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Acute Toxicity Evaluation of Non-Innocent Oxidovanadium(V) Schiff Base Complex

Lidiane M. A. Lima [1], Heide Murakami [2], D. Jackson Gaebler [2], Wagner E. Silva [1], Mônica F. Belian [1], Eduardo C. Lira [3,*] and Debbie C. Crans [2,4]

[1] Departamento de Química, Universidade Federal Rural de Pernambuco, Recife-PE 52171-900, Brazil; lidianelimaa@gmail.com (L.M.A.L.); wesdqr@gmail.com (W.E.S.); mfbelian@gmail.com (M.F.B.)
[2] Department of Chemistry, Colorado State University, Fort Collins, CO 80523, USA; heideam@gmail.com (H.M.); jackson.Gaebler@rams.colostate.edu (D.J.G.); debbie.crans@coloState.edu (D.C.C.)
[3] Centro de Biociências, Departamento de Fisiologia e Farmacologia, Universidade Federal de Pernambuco, Recife-PE 50670-901, Brazil
[4] Cell and Molecular Biology Program, Colorado State University, Fort Collins, CO 80513, USA
* Correspondence: eduardo.clira2@ufpe.br; Tel./Fax: +55-(81)-996368873

Abstract: The vanadium(V) complexes have been investigated as potential anticancer agents which makes it essential to evaluate their toxicity for safe use in the clinic. The large-scale synthesis and the acute oral toxicity in mice of the oxidovanadium(V) Schiff base catecholate complex, abbreviated as [VO(HSHED)dtb] containing a redox-active ligand with tridentate Schiff base (HSHED = N-(salicylide neaminato)-N′-(2-hydroxyethyl)-1,2-ethylenediamine) and dtb = 3,5-di-(t-butyl)catechol ligands were carried out. The body weight, food consumption, water intake as well biomarkers of liver and kidney toxicity of the [VO(HSHED)dtb] were compared to the precursors, sodium orthovanadate, and free ligand. The 10-fold scale-up synthesis of the oxidovanadium(V) complex resulting in the preparation of material in improved yield leading to 2–3 g (79%) material suitable for investigating the toxicity of vanadium complex. No evidence of toxicity was observed in animals when acutely exposed to a single dose of 300 mg/kg for 14 days. The toxicological results obtained with biochemical and hematological analyses did not show significant changes in kidney and liver parameters when compared with reference values. The low oral acute toxicity of the [VO(HSHED)dtb] is attributed to redox chemistry taking place under biological conditions combined with the hydrolytic stability of the oxidovanadium(V) complex. These results document the design of oxidovanadium(V) complexes that have low toxicity but still are antioxidant and anticancer agents.

Keywords: oxidovanadium(V); vanadium Schiff base coordination complex; low acute toxicity

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1. Introduction

A wide range of vanadium(IV) and (V) coordination complexes and salts display desirable biological effects such as antidiabetic and anticancer agents [1–19]. Vanadium coordination complexes such as bis(maltolato)oxidovanadium(IV) (BMOV) [5,9] and bis(allixinato)oxidovanadium(IV) ([$VO(alx)_2$]) (Figure 1) [20] have been found to result in glucose-lowering levels in streptozotocin (STZ)-induced rats [21–26]. Furthermore, vanadium Schiff base complexes such as dioxidovanadium(V)dipicolinate ([VO_2dipic]$^-$) [18,27,28] and V(V)-catecholate substituted complexes as [VO(HSHED)dtb] [19] and [VO(naph-L-Pheol-im)(8HQ)] [1] have demonstrated anticancer properties against human ovarian, prostate and brain cells as well as enhancing the effects of oncolytic viruses (Figure 1).

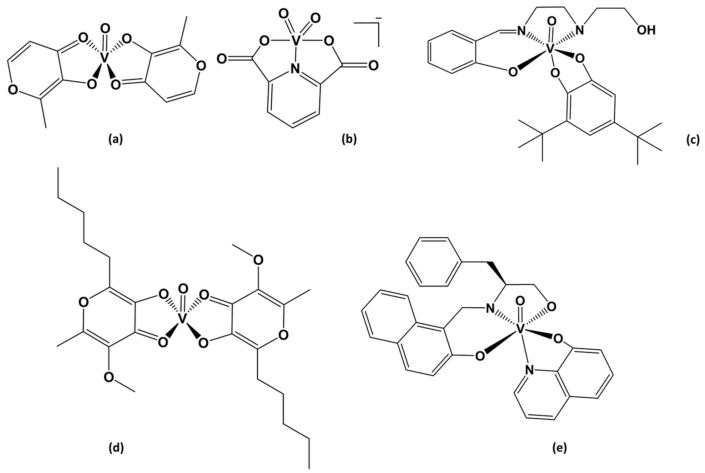


Figure 1. Structures of vanadium complexes with antidiabetic and/or anticancer properties, (**a**) bis(maltolato)oxidovanadium(IV) [BMOV], (**b**) dioxidovanadium(V)dipicolinate $[VO_2(dipic)]^-$, (**c**) V(V)-catecholate substituted [VO(HSHED)dtb] (Hshed = N-(salicylideneaminato)-N′-(2-hydr oxyethyl)-1,2-ethylenediamine and dtb = 3,5-di(t-butyl)catechol), (**d**) bis(allixinato) oxidovanadium(IV) $[VO(alx)_2]$, and (**e**) V(V)-Schiff base substituted [VO(naph-L-Pheol-im)(8HQ)] (L-pheol-im = L-phenylalaninol, 8HQ = hydroxyquinoline).

