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Novel Tacrine Derivatives that Block Neuronal Calcium Channels

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Abstract—A new series of tacrine (9-amino-1,2,3,4-tetrahydroacridine) derivatives were synthesized and their effects on $^{45}Ca^{2+}$ entry into bovine adrenal chromaffin cells stimulated with dimethylphenylpiperazinium (DMPP) or K^+, studied. At 3 μM, compound **1** did not affect $^{45}Ca^{2+}$ uptake evoked by DMPP. Compounds **14**, **15** and **17** inhibited the effects of DMPP by 30%. Compounds **3**, **9** and tacrine blocked the DMPP signal by about 50%. Compounds **5** and **12** were the most potent blockers of DMPP-stimulated $^{45}Ca^{2+}$ entry (90%); the rest of the compounds inhibited the effects of DMPP by 70–80%. Compounds **1**, **3**, **4**, **8**, **10**, **11**, **13**, **16**, **17** and tacrine inhibited $^{45}Ca^{2+}$ uptake induced by K^+ about 20%. Compounds **6**, **14** and **15** inhibited the K^+ effects by 10% or less. Compounds **7**, **9**, **12** and **18** blocked the K^+ signal by 30% and, finally, compounds **2** and **5** inhibited the K^+-induced $^{45}Ca^{2+}$ entry by 50%. None of the new compounds was as effective as diltiazem ($IC_{50}=0.03$ μM) in causing relaxation of the rat aorta precontracted with 35 mM K^+; the most potent was compound **7** ($IC_{50}=0.3$ μM). Compounds **5**, **6**, **8**, **9**, **10** and **13** had IC_{50}s around 10 μM and compounds **3**, **4**, **11** and **12** around 20 μM. Blockade of Ca^{2+} entry through neuronal voltage-dependent Ca^{2+} channels, without concomitant blockade of vascular Ca^{2+} channels, suggests that some of these compounds might exhibit neuroprotectant effects but not undesirable hemodynamic effects. © 2002 Elsevier Science Ltd. All rights reserved.

Introduction

Neuronal nicotinic acetylcholine receptors (nAChRs) participate in the physiological regulation of different cerebral functions like modulation of pain signals,[1] regulation of cytosolic calcium signals, $[Ca^{2+}]_c$, and processes related to learning and memory.[2–4] For this reason, compounds that modulate nAChRs could have potential therapeutic interest in analgesia, neuroprotection, stroke and Alzheimer's disease (where the reduction in the number of functional nAChRs in patients seems to be closely related to neurological symptoms).[5] In this respect, it has been shown that a non-selective nAChR agonist such as nicotine exerts a neuroprotective action through the synthesis of nervous growth factors or the reduction of the neurotoxicity elicited by glutamate.[6–8] The implication of nAChR is reinforced by the fact that at present, the only pharmacological therapy available, showing some efficacy in the treatment of Alzheimer's disease, is that addressed to the improvement of nicotinic neurotransmission, in order to compensate the deficit of cerebral ACh.[9] Despite their growing importance, there are still few ligands and drugs to distinguish between the multiple nAChR subtypes.[10,11] New compounds acting on a specific subtype of these receptors would help to clarify their physiological role and may eventually lead to novel therapeutic strategies to treat neuropsychiatric disorders.

Tacrine (9-amino-1,2,3,4-tetrahydroacridine) has been the first drug that proved to have a beneficial effect on cognition in patients with Alzheimer's disease.[12,13] Unfortunately, this drug is not used anymore due to the fact that doses therapeutically effective produce in many cases hepatotoxicity.[14] The beneficial effects of tacrine have been mainly attributed to its anticholinesterase action;[15] however, the compound also inhibits voltage-dependent Ca^{2+} channels in dorsal root ganglion cells[16] and the binding of ligands to nicotinic receptors.[17,18]

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In addition to nAChRs, there is general agreement that Ca^{2+} ions play a crucial role in cellular disintegration mechanisms and neuronal death.[19,20] A sustained elevation of intracellular Ca^{2+} caused by the entry of that cation, possibly through a specific channel subtype,[21] determines a Ca^{2+} overload and the death of cells. This study was planned to look for new tacrine derivatives having a double pharmacological receptor target, trying to improve their potential neuroprotectant actions, that is neuronal nAChR and neuronal Ca^{2+} channels. We selected the bovine chromaffin cell as a biological model to test the effects of the new compounds because several subtypes of neuronal nAChRs (α_3, α_5, α_7, β_4)[11] and Ca^{2+} channels (L, N, P/Q, R)[22] are expressed by this cell. We present here the synthesis and biological effects on nAChRs and Ca^{2+} channels, of 18 of such novel derivatives, and their comparison with tacrine. The ability to inhibit the enzymes acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) of some of these compounds has already been studied[23] showing an apparent selectivity to block the AChE enzymatic activity; this suggests that these compounds might lack the hepatotoxic effects of tacrine, due to the preferential inhibition of BuChE of this drug.

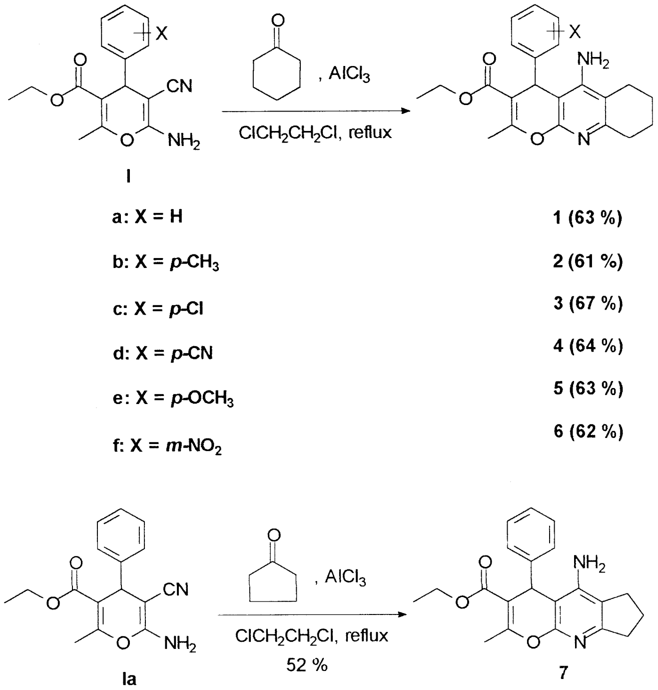
Chemistry

The synthesis of compounds **1** to **7**, previously described,[24] has been performed as shown in Scheme 1. Starting from known compounds of type **I**[25] and according to the standard Friedländer reaction protocols,[26] compounds **1**–**7** have been obtained in good yield.

The synthesis of compounds **8**–**13** has been undertaken in order to determine some structure–activity relationships, on the basis of the interesting activity found for compound **5** (Scheme 1). The size of the cycloalkyl fused ring (from cyclopentane to cycloheptane) and position of the methoxy group at the aromatic ring (from *para* in **5** to *ortho* and *meta*) were modified. Structures were on agreement with analytical and spectral data.[24] Aromatic CH and quaternary carbons were assigned with HMBC assays. Compounds **8** to **13** have an OCH_3 group at the aromatic ring showing chemical shifts between 3.69 and 3.97 ppm in ^1H NMR. The position of the OCH_3 was elucidated in each case by their assignment in HMBC spectra. Cycloalkane-fused ring signals are according with the expected spectral data for cyclohepta-, cyclohexa- and cyclopentane moieties.

The synthesis of these compounds has been performed as previously described,[24] as shown in Schemes 2 and 3. Starting from known compound **II**,[27] easily available by ethyl acetoacetate ester addition to *o*-methoxybenzylydenemalononitrile **III**,[28] and final standard Friedländer reaction, according to the usual protocol,[24,26] compounds **8**–**10** were obtained in reasonable yield. The same sequence starting from intermediate **IV**,[28] via 2-amino-3-cyano-4*H*-pyran **V**,[27] gave compounds **11**–**13**, the series of compounds with a *meta*-methoxy substituent at the aromatic ring (Scheme 3).

The furan-like tacrine-related compounds **14**, **15**, **16** and **17** have also been prepared following the method previously reported.[24] As shown in Scheme 4, starting from the known and readily available 2-amino-4,5-diaryl-3-cyano substituted furans **VI**,[29] **VII**[30] and **VIII**,[30] respectively, the desired compounds have been synthesized in low to good yield. The analytical and spectroscopic data of these compounds are in good agreement with these structures and with the described data for analogous compounds. Quaternary carbons are assigned with both



Scheme 1. Synthesis of compounds **1**–**7**.

