

Vanadium(IV) complexes inhibit adhesion, migration and colony formation of UMR106 osteosarcoma cells

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Abstract Vanadium is a trace element widely distributed in the environment. In vertebrates it is mainly stored in bone tissue. The unique cellular environment in the bone and the variety of interactions that mediate cancer metastasis determine that certain types of cancer, such as breast and prostate cancer, preferentially metastasize in the skeleton. Since this effect usually signifies serious morbidity and grave prognosis there is an increasing interest in the development of new treatments for this pathology. The present work shows that vanadium complexes can inhibit some parameters related to cancer metastasis such as cell adhesion, migration and clonogenicity. We have also investigated the role of protein kinase A in these processes.

Keywords Vanadium · Cancer · Metastasis · Osteosarcoma

Introduction

Vanadium is a trace element widely distributed in the environment [24]. After being orally absorbed, vanadium is rapidly distributed in tissues (lungs, kidneys, spleen and muscle), and it is mainly stored in bone [29]. It has been demonstrated that vanadium can exert specific effects on skeletal tissue such as to induce bone and teeth mineralization [24], to stimulate osteoblast proliferation and differentiation as well as collagen synthesis [2, 6]. There are also

evidences that vanadium is accumulated in higher amounts in neoplastic cells than in non-transformed ones [15]. Recent studies indicate the role of vanadium as a chemical preventive agent in some tumoral animal models, human cancer cell lines and also in certain xenografted human carcinomas [20, 27]. On the other hand, using in vitro models it has been shown that some vanadium complexes displayed antitumoral actions [3, 8, 9, 11, 15, 22].

Breast and prostate cancer preferentially metastasize in the skeleton, and this effect usually signifies serious morbidity and grave prognosis. The unique cellular environment in the bone and the variety of interactions that mediate cancer metastasis growth are just being identified [31]. Although the precise molecular mechanism that governs bone metastasis has not been clearly identified yet, it has been suggested that bone provides the microenvironment that allows cancer cells to arrest, growth, colonize and survive [31]. In the neoplastic process there are three key events that determine tumour growth and dissemination. These processes are cell adhesion to the extracellular matrix (ECM), cell clonogenicity and cell migration from the tumour mass to invade the ECM and colonize distant tissues in the host [1, 31]. Under this perspective, those drugs which are able to affect these key events may be useful in antitumoral treatments.

Previous results from our research group have shown that vanadium(IV) derivatives with some non-steroidal anti-inflammatory drugs such as naproxen, as well as some vanadyl(IV) complexes with mono- and disaccharides (like glucose and trehalose), displayed antitumoral properties in an osteosarcoma in vitro model [3, 12, 13, 22]. The mechanism of cytotoxicity in the UMR106 osteosarcoma cells seems to be related to free radical production as well as to an increase in apoptosis levels [4, 22, 23]. The purposes of the present work are to investigate the effect of three

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vanadium complexes: one with glucose (GluVO), other with trehalose (TreVO) and the last one with aspirin (VOAspi), on adhesion, migration and colony formation in the osteosarcoma UMR106 cell line. We have also investigated the role of protein-kinase A (PKA) in the anticarcinogenic properties of these vanadium compounds.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM), trypsin-EDTA and fetal bovine serum (FBS) were from Gibco (Life Technology, Buenos Aires, Argentina), and tissue culture disposable material was from Orange Scientific (Buenos Aires, Argentina). All other chemicals and reagents were obtained from commercial sources and were of analytical grade. Vanadyl(IV) complexes were synthesized as previously described and characterized by infrared spectroscopy as previously reported [3, 12, 14]. The FT-IR results indicate that the samples of the complexes have chemical purity. For experiments, fresh solutions of TreVO and GluVO were made by dissolving the complexes in water. On the other hand, VOAspi was dissolved in a mixture of ethanol and distilled water (1:1).

Cell culture and incubations with vanadium compounds

UMR106 rat osteosarcoma cells were grown in DMEM containing 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in 5% CO₂ atmosphere [5]. Cells were seeded on 75 cm² flasks and sub-cultured using trypsin-EDTA. For experiments, osteoblast-like cells were placed on multi-well plates and incubated in 10% FBS media supplemented with the addition of different doses of vanadium compounds during the periods of time indicated in the legends of the figures.