Due to the prospective application of various compounds as therapeutic agents, significant effort has been directed toward demonstrating that such compounds have no toxic effects in vivo and in vitro [29–31]. In the case of vanadium compounds, few studies have been carried out to determine the toxicity of the coordination complexes [32], although literature reports exist for vanadium salts and simple vanadium oxides [32–34]. It has been known that an excess of vanadate induces toxic effects in cells by oxidative stress increasing [35–37]. In vivo and in vitro studies show that high levels of reactive oxygen species are often implicated in vanadium deleterious effects [38–41]. However, the ability of V-complexes to inhibit protein phosphatases enhances their potential application as a therapeutic agent and has been an area of extensive research [42].

The presence of catechol-moieties is an established feature of leading anticancer agents used in the clinic including doxorubicin, daunorubicin, and mitomycin C (quinone-based compounds) [43–45]. Furthermore, the Schiff base vanadium complexes containing catecholates display a particularly interesting redox-chemistry in cell environments and have been investigated due to antidiabetic [46,47] and anticancer properties [19,48–53], however, there is no study with a focus on their toxicity. Thus, the evaluation of the toxicity of vanadium Schiff base catecholate complexes in vivo and in vitro is fundamentally important for potential medicinal applications [54].

In this manuscript, the focus is to evaluate the toxicity of the vanadium Schiff base di-t-butyl substituted catecholate complex—[VO(HSHED)dtb]—compared with the vanadate and the free catecholate ligand. The toxicological analysis was performed as recommended by the OECD (Organization for Economic Cooperation and Development) 423 guidelines characterizing the acute toxicity in mice.

2. Results

2.1. Synthesis of [VO(HSHED)dtb] Complex

Considering that the amount of material to carry out in vivo animal studies was 100 times greater than the scale of the vanadium catecholate previously prepared, it was necessary to scale up the reactions to prepare the target compounds for the biological studies. Furthermore, considering that V(V)-Schiff base vanadium complexes are non-innocent coordination complexes and thus redox-active, attempts to change the reaction scale was non-trivial. Impure side products were avoided maintaining the reaction under argon atmosphere and the solvent to reactant ratio was kept as that described in the

originally reported reaction [19,55,56]. Increasing the literature reported milligrams starting material reactions by a factor of about 7–10 but keeping the reactant–solvent ratios (1:70) constant, gram–scale of the target compound was obtained in each run. The major change to the scaled-up reactions was increasing the reaction time from 3 h to 48 h. This increased the amounts of products, from 200 mg to 2 g for each reaction. Interestingly, despite the scale-up of the reactions, the yields increased to 79% from the first reported 40% [55]. The improved yield is due to the longer reaction times in addition to minimizing oxidation by keeping the reaction under argon. ^{51}V NMR of the scaled-up reaction product was identical to the reported previously [55,56].

2.2. Stability of [VO(HSHED)dtb] Complex

Studies were previously carried out with [VO(HSHED)dtb] and were found to remain stable for few hours in cell culture media so the compound could be taken up by cells [19,56]. Even though the compound has low water solubility, it was found to be readily absorbed into cells from cell culture studies [56].

Furthermore, a low concentration was observed in membrane model studies. Although upon extended periods of time (5–24 h) contact with water will result in complex hydrolysis. The compound hydrolyzes to form starting materials, vanadate, catechol, Schiff base ligand which then degrades into salicylaldehyde and amine as reported previously [19,56]. However, the [VO(HSHED)dtb] has a longer lifetime than other Schiff base catecholate vanadium complexes and remains intact for a few hours before hydrolysis in various aqueous environments.

The experimental design called for administration of [VO(HSHED)dtb] by oral gavage, the concerns with compound solubility are less. We point to the fact the hydrophobic nature of this compound would increase the absorption of the complex once it was administered. Furthermore, if the solutions are freshly prepared and used immediately the amount of hydrolysis is minimized. That is, this mode of administration would allow delivery of compounds with low water solubility and lower stability because compounds would be delivered directly to the stomach of the animal.

To determine how much of the [VO(HSHED)dtb] was intact during the treatment, the UV-Vis (Ultraviolet-visible spectroscopy) under the conditions the compound was administered for the toxicological studies were investigated. In Figure 2, is shown the UV-Vis spectra of [VO(HSHED)dtb] dissolved in 5%, 10%, and 20% Tween 80 compared to a spectrum where the compounds were dissolved in DMSO and added to an aqueous solution. We carried out these studies as a function of time ranging from t = 0 (black curves) to t = 24 h (turquoise curves) at three different Tween 80 concentrations. As observed in Figure 2 all samples were sufficiently stable for the short periods of time needed for the administration, and importantly the addition of Tween 80 did not reduce the stability of the compounds significantly. However, significant amounts of compound decomposed after 24 h, particularly in the presence of the Tween 80. We conclude that Tween 80 destabilizes [VO(HSHED)dtb] but that over 24 h of time the presence of Tween 80 facilitates the complete hydrolysis and more rapidly than the control aqueous solution.