HMBC assays and theoretical chemical shifts calculated for furan moieties.

The synthesis of **18** (Scheme 5) has been achieved from **IX,** following the general procedure for the synthesis of the tacrine analogues (see Experimental), structure was on agreement with analytical and spectral data.[24]

Pharmacology

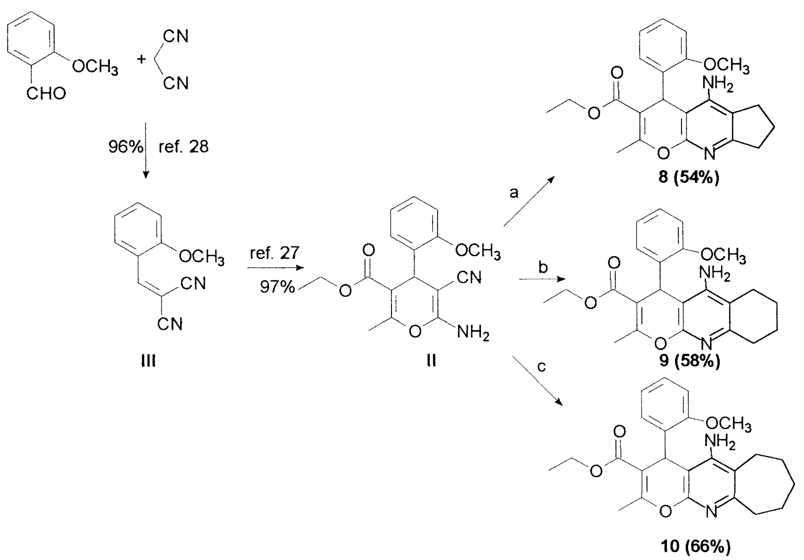
The entry of $^{45}Ca^{2+}$ through Ca^{2+} channels was stimulated by direct depolarization of bovine chromaffin cells with high K^+ concentration, or by indirect cell depolarization with the nAChR agonist DMPP. Concentrations of K^+ (70 mM) and DMPP (100 μM) producing similar increments of $^{45}Ca^{2+}$ entry were selected.[31,32]

Experiments with rat aorta were performed precontracting the preparations with addition to the Krebs-bicarbonate solution of a concentrated solution of KCl, to get a final concentration of 35 mM K^+. Once the contraction reached a plateau, the different drugs were added in a cumulative manner.[33]

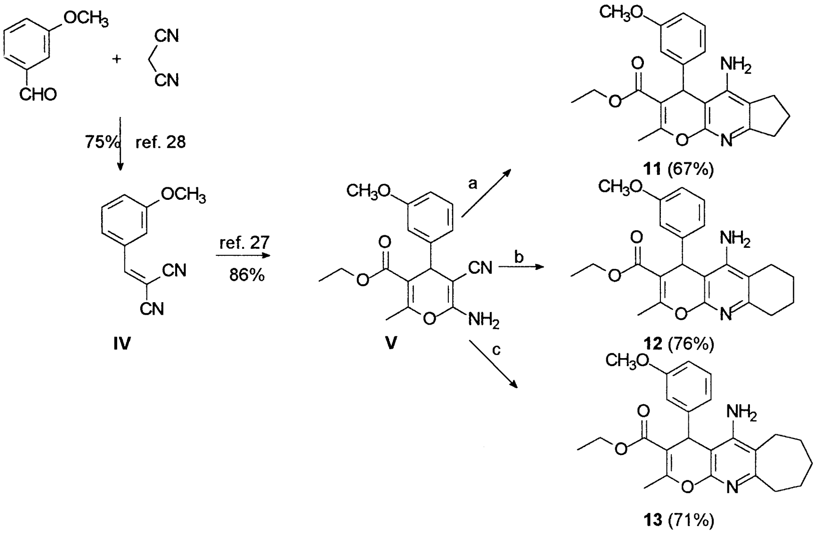
Results

$^{45}Ca^{2+}$ uptake into bovine chromaffin cells stimulated by DMPP or high K^+ concentration

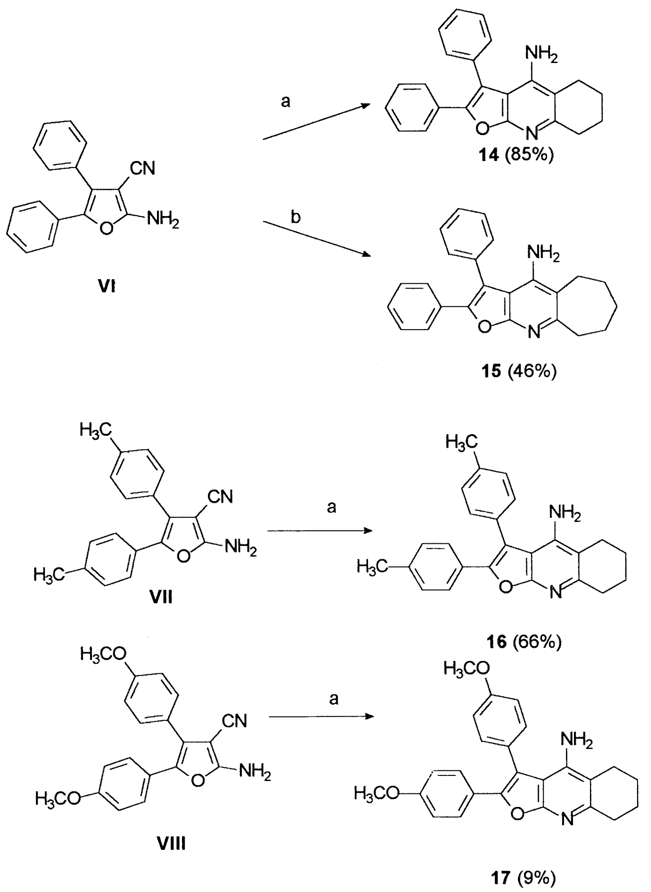
Stimulated $^{45}Ca^{2+}$ uptake was studied by two means, activation of nAChR by the agonist DMPP, or by high K^+ concentration. Usually, experiments were performed in parallel with the two stimulating agents, using 96-well plates.[31]



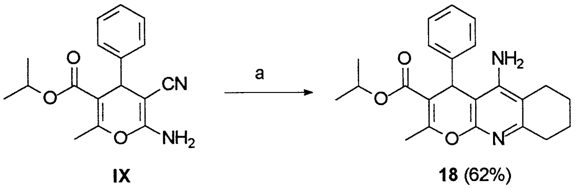
Scheme 2. Synthesis of compounds **8**–**10**. Reagents: (a) cyclopentanone, $AlCl_3$, $ClCH_2CH_2Cl$, reflux; (b) cyclohexanone, $AlCl_3$, $ClCH_2CH_2Cl$, reflux; (c) cycloheptanone, $AlCl_3$, $ClCH_2CH_2Cl$, reflux.



Scheme 3. Synthesis of compounds **11**–**13**. Reagents: (a) cyclopentanone, $AlCl_3$, $ClCH_2CH_2Cl$, reflux; (b) cyclohexanone, $AlCl_3$, $ClCH_2CH_2Cl$, reflux; (c) cycloheptanone, $AlCl_3$, $ClCH_2CH_2Cl$, reflux.



Scheme 4. Synthesis of compounds **14**–**17**. Reagents: (a) cyclohexanone, $AlCl_3$, $ClCH_2CH_2Cl$, reflux; (b) cycloheptanone, $AlCl_3$, $ClCH_2CH_2Cl$, reflux.



Scheme 5. Synthesis of compound **18**. Reagents: (a) cyclohexanone, $AlCl_3$, $ClCH_2CH_2Cl$, reflux.

