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Comparison of the Effect of VOSO_4 , Na_3VO_4 and NaVO_3 on Proliferation, Viability and Morphology of H35-19 Rat Hepatoma Cell Line

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This study presents the investigation and comparison the influence of VOSO_4 [V(IV)], Na_3VO_4 and NaVO_3 [V(V)] in the range of 0.5–20.0 μM on the rat hepatoma cell line H35-19. The cells were tested with crystal violet (N-hexamethylpararosaniline), and counted in a Bürker chamber to determine their rate of proliferation, while the survival level was established with neutral red and MTT [bromide 3-(4,5-dimethyltioazo-2)-2,5-diphenyl-tetrazole]. Parallel independent pathomorphological studies with electron microscopic examinations were done. We found progressive growth inhibition of rat hepatoma H35-19 cells within the range 0.5–20.0 concentrations of three vanadium salts. The most effective (and/or toxic) was NaVO_3 , whereas VOSO_4 showed a relatively mild action. As compared with metavanadate or vanadyl sulphate and especially organic vanadium derivatives, previously studied by the same authors under similar experimental conditions, sodium orthovanadate showed an intermediate effect. Electron microscopic examinations confirmed these results. Vanadium salts in low concentration in medium (0.5 μM) were observed to normalize cell morphology. Higher doses of vanadium salts (greater than 2.5 or 5.0 μM) resulted in damaging cell organelles and the more cytotoxic the compounds seemed to be.

Introduction

Vanadium salts have high biological significance. In low concentrations, vanadium is an essential element for development and growth for some organisms from algae to vertebrates (e.g. chickens or rats). Deficiency of vanadium as a microelement in nutrition caused growth inhibition, disorders of generative function, metabolism of the thyroid or

bones, mineralization, as well as, disturbances of metabolic pathways of lipids and carbohydrates [2, 10, 14]. Vanadium derivatives play important role in normalization of some metabolic alterations found both in model diabetes in animals [3, 8, 24, 25, 27, 28, 31] and in human diabetic volunteers with diabetes type 1 or 2 [1, 11, 13, 16, 19, 29, 32].

In the last few years, reports were published describing the anti-tumor effect of vanadium salts [4, 7, 12, 21, 23, 34] or – in contrary, presenting the element as promoting carcinogenesis [17, 26, 30, 36, 37]. Vanadium compounds may cause lipid peroxidation, erythrocyte hemolysis, reprotoxic, aneuploidogenous and genotoxic effects (induced DNA strand breaks, chromosomal aberrations) or induction of neoplastic progression. These effects seemed to be dependent on the type of cells, vanadium derivative and experimental conditions (dose, route of application, duration of experiment)

Take into account these facts; we decided investigate the effect of three vanadium salts more commonly used in animals or human treatment: vanadyl sulphate (VOSO_4), sodium orthovanadate (Na_3VO_4) and sodium metavanadate (NaVO_3), on proliferation, viability and morphology rat hepatoma cell line H35-19. In these compounds, vanadium shows V (IV) valence as sulphate, or V(V) in vanadates. The employed cell line seemed to be very a good model, because these cells show high morphological and functional similarities to hepatocytes from regenerating control rat liver.

Material and Methods

Reagents

Sodium orthovanadate, DMEM, F12, glucose, L-glutamine, trypsin, tylosine, EDTA, albumin, penicillin, streptomycin, neutral red, crystal violet (N-hexamethylpararosa-

niline), MTT [bromide 3-(4,5-dimethylthioazo-2)-2,5-diphenyl-tetrazole], glutaraldehyde 8% or 25% were obtained from Sigma Chemical Company St Louis USA, vanadyl sulphate hydrate comes from Aldrich Chem. Comp Inc, sodium metavanadate 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 2 ml, Falcon bottles 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 others reagent at analytical grade came from POChem Gliwice, Poland.

