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Spectroscopic Characterization of a VO^{2+} Complex of Oxodiacetic Acid and its Bioactivity on Osteoblast-like Cells in Culture

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Abstract The oxovanadium(IV) complex of oxodiacetic acid (H_2oda) of stoichiometry [VO(oda)$(H_2O)_2$], which presents an unprecedented tridentate OOO coordination, was thoroughly characterized by infrared, Raman, electronic, and electron paramagnetic resonance spectroscopies. The biological activity of the complex on the cell proliferation and differentiation was tested on osteoblast-like cells (MC3T3E1 osteoblastic mouse calvaria-derived cells and UMR106 rat osteosarcoma-derived cells) in culture. The complex caused inhibition of cellular proliferation in both osteoblast-like cells in culture, but the cytotoxicity was stronger in the normal (MC3T3E1) than in the tumoral (UMR106) osteoblasts. The effect of the complex in cell differentiation was tested through the specific activity of alkaline phosphatase of the UMR106 cells because they expressed a high activity of this enzyme. What occurs with other vanadium compounds [VO(oda)$(H_2O)_2$] is an inhibitory agent of osteoblast differentiation.

Keywords Oxovanadium(IV) · Oxodiacetic acid · Vibrational spectra · Electronic spectra · EPR spectra · Osteoblasts · Cells in culture

Introduction

The biodistribution, toxicology, and biological effects of vanadium, as well as its pharmacological activity, are areas of increasing research interest. Although numerous biochemical and physiological functions have been suggested for this element, and despite the magnitude of the knowledge so far accumulated, vanadium still does not have a clearly defined role in the higher forms of life [1–3]. Once in the body of higher animals, the element is distributed among different tissues and stored mainly in bone, kidney, and liver [1, 4].

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Vanadium compounds display potential capacity to regulate different physiological processes such as cell proliferation and differentiation, as well as cellular metabolism [5, 6], and have shown beneficial effects in the treatment of diabetic experimental rat models [7–9] and in clinical trials of diabetic patients [10, 11]. Besides, a number of vanadium compounds also present antitumoral properties in different systems [12–14]. On the other hand, toxic effects of vanadium are also well known [15–17] and this is the reason for the study of side effects and cytotoxicity of each new vanadium derivative with a potential pharmacological ability.

Recently, del Rio et al. [18] prepared an oxovanadium (IV) complex of oxodiacetic acid ($H_2oda{=}O(CH_2COOH)_2$) of stoichiometry $[VO(oda)(H_2O)_2]$, determined its crystal structure, and investigated its antiapoptotic activity in insulin-producing cells. The aim of the present study was to thoroughly investigate the general spectroscopic properties of this new complex and its effects on cell proliferation and differentiation in two osteoblast-like cell lines in culture. The culture model used in the present work involves MC3T3E1 cells (nontransformed osteoblasts derived from mouse calvaria) and UMR106 cells (tumoral osteoblasts derived from a rat osteosarcoma).

Materials and Methods

Reagents and Tissue Culture Materials Oxovanadium (IV) acetylacetonate were purchased from Fluka and oxodiacetic acid from Aldrich. Crystal violet, *p*-nitrophenylphosphate (*p*-NPP), glycine, $MgCl_2$, and all the other chemicals used in the biological activity experiments were of analytical grade from Sigma Chemical Co. Tissue culture materials were purchased from Corning (Princeton, NJ, USA) and Dulbecco's Modified Eagles Medium (DMEM) and DMEM low glucose and trypsin–EDTA from Gibco (Gaithersburg, MD, USA), and fetal bovine serum (FBS) from GibcoBRL (Life Technologies, Germany).

Synthesis of the Complex The complex $[VO(oda)(H_2O)_2]$ was prepared by reaction of 20 mmol (5.3 g) of oxovanadium (IV) acetylacetonate with 20 mmol (2.7 g) of oxodiacetic acid in 60 ml of distilled water. The mixture was heated at reflux during ca. 3 h. The hot solution was filtered and concentrated to half is volume over a water bath. After cooling, the complex crystallized in form of small blue crystals, which were collected by filtration, recrystallized from water, washed with cold acetone and diethyl ether, and finally dried in air [18]. The purity was confirmed by elemental chemical analysis (calc. for $C_4H_8O_8V$: C, 20.43; H, 3.40; and V, 21.68%. Found: C, 20.48; H, 3.52; V, and 21.50%).

