European Journal of Medicinal Chemistry 46 (2011) 1–10

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Synthesis, biological assessment and molecular modeling of new dihydroquinoline-3-carboxamides and dihydroquinoline-3-carbohydrazide derivatives as cholinesterase inhibitors, and Ca channel antagonists

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ARTICLE INFO

Article history:
Received 28 May 2010
Received in revised form
31 July 2010
Accepted 25 August 2010

Keywords:
Alzheimer's disease
Dihydroquinoline-3-carboxamides
Dihydroquinoline-3-carbohydrazides
hexahydropyrimido[5,4-*c*]quinoline-2,
5-diones
AChE
BuChE
Molecular modeling
Ca channel antagonism

ABSTRACT

The synthesis, biological evaluation, and molecular modeling of new 4-hydroxy-2-oxo-1,2-dihydroquinoline-3-carboxamides(**4**), 4-hydroxy-2-oxo-1,2-dihydroquinoline-3-carbohydrazide (**6**), and some hexahydropyrimido[5,4-*c*]quinoline-2,5-diones (**9**) produced earlier by our laboratory, as AChE/BuChE inhibitors, is described. From these analyses compound **4c** resulted equipotent regarding the inhibition of cholinesterases'; inhibitors **6k**, **9a**, **9b** were selective for AChE, whereas product **4d** proved selective for BuChE. Docking analysis has been carry out in order to identify the binding mode in the active site, and to explain the observed selectivities. Only compound **9a** has been shown to decrease K^+-induced calcium signals in bovine chromaffin cells.

1. Introduction

Alzheimer's disease (AD), the first most common form of senile dementia, affects nearly 50% of adults over the age of 85. Once contracted, AD damages the brain progressively and relentlessly. The average survival time after being diagnosed with this illness is about 8–10 years [1]. Because of the complex pathophysiology of AD, which involves many pathways, development of a satisfactory therapy is problematical. The main therapy targets are diffuse loss of neurons, reduced levels of the neurotransmitter acetylcholine (ACh), deposits of β-amyloid (Aβ) plaques, and neurofibrillary tangles [2].

One of the current therapeutic strategies is to reduce the oxidative stress involved in cellular death. Senile plaques release free radicals which are extremely toxic [3]. The accumulation of reactive oxygen species (ROS) results in damage to major cell components, such as the nucleus, mitochondrial DNA, membranes, and cytoplasmic proteins [4]. Oxidative stress is therefore included in all the pathophysiological hypotheses for AD, and studies have shown the efficacy of several antioxidant compounds. Three free radical-scavenging drugs [vitamin E (α-tocopherol), selegiline (a monoamine oxidase B inhibitor), and *Ginkgo biloba* extract (EGb 761, Tanakan)], used for therapeutic purposes in different fields, have also been examined in AD clinical studies and their beneficial effects were demonstrated [4].

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doi:10.1016/j.ejmech.2010.08.054

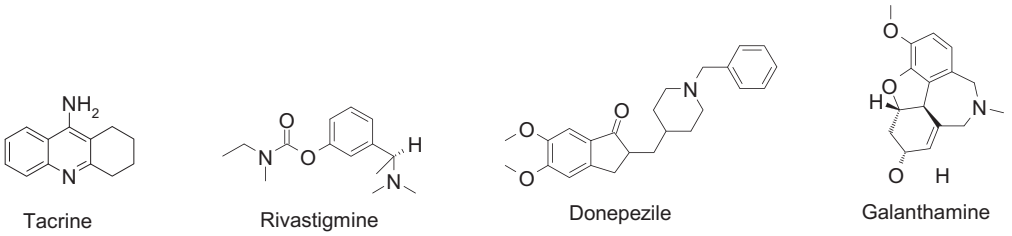


Fig. 1. Chemical structure of tacrine, rivastigmine, donepezile and galanthamine.

Another therapeutic strategy is to restore the brain acetylcholine (ACh) level, as this neurotransmitter is important for the regulation of the memory and the learning process [5]. Two cholinesterases: acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) hydrolyze ACh. Recent evidence suggests that AChE plays also a non-cholinergic role in the development of AD [6,7], as it works as a chaperone molecule, accelerating the Aβ peptide deposition, and the aggregation of Aβ into insoluble fibrils [8,9]. The inhibition of these enzymes could therefore improve AD symptoms.

Tacrine (Cognex®) was the first AChE inhibitor approved for the treatment of AD [10]. Due to its toxicity [11], other substances have been developed, such as rivastigmine (Exelon®) [12], donepezil (Aricept®)[13,14] and galanthamine (Reminyl®)[15] (Fig. 1).

New AChE and BuChE inhibitors must be designed in order to clarify the structural characteristics and functions of BuChE and AChE. The structures of these enzymes are similar: they share 65% amino acid sequences at the molecular level [8]. Both have the same active sites, i.e., a mid gorge interaction site and a peripheral anionic site (PAS) [16–18]. They differ at the acyl-binding pocket level: AChE contains amino acid residues Phe288 and Phe290, and BuChE contains Leu286 and Val288 [18].

Due to its multifactorial pathogenesis, the current strategy for the development of new drugs for AD is focusing on multipotent molecules acting in a complementary manner, in different neural and biochemical targets, which could be more efficacious for AD patients [19,20].

Many compounds containing coumarin or quinoline rings have been studied for their biological activity in AD. They are used as radical scavengers, such as vitamin E (α tocopherol), as copper or iron chelators such as clioquinol, or as inhibitors of AChE such as tacrine.

Following our studies on the synthesis of quinolines carried out in our laboratory [21], we now report the synthesis of new 4-hydroxy-2-oxo-1,2-dihydroquinoline-3-carboxamides and new 4-hydroxy-2-oxo-1,2-dihydroquinoline-3-carbohydrazides.

These new compounds and some of our previously designed hexahydropyrimido[5,4-*c*]quinoline-2,5-diones [21] were tested as AChE and BuChE inhibitors.

Finally, and thanks to molecular docking, we have identified the interactions with AChE and BuChE.

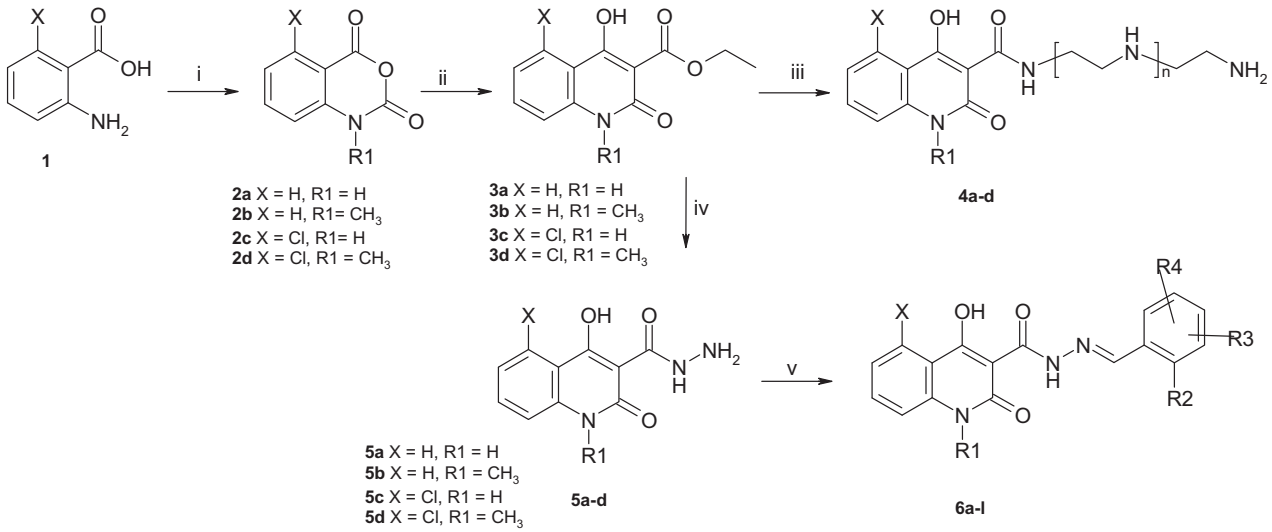
2. Results and discussion

2.1. Chemistry

The synthesis of new compounds 4-hydroxy-2-oxo-1,2-quinoline-3-carboxamides **4a**–**d** and 4-hydroxy-2-oxo-1,2-quinoline-3-carbohydrazides **6a**–**l** was easily achieved, in good chemical yield (see Scheme 1).

Compounds **4a**–**d** were prepared in two steps by reaction of isatoic anhydride (**2a**) and its derivatives **2b**, bearing a *N*-methyl group, and **2c** bearing a chloro substitutent at C-5 with diethyl malonate, under basic conditions, *via* intermediates **3a**–**c**, followed by treatment with tetraethylenetetramine and dithylenetetramine in xylene at reflux [22] (Table 1).