In 133 separated wells from 34 different cell cultures, the $^{45}Ca^{2+}$ taken up in 5 s by resting cells amounted to 370±13 cpm 2×10^{5} cells^{-1}. DMPP (100 μM for 5 s) elevated this figure to 1471±56 cpm 2×10^{5} cells^{-1} and K^+ (70 mM for 5 s) to 1874±90 cpm 2×10^{5} cells^{-1} (Fig. 1A). These figures correspond respectively to 0.31±0.01, 1.32±0.05 and 1.58±0.07 fmol of total Ca^{2+} ($^{40}Ca^{2+}$ + $^{45}Ca^{2+}$) taken up by a single cell in resting or depolarizing conditions. Figure 1B shows the effect of tacrine (reference compound) on net $^{45}Ca^{2+}$ uptake. Tacrine (3 μM) reduced the net DMPP-stimulated $^{45}Ca^{2+}$ uptake from 1.01±0.04 to 0.51±0.05 fmol cell^{-1}. Net K^+-stimulated $^{45}Ca^{2+}$ uptake was not significantly modified by tacrine (1.27±0.07 to 1.53±0.17 fmol cell^{-1}).

We selected 3 μM as a test concentration because in preliminary experiments some compounds blocked completely the DMPP signal and others did not affect it. In addition, blockade of L and non-L subtype of Ca^{2+} channels activated by cell depolarization were blocked by ω-toxins and organic compounds in this low micromolar range of concentrations, as previously shown.[34]

Effects of tacrine derivatives on DMPP-evoked $^{45}Ca^{2+}$ uptake into chromaffin cells

At the concentration of 3 μM, most of the compounds were much more efficacious blocking DMPP- than K^+-induced $^{45}Ca^{2+}$ entry (Table 1). $^{45}Ca^{2+}$ uptake evoked by DMPP was not affected by compound **1.** Compounds **14**, **15** and **17** inhibited the effects of DMPP by 30%. Compounds **3**, **9**, **16** and tacrine blocked the signal of DMPP about 50%. Compounds **5** and **12** were the most potent blockers of DMPP-stimulated $^{45}Ca^{2+}$ entry (90%) and the rest of the compounds inhibited the effects of DMPP by 70–80%. Table 1 and Figure 2 show the effect of the new compounds and tacrine (**T**) on $^{45}Ca^{2+}$ uptake induced by 100 μM DMPP (□) or 70 mM K^+ (■). The compounds in Figure 2 are classified in order of growing potency for DMPP. Since compounds **8**, **11**, **12** and **13** scarcely blocked the K^+-evoked $^{45}Ca^{2+}$ entry through Ca^{2+} channels, the possibility exists that these compounds are acting directly on the nAChR. Nevertheless, further experiments using patch-clamp techniques for the direct recording of nAChR-associated inward currents are necessary to prove this hypothesis.

Effects of tacrine derivatives on K^+-evoked $^{45}Ca^{2+}$ uptake into chromaffin cells

Compounds **1**, **3**, **4**, **8**, **10**, **11**, **13**, **16**, **17** and tacrine inhibited $^{45}Ca^{2+}$ uptake induced by K^+ about 20%. Compounds **6**, **14** and **15** inhibited the K^+ effects by 10% or less. Compounds **7**, **9**, **12** and **18** blocked the K^+ signal by 30% and, finally, compounds **2** and **5** inhibited K^+-induced $^{45}Ca^{2+}$ entry by 50%. Table 1 and Figure 3 show the effect of all the compounds [18 new compounds plus tacrine (**T**)] on $^{45}Ca^{2+}$ uptake induced by 70 mM K^+ (■) or 100 µM DMPP (□). The compounds in Figure 3 are classified in order of growing potency for K^+. Data about the effect of diltiazem on K^+-evoked $^{45}Ca^{2+}$ uptake are included in Table 1 although it was used mainly as a standard L-type Ca^{2+} channel blocker in the experiments on rat aorta.

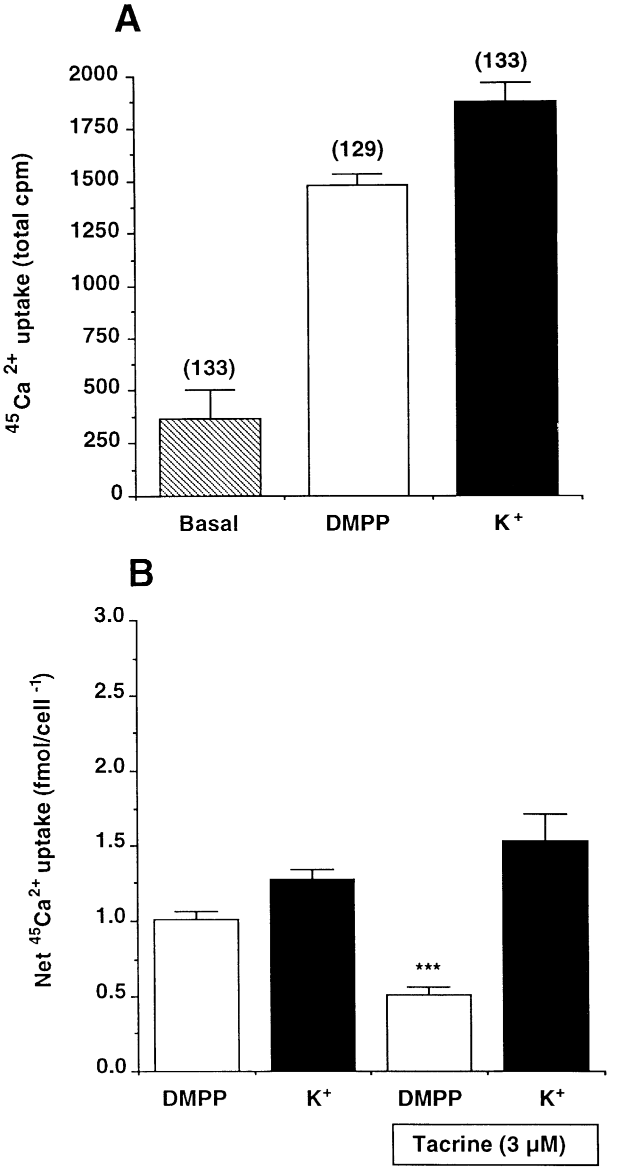


Figure 1. $^{45}Ca^{2+}$ uptake into resting or stimulated chromaffin cells. In resting conditions (Basal □), cells were incubated for 5 s in a normal Krebs-Hepes solution containing 5 µCi mL−1 $^{45}Ca^{2+}$ and 1 mM $^{40}Ca^{2+}$. Stimulated cells were exposed for 5 s to a normal Krebs-Hepes solution containing $^{40}Ca^{2+}$ plus $^{45}Ca^{2+}$ and 1,1-dimethyl-4-phenylpiperazinium (DMPP, 100 µM, □) or high K+ concentration (70 mM K+, ■). The actual cpm of $^{45}Ca^{2+}$ taken up are shown. B. Net $^{45}Ca^{2+}$ uptake (fmol $cell^{-1}$) by chromaffin cells stimulated with DMPP (□) or K+ (■) in the absence or presence of tacrine (3 µM). Data are means ± SEM of the number of individual wells shown in parentheses on top of each column. $***p < 0.001$ with respect to control without tacrine.

Those compounds blocking K^+-induced $^{45}Ca^{2+}$ entry by 30% or more, and compound **1** that does not affect at all DMPP-evoked $^{45}Ca^{2+}$ uptake were selected for a more detailed study and dose–response curves were carried out (Fig. 4). For most of the compounds the maximum effect was reached at the concentration of 10 µM (40–60%). Only compound **5** increased its blocking effect at 30 µM (62%).

Effects of tacrine derivatives on isolated rat aorta precontracted with 35 mM K^+

Bovine chromaffin cells express four different subtypes of voltage-dependent Ca^{2+} channels: L, N, P/Q and R.[22] Pharmacological dissection of those channels, using $^{45}Ca^{2+}$ uptake techniques,[34] indicate that the proportion of the L type is around 20%. It could be possible that those compounds blocking the K^+-induced $^{45}Ca^{2+}$ entry by 20% were also acting on the cardiovascular L-type Ca^{2+} channels. Hence, these compounds (and also the other four compounds producing a higher blockade) were tested on the rat aorta, which was precontracted with 35 mM K^+, a depolarizing stimulus enough to open L-type Ca^{2+} channels, as proven by the fact that dihydropyridines, specific L-type channel blockers, completely block the K^+-induced contraction of this vessel.[35]

Table 1. Effects of tacrine derivatives on $^{45}Ca^{2+}$ uptake induced by 70 mM K+ or by the nicotinic agonist DMPP (100 µM) into bovine chromaffin cells. All drugs were tested at the concentration of 3 µM

Compound	70 mM K^+		100 mM DMPP	
	% Inhibition	*n*	% Inhibition	*n*
1	23.3±5.4	16	0 (+1)±20.1	16
2	47.8±5.0	16	70.3±2.4	16
3	20.4±6.1	16	44.5±3.3	16
4	17.7±3.7	11	70.7±2.1	11
5	49.7±6.2	12	90.2±2.6	12
6	13.4±5.4	11	67.6±1.4	11
7	34.9±3.0	12	77.1±3.2	12
8	24.9±1.7	12	81.1±0.5	12
9	27.8±7.3	21	53.9±11.6	23
10	22.7±9.8	23	67.3±6.1	21
11	22.3±3.1	16	81.5±2.1	12
12	31.3±4.2	16	88.5±2.2	12
13	17.9±3.9	16	85.2±4.8	12
14	3.1±4.4	12	26.5±4.8	12
15	9.6±3.8	12	35.7±2.3	12
16	20.7±9.4	16	46.1±7.5	16
17	21.3±6.3	14	35.0±6.6	14
18	33.8±2.3	12	72.3±1.4	12
Tacrine	17.0±6.7	15	55.3±2.6	14
Diltiazem	36.2±4.9	12	91.6±1.8	12

Data correspond to the mean ± SEM of the number of individual wells shown in *n*, from at least three different batches of cells.

