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Salicylanilide diethyl phosphates as cholinesterases inhibitors

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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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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].

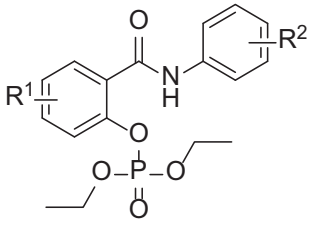


Fig. 1. Salicylanilide diethyl phosphates **1**–**27** (diethyl [2-(phenylcarbamoyl)phenyl] phosphates; R^1 = 4-Cl, 5-Cl, 4-Br; R^2 = 3-Cl, 4-Cl, 3,4-diCl, 3-Br, 4-Br, 3-F, 4-F, 3-CF_3, 4-CF_3).

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

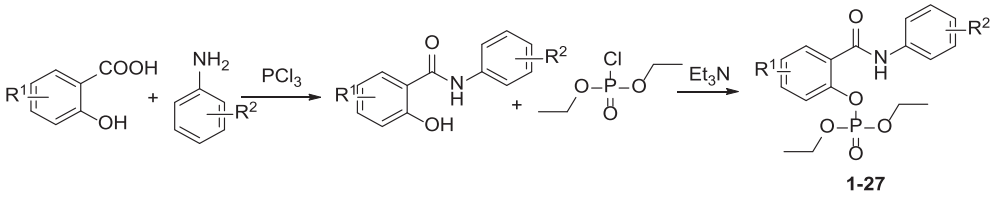
3.1. Chemistry

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



Scheme 1. Synthesis of diethyl [(2-phenylcarbamoyl)phenyl] phosphates **1–27** (Et_3N = triethylamine; R^1 for esters = 4-Cl, 4-Br, 5-Cl, R^2 = 3-Cl, 4-Cl, 3,4-diCl, 3-Br, 4-Br, 3-F, 4-F, 3-CF_3, 4-CF_3)

Table 1
IC_{50} values against acetylcholinesterase and butyrylcholinesterase.

Code	R	R^1	IC_{50} for AChE (μM)	IC_{50} for BChE (μM)	Selectivity to BChE
Group 1					
1	4-Br	3-F	69.8 ± 3.4	68.3 ± 3.7	1.0
2	4-Br	3-Cl	79.9 ± 0.3	24.4 ± 3.3	3.3
3	4-Br	4-Cl	45.5 ± 1.2	19.9 ± 0.8	2.3
4	4-Br	3,4-di-Cl	55.2 ± 0.3	49.6 ± 1.5	1.1
5	4-Br	3-CF_3	46.1 ± 1.9	14.3 ± 3.7	3.2
6	4-Br	4-CF_3	56.7 ± 0.9	10.3 ± 0.1	5.5
7	4-Br	3-Br	70.3 ± 1.7	18.0 ± 0.0	3.9
8	4-Br	4-Br	50.8 ± 1.2	13.0 ± 1.7	3.9
9	4-Br	4-F	53.7 ± 0.4	22.7 ± 1.1	2.4
10	4-Cl	3-Cl	64.3 ± 2.6	28.1 ± 2.5	2.3
11	4-Cl	4-Cl	60.9 ± 4.4	42.1 ± 1.0	1.4
12	4-Cl	3-F	61.1 ± 2.1	31.6 ± 1.0	1.9
13	4-Cl	4-F	48.5 ± 0.3	20.5 ± 0.1	2.4
14	4-Cl	4-Br	59.2 ± 0.7	40.2 ± 2.4	1.5
15	4-Cl	3,4-di-Cl	80.9 ± 2.2	40.3 ± 4.0	2.0
16	4-Cl	3-Br	63.3 ± 4.3	16.9 ± 0.3	3.7
17	4-Cl	3-CF_3	51.5 ± 1.8	24.8 ± 0.7	2.1
18	4-Cl	4-CF_3	**41.6 ± 0.3**	20.9 ± 1.1	2.0
Group 2					
19	5-Cl	3-Cl	**41.1 ± 0.5**	**3.68 ± 0.1**	11.2
20	5-Cl	3-Br	**35.4 ± 0.5**	**1.77 ± 0.1**	20.0
21	5-Cl	3-F	60.2 ± 3.5	11.5 ± 0.3	5.2
22	5-Cl	4-F	80.8 ± 5.9	**4.43 ± 0.2**	18.2
23	5-Cl	4-Br	55.1 ± 0.5	8.76 ± 0.5	6.3
24	5-Cl	4-Cl	60.9 ± 0.8	**5.01 ± 0.5**	12.2
25	5-Cl	3,4-di-Cl	45.5 ± 4.4	**0.903 ± 0.003**	50.4
26	5-Cl	3-CF_3	71.9 ± 7.2	**3.53 ± 0.05**	20.4
27	5-Cl	4-CF_3	86.3 ± 4.9	9.84 ± 0.06	8.8
Rivastigmine			501 ± 3.08	19.95 ± 0.20	–
Galantamine			4 ± 0.13	7.96 ± 0.59	–

AChE and BChE inhibition is expressed as the mean ± SD (n = 3 experiments). The best results for each enzyme are shown in bold.
Selectivity to BChE: IC50 for AChE/IC50 for BChE.

