In vitro Studies of Antitumor Activity of Vanadium Complexes with Orotic and Glutamic Acids

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Estudos in vitro da Atividade Antitumoral de Complexos de Vanádio com Ácidos Órotico e Glutâmico

Estudios *in vitro* de la Actividad Antitumoral de Complejos de Vanadio con Ácidos Orótico y Glutámico

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Abstract

Introduction: Three vanadium complexes with orotic and glutamic acids, in their anion forms, were prepared and their *in vitro* cytotoxicity toward human lung fibroblasts (MRC-5), human hepatocellular carcinoma (HepG2) and human colorectal adenocarcinoma (Caco-2) are reported. **Objective:** Describe the synthesis and characterization of new vanadium complexes with orotic and glutamic acids, and test its antitumor activity against HepG2 and Caco-2. **Method:** The complexes were formulated as VO (oro), VO (α-glu) and VO (γ-glu) based on chemical, thermogravimetric analyses and infrared spectra. **Results:** Resazurin assay demonstrates its cytotoxicity against the HepG2 and Caco-2 cell lines with the IC_{50} ranging from 7.90 to 44.56 μmol.L^{-1}. The cytotoxicity profiles indicate that the tumoral lines show more activity than the cells MRC-5, with selectivity indexes ranging from 1.58 to 8.96. **Conclusion:** The three complexes had better *in vitro* activity than cisplatin for both normal and cancer cell lines. The IC_{50} values are two to six times better for the cancer cell lines and five to seven times better for the normal cell lines. This study indicates that the complexes obtained are promising candidates for antitumor drugs.

Key words: Carcinoma, Hepatocellular; Vanadium Compounds; Drug Screening Assays, Antitumor.

Resumo

Introdução: Foram preparados três complexos de vanádio com ácidos orótico e glutâmico, em suas formas aniônicas, e foi testada sua citotoxicidade *in vitro* para fibroblastos pulmonares humanos (MRC-5), carcinoma hepatocelular humano (HepG2) e adenocarcinoma colorretal humano (Caco-2). **Objetivo:** Descrever a síntese e caracterização de novos complexos de vanádio com ácidos orótico e glutâmico e testar sua atividade antitumoral contra HepG2 e Caco-2. **Método:** Os complexos foram formulados como VO (oro), VO (α-glu) e VO (γ-glu) com base em análises químicas, termogravimétricas e espectros no infravermelho. **Resultados:** O ensaio de resazurina demonstrou sua citotoxicidade contra as linhagens celulares HepG2 e Caco-2 com o IC_{50} variando de 7,90 a 44,56 μmol.L^{-1}. Os perfis de citotoxicidade indicam que as linhas tumorais apresentam maior atividade que as células MRC-5, com índices de seletividade variando de 1,58 a 8,96. **Conclusão:** Os três complexos tiveram melhor atividade *in vitro* do que a cisplatina, tanto para linhagens celulares normais como cancerosas. Os valores de IC_{50} são de duas a seis vezes melhores para as linhagens celulares cancerosas e de cinco a sete vezes melhores para as linhagens celulares normais. Este estudo indica que os complexos obtidos são promissores candidatos a fármacos antitumorais.

Palavras-chave: Carcinoma Hepatocelular; Compostos de Vanádio; Ensaios de Seleção de Medicamentos Antitumorais.

Resumen

Introducción: Tres complejos de vanadio con ácidos orótico y glutámico, en sus formas aniónicas, fueram preparados. Su citotoxicidad *in vitro* hacia los fibroblastos pulmonares humanos (MRC-5), el carcinoma hepatocelular humano (HepG2) y el adenocarcinoma colorrectal humano (Caco-2) son reportados. **Objetivo:** Los principales objetivos de este trabajo son describir la síntesis y caracterización de nuevos complejos de vanadio con ácidos orótico y glutámico y probar su actividad antitumoral contra el HepG2 y el Caco-2. **Método:** Los complejos fueron formulados como VO (oro), VO (α-glu) y VO (γ-glu) basados en análisis químicos, termogravimétricos y espectros infrarrojos. El ensayo de resazurina demuestra su citotoxicidad contra las líneas celulares HepG2 y Caco-2 con el IC_{50} que van de 7,90 a 44,56 μmol.L^{-1}. Los perfiles de citotoxicidad indican que las líneas tumorales presentan mayor actividad que los MRC-5, con índices de selectividad que van de 1,58 a 8,96. **Conclusión:** Los tres complejos tuvieron mejor actividad *in vitro* que el cisplatino, tanto para líneas celulares normales como para líneas celulares cancerosas. Los valores del IC_{50} son de dos a seis veces mejores para las líneas celulares de cáncer y de cinco a siete veces mejores para las líneas celulares normales. Este estudio indica que los complejos obtenidos son candidatos prometedores para fármacos antitumorales.