Fresh stock solutions of the complex and of the free ligand were prepared in distilled water at a 100-mM concentration and diluted according to the experimental necessities.

Spectroscopic Characterization Electronic absorption spectra were measured with a Hewlett-Packard 8452 diode-array spectrophotometer, using 10-mm quartz cells. X-band (9.5 GHz) electron paramagnetic resonance (EPR) measurements were carried out on powders and diluted aqueous solutions using a Bruker ELEXSYS E580 spectrometer, equipped with an Oxford Cryosystems low-temperature device. The samples were put in quartz tubes (3 mm internal diameter), cooled at liquid nitrogen temperature, and then inserted into the microwave cavity.

Infrared spectra were obtained with a Bruker IFS 66 Fourier Transform Infrared (FTIR) instrument using the KBr pellet technique. Raman spectra were measured on powdered samples using the FRA 106 Raman accessory of the same FTIR instrument. Radiation of

1,064 nm from a Nd:YAG solid-state laser was used for excitation. Spectral resolution was ± 4 cm^{-1} for both spectral measurements.

Cell Culture MC3T3E1 osteoblastic cells derived from mouse calvaria and UMR106 tumoral cells derived from a rat osteosarcoma were grown in DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% (v/v) FBS at 37°C, 5% CO_2. When 70–80% confluence was reached, cells were subcultured using 0.1% trypsin 1 mM EDTA in Ca(II)/Mg(II)-free phosphate-buffered saline (PBS) (11 mM KH_2PO_4, 26 mM Na_2HPO_4, 115 mM NaCl, pH 7.4) [19, 20]. In the experiments, cells were grown in multiwell plates. When cells reached 70% confluence, the monolayers were washed twice with DMEM and then incubated in different conditions according to the experiments.

Cell Proliferation Assay A mitogenic bioassay was carried out as described by Okajima et al. [21], with some modifications. Briefly, cells were grown in 48-well plates. When cells reached 60% confluence, the monolayers were washed twice with serum-free DMEM and incubated with different concentrations of $[VO(oda)(H_2O)_2]$ (2.5–100 μM). Then, the monolayers were washed with PBS and fixed with 5% glutaraldehyde/PBS at room temperature for 10 min. After that they were stained with 0.5% crystal violet/25% methanol for 10 min. 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/well 0.1 M glycine/HCl buffer, pH 3.0/30% methanol and transferred to test tubes. Absorbance was read at 540 nm after a convenient sample dilution. We have previously shown that under these conditions, the colorimetric bioassay strongly correlated with cell proliferation measured by cell counting in Neubauer chamber [19, 20].

Cell Differentiation Assay Alkaline phosphatase-specific activity (ALP) has been used as a marker of osteoblast phenotype [5, 19, 20]. Cells were grown in 24-well plates until 70–80% confluence, and the monolayers were washed twice in serum-free DMEM. Then, the cells were incubated overnight with serum-free DMEM and different doses of $[VO(oda)(H_2O)_2]$ (2.5–100 μM). The cell layer was then washed with PBS and solubilized in 0.5 ml 0.1% Triton X-100. Aliquots of the total cell extract (10–20%) were used for protein determination by Bradford's technique [22]. Measurement of alkaline phosphatase activity was carried out by spectrophotometric determination of initial rates of hydrolysis of *p*-NPP to *p*-nitrophenol (*p*-NP) at 37°C for 10 min. The mixture of reaction was prepared as follows: 10 μl of cell extract in 800 μl of buffer glycine (55 mM glycine, 0.55 mM $MgCl_2$, pH 10.5). The reaction was initiated by addition of 100 μl of solution of substrate, 5 mM *p*-NPP in buffer glycine. The production of *p*-NP was determined by the absorbance at 405 nm. Under these experimental conditions the product formation was linear for 15 min.

Statistical Methods At least three independent experiments were performed for each experimental condition. Results are expressed as the mean±SEM. Statistical differences were analyzed using Student's *t* test.

