Tyrosinase and Carbonic Anhydrase Enzymes Inhibition Studies of Vanadium(V) Complexes

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Summary: Present study endeavors synthesis of series of vanadium(V) hydrazide complexes and its enzyme inhibition studies. Octahedral structure of complexes has been evaluated previously using conductance measurements, spectroscopic techniques involving IR, ^{1}H-NMR and ^{13}C-NMR, elemental analysis using CHN technique. Complexes **1c-12c** have found to exhibit monomeric form with hydrazides behaving as bidentate ligand coordinating by their N and O atoms, while two oxygen atoms have also been found to show attachment with the metal centre. This study includes activity of vanadium(V) complexes to inhibit tyrosinase and carbonic anhydrase enzymes. For inhibition of carbonic anhydrase all, while for tyrosinase most of the hydrazide ligands were found to be inactive. Vanadium(V) complexes with these hydrazides have found to bear variable degree of carbonic anhydrase and tyrosinase inhibition activity. Some of the vanadium(V) hydrazide complexes were found to be potent inhibitors of tyrosinase enzyme and carbonic anhydrase as well.

Keywords: Vanadium(V) Hydrazide Complex, Enzyme Inhibition, Tyrosinase, Carbonic Anhydrase.

Introduction

In last two decades vanadium coordination chemistry has grasp the interest of chemists due to its importance from catalysis and medicinal point of view [1-3]. Oxo compounds of vanadium (IV) and (V) show insulin-mimetic activity [4, 5].

Research over the years revealed the presence of vanadium in both the terrestrial and aquatic environments in biological systems [6]. It is also found to be active in vanadium dependent haloperoxidase and nitrogenase enzymes [7]. A number of vanadium species are found to be effective as external cofactors, while inhibits enzymes (lipoprotein lipase, glyceraldehyde-3-phosphate dehydrogenase, tyrosine phosphorylase, glucose-6-phosphate dehydrogenase, adenylate cyclase, cytochrome oxidase and glycogen synthase) function and stimulates the function of (H^+/K^+-ATPase, phosphofructokinase, myosin ATPase, dynein, Na^+-K^+-ATPase, adenylate kinase and choline esterase) [8, 9]. Vanadium in two of its oxidation states *i.e.* V(IV) and V(V) is of great interest biologically as it is found in form of anionic as well as cationic complexes at physiological pH range (pH 2-8). These oxidation states are physiologically relevant and *in vivo* found to show inter conversion into each other and distribution in intra-cellular and extra-cellular fluids.

Generally hydrazides (R-CO-NH-NH_2) are organic compounds possessing activity as antifungal, antitumoral and antibacterial agents and thus considered as biologically active. This activity originates the interest for their search. Isonicotinic acid hydrazide is used for prevention and treatment of mycobacterium tuberculosis as first-line medication [10-12]. Hydrazides readily coordinate with the transition metals to form complexes and this capability is used to enhance their biological activity as their activity increases after complexation with transition metals [10-13].

Tyrosinase, which is Cu-containing enzyme, belongs to monooxygenase enzyme family. A series of enzymatic as well as non enzymatic reactions is involved in the formation of melanin pigments [14, 15], where tyrosinase enzyme acts as a catalyst. Activity of tyrosinase, as producer of dopamine which is used in Parkinson's disease treatment, makes it viable to be applicable in pharmaceutical industry. In melanoma patients it has also been recognized as marker [16] and prodrugs activation, it serves as a target [17]. Food proteins can be modified using cross-linking effects of tyrosinase giving rise to its worth for food industry [15, 18-20].

Hazardous radiations (UV, X-ray and gamma ray) can be prevented by using synthetic

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melanin which is also used for antioxidants, cation exchangers, drug carriers, antiviral agents. Thus the tyrosinase enzyme shows a versatile potential to be applicable in agricultural, food and medicine industry and for environmental and analytical purposes as well [21-23].