Cell culture

The rat hepatoma cell line H35-19 was obtained from Institute of Immunology and Experimental Therapy Wroclaw, Poland. The stock cultures were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% FBS, 2 mM L-glutamine, 0.45% glucose, penicillin (100 units/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$) at 37°C in atmosphere with saturated water vapor and 5% CO_2 in 25 cm^2 Falcon bottles. The cells were passage two times per week with using 0.05% trypsin solution with 0.02% EDTA in buffered physiological salt (PBS) without Ca and Mg cations. The cell line was cultivated for a minimum of two passages after thawing prior to experimentation. To investigation of proliferation or viability, H35-19 cells were seed at 96-well plate at a density 1×10^4 cells/well in 200 μl DMEM with 5% FBS and 2 mM glutamine and 0.45% glucose and antibiotics. After 24 h the medium was replaced by serum free DMEM/F12 (1:1) [supplemented with 5% albumin, 5 $\mu\text{g}/\text{ml}$ transferrin, 0.3 mg/ml L-glutamine, 10 $\mu\text{g}/\text{ml}$ tylosine, 2 ng/ml of sodium selenite and 100 units/ml of penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin] with VOSO_4 or Na_3VO_4 or NaVO_3 (in concentration within 0.5, 2.5, 5.0, 10.0, 15.0 and 20 μmoles). Initial experiments determined the optimum time of cells incubation with vanadium salts as 48 h. Triplicate independent experiments in six repetitions were used in all experiments.

Estimation of proliferation rate by crystal violet and Bürker chamber counting:

a) crystal violet (CV) staining by the modified method described by Gillies et al.[15] As the "control" serve cells cultured in defined DMEM/F12 medium without the addition of vanadium salts in six wells, immediately fixed with 80% methanol. After 48 h incubation in 37°C the "experimental cells" (i.e. cells exposed to vana-

dium) were fixed as previously described (80% methanol) and control and experimental cells were dyed with 0.5% crystal violet. After 2 minutes, the dye was extracted with 1.1% sodium citrate in 50% methanol. Half an hour (30 min) later, the absorbance was measured at 540 nm with spectrofluorimeter Spectra Fluor Plus (Tecan) plate counter. The results were monitored by Magellan 3 program.

b) Bürker chamber counting (BC). Following planting of H35-19 cells in 96-well plates and incubation with three vanadium salts in appropriate concentrations, after 48 h, as described previously, the medium was discarded and 0.05% trypsin solution (0.02 ml/well) was added. Detachment of the cells was monitored under light microscope and then counted in Bürker chamber.

Estimation of cells viability

a) MTT staining by the method of Mosmann [22] method (MTT). According to this method, the control was cells cultured in defined DMEM/F12 in six wells immediately fixed with 80% methanol. After 48 h, the medium was replaced by 150 $\mu\text{l}/\text{well}$ of MTT (in concentration 0.5 mg/ml DMEM), followed by 3 h incubation with the dye to allow MTT to form formazon crystals by reacting with metabolic active cells. The formazon crystals were extracted and solubilised with concentrated isopropanol. Absorbance was measured at 570 nm with Spectra Fluor Plus (Tecan). The percentage of survival was calculated using the formula:

$$\% \text{ survival} = [\text{live cell number}(\text{test})] / [\text{live cell number}(\text{control})] \times 100.$$

c) Neutral red staining according to Borenfreund and Puerner [5] (NR) The amounts of neutral red transported by pinocytosis into the cells and stored in their lysosomes, were proportional to the number of cells with normal physiology. Under the above-described standard conditions, after 48 h, the medium with vanadium salts was discarded and 200 $\mu\text{l}/\text{well}$ of neutral red in DMEM (at the concentration 40 $\mu\text{g}/\text{ml}$) were added to each well. After 3 h incubation, the cells were fixed with 1% CaCl_2 in 0.5% formaldehyde during 1 min. Subsequently, the dye was extracted with 1% acetic acid in 50% ethanol and after 15 min the absorbance was measured at 540 nm with Spectra Fluor Plus (Tecan).

