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Vanadium and cancer treatment: Antitumoral mechanisms of three oxidovanadium(IV) complexes on a human osteosarcoma cell line

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ABSTRACT

We report herein the antitumor actions of three oxidovanadium(IV) complexes on MG-63 human osteosarcoma cell line. The three complexes: VO(oda), VO(oda)bipy and VO(oda)phen (oda = oxodiacetate), caused a concentration dependent inhibition of cell viability. The antiproliferative action of VO(oda)phen could be observed in the whole range of concentrations (at 2.5 μM), while VO(oda)bipy and VO(oda) showed a decrease of cell viability only at higher concentrations (at 50 and 75 μM, respectively) ($p<0.01$). Moreover, VO(oda)phen caused a decrease of lysosomal and mitochondrial activities at 2.5 μM, while VO(oda) and VO(oda)bipy affected neutral red uptake and mitochondrial metabolism at 50 μM ($p<0.01$). On the other hand, no DNA damage studied by the Comet assay could be observed in MG-63 cells treated with VO(oda) at 2.5–10 μM. Nevertheless, VO(oda)phen and VO(oda)bipy induced DNA damage at 2.5 and 10 μM, respectively ($p<0.01$). The generation of reactive oxygen species increased at 10 μM of VO(oda)phen and only at 100 μM of VO(oda) and VO(oda)bipy ($p<0.01$). Besides, VO(oda)phen and VO(oda)bipy triggered apoptosis as determined by externalization of the phosphatidylserine. The determination of DNA cleavage by agarose gel electrophoresis showed that the ability of VO(oda)(bipy) is similar to that of VO(oda), while VO(oda)(phen) showed the highest nuclease activity in this series. Overall, our results showed a good relationship between the bioactivity of the complexes and their structures since VO(oda)phen presented the most potent antitumor action in human osteosarcoma cells followed by VO(oda)bipy and then by VO(oda) according to the number of intercalating heterocyclic moieties.

1. Introduction

Vanadium is a widespread element in the environment and it is present at trace concentrations in biological systems [1,2]. This metal belongs to the first transition series and its chemical properties are very interesting since it can form a great variety of compounds in different oxidation states. From a biological point of view the more relevant valences of vanadium are III, IV, and V. Vanadium(III) and (IV) are unstable at physiological pH and in the presence of oxygen. Nevertheless, the stability of V(IV) coordination compounds depends on the ligands. An example of a particularly stable V(IV) compound is the complex formed by V(IV)O with transferrin [3].

Vanadium(IV) is easily oxidized to vanadium(V) under physiological conditions, and the vanadium(V) species are found as vanadate anions in biological systems [4]. For many years vanadium compounds have attracted the scientific interest due to the complexity of its chemical behavior, its different oxidation states and the great versatility of coordination sphere around the metal center [4,5]. Besides, vanadium compounds display interesting effects at pharmacological doses both in vivo and in vitro systems [6–11]. Nevertheless, some toxic effects have also been reported [12].

Although the mechanisms of vanadium's toxicity are still poorly understood, it has been reported that this metal can regulate cell growth. It behaves as a growth factor mimetic agent [13] through its action upon signal transduction pathways [14,15] and promotes morphological alterations in the cells [16,17]. In some cases it also induces antimetastatic behavior, decreasing cell spreading and adhesion to the extracellular matrix as well as cell migration and clonogenicity [18,19].

On the other hand, it has also been demonstrated that vanadium compounds induce the generation of reactive oxygen species (ROS) which play an important role in its adverse biological effects [20,21]. Previously reported studies have shown that vanadium compounds promote apoptosis and convey the cells to death through the increasing of ROS levels and disturbance of the redox status, especially by alteration of mitochondria functions in the cells [22–24].

Numerous studies have been carried out in order to get a deeper insight into the putative mechanisms of action of vanadium derivatives. Studies with oxidovanadium(IV) complexes have demonstrated that

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some compounds cause substantial single strand breaks in DNA and produce lipid peroxidation [20,25].

Various organically chelated vanadium compounds are more potent to facilitate pharmacological actions than the simple vanadium salts [26–28]. This is attributed, among other factors, to the poor absorption of some vanadium species in the gastrointestinal tract. For this reason, the use of organic ligands would enhance the lipophilicity of vanadium, increasing the gastrointestinal absorption, and thereby decreasing the dose of vanadium required to produce its biological or pharmacological effects [29–32]. In this sense, the synthesis of vanadium complexes with ligands that hold multiple donor atoms is of considerable interest in vanadium biochemistry and in relation to their potential therapeutic applications [33–35].

Strong chelating ligands are very important in living systems facilitating the uptake and transport of metals [4,36]. In this process, the role of low-molecular-mass compounds involving multidentate oxygen donors is of great value [36,37]. A model compound useful for this subject is oxodiacetate (oda), $O(CH_2COO^-)_2$, a very versatile ligand since the O donor atoms can be placed on different orientations. Oda holds an OOO donor group and can coordinate metal ions by forming chelate rings [38].

Taking into account that vanadium compounds once absorbed in mammals are storage mainly in bones with a half-life of ca. 5 days [2], it is of great scientific interest to investigate the effects of vanadium in hard tissues.

As part of a project related to vanadium compounds with potential pharmacological applications, the present study deals with the effects of an oxidovanadium(IV) complex with oda and two ternary complexes of oxidovanadium(IV) with oda and 2.2′-bipyridyl, VO(oda)bipy and with o-phenanthroline, VO(oda)phen (Fig. 1) on a human osteosarcoma cell line (MG-63). We have investigated and reported herein the action of these compounds on the proliferation of this tumoral cell line and the putative mechanisms involved in their antiproliferative effects. In particular, we focus our investigation on the role of oxidative stress, its effects on cytotoxicity, mainly at lysosomes and mitochondria metabolism, as well as on the morphological transformations and apoptosis. Finally, we have investigated the effects of these compounds on plasmidic DNA as a model for the interactions of these compounds with cellular DNA.

2. Materials and methods

2.1. Materials

Tissue culture materials were purchased from Trading New Technologies (Buenos Aires, Argentina). Dulbecco's modified Eagles medium (DMEM), fetal bovine serum (FBS) from GBO Argentina SA (Buenos Aires, Argentina); TrypLE™ was provided by Gibco (Gaithersburg, MD, USA); crystal violet, propidium iodide, neutral red (NR) and (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium-bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dihydrorhodamine 123 (DHR) and annexin V were from Molecular Probes (Eugene, OR, USA). Plasmid DNA (pA1) used for gel electrophoresis experiments was purchased from Bluescribe, Stratagene, UK. VIVO(acac)2 was from Aldrich (98%); 3-(N-morpholino)propanesulfonic acid (MOPS, 99.5%) and sodium benzoate (≥99.9%) were from Fluka; dipotassium hydrogen phosphate (99%) and hydrogen peroxide (30%) were purchased from Panreac; sodium azide (99%) was from HiMedia; and dimethyl sulfoxide (DMSO, ≥99.7%) was from Fisher Scientific.

2.2. Methods

2.2.1. Preparation of VO(oda), VO(oda)bipy, VO(oda)phen and their solutions

VO(oda), VO(oda)bipy and VO(oda)phen were synthesized and characterized according to literature [38,39].

Fresh stock solutions of the complexes (VO(oda)bipy and VO(oda)phen) were prepared in DMSO at 20 mM and diluted according to the concentrations indicated in the legends of the figures. Moreover, VO(oda) solutions was prepared in distilled water at 10 mM and then diluted according to the concentrations indicated in the legends of the figures.

2.2.2. Stability of the complexes in solution

To test the stability of VO(oda), VO(oda)bipy and VO(oda)phen under different experimental conditions used in this work, we analyzed the UV–visible spectra of different solutions of the complexes. VO(oda) 10 mM in distilled water and DMEM, VO(oda)bipy 10 mM and VO(oda)phen 5 mM solutions in DMSO were prepared. The electronic spectra were recorded at times ranging from 0 to 24 h. The rate of decomposition of the complexes was spectrophotometrically measured.

2.2.3. Cell culture and incubations

MG-63 human osteosarcoma cell line was grown in DMEM containing 10% FBS, 100 IU/mL penicillin and 100 μg/mL streptomycin at 37 °C in 5% CO_2 atmosphere. Cells in a 75 cm2 flask were grown until they reach 70–80% of confluence. Then, the cells were subcultured using TrypLE TM. For experiments, cells were grown in multi-well plates. After 24 h the monolayers were washed with DMEM and were incubated under different conditions according to the experiments.