CO_2 is a vital component of living organisms. Trapping of CO_2 in cell is caused by its conversion into HCO_3^-. The process of interconversion of CO_2 and HCO_3^- is slow at physiological pH ranges. It gives rise to the need of an enzyme to speed up the reaction such as carbonic anhydrase (Zn-containing enzyme), catalyzing dehydration of HCO_3^- and hydration of CO_2. Carbonic anhydrase (CA) reversibly catalyses reaction of formation of carbonic acid [24]. Carbonic acid is formed by combination of CO_2 with the water, in tissues acquiring higher concentration of CO_2 and exists in form of HCO_3^-, H^+ in the living body. This H_2CO_3 again dissociates into CO_2 and H_2O again in lungs where the concentration of CO_2 is low [25]. CA has been considered to be the diagnostic marker of tumor. A number of compounds have been identified as activators or inhibitors of CA activity [26-31]. In the treatment of different diseases such as obesity, cancer and glaucoma, CA inhibitors are emerging to be used as remedy [26].Glaucoma is treated by the drugs such as dichlorphenamide, methazolamide, and acetazolamide which target the enzyme carbonic anhydrase [25].

CA isozymes are also ascribed to a wide range of diseases like obesity, edema, osteoporosis, glaucoma, epilepsy, duodenal and gastric ulcers. CA inhibitors are designed with clinical applications to target the activity of CA isozymes and treat such diseases. Among the inhibitors, the unsubstituted sulfonamides and the metal-coordinating anions constitute two main classes [32]. Sulfonamides, the most powerful CAIs, bind to the enzymatic Zn^{+2} either by substitution (by replacement of non-protein ligand *i.e.* H_2O or OH^-) or addition reaction (addition to coordination sphere forming trigonal-bipyramidal geometry). The thiocyanate binds to Zn^{+2} center through the deprotonated nitrogen atom of sulfonamide group and form a trigonal-bipyramidal adducts [33-35]. Three components of CAIs are involved in the binding with enzymes; a head group that coordinates to Zn^{+2} active site of enzyme, an organic linker that binds non covalently (hydrophobic, electrostatic or hydrogen bond) to enzyme surface and a tail which is compliant to structural alterations [36].

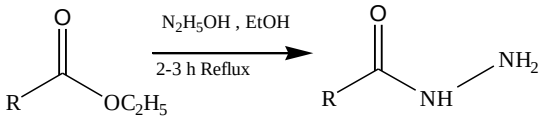
Experimental

Materials and Methods

Metal salt (NH_4VO_3) and all the chemicals used in this study were of reagent grade and purchased from Merck and Sigma Aldrich companies. Before use all the solvents were purified by using standard protocols. Prior to use distilled water was deionized using ELGA Cartridge Type C114 containing deionizer. Conductivity of vanadium(V) hydrazide complexes was measured by calibrated Hanna (HI-8633) conductivity meter (Romania). In order to calibrate the instrument KCl solution was used to dip the probe and the temperature was adjusted to 18±1 ^oC. Synthesized Vanadium(V) complex was digested in concentrated HNO_3 by heating. Vanadium content in the sample was then estimated using standard iodometric analysis, in which particular volume of digested sample was titrated against standard $Na_2S_2O_3$ using KI as an iodine source and starch as an indicator [37]. Perkin Elmer 2400 series II CHN/S analyzer was used to analyze carbon, hydrogen and nitrogen. Shimadzu 460 IR spectrometer was used to record Infrared spectra of all the synthesized ligands and their V(V) complexes, in the range of 4000-400 cm^{-1} using KBr disks. ^{1}H-NMR and ^{13}C NMR spectra were recorded in DMSO solvent at 300 MHz on a Bruker AVANCE AV 300 spectrophotometer.