Palabras clave: Carcinoma Hepatocelular; Compuestos de Vanadio; Ensayos de Selección de Medicamentos Antitumorales.

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INTRODUCTION

Since the discovery of cisplatin inhibition of cell division[1,2] and the evidence of antitumor activity of platinum complexes[3], a search for new complexes analogous to cisplatin that exhibit high selectivity and action against tumor cells started to be developed. Studies have been proving the effectiveness of cisplatin, which, when administered together with other approved drugs, has an efficient effect on metastatic testicular tumors[4-6], metastatic ovarian tumors[7-9], advanced bladder carcinoma[10], head and neck spinocell carcinomas[11,12], esophageal, small and non-small cell lung, breast, cervical, stomach, prostate cancers, Hodgkin's and non-Hodgkin's lymphomas, neuroblastoma, sarcomas, multiple myeloma, melanoma and mesothelioma[13].

Cisplatin inhibits DNA synthesis by producing inter- and intrafite cross-links. Syntheses of proteins and RNA are also inhibited, but on a smaller scale[14]. Residues of platinum complexes are slowly eliminated by the body with a half-life of five days or more. Platinum metal concentrations are high in liver, prostate and kidneys, low in bladder, muscles, testicles, pancreas and spleen, and lower in intestines, adrenals, heart, lungs, brain and cerebellum. The metal remains in the tissues for up to 180 days after the last administration. With the exception of intracerebral, platinum concentrations in tumors are generally lower than concentrations in the organ where the tumor is located[14]. Many cancers, however, respond initially to platinum treatment, but drug resistance often occurs if the tumor reappears. Resistance to cisplatin is associated with three molecular mechanisms: increased DNA repair, altered cellular accumulation, and increased drug inactivation[15].

Besides cisplatin, other platinum complexes currently in use for cancer treatment, which are approved by the US Food and Drug Administration (FDA), are carboplatin and oxaliplatin, and according to a review article from 2014, nedaplatin, lobaplatin, heptaplatin and satraplatin were under clinical trials in the United States[16].

In addition to the platinum complexes, other metal complexes have been used and some of them, such as vanadium[17,18], molybdenum[19], ruthenium, gold, palladium, silver and copper, among others[20], have been shown effective against cancer cells.

Vanadium is considered an essential element for many living organisms and biological processes, in which its complexes are involved in different oxidation states. Vanadium compounds, especially organic derivatives, have action proposed for the treatment of cancer, and other conditions, such as diabetes and diseases caused by parasites. Research on therapeutic properties of vanadium has considerably increased in the last 15 years, although they are not well understood. According to Pessoa et al.[21], complexes that have been reported to have potential action as antitumor agents are Metvan [$V^{IV}O$ (SO_4) $(4,7\text{-Mephen})_2$], a vanadocene dichloride [$(\eta^5\text{-Cp})_2V^{IV}Cl_2$], a V-cysteine complex, a semicarbazone derivative and [VO $(acac)_2$].

The main goals of this work are to describe the synthesis and characterization of new vanadium complexes with orotic and glutamic acids, and test its antitumor activity against human hepatocellular carcinoma (HepG2) and human colorectal adenocarcinoma (Caco-2).

This study is important to know more about vanadium chemistry and obtain less expensive metal complexes than the platinum ones currently used for cancer treatment, and to propose new candidates for antitumor drugs.

METHOD

Glutamic acid, orotic acid and vanadyl sulfate were purchased from Sigma Aldrich, Acros Organics and Alfa Aesar, respectively. All reagents were used as received without further purification.

The compounds were characterized using the following equipment:

Fourier-transform infrared spectroscopy (FTIR): Absorption spectrophotometer in the medium infrared region with Fourier transform – VERTEX 70 from Bruker. Detector: DLaTGS. Reading range: 4000 to 400 cm^{-1}. Accessory ATR (total reflectance attenuated).

CHNS/O Elemental analysis: CHNS/O Elemental Analyzer 2400 series II from Perkin Elmer. Analyses sample composition (CHNS) through combustion in pure oxygen medium.