2.2.4. Cell viability: Crystal violet assay

A mitogenic bioassay was carried out as described by Okajima et al. [40] with some modifications. Briefly, the cells were grown in 48-well plates. After 24 h, the monolayers were washed twice with serum-free DMEM and incubated with medium alone (basal) and with different concentrations of the complex (2.5–100 μM), Then, the monolayers were washed with PBS and fixed with 5% glutaraldehyde/PBS at room temperature for 10 min. Then, they were stained with 0.5% crystal violet/25% methanol for 10 min. After that, the dye solution was discarded and the plate was washed with water and dried. The dye taken up by the cells was extracted using 0.5 mL per well 0.1 M glycine/HCl buffer, pH 3.0, in 30% methanol and transferred to test tubes. Absorbance was read at 540 nm after a convenient sample

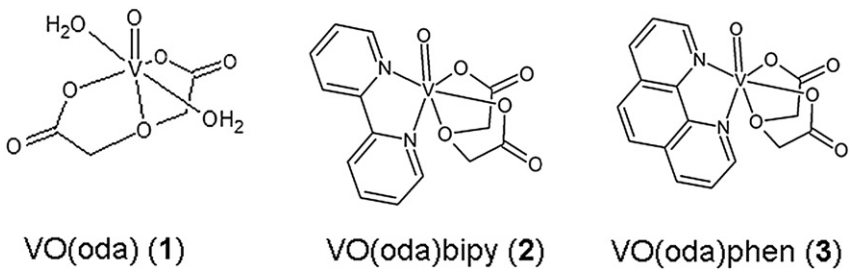

Fig. 1. Schematic molecular structures of VO(oda) (**1**), VO(oda)bipy (**2**) and VO(oda)phen (**3**). Derived from [39] with some modifications.

dilution. We have previously shown that under these conditions the colorimetric bioassay strongly correlated with the cell proliferation measured by cell counting in a Neubauer chamber [13].

2.2.5. Cytotoxicity study

2.2.5.1. Neutral red uptake assay. The NR accumulation assay was performed according to Borenfreund and Puerner [41]. Cells were plated in 96 well culture plates (2.5 x10^4 cell/well). Cells were treated with different concentrations of the complexes for 24 h at 37 °C in 5% CO_2in air. After treatment, the medium was replaced by one containing 100 lg/mL NR dye and cells were incubated for an additional period of 3 h. Then, NR medium was discarded, the cells were rinsed twice with warm (37 °C) PBS (pH 7.4) to remove the non-incorporated dye, and 100 μL of 50% ethanol, 1% acetic acid solution was added to each well to fix the cells releasing the NR into solution. The plates were shaken for 10 min, and the absorbance of the solution in each well was measured in a Microplate Reader (7530, Cambridge technology, Inc, USA) at 540 nm, and compared with the control wells (untreated cells). Optical density was plotted as percentage of control.

2.2.5.2. MTT (methyl tetrazolium) assay. The MTT assay was based on a report previously described by Mosmann [42]. Briefly, cells were seeded in a 96-multiwell dish, allowed to attach for 24 h and treated with different concentrations of the complexes for 24 h. After this treatment, the medium was changed and cells were incubated with 0.5 mg/mL MTT under normal culture conditions for 3 h. Cell viability was marked by the conversion of the tetrazolium salt MTT to a colored formazan by mitochondrial dehydrogenases. Color development was measured spectrophotometrically in a Microplate Reader (7530, Cambridge technology, Inc, USA) at 570 nm after cell lysis in DMSO (100 μL/well). Cell viability is shown graphically as percent of the control value.

2.2.6. Cell morphology

Cells were grown on glass coverslips and incubated with the complexes at different concentrations in serum-free DMEM for 24 h. Then, the cells were fixed and stained with Giemsa [16,24,43]. Samples were observed under light microscopy and pictures were taken for further evaluation.

2.2.7. Genotoxicity study: Single cell gel electrophoresis (SCGE) assay

For detection of DNA strand breaks the single cell gel electrophoresis (comet) assay was used in the alkaline version, based on the method of Singh et al., 1988 [44] with minor modifications. This method has reached the consensus at the International Workshop on Genotoxicity Test Procedures [45]. Under alkaline conditions, DNA loops containing breaks loose supercoiling, unwind and are released from the nucleus and form a comet tail after gel electrophoresis. For this experiment, 2×10^4 cells were seeded in a twelve-well plate; 24 h later the cells were incubated for 24 h with various concentrations of three complexes. After treatment, the cells were suspended low melting point agarose (pH 7.4) and immediately pipetted onto a frosted glass microscope slide precoated with a layer of normal melting point agarose to promote a firm attachment of the second layer. After these layers had solidified at 4 °C for 10 min, slides were immersed in ice-cold freshly prepared lysis solution for 1 h at 4 °C in order to lyse the cells, remove cellular proteins and to permit DNA unfolding. Immediately after, electrophoresis was performed for 30 min at 25 V. Afterwards, slides were neutralized and stained with Syber Green. Analysis of the slides was performed in an Olympus BX50 fluorescence microscope. Cellular images were acquired with the Leica IM50 Image Manager (Imagic Bildverarbeitung AG). A total of 50 randomly captured cells per experimental point of each experiment was used to determine the tail moment (product of tail length by tail DNA percentage) using a free comet scoring software (Comet Score version 1.5). Independent experiments were repeated twice.

2.2.8. Mechanism of action

2.2.8.1. Effect of oxidovanadium complexes on the oxidative stress: Determination of reactive oxygen species (ROS) production. The effect of the complexes on the oxidative stress was evaluated by measurement of the intracellular production of ROS after incubation of the MG-63 cell monolayers with different concentrations of the complexes during 24 h at 37 °C. ROS generation was determined by oxidation of DHR-123 to rhodamine by spectrofluorescence as we have previously described [24].

2.2.8.2. Effect of oxidovanadium complexes on apoptosis: Measurement of the exposure of phosphatidyl serine (PS) with Annexin V-FITC/PI by flow cytometry. Cells in early and late stages of apoptosis were detected with Annexin V-FITC and propidium iodide (PI) staining. Cells were treated with 100 μM of the three complexes and incubated for 12 and 24 h prior to analysis. For the staining, cells were washed with PBS and adjusted to a concentration of 1×10^6 cell/mL in 1× binding buffer. To 100 μL of cell suspension, 2.5 μL of Annexin V-FITC and 2 μL PI (250 μg/mL) were added and incubated for 15 min at room temperature prior to analysis. Cells were analyzed using flow cytometer (BD FACS Calibur™) and FlowJo 7.6 software. For each analysis 10,000 counts, gated on a FSC vs SSC dot plot, were recorded. Four subpopulations were defined in the dot plot: the undamaged vital (Annexin V−/PI−), the vital mechanically damaged (Annexin V/PI+), the apoptotic (Annexin V+/PI−), and the secondary necrotic (Annexin V+/PI+) subpopulations.

2.2.8.3. Effect of oxidovanadium complexes on a plasmidic model of DNA cleavage by agarose gel electrophoresis (AGE) of plasmid DNA. Cleavage of plasmid DNA (pA1) by the three complexes was examined by agarose gel electrophoresis under inorganic (phosphate buffer) and 3-(N-morpholino) propanesulfonic acid (MOPS) media at physiological pH according to a previous report [46].

DNA cleavage activity of the complexes was evaluated by the amount of supercoiled plasmid DNA (Sc) converted into nicked circular DNA (Nck) and linear DNA (Lin). 1% agarose gel in 0.5× TBE was prepared as described elsewhere [46]. A mixture of 10 mM phosphate buffer and 50 μM of VO(acac)2 incubated with pA1 was used as a reference of the linearized DNA form since this form appeared to be on the same position as the one obtained by digestion of pA1 with the restriction enzyme *Hind*III. First, the complexes were tested at different metal:bp ratios: 0.33, 0.67, 1.7, 3.3 and 6.7 which correspond to 5, 10, 25, 50 and 100 μM concentrations, respectively. A 200 μM stock solution of the complexes in MilliQ® water for VO(oda) and in DMSO for VO(oda)bipy and VO(oda)phen were freshly prepared for each experiment. After verifying the concentration effect on the nuclease activity of the complexes, they were studied in the presence of scavengers (NaN3, NaBz and DMSO); stock solutions, prepared in water, were of 400 mM and H_2O_2 (stock solution) 1 mM. The control samples were prepared in the absence of metal complex. After samples were digested at 37 °C for 1 h, the reaction was quenched by addition of 5 μL of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in water); the samples were loaded onto 1% agarose gel in 0.5× TBE (89 mM Tris-borate, 1 mM EDTA, pH 8.3) containing ethidium bromide (3 μg/mL). The electrophoresis was carried out for 3 h at 100 V. Gels were exposed to UV light and photographed using AlphaImager from Alpha Innotech. Gel images were then analyzed: peak areas of each DNA form were measured using1D-Multi densitometry (AlphaEaseFCTM Software, Alpha Innotech), proceeding with corresponding percentage calculations. Peak areas for the Sc form were always corrected by factor 1.47 to account for its weak binding capacity to ethidium bromide [47]. Typically, experiments were repeated two times and a gel image of best quality was presented.

2.2.9. Statistical analysis

At least three independent experiments were performed for each experimental condition in all the biological assays. The results are expressed as the mean ± the standard error of the mean (SEM). Statistical differences were analyzed using the analysis of variance method (ANOVA) followed by the test of least significant difference (Fisher).