Cell adhesion assay

The adhesion assay was performed as it was previously reported [21]. Briefly, cells were plated in DMEM-10% FBS on 24 well plates, at a seeding density of 10⁵ cell/ml, in the presence of different doses of vanadium compounds. Cells were allowed to adhere for 1 h at 37°C. Each well was then washed with phosphate-buffered saline (PBS), fixed with methanol for 10 min and stained with Giemsa. The number of adhered cells was evaluated by microscopy, counting several representative fields per well. Cell spreading was assessed by the observation of stained cells that presented more than one corner, in several representative microscopic fields per well.

Localization of actin fibres by immunofluorescence analysis

Cells were plated in DMEM-10% FBS in the presence or absence of different vanadium compounds on coverslips, allowed to attach for 1 h at 37°C. After incubation, cells were fixed with 4% *p*-formaldehyde in PBS for 15 min, permeabilized with cold methanol for 4 min, and stained with fluorescein-labelled pallodoin (1:100) for 1 h at room temperature. Coverslips were mounted in a Vectastain mounting liquid, and images were recorded and analysed using a Nikon-5000 fluorescence microscope and a digital camera.

Cell migration assay

Cell migration was assessed by an in vitro wound assay [1]. Confluent monolayers of UMR106 cells were wounded by scratching lines with a yellow pipette tip. After washing with culture media, tumoral cells were incubated for 12 h in DMEM—10% FBS, with or without vanadium complexes. After this incubation period, the monolayers were fixed and stained with Giemsa. The migration distance was evaluated with a calibrated ocular, assessing the distance between the borders of the wound.

Clonogenicity assay

UMR106 cells were seeded in DMEM—10% FBS at a density of 100 cells/well and allowed to adhere for 24 h. Cells were cultured with different doses of vanadium complexes during 10 days, replacing the media every day. After this culture period, the cells were fixed and stained with Giemsa. Colony formation units were evaluated counting the number of colonies/well in a 5× magnification field. Only colonies with more than 10 cells were evaluated [1].

Stability studies

The stability studies were carried out by measuring the UV-visible spectra of vanadium complexes. Briefly, 20 mM concentration of vanadium complexes were dissolved in water or in DMEM, and the visible spectra of the solutions were measured for different periods of time. Complex degradation was evaluated by measuring the decrease in the intensity of the characteristic bands of absorbance for each compound as previously reported [3, 12, 14]. After that, the absorbance of the solutions was graphicated against the time, and the resulting linear graphics were analysed to obtain kinetic parameters ($t_{1/2}$: lapse of time to cause a diminish of one-half of concentration; Kd: degradation constant). When no linear graphics were obtained, the first derivative was plotted and the kinetic parameters were obtained.

Statistical analysis

Three independent experiments were run for each experimental condition. Results are expressed as the mean \pm SEM. Statistical analysis of the data was performed by Student's *t* test.

Results

Effect of vanadium complexes on osteosarcoma cell adhesion and spreading

The investigation of cell adhesion and spreading are important events in cell survival. In particular, in tumoral cells these processes are the first series of events fundamental for cancer metastasis. In a first series of experiments we evaluated the action of vanadium(IV) complexes on cell adhesion/spreading in a model of osteosarcoma cells in culture. Treatment with vanadium(IV) complexes for 1 h, inhibited both parameters in a dose-dependent manner (Fig. 1). GluVO and TreVO showed the maximal response at 5 μ M. Both vanadyl complexes diminished cell adhesion nearly 40% ($P < 0.001$, differences vs. basal) (Fig. 1a), while at this vanadium concentration cell spreading was inhibited 55% of basal ($P < 0.001$) (Fig. 1b). On the other hand, VOAspi showed a weaker effect than GluVO and TreVO on cell adhesion and spreading, decreasing these parameters by about 25% of the basal at 5 μ M ($P < 0.001$), in this model of tumoral cells. Concentrations over 5 μ M of the three complexes were also tested. However, these concentrations caused deleterious effects related to cell death that interfere with the adhesion process. The inhibitory effect of the three vanadium compounds on cell adhesion and spreading was completely reversed by the treatment with 0.5 μ M of PKA inhibitor H89 (Table 1).