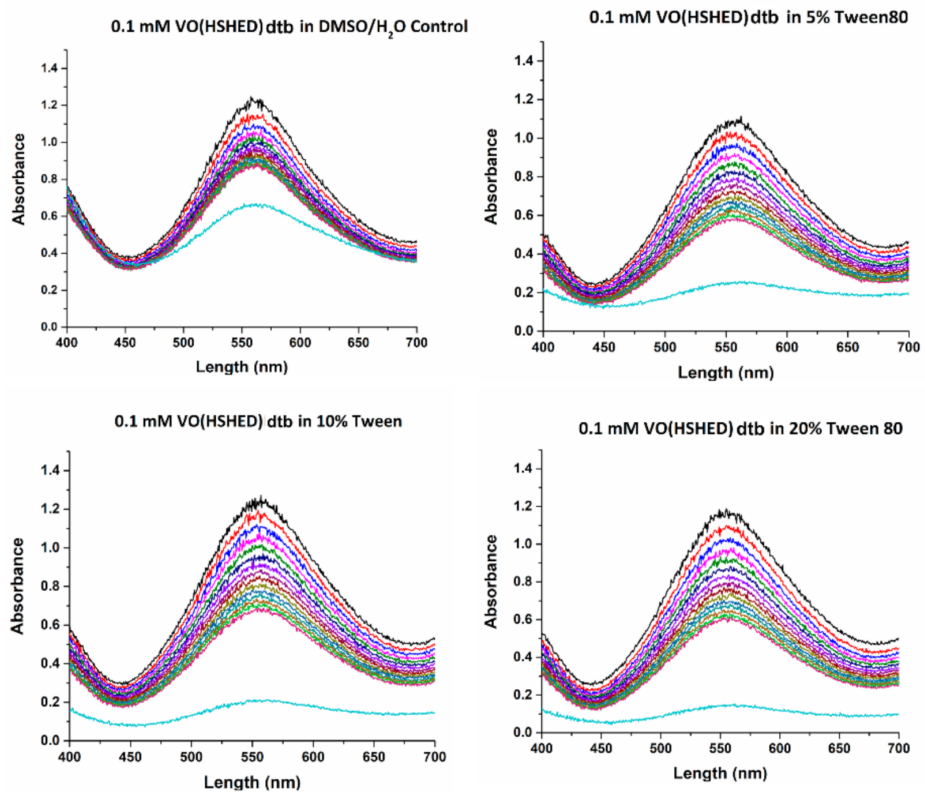


Figure 2. The UV-Vis spectra are shown of 0.1 mM [VO(HSHED)dtb] in DMSO, H_2O, and in 5%, 10%, and 20% Tween 80 at various time increments, from t = 0 h to t = 24 h.

Administration of compounds in animals is always subjected to the pharmacokinetic and ADME (Absorption, Distribution, Metabolism, and Excretion) as well as the pharmacodynamic, and as such it is of interest what forms of the compounds are present. However, since the compounds are rapidly distributed making concentrations low, and it is non-trivial to measure since this compound undergoes both hydrolytic and redox chemistry [19,56], methods are being developed to measure such different forms [57,58]. However, at present it seems pertinent and more profitable for the determination of whether the compound is toxic, to measure the impact on toxicity markers. Such an approach is particularly beneficial if these parameters are compared to marker formation in animals treated with the potentially toxic hydrolysis products, vanadate, and catecholate.

2.3. Acute Toxicity in Mice

Neither animal mortality, severe toxicity nor detrimental side effects were observed during the entire 14 days experimental period in all groups, except for the very high concentration (2000 mg/kg) of [VO(HSHED)dtb]. At the high dose administration of vanadium(V) Schiff base complex resulted in the death of all animals during the first 24 h, and as a result, it was not possible to collect the data on the 14th day. In contrast, no signs of toxicity were observed after the administration of [VO(HSHED)dtb) in a single dose of 300 mg/kg, i.e., no-observed adverse-effect level (NOAEL). Neither behavior alterations like lethargy, sleep, tremors, salivation, convulsion, nor common side effects for V-compounds, such as diarrhea, were observed in all groups for 14 days [59].

The acute toxicity was performed using OECD 423 guideline. The OECD is an international organization that works to build better policies for better lives, and they have developed protocols for animal studies [60]. This guideline allows the characterization of substances according to the Globally Harmonized System (GHS) for the classification of chemicals that cause acute toxicity. The classification of the test substance is based on the determination of the mortality dose(s), when there are no effects observed this concentration

will be the highest dose tested by gavage in a single dose. Thus, the compounds could be rated as categories 1 to 5, from the most to least toxic, according to the LD_{50} (median lethal dose) values in mg/kg b.w from each category [60]. [VO(HSHED)dtb] and 3,5-di(t-butyl)catechol was found to be category 4, with median lethal dose LD_{50} estimated to be between 300 mg/kg and 2000 mg/kg b.w. and orthovanadate is more toxic than both (category 3) with LD_{50} estimated to be between 50 mg/kg and 300 mg/kg b.w. Furthermore, the use of female *Swiss* mice was chosen in this study because literature surveys of conventional LD_{50} tests show that usually there is little difference in sensitivity between the sexes, but in these cases where differences are observed, females are generally slightly more sensitive [61].

2.4. Clinical Observations

The observations were made on the animals treated with the vanadium coordination complex, orthovanadate salt, the catechol ligand, and control group. Figure 3 shows that there is no statistical difference in body weight, food consumption, and water intake between the control treated with water and treated groups ($p > 0.05$) throughout 14 days and at the end of treatment (Table 1).