3. Results

3.1. Stability study

The electronic absorption spectrum of VO(oda) is determined in distilled water and DMEM. In this spectrum, two d–d transitions were observed at 792 and 618 nm. The electronic absorption spectra of VO(oda)bipy and VO(oda)phen were determined in DMSO solution due to the solubility of these compounds. The former showed d–d transitions at 650 nm ($\varepsilon = 13\ M^{-1}\ cm^{-1}$), and 440 nm ($\varepsilon = 5\ M^{-1}\ cm^{-1}$). For VO(oda)phen, the electronic absorption spectrum showed d–d transitions at 580 nm ($\varepsilon = 12\ M^{-1}\ cm^{-1}$), 820 nm ($\varepsilon = 21\ M^{-1}\ cm^{-1}$) and 450 nm ($\varepsilon = 40\ M^{-1}\ cm^{-1}$). The solution spectra were stable for the three complexes at least during 24 h.

3.2. Effect of VO(oda), VO(oda)bipy and VO(oda)phen on osteoblast-like cell viability

The action on the cellular viability of the three chelate complexes of oxidovanadium(IV) cation with oxygen donor centers such as oxodiacetate and related derivatives was determined on MG-63 human osteosarcoma cell line. The three complexes caused a concentration dependent inhibition of cell growth. The more deleterious compound was VO(oda)phen and its antiproliferative action could be observed in the whole range of concentration (Fig. 2). On the contrary, in the evaluation of VO(oda)bipy the damage was observed only in the higher concentration range (50–100 μM). For the parent compound, VO(oda) the inhibitory effect on cell viability was observed only between 75 and 100 μM. The percentage of survival cells at 100 μM for each complex was: 39% for VO(oda)phen, 70% for VO(oda)bipy and 84% for VO(oda) as can be seen from Fig. 2. Moreover, the IC_{50} values in MG-63 human osteosarcoma cell line were 58 μM for VO(oda)phen while they were greater than 100 μM for the other to complexes.

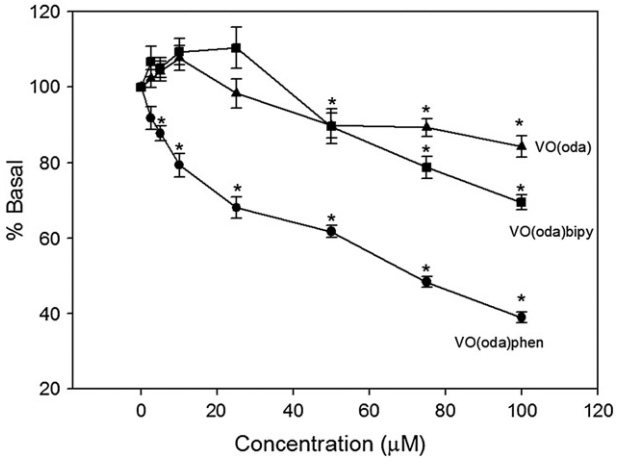

Fig. 2. Effects of VO(oda), VO(oda)bipy and VO(oda)phen on the viability of MG-63 human osteosarcoma cell line by the crystal violet assay. Cells were incubated in serum-free DMEM alone (basal) or with different concentrations (2.5–100 μM) of the compounds at 37 °C for 24 h. Then the monolayer was stained with crystal violet dye according to the Materials and methods section. The results are expressed as the percentage of the basal level and represent the mean ± SEM (n = 18). *Significant difference in comparison with the basal level ($p < 0.01$).

According to these results, VO(oda)phen was the more potent antiproliferative compound in the human osteosarcoma cells. In comparison oxidovanadium(IV) chloride only provoked a slight inhibitory effect at 100 μM in MG-.63 cells ($IC_{50} > 100$ μM), previously reported by León and Col [48].

Taking into consideration the intense scientific search of new potentially active compounds for cancer treatment, we decided to focus the investigation of the cytotoxicity and genotoxicity of the complexes on MG-63 cells.

To get a better knowledge of the cytotoxic effects of the complexes, we investigated their action on cellular metabolism through the neutral red (NR) uptake and the MTT assays.

3.3. Cytotoxicity induction in osteosarcoma MG-63 cells in culture

3.3.1. Effects of the complexes on the lysosomal metabolism: Neutral red assay

The lysosome of viable cells has great affinity for the vital dye neutral red (NR). When the cellular metabolism is altered by different factors, the ability of the lysosomes to uptake NR is diminished. For this reason, the NR assay is a means of measuring living cells. The method was originally developed by Borenfreund and Puerner [41]. The cytotoxic

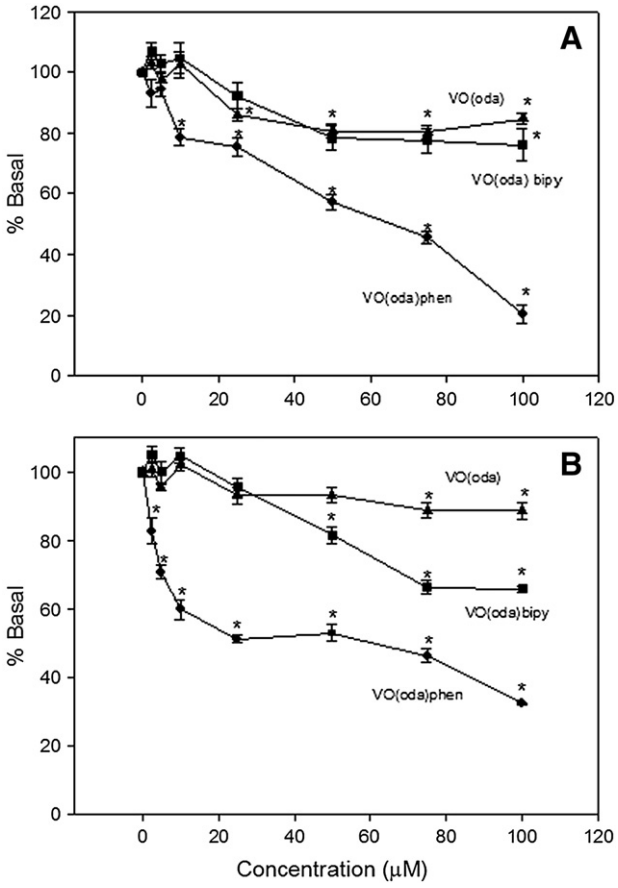

Fig. 3. Comparative effects of VO(oda), VO(oda)bipy and VO(oda)phen on the lysosome and mitochondria metabolism of the osteosarcoma MG-63 cell line using the neutral red uptake assay (NR) and MTT assay. A. Tumoral cells were incubated with different concentrations (2.5–100 μM) of the complexes for 24 h at 37 ° C. After incubation, cell viability was determined by the uptake of neutral red. The dye taken up by the cells was extracted and the absorbance read at 540 nm. B. Evaluation of the mitochondrial succinate dehydrogenase activity by the MTT assay in MG-63 cells in culture. Osteosarcoma cells were incubated with different concentrations (2.5–100 μM) of VO(oda), VO(oda)bipy and VO(oda)phen for 24 h at 37 ° C. After incubation, cell viability was determined by the MTT assay. All the results are expressed as % basal and represent the mean ± SEM, n = 18, *significant difference in comparison with the basal level ($p < 0.01$).

effects of VO(oda), VO(oda)bipy and VO(oda)phen affected the function of lysosomes in MG-63 cells. This effect can be observed in Fig. 3A. VO(oda) and VO(oda)bipy decreased NR uptake at 50 μM while VO(oda)phen caused a stronger decrease of lysosome activity in the whole range of concentration ($p < 0.01$). As can be seen, a statistically significant decrease in the uptake of the dye by the lysosomes of the cells in culture could be determined.

3.3.2. MTT assay

The MTT assay determines the activity of viable cells that are able to reduce the tetrazolium dye, MTT, to formazan, a compound of purple color. Other closely related tetrazolium dyes can also be used [42]. The reduction of the dye determines mitochondrial activity. Since for most cell populations the total mitochondrial activity is related to the number of viable cells, this assay is broadly used to measure the in vitro cytotoxic effects of drugs.

Results of this test can be seen in Fig. 3B. The three compounds affected mitochondria enzyme activity causing a decrease that was stronger for VO(oda)phen in the whole range of concentrations ($p < 0.01$). In the case of VO(oda)bipy and VO(oda) the inhibition of cell metabolism was statistically significant at 50 μM ($p < 0.01$).

3.4. Morphological changes

To further investigate the cytotoxicity of oxidovanadium(IV) complexes in MG-63 osteoblasts in culture, we determined the effect of the three complexes on the morphology of these cells by light microscopy.

Fig. 4 shows the morphological features of MG-63 osteoblast-like cells and the effects caused by 10, 25 and 50 μM of the three complexes.

