Design and activity of cationic fullerene derivatives as inhibitors of acetylcholinesterase

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Four different regioisomers of cationic bis-N,N-dimethylfulleropyrrolidinium salts have been prepared and evaluated as inhibitors of the enzymatic activity of acetylcholinesterase. These fullerene-based derivatives were found to be noncompetitive inhibitors of acetylthiocholine hydrolysis. Molecular modelling was used to describe the possible interactions between the fullerene cage and the amino acids surrounding the cavity of the enzyme. The cationic C_{60} derivatives used in this study represent a new class of molecules potentially able to modulate the enzymatic activity of acetylcholinesterase.

Introduction

Different classes of fullerene derivatives have shown interesting potential in biomedical applications.[1] The biological properties of fullerenes include photocleavage,[2] antiapoptotic activity,[3] neuroprotection,[4] drug and gene delivery,[5] antioxidation,[6] chemotaxis,[7] antibacterial activity,[8] and enzyme inhibition.[9] Concerning the development of enzyme inhibitors based on a C_{60} core, only a few examples have been reported.[9] It has been demonstrated that the structure of C_{60} is highly complementary to the HIV protease (HIVP).[9a,10] As a consequence, a series of fullerene derivatives has been designed and found to bind to the active site of HIVP, which is characterised by a hydrophobic pocket. Similarly, an antibody raised against a series of fullerene conjugates presented a non polar binding site highly specific for C_{60}.[11]

Another family of enzymes that contain a hydrophobic cavity with the ideal dimensions to accommodate a fullerene moiety is that of cholinesterases.[12] Within these serine esterases, acetylcholinesterase (AChE) plays an important role in the regulation of functions of central and peripheral nervous systems.[13] AChE hydrolyses the cationic neurotransmitter acetylcholine (ACh).[14] Dysfunctions on the level of ACh have a direct implication in the development of neurodegenerative disorders such as Alzheimer's disease (AD).[15] Therefore, selective ligands have been developed over the years because of the pharmacological and toxicological importance of AChE. The active site of AChE has been described at the atomic resolution by X-ray crystallography of the protein alone or complexed to different types of inhibitors.[16] It comprises the principal site, constituted by the catalytic triad Ser200, Glu327 and His440, which is located at the bottom of a gorge approximately 20 Å deep, and the peripheral site, near the edge of the cavity. The two sites, about 15 Å apart, are surrounded by the rings of 14 conserved aromatic residues.[17] The current inhibitors present different chemical structures, ranging from simple cationic compounds to bis-quaternary salts able to interact with both principal and peripheral binding sites.[18,19] In particular, the dual-site inhibitors are able to form an important cation–π interaction with Trp279 and Trp84 at the top and the bottom of the hydrophobic pocket, respectively. On the basis of these chemico-physical characteristics of the enzyme cavity and the search of efficient ligands, cationic fullerene derivatives may mimic the action of bis-quaternary salts and become a new class of potential inhibitors of AChE.

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Results and discussion

Design and synthesis of AChE fulleropyrrolidine inhibitors

The most effective, past inhibitors of AChE were characterised by the molecular structures bearing tertiary amines or quaternary ammonium salts, which interacted with the catalytic triad and Trp84 at the base of the gorge.[18,20,21] After a demonstration that the peripheral site at the rim of the cavity played also an important role in the enzymatic activity, new generations of inhibitors able to simultaneously block the two binding sites have been conceived and developed.[19,22] In fact, bivalent ligands enhance the affinity for the target.[21f] For example, decamethonium is a symmetric bis-quaternary ammonium salt that inhibits AChE by spanning the distance of nearly 15 Å between Trp84 and Trp279.[19a] The dimension of the C_{60} sphere (~7.0 Å diameter) and the possibility of its double functionalization[23] suggested the idea that fullerene modified with the appropriate functionalities could accommodate into the hydrophobic core of AChE and display an inhibitory action. Indeed, cationic bis-*N*,*N*-dimethylfulleropyrrolidinium salts **1–4** (Fig. 1) can be considered very promising compounds because the aromatic fullerene cage perfectly fits into the hydrophobic core of AChE while the *N*,*N*-dimethylpyrrolidinium groups located around the sphere may tackle both the main and secondary active sites. The C_{60} cage would not only act as a spacer between the quaternary ammonium moieties but might play an active role in the target recognition process by establishing its own interaction with the enzyme.