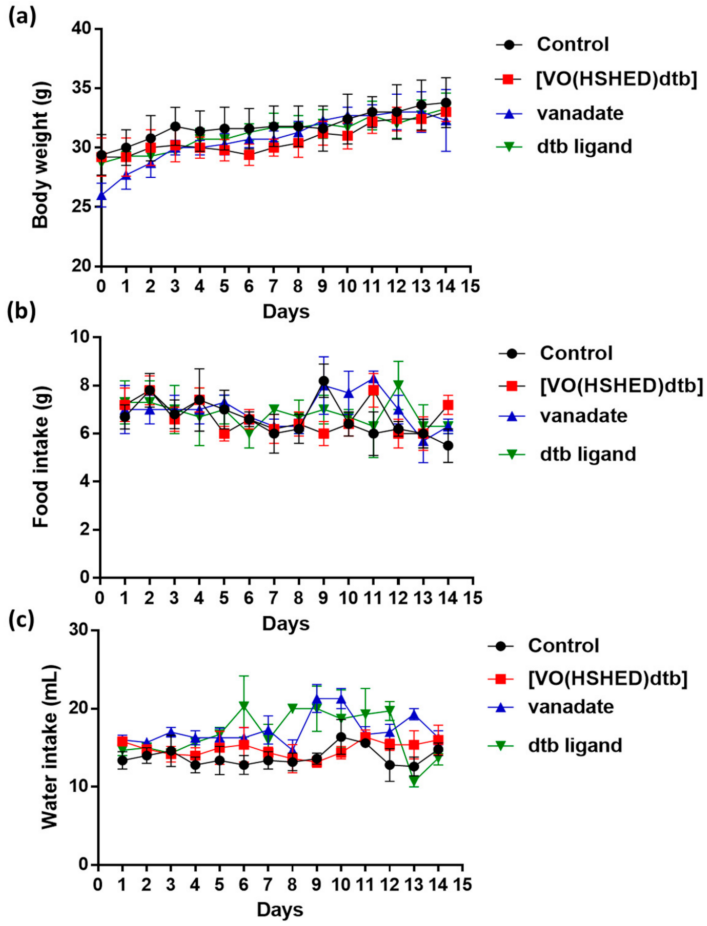


Figure 3. Effects on (**a**) body weight, (**b**) food intake, and (**c**) fluid intake throughout 14 days in mice administered acute levels of 300 mg/kg [VO(HSHED)dtb], 50 mg/kg orthovanadate and 300 mg/kg dtb ligand (3,5-di(t-butyl)catechol) groups by oral gavage. The data are presented as the mean ± SD ($n = 5$).

Table 1. Effects by acute oral administration of 300 mg/kg [VO(HSHED)dtb], 50 mg/kg orthovanadate, and 300 mg/kg dtb ligand, in different mice groups with regards to body weight, weight gain, food intake, and water intake at day 14.

	Study Group			
Parameter	**Control**	**[VO(HSHED)dtb]**	**Orthovanadate**	**3,5-di(t-butyl)catechol**
Body weight (g)	31.8 ± 1.8	30.7 ± 1.0	30.8 ± 0.9	31.2 ± 0.7
Weight gain (g)	0.3 ± 0.0	0.3 ± 0.1	0.2 0 ±.1	0.2 ± 0.1
Food intake (g/24 h)	6.8 ± 0.3	6.7 ± 0.3	7.0 ± 0.1	6.8 ± 0.3
Water intake (mL/24 h)	13.8 ± 0.8	15.0 ± 0.6	17.3 ± 0.5 *	16.7 ± 1.5

The data are presented as the mean ± SD (n = 5). Values are statistically significant at * $p < 0.05$ compared to the control group.

Once the [VO(HSHED)dtb] was found to cause no changes in clinical parameters monitored at the 300 mg/kg level, the biochemical and hematological parameters were monitored to investigate further toxic effects of the compounds as shown in Tables 2 and 3. Furthermore, the macroscopic analysis did not show any changes in tissue weight such as liver, kidney, heart, spleen, and lung in animals treated with 300 mg/kg [VO(HSHED)dtb] for the 14 days as shown by the data summarized in Table 4.

Table 2. Effects by acute oral administration of 300 mg/kg [VO(HSHED)dtb], 50 mg/kg orthovanadate, and 300 mg/kg dtb ligand in different mice groups with regard to AST/ALT ratio, albumin, total proteins, globulin, and A/G ratio parameters at day 14.

	Study Group			
Biochemical Parameters	**Control**	**[VO(HSHED)dtb]**	**Orthovanadate**	**3,5-di(t-butyl)catechol**
AST/ALT ratio	1.1 ± 0.01	0.9 ± 0.02	1.0 ± 0.01	1.1 ± 0.01
Albumin (g/dL)	2.2 ± 0.2	2.1 ± 0.1	2.1 ± 0.1	2.1 ± 0.1
Total proteins (g/dL)	5.7 ± 0.4	5.5 ± 0.2	6.5 ± 0.6	5.8 ± 0.3
Globulin (g/dL)	3.5 ± 0.3	3.4 ± 0.2	4.3 ± 0.5 *	3.8 ± 0.3
A/G ratio	0.6 ± 0.01	0.6 ± 0.01	0.5 ± 0.02	0.6 ± 0.02

The data are presented as the mean ± SD (n = 5). Values are statistically significant at * $p < 0.05$ compared to the control group.

Table 3. Effects by acute oral administration of 300 mg/kg [VO(HSHED)dtb], 50 mg/kg orthovanadate, and 300 mg/kg dtb ligand in different mice groups with regard to hematological parameters at day 14.

	Study Group			
Hematological Parameters	**Control**	**[VO(HSHED)dtb]**	**Orthovanadate**	**3,5-di(t-butyl)catechol**
Red blood cell (10^{12}/L)	9.5 ± 0.2	9.3 ± 0.2	8.9 ± 0.2	9.7 ± 0.3
Mean corpuscular volume (Fl)	57.6 ± 0.2	56.2 ± 0.8	57.0 ± 0.6	56.3 ± 0.3
Hemoglobin (g/dL)	12.4 ± 0.5	12.5 ± 0.1	12.3 ± 0.1	13.4 ± 0.2
Mean corpuscular hemoglobin (ρg)	13.5 ± 0.5	15.4 ± 2.0	13.7 ± 0.3	13.8 ± 0.1
mean corpuscular hemoglobin concentration (g/dL)	23.5 ± 0.6	23.9 ± 0.2	23.9 ± 0.2	24.4 ± 0.1
Hematocrit (%)	52.6 ± 0.9	52.5 ± 0.5	51.6 ± 0.8	55.0 ± 1.6
White blood cell (10^{9}/L)	12.6 ± 1.3	8.6 ± 1.3 *	12.7 ± 1.9	10.9 ± 1.1
Lymphocytes (%)	82.6 ± 0.8	81.8 ± 0.9	84.3 ± 1.5	83.2 ± 1.3

The data are presented as the mean ± SD (n = 5). Values are statistically significant at * $p < 0.05$ compared to the control group.

