

## RESEARCH ARTICLE

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**Effect of ammonium vanadate on viability and proliferation of human and animal tumor and non-tumor cells****ABSTRACT**

The aim of the study presented was to evaluate the effect of ammonium vanadate ( $\text{NH}_4\text{VO}_3$ ) on viability and proliferation of cultured tumor and non-tumor cells. Permanent cell lines obtained from some of the most common human cancers were used as model systems: MCF-7 (breast cancer), HeLa (carcinoma of the uterine cervix), HepG2 (hepatoma). The non-tumor cell lines established from human embryos (MRC-5 and Lep-3) and bovine kidney (MDBK) were also included in the experiments. The investigations were performed by thiazolyl blue tetrazolium bromide (MTT) test, neutral red uptake cytotoxicity assay (NR) and crystal violet staining (CVS). The results obtained revealed that applied at concentrations of 0.1, 0.5, 1, 5, 10 and 20  $\mu\text{g/ml}$  for 24 h, 48 h and 72 h,  $\text{NH}_4\text{VO}_3$  decreased significantly (in a time- and concentration-dependent manner) the viability and proliferation of human tumor (MCF-7, HeLa, HepG2) and non-tumor (MRC-5, Lep-3) cells whereas bovine kidney MDBK cells seem to be relatively more resistant. A positive correlations between the data coming from MTT, NR and CVS methods were observed.

**Key words:** Ammonium vanadate, tumor and non-tumor cell lines, cytotoxicity assays

**Introduction**

The interest in biological activity of vanadium and its compounds has increased significantly during the recent years. This fact could be explained by several reasons: i) vanadium is widely distributed in soils, water, plant and animal tissues; ii) it has considered to be among the 40 essential micronutrients that are required in small amounts for normal metabolism; iii) the daily human exposure to vanadium compounds due to the wide application of this metal in current industry; iv) 66 000 tons of vanadium are released into atmosphere each year (Alexandrova, 1999; Mukherjee *et al.*, 2004). Among the most intriguing properties of vanadium are its insulin mimetic action and

anticancer potential. Vanadium in several animal cancer models provides protection against all stages of carcinogenesis – initiation, promotion, and progression (Evangelou, 2002; Kostova, 2009; Bishayee *et al.*, 2010). The aim of our study was to evaluate the influence of ammonium vanadate on viability and proliferation of cultured human and animal tumor and non-tumor cells using cytotoxicity assays with different cell targets and mechanism(s) of action.

**Materials and Methods****Chemicals and other materials**

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco-Invitrogen (UK). Dimethyl sulfoxide (DMSO), neutral red, crystal violet

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and trypsin were obtained from AppliChem (Germany); thiazolyl blue tetrazolium bromide (MTT) is from Sigma-Aldrich Chemie GmbH (Germany). All other chemicals of the highest purity commercially available were purchased from local agents and distributors. All sterile plastic and syringe filters were from Orange Scientific (Belgium).

**Compound**

Ammonium vanadate (Valerus) was dissolved initially in bidistilled water and sterilized by filtration (diameter of pores 0.2  $\mu\text{m}$ ) and then diluted in culture medium. The concentration of the compound in stock solution was 1 mg/ml.

**Cell cultures and cultivation**

Permanent cell lines (Cell Culture Collection of IEMPAM-BAS) obtained from some of the most common human cancers were used as model systems in our study: MCF-7 (breast cancer), HeLa (carcinoma of the uterine cervix), HepG2 (hepatoma). The non-tumor cell lines established from human embryos (MRC-5 and Lep-3) and bovine kidney (MDBK) were also included in the experiments.

The cells were grown as monolayer cultures in DMEM medium, supplemented with 5-10% FBS, 100 U/mL penicillin and 100 g/mL streptomycin. The cultures were maintained at 37 °C in a humidified CO<sub>2</sub> incubator (Thermo scientific, Hepa class 100). For routine passages adherent cells were detached using a mixture of 0.05% trypsin and 0.02% EDTA. The experiments were performed during the exponential phase of cell growth.