Electron microscopy examination

After passaging, H35-19 cells were planted in the amount of 5×10^6 /plate on plates 10 cm in diameter and 15 ml DMEM with 5% FBS and 2 mM glutamine and 0.45% of glucose, penicillin (100 units/ml) and streptomycin (100

$\mu\text{g/ml}$)] was added to each plate. After 24 h the medium was replaced by standard defined serum free medium DMEM/F12 (1:1) with investigated concentration (from 0.5–20.0 μM) of three 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 in 37°C, 14 ml of the liquid was discarded from each plate, the cells were scraped away from the plate and placed in 2 ml Eppendorf tubes with approximately 1 ml of the liquid. The tubes were centrifuged in MPW-360 centrifuge at 1000 rev/min for 2 min. After discarding supernatant, the cells in the form of pellets were underlayered with 2 ml 4% glutaraldehyde at 4°C and allowed to remain in 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 fixed 120 min according to the method of Karnovsky [18]. Subsequently, the samples postfixed in 1% osmium tetroxide. After dehydration in graded concentrations of ethyl alcohol and propylene oxide, the tissue was embedded in the Spurr medium. The samples were sectioned with an ultramicrotome Reichert Ultracut S using a diamond knife. Semi-thin sections were stained with methylene blue and ultra-thin sections with 8% uranyl acetate dissolved in 50% methanol and then in lead citrate according to Venable and Coggeshal [33]. All studies were performed under electron microscope Zeiss EM 900 operating at 80 kV.

Statistical analysis:

The statistical analysis of the results was performed by Student's t-test computer program Microsoft Excel 2000. Only the results with the differences in which $p < 0.05$, were regarded as statistically significant.

Results

The inhibition of proliferation H35-19 cells by VOSO_4 , Na_3VO_4 or NaVO_3 was determined by two methods i.e. staining with crystal violet (CV) and Bürker chamber (BC) counting. Their results showed that within the concentration range of 0.5–2.5 μmoles the differences between control and experimental cells were lower than 1%, between 5.0–15.0 μmoles up to 3% for three vanadium salts. The percentage of viability H35-19 was studied by staining with MTT or neutral red (NR). The difference between the results obtained by these two last methods, were as follows: at the concentration 0.5–2.5 μmoles – approximately 1% and within 5.0–15.0 μmoles vanadium in medium – up to 2% for all vanadium salts. For the resin on Fig. 1 and Fig. 2 the

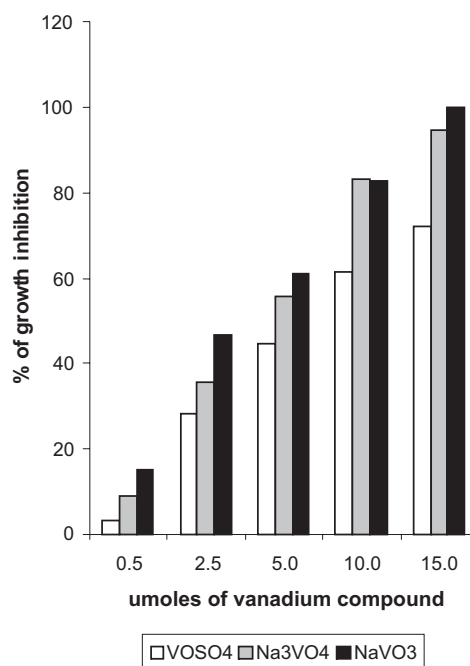


Fig. 1. Influence of three vanadium compounds on H35-19 hepatoma cell line growth inhibition. Reaction with crystal violet (CV) or Bürker chamber counting (BC).