Products **6a**–**l** (Table 2), were prepared as follows: hydrazine was added to compounds **3a**–**d** to obtain intermediates **5a**–**d**, and then submitted to reaction with differently substituted benzaldehyde derivatives in dimethyl sulfoxide [23]. This procedure gave



Scheme 1. Synthesis of designed inhibitors **4a**–**d** and **6a**–**l**. Reagents and conditions: (i) triphosgene, dioxane, 3 h, 0 °C; (ii) diethyl malonate, NaH, DMF, 2 h, 30, 100 °C; (iii) triethylenetetramine or diethylenetetramine, xylene, 2 h, 150 °C; (iv) hydrazine, MeOH, 30 min, 100 °C; (v) corresponding benzaldehyde, *o*-phosphoric acid, DMSO, 3 h, 100 °C.

Table 1
Description of compounds **4a**–**d**.

	X	*n*	R_1
4a	H	1	H
4b	H	1	CH_3
4c	H	2	H
4d	H	2	CH_3

Table 2
description of compounds **6a**–**l**.

	X	R_1	R_2	R_3	R_4
6a	H	H	OH	4-OH	H
6b	H	H	OH	5-CH_3	H
6c	H	H	OH	5-OCH_3	H
6d	H	CH_3	OH	4-OH	H
6e	H	CH_3	OH	5-CH_3	H
6f	H	CH_3	OH	5-OCH_3	H
6g	H	H	NO_2	H	H
6h	H	CH_3	NO_2	H	H
6i	H	H	Cl	3-NO_2	6-Cl
6j	H	CH_3	Cl	3-NO_2	6-Cl
6k	Cl	H	OH	4-OH	H
6l	Cl	CH_3	OH	4-OH	H

compounds **6a**–**c**, as a mixtures of *E* and *Z*-isomers in an *E*: *Z*: 9:1, 7:3 and 4:1 ratio, respectively, whereas the target molecules **6d**–**l** were isolated as pure *E* isomers.

The ^1H and ^{13}C NMR spectroscopic data are in good agreement with values found for related compounds, and the assignments are based on standard NMR experiments (^1H–^1H NOESY, HMQC, HMBC).

The *E* configuration compounds **6** were characterized in the 2D-NMR (^1H–^1H) spectra by NOESY experiments, and analyzing by the NOE effects on the hydrogens for the N*H* amide of carbohydrazide moiety, and the C*H* of imines.

The synthesis procedure for racemic compounds **9a**–**b**, using Biginelli's multicomponent method [21] (Scheme 2) was the same as that for **9c**–**h** (see Table 3).

The structures of **9a**–**b** were assigned from FTIR, ^1H NMR spectra and elemental analysis.

2.2. Pharmacological evaluation

2.2.1. Studies of AChE/BuChE inhibition

Using Ellman's method [24], compounds **4** were tested as inhibitors of AChE from commercial *Electrophorus electricus* (*Ee*AChE) (Sigma), and from horse serum BuChE (*eq*BuChE) (Sigma). For comparative purposes, tacrine was used as reference compound. The AChE inhibitory results are summarized in Table 4. None of the test compounds showed higher AChE and BuChE inhibition than tacrine (IC_{50} 0.027 μM and 0.0052 μM, respectively). In this assay, compound **9b** (IC_{50} 6 μM) was the most active for AChE inhibition and compound **4d** (IC_{50} 5.2 μM) for BuChE.

As shown in Table 4, from the target compounds **4**, only **4c** and **4d** were active, **4c** being almost equipotent for AChE and BuChE, while compound **4d** was totally selective for BuChE. Regarding structure–activity relationships, very interestingly, compound **4c** with an N–H bond was an AChE inhibitor, while the *N*-methylated derivative **4d** was a selective BuChE inhibitor. This result proved that this position (the N–H bond) was significant for ChE inhibition. However, compound **4c** is equipotent showing similar AChE and BuChE inhibition. Only compounds with the longest chain were active, underlining again the importance of this functional motif.

For compounds **6a**–**l**, only compound **6k** showed an acceptable AChE inhibition, showing that the replacement of a hydrogen by a chloride was therefore favorable, and critical for the biological activity. We also observed that, in this case, replacement of an *N*–H bond by *N*–Me group did not produce higher activity. Phenyl substitution did not appear to have any influence on the potency of activity.

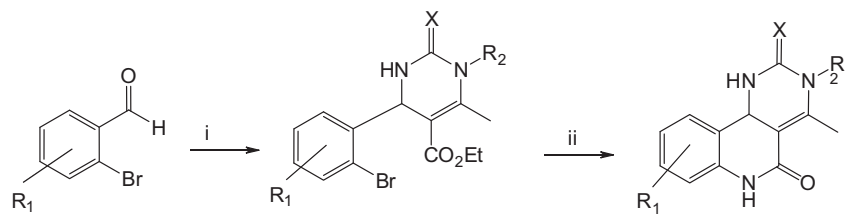
Finally, compounds **9a** and **9b** were the best AChE inhibitors in this study, being also almost inactive for BuChE. Note also that when the nitro substituent was replaced by a chlorine atom at the same position, no inhibition was observed (Table 4).

2.2.2. Studies of molecular modeling

The docking study of compound **4c** provided a large variety of solutions. The ligand was placed at both the catalytic center (CC) and the PAS (Fig. 2). In both cases, the terminal amine group, protonated at physiological pH, was hydrogen-bonded with the lateral chain of Glu202.

At the CC: in the best docking pose, as expected, the aromatic core was stacked against the indole and phenol moieties of Trp86 and Tyr337, respectively. The NH group of the quinoline formed a hydrogen bond with Asp74. This interaction was not possible for the *N*-methyl analogue (**4d**) which would of course destabilize the complex due to strong steric interactions; this explains the decreased activity. Hydrophobic interactions were observed with Trp439. The flexibility of the ligand chain allows a variety of conformations where additional interactions may be established. This small cluster (11 occurrences) was ranked in energy in position 1 and showed a binding energy of −1.68 kcl/mol.

At the PAS: the ligand was also placed at the PAS by the usual pi-stacking interactions with Trp286 and Tyr341. The hydroxyl group was out-of-plane because of an H-bond to Asp74 and Tyr72 side chains. The carbonyl moiety of the acyclic amide also breaks co-planarity with the aryl ring because the carbonylic oxygen also interacted with Tyr124 by H-bonding (see Fig. 2). While the protonated group interacted with Glu202, the chain formed additional H-bonds with residues at the tunnel connecting the CC and PAS, as for Tyr124, Tyr341, Tyr337, Ser125 and Gly121. This cluster

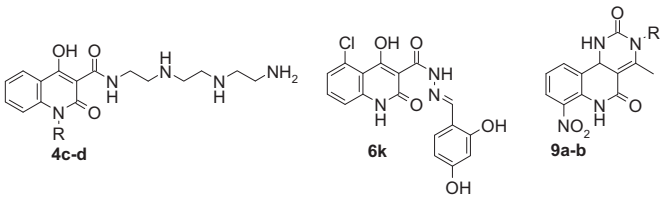


Scheme 2. Synthesis of designed inhibitors **9a**–**h**. (i) H_2NCXNR, H_3BO_3, AcOH, ethyl acetoacetate; (ii) NH_3 28%, 250 °C. 10 bars.

Table 3
Description of compounds **9a**–**h**.

	X	R_1	R_2
9a	O	7-NO2	Me
9b	O	7-NO2	Et
9c	O	10-Cl	Me
9d	O	10-Cl	Et
9e	O	7-Cl	Me
9f	O	7-Cl	Et
9g	S	10-Cl	Et
9h	S	7-Cl	Me

Table 4
Inhibition of AChE and BuChE (IC_{50}) by compounds **4c**–**d**, **6k** and **9a**–**b**.



Compd.	R	IC_{50}[a] (μM) *Ee*AChE[b]	IC_{50}[a] (μM) *eq*BuChE[c]	Selectivity[d]
Tacrine		0.027 ± 0.002	0.0052 ± 0.0002	0.193
4c	H	13 ± 3	19 ± 3	2
4d	Me	>30	5.2 ± 0.3	0.173
6k		12 ± 1	>30	>3
9a	Me	8 ± 1	>30	>4
9b	Et	6 ± 1	>30	>5

[a] IC_{50} values are means ± SEM of at least three independent measurements.
[b] *Ee*, electric eel.
[c] *eq*, equine.
[d] *eq*BuChE/EeAChE.

(7 occurrences, ranked 4) showed a slightly less favorable binding energy (−1.23 kcal/mol).

For compound **6k**, all the solutions, albeit showing slightly different conformations, placed the resorcinol scaffold at the PAS stacked between Trp286 and Tyr341 (Fig. 3). In the lowest-energy cluster (68 occurrences, binding energy −1.81 kcal/mol), the quinoline was placed perpendicular to the aromatic side chain of Trp86 which precluded the expected pi-stacking interactions. The indole, however, created van der Waals forces with the amide proton while the hydroxyl group was H-bonded to Tyr124 and Gly121, thus stabilizing the complex. The acetohydrazide linker formed an H-bond with Tyr337.

Finally, we observed the formation of an H-bond between the chloride atom of the ligand and the hydroxyl group of Ser203. Closely-related compound **6i** was inactive. Because it had no chloride substitute, the interaction with Ser203 did not take place.

To perform the docking analysis of compound **9b**, both enantiomers were taken into account. Surprisingly, only one docked pose was found for each one (Fig. 4).