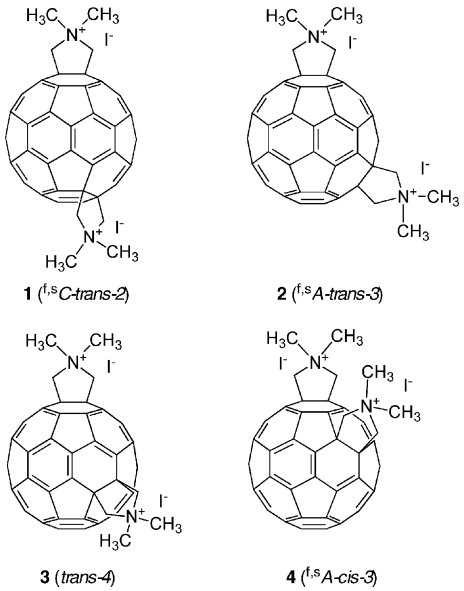

Fig. 1 Molecular structures of bis-*N*,*N*-dimethylfulleropyrrolidinium salts **1–4.** For simplicity, only one enantiomer of chiral *trans*-2 (**1**), *trans*-3 (**2**) and *cis*-3 (**4**) is illustrated.[24b]

The synthesis of fullerene bis-adducts **1–4**, using the 1,3-dipolar cycloaddition reaction of azomethine ylides to C_{60}, was previously reported.[24] C_{60} was reacted with sarcosine and paraformaldehyde in toluene. The mono-adduct and the different regioisomeric bis-adducts were separated by repeated medium-pressure column chromatography, preparative TLC and semipreparative HPLC. The most abundant bis-adducts were methylated with methyl iodide, affording compounds **1–4** (Fig. 1), which were used for the study of inhibition activity against AChE. The three derivatives **1**, **2** and **4** are chiral due to their double addition pattern.[24b] The separation of the single enantiomers was beyond the scope of this work, and the AChE inhibition experiments have been performed using the racemic mixtures.

Study of the inhibition of AChE activity

The inhibitory activity of the four cationic fulleropyrrolidinium regioisomers was evaluated on AChE from *Electrophorus electricus*.[18] The determination of the type of inhibitory mechanism can be directly correlated to the action of the inhibitor and its interaction with the enzyme active site. The initial hydrolysis rates ($\Delta A/\Delta t$) were plotted *versus* the concentration of acetylthiocholine iodide (ATCI), at different concentrations of inhibitors (Fig. 2).

The different curves were calculated using the typical equations for the competitive and noncompetitive inhibition process by a non linear regression method (Sigmaplot, Chicago, Il). The K_i values and the corresponding square correlation coefficients (R^2) are reported in Table 1. The discrimination between competitive or noncompetitive action of the inhibitors was assessed by comparing the values of R^2 using eqn (1) and (2) (see Experimental section).

We wanted first to test the enzyme specificity using edrophonium chloride. This competitive inhibitor was employed in our study as the reference compound.[25] We found an inhibition constant of 0.054 μM which was in full agreement with the previous data

Table 1 AChE enzymatic inhibition of bis-*N*,*N*-dimethylfulleropyrrolidinium salts

Entry	K_M/μM	K_i/μM[a]	R^{2b}
1 (*trans*-2)	76.9 ± 9.4	15.6 ± 1.7	0.950
2 (*trans*-3)	64.4 ± 4.7	31.4 ± 1.4	0.988
3 (*trans*-4)	80.9 ± 10.6	27.8 ± 2.8	0.959
4 (*cis*-3)	76.3 ± 11.0	17.7 ± 1.8	0.963
Edrophonium chloride	68.9 ± 12.3	0.054 ± 0.009	0.967

[a] The inhibition constants K_i are noncompetitive for **1–4** and competitive for edrophonium chloride. [b] R^2 corresponds to the square of the correlation coefficient.