Results and Discussion

Structural Characteristics of $[VO(oda)(H_2O)_2]$

The crystal structure of the complex was determined by single crystal x-ray diffractometry [18]. The coordination geometry around the metal center is a distorted octahedron. The two water

molecules are mutually *trans* whereas the oxodiacetate ligand is meridionally distributed, occupying three coordination positions through two carboxylate O atoms and the ethereal O atom, generating the interesting and unprecedented tridentate OOO coordination [18]. The remaining meridional position is occupied by the oxo-atom of the VO^{2+} cation. V–O distances ranged between 1.590(7) Å for the V=O bond, 2.053(3) Å for the water ligands, 2.016(3) Å for the carboxy group, and 2.180(6) Å for the ethereal O atom [18].

Electronic Absorption Spectrum of $[VO(oda)(H_2O)_2]$

The electronic absorption spectrum of the complex, measured in aqueous solution, shows d–d transitions at 618 nm (ε=12.5 l mol^{-1} cm^{-1}) and 792 nm (ε=28.9 l mol^{-1} cm^{-1}). A third, strongest, band is observed at 212 nm (ε=2393 l mol^{-1} cm^{-1}) with a shoulder at 240 nm (ε=870 l mol^{-1} cm^{-1}). This spectrum is in agreement with the simple M.O. model proposed by Ballhausen and Gray for the $[VO(H_2O)_5]^{2+}$ complex [23, 24]. In this model, the two lower energy bands are assigned to the $b_2 \rightarrow e$ (792 nm) and $b_2 \rightarrow b_1$ (618 nm) transitions, whereas the one found as a shoulder at 240 nm is probably the $b_2 \rightarrow a_1$ transition, partially overlapped with the stronger charge transfer band observed at 212 nm.

In the aquo complex, $[VO(H_2O)_5]^{2+}$, the first two d–d transitions are observed at 760 and 625 nm [23]. As the 10-Dq parameter can be directly determined from the position of the $b_2 \rightarrow b_1$ transition [23, 24], it is clear that the crystal field strength generated in the present case is comparable to that found in the aquo complex. This behavior suggests that the σ-donating properties of the oxodiacetate ligand are comparable to those of water.

EPR Spectrum of $[VO(oda)(H_2O)_2]$

The spectra were measured at 4.3 K and show a characteristic axial symmetry. When the sample is diluted in water the EPR signal is considerably broaden, showing the weakening of the spin–spin interaction responsible for the collapse of the hyperfine interaction that generate the typical multiplet of oxovanadium(IV) complexes arising from the spin electronic (S=1/2) and ^{51}V nuclear spin (I=7/2) interaction [25].

To obtain the best EPR parameters the spectrum was also simulated using the EasySpin package programs [26]. Determined g values were $g_\perp = 2.0070$, $g_\parallel = 1.9180$, and $\langle g \rangle = 1.9773$. These values are similar to those measured in a great number of VO^{2+} complexes [25, 27] and, as also in these cases, the anisotropy of the g values is relatively small and the average $\langle g \rangle$ value lies close to the free electron value, although slightly lower.

Vibrational Spectrum of $[VO(oda)(H_2O)_2]$

To analyze the IR and Raman spectra of the complex, we have also recorded the respective spectra of the free ligand under the same experimental conditions. The obtained spectral data, together with the proposed assignments, are shown in Table 1. The assignments are based on information provided by well-known standard reference texts [28, 29] and are briefly commented as follows:

– Typical vibrational frequencies of the "free" acid, such as the stretching vibrations of the OH group and of the C=O and C–O moieties of the carboxylate group, are found in the usual ranges for these motions. This is also true for the three vibrations related to the ethereal function (ν_{as}(C–O–C), ν_s(C–O–C), and δ(C–O–C)), from which the symmetric stretching mode is the strongest band in the Raman spectrum.

