Bioorganic & Medicinal Chemistry 19 (2011) 7063–7069

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Inhibition of rat synaptic membrane Na^+/K^+-ATPase and ecto-nucleoside triphosphate diphosphohydrolases by 12-tungstosilicic and 12-tungstophosphoric acid

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ARTICLE INFO

Article history:
Received 22 July 2011
Revised 30 September 2011
Accepted 4 October 2011
Available online 13 October 2011

Keywords:
12-Tungstosilicic acid
12-Tungstophosphoric acid
Monolacunary Keggin anion
$Na_2WO_4 \times 2H_2O$
Na^+/K^+-ATPase
Ecto-nucleoside triphosphate diphosphohydrolases
Simultaneous exposure

ABSTRACT

The in vitro influence of Keggin structure polyoxotungstates, 12-tungstosilicic acid, $H_4SiW_{12}O_{40}$ (WSiA) and 12-tungstophosphoric acid, $H_3PW_{12}O_{40}$ (WPA), and monomer $Na_2WO_4 \times 2H_2O$ on rat synaptic plasma membrane (SPM) Na^+/K^+-ATPase and E-NTPDase activity was studied, whereas the commercial porcine cerebral cortex Na^+/K^+-ATPase served as a reference. Dose-dependent Na^+/K^+-ATPase inhibition was obtained for all investigated compounds. Calculated IC_{50} (10 min) values, in mol/l, for SPM/commercial Na^+/K^+-ATPase, were: $3.4 \times 10^{-6}/4.3 \times 10^{-6}$, $2.9 \times 10^{-6}/3.1 \times 10^{-6}$ and $1.3 \times 10^{-3}/1.5 \times 10^{-3}$ for WSiA, WPA and $Na_2WO_4 \times 2H_2O$, respectively. In the case of E-NTPDase, increasing concentrations of WSiA and WPA induced its activity reduction, while $Na_2WO_4 \times 2H_2O$ did not noticeably affect the enzyme activity at all investigated concentrations (up to 1×10^{-3} mol/l). IC_{50} (10 min) values, obtained from the inhibition curves, were (in mol/l): 4.1×10^{-6} for WSiA and 1.6×10^{-6} for WPA. Monolacunary Keggin anion was found as the main active molecular species present under physiological conditions (in the enzyme assays, pH 7.4), for the both polyoxotungstates solutions (1 mmol/l), using Fourier transform infrared (FT-IR) and micro-Raman spectroscopy. Additionally, commercial porcine cerebral cortex Na^+/K^+-ATPase was exposed to the mixture of $Na_2WO_4 \times 2H_2O$ and WSiA at different concentrations. Additive inhibition effect was achieved for lower concentrations of $Na_2WO_4 \times 2H_2O$/WSiA ($\leqslant 1 \times 10^{-3}/4 \times 10^{-6}$ mol/l), while antagonistic effect was obtained for all higher concentrations of the inhibitors.

1. Introduction

Polyoxometalates (POMs) are polyanionic oligomeric aggregates of transition metal ions, such as tungsten, molybdenum, vanadium, etc. held together by oxygen bridges, with a high density of negative charge. They are relatively stable, some even highly stable in aqueous solutions at biological pH values.[1,2] In addition to applications in catalysis, separations, analysis, and as electron-dense imaging agents, some of these complexes have been shown to exhibit biological activity in vitro as well as in vivo ranging from anti-cancer, antibiotic, and antiviral to antidiabetic effects.[2–6] Due to their negative charges they bear resemblance to nucleotides and therefore affect activity of nucleotide-dependent enzymes.[7–12]

Na^+/K^+-ATPase (sodium pump) belongs to the P-type ATPase family, the members of which are able to utilize the energy of ATP to transport ions against their electrochemical gradient. Na^+/K^+-ATPase is a cell membrane located enzyme that establishes and maintains the high internal K^+ and low internal Na^+ concentrations, characteristic and essential for normal cellular activities of most animal cells.[13,14] The activity of this enzyme is very sensitive to the presence of some metal ions and organic compounds of various structures, especially some drugs and pesticides.[15–18] Moreover, recent studies show that in addition to pumping ions, Na^+/K^+-ATPase interacts with neighboring membrane proteins and organized cytosolic cascades of signaling proteins to send messages to the intracellular organelles.[19,20] Thus, this function of sodium pump as a receptor and signaling mediator suggests that Na^+/K^+-ATPase has pivotal role in cancer cell migration and supports the view that Na^+/K^+-ATPase could be an important target for development of anti-cancer drugs.[21–24]

Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; E-NTPDases, ecto-nucleoside triphosphate diphosphohydrolases; FT-IR, Fourier transform infrared; POMs, polyoxometalates; SPM, synaptic plasma membrane; WPA, 12-tungstophosphoric acid; WSiA, 12-tungstosilicic acid.