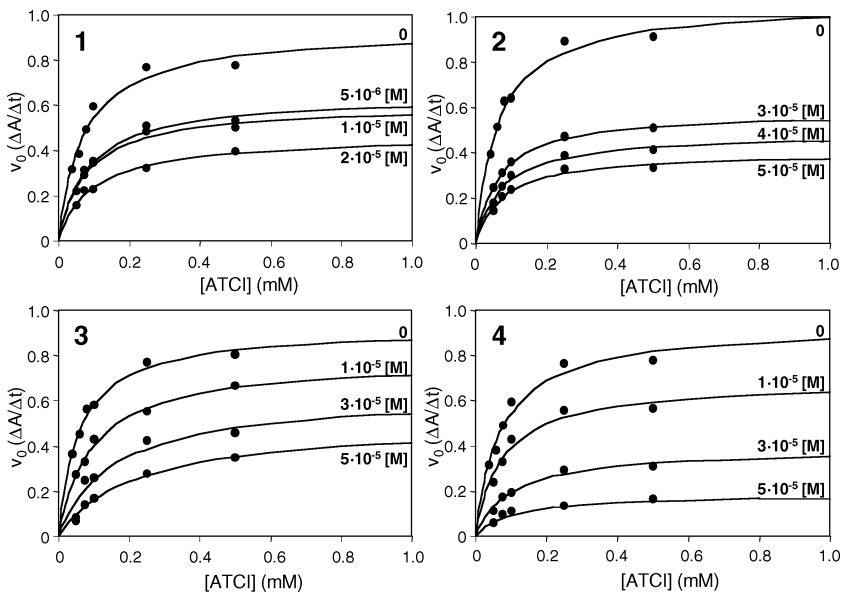

Fig. 2 Kinetics analyses of AChE inhibition by cationic bis-*N*,*N*-dimethylfulleropyrrolidinium salts **1–4**. The initial hydrolysis rates are plotted against the concentration of the substrate. The concentrations of each inhibitor are reported on the theoretical curves.

reported in the literature (0.050 μM) (Table 1).[25] All fulleropyrrolidinium salts **1–4** exhibited instead a noncompetitive inhibition as clearly indicated by a decrease of V_{max} values with increasing amount of substrate, while the values of K_M remained almost unchanged (Table 1). This is a common behaviour for AChE noncompetitive inhibitors that do not affect the affinity of the enzyme towards the substrate.[26] The fullerene derivatives appear not to directly interact with the catalytic triad but partially block the binding gorge. These fullerene-based ligands are probably not able to enter the gorge as deep as other double cationic inhibitors,[18,22] and this could explain their noncompetitive activity. K_i values for the four fulleropyrrolidinium salts are in the micromolar range (Table 1). These values are similar to those reported for other noncompetitive molecules.[27] The comparison between the affinity data of compounds **1–4** suggested that the relative position of the *N*,*N*-dimethylpyrrolidinium moieties did not produce a remarkable variation on the inhibition activity. In the case of the *trans* isomers **1–3** the distance between the cationic nitrogens is comprised between 10.0 and 11.5 Å (Table 2). The best inhibitor is compound **1** (*trans*-2) where the functional groups are further away. This spatial arrangement is likely in favour of a better orientation towards Trp84 and Trp279, mimicking other bis-quaternary salts, unless other residues affect this binding driving the cationic groups in a different direction (*vide infra*). On *cis* isomer **4**, the two charged groups are very close (7.45 Å) and located on the same side of the C_{60} cage. This conformation does not permit the quaternary salts to orient towards both the principal and secondary binding site. The inhibition value was almost identical to that of compound **1**, reinforcing the finding of a noncompetitive mechanism of AChE inhibition displayed by this new class of ligands. These results should be considered carefully, but they seem to indicate that not all the cationic groups on C_{60} could completely reach the binding sites of the AChE gorge. To explain this behaviour and to disclose the possible interactions exerted by the bis-*N*,*N*-dimethylfulleropyrrolidinium salts inside the hydrophobic cavity of AChE, we have subsequently carried out a docking analysis.

