Inhibition of acetylcholinesterase by an alkylpyridinium polymer from the marine sponge, *Reniera sarai*

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**Abstract**

Large polymeric 3-alkylpyridinium salts have been isolated from the marine sponge *Reniera sarai*. They are composed of N-butyl(3-butylpyridinium) repeating subunits, polymerized head-to-tail, and exist as a mixture of two main polymers with molecular weights without counterion of about 5520 and 18900. The monomer analogue of the inhibitor, N-butyl-3-butylpyridinium iodide has been synthesized. This molecule shows mixed reversible inhibition of acetylcholinesterase. The polymers also act as acetylcholinesterase inhibitors and show an unusual inhibition pattern. We tentatively describe it as quick initial reversible binding, followed by slow binding or irreversible inhibition of the enzyme. This kinetics suggests that there are several affinity binding sites on the acetylcholinesterase molecule where the polymer can bind. The first binding favors binding to other sites which leads to an apparently irreversibly linked enzyme-inhibitor complex. © 1998 Elsevier Science B.V. All rights reserved.

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**1. Introduction**

Several recent studies of marine sponges have shown that these simple marine organisms are real ‘chemical factories’, producing a large number of biologically active substances. They may act as repellents or antifouling substances and help these sessile organisms in territorial competition. Most often, these compounds have been found to be polyketides, terpenoids and peptides. In recent years, several 3-alkylpyridinium salts endowed with potent biological activities have been isolated from various marine sponges. Amphitoxins, isolated from the sponge *Amphimedon compresa* [1], show antifeeding activity, and halitoxins from several sponges of the order *Haliclonia* [2] are cytotoxic, hemolytic and lethal to fish and mice. Cyclostelletamines, cyclic pyridinium alkaloids from the sponge *Stelleta maxima* [3], inhibit binding of methyl quinuclidinyl benzylate to muscarinic acetylcholine receptors, and finally polymeric compounds from the sponge *Callyspongia fibrosa* [4] act as inhibitors of the epidermal growth factors. All
these compounds are only slightly water soluble and their potential acetylcholinesterase-inhibitory activity has not been reported.

Acetylcholinesterase (AChE, EC 3.1.1.7) is the protein responsible for the hydrolysis of the neurotransmitter acetylcholine in the nervous system synapses. This takes place at the bottom of a 20 Å deep enzyme gorge [5]. The choline is recognized by a so-called anionic binding site and the reaction is performed via a serine, activated by a catalytic triad [6]. At the rim of the gorge, another choline binding site exists – the peripheral anionic site. Ligand binding at this site destabilizes binding of the substrate [7], modulates activity [8] through conformational changes [9] and hence is responsible for inhibition by excess substrate. Several reversible inhibitors have been used to study these two sites. Some of them, such as edrophonium, bind to the anionic site, and others, such as propidium, bind to the peripheral anionic site. Decamethonium, which has two quaternary nitrogens, binds to both sites [10].

In our laboratory, new polymeric pyridinium compounds (poly-APS) have been isolated from the marine sponge *Reniera sarai* [16]. The isolated material is a mixture of two polymers composed of 29 and 99 repeating N-butyl(3-butylpyridinium) units (Fig. 1). They are soluble in water and show strong anti-cholinesterase activity [11]. Since the pattern of AChE inhibition by poly-APS appeared to be rather unusual and obviously included several mechanisms, we report the inhibition kinetics and propose a model to explain them.

2. Experimental procedures

2.1. Isolation of the inhibitor

Specimens of *R. (Haliclona) sarai* (Pulitzer-Finali) were collected by scuba diving in the Northern Adriatic Sea. Poly-APS were isolated as described by Šepčić et al. [11]. Poly-APS are a mixture of two polymers with molecular masses of 5520 and 18 900 Da, according to MALDI-TOF spectroscopy, which corresponds to a minimum of 29 and a maximum of 99 monomer units, respectively. Since it was impossible to determine the exact ratio between them, the kinetic constants were estimated assuming a 1:1 ratio between the two polymers, giving a molecular mass of 12 kDa for molar concentration calculations.