MG-63 cells showed fibroblastic characteristics with very well-stained cytoplasm and oval nuclei. The monolayer exhibited multiple connections between cells (first panel). As can be seen from the figure, VO(oda) caused a slight decrease in the cell number per field at 10 μM. Besides, a reduction in the cytoplasms could be seen with quite good conservation of the nuclear morphology. At 25 and 50 μM, a marked decrease in the number of cells per field was observed with pronounced alterations in the cytoplasms and the nuclei. When MG-63 cells were treated with VO(oda)bipy, the observed effects were similar to that seen in VO(oda)-treated cells, with a stronger effect at higher concentrations. On the other hand, VO(oda)phen produced a much deeper influence in the morphological features of MG-63 cells: at 10 μM apoptotic cells were distinguished for their fragmented nuclei, and at higher concentrations, the cell number per field decreased dramatically. Modifications in the number of cells and morphological changes increased with the complex concentration.

3.5. Genotoxicity effects of the complexes on MG-63 cells

After the evaluation of the cytotoxic effects of the three oxidovanadium(IV) complexes on the human osteosarcoma MG-63 cells, we investigated the actions of the complexes on the genotoxicity by the single cell gel electrophoresis (comet) assay, a useful technique for studying DNA damage.

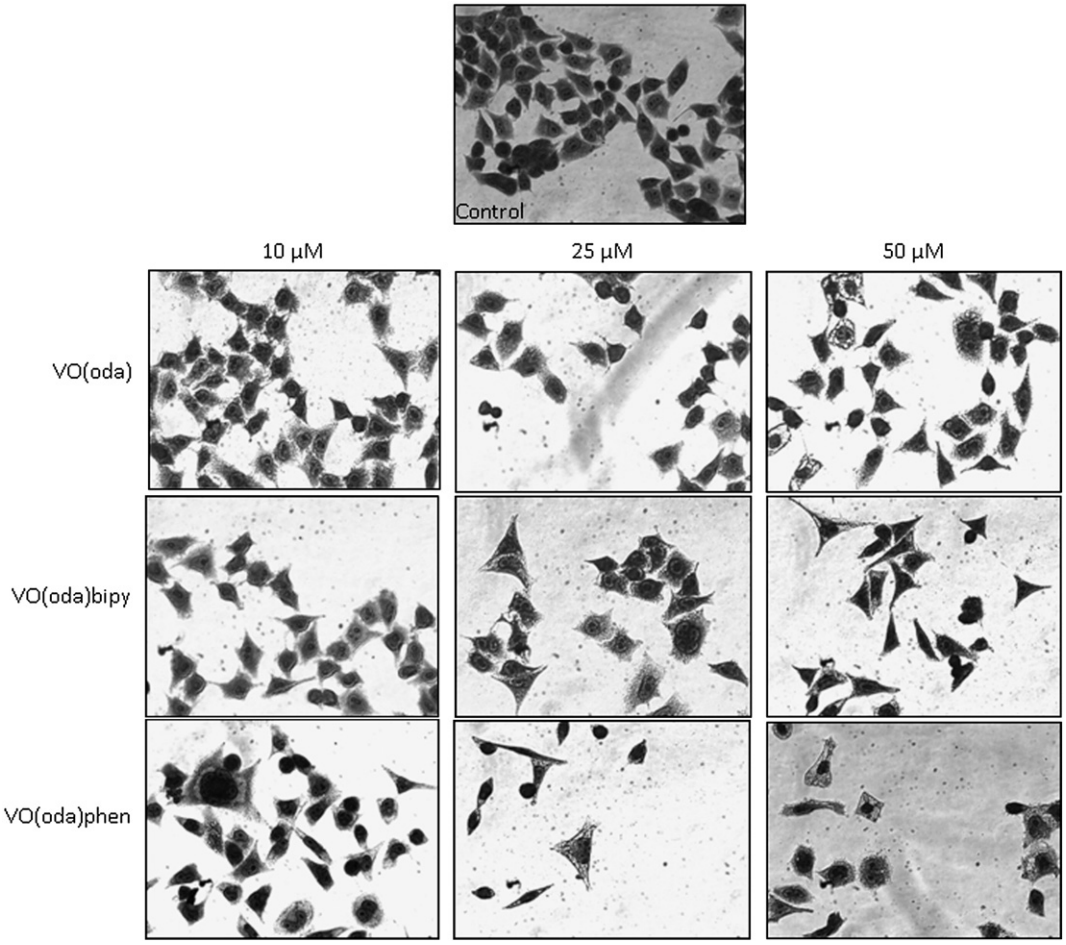

Fig. 4. Effects of VO(oda), VO(oda)bipy and VO(oda)phen on MG-63 human osteosarcoma cell line morphology by Giemsa staining. Tumoral osteoblasts were incubated for 24 h without drug addition (basal, first panel), or with different concentrations (10, 25 or 50 μM) of VO(oda), VO(oda)bipy and VO(oda)phen. After incubation, the cells were stained with Giemsa and observed under the microscope. Magnification 400×.

3.5.1. Induction of DNA damage assay by comet assay

The comet assay was carried out to elucidate the genotoxic effect of the complexes. We evaluated the Tail Moment parameter as the genotoxic endpoint, which is defined as the Tail Length × DNA amount in the tail.

In Fig. 5, it can be seen that no comets could be observed in MG-63 cells treated with VO(oda) in the studied concentration range (2.5–10 μM). However, VO(oda)phen induced DNA damage in MG-63 cells from 2.5 to 10 μM. The increase in the Tail Moment at 5 and 10 μM is higher than the DNA damage generated by a pulse of 20 min of 1 μg/mL of bleomycin, employed as positive control in this assay. On the other hand, comets could be observed in MG-63 cells treated with VO(oda) bipy only at the highest concentration studied (10 μM).

3.6. Mechanism of action

The antitumoral action of the complexes may be mediated through different mechanisms. To get a more exhaustive knowledge of the possible mechanisms involved in the cell death triggered by the oxidovanadium(IV) complexes, we investigated the oxidative stress through the action of the complexes on ROS levels, the apoptosis process and finally, their action on DNA cleavage using a model of plasmidic DNA and correlating the results with those of genotoxicity.

3.6.1. Determination of reactive oxygen species: Intracellular ROS production

The reactive oxygen species mediate different mechanisms of actions in cells in culture. The determination of ROS in our model system of human osteosarcoma cells was carried out using DHR-123 that is not a fluorescent compound. This probe determines the levels of intermediate peroxynitrite, hydroxyl radicals and hydrogen peroxide [49–51]. In the cells, DHR-123 was oxidized to rhodamine123 in the presence of oxidizing drugs. Because fluorescent oxidation products may be produced only in metabolically active cells, DHR-123 can also be used as a viability indicator. Cell extracts for rhodamine measurements were obtained and processed as previously described, using fluorescence spectroscopy [24].

As can be seen in Fig. 6, VO(oda)phen induced a concentration-dependent oxidative stress in MG-63 cell line. Significant differences vs. basal could be observed at 10 μM. This complex increases a 300%