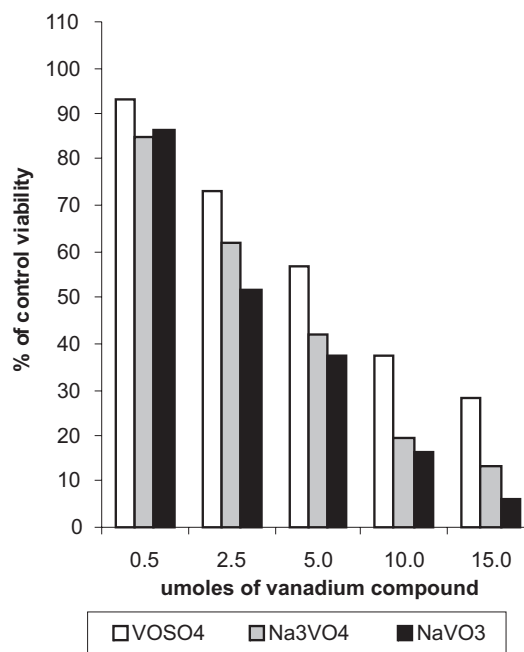


Fig. 2. Influence of three vanadium compounds on percentage of control viability of H35-19 rat hepatoma cell line. Study with MTT or neutral red (NR) reactions.

mean values for the pairs of the two methods are shown. In the four investigated methods, when vanadium concentration values reached or exceeded 2.5 μmoles , the difference between control and experimental cells were statistically significant: for 2.5 μmoles $p < 0.05$, for 5.0 μmoles $0.001 < p < 0.01$ and at 10.0 and 15.0 μmoles $p < 0.001$ for three vanadium salts (data not shown in the graphs).

TABLE 1

Influence of three vanadium salts on growth inhibition or viability of H35-19 cells

Vanadium salt	Vanadium valence	Vanadium concentration in medium	% of cells H35-19 viability	Vanadium concentration in culture	% inhibition of proliferation
VOSO ₄	V(IV)	5.0–7.5 μmoles	50%	15 μmoles	70%
Na ₃ VO ₄	V(V)	5.0 μmoles	50%	15 μmoles	90%
NaVO ₃	V(V)	2.5 μmoles	50%	15 μmoles	95–100%

The cells line was tested with crystal violet (CV) and counted in a Bürker chamber (BC) to determine their rate of proliferation, while the survival level was established with neutral red (NR) and MTT [bromide 3-(4,5-dimethylthiazolo-2)-2,5-diphenyltetrazole].

VOSO₄ was relatively less toxic; the 50% of growth inhibition was reached at 5.0–7.5 μmoles of vanadium, whereas the same effect was achieved at c. 2.5 μmoles of NaVO₃. Vanadium at the concentration 15.0 μmoles in the medium, inhibited proliferation of H35-19 in 70%, 90% or 95–100% of the cells for VOSO₄, Na₃VO₄ or NaVO₃ respectively. At the concentration 20 μmoles in the case of all investigated vanadium compounds, 95–100% inhibition of proliferation was achieved as compared with the control cells. Some parameters are presented in Table 1.

In the control group consisting of rat hepatoma H-35-19 cells cultured *in vitro*, the investigators observed poorly differentiated cells. Single cells were characterized by organelles-poor cytoplasm. The most frequent observation included short endoplasmic reticulum canaliculi and single mitochondria. Large cellular nuclei, often with two nucleoli,

were surrounded by a nuclear envelop with a highly irregular outline, what resulted in numerous indentations or pseudoindentations of the envelop. No Golgi structures were noted in this group (Fig. 3). Figures 4–6 show the influence of low concentration (0.5 μmoles) of vanadium salts on morphology of H35 cells, when the compounds improved these cells organization. At 2.5 μmoles or greater vanadium concentration in the medium, the cells damage was observed. This effect was evaluated parallel with increased vanadium concentrations and achieved maximum at c. 15 μmoles in medium. Data not shown.