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doi:10.1016/j.bmc.2011.10.008

The ecto-nucleoside triphosphate diphosphohydrolases, E-NTPDases, (ecto-ATPase) which do not belong to the P-type ATPase family, represent plasma membrane bound enzymes that, in the presence of divalent cations (Ca^{2+} or Mg^{2+}), hydrolyses extracellular nucleotides (ATP and ADP) because of the outward orientation of its active site.[25,26] Since extracellular adenosine and adenine nucleotides induce various cellular responses (through activation of P1 and P2 receptors), E-NTPDases represent the major part of purinergic signaling.[8,26] Moreover, inhibition of E-NTPDases may explain or contribute to some observed in vitro and in vivo polyoxometalate effects, including anti-cancer activity, protection against viral, bacterial and protozoa infections, antidiabetic activity.[7,27,28]

The aim of this work was the investigation of the in vitro effect of two representative polyoxotungstates possessing Keggin structure: 12-tungstosilicic acid, $H_4SiW_{12}O_{40}$ (WSiA) and 12-tungstophosphoric acid, $H_3PW_{12}O_{40}$ (WPA) (Fig. 1), that are not cytotoxic,[29] on Na^+/K^+-ATPase, and E-NTPDases activity using rat synaptic plasma membrane (SPM) as a model system, while the commercial porcine cerebral cortex Na^+/K^+-ATPase served as a reference. Na^+/K^+-ATPase and E-NTPDases were chosen because of their key role in normal functioning most cells of higher eukaryotic organisms[26,30] as well as in development and progression of different cancers[23] and, on the other hand, of the known influence of POMs on nucleotide-dependent enzymes[9–11,31,32] as well as their anti-cancer activity. In order to determine the truly active species in water at physiological pH, the stability and molecular speciation of WPA and WSiA under enzyme assay conditions, using FT-IR and micro-Raman spectroscopy, was screened. In addition, the influence of $Na_2WO_4 \times 2H_2O$, the starting monomer in the POMs synthesis, on Na^+/K^+-ATPase and E-NTPDases activity was studied, as well as its simultaneous effect, in combination with WSiA, on the commercial Na^+/K^+-ATPase.

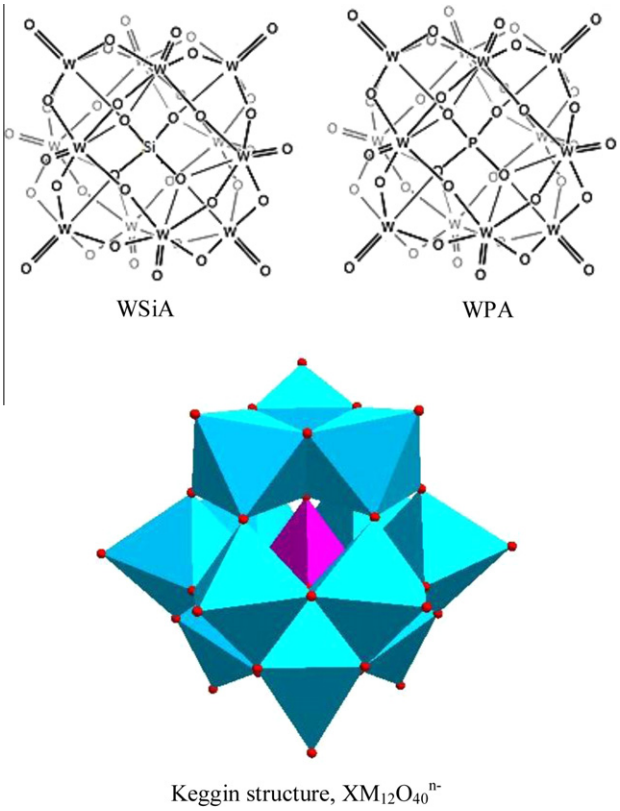

Figure 1. The chemical structures of WSiA and WPA.

2. Material and methods

2.1. Chemicals

All chemicals were of analytical grade. Na^+/K^+-ATPase from porcine cerebral cortex and ATP were purchased from Sigma Chemicals Co. (Germany), as well as some chemicals for medium assay (magnesium chloride and Tris–HCl). The specific Na^+/K^+-ATPase activity was 25.8 μmol Pi/h/mg protein (Pi—inorganic orthophosphate). Other medium assay chemicals (sodium chloride, potassium chloride), chemicals for determination of Pi (stannous chloride and ammonium molybdate) were from Merck (Germany). $Na_2WO_4 \times 2H_2O$ and WSiA were commercially available (Fluka, Germany), while WPA was prepared according to the literature method[33] and confirmed by infrared spectroscopy. Both acids were recrystallized prior to use and heated about 10 min at 80 °C in order to get stable acid hexahydrate (WPA·$6H_2O$, WSiA·$6H_2O$).

2.2. Preparation of 12-tungstosilicic acid and 12-tungstophosphoric acid solutions

Stock solutions of POMs (10^{-2} mol/l, pH ≈ 1) were prepared daily, by solving the solid compounds in water shortly before use, while prepared $Na_2WO_4 \times 2H_2O$ (10^{-2} mol/l, pH ≈ 7) stock solution was kept frozen. Working solutions were prepared daily by diluting the stock solutions to desired concentrations. Preliminary studies showed that the presence of POMs (at 5×10^{-4} mol/l and lower concentrations) did not change pH of the medium assay (pH 7.4). As shown in our earlier studies, the investigated solutions of 12-tungstosilicid and 12-tungstophosphoric acid are stable at pH 7.4 and at 37 °C even in concentration of 5×10^{-2} mol/l.[34,35]

2.3. Fourier transform infrared and Raman spectroscopy

Solid samples for IR measurements were obtained after evaporation of solvent from the aliquot of 1 mL of analyzed solutions. The FT-IR spectra of the solid residua were recorded on Thermo Nicolet 6700 spectrophotometer using the KBr pellets technique, in the wavenumber range from 4000 to 400 cm^{-1}, at room temperature.

