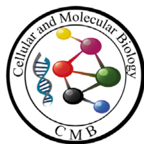

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Original Research

In vitro effects of novel type M-V-O derivatives on Xanthine Oxidase

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Abstract: Xanthine Oxidase (XO) is related with different diseases such as vascular, gout, nephropathy and renal stone diseases that are relevant to high uric acid levels and oxidative stress. Some common natural inhibitors of xanthine oxidase are known as rosmarinic acid and apigenin. With this study, we aimed to determine inhibitory effects of originally synthesized new generation transition metal vanadates on Xanthine Oxidase (XO) from bovine milk. Because, Xanthine oxidase inhibitors are typically used in the treatment of gout and nephropathy and renal stone diseases linked to hyperuricaemia. We found considerable IC_{50} constants for inhibition of XO. Among the synthesized compounds, Cu–V–O was found to be the most active (IC_{50} = 7.119 mM) for inhibition of XO.

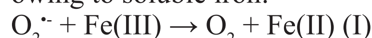
Key words: Xanthine Oxidase; Vanadium; Transition metal; Inhibition; IC_{50}.

Introduction

Since archaic ages, metal containing compounds have been drawing much attention due to usage for the treatment of a wide range of diseases. One of the characteristics of metals is their potential to undergo redox processes, as determined by their redox potentials. Among them, transition metal ions are generally can button between several oxidation states (1). They display significant acts in a series of biological oxidation incidents such as dehalogenations; hydroxylations; dehydrogenations; epoxidations; sulfoxidations; oxidative deaminations and dehalogenations; alcohol and aldehyde oxidations; and N, S and O-dealkylations. They also play critical roles in the oxidative processes employed in chemical industry and laboratory synthesis. In the presence of a specific ligand, the oxidation properties of the transition metal ion are highly controllable (2–5). Nevertheless, all oxidation conditions are not formed under physiological conditions in the organism. Because of the redox activity of transition metals they can be candidates to distortion of the sensitive cellular redox homeostasis, which is vital for health and survival of the body (6–9).

XO catalyses the hydroxylation of hypoxanthine and of xanthine to produce uric acid and superoxide anions. The second one has been connected to post ischemic tissue injury and edema along with vascular permeability (10,11). XO can also oxidize of synthetic purine drugs, such as antileukemic 6-mercaptopurine, by means of oxidization reaction (11). Controlling the activities of XO may be helpful to the therapy of some correlative diseases (e.g., gout). Nowadays, transition metal compounds, as a potent inhibitor of XO to cure gout has already been known. But studies on inhibitory activity regarding the XO of metal compounds are very few in the literature (12).

In the last fifty years, it has been investigated that transition metals exhibited a catalytic role against free radicals. As a suggestion, a superoxide radical anion ($0_2^{\bullet-}$) can be transform to the highly reactive hydroxyl radical ($^{\bullet}OH$) by the following Haber-Weiss Reaction owing to soluble iron:



$O_2^{\bullet-} + Fe(III) \rightarrow O_2 + Fe(II)$ (I)

$Fe(II) + H_2O_2 \rightarrow Fe(III) + OH^- + {}^{\bullet}OH$ (2)

This soluble iron which is insoluble under physiological conditions is bound to biological molecules, and can undergo cyclic reduction and reoxidation to dispose of free radical induced damage. Due to the generation of reactive oxygen species by XO, transition metal compounds are good candidates to reduce undesired damage (13).

Xanthine oxidoreductase is in two functionally different forms, xanthine dehydrogenase (XDH) and xanthine oxidase (XO). A part of the enzyme comprises in an NAD^+- dependent dehydrogenase form that generates NADH and urate under normal circumstances. The dehydrogenase can be converted to an oxygen-dependent oxidase, which generates reactive oxygen species (oxygen radicals and/or hydrogen peroxide) and urate under varied (patho) physiological circumstances (14). The physiological role of the enzymes has not been fully understood yet. While some authors attributed a bactericidal function to xanthine oxidoreductase (15–17), the others suggested an anti-oxidant function dependent on the production of urate (18–23).

In this research, various transition metal vanadates were synthesized and their effects on XO purified from bovine milk were evaluated. The reason to select different transition metals is to observe the redox potential differences.

Materials and Methods

Materials

Sepharose 4B, L-tyrosine, benzamidine, and protein assay reagents were obtained from Sigma Chem Co. (Milan, Italy) All other chemicals obtained from Sigma and Merck (Istanbul, Turkey) were used without further purification.