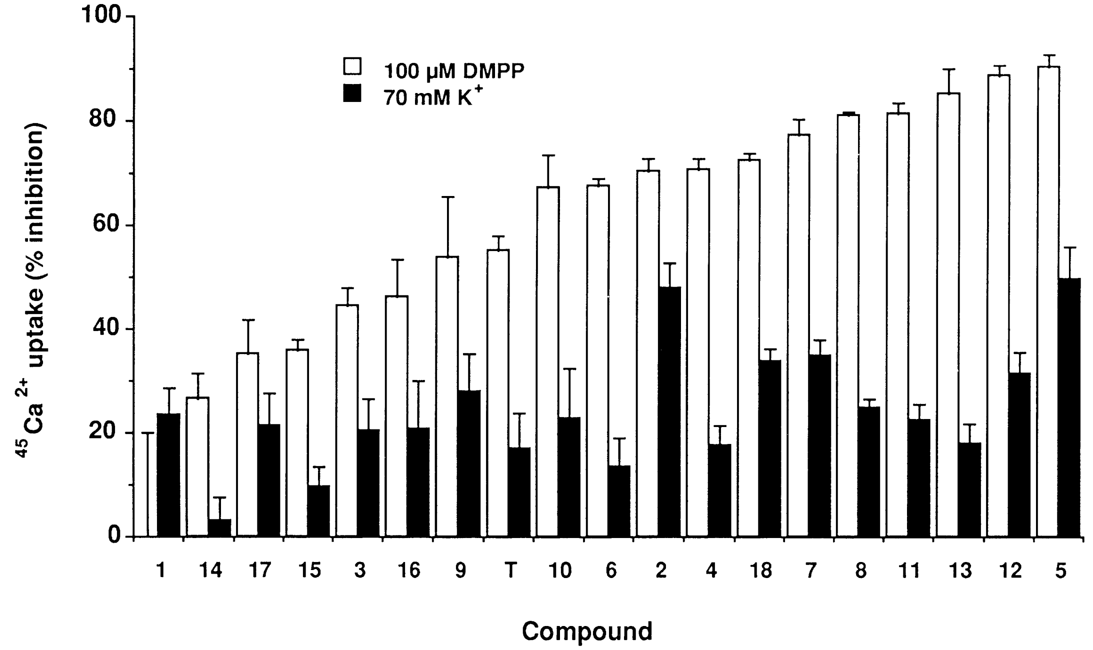


Figure 2. $^{45}Ca^{2+}$ uptake into cultured bovine chromaffin cells. Evoked Ca^{2+} uptake was studied by exposing the cells for 5 s to 100 µM DMPP (□) or 70 mM K+ (■). All compounds were present since 15 min before and during the stimulation period and are arranged in order of growing potency to block DMPP-evoked Ca^{2+} uptake. Data are means ± SEM of individual wells from at least three different experiments in quadruplicate.

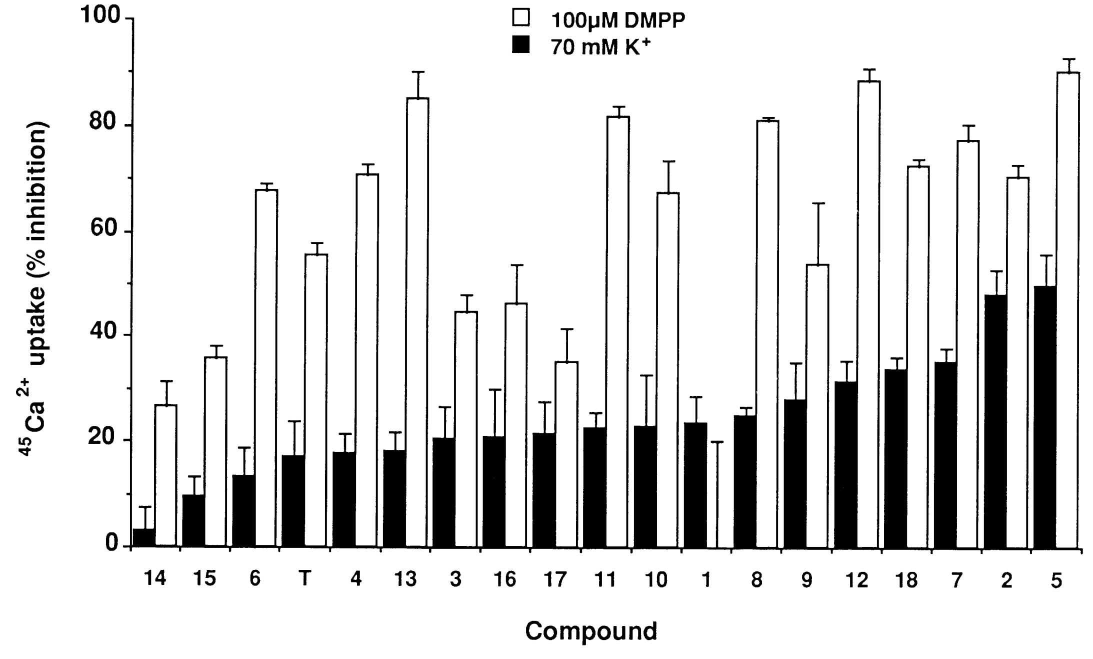


Figure 3. $^{45}Ca^{2+}$ uptake into cultured bovine chromaffin cells. Evoked Ca^{2+} uptake was studied by exposing the cells for 5 s to 70 mM K+ (■) or 100 µM DMPP (□). All compounds were present since 15 min before and during the stimulation period, and are arranged in order of growing potency for the K^+-evoked Ca^{2+} uptake. Data are means ±SEM of at least three different experiments in quadruplicate.

The effect of all the compounds on rat aorta was compared with that of diltiazem, a well-known L-type Ca^{2+} channel blocker widely used in clinic as a cardioprotectant;[36] the results are summarized in Table 2. At the maximum concentration tested (30 µM), compound **16** produced a relaxation of only 5% with respect to a control tissue. Compound **14** relaxed the aorta by 16% and compounds **1**, **2**, **15** and **17** induced a relaxation of about 40–50%. The rest of the compounds and diltiazem produced a relaxation above 50%, enough to calculate the IC_{50} (inhibitory concentration that causes 50% of the maximum effect). None of the new compounds was as effective as diltiazem ($IC_{50} = 0.03$ µM). The most potent was compound **7**, with an IC_{50} of 0.3 µM. Compound **5** has an IC_{50} around 2 µM. Compounds **6**, **8**, **9**, **13** and **18** have IC_{50}'s around 10 µM and compounds **3**, **4**, **11** and **12** around 20 µM.