(*S*)-**9b** (binding energy −4.17 kcal/mol), the only binding mode we found, placed the ligand in the CC. Besides the expected pi-stacking interactions of the aromatic core with Trp86, the ligand established two H-bonds: one between the dihydropyrimidinone proton and Glu202, and the other between the nitro group and the hydroxyl group of Tyr337. Two additional H-bonds were formed between the carboxylic oxygen atoms and Tyr133 and Ser125.

(*R*)-**9b** (binding energy −3.90 kcal/mol) the simulations also placed the ligand exclusively in the CC where the aromatic scaffold occupied the same position as its enantiomer, i.e., forming pi-stacking interactions with Trp86. An H-bond between the nitro group (without conformational deviation from co-planarity) and Tyr449, and carbonyl and NH of dihydropyrimidinone with Asp74 NH-backbone and side chain, respectively, were also detected.

Both enantiomers showed similar low conformational distortion upon binding while enantiomer (*S*) gives rise to a slightly lower, more favourable, binding energy than (*R*) enantiomer, probably as a result of a better optimization of the potential H-bond interactions that the ligand can establish with the enzyme. Hence, we could hypothesized that conformer (*S*) would bind preferentially, although this conclusion must be taken with caution.

2.2.3. *Ca channel antagonism*

On the other hand, it is known that Ca^{2+} overload is the main factor that triggers the processes leading to cell death. Thus, it has been shown that calcium dysfunction, involved in the pathogenesis of AD, augments Aβ formation and τ hyperphosphorylation [25,26] Moreover, calcium entry through L-type Ca^{2+} channels (Cav 1.1–1.4) causes both calcium overload and mitochondrial

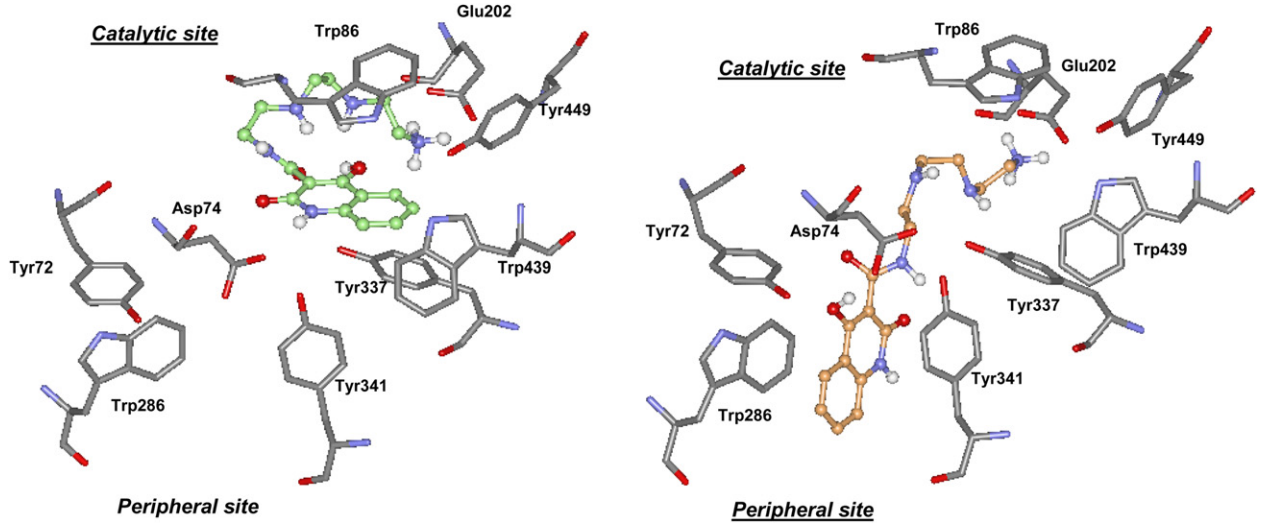


Fig. 2. Docking of compound **4c** in the two regions of interest: catalytic center (left) and in the peripheral anionic site (right).

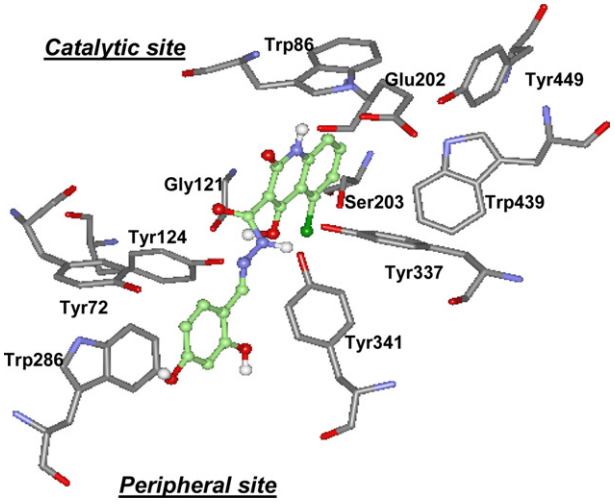


Fig. 3. Docking of compound **6k**.

disruption, which leads to the activation of the apoptotic cascade and cell death [27] In fact, nimodipine, a neuronal L-type Ca^{2+} channel blocker, protects neurons from death evoked by focal cerebral ischemia [28]. Since 1,4-dihydropyridines (DHPs) are compounds that selectively block L-type voltage-dependent Ca^{2+} channels (VDCC), hybrid molecules that combine an AChEI and a DHP, such as tacrine (Fig. 1) and nimodipine, might represent a promising approach to the treatment of AD. Support to this therapeutic strategy comes from the fact that bis(7)-tacrine attenuates β-amyloid neuronal apoptosis by regulating L-type calcium channels [28]. Besides inhibition of AChE and blockade of VDCC, compounds able to prevent the oxidative stress might have increased therapeutical value, since oxidative damage precedes the appearance of other pathological hallmarks of AD [29–32].

To explore the potential of the selected compounds (**4c**, **4d**, **6k**, **9a** and **9b**) as putative voltage-dependent calcium channels antagonist, changes in cytosolic Ca^{2+} signals ($[Ca^{2+}]_c$) elicited by the application of a depolarizing solution containing high K^+ (70 mM) were evaluated in bovine chromaffin cell populations.

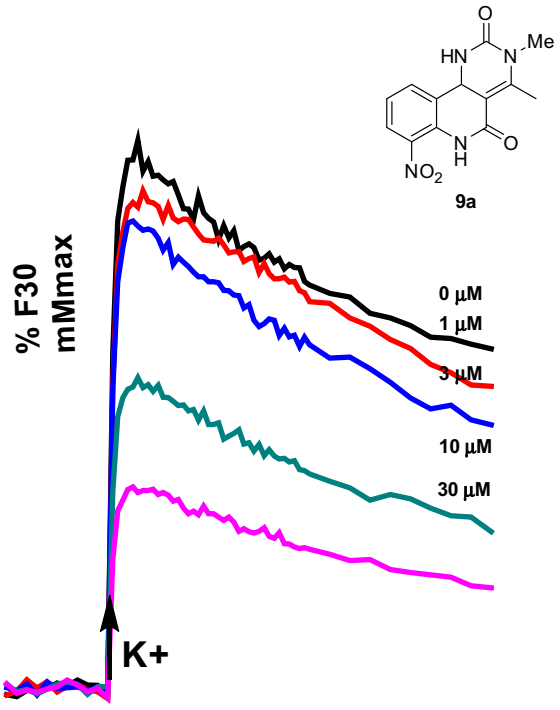


Fig. 5. Representative recordings of the increase of $[Ca^{2+}]_c$ in bovine chromaffin cell populations stimulated with a depolarizing solution containing high K^+ (70 mM), added as indicated by the arrow, in the absence or in the presence of increasing concentrations of compound **9a**, incubated since 10 min before the K^+ challenge. Data are expressed as % of F_{max}.