Table 1 Assignment of the Vibrational Spectra of Oxodiacetic Acid (H_2oda) and its Oxovanadium(IV) Complex, $[VO(oda)(H_2O)_2]$

$O(CH_2–COOH)_2$ (H_2oda)		$[VO(oda)(H_2O)_2]$		Assignments
IR	Raman	IR	Raman	
3032 vs, br				ν(OH)
		3057 vs, br		ν(OH)water
	2974 s		2989 s	$\nu_{as}(CH_2)$
2935 w	2937 vs	2898 sh	2924 s	$\nu_s(CH_2)$
2665vw,2559vw				(?)
2480 vw				(?)
1734 vs/1700 sh	1661 m			ν(C=O)
		1591 vs	1608 m	$\nu_{as}(COO^-)$
1468 w/1419 vs	1430 s	1468 m/1425 vs	1493 s/1402 s	$\delta(CH_2)$
1309 m/1248 vs	1309 m			δ(C–OH)
		1354 vs/1308 vs	1354 s/1307 m	$\nu_s(COO^-)$
1200 vs				ν(C–O)
1149 vs	1161 w	1138 vs	1142 m	ν_{as}(C–O–C)
1065 s/972 s	1060 w/970 m	1043 m		ν(CC)
		995 s	991 vs	ν(V=O)
914 vs				$\delta(OH^{\bullet\bullet\bullet}O)$
		947 m	959 s	ν(CC)
889 s	890 vs	891 w/843 s	900 w	ν_s(C–O–C)
	869 m			ν(CC)
726 s		735 s		ν(CC)
694 s/661 sh		681 w	666 m	$\rho(CH_2)$
		598 s/586 sh	588 m	
575 m/538 s	568 m			
522 s				
505 m	501 m	503 s	498 m	δ(C–O–C)
		422 s	406 m	ν(V–O)
	370 w			
			304 s/263 s	

vs = very strong, s = strong, m = medium, w = weak, vw = very weak, w = weak, br = broad, sh = shoulder

- The two stretching modes of the CH_2 groups could be clearly identified in the Raman spectrum of the acid, whereas only the symmetric one is detected as a weak IR shoulder at the low energy side of the strong and broad ν(OH) band. Also, bending vibrations related to this moiety could be identified in both the IR and Raman spectra. Some very weak IR bands found between 2480 and 2665 cm^{-1} are probably combination or overtone bands.
- In the case of the complex, the carboxylate groups show the typical spectroscopic behavior expected from a monodentate interaction of this moiety with the metallic center, i.e., the antisymmetric stretching vibration presents a lower energy than the original ν(C=O) vibration, whereas the symmetric mode lies somewhat higher than the ν(C–O) mode [30]. The doublet structure of this vibration in the complex suggests that both V-carboxylate bonds are not strictly equivalent. Besides, both carboxylate stretchings also show an energy difference (about 260 cm^{-1}), which is characteristic for monodentate binding [30].
- Regarding the motions of the ethereal O atom, the ν_{as}(C–O–C) vibration is clearly displaced to lower energies after interaction of its O atom with the metal center,

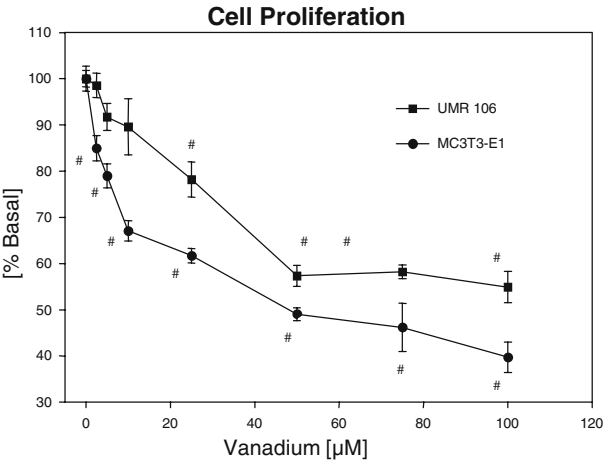

Fig. 1 Effect of $[VO(oda)(H_2O)_2]$ on MC3T3E1 and UMR106 osteoblast-like cell proliferation. Cells were incubated in serum-free DMEM alone (basal) or with different concentrations of the complex at 37°C for 24 h. Results are expressed as percent basal and represent the mean±SEM, $n=9$; # $p<0.001$

whereas the corresponding symmetric stretching and the deformational modes are less affected. Also, most of the CH_2 vibrations show only minor changes after coordination.