Synthesis of Hydrazide Ligands

Synthesis of hydrazide ligands was carried out by following reported method [38-40]. 50 mmol of hydrated hydrazine was added to 75 mL of ethanol, 10 mmol of ester was then added to the same solution. The mixture was then refluxed for 2-3 h. Monitoring of ligand synthesis was carried out by TLC analysis. Washing of the product was carried out with hexane. Methanol was then used for recrystallization of the product, which was then dried in air to obtain ligands (Scheme-1).



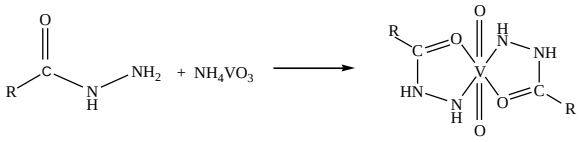
Ester Benzohydrazide Ligand (**1-12**)

Scheme-1: Synthesis of hydrazide ligands **(1-12)**.

Synthesis of Vanadium(V) Complexes

In order to prepare metal solution, 5 mmol of ammonium metavanadate (NH_4VO_3) was

dissolved in 30 mL of distilled water with constant stirring by a magnetic stirrer over a period of 5-6 h. pH of this solution was then maintained to 2.5 by H_2SO_4 and NaOH. For preparation of ligand solution, 10 mmol of hydrazide was simply dissolved in 30 mL of methanol. Complexation was then carried out by dropwise mixing of aqueous solution of metal to the methanolic solution of ligand, which resulted in the formation of brown precipitates of V(V) complex (Scheme-2). These precipitates were then filtered by Whatman 41 filter paper, washed with water and dried in air to get dry product. Characterization data of all synthesized vanadium(V) complexes (**1c**-**12c**) is already reported [41].



1	R = C_6H_6	**7**	R = 2-NH_2-C_6H_4
2	R = 2-F-C_6H_4	**8**	R = 3-NH_2-C_6H_4
3	R = 4-Cl-C_6H_4	**9**	R = 4-OCH_3-C_6H_4
4	R = 4-Br-C_6H_4	**10**	R = 3-Pyridyl
5	R = 3-I-C_6H_4	**11**	R = 4-Pyridyl
6	R = 4-I-C_6H_4	**12**	R = C_6H_5-CH_2

Scheme-2: Synthesis of Vanadium(V) Hydrazide Complexes.

Tyrosinase Enzyme Inhibition Assay

Principle

The assay was performed by targeting Raper-Mason pathway of melanin biosynthesis [42] in initial step. Dopachrome is formed as an intermediate when L-tyrosine oxidized catalytically, and this dopachrome is orange-red in color. Quantification of this enzyme activity is carried out spectrophotometrically by using quantity of dopachrome formed, at wavelength of 480 nm. Rate of formation of dopachrome is affected by the rate at which substrate (L-tyrosinase) is oxidized which is ultimately decreased by the introduction of tyrosine inhibitors. This inhibition may be induced either by reduction of *o*-quinone to diphenol or by decrease in O_2 consumption for the reaction [43,44]. Determination of inhibition potential of test compounds is then carried out by decrease in dopachrome optical density at 480nm.

Method

Enzyme inhibition assay for tyrosinase enzyme was performed using modified procedure reported by Kim, 2005 [45]. Incubation of reaction mixture containing enzyme 60 units, test compound 10 µL and buffer in volume of 150 µL in each well was performed at 30˚C for 15 minutes. A preread was then followed at 480 nm and then 20 µL of substrate were added to each well. Reaction mixture was re-incubated for 30 minutes and absorbance was recorded at 480 nm. Kojic acid was used as control for this assay.

Carbonic Anhydrase Enzyme Inhibition Assay

Principle

In this assay colorless 4-nitrophenylacetate (4NPA) used as substrate, by the reaction with carbonic anhydrase enzyme this substrate is hydrolyzed into 4-nitrophenol (yellow in color) and monitored spectrophotometrically [46]. The enzyme inhibition is investigated at wavelength of maximum absorbance of *p*-nitrophenol (400 nm) by monitoring decrease in absorbance, in the presence of inhibitor [47].