2.2. Synthesis of the monomer analogue

Five grams (36.9 mmol) of 3-butylpyridine and 18 g (99.6 mmol) of butyliodide were added to 10 ml of anhydrous diethyl ether. The mixture was put on a magnetic stirrer and kept at room temperature for 3 days, until the reaction was finished as checked with TLC. The monomer analogue, N-butyl-3-butylpyridinium iodide, occurred as a brownish oily precipitate. It was washed five times with anhydrous ether in order to remove the starting reagents. The remaining ether was removed at reduced pressure and the total mass of compound synthesized was 3.2 g (27% yield). The purity of the final compound was checked by 1H-NMR. The spectrum gave the following: δ in ppm: 0.7 (t, 3H), 0.9 (t, 3H), 1.3 (m, 4H), 1.65 (m, 2H), 1.7 (m, 2H), 2.66 (t, 2H), 4.65 (t, 2H), 8.04 (dd, 1H), 8.45 (d, 1H), 9.18 (d, 1H). The product was kept at 4°C until use, and then dissolved in methanol.

2.3. Modeling

We made a three-dimensional model of the monomer analogue and of poly-APS containing 29 monomer units. The modelization was performed in water with five layers and chloride as counteranion. Molecular dynamics and energy minimization calculations were carried with the program Biosym and Discover. AChE coordinates were taken from the structure of *Torpedo californica* AChE determined by X-ray crystallography [5].

2.4. Inhibition kinetics

AChE activity was measured using acetylthiocholine (ATCh) as substrate in 100 mM sodium phosphate buffer pH 8.0 at 25°C using electric eel AChE as source of enzyme (Sigma, 1–10 U/ml). Hydrolysis of acetylthiocholine iodide was followed at 412 nm using the method of Ellman et al. [12]. For the monomer analogue, kinetics experiments were performed in 5% methanol (at this concentration the methanol had no significant effect on the enzyme) to ensure the solubilization of the inhibitor. $V_{\text{max}}$
and $K_m$ and inhibitory constants for the monomer analogue were determined by non-linear regression. Kinetics experiments with poly-APS were performed as for their monomer analogue. Additionally, the dilution method of Aldridge [13] was used to estimate irreversible inhibition. Briefly, different concentrations of poly-APS were incubated with enzyme from 0 to 180 min. The mixture was then diluted 40× in 100 mM phosphate buffer, pH 8.0. Recording of the initial velocity at various times after dilution (from 5 s to 2 h) allowed the estimation of the remaining activity.

2.5. Effect of the inhibitors on 5-thio-2-nitrobenzoic acid

DTNB (5,5-dithio-bis(2-nitrobenzoic acid)) was used to follow the hydrolysis of the substrate by AChE. DTNB reacts with the thiocholine, releasing 5-thio-2-nitrobenzoic acid (TNB) which absorbs at 412 nm. To check whether inhibitors interfere with TNB, DTNB was reduced with 2-mercaptoethanol and the solution diluted with 100 mM phosphate buffer pH 8.0. Different concentrations of poly-APS or its monomer analogue were added to a reduced DTNB solution (TNB) at a concentration of 37 μM and the decrease in absorption was monitored between 220 and 800 nm. N-Butyl-3-butylpyridinium iodide induces dose-dependent bleaching of TNB which was detectable at 412 nm above 70 μM and which reached 50% at 800 μM. We tentatively assigned this effect to a π-π interaction between the aromatic rings of the pyridinium and thionitrobenzoic acid. Poly-APS did not induce any bleaching in the 0–23 μM range. As a consequence, inhibition was performed below 50 μM or 10 μM for the monomer analogue and poly-APS, respectively.

2.6. Fluorescence assays

Binding of propidium to the enzyme was followed according to Taylor and Lappi [14] at various concentrations of ligand: 60–300 nM. Fluorescence was detected by excitation of the enzyme-propidium complex at 290 nm, monitoring the emission at 602 nm. Two concentrations of monomer analogue (100 and 200 μM) were used to study the displacement.

3. Results

3.1. Inhibitor structures

We synthesized the monomer analogue of poly-APS, N-butyl-3-butylpyridinium iodide, which is a brownish oily compound with molecular mass of 319 Da. It is stable at room temperature and soluble in methanol. Its UV spectrum is characteristic of pyridinium salts, with a maximum absorption at 266 nm. The structure of the compound is shown in Fig. 1. Modelization, minimization and molecular dynamics of the poly-APS with 29 residues with chloride as counterion in water gives a polymer structure which is larger than the AChE diameter (Fig. 2).