Docking inside AChE active site

The molecular modelling study was performed using the crystal structure coordinates of AChE from *Torpedo californica*.[20] This acetylcholinesterase is structurally homologous to that from *Electrophorus electricus* used for the determination of the fulleropyrrolidine inhibitory activity.[28–30] The simulations were performed on ^{f,s}C-*trans*-2 (**1**), *trans*-4 (**3**) and ^{f,s}A-*cis*-3 (**4**) isomers. The fullerene ligands were readily synthesised as racemates. The modelling however required analysis of each enantiomer separately, because the different isomers could interact differently with the chiral environment of the AChE gorge. In the case of *trans*-2, the calculation was done on both chiral regioisomers. Each derivative was put manually into the active site of the enzyme close to the catalytic triad. The gorge width calculated on the center of mass of Trp279 and Gly335 has an average of 12.0 Å.[27c] The two quaternary pyrrolidinium groups were oriented towards the principal and the peripheral binding site, respectively. After a first minimization under fixation of the enzyme backbone, the contact residues between the C_{60} ligand and the active site residues of the receptor within a distance of 5.0 Å were determined. Subsequently, dynamics and minimization using the frames corresponding to the most stable lower energy conformation state were run, enabling the fullerene derivatives to relax inside the enzyme active site. Water molecules in the gorge were not taken into consideration during the calculations.[21c] It is reasonable to assume that these molecules, which present highly reduced hydrogen bond coordination, are rapidly displaced.[16a] This phenomenon is also known as the desolvation effect. Desolvation of hydrophobic surfaces was shown to improve for example the binding affinity in the case of fullerene inhibitors of HIV-1 protease.[10a] Any significant alteration in the structure of the enzyme was observed after the minimization analysis in comparison to the original crystal structure. Fig. 3 shows the molecular structures of the complexes between the residues of the AChE active pocket (black) and the regioisomers **1**, **3** and **4** (cyan). The catalytic triad is displayed in magenta while the two key residues at the principal (Trp84) and secondary (Trp279) binding sites are shown in blue. The C_{60} derivatives present a high steric hindrance and a reduced conformational flexibility. The elements introduced around the fullerene cage can be oriented in a limited number of ways inside the AChE active channel. All three isomers are characterised by close proximity between the bottom dimethylpyrrolidinium group and Trp84 while the second cationic group remains at a distance greater than 10 Å from Trp279 (Table 2). The lack of the second cation–π interaction, typical of the bis-quaternary inhibitors,[31] is compensated by a series of salt bridges between the two ammonium groups and various aspartic and glutamic residues. In particular, the bottom pyrrolidine of the *trans* isomers is very close to Glu199 (4.01–4.27 Å) and that at the top is involved in bonding with Asp72 and Glu82 (4.44–4.91 Å). The same interactions are less strong for the *cis* isomer where the distance between the positive charges and the carboxylate side chains are comprised between 5.0 and 6.25 Å. We could also evidence stabilisation by a cation–π interaction

Table 2 AChE docking parameters of bis-*N*,*N*-dimethylfulleropyrrolidinium salts

Entry	$N^+(CH_3)_2 \cdots N^+(CH_3)_2$/Å[a]	W84 $\cdots$ $N^+(CH_3)_2$/Å	van der Waals/kcal mol^{-1}[b]	Electrostatic/kcal mol^{-1}[b]
1 (^{f,s}C-*trans*-2)	10.83	4.55	−107	−613
1 (^{f,s}A-*trans*-2)	11.42	4.58	−131	−603
3 (*trans*-4)	10.01	4.20	−113	−613
4 (^{f,s}A-*cis*-3)	7.45	4.25	−102	−600
4 (^{f,s}A-*cis*-3) 180° *y*-rot[c]	7.17	nd[d]	−120	−557

[a] The distance between the two pyrrolidine nitrogens was measured after the minimization of the ligand into the active site of AChE. [b] Energy of interaction between the enzyme and the inhibitor (calculated as continuous evaluation energy). [c] rot = rotation. [d] nd = not determined.

