Vanadium compounds affect growth and morphology of human rhabdomyosarcoma cell line

Wojciech Dąbroś[1], Anna Adamczyk[2], Katarzyna Ciurkot[2], Anna M. Kordowiak[2]

[1]Chair and Department of Pathomorphology, Faculty of Medicine *Collegium Medicum* Jagiellonian University, Cracow, Poland
[2]Department of General Biochemistry, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Cracow, Poland

Rhabdomyosarcoma (RMS) cells were incubated with four vanadium compounds: cations BMOV and vanadyl sulphate, and anions ortho- and metavanadate. Growth inhibition of RMS cells in the culture was determined by two staining methods: with N-hexamethylpararosaniline (crystal violet = CV) or bromide 3-(4,5-dimethylthiazol-2)-2,5-diphenyltetrazolium (MTT). After 48 h incubation with 10-40 μM for $NaVO_3$ or 20-40 μM for the other three vanadium salts, the results were statistically significantly lower ($0.001 < p < 0.01$) as compared to the controls (without vanadium in the medium). A vanadium concentration higher than 40 μM resulted in cell destruction or death in all cells. A comparison with our previously obtained results showed the greatest sensitivity of rat hepatoma H 35-19 cells in comparison to four human cancer cell lines (A549, DU145, HTB, RMS). Investigations of human cancer cells demonstrated that the highest resistance to orthovanadate was characteristic of RMS (c.40 μM) and HTB (c. 20 μM).
Electron microscopic examination showed pleomorphic nuclei with visible amounts of heterochromatin and large nucleoli, characteristic of RMS cells. Cells at various stages of differentiation were observed.

Key words: human cancer cells, autocrine growth and morphology of RMS cells, vanadyl sulphate ($VOSO_4$), BMOV = bis(maltolato)oxovanadium, sodium orthovanadate (Na_3VO_4), sodium metavanadate ($NaVO_3$).

Introduction

Vanadium (atomic number 23) is a period 4, VB group element, which appears in compounds at different stages of oxidation (-III, -I, 0 and from +I to +V). According to Aureliano and Gandara 2005 [1] and Soares *et al.* 2006 [2], inside various types of cells, 98% of vanadium appears as vanadyl ions [V(IV)], whereas vanadate forms not only monomers but also dimers, tetramers, pentamers and decamers that interact with proteins. The majority of mammalian tissues contain approximately 20 nM of vanadium; the tissue deposit decreases in the following descending order: bone > liver = kidney = spleen > blood > muscle > brain. In living organisms, vanadium affects the growth, development and differentiation of some species, and as it has been known since 1899, it acts as an insulin-mimetic, antidiabetic compound, while since the second half of the 20th century, vanadium has been acknowledged as a "drug", affecting such mitogenic cell responses as apoptosis, proliferation and neoplastic transformation. Vanadium compounds are capable of operating as pro- or antineoplastic agents, i.e. of inducing growth or inhibiting proliferation and survival of neoplastic cells. They act on both animal and human cells, affecting not only neoplastic, cancer cells but also normal cells. The results of vanadium activity have been reported as those of anti-tumour drugs by many investigators (Ban *et al.* 2000 [3], Evangelou *et al.* 1997 [4], Molinuevo *et al.* 2004 [5], Scrivens *et al.* 2003 [6], Zhang *et al.* 2001 [7], Wozniak and Blasiak 2004 [8]), similarly as in our preliminary experiments (Holko

et al. 2008 [9], Kordowiak *et al.* 2007 [10] Klein *et al.* 2008 [11]); however, some authors have not confirmed this effect and suggest a contrary action of this element (Sakai 1997 [12], Ding *et al.* 1999 [13], Rodriguez-Mercado *et al.* 2003 [14], Shi *et al.* 2004 [15], Zhang *et al.* 2002 [16]).

This paper presents a comparison between the influence of two inorganic salts, orthovanadate and metavanadate [V(V)] and BMOV(bis (maltolato)oxovanadium), an organic vanadyl derivative, and vanadium sulphate [V(IV)] on growth, survival and morphology of the human cancer RMS cell line.