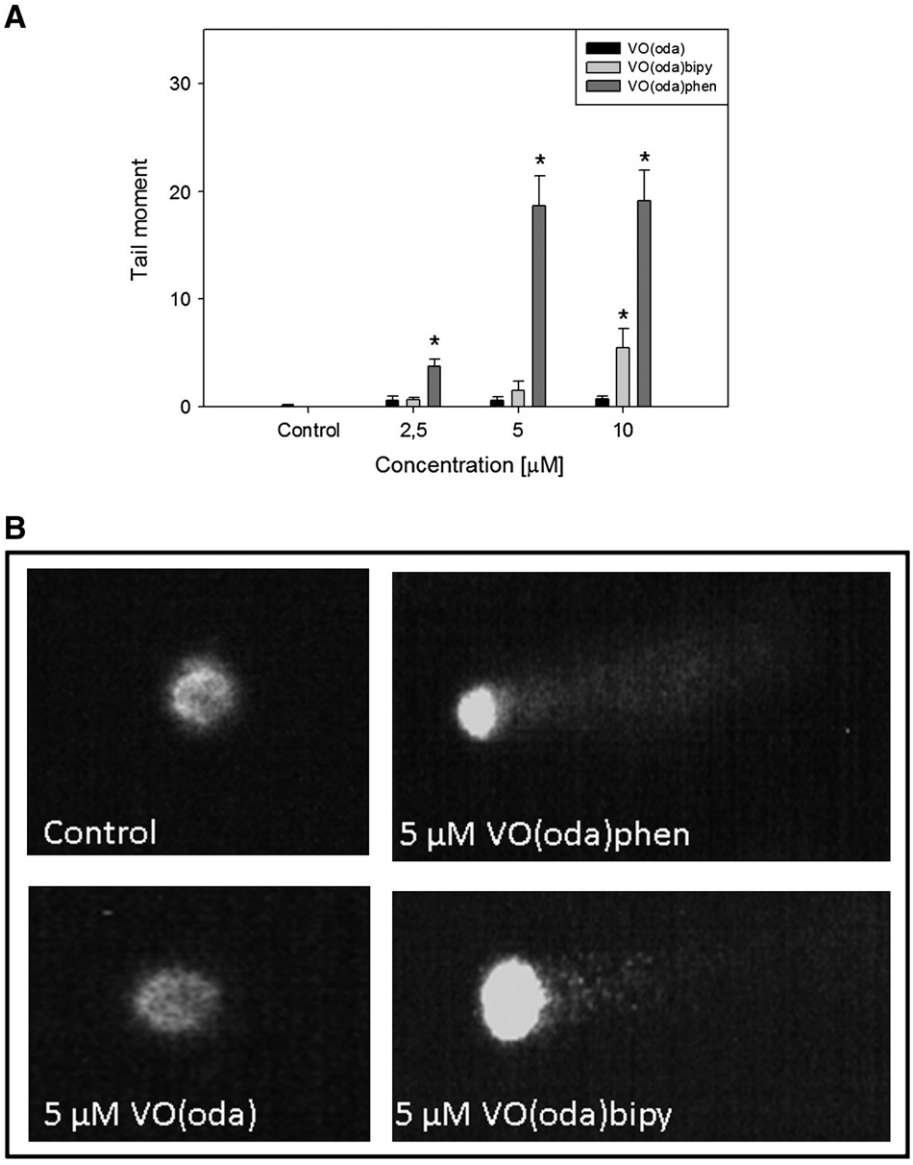

Fig. 5. Genotoxicity effect of VO(oda), VO(oda)bipy and VO(oda)phen on MG-63 cells evaluated by the comet assay. Osteosarcoma cells were incubated with different concentrations (2.5, 5 and 10 μM) of the complexes for 24 h at 37 º C. Results are presented as mean Tail moment ± s (standard error of the mean). *Significant differences versus control, $p < 0.01$. BLM, bleomycin (1 μg/mL, positive control).

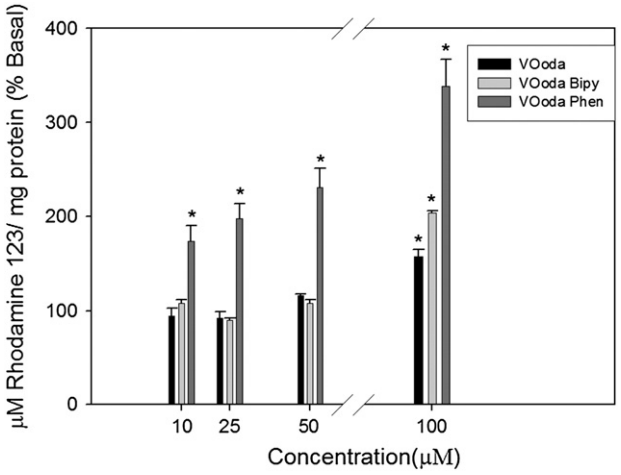

Fig. 6. Induction of ROS by VO(oda), VO(oda)bipy and VO(oda)phen in MG-63 human osteosarcoma cell line. Cells were incubated with growing concentrations of three complexes at 37 °C for 24 h. ROS production in the cells was evaluated through the oxidation of DHR-123 to rhodamine123. Results represent the mean ± SEM, n = 12, *significant differences vs. control $p < 0.01$.

the level of ROS over basal ($p < 0.01$). Nevertheless only at 100 µM of VO(oda) and VO(oda)bipy could be observed an increase in ROS level. These results agree to cell viability and cytotoxicity results.

3.6.2. Apoptosis

Apoptosis is a physiological process of cell death enhanced in the presence of injuring agents. Apoptosis determines various modifications in cell structure, mainly at the cellular membrane level. One of the first alterations that can be defined is the externalization of the phosphatidylserine (PS), at the outer plasma membrane leaflet. Annexin V-FITC is a fluorescent probe with high affinity for PS allowing its determination by fluorescence assays.

Fig. 7 displays the quantification of apoptotic cells determined by flow cytometry in MG-63 cells incubated with 100 µM of VO(oda), VO(oda)bipy and VO(oda)phen for 12 and 24 h. As can be seen MG-63 cells showed 4% of apoptotic cells under basal conditions. This percentage increased to 23% and 37% over basal at 100 µM of VO(oda)bipy and VO(oda)phen, respectively ($p < 0.01$). In the case of VO(oda) no statistical differences in comparison with control was observed at $p < 0.01$. On the contrary, at 12 h of incubation no apoptotic effects could be detected for the complexes. These results demonstrated a stronger deleterious action of VO(oda)phen than VO(oda)bipy and VO(oda). The order of activity is the following: VO(oda)phen > VO(oda)bipy > VO(oda). These results are in agreement with those of cell viability, cytotoxicity studies and intracellular ROS production.

3.6.3. Plasmid DNA cleavage

Considering as a whole the results of ROS production, comet assay and apoptosis we decided to investigate the interaction of the three complexes with an in vitro DNA plasmid model (pDNA). In particular this interaction was assayed through the determination of DNA cleavage by AGE. The efficiency by which a nuclease cleaves supercoiled DNA can be measured by the decrease of the intensity of the supercoiled (Sc) DNA band after incubation, relative to the native DNA control. Single strand cleavage of pDNA increases the concentration of the nicked form (Nck) of pDNA, and is seen by the higher intensity of this band. Strong nucleases will cleave the double strand, generating a sharp band of linear DNA. Nuclease activity of vanadium compounds has been found to depend on the nature of the pH buffer [46] so two buffers were tested: phosphate and MOPS. The nuclease activity of VO(oda)bipy and VO(oda)phen under phosphate buffer is shown in Fig. 8A and B respectively. The nuclease activity of VO(oda) has been previously reported [52]. The results showed that VO(oda)bipy was active for DNA cleavage, but to a similar extent than VO(oda) and much lower than VO(oda)phen (Fig. 8A). Besides, VO(oda)phen cleaved DNA very efficiently. Strong cleavage, including linearization, could be observed for complex concentrations as low as 5 µM, corresponding to a metal:bp ratio of ri = 0.3. The nuclease activity of VO(oda)phen under phosphate buffer was higher than that of VO(oda)bipy and VO(oda), as it can be seen in Fig. 8B. Fig. 8C displays the nuclease activity of the three complexes and that of VO(acac) which was the positive control in the whole range of concentrations. As can be seen, the DNA cleavage ability of VO(oda)bipy is similar to that of VO(oda), while VO(oda)phen has a much higher nuclease activity. Quantification of the nuclease activity in phosphate buffer can be observed in Table 1 (−ln(%Sc) vs concentration). Besides, the calculations of the absolute slope values of the lines for phosphate buffer in Fig. 8C also demonstrate the potency order for this activity (intervals for 95% confidence): VO(oda)phen [0.3 ± 0.4] > VO(oda)bipy [0.018 ± 0.002] > VO(oda) [0.021 ± 0.008]. For $VO(acac)_2$, considered as a positive control [45], this value is 0.031 ± 0.004, showing a much lower nuclease activity than VO(oda)phen. For VO(oda)phen under MOPS the values of −ln(%Sc) vs concentration diverge somewhat from linearity because, simultaneously to the cleavage of the Sc, cleavage of the Nck into Lin form also takes place.

In order to get a deeper insight into the antiproliferative effects and its relationship with the generation of ROS and plasmid DNA cleavage, VO(oda)bipy and VO(oda)phen were tested in the presence of scavengers of singlet oxygen (NaN_3) and of free radicals (NaBz, DMSO). Since H_2O_2 plays an important role in hydroxyl radical formations, it was tested with both complexes to see its effect on their DNA cleavage activity. VO(oda)bipy effectively linearized pA1 pDNA at 100 µM under phosphate buffer (lane11, Fig. 9A). Oxodiacetate and bipyridyne, which were added for comparison, did not induce DNA degradation (lanes 12 and 13). All the three scavengers inhibited the complex activity in the same manner, showing that the ROS involved are not singlet oxygen but rather hydroxyl radicals. Besides, H_2O_2 increased strongly the DNA cleavage leading to the complete degradation of the native pDNA into nicked and linear forms. Similar results were previously reported for VO(oda) [52].

In the case of VO(oda)phen the effect of scavengers was very different. The inhibition of DNA cleavage was less efficient. NaN_3 (lane 7, Fig. 9B) scavenged the most, followed by NaBz and DMSO (lanes 8 and 9). Moreover, the hydrogen peroxide caused a synergistic effect with the complex dramatically increasing the nuclease activity and leading to a degradation of the linear form, visible as a smear of its band (lane 10). Oxodiacetate and phenanthroline, added for comparison, do not show any effect on DNA breakage (lanes 12 and 13).

4. Discussion

Vanadium compounds are a group of drugs with potential pharmacological effects [53,54]. Among organic ligands for vanadium ions, strong chelating ligands are very important in the investigation of the interactions between metal ions and cellular components. Living organisms need different metal ions to accomplish their vital functions and for the maintenance of the homeostasis. The metals are transported and stored mainly by low molecular mass compounds involving multidentate oxygen donors [36,55]. Nevertheless, some proteins such as transferrin also play an important role in the transportation of vanadium(IV) compounds [3]. Moreover, metallodrug interactions with cell membranes can be relevant for the delivery and transport into the cell. For other vanadium compounds it has been demonstrated that the interaction with the cellular membrane components can affect the signal transduction pathways [56]. Crans and coworkers have shown the possibility that a negatively charged organic molecule can penetrate the lipid interface and reside in nonpolar locations [57].