Discussion

We have shown in this study that, in general, the novel tacrine derivatives were much more efficacious and potent in blocking the DMPP-mediated $^{45}Ca^{2+}$ uptake (and hence nAChRs), than K^+-evoked $^{45}Ca^{2+}$ uptake (and hence voltage-dependent Ca^{2+} channels). The fact that these novel derivatives did not produce full blockade of DMPP-induced $^{45}Ca^{2+}$ entry into the cells suggests that like tacrine,[37] behave as non-competitive inhibitors of nAChR. With these data, it is difficult to establish a structure–activity relationship, but some approaches can be done. For instance, compounds **11**, **12** and **13**, with a *meta*-methoxy substituent at the aromatic ring, behaved as excellent blockers of DMPP-mediated $^{45}Ca^{2+}$ uptake, but they scarcely inhibited the K^+-stimulated $^{45}Ca^{2+}$ uptake. On the other hand, compounds **5**, **8**, **9** and **10**, with a methoxy substituent in other position at the aromatic ring, were good blockers of either DMPP- or K^+-evoked $^{45}Ca^{2+}$ uptake. Changes in lateral chains can also affect the DMPP-evoked $^{45}Ca^{2+}$ entry, as suggested by focusing the structural changes and activity involved in compounds **1**, **7** and **18**. The furan-like tacrine-related compounds **14**, **15**, **16** and **17** were the poorest blockers of the DMPP response.

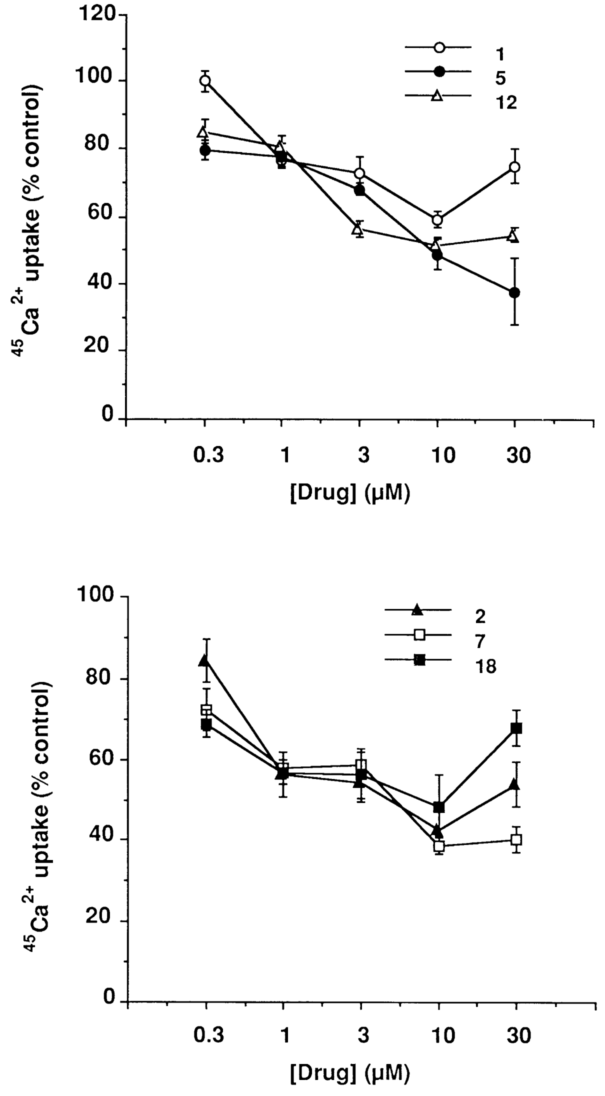


Figure 4. Dose–response curves for K^+-stimulated $^{45}Ca^{2+}$ uptake into cultured bovine chromaffin cells. Evoked Ca^{2+} uptake was studied by exposing the cells for 5 s to 70 mM K^+. The selected compounds were present since 15 min before and during the stimulated period. Data are means ± SEM of at least three different experiments in quadruplicate.

Concerning the effects on K^+-stimulated $^{45}Ca^{2+}$ uptake, we found that compounds blocking this response more than 25% (i.e., compounds **2**, **5**, **9**, **12** and **18**) have a cyclohexane moiety as cycloalkane-fused ring. Furthermore, all of them bear electron-donating groups showing an electronic rich charge at their aromatic rings. On the other hand, compounds **3**, **4** and **6**, having electron-withdrawing groups at their aromatic rings, show a poorer blockade of K^+ responses, about 20% or less.

None of the furan-like tacrine-related compounds blocked more than 20% the K^+ response. In this context, it seems that the blockade of nicotinic receptors depends more on steric changes of the substituents, while voltage-gated Ca^{2+} channel blockade is more affected by changes in the electronic structure of the substituents.

An interesting problem emerges from the data on K^+-evoked $^{45}Ca^{2+}$ uptake. As stated above, only a fraction of 20% of the total $^{45}Ca^{2+}$ uptake taken up by cells is associated to L-type Ca^{2+} channels; the rest is associated to N and P/Q-subtypes of Ca^{2+} channels.[34] This means that compounds **2**, **5**, **7**, **12** and **18**, that block 30–50% $^{45}Ca^{2+}$ uptake, must block at least partially, L-type as well as non-L-type Ca^{2+} channels. Since non-toxin/non-peptide compounds that selectively block N or P/Q channels are not available,[38] tacrine derivatives of the type of compounds **2**, **5**, **7**, **12** and **18** could serve as structural models for further search for selective N- or P/Q-type of Ca^{2+} channels. These channels mediate the release of several neurotransmitters at brain synapses and thus, non-toxin/non-peptide com-

Table 2. Effects of tacrine derivatives on isolated rat aorta precontracted with 35 mM K+

Compound	Maximum relaxation (% control)	*n*	IC_{50} (μM)
1	51±8	4	—
2	48±6	7	—
3	77±8	5	16
4	97±3	5	21
5	95±3	7	1.6
6	92±3	4	9.2
7	98±2	6	0.3
8	98±2	5	8.4
9	94±3	6	7.1
10	90±4	5	3.4
11	98±1	5	18
12	88±5	5	17
13	95±2	5	11
14	16±8	7	—
15	37±8	4	—
16	5±4	4	—
17	41±7	4	—
18	55±9	7	13
Diltiazem	96±2	8	0.03

Data correspond to mean ± SEM of the number of aorta segments shown in *n*.

pounds blocking selectively N or P/Q Ca^{2+} channels might have therapeutic potential for various neurological or psychiatric disorders. Compound **5** might be the prototype of a novel strategy to treat Alzheimer's disease. As stated in the Introduction, the pharmacological inhibition of AChE is the only strategy available to delay the impairment of cognition function in Alzheimer patients.[12,13,15] It is interesting, however, that galantamine, that causes a poor inhibition of AChE (i.e., 10-fold less potent than tacrine),[39] also delay the cognitive impairment of Alzheimer patients.[40] This beneficial action has been attributed to an additional effect, other than enzyme inhibition, that is a direct allosteric action of galantamine on neuronal nAChRs.[41]

A similar, but slightly different concept might be exemplified by compound **5** that, as galantamine, is 10-fold less potent than tacrine as AChE inhibitor.[23] However, as galantamine, this compound has an additional effect, that is blockade of Ca^{2+} entry into chromaffin cells. Cell Ca^{2+} overload is a well characterized signal that leads to neuronal degeneration and death.[19,20] It is, therefore, likely that blockers of L-, N- and P/Q-type of Ca^{2+} channels, by preventing Ca^{2+} overload, favor cell survival and pharmacological neuroprotection. This is the case for the 'wide spectrum' Ca^{2+} channel blocker lubeluzole,[34,42] that offers neuroprotection against cell damage caused by ischemia-reperfusion in in vitro[21] and in vivo[43] models. This could also be the case for compound **5** and similar derivatives. We are presently designing experiments to test this possibility.

It is desirable that if used as neuroprotectant, a drug should be devoid of peripheral cardiovascular effects. Compound **5** had an IC_{50} to relax the rat aorta strips 50-fold higher than diltiazem (Table 2). Other compounds blocking $^{45}Ca^{2+}$ entry had even much less potency than diltiazem, that is compound **18** had an IC_{50} of 13 μM while diltiazem had an IC_{50} of 0.03 μM, 433-fold smaller. Thus, it is unlikely that if used as neuroprotectants in vivo, these compounds display any major hemodynamic effects.

Conclusions

We have synthesized a series of tacrine derivatives that behave as ligands of neuronal nAChRs as well as neuronal voltage-dependant Ca^{2+} channels; though weak, some of these compounds retain the ability to inhibit AChE. We raise the hypothesis that this pharmacological profile might be compatible with a neuroprotectant action, that might be therapeutically useful in neurodegenerative diseases as well as in ischemic-reperfusion neuronal damage (stroke).