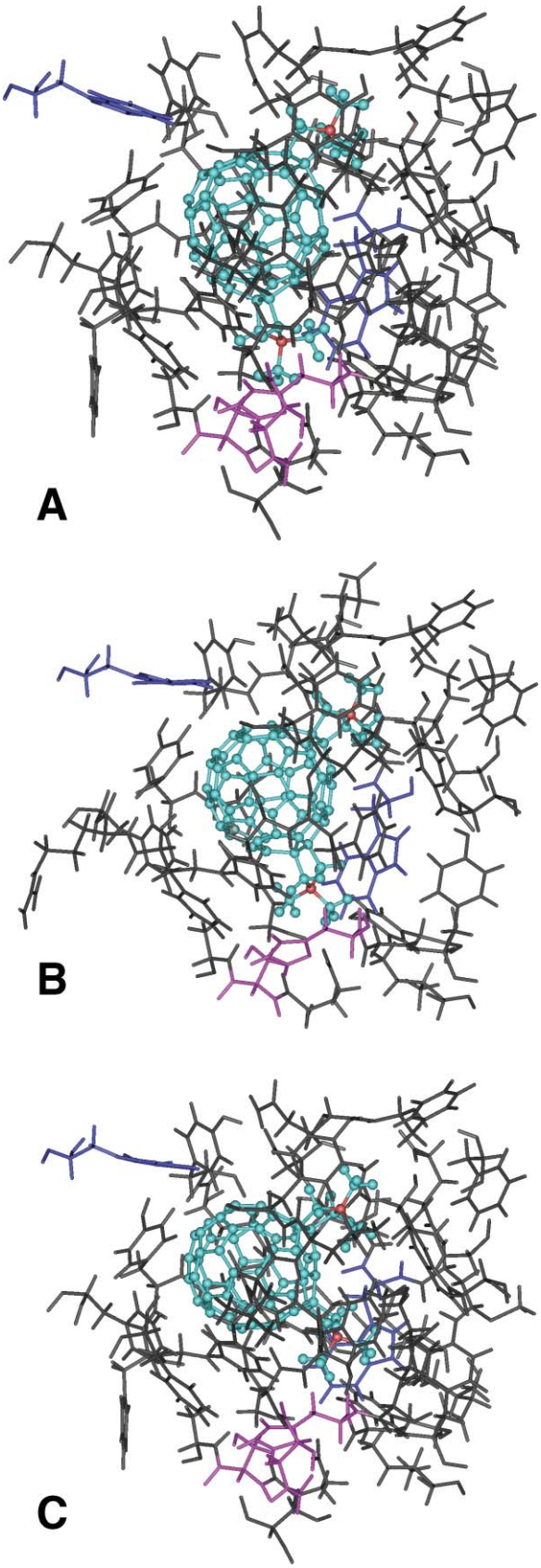

Fig. 3 Ball and stick molecular model representation of the AChE hydrophobic pocket complexed with bis-*N*,*N*-dimethylfulleropyrrolidinium salts ^{f,s}C-*trans*-2 (**1**) (A), *trans*-4 (**3**) (B), and ^{f,s}A-*cis*-3 (**4**) (C).

bridging the top pyrrolidinium nitrogen with Trp432. In addition, the hydrophobic surface of the C_{60} is surrounded and stabilized by a high number of π–π contacts with the aromatic side chains of the residues located near the peripheral (Tyr70, Phe75, Tyr121, Tyr334) and main (Phe288, Phe290, Phe330, Phe331) binding domains. In fact, nearly 70% of the surface of the AChE cavity is covered by aromatic rings.[17] The favourable interactions by van der Waals forces are also supported by the values of the non covalent complex stabilization energy (Table 2). Simulation using the second enantiomer of the other chiral *trans*-2 derivative provided the same way of binding of its mirror image (not shown). Molecular modelling clearly showed there were no deep differences between the two enantiomers. The enzyme seemed to show an inherent preference for either of the two molecules. It is worth noting that the bis-*N*,*N*-dimethylpyrrolidinium moieties might not be large enough to influence the stereospecific preference among the two components of the racemate.[32]

Although enantiomeric selectivity is a key issue in the development of new drugs and inhibition of racemates can be less effective than inhibition of the preferred enantiomer, examples of non selectivity between enantiomers have already been described for other classes of AChE ligands.[22a]

Due to a difficulty in the bulky C_{60} sphere rotating and reorienting into the enzyme cavity, we could not exclude that the fulleropyrrolidinium salts might display alternative modes of binding. In fact, the *cis* isomer could adopt different orientation inside the binding pocket, stabilised by favourable interactions. A simulation was repeated after rotation of 180° around the *y*-axis. We found that the two quaternary ammonium groups pointed towards Trp279 at an ideal distance for the formation of a bidentate cation–π bond (Fig. 4). We measured a distance of 4.31 and 5.07 Å for the two pyrrolidinium nitrogens from the indole ring of Trp279. The alternative stable complex derived from this orientation supports the good affinity of the *cis*-3 C_{60} derivative in comparison to *trans*-3 and *trans*-4 despite the fact that the ammonium groups are spatially located at a shorter distance (Table 2).

