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Polyoxometalates as inhibitors of P-type ATPases and the role of polyphenols

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Abstract

Polyoxometalates (POMs) have been described to exhibit anti-tumor, anti-bacterial and anti-virus activity, although their mechanisms of action are still to be determined. POMs' mechanisms of action as anti-cancer and anti-bacterial activities were linked, at least in part, to the inhibition of P-type ATPases, such as the Ca^{2+}-ATPase. In the present study, we analyzed the effects of W-based POMs (P_2W_{18}) in the activity of Ca^{2+}-ATPase from the sarcoplasmic reticulum in the presence of polyphenols (quercetin) and antioxidants (glutathione). It was observed that P_2W_{18} inhibits the Ca^{2+}-ATPase activity through a mixed type inhibition, and with IC_{50} values of 0.6 µM, which is about 30 and 70 times lower than for V_{10} (15 µM), and Nb_{10} (35 µM). While no effect was observed upon quercetin addition, the presence of the intracellular antioxidant GSH (2 mM) induced a reduction of the inhibition from 60% to 20%, for 1 µM of P_2W_{18}, and increased the IC_{50} value up to about two-fold, from 0.6 to 1.2 µM. In sum, the presence of polyphenols and antioxidants should be taken into account in the studies of POMs inhibition of calcium pumps, which are well-known drugs targets, once the different effects for different polyoxometalates can be determined in processes associated with ion homeostasis.

Introduction

The number of references concerning polyoxometalates doubled in the last seven years (2010-2016) when compared to the similar period between 2003 and 2009, from 2060 to 3888. Polyoxometalates (POMs) are of great interest against virus, bacteria and tumor cells, besides others biological activities (Sun *et al*., 2016; Blazevic *et al*, 2016, Trevino *et al*, 2016). The POMs effects on tumor proliferation might, at least in part, be due to the inhibition of specific enzymes, such as alkaline phosphatases, ecto-nucleotidases, as well as P-type ATPases, although the mechanism of action is still unlear (Stephan *et al*, 2013; Aureliano *et al*, 2013; Aureliano, 2016). However, the majority of the studies on the biological effect of POMs do not account for the presence of antioxidants in the medium. Polyphenols are well known to present several roles in toxicology and diseases. Moreover, flavonoids were also described to be responsible for several cellular

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protective actions (Lagoa *et al*, 2017). Previously, it was observed that quercetin and kaempferol prevents protein cysteine oxidation induced by the isopolyoxometalate decavanadate (V_{10}), but does not prevent the calcium ATPase (Ca^{2+}-ATPase) inhibition (Fraqueza *et al*, 2012). Ca^{2+}-ATPase from sarcoplasmic reticulum is involved in calcium homeostasis and is associated with several processes such as muscle relaxation, cell death (apoptosis and necrosis) and diabetes. Ca^{2+}-ATPase, as are others P-type ATPases, is a known target of drugs and it was suggested to be an excellent model to study the effects of POMs such as decavanadate (V_{10}) (Aureliano *et al*, 2013; Aureliano, 2016). In the present study, we analysed the effect of another type of POM, such as the Wells-Dawson type $K_6[\alpha\text{-}P_2W_{18}O_{62}].14H_2O$ (abbreviated P_2W_{18}) in the Ca^{2+}-ATPase activity and its putative reversion by antioxidants and polyphenols, showing for the first time that the mechanism of action of POMs can be counteracted by the activity of antioxidants like polyphenols or glutathione (Fig. 1).

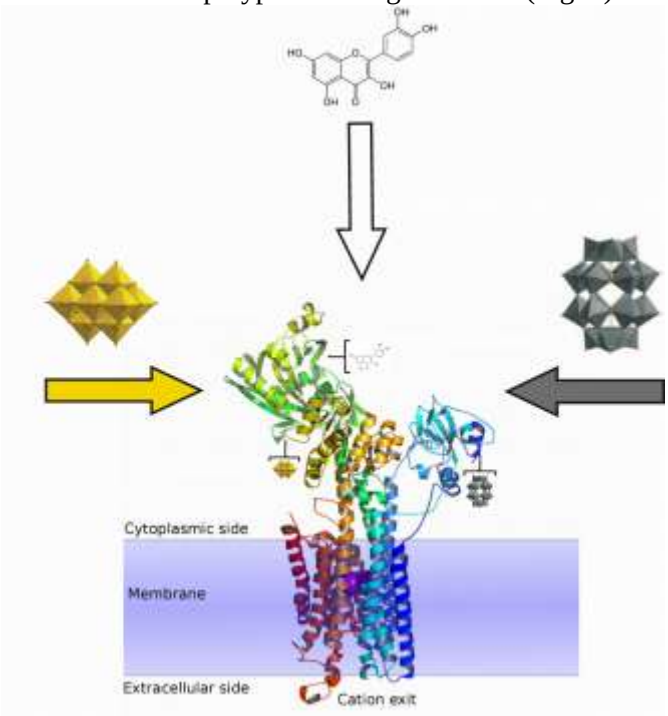

Figure 1. Polyoxometalates (V_{10}, yellow and P_2W_{18} in gray), pumps and polyphenols (quercetin) can be associated in several biological processes such as in calcium homeostasis. POMs are potential agents for biomedical applications.