Thermogravimetric analysis (TG/DTA): Seiko thermal analysis equipment. Model TG / DTA 6200. Conditions of analysis: synthetic air, heating rate of 10 °C per min and alumina crucible.

X-ray diffractometry: X-ray diffractometer SIEMENS, model D500, DIFFRAC PLUS XRD Commander. Cu-Kα radiation with scintillation detector and graphite monochromator. Analysis conditions: 2θ = 5-70°, 0.02° and t = 2 s.

The complexes were obtained by the direct reaction between the ligand and the metal salt solubilized in water or ethanol. Adjustment of the pH for deprotonation was processed using NaOH or KOH. The reactions were conducted under reflux and stirring.

Oxovanadium orotate, VO (oro), has been described in the literature but there were no studies of its antitumor behavior. The complex was prepared following the procedure described in the literature with slight

modifications, by mixing a solution of $VOSO_4 \cdot 3H_2O$ (1 mmol) in H_2O with a solution of orotic acid (2 mmol) in H_2O. The mixture was refluxed for 45 min and water was evaporated until a blue compound precipitated. The precipitate was then filtered off, washed with cold ethanol and dried in vacuum over P_2O_5[22]. Results for the CHN elemental analysis are similar to those reported in the literature[22], confirming composition of the complex with molecular formula $[VO(C_5H_4N_2O_4)_2]$, abbreviated as VO (oro). Experimental and calculated values: % C = 29.98(31.8), % H=2.17 (1.6) and % N=13.64 (14.9). Molar mass of the complex is: 377.0 $g.mol^{-1}$.

The oxovanadium glutamate complex, VO (α-glu), was obtained by the reaction between glutamic acid and NaOH, followed by the addition of $VOSO_4$. The obtained complex was a blue, poorly hygroscopic and water-soluble powder. The blue color of the compound is an indicative of the presence of VO^{2+} ions in its composition. Results of elemental analysis (exp. and calc.) for Na [VO (OH) $(C_5H_7NO_4)$] are: % C=22.52 (23.83), % H=3.42 (3.20), and % N=5.28 (5.23). These results are consistent with a 1:1 (M:L) complex with a Na^+ ion in γ-COO^-. Molar mass of the complex is: 252.0 $g.mol^{-1}$.

The oxovanadium glutamate complex, VO (γ-glu), obtained by the reaction between glutamic acid and NaOH, followed by the addition of $VOSO_4$, using a higher pH range. The complex obtained was a yellow, poorly hygroscopic and water-soluble powder. The yellow color of the compound is an indicative of the presence of VO^{3+} ions in its composition. Results of elemental analyses (exp. and calc.) for Na $[VO(OH)_2(C_5H_7NO_4)]$ are: % C= 22.32 (22.74), % H=3.37 (3.62), and % N=5.21 (5.28). These results are consistent with a 1:1 molar ratio (M:L) for the complex with a Na^+ ion in γ-COO^-. Molar mass of the complex is: 269.0 $g.mol^{-1}$.

Cell screening was performed using tumor and nontumor cell lines in order to compare the selectivity of the test substances. The following strains were obtained from the Laboratory of Mycobacteriology and the Laboratory of Mutagenesis of the Faculty of Pharmaceutical Sciences, São Paulo State University (UNESP). Cell lines were stored in liquid nitrogen.

HepG2 (ATCC HB-8065™), human hepatocellular carcinoma;

- Caco-2 (BCRJ # 0059), human colorectal adenocarcinoma;
- MRC-5 (ATCC® CCl-171™), normal human lung fibroblasts.

The strains were grown in DMEM medium (Dulbecco's modification of Eagle's medium – Sigma-Aldrich) and supplemented with 10% of fetal bovine serum (FBS-Gibco).

The cell lines were incubated under standard conditions (37 °C, 5% CO_2 atmosphere) of cell culture in 75 cm^2 culture bottles containing culture medium supplemented with FBS (Gibco). Cells were collected from the culture bottles using 3 mL 0.25% trypsin-EDTA (Gibco), centrifuged at 2000 rpm for 4 min and quantified in double chamber automatic cell counter (TC20 Automated Cell Counter Bio-RAD). Then, cells were seeded in 96-well microtiter plates with a cell concentration of 1.5×10^4 cells/well, which was incubated under conditions as described above for 24 h to allow cells adhesion. Solutions of the complexes were prepared to perform eight dilutions in microplates, in order to obtain concentrations from 0.97 to 125 μg mL^{-1}. The wells were prepared with cells without any treatment and wells with culture medium with sodium azide (7.81 to 1000 μg mL^{-1}) to conduct the negative and positive controls, respectively. A control of the redox reaction from the complexes was carried out in which the complexes and the resazurin solution were added. After the treatments, cells were again incubated for 24 h. Subsequently, 50 μL of 0.01% resazurin hydrochloride (Sigma) were added. The plates were again incubated for 1 to 4 h, according to the cell line. Fluorescence reading was performed on a microplate reader in excitation filters at wavelengths of 530.59 nm. The results were expressed by the IC_{50} according to the US Food and Drug Administration (FDA), which represents the required concentration of the drug capable of inhibiting 50% of cell growth.

