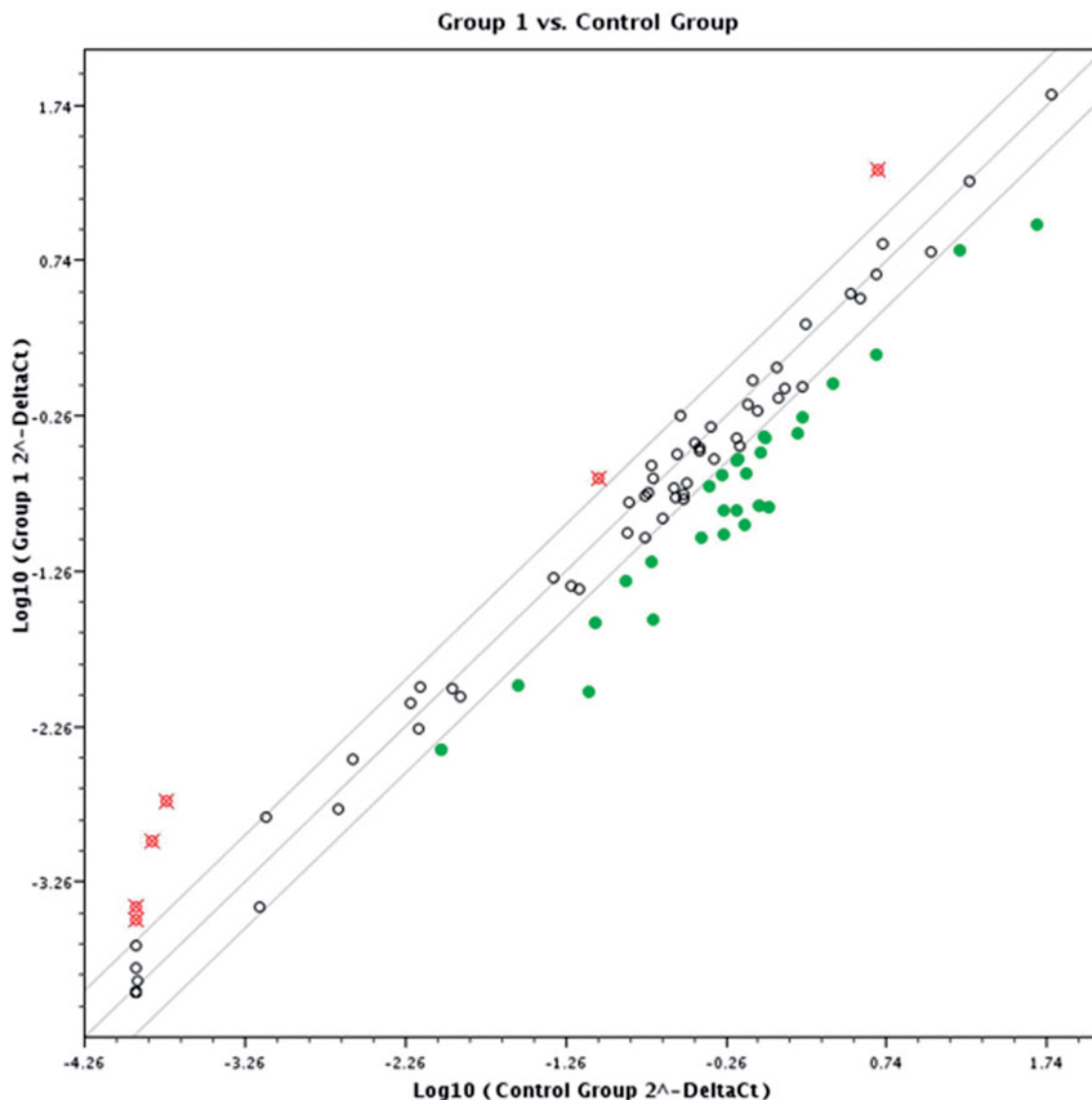


Figure 1. Scatter plot diagram comparing expression profiles of the cells treated with 1 mM of  $K_2(B_3O_3F_4OH)$  and untreated melanoma cells. Empty dots represent genes whose expression did not change significantly in response to treatment. Filled dots represent genes that have been down-regulated and crossed dots genes with two or more fold up-regulation.

