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The Electronic Influence on the Active Site-Directed Inhibition of Acetylcholinesterase by *N*-aryl-Substituted Succinimides

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Abstract: A computational docking approach, in combination with the Hammett relationship, has been employed to evaluate the electronic influence of substituents on ligand binding and the active site-directed inhibitory potency on acetylcholinesterase using nine *N*-aryl-substituted succinimides. Our results indicate that electron-withdrawing groups attached to benzene moiety of the compounds favor the inhibitory potency while electron-donating groups do not. This fact was confirmed by performing kinetic experiments on acetylcholinesterase from *Electrophorus electricus;* the experiments showed that *para*-substituted-NO_2 compound inhibits better than *para*-substituted-OMe and –H derivatives. This approach may be useful for the rationalization of drugs design, as well as the mechanism of the active site.
Key words: Acetylcholinesterase, Hammett Relationship, Docking, *N*-aryl-substituted-succinimides

Resumen: La aproximación por *docking,* en combinación con la relación de Hammett han sido empleadas conjuntamente para evaluar la influencia electrónica de sustituyentes sobre la unión del ligante y la potencia inhibitoria de la enzima acetilcolinesterasa de 9 *N*-aril-succinimidas sustituidas. Nuestros resultados indican que grupos electro-atractores favorecen su potencia inhibitoria, mientras que los electro-donadores no lo hacen. Este hecho fue confirmado experimentalmente sobre AChE de *Electrophorus electricus;* donde el grupo *p*–NO_2 la inhibe de manera más potente que aquellos derivados con grupos *p*-OMe y –H. Esta aproximación podría ser utilizada para racionalizar tanto el diseño de fármacos, así como el mejor entendimiento del mecanismo de unión sobre el sitio activo.
Palabras Clave: Acetilcolinesterasa, relación de Hammett, Docking, *N*-aril-Succcinimidas sustituidas.

Introduction

Acetylcholinesterase (AChE) plays a central role in the hydrolysis of its natural substrate, acetylcholine, a neurotransmitter of the central and peripheral nervous system. For many years, there has been interest in the inhibition of AChE, as it is a promising drug-design target in the palliative treatment of the Alzheimer's disease [1,2]. AChE is the only target that has provided the few palliative drugs presently marketed for the treatment of the Alzheimer's disease. Although quantitative structure-activity relationship analyses as well as kinetic and computational studies have been perfomed on the AChE inhibition mechanism [3], it is still uncertain how the pure electronic effect of the substituents attached to the inhibitor's skeleton influence the overall active-site directed inhibitory activity on AChE. On this basis, in this contribution we have demonstrated the usefulness of the computational docking approach in combination with the Hammett relationship [4], to gain insight into the electronic influence of substituents on ligand binding and the active site-directed inhibitory potency on AChE.

Previous studies made by our workgroup have revealed that *N*-aryl-succinimides[5-8]: a) are active-site inhibitors of AChE, interacting with W86 [9] via π-π contacts; b) are simply structured (two substituents on a benzene ring may allow us to better evaluate the electronic effect of substituents on the inhibitory potency); and c) behave as reversible inhibitors, facilitating the understanding of the interactions involved in the binding mechanism [5-8]. Based on the three characteristics mentioned above, we used these compounds as study prototypes to carefully model a family of nine *N*-aryl-substituted-succinimides (Chart 1). Those compounds were further docked into three different AChE structures employing the well-known docking program AutoDock [10]. This program was chosen because with the advent of the Lamarckian Genetic Algorithm and a very successful empirical free energy function based on the AMBER force field, it is able to perform very efficient docking of ligands. Finally, the calculated inhibition constants for each ligand and source were correlated with the Hammett constant of each substituent. The outcomes of these

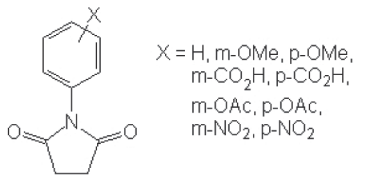

Chart 1. Structure of the *N*-aryl-substituted-succinimides studied.

simulations would provide valuable insights on the electronic influence of substituent in the inhibition of the AChE, which was experimentally demostrated by inhibition on enzyme in presence of *para*-substituted aryl-succinimides (-NO_2, -OMe, -H). Both the simulation and enzymatic assays constitute a powerful tool for the rational design of new inhibitors as well as to better understand the nature of molecular recognition on the AChE and other targets.