**Cytotoxicity assays**

The cells were seeded in 96-well flat-bottomed microplates at a concentration of  $1 \times 10^4$  cells/well. After the cells were grown for 24 h to a subconfluent state (~ 60-70%), the culture medium was removed and changed media modified with different concentrations (0.1, 0.5, 1, 5, 10 and 20  $\mu\text{g/ml}$ ) of NH<sub>4</sub>VO<sub>3</sub>. Each solution was applied into 4 to 6 wells. Samples of cells grown in non-modified medium served as controls. After 24 h, 48 h and 72 h of incubation, the effect of the compound on cell viability and proliferation was examined by MTT (thiazolyl blue tetrazolium bromide) test (Mossmann, 1983), neutral red uptake cytotoxicity assay (NR) (Borenfreund & Puerner, 1985) and crystal violet staining (CVS) (Saotome *et al.*, 1989). Optical density was measured at 540 nm using an automatic microplate reader

(TECAN, Sunrise™, Austria). Relative cell viability, expressed as a percentage of the untreated control (100% viability), was calculated for each concentration. “Concentration – response” curves were prepared and the effective concentrations of the compound - CC<sub>50</sub> (causing a 50% reduction of cell viability) and/or CC<sub>90</sub> (causing a 90% reduction of cell viability) were estimated (where possible) from these curves using Origin 6.1. All data points represent an average of three independent assays.

**Statistical analysis**

The data are presented as mean  $\pm$  standard error of the mean. Statistical differences between control and treated groups were assessed using one-way analysis of variance (ANOVA) followed by Dunnett post-hoc test and Origin 6.1™.

**Results**

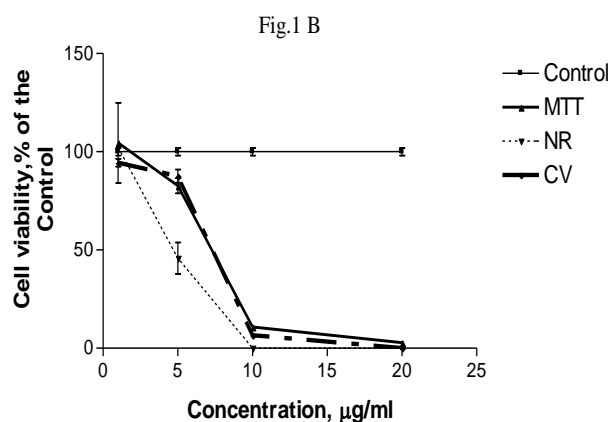
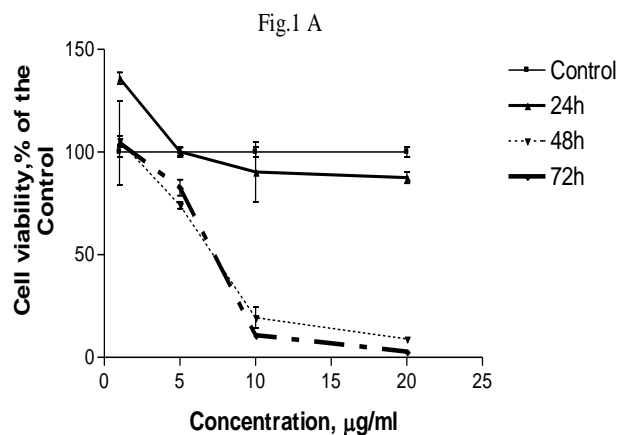
The influence of NH<sub>4</sub>VO<sub>3</sub> on viability and proliferation of cultured tumor and non-tumor cells was evaluated by three cytotoxicity assays - thiazolyl blue tetrazolium bromide (MTT) test, neutral red uptake cytotoxicity assay (NR) and crystal violet staining (CVS). The data obtained are summarized in Figures 1-3 and Tables 1 and 2. They revealed that:

1) Applied at a concentration range of 0.1 - 20  $\mu\text{g/ml}$  NH<sub>4</sub>VO<sub>3</sub> reduced significantly the viability and proliferation of human tumor (MCF-7, HeLa, HepG2) and non-tumor (MRC-5, Lep-3) cells whereas bovine kidney MDBK cells seemed to be more resistant. CC<sub>50</sub> of ammonium vanadate for MDBK cells was not determined because at all concentrations tested the cell viability was > 50%.

