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Original article

p–Aminobenzoic acid derivatives as acetylcholinesterase inhibitors

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Abstract

Because Alzheimer's disease (AD) is a medical problem characterized by progressive loss of memory and cognition that is associated with a deficient cholinergic system, this work aims to evaluate some *p*-aminobenzoic acid (PABA) derivatives as acetylcholinesterase inhibitors in vitro, in continuation with our last studies. The assayed compounds are low toxic, simple-structured and low cost.

Keywords: Acetylcholine; Acetylcholinesterase; Amides; Imides; Enzymatic inhibitors; Molecular docking

1. Introduction

Acetylcholinesterase (AChE; EC 3.1.1.7) terminates signaling at cholinergic synapses by rapid hydrolysis of the neurotransmitter acetylcholine (ACh). The crystal structure of human AChE shows a narrow gorge of 20 Å in depth, where is located the catalytic site (Ser203, His447 and Glu334) and an anionic subsite, in which the residue Trp86 plays a crucial role not only in the catalytic efficiency of the enzyme but also in recognizing and binding several number of active-site directed inhibitors [1,2].

The AChE has received important attention as a drug-design target for the palliative treatment of the Alzheimer's disease (AD). In fact, it is the only target that has provided the few palliative drugs presently marketed for the treatment of the AD [3,4]. Unfortunately, the therapeutic applications of the most common AChE inhibitors has been restricted due to they highly toxicity, as tacrine [5], short half-life, as rivastigmine [6], and purpuric rash and high-cost, as E2020 [7,8]. In our last studies, we have focused our attention not only in develop potent AChE inhibitors, but also in their low toxicity and cost as well as high biodisponibility, focusing on the inverse relationship between potency and toxicity (high potency/low toxicity) [9,10]. In connection with our original interest in the design and development of arylamides and arylimides structurally related with ACh, in this work we report the synthesis, anticholinesterase activities and docking studies of two arylamides (**1a**, **2a**) and two arylimides (**1b**, **2b**) derived from the *p*-aminobenzoic acid (PABA). In last studies we found that substituted arylamides and arylimides presented AChE inihibitory activity [9,10]. Through experimental and structure–activity relationship analyses we have observed that (a) *para*-substituted derivatives, in comparison with *meta*- and *ortho*-derivatives, showed the best inhibitory activity; this behavior is given due to the decrease of the steric interactions in the diffusion of molecules towards the active site, particularly the maleic anhydride derivatives and (b) arylamides and arylimides containing hydroxy and methoxy substituents in their structure are low potent, whereas amino and halogen derivatives are highly toxic; thereby, this kind of inhibitors are no suitable for therapeutic uses. In addition, the use of carboxylic group has shown good results on the inhibitory potency and low toxicity. In this sense, taking into account the observations made above we supposed that the structural combination of the *para* position in the benzene ring of our compounds and the presence of carboxylic group would give successful results in the improvement of high potency/low toxicity relationship than those we have reported in last studies. Following these considerations, we

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report the synthesis, inhibitory activity on AChE, molecular dynamics, docking and toxicity studies of four PABA derivatives.