Methods and modeling protocol

Protein setup. In this study, three different structures of AChE were employed. The X-ray structures were taken from the Protein Data Bank (http://www.rcsb.org/pdb/) under 2ACE for *Torpedo californica* (TcAChE), 1DX4 for *Drosophila melanogaster* (DmAChE) and 1B41 for human (hAChE) entries. Ligand and solvent molecules were removed from the original PDB files. All hydrogen atoms were added to three structures and then minimized by using 5000 steps of conjugate gradients protocol. NAMD 2.5 [11] and the AMBER [12] force field were used for minimization. After minimization, nonpolar hydrogen atoms were merged and Kollman united atom charges [13] were added. Finally, fragmental volumes and solvation parameters were added to the macromolecules using the ADDSOL utility of AutoDock. The grid maps representing the protein in the actual docking process were calculated with the aid of AutoGrid. The set up of the grids were performed with 60 points in each dimension, with a spacing of 0.375 Å between the grid points.

Ligands setup. In order to accurately reproduce the electron-donating or electron-withdrawing effects of the substituents on the aromatic ring on the compounds studied here, high-level quantum mechanics calculations were applied to the ligands. The relevant quantum indices for the nine *N*-aryl-substituted-succinimide compounds were obtained using the density functional theory (DFT). These DFT calculations were computed using the hybrid B3LYP approach [14]. The basis set employed for this level of theory was 6-31+G(d,p). These calculations were carried out using the program Gaussian 98 [15] and the direct Self Consistent Field (SCF) criteria. The feed starting geometries were obtained by performing a rough conformational search and minima localization with the Dreiding force field. Geometry optimizations were computed on a dual core Xeon Intel based blade server with an overall 16 cores set up in parallel. The vibrational analyses were done at the same level of theory with the same basis set (B3LYP/6-31+G(d,p)// B3LYP/6-31+G(d,p)), and the resulting Hessian matrix showed no negative frequencies. The Mulliken method was employed to derive the partial charges on the atoms of the nine ligands.

Automatic Docking simulations. Docking simulations were performed with the version 3.0.5 of the program AutoDock [10]. This program has the advantage that allows full flexibility to the ligand, allowing it to find its optimal orientation/conformation in the protein. Docking simulations were carried out using the Lamarckian Genetic Algorithm, with an initial population of 75 randomly placed individuals, a maximum number of 2.5×10^6 energy evaluations, a mutation rate of 0.02, a crossover rate of 0.80 and an elitism value of 1. For the local search, the pseudo-Solis and Wets algorithm was applied using a maximum number of 300 iterations per local search. The probability of performing local search on an individual in the population was 0.06, and the maximum number of consecutive successes of failures before doubling or halving the local search step size was 4. 100 independent runs were carried out for each ligand and for each AChE. Resulting docking orientations within 1.0 Å in the root-mean square deviation (RMSD) tolerance of each other were clustered together and represented by the result with the most favorable free energy of binding.

Kinetics assays

The synthesis of *N*-arylsuccinimides (*p*-NO_2, -OMe and –H) were prepared and identified as those previously reported by our workgroup [7, 25]. The inhibitory effects of the compounds obtained here were tested on AChE *in vitro* by using the modified Bonting and Featherstone's colorimetric method [25]. AChE (EC 3.1.1.7 of *Electrophorus electricus*, Sigma-Aldrich) and acetylcholine iodide (ACh^+I^-, Merck) were used as received. The optimum concentration of AChE was 0.1U/mL and was diluted in phosphate buffer (0.1M, pH = 8.0). One unit of this enzyme hydrolyzes 1.0 mmol of ACh per min at pH = 8.0 at 37°C. ACh iodide was used as a substrate at several concentrations (0.1, 0.2, 0.4, 0.8, 1.6, 2.4, 3.2, 4.8 and 6.4 mM), which are slightly above and below the K_m of AChE catalytic activity. The ligands were dissolved in 1.0 mL (0.1M, Stock solution), and eventually were prepared five solutions dissolved in phosphates buffer (0.1M, pH = 8.0) at several concentrations (1×10^{-7} to 1×10^{-3} M). Neostigmine (Neostigmine bromide, Sigma-Aldrich) was dissolved in phosphate buffer (0.1M, pH = 8.0) and tested as positive control at several concentrations (1×10^{-9} to 1×10^{-5} M).