Material and methods

Reagents

Sodium orthovanadate (Na_3VO_4), DMEM, F12, glucose, L-glutamine, trypsin, tylosine, EDTA, albumin, penicillin, streptomycin, N-hexamethylpararosaniline (crystal violet = CV) or bromide 3-(4,5-dimethylthiazol-2)-2,5-diphenyltetrazolium (MTT), glutaraldehyde 8% or 25% were obtained from Sigma Chemical Company St Louis USA, vanadyl sulphate ($VOSO_4$) hydrate comes from Aldrich Chem. Comp Inc, sodium metavanadate ($NaVO_3$) comes from Fluka, bovine serum and physiological buffered saline (PBS) came from WSS Lublin Poland: fetal bovine serum (FBS) was obtained from Biowest, South American Origin. For electron microscopy, Spurr epoxy resin from Pellco Co. and formaldehyde and osmium tetroxide were purchased from Polysciences Inc., test tubes 15 and 50 ml, Eppendorf tubes 1.5 ml, Falcon bottles of areas 25 and 75 cm, 96-well plates, 10 cm plates, sterilized filters with pore size 0.22 μm were obtained from Technoplastic Products AG, Switzerland. All other reagent at analytical grade came from POChem Gliwice, Poland. Bis(maltolato)oxovanadium (BMOV) was synthesized and characterized in Chemical Faculty UJ.

Cell culture

The human cancer rhabdomyosarcoma (RMS) cell line was obtained from the Institute of Immunology and Experimental Therapy, Wroclaw, Poland.

Cell proliferation assays

This investigation was conducted employing two staining methods: N-hexamethylpararosaniline (crystal violet = CV) or bromide 3-(4,5-dimethylthiazol-2)-2,5-diphenyltetrazolium (MTT). The cells were seeded on 24-well plates at a density of 12×10^3 per well in 0.8 ml DMEM. After 24 h the medium was replaced by serum-free medium and the cells were exposed to from 0 (control without vanadium) to 60 μM of the four vanadium salts. Within 48 h of incubation with two inorganic salts, Na_3VO_4, $NaVO_3$ [V(V) as anions], or BMOV (an organic vanadyl derivative, i.e. bis(maltolato)oxovanadium), $VOSO_4$ where [V(IV) as cations] from 0 (control without vanadium) up to 60 μM in serum-free medium, the mean values of IC50 of these two tests for RMS were calculated. This paper presents a comparison between the influence of these four vanadium compounds on growth, survival and morphology of the human RMS cell line. The methods of Gillies *et al.* 1986 [17] and Mosmann 1983 [18] were used to determine proliferation or survival of these cells. Each experiment was repeated at least seven times in threefold irrespectived tests.

Statistical analysis

The results are expressed as mean ± standard error (SEM). Differences between the vanadium-treated cells and control cells were evaluated statistically using Wilcoxon's matched pair test according to Statsoft Statistica program (Motulsky 1996 [19] Statsoft Statistica manuals [20]). P values lower than 0.05 were considered significant. CV and MTT results were used to obtain a hypothetical dose-dependent curve. The statistical analysis of the obtained dose-dependent curves was performed by MANOVA according to the Statsoft Statistica software.

Electron microscopic examination

After passaging, RMS cells were plated (in the amount of 5×10^6/plate) on plates 10 cm in diameter and 15 ml of DMEM with 5% FBS and 2 mM glutamine and 0.45% glucose, penicillin (100 units/ml) and streptomycin (100 μg/ml) was added to each plate. After 24 h the medium was replaced by standard defined serum-free medium DMEM/F12 (1 : 1) with the investigated concentration of four vanadium salts (see above). In the case of control cells, the medium consisted solely of DMEM/F12 without the addition of vanadium compounds. After 48 h at 37°C, 14 ml of the liquid was discarded from each plate, the cells were scraped away from the plate and placed in 1.5 ml Eppendorf tubes with approximately 1 ml of the liquid. The tubes were centrifuged in an MPW-360 centrifuge at 1000 rev/min for 1 min. After discarding the supernatant, the cells in the form of pellets were underlayered with 1.5 ml of 4% glutaraldehyde at 4°C and allowed to remain at the same temperature for approximately 60 min. Subsequently, the samples were transferred to the Chair of Pathomorphology, Collegium Medicum Jagiellonian University. After centrifuging, all the samples were washed in buffer. Subsequently, the samples were postfixed in 1% osmium tetroxide. After dehydration in graded concentrations of ethyl alcohol and propylene oxide, the tissue was embedded in Spurr medium. The samples were sectioned with an ultramicrotome Reichert Ultracut S using a diamond knife. Semi-thin sections were stained with methylene