Unfortunately, but very interestingly, only compound **9a** was active in this experiment. Fig. 5 shows a representative experiment carried out in bovine chromaffin cells in the absence (control) and in the presence of increasing concentrations (1–3–10–30 μM) of compound **9a**. The application of a high K^+ (70 mM) solution elicited a sharp increase in $[Ca^{2+}]_c$ that reached a plateau and then tended to slowly decline along the 40 s of the recording. Incubation of the cells with compound **9a** induced a concentration-dependent decrease of $[Ca^{2+}]_c$ signal. Fig. 6 shows average data obtained by

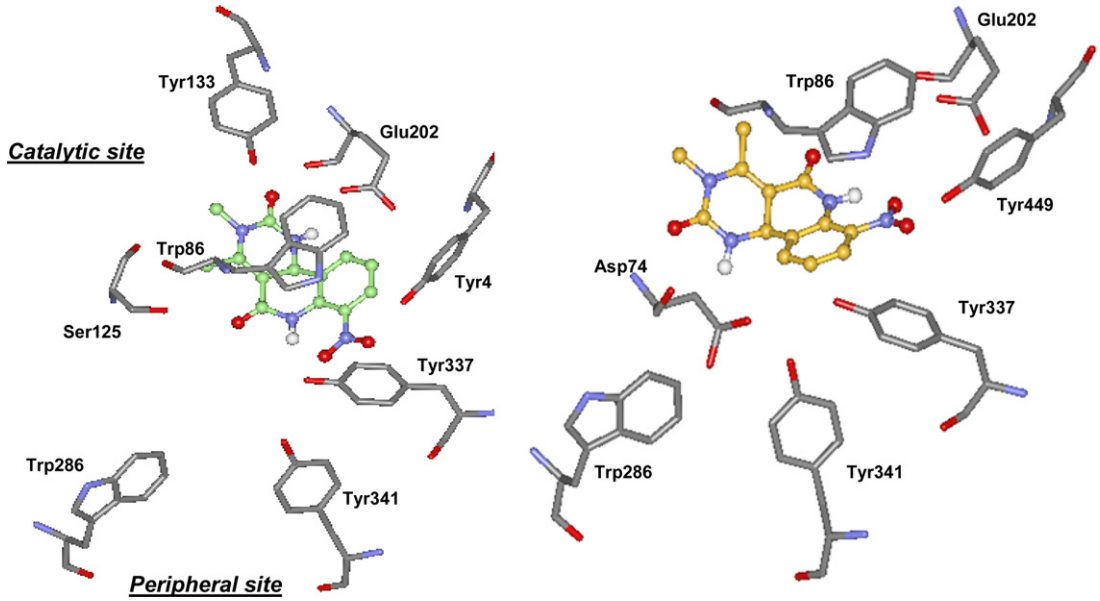


Fig. 4. Docking of compounds (*S*)-**9b** and (*R*)-**9b**.

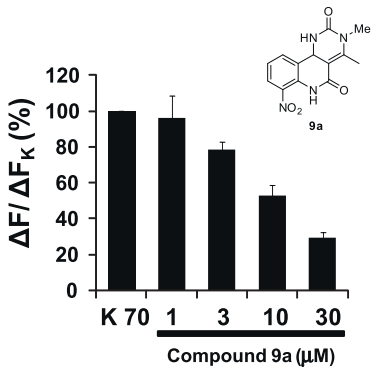


Fig. 6. Averaged data obtained in nine dishes (from three different cell cultures) upon application of the depolarizing solution containing 70 mM K^+ in the absence or in the presence of increasing concentrations of compound **9a** as indicated. Data have been normalized with respect to the initial response to K^+.

using this type of protocol in 9 cells from three different cultures and four different concentrations of the compound (1, 3, 10, and 30 μM); a clear concentration-dependent blockade of the K^+-elicited $[Ca^{2+}]_c$ signal was observed.

3. Conclusions

To summarize, we have reported the synthesis of new 4-hydroxy-2-oxo-1,2-quinoline-3-carboxamides **4a**–**d** and 4-hydroxy-2-oxo-1,2-quinoline-3-carbohydrazides **6a**–**k**. The AChE and BuChE inhibition of these molecules and some previously synthesized hexahydropyrimido [5,4-*c*]quinoline-2,5-diones were evaluated and compared to tacrine. None of the assayed compounds showed higher activity than tacrine. The most potent compounds were **9a**–**b**, and compounds **4c** and **6k** showed moderate activity.

Most of these compounds were extremely selective for AChE, but we observed that compound **4c** showed equipotent, moderate activity for both AChE and BuChE. When the N–H bond of compound **4a** was replaced by a *N*-methyl group in **4d**, a more selective interaction for BuChE occurred.

While molecular modeling showed that the NH group of quinoline formed a hydrogen bond with Asp74, this was not possible for the *N*-methyl analogue. The chlorine atom of compound **6k** and the hydroxyl group of Ser203 also formed a hydrogen bond. Finally, only one docked pose was found for each enantiomer of compound **9b**. The only binding mode we observed placed the ligand in the CC.

Finally, it has been shown that only compound **9a** decreased the decrease K^+-induced calcium signals in bovine chromaffin cells. These results suggest that these compounds behave as a voltage-dependent calcium channel antagonist, and consequently could be useful for a multi-target directed ligand [33] approach for drug design in AD, in view of its significant AChE inhibitory profile[34].

4. Experimental

4.1. General methods

Reactions were monitored by TLC using precoated silica gel aluminum plates containing a fluorescent indicator (Macherey–Nagel). Detection was done with UV (254 nm). Melting points were determined on a Kofler block and were uncorrected. Infrared spectra were recorded on a Shimadzu FTIR-8201 PC spectrometer in KBr (ν in cm^{-1}). ^1H NMR spectra were recorded on a Bruker AC 300 spectrometer. Microanalyses were carried out by the Service Central d'Analyses, CNRS, Vernaison (France). All reagents were pure analytical grades and used without further purification.

4.2. 5-Chloroisatoic anhydride (**2c**)

2-Amino-6-chlorobenzoic acid (1 g, 6 mmol, 1 eq) was suspended in 1,4-dioxane (60 mL) under 0 °C. Triphosgene (1.4 g, 5 mmol, 0.8 eq) was added and the solution stirred for 3 h at a temperature under 20 °C. The solid was collected by filtration and washed with water to give product **2c** (0.95 g, 82%): mp > 260 °C; IR (KBr) υ 3436, 1774, 1701 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 11.85 (s, 1H), 7.66 (t, J = 8.3 Hz, 1H), 7.31 (d, J = 7.9 Hz, 1H), 7.10 (d, J = 8.3 Hz, 1H). Anal. Calcd. for $C_8H_4ClNO_3$: C,48.63; H, 2.04; Cl, 17.94; N, 7.09. Found: C, 48.67; H, 2.06; Cl, 17.85; N, 7.18.

4.3. N-Methyl-5-chloroisatoic anhydride (**2d**)

Iodométhane (2 mL, 4.6 mmol, 4 eq) and DIPEA (1 mL, 0.74 mol) were suspended in DMA (10 mL) for 10 min at RT. Then 5-chloroisatoic anhydride (200 mg, 1.01 mmol, 1eq) was added and the reaction mixture was stirred at for 5 H at 40 °C. The resulted product 2d was collected by filtration and washed with water (150 mg, 70%). mp = 224 °C, IR (KBr) υ 1774, 1716, 1593 cm^{-1} ^1H NMR (DMSO-d_6, 300 MHz) δ 7.78 (t, J = 8.1 Hz, 1H), 7.42 (m, 2H), 3.45 (s, 3H). Anal. Calcd for $C_9H_6ClNO_3$: C, 51.08; H, 2.86; Cl, 16.75; N, 6.62; O, 22.68. Found: C, 51.12; H,2.36; Cl,16.71; N,6.58.

4.4. General method of preparation of compounds **3a**–**d**

The corresponding anhydride isatoic (1 eq) was suspended in DMF (10 mL) at 0 °C. Sodium hydride (2 eq) and diethyl malonate (5 eq) were added slowly. Then the reaction mixture was heated at 85 °C for 5 h. Then, 10 mL of water were added and the mixture was acidified with concentrated hydrochloric acid. The resulting solid was filtered, washed with water and dried, yielding the desired compound.

4.4.1. Ethyl 4-hydroxy-2-oxo-1,2-dihydroquinoline-3-carboxylate (**3a**)

Following the General procedure in section 5.3, the reaction of compound **2a** (1 g, 6.13 mmol) with NaH (0.29 g, 12.3 mmol), and diethyl malonate (4.91 g, 30.7 mmol) gave product **3a** (1 g, 70%): mp 134 °C; IR (KBr) υ 3406, 3193, 1658, 1604 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 11.47 (s, 1H), 7.94 (d, J = 8.1 Hz, 1H), 7.62 (t, J = 7.2 Hz, 1H), 7.27 (d, J = 8.1 Hz, 1H), 7.20 (t, J = 7.5 Hz, 1H), 4.34 (q, J = 6.9 Hz, 2H), 1.31 (t, J = 7.2 Hz, 3H). Anal. Calcd. for $C_{12}H_{11}NO_4$: C, 61.80; H, 4.75; N, 6.01. Found: C, 61.72; H, 4.78; N, 6.10.

4.4.2. Ethyl 4-hydroxy-1-methyl-2-oxo-1,2-dihydroquinoline-3-carboxylate (**3b**)

Following the General procedure in section 5.3, the reaction of compound **2b** (1 g, 5.65 mmol) with NaH (0.27 g, 11.3 mmol), and diethyl malonate (4.52 g, 28.2 mmol) gave product **3b** (0.56 g, 41%): mp 104 °C; IR (KBr) υ 1631, 1593, 1562 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 8.05 (d, J = 7.8 Hz, 1H), 7.45 (t, J = 7.5 Hz, 1H), 7.52 (d, J = 8.4 Hz, 1H), 7.31 (t, J = 7.2 Hz, 1H), 4.33 (q, J = 7.2 Hz, 2H), 3.54 (s, 3H), 1.30 (t, J = 7.2 Hz, 3H). Anal. Calcd. for $C_{13}H_{13}NO_4$: C, 63.15; H, 5.30; N, 5.67. Found: C, 63.24; H, 5.27; N, 5.61.

