Eur. J. Med. Chem. 35 (2000) 913–922

PII: S0223-5234(00)01166-1/FLA

Original article

N-Benzylpiperidine derivatives of 1,2,4-thiadiazolidinone as new acetylcholinesterase inhibitors

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Received 31 January 2000; revised 20 March 2000; accepted 26 April 2000

Abstract – A new family of 1,2,4-thiadiazolidinone derivatives containing the *N*-benzylpiperidine fragment has been synthesised. The acetylcholinesterase (AChE) inhibitory activity of all compounds was measured using Ellman's method and some of them turned out to be as potent as tacrine. Furthermore, compound **13** was as active as tacrine in reversing the blockade induced by tubocurarine at rat neuromuscular junction. Additionally, receptor binding studies provided new lead compounds for further development of α_2-adrenergic and sigma-receptor antagonists. Molecular dynamic simulation using X-ray crystal structure of AChE from *Torpedo californica* was used to explain the possible binding mode of these new compounds. © 2000 Éditions scientifiques et médicales Elsevier SAS

thiadiazolidinones / acetylcholinesterase inhibitors / α_2-adrenoceptor antagonists / Alzheimer's disease / molecular modelling

1. Introduction

Alzheimer's disease (AD) is a complex and multifaceted neurodegenerative disease characterised by cognitive and behavioural abnormalities [1, 2]. The primary cognitive deficit has been correlated with extensive cholinergic disfunction and the efficacy of cholinergic therapies in this disease validates and supports the cholinergic hypothesis of AD [3, 4]. To date, acetylcholinesterase inhibitors such as tacrine [5] and donepezil [6], have shown a clinical efficacy in 20–30% of patients and it is the only drug group that has been approved for symptomatic treatment of AD [7]. However, the limited efficacy of pure cholinergic therapies suggests that other neurotransmitter systems may be involved in this disease. This evidence favours the hypothesis that a mechanism-based therapy which activates more than one neurotransmitter system might be beneficial in the treatment of AD [8].

Following our work in this field [9, 10], we report here the synthesis, biological evaluation and receptor binding properties of novel *N*-benzylpiperidine derivatives of 1,2,4-thiadiazolidinones (I in *figure 1*). Some of them exhibited both acetylcholinesterase inhibition and α_2-adreneceptor antagonist properties. Molecular dynamic simulation is also presented to explore a possible binding mode of the lead compound **13** to the X-ray crystal structure of AChE from *Torpedo californica*.

2. Chemistry

N-benzylpiperidine and *N*-benzylpiperazine derivatives of 1,2,4-thiadiazolidinones prepared for this study were initially synthesised following a pathway which is based on the reactivity of *N*-alkyl-S-[*N*′-(chlorocarbonyl)-amino]isothiocarbamoyl chlorides

Abbreviations: AChE, acetylcholinesterase; AD, Alzheimer's disease; BChE, butyrylcholinesterase.

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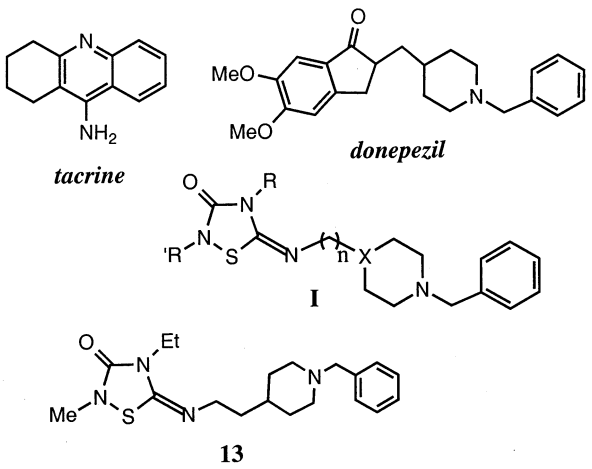

Figure 1. *N*-benzylpiperidine derivatives of 1,2,4-thiadiazolidinones.

[11]. Here, the key synthetic step was nucleophilic susbtitution by primary alkyl amines on the oxathiadiazolium salts **1–4** (*figure 2*). Reactivity of these heterocyclic salts with aryl amines had previously been studied in our group [12]. Chlorination of alkylisothiocyanates in an inert atmosphere and subsequent reaction with *N*-alkylisocyanates yields sparingly soluble 3-oxathiadiazolium salts via the intermidate iminochloromethylsulfenyl chloride. Heterocyclic salts **1–4** are exceptionally reactive white solids, which fume heavily in moist air yielding after evolution of hydrogen chloride, the 1,2,4-thiadiazolidin-3,5-diones **5–8**. Only when salts were not isolated and manipulated under a dry nitrogen atmosphere, could they easily be converted in thiadiazolidinones **9–18** by reaction with the appropriate primary alkyl amines in a 1:1 salt:amine ratio. The reaction was completed in the presence of two molar equivalents of triethylamine which neutralised the hydrogen chloride evolved in the medium.

The structures of all new compounds were elucidated according to analytical and spectroscopic data (see Section 5). Unequivocal assignment of all chemical shifts (^{1}H- and ^{13}C-NMR), including the nine spin system of piperidine moiety, was done using bidimensional experiments such as COSY or HMQC for one bond correlation. The methylene protons of piperidine ring were diasterotopic with geminal, axial and equatorial couplings. A symmetry axis is present in the hexagonal saturated heterocycle which facilitates the chemical shifts assignation.