Table 4. Effects by acute oral administration of 300 mg/kg [VO(HSHED)dtb], 50 mg/kg orthovanadate, and 300 mg/kg dtb ligand in different mice groups with regard to the heart, kidney, liver, spleen, and lung weight at day 14.

	Study Group			
Organs (g/100 g b.w)	**Control**	**[VO(HSHED)dtb]**	**Orthovanadate**	**3,5-di(t-butyl)catechol**
Heart	0.54 ± 0.03	0.53 ± 0.02	0.59 ± 0.04	0.60 ± 0.07
Kidney	5.75 ± 0.29	6.23 ± 0.38	6.11 ± 0.11	5.50 ± 0.54
Liver	1.36 ± 0.10	1.52 ± 0.08	1.70 ± 0.20 *	1.61 ± 0.18
Spleen	0.48 ± 0.05	0.47 ± 0.03	0.66 ± 0.06 *	0.59 ± 0.13
Lung	0.88 ± 0.11	0.79 ± 0.03	0.86 ± 0.14	0.75 ± 0.15

The weight of body organs was measured in mice after 14 days of treatment. The data are presented as the mean ± SD (n = 5). Values are statistically significant at * $p < 0.05$ compared to the control group.

2.5. Hematology and Biochemical Analysis

Treatment with neither the dtb (3,5-di(t-butyl)catechol) ligand nor orthovanadate administration caused any alteration in biochemical and hematology parameters, in contrast to treatment with [VO(HSHED)dtb] which slightly decreased the WBC (white blood cell) level ($p < 0.05$) as shown in Table 3.

The alanine and aspartate aminotransferases (abbreviated ALT and AST, respectively) are commonly used as a marker in the diagnosis of liver injury and disease [62]. High levels of serum ALT activity reflects damage to hepatocyte and is considered to be a highly sensitive and fairly specific preclinical and clinical biomarker of hepatotoxicity [63]. In the present study, there were no significant changes in hepatic enzymes as shown in Figure 4, suggesting that the [VO(HSHED)dtb] did not cause any damage to the hepatic functions. In addition, hepatic-cellular damage leads to a reduction in albumin accompanied by a relative increase in globulins, which decreases the A/G ratio [64]. As shown in Table 2, no experimental significant difference between the control group and 300 mg/kg [VO(HSHED)dtb] ($p > 0.05$) at protein levels suggested the vanadium-catecholate complex did not cause hepatotoxicity in vivo.

As shown in Figure 4, no significant changes were observed in kidney biomarkers such as BUN (blood urea nitrogen) and creatinine which suggested that [VO(HSHED)dtb] did not cause nephrotoxicity [65]. However, the treatment with the free catechol ligand did increase significantly creatine levels ($p < 0.05$) documenting some toxicity is observed of the free ligand at 300 mg/kg single dose. These results provide evidence that the vanadium Schiff base catecholate complex does not exert toxicity in vivo up to therapeutic doses.

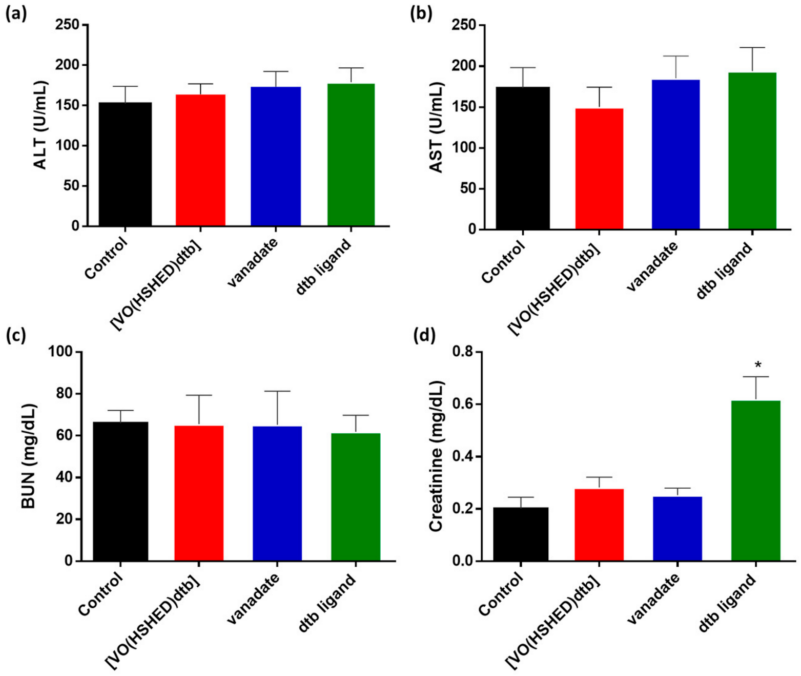


Figure 4. Effects by acute oral administration of 300 mg/kg [VO(HSHED)dtb], 50 mg/kg orthovanadate, and 300 mg/kg dtb ligand on mice groups with regard to biomarkers (**a**) ALT (Alanine aminotransferase), (**b**) AST (aspartate aminotransferase), (**c**) BUN (blood urea nitrogen), and (**d**) plasmatic creatinine levels on day 14. The data are presented as the mean ± SD (n = 5). Values are statistically significant at * $p < 0.05$ compared to the control group.