All the results were obtained from three independent experiments and Anova followed by Tukey's test were used for statistical analysis.

RESULTS AND DISCUSSION

Characterization of the complex formed by the reaction between orotic acid $(C_5H_4N_2O_4)$ and vanadilsulfate $(VOSO_4 \cdot 3H_2O)$ occurred by analysis of the main bands of the IR spectra of the free ligand and the complex. The main change in the IR spectra that shows the formation of the complex is the displacement of the characteristic bands of the carboxylate group, which appears at 1700 cm^{-1} for the free orotate ligand and 1651 cm^{-1} for the complex[22].

Thermogravimetric analysis for VO (oro) showed V_2O_5 as the final residue. XDR of the residue obtained at 600 °C is compatible even with the low signal intensity, due to the low acquisition time used in the analysis, with the crystallographic data sheets of vanadium pentoxide.

Figure 1 shows the spectra of the VO (α-glu) complex and monosodium glutamate. In these spectra, ν (NH) bands between 3500 and 3000 cm^{-1} are observed. It is also possible to identify in this region the weak ν (OH)

band for the complex and for monosodium glutamate. In the region 1750-500 cm^{-1}, the main bands for the three compounds are showed, and the changes in frequencies can be observed in the region 1500-1100 cm^{-1}, where the absorptions of ν (COO^-) (for the salt and the complex) and ν (COOH) (for glutamic acid) groups occur.

The thermogravimetric analysis for VO (α-glu) shows mass losses and formation of V_2O_5 as the final residue with a mass equal to 39.97% of the initial mass. Calculating the percentage of vanadium in the final residue, a value of 21.85% was obtained. This value is close to the calculated one, equal to 19.55%, thus confirming the composition of the complex.

FTIR spectra for VO (α-glu) and monosodium glutamate were included in this article for comparison purposes (Figure 1). FTIR spectra for the other two complexes and their salts were not included here, since they are very similar to VO (α-glu) and its salt spectra. The same occurs with the respective acids.

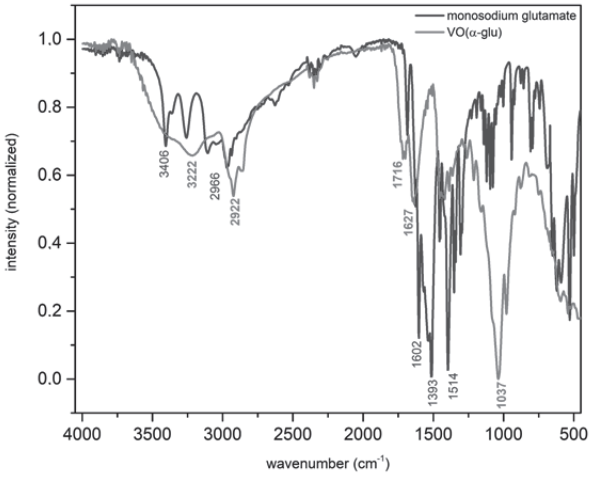
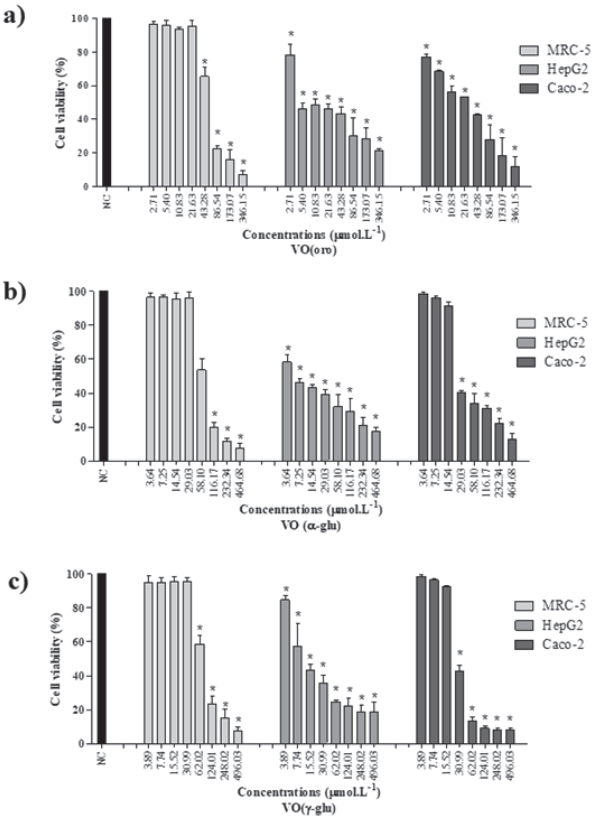