Method

Enzyme inhibition assay for carbonic anhydrase enzyme was carried out using reported procedure [48]. Total volume of reaction mixture was maintained upto 200 µL comprising 140 µL (20 mM HEPES(bioworld: cat#40820000-1) tris (Invitrogen : cat# 15504-020) buffer of pH 7.4, 20 µL (0.1 - 0.2 mg/ ml in deionized water) of enzyme, 20 µL (0.5 mg/ml in DMSO) of test compound. Reaction mixture was then assimilated. Incubation was carried out for 15 minutes at 25˚C. Reaction mixture was then preread at 400 nm. Further substrate 20 µL volume of substrate *i.e*. 4-nitrophenylacetate (0.7 mM in ethanol) was added and incubated again at 25˚C for 30 minutes. After 30 minutes analysis of reaction mixture was carried out at 400 nm on ELISA Reader SPECTRA-Max 340 spectrophotometer, Molecular Devices (USA). All analysis was carried out in triplicates. Acetazolamide (ACZ) was used as standard for this assay.

Determination of % Inhibition and IC_{50}

The %inhibition for enzyme inhibition assay was calculated using following formula. Serial dilution method was applied on original solutions in order to calculate IC_{50}.

% inhibition = (Ac – As) x100 / Ac

where, As = absorbance of test compound and Ac = absorbance of control

Table-1: Tyrosinase and carbonic anhydrase inhibition activity of hydrazides **1-12** and their V (V) complexes **1c-12c**.

Compound	IC_{50} (µM)		Compound	IC_{50} (µM)	
	Tyrosinase	Carbonic Anhydrase		Tyrosinase	Carbonic Anhydrase
1	**32.0**	**NA**	**7**	**45.8**	**NA**
1c	**5.3**	**0.14**	**7c**	**10.5**	**0.10**
2	**NA**	**NA**	**8**	**NA**	**NA**
2c	**NA**	**NA**	**8c**	**7.3**	**0.10**
3	**36.8**	**NA**	**9**	**38.8**	**NA**
3c	**150.0**	**0.09**	**9c**	**6.0**	**1.3**
4	**NA**	**NA**	**10**	**NA**	**NA**
4c	**147.3**	**0.07**	**10c**	**15.6**	**56**
5	**NA**	**NA**	**11**	**NA**	**NA**
5c	**144.8**	**0.10**	**11c**	**20.0**	**38**
6	**NA**	**NA**	**12**	**NA**	**NA**
6c	**120.4**	**0.08**	**12c**	**86.9**	**NA**
NH_4VO_3	**NA**	**NA**	**Kojic acid***	**25.0**	**-**
			Acetazolamide**	**-**	**0.13**

NA = Not active
*Standard inhibitor of tyrosinase
**Standard inhibitor of carbonic anhydrase

Results and Discussion

Tyrosinase Enzyme Inhibition Studies of Hydrazide Ligands and their Vanadium(V) Complexes

All ligands and their synthesized V(V) hydrazide complexes were evaluated for their enzyme inhibition activity against tyrosinase enzyme and the activity is summarized in Table-1, Fig. 1. Eight out of twelve ligands that is **2-6**, **8 10** and **11** were found to show no activity while rest of the ligands show variable degree of enzyme inhibition with IC_{50} values ranging between 32 to 86.9 µM. Kojic acid was used as standard in this assay.

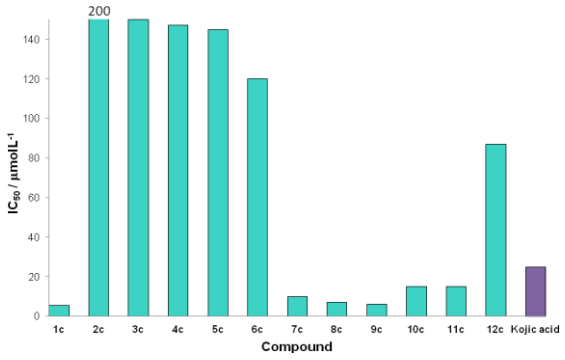


Fig. 1: Tyrosinase inhibition activity of vanadium (V) hydrazide complexes **1c-12c**.