3. Discussion

The evaluation of the toxic effects of an oxidovanadium(V) Schiff base complex was carried out as a result of the recently discovered anticancer effects of this complex. This complex has anticancer activity against prostate, brain, and breast cancer cells [19,56], however, no previous toxicity studies of [VO(HSHED)dtb] in vivo have been reported. Previous toxicity studies were mainly carried out with simple salts and oxides, including metavanadate and orthovanadate, vanadyl sulfate, and vanadium pentoxide [32,66,67]. A few coordination complexes have also been investigated, and those include vanadium dipicolinate complexes [28,68]. This [VO(HSHED)dtb] complex was chosen because it is a non-innocent vanadium complex and thus can undergo redox chemistry both at the ligand and the metal site. This chemical reactivity is thus fundamentally different from the vanadium coordination compounds and salts that have previously been subjected to toxicity studies. Furthermore, the [VO(HSHED)dtb] compound deviates from these previous compounds in that it is more hydrolytically stable, and that results in it being significantly more readily taken up by cells. Finally, it was recently reported with anticancer properties that exceeded cisplatin [19].

In order to generate enough materials for toxicological studies, the preparation of the redox-active target compound was scaled up to a 2 g scale. The preparation of the compound was done without loss in yields. This was accomplished by keeping the reaction under argon, keeping the same substrate to solvent ratio (1:70) but increasing the reaction time. This approach saved much time in preparation of the 20-plus gram pure vanadium complex needed for the animal studies and suggests that although these compounds are not trivial to prepare there is potential for scale-up.

The toxicity of vanadium(IV) and (V) compounds with antidiabetic effects have been evaluated after acute parenteral administration but the effects are less compared to oral administration [69]. This is presumably because the compound is poorly absorbed by the gastrointestinal tract [9,69]. Nevertheless, attempts have been made to develop vanadium derivatives with biological effects and low toxicity like the coordination complex

[bis(maltolato)oxidovanadium(IV)] (BMOV) [5,70]. This complex has high lipophilicity and was selected for clinical trials where it demonstrated glucose-lowering effects in animals and humans [5]. Similarly, as reported previously, the hydrophobic nature of this [VO(HSHED)dtb] would enhance the absorption of the complex and exert its anticarcinogenic effects [56].

Acute toxicity evaluation of [VO(HSHED)dtb] using a dose of 300 mg/kg did not induce mortality or symptoms of severe toxicity throughout the period of the experiment. Administration of oxidovanadium(V) Schiff base complex did not affect hematological indices, hepatic, and renal biomarkers levels. Furthermore, the most common side-effects of vanadium treatment, such as weight loss and diarrhea [59], were not observed after acute exposure to a dosage of 300 mg/kg [VO(HSHED)dtb] for 14 days.

Vanadium compounds are known to often convert to other species upon administration in biological environments [71]. The toxicity of the [VO(HSHED)dtb] should be determined and compared to the effects of its components, i.e., free ligand catechol, Schiff base, and vanadate before applications as a therapeutic compound [31,72–74]. The hydrolysis of the complex and the Schiff base generates the salicylaldehyde and the amine [19,56]. Since previous works have reported that simple aldehyde and amine-based compounds are safe and used for designing new drugs [75,76], accordingly, to investigate the most toxic component of the ligands we focused on catechols and designed an experiment investigating the toxicity of the catechol.

Although the parent catechol is known to show some toxicity, the 3,5-di(t-butyl)catechol is much less toxic with an LD_{50} value in mice of 1.040 g/kg b.w. by OG (oral gavage) [77]. However, our results showed a slight increase of creatinine levels in animals treated with 3,5-di(t-butyl) catechol suggesting nephrotoxicity. Thus, both [VO(HSHED)dtb] and 3,5-di(t-butyl)catechol were rated at the same toxicity level according to the Globally Harmonized System (GHS) with LD_{50} values between 300 and 2000 mg/kg.

The sodium orthovanadate salt has been reported to cause insulin-mimetic activity [9,10], reduction in glucose levels in STZ-diabetic animals [9,10] and be potent phosphatase inhibitors [2,3], and previous toxic studies showed LD_{50} values in rats of 36.3 mg/kg and 330 mg/kg [74]. Similar experiments with $NaVO_3$ showed LD_{50} values in mice of 74.6 mg/kg and 35.9 mg/kg for oral and intraperitoneal (i.p) administration, respectively [32,34,78]. The toxicity of the [VO(HSHED)dtb] was found to be lower than vanadate. This result confirmed some previous reports that have shown the vanadium will be less toxic when coordinated to a ligand [5–8,79]. However, other complexes show similar effects between the vanadium coordination complex and salt which is explained the vanadium compound readily hydrolyze under physiological conditions [71].

Attributing the biological effects to coordination complexes is less trivial than simple organic compounds, because depending on the metal complex and the conditions such complexes may undergo ligand exchange, hydrolysis, or formation of oxidation products [80]. Therefore, it is important that the stability of the compounds under investigation be determined under the conditions of the biological system [71,80–83]. In this study, the stability of the [VO(HSHED)dtb] in the solutions used for oral gavage treatments was characterized. Since [VO(HSHED)dtb] has limited solubility aqueous solution and to be able to solubilize enough for oral gavage administration, 10% Tween 80 was used. Accordingly, we measured out the stability of [VO(HSHED)dtb] in 5%, 10%, and 20% Tween, and we found that the Tween barely affected the stability of [VO(HSHED)dtb]. The toxicity results we obtained showed that the [VO(HSHED)dtb] was less toxic, and this is consistent with the interpretation that the compound was intact for some time after administration.

Vanadium compounds undergo redox chemistry under physiological conditions, can act as a strong pro-oxidant, and interact synergistically with other oxidants enhancing oxidative stress [84,85]. These findings are consistent with redox chemistry taking place under biological conditions and may impact the low toxicity properties of the [VO(HSHED)dtb]. These results are very encouraging because they demonstrate that low toxicity of a vanadium complex is possible and importantly lower toxicity than the salt ($H_2VO_4^-$) and hence

lend support for future potential applications on cancer therapy. These findings suggest that it will be possible to design vanadium complexes that have even lower toxicity even when containing redox-active components.