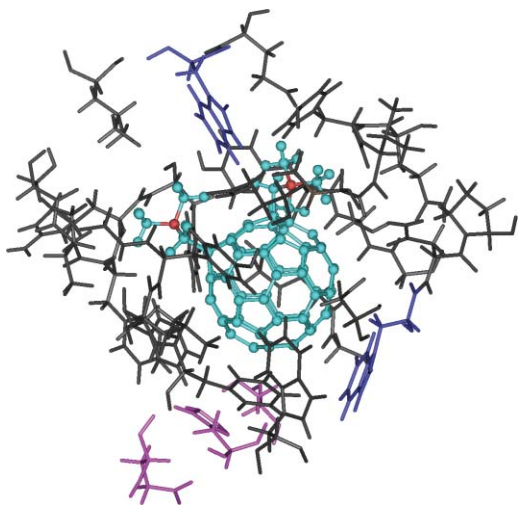

Fig. 4 Molecular model of ^{f,s}A-*cis* (**4**) inside the active site of AChE after 180° rotation of its minimized analogue around the *y*-axis.

The prediction of the binding geometry and the analysis of the ligand–receptor interactions permitted us to identify the possible modes of binding of the fulleropyrrolidinium salts and to explain the inhibition mechanism derived by the kinetics. The cationic bis-fulleropyrrolidinium salts are probably not able to enter into the hybrophobic channel as deep as decamethonium or other bivalent ligands because they are sterically hindered.[31] For this reason, similar to other types of inhibitors, they are noncompetitive.[27] However, the simulations are not conclusive and should be considered as the initial step to provide possible structures of the complex between AChE and the fullerene derivatives. As other large quaternary dual inhibitors were able to crystallise into the active cavity of the protein,[19a] we believe that this behaviour could be exerted also by the fullerene derivatives. It has recently been shown that a certain flexibility of the loops at the neck of the gorge permitted the binding of bulky inhibitors such as galanthamine-based derivatives.[33] Indeed, enzymes are dynamic molecular machines characterised by intrinsic motions which are fundamental for their function.[34]

Finally, the description of the complex contacts may suggest possible modifications on the surface of the fullerene that can potentially improve the binding properties as well as modulate the solubility and bioavailability of this new class of inhibitors.

Conclusions

In this study, we have designed a series of cationic bis-*N*,*N*-dimethylfulleropyrrolidines and demonstrated that they were able to inhibit the enzymatic activity of acetylcholinesterase. The inhibition process was noncompetitive and the values of the affinity constants were found in the micromolar range. The possible mechanism of ligand–receptor interaction was subsequently analysed by molecular simulations. Each bis-quaternary salt was docked into the active site of the enzyme and the models showed that the aromatic fullerene core perfectly fits the hydrophobic cavity of AChE, while the ammonium groups can interact with the side chains of the key amino acid residues of the principal or peripheral binding sites.

On the basis of our results, these new compounds might be potential candidates for the modulation of acetylcholinesterase activity.

Experimental

Chemistry

All reagents and solvents were obtained from commercial suppliers and used without further purification. C_{60} was purchased from Bucky-USA (Houston, TX). Cationic bis-*N*,*N*-dimethylfulleropyrrolidinium salts **1–4** (Fig. 1) were prepared as described in the literature.[24]

