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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Polyoxometalates as potent inhibitors for acetyl and butyrylcholinesterases and as potential drugs for the treatment of Alzheimer's disease

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Abstract Polyoxometalates (POMs) show significant importance in medicine due to their enzyme inhibition, antiviral and anticancer properties. In this study, some polyoxotungstates were identified as potent inhibitors of acetyl and butyrylcholinesterases. Compounds $[H_2W_{12}O_{42}]^{10-}$ and $[TeW_6O_{24}]^{6-}$ have the most potent acetylcholinesterase activity, exhibiting IC_{50} values of 0.29 ± 0.01 and 0.31 ± 0.01 μM, respectively. Whereas, compound $[(O_3PCH_2PO_3)_4W_{12}O_{36}]^{16-}$ was a potent and selective inhibitor of butyrylcholinesterase with IC_{50} value of 0.18 ± 0.05 μM. In general, POMs were found to be effective cholinesterase inhibitors in terms of efficiency as well as selectivity and represent non-classical cholinesterase inhibitors.

Keywords Acetylcholinesterase · Anti-Alzheimer · Butyrylcholinesterase · Enzyme Inhibition · Polyoxometalates

Introduction

Alzheimer's disease (AD) is the most common cause of dementia in elderly people (Samadi *et al*., 2011). It is characterized by profound memory impairments, emotional disturbance, and personality changes (Rouleau *et al*., 2011). The cholinergic hypothesis postulates that memory impairments in patients with AD result from a decrease in hippocampus and cortical levels of the neurotransmitter acetylcholine (Komloova *et al*., 2011). Cholinesterase inhibitors are used in the treatment of Alzheimer's disease by increasing the level of acetylcholine in the brain (Rouleau *et al*., 2011). However, clinical use of cholinesterase inhibitors is often limited because of their adverse effects and loss of efficacy on long-term use (Tasso *et al*., 2011). The selective inhibitors of acetyl and butyrycholinesterase are reported to increase the level of acetylcholine in the brain and also reduce the formation of abnormal amyloid. However, the other approach is the development of dual inhibitors for acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) as BChE activity seems to correlate with AChE activity in AD and a cognitive improvement could be reached (Alptuzun *et al*., 2010). Cholinesterase inhibitors such as galanthamine, donepezil, rivastigmine, and huperzine increase the brain acetylcholine levels by preventing the degradation of released neurotransmitter, thereby enhancing neurotransmission at cholinergic synapses (Marco *et al*., 2004; Korabecny *et al*., 2010). Therefore, it is highly desirable to discover potent and highly selective inhibitors of AChE and BChE. Polyoxometalates (POMs) are anionic complexes of transition-metal oxide clusters, possessing extremely rich diversity in composition, structure and electronic properties (Acerete *et al*., 1990; Kortz *et al*., 1994; Contant *et al*., 2007; Dong *et al*., 2011b). Some of the POMs also exhibit high thermodynamic and kinetic stability in aqueous solutions at biologic pH (Sarafianos *et al*., 1996; Dong *et al*., 2011b). Some features of POMs namely polarity, surface charge distribution, and shape can be tuned at the molecular level to enhance the selectivity and reactivity of POMs towards target proteins. In addition to well-developed applications in catalysis, separations, analysis, and as electron-dense