In case of V(V) complexes, some show excellent activity for enzyme inhibition while rest show moderate to weak activity against tyrosinase inhibition assay. Complex **1c** with hydrazide possessing unsubstituted phenyl ring shows good activity with IC_{50} 5.3 µM, while its ligand shows lower activity with IC_{50} of 32.0 µM which is compatible with the standard IC_{50} 25.0±0.26 µM. Complexes with hydrazides substituted with halogens (F^-, Cl^-, Br^-, I^-) show poor activity against tyrosinase enzyme. These results are in accordance with the view that substitution in the benzene ring decreases enzyme inhibition activity of the complex [49]. Free halide ions have ability to bridge with Cu present at the active site of tyrosinase, inhibiting the enzyme activity in a non competitive manner [50]. It can be concluded from the higher IC_{50} values of complexes **2c-6c** that the addition of these electron withdrawing groups to the ring doesn't create a positive enzyme inhibition effect in the V(V) complexes but the electronegativity effect is diminished by increase in the size of substituents as in case of **4c** and **5c** with bromo and iodo groups as substituents respectively, and this substitution gives rise to a positive effect on enzyme inhibition activity of V(V) complexes.

Activity of aromatic aldehydes as tyrosinase inhibitors was reported by Kubo & Kinst-Hori, 1998; Kubo & Kinst-Hori, 1999; Lee *et al*., 2000 [51-53]. Studies reveal the importance of substitution in benzyl ring and consider these substituents to be responsible for varying the activity of compounds. Authors presented the tyrosinase inhibition activity of aromatic aldehydes and its enhancement by the addition of an electron donating group. Comparing the results of V(V) hydrazide complexes in the same context it is found that complexes **7c** and **8c** with electron donating amino group at *ortho* and *meta* positions respectively, and complex **9c** with *para* substituted methoxy group show good activity with IC_{50} 10.5, 7.3 and 6.0 µM respectively. Concluding these results, suggestion can be made that these complexes with electron donating effect possesses affinity to bind the enzyme, thus inhibiting its activity.

Complexes **10c** and **11c** with hydrazides possessing pyridyl ring instead of benzene ring show

good activity as tyrosinase inhibitors with IC_{50} values 15 and 20 µM, respectively. These results are in agreement with the conclusions made by Yi *et al*., 2009. Complex **12c** with hydrazide having an alkyl group between phenyl ring and carbonyl group show poor activity with IC_{50} value of 86.9 µM.

Carbonic Anhydrase Enzyme Inhibition Studies of Hydrazide Ligands and their Vanadium(V) Complexes

Synthesized hydrazide ligands, vanadium (V) hydrazide complexes and metal salt were evaluated for CA inhibition activity and summarized in Table-1, Fig. 2. Hydrazides and metal salt were found to be inactive against carbonic anhydrase enzyme while vanadium(V) complexes showed excellent, moderate or poor degree of inhibition.

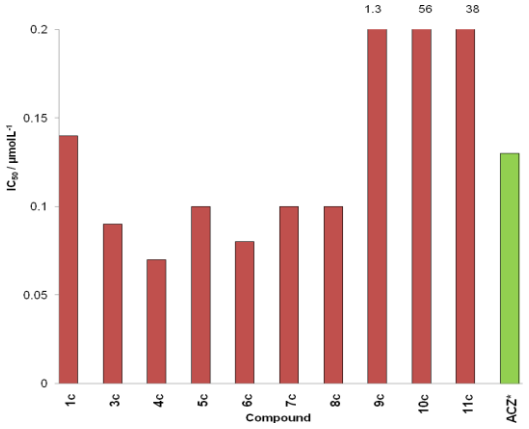


Fig. 2: Carbonic Anhydrase inhibition activity of vanadium(V) hydrazide complexes **1c**-**12c**.