3.2. Inhibition by the monomer analogue

At concentrations lower than 70 μM, to avoid any interference with the Ellman’s reagent (see Section 2), the monomer analogue showed reversible inhibition of AChE. The Eadie-Hofstee plot shows that the inhibition is of the mixed competitive and non-competitive type (Fig. 3a), allowing us to hypothesize that the monomer analogue binds to the peripheral anionic site and to the catalytic anionic site. Data
were analyzed according to Eq. 1 corresponding to Scheme 1: the inhibition constants were estimated by non-linear regression to $K_i = 14 \mu M$, the competitive inhibitory constant, and to $K'_i = 140 \mu M$, the non-competitive inhibitory constant.

$$v = \frac{V_{max}}{K_s \left(1 + \frac{[I]}{K_i}\right) + \left(1 + \frac{[I]}{K'_i}\right)}$$  \hspace{1cm} (1)

Monomer displaced propidium which is a ligand specific for the peripheral anionic site, this displacement corroborates the binding of the monomer analogue at the peripheral anionic site (Fig. 3b).

3.3. Inhibition by poly-APS

Plots of substrate metabolization by AChE versus time in presence of poly-APS were not linear (Fig. 4), in contrast to the inhibition by the monomer analogue which was time independent; this suggests that there is another component in the inhibition which is slow binding and can be assigned to the polymeric

![Fig. 2. The minimized poly-APS in water with chloride as counterion and the T. californica AChE. Note that the two molecules are drawn at the same scale.](image)

![Fig. 3. AChE inhibition by N-butyl-3-butylpyridinium iodide.](image)
structure of poly-APS. This pattern has been observed with the electric eel AChE (Fig. 4) and with the Drosophila enzyme (not shown). Furthermore, following the inhibition, the activity of the enzyme is not completely restored by dilution of the enzyme-inhibitor mixture (Fig. 5). Recovered activity reaches a plateau at a level which depends on the previous incubation time of the enzyme with the inhibitor, showing the presence of an irreversible component in the enzyme inhibition. Most slow binding inhibition can be described by slow isomerization from the initial enzyme-inhibitor complex E·I to a final tight-binding complex [15,16]. Equations used to analyze such inhibitions suppose that the inhibition is competitive and the existence of only one isomerization way. But our preliminary results did not corroborate this supposition. So, we tried to analyze independently the first reversible inhibition, the slow binding components and the irreversible inhibition reminding that separation of individual components contributed to some extent to experimental errors in determination of constants.

3.4. Reversible inhibition

This inhibition was estimated in the presence of substrate during the first 15 s in order to minimize the influence of the slow binding and irreversible components. At low concentrations of substrate (25–500 μM), the enzyme displays a Michaelian behavior with a $K_m$ estimated to 42 μM. We observed non-competitive inhibition with a $K_i = 11 \pm 3$ nM suggesting that inhibition did not originate from the binding onto the catalytic anionic site (Fig. 6a). At high concentrations of substrate (1–30 mM), the enzyme is inhibited by the binding of a substrate

![Fig. 4. Time course of AChE inhibition by poly-APS. AChE was incubated simultaneously with 1 mM ATCh and various concentrations of inhibitor: 25 nM (○), 11.5 nM (●), 9.2 nM (▲), 4.6 nM (◇) and 0 nM (□). The activity was recorded for 1 h. The concentration of thiocholine produced ([P]) is in moles per liter.](image)

![Fig. 5. Time course of AChE recovery following inhibition by poly-APS. The inhibitor (460 nM) was preincubated in the presence of AChE (0.2 nM) for various times: 0.75 min (○); 4 min (●); 10 min (◇); 20 min (▲); 60 min (◇); 90 min (●) and 180 min (▲). Then the mixture was diluted and the residual activity recorded after various times (0–75 min).](image)
molecule to the peripheral anionic site. At these substrate concentrations, poly-APS competed with substrate inhibition (Fig. 6b) enforcing the hypothesis that poly-APS binds on the peripheral anionic site.

3.5. Slow binding reversible inhibition

The $k_{\text{bind}}$ for the slow binding reversible part of inhibition was determined by incubating enzyme at various inhibitor concentrations and at various times but less than 5 min to minimize the irreversible component. Aliquots were 40-fold diluted to eliminate fast binding reversible component and the remaining activity was immediately recorded (Fig. 7a). The rate of reaction ($v$) is in $\mu$moles of thiocholine produced per min and concentration of substrate is in $\mu$moles per liter.

