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Sodium Orthovanadate Affects Growth of Some Human Epithelial Cancer Cells (A549, HTB44, DU145)

Andrzej KLEIN, Przemyslaw HOLKO, Janusz LIGEZA, and Anna M. KORDOWIAK

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Within the concentration range of 1-20 μM, orthovanadate (Na_3VO_4) demonstrated a time and dose-dependent inhibition of autocrine growth of the human carcinoma cell lines A549 (lung), HTB44 (kidney) and DU145 (prostate), as compared to appropriate controls (without Na_3VO_4). The investigation was conducted by two methods: staining with N-hexa-methylpararosaniline (crystal violet=CV) or bromide3-(4,5-dimethyltio-azo-2)-2,5-diphenyl-tetrazole (MTT). In 5, 10 and 20 μM of Na_3VO_4 in serum-free medium, the mean values of these two tests for A549 were approximately 40%, 45% or 65% as compared to the appropriate controls. HTB44 had the greatest opportunity (statistically insignificant) at lower vanadium concentrations (up to 10 μM), whereas at 20 μM growth inhibition of these cells was approximately 50% of the controls. DU145 showed approximately 33%, 65% and 98% growth inhibition for 5, 10 and 20 μM of Na_3VO_4, respectively Additionally, hypothetical curves obtained by a MANOVA test based on the CV results after 72 h incubation with Na_3VO_4 in serum-free medium, and an example of a time-dependent effect of Na_3VO_4 on A549 cells, were also presented.

Sodium orthovanadate was also examined for its cytotoxic capabilities, especially its ability to induce tumor cell apoptosis; the results were compared with the effect of paclitaxel. The target cells were dyed by differential staining (HOECHST33258 and propidium iodide) after 3 h and 24 h (DU145) or 3 h and 72 h (A549) of incubation with the vanadium compound. Contrary to the two cancer cell lines (viable, apoptotic or necrotic in experimental conditions), the renal HTB44 cells were insensitive up to 15 μM Na_3VO_4 concentrations. After 3 h incubation with Na_3VO_4, both lung (A549) and prostate (DU145) cancer cells showed a slight but significant reduction in the percentage of viable cells, and an increased amount of apoptotic cells. In contrast to the lung cells, DU145 prostate cells after 24 h were more sensitive to paclitaxel than to sodium orthovanadate. In the case of lung cells, the time of incubation was prolonged (to 72 h) to allow for a study of the effect of orthovanadate in greater detail. After 72 h of incubation with Na_3VO_4 or paclitaxel, A549 showed a similar level of viable cells (25-32% of total cultured cells); however, the percentage of apoptotic cells was higher in the case of A549 cells – ca 36% for both drugs, but the concentration of Na_3VO_4 was significantly greater than paclitaxel levels.

Key words: Human epithelial cancer cells, autocrine growth, orthovanadate (Na_3VO_4 or OV).

Andrzej KLEIN, Przemyslaw HOLKO, Janusz LIGEZA, Anna M. KORDOWIAK, Department of General Biochemistry, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Gronostajowa 7, 30-387 Kraków, Poland.
E-mail: anna@kordowiak.pl

One of the dietary microelements, vanadium, is essential for the development and growth of some organisms (ALMEDEIDA *et al*. 2001; DOMINGO 1996; FRENCH & JONES 1993). Both organic and inorganic vanadium complexes show numerous biological activities (CRANS *et al*. 2004; MUKHERJEE *et al*. 2004), often with contrasting effects depending on the type of vanadium ligands, total dose, route and duration of administration, and animal strain or cell line used in experiment. The objective of investigations and the final answer to scientific questions are both very important. The ability to normalize some clinical and biochemical symptoms of diabetes (both in human patients and experimental animals) is the best known and most widely investigated effect of vanadium (THOMPSON & ORVIG 2006; DOMINGO 2000; GOLDFINE *et al*. 2000; DABROS *et al*. 2006; KORDOWIAK *et al*. 2002; KORDOWIAK *et al*. 2004).