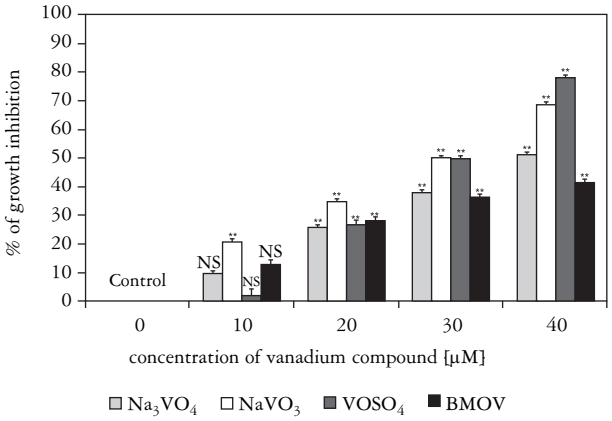

Fig. 1. The growth inhibition of RMS cancer cell line by four vanadium compounds determined by modified crystal violet (CV) staining method and MTT test after 48 h of incubation in serum-free medium (DMEM/F12). The results are presented as mean values of the two methods. NS – non-significant ($p > 0.05$) in comparison with control sample (without vanadium salts)
** $0.001 < p < 0.01$

blue and ultra-thin sections with 8% uranyl acetate dissolved in 50% methanol and then in lead citrate according to Venable and Coggeshal 1965 [21]. All studies were performed under the electron microscope Zeiss EM 900 operating at 80 kV.

Results

The effect of vanadium cations (i.e. BMOV and $VOSO_4$) or anions (Na_3VO_4 and $NaVO_3$) on autocrine growth of rhabdomyosarcoma (RMS) human childhood malignancies is shown in Fig 1. The figure summarizes the mean values ± SD obtained by two different methods: CV (crystal violet) and MTT. Statistically significant values as compared with the control within 20-40 μM vanadium ion concentration were obtained. Lower than 20 μM vanadium ion concentration (excluding $NaVO_3$ which obtained the value at 10 μM) were not statistically significant; greater than 40 μM, almost all cells demonstrate destruction or death and are not shown. Table I showed comparison of the influence on RMS cell line growth inhibition by four investigated vanadium compounds within the investigated concentration.

Table II presents the mean values of IC_{50} from the CV and MTT study. The last result was important, because within these concentrations the cells were prepared for morphological investigations. Results of the study are shown in Figs. 2-6. Ultrastructurally, RMS cells did not show the presence of contractile filaments, either thick or thin. In the whole investigated group the time of cells' incubation was 48 h and cells were observed under primary magnification of EM 4500×. Control (without vanadium compounds) surviving cells demonstrated a large number of small vacuoles and small cytoplasmic fissures. In groups submitted to action of both V(V) compounds there were encountered smooth cell membranes often lacking cytoplasmic processes or microvilli. Scarce large vacuoles at times were filled with biological material. In cells submitted to $NaVO_3$ influence, large fissures in the perinuclear zone and smaller cytoplasmic ones were observed. Cells subjected to vanadium derivatives V(IV) had less or more plicated cell membranes often with preserved microvilli. Large vacuoles (often digestive) appeared in cells submitted to 45 μM BMOV action; after influence of c. 30 μM $VOSO_4$ the cells demonstrated fairly small cytoplasmic vacuoles. A predominantly characteristic observation in RMS cells was chiefly pleomorphic nuclei with a visible amount of heterochromatin and large nucleoli. In all investigated groups a large number of markedly damaged or dead cells were noted.

Discussion

Vanadium compounds in low concentrations (c. ng/g of food) act as one of the essential microelements for some species from algae to vertebrates, such as chicken or rat, but not for humans, who daily receive the element with food and fluid intake. The mean vanadium concentration in blood, ranging from 0.032 to 0.095 ng/ml in the general population, reaches a much higher level (even 1000-fold) in people exposed to high vanadium concentration levels in the air, e.g. industrial workers [22]. The molecular mechanism of intracellular vanadium action is not yet fully understood.

Table I. Comparison of influence on RMS cell line growth inhibition by four investigated vanadium compounds

VANADIUM CONCENTRATION [μM]	% OF RMS GROWTH INHIBITION IN COMPARISON WITH CONTROL			
	ANIONS		CATIONS	
	Na_3VO_4	$NaVO_3$	$VOSO_4$	BMOV
10	9.4 ±1.2	20.8 ±1.0	1.8 ±2.4	12.7 ±1.8
20	25.7 ±0.9	34.7 ±1.1	26.8 ±1.5	27.9 ±1.5
30	38.0 ±0.9	50.0 ±1.0	49.8 ±1.1	36.0 ±1.5
40	51.2 ±0.8	68.6 ±1.0	78.0 ±1.3	41.5 ±1.3

While vanadium's influence as an insulin-mimetic agent acting towards normalization of metabolism of lipids and carbohydrates is relatively well known [23], intensive investigations of this microelement's effect on proliferation and viability of cells (including cancer cell lines) are in progress. Vanadium derivatives as phosphate analogues inhibited protein tyrosine phosphatases and activated kinases [2, 19, 25, 26], influenced gene expression, DNA repair mechanisms, cell growth, apoptosis, and acted as pro-cancerogenic [e.g. 24-26] or anti-cancerogenic "drugs" [15, 27, 28].