Methods

Synthesizing of the compounds

Transition metal oxides and vanadium (V) oxide that were supplied by Sigma Aldrich were analytically pure. Transition metals, used in study, have different redox potential due to the differences of their metal characters, electronegative properties and simple molecular structures. Each metal oxide was weighed out in a suitable molar ratio to react same quantity of vanadium (V) oxide, and then homogenized in an agate grout. The mixtures were put into a porcelain pot for heating in home type microwave oven. Afterwards, all substances were subjected to microwave heat throughout 10 min and then homogenized over again respectively. The samples were heated at 700 °C for 2 h for the crystallization. After that, eventual product was ready before the characterization process. The X-ray powder diffraction was used for characterization process of the samples. The powder X-ray diffraction (XRD) patterns were saved by using PANanalytical X'Pert PRO diffractometer with Cu Kα (1.5406A°, 45 kV and 30 mA) radiation.

Purification protocol of enzyme

Fresh bovine milk that has no preservative substances was kept at a refrigerator at to 4 °C for 15 h. Then, EDTA and toluene were added to it for ensuring last concentrations of 2 mM and 3% (v/v), respectively. The milk was stirred by using a blender for 30 min at max speed in ambient temperature. During that time, the temperature of the milk is raised from 4 to 45 °C. After decreasing the stirred milk temperature to about 4 °C, the stirring process recurred and the sample was leached. We ensured this sample to reach to 38% saturation by adding of solid ammonium sulphate (24). The suspension was centrifuged at 15000 rpm for 30 min, and we discarded the sediment that had been formed. The supernatant was ensured to reach to 50% saturation by using solid ammonium sulphate. We collected sediment formed by centrifugation at 15000 rpm for 60 min and ensured to be decomposed into 0.1 M Tris-HCl, pH=7.6. The affinity column including sepharose-4B-L-tyrosine-p-amino benzamidine was equilibrated in 0.1 M glycine, 0.1 M NaCl, pH=9.0 (25). Then we applied the sample to the affinity gel, and washed with 0.1 M glycine, pH=9.0. XO, and then we eluted it with 25 mM benzamidine in 0.1 M glycine, 0.1 M NaCl, pH= 9.0 (26). 1.5 mL fractions of it were collected, and we measured absorbances at 280 nm.

Measurements of enzyme activity

We designated xanthine oxidase activity at 37 °C according to the modified method of Massey et al. (27). We observed the transformation of xanthine to uric acid by monitoring the change in absorbance at 295 nm and by using a CARY 1E, UV-Visible Spectrophotometer-VARIAN spectrometer (ε_{292} = 9.5 mM^{-1} cm^{-1}). The reaction mixture that was at 37 °C included 50 mM Tris-HCl, pH=7.6, and 0.15 mM xanthine. We started the experiment by adding the enzyme. A unit of enzyme activity was described as the quantity of enzyme that transforms one μmol of xanthine to uric acid per min under specified conditions (25).

Kinetic studies of XO

We added different concentrations to the enzyme activity to study the inhibition of some transition metal vanadate derivatives. Following the oxidation of xanthine, XO enzyme activity with transition metal vanadate derivatives was tested three times. We designated proportional values of activity belonging to xanthine oxidase for seven different concentrations of transition divanadate derivatives by regression analysis using Microsoft Office Excel. We accepted XO enzyme activity that does not have a transition metal vanadate as 100% active. The graphs as follows indicate the inhibitor concentration led up to 50% inhibition (IC_{50} values) on the enzyme (25).

Results and discussion

In Figure. 1, powder XRD patterns of transition metal vanadates; Ti─V─O, Cr─V─O, Mn─V─O, Fe─V─O, Co─V─O, Ni─V─O, Cu─V─O, and Zn─V─O, are shown. When the patterns were compared with the International Centre for Diffraction Data (ICDD) cards, they are in line with the target compounds containing one of the transition metals, vanadium and oxygen. These types of compounds have been synthesized for the first time via microwave method, which clearly differentiates this study from previous ones.

Xanthine oxidoreductase (XOR) is a molybdenum containing enzyme (28) that has a key role in the metabolism (29). It catalyses the hydroxylation reaction on sp^2─hybridized carbon centers on a variety of substrates with the inclusion of purines, aldehydes and other heterocyclic compounds (30). Also, it's one of the most important roles in metabolism are NAD^+ regeneration, iron absorption, mobilization and reduction of nitrates (31–35). Due to such important functions of XO, there are some studies in the literature concerning XO inhibition in terms of various compounds such as flavonoids (36), and different complexes (10). In a similar study to ours, Yu-Guang Li et al. presented IC_{50} values about XO inhibitors (10). Yet our research is still unique in the sense that our synthesized compounds have metal─vanadium─oxygen structure and there is no

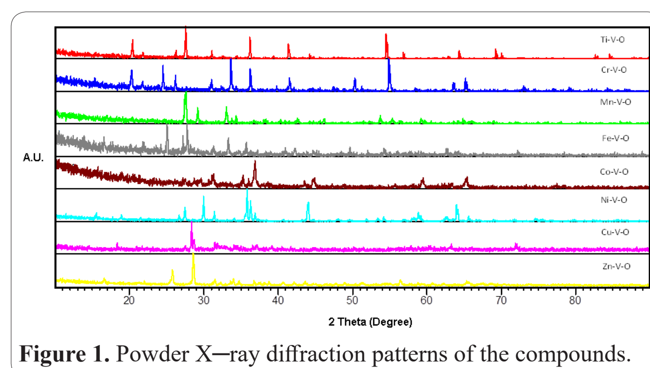


Figure 1. Powder X─ray diffraction patterns of the compounds.