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imaging agents, POMs are emerging as useful materials for a variety of potential applications in the biologic and medical fields (Fukuma *et al*., 1991; Barnard *et al*., 1997; Dan *et al*., 2002; Dong *et al*., 2011b; Geisberger *et al*., 2011a; Guo *et al*., 2011; Sartorel *et al*., 2011). They possess potential antibacterial (Inoue *et al*., 2006a; Inoue *et al*., 2006b), anticancer (Dong *et al*., 2011a), antiviral (De Clercq, 1995; Damonte, 1996; De Clercq, 1997, Shigeta *et al*., 2006; Flutsch *et al*., 2011), and enzyme inhibition (Sarafianos *et al*., 1996; Judd *et al*., 2001; Sun *et al*., 2010; Flutsch *et al*., 2011) properties. Detailed anticancer properties of POMs have been reviewed in an article published by Yanagie *et al*. (2006). Several research groups have reported detailed studies on the interaction between POMs and their target proteins. In in vitro and molecular modeling studies, it was found that polyanion $[\alpha\text{-}PTi_2W_{10}O_{40}]^{7-}$ exhibits potent anti-SARS (severe acute respiratory syndrome) activity (Hu *et al*., 2007) as it binds in the active site of the enzyme. In another study (Prudent *et al*., 2008, 2010), it was observed by in vitro assays and molecular modeling studies that POMs inhibited the protein kinase CK2 enzyme with IC_{50} values in a nanomolar range. Until now, POMs are the most potent and selective inhibitors of NTPDases enzymes (Müller *et al*., 2006; Kohler *et al*., 2007; Wall *et al*., 2008). Recently, there is an increasing interest in developing nanomedicine for the treatment of cancer using chitosan-POMs (Geisberger *et al*., 2011a, b; Guo *et al*., 2011). In a recent study (Geng *et al*., 2011), it was observed that POMs are the potential inhibitors of the aggregation of amyloid β peptides which is associated with AD. Polyoxometalates have been investigated as inorganic drug candidates because of their high solubility in water and high structure selectivity on biologic targets (Hu *et al*., 2007; Prudent *et al*., 2008, 2010). The objective of present study was to investigate the potential of novel polyoxotungstates as inhibitors of acetyl and butyrylcholinesterases. All the tested compounds were potent inhibitors of the cholinesterases, some of the investigated compounds have IC_{50} values in a submicromolar range and the inhibitory mode of the POMs is non-competitive.

Materials and methods

Acetylcholinesterase (AChE) (EC 3.1.1.7, type VI-S from Electric eel), butyrylcholinesterase (BChE) (EC 3.1.1.8, from horse serum), acetylthiocholine iodide (ATCI), *S*-butyrylthiocholine chloride (BTCCl), 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), neostigmine methylsulfate, and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich (Steinheim, Germany). Chemicals used in the synthesis of POMs were obtained commercially and used without additional purification. For the cation exchange, Dowex 50WX8 (AppliChem) was used.

General procedures for the synthesis of polyoxometalates

The hydrated salts of the polyoxoanions $[H_2W_{12}O_{40}]^{6-}$ (**1**), $[P_6W_{18}O_{79}]^{20-}$ (**2**), $[P_8W_{48}O_{184}]^{40-}$ (**3**), $[(O_3POPO_3)_4W_{12}O_{36}]^{16-}$ (**4**), $[(O_3PCH_2PO_3)_4W_{12}O_{36}]^{16-}$ (**5**), $[H_2W_{12}O_{42}]^{10-}$ (**6**), and $[TeW_6O_{24}]^{6-}$ (**7**) were synthesized according to the procedures described below and identified by FT-IR, NMR, and AA spectroscopy.

$Na_6[H_2W_{12}O_{40}]\cdot 2H_2O$ (**Na-1**): In order to obtain the compound, sodium ion-exchange through a column of Dowex 50WX8 was performed on the commercially available ammonium (meta)-tungstate $(NH_4)_6[H_2W_{12}O_{40}]$ (Fluka). The addition of ethanol to the resulting solution led to the precipitation of the final product.

$Na_{20}[P_6W_{18}O_{79}]\cdot 37H_2O$ (**Na-2**): The compound was synthesized according to the literature procedure (Acerete *et al*., 1990). Slow addition of glacial acetic acid (8.4 mL) to a solution of 50 g of $Na_2WO_4\cdot 2H_2O$ and 3.5 mL of 85 % H_3PO_4 in 50 mL of water, followed by evaporation at 100 °C to a final volume of ca. 50 mL gives upon cooling a good yield (~90 % based on Na_2WO_4) of **Na-2** as a white crystalline precipitate.