In the anti-tumour activity of vanadium, two activity levels may be distinguished. The former consists in a cytoprotective effect exerted upon experimentally induced cancers in animal models. Administration of vanadium derivatives results in a decreased rate of proliferation and/or mass and number of newly developing tumours. Chakraborty *et al.* [29-32] believe that anti-tumour vanadium activity consists in induction and/or stabilization of liver enzymes, as well as inhibition of activity of γ-glutamyl transpeptidase and placental glutathione S-transferase. The other mechanism involves decreasing the proliferation activity of specific tumour cell lines. The latter stage is subject to extensive studies. Having entered the cell, vanadium compounds are believed to affect nuclear DNA, resulting in formation of cross-links between DNA strands, which hinders or even completely prevents DNA replication, thus sending the cells on the path to apoptosis. In the opinion of Young and Wang [28], the vanadium-selenium complex exhibits multidirectional activity,

Table II. IC_{50} values obtained by CV and MTT staining methods for four vanadium compounds

VANADIUM COMPOUND	$IC_{50} \pm SD$ [μM]
$NaVO_3$	27.6 ±1.5
Na_3VO_4	39.4 ±0.6
$VOSO_4$	28.2 ±0.2
BMOV	46.2 ±0.9

causing inhibition of S189 and H22 life cycle in S and G2/M phase; the mechanism underlying the process depends on Ca^{2+} and Mg^{2+} accumulation in the cell, an increase in the amount of free radicals, a decrease of pH in mitochondrial membranes and a decreased potential of these membranes. Barrio *et al.* [33] demonstrated antiproliferative abilities of the vanadium-trehalose complex, which depended on the concentration employed (UMR106 cell line) and appeared at 50-100 μM. ERK kinase stimulation in the cells was also noted, with the activity dependent upon the concentration of the compound and time of exposure. Molinuevo *et al.* [27] observed vanadium (IV) complex-evoked inhibition of adhesion, migration and colony forming in the osteosarcoma UMR cell line.

Our previous investigations showed the effect of vanadium cation (as vanadyl sulphate) as well as anion (orthovanadate) on autocrine growth and viability of the rat hepatoma cell line H35-19 [10], similarly as on some human carcinoma cell lines (lung, kidney and prostate) [9, 11]. It has seemed interesting to study

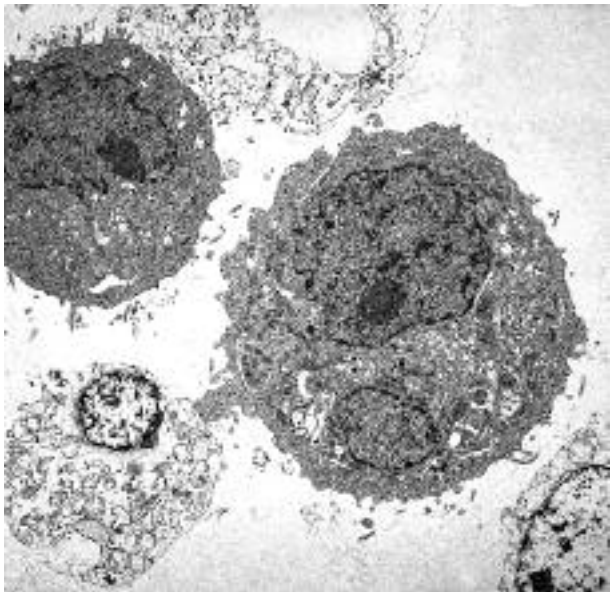

Fig. 2. RMS controls (C), the surviving cells as a rule demonstrated a large number of small vacuoles and small cytoplasmic fissures. The membrane of these cells showed cytoplasmic processes and microvilli. In the vicinity of the surviving cells, a high number of dead cells were seen. Initial magnification 4500×