### Trypan blue exclusion assay

For the cytotoxicity analysis trypan blue exclusion assay was performed after 48 h of incubation of melanoma cell cultures with  $K_2(B_3O_3F_4OH)$ . Each treatment was carried out in triplicates. Cells were harvested by trypsinization and the cell viability (%) was determined as (number of viable cells/total number of viable + non-viable cells)  $\times$  100. ANOVA and Newman-Keuls multiple comparison test in WINKS 4.5 Professional software (TexaSoft, Cedar Hill, TX) were used for statistical analysis and pairwise comparisons. Statistical significance threshold was fixed at 0.05.

### RNA isolation and RT PCR

Upon the treatment of melanoma cells by  $K_2(B_3O_3F_4OH)$  in the above-mentioned manner, total RNA was extracted from harvested cells using *RNA isolation kit* (QIAGEN GmbH, Hilden, Germany). The quality and quantity of isolated RNA was estimated using RNA fluorometric assay on Qubit 2.0 (Life Technologies, London, United Kingdom). Additional purification step was introduced to eliminate any residual genomic DNA that might cause false positive signals. RT<sup>2</sup> First strand kit (Qiagen

GmbH, Hilden, Germany) was used for generation of cDNA for further genetic analysis according to manufacturer's protocol (RT<sup>2</sup> Profiler PCR Array Handbook, Qiagen N. V., Venlo, Limburg, Netherlands).

### Gene expression analysis

Real Time PCR is a highly sensitive and reliable method for gene expression analysis at cell transcriptome level. It is a convenient method for simultaneous analysis of number of different genes in the same sample. Human Cancer Drug Targets RT<sup>2</sup> Profiler<sup>TM</sup> PCR method is used according to manufacturer's instructions (RT<sup>2</sup> Profiler PCR Array Handbook, Qiagen N. V., Venlo, Limburg, Netherlands) for a multigene profiling of expression of 84 genes (Table 2) on ABI 7300 real-time PCR apparatus (Applied Biosystems, Foster City, CA). The three cell populations used in the experiment (cell culture control sample and two cell cultures treated with  $K_2(B_3O_3F_4OH)$  in concentrations of 0.1 mM and 1 mM) were subsequently analyzed in three identical 96 wells array formats. The quality check up (false positive and false negative signals evaluation) was based on positive and negative controls included on arrays, as well as gDNA contamination probes.