The same solid residua were analyzed by micro-Raman spectroscopy using Thermo DXR Raman microscope. 532 nm laser excitation line was used with a constant power of 10 mW and exposure time of 10.00 s.

The Raman spectra of freshly prepared solutions were recorded in situ, using the fiber optic probe with the 780 nm laser excitation line, with a constant power of 150 mW, exposure time of 60.00 s and 5 scans. The pH of solution was controlled before and after recording spectra and it was not changed.

2.4. Synaptic plasma membrane preparation

SPMs were isolated from the whole brain of 3-month-old male Wistar albino rats from the local colony. Animals were kept under controlled illumination (lights on: 5:00 am–5:00 pm) and temperature (23 ± 2 °C), and had free access to food and water. The Guiding Principles for the Care and Use of Animals based upon Helsinki Declaration (1964) and Protocol of the 'Vinča' Institute on Care and Treatment of Laboratory Animals were strictly followed. After decapitation with a guillotine (Harvard Apparatus), brains were rapidly excised and pooled (6/pool) for immediate preparation of SPM isolated according to the method of Cohen et al.,[36] as modified by Towle and Sze.[37] The mitochondrial contamination and protein content were determined according to the standard procedure.[38] SPMs were stored at −70 °C until used.

2.5. ATPase assays

The standard assay medium for investigation of Na^+/K^+-ATPase activity contained (in mmol/l): 50 Tris–HCl (pH 7.4), 100 NaCl, 20 KCl, 5 $MgCl_2$, 2 ATP and 125 mg/l SPM proteins (i.e. 290 mg/l commercial porcine cerebral cortex proteins) in a final volume of 200 μl. Assay for SPM ecto-ATPase activity contained (in mmol/l): 50 Tris–HCl (pH 7.4), 5 $MgCl_2$, 2 ATP and 125 mg/l SPM proteins. After preincubation for 10 min at 37 °C in the absence (control) or in the presence of investigated compounds (separate and simultaneous), the reaction was initiated by addition of ATP and stopped after 10 min by adding 22 μl ice cold of 3 mol/l $HClO_4$ and immediate cooling on ice. The released Pi liberated from the hydrolysis of ATP was determined by a modified spectrophotometric method.[39] The spectrophotometric measurements were performed on a Perkin Elmer Lambda 35 UV–vis spectrophotometer. The activity obtained in the presence of Mg^{2+} alone was attributed to E-NTPDases activity. SPM Na^+/K^+-ATPase activity was calculated by subtracting the E-NTPDases activity from the total ATPase activity in the presence of Na^+, K^+ and Mg^{2+} ions. The results are expressed as mean percentage enzyme activity compared to the corresponding control value ± S.E.M. of at least three independent experiments done in triplicate.

2.6. Statistical analysis

Analysis of variance (One way ANOVA) was used to compare the mathematical sum of inhibitions caused by single exposure to investigated inhibitors with inhibitions induced by exposure to both compound in combination (simultaneously). When a significant continuous probability distribution (F value) (P <0.05) was obtained, post hoc test Bonferoni was used to determine differences.

3. Results

3.1. Molecular species of 12-tungstosilicic acid and 12-tungstophosphoric acid under physiological conditions

The speciation of WSiA and WPA under the enzyme assay conditions (physiological pH 7.4) was investigated by two common, complementary spectroscopic methods: FT-IR and micro-Raman spectroscopy.

The FT-IR spectra of solid residue of following solutions:

- 1 mmol/l WSiA/WPA water solution, pH 1 (WSiA/WPA, pH 1)
- 1 mmol/l WSiA/WPA water solution, pH 7.4 (WSiA/WPA, pH 7.4)
- Na^+/K^+-ATPase assay containing (in mmol/l): 50 Tris–HCl (pH 7.4), 100 NaCl, 20 KCl, 5 $MgCl_2$, 2 ATP and 290 mg/l commercial porcine cerebral cortex proteins (solution 1)
- 1 mmol/l WSiA/WPA in the presence of the Na^+/K^+-ATPase assay components, pH 7.4 (solution 2/solution 3)

were recorded and presented in Figure 2.

As may be seen in Figure 2a, FT-IR spectrum of solution 1 possesses vibration bands corresponding Tris–HCl with the main bands at 1133, 1035, 903, 629 and 599 cm^{-1} and this spectrum is identical with spectrum of incubation mixture. The bands originating from ATP and Na^+/K^+-ATPase are not observed because of their low concentrations. However in FTIR spectra of solution 3 with WPA, new bands appear. Band at 954 cm^{-1} corresponds to W–O_d, band at 900 cm^{-1} corresponds to W–O_b–W and bands at 814 and 735 cm^{-1} correspond to W–O_c–W vibrations. P–O vibrations at 1100 and 1042 cm^{-1} are overlaped with bands of solution 1. The spectra of solution 3 does not corresponding to the Keggin WPA anion, which is stable at very acid solution of about pH ~1 (WPA, pH 1 spectrum) but is identical with spectrum of monolacunary Keggin anion (WPA, pH 7.4 spectrum), obtained by removal one WO unit from the parent acid.[37,38] Also, bands characteristic for monolacunary Keggin anion (WSiA, pH 7.4) are present in FTIR spectra of solution 2 which contains WSiA (Fig. 2b).

The Raman spectra of the same samples are presented in Figure 3 for solid residua of WPA, Figure 3a and WSiA, Figure 3b.