Cells treated with 0.5 μmoles of vanadyl sulphate demonstrated some degree of morphological normalization. The most striking change – as compared to the controls – involved the nuclei, with the smooth surface of their nuclear envelop. A higher degree of organelle variation was noted

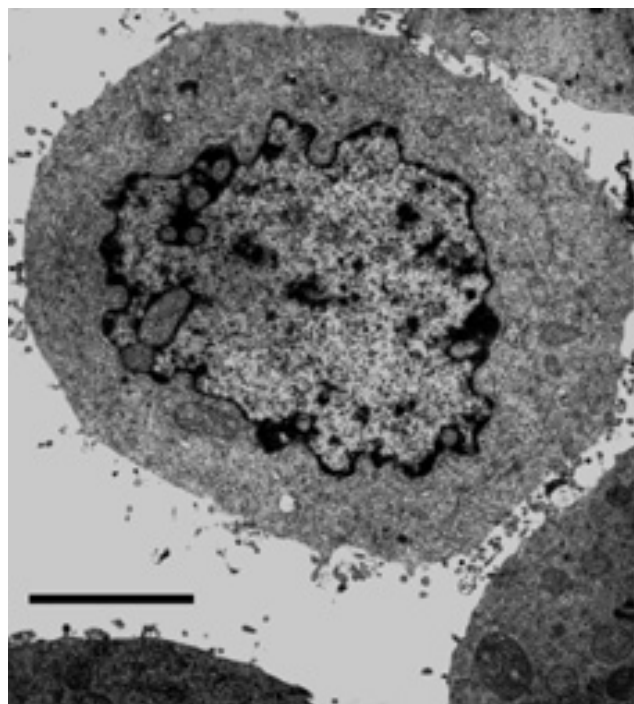


Fig. 3. Control H35-19 hepatoma cells. A single cell with a large, plicated nucleus and scant cytoplasm. The bar represents 5 μm.

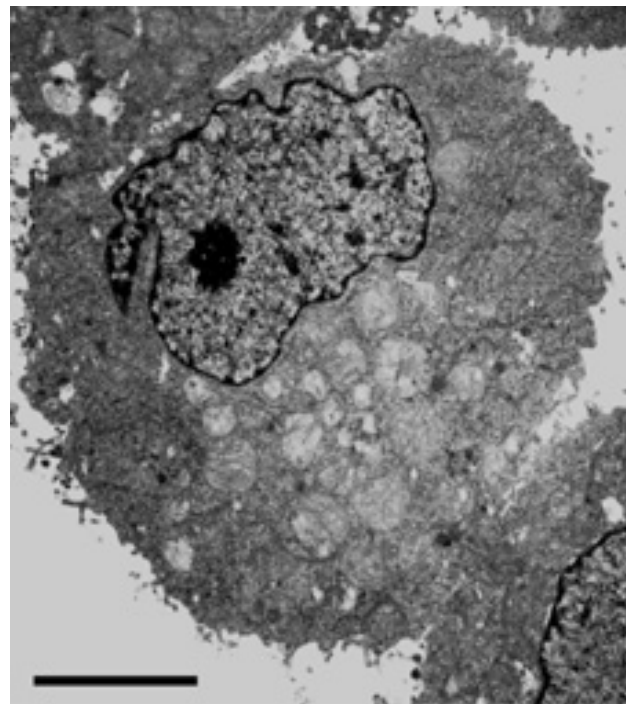


Fig. 4. Experimental H35-19 hepatoma cells with 0.5 μM vanadyl sulphate. A cell with a large, smoothed-out nucleus and numerous organelles in the cytoplasm. The bar represents 5 μm.