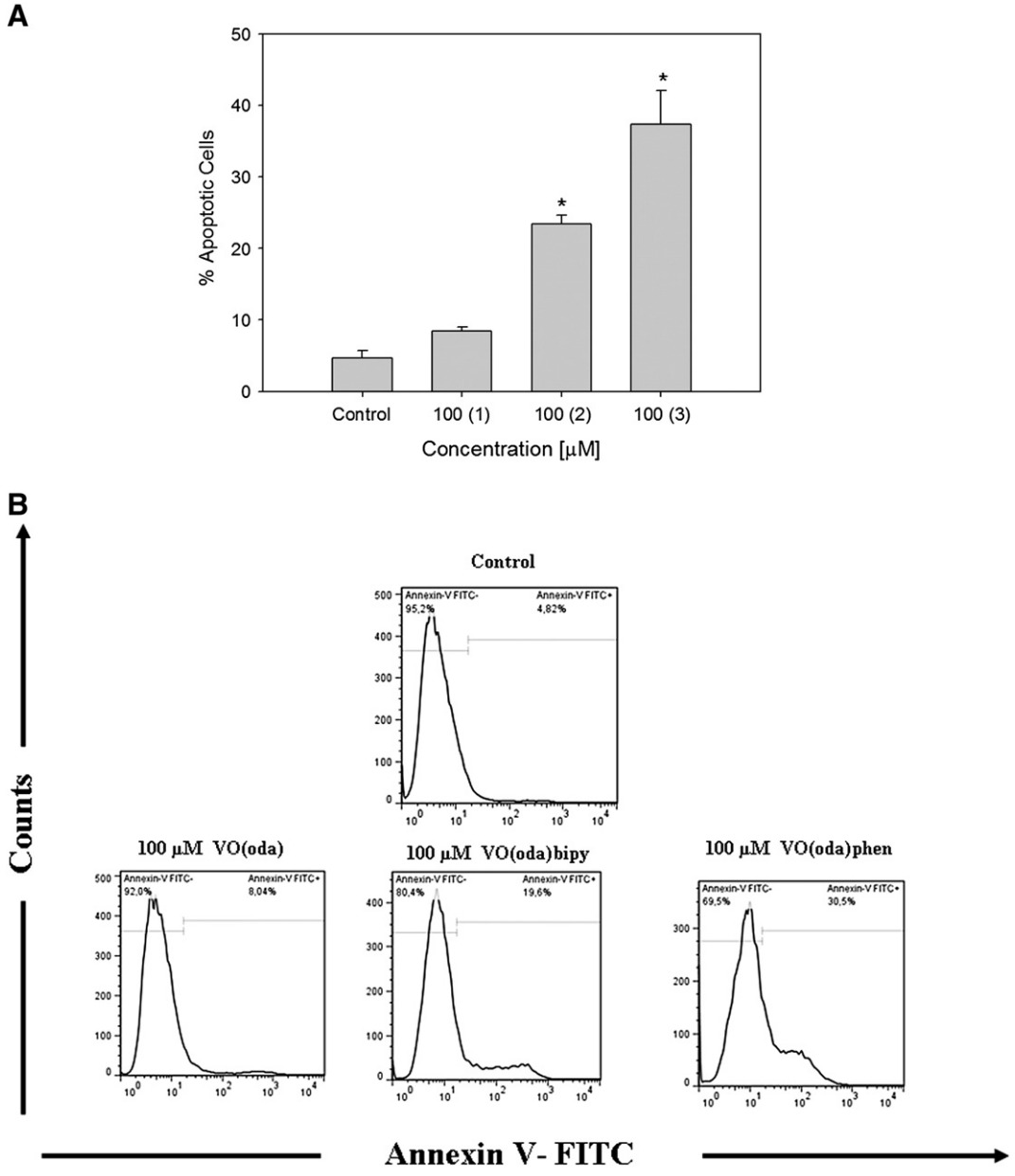

Fig. 7. A. Effect of VO(oda), VO(oda)bipy and VO(oda)phen on the externalization of PS by flow cytometry in MG-63 cells. The cells were incubated with 100 μM of the complexes during 24 h. Graphical bars show the percentage of Annexin V (+) cells. Results are expressed as the mean ± SEM, n = 9, *significant differences vs. control ($p < 0.01$). B. Histograms are representative of three independent experiments. The number indicates cells Annexin V (+). For each analysis 10,000 counts, gated on a FSC vs SSC dot plot, were recorded.

Besides, it has been shown that some vanadium compounds suffer hydrolysis processes after administration and that other complexes can form with cellular components [58]. Due to these processes it is very difficult to establish the different active species that underlie the biological effects of vanadium compound and it is clear that the effects of the coordination complexes are difficult to interpret.

In the vertebrates, after absorption and distribution among different tissues, vanadium is mainly stored in bone [2]. We have previously reported that osteoblasts in culture vanadium complexes regulate cellular proliferation and differentiation [13,15,17,21,24].

In the present study we have addressed the investigation of the biological effects of three oxidovanadium(IV) complexes with oxodiacetate (oda) or (oda) and bipy or (oda) and phen. First we have determined the stability of the complexes in solution measured spectrophotometrically during 24 h. The obtained spectra were relatively simple and typical of V(IV) complexes. Although their symmetry is lower than C_{4v}, in a first approximation they can be analyzed on the basis of the simple M.O. model proposed by Ballhausen and Gray for the $[VO(H_2O)_5]^{2+}$ complex [59–61]. According to this model, the lower energy bands of VO(oda)bipy (855 nm) was assigned to the $b_2 \rightarrow e$ transition, the band at 650 nm was assigned to the $b2 \rightarrow b1$ transition, whereas the other one, found at 440 nm could be assigned to the $b2 \rightarrow a1$ transition. For VO(oda)phen, the two lower energy bands were assigned to the $b2 \rightarrow e$ (820 nm) and $b2 \rightarrow b1$ (580 nm) transitions, whereas the one, found at 450 nm could be assigned to the $b2 \rightarrow a1$ transition. In the precursor complex, VO(oda), the first two d–d transitions were observed at 792 and 618 nm, respectively. The spectra did not show any significant change during a 24 h period, attesting the stability of the complexes in the study conditions.

To study the biological and pharmacological actions of VO(oda), VO(oda)bipy and VO(oda)phen, we have investigated the complexes' bioactivity, cyto- and genotoxicity, and the mechanisms of actions