Bands of monolacunary Keggin anion of WPA and WSiA are present in spectra at physiological pH 7.4, confirming results obtained from FT-IR spectra.[40,41] However, in spectra of solid residue of solutions WSiA, pH 7.4 and 2, Keggin structure of parent WSiA acid (WSiA, pH 1 spectrum) can be observed in traces, indicating higher stability of WSiA at physiological conditions compared to WPA acid.

In order to prove that solid residua recovered intact after evaporation, the Raman spectra were recorded in situ from solutions by fiber optic probe. The obtained spectra are given in Figure 4a for WPA and in Figure 4b for WSiA.

In spectra of solution WPA, pH 7.4 and solution 3 the main band at 983 (ν_{as} W–O_d), characteristic for monolacunary anion, confirms unchanged form of WPA present in solutions at physiological conditions. The same is observed for solution with addition of WSiA, but here the traces of the parent WSiA Keggin anion (WSiA, pH 1) are also noticed, in accordance with higher stability of WSiA.[34]

3.2. The in vitro influence of $Na_2WO_4 \times 2H_2O$ and polyoxo-metalates on Na^+/K^+-ATPase activity

The separate influence of $Na_2WO_4 \times 2H_2O$, WSiA and WPA on SPM and commercial porcine cerebral cortex Na^+/K^+-ATPase activity was investigated in the concentration range from 1×10^{-8} to 1×10^{-3} mol/l, by in vitro exposure to the enzymes. The results show that increasing concentrations of investigated compounds induce inhibition of enzymatic activity in a concentration-dependent manner in all cases (Figs. 5a and b). The dependence of enzyme activity, expressed as a percentage of the control value (obtained without inhibitor), on inhibitor concentration fits a sigmoidal function (Eq. (1)) for both enzymes.

$$y = \frac{A_1 - A_2}{1 + (x/x_0)^p} + A_2 \quad (1)$$

where x is inhibitor concentration in mol/l, x_0 is equal to IC_{50} value and y is enzyme activity (percentage of control).

The inhibition parameters, the concentrations of investigated compounds with capability to inhibit 50% of the enzyme after given exposure time (IC_{50} values) and Hill coefficient, n_H, determined using the Hill method (Eq. 2) (Fig. 5a(inset) and 5b(inset)) are summarized in Table 1,

$$\log\left(\frac{\%\ activity}{100 - \%\ activity}\right) = -n\log[I] + n\log IC_{50} \quad (2)$$

where n is Hill coefficient and [I] is inhibitor concentration.

It is clearly apparent that in both cases (Figs. 5a and b) inhibitor efficiency of polyoxometalates and Na_2WO_4 is quite different. At the concentration of 1×10^{-5} mol/l polyoxometalates inhibit both SPM and commercial Na^+/K^+-ATPase up to 75%, while the effect of the same concentration of Na_2WO_4 on the enzyme activity is negligible. IC_{50} (10 min) of the enzyme activity is achieved at $(3.6 \pm 0.5) \times 10^{-6}$ mol/l of WSiA for SPM Na^+/K^+-ATPase, $(3.8 \pm 0.3) \times 10^{-6}$ mol/l of WSiA for commercial Na^+/K^+-ATPase and $(3.0 \pm 0.4) \times 10^{-6}$ mol/l of WPA for SPM Na^+/K^+-ATPase, $(3.1 \pm 0.4) \times 10^{-6}$ mol/l of WPA for commercial Na^+/K^+-ATPase, while the same effect is observed at several orders of magnitude higher concentration of Na_2WO_4: $>1 \times 10^{-3}$ mol/l, for both enzymes (Figs. 5a and b) (Table 1).