3. Results and discussion

3.1. AChE inhibition determinations

To determine the AChE activity, compounds **9–18** were assayed by the method of Ellman et al. [13] on AChE from bovine erythrocytes. *Table I* summarises the results. As *table I* shows, some of the compounds inhibit the AChE at μM range, compound **13** being as active as tacrine. Thereby, this compound was also assayed in AChE from human serum and butyrylcholinesterase (BChE) from human serum. Experimental data showed

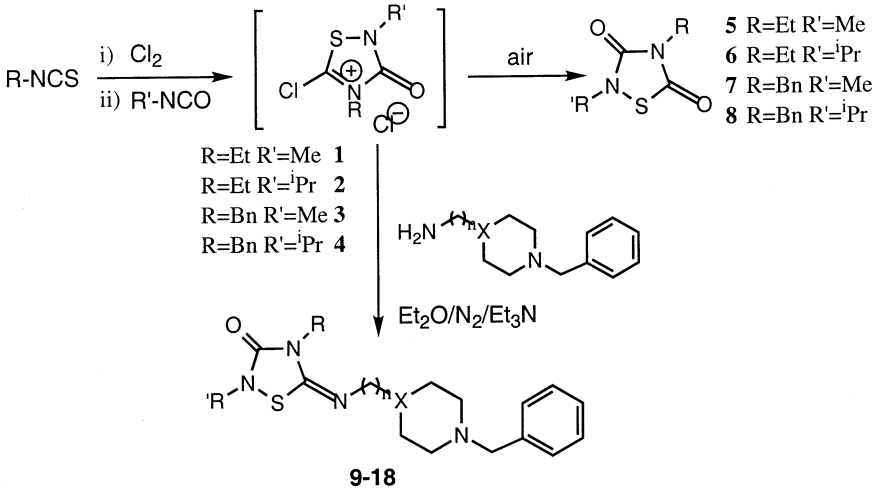

Figure 2. Synthesis of compounds **9–18**.

Table I. Acetylcholinesterase, α_{2A}, α_{2B}, α_{2C} and σ receptor binding properties of thiadiazolidinones **9–18**.

Compound	R	R′	*n*	X	AChE IC_{50} (μM)[a]	α_{2A} IC_{50} (μM)[b]	α_{2B} IC_{50} (μM)[b]	α_{2C} IC_{50} (μM)[b]	σ IC_{50} (μM)[c]
9	Et	^{i}Pr	0	CH	–	–	–	–	0.015
10	Et	^{i}Pr	2	CH	1.04 ± 0.11	–	–	–	0.019
11	Et	^{i}Pr	2	N	9.66 ± 2.66	–	–	3.16	0.039
12	Et	Me	0	CH	31.1 ± 6.6	–	–	–	0.199
13	Et	Me	2	CH	0.14 ± 0.02	–	–	–	0.079
14	Et	Me	2	N	1.81 ± 0.23	–	0.79	–	0.630
15	Bn	^{i}Pr	0	CH	19.9 ± 5.1	1.25	1.25	1.58	0.050
16	Bn	^{i}Pr	2	CH	1.57 ± 0.11	1	2.51	1	0.031
17	Bn	^{i}Pr	2	N	27.3 ± 4.2	0.31	3.16	0.39	0.063
18	Bn	Me	2	CH	4.06 ± 0.3	–	–	–	–

[a] All values are expressed as mean ± standard error of the mean of at least four experiments. IC_{50}: 50% inhibitory concentration of acetylcholinesterase activity (μM). References values: tacrine $IC_{50} = 0.20 \pm 0.03$, donepezil [14] $IC_{50} = 0.04 \pm 0.01$.
[b] IC_{50}: 50% inhibitory concentration of radioligand binding of α_{2A}, α_{2B}, and α_{2C} adrenergic activity respectively ([^{3}H]-Rauwolscine, human CHO cells).
[c] IC_{50}: 50% inhibitory concentration of radioligand binding of guinea pig haloperidol-sensitive σ_1 receptor ([^{3}H]-Haloperidol, guinea pig medulla oblongata).

that compound **13** had an $IC_{50} = 0.014 \pm 0.002$ μM on human erythrocyte AChE and 8.55 ± 0.43 μM on BChE, respectively. Therefore, the ratio BChE/AChE was 610, showing a strong selectivity for human AChE. Compound **13** was further analysed in a cholinergic synapse, such as skeletal neuromuscular junction, where its ability to reverse the tubocurarine-induced neuromuscular blockade, a well-known effect of AChE inhibitors [15], was tested. The antagonism index (AI_{50}) was 0.36 μM for thiadiazolidinone **13** whereas tacrine showed a value for the AI_{50} of 71.7 μM. The discrepancy between the potency of compound **13** and tacrine may be due to several factors. First, compound **13** may be devoid of some unspecific effects that limit the pharmacological activity of tacrine, such as blockade of sodium channels [16]. Second, compound **13** may have additional unknown actions that may enhance further its facilitatory effect on cholinergic neurotransmission. Although we favour the first hypothesis, at present experimental data are lacking to confirm or refuse any explanation. Work in progress will help to elucidate which mechanism may explain the findings described above.

In connection with the AChE inhibitory activity, some qualitative structure–activity relationships can be derived from the above data: the presence of a dimethylene spacer between the *N*-benzylamine and the thiadiazolidinone seems to be important, piperidine derivatives were more active than the piperazine analogues, and a small alkyl group in position 2 of the heterocyclic moiety is favourable for AChE inhibition.

Due to the fact that other neurotransmitter systems are involved in neurodegenerative processes, we also investigated the receptor-binding in vitro profile of these new compounds for several neurotransmitter receptor subtypes (adrenaline, serotonine, dopamine, acetylcholine, opioid...), ion channel-binding sites and monoamine transporters. Assay conditions were as reported by Schotte et al. [17]. The IC_{50} found in these assays was in all cases at mM level except for α_2-adrenoceptor subtype and sigma-receptors where IC_{50} values were comprised between μM and nM concentrations. These values are summarised in *table I*.

The use of α_2-adrenoceptor antagonists have been postulated to be a new approach to overcome the progression of neurodegenerative pathologies [18], while recent studies proposed that a noradrenergic activation through pre-synaptic α_2-adrenoceptor blockade may potentiate cholinergic activity in the formation of a long-term memory trace [19]. Moreover, the design of selective sigma-receptor ligands is a very active field in the search of new drugs for psychotic disorders [20].

Consequently, the pharmacological profile found in the new thiadiazolidinones together with antiacetylcholinesterase activity, is promising for the development of more potent compounds with dual action on AChE and α_2-receptors.

3.2. Molecular modelling binding model

In order to shed some light on the possible interaction of this new lead compound with AChE inhibiton, which

allows the rational design of more potent inhibitors, a molecular modelling study was performed.