![Fig. 6. Reversible inhibition in the presence of ACh. Poly-APS were mixed with ACh and AChE. Activity was recorded for 15 s. The tangent at the origin was estimated. An Eadie-Hofstee plot (a) was drawn to see the effect on $K_m$ and $V_{\text{max}}$. Three concentrations of poly-APS were used: 11.5 ($\Delta$), 25 ($\blacksquare$) and 34.5 ($\bullet$) nM. A Haldane plot (b) was drawn to see the effect on $K_s$. The concentrations of poly-APS used were: 115 ($\Delta$), 230 ($\blacksquare$) and 345 ($\bullet$) nM. The rate of reaction ($v$) is in $\mu$moles of thiocholine produced per min and concentration of substrate is in $\mu$moles per liter.]

![Fig. 7. Slow binding reversible inhibition of AChE by Poly-APS. To determine the $k_{\text{obs/bind}}$ (a), the enzyme was incubated with the inhibitor (460 nM) for various times (from 0.5 to 4 min), then diluted and the remaining activity immediately recorded. To determine the $k_{\text{rec}}$ (b), 0.057 nM of enzyme was preincubated with the inhibitor (460 nM) for 10 min. The mixture was then diluted and incubated for various times (0.5–20 min) before recording the remaining activity. $[E'P]_0$ and $[E'P]_t$ are the estimated amounts of inhibited enzyme by the slow binding component at time $t=0$ and $t$.]

Fig. 6. Reversible inhibition in the presence of ATCh. Poly-APS were mixed with ATCh and AChE. Activity was recorded for 15 s. The tangent at the origin was estimated. An Eadie-Hofstee plot (a) was drawn to see the effect on $K_m$ and $V_{\text{max}}$. Three concentrations of poly-APS were used: 11.5 ($\Delta$), 25 ($\blacksquare$) and 34.5 ($\bullet$) nM. A Haldane plot (b) was drawn to see the effect on $K_s$. The concentrations of poly-APS used were: 115 ($\Delta$), 230 ($\blacksquare$) and 345 ($\bullet$) nM. The rate of reaction ($v$) is in $\mu$moles of thiocholine produced per min and concentration of substrate is in $\mu$moles per liter.

The reaction followed pseudo-first-order kinetics and we estimated $k_{\text{bind}}$ as $65 \pm 12$ mmol$^{-1}$ 1 min$^{-1}$. Restoration of the activity also depends on the time after dilution showing the slow binding reversible component in the inhibition. To tentatively estimate the rate of recovery, $k_{\text{rec}}$, the enzyme was preincubated with
the inhibitor for 10 min. Then the reaction mixture was diluted and the recovered activity was measured at different times. Twenty minutes after dilution, a plateau was reached which corresponds to the enzyme reversibly inhibited ([E\textsubscript{W}I]+[E\textsubscript{I}]) for fast and slow binding components. Variation of [E\textsubscript{I}] with time followed first-order kinetics and we estimated \( k_{\text{rec}} \) as 0.21 ± 0.06 min\(^{-1} \) and \( K_i = k_{\text{rec}}/k_{\text{bind}} = 3.23 \) μM (Fig. 7b).

3.6. Irreversible inhibition of AChE by poly-APS

Only a fraction of the enzyme activity can be recovered after elimination of the inhibitor. As the amount of recovery depends on the incubation time, we concluded that the enzyme is irreversibly blocked by the inhibitor (Fig. 5). To analyze this irreversible inhibition, the enzyme was incubated with the inhibitor for various times and with various inhibitor concentrations, the reaction mixture was diluted and postincubated for 60 min before measuring the remaining activity. After 20 min, the inhibition follows a pseudo-first-order kinetics (Fig. 8). The \( k_i \) was estimated to be 388 ± 47 mmol\(^{-1}\) 1 min\(^{-1} \) and was in the same range when time or inhibitor concentration was used as variable. In the first 20 min, inhibition presents non-linear kinetics with time. Rate of inhibition decreased with the time (Fig. 8), suggesting that the concentration of the enzyme species which could be irreversibly inhibited diminishes during the first 20 min. If irreversible inhibition would result from isomerization of a slow binding inhibited form, rate of irreversible inhibition would increase in the first 20 min. As we observed an decrease, we can hypothesize that there was a competition between slow binding and irreversible inhibition in the first 20 min.