Studies of cytoskeleton actin by fluorescence microscopy

To investigate the mechanism of the inhibitory effect of vanadium complexes on cell adhesion, we analysed their action over actin fibres. Untreated cells (control, Fig. 2a) showed polymerisation of actin into stress fibres as well as cytoplasm extensions indicative of correct adhesion to the culture plate. Treatment with 5 μ M of vanadium complexes for 1 h (Fig 2b–d) decreased the formation of stress fibres. A few number of cells were also found in the dishes, consistent with the results of the adhesion assay (Fig. 1). The remaining cells showed an altered round and condensed morphology with no evidence of actin fibres. It can also be observed that a tendency of the cells to agglutinate, an effect that was more evident after VOAspi treatment (Fig. 2d). The inhibitory effect of vanadium compounds on

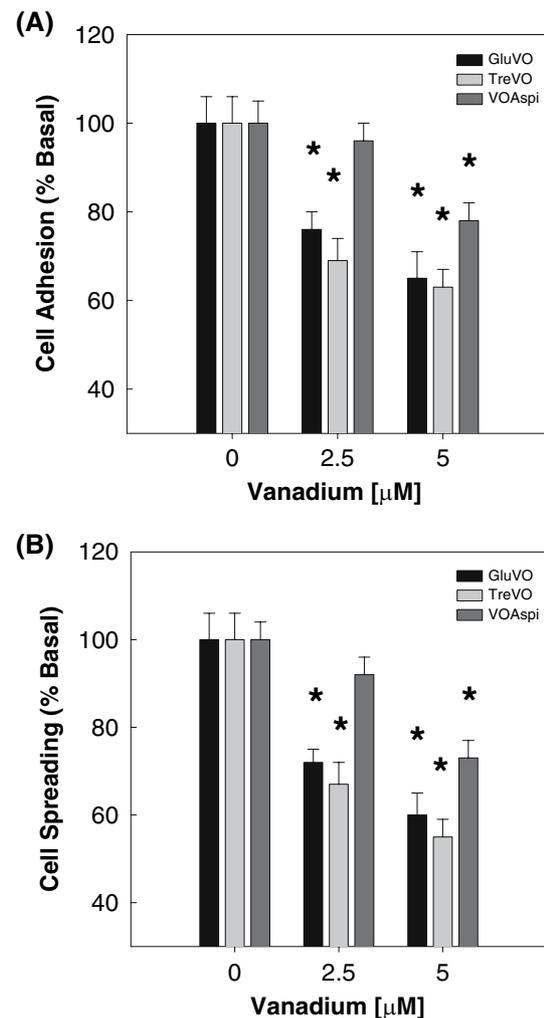


Fig. 1 Effect of vanadium compounds on cell adhesion (a) and spreading (b). UMR106 cells were allowed to adhere for 1 h in the presence of the doses of vanadium complexes as indicated in the figures. Then, the number of adhering cells (a) and the number of spreading cells (b) per field were evaluated. Results are expressed as % Basal and represent the Media \pm SEM of three independent experiments, * $P < 0.001$

the formation of stress fibres was partially prevented by the co-treatment with the protein-kinase A inhibitor, H89 (Fig. 2e, f). These results suggest that in UMR106 osteosarcoma cells, vanadium complexes inhibit actin polymerisation, at least in part, by suppressing the PKA mediated phosphorylation.

Evaluation of the migration capacity of UMR106 cells

In the invasion process, cell migration to surrounding tissues is a vital important process. The effect of vanadium(IV) complexes on the migration of osteosarcoma cells was assessed using in vitro wound assay. In the control untreated-culture, immediately after scratching the monolayer, the wound borders were well defined. After

