Bioorganic Chemistry 58 (2015) 48–52

Contents lists available at ScienceDirect

Bioorganic Chemistry

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

Salicylanilide diethyl phosphates as cholinesterases inhibitors

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ARTICLE INFO

Article history:
Received 18 August 2014
Available online 13 November 2014

Keywords:
Acetylcholinesterase
Butyrylcholinesterase
Enzyme inhibition
Organophosphate
Salicylanilide diethyl phosphate

ABSTRACT

Based on the presence of dialkyl phosphate moiety, we evaluated twenty-seven salicylanilide diethyl phosphates (diethyl [2-(phenylcarbamoyl)phenyl] phosphates) for the inhibition of acetylcholinesterase (AChE) from electric eel (*Electrophorus electricus* L.) and butyrylcholinesterase (BChE) from equine serum. Ellman's spectrophotometric method was used. The inhibitory activity (expressed as IC_{50} values) was compared with that of the established drugs galantamine and rivastigmine. Salicylanilide diethyl phosphates showed significant activity against both cholinesterases with IC_{50} values from 0.903 to 86.3 μM. IC_{50}s for BChE were comparatively lower than those obtained for AChE. All of the investigated compounds showed higher inhibition of AChE than rivastigmine, and six of them inhibited BChE more effectively than both rivastigmine and galantamine. In general, derivatives of 4-chlorosalicylic acid showed enhanced activity when compared to derivatives of 5-halogenated salicylic acids, especially against BChE. The most effective inhibitor of AChE was *O*-{5-chloro-2-[(3-bromophenyl)carbamoyl]phenyl} *O*,*O*-diethyl phosphate with IC_{50} of 35.4 μM, which is also one of the most potent inhibitors of BChE. *O*-{5-Chloro-2-[(3,4-dichlorophenyl)carbamoyl]phenyl} *O*,*O*-diethyl phosphate exhibited *in vitro* the strongest inhibition of BChE (0.90 μM). Salicylanilide diethyl phosphates act as pseudo-irreversible cholinesterases inhibitors.

1. Introduction

Acetylcholine (ACh) is a cholinergic neurotransmitter interacting with either nicotinic or muscarinic receptors thereby affecting function of postsynaptic cells. Signalling action of ACh is terminated by the action of acetylcholinesterase (AChE; E.C. 3.1.1.7) and butyrylcholinesterase (BChE; E.C. 3.1.1.8), which control ACh level by its hydrolysis. Both enzymes are widely distributed throughout the body; however, AChE remains the major cholinesterase within the human brain. BChE does not possess the same affinity for ACh as AChE does [1,2].

Inhibitors of cholinesterases (ChE) have been used in the treatment of various diseases, e.g., myasthenia gravis, Alzheimer's disease (AD) and some other dementias [3], parasitic infections [4], glaucoma, obstipation or to antagonize muscle relaxation [5]. Pesticides or chemical warfare nerve agents belong to their other applications [3,5].

The number of patients suffering from AD increased continuously during the last decades. One typical characteristic feature of AD is a decreased level of ACh. The changes result in a cognitive impairment. Based on cholinergic hypothesis, inhibition of ChE represents one of the major pharmacological interventions for AD. During the progress of AD, BChE activity is increased. Drugs which have been introduced into the treatment of AD are non-selective (rivastigmine) or AChE-selective inhibitors (galantamine or donepezil) [1,5]. In addition to the increased level of ACh, recent findings indicate that cholinesterase inhibitors can attenuate neuronal damage and protect them from cellular death, and therefore might affect AD pathogenesis and delay the progression. This mechanism seems to be complex [6].

Compounds based on phosphoric moieties are well-known anticholinesterases agents including substituted diethyl phenyl phosphates, e.g., paraoxon. They cause irreversible inhibition of both AChE and BChE [5,7]. Organophosphorus-based molecules act as inhibitors of the esteratic part of ChE by interaction with serine providing stable esters. Organophosphorus compounds create stable, covalently bound adducts with spontaneous dissociation once covalently connected with serine hydroxyl [3].

Organophosphates have been also investigated as potential insecticides [8–10], herbicides [11], or antifungal agents [12].

Abbreviations: ACh, acetylcholine; ATCh, acetylthiocholine; AD, Alzheimer's disease; AChE, acetylcholinesterase; BChE, butyrylcholinesterase; ChE, cholinesterases; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; TNB, 5-thio-2-nitrobenzoic acid.

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http://dx.doi.org/10.1016/j.bioorg.2014.11.005
0045-2068/

A long-acting irreversible inhibitor of AChE metrifonate (trichlorfon) has been evaluated as potential drug for the treatment of AD [13]. Metrifonate is a prodrug which is activated non-enzymatically into dichlorvos (2,2-dichlorovinyl dimethyl phosphate) [4].

Salicylanilide-like derivatives have exhibited a wide range of interesting biological activities [14–20]. Previously, two groups of salicylanilide derivatives have been reported as cholinesterases inhibitors – salicylanilide *N*-alkyl carbamates [21] and, more importantly, *O*,*O*-diethyl thiophosphates (phosphorothioates) which were described as potent inhibitors of both AChE and BChE with IC_{50} values in the micromolar range [22]. The fact that organophosphate pesticides acting *via* ChE inhibition are more toxic than their thioforms [3] inspired us to the evaluation of salicylanilide diethyl phosphates (oxygen-isosteres of salicylanilide diethyl thiophosphates [22]) against both AChE a BChE.