Figure 1. Absorption spectra in the IR region for VO(α-glu) and monosodium glutamate

The two cancer cell lines used in this work were chosen because they are easy to be purchased, widely cited in the literature showing good response when tested with cisplatin. As one of the objectives of this work was to compare the activity of vanadium complexes with cisplatin-based chemotherapeutic agents, cell lines that are easy to handle and readily available were chosen.

Analyzing the tests with VO (oro) and MRC-5, HepG2 and Caco-2 lines, it is possible to note that between the concentrations from 2.71 to 21.63 μmol.L^{-1} the complex has none activity against MRC-5 cells and induces cell death of Caco-2 and HepG2 at concentration of 5.40 μmol.L^{-1} (Figure 2a). For HepG2 cells, it is possible to observe the decrease in cell viability to values lower than 50% already at the concentration of 5.40 μmol.L^{-1}, while cell viability of the MRC-5 cells is not altered. For cells of the Caco-2 line between 2.71 μmol.L^{-1} and values lower than 43.28 μmol.L^{-1}, the cell viability values show a decrease ranging from 75 to 50%, and for 43.28 μmol.L^{-1} the value of cell viability is below 50%. For concentrations above 43.28 μmol.L^{-1} the action is similar in all strains, showing that at these concentrations death of all cell types (normal or cancerous) occurs. Such results are interesting to demonstrate the antitumor action of the VO (oro) complex and its potentiality. Some authors present the use of some vanadium salts and complexes for mimicking insulin[16,23].



NC: negative control (DMEM with 10% SBF - 100% cell viability); PC: positive control (sodium azide, 200 μg mL^{-1} - 27%, 18%, 22% cell viability of MRC-5, HepG2 and Caco-2 respectively). Results obtained from three independent experiments. *Statistically different from NC ($p < 0.05$, ANOVA, followed by Tukey's test).

Figure 2. Cell viability determined by the resazurin assay in MCR-5, HepG2 and Caco-2 cells treated for 24 h with different concentrations of (a) VO (oro), (b) VO (α-glu) and (c) VO (γ-glu)

The antitumor activity of VO (α-glu) in HepG2 cells is almost 15 times higher than the activity of the vanadium salt, $VOSO_4$ (Figure 2b). It is possible to observe that VO (α-glu) causes the death of 50% of Caco-2 cells at concentrations as low as 7.25 μmol.L^{-1}. Comparing with

cisplatin, this result is 15 times better (7.90 μmol.L^{-1} for VO (α-glu) and 120.9 μmol.L^{-1} for cisplatin) showing high selectivity index. For HepG2 cell line, VO (α-glu) IC_{50} is 29.03 μmol.L^{-1} and cisplatin IC_{50} is 15.9 μmol.L^{-1}, a two times better result when compared to VO (α-glu) and cisplatin.

For the VO (γ-glu) complex, IC_{50} was 13.32 and 27.77 μmol.L^{-1} for HepG2 and Caco-2 lines, respectively (Figure 2c). This complex presented the best IC_{50} value for the MRC-5 and good values for Caco-2 and HepG2.

For the Caco-2 line, VO (oro) presented the best antitumor activity with an IC_{50} value equal to 19.79 μmol.L^{-1}, followed by VO (γ-glu) with a value of 27.77 μmol.L^{-1} and by VO (α-glu) with a value of 44.56 μmol.L^{-1}. $VOSO_4$ presented an IC_{50} equal to 53.55 μmol.L^{-1} and therefore almost three times higher than the value for VO (oro) and almost twice as much as the value for VO (γ-glu) (Table 1).