Molecular dynamic simulation was carried out to investigate the possible binding mode of derivative **13** and AChE. The publication of the X-ray structure of AChE [21] from *T. californica* makes possible this study. The results of simulations allowed the identification of the key features responsible for the enzyme activity of this thiadiazolidinone inhibitor. The binding mode and the key interactions are illustrated in *figures 3* and *4*, respectively. These data suggest that inhibitor **13** binds to the aromatic gorge of AChE, hindering the access of the natural substrate into the catalytic pocket of the enzyme.

The inhibitor-enzyme binding was stabilised by two hydrogen bonds: one is formed between the positively charged piperidine of **13** and the negatively charged carboxylate side chain of Asp-72. This H-bonded interaction probably serves to anchor the location of the piperidine within the gorge. And the other one, between the carbonyl oxygen of thiadiazolidinone heterocycle and the backbone N–H of Phe 288, which is found in the floor of the gorge.

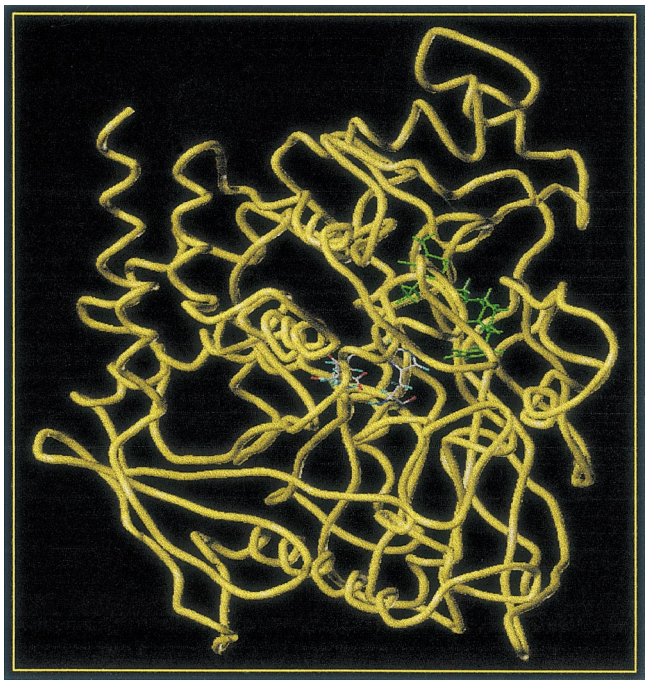

Figure 3. AChE binding model for thiadiazolidine **13**. Legend: yellow, AChE backbone; green, thiadiazolidinone **13**; other colours, AChE catalytic triad.

Compound **13** is also stabilised by hydrophobic interactions. It is known that the gorge leading to the active site is lined with aromatic residues, which constitute 40% of the residues present in this region [21]. Inhibitor **13** takes advantage of this hydrophobic lining by making specific contacts. The *N*-benzyl substituent of **13** forms an off-centre π-stacking interaction with the indole side chain of Trp-84. The arrangement of the rings is roughly parallel with an average centre–centre distance between the plane of the indole and the benzyl ring of 4.4 Å. This distance is near the minimun (4.5 Å) reported for a planar π-stacked interaction between two benzene rings in the gas phase, with a similar off-centre geometry [22]. Trp-84 has been implicated in the binding of acetylcholine and other quaternary ligands as tacrine [21]. In addition to the important interaction of Trp-84 with the *N*-benzyl substituent of **13**, we find that the phenyl ring of Phe-330 interacts with the phenyl ring of **13** via an edge-on configuration [22]. Furthermore, a parallel disposition between the indole side chain of Trp-279, which is located at the entrance to the gorge in the peripheral binding site, and the heterocycle present in the inhibitor is observed at an average distance of 3.84 Å. This geometry allows the formation of an off-centre π-stacking interaction. The recent observations concerning the effect of peripheral-site ligands on AChE-enhanced amyloid deposition [23], raise the possibility that thiadiazolidinone **13**, which our model clearly show as stacking against Trp-279, might also moderate the rate of fibril formation.

This model is in agreement with the biological results found since it shows a profund effect on potency changing the length of the chain that connects the piperidine to the thiadiazole ring (*table I*, compound **12** vs compound **13**). This is consistent with our proposed binding model since shortening the spacer would lose potential hydrogen-bonding and hydrophobic interactions. Moreover, this fact could confirm that the *N*-benzylpiperidine fragment is ideally situated and the nature of the spacer could control the positioning of the thiadiazolidinone within the gorge.

Simultaneously to the ellaboration of the above described model, the 3D crystal structure of AChE complexed with donepezil was reported [24, 25]. The same key aminoacids (Trp-84, Phe-330, Asp-72, Trp-279 and Phe-288) are involved in the binding interactions. Moreover, conclusions derived from Kryger's study [25] concerning the structural features of new anti-AD drugs, are present in the new family of drugs here described where the chiral centre of donepezil has been eliminated.

