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Alkynyl and β-ketophosphonates: Selective and potent butyrylcholinesterase inhibitors

Valeria Cavallaro *, Yanina F. Moglie *, Ana P. Murray, Gabriel E. Radivoy

Instituto de Química del Sur, INQUISUR (CONICET-UNS), Departamento de Química, Universidad Nacional del Sur, Avenida Alem 1253, 8000 Bahía Blanca, Argentina

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ABSTRACT

A series of thirty-three alkynyl and β-ketophosphonates were evaluated for their in vitro acetyl- and butyryl-cholinesterase (AChE and BChE) inhibitory activities using Ellman's spectrophotometric method. None of the examined compounds inhibited AChE activity at tested concentrations while twenty-nine of them showed significant and selective inhibition of BChE with IC_{50} values between 38.60 μM and 0.04 μM. In addition, structure-activity relationships were discussed. The most effective inhibitors were the dibutyl *o*-methoxyphenyl alkynylphosphonate **3dc** and dibutyl *o*-methoxyphenyl β-ketophosphonate **4dc**. Activities of most potent compounds were also compared with a commercial organophosphorus compound. These results could inspire the design of new inhibitors with stronger activity against BChE.
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1. Introduction

Cholinesterases (ChEs) belong to the serine hydrolase family and therefore possess an αβ-hydrolase fold structure [1]. Their main function is to hydrolyze the neurotransmitter acetylcholine (ACh) to choline and acetic acid, terminating impulse transmission at cholinergic synapses, which is an essential process for the restoration of cholinergic neurons [2]. There are two major forms of ChEs in vertebrates: acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BChE, EC 3.1.1.8), also called pseudo-cholinesterase or nonspecific cholinesterase [3]. These two enzymes share about 54% amino acid sequence identity, but differ in their specificity towards various substrates and inhibitors [4]. Their crystal structures have revealed similar architecture, with one catalytic triad located at the bottom of a deep gorge [5,6]. Compounds with the ability of reversibly or irreversibly inhibit ChEs, restrict the enzymes from breaking down ACh, increasing the amount of neurotransmitter available for neuronal and neuromuscular transmission. ChEs inhibitors have been successfully used in the treatment of various diseases, such as myasthenia gravis, Alzheimer's disease (AD) and some other dementias [7–9], parasitic infections, glaucoma, obstipation or to antagonize muscle relaxation [6,10]. Other important applications include their use as insecticides, herbicides, antifungal agents and chemical warfare nerve agents [11–13].

Organophosphorus compounds (OPs) are some of the most powerful and well-studied ChEs irreversible inhibitors [6,14,15]. The chemical warfare agents VX and Sarin, the insecticide malathion and the potential drug for AD metrifonate are examples of this group of inhibitors. The electrophilic phosphorous atom of OPs can react with the hydroxyl group of the serine residue in the active site through nucleophilic attack, which leads to the enzymes' phosphorylation and deactivation. Even though most of these compounds are highly toxic, investigations on the ChEs inhibition of OPs is widely appreciated [16–18]. Less toxic forms of these agents have the potential to serve as therapeutic alternatives. In addition, insect and fungus chemical resistance requires new insecticides for agricultural uses. Therefore, the search for new and potent ChEs inhibitors with phosphorus moiety in its structure is an ongoing quest mobilizing the scientific community around the world.

Phosphonates are well known as non-hydrolyzable analogs of biological phosphates and are of widespread interest in synthetic organic chemistry [19]. In a previous work some of us reported a new simple and mild protocol for the direct synthesis of alkynylphosphonates and β-ketophosphonates from terminal alkynes catalyzed by Cu_2O or copper nanoparticles supported on zinc oxide (CuNPs/ZnO), respectively [20,21]. In this study, we present our results on the evaluation of their AChE and BChE inhibitory activities. In addition, structure-activity relationships are discussed. Since differences in the inhibition potency of organophosphorus agents are a manifestation of differing molecular properties of the inhibitors involved in the interaction with the active site of the enzyme, we were interested in studying the inhibition potency

* Corresponding authors.
E-mail addresses: valeria.cavallaro@uns.edu.ar (V. Cavallaro), ymoglie@uns.edu.ar (Y.F. Moglie).

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of a wide variety of new phosphonates, whose synthesis and biological activity is reported here for the first time. Moreover, activities shown by the most potent of these phosphonates were compared with that of a commercial organophosphorus compound.

2. Results and discussion

A library of twenty previously reported alkynyl- and β-ketophosphonates were tested for their ChEs inhibition using Ellman's method [22]. The effectiveness of the inhibitors is expressed as IC_{50}, representing the concentration of an inhibitor required for 50% inhibition of the enzyme. For this study, compounds with IC_{50} values over 50 μM were considered to be inactive. Tacrine, a well-known ChEs inhibitor and FDA approved drug for AD treatment, was used as reference inhibitor [23].

We also prepared and tested a series of thirteen new phosphonates (entries 5, 10, 15–19, Table 1, and entries 4, 8–10, Table 2), which allowed us to study the influence of the substitution pattern at the aromatic moiety on enzyme inhibition. Alkynylphosphonates were prepared in good yields (64–97%) by Cu_2O-catalyzed cross-coupling of a dialkylphosphite and the appropriate terminal alkyne, using acetonitrile as solvent at 70 °C (Scheme 1). On the other hand, β-ketophosphonates were obtained also in good yields (60–97%) by direct reaction of a dialkylphosphite and terminal alkynes catalyzed by CuNPs/ZnO using acetonitrile as reaction media at 70 °C (Scheme 2).