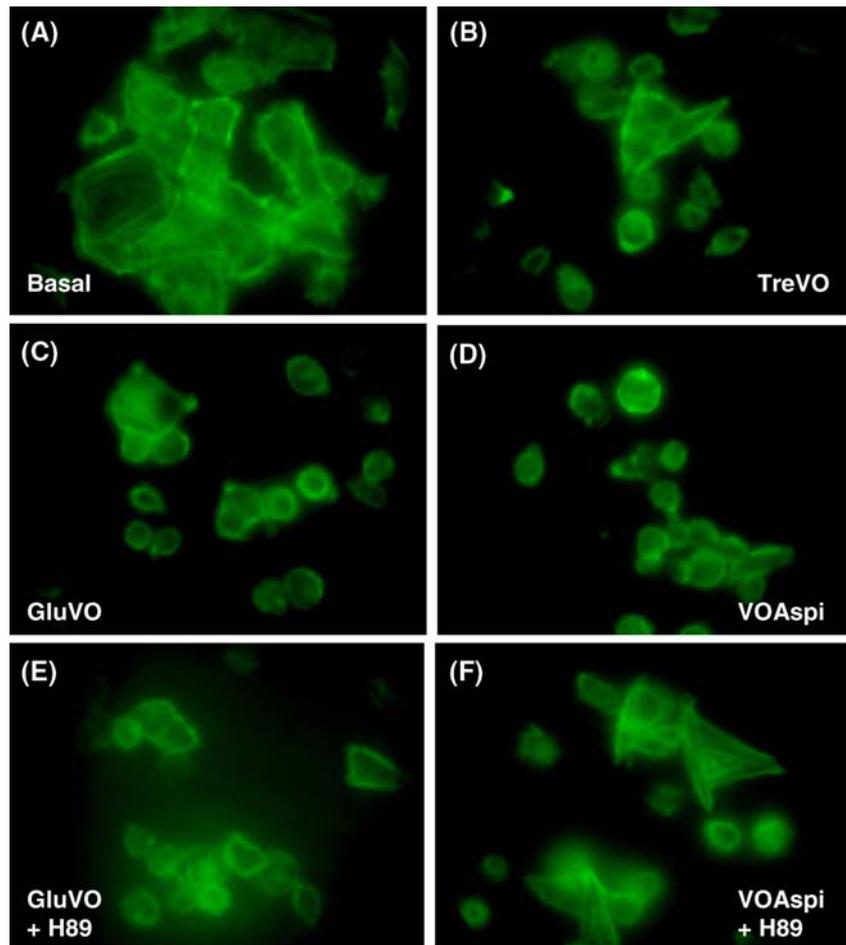
Table 1 PKA inhibitor H89 reverses the inhibitory action of vanadium compounds on cell adhesion and spreading

	Basal		GluVO		TreVO		VOAspi	
		+0.5 μ M H89	5 μ M	+0.5 μ M H89	5 μ M	+0.5 μ M H89	5 μ M	+0.5 μ M H89
Adhesion	100 \pm 8	105 \pm 7	71 \pm 5**	99 \pm 7	78 \pm 7**	91 \pm 6	60 \pm 5*	88 \pm 6
Spreading	100 \pm 8	105 \pm 8	52 \pm 6**	81 \pm 8	71 \pm 7**	84 \pm 7	42 \pm 5*	81 \pm 6

UMR106 cells were allowed to adhere for 1 h in the presence of 0.5 μ M H89 alone or/and plus 5 μ M vanadium complexes. Then, the number of adhering cells and the number of spreading cells per field were evaluated. Results are expressed as % Basal and represent the Media \pm SEM of three independent experiments

* $P < 0.001$, ** $P < 0.01$

Fig. 2 Localization by Immunofluorescence of actin fibres. UMR106 monolayers were treated for 1 h with 5 μ M vanadium compounds (b–d), a basal condition was also included (a) for comparative reasons. The action of vanadium complexes was partially reversed by the treatment with 0.5 μ M H89 (e, f). Immunofluorescence assay was performed as described in [Materials and methods](#) (Magnification 100 \times)



12 h of culture, cells migrated to the centre of the wound to repair the damage. Figure 3a showed that under basal conditions, UMR106 cells have the capacity to migrate into the wound, exhibiting cytoplasm protrusions to the edge of migration. Treatment with 5 μ M GluVO significantly suppressed migration, the cells did not show repair capacity, remaining in the border of the wound (Fig. 3b). The quantification of cell migration (Table 2a) shows that the treatment of UMR106 osteosarcoma cells with GluVO, TreVO and VOAspi significantly inhibited cell migration in a dose-dependent fashion. The role of PK-A was investigated treating the osteoblast-like cells with 0.5 μ M of H89, a PKA

inhibitor. As it is shown in Table 2b, the inhibitory effect of vanadium compounds on cellular migration was partially or totally prevented by the addition of H89 for GluVO or the other two complexes, respectively.