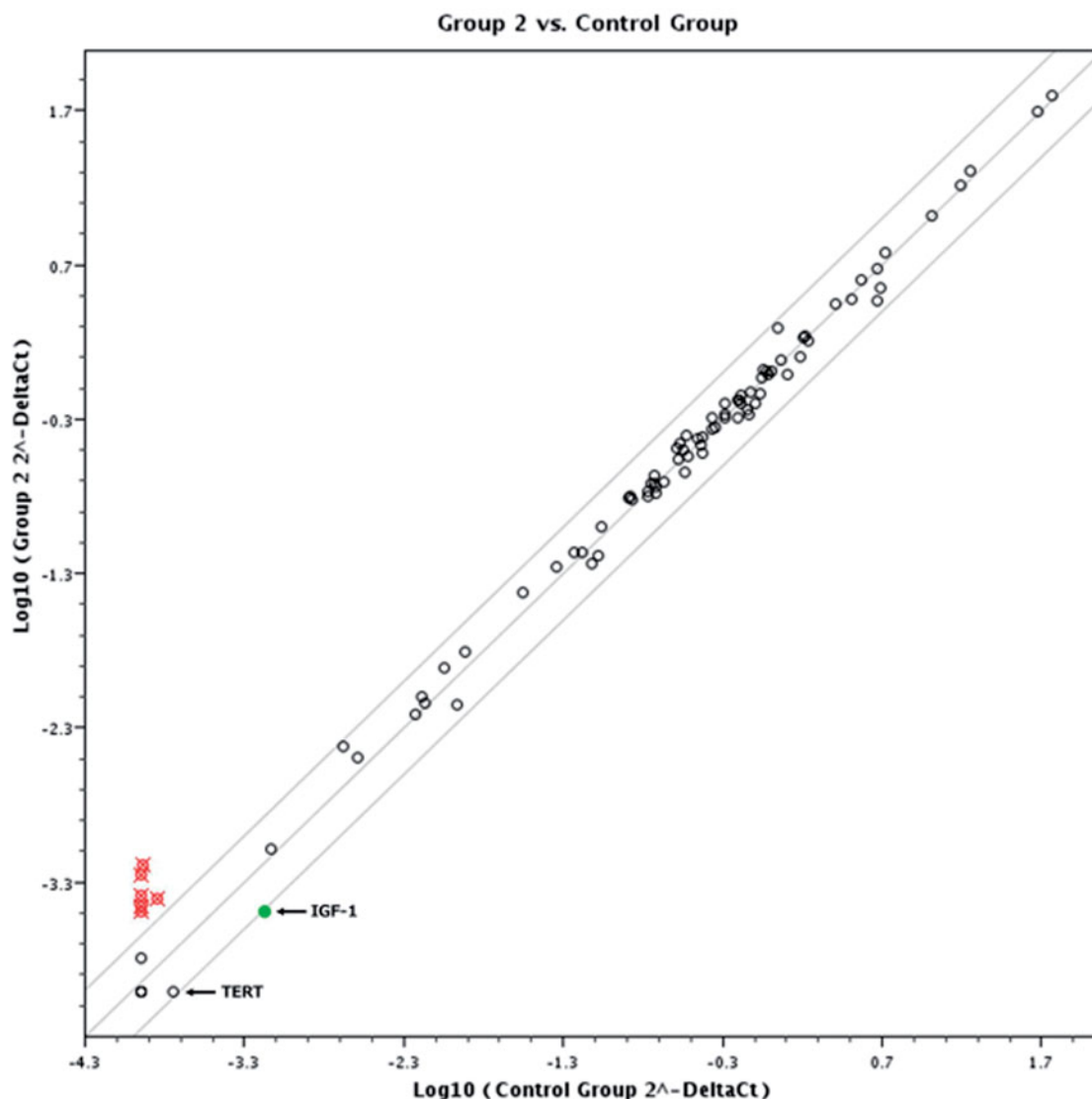


Figure 2. Scatter plot diagram comparing expression profiles of the cells treated with 0.1 mM of  $K_2(B_3O_3F_4OH)$  and untreated melanoma cells. Empty dots represent genes whose expression did not change significantly in respect to treatment. Filled dots represent genes that have been down-regulated and crossed dots genes with two or more fold up-regulation.

Genetic data were collected during the real-time PCR procedure in a form of Ct value or the order number of PCR cycle when target (gene) signal amplification reached exponential phase. All samples that had ct value 35 and higher were considered as null gene expression. Fold change in gene expression between melanoma cell culture treated with  $K_2(B_3O_3F_4OH)$  (0.1 mM and 1 mM) and control (untreated cells) and between two treated samples was calculated using  $2^{(-\Delta\Delta CT)}$  Method (www.SABiosciences.com).

## Results and discussion

### Cytotoxic effects

Exposure of GR-M cells to  $K_2(B_3O_3F_4OH)$  induced decrease in viable cell number in a concentration dependent manner. Data, presented in Table 1, are expressed as mean  $\pm$  standard deviation (SD). Analysis of variance revealed that the decrease of cell viability was significant ( $p=0.006$ ) in both treatments as compared to controls, while there were no significant differences between treatments with two different concentrations of  $K_2(B_3O_3F_4OH)$ .

### Gene expression effects

Prior to results readout, quality control assessment of the experiment was done. All gDNA controls were found to be above expected threshold level of 35 (below positive amplification), suggesting that any false positive signal in this experiment due to sample contamination with genomic DNA is unlikely. The cycle number deviation between triplicated signals from house-keeping genes (reference genes) was  $20 \pm 0.3$  and within the acceptable range ( $20 \pm 2.0$  cycle), indicating that no PCR inhibitors were present in amplification runs.

Gene expression was evaluated for each well and Ct values were exported for further data analysis. The control sample (untreated melanoma cells), that was used as the gene expression reference, had a measurable expression of all 84 tested genes.