Materials and methods

Chemicals and reagents

$K_6[\alpha\text{-}P_2W_{18}O_{62}].14H_2O$ (abbreviated P_2W_{18}) 1 mM stock solution was prepared in water considering a MW of 4849.83 g/mol. P_2W_{18} is soluble in water up to 10 mM. Analytical solutions of quercetin and glutathione were prepared from reagents obtained from Sigma-Aldrich (Portugal).

Preparation of Ca^{2+}-ATPase from sarcoplasmic reticulum

Sarcoplasmic reticulum (SR) vesicles containing Ca^{2+}-ATPase were prepared from freshly obtained rabbit skeletal muscle and suspended in 0.1 M KCl, 10 mM HEPES (pH 7.0), diluted 1:1 with 2.0 M sucrose and frozen in liquid nitrogen prior to storage at –80 °C, as described elsewhere (Fraqueza *et al*., 2012).

Protein concentrations were determined spectrophotometrically at 595 nm, by the Bradford method, using bovine serum albumin as a standard in the presence of 0.125% of sodium dodecyl sulphate. The SR Ca^{2+}-ATPase analyzed by SDS polyacrylamide gel electrophoresis comprised at least 70% of the total protein in the SR vesicles. The SERCA-1, (sarcoplasmic, or endoplasmic reticulum Ca^{2+}-ATPase-1) was the predominant isoform in the SR preparations (Fraqueza *et al*., 2012).

Kinetic studies

Inhibition of Ca^{2+}-ATPase activity by P_2W_{18} was measured spectrophotometrically at 340 nm and 25 °C, using the coupled enzyme pyruvate kinase/lactate dehydrogenase assay under the following conditions: 25 mM HEPES (pH 7.0), 100 mM KCl, 5 mM $MgCl_2$, 50 μM $CaCl_2$, 2.5 mM ATP, 0.42 mM phosphoenolpyruvate, 0.25 mM NADH, 18 IU lactate dehydrogenase and 7.5 IU pyruvate kinase. The experiments were initiated after the addition of 10 μg/ml calcium ATPase, in the presence and/or absence of 4% (w/w) of calcium ionophore A23187. The inhibitor was added previous to the addition of the ATPase.

Results and discussion

In the present study, we observed that P_2W_{18}, a Wells-Dawson type POM, also inhibits the calcium ATPase activity and with a lower IC_{50} value (0.6 μM) than the ones observed for decavanadate (V_{10}, 15 μM) and for the isostructural and isoelectronic decaniobate, (Nb_{10},

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35 μM), both included in the Lindqvist type POMs. Therefore, P_2W_{18} showed to be the most potent Ca^{2+}-ATPase inhibitor so far described in the literature, with IC_{50} values of inhibition about 30 and 70 times lower than the ones reported for V_{10} and Nb_{10}, respectively (Fraqueza *et al*., 2012). It was reported that some POMs have lower IC_{50} values of inhibition for the Ca^{2+}-ATPase activity than several common drugs targeting the P-type ATPases (Aureliano *et al*, 2013), such as chlorpromazine (IC_{50} = 62.5 μM), omeprazole (IC_{50} = 30-50 μM), curcumin (IC_{50} = 15 μM) or celcoxib (IC_{50} = 35 μM). In that sense, POMs have been suggested as potential agents for therapeutic applications (Aureliano *et al*, 2013).

Previous studies reported that both V_{10} and Nb_{10} are Ca^{2+}-ATPase non-competitive inhibitors (Fraqueza *et al*, 2012). However, in this study, we observed that the Wells-Dawson type P_2W_{18} presented a mixed type of inhibition, considering the concentration that inhibits 50% of the enzymatic activity (0.6 μM) and the traditional Lineweaver-Burke analysis shown in Fig. 2. The mixed inhibition exhibited by P_2W_{18} suggests that the POM may bind to the Ca^{2+}-ATPase whether or not the enzyme has already bound substrate, implying the existence of two distinct protein binding sites for P_2W_{18}.