Moreover, vanadium complexes are used as antitumor drugs (BAN *et al*. 2000; EVANGELOU *et al*. 1997; MOLINUEVO *et al*. 2004; ZHANG *et al*. 2001;

WOZNIAK & BLASIAK 2004; SCRIVENS *et al*. 2003), however, other authors (e.g. ZHANG *et al*. 2002; RODRIGUEZ-MERCADO *et al*. 2003, SHI *et al*. 2004; SAKAI 1997; DING *et al*. 1999) do not agree with this point of view and have suggested a contrary action of different vanadium compounds. In previous experiments we noted that three organic vanadium [V(IV)] derivatives: bis(maltolato)oxovanadium(IV), bis(kojato)oxovanadium(IV) and bis(2,2'–bipy- ridine)oxovanadium(IV) sulphate resulted in similar, progressive H-35 cell growth and proliferation rate inhibition in the range 0.5-5.0 μM of appropriate vanadium salts in the medium (DABROS *et al*. 2003; DABROS *et al*. 2004). Vanadyl sulphate [V(IV)] or vanadate[V(V)], however, in concentrations approximately six times greater than that of organic complexes, induced a similar effect (KORDOWIAK *et al*. 2007).

It seemed interesting to study the effect of two inorganic vanadium salts: vanadyl sulphate and orthovanadate (LIGEZA *et al*. 2006), on three human epithelial cancer cell lines (A549, HTB44, DU145). Vanadyl sulphate triggers the autocrine growth of these cells in a time- and dose-dependent manner (HOLKO *et al*. 2008). This paper discusses the influence of sodium orthovanadate under experimental conditions in which the vanadium compound in the form of an anionic ion [V(V)] acts as an analog of phosphorus compounds and participates in metabolic pathways of lipids and carbohydrates (ALMEDEIDA *et al*. 2001; DOMINGO 1996), affecting the activities of kinases and phosphorylases (BAN *et al*. 2000; SAKAI 1997; SCRIVENS *et al*. 2003).

Material and Methods

2.1 Reagents

Dulbecco's Modified Eagle's Medium (DMEM), Minimal Essential Medium (MEM), F12 medium, glucose, L-glutamine, trypsin, tylosine, EDTA, albumin, penicillin, streptomycin, crystal violet (CV) (N-hexamethylpararosaniline), MTT [bromide3-(4,5-dimethyltio-azo-2)-2,5-diphenyl-tetrazole], HOECHST 33258 (bisbenzimid) and propidium iodide (PI) were purchased from Sigma Chemical Company (St Louis, USA). Sodium orthovanadate was obtained from Aldrich Chem. Comp. Inc. Bovine serum (FBS) was obtained from Biowest, South American Origin. All other reagents were purchased as analytical grade from Polish Reagents POChem Gliwice, Poland.

2.2 Cell Culture

The human tumor epithelial cell lines A549 (lung), HTB44 (kidney) and DU145(prostate) were used as target cells. The A549, HTB-44 cells were obtained from Institute of Immunology and Experimental Therapy Wrocław, Poland. DU145 were purchased from American Type Culture Collection (ATCC). The stock cultures for DU145, A549 were maintained in DMEM supplemented with 10% FBS, 2mM L-glutamine, 0.45% glucose, penicillin (100 units/ml) and streptomycin (100 μg/ml). HTB-44 cells stock culture was maintained in MEM supplemented with 1 mM sodium pyruvate and 1% non-essential amino acids. The cells were passaged 2-3 times per week using 0.05% trypsin solution with 0.02% EDTA in buffered physiological salt (PBS) without Ca^{2+} and Mg^{2+}.