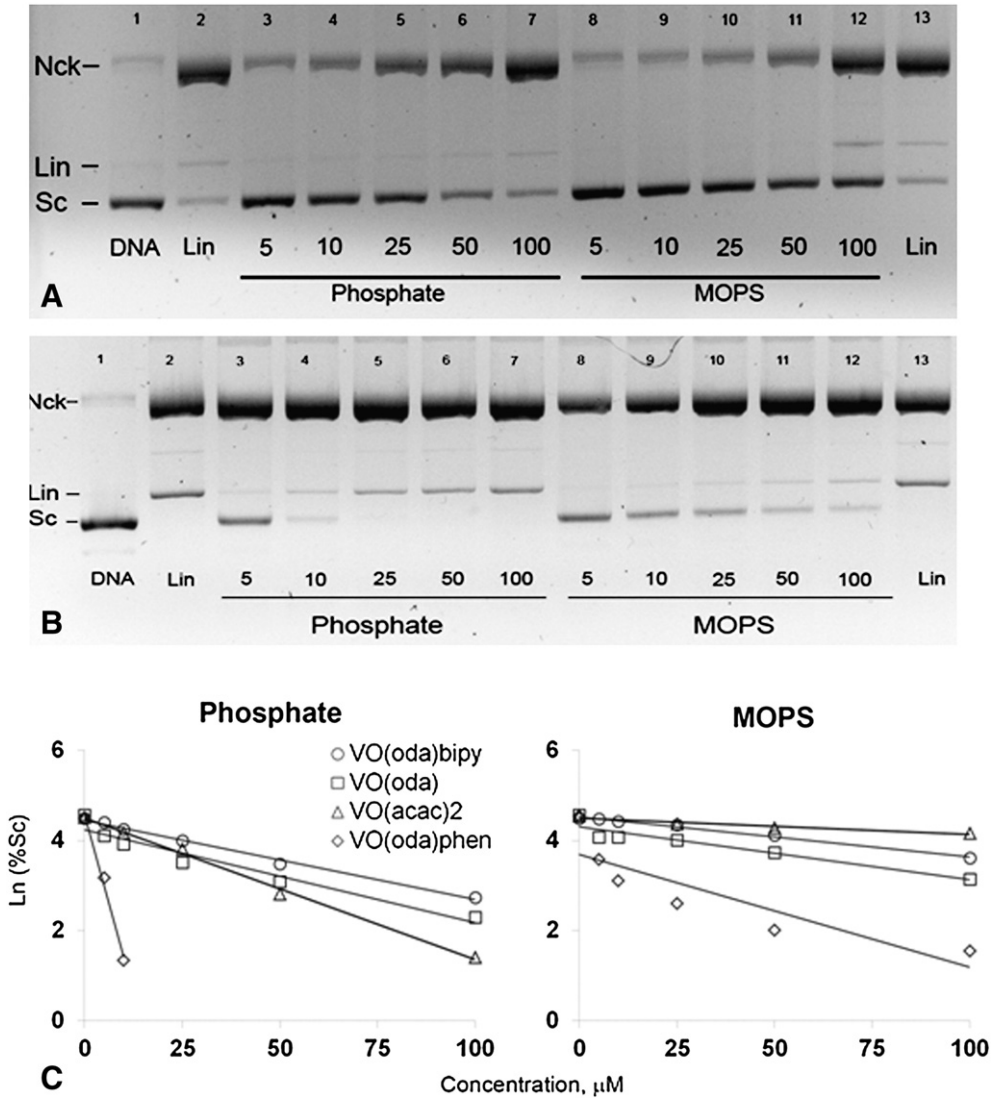

Fig. 8. A. AGE images of pA1 pDNA digested with VO(oda)bipy (**2**) at 5, 10, 25, 50 and 100 μM (ri = 0.33, 0.67, 1.7, 3.3 and 6.7, respectively). Samples 3–7 were digested under phosphate buffer; samples 8–12 were digested under MOPS buffer. Buffer concentration was 10 mM (pH 7.4). "DNA" and "Lin" represent controls for the supercoiled (Sc) and linear (Lin) forms of DNA. Assigned numbers for the samples 3–12 indicate complex concentration in μM. B. AGE images of pA1 pDNA digested with VO(oda)phen (3) at 5, 10, 25, 50 and 100 μM (ri = 0.33, 0.67, 1.7, 3.3 and 6.7, respectively). Samples 3–7 were digested under phosphate buffer; samples 8–12 were digested under MOPS buffer. Buffer concentration was 10 mM (pH 7.4). "DNA" and "Lin" represent controls for the supercoiled (Sc) and linear (Lin) forms of DNA. Assigned numbers for the samples 3–12 indicate complex concentration in μM. C. Comparison of the nuclease activity of VO(oda), VO(oda)bipy and VO(oda)phen (**1**–**3**) under phosphate and MOPS buffers, represented as the slope of ln(%Sc) vs. complex concentration. $VO(acac)_2$ was included as a positive control.

involved in their antiproliferative effects in the human osteosarcoma cell line, MG-63.

We found that the three complexes: VO(oda), VO(oda)bipy and VO(oda)phen induced inhibition of cell viability but with some differences ($p < 0.01$). According to the viability experiments with the crystal violet assay and the IC_{50} values, the inhibitory potency for the three complexes in MG-63 cell line is, in decreasing order: VO(oda)phen > VO(oda)bipy > VO(oda). We have previously shown that the mouse calvaria MC3T3-E1 cell line showed more sensitivity to VO(oda) and VO(oda)phen complexes than the rat tumoral osteoblasts UMR106 [62,63]. Similar effects were previously reported by our group for other vanadium compounds [13,16,21,24]. Besides, comparing the inhibition of cell viability among the three osteoblastic cell lines: MG-63, UMR106 and MC3T3-E1, it can be seen that the cytotoxicity is a complex phenomenon and that the origin of the cell lines also plays a role. Quantifying cell viability is crucial for understanding cellular responses, cancer biology and compound toxicity.

To better understand the cytotoxic effects of the complexes on MG-63 human osteosarcoma cells, we next investigated the effect of the complexes on two parameters sensitive towards drug toxic effects: the metabolism of lysosomes and mitochondria which are key organelles for the cells. Viable cells will take up the neutral red (NR)

Table 1
Values of −ln(%Sc) corresponding to 0, 5, 10, 25, 50 and 100 μM complex concentration under phosphate buffer. Sampe with complex concentration '0' is the one with plasmid DNA and no complex added.

Concentration, μM	VO(oda), 1	VO(oda)(bipy), 2	VO(oda)(phen), 3	$VO(acac)_2$
0	4.571	4.491	4.495	4.513
5	4.105	4.398	3.169	–
10	3.918	4.248	1.346	4.168
25	3.515	4.000	*	3.805
50	3.091	3.485	*	2.808
100	2.299	2.733	*	1.405

*Sc not detected due to complete degradation into Nck and Lin.

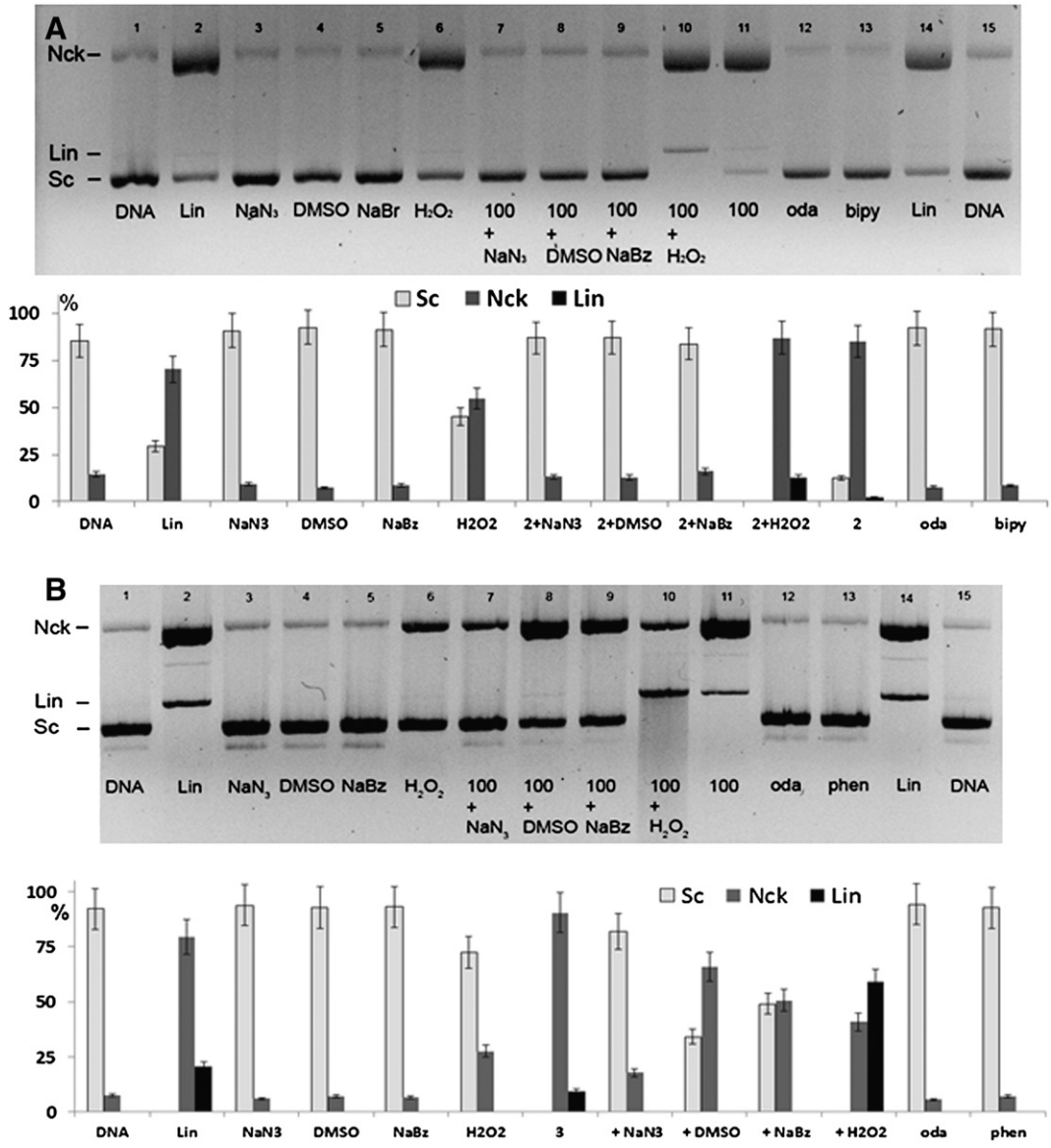

Fig. 9. A. Effect of scavengers and H_2O_2 on the nuclease activity of VO(oda)bipy (**2**) at 100 μM (ri = 6.7) in phosphate buffer. 'NaN_3', 'NaBz', 'DMSO' and 'H_2O_2' are the controls prepared in the absence of the complex. B. Effect of scavengers and H_2O_2 on the nuclease activity of VO(oda)phen (**3**) at 100 μM (ri = 6.7) in phosphate buffer. 'NaN_3', 'NaBz', 'DMSO' and 'H_2O_2' are the controls prepared in the absence of the complex.

dye by active transport and incorporate the dye into the lysosomes, whereas non-viable cells will not take up the dye. The probe NR has a great affinity for the metabolically active lysosomes in the cells. If a drug impairs this metabolic activity, the capacity of lysosomes to uptake NR decreases. The obtained results with NR dye agree quite well with the effects of the complexes on the MG-63 osteoblast viability. On the other hand, the MTT cell viability assay is widely used in determining drug sensitivity profiles in primary screening of potential chemotherapeutic drugs. The inhibition pattern is similar to that observed for the NR uptake assay, showing that VO(oda)phen was more deleterious complex of this series.