Vanadium compounds inhibited colony formation

Finally we evaluated the ability of GluVO, TreVO and VOAspi to inhibit UMR106 cell-colony formation. Treatment with vanadyl(IV) complexes for 10 days inhibited the formation of colonies in a dose-dependent manner (Table 3). However, the inhibition caused by GluVO and

Fig. 3 Vanadium complexes inhibit monolayer wound healing in UMR106 cells. In basal conditions (a) UMR106 cells are able to heal the wound, while the treatment with GluVO for 12 h inhibits the repair process by inhibiting cell migration. Cell monolayers were fixed and stained with Giemsa, and cell migration was evaluated by light microscopy (Magnification 10×)

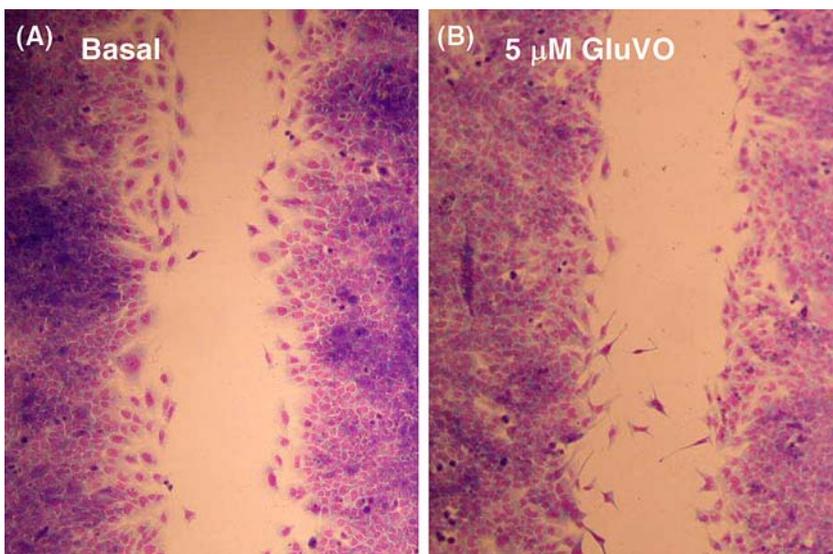


Table 2 The PKA inhibitor H89 reverses inhibition of cell migration by vanadium complexes

Cell migration (A)			
(μM)	GluVO	TreVO	VOAspi
0	100 ± 0	100 ± 1	100 ± 6
2.5	62 ± 11***	71 ± 5**	65 ± 9
5	57 ± 12***	44 ± 5*	36 ± 13**
Cell migration (B)			
	+0.5 μM H89		
Basal	100 ± 2	96 ± 0	
5 μM GluVO	53 ± 6	73 ± 6**	
5 μM TreVO	74 ± 4	89 ± 10	
5 μM VOAspi	48 ± 4	85 ± 4*	

UMR106 cells were scratched with a yellow tip and allowed to heal the wound for 12 h in the absence or presence of different doses of vanadium complexes (A). To investigate the role of PKA in the inhibition of cell migration, the assay was carried out in the presence of 5 μM vanadium derivatives plus 0.5 μM H89 (B). Results are expressed as % Basal and represent the Media ± SEM of three independent experiments

(A) Differences versus basal (0 μM Vanadium complexes): **P* < 0.001, ***P* < 0.02, ****P* < 0.01

(B) Differences versus treated cells: **P* < 0.001, ***P* < 0.05

VOAspi was stronger than that induced by treatment with TreVO.