Experimental

Chemistry

Reactions were monitored by TLC using precoated silica gel aluminium plates containing a fluorescent indicator (Merck, 5539). Detection was done by UV (254 nm) followed by charring with sulfuric-acetic acid spray, 1% aqueous potassium permanganate solution or 0.5% phosphomolybdic acid in 95% EtOH. Anhydrous Na_2SO_4 was used to dry organic solutions during workups and the removal of solvents was carried out under vacuum with a rotary evaporator. Flash column chromatography was performed using silica gel 60 (230–400 mesh, Merck) and hexane–ethyl acetate mixtures as eluent, unless otherwise stated. ^1H and ^{13}C 1D and 2D NMR spectra were recorded with a Varian VXR-300S and Varian Gemini 200 spectrometer, using tetramethylsilane as internal standard. The synthesis of compounds **1** to **7** has been previously described.[24]

General method for the synthesis of tacrine analogues

Synthesis of compounds 8–18. In a typical experiment, aluminium chloride (1.1 eq) was suspended in dry 1,2-dichloroethane (10 mL/mmol) at room temperature under argon. The corresponding 2-amino-3-cyano-4*H*-pyran (**II**,[27] **V**[27] or **IX**[27]) or 2-amino-3-cyanofuran (**VI**,[29] **VII**[30] or **VIII**[30]) (1 equiv) and the ketone (cyclopentanone, cyclohexanone or cycloheptanone; 1.1 equiv) were added. The reaction mixture was heated under reflux (2–8 h). When the reaction was over (TLC analysis), a mixture of THF/H_2O (2:1) was added dropwise at rt. An aqueous solution of sodium hydroxide (10%) was added dropwise to the mixture until the aqueous solution was basic. After stirring for 30 min, the mixture was extracted twice with ethyl acetate. The organic layer was washed with brine, dried over anhydrous sodium sulfate, filtered and the solvent was evaporated. The solid was purified by silica gel flash chromatography and recrystalized from ethyl acetate–hexane mixtures to give pure compounds **8**–**18**, respectively. These compounds showed good elemental analysis in agreement with their structures.

Ethyl 5-amino-4,6,7,8-tetrahydro-4-(*o*-methoxyphenyl)-2-methylcyclopenta[*e*]pyrano[2,3-*b*]pyridine-3-carboxylate (8). Following the General method, compound **II** (100 mg, 0.31 mmol) [$AlCl_3$ (51 mg, 0.38 mmol), $ClCH_2CH_2Cl$ (3 mL), cyclopentanone (33.8 μL, 0.38 mmol)] afforded product **8** (66 mg, 54%): mp 170–172 °C; IR (KBr) ν 3400, 3310, 3190 (Ar–NH_2 st), 1690 (COOEt), 1630, 1590, 1570, 1460 (Ar C=C, C=N st), 740 cm^{-1} (Ar δ_{oop}); UV–vis (MeOH) λ_{max} (log ε): 274 (4.0), 245 (4.2), 207 (4.5); ^1H NMR ($CDCl_3$, 300 MHz) δ 7.22–6.85 (m, 4H, C_6H_4), 5.32 (s, 1H, H4), 4.72 (s, 2H, NH_2), 4.03 (q, 2H, $J = 7.2$ Hz, $CO_2CH_2CH_3$), 3.97 (s, 3H, CH_3O), 2.81 (m, 2H, H8), 2.55 (m, 2H, H6), 2.53 [s, 3H, C(2)-CH_3], 2.05 (m, 2H, H7), 1.14 (t, 3H, $J = 7.2$ Hz, $CO_2CH_2CH_3$); ^{13}C NMR ($CDCl_3$, 75.4 MHz) δ 166.8 (C=O), 161.2, 161.0 (C2, C9a), 156.4 (C2′), 154.5 (C8a), 148.7 (C5), 133.2 (C1′), 130.2 (C4′), 127.8 (C6′), 122.0 (C5′), 117.2 (C5a), 110.3 (C3′), 106.4 (C3), 100.0 (C4a), 60.0 ($CO_2CH_2CH_3$), 55.7 (C4), 34.1 (C8), 29.9 (OCH_3), 27.0 (C6), 22.2 (C7), 19.5 [C(2)-CH_3], 14.0 ($CO_2CH_2CH_3$). MS (API-ES+) *m*/*z* 381 [$(M+H)^+$, 100]. Anal. calcd for $C_{22}H_{24}N_2O_4$: C, 69.46; H, 6.36; N, 7.36. Found: C, 69.46; H, 6.55; N, 7.40.

Ethyl 5-amino-6,7,8,9-tetrahydro-4-(*o*-methoxyphenyl)-2-methyl-4*H*-pyrano[2,3-*b*] quinoline-3-carboxylate (9). Following the General method, compound **II** (100 mg, 0.31 mmol) [$AlCl_3$ (50.9 mg, 0.38 mmol), $ClCH_2CH_2Cl$ (3 mL), cyclohexanone (32.5 μL, 0.38 mmol)] afforded product **9** (73 mg, 58%): mp 183–184 °C; IR (KBr) ν 3400, 3320 ($Ar–NH_2$ st), 1680 (COOEt), 1640, 1580, 1550, 1470 (Ar C=C, C=N st), 735 cm^{-1} (Ar δ_{oop}); UV–vis (MeOH) λ_{max} (log ε): 273 (3.9), 244 (4.2), 205 (4.5); ^1H NMR ($CDCl_3$, 300 MHz) δ 7.20–6.83 (m, 4H, C_6H_4), 5.30 (s, 1H, H4), 4.75 (s, 2H, NH_2), 4.02 (q, 2H, $J=7.0$ Hz, $CO_2CH_2CH_3$), 3.96 (s, 3H, CH_3O), 2.68 (m, 2H, H9), 2.52 [s, 3H, C(2)–CH_3], 2.20 (m, 2H, H6), 1.75 (m, 4H, H7, H8), 1.12 (t, 3H, $J=7.0$ Hz, $CO_2CH_2CH_3$); ^{13}C NMR ($CDCl_3$, 75.4 MHz) δ 166.9 (C=O), 161.3 (C2), 154.6 (C2′), 154.2 (C10a), 153.0 (C9a), 150.6 (C5), 133.1 (C1′), 130.2 (C4′), 127.8 (C6′), 122.0 (C5′), 112.7 (C5a), 110.3 (C3′), 106.4 (C3), 99.8 (C4a), 59.9 ($CO_2CH_2CH_3$), 55.7 (C4), 32.3 (C9), 29.9 (OCH_3), 22.9, 22.5, 22.4 (C6, C7, C8), 19.6 [C(2)–CH_3], 14.0 ($CO_2CH_2CH_3$). MS (API-ES+) *m/z* 395 [$(M+H)^+$, 100]. Anal. calcd for $C_{23}H_{26}N_2O_4$: C, 70.03; H, 6.64; N, 7.10. Found: C, 69.75; H, 6.97; N, 6.87.

Ethyl 5-amino-4,6,7,8,9,10-hexahydro-4-(*o*-methoxyphenyl)-2-methylcyclohepta[*e*] pyrano[2,3-*b*]pyridine-3-carboxylate (10). Following the general method, compound **II** (100 mg, 0.31 mmol) [$AlCl_3$ (50.9 mg, 0.38 mmol), $ClCH_2CH_2Cl$ (3 mL), cycloheptanone (45.2 μL, 0.38 mmol)] afforded product **10** (86 mg, 66%): mp 142–143 °C; IR (KBr) ν 3400, 3360 ($Ar–NH_2$ st), 1690 (COOEt), 1630, 1550, 1440 (Ar C=C, C=N st), 735 cm^{-1} (Ar δ_{oop}); UV–vis (MeOH) λ_{max} (log ε): 274 (4.1), 242 (4.3), 204 (4.6); ^1H NMR ($CDCl_3$, 300 MHz) δ 7.21–6.83 (m, 4H, C_6H_4), 5.28 (s, 1H, H4), 4.78 (s, 2H, NH_2), 4.03 (q, 2H, $J=7.2$ Hz, $CO_2CH_2CH_3$), 3.95 (s, 3H, CH_3O), 2.83 (m, 2H, H10), 2.52 [s, 3H, C(2)–CH_3], 2.43 (m, 2H, H6), 1.60 (m, 6H, H7, H8, H9), 1.13 (t, 3H, $J=7.2$ Hz, $CO_2CH_2CH_3$); ^{13}C NMR ($CDCl_3$, 75.4 MHz) δ 166.8 (C=O), 161.1 (C2), 159.5 (C2′), 154.7 (C11a), 153.8 (C10a), 149.7 (C5), 133.0 (C1′), 130.4 (C4′), 127.8 (C6′), 121.9 (C5′), 118.0 (C5a), 110.2 (C3′), 106.5 (C3), 100.6 (C4a), 59.9 ($CO_2CH_2CH_3$), 55.6 (C4), 38.4 (C10), 32.1 (C6), 30.2 (OCH_3), 26.7, 26.0, 25.7 (C7, C8, C9), 19.5 [C(2)–CH_3], 14.0 ($CO_2CH_2CH_3$). MS (API-ES+) *m/z* 409 [$(M+H)^+$, 100]. Anal. calcd for $C_{24}H_{28}N_2O_4$: C, 70.57; H, 6.91; N, 6.86. Found: C, 70.35; H, 6.80; N, 7.13.