The enzymatic inhibition measurements were carried out with and without ligands at the differents substrate concentrations mentioned. At the end of each incubation (25 min at 37°C), the reaction was stopped by adding 48 μL of alkaline hydroxylamine (0.048 mmol). Then, 0.08 mL aliquots were mixed with 1.5 mL of $FeCl_3$ 0.05M dissolved in 0.5N HCl, and centrifuged at 3 min, 3000 rpm at 4°C. Finally the absorption measurements were made at λ_{max} = 500 nm.

The K_m and V_{max} values were determined with Lineweaver-Burk's method because the data had Michaelis-Menten behavior. The K_i values for both compounds and neostigmine were determined with Schild's method. All results were reported as mean ± standard error of the mean (SEM) and analyzed with '*t* Student' lineal regression analysis and $p < 0.05$ were taken as significance data. The experiments were repeated from 3 to 5 times.

Results and discussion

Docking experiments showed that all inhibitors docked to the AChE ligand binding site of the three structures, in agreement with previous reports [5-8]. In all cases, W86 played an important role in the recognition mechanism by the enzyme.

As presented in table 1, the calculated inhibition constants (K_i), as well as pK_i values were correlated with each electronic substituent constant (σ). The plots of the pK_i versus σ were obtained (Figure 1, Table 1), and a linear trend for the three sources here studied was observed. Similarly, the experimental data were correlated with Hammett sigma (σ) for each substituent (Table 2).

The correlation obtained indicates that the inhibition on each AChE source depends on the electronic effect of the substituents of the inhibitors. The plots for each AChE source were built according to the Hammett equation:

$$pK_i = \rho\sigma + C$$

where ρ is a constant of the sensitivity of the inhibitory potency. Thus, as observed in table 1, the equation obtained by linear regression for each enzyme structure showed a positive slope value, indicating that electron-withdrawing substituents in the inhibitors favor the binding mechanism by AChE and therefore the binding free energy with the enzyme is more favorable. Considering that the mechanism in which AChE recognizes *N*-aryl-substituted-succinimides, the behavior showed by these compounds can be rationalized as follows: in all cases, it was observed that the inhibitors did dock in the active site of the enzyme, and directly interacted with W86 via π-π interactions. Since the free energy of binding evaluation included in the AutoDock software takes into account the van der Waals, electrostatic, torsional, solvation and hydrogen bond terms, and the electronic effect of substituents lays only on the aromatic ring moiety of the compounds, we suggest that the major contribution to the binding free energy and thus to the inhibition constants should come from the short-range van der Waals (dispersion) and electrostatic terms, which can be effectively parameterized with the AMBER force field [16]. Furthermore, as solvation must be taken into account to predict the correct intermolecular geometry of the aromatic-aromatic complexes [17], it was also considered as it is included in the AutoDock function [10,18].

In terms of the experimental results, these suggested a similar behavior (Table 2); where the *para*-NO_2-phenylsuccinimide (electron-withdrawing substituent) showed a better affinity compared to *para*-OMe-phenylsuccinimide (electron-donating substituent) and phenylsuccnimide (-H). Evidently this experimental outcome are not similar magnitude to those calculated through docking approaches that are performed with the rigid protein also set up parameters as pH, temperature, solvent media, ionic strength, etc. (see docking section) are not equal to those employed for the experimental conditions (see experimental section).

Experimental and theoretical evidence have shown that, in the ground state, van der Waals and electrostatic interactions play an important role in the stability of aromatic-aromatic complexes [19,20]. Hence, the formation and stabilization of complexes may be facilitated by electron-withdrawing groups by reducing the repulsive interactions between the aromatic rings of the inhibitors and the residue W86 (Figure 2), while electron-donating groups may increase the repulsive interac-