IC_{50}s for both enzymes comparable. Similarly, Kaboudin et al. [30] found that *S*-substituted-*O*,*O*′-diethyl phosphorothioates behaved more selectively towards BChE.

The most effective inhibitor of AChE is *O*-{5-chloro-2-[(3-bromophenyl)carbamoyl]phenyl} *O*,*O*-diethyl phosphate **20** with an IC_{50} of 35.4 μM, which is also the second-best inhibitor of BChE (IC_{50} = 1.77 μM) of all evaluated compounds. *O*-{5-Chloro-2-[(3,4-dichlorophenyl)carbamoyl]phenyl} *O*,*O*-diethyl phosphate **25** exhibited the best *in vitro* inhibition of BChE, with an IC_{50} lower than 1 μM (0.903 μM).

O-(5-Chloro-2-{[(4-trifluoromethyl)phenyl]carbamoyl}phenyl *O*,*O*-diethyl phosphate **27** was assayed as the least potent inhibitor of AChE (IC_{50} = 86.3 μM) and the least effective inhibitor of BChE is *O*-{4-bromo-2-[(3-fluorophenyl)carbamoyl]phenyl} *O*,*O*-diethyl phosphate **1** (IC_{50} = 68.3 μM).

The activities of phosphates **1–27** were compared with those of galantamine and rivastigmine which are clinically used for the treatment of dementia. All tested compounds **1–27** showed greater inhibition of AChE than rivastigmine but not galantamine, and six of them (**19**, **20**, **22**, **24–26**) inhibited BChE more effectively than both drugs.

Members of **Group 2** (5-chloro derivatives synthesized from 4-chlorosalicylic acid) are more effective inhibitors of BChE (IC_{50}s form 0.9 to 11.5 μM) than derivatives of **Group 1** (10.3–68.3 μM). Similar to the trend shown for acetylcholinesterase inhibition, there was no observed effect of salicylic ring substitution by 4-chlorine or 4-bromine on the power of inhibition. In general, there is no pronounced effect of aniline substitution by various substituents at different positions on both cholinesterase inhibitions, only particular aspects were identified. For both AChE and BChE, esters containing 4-substituted anilines exhibited higher activity then isomeric 3-substituted anilines in **Group 1**. The same relationship but only for BChE was found in **Group 2** with one exception (fluorinated molecules **21** and **22**). For inhibition of both ChE in **Group 1**, anilines bearing trifluoromethyl group confer a slightly better activity.

IC_{50}s of all derivatives **1–27** for AChE are within a comparatively narrow concentration range (35–86 μM). The situation for BChE is similar although there is a **Group 2** which contains more potent compounds. Based on literature [21] and our experiments (Section 3.2.1), we presume that this is a consequence of the mechanism of action – phosphorylation of serine localized in the active

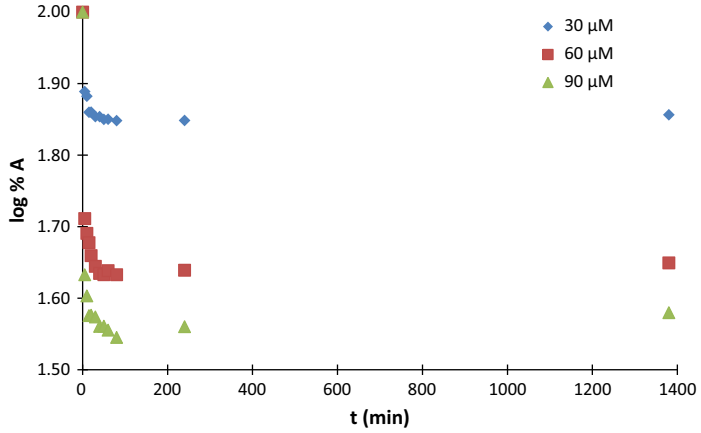


Fig. 2. The dependence log % *A* vs. time. Enzyme: acetylcholinesterase, derivative: **24**, concentration of inhibitor: 30 μM (rhomb), 60 μM (square), 90 μM (triangle).

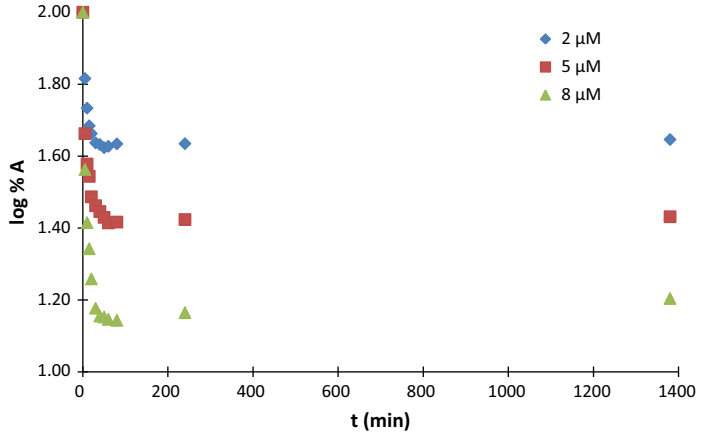


Fig. 3. The dependence log % *A* vs. time. Enzyme: butyrylcholinesterase, derivative: **24**, concentration of inhibitor: 2 μM (rhomb), 5 μM (square), 8 μM (triangle).