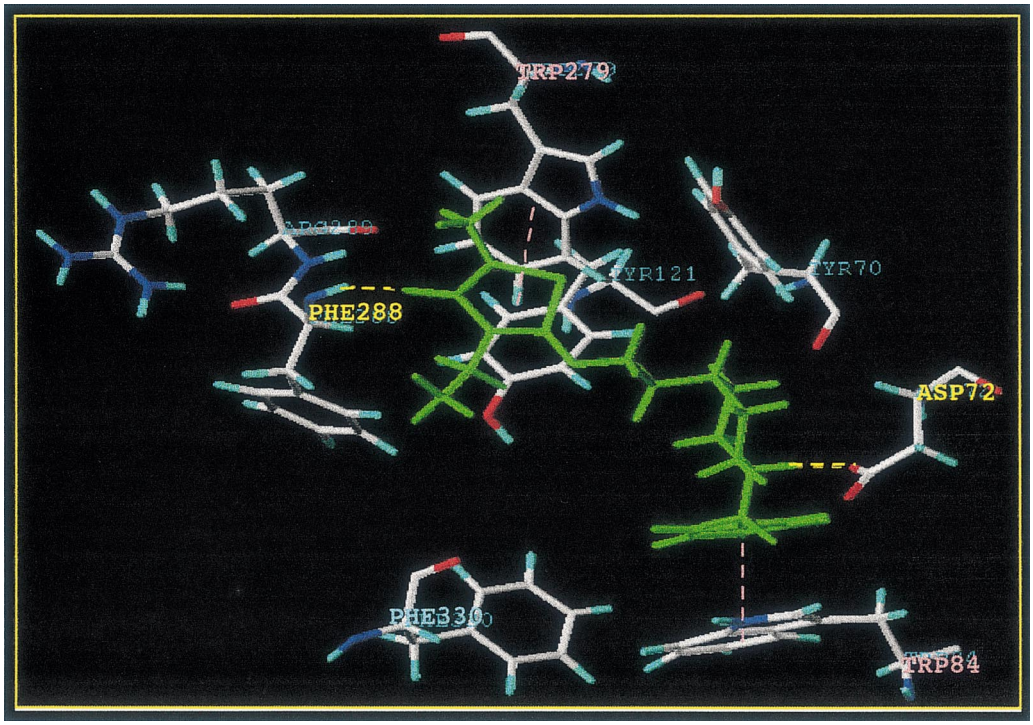

Figure 4. AChE-thiadiazolidine **13** key interactions. Legend: yellow, hydrogen–bonding interactions; pink, hydrophobic interactions.

4. Conclusions

A novel family of inhibitors of AChE derived from thiadiazolidinone moiety bearing the *N*-benzylpiperidine fragment has been synthesised. The lead compound has shown to be more active than tacrine in reversing the neuromuscular blockade induced by tubocurarine. Additionally, receptor binding studies provided new lead compounds for further development of α_2-adrenergic and sigma-receptor antagonists. Considering the conclusions derived from the molecular modelling and 3D crystal structure of donepezil-AChE complex [21], the 'empty' spaces within the gorge of AChE will be used in the near future to add structural features that enhance the α_2-selective binding of the new reported drugs.

The results found in the present study suggest that this thiadiazolidinone family may be considered as new lead compounds for further drug development in the search of an Alzheimer's disease treatment.

5. Experimental protocols

5.1. Molecular modelling

Molecular dynamics simulations were carried out using the Tripos force field on a Silicon Graphics Iris-4D computer. Structure of inhibitor **13** was built up using the standard parameters within SYBYL molecular modelling program [26]. A full geometry optimization was followed using the semiempirical method AM1 [27]. The coordinates of the protein were obtained from the X-ray structure of AChE isolated from *T. californica*, as deposited in the Brookhaven Protein Data Bank (entry 2ACE). Crystallographic water molecules as well as a modelled acetylcholine molecule were deleted. Polar and aromatic hydrogen atoms, in addition to any missing heavy atoms, were added to the protein using the SYBYL program bonds. Initial docking of **13** within the gorge leading to the active site was accomplished by locating hydrogen-bonding sites on the protein that could complement those found on the inhibitor. The

initial ligand/AChE complex was minimised using the above-mentioned force field until converged (defined as an energy gradient of 0.001 kcal · mol^{-1} Å^{-1} or less) applying the Powell algorithm. Molecular dynamics simulations were carried out by heating the system over 10 ps from 0 to 300 K, with a time step of 0.001 ps. The system was then equilibrated for 30 ps, and a constant temperature dynamics simulation was then performed for 50 ps. The simulation trajectory was recorded every 0.1 ps.

5.2. Synthesis

Melting points were determined with a Reichert–Jung Thermovar apparatus and are uncorrected. Flash column chomatography was carried out at medium pressure using silica gel (E. Merck, Grade 60, particle size 0.040–0.063 mm, 230–240 mesh ASTM) with the indicated solvent as eluent. ^{1}H-NMR spectra were obtained on Varian XL-300 and Gemini-200 spectrometers working at 300 and 200 MHz, respectively. Typical spectral parameters were: spectral width 10 ppm, pulse width 9 μs (57°), data size 32 K. N.O.e difference spectra were measured under the same conditions, using a presaturation time of 3 s. ^{13}C-NMR experiments were carried out on the Varian Gemini-200 spectrometer operating at 50 MHz. The acquisiton parameters were: spectral width 16 kHz, acquisition time 0.99 s, pulse width 9 μs (57°), data size 32 K. Chemical shifts are reported in δ values (ppm) relative to internal Me_4Si and J values are reported in Hertz. Elemental analyses were performed by the analytical departement at C.N.Q.O. (CSIC) and the results obtained were within $\pm 0.4\%$ of the theoretical values.

5.2.1. General procedure for the synthesis of thiadiazolium salts and 2,4-dialkyl-1,2,4-thiadiazolidine-3,5-diones

Chlorine was added slowly to a solution of alkylisothiocyanate in dry hexane (25 mL) at -15 to -10 °C. Chlorine was generated by the addition of HCl 35% to MnO_4K. The temperature of the reaction mixture was carefully controlled during the addition step. At this point, *N*-alkyl-*S*-chloroisothiocarbamoyl chloride was formed. Afterwards, alkylisocyanate was added. The mixture was stirred at room temperature for 12 h and the white solid, which fumes heavily in moist air, was separated in a dry nitrogen atmosphere by suction filtration, to provide thiadiazolium salts **1–4**. These compounds were used in the next synthetic step without further purification. A solution of each individual salts in EtOH (20 mL) was stirred at room temperature for 12 h. The solvent was evaporated under reduced pressure and the residue was purified by silica gel column using as eluent mixtures of solvents in the proportions indicated for each particular case.

5.2.1.1. 4-Ethyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione **5**

Reagents: Ethylisothiocyanate (0.56 mL, 4.8 mmol), HCl 35% (3 mL), MnO_4K (0.5 g), methylisocyanate (0.34 mL, 5.8 mmol). Purification: CH_2Cl_2:MeOH (50:1). Compound **5** was obtained as an orange oil; ^{1}H-NMR ($CDCl_3$) δ: 1.19 (t, 3H, $J = 6.9$ Hz, CH_2CH_3), 3.13 (s, 3H, CH_3), 3.68 (q, 2H, $J = 6.9$ Hz, CH_2CH_3); ^{13}C-NMR ($CDCl_3$) δ: 13.10 (CH_2CH_3), 31.29 (CH_3), 46.54 (CH_2CH_3), 153.23 (3-*C*=O), 165.52 (5-*C*=O). Anal. ($C_5H_8N_2O_2S$) C, H, N.