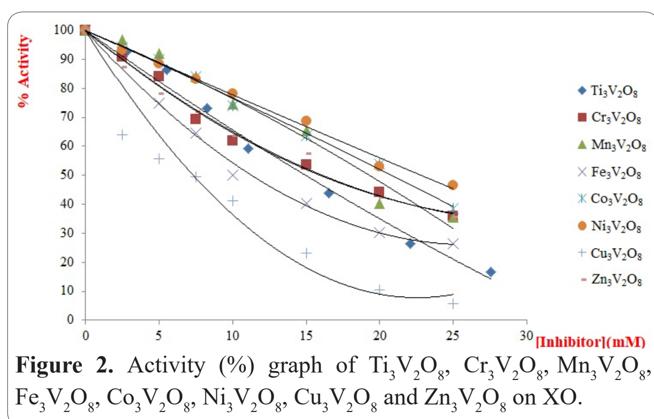


Figure 2. Activity (%) graph of $Ti_3V_2O_8$, $Cr_3V_2O_8$, $Mn_3V_2O_8$, $Fe_3V_2O_8$, $Co_3V_2O_8$, $Ni_3V_2O_8$, $Cu_3V_2O_8$ and $Zn_3V_2O_8$ on XO.

similar or any other report about bioactivity of M─V─O for the reason of complex novelty.

By our recent research, transition metal vanadate compounds inhibited XO as seen in Figure. 2. Our spectrophotometrically measure results showed that uric acid production decreased similarly to the relevant literature (27). Inhibition effect of the transition metal vanadates was found to be in the 7.119–22.801mM range as listed in Table 1. Results of present study pointed out that the new transition metal vanadate compounds which are synthesized by microwave method at first have a powerful inhibition and they have close values to that of allopurinol, the well-known standard inhibitor of XO, by the IC_{50} value of 7.4±0.07 mM (37).

The ability of Cu─V─O complex to inhibit XO exhibits the strongest among the other seven *M*─V─O (Ti─V─O, Cr─V─O, Mn─V─O, Fe─V─O, Co─V─O, Ni─V─O, and Zn─V─O). The best inhibition effect is from the copper compounds (Cu─V─O), which presumably because of its molecular structure, and is better in allowing the inhibitor interacts with the active site of XO or substrate of XO than the others. On the other hand, apart from Cu─V─O, the other seven *metal*─vanadium─oxygen compounds weak inhibitory effects and interaction with XO or substrate of XO probably due to their metallic character like redox potential differences.

As we know that allopurinol, a powerful inhibitor of XO, is structurally similar to xanthine and is oxidized by the enzyme to give oxyallopurinol, which binds tightly to the active centre of XO and therefore causes strong inhibition (38). We consider that the new generation transition metal vanadate complexes might also inhibit the enzyme activity by binding the complex to the active centre of the enzyme, with metal ions binding to the oxygen atom, and with $V_2O_8^{-6}$ structures binding to the Mo ion in the active centre. The mechanisms of the inhibitory activity require to be further investigated.

Table 1. IC_{50} values of $Ti_3V_2O_8$, $Cr_3V_2O_8$, $Mn_3V_2O_8$, $Fe_3V_2O_8$, $Co_3V_2O_8$, $Ni_3V_2O_8$, $Cu_3V_2O_8$ and $Zn_3V_2O_8$.

Compound	IC_{50} Values (mM)
$Ti_3V_2O_8$	14.658±1.099
$Cr_3V_2O_8$	15.725±1.092
$Mn_3V_2O_8$	19.128±0.296
$Fe_3V_2O_8$	11.087±1.922
$Co_3V_2O_8$	20.875±1.313
$Ni_3V_2O_8$	22.801±1.414
$Cu_3V_2O_8$	7.119±0.721
$Zn_3V_2O_8$	16.348±1.493

Reduction of side effects of XO inhibitors is very important and necessary for their improvement. As it can be seen from the literature, allopurinol has several adverse effects. Moreover, the cellular and molecular mechanisms of these adverse effects are far from being understood. It can be found in some latest studies that the renal toxicity of allopurinol is associated with impairment of pyrimidine metabolism (39). However, reassuring findings, such as the new series of XO inhibitors mentioned in this study, may play a key role for a number of therapeutic indications for XO inhibition. The aim of this article is to provide a critical aspect towards the effects of new type XO inhibitors, called transition metal vanadates, and also to examine the new existing therapeutic strategies presented by this promising approach.

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