2.3 Cell proliferation and metabolic activity assays

The target cells were seeded on 96-well plates at a concentration of $3 \cdot 10^3$ cell/well in (DMEM or MEM), supplemented with 100 I.U./ml penicillin and 100 mg/ml streptomycin in the presence of 10%FBS. Following 24h incubation the culture medium was replaced with serum-free DMEM/F12 (1:1) supplemented with 5% albumin, 5 μg/ml transferrin, 0.3 mg/ml L-glutamine, 10 μg/ml tylosine, 2 ng/ml of sodium selenite and 1000 units/ml of penicillin and 100 mg/ml streptomycin. After the following 48h, the medium was replaced twice by serum-free DMEM/F12, and next with the same liquid containing Na_3VO_4 (in concentration range 0.5-20 μM). The incubation was continued for the subsequent 72 h at 37 ^{o}C. The modified crystal violet staining method (CV) (GILLIES *et al*. 1986) and the MTT tetrazolium assay (MTT) (Mosmann 1983) were used to determine the effect of the vanadium compound on the proliferation together with metabolic activity of target cells. The absorbance was measured using a Tecan multiscan plate reader. Ten replicate wells were used for each experiment. The results were monitored by the Magellan 3 program. The influence of the vanadium compound was expressed as a decrease in cell growth relative to the control. MTT and CV results are presented as: % of control growth (Fig 2): $(A_i\text{-}A_0)/(A_c\text{-}A_0)$ x 100, or % of growth inhibition (% GI) = 1 - $(A_i\text{-}A_0)/(A_c\text{-}A_0)$ x 100; A_0, A_c, A_i – average values of absorbance at 540 nm (CV), 570 nm (MTT) of control sample at the start of experiment (A_0), the control sample after 72 h of incubation (A_c) and after 72h incubation with Na_3VO_4 (A_i).

2.4.Assessment of cell viability

A differential staining method was used to investigate the effect of Na_3VO_4 on viability of the cancer cell lines. The cells were seeded on 24-well plates at a density of $12 \cdot 10^3$ per well in 0.8 ml DMEM or MEM with 10% FBS. After 48 h, the medium was replaced twice by serum-free medium, and the cells were exposed to 20 μM concentration of Na_3VO_4 After 3 h, 24 h, 72 h and 120 h of incubation the cells were stained with HOECHST 33258 and propidium iodide (PI) (at concentration 5 μg/ml and 1 μg/ml, respectively). After 15 min the investigated cultures were directly examined on plates with an epifluorescence microscope (Olympus IX-50) equipped with appropriate filters. Two excitation filters were used: one allowed for excitation of both dyes, and the other only for PI excitation. The Image J software was used for image processing (merging RGB channels, enhancing contrast and sharpening) and the quantitative analysis of the processed pictures (cell counting). This allowed for an estimation of the fraction of necrotic cells (PI/DNA signal), viable cells (HOECHST33258/DNA signal) and apoptotic cells (HOECHST33258/DNA signal with morphological changes). 50 nM paclitaxel (BERGSTRALH & TING 2006) was used as the positive control in proapoptotic examination of Na_3VO_4. Each experiment was repeated at least six times; images that presented more than 100-600 cells were used in the quantitative analysis.

2.5 Statistical analysis

The results were expressed as mean ± standard error (SEM). Differences between the vanadium-treated cells and control cells were evaluated statistically using the Wilcoxon's matched pair test according to the Statsoft Statistica program (MOTULSKY 1996, Statsoft Statistica manuals). P values lower than 0.05 were considered significant. CV and MTT results were used to obtain a hypothetical dose dependent curve (Logistic model origin MANUAL), the equation describing the curve was used to calculate the IC_{50} value. The statistical analysis of the obtained dose-dependent curves was performed by MANOVA according to the Statsoft Statistica software.

Table 1

Time-dependent influence of orthovanadate on growth of investigated human carcinoma cell lines. The sodium orthovanadate concentration required for 50 % inhibition of control's growth (IC_{50}) were obtained from crystal violet staining results (CV) and MTT reduction method results

Cell lines	Time of incubation [h]	IC_{50} SD [μM]	
		CV	MTT
A549 (lung)	72	9.55±0.81	9.59±2.59
	120	2.89±0.42	2.61±0.31
HTB44 (kidney)	72	19.50±1.50	20.03±2.13
	120	>15.0	14.63±5.88
DU145 (prostate)	72	9.37±0.42	5.06±1.78

Results

The exposure of the A549, HTB44 and DU145 cells to the orthovanadate resulted in time and dose-dependent suppression of proliferation as compared to appropriate controls (without the vanadium in the culture medium). The dependence on time is presented in Table 1. The effects of sodium orthovanadate (Na_3VO_4) on autocrine growth of three human cancer epithelial cell lines: A549, HTB44 and DU145 are shown in Figure 1.