Carbonic anhydrase inhibitors may be zinc-binding or non zinc-binding. Zinc-binding CA inhibitors are either added to Zn metal center as in case of thiocyanates CAIs, converting the normal tetrahedral geometry into a trigonal bipyramidal one. Sulphonamide CAIs in contrast, replace a non-protein ligand (*i.e*. hydroxide ion or water) from the normal enzyme carbonic anhydrase structure, whereas the geometry is retained to tetrahedral [54-57].

Complex **1c** with unsubstituted hydrazide shows good inhibition of enzyme carbonic anhydrase with 0.14 µM IC_{50}. Complexes **3c** (IC_{50} = 0.09 µM), **4c** (IC_{50} = 0.07 µM) and **6c (**IC_{50} = 0.08 µM) having substitution of halo groups on *para* positions show excellent activity against enzyme carbonic anhydrase with IC_{50} value much better than that of ACZ which is used as standard carbonic anhydrase inhibitor in this assay.

These results are consistent with the results obtained through the study carried out by Pichake *et al*., 2014, [58] in order to evaluate human carbonic anhydrase inhibitors. In this study importance of nature of substitution on *para* position was discussed.

Complex **2c** with fluoro substitution at *ortho* position was found to show no CA enzyme inhibition activity. Substitution of amino group at *ortho* (complex **7c)** or *meta* (**8c)** positions with IC_{50} = 0.10 µM also found to have good activity which are compatible with IC_{50} = 0.13 µM of ACZ for carbonic anhydrase inhibition. Substitution of methoxy group at *para* position was found to generate a negative effect as the carbonic anhydrase inhibition is poor with IC_{50} value for complex **9c** having methoxy substitution at *para* position was found to be 1.3 µM.

Complexes with hydrazide having either pyridyl ring instead of phenyl ring or bearing an additional alkyl ($-CH_2$) group between benzene ring and carbonyl of hydrazide, show poor and no activity with IC_{50} values 56 µM and 38 µM for complexes **10c and 11c** respectively while complex **12c** is inactive.

It can be concluded from these results, that the electron donating halo groups by substituted on the benzene ring of hydrazide activate the vanadium (V) complexes to behave as carbonic anhydrase inhibitor. The case of complex **2c** having fluoro substitution at *ortho* position is contrasting, being not active. On the other hand substitution of electron-withdrawing group on benzene ring with hydrazide, make the vanadium(V) complexes inactive, as in complexes **9c-11c**. The reason may be the stability of deprotonated form. As deprotonated form of sulphonamide have already being reported to show coordination with Zn(II) of cabonic anhydrase enzyme and to take part in hydrogen bonding through NH moiety with O of Thr 199 [59, 60, 61].

Conclusion

During the course of this study hydrazide ligands and vanadium(V) complexes of these hydrazides were synthesized. Structure elucidation of these hydrazides and vanadium(V) complexes was carried out using different techniques. On the basis of results obtained by these techniques it is suggested that hydrazides serve as bidentate ligand where N and O atoms have found to be the coordinating with metal centre. These hydrazide ligands are anionic in nature.

Furthermore synthesized vanadium(V) hydrazide complexes have found to exhibit 1:2 molar metal to ligand ratio as found by the results of IR and NMR studies. Tyrosinase and carbonic anhydrase enzymes inhibition studies show that ligands are not good inhibitors of these enzymes. In case of vanadium(V) hydrazide complexes variable degree of inhibition potential have found to be exhibited by these complexes which is in agreement with the thought that the nature and position of the substituted group of benzene ring of hydrazide plays an important role in originating the potency of these complexes as inhibitors of considered enzymes *i.e*. tyrosinase and carbonic anhydrase. It is worth mentioning here that V(V) complexes are more active for these enzymatic activities reported here. Metal salt is found to be inactive against these enzymes, which demonstrates the different interactions of metal and its complexes with metalloenzymes.

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