4.4.3. Ethyl 5-chloro-4-hydroxy-2-oxo-1,2-dihydroquinoline-3-carboxylate (**3c**)

Following the General procedure in section 5.3, the reaction of compound **2c** (1 g, 5.06 mmol) with NaH (0.24 g, 10.1 mmol), and diethyl malonate (4.05 g, 25.3 mmol) gave product **3c** (0.21 g, 16%): mp >260 °C; IR (KBr) υ 2927, 2850, 1685, 1207 cm^{-1} ^1H NMR (DMSO-d_6, 300 MHz) δ 11.62 (s, 1H), 7.51 (s, 1H), 7.23 (m, 2H), 4.35

(m, 2H), 1.28 (m, 3H). Anal. Calcd. for $C_{12}H_{10}ClNO_4$: C, 53.85; H, 3.77; Cl, 13.25; N, 5.23. Found: C, 53.72; H, 3.81; Cl, 13.18; N, 5.31.

4.4.4. Ethyl 5-chloro-4-hydroxy-1-methyl-2-oxo-1,2-dihydroquinoline-3-carboxylate (**3d**)

Following the procedure section 5.3, the reaction of compound **2d** (602 mg, 2.84 mmol) with NaH (135 mg, 5.62 mmol), and diethyl malonate (1.7 mL, 11.1 mol), after 5 H, gave product 3d (177 mg, 22%). IR (KBr) υ 2977, 2930, 1730, 1640, 1594, 1567, 1525 cm^{-1} ^1H NMR (DMSO-d_6, 300 MHz) δ 13.84(s, 1H), 7.67 (t, $J = 8.1$ Hz, 1H), 7.52 (d, $J = 8.7$ Hz, 1H), 7.36 (d, $J = 7.8$ Hz, 1H), 4.35 (q, $J = 6.9$ Hz, 2H), 3.55 (s, 3H), 1.31 (t, $J = 7.2$ Hz, 3H). Anal. Calcd. For $C_{13}H_{12}ClNO_4$: C, 55.43; H, 4.29; Cl, 12.59; N, 4.97; O, 22.72. Found: C, 55.34; H, 4.21; Cl, 12.69; N, 4.89.

4.5. General method for compounds **4a**–**d**

The corresponding amine (2.4 eq) was suspended in xylene (20 mL), then the corresponding quinoline (1 eq) was added. The resulting mixture was heated at reflux for 2 h and then was allowed to cool to room temperature. The compound was filtered and washed with ether.

4.5.1. N-{2-[(2-Aminoethyl)amino]ethyl}-4-hydroxy-2-oxo-1,2-dihydroquinoline-3-carboxamide (**4a**)

Following the General procedure in section 5.4, the reaction of diethylenetriamine (0.54 g, 5.23 mmol) with compound **3a** (1 g, 4.29 mmol) gave product **4a** (0.91 g, 73%): mp >260 °C; IR (KBr) υ 1651, 1608, 1542 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 9.76 (s, 1H), 7.03 (t, $J = 7.9$ Hz, 1H), 6.65 (d, 7.3 Hz, 1H), 6.35 (t, $J = 7.9$ Hz, 1H), 6.25 (t, $J = 7.3$ Hz, 1H), 4.55 (m, NH), 1.87–1.60 (m, 8H). Anal. Calcd. for $C_{14}H_{18}N_4O_3$: C, 57.92; H, 6.25; N, 19.30. Found: C, 57.88; H, 6.26; N, 19.41.

4.5.2. N-{2-[(2-Aminoethyl)amino]ethyl}-4-hydroxy-1-methyl-2-oxo-1,2-dihydroquinoline-3-carboxamide(**4b**)

Following the General procedure in section 5.4, the reaction of diethylenetriamine (0.50 g, 4.85 mmol) with compound **3b** (1 g, 4.05 mmol) gave product **4b** (1.09 g, 89%): mp 162 °C; IR (KBr) υ 3305, cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 9.46 (s, 1H), 7.04 (d, $J = 7.8$ Hz, 1H), 6.62 (t, $J = 8.3$ Hz, 1H), 6.41 (d, $J = 8.4$ Hz, 1H), 6.19 (t, $J = 7.3$ Hz, 1H), 2.42 (s, 3H), 1.68–1.47 (m, 8H). Anal. Calcd. for $C_{15}H_{20}N_4O_3$: C, 59.20; H, 6.62; N, 18.41. Found: C, 59.31; H, 6.58; N, 18.34.

4.5.3. N-[2-({2-[(2-Aminoethyl)amino]ethyl}amino)ethyl]-4-hydroxy-2-oxo-1,2-dihydroquinoline-3-carboxamide (**4c**)

Following the General procedure in Section 5.4, the reaction of triethylenetetramine (0.75 g, 5.13 mmol) with compound **3a** (0.5 g, 2.23 mmol) gave product **4c** (0.62 g, 84%): mp >260 °C; IR (KBr) υ 2839, 2742, 1651, 1624 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 10.82 (s, 1H), 7.92 (t, $J = 7.7$ Hz, 1H), 7.51 (t, $J = 8.3$ Hz, 1H), 7.26 (m, 1H), 7.14 (m, 1H), 4.23 (s, 3H), 2.65 (m, 12H). Anal. Calcd. for $C_{16}H_{23}N_5O_3$: C, 57.64; H, 6.95; N, 21.01. Found: C, 57.70; H, 6.92; N, 21.06.

4.5.4. N-[2-({2-[(2-Aminoethyl)amino]ethyl}amino)ethyl]-4-hydroxy-1-methyl-2-oxo-1,2-dihydroquinoline-3-carboxamide (**4d**)

Following the procedure in section 5.4, the reaction of triethylenetetramine (0.88 g, 6.02 mmol) with compound **3b** (1 g, 4.05 mmol) gave product **4d** (0.09 g, 10%); mp >260 °C; IR (KBr) υ cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 10.48 (s, 1H), 7.82 (d, $J = 7.8$ Hz, 1H), 7.66 (t, $J = 7.2$ Hz, 1H), 7.45 (d, $J = 8.4$ Hz, 1H), 7.19 (t, $J = 7.2$ Hz, 1H), 3.43 (m, 8H), 2.79 (s, 2H), 2.68 (s, 3H), 2.50 (s, 3H). Anal. Calcd. for $C_{17}H_{25}N_5O_3$: C, 58.77; H, 7.25; N, 20.16. Found: C, 58.84; H, 7.21; N, 20.05.

4.6. General method for compounds **5a**–**d**

The quinoline-3-carboxylate (1 eq) and its derivatives were suspended in methanol (20 mL). Hydrazine (1.5 eq) was added and the mixture was heated at 100 °C for 30 min. The precipitated compound was collected by filtration and used without further purification.

4.6.1. 4-Hydroxy-2-oxo-1,2-dihydroquinoline-3-carbohydrazide (**5a**)

Following the General procedure in section 5.5, the reaction of hydrazine (0.52 g, 16.3 mmol) with compound **3a** (2 g, 8.58 mmol) gave product **5a** (1.57 g, 84%): mp >260 °C; IR (KBr) υ 3167, 1674, 1616, 1531 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 11.89 (s, 1H), 10.97 (s, 1H), 7.97 (d, $J = 7.9$ Hz, 1H), 7.68 (t, $J = 7.2$ Hz, 1H), 7.36 (d, $J = 8.3$ Hz, 1H), 7.29 (t, $J = 7.3$ Hz, 1H), 2.79 (s, 2H). Anal. Calcd. for $C_{10}H_9N_3O_3$: C, 54.79; H, 4.14; N, 19.17. Found: C, 54.85; H, 4.08; N, 19.90.

4.6.2. 4-Hydroxy-1-methyl-2-oxo-1,2-dihydroquinoline-3-carbohydrazide (**5b**)

Following the General procedure in section 5.5, the reaction of hydrazine (0.48 g, 15 mmol) with compound **3b** (2 g, 8.10 mmol) gave product **5b** (1.20 g, 64%): mp >260 °C; IR (KBr) υ 3328, 3240, 1647, 1589 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 11.00 (s, 1H), 8.01 (d, $J = 7.2$ Hz 1H), 7.81 (t, $J = 7.3$ Hz, 1H), 7.62 (d, $J = 8.6$ Hz, 1H), 7.38 (t, $J = 7.3$ Hz, 1H), 4.95 (s, 2H), 3.63 (s, 3H). Anal. Calcd. for $C_{11}H_{11}N_3O_3$: C, 56.65; H, 4.75; N, 18.02. Found: C, 56.52; H, 4.79; N, 18.11.

4.6.3. 5-Chloro-4-hydroxy-2-oxo-1,2-dihydroquinoline-3-carbohydrazide (**5c**)

Following the procedure in section 5.5, the reaction of hydrazine (0.27 g, 8.4 mmol) with compound **3c** (0.5 g, 1.87 mmol) gave product **5c** (0.43 g, 91%): mp 106 °C; IR (KBr) υ 2924, 2854, 1670, 1546 cm^{-1} ^1H NMR (DMSO-d_6, 300 MHz) δ 12.68 (s, 1H), 11.12 (s, 1H), 7.89 (s, 1H), 7.41 (m, $J = 8.6$ Hz, 1H), 7.38 (t, $J = 7.3$ Hz, 1H), 4.95 (s, 2H), 3.63 (s, 3H). Anal. Calcd. for $C_{10}H_8ClN_3O_3$: C, 47.35; H, 3.18; Cl, 13.98; N, 16.57. Found: C, 47.31; H, 3.20; Cl, 13.95; N, 16.63.