$Na_{33}H_7[P_8W_{48}O_{184}]\cdot 92H_2O$ (**Na-3**): The acidic sodium salt of $[P_8W_{48}O_{184}]^{40-}$, **NaH-3**, was obtained from $K_{28}Li_5H_7[P_8W_{48}O_{184}]\cdot 92H_2O$ (prepared as reported previously (Contant *et al*., 2007) by a similar ion-exchange procedure as described for **Na-1**.

$Na_{16}[(O_3POPO_3)_4W_{12}O_{36}]\cdot 38H_2O$ (**Na-4**) and $Na_{16}[(O_3PCH_2PO_3)_4W_{12}O_{36}]\cdot 16H_2O$ (**Na-5**): The compounds were synthesized according to the literature procedure (Kortz *et al*., 1994) by addition of 50 mL of 0.5 M sodium diphosphate for **Na-4** or methylenediphosphonic acid for **Na-5** to 150 mL of 0.5 M sodium tungstate solution in H_2O at room temperature, followed by adjustment of pH to 4 for **Na-4** or 6 for **Na-5** with 12 M HCl. The addition of dimethyl sulfoxide to the resulting solutions led to the precipitation of the final products.

$Na_{10}[H_2W_{12}O_{42}]\cdot 27H_2O$ (**Na-6**): 5 g of $Na_2WO_4\cdot 2H_2O$ was dissolved in 10 ml H_2O then HCl (~10 %) was added with magnetic stirring until pH 7.4. Then, the resulting solution was filtered and allowed to crystallize at room temperature. Colorless crystals of the title compound were obtained after several days, filtered off, and air dried.

$Na_6[TeW_6O_{24}]\cdot 22H_2O$ (**Na-7**): The compound was synthesized according to the modified literature procedure. 5.00 g of $Na_2WO_4\cdot 2H_2O$ (15.15 mmol) and 0.60 g of $Te(OH)_6$ were dissolved in 100 ml H_2O. The pH was adjusted to 5.0 by adding several drops of 1 M HCl. The resulting solution was heated at 100 °C until 75 % of

volume. Then, it was cooled to room temperature, filtered, and allowed to crystallize at room temperature. Colorless crystals of **Na-7** were obtained after ca. 1 week, filtered off, and air dried.

Enzyme assays and inhibition studies

Assays of acetyl and butyrylcholinesterases were performed according to the Ellman's method described previously (Ellman *et al*., 1961). The assays in 96-microtiter-well plates were conducted with some modifications as in Ingkaninan *et al*. (2003). Briefly, assays of the enzyme inhibition activities of acetyl and butyrylcholinesterases were performed as follows. The reaction mixtures (100 µl), containing 20 µl of buffer (containing: 50 mM Tris–HCl buffer (pH 8.0), 0.1 M NaCl, 0.02 M $MgCl_2{\cdot}6H_2O$), 50 µl of 3 mM DTNB, and 10 µl of test compounds in water were added. Then, 10 µl of AChE or BChE (0.5 and 3.4 U per mg, respectively) in 50 mM Tris–HCl buffer containing 0.1 % (w/v) BSA (pH 8) was added and incubated at 25 °C for 10 min. Reaction was started by adding 10 µl of 10 mM ATCI for AChE or butyrylthiocholine chloride for BChE assay and again incubated for 15 min at 25 °C. Initially, the compounds were screened at 1 mM concentrations. All the investigated compounds exhibited more than 50 % inhibition at 1 mM concentrations. Therefore, six to seven different concentrations of each test compound were used to measure the inhibitory potency for AChE and BChE. Each concentration was tested in triplicates in three independent assays. The change in absorbance was measured at 405 nm using a micro plate reader (Bio-Tek ELx 800^{TM}, Instruments, Inc. USA). IC_{50} values were calculated by means of a non-linear curve fitting program PRISM 5.0 (GraphPad, San Diego, California, USA). A parallel control with no inhibitor in the mixture allowed adjusting activities to be measured at various times. Neostigmine and POMS were dissolved in water and then diluted in Tris hydrochloride buffer (pH 8.0) containing 0.1 M sodium chloride and 0.02 M magnesium chloride to provide a final concentration range.