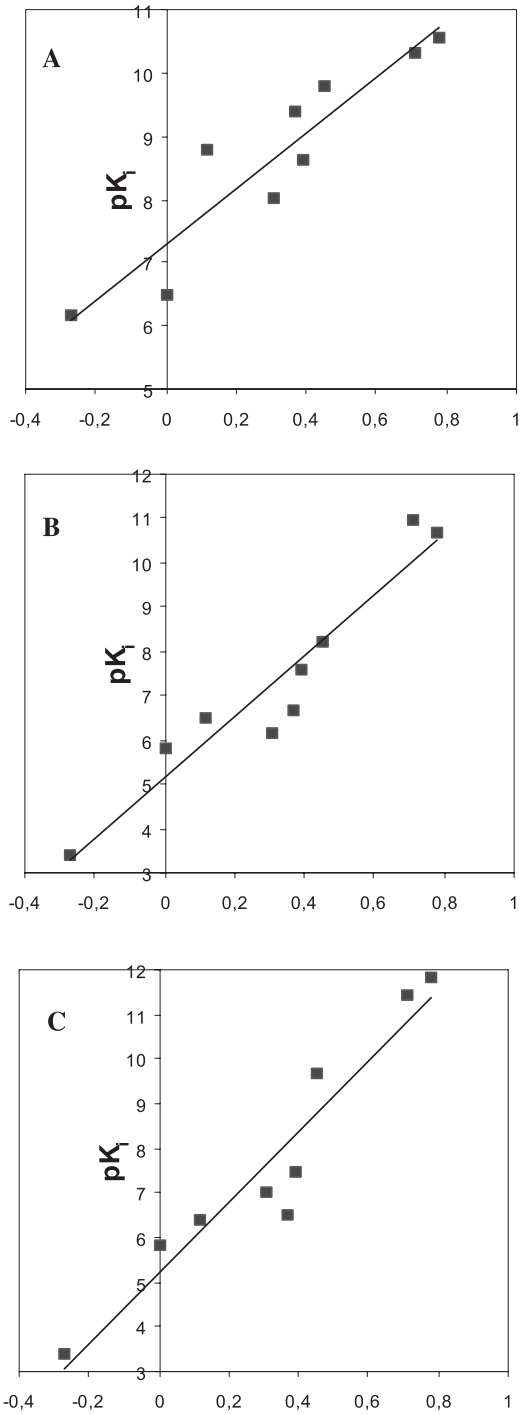

Fig. 1. Plots of the pK_i values vs σ. The relationship is shown to be linear. (A) *Torpedo californica* AChE; (B) *Drosophila melanogaster* AChE; (C) Human AChE.

Table 1. Calculated inhibition constants for the three different AChE structures.

Substituent	s[a]	*Torpedo californica*[b]		*Drosophila melanogaster*[b]		Human[b]	
		K_i (nM)	pK_i	K_i (nM)	pK_i	K_i (nM)	pK_i
***p*-OMe**	-0.27	695	6.158	387000	3.412	400000	3.398
H	0.00	337	6.472	1520	5.818	1400	5.854
***m*-OMe**	0.12	1.61	8.793	339	6.470	424	6.373
***p*-OAc**	0.31	9.73	8.012	697	6.157	101	6.996
***m*-COO^-**	0.37	0.416	9.381	209	6.680	313	6.504
***m*-OAc**	0.39	2.27	8.644	25.7	7.590	32.4	7.489
***p*-COO^-**	0.45	0.167	9.777	5.69	8.245	0.219	9.660
***m*-NO_2**	0.71	0.0475	10.323	0.011	10.959	0.00353	11.452
***p*-NO_2**	0.78	0.0283	10.548	0.0211	10.676	0.00142	11.848

[a]Values of σ for different substituents were taken from Leffler and Grunwald [24]
[b]According to the Hammett relationship, $pK_i = \rho\sigma + C$, where ρ is a constant of sensitivity of inhibitory potency, we obtained the following parameters of the fit: (a)*T. californica*: ρ = 4.38, *C* = 7.29, $\rho^2 = 0.861$; (b) *D. melanogaster*: ρ = 6.87, *C*=5.15, $\rho^2 = 0.913$; (c) Human: ρ = 7.92, *C* = 5.22, $\rho^2 = 0.906$.

Table 2. Enzymatic evaluation of the inhibition constants for the *para* substituted *N*-phenylsuccinimides substituted with –OMe (electron-donating), -NO_2 (electron-withdrawing) and –H (proton) on AChE of *Electrophorus electricus.*

Substituent	σ[a]	*Electrophorus electricus* $K_i \pm$ SEM (nM)	*Electrophorus electricus* pKi
p-OMe	-0.27	30750.74 ± 5061.07*	4.51 ± 0.06*
-H	0.00	991000 ± 76200*	3.00 ± 0.03*
p-NO_2	0.78	5514.22 ± 816.88 *	5.26 ± 0.06*
Neostigmine	——-	7.28 ± 4.76*[+]	8.13 ± 0.21*
Substrate only (Acetylcholine)	——-	K_m = 0.2146 ± 0.0161* mM	V_{max} = 0.2291 ± 0.0220* mmol/U.min

[a] Values of σ for different substituents were taken from Leffler and Grunwald [24]
[+] The inhibition constant reported is found from 9.0 to 20.0 nM [26-27]
* Significance data with $p < 0.05$.