2. Chemistry

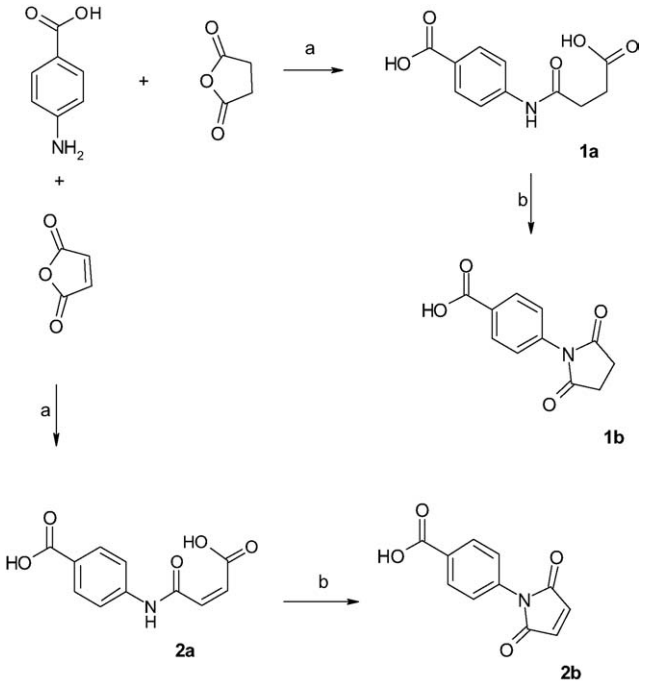
The synthesis of arylamides and arylimides was achieved as previously reported [9,10], and consists briefly as follows: The reaction between PABA and succinic and maleic anhydride, in presence of tetrahydrofurane at room temperature, was carried out, obtaining the respective arylamides, **1a** and **2a**. These arylamides were transformed to the corresponding arylimides, **1b** and **2b**, by heating them in acetic anhydride with an equimolecular amount of sodium acetate (Scheme 1). The characterization and purity criteria were followed by comparison with the previously reported data [11].

3. Biological evaluation

In vitro acetylcholinesterase (from bovine erythrocyte) and butyrylcholinesterase (from human serum) inhibitory activities of four compounds were determined employing the modified Bonting and Featherstone's colorimetric method [12]. In addition, for compounds **2a** and **2b**, in which structure is present an α–β unsaturation, was observed an irreversible behavior, evaluated through the method proposed by Kitz and Wilson [13].

4. Acute toxicity evaluation

The mean lethal dose (LD_{50}) was determined for the four compounds in CD1 male mice with weights varying between 20 and 25 g. Each derivative of *p*-ABA was applied intraperitoneally, using vegetable oil as a vehicle (the hundredth part of the animal weight). The behavior of **1a**, **1b**, **2a** and **2b** as well as the cholinergic symptoms were observed during the first few minutes after the administration of these compounds and the numbers of deaths were registered at 24 h.



Scheme 1. The synthesis pathway of the compounds here presented. (a) THF, 25 °C, 1 h. (b) Ac_2O, AcONa, 80 °C, 3 h.

5. Results, discussion and molecular modeling

As expected, four compounds showed AChE inhibitory activity. Results of inhibition kinetics are summarized in Table 1. Interestingly, these molecules in comparison with the *m*-aminobenzoic acid (*m*-ABA) acid derivatives reported by our workgroup, have presented an improvement in the inhibitory activity. Originally, the inhibitory potency of the compounds derived from the *m*-ABA was correlated with three main factors: (a) the lipophilicity of each molecule, (b) the degrees of freedom of the dihedral angles and (c) the electronic contribution of the π orbitals in each molecule. In the particular case of these compounds, only points (b) and (c) were taken into account for our analysis purposes.

In a last paper we have demonstrated the utility of molecular dynamics simulations on the inhibitors in rationalizing the inhibitory potency, since the molecules in solution are found as dynamic populations and not just a static conformation [10]. Hence, to better understand the conformational behavior of the compounds in water and in the receptor, we carried out molecular dynamics simulations of each molecule in water and docking simulations on human AChE.

To carry our molecular dynamics simulations for the four molecules, we build a water box cluster of 20 Å in each dimension. Before simulations, the system was minimized and relaxed, employing the MM+ force field implemented in the Hyperchem software [14]. To the relaxed system, molecular dynamics was applied, with $T = 310$ K, integration time step of 100 ps, step size equal to 0.001 ps. Total potential energy of each step was collected and used for further analysis. At the end of each simulation, we observed that compounds **1a** and **2a** are found in their extended form starting from the amidic bond, as judged by the potential energy ranking; this phenomena is given because exists an important number of repulsive forces between the benzene ring and the oxygen atoms from the amidic fragment, and the best way to low the energetic barrier in order to reach the lowest energy is keeping the extended form. In the case of molecule **1b**, we found