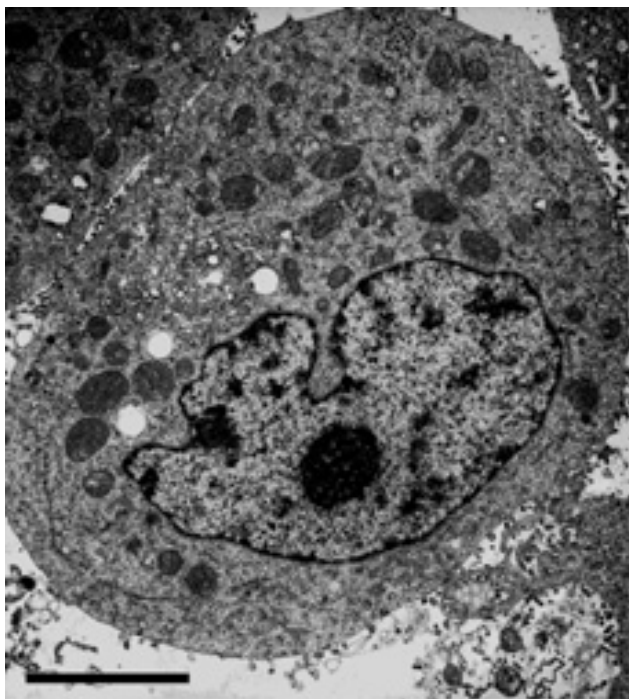


Fig. 5. Experimental H35-19 hepatoma cells with 0.5 μM sodium orthovanadate. Numerous organelles fill the cytoplasm of a hepatoma cell. Note the double membrane in the perinuclear zone that corresponds to a Golgi structure. The bar represents 5 μm .

within the cytoplasm. Single membranous structures were seen both within hepatoma cells and in their vicinity (Fig. 4). In the subsequent experimental group of hepatoma cells, which were subjected to the effect of 0.5 μmoles sodium orthovanadate, the morphology showed almost no differences as compared to normal hepatocytes. In a few instances only, the nuclei demonstrated indentations of the nuclear envelop. Nucleoli with apparent trabecular contents were observed. In this group, the investigators observed stacks of short cisterns that formed arrangements resembling a Golgi structure undergoing organization (Fig. 5). The ultimate experimental group, where the culture medium was enriched with 0.5 μmoles of sodium metavanadate, the ultrastructure of hepatoma cells was typical of differentiated cells. Similarly as in the previous group, the authors observed abundant organelles, including small compartments of Golgi structures undergoing the process of organization (Fig. 6).

Discussion

It has been commonly believed that the type of molecular response by the cells subjected to the vanadium derivatives depends on potential redox, cytoplasmic pH, generation of oxygen reactive forms, especially hydrogen peroxide (cytotoxic effect often inhibited by catalase, and intracellular level of NADPH and glutathione). Moreover vana-

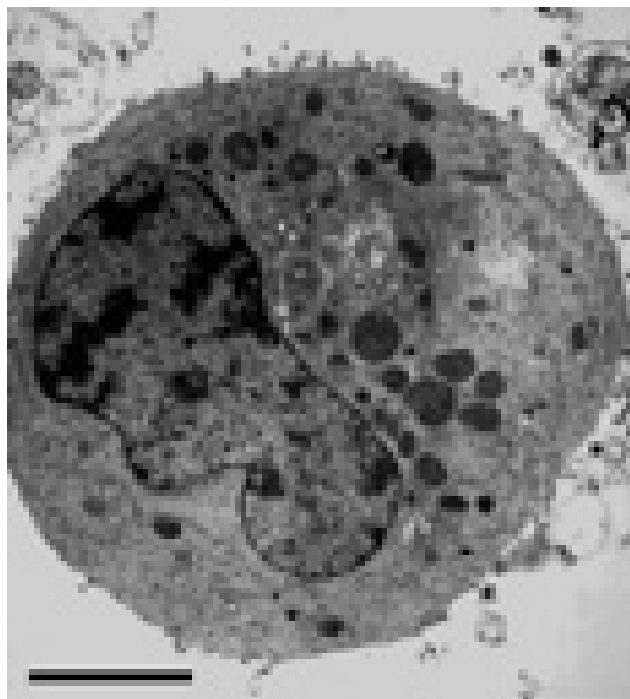


Fig. 6. Experimental H35-19 hepatoma cells with 0.5 μM sodium metavanadate. A well differentiated cell. Apart from organelles described in the previous group, electron-dense lysosome-like structures and more numerous compartments of smooth endoplasmic reticulum. The bar represents 5 μm .