Kinetic studies of AChE and BuChE

Kinetic studies of AChE and BuChE were performed by Ellman's method as described above. The most potent inhibitor of AChE **Na-6** was selected for the determination of inhibitory mechanism of AChE and BuChE. Kinetic characterization of the hydrolysis of ATCI for AChE or BTCCl for BChE was carried out spectrometrically at 405 nm. A parallel control was run with the assay solution without inhibitor. The inhibition was evaluated by Lineweaver–Burk plot for inhibitor concentration (0, 0.05 and 0.15 µM) and substrate concentrations range between 0.1 and 0.4 mM for ATCI and BTCCl. The type of inhibition was calculated from the Michaelis–Menten equation by Lineweaver–Burk plot (double reciprocal plot). Graphs were plotted by means of PRISM 5.0 (GraphPad, San Diego, California, USA).

Results and discussion

Seven polyoxotungstates (**1**–**7**) having different size, charge, and shape were synthesized as hydrated salts and investigated for their potency to inhibit acetylcholinesterase and butyrylcholinesterase. Enzyme inhibition experiments were performed with aqueous solutions of compounds dissolved in 50 mM Tris hydrochloride buffer (pH 8.0). The results are summarized in Table 1. All the compounds showed high activities, and few of them exhibited good selectivity for AChE and BChE inhibition. Compounds **Na-6** (IC_{50} 0.29 ± 0.01) and **Na-7** (IC_{50} 0.31 ± 0.01) were found to be the most potent inhibitors of acetylecholinesterase. All other compounds **Na-1**–**Na-5** showed significant activities on AChE and BChE. Compound **Na-2** was least active on both enzymes. In general,

Table 1 Potency of polyoxotungstates as inhibitors of AChE and BChE obtained by spectrophotometric assays

No.	Formula of polyoxoanion	Charge at pH 8.0	Stability at pH 8.0	M_r (g/mol) of anions	IC_{50} (µM) ± SEM	
					AChE	BChE
Na-1	$[H_2W_{12}O_{40}]^{6-}$	**−6**	**+**	2848.2	2.30 ± 0.44	1.56 ± 0.46
Na-2	$[P_6W_{18}O_{79}]^{20-}$	**−20**	**+**	4759.1	8.71 ± 0.6	1.71 ± 0.82
Na-3	$[P_8W_{48}O_{184}]^{40-}$	**−40**	**+**	12016.5	4.47 ± 0.2	0.52 ± 0.04
Na-4	$[(O_3POPO_3)_4W_{12}O_{36}]^{16-}$	**−16**	**+**	3478.0	3.51 ± 1.84	0.18 ± 0.05
Na-5	$[(O_3PCH_2PO_3)_4W_{12}O_{36}]^{16-}$	**−16**	**+**	3470.1	5.04 ± 1.06	0.18 ± 0.05
Na-6	$[H_2W_{12}O_{42}]^{10-}$	**−10**	**+**	2880.2	0.29 ± 0.01	0.57 ± 0.09
Na-7	$[TeW_6O_{24}]^{6-}$	**−6**	**+**	1614.7	0.31 ± 0.01	0.46 ± 0.01

IC_{50} values represent the concentration of inhibitor required to decrease enzyme activity by 50 % and are the means of three independent measurements, each performed in triplicate (*SEM* standard error of the mean)

POMs were found to be more potent on BChE than AChE. The activities of POMs **Na-3**–**Na-7** on BChE were in nanomolar range. The mechanism of inhibition of POMs was determined for AChE and BChE using the most potent compound **Na-6**. Figure 1 shows Lineweaver–Burk plots for compound **Na-6** for AChE and BChE. Both of the plots visualize the non-competitive mechanism of inhibition. It was also evident from the previous studies that interaction of POMs with proteins is electrostatic in nature (Judd *et al*., 2001; Zhang *et al*., 2007). Therefore, it is expected that POMs bind electrostatically to the AChE and BChE and change the active site of the enzyme, which results in non-competitive inhibition of the enzymatic activity.