5.2.1.2. 4-Benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione **7**

Reagents: Benzylisothiocyanate (0.43 mL, 3.2 mmol), HCl 35% (1.5 mL), MnO_4K (0.25 g), methylisocyanate (0.16 mL, 2.7 mmol). Purification: CH_2Cl_2:MeOH (100:1). Compound **7** was obtained as an orange oil; ^{1}H-NMR ($CDCl_3$) δ: 3.20 (s, 3H, CH_3), 4.18 (s, 2H, CH_2Ph), 7.30 (m, 5H, *H*-Ph); ^{13}C-NMR ($CDCl_3$) δ: 31.29 (CH_3), 46.23 (CH_2Ph), 154.20 (3-*C*=O), 163.50 (5-*C*=O). Anal. ($C_{10}H_{10}N_2O_2S$) C, H, N.

5.2.1.3. 4-Benzyl-2-isopropyl-1,2,4-thiadiazolidine-3,5-dione **8**

Reagents: Benzylisothiocyanate (0.43 mL, 3.2 mmol), HCl 35% (1.5 mL), MnO_4K (0.25 g), isopropylisocyanate (0.26 mL, 2.7 mmol). Purification: CH_2Cl_2:MeOH (100:1). Compound **8** was obtained as an orange oil; ^{1}H-NMR ($CDCl_3$) δ: 1.24 (d, 6H, $J = 6.2$ Hz, $CH(CH_3)_2$), 4.27 (s, 2H, CH_2Ph), 4.63 (m, 1H, $J = 6.2$ Hz, $CH(CH_3)_2$), 7.26 (m, 5H, *H*-Ph); ^{13}C-NMR ($CDCl_3$) δ: 21.16 ($CH(CH_3)_2$), 46.23 (CH_2Ph), 47.84 ($CH(CH_3)_2$), 152.21 (3-*C*=O), 166.10 (5-*C*=O). Anal. ($C_{12}H_{14}N_2O_2S$) C, H, N.

5.2.2. General procedure for the synthesis of 5-alkylimino-1,2,4-thiadiazolidine-3-ones

To a solution of the corresponding thiadiazolium salt **1–4** previously obtained in Et_2O (40 mL) under nitrogen atmosphere, the corresponding primary amine and triethylamine were added. The resulting mixture was stirred at room temperature for 12 h, and then filtered. The filtrate was evaporated to dryness in vacuo and the

residue purified by silica gel column chromatography using the appropiate solvent.

5.2.2.1. 4-Ethyl-5-[imino-[1-(phenylmethyl)-4-piperidinyl]]-2-isopropyl-1,2,4-thiadiazolidin-3-one ***9***

Reagents: 5-chloro-4-ethyl-2-isopropyl-3-oxo-1,2,4-thiadiazolium chloride **2** (0.76 g, 3.1 mmol), 4-amino-1-benzylpiperidine (0.58 g, 3.1 mmol), triethylamine (0.62 g, 6.2 mmol). Purification: AcOEt:hexane (1:2). Yield: 0.55 g (50%) of colourless oil; ^{1}H-NMR ($CDCl_3$) δ: 1.15 (t, 3H, CH_2CH_3), 1.17 (d, 6H, $CH(CH_3)_2$), 1.65 (m, 4H, 3-*H*pip.), 2.10 (m, 2H, 2_{ec}-*H*pip.), 2.76 (m, 2H, 2_{ax}-*H*pip.), 2.59 (c, 1H, 4-*H*pip.), 3.47 (s, 2H, CH_2Ph), 3.70 (q, 2H, CH_2CH_3), 4.55 (m, 1H, $CH(CH_3)_2$), 7.23 (m, 5H, *H*-Ph); ^{13}C-NMR ($CDCl_3$) δ: 12.48 (CH_2CH_3), 20.98 ($CH(CH_3)_2$), 32.68 (3-*C*pip.), 38.34 (CH_2CH_3), 46.80 ($CH(CH_3)_2$), 51.77 (2-*C*pip.), 61.13 (4-*C*pip.), 63.15 (CH_2Ph), 126.92 (C*p*), 128.15 (C*o*), 129.08 (C*m*), 138.50 (C*i*), 146.07 (5-C), 154.66 (C=O). Anal. ($C_{19}H_{28}N_4OS$) C, H, N.

5.2.2.2. 4-Ethyl-5-[imino-[1-(phenylmethyl)-4-piperidinyl]ethyl]-2-isopropyl-1,2,4-thiadiazolidin-3-one ***10***

Reagents: 5-chloro-4-ethyl-2-isopropyl-3-oxo-1,2,4-thiadiazolium chloride **2** (0.38 g, 1.5 mmol), 4-aminoethyl-1-benzylpiperidine [28] (0.34 g, 1.5 mmol), triethylamine (0.31 g, 3.1 mmol). Purification: CH_2Cl_2:MeOH (20:1). Yield: 0.12 g (20%) of colourless oil; ^{1}H-NMR ($CDCl_3$) δ: 1.14 (t, 3H, CH_2CH_3), 1.15 (d, 6H, $CH(CH_3)_2$), 1.25 (m, 3H, 4-*H*pip. and 3_{ax}-*H*pip.), 1.53 (q, 2H, CH_2CH_2N), 1.59 (m, 2H, 3_{ec}-*H*pip.), 1.88 (t, 2H, 2_{ax}-*H*pip.), 2.80 (d, 2H, 2_{ec}-*H*pip.), 2.96 (t, 2H, CH_2CH_2N), 3.41 (s, 2H, CH_2Ph), 3.68 (q, 2H, CH_2CH_3), 4.50 (m, 1H, $CH(CH_3)_2$), 7.21 (m, 5H, *H*-Ph); ^{13}C-NMR ($CDCl_3$) δ: 12.51 (CH_2CH_3), 20.84 ($CH(CH_3)_2$), 32.05 (3-*C*pip.), 33.61 (4-*C*pip.), 37.15 (CH_2CH_2N), 38.12 (CH_2CH_3), 46.80 ($CH(CH_3)_2$), 50.68 (CH_2CH_2N), 53.68 (2-*C*pip.), 63.34 (CH_2Ph), 126.83 (C*p*), 128.01 (C*o*), 129.15 (C*m*), 138.14 (C*i*), 147.80 (5-C), 154.52 (C=O). Anal. ($C_{21}H_{32}N_4OS$) C, H, N.