dium compounds also affect numerous enzymes, participating in regulation of cell cycles and responsible for metabolic pathways [6, 9, 35]. However, the particular and universal molecular mechanism of vanadium action is not yet known. Over the past 30 years, it was demonstrated that vanadium complexes, even simple inorganic salts such as vanadyl sulphate [V (IV)] or sodium vanadate [V (V)], exhibit a strong pharmacological activity as “drugs”. As first, the above compounds were shown to exert an insulin-mimetic, anti-diabetic effect; subsequently their anticarcinogenic activity was demonstrated. However, the latter effect was more controversial, because experiments according to some authors showed antitumor activity, whereas other investigators reported carcinogenesis-promoting properties, what was stressed in the Introduction.

The authors of the present paper performed parallel and independent investigations of the physiological status of the cells and their morphology in order to detect possible changes in their intracellular structure. Such observations were performed for all the three investigated concentration of three vanadium salts. Low concentrations, starting from 0.5 μM were found to “improve” the structure of these cells, whereas at the level of 5 μM , as much as 50% of the cells were destroyed. In view of the restricted volume of the paper, the section on the Results presented photographs of the most characteristic changes of intracellular structure, when vanadium concentrations were low (0.5 μmoles). It should be emphasized, how-

ever, that at all the investigated concentration ranges, i.e. 0.5–20.0 μM , the authors observed a correlation between the results of biochemical and morphological studies. Conclusions drawn based on these experiments are in accord, i.e. in the presented experiments, the lowest effectiveness (but at the same time the lowest toxicity) in the case of H35 cells was characteristic of vanadyl sulphate [V (IV)], while the strongest effect consisting in inhibiting cellular proliferation was exhibited by metavanadate [V(V)]. Orthovanadate (in concentration 0.5 μmoles), where vanadium also shows [V(V)] valence, occupies an intermediate place with respect to its effectiveness. In the experimental group, i.e. in groups subjected to the effect of vanadium salts, hepatoma cells characterized by morphological properties showing a greater degree of variation as compared to the controls, in the majority of cases formed clusters composed of 2–4 cells. At the junction of two/three adherent cells, the authors noted structures resembling biliary canaliculi. It seems this phenomenon denotes the beginning of cellular polarization, which is so strongly characteristic of the normal rat liver. It should be strongly stressed, however, that these phenomena occur in cells cultured *in vitro* and cannot be uncritically translated into the biology of cancer cells *in situ*. Preliminary studies on the effect of these vanadium salts on other lines of epithelial human tumors cells, such as A549 (lung carcinoma) HTB44 (renal carcinoma) or DU145 (prostate carcinoma) have demonstrated a similar effect (Ligeza et al. [20]); nevertheless, detailed investigations, including in particular changes in the morphology of such cells, must be carried out in the future.

Conclusions

Three inorganic vanadium salts: vanadyl sulphate (VOSO_4), sodium orthovanadate (Na_3VO_4) and sodium metavanadate (NaVO_3) inhibited proliferation and viability of the rat hepatoma cell line H35-19. At low concentrations in the medium, i.e. 0.5–1.0 μmoles , they improved the morphology and viability of these cells, whereas at the level of 2.5 μmoles or higher, vanadium acted as a cellular growth inhibitor. The strongest effect was demonstrated by NaVO_3 , the lowest VOSO_4 , whereas Na_3VO_4 showed an intermediate effect. This conclusion attributed both biochemical and morphological study. This effect may be associated with toxicity of these compounds.

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