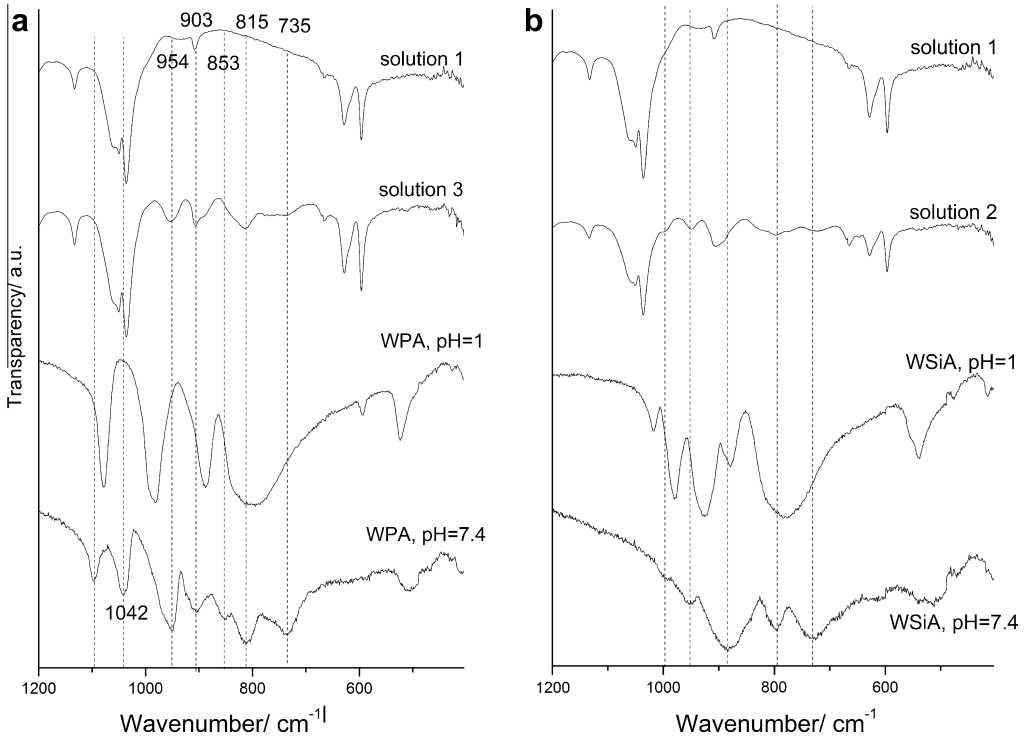

Figure 2. FTIR spectra of solid residua from: (a) solutions 1 and 3, WPA evaporated from 1 mmol/l water solution of pH 1, WPA evaporated from 1 mmol/l water solution of pH 7.4; (b) solutions 1 and 2, WSiA evaporated from 1 mmol/l water solution of pH 1, WSiA evaporated from 1 mmol/l water solution of pH 7.4.

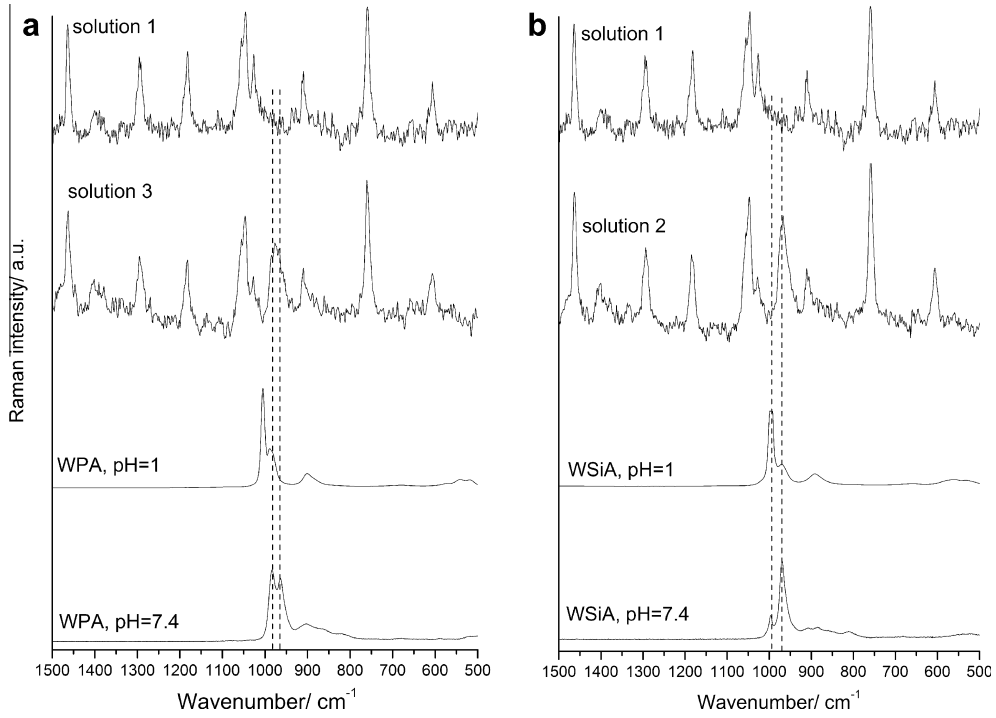

Figure 3. Raman spectra of solid residua from: (a) solutions 1 and 3, WPA evaporated from 1 mmol/l water solution of pH 1, WPA evaporated from 1 mmol/l water solution of pH 7.4; (b) solutions 1 and 2, WSiA evaporated from 1 mmol/l water solution of pH 1, WSiA evaporated from1 mmol/l water solution of pH 7.4.

3.3. The in vitro influence of $Na_2WO_4 \times 2H_2O$ and polyoxometalates on synaptic plasma membrane ecto-ATPase activity

The influence of $Na_2WO_4 \times 2H_2O$, WSiA and WPA on SPM ecto-ATPase activity was investigated by in vitro exposure to the enzyme in the same concentration range as Na^+/K^+-ATPase (from 1×10^{-8} to 1×10^{-3} mol/l). The results (Fig. 6) show that increasing concentrations of both investigated polyoxometalates (WSiA and WPA) induce inhibition of enzymatic activity in a concentration-dependent manner.

It is obvious that WSiA and WPA show similar inhibitor potencies. IC_{50} (10 min) value of the ecto-ATPase activity, according to