Pairwise comparison results in scatter diagram show clear difference in gene expression pattern between control and cells treated with  $K_2(B_3O_3F_4OH)$  in concentration of 1 mM (Figure 1) and 0.1 mM (Figure 2) and between cells treated with different  $K_2(B_3O_3F_4OH)$  concentrations (Figure 3). If the threshold is set to two fold change in gene expression and higher, several genes'

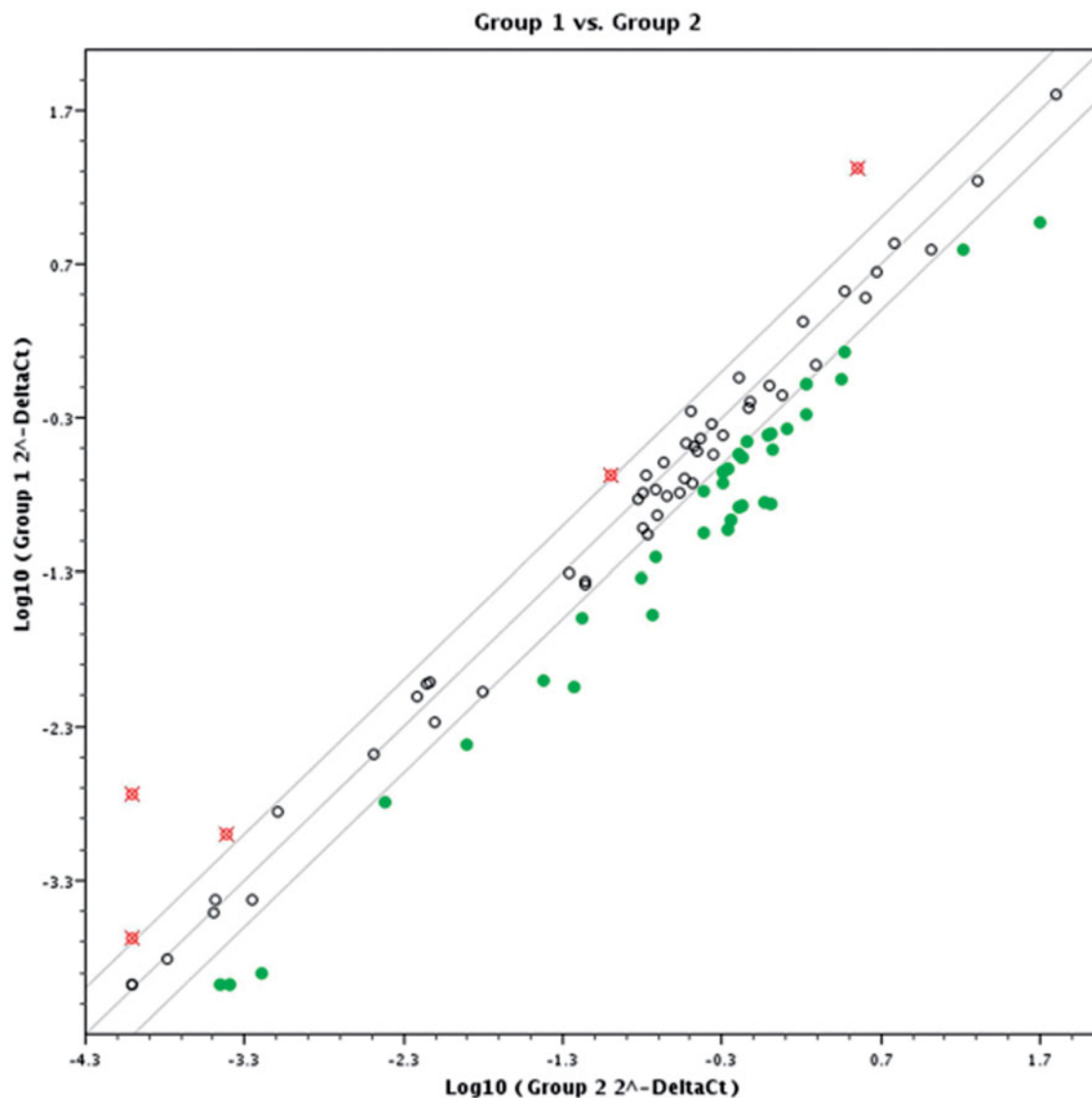


Figure 3. Scatter plot diagram comparing expression profiles of the cells treated with 1 mM and 0.1 mM of  $K_2(B_3O_3F_4OH)$ . Empty dots represent genes whose expression did not change significantly in respect to treatment. Filled dots represent genes that have been down-regulated and crossed dots genes with two or more fold up-regulation.

expression is clearly grouped as those affected by the treatment (Table 1).