4.6.4. 5-Chloro-4-hydroxy-1-methyl-2-oxo-1,2-dihydroquinoline-3-carbohydrazide (**5d**)

Following the procedure section 5.5, the reaction of hydrazine (40 μL, 1.26 mmol) with compound **3d** (177 mg, 0.63 mmol) after 20 H gave product **5d** (40 mg, 24%). mp °C; IR (KBr) υ 3320, 3211, 1608 cm^{-1} ^1H NMR (DMSO-d_6, 300 MHz) δ 18.10 (s, 1H), 11.15 (s, 1H), 7.68 (d, $J = 1.2$ Hz, 1H), 7.61 (d, $J = 5.1$ Hz, 1H), 7.41 (s, 1H), 4.98 (s, 2H), 3.63 (s, 3H). Anal. Calcd. for $C_{11}H_{10}ClN_3O_3$: C, 49.36; H, 3.77; Cl, 13.25; N, 15.70; O, 17.93. Found: C, 49.41; H, 3.78; Cl,13.19; N, 15.78.

4.7. General method for compounds **6a**–**l**

The corresponding quinoline-3-carboxyhydrazides **5a**–**c** were stirred with 2,4-dihydroxybenzaldehyde or its derivatives in dimethyl sulfoxide and four drops of orthophosphoric acid for 15 min at room temperature. The mixture was then heated at 100 °C for 1 h. The compound was collected by filtration and washed with water.

4.7.1. N'-[(E)-(2,4-Dihydroxyphenyl)methylidene]-4-hydroxy-2-oxo-1,2-dihydroquinoline-3-carbohydrazide (**6a**)

Following the General procedure in section 5.6, the reaction of 2,4-dihydroxybenzaldehyde (0.31 g, 2.3 mmol) with compound **5a** (0.5 g, 2.3 mmol) gave product **6a** (0.75 g, 97%): mp >260 °C; IR

(KBr) υ 3205, 1658, 1558 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 13.19 (s, 1H), 12.01 (s, 1H), 11.08 (s, 1H), 10.10 (s, 1H), 8.51 (s, 1H), 7.96 (d, J = 7.9 Hz, 1H), 7.66 (t, J = 7.7 Hz, 1H), 7.34 (t, J = 7.3 Hz, 2H), 7.27 (t, J = 7.7 Hz, 1H), 6.34 (d, J = 8.4 Hz, 1H), 6.29 (s, 1H). Anal. Calcd. for $C_{17}H_{13}N_3O_5$: C, 60.18; H, 3.86; N, 12.38. Found: C, 60.24; H, 3.84; N, 12.31.

4.7.2. 4-Hydroxy-N'-[(1E)-(2-hydroxy-5-methylphenyl) methylene]-2-oxo-1,2-dihydroquinoline-3-carbohydrazide (**6b**)

Following the General procedure in section 5.6, the reaction of 2-hydroxy-5-methyl-benzaldehyde (0.15 g, 1.10 mmol) with compound **5a** (0.25 g, 1.14 mmol) gave product **6b** (0.31 g, 81%): mp >260 °C, IR (KBr) υ 3001, 1651, 1612, 1581, 1546 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 13.65 (s, 1H), 8.87 (s, 1H), 8.27 (d, J = 7.9 Hz, 1H), 7.97 (t, J = 7.5 Hz, 1H) 7.67 (s, 1H), 7.57 (t, J = 7.5 Hz, 1H), 7.39 (d, J = 8.1 Hz, 1H), 7.11 (t, J = 8.6 Hz, 1H), 2.75 (s, 3H). Anal. Calcd. for $C_{18}H_{15}N_3O_4$: C, 64.09; H, 4.48; N, 12.46. Found: C, 64.12; H, 4.46; N, 12.38.

4.7.3. 4-Hydroxy-N'-[(1E)-(2-hydroxy-5-methoxyphenyl) methylene]-2-oxo-1,2-dihydroquinoline-3-carbohydrazide (**6c**)

Following the General procedure in section 5.6, the reaction of 2-hydroxy-5-methoxy-benzaldehyde (0.28 g, 1.84 mmol) with compound **5a** (0.40 g, 1.83 mmol) gave product **6c** (0.59 g, 92%): mp >260 °C; IR (KBr) υ 3355, 1670, 1651, 1577, 1542 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 13.41 (s, 1H), 10.43 (s, 1H), 8.92 (s, 1H), 8.58 (s, 1H), 7.98 (d, J = 7.9 Hz, 1H), 7.68 (t, J = 7.9 Hz, 1H), 7.36 (d, J = 8.3 Hz, 1H), 7.23 (m, 1H), 7.12 (d, J = 2.8 Hz, 1H), 6.90 (m, 3H), 3.69 (s, 3H). Anal. Calcd. for $C_{18}H_{15}N_3O_5$: C, 61.19; H, 4.28; N, 11.89. Found: C, 61.26; H, 4.25; N, 11.82.

4.7.4. N'-[(E)-(2,4-dihydroxyphenyl)methylidene]-4-hydroxy-1-methyl-2-oxo-1,2-dihydroquinoline-3-carbohydrazide (**6d**)

Following the General procedure in section 5.6, the reaction of 2,4-dihydroxybenzaldehyde (0.15 g, 1.1 mmol) with compound **5b** (0.25 g, 1.1 mmol) gave product **6d** (0.30 g, 79%): mp >260 °C; IR (KBr) υ 3460, 1624, 1581 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 13.25 (s, 1H), 11.15 (s, 1H), 10.10 (s, 1H), 8.58 (s, 1H), 8.12 (d, J = 7.7 Hz, 1H), 7.83 (t, J = 7.7 Hz, 1H), 7.64 (d, J = 8.6 Hz, 1H), 7.41 (d, J = 7.5 Hz, 1H), 7.35 (d, J = 8.6 Hz, 1H), 6.38 (d, J = 8.4 Hz, 1H), 6.33 (s, 1H), 3.66 (s, 3H). Anal. Calcd. for $C_{18}H_{15}N_3O_5$: C, 61.19; H, 4.28; N, 11.89. Found: C, 61.25; H, 4.23; N, 11.84.

4.7.5. 4-Hydroxy-N'-[(1E)-(2-hydroxy-5-methylphenyl) methylene]-1-methyl-2-oxo-1,2-dihydroquinoline-3-carbohydrazide (**6e**)

Following the General procedure in section 5.6, the reaction of 2-hydroxy-5-methyl-benzaldehyde (0.15 g, 1.10 mmol) with compound **5b** (0.25 g, 1.07 mmol) gave product **6e** (0.34 g, 93%): mp >260 °C; IR (KBr) υ 2985, 1624, 1589, 1558, 1519 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 12.55 (s, 1H), 9.95 (s, 1H), 7.81 (s, 1H), 7.30 (d, J = 7.7 Hz, 1H), 7.00 (t, J = 8.1 Hz, 1H), 6.82 (d, J = 8.6 Hz, 1H), 6.58 (d, J = 7.5 Hz, 1H), 6.53 (s, 1H), 6.30 (d, J = 8.3 Hz, 1H), 6.01 (d, J = 8.3 Hz, 1H), 2.83 (s, 3H), 1.41 (s, 3H). Anal. Calcd. for $C_{19}H_{17}N_3O_4$: C, 64.95; H, 4.88; N, 11.96. Found: C, 64.92; H, 4.86; N, 11.95.

4.7.6. 4-Hydroxy-N'-[(1E)-(2-hydroxy-5-methoxyphenyl) methylene]-1-methyl-2-oxo-1,2-dihydroquinoline-3-carbohydrazide (**6f**)

Following the General procedure in section 5.6, the reaction of 2-hydroxy-5-methoxy-benzaldehyde (0.16 g, 1.05 mmol) with compound **5b** (0.25 g, 1.07 mmol) gave product **6f** (0.33 g, 84%): mp >260 °C; IR (KBr) υ 2966, 1643, 1562, 1535 cm^{-1}: ^1H NMR (DMSO-d_6, 300 MHz) δ 13.49 (s, 1H), 10.51 (s, 1H), 8.65 (s, 1H), 8.15 (d, J = 7.5 Hz, 1H), 7.82 (d, J = 7.0 Hz, 1H), 7.65 (d, J = 8.1 Hz, 1H), 7.40 (t, J = 7.2 Hz, 1H), 7.15 (s, 1H), 6.92 (m, 2H) 3.74 (s, 3H), 3.67 (s, 3H). Anal. Calcd. for $C_{19}H_{17}N_3O_5$: C, 62.12; H, 4.66; N, 11.44. Found: C, 62.19; H, 4.63; N, 11.41.