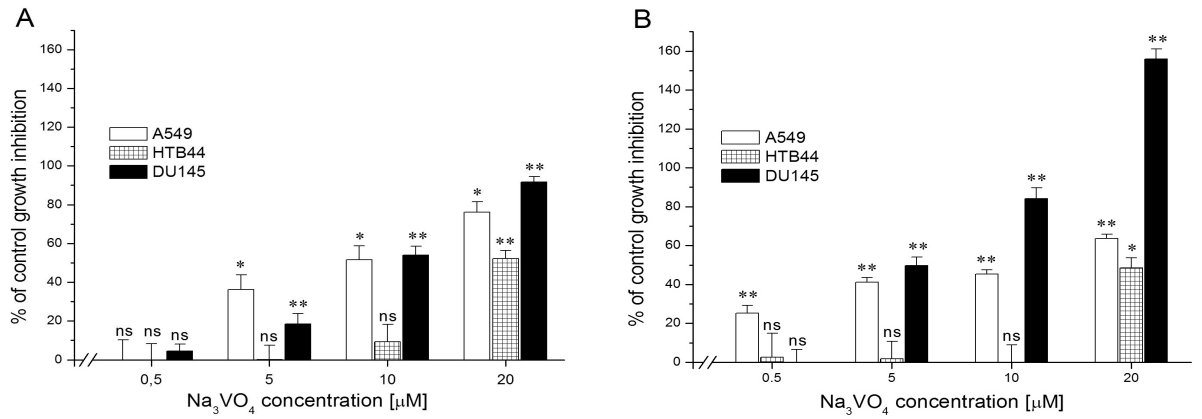

Fig. 1. The percent of control growth inhibition of cancer cell lines (A549, HTB44, DU145) by Na_3VO_4 determined by modified crystal violet staining method (A) and MTT test (B) after 72h of incubation in serum-free medium (DMEM/F12). ns – non significant (P>0.05) in comparison with the control sample (without Na_3VO_4). * 0.01<P <0.05; ** 0.001<P<0.01. The bar, (seen last in part B), which represents an orthovanadate concentration greater than 100% in 20 μM after 72 h, indicates that despite growth inhibition, appropriate doses of the salt exert a cytotoxic effect (at the time of incubation the cell count is less than at the time of vanadium addition).

The results obtained by two different methods, crystal violet (A) and MTT (B) were dependent on the type of investigated cells. In the case of A549 cells, both methods showed a similar effect of Na_3VO_4. The percent of control growth inhibition (%GI) of A549 cells at 10 μM and 20 μM concentrations of vanadium were about 45% and 65% respectively, regardless of the method used. The level of growth inhibition of the DU145 cells determined by the CV method (54% and 91%, at the concentration of 10 μM and 20 μM, respectively) was much lower than that demonstrated in the MTT test (84% and 156% at concentration 10 μM and 20 μM, respectively). No statistically significant differences (in comparison with the control samples) were observed at the vanadate concentration of 0.5 μM determined by the CV method, while MTT indicated 25% inhibition of cell growth of A549. A significant effect of orthovanadate on the growth of the HTB44 cells was observed at a concentration of 20 μM. The values of the percentage of growth inhibition for the HTB44 cells incubated with 20 μM vanadium were 52% and 49% for CV and MTT methods, respectively.

Hypothetical curves obtained from MTT and CV results and tested by MANOVA are presented in Figure 2. Figure 2A shows hypothetical dose-response curves obtained from CV results after 72h incubation with Na_3VO_4 in serum-free medium. These confirm results of MTT and CV, showing the dependences of the type of examined cells on the magnitude of vanadium impact across the full of range of vanadium concentration. Figure 2B shows an example of a time-dependent effect of Na_3VO_4 on the A549 cells. If the time of action is prolonged, the curves move downwards toward low Na_3VO_4 concentrations, against minimum differences in the incline of these curves. This indicates that the time-dependence observed in MTT and CV analyses is statistically significant.