Inhibition assays

Inhibition activity of AChE was determined using the standard method of Ellman.[35] AChE from *Electrophorus electricus* was obtained from Sigma (V–S type, C-2888). Lyophilised AChE was reconstituted in 0.1 M phosphate buffer at pH 7.2 (572 U mL^{-1}). Enzyme stock solution was prepared by taking 15.3 μL and diluting in 1 mL of the same buffer. One enzymatic unit (U) was defined as the amount of enzyme which hydrolyses 1 μmol of ATCI per minute. We initially determined the concentration of AChE necessary to hydrolyse 50% of the substrate in about 2 minutes. This value corresponded to 0.175 U ml^{-1}. Self-hydrolysis of ATCI was verified at concentrations of ATCI between $1{\cdot}10^{-5}$ and $1{\cdot}10^{-3}$ M in the presence of a constant amount of 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) ($2.5{\cdot}10^{-3}$ M). No ATCI appreciable hydrolysis in the absence of the enzyme was observed in this range. The inhibition activity was evaluated by preparing a reaction mixture containing 10 μL of AChE solution (8.75 U mL^{-1} of 0.1 M phosphate buffer, pH 7.2), 250 μL of a solution of DTNB (stock solution $5{\cdot}10^{-3}$ M in 0.1 M phosphate buffer, pH 7.2), a variable volume of the buffer solution of ATCI (stock solution $5{\cdot}10^{-3}$ M in 0.1 M phosphate buffer, pH 7.2), and a variable volume of the buffer solution of the inhibitor, namely bis-*N*,*N*-dimethylfulleropyrrolidinium salts **1–4** and edrophonium chloride. The final volume of the mixture was adjusted to 0.5 mL with the buffer solution. The enzyme was added as the last component. Edrophonium chloride, the competitive inhibitor that was taken as a reference, was used in concentrations between $2{\cdot}10^{-7}$ and $2{\cdot}10^{-6}$ M, while the fullerene derivatives varied in the range $5{\cdot}10^{-6}$ and $5{\cdot}10^{-5}$ M. Bis-*N*,*N*-dimethylfulleropyrrolidinium salts **1–4** (about 1 mg) were initially dissolved in 50 μL of pure DMSO. Subsequently, 25 μL were diluted in 975 μL of 0.1 M phosphate buffer at pH 7.2 to obtain a final concentration of about $4.5{\cdot}10^{-4}$ M. This stock solution was further diluted for each experiment according to the final concentration of required inhibitor. The final concentration of DMSO was less then 0.5%. This amount of DMSO did not alter the activity of AChE. The kinetics analyses were conducted for 4 minutes at 23 °C following the variation of the absorbance ($\Delta A/\Delta t$) at 412 nm. At this wavelength the absorbance of fullerene derivatives at the highest inhibitor concentration is negligible. The experiments were performed on a UV–Vis Anthelie Advanced V2.5 spectrophotometer.

Data analysis

Initial rate (v_0) corresponding to the variation of the absorbance within the first 10 seconds was plotted against the concentration of ATCI. Determination of the type of inhibition was based on the Michaelis–Menten equation. Graphs were plotted using Sigma Plot Multiple Function Non Linear Regression. Michaelis–Menten parameters K_M and V_{max} were calculated from the reaction rate as a function of the substrate concentration. Apparent K_i constants were calculated by using eqn (1) and (2), that describe a competitive and a noncompetitive inhibition process, respectively.[36]

$$v_0 = \frac{V_{max} \cdot [S]}{K_M \cdot \left(1 + \frac{[I]}{K_i}\right) + [S]} \quad (1)$$

$$v_0 = \frac{V_{max} \cdot [S]}{(K_M + [S]) \cdot \left(1 + \frac{[I]}{K_i}\right)} \quad (2)$$

V_{max} and K_M were obtained in the absence of inhibitor, while K_i was calculated in the presence of the inhibitors. [I] and [S] represent the concentration of inhibitors

(bis-*N*,*N*-dimethylfulleropyrrolidinium salts **1–4** and edrophonium chloride) and ATCI, respectively.

Docking studies

Docking was performed using the crystal structure coordinates 1QTI from *Torpedo californica*.[20] Fullerene derivatives **1**, **3** and **4** were manually placed into the active site of the enzyme. After a first minimization using a conjugate gradient under fixation of the enzyme backbone, the contact residues within 5.0 Å between the C_{60} derivatives and the amino acids of the active site were determined. All residues outside the contact pocket and the backbone (Cα, NH and CO) of the residues within a distance of 5.0 Å were fixed to perform 100 psec dynamics at 300 K after an equilibration step of 10 psec. Conformations were collected every 1000 fsec. The frames corresponding to the minimum were then selected for the minimization by a conjugate gradient algorithm (Accelrys, San Diego, CA) enabling the fullerene derivative to relax inside the enzyme active site.

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