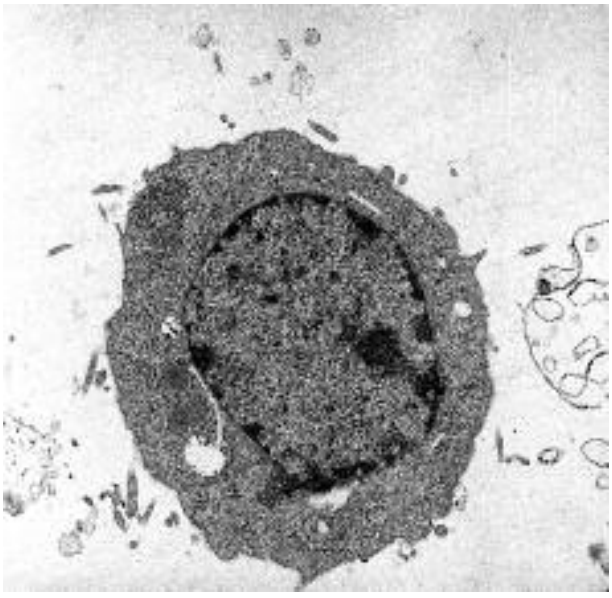

Fig. 3. Group treated with 40 μM Na_3VO_4, 48 hours of incubation, there were encountered cells with both plicated and smooth cell membranes, often lacking cytoplasmic processes or microvilli. The cytoplasm contained scarce large vacuoles, at times filled with biologically foreign material. Initial magnification 4500×

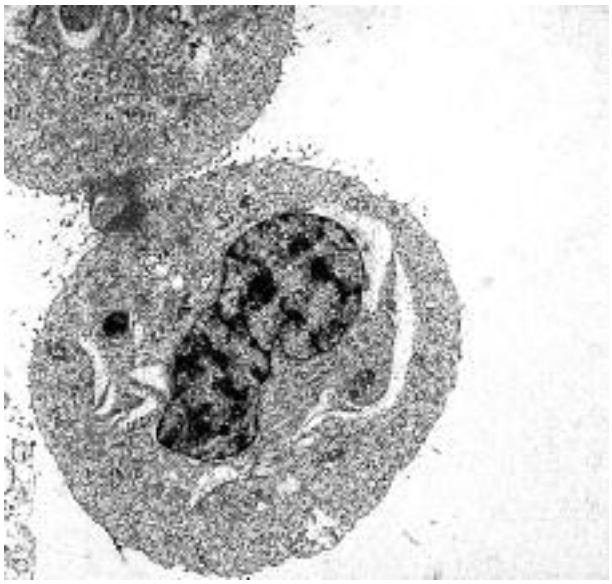

Fig. 4. Group treated with 28 μM $NaVO_3$, 48 hours of incubation. In the surviving cells, the plasma membranes were generally smooth. In addition to large vacuoles, there were observed large fissures in the perinuclear zone and smaller ones in the cytoplasm. Initial magnification 4500×

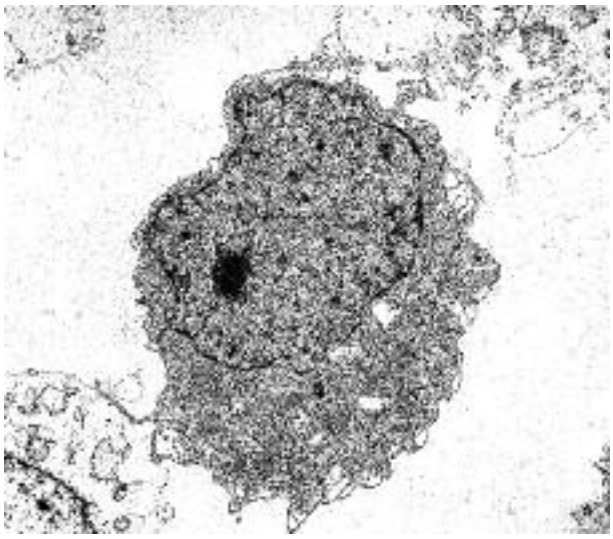

Fig. 5. Group treated with 28 μM $VOSO_4$, 48 hours of incubation. A large number of dead cells were observed. The surviving cells showed plicated cell membrane with preserved microvilli. The cells demonstrated fairly small cytoplasmic vacuoles. Initial magnification 4500×