4. Discussion

The AChE-inhibitory compounds, isolated from the marine sponge \textit{R. sarai}, are alkylpyridinium salts, recently isolated from several marine sponges. Natural AChE inhibitors are common, but only few have been isolated from marine organisms. Among them, we can only mention onchidal, an irreversible inhibitor from the mollusk \textit{Onchidella binneyi} [17] and a pseudozoanthoxanthin-like compound from the encrusting coral \textit{Parazoanthus axinellae} [18]. Synthetic alkylpyridinium compounds have recently been described as anti-cholinesterase agents [19]. The anti-cholinesterase activity of the \textit{R. sarai} extract is due to two compounds of this family, two large polymeric 3-alkylpyridinium compounds (poly-APS). The degree of polymerization and the length of the alkyl chains in pyridinium compounds may play an important role in their inhibitory activity since the

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**Fig. 8.** Irreversible AChE inhibition by poly-APS. AChE was incubated in the presence of inhibitor (140 (○) and 460 nM (■)) for different periods of time, from 1 to 60 min. The mixture was diluted and incubated for 1 h before addition of ATCh to record restored activity.
monomer analogue has a significantly lower inhibitory activity and does not show the same pattern of inhibition. The effect of polymerization on biological activity has already been reported for inhibitors of epidermal growth factors [3] and for cytotoxic compounds [20]. With poly-APS, polymerization results in a new type of inhibition presenting a kinetic behavior composed of different inhibition patterns. We tentatively determined the constants of each inhibition and proposed a mechanism of action for the polymer.

Several experiments suggest that one binding site responsible for the inhibition of AChE by poly-APS is a peripheral site. The N-butyl-3-butylpyridinium iodide, the monomer analogue of the inhibitor, is a reversible inhibitor of AChE with mixed competitive and non-competitive inhibition kinetics, suggesting that binding can occur at the anionic site inside the enzyme gorge, and at the peripheral anionic site. The latter binding is in accordance with the displacement of propidium from the peripheral anionic site by the monomer, seen through a decrease of the fluorescence of the enzyme-propidium complex. Binding to the two anionic sites differs from the results published by Whiteley and Ngwenya [19] who found only one binding site for alkylated pyridinium salts, but we should note that they used methyl or ethyl pyridinium and not N-butyl-3-butylpyridinium. Several lines of evidence suggest that poly-APS bind like the monomer analogue to a peripheral site. (1) Reversible inhibition of poly-APS showed a clear non-competitive pattern at low substrate concentrations. (2) Poly-APS is able to displace the substrate molecule bound to the peripheral anionic site and responsible for the inhibition of AChE by excess substrate. (3) Inhibition of the enzyme by poly-APS is partially protected by propidium, a ligand specific for the peripheral anionic site. All these data are consistent with the binding of poly-APS at the rim of the active site gorge.

Besides this binding, there is a corpus of evidence which suggests that poly-APS also bind to other sites. (1) The poly-APS molecules are very large, above 1000 Å in their unfolded conformation; their length considerably exceeds the diameter of a single cholinesterase molecule (Fig. 2). Thus, theoretically one inhibitor molecule could bind all the sites of the enzyme at once. Among them, there is the catalytic anionic site suitable for binding of the monomer analogue and several aromatic and charged residues at the external surface of the enzyme. They might allow the binding of the repetitive positively charged aromatic residues of poly-APS via ionic-π interactions. (2) The reversible inhibition has a slow binding component, and this type of inhibition may be explained by isomerization due to multisite binding. (3) Irreversible inhibition is usually due to covalent binding, but poly-APS do not bear any reactive groups. One hypothetical way to achieve this type of inhibition is a multisite binding leading to denaturation and precipitation of the enzyme as it can occur for example in antibody-protein interactions.

Poly-APS inhibit AChE and show an unprecedented inhibition pattern composed of three different inhibition patterns. All the results obtained for inhibition of acetylcholinesterase may be presented in the simplified model shown on Scheme 2, where E represents the free enzyme, E-I the fast reversible complex, E-İ the result of the slow bindings and E-I the result of the irreversible inhibition. Most probably poly-APS can bind to several sites and first a fast reversible binding takes place at one of these binding sites. Then, the polymeric structure of the inhibitor allows the binding and stabilization of other pyridinium residues at other binding sites. At least one combination of binding sites results to an apparent irreversible interaction between the two molecules.

Production of biologically active molecules is very important for marine sessile invertebrates and a great number of these compounds have recently been isolated from them. As these organisms lack a locomotory system, chemical defense helps them to survive. Bioactive molecules are involved in territorial competition, they prevent fouling of other micro- and
macro-organisms on the surface of the animal, and finally act in defense strategy against a variety of predators. The surface of *R. sarai* is never fouled with other organisms and is covered with considerable amounts of poly-APS which can interact with proteins such as AChE, and display hemolytic and cytotoxic activities [21]. These facts lead us to hypothesize that these organic polymers play an important role in the sponge defense strategy as was recently suggested for other alkylpyridinium compounds [1,22].

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