(I) The treatment with 1 mM concentration of  $K_2(B_3O_3F_4OH)$  induced change in expression pattern of 36 genes from the tested panel of human cancer drug targets or 43% of all analyzed genes.

(II) Six genes were up-regulated in 1 mM concentration: ERBB3, ESR1, ESR2, HSP90B, PARP2 and TERT, and 30 genes were down-regulated under equal treatment conditions (Table 2). Negative deregulation of TOP2A, PDGFRA, KDR, GRB2 and cathepsins was observed.

(III) Treatment with 0.1 mM concentration of  $K_2(B_3O_3F_4OH)$  caused less effect on targeted genes expression profile (eight genes). Genes from the family of hormone receptors (ERBB3, ERBB4, ESR1, ESR2, PGR) and PRKCB (Protein kinase C-beta) were up-regulated in the range between 2.5 and 5-fold.

(IV) Interestingly, two genes, encoding for IGF1 and TERT were significantly down-regulated. Assessment of differential gene expression pattern between two different concentrations of  $K_2(B_3O_3F_4OH)$  revealed that: TERT gene was significantly up-regulated and IGF-1 was not deregulated at all under 0.25 mg/ml treatment.

Dysregulation of the IGF system in tumor cells has been previously qualified as a key mechanism by which the balance of pro-survival and pro-apoptotic signaling shifts in favor of survival. This pro-survival predisposition of tumors may also have a major negative impact on the anti-tumor therapies that are used in clinical practice that rely on apoptosis: cytotoxic chemotherapy, biological therapies, hormonal therapies and radiation therapy. It has been observed that patient with melanoma exhibit significantly higher serum concentrations of IGF-1 which has also been associated with increased risk of lymph metastasis<sup>8</sup>. Clinical trials have shown that patients with tumors expressing IGF-1 receptors are much less likely to obtain a clinical response to neoadjuvant therapy than non-expressors of IGF-1<sup>9</sup>. From this perspective, blocking IGF system signaling has the potential for numerous clinically useful effects, including increasing the proportion, extent and duration of clinical responses from cytotoxic therapies when used in combination. Other studies showed the potential of IGF/IGF-IR pathway inhibition in antitumor and treatment of autoimmune disorders<sup>10,11</sup>.

Another notable effect of  $K_2(B_3O_3F_4OH)$  treatment at concentration of 0.1 mM is its downregulating effect on TERT gene

that is involved in telomerase activity. Telomerase is active in the majority of cancer cell types and is virtually absent in somatic cells with rare exceptions. This difference allows us to consider inhibition of telomerase activity as a possible approach to antitumor therapy<sup>12,13</sup>.

Our results are in favor of further assessment of antitumor targets deregulated by  $K_2(B_3O_3F_4OH)$  at gene expression level and proof of its inhibitory effect on various cancers via defined molecular targets and implicated pathway analysis – i.e. Ras/Raf/MEK/ERK/MAPK (Ras pathway) and PI3K/AKT (AKT pathway) that are mean of oncogenic mediation of IGF-1 signaling or telomerase components: the reverse transcriptase (hTERT), which is a catalytic subunit, and telomerase RNA (hTR) availability and interactions<sup>14</sup>.

## Conclusion

This work represents first evaluation of antitumor effects of  $K_2(B_3O_3F_4OH)$  on expression of 84 genes that encode for proteins qualified as common anti-cancer drug targets. Tested substance shows high potency, polyvalence and observable effects on gene expression of a number of tumor associated proteins and their implicated pathways. Both tested concentrations (1 and 0.1 mM) significantly inhibit cell proliferation of melanoma cells and specifically deregulate genes associated with protumor activity revealing potential antitumor molecular targets of  $K_2(B_3O_3F_4OH)$ . Targeted pathway assays upstream from deregulated genes (suggestively TERT and IGF-1) should be challenged in order to explain molecular mechanism action of  $K_2(B_3O_3F_4OH)$  in detail.

## Declaration of interest

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