Table 1 shows that IC_{50} values for cells of the normal line MRC-5 is larger than for the vanadium salt. This result indicates that the metal ions complexation with the proposed ligands caused greater selectivity and less cytotoxicity for normal cells. Concentrations to cause the death of 50% of the cells are at least two times higher for VO (oro) and three times higher for the other two glutamate complexes. When compared with cisplatin, the results of IC_{50} for the MRC-5 cell line show an excellent range of values from 54.69 to 73.72, 5 to seven times greater than the cisplatin value[24].

Taking into account the differences in the values, it is possible to propose a range of use for each complex. Since the cytotoxicity for tumor cells is higher than the cytotoxicity for normal cells, it is possible to calculate the selectivity index of each compound for each cell line, as shown in Table 2.

Table 1 shows the three complexes are efficient against liver HepG2, with IC_{50} values ranging from 7.90 for VO (α-glu), 13.32 for VO (oro) to 14.31 for VO (γ-glu). When compared to cisplatin complexes for other types of HepG2, whose IC_{50} ranges from 14.1 (HLE) to 673.5 (SNU-387), it can be observed that the three obtained complexes present an optimal performance, which can make them good candidates for cancer treatment in the future[24].

Table 2. Complex selectivity indexes for the cell lines tested

Selectivity indexes		
	HepG2	Caco-2
cisplatin	1.20	0.08
VO (α-glu)	8.96	1.58
VO (γ-glu)	5.15	2.65
VO (oro)	4.10	2.76

For human colorectal adenocarcinoma cells (Caco-2), the situation is slightly different, since the three IC_{50} value range from 19.79 for VO (oro), 27.77 for VO (γ -glu), to 44.56 for VO (α-glu). When compared to cisplatin complexes for other types of colon and rectum adenocarcinoma cells, whose IC_{50} ranges from 0.6 (SK-CO-1) to 15,153.6 (COLO-678)[27].

CONCLUSION

One complex of vanadium with orotic acid, VO (oro), and two complexes of vanadium with glutamic acid, VO (α-glu) and VO (γ-glu), were obtained. The three complexes showed selectivity indexes ranging from 1.5 to 9.0 and IC_{50} ranging from 7.90 to 44.56 μmol.L^{-1}. $VOSO_4$ has lower IC_{50} for normal cells and it is at least 1.5 times higher for tumor cells than the three complexes described in this work. These findings have provided new insights for the development of tumor selective vanadium (IV, V) complexes based anticancer drugs.

The three complexes had better *in vitro* activity than cisplatin for both normal and cancer cell lines. Values are two to seven times better and further investigation should be developed including *in vivo* tests. The IC_{50} values are two to six times better for the cancer cell lines and five to seven times better for the normal cell lines.

The major obstacle of using vanadium as a substitute for insulin is its toxic effects, such as hematological and biochemical changes, abnormalities in reproduction, and functional lesions in liver, kidneys, bones, spleen and

Table 1. IC_{50} for the three complexes, ligands and vanadium metal salt for the tested cell lines

IC50			
	MRC-5	HepG2	Caco-2
cisplatin	9.5[25]	15.9[26]	120.9 ± 7.0[27]
$VOSO_4 \cdot 3H_2O$	26.77 ± 0.70	110.78 ± 32.76	53.55 ± 3.50
VO (α-glu)	70.79 ± 7.50	7.90 ± 1.87	44.56 ± 4.21
VO (γ-glu)	73.72 ± 0.27	14.31 ± 0.05	27.77 ± 0.10
VO (oro)	54.69 ± 0.15	13.32 ± 0.04	19.79 ± 0.05
glutamic acid	> 6800	> 6800	> 6800
orotic acid	> 6400	> 6400	> 6400

leukocytes. In addition, it is important to highlight that platinum is a noble metal, rare in the Earth's crust, and is much more expensive than vanadium, which stimulates research on new complexes other than platinum for cancer treatment.

Since vanadium-based complexes have higher selectivity index and better *in vitro* results than cisplatin, it seems feasible and interesting to continue studies for *in vivo* test phases.

CONTRIBUTIONS

Antonio Carlos Massabni participated of the conception, methodology, investigation, wording, review and editing of the manuscript, resources and supervision. Flavia Aparecida Resende Nogueira participated of the methodology, investigation, wording, review and editing of the manuscript. Filipe Boccato Payolla participated of the investigation, wording, review and editing of the manuscript. Nadia Andrade Aleixo participated of the investigation. All the authors approved the final version of the manuscript.

CONFLICT OF INTEREST

We have no conflict of interest to declare.

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