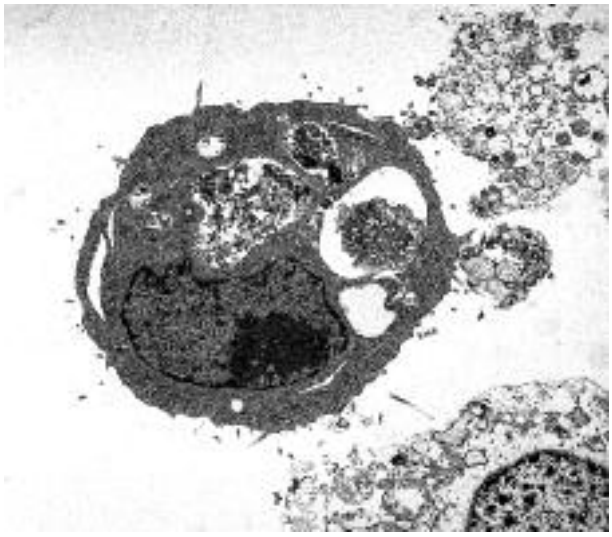

Fig. 6. Group treated with 45 μM BMOV, 48 hours of incubation. The cells were characterized by more or less plicated membranes. The cytoplasm showed large vacuoles; often these were digestive vacuoles. The cytoplasm also demonstrated the presence of small fissures. Initial magnification 4500×
In all the investigated groups, a large number of markedly damaged or dead cells were noted.

in particular the effect of four vanadium compounds (as two cations, $VOSO_4$ and BMOV [V(IV)], and two anions, Na_3VO_4 and $NaVO_3$ [V(V)]) on cell growth and viability, and to carry out a parallel investigation of the morphology of rhabdomyosarcoma, a human cancer cell line. Rhabdomyosarcoma (RMS) arises mainly in the head and neck region, genitourinary tract and extremities; it is the most common childhood malignancy. Permanent activation of signal transduction pathways promotes RMS growth.

Within the cell, VO^{2+} cation was a dominant form; when bound to an organic ligand (BMOV), it was more absorbable and less toxic than $VOSO_4$ [34]. Both [V(IV)] compounds at concentrations of approximately 10 μM resulted in a statistically significant decrease in proliferation rate of the investigated cells; when the concentration increased above 20 μM, they depressed respiratory activity in RMS. The half maximal inhibitory concentration (IC50) of $VOSO_4$ metavanadate was lower by 1/3 (28 μM) as compared to BMOV (47 μM). Orthovanadate showed an intermediate value (40 μM). The compounds exerted a dose and incubation time-dependent cytotoxic effect on the investigated cell line; the onset of proapoptotic activity was noted after 24-48 hours of incubation, while longer incubation periods resulted in predominance of a necrotic effect.

Electron microscopic study confirmed a relatively high number of dead cells. Four vanadium derivatives resulted in ultrastructural changes in RMS cells. In the case of V(V), the cell membranes were smoothened out, with obliteration of the cytoplasmic processes and microvilli, while the vacuoles were large; following exposure to $NaVO_3$, large fissures were seen around the nucleus and smaller ones in the cytoplasm. As the effect of vanadium V(IV) derivatives, the cell membranes

were plicated to a greater or lesser degree (similarly as in the control cells) and the microvilli were preserved. Large, scant and often digestive vacuoles were observed in the cells subjected to the effect of c. 50 μM BMOV, while following the application of c. 30 μM $VOSO_4$, small cytoplasmic vacuoles were noted, similarly as in the controls. Ultrastructurally, RMS cells did not show the presence of contractile filaments, either thick or thin. As it follows from the literature, single thin filaments observed at times in early-stage rhabdomyoblasts are not present in RMS cells. Also characteristic arrangements of single thick filaments surrounded by thin filaments are almost never encountered in poorly differentiated rhabdomyoblasts, and even less so in RMS [35].

Similarly as in our investigations, embryonal RMS often manifests cells the morphology of which is difficult to fully diagnose. In such situations, establishing the diagnosis is facilitated by clinical data, as well as by histopathology results. In the case of cultured cells, we are sure of both the origin and the cell line. The presence of cell dedifferentiation (especially in consecutive passages) is a well-known phenomenon. A predominantly characteristic observation in RMS cells was chiefly pleomorphic nuclei with a visible amount of heterochromatin and large nucleoli. Similarly as in other tumour cell lines, cells in various differentiation stages were also noted. In the present investigations, no significant difference was observed between particular experimental groups, while the cells originating from these groups were markedly more damaged, most likely in consequence of vanadium activity. In RMS cells, practically no cytoskeleton elements were noted in either group, control or experimental, in contrast to the results of similar investigations carried out in human lung cancer cells of the A549 line, where cytoskeletal elements were seen as bundles or single intermediate filaments or microtubules (paper being prepared for publication).