2) A positive correlations between the data coming from MTT (which reflects damage to mitochondria), NR (indicates damage to lysosomes and Golgi apparatus) and CVS (shows the growth rate reduction reflected by the colorimetric determination of the stained cells) methods were observed.

3) The human cell lines established from healthy embryos (Lep-3, MRC 5) and malignancies (MCF-7, Hep G2, HeLa) showed similar chemosensitivity to NH<sub>4</sub>VO<sub>3</sub>. This is not surprising because it is well known that cancer and embryo cells share some common characteristics including low state of differentiation and high proliferative potential.

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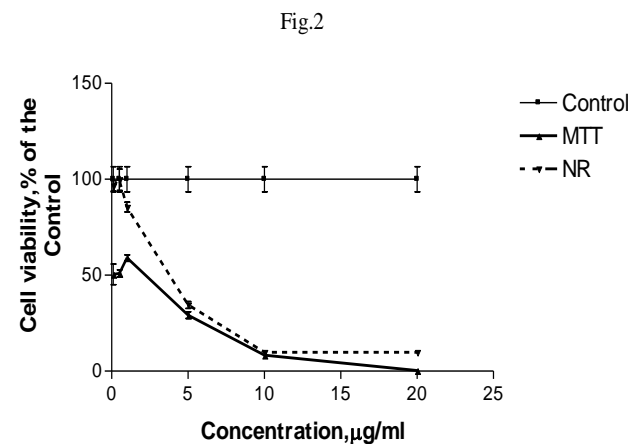


**Figure 1.** Effect of  $\text{NH}_4\text{VO}_3$  on viability and proliferation of human HeLa cells. The compound was applied at concentrations of 0.1, 0.5, 1, 5, 10 and 20  $\mu\text{g/ml}$ . (A) The investigations were carried out by MTT test after for 24 h, 48 h and 72 h. (B) The evaluation was performed by thiazolyl blue tetrazolium bromide (MTT) test, neutral red uptake cytotoxicity assay (NR) and crystal violet staining (CVS) on 72 h.

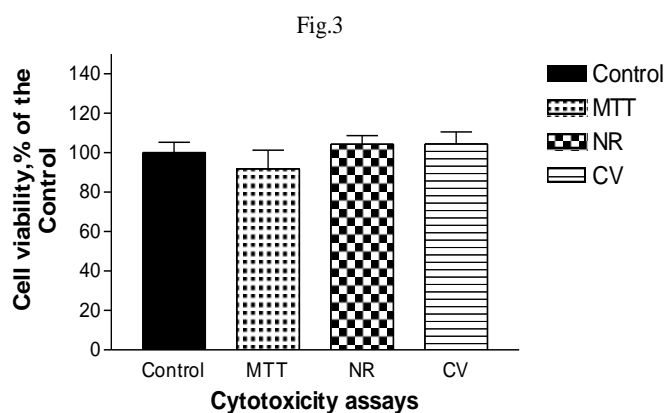
## Discussion

In this study we present data about the ability of ammonium vanadate to decrease in a time- and concentration-dependent manner the viability and proliferation of cultured human cells from cancers of the breast (MCF-7), uterine cervix (HeLa), liver (HepG2) as well as from healthy embryos (MRC-5, Lep-3). In contrast to human tumor and non-tumor cells bovine kidney MDBK cells have been found to be less sensitive to cytotoxic/cytostatic effects of  $\text{NH}_4\text{VO}_3$ .

The data about influence of vanadium compounds on cultured tumor and non-tumor cells have also been reported by other research groups. Applied at concentrations of 100, 175 and 250  $\mu\text{M}$  for 36 h ammonium vanadate has been shown to induce apoptosis in MCF-7 cells in a concentration-dependent manner (Ray et al., 2007).



**Figure 2.** Effect of  $\text{NH}_4\text{VO}_3$  on viability and proliferation of human HepG2 hepatoma cells. The compound was applied at concentrations of 0.1, 0.5, 1, 5, 10 and 20  $\mu\text{g/ml}$  for 72 h. The investigations were performed by thiazolyl blue tetrazolium bromide (MTT) test and neutral red uptake cytotoxicity assay (NR).