Table 1
Kinetic data for PABA derivatives

Compound	K_i AChE (nM)	K_i BChE (nM)	Inhibition
2b	52.6 ± 9	83.8 ± 16	Irreversible
2a	100 ± 31	411 ± 13	Irreversible
1a	150 ± 45	736 ± 30	Reversible
1b	243 ± 10	117 ± 23	Reversible
m-ABA derivatives	33–357	n/d	Mixed
E2020	5	–	Reversible
Tacrine	10	7	Reversible

that the bond between the aromatic carbon from the benzene ring and the nitrogen from the five-membered ring behave as a free rotor, giving *n* different conformations in water media. Finally, compound **2b** remains rigid along the time, since this compound presents a high population of π electrons in its structure, and the resonance effect goes from the carboxylic fragment until the five-membered ring. The information obtained from molecular dynamics simulations showed that, in an aqueous media, compounds should present a specific number of conformations in which the molecules start their diffusion to the active site. In this manner, the molecule **2b** will start it diffusion toward the active-site easier than the other three molecules, due to the fact that this compound remains quite rigid in water. In the case of compounds **1a** and **2a**, they present an amidic fragment, which must adopt different conformations, but due to the steric clashes between oxygen and carbon atoms, this fragment will adopt the extended form, giving minimal steric interactions in the diffusion of the molecule toward the active site. Finally, as molecule **1b** adopts *n* number of conformations between the benzene and five-membered rings, should increase their contacts with the residues of the enzyme, decreasing its diffusional efficiency and diminishing its anticholinesterase activity. Inhibitory potency data presented in Table 1 support the asseverations made above.

In previous studies we found that, experimentally, these kind of compounds are active-site directed, since they compete with edrophonium for the binding site. Edrophonium, as well as other AChE inhibitors, does bind to the residue Trp86, the principal member of the Anionic Site (AS). The binding mechanism between this residue and the inhibitors is carried out via π–π interactions, which have been determined as fundamental in many ligand–receptor binding phenomena. Thereby, we carried out computational docking simulations to support the fact that these inhibitors bind to residue Trp86. Docking simulations were carried out with the latest version of AutoDock software (3.0.5) [15]. The building and optimization of the structures of four compounds and the optimization of the human AChE X-ray structure (PDB code 1B41) were made with the SYBYL package [16]. Docking simulations were effectuated employing the Lamarckian Genetic Algorithm, with an initial population of 75 randomly placed individuals, a maximum number of 2.5×10^6 energy evaluations and a maximum number of iterations in the pseudo Solis and Wets local search of 300. Fifty independently docking runs were carried out for each ligand. Resulted docked orientations within 1.0 Å were clustered together, and the best cluster was used for further analysis. The best docked conformations of four compounds are shown in the Fig. 1.

As experimentally obtained, four compounds directly interact with the residue Trp86. In all cases, the carboxylic fragment present in the molecules is found in direction to the catalytic triad, and the rest of the molecule is observed in direction to the gorge's entrance. As observed in the Fig. 1, compounds **1a** (red) and **1b** (blue) are attached to the Trp86 via π–π interactions; in both cases, it is observed that