site of enzyme. For this action, the phosphate part of molecule is the key fragment of salicylanilide diethyl phosphates, while salicylanilide core serves mainly as a carrier of this phosphorylating moiety. Theoretically, the differences in IC_{50}s may consist in the fact that salicylanilide moiety influences probably the orientation and binding of the inhibitor in the active site of enzyme and its electronic and/or steric effects may have an impact on subsequent reaction of phosphorus with enzyme hydroxyl group. Keeping in mind that hydrophobicity favours the entering of inhibitors into the active site gorge of the enzyme [21], the differences in lipophilicity may also play some role.

Surprisingly, salicylanilide diethyl phosphates **1**–**27** showed significantly weaker inhibition of AChE than their sulphur isosters, salicylanilide diethyl thiophosphates (phosphorothioates). For BChE, the average activity was higher in the case of thiophosphates, but six phosphates (**19**, **20**, **22**, **24**, **25**, and **26**) exhibited lower IC_{50} values than all thiophosphates [22].

Salicylanilide diethyl phosphates **1** demonstrated comparable or slightly higher IC_{50} values for AChE than salicylanilide *N*-alkyl carbamates [21].

3.2.1. Type of cholinesterases inhibition determination

We also investigated the type of inhibition for both AChE and BChE. For this advance experiment, we selected one derivative with higher inhibitory activity (**24**; **Group 2**), one with poorer IC_{50}s (**15**; **Group 1**) and diethyl phosphate **27** (**Group 2**) as a representative of derivatives with an intermediate activity towards BChE within this series.

We used the procedure described in Section 2.3, which enables to distinguish reversible, pseudo-irreversible and irreversible inhibition on the basis of the changes of enzyme activity in presence of inhibitor. For reversible inhibition, the activity of enzyme goes down immediately but the inhibiting molecule is bound to the enzyme for a short period of time, after which the inhibitor and enzyme molecules dissociate and enzyme activity is restored. During pseudo-irreversible inhibition, the inhibitor binds to the enzyme molecule, but the bond is broken down more slowly, delaying the return of enzymatic activity to initial state. In the case of irreversible inhibitors, the reaction of enzyme and inhibitor is not instantaneous. Instead, there is a time-dependent decrease in enzymatic activity. Irreversible inhibitors decrease enzyme activity successively and the dependence log % *A* vs. time in presence of such inhibitor is linear. These inhibitors bind permanently to the enzyme, and thus the enzyme does not become available again [31,32].

For investigation of cholinesterases type of inhibition we chose three salicylanilide derivatives mentioned above (**15**, **24**, **27**). For each of them three different concentrations were chosen according to the value of IC_{50}. The first concentration was lower than IC_{50}, the second was close to IC_{50} and the third was higher than IC_{50}.

All obtained dependences log % *A* vs. time for three chosen derivatives and both enzymes show very similar course. First the dependence log % *A* vs. time in presence of inhibitor shows decreasing course. This decreasing of enzyme activity is stopped after approximately 30 min and longer incubation period does not lead to further drop of enzyme activity. Moreover, after

240 min of incubation the activity slightly increases. The dependences log % *A* vs. time in presence of derivative **24** are presented in Fig. 2 (inhibition of AChE) and Fig. 3 (inhibition of BChE) as examples.

The results obtained for remaining two salicylanilide diethyl phosphates **15** and **27** showed identical trends and curves (data not shown).

In our opinion, the obtained results showed that three investigated salicylanilide diethyl phosphates do not act as irreversible nor reversible inhibitors. Both ChE are inhibited by chosen derivatives for longer period, but none of observed dependences log % *A* vs. time shows the typical course for irreversible inhibition (i.e. the linear decrease of log % *A* in time). Owing to the longer period of enzyme inhibition, we can conclude that tested salicylanilide derivatives act as pseudo-irreversible inhibitors.

4. Conclusions

A series of twenty-seven salicylanilide diethyl phosphates as representatives of organophosphorus compounds were evaluated against acetylcholinesterase and butyrylcholinesterase. All of the compounds shared significant inhibition of both cholinesterases with IC_{50} values in the micromolar range. It was observed that the activity against butyrylcholinesterase was comparatively higher than those obtained for acetylcholinesterase. Presented derivatives showed a clear superiority to rivastigmine against acetylcholinesterase and six of them exhibited more potent inhibition of butyrylcholinesterase than both rivastigmine and galantamine. In general, derivatives of 4-chlorosalicylic acid showed better inhibition results. The mechanism of cholinesterases inhibition was determined experimentally as pseudo-irreversible.

Conflict of interest

The authors declare no conflict of interest.

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