4.7.7. 4-Hydroxy-N'-[(1E)-(2-nitrophenyl)methylene]-2-oxo-1,2-dihydroquinoline-3-carbohydrazide (**6g**)

Following the General procedure in section 5.6, the reaction of 2-nitrobenzaldehyde (0.31 g, 2.05 mmol) with compound **5a** (0.20 g, 0.91 mmol) gave product **6g** (0.18 g, 56%): mp >260 °C; IR (KBr) υ 3328, 3298, 3031, 1635, 1604, 1554 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 16.45 (s, 1H), 13.56 (s, 1H), 12.14 (s, 1H), 8.81 (s, 1H), 8.05 (m, 3H), 7.86 (m, 1H), 7.74 (m, 2H), 7.39 (m, 2H). Anal. Calcd. for $C_{17}H_{12}N_4O_5$: C, 57.96; H, 3.43; N, 15.90. Found: C, 57.92; H, 3.45; N, 15.94.

4.7.8. 4-Hydroxy-1-methyl-N'-[(1E)-(2-nitrophenyl)methylene]-2-oxo-1,2-dihydroquinoline-3-carbohydrazide (**6h**)

Following the General procedure in section 5.6, the reaction of 2-nitrobenzaldehyde (0.78 g, 5.16 mmol) with compound **5b** (0.20 g, 0.86 mmol) gave product **6h** (0.29 g, 92%): mp >260 °C; IR (KBr) υ 1670, 1566, 1523 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 8.67 (s, 1H), 8.15 (s, 1H), 8.11 (d, J = 7.6 Hz, 1H), 8.07 (s, 1H), 7.83 (t, J = 6.9 Hz, 1H), 7.67 (m, 2H), 7.48 (s, 1H), 7.26 (s, 1H), 3.58 (s, 3H). Anal. Calcd. for $C_{18}H_{14}N_4O_5$: C, 59.02; H, 3.85; N, 15.29. Found: C, 59.11; H, 3.82; N, 15.25.

4.7.9. N'-[(1E)-(2,6-dichloro-3-nitrophenyl)methylene]-4-hydroxy-2-oxo-1,2-dihydroquinoline-3-carbohydrazide (**6i**)

Following the General procedure in section 5.6, the reaction of 2,6-dichloro-3-nitro-benzaldehyde (1 g, 4.55 mmol) with compound **5a** (0.50 g, 2.30 mmol) gave product **6i** (0.87 g, 92%): mp >260 °C; IR (KBr) υ 3298, 3093, 1735, 1620, 1596, 1539 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 13.92 (s, 1H), 11.80 (s, 1H), 8.60 (s, 1H), 8.16 (d, J = 8.5 Hz, 1H), 8.01 (d, J = 8.1 Hz, 1H), 7.88 (d, J = 8.7 Hz, 1H), 7.66 (s, 1H), 7.35 (s, 1H), 7.26 (s, 1H). Anal. Calcd. for $C_{17}H_{10}Cl_2N_4O_5$: C, 48.48; H, 2.39; Cl, 16.83; N, 13.30. Found: C, 48.44; H, 2.37; Cl, 16.86; N, 13.25.

4.7.10. N'-[(1E)-(2,6-dichloro-3-nitrophenyl)methylene]-4-hydroxy-1-methyl-2-oxo-1,2-dihydroquinoline-3-carbohydrazide (**6j**)

Following the General procedure in section 5.6, the reaction of 2,6-dichloro-3-nitro-benzaldehyde (0.78 g, 3.55 mmol) with compound **5b** (0.30 g, 1.28 mmol) gave product **6j** (0.51 g, 91%): mp 230 °C; IR (KBr) υ 3058, 1635, 1577, 1558, 1519 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 8.66 (s, 1H), 8.15 (d, J = 7.2 Hz, 2H), 7.87 (d, J = 8.7 Hz, 1H), 7.79 (s, 1H), 7.60 (s, 1H), 7.36 (s, 1H), 3.64 (s, 3H). Anal. Calcd. for $C_{18}H_{12}Cl_2N_4O_5$: C, 49.67; H, 2.78; Cl, 16.29; N, 12.87. Found: C, 49.71; H, 2.76; Cl, 16.31; N, 12.82.

4.7.11. 5-Chloro-N'-[(E)-(2,4-dihydroxyphenyl)methylidene]-4-hydroxy-2-oxo-1,2-dihydroquinoline-3-carbohydrazide (**6k**)

Following the General procedure section 5.6, the reaction of 2,4-dihydroxybenzaldehyde (0.04 g, 0.20 mmol) with compound **5c** (0.05 g, 0.20 mmol) gave product **6k** (0.04 g, 54%): mp >260 °C; IR (KBr) υ 3159, 2896, 1639, 1589, 1554 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 13.10 (s, 1H), 11.97 (s, 1H), 10.84 (s, 1H), 9.87 (s, 1H), 8.35 (s, 1H), 7.39 (t, J = 8.1 Hz, 1H), 7.17 (d, J = 7.8 Hz, 2H), 7.11 (d, J = 8.8 Hz, 2H), 6.15 (d, J = 8.4 Hz, 1H), 6.11 (s, 1H). Anal. Calcd. for $C_{17}H_{12}ClN_3O_5$: C, 54.63; H, 3.24; Cl, 9.49; N, 11.24. Found: C, 54.58; H, 3.21; Cl, 9.52; N, 11.27.

4.7.12. 5-Chloro-N'-[(E)-(2,4-dihydroxyphenyl)methylidene]-4-hydroxy-1-methyl-2-oxo-1,2-dihydroquinoline-3-carbohydrazide (**6l**)

Following the procedure section 5.6, the reaction of 2,4-dihydroxybenzaldehyde (20 mg, 0.14 mmol) with compound **5d** (40 mg,

0.15 mmol) after 1H gave product **6l** (23 mg, 39%). mp >260 °C; IR (KBr) υ 1610, 1558 cm^{-1} ^1H NMR (DMSO-d_6, 300 MHz) δ 17.74 (s, 1H), 13.34 (s, 1H), 11.08 (s, 1H), 10.09 (s, 1H), 8.61 (s, 1H), 7.73 (d, $J = 6.9$ Hz, 1H), 7.65 (d, $J = 7.8$ Hz, 1H), 7.45 (d, $J = 5.4$ Hz, 1H), 7.37 (d, $J = 8.1$ Hz, 1H), 6.38 (d, $J = 8.7$ Hz, 1H), 6.34 (s, 1H), 3.67 (s, 3H). Anal. Calcd. for $C_{18}H_{12}ClN_3O_5$: C, 55.75; H, 3.64; Cl, 9.14; N, 10.84; O, 20.63. Found: C, 55.81; H, 3.57; Cl, 9.21; N, 10.75.

4.8. 2-Bromo-3-nitrobenzaldehyde (**7**)

Chromium (VI) oxide (1.86 g, 6.94 mmol, 2.7 eq) was dissolved in acetic anhydride (30 mL), and the mixture was stirred until the solid was completely dissolved. On the other hand, 2-bromo-3-nitrotoluene (1.5 g, 18.7 mmol, 1 eq) was suspended in acetic anhydride (12 mL). Then sulphuric acid was added dropwise to this solution at 0 °C. The chromium oxide solution was added dropwise during 1h 30 min at 0 °C. The reaction mixture was allowed to room temperature and stirred 3 h. The solution was neutralised carefully with ice water. The resulting solid **7** (0.63 g, 39%) was filtered and used without further purification. **7**: mp 92 °C; IR (KBr) υ 1697, 1539, 1519 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 14.24 (s, 1H), 8.26 (d, $J = 7.8$ Hz, 1H), 8.07 (d, $J = 7.8$ Hz, 1H), 7.79 (t, $J = 8.1$ Hz, 1H). Anal. Calcd. for $C_7H_4BrNO_3$: C, 36.55; H, 1.75; Br, 34.74; N, 6.09. Found: C, 36.60; H, 1.74; Br, 34.81; N, 6.04.

4.9. General method for compounds ***8a–b***

A solution of compound **7** (1 eq), ethyl acetoacetate (1.5 eq), urea derivatives (1.8 eq) and H_3BO_3 (0.75 eq), in glacial acetic acid (10 mL) is heated at 100 °C, while stirring for 2 h. Then it was cooled to room temperature, and poured into a mixture of ice water (50 mL) and ethanol. The product was collected by filtration and recrystallised from EtOH (95%), to give the pure product.

4.9.1. Ethyl 4-(2-bromo-3-nitrophenyl)-1-ethyl-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (***8a***)

Following the General procedure section 5.8, the reaction of ethyl acetoacetate (0.81 g, 6.23 mmol) with ethylurea (0.66 g, 7.49 mmol), boric acid (0.38 g, 3.28 mmol) and compound **7** (1 g, 4.34 mmol) gave product **8a** (1.10 g, 61%): mp 224 °C; IR (KBr) υ 3228, 1708, 1674, 1624, 1535 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 8.12 (s, 1H), 7.89 (d, $J = 6.8$ Hz 1H), 7.6 (m, 2H), 5.75 (s, 1H), 3.97 (d, $J = 6.1$ Hz, 2H), 3.79 (m, 2H), 2.63 (s, 1H), 1.19 (s, 3H), 1.03 (t, $J = 6.6$ Hz, 3H). Anal. Calcd. for $C_{16}H_{18}BrN_3O_5$: C, 46.62; H, 4.40; Br, 19.38; N, 10.19. Found: C, 46.67; H, 4.38; Br, 19.35; N, 10.07.