Ethyl 5-amino-4,6,7,8-tetrahydro-4-(*m*-methoxyphenyl)-2-methylcyclopenta[*e*]pyrano[2, 3-*b*]pyridine-3-carboxylate (11). Following the General method, compound **V** (100 mg, 0.31 mmol) [$AlCl_3$ (51 mg, 0.38 mmol), $ClCH_2CH_2Cl$ (3 mL), cyclopentanone (33.8 μL, 0.38 mmol)] afforded product **11** (74 mg, 76%): mp 146–147 °C; IR (KBr) ν 3400, 3350, 3190 ($Ar–NH_2$ st), 1690 (COOEt), 1630, 1590, 1570, 1460 (Ar C=C, C=N st), 750 cm^{-1} (Ar δ_{oop}); UV–vis (MeOH) λ_{max} (log ε): 276 (4.4), 245 (4.5), 202 (5.2); ^1H NMR ($CDCl_3$, 200 MHz) δ 7.22–6.65 (m, 4H, C_6H_4), 4.84 (s, 1H, H4), 4.12 (q, 2H, $J=7.2$ Hz, $CO_2CH_2CH_3$), 4.02 (s, 2H, NH_2), 3.72 (s, 3H, CH_3O), 2.86 (m, 2H, H8), 2.57 (m, 2H, H6), 2.44 [s, 3H, C(2)–CH_3], 2.07 (m, 2H, H7), 1.25 (t, 3H, $J=7.2$ Hz, $CO_2CH_2CH_3$); ^{13}C NMR ($CDCl_3$, 50.3 MHz) δ 166.8 (C=O), 162.1 (C2), 159.8 (C9a, C3′), 156.4 (C8a), 148.5 (C5), 145.6 (C1′), 129.5 (C5′), 120.8 (C6′), 118.1 (C5a), 114.6 (C4′), 111.9 (C2′), 106.4 (C3), 99.8 (C4a), 60.3 ($CO_2CH_2CH_3$), 55.1 (C4), 38.5 (OCH_3), 34.2 (C8), 27.0 (C6), 22.2 (C7), 19.6 [C(2)–CH_3], 14.2 ($CO_2CH_2CH_3$). MS (API-ES+) *m/z* 381 [$(M+H)^+$, 100]. Anal. calcd. for $C_{22}H_{24}N_2O_4$: C, 69.46; H, 6.36; N, 7.36. Found: C, 69.77; H, 6.35; N, 7.36.

Ethyl 5-amino-6,7,8,9-tetrahydro-4-(*m*-methoxyphenyl)-2-methylpyrano[2,3-*b*] quinoline-3-carboxylate (12). Following the General method, compound **V** (100 mg, 0.31 mmol) [$AlCl_3$ (50.9 mg, 0.38 mmol), $ClCH_2CH_2Cl$ (3 mL), cyclohexanone (32.5 μL, 0.38 mmol)] afforded product **12** (96 mg, 76%): mp 142–143 °C; IR (KBr) ν 3400, 3320, 3210 ($Ar–NH_2$ st), 1680 (COOEt), 1640, 1580, 1550, 1470 (Ar C=C, C=N st), 750 cm^{-1} (Ar δ_{oop}); UV–vis (MeOH) λ_{max} (log ε): 274 (4.0), 242 (4.2), 207 (4.6); ^1H NMR ($CDCl_3$, 200 MHz) δ 7.26–6.69 (m, 4H, C_6H_4), 4.83 (s, 1H, H4), 4.12 (q, 2H, $J=7.1$ Hz, $CO_2CH_2CH_3$), 4.08 (s, 2H, NH_2), 3.74 (s, 3H, CH_3O), 2.74 (m, 2H, H9), 2.44 [s, 3H, C(2)–CH_3], 2.25 (m, 2H, H6), 1.75 (m, 4H, H7, H8), 1.26 (t, 3H, $J=7.1$ Hz, $CO_2CH_2CH_3$); ^{13}C NMR ($CDCl_3$, 50.3 MHz) δ 166.7 (C=O), 159.7, 159.6 (C2, C3′) 154.1 (C10a), 153.8 (C9a), 150.3 (C5), 145.4 (C1′), 129.4 (C5′), 120.8 (C6′), 114.6 (C4′), 113.4 (C5a), 111.8 (C2′), 106.3 (C3), 99.4 (C4a), 60.1 ($CO_2CH_2CH_3$), 55.0 (C4), 38.4 (OCH_3), 32.3 (C9), 22.7, 22.4, 22.2 (C6, C7, C8), 19.5 [C(2)–CH_3], 14.1 ($CO_2CH_2CH_3$). MS (API-ES+) *m/z* 395 [$(M+H)^+$, 100], 381 (2). Anal. calcd for $C_{23}H_{26}N_2O_4$: C, 70.03; H, 6.64; N, 7.10. Found: C, 69.85; H, 6.78; N, 6.90.

Ethyl 5-amino-4,6,7,8,9,10-hexahydro-4-(*m*-methoxyphenyl)-2-methylcyclohepta[*e*] pyrano[2,3-*b*]pyridine-3-carboxylate (13). Following the general method, compound **V** (100 mg, 0.31 mmol) [$AlCl_3$ (50.9 mg, 0.38 mmol), $ClCH_2CH_2Cl$ (3 mL), cycloheptanone (45.2 μL, 0.38 mmol)] afforded product **13** (92 mg, 71%): mp 152–153 °C; IR (KBr) ν 3450, 3340 ($Ar-NH_2$ st), 1695 (COOEt), 1630, 1610, 1550, 1440 (Ar C=C, C=N st), 745 cm^{-1} (Ar δ_{oop}); UV–vis (MeOH) λ_{max} (log ε): 275 (4.0), 210 (4.6), 208 (4.5) 206 (4.5); ^1H NMR ($CDCl_3$, 200 MHz) δ 7.19–6.66 (m, 4H, C_6H_4), 4.79 (s, 1H, H4), 4.15 (m, 4H, NH_2, $CO_2CH_2CH_3$), 3.69 (s, 3H, CH_3O), 2.84 (m, 2H, H10), 2.40 [m, 5H, C(2)–CH_3, H6], 1.60 (m, 6H, H7, H8, H9), 1.23 (t, 3H, $J=7.1$Hz, $CO_2CH_2CH_3$); ^{13}C NMR ($CDCl_3$, 50.3 MHz) δ 166.7 (C=O), 160.3 (C2), 159.7, 159.5 (C3′), (C11a), 153.7 (C10a), 149.4 (C5), 145.3 (C1′), 129.4 (C5′), 120.8 (C6′), 118.0 (C5a), 114.5 (C4′), 111.9 (C2′), 106.4 (C3), 100.2 (C4a), 60.1 ($CO_2CH_2CH_3$), 55.0 (C4), 38.6 (C10), 38.5 (OCH_3), 32.0, 26.7, 26.0, 25.6 (C6, C7, C8, C9), 19.5 [C(2)–CH_3], 14.1 ($CO_2CH_2CH_3$). MS (API-ES+) *m/z* 409 [$(M+H)^+$. Anal. calcd for $C_{24}H_{28}N_2O_4$: C, 70.57; H, 6.91; N, 6.86. Found: C, 70.53; H, 6.77; N, 7.10.