Sodium orthovanadate was also examined for its cytotoxic potential, especially its ability to induce tumor cell apoptosis. The target cells were stained after 3 h and 24 h (DU145) or 3 h and 72 h (A549) of incubation with the vanadium compound. The renal HTB44 cells were insensitive to the investigated Na_3VO_4 concentrations, therefore, Figures 3 and 4 illustrate only the results for 20 μM of the vanadium in comparison with 50 nM of paclitaxel, a known cytotoxic drug (BERGSTRALH & TING 2006) with proapoptotic action for two cancer cell lines: (A549 and DU145 – viable, apoptotic or necrotic under the experimental conditions). Apoptotic cells were clearly distinguishable by their characteristic morphology (cytoplasmic blebbing, cell shrinkage, nuclear condensation and fragmentation). Such morphological alterations were found in all the investigated cell cultures; how-

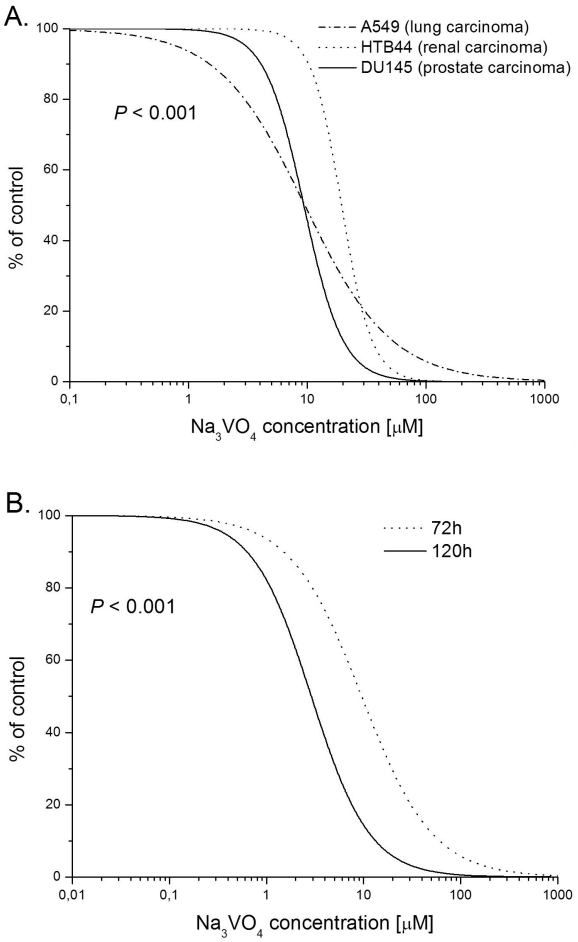

Fig. 2. Hypothetical dose- response curves obtained from the results of CV. (A) Dose- response curves for the sodium orthovanadate effect on carcinoma cells after 72 h incubation in a serum- free medium. (B) Time-dependence of sodium orthovanadate effect on the A549 cells.

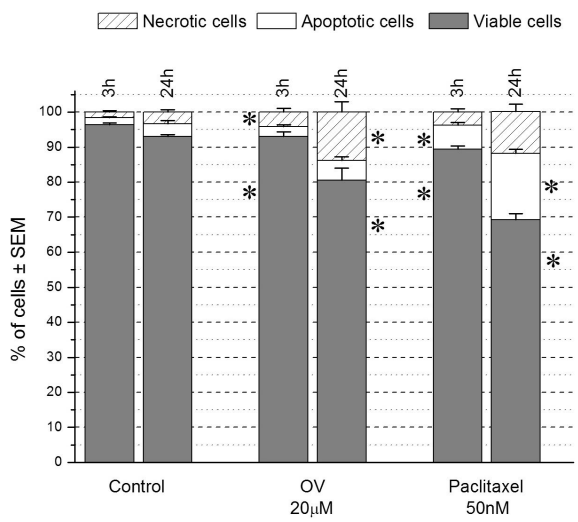

Fig. 3. The cytotoxic effect of sodium orthovanadate (OV) on cell viability of the DU145 prostate carcinoma cell line determined by differential staining (HOECHST 33258/PI) after 3 h and 24 h of incubation with 20 μM OV.
* $0.01<P<0.05$ as compared to the controls (without Na_3VO_4 or paclitaxel).