4.9.2. Ethyl 4-(2-bromo-3-nitrophenyl)-1,6-dimethyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (***8b***)

Following the General procedure section 5.8, the reaction of ethyl acetoacetate (0.81 g, 6.23 mmol) with methylurea (0.64 g, 8.62 mmol), boric acid (0.38 g, 3.28 mmol) and compound **7** (1 g, 4.34 mmol) gave product **8b** (0.72 g, 42%): mp 165 °C; IR (KBr) υ 3217, 1708, 1681, 1635, 1542 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 8.11 (d, $J = 2.7$ Hz, 1H), 7.84 (dd, $J = 6.6$, 2.7 Hz, 1H), 7.60 (t, $J = 5.4$ Hz, 2H), 5.71 (s, 1H), 3.93 (q, $J = 7.2$ Hz, 2H), 3.18 (s, 3H), 2.57 (s, 3H), 0.99 (t, $J = 7.2$ Hz, 3H). Anal. Calcd. for $C_{15}H_{16}BrN_3O_5$: C, 45.24; H, 4.05; Br, 20.07; N, 10.55. Found: C, 45.21; H, 4.07; Br, 20.12; N, 10.51.

4.10. General method for compounds ***9a–b***

The corresponding dihydropyrimidoquinoline (1 eq) was suspended in of an aqueous ammonia solution (28%) (90 mL) in a pressure vessel. The reaction was heated (250 °C) under pressure (10 bar). After 20 h, the reaction was allowed to cool at room temperature and at atmosphere pressure. Then the reaction was filtered and the filtrate was neutralized with concentrated hydrochloride acid. The resulting solid was filtered and washed with water.

4.10.1. 3,4-Dimethyl-7-nitro-6,10b-dihydropyrimido[5,4-c] quinoline-2,5(1H,3H)-dione (***9a***)

Following the General procedure section 5.9, the reaction of dihydropyrimidoquinoline (0.5 g, 1.15 mmol) gave product **9a** (0.08 g, 24%): mp 188 °C; IR (KBr) υ 3359, 2854, 1678, 1634 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 12.25 (s, 1H), 8.12 (d, $J = 3.3$ Hz, 1H), 7.95 (d, $J = 4.0$ Hz, 1H), 7.68 (d, $J = 5.0$ Hz, 2H), 5.75 (d, $J = 2.9$ Hz, 1H), 3.25 (s, 3H), 2.67 (s, 3H). Anal. Calcd. for $C_{13}H_{12}N_4O_4$: C, 54.17; H, 4.20; N, 19.44. Found: C, 54.06; H, 4.22; N, 19.49.

4.10.2. 3-Ethyl-4-methyl-7-nitro-6, 10b-dihydropyrimido[5,4-c] quinoline-2,5(1H,3H)-dione (***9b***)

Following the General procedure section 5.9, the reaction of dihydropyrimidoquinoline (0.8 g, 1.94 mmol) gave product **9b** (0.09 g, 15%): mp 204 °C; IR (KBr) υ 3197, 3070, 2981, 1678, 1624, 1535 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 12.17 (s, 1H), 7.95 (s, 1H), 7.84 (s, 1H), 7.56 (d, $J = 17.6$ Hz, 2H), 5.63 (s, 1H), 3.72 (m, 2H), 2.58 (s, 3H), 1.12 (s, 3H). Anal. Calcd. for $C_{14}H_{14}N_4O_4$: C, 55.63; H, 4.67; N, 18.53. Found: C, 55.60; H, 4.68; N, 18.51.

4.11. Pharmacological studies

4.11.1. Measurement of AChE activity

The AChE inhibitory activity of the compounds was determined using Ellman's method [24], using AChE from *E. electricus* (Sigma) and acetylthiocholine iodide (0.35 mM) as a substrate. The reaction took place in the final volume of 3 mL of a phosphate-buffered solution at pH 8, containing 0.035 U/mL of *Ee*AChE and 0.35 mM of 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), which produced yellow anion 5-thio-2-nitrobenzoic acid. Inhibition curves were made by incubating with the different compounds for 15 min; a sample without any compound was always used to determine the 100% of enzymatic activity. After the 15 min incubation period, the production of colour, as an indicator of enzymatic activity, was evaluated by measuring absorbance at 412 nm in a spectrophotometer plate reader (iEMS Reader MF, Labsystems). Data are means ± SEM of at least three different experiments in quadruplicate.

4.11.2. Measurement of BuChE activity

The BuChE inhibitory activity of the compounds was determined using Ellman's method [24], using horse serum BuChE from Sigma, and butyrylthiocholine iodide (0.5 mM) as a substrate. The reaction took place in the final volume of 3 mL of a phosphate-buffered solution at pH 8, containing 0.05 U/mL of eBuChE and 0.35 mM of 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), which produces the yellow anion 5-thio-2-nitrobenzoic acid. Inhibition curves were made by incubating the different compounds for 15 min; a sample without any compound was always used to determine the 100% of enzymatic activity. After the 15 min incubation period, the production of colour, as an indicator of enzymatic activity, was evaluated by measuring absorbance at 412 nm in a spectrophotometer plate reader (iEMS Reader MF, Labsystems). Data are means ± SEM of at least three different experiments in quadruplicate.

4.11.3. Molecular modeling

Docking studies with the program AutoDock (version 4.0). A docking study using AutoDock was performed to explore the binding of the compounds to AChE. Our objectives were to

determine the binding site and mode of the molecule, and therefore two regions of the target protein were scanned: the CC and the PAS. Thus, the crystal structure of the hAChE–fasciculim complex (PDB code 1B41) was selected as a model. It showed the key aminoacids in the same sequence as *Ee*AChE (PDB code 1C2B) in the region of interest.

Affinity grid files on the molecule target were generated using the auxiliary program AUTOGRID. The dimensions of the grid were $20 \times 20 \times 20$ Å^3 centered on the Trp86 and Trp286 for the catalytic and peripheral sites (CC and PAS), respectively, with grid points separated by 0.30 Å. The original Lennard-Jonnes and hydrogen-bonding potentials provided by the program were used. The flexible docking of the ligand structures was done by the Lamarckian genetic algorithm (LGA), searching for favourable bonding conformations of the ligands at the sites of the target protein. After docking, the 100 solutions were clustered in groups with rms deviations of less than 1.0 Å. The clusters were ranked by the lowest-energy representative of each cluster.

Geometry of the ligands was get as follows. First of all, we performed a preliminary conformational search with Spartan program (MMFF94 force field, systematic search algorithm) in order to detect the possible intramolecular H-bonds and estimate their relative strength. The lowest-energy conformers showed co-planar conformation of the aromatic substituents and intra-molecular H-bonds (between OH and O and between =O and NH in **4c** and **6k**). These conformers were submitted to DFT optimization the ab initio quantum chemistry program Gaussian03 and the B3LYP/3-21G* basis set. Partial atomic charges were then obtained using the RESP methodology with the 6-31G* basis set. Different conformers of the ligands were docked using the LGA algorithm implemented in AutoDock, by randomly changing the torsion angles and overall orientation of the molecule.

Our interest was focused on the CC and PAS regions of the enzyme and hence, we selected the docked structures of lowest-energy in the most populated cluster, that in general correspond to the 1–4 cluster ranked in energy.

4.11.4. Isolation and culture of bovine chromaffin cells

Bovine chromaffin cells were isolated from adrenal glands of adult cows, following standard methods [34] with some modifications [35]. Cells were suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% foetal calf serum, 10 μM cytosine arabinoside, 10 μM fluorodeoxyuridine, 50 IU ml^{-1} penicillin and 50 μg/ml streptomycin. Cells were plated at a density of 2×10^5 cells per well into 96-well plates. Cells were kept for 2 days at 37 °C in a water-saturated incubator, with a 5% CO_2/95% air atmosphere. Experiments were performed at room temperature (24+2 °C).

4.11.5. Measurement of $[Ca^{2+}]_c$ in bovine chromaffin cells

Cells were loaded with Krebs-HEPES (in mM: 144 NaCl, 5.9 KCl, 1.2 $MgCl_2$, 2 $CaCl_2$, 11 D-glucose, 10 HEPES, pH 7.4) containing 10 μM fluo-4 AM, and 0.2% pluronic acid for 45 min at 37 °C in the dark. After this incubation period, cells were washed twice with Krebs-HEPES at room temperature in the dark. Changes in fluorescence (excitation 485 nm, emission 520 nm) were measured using a fluorescent plate reader (Fluostar, BMG Lab technologies). Basal levels of fluorescence were monitored before adding drugs by using an automatic dispenser. After stimulation with a depolarizing solution containing 70 mM K^+, changes in fluorescence were measured for 40 s.

To normalize fluo-4 signals, responses from each well were calibrated by measuring maximum and minimum fluorescence values. At the end of each experiment, addition of 3% Triton X-100 (F_{max}) was followed by addition of 1 M Cl_2Mn (F_{min}). Data were calculated as a percentage of $F_{max} - F_{min}$.

Acknowledgements

We thank the 'Conseil Regional de Franche-Comte' for their financial support. LG thanks Ministerio de Educación y Ciencia (SAF 2007-65181) for support.

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