All compounds, except **3jb** and **3mb**, displayed potent inhibitory activity against BChE at micromolar and sub-micromolar range (IC_{50} = 38.60–0.04 μM). The inhibition was found to be highly selective since none of the phosphonates were active against AChE at tested concentrations. BChE inhibitory activity results are summarized in Tables 1 and 2.

In order to explain the selectivity of our phosphonates towards BChE main differences in the ligand binding sites in both enzymes should be considered, i.e., active site and peripheral anionic site (PAS). It is known that the active site size in BChE is larger than in AChE [5,24,25]. Therefore, BChE can accommodate ligands with larger molecular structures. In addition, in the PAS and along the gorge structure, there are many hydrophobic amino acids, being predominantly aromatic in AChE and mainly aliphatic in BChE [26,27]. Since tested compounds could be considered of medium size when compared with other inhibitors, the larger active site in BChE does not provide an explanation for the observed selectivity. On the contrary, it could be hypothesized that the interactions between our compounds and the aromatic residues in AChE's PAS would be reducing favorable interactions with the active site residues, consequently avoiding AChE inhibition.

It is important to note that, unlike results obtained in this work, several authors have reported that even though OPs behave more selectively towards BChE, most of them elicit inhibition of AChE [28–31].

2.1. Evaluation of alkynylphosphonates as BChE inhibitors

Based on the IC_{50} values obtained and the nature of R^1 and R^2 substituents in the alkynylphosphonates, some trends in terms of structure-activity relationships could be envisaged (Table 1). A clear difference in activity was observed between compounds **3aa** and **3ab** (R^2 = Me and Et, respectively) when compared with compound **3ac** (R^2 = Bu), the latter being six/ten times more potent than its congeners. This variation on activity could be attributed to the increase in the hydrophobicity of the R^2 chain. Comparable results were obtained by Nakayama and co-workers for dialkylphosphates where, increasing the length of the *n*-alkyl moiety of substituents attached to the oxygen produced an increase in their affinity with ChEs [32].

According to the obtained IC_{50} values for compounds **3ab** and **3ib** it could be assumed that BChE active site can interact positively with both alkyl chains and phenyl groups attached to the carbon-carbon triple bond. These results led us to think whether these interactions would depend on the alkyl chain length. Therefore, we synthetized compound **3jb** with an alkyl chain four carbon atoms larger, but resulted to be inactive against BChE. Then, it could be hypothesized that compound **3jb** with a larger R^1 substituent could not fit well in the enzyme active site, consequently diminishing its activity.

With regard to electronic effects, electron-withdrawing groups in the phenyl ring showed a detrimental effect on inhibition as it is illustrated with the IC_{50} values of compounds **3gb** and **3hb** (27.60 μM and 14.50 μM, respectively) when compared with the unsubstituted phosphonate **3ab**. Moreover, a chlorine atom

Table 1
BChE inhibitory activity of alkynylphosphonates expressed as IC_{50} (μM).

Entry	R^1	R^2	Compound[a]	IC_{50} (μM)[b]
1	Ph (**1a**)	Me (**2a**)	**3aa**	6.60 ± 1.22
2	Ph	Et (**2b**)	**3ab**	10.10 ± 1.48
3	*m*-CH_3-Ph (**1b**)	Et	**3bb**	3.80 ± 0.77
4	*p*-CH_3-Ph (**1c**)	Et	**3cb**	4.70 ± 1.15
5	*o*-OCH_3-Ph (**1d**)	Et	**3db**	2.17 ± 0.47
6	*p*-OCH_3-Ph (**1e**)	Et	**3eb**	10.50 ± 2.14
7	*p*-$N(CH_3)_2$-Ph (**1f**)	Et	**3fb**	1.90 ± 0.11
8	*m*-Cl-Ph (**1g**)	Et	**3gb**	27.60 ± 4.98
9	*m*-CF_3-Ph (**1h**)	Et	**3hb**	14.50 ± 5.54
10	CH_3-$(CH_2)_5$- (**1i**)	Et	**3ib**	9.50 ± 0.81
11	CH_3-$(CH_2)_9$- (**1j**)	Et	**3jb**	>50.0
12	HO-CH_2- (**1k**)	Et	**3 kb**	20.40 ± 6.57
13	HO-$(CH_2)_2$- (**1l**)	Et	**3 lb**	4.50 ± 0.44
14	Cl-$(CH_2)_3$- (**1m**)	Et	**3mb**	>50.0
15	Ph	Bu (**2c**)	**3ac**	1.60 ± 0.30
16	*m*-CH_3-Ph	Bu	**3bc**	0.31 ± 0.04
17	*p*-CH_3-Ph	Bu	**3 cc**	4.50 ± 1.06
18	*o*-CH_3-Ph (**1n**)	Bu	**3nc**	1.52 ± 0.06
19	*o*-OCH_3-Ph	Bu	**3dc**	0.08 ± 0.01
20	3,4-OCH_3-Ph (**1o**)	Bu	**3oc**	0.21 ± 0.06
21	Tacrine	–	–	0.004 ± 0.001

[a] Reaction conditions: alkyne (0.5 mmol) added to a suspension of dialkyl phosphite (0.7 mmol) and Cu_2O (14 mol%) in MeCN (2 mL), stirred overnight at 70 °C under air.
[b] BChE inhibitory activity was measured in vitro by the spectrophotometric method developed by Ellman with slight modifications [20].

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