A comparison of the present results with the results obtained previously by our investigative team demonstrated the highest sensitivity to vanadates and vanadyl sulphate (IC50 up to 5 μM) of rat hepatoma cells (H35-19) as compared to the four investigated lines of human cells (A549 lung, DU145 prostate, HTB kidney and RMS). The highest resistance to the effect of Na_3VO_4 was seen in RMS cells (approx. 40 μM) and HTB cells (20 μM), while the A549 and DU145 lines reached IC50 already at several micromoles (5-10 μM). Under the effect of $VOSO_4$, both the cell lines achieved IC50 at a concentration value that was 50% lower as compared to RMS (15 μM versus 30 μM). The lowest effect was exerted on RMS cells by BMOV, since the concentration that needed to be employed reached up to 50 μM. Analysing the resultant values, one may state that rat hepatoma cells are more sensitive to the effect of inorganic vanadium salts in comparison to the four investigated lines of human cancer cells. The highest resistance to the effect of these compounds was seen in the HTB and RMS cell lines.

In the investigated RMS line, a similar effect of approximately 50% growth inhibition was evoked by metavanadate and vanadyl sulphate, with IC50 occurring at < 30 μM. The weakest effect was exerted by BMOV at c. 50 μM, while orthovanadate showed an intermediate effect at c. 40 μM. These findings are generally in agreement with data reported by other authors investigating the properties of this microelement. In the case of $NaVO_3$, one should take into consideration the highest toxicity of this derivative, while BMOV showed the mildest effect both *in vivo* and *in vitro* [36].

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References

1. Aureliano M, Ureliano M, Gandara RMC. Decavanadate effects in biological systems. J Inorg Biochem 2005; 99: 979-985.
2. Soares SS, Martins H, Aureliano M. Vanadium distribution following decavanadate administration. Arch Environ Contam Toxicol 2006; 50: 60-64.
3. Ban J, Maysinger D, Kova V, et al. Molecular mechanisms involved in the antiproliferative action of protein tyrosine phosphatase inhibitor potassium bisperoxo(1,10-phenanthroline) oxovanadate. Life Sciences 2000; 68: 165-175.
4. Evangelou A, Karkabounas S, Kalpouzos G, et al. Comparison of the therapeutic effects of two vanadium complexes administered at low doses on benzo[a]-pyrene–induced malignant tumors in rats. Cancer Letters 1997; 119: 221-225.
5. Molinuevo MS, Barrio DA, Cortizo AM, et al. Antitumoral properties of two new vanadyl(IV) complexes in culture: role of apoptosis and oxidative stress. Cancer Chemother Pharmacol 2004; 53: 163-172.
6. Scrivens PJ, Alaoui-Jamali MA, Giannini G, et al. Cdc25A- inhibitory properties and antineoplastic activity of bisperoxyvanadium analogues. Mol Cancer Ther 2003; 2: 1053-1059.
7. Zhang Z, Huang C, Li J, et al. Vanadate-induced cell growth regulation and the role of reactive oxygen species. Arch Biochem Biophys 2001; 392: 311-320.
8. Wozniak K, Blasiak J. Vanadyl sulphate can differentially damage DNA in human lymphocytes and HeLa Wells Arch Toxicol 2004; 78: 7-15.
9. Holko P, Ligeza J, Kisielewska J, et al. The effect of vanadyl sulphate (VOSO4) on autocrine growth of human epithelial cancer cell lines. Pol J Pathol 2008; 59: 3-8.
10. Kordowiak AM, Klein A, Goc A, et al. Comparison of the effect of VOSO4, Na3VO4 and NaVO3 on proliferation, viability and morphology of H35-19 rat hepatoma cell line. Pol J Pathol 2007; 58: 51-57.
11. Klein A, Holko P, Ligęza J, et al. Sodium orthovanadate affects growth of some human epithelial cancer ells (A549, DU145, HTB44). Folia Biol Krakow 2008; 56: 115-121.
12. Sakai A. Orthovanadate, an inhibitor of protein tyrosine phosphatases acts more potently as a promoter than an inhibitor in the BALB/3T3 cell transformation. Carcinogenesis 1997; 18: 1395-1397.