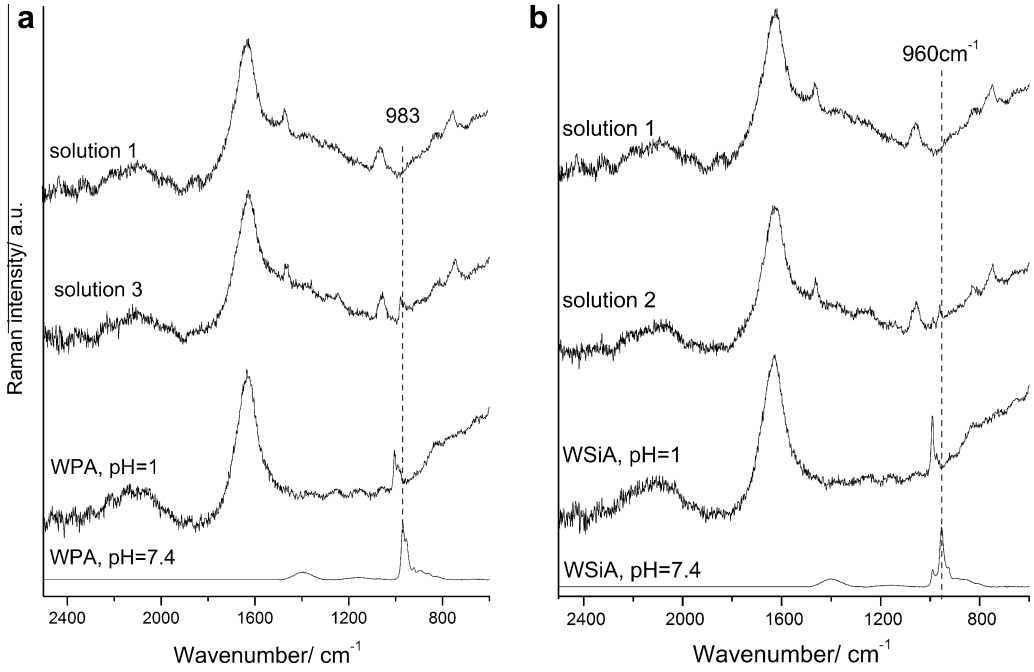

Figure 4. Raman spectra of: (a) solutions 1 and 3, 1 mmol/l water solution of WPA pH 1, 1 mmol/l water solution of WPA pH 7.4; (b) solutions 1 and 2, 1 mmol/l water solution of WSiA pH 1, 1 mmol/l water solution of WSiA pH 7.4.

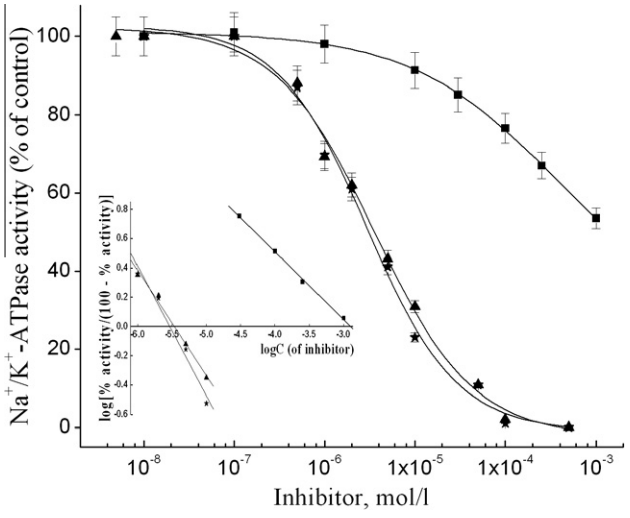

Figure 5a. The concentration-dependent inhibition of SPM Na^+/K^+-ATPase by $Na_2WO_4 \times 2H_2O$ (square), WSiA (triangle) and WPA (asterisk). The values are expressed as mean ± S.E.M. Inset: Hill analysis of inhibition of SPM Na^+/K^+-ATPase activity induced by $Na_2WO_4 \times 2H_2O$ (square), WSiA (triangle) and WPA (asterisk).

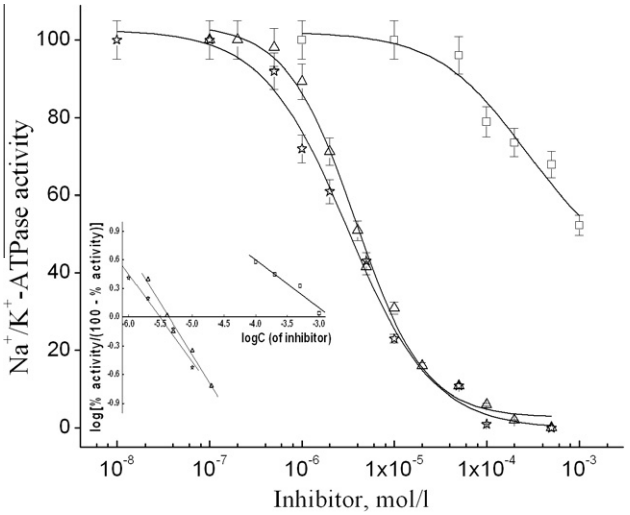

Figure 5b. The concentration-dependent inhibition of commercially available Na^+/K^+-ATPase by $Na_2WO_4 \times 2H_2O$ (open square), WSiA (open triangle) and WPA (open asterisk). The values are expressed as mean ± S.E.M. Inset: Hill analysis of inhibition of commercially available Na^+/K^+-ATPase activity induced by $Na_2WO_4 \times 2H_2O$ (open square), WSiA (open triangle) and WPA (open asterisk).

sigmoidal function, is achieved at $(3.4 \pm 0.1) \times 10^{-6}$ mol/l of WSiA and $(1.7 \pm 0.2) \times 10^{-6}$ mol/l of WPA (Table 1). On the contrary, $Na_2WO_4 \times 2H_2O$ up to concentration of 1 mmol/l did not cause a marked reduction of SPM ecto-ATPase activity (Fig. 6). The inhibition parameters, the IC_{50} (10 min) value and Hill coefficient n_H, determined using the Hill method (Eq. 2) (Fig. 6(inset)), are in good agreement with the values obtained by sigmoidal fitting (Table 1).