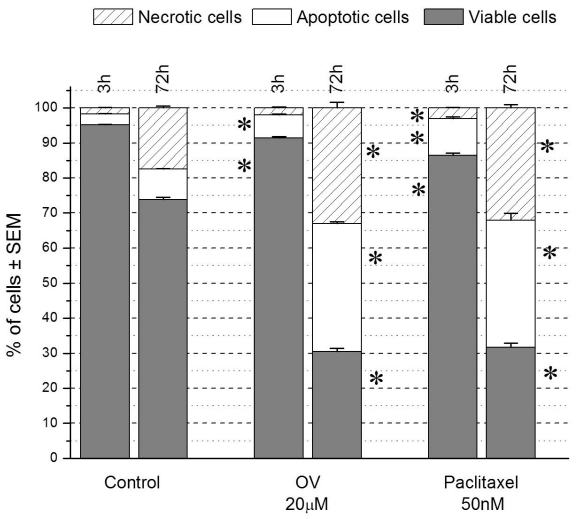

Fig 4. The cytotoxic effect of sodium orthovanadate (OV) on cell viability of the A549 lung carcinoma cell line determined by differential staining (HOECHST 33258/PI) after 3h and 72h of incubation with 20 μM OV.
* $0.01<P<0.05$ as compared to the controls (without Na_3VO_4 or paclitaxel).

ever, in the case of control cultures they did not involve more than 10% of the total cell population. After 3 h incubation with orthovanadate both lung (A549) and prostate (DU145) cancer cells showed slight but significant reductions in the percentage of viable cells and an increased number of apoptotic (A549) or necrotic (DU145) cells. In contrast to the lung cells, prostate cells DU145 were more sensitive to paclitaxel than to sodium orthovanadate. After 24 h of incubation with orthovanadate, 81% of the prostate cancer cells survived (Fig. 3). Incubation of these cells with paclitaxel caused a 30% decrease in viability. The time of incubation was prolonged in the case of lung cells (these cells grow slower than DU 145), to allow for a more detalied investigation of the effect of orthovanadate. After 72 h of incubation with orthovanadate or paclitaxel, the A549 cells showed a similar percentage of viable cells (25-32% of the total cultured cells) (Fig. 4). The percentage of apoptotic cells in the case of A549 reached approximately 36% for both drugs.

4. Discussion

Although the particular mechanisms of the effect of vanadium on living cells has not been elucidated to date, several possibilities have been proposed by investigators, such as inhibition of protein phosphatases (ZHANG *et al*. 2003; VINALS *et al*. 2001), an increased activity of phosphodiesterases (KAWABE *et al*. 2006), protein kinases involved in cell growth and development (BAN *et al*. 2000; ZHANG *et al*. 2003; SCRIVENS *et al*. 2003; DING *et al*. 1999; VINALS *et al*. 2001), DNA damage (ZHANG *et al*. 2002; RODRIGUEZ-MERCADO *et al*. 2003; WOZNIAK & BLASIAK 2004; IVANCSITS *et al*. 2002), changes in genes and synthesis of regulatory proteins (CAPELLA *et al*. 2002; VINALS *et al*. 2001) or generation of reactive oxygen species and oxidative stress (MOLINUEVO *et al*. 2004; ZHANG *et al*. 2001; ZHANG *et al*. 2003; SHI *et al*. 2004; CAPELLA *et al*. 2002; DING *et al*. 1999). These suggestions address many different sites and modes of action, some of them being synergistic; however, certain authors hold their own views that some of these effects are mutually exclusive. For example, SCRIVENS *et al*. (2003) suggest that the anti-neoplastic potential of bisperoxyvanadium analogues is associated with phosphatase inhibition in the entire cells and does not appear to result from DNA damage or oxidative stress. Contrary to this opinion, IVANCSITS *et al*. (2002) propound that VO_4^{3-} is a phosphate analogue and thus may interfere with phosphate-containing enzymes, being involved in DNA repair mechanisms. According to these authors, vanadate is genotoxic per se and may act as an indirect genotoxic agent. *In vitro* vanadate may effectively induce DNA breaks in human fibroblasts (IVANCSITS *et al*. 2002).

In our opinion, the mode of action of vanadium derivatives depends on the employed models and experimental conditions, total doses and time of action, as well as on the kinds of complexes. The latter issue is an interesting problem associated with differences in the mode of action of various vanadium derivatives, i.e. the effect of the vanadium-complexing ligands themselves on the structure and function of intracellular organelles (DABROS & KORDOWIAK 2007). The employed ligands affect the cells in a manner that is entirely different to the effect of complete (vanadium including) complexes; moreover, they alone evoke different changes, e.g. in intracellular structures. In addition, if the degree of the effects of various vanadium compounds are analyzed, differences in their absorption should be taken into consideration, the ease of dissociation in the environment in which they act (e.g. the gastrointestinal tract, tissue, cultured cells), as well as changes in the valence of vanadium that crosses the cellular membrane barrier [e.g. from V(IV) to V(V)]. Of great significance is also the model system employed in the experiments. For example, two chemical analogues, such as bis(maltolato)oxovanadium (BMOV) and bis(kojato)oxovanadium (BKOV), are diametrically different in their effect as antidiabetic "drugs" *in vivo*, but have the same effect on growth and proliferation when added to the culture medium of H35-19 cells (DABROS *et al*. 2004 a, b).