13. Ding M, Li JJ, Leonard SS, et al. Vanadate- induced activation of activator protein 1: role of reactive oxygen species. Carcinogenesis 1999; 20: 663- 668.
14. Rodríguez-Mercado JJ, Roldán-Reyes E, Altamirano-Lozano M. Genotoxic effects of vanadium (IV) in human peripheral blood cells. Toxicol Lett 2003; 144: 359-369.
15. Shi X, Hudson LG, Liu KJ. Oxidative stress and apoptosis in metal ion-induced carcinogenesis. Free Radical Biol Med 2004; 37: 582-593.
16. Zhang Z, Huang C, Li J, et al. Vanadate-induced cell growth arrest is p53-dependent through activation of p21 in C141 cells. J Inorg Biochem 2002; 89: 142-148.
17. Gillies RJ, Didier N, Denton M. Determination of cell number in monolayer cultures Anal Biochem 1986; 59: 109-113.
18. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983; 65: 55-63.
19. Motulsky H. Analyzing data with GraphPad Prism. 1996 San Diego SA, Graph Pad Software Inc.
20. Statsoft Statistica's manuals: http://www.statsoft.com
21. Venable JH, Coggeshall RA. Simplified lead citrate stain for use in electron microscopy. J Cell Biol 1965; 25: 407-408.
22. Lin TS, Chang CL, Shen FM. Whole blood vanadium in Taiwanese College Students. Bull. Environ. Contam Toxicol 2004; 73: 781-786.
23. Shechter Y, Goldwaser I, Mironchik M, et al. Historic perspective and recent developments on the insulin-like actions of vanadium; toward developing vanadium-based drugs for diabetes. Coord Chem Rev 2003; 237: 3-11.
24. Noutsopoulos D, MarKopoulos G, Koliou M, et al. Vanadium induces VL30 retrotransposition at an unusually high level: a possible carcinogenesis mechanism. J Mol Biol 2007; 374: 80-90.
25. Tang H, Sun Y, Xiu Q, et al. Cyclooxygenase-2 induction requires activation of nuclear factor of activated T-cells in Beas-2B cells after vanadium exposure and plays an anti-apoptotic role. Arch Biochem Biophys 2007; 468: 92-99.
26. Valko M, Rhodes CJ, Moncol J, et al. Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chem Biol Interact 2006; 160: 1-40.
27. Molinuevo MS, Cortizo AM, Etcheverry SB. Vanadium (IV) complexes inhibit adhesion, migration and colony formation of UMR106 osteosarcoma cells. Cancer Chemother Pharmacol 2008; 61: 767-773.
28. Yang J, Wang Z. The antitumor effects of selenium compound Na5SeV5O18.3H20 in K562 cell. Arch Pharm Res 2006; 29: 859-865.
29. ChakrabortyT, Chatterjee A, Dhachinamoorthi D, et al. Vanadium limits the expression of proliferating cell nuclear antigen and inhibits early DNA damage during diethylnitrosamine-induced hepatocellular preneoplasia in rats. Environ Mol Mutagen 2006; 47: 603–615.
30. Chakraborty T, Chatterjee A, Rana A, et al. Carcinogen-induced early molecular events and its implication in the initiation of chemical hepatocarcinogenesis in rats: Chemopreventive role of vanadium on this process. Biochim Biophys Acta 2007; 1772: 48-59.
31. Chakraborty T, Chatterjee A, Rana A, et al. Suppression of early stages of neoplastic transformation in a two-stage chemical hepatocarcinogenesis model: supplementation of vanadium, a dietary micronutrient, limits cell proliferation and inhibits the formations of 8-hydroxy-2'-deoxyguanosines and DNA strand-breaks in the liver of sprague-dawley rats. Nutr Cancer 2007; 59: 228-247.
32. Chakraborty T, Chatterjee A, Saralaya MG, et al. Chemopreventive effect of vanadium in a rodent model of chemical hepatocarcinogenesis: reflections in oxidative DNA damage, energy-dispersive X-ray fluorescence profile and metallothionein expression. J Biol Inorg Chem 2006; 11: 855-866.
33. Barrio DA, Williams PAM, Cortizo AM, et al. Synthesis of a new vanadyl (IV) complex with trehalose (TreVO): insulin-mimetic activities in osteoblast-like cells in culture J Biol Inorg Chem 2003; 8: 459-468.
34 Thompson KH, Orvig C. Vanadium in diabetes: 100 years from phase 0 to phase I. J Inorg Biochem 2006; 100: 1925-1935.
35. Dickersin GR. Diagnostic electron microscopy text/atlas. 2nd ed. Springer-Verlag, New York 2000.
36. Domingo JL. Vanadium and diabetes. What about vanadium toxicity? Mol Cell Biochem 2000; 203: 185-187.

Address for correspondence

Wojciech Dąbroś PhD
Chair of Pathomorphology
Faculty of Medicine
Collegium Medicum Jagiellonian University
ul. Grzegórzecka 16
31-531 Kraków, Poland
e-mail: mpdabros@cyf-kr.edu.pl