– The coordinated water presents a very strong and broad band related to the O–H stretching vibrations. The corresponding deformational modes are surely overlapped by the strong and broadened $\nu_{as}(COO^-)$ IR band centered at 1,591 cm^{-1}.
– The characteristic ν(V=O) breathing was easy to identify because it generates the strongest Raman band in the spectrum of the complex, and appears in the usually expected region [24]. The assignment of metal-to-ligand bands in the complex is more difficult and we have only tentatively identified one pair (422 cm^{-1} [IR], 406 cm^{-1} [Raman]).

Effect of $[VO(oda)(H_2O)_2]$ on the Osteoblast-like Cell Proliferation

Figure 1 shows the effect of different $[VO(oda)(H_2O)_2]$ concentrations on the proliferation of MC3T3E1 and UMR106 osteoblast-like cells, determined by the crystal violet bioassay.

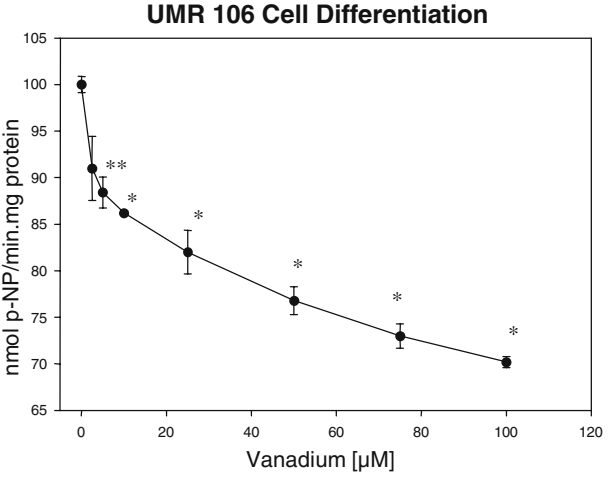

Fig. 2 Effect of $[VO(oda)(H_2O)_2]$ on osteoblast-like cell differentiation. UMR106 cells were incubated either in serum-free DMEM alone (basal) or with different concentrations of $[VO(oda)(H_2O)_2]$ at 37°C for 24 h. Basal activity of osteoblast alkaline phosphatase was 1.2 μmol pNP/min × mg protein. Results are expressed as percent basal and represent the mean±SEM, $n=9$; *$p<0.001$, **$p<0.002$

After a 24-h culture, the complex significantly inhibited UMR106 cell proliferation in the range of 2.5–100 μM ($p<0.001$). A stronger inhibitory effect was observed on the nontransformed MC3T3E1 line in which it was exerted in the whole range of tested concentrations (2.5–100 μM) ($p<0.001$). The vanadium complex caused cytotoxicity on both osteoblastic lines, although this effect was stronger in the MC3T3E1 cells than in the UMR106 line. Moreover, the free ligand did not exert any effect on neither cell lines in the whole range of tested concentrations. In previous works we have reported that oxovanadium(IV) cation stimulated UMR106 proliferation in the same range [19, 31]. In MC3T3E1 cells, the VO^{2+} cation was an inhibitory agent in the range of 10–100 μM.

Effect of $[VO(oda)(H_2O)_2]$ on the Osteoblastic Differentiation

The effect of the complex on osteoblast-like cell differentiation was assessed by ALP, as it was described under "Materials and Methods." As can be seen from Fig. 2, $[VO(oda)(H_2O)_2]$ significantly inhibited UMR106 cell differentiation in the range of 10–30% of basal ($p<0.001$). These results are in agreement with previous studies in which different oxovanadium(IV) compounds were inhibitors of the osteoblastic differentiation [19, 31]. Because the MC3T3E1 cells do not express measurable ALP levels after short culture periods (3 days) [32], the effect of $[VO(oda)(H_2O)_2]$ was not assessed in this cell line.

To conclude, $[VO(oda)(H_2O)_2]$ displayed a typical inhibitory effect on alkaline phosphatase specific activity, one of the marker of osteoblast differentiation. Moreover, it exerted a stronger inhibitory effect on nontransformed than in tumoral osteoblasts. These results point to the fact that the complex did not show promising properties to be further investigated as a possible antitumoral agent in this model of bone-related cells in culture.

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