Stability studies

The stability studies show that vanadium complexes were much more stable in culture media than in water (Table 4). We found that in DMEM all the three compounds presented an exponential decay in the concentration as measured by

Table 3 Inhibition of colony formation by vanadium complexes

Colony formation			
(μM)	GluVO	TreVO	VOAspi
0	12 ± 1	13 ± 1	12 ± 1
2.5	5 ± 0*	11 ± 0.4	4 ± 1*
5	1 ± 0*	9 ± 0.3**	1 ± 0*

After 10 days of culture, vanadium compounds inhibited UMR106 cell-colony formation, in a dose–response fashion. Results are expressed as % Basal and represent the Media ± SEM of three independent experiments

P* < 0.001, *P* < 0.002. Differences are versus basal condition (0 μM Vanadium complexes)

the spectra of the fresh solutions. We can make a ranking of stabilities for the three compounds both in water and in DMEM. In water the relative stabilities were: GluVO>TreVO>VOAspi, whereas in DMEM it was: TreVO>GluVO>VOAspi.

Discussion

Over the past two decades, it has been reported that vanadium derivatives exhibiting antitumoral properties become an interesting group of non-platinum antineoplastic drugs [10, 15]. In particular, because vanadium is mainly accumulated in bone, it seems reasonable to think that its derivatives could be useful in the treatment of bone-tumour metastasis. On the other hand, recent studies demonstrate that vanadium behaves as an osteogenic agent, promoting the mineralization process in bone [16]. Our group has demonstrated that high concentrations of vanadium compounds exert deleterious effects on osteoblast-like cells in

Table 4 Stability studies

	H ₂ O			H ₂ O		
	Kd (h ⁻¹)	t _{1/2} (h)	Degradation kinetic	Kd (h ⁻¹)	t _{1/2} (h)	Degradation kinetic
The stability of vanadium complexes in solution was spectrophotometrically evaluated. Kd: constant of degradation; t _{1/2} : half life time	GluVO	0.101	6.9	0.055	12.6	Exponential decay
	TreVO	0.157	4.4	0.014	50.2	Exponential decay
	VOAspi	0.250	2.8	0.108	3.4	Exponential decay

culture. These toxic effects are mainly related to reactive-oxygen species production [4, 6, 22]. In the present work we studied the antineoplastic properties of three vanadium(IV) complexes with biologically relevant ligands. We found that vanadium derivatives inhibited both cell adhesion and spreading (Fig. 1). Cell adhesion to the extracellular matrix (ECM) is a complex event that depends on multiple factors such as the correct cytoskeleton organization as well as the interaction of the actin fibres with adhesion molecules (i.e. integrins) [7, 17, 19]. This interaction eventually triggers an intracellular signalling pathway that controls cell shape and motility as well as cell proliferation [17]. Multiple tyrosine kinases and phosphotyrosine proteins have been identified to be involved in these processes. Besides, protein tyrosine phosphatases (PTPases) have been related to cell adhesion [30].

The inhibitory effect on cell adhesion caused by vanadium(IV) compounds could be partially explained by two phenomena. First, vanadium is a well-known enhancer of phosphoprotein levels, especially for those proteins that are phosphorylated on tyrosine residues by inhibiting PTPases and/or activating tyrosine kinases [26, 28]. It has been demonstrated that with a high level of tyrosine phosphorylation there is a rapid reorganization of the actin cytoskeleton [25]. Second, it has been demonstrated that agonists of cAMP/PKA signalling cascade may cause significant changes in the cytoskeleton architecture such as dissolution of stress fibres and induction of morphological changes in different cell types [17, 18]. Moreover, cAMP/PKA regulates the disassembly of focal adhesion and also causes the dissolution of actin filaments as well as a decrease in the phosphotyrosine content of paxillin, affecting in last instance cell adhesion as well as motility [17]. There are results indicating that vanadium can enhance PKA activity both in vivo and in vitro [18, 26].

Moreover, our results show that the PKA pathway could mediate the vanadium-induced inhibition of stress fibres-formation, because the treatment of the osteosarcoma cells with the PKA-inhibitor H89, partially reversed the observed effect (Table 1). In consequence, both, cell migration and clonogenicity, are diminished (Fig. 3; Tables 2a, b, 3).

In conclusion our study demonstrates that the three vanadium(IV) derivatives markedly inhibit the key events of tumour cell dissemination: cell adhesion, migration and clonogenicity. We have also demonstrated that there is a

PKA-dependent mechanism involved in the antitumoral action of GluVO, TreVO and VOAspi.

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