Salicylanilide diethyl phosphates (Fig. 1) were reported as potential antimicrobial agents against both drug-susceptible and resistant strains of *Mycobacterium tuberculosis*, atypical mycobacteria, Gram-positive bacteria and some fungal species. Additionally, they share alleviated cytotoxicity when compared to parent salicylanilides [23].

2. Materials and methods

2.1. Chemistry

The synthetic pathway for salicylanilide diethyl phosphates **1**–**27** (diethyl [(2-phenylcarbamoyl)phenyl] phosphates) was published previously by our group [23]. They were obtained by a quite simple procedure (Scheme 1). First, salicylanilides were prepared by the reaction of appropriate salicylic acids with anilines in the presence of phosphorus trichloride under microwave irradiation [14]. In the next step, salicylanilide triethyl ammonium salts generated *in situ* were esterified with diethyl chlorophosphate at ambient temperature [23].

2.2. Determination of IC_{50} for cholinesterases

The IC_{50} values were determined using the spectrophotometric Ellman's method, which is a simple, rapid and direct method to determine the SH and –S–S– group content in proteins [24]. This method is widely used for the evaluation of cholinesterase activity and screening the efficiency of ChE inhibitors. Cholinesterase activity is measured indirectly by quantifying the concentration of the 5-thio-2-nitrobenzoic acid (TNB) ion formed in the reaction between the thiol reagent 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) and thiocholine, a product of substrate hydrolysis (*i.e.*, acetylthiocholine, ATCh) by cholinesterases [25]. All of the tested compounds were dissolved in 0.01 M dimethyl sulphoxide and then diluted in demineralised water to 0.001 M and 0.0001 M. Ellman's method was modified slightly according to Zdrazilova et al. [26].

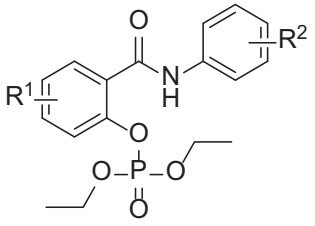

Fig. 1. Salicylanilide diethyl phosphates **1**–**27** (diethyl [2-(phenylcarbamoyl)phenyl] phosphates; R^1 = 4-Cl, 5-Cl, 4-Br; R^2 = 3-Cl, 4-Cl, 3,4-diCl, 3-Br, 4-Br, 3-F, 4-F, 3-CF_3, 4-CF_3).

Acetylcholinesterase was obtained from electric eel (*Electrophorus electricus* L.) and butyrylcholinesterase was from equine serum. Rivastigmine and galantamine were involved as reference drugs.

2.3. Investigation of inhibition type

Three salicylanilide diethyl phosphate derivatives (**15**, **24**, **27**; see Table 1) were used for investigation of mechanism of cholinesterases inhibition. For each of them three different concentrations of inhibitor were chosen according to their IC_{50} values. The purpose was to observe the effect of inhibitor on enzyme activity (A) in time. On this basis, it is possible to distinguish reversible and irreversible inhibition [27,28].

The enzyme activity was determined using spectrophotometric Ellman's method. Pursuant the procedure described in [29], the determination was performed subsequently: The reaction mixture containing phosphate buffer, AChE or BChE and chosen salicylanilide derivative (in one of the chosen concentrations) was prepared and intensively stirred. In given times (5, 10, 15, 20, 30, 40, 50, 60, 80, 240 and 1380 min), DTNB and ATCh were added to the sample withdrawn from reaction mixture, quickly mixed and absorbance was measured. Consequently the enzyme activity was determined. Based on knowledge of enzyme activity in absence of inhibitor (i.e. 100% activity), the percentages of residual enzyme activity in presence of inhibitor were calculated. Then the dependence of logarithm of percentage of residual enzyme activity (log % A) vs. time was constructed. Based on these kinetic data, it is possible to distinguish reversible, pseudo-irreversible and irreversible inhibition.

3. Results and discussion

3.1. Chemistry

Salicylanilides were obtained with the efficiency about 80–95%. The general yield of salicylanilide diethyl phosphates **1**–**27** (Table 1) ranged from 11% up to 78% [23]. Some comparatively low yields were caused by isolation and purification process, while the reactions were monitored repeatedly by thin layer chromatography till all reactions were complete (until 2 h for all compounds).

3.2. In vitro cholinesterases inhibition

The ability of the investigated salicylanilide derivatives **1**–**27** to inhibit AChE from electric eel and BChE from equine serum was screened *in vitro* using modified Ellman's method. The effectiveness of the inhibitors is expressed as IC_{50}, representing the concentration of an inhibitor required for 50% inhibition of the enzyme. The obtained results were compared with rivastigmine and galanthamine (Table 1). These standards were chosen due to their different structures. Rivastigmine is an acylating pseudo-irreversible carbamate inhibitor that inhibits AChE as well as BChE, while galanthamine acts as a non-acylating competitive reversible inhibitor. Furthermore, it modulates allosterically nicotinic ACh receptors. The choice of these drugs with different mechanism of action can provide relevant results.

With respect to the inhibition of cholinesterases, all tested phosphates **1**–**27** exhibited good inhibitory activity, with IC_{50} values from 0.903 to 86.3 μM (Table 1). Salicylanilide diethyl phosphates **1**–**27** could be divided into two groups based on their substitution and activity. **Group 1** includes derivatives of 5-bromosalicylic and 5-chlorosalicylic acids **1–18** and **Group 2** includes 4-chlorosalicylic acid derivatives **19**–**27**. In general, most of the tested compounds inhibited butyrylcholinesterase more effectively than acetylcholinesterase, sometimes by several fold; only for **1** are