5.2.2.3. 4-Ethyl-5-[imino-[1-(phenylmethyl)-4-piperazinyl]ethyl]-2-isopropyl-1,2,4-thiadiazolidin-3-one ***11***

Reagents: 5-chloro-4-ethyl-2-isopropyl-3-oxo-1,2,4-thiadiazolium chloride **2** (0.38 g, 1.5 mmol), 4-aminoethyl-1-benzylpiperazine [29] (0.37 g, 1.5 mmol), triethylamine (0.31 g, 3.1 mmol). Purification: CH_2Cl_2:MeOH (40:1). Yield: 0.14 g (22%) of colourless oil; ^{1}H-NMR ($CDCl_3$) δ: 1.13 (t, 3H, CH_2CH_3), 1.16 (d, 6H, $CH(CH_3)_2$), 2.44 (m, 4H, 2-*H*pip.), 2.49 (m, 4H, 3-*H*pip.), 2.60 (t, 2H, CH_2CH_2N), 3.12 (t, 2H, CH_2CH_2N), 3.44 (s, 2H, CH_2Ph), 3.67 (q, 2H, CH_2CH_3), 4.53 (m, 1H, $CH(CH_3)_2$), 7.20 (m, 5H, *H*-Ph); ^{13}C-NMR ($CDCl_3$) δ: 12.54 (CH_2CH_3), 20.84 ($CH(CH_3)_2$), 38.14 (CH_2CH_3), 46.81 ($CH(CH_3)_2$), 51.14 (CH_2CH_2N), 52.91 (3-*C*pip.), 53.45 (2-*C*pip.), 58.65 (CH_2CH_2N), 62.99 (CH_2Ph), 126.94 (C*p*), 128.10 (C*o*), 129.12 (C*m*), 137.95 (C*i*), 148.90 (5-C), 154.48 (C=O). Anal. ($C_{20}H_{31}N_5OS$) C, H, N.

5.2.2.4. 4-Ethyl-5-[imino-[1-(phenylmethyl)-4-piperidinyl]]-2-methyl-1,2,4-thiadiazolidin-3-one ***12***

Reagents: 5-chloro-4-ethyl-2-methyl-3-oxo-1,2,4-thiadiazolium chloride **1** (0.24 g, 1.1 mmol), 4-amino-1-benzylpiperidine (0.29 g, 1.1 mmol), triethylamine (0.22 g, 2.2 mmol). Purification: CH_2Cl_2:MeOH (50:1). Yield: 0.09 g (18%) of colourless oil; ^{1}H-NMR ($CDCl_3$) δ: 1.14 (t, 3H, CH_2CH_3), 1.65 (m, 4H, 3-*H*pip.), 2.07 (m, 2H, 2_{ec}-*H*pip.), 2.73 (m, 2H, 2_{ax}-*H*pip.), 2.52 (c, 1H, 4-*H*pip.), 3.01 (s, 3H, CH_3), 3.44 (s, 2H, CH_2Ph), 3.69 (q, 2H, CH_2CH_3), 7.23 (m, 5H, *H*-Ph); ^{13}C-NMR ($CDCl_3$) δ: 12.33 (CH_2CH_3), 31.92 (3-*C*pip.), 32.53 (CH_3), 38.74 (CH_2CH_3), 51.56 (2-*C*pip.), 60.01 (4-*C*pip.), 63.02 (CH_2Ph), 126.84 (C*p*), 128.05 (C*o*), 128.95 (C*m*), 138.36 (C*i*), 145.33 (5-C), 155.54 (C=O). Anal. ($C_{17}H_{24}N_4OS$) C, H, N.

5.2.2.5. 4-Ethyl-5-[imino-[1-(phenylmethyl)-4-piperidinyl]ethyl]-2-methyl-1,2,4-thiadiazolidin-3-one ***13***

Reagents: 5-chloro-4-ethyl-2-methyl-3-oxo-1,2,4-thiadiazolium chloride **1** (0.68 g, 3.1 mmol), 4-aminoethyl-1-benzylpiperidine [28] (0.69 g, 3.1 mmol), triethylamine (0.64 g, 6.3 mmol). Purification: CH_2Cl_2:MeOH (40:1). Yield: 0.21 g (19%) of colourless oil; ^{1}H-NMR ($CDCl_3$) δ: 1.15 (t, 3H, CH_2CH_3), 1.29 (m, 3H, 4-*H*pip. and 3_{ax}-*H*pip.), 1.52 (q, 2H, CH_2CH_2N), 1.60 (m, 2H, 3_{ec}-*H*pip.), 1.93 (t, 2H, 2_{ax}-*H*pip.), 2.85 (d, 2H, 2_{ec}-*H*pip.), 2.93 (t, 2H, CH_2CH_2N), 3.04 (s, 3H, CH_3), 3.47 (s, 2H, CH_2Ph), 3.68 (q, 2H, CH_2CH_3), 7.22 (m, 5H, *H*-Ph); ^{13}C-NMR ($CDCl_3$) δ: 12.58 (CH_2CH_3), 31.86 (CH_3), 32.00 (3-*C*pip.), 33.49 (4-*C*pip.), 37.08 (CH_2CH_2N), 38.74 (CH_2CH_3), 50.79 (CH_2CH_2N), 53.82 (2-*C*pip.), 63.20 (CH_2Ph), 127.16 (C*p*), 128.20 (C*o*), 129.40 (C*m*), 137.50 (C*i*), 147.30 (5-C), 155.50 (C=O). Anal. ($C_{19}H_{28}N_4OS$) C, H, N.