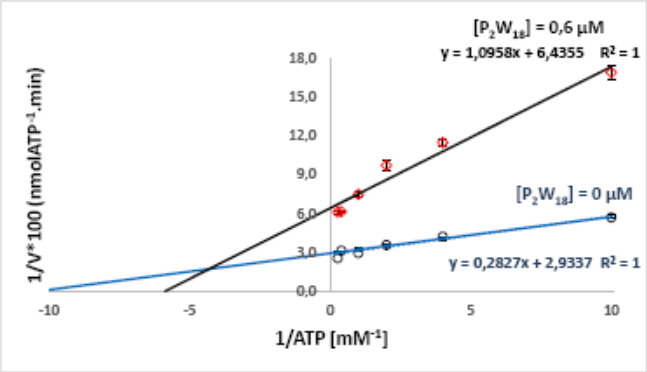

Figure 2. Lineweaver-Burk plot of Ca^{2+}-ATPase activity in the absence (blue) and in the presence (orange) of 0.6 μM of the polyoxometalate P_2W_{18}, used for determining the type of enzyme inhibition. The Wells-Dawson type P_2W_{18} presented a mixed type of inhibition. Data are plotted as means ± SD. The results shown are the average of triplicate experiments.

Furthermore, it was observed that the presence of physiological concentrations (2 mM) of the intracellular antioxidant glutathione (GSH) induces a reduction of the inhibition of the Ca^{2+}-ATPase activity, for 1 μM of P_2W_{18}, from about 60% to 20% (Fig. 3); the P_2W_{18} IC_{50} increases up to about two-fold, from 0.6 to 1.2 μM, as shown in Fig. 3.

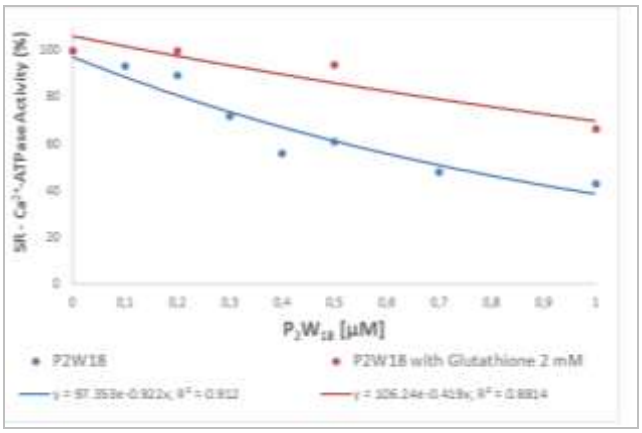

Figure 3. Inhibition of Ca^{2+}-ATPase activity by the Wells-Dawson type polyoxometalate P_2W_{18}, in the absence (blue) and in the presence of 2 mM glutathione (orange). Upon GSH addition, it was observed an increasing of the IC_{50} value up to about two-fold, from 0.6 to 1.2 μM.

Nevertheless, it was observed that the addition of quercetin (10 μM) to the reaction medium does not prevent or affect the inhibition of the Ca^{2+}-ATPase activity by P_2W_{18} (not shown). Therefore, POMs inhibition of enzymes that are well known drugs targets, like P-type ATPases, may be dependent on the presence of polyphenols or intracellular antioxidants.

Conclusions

To our knowledge, P_2W_{18} is the most potent polyoxometalate so far described in the literature to have the capacity to induce the inhibition of the Ca^{2+}-ATPase activity, with IC_{50} values of inhibition, about 30 and 70 times lower (IC_{50} = 0.6 μM) than the ones previously described for V_{10} (15 μM) and for Nb_{10} (35 μM). In fact, some POMs have lower IC_{50} values than several drugs regarding the Ca^{2+}-ATPase activity, such as chlorpromazine (IC_{50} = 62.5 μM), omeprazole (IC_{50} = 30-50 μM), curcumin (IC_{50} = 15 μM) or celcoxib (IC_{50} = 35 μM). Thus, POMs can be considered as a putative therapeutic agent. P_2W_{18} shows to behave as a mixed type Ca^{2+}-ATPase inhibitor, whereas Nb_{10} and V_{10} were described as non-competitive inhibitors. P_2W_{18} inhibition of Ca^{2+}-ATPase activity is reversed by GSH, but not by quercetin. Conversely, the inhibition of Ca^{2+}-ATPase activity by V_{10} is not reversed by GSH or quercetin, suggesting a different mode of interaction with the protein for V_{10} and P_2W_{18}. In sum, the presence of polyphenols and antioxidants should be taken into account in the study of the Ca^{2+}-ATPase inhibition by POMs. Thus, different effects for different POMs analyzed (V_{10} and P_2W_{18}) can be

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observed regarding the mode of interaction and the extension of inhibition of the Ca^{2+}-ATPase activity in the absence or in the presence of flavonoids and antioxidants. We propose that the present studies will contribute for the understanding of putative biomedical applications of polyoxometalates**.**

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