3.4. Effects of simultaneous exposure to 12-tungstosilicic acid and Na_2WO_4 on Na^+/K^+-ATPase activity

The effect of simultaneous exposure to combinations of WSiA and $Na_2WO_4 \times 2H_2O$ on the commercially purified Na^+/K^+-ATPase activity was studied by using the mixtures of the investigated compounds. The chosen concentrations of WSiA (1×10^{-6}–5×10^{-6} mol/l) and Na_2WO_4 (1×10^{-4}–1×10^{-3} mol/l) produced by individual exposure around 50% of inhibition or less. The inhibition obtained by exposure to several combinations was compared to the inhibition in the presence of each single inhibitor (Table 2). An antagonistic effect is defined as a statistically significant ($P < 0.05$) difference between the inhibitions caused by simultaneous exposure and mathematical sum of the values of WSiA and Na_2WO_4 inhibitions assayed separately, where the former is lower than latter. If simultaneously induced inhibition is equally to the calculated sum of the values of single enzyme activity reductions, the inhibition effect is additive.

Table 1
The inhibition parameters (IC_{50} values (10 min) and Hill coefficients n_H) of $Na_2WO_4 \times 2H_2O$, WSiA and WPA for SPM Na^+/K^+-ATPase, ecto-ATPase and commercially purified Na^+/K^+-ATPase, obtained by Hill analysis and fitting the experimental data by sigmoidal function

Compound	Enzyme	Sigmoidal fitting	Hill analysis	
		IC_{50} (10 min), mol/l	IC_{50} (10 min), mol/l	n_H
$Na_2WO_4 \times 2H_2O$	Na^+/K^+-ATPase	$>1.0 \times 10^{-3}$	1.3×10^{-3}	0.5 ± 0.1
	ecto-ATPase	—	—	—
	Commercial Na^+/K^+-ATPase	$>1.0 \times 10^{-3}$	1.5×10^{-3}	0.5 ± 0.1
WSiA	Na^+/K^+-ATPase	$(3.6 \pm 0.5) \times 10^{-6}$	3.4×10^{-6}	0.7 ± 0.1
	ecto-ATPase	$(3.4 \pm 0.1) \times 10^{-6}$	4.1×10^{-6}	1.4 ± 0.1
	Commercial Na^+/K^+-ATPase	$(3.8 \pm 0.3) \times 10^{-6}$	4.3×10^{-6}	1.1 ± 0.1
WPA	Na^+/K^+-ATPase	$(3.0 \pm 0.4) \times 10^{-6}$	2.9×10^{-6}	0.9 ± 0.1
	ecto-ATPase	$(1.7 \pm 0.2) \times 10^{-6}$	1.6×10^{-6}	1.0 ± 0.1
	Commercial Na^+/K^+-ATPase	$(3.1 \pm 0.4) \times 10^{-6}$	3.1×10^{-6}	0.9 ± 0.1

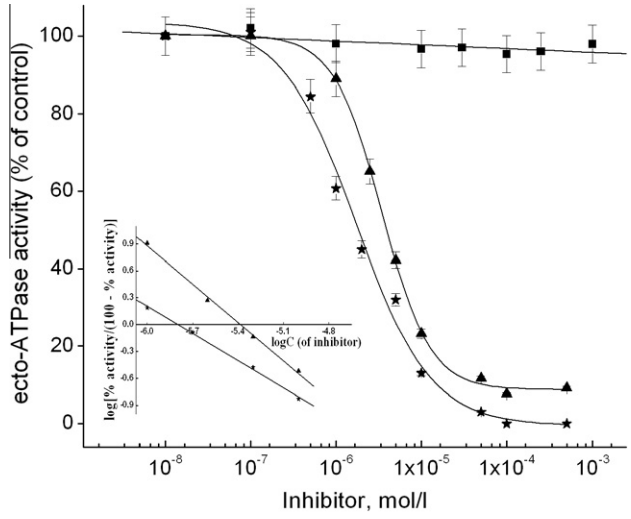

Figure 6. The concentration-dependent inhibition of SPM ecto-ATPase by $Na_2WO_4 \times 2H_2O$ (square), WSiA (triangle) and WPA (asterisk). The values are expressed as mean ± S.E.M. Inset: Hill analysis of inhibition of SPM E-NTPDases-ATPase activity induced by WSiA (triangle) and WPA (asterisk).

Table 2
Inhibition of commercial Na^+/K^+-ATPase activity induced by simultaneous exposure of $Na_2WO_4 \times 2H_2O$ and WSiA

$Na_2WO_4 \times 2H_2O$ (mol/l)	Inhibition (%)			
	WSiA (mol/l)			
	1×10^{-6} (10.7)	2×10^{-6} (28.7)	4×10^{-6} (49.0)	5×10^{-6} (58.0)
1×10^{-4} (21.0)	29.0	45.4	68.8	55.0
2×10^{-4} (26.4)	34.4	54.6	42.3	51.0
5×10^{-4} (32.1)	42.7	36.3	40.2	53.0
1×10^{-3} (47.7)	57.0	45.3	65.0	68.0

Values in paranthesis represent the enzyme inhibition obtained during separate exposure to $Na_2WO_4 \times 2H_2O$ or WSiA.
Note: Measurement error is ±5%.