5.2.2.6. 4-Ethyl-5-[imino-[1-(phenylmethyl)-4-piperazinyl]ethyl]-2-methyl-1,2,4-thiadiazolidin-3-one ***14***

Reagents: 5-chloro-4-ethyl-2-methyl-3-oxo-1,2,4-thiadiazolium chloride **1** (0.23 g, 1.1 mmol), 4-aminoethyl-1-benzylpiperazine [29] (0.24 g, 1.1 mmol), triethylamine (0.22 g, 2.2 mmol). Purification: CH_2Cl_2:MeOH (40:1). Yield: 0.05 g (13%) of colourless oil; ^{1}H-NMR ($CDCl_3$) δ: 1.15 (t, 3H, CH_2CH_3), 2.44 (m, 4H, 2-*H*pip.), 2.48 (m, 4H, 3-*H*pip.), 2.59 (t, 2H, CH_2CH_2N), 3.04 (s, 3H, CH_3), 3.06 (t, 2H, CH_2CH_2N), 3.44 (s, 2H, CH_2Ph), 3.68 (q, 2H, CH_2CH_3), 7.22 (m, 5H, *H*-Ph); ^{13}C-NMR ($CDCl_3$) δ: 12.52 (CH_2CH_3), 31.85 (CH_3), 38.64 (CH_2CH_3), 50.87 (CH_2CH_2N), 52.87 (3-*C*pip.), 53.45 (2-*C*pip.), 58.60 (CH_2CH_2N), 62.97 (CH_2Ph), 126.93 (C*p*), 128.09 (C*o*), 129.11 (C*m*), 137.94 (C*i*), 148.23 (5-C), 155.40 (C=O). Anal. ($C_{18}H_{27}N_5OS$) C, H, N.

5.2.2.7. 4-Benzyl-5-[imino-[1-(phenylmethyl)-4-piperidinyl]]-2-isopropyl-1,2,4-thiadiazolidin-3-one ***15***

Reagents: 5-chloro-4-benzyl-2-isopropyl-3-oxo-1,2,4-thiadiazolium chloride **4** (0.33 g, 1 mmol), 4-amino-1-benzylpiperidine (0.20 g, 1 mmol), triethylamine (0.20 g, 2 mmol). Purification: CH_2Cl_2:MeOH (50:1). Yield: 0.08 g (18%) of colourless oil; ^{1}H-NMR ($CDCl_3$) δ: 1.09 (d, 6H, $CH(CH_3)_2$), 1.67 (m, 4H, 3-*H*pip.), 2.13 (m, 2H, 2_{ec}-*H*pip.), 2.70 (m, 2H, 2_{ax}-*H*pip.), 2.69 (c, 1H, 4-*H*pip.), 3.46 (s, 2H, CH_2Ph), 4.79 (s, 2H, CH_2Ph), 7.30 (m, 10H, *H*-Ph); ^{13}C-NMR ($CDCl_3$) δ: 20.92 ($CH(CH_3)_2$), 32.51 (3-*C*pip.), 46.29 (CH_2Ph), 46.89 ($CH(CH_3)_2$), 51.40 (2-*C*pip.), 60.30 (4-*C*pip.), 63.20 (CH_2Ph), 126.94, 127.49 (C*p*), 128.12 (C*o*), 129.07 (C*m*), 136.67, 138.50 (C*i*), 146.70 (5-C), 154.58 (C=O). Anal. ($C_{24}H_{30}N_4OS$) C, H, N.

5.2.2.8. 4-Benzyl-5-[imino-[1-(phenylmethyl)-4-piperidinyl]ethyl]-2-isopropyl-1,2,4-thiadiazolidin-3-one ***16***

Reagents: 5-chloro-4-benzyl-2-isopropyl-3-oxo-1,2,4-thiadiazolium chloride **4** (0.35 g, 1.1 mmol), 4-aminoethyl-1-benzylpiperidine [28] (0.24 g, 1.1 mmol), triethylamine (0.22 g, 2.2 mmol). Purification: CH_2Cl_2:MeOH (40:1). Yield: 0.12 g (25%) of colourless oil; ^{1}H-NMR ($CDCl_3$) δ: 1.18 (d, 6H, $CH(CH_3)_2$), 1.23 (m, 3H, 4-*H*pip. and 3_{ax}-*H*pip.), 1.50 (q, 2H, CH_2CH_2N), 1.59 (m, 2H, 3_{ec}-*H*pip.), 1.86 (t, 2H, 2_{ax}-*H*pip.), 2.80 (d, 2H, 2_{ec}-*H*pip.), 2.97 (t, 2H, CH_2CH_2N), 3.44 (s, 2H, CH_2Ph), 4.56 (m, 1H, $CH(CH_3)_2$), 4.80 (s, 2H, CH_2Ph), 7.25 (m, 10H, *H*-Ph); ^{13}C-NMR ($CDCl_3$) δ: 20.90 ($CH(CH_3)_2$), 32.03 (3-*C*pip.), 33.34 (4-*C*pip.), 37.09 (CH_2CH_2N), 46.14 (CH_2Ph), 46.94 ($CH(CH_3)_2$), 50.43 (CH_2CH_2N), 53.68 (2-*C*pip.), 63.38 (CH_2Ph), 126.84, 127.44 (C*p*), 128.03, 128.19 (C*o*), 128.57, 129.19 (C*m*), 136.55, 138.17 (C*i*), 147.30 (5-C), 154.62 (C=O). Anal. ($C_{26}H_{34}N_4OS$) C, H, N.

5.2.2.9. 4-Benzyl-5-[imino-[1-(phenylmethyl)-4-piperazinyl]ethyl]-2-isopropyl-1,2,4-thiadiazolidin-3-one ***17***

Reagents: 5-chloro-4-benzyl-2-isopropyl-3-oxo-1,2,4-thiadiazolium chloride **4** (0.35 g, 1.1 mmol), 4-aminoethyl-1-benzylpiperazine [29] (0.24 g, 1.1 mmol), triethylamine (0.22 g, 2.2 mmol). Purification: CH_2Cl_2:MeOH (40:1). Yield: 0.13 g (27%) of colourless oil; ^{1}H-NMR ($CDCl_3$) δ: 1.15 (d, 6H, $CH(CH_3)_2$), 2.42 (m, 4H, 2-*H*pip.), 2.43 (m, 4H, 3-*H*pip.), 2.59 (t, 2H, CH_2CH_2N), 3.10 (t, 2H, CH_2CH_2N), 3.43 (s, 2H, CH_2Ph), 4.54 (m, 1H, $CH(CH_3)_2$), 4.78 (s, 2H, CH_2Ph), 7.28 (m, 10H, *H*-Ph); ^{13}C-NMR ($CDCl_3$) δ: 20.92 ($CH(CH_3)_2$), 46.10 (CH_2Ph), 46.93 ($CH(CH_3)_2$), 50.88 (CH_2CH_2N), 52.88 (3-*C*pip.), 53.40 (2-*C*pip.), 58.60 (CH_2CH_2N), 62.96 (CH_2Ph), 126.93, 127.43 (C*p*), 128.09, 128.19 (C*o*), 128.49, 129.11 (C*m*), 136.49, 137.95 (C*i*), 148.37 (5-C), 154.56 (C=O). Anal. ($C_{25}H_{33}N_5OS$) C, H, N.