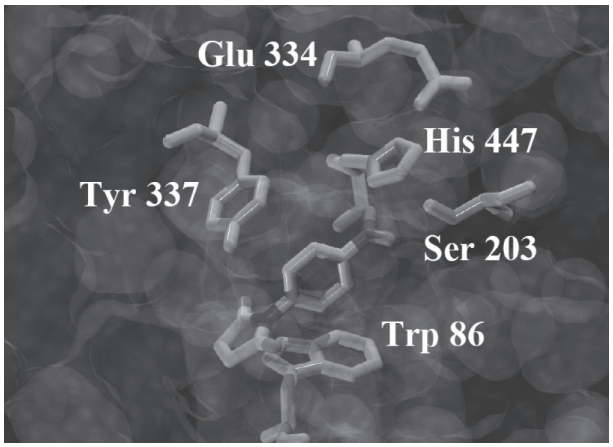

Fig. 2. *p*-NO_2 compound binding on human AChE.

tions in the ground state; consequently, a better inhibition potency may be achieved in presence of electron-withdrawing groups, as observed in the plots herein obtained.

The correlations suggest that the three AChE source studied here use repulsive-dispersion interactions to stabilize the complex inhibitor-enzyme. In this case, the electronic contribution of the substituents seemed to play a role in the strength of the π-π interactions between W86 and the inhibitors. Thus, while electron-donating groups increase the electronic density of the aromatic moiety of the inhibitors, while electron-withdrawing groups reduce the electronic density around the aromatic ring. In the latter, van der Waals and dispersion-attraction favor the overlapping of the electronic clouds of W86 and the aromatic ring of the inhibitors. Moreover, electrostatic forces might define the geometry of the complexes via electrostatic repulsion or attraction of point charges on both W84 and the inhibitors.

Our calculations correlate well with the experimental data reported recently by Hunter and co-workers. These authors used supramolecular systems as a model for π–π stacking orientations. Careful substitutions were made on one of the aromatic moieties and then the stacking free energies were evaluated. Finally, they correlated those energies with the Hammett constant and a linear slope was observed [21]. These observations support the theoretical data presented here.

We also observed a similarity between the type of slopes obtained for the three AChE sources. This suggests that the binding mechanism and the effect of the substituents on the AChE could conserve along the phylogenetic tree. In addition, different ρ values calculated for the three structures ($\rho_{T.Californica}$=4.38, $\rho_{D.\ Melanogaster}$=6.87, ρ_{human}=7.92) might reflect the level of evolution of the binding mechanism of the three species (i.e, their capacity to become more selective to ligands by the anionic site). Therefore, *?* values might serve as an indicator of specialization of the binding mechanism by the enzyme at the active site. Yet at this point it is not very clear what could be the most suitable interpretation of different *?* values from different species, and more work needs to be performed in order to address this issue.

In summary, the strong correlation between pK_i and σ values demonstrates that the electronic influence of substituents on the inhibitors plays a crucial role in the stabilization and inhibitory potency on the active site-directed inhibition of AChE. This fact was demonstrated with the experiments carried out in this work, because that the theoretical and the experimental data showed the similar trend in both cases. This observation supports our hypothesis of the importance of the, electronic influence of the ligands in the recognition mechanism by AChE.

These results would be helpful in the rational design of potent AChE inhibitors; moreover, a better understanding of the molecular recognition mechanism by AChE could be extended to other biological systems where aromatic interactions are involved in the molecular recognition process. In addition, the experimental data suggests that the electronic influence of aryl-succinimides derivatives are important like show the evaluations results.

As the model presented here only allows flexibility of the ligand (semi-flexible docking) the binding process and stability of the complexes may not be as optimal as it was expected due to the lack of a thorough conformational space sampling; moreover, as ligands may bind to conformations that occur only rarely in the dynamics of the receptor, giving a multivalent attachment of the ligand to the receptor [22,23], future studies will include molecular dynamic simulations to allow the enzyme to be flexible. More accurate energy sampling methods (i.e., MM/PBSA) will also be used for a detailed study of the electronic influence of substituents on the active site-directed inhibitory potency on AChE.

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