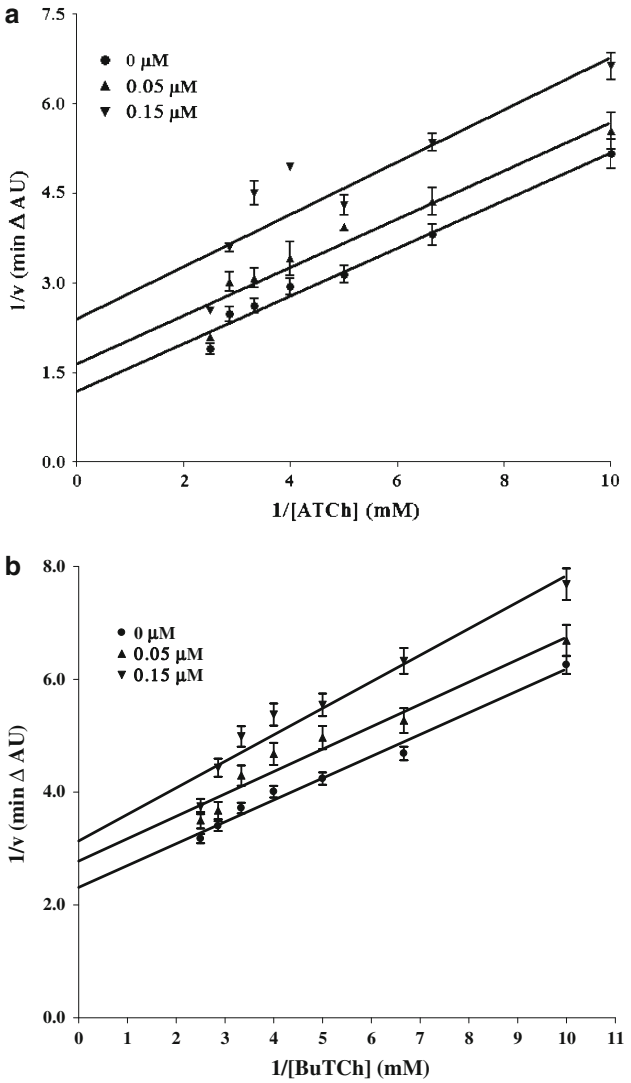

Fig. 1 Steady state inhibition by **Na-6** of (**a**) AChE hydrolysis of ATCI and (**b**) BChE hydrolysis of BTCCl. Figure shows the Lineweaver–Burk plots of the reciprocal values of initial velocities versus the reciprocal values of seven fixed ATCI and BTCCl concentrations, in the absence and in the presence of 0.05 and 0.15 μM concentrations of inhibitor, **Na-6**. The plots showed non-competitive type of inhibition for both AChE and BChE

Conclusion

In conclusion, we identified polyoxotungstates as a novel class of acetyl and butyrylcholinesterase inhibitors. All of the tested compounds showed high activities, and few of them exhibited good selectivity for AChE and BChE inhibition. To the best of our knowledge, it is the first study which shows that POMs can inhibit cholinestrases enzymes. In future, in vivo studies can be performed by encapsulation of POMs into non-toxic matrices like chitosan which is an effective drug carrier with high biocompatibility. We hope that this study will prompt the design and screening of more POMs as therapeutic agents for Alzheimer's disease.

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