**Figure 3.** Effect of  $\text{NH}_4\text{VO}_3$  on viability and proliferation of bovine kidney MDBK cells. The compound was applied at a concentration of 10  $\mu\text{g/ml}$  for 72 h. The investigations were performed by thiazolyl blue tetrazolium bromide (MTT) test, neutral red uptake cytotoxicity assay (NR) and crystal violet staining (CVS).

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**Table 1.** Cytotoxicity ( $CC_{50}$  and  $CC_{90}$ ,  $\mu\text{g/ml}$ ) of ammonium vanadate ( $\text{NH}_4\text{VO}_3$ ) against human tumor HeLa, MCF-7 and HepG2 cell lines.

Cell line	HeLa					MCF-7					HepG2	
	MTT			NR	CVS	MTT			NR	CVS	MTT	NR
Assay	24h	48h	72h	72h	72h	24h	48h	72h	72h	72h	72h	72h
CC50	-	7.4	7.0	4.4	7.2	-	-	7.7	8.4	-	2.1	3.8
CC90	-	19.0	10.0	9.5	9.5	-	-	18.5	-	-	9.5	9.5

**Legend:** MTT = thiazolyl blue tetrazolium bromide (MTT) test, NR = neutral red uptake cytotoxicity assay; CVS = crystal violet staining

**Table 2.** Cytotoxicity ( $CC_{50}$  and  $CC_{90}$ ,  $\mu\text{g/ml}$ ) of ammonium vanadate ( $\text{NH}_4\text{VO}_3$ ) against non-tumor human Lep-3 and MRC-5 cell line.

Cell line	Lep-3					MRC-5	
	MTT			NR	CVS	MTT	NR
Assay	24h	48h	72h	72h	72h	72h	72h
CC50	-	19.2	8.0	-	-	1.0	7.8
CC90	-	-	17.0	2.6	4.4	14.0	-

**Legend:** MTT = thiazolyl blue tetrazolium bromide (MTT) test, NR = neutral red uptake cytotoxicity assay; CVS = crystal violet staining

The results obtained by Fu et al., 2008 indicate that vanadium compounds can block cell cycle progression at the G1/S phase in human hepatoma HepG2 cells via a highly activated extracellular signal-regulated protein kinase signal. Something more, vanadium compounds have been shown to discriminate HepG2 hepatoma cells and normal hepatic cells by differential regulation of reactive oxygen species (Wang et al., 2010).

It has been found by Wozniak & Blasiak, 2004 that vanadyl sulfate can be genotoxic for human normal (lymphocytes) and cancer (HeLa) cells expressing higher genotoxic potential for HeLa cells than for healthy lymphocytes.

The mechanisms underlying the antitumour properties of vanadium remains unclear. Some of the following actions are probably related to its anticancer properties: 1) Protective effect against the induction of DNA strand breaks and chromosome aberrations by potent hepatocarcinogens; 2) Inhibition of metabolic activation of the procarcinogen, leading to reduced generation and/or binding of the ultimate carcinogen to DNA; 3) Elevated detoxification of the precarcinogen and/or its reactive metabolites through specific

induction of activities of some of the xenobiotic biotransforming enzymes; 4) Inhibition of DNA polymerases, nucleotidyl transferases and phosphotransferases; 6) Effect on the immune system (Alexandrova et al., 2002; Evangelou, 2002; Alexandrova & Alexandrov, 2004; Kostova, 2009; Bishayee et al., 2010). Vanadium may also exert inhibitory effects on cancer cell metastatic potential through modulation of cellular adhesive molecules, and reverse antineoplastic drug resistance (Evangelou, 2002).

The anticancer properties of vanadium, in combination to its relatively low toxicity, established also, by its administration in humans, suggest this element as a promising antineoplastic agent. Something more, vanadium compounds have been expected to offer a different alternative for cancer chemotherapy which do not follow mechanism of action of the platinum complexes. Additional experiments are required to clarify better the mechanism(s) of action and cellular targets of vanadium, to identify the most perspective anticancer vanadium compounds and to determine the spectrum of their antitumor activity.

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