Numerous publications based on the morphological alterations induced by different chemical compounds on cellular cultures have shown that these changes are good markers to complement the toxicity studies. The morphological alterations have demonstrated that these changes and the modifications in the number of cells per field increased with the concentration of the complexes correlating with the viability and cytotoxicity results as it has been previously reported for other vanadium derivatives [17,23,52]. Moreover, all the previous results correlate with the genotoxic actions of the complexes. The single cell gel electrophoresis (comet) assay that detects single and double strand DNA breaks was used to analyze the genotoxic effects of the three complexes in MG-63 cells. Under alkaline conditions, additional DNA structures are detected as DNA damage: AP sites (abasic sites missing either a pyrimidine or purine nucleotide) and sites where excision is taking place. This method is a simple and sensitive procedure for studying DNA damage and repair processes. The assay has manifold applications in fundamental research for DNA damage and repair, in genotoxicity testing of novel chemicals and pharmaceuticals, environmental biomonitoring, and human population monitoring.

In this paper, we have reported a significant genotoxic effect of VO(oda)bipy and VO(oda)phen in MG-63 cells at 2.5 and 10 μM onwards, respectively. On the contrary, no statistically significant DNA damage could be detected in VO(oda)-treated MG-63 cells with this method. However, employing the comet assay we have previously reported the genotoxic effect of VO(oda) in tumoral Caco-2 cells and this effect agreed with the result on plasmidic DNA causing single and double strand cleavage [52]. This cell line dependent effect was also observed

for hydroxylamido/amino acid complexes of oxidovanadium(V) in osteoblast-like cell lines, in which the genotoxic effect was only appreciated in the normal phenotype MC3T3-E1 osteoblast-like cells and not in the tumoral ones [24]. According to our findings, DNA damage was also reported in human blood leukocytes exposed to vanadium oxides in vitro. It was observed that the genotoxic effect of vanadium can be produced by any of its more usual oxidation states in biological systems. However, vanadium(IV) induces double-strand breaks, and it is believed that when these lesions are linked, they produce structural chromosomal aberrations [64]. On the other hand, in vivo experiments showed the genotoxicity of tetravalent vanadium. Oxidovanadium sulfate in male CD1 mice produced an increase in the incidence of micronucleated blood reticulocytes and bone marrow polychromatic erythrocytes, in addition to DNA lesions detectable by comet assay [65]. In this study, the complex with phenanthroline showed a much stronger effect than the one with bipyridine. This effect could be explained based on the DNA binding affinity as it was demonstrating for a series of mononuclear mixed ligand copper(II) complexes. The complex with phenanthroline displayed stronger DNA binding affinity and oxidative DNA cleavage ability than the complex with bipyridine [66]. Moreover, casiopeinas, a very interesting group of copper compounds with phenanthroline and bipyridine ligands, that displayed antitumoral properties, have also shown genotoxic effects in HeLa cells and lymphocytes by SGE assay [67]. Moreover, cobalt(III) complexes with bipyridine or phenanthroline affected the viability of MCF-7 breast cancer cells as determined by the MTT assay. The complex with phenanthroline seemed to be more potent and caused DNA damage studied by the comet assay [68].

After investigating the putative mechanisms of action involved in the antitumoral effects of the three oxidovanadium(IV) complexes reported herein, we have shown that the oxidative stress may play a key role in the toxic effect of the complexes as it has been previously shown for other oxidovanadium(IV) complexes [21,23,24,52]. VO(oda)phen caused the strongest oxidative stress on MG-63 cells in this series of complexes. This result is in parallel with the viability, cyto- and genotoxicity studies. As a whole, these results suggest that the generation of ROS may be, at least partly, an important mechanism of action involved in deleterious effects of these complexes.

Apoptosis is considered a physiological mechanism of cell death, inherent to cellular development, which is triggered by different endogenous or exogenous factors [69]. These factors may be recognized by receptors in the cell surface and may cause a chain activation of cytoplasm proteins. As a consequence, a genetic program that leads to cell death is triggered. This process is accompanied by a characteristic of morphological changes on the nucleus and the cytoplasm of the cells. Independent of the cellular type and the nature of the trigger agent, the externalization of PS is always present in the earlier apoptotic events.

Our flow cytometry results showed an increment of the apoptotic cells over basal in the presence of 100 μM VO(oda)bipy and VO(oda)phen (23% and 37%, respectively). Apoptosis can be activated by ROS, as it has been previously reported for different cell types [70]. In particular, vanadium-induced oxidative stress leads different cellular types to death by apoptotic and/or necrotic processes [19,21,71–74]. Moreover, reports on simple inorganic vanadium compounds such as orthovanadate, $NaVO_3$, $VOSO_4$, and oxidovanadium complexes with organic ligands, induced oxidative stress and thus caused an increase in different apoptotic mechanisms in different cell types [75,76].

Since the discovery of the DNA intercalation process by Lerman in 1961 [77], thousands of organic, inorganic octahedral (particularly ruthenium(II) and rhodium(III)) and square planar (particularly platinum(II)) compounds have been developed as potential anticancer agents [78]. The design and synthesis of new drugs is focused on multiple intercalators which have intercalating groups linked via a variety of ligands, and synergistic drugs, which combine the anticancer properties of intercalation with other functionalities. Advances in this subject mean that the process of DNA intercalation may be examined in greater detail to obtain important information on structure–activity relationships. In general, metal complexes upon binding to DNA are stabilized through a series of weak interactions such as the л-stacking interactions of aromatic heterocyclic groups between the base pairs (intercalation), hydrogen bonding and van der Waals interactions of functional groups bound along the groove of the DNA helix [79].

Phenanthroline is a DNA intercalator and as such it is not surprising that it increases the reactivity of VO(oda) towards DNA. The presence of phen may allow the binding of the complex to DNA through intercalation, and may also contribute to the reactivity by deforming the DNA double chain thus making it more susceptible to attack. We would expect the bipy ligand to provide a similar effect, but apparently the addition of bipy does not greatly affect the reactivity of VO(oda).

The molecular structures of VO(oda)phen and of VO(oda)$(MeO)_2$-bipy (MeO = methoxy), a derivative of VO(oda)bipy, are very similar [39], with the bidentade ligand bipy/phen bound equatorially and the tridentate oda occupying the remaining axial and two equatorial positions. The intercalating properties of phen make its complexes more efficient in DNA cleavage than their equivalent complexes with bipy [80,81].

It would be expected that bipy would allow some level of intercalation, and that it would increase the nuclease activity of VO(oda), but the results show that, if there is such an increase, it is negligible. On the contrary, the results obtained by plasmid DNA cleavage for VO(oda)bipy and VO(oda) do not correlate to the viability, genotoxicity and apoptosis studies in the cell cultures. In fact, at cellular levels there exist slight differences between VO(oda) and VO(oda)bipy. Nevertheless, the plasmid DNA cleavage results have shown a relation to the generation of ROS, interaction of cellular DNA and antiproliferative effects for VO(oda)phen.

5. Concluding remarks and perspectives

In this paper we have thoroughly investigated the putative mechanisms of action of three oxidovanadium(IV) complexes with multiple oxygen donor ligands such as oxodiacetate (oda) and related derivatives with 2,2'bipyridine and 1,10-phenanthroline. The complexes VO(oda), VO(oda)bipy and VO(oda)phen interact very strongly with DNA and ultimately cause DNA degradation. Moreover, the interactions of these complexes with cellular organelles such as lysosomes and mitochondria demonstrate some of the cytotoxicity mechanisms involved in their actions on tumoral cells. Moreover, the genotoxic effects give a deeper insight into the antiproliferative actions and apoptotic death mechanisms that underlie the deleterious effects of the complexes on the MG-63 human osteosarcoma cell line. Finally, a positive correlation has been found between the presence of different numbers of heterocyclic moieties in the ligands and the antitumoral properties.

Abbreviation list

AGE	agarose gel electrophoresis
bipy	2,2 bipyridine
DHR	dihydrorhodamine
DMEM	Dulbecco's modified Eagles medium
FBS	Fetal bovine serum
FITC	fluorescein isothiocyanate
FSC	forward scatter
IC_{50}	half maximal inhibitory concentration
Lin DNA	linear DNA
MOPS	3-(N-morpholino)propanesulfonic acid
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium-bromide)
NaBz	sodium benzoate
Nck DNA	nicked DNA
oda	oxodiacetate

pDNA	plasmidic deoxyribonucleic acid
phen	phenantroline
PI	propidium iodide
PS	phosphatidyl serine
NR	neutral red
ROS	reactive oxygen species
Sc DNA	supercoiled DNA
SEM	standard error of the mean
TBE	Tris/Borate/EDTA

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