4. Material and Methods

4.1. Reagents and Chemical Analysis

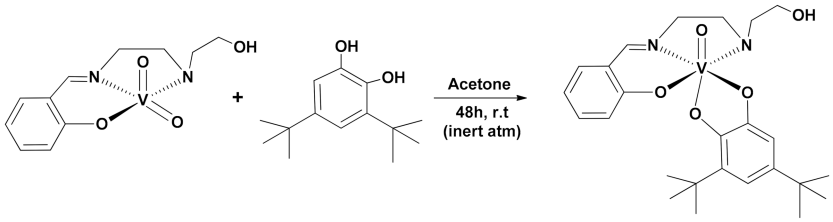
Catechol, 3,5-di(t-butyl)catechol, salicylaldehyde, N-(2-hydroxyethyl) ethylenediamine, vanadyl sulfate were purchased from Sigma Aldrich. Chemicals were used without purification. HPLC (high performance liquid chromatography) grade solvents were used for the synthesis and characterization of the oxidovanadium(V) complex. The compounds were characterized in the Colorado State Central Instrumentation Facility. Nuclear Magnetic Resonance (NMR) measurements were performed using a Bruker spectrometer at 78.9 MHz for ^{51}V and 400.13 MHz for ^{1}H, using 4096 scans and a window from −53 to −1043 ppm as reported previously [86–88]. V chemical shifts were measured in parts per million from $VOCl_3$ as external standard at 0.00 ppm with upfield shifts considered negative.

4.2. Synthesis of [VO_2(HSHED)] Complex

The precursor [VO_2(HSHED)] complex was synthesized using a condensation reaction between salicylaldehyde and N-(2-hydroxyethyl)ethylenediamine followed by a coupling reaction to vanadyl sulfate using previously reported methods [89,90]. The NMR spectra have shown the complex to have ^{51}V NMR chemical shifts consistent with the literature (−529 ppm vs. $VOCl_3$) [89].

4.3. Synthesis of [VO(HSHED)dtb] Complex

To synthesize [VO(HSHED)dtb] complex, the 3,5-di(t-butyl)catechol (1.11 g, 5.00 mmol) was added to a solution of [VO_2(HSHED)] (1.45 g, 5.00 mmol) and stirred in 350 mL of acetone for 48 h under an argon atmosphere (Scheme 1) [89,90]. A dark purple solution formed after 10 min. After 48 h, the solution was concentrated to dryness, and then the solution was vacuum filtered. A minimal amount of acetone (27 mL) was used to dissolve the crude product, followed by the addition of 300 mL of hexane. The solution was left to precipitate overnight at −20 °C. The precipitate was filtered, washed with cold hexane (75 mL), and dried on a pump for 4 days. Yield 1.90 g (79%). The solid has similar characteristics as reported [VO(HSHED)dtb] [19,89,91].



Scheme 1. Synthesis of [VO(HSHED)dtb] using precursor [VO_2(HSHED)] complex and 3,5-di(t-butyl) catechol.

4.4. Stability of [VO(HSHED)dtb] Complex

The stability of the [VO(HSHED)dtb] was measured using UV-Vis spectroscopy. [VO(HSHED)dtb] was dissolved in DMSO and added to an aqueous solution at the final concentration of 0.1 mM. The UV-Vis spectra were recorded at t = 0 h, and every 15 s following, and then again at 24 h. Corresponding experiments were done with [VO(HSHED)dtb] dissolved in 5%, 10% and 20% Tween 80 to a final concentration of 0.1 mM.

4.5. Animals

The protocols for these experiments were approved by the Animal Ethics Committee of the Universidade Federal de Pernambuco (process number #004-2019) and conducted in accordance with the Ethical Principles in Animal Research. Twenty-five female Swiss mice (30 ± 5 g) were obtained from the Laboratory of the immunopathology of Keizo Asami (LIKA) at Universidade Federal de Pernambuco (UFPE) and the animal experiments were performed at the Laboratory of Neuroendocrinology and Metabolism at UFPE. The mice were housed in individual cages with a 1–12 light-dark cycle, 22 ± 3 °C, and were given free access to water and conventional lab chow diet ad libitum (Purina, Labina®, Ribeirão Preto, Brazil) during the study period. All experiments were performed between 8:00 and 10:00 am.

4.6. Acute Toxicity in Mice

Acute oral toxicity effect of [VO(HSHED)dtb] was performed in accordance with the Organization for Economic Cooperation and Development (OECD) [60]. After 5 days of acclimatization in individual cages, non-pregnant and nulliparous female mice fasted overnight before the administration of compounds. The [VO(HSHED)dtb] was administered by gavage in mice at a single dose using animal feeding needles (100 µL/100 g b.w). The [VO(HSHED)dtb] was dissolved in warm water (50 °C) with 10 µL of 10% the surfactant Tween 80 (v/v) because of its poor solubility in water. Animals were randomly divided into 5 groups with 5 animals each: (a) negative control group which received only vehicle (distilled water), (b) group treated with low dose 300 mg/kg of [VO(HSHED)dtb], (c) group treated with high dose 2000 mg/kg of [VO(HSHED)dtb], (d) positive control group treated with 50 mg/kg of sodium orthovanadate, and (e) free ligand group treated with 300 mg/kg of 3,5-di(t-butyl)catechol.