The previously observed growth inhibition of the rat hepatoma cancer cell line H35-19 by organic and inorganic vanadium compounds (DABROS *et al*. 2003; DABROS *et al*. 2004, KORDOWIAK *et al*. 2007) prompted us to carry out experiments with three human epithelial cancer cell lines. The results with vanadyl sulphate (HOLKO *et al*. 2008) were promising, therefore we decided to study the effects of orthovanadate (the second vanadium salt most often used in human volunteers CRANS *et al*. 2004; THOMPSON & ORVIG 2006) on the human carcinoma cell lines i.e. A549 (lung), HTB44 (kidney) and DU145 (prostate), In previous investigations we observed growth inhibition in a concentration range of 1-20 μM Na_3VO_4 (LIGEZA *et al*. 2006). The viability of the three cell lines investigated under the same conditions was impaired by this vanadium salt in 87%, 56% and 49% respectively. Based on the experimental results used for the MANOVA test, we obtained a hypothetical time- and dose-response, logistic models of two curves. These curves showed a significant difference in the response of cancer cells to an increased concentration or a prolonged time of action of the drug. The development of hypothetical curves confirmed the variability of the response of various cell lines to the same orthovanadate concentration values (Fig. 2A) and the statistical significance of the prolonged exposure time of these cells to the investigated vanadium compound (Fig. 2B). Based on the hypothetical curves, the authors obtained the values of $IC_{50} \pm SD$ presented in Table 1. In all the employed concentrations, the greatest value was demonstrated by the renal HTB44 cell line.

This study additionally presents a comparison of the effect of the vanadium salt or paclitaxel on the viability of the investigated cells, determined by differential staining (HOECHST33258, PI) method after 3 h and 24 h or 3h and72 h of incubation in a serum free medium and compared to appropriate controls incubated under the same experimental conditions without Na_3VO_4 or paclitaxel in the medium. As follows from our observations, the action of the vanadium salt has a stronger influence on the lung [A549] and prostate [DU145] cell lines than on the renal [HTB44] carcinoma cells. The renal cells showed the greatest opportunity against orthovanadate under the same experimental conditions. An exact comparison of the effect of orthovanadate on the lung and prostate human carcinoma cell lines was performed after the same duration of action of the drug, i.e. 3h. After this time, the A549 cell line, in contrast to DU 145 cells, showed a significantly higher percent of apoptotic cells as compared with appropriate controls. In order to perform a more accurate assessment of the action of orthovanadate on the lung A549 carcinoma cells and to compare it to the effect of paclitaxel, a well-known specific inductor of apoptosis (BERGSTRALH & TING 2006), the time of incubation was increased three-fold (from 24h to 72h). In this case the effect of both drugs was similar, however, Na_3VO_4 had to be used in a concentration that was approximately 400 times higher than the level of paclitaxel. Similar effects were previously obtained with vanadyl sulphate in the same experimental conditions (HOLKO *et al*. 2008), but the $VOSO_4$ concentration in the serum free medium must be 1.5 times higher as compared to orthovanadate to induce similar effects in the investigated cells.

In conclusion, orthovanadate (and previously studied vanadyl sulphate) is capable of inhibiting *in culture* the growth of some human epithelial cancer cells. However, vanadium salts must be used in concentrations higher than those of paclitaxel; these are also capable of inducing death of cancer cells by apoptosis. The authors suggest that by induction of oxidative stress and/or inhibition of phosphatases, orthovanadate affects the activities of protein kinases, which are adjusted during cell growth. To confirm this theory, detailed investigations must be carried out in the future. An additional question is whether the anti-proliferative effect of some vanadium compounds will allow for employing such compounds as "auxiliary drugs" in certain types of cancer.

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