As can bee seen from Table 2, during the simultaneous exposure of enzyme to WSiA and Na_2WO_4 in various concentration ratios, additive inhibition effects are observed at lower concentrations of the inhibitors (in the presence of WSiA (mol/l)/Na_2WO_4(mol/l) at concentrations: $1 \times 10^{-6}/1 \times 10^{-4}$, $1 \times 10^{-6}/2 \times 10^{-4}$, $1 \times 10^{-6}/5 \times 10^{-4}$, $1 \times 10^{-6}/1 \times 10^{-3}$, $2 \times 10^{-6}/1 \times 10^{-4}$, $2 \times 10^{-6}/2 \times 10^{-4}$ and $4 \times 10^{-6}/1 \times 10^{-4}$), while a statistically significant antagonistic inhibition is obtained for all higher concentrations of the inhibitors.

4. Discussion

In the present study we investigated the in vitro effect of two representative polyoxometalates (12-tungstosilicilic and 12-tungstophosphoric acids) on the activity of rat synaptic plasma membrane Na^+/K^+-ATPase and E-NTPDase. The obtained FT-IR and Raman spectra (Figs. 2–4) show that investigated POMs in the incubation medium (in the presence of commercial enzyme and its substrate-ATP) form monolacunary Keggin anion, the main molecular species present in the physiological conditions. The species arisis by removal of one WO unit from Keggin anion thus forming very unstable form which reacts with various ligands.

The obtained results (Table 1 and Fig. 5) show that WPA and WSiA exibit concentration-dependent inhibitory effect on the activity of Na^+/K^+-ATPase. It is obvious that sodium pumps from both model systems show similar sensitivity toward the investigated compounds. These results are in agreement with previously reported findings that POMs inhibit several nucleotide-dependent enzymes,[9,12,42,43] while the data about the influence of of polyoxotungstates on Na^+/K^+-ATPase activity are scant. The obtained results for Na^+/K^+-ATPase are consistent with previously published inhibition of Ca^{2+}-ATPase (belongs to, as well as Na^+/K^+-ATPase, E1E2 ATPase) by vanadium coordination complexes, and the influence of decavanadate on Ca^{2+}-ATPase and Na^+/K^+-ATPase activity. The obtained IC_{50} values indicate that polyoxotungstates are more potent inhibitors than the vanadium coordination complexes (approximately 10–100 times), while decavanadate IC_{50} values are lower from one to two orders of magnitude.[12,44]

The obtained results (Table 1 and Fig. 6) show that activity of SPM E-NTPDase was inhibited in dose-dependent manner by WPA and WSiA in the investigated concentration range. The obtained sensitivity of ecto-ATPase toward WPA and WSiA (Table 1) confirms previously reported findings that polyoxometalates are generally more potent than standard E-NTPDase inhibitors.[7] Although three different isoforms of rat E-NTPDase have been known (NTPDase 1, 2 and 3),[7] the obtained monophasic inhibition curves (Fig. 6) do not indicate the heterogeneity of the polyoxotungstate binding sites, which is in agreement with previously published findings that $H_3PW_{12}O_{40}$ is a non-selective inhibitor of all three rat recombinant NTPDases.[7] In comparison with previously investigated inhibitors (different metal ions, decavanadate),[12,17,39] the investigated polyoxotungstates are a few orders of magnitude more potent inhibitors of SPM E-NTPDase. Unlike the polyoxotungstates, $Na_2WO_4 \times 2H_2O$ did not affect ecto-ATPase activity up to concentration 1 mmol/l (Fig. 6). On the other side, $Na_2WO_4 \times 2H_2O$ inhibited SPM and commercial Na^+/K^+-ATPase at concentration higher than 1×10^{-5} mol/l, although with lower inhibitor efficiency than the polyoxotungstates (IC_{50} $>1 \times 10^{-3}$ mol/l) (Fig. 5), suggesting that orthotungstate, WO_4^{2-}, (similarly to orthovanadate) probably affects phosphorylation step in the enzyme cycle of P-type ATPase.[42,45,46]

The additive inhibition of commercial Na^+/K^+-ATPase activity obtained by simultaneous exposure to low concentrations of Na_2WO_4 and WSiA (Table 2) suggests that both inhibitors compete for the same set of inhibitory binding sites and there is an excess of these sites over the concentration ranges examined.[47] The observed antagonistic inhibition, in the presence of mixtures of the inhibitors at higher concentrations (Table 2), indicates effectively competing for a limited number of inhibitory binding sites on Na^+/K^+-ATPase.[47] These effects might be assigned to binding the polyoxotungstates, due to analogy in charge to ATP,[7] and Na_2WO_4, probably affecting phosphorylation step because of similarity to orthophosphate and orthovanadate,[42,45,46,48] to the same, that is active site of this P-type ATPase.

It could be summarized that the investigated polyoxotungstates are potent inhibitors of the chosen ATPases and belong to the most potent E-NTPDase inhibitors described to date. Considering the role of Na^+/K^+-ATPase and E-NTPDase in normal cell functioning as well as in the development of pathological states, the inhibition of the selected enzymes by POMs may contribute to (in vitro and in vivo) observed anti-cancer, anti-viral and anti-bacterial activity of polyoxometalates.

Acknowledgment

This work was financially supported by Ministry of Science and Technological Development of the Republic of Serbia (Projects No. 172023 and 172043).

Supplementary data

Supplementary data (Infrared and Raman wave numbers for the solid residua of the investigated solutions) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.10.008.

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