Since there is no toxicological information about [VO(HSHED)dtb], in accordance with OECD 423 [60], [VO(HSHED)dtb] was evaluated at 2 doses and vanadate and free ligand groups were used in single dose because acute toxicity tests have been previously described in the literature [74,77]. After the administration period, animals were observed for mortality and clinical symptoms of toxicity daily for 14 days. The symptoms of toxicity analyzed were alterations on skin and eyes, mucous membrane toxic effects and behavior patterns, lethargy, sleep, diarrhea, tremors, salivation, convulsion, coma, motor activity, hypo-activity, abdominal rigidity, breathing difficulty, cyanosis, and death following the Hippocratic screening protocol. The consumption of water and feed, as well as the body weight of each animal, were recorded daily. In addition, animals were daily observed for general health conditions and clinical evidence of toxicity [92]. On the 14th day of experiment, fasting mice were anesthetized using ketamine (90% b.w) and xilazin (10% b.w), and the blood was drawn from the retro-orbital route with or without heparin for hematological and serum biochemical analysis, respectively. The following organs were removed, cleaned, weighed on an analytical scale, and macroscopically analyzed: liver, kidney, spleen, heart, and lung [93].

4.7. Biochemical Analysis: Determination of Serum Biomarkers for Liver and Kidney Functions

The blood samples on the 14th day were collected in dry tubes, were centrifuged (3000 rpm, 15 min) and the obtained serums were analyzed for liver and kidney functions by colorimetric assay using commercial kits (Lab Test Diagnostic SA, Santa Lagoa, Brazil). Optical densities were measured by spectrophotometry (Varioskan TM Lux multimode microplate reader, Thermo Scientific®, Waltham, MA, USA) at wavelengths specific for each biochemical parameter described on datasheets [93]. Baseline measurements were obtained by comparing the optical densities of the samples with the respective standards, available in the kits. Biochemical analysis was performed for determining the following biomarkers parameters: alanine (ALT) and aspartate aminotransferase (AST), total protein (TP), albumin (ALB), AST/ALT and albumin/globulin ratio (A/G), blood urea nitrogen (BUN), and creatinine (CRE). Globulin was obtained from the difference between total

protein and albumin [94]. Data were expressed by U/mL (AST and ALT) and mg/dL for the others.

4.8. Hematology Analysis

Hematological parameters included red blood cells (RBC), mean corpuscular volume (MCV), hemoglobin (Hb), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), hematocrit (HCT), white blood cells (WBC), lymphocytes (LYM) were performed using a multiparameter hematology analyzer (The Sysmex® XE-2100D, Sysmex®, Curitiba, Brazil) designed for hematology testing samples with ethylenediaminetetraacetic acid (EDTA).

4.9. Statistical Analysis

In vivo data was expressed as mean ± standard deviation (SD). The one-way analysis of variance (ANOVA) was employed to analyze the data between treated groups and their respective control groups followed. Tukey's Multiple Comparison Test was used to analyze the statistical comparisons. The p values less than 0.05 were considered statistically significant among the groups. Graph Pad Prism® (GraphPad Software, San Diego, CA, USA), version 5.0 software was used for all statistical analysis.

5. Conclusions

In summary, the administration of [VO(HSHED)dtb] complex in mice did not show any signs of toxicity up to a dose of 300 mg/kg. The complex was found to be less toxic than orthovanadate salt consistent with the compound being at least partially intact during the administration. The hematology, liver, and kidney biomarkers parameters demonstrate that the vanadium Schiff base complex exerts neither hepatotoxicity nor nephrotoxicity in mice. Although this compound contains both vanadium and a catechol which individually are known to be redox-active, and exert some toxicity, administration of this complex was tolerated at a low level (0.6 mol L^{-1}, 300 mg/kg). It was found to be toxic at a high level (4.0 mol L^{-1}, 2000 mg/kg) giving it an estimated LD_{50} between 300 and 2000 mg/kg, the latter being significantly higher than the usual therapeutic doses.

The low toxicity is attributed to the redox properties obtained when combining the redox-active ligand 3,5-di(tert-butyl)catechol with the hydrolytic stability of the [VO(HSHED)dtb], which prevent the formation of the vanadate and catechol ligand. The stability test in aqueous media with Tween 80 demonstrated that although the complex may not be stable in an aqueous solution for more than a few hours, this stability is not changed significantly in the presence of Tween 80 used for the administration of the compound. Hence, the compound stability is sufficient for the complex to exerts its action. Therefore, these studies are very encouraging and demonstrate that a vanadium compound such as the [VO(HSHED)dtb] complex does not exert toxicity to the same degree as vanadate even in a complex with a ligand that has potential for toxicity.

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Abbreviations

ADME	Absorption, Distribution, Metabolism and Excretion
ALB	albumin
ALT	alanine aminotransferase
Alx	allixinate
ANOVA	one-way analysis of variance
AST	aspartate aminotransferase
BMOV	bis(maltolato)oxidovanadium(IV)
BUN	blood urea nitrogen
CRE	creatinine
Dipic	dipicolinate
Dtb	3,5-di(t-butyl)catechol
EDTA	ethylenediamine tetraacetic acid
GHS	Globally Harmonized System
GLB	globulin
Hb	hemoglobin
HCT	hematocrit
HPLC	high performance liquid chromatography
i.p	intraperitoneal administration
LD_{50}	median lethal dose
LIKA	Laboratory of the immunopathology of Keizo Asami
L-Pheol-im	L-phenylalaninol
LYM	lymphocytes
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration
NMR	nuclear magnetic ressonance
NOAEL	no-observed adverse-effect level
O.G	oral gavage
OECD	Organization for Economic Cooperation and Development
RBC	red blood cells
Rpm	rotation per minute
SD	standard deviation
STZ	streptozotocin
TP	total protein
UV-Vis	Ultraviolet-visible spectroscopy
WBC	white blood cells
8HQ	hydroxyquinoline

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