5.2.2.10. 4-Benzyl-5-[imino-[1-(phenylmethyl)-4-piperidinyl]ethyl]-2-methyl-1,2,4-thiadiazolidin-3-one ***18***

Reagents: 5-chloro-4-benzyl-2-methyl-3-oxo-1,2,4-thiadiazolium chloride **3** (0.33 g, 1.2 mmol), 4-aminoethyl-1-benzylpiperidine [28] (0.26 g, 1.2 mmol), triethylamine (0.24 g, 2.4 mmol). Purification: CH_2Cl_2:MeOH (40:1). Yield: 0.30 g (60%) of colourless oil; ^{1}H-NMR ($CDCl_3$) δ: 1.23 (m, 3H, 4-*H*pip. and 3_{ax}-*H*pip.), 1.48 (q, 2H, CH_2CH_2N), 1.55 (m, 2H, 3_{ec}-*H*pip.), 1.85 (t, 2H, 2_{ax}-*H*pip.), 2.78 (d, 2H, 2_{ec}-*H*pip.), 2.92 (t, 2H, CH_2CH_2N), 3.05 (s, 3H, CH_3), 3.44 (s, 2H, CH_2Ph), 4.79 (s, 2H, CH_2Ph), 7.25 (m, 10H, *H*-Ph); ^{13}C-NMR ($CDCl_3$) δ: 31.90 (3-*C*pip.), 31.97 (CH_3), 33.23 (4-*C*pip.), 36.99 (CH_2CH_2N), 46.58 (CH_2Ph), 50.30 (CH_2CH_2N), 53.59 (2-*C*pip.), 63.28 (CH_2Ph), 126.78, 127.47 (C*p*), 127.98, 128.14 (C*o*), 128.56, 129.10 (C*m*), 136.38, 138.13 (C*i*), 146.60 (5-C), 155.41 (C=O). Anal. ($C_{24}H_{30}N_4OS$) C, H, N.

5.3. Biological methods

5.3.1. AChE inhibition

AChE inhibitory activity was evaluated spectrophotometrically at 25 °C by the method of Ellman [13], using AChE from bovine erythrocytes and acetylthiocholine

iodide (0.53 mM) as substrate. The reaction took place in a final volume of 3 mL of 0.1 M phosphate-buffered solution (pH 8.0), containing 0.025 unit of AChE and 333 μM 5,5′-dithiobis(2-nitrobenzoic) acid (DTNB) solution used to produce the yellow anion of 5-thio-2-nitrobenzoic acid. Inhibition curves with different derivatives were performed in triplicate by incubating with at least 12 concentrations of inhibitor for 15 min. One triplicate sample without inhibitor was always present to yield the 100% of AChE activity. The reaction was stopped by the addition of 100 μL of 0.01 M eserine, and the colour production was measured at 412 nm. BChE inhibitory activity determinations were carried out similarly, using human serum BChE and butyrylthiocholine instead of AChE and acetylthiocholine.

The drug concentration producing 50% of AChE or BChE activatory inhibition (IC_{50}) was calculated. Results are expressed as mean ± SEM of at least four experiments. DTNB, acetylthiocholine, butyrylthiocholine, and the enzymes were purchased from Sigma, and eserine from Fluka.

5.3.2. Neuromuscular studies

Right and left phrenic nerve-hemidiaphragm removed from male Sprague–Dawley rats (250–300 g) were used. Details of the experimental procedures have been previously described [30]. Briefly, rats were lightly anaesthetised with ether and decapitated. After quick dissection, each phrenic-hemidiaphragm preparation was suspended in organ baths of 75 mL volume with Krebs–Henseleit solution of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11.1. The preparation was bubbled with 5% CO_2 in oxygen and the temperature was maintained at 25 ± 1 °C. Drug effects of AChE inhibitory compounds on neuromuscular junction were assessed as the ability of reversing the partial blockade induced by tubocurarine in indirectly elicited twitch responses. The twitches were obtained by stimulating the phrenic nerve with square pulses of 0.5 ms duration at 0.2 Hz and a supramaximal voltage. Neuromuscular blockade was obtained with the addition of tubocurarine (1–1.5 μM). Study drugs were added when a reduction of twitch response to 70–80% control values was obtained and the effect of each drug was evaluated during 15 min of exposure. To avoid the possible carry-over effects, only one concentration of inhibitor was tested on each preparation. Several drug concentrations were used for each AChE inhibitor. To evaluate the reversal effect of each drug, the antagonism index (AI or % antagonism [31] was determined for each concentration and the AI_{50} (μM drug concentration that inhibits a 50% tubocurarine blockade) was calculated. All results were expressed as mean ± SEM.

5.3.3. Receptor-binding investigation

The binding affinities of thiadiazolidinones derivatives **9**–**18** were measured in more than thirty radioligand-binding assays, including neurotransmitter receptor subtypes, receptor for peptide- and lipid- derived factors, ion channel-binding sites and monoamine transporters, following the conditions described by Schotte [17].

For the cloned human receptors α_{2A}, α_{2B} and α_{2C}, the receptor DNA was amplified by PCR, cloned in a plasmid, and sequenced as previously described [32].

Acknowledgements

This work was financially supported by CICYT (project no. SAF 96/107 and SAF 99/98) and by CAM (project no. 8.5/45.1/99). Receptor-binding profile assays are kindly acknowledged to Janssen Pharmaceuticals.

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