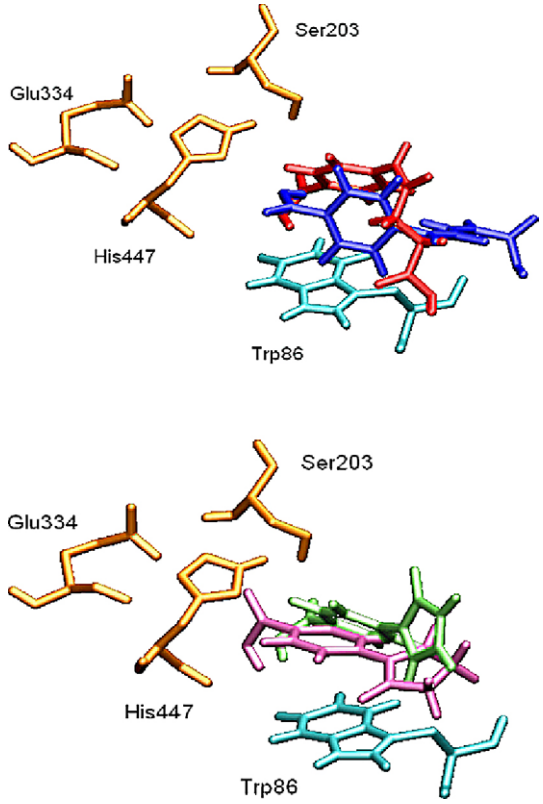


Fig. 1. Graphical representation of the binding modes of four PABA derivatives on AChE. (**1a**, red; **1b**, blue; **2a**, pink; **2b**, green).

the amidic fragment remains in its extended conformation, as predicted by the molecular dynamics simulations. In the case of **1a**, in comparison with **1b**, the amidic fragment is accommodated towards Trp86; this orientation in the active-site does not allow the benzene ring of **1a** to adequately interact with Trp86 due to the van der Walls forces applied from the amidic fragment and consequently, the inhibitory activity is diminished. For compounds **2a** (pink) and **2b** (green), it is observed that the conformation adopted by **2a** (extended) seems to be more stable than the conformation of **2b**, but in molecular dynamics simulations we found that the rotatable bond between the benzene ring and the imidic fragment is considered as a free rotor. So, the five-membered ring of **2a** may interfere with the π–π interactions needed for the stability of the complex. As **2b** is more rigid and possess only π orbitals along the molecule, the orientation adopted by this molecule will be more stable, due to the increasing in the π–π interactions and diminishing the steric clashes with the residue Trp86.

Combining both techniques, we can determine why **2b** is the most potent AChE inhibitor, while **1b** is less potent than the other three. In the molecular dynamics simulations we found that compound **2b** remains rigid, and in the docking procedures the adopted conformation allows a better interaction with residue Trp86. On the other hand, **1b** presents a fragment, which can be found in *n* different conformations, avoiding a good diffusion of the molecule through the enzyme's gorge and giving to the ligand–receptor complex low stability.

Hence, is demonstrated that the compounds here presented have a direct relationship between the inhibitory potency on AChE and the steric and electronic effects, judged by the data obtained in both molecular dynamics and docking simulations. The utility of the combination of the molecular dynamics and docking of the ligand may be summarized as follows: (a) first, the behavior of the compound in water let us know the molecular topology at the entrance to the active site, obtaining an approximation of the best conformational state and (b) flexible docking simulations let us know how the molecules would behave inside the active site.

Acute toxicity assays shown that no deaths were observed after applying **1a**, **1b** and **2a** at a dose > 1000 mg/kg of body weight. The toxicity of those compounds was found in low relation to that reported in the literature (sarin, 0.10 mg/kg; soman, 0.042 mg/kg; tabun, 0.28 mg/kg) [17].

Finally, the inhibition kinetics of four compounds on butyrylcholinesterase showed that none of them are significantly selective to the AChE. This is due to the fact that both enzymes possess a similar recognition and binding mechanisms at the active site. We emphasize in the fact that, in principle, the main strategy of our work was to find not also potent compounds, but also low toxic and cost and high biodisponibility.

In summary, we found that PABA derivatives here reported, in comparison with the *meta* derivatives reported by our group, showed an improvement in the inhibitory activity, keeping also their low toxicity, following the relationship high potency/low toxicity, as initially expected. Hence, these inhibitors should be helpful starting materials for the synthesis of new compounds as candidates to be potent AChE inhibitors.

In Further studies, we will search for the selectivity for AChE and improve the relationship high potency/low toxicity of these kind of compounds, and will be reported in due course.

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