4-Amino-5,6,7,8-tetrahydro-2,3-diphenylfuro[2,3-*b*]quinoline (14). Following the General method, compound **VI** (100 mg, 0.38 mmol) [$AlCl_3$ (56.3 mg, 0.42 mmol), $ClCH_2CH_2Cl$ (2.3 mL), cycloheptanone (44 μL, 0.42

mmol)] afforded product **14** (111 mg, 85%): mp 209–211 °C; IR (KBr) ν 3500, 3400 (Ar–NH_2 st), 1620, 1600, 1450, 1435 (Ar C=C, C=N st), 765, 690 cm^{-1} (Ar δ_{oop}); UV–vis (MeOH) λ_{max} (log ε): 275 (4.0), 210 (4.6), 206 (4.5); ^1H NMR ($CDCl_3$, 200 MHz) δ 7.60–7.15 (m, 10 H, 2 C_6H_5), 4.07 (s, 2H, NH_2), 2.93 (m, 2H, H8), 2.39 (m, 2H, H5), 1.86 (m, 4H, H6, H7); ^{13}C NMR ($CDCl_3$, 50.3 MHz) δ 159.9 (C9a), 153.1 (C8a), 146.3 (C4), 145.9 (C2), 133.7, 130.4, 130.1, 129.3, 128.4, 128.3, 127.7, 126.0 (2 C_6H_5), 115.7 (C4a), 110.4 (C3a), 105.1 (C3), 33.2 (C8), 22.8 (C5, C6, C7). MS (API-ES+) *m*/*z* 341 [$(M+H)^+$, 100]. Anal. calcd for $C_{23}H_{20}N_2O$: C, 81.15; H, 5.92; N, 8.23. Found: C, 80.95; H, 6.08; N, 8.31.

4-Amino-6,7,8,9-tetrahydro-2,3-diphenyl-5*H*-cyclohepta[*e*]-furo[2,3-*b*]pyridine (15). Following the General method, compound **VI** (100 mg, 0.38 mmol) [$AlCl_3$ (56.3 mg, 0.42 mmol), $ClCH_2CH_2Cl$ (3.8 mL), cycloheptanone (50 μL, 0.42 mmol)] afforded product **15** (63.5 mg, 46%): mp 199–201 °C; IR (KBr) ν 3480, 3350 (Ar–NH_2 st), 1640, 1600, 1585, 1470, 1440 (Ar C=C, C=N st), 760, 690 cm^{-1} (Ar δ_{oop}); UV–vis (MeOH) λ_{max} (log ε): 271 (5.9), 200 (5.6); ^1H NMR ($CDCl_3$, 200 MHz) δ 7.54–7.18 (m, 10 H, 2 C_6H_5), 4.09 (s, 2H, NH_2), 3.04 (m, 2H, H9), 2.59 (m, 2H, H5), 1.73 (m, 2H, H8), 1.64 (m, 2H, H7), 1.60 (m, 2H, H6); ^{13}C NMR ($CDCl_3$, 50.3 MHz) δ 159.9 (C10a), 159.3 (C9a), 146.3 (C4), 145.5 (C2), 133.6, 130.4, 130.2, 129.3, 128.4, 128.3, 127.6, 126.1 (2 C_6H_5), 116.1 (C4a), 115.9 (C3a), 105.8 (C3), 39.1 (C9), 33.2 (C5), 27.2 (C8), 26.6 (C7), 25.2 (C6). MS (API-ES+) *m*/*z* 355 [$(M+H)^+$, 100]. Anal. calcd for $C_{24}H_{22}N_2O$: C, 81.33; H, 6.26; N, 7.90. Found: C, 80.14; H, 5.90; N, 7.75.

4-Amino-5,6,7,8-Tetrahydro-2,3-di(*p*-tolyl)furo[2,3-*b*]quinoline (16). Following the General method, compound **VII** (200 mg, 0.69 mmol) [$AlCl_3$ (101.7 mg, 0.76 mmol), $ClCH_2CH_2Cl$ (4.2 mL), cyclohexanone (79 μL, 0.76 mmol)] afforded product **16** (170 mg, 66%): mp 235–238 °C; IR (KBr) ν 3460, 3400, 3320 (Ar–NH_2 st), 1630, 1600, 1590, 1520, 1500, 1460 (Ar C=C, C=N st), 815 cm^{-1} (Ar δ_{oop}); UV–vis (MeOH) λ_{max} (log ε): 321 (4.2), 205 (4.5); ^1H NMR ($CDCl_3$, 200 MHz) δ 7.44–7.02 (m, 8H, 2 C_6H_4), 4.09 (s, 2H, NH_2), 2.92 (m, 2H, H8), 2.44 (s, 3H, CH_3), 2.38 (m, 2H, H5), 2.29 (s, 3H, CH_3), 1.85 (m, 4H, H6, H7); ^{13}C NMR ($CDCl_3$, 50.3 MHz) δ 159.8 (C9a), 152.6 (C8a), 151.8 (C4), 146.2 (C2), 138.0, 137.5, 130.6, 130.0, 129.9, 129.0, 127.7, 125.9 (2 C_6H_4), 114.8 (C4a), 110.3 (C3a), 105.2 (C3), 33.2 (C8), 22.9, 22.8 (C5, C6, C7), 21.3, 21.2 (2 CH_3). MS (API-ES+) *m*/*z* 369 [$(M+H)^+$, 100]. Anal. calcd for $C_{25}H_{24}N_2O$: C, 81.49; H, 6.56; N, 7.60. Found: C, 81.15; H, 6.58; N, 7.53.

4-Amino-5,6,7,8-tetrahydro-2,3-di(*p*-methoxyphenyl)-furo[2,3-*b*]quinoline (17). Following the General method, compound **VIII** (140 mg, 0.43 mmol) [$AlCl_3$ (64 mg, 0.48 mmol), $ClCH_2CH_2Cl$ (2.6 mL), cyclohexanone (50 μL, 0.48 mmol)] afforded product **17** (16 mg, 9%): mp 176–179 °C; IR (KBr) ν 3460, 3410 (Ar–NH_2 st), 1620, 1590, 1520, 1500, 1460 (Ar C=C, C=N st), 830 cm^{-1} (Ar δ_{oop}); UV–vis (MeOH) λ_{max} (log ε): 275 (4.0), 210 (4.6), 206 (4.5); ^1H NMR ($CDCl_3$, 200 MHz) δ 7.50–7.36 [AA′-BB′ system, 4H, C(2)-C_6H_5], 7.17–6.75 [AA′–BB′ system, 4H, C(3)–C_6H_5], 4.06 (s, 2H, NH_2), 3.88 (s, 3H, OCH_3), 3.77 (s, 3H, OCH_3), 2.92 (m, 2H, H8), 2.39 (m, 2H, H5), 1.86 (m, 4H, H6, H7); ^{13}C NMR ($CDCl_3$, 50.3 MHz) δ 159.7 (C9a), 159.5 (C8a), 159.1 (C4), 152.4 (C2), 146.2, 146.1, 139.3, 127.4, 125.6, 123.3, 114.7, 113.8 (2 C_6H_4), 113.5 (C4a), 110.4 (C3a), 105.4 (C3), 55.3, 55.2 (2 OCH_3), 33.1 (C8), 22.9, 22.8 (C5, C6, C7). MS (API-ES+) *m*/*z* 401 [$(M+H)^+$, 100]. Anal. calcd for $C_{25}H_{24}N_2O_3$: C, 74.98; H, 6.04; N, 6.99. Found: C, 74.63; H, 6.05; N, 6.73.

Isopropyl 5-amino-6,7,8,9-tetrahydro-2-methyl-4-phenyl-4*H*-pyrano[2,3-*b*]quinoline-3-carboxylate (18). Following the General method compound **IX** (100 mg, 0.34 mmol) [$AlCl_3$ (54 mg, 0.40 mmol), $ClCH_2CH_2Cl$ (4 mL), cyclohexanone (59 μL, 0.57 mmol)] afforded product **18** (78.3 mg, 62%): mp 197–200 °C; IR (KBr) ν 3450, 3360, 3260 (Ar–NH_2 st), 1690 (COOEt), 1640, 1600, 1570, 1450 (Ar C=C, C=N st); UV–vis (MeOH) λ_{max} (log ε): 271 (4.0), 241 (4.2), 206 (4.5); ^1H NMR ($CDCl_3$, 300 MHz) δ 7.34–7.16 (m, 5H, C_6H_5), 5.00 [h, 2H, $J = 6.2$ Hz, $CO_2CH(CH_3)_2$], 4.83 (s, 1H, H4), 4.05 (s, 2H, NH_2), 2.75 (m, 2H, H9), 2.46 [s, 3H, C(2)–CH_3], 2.26 (m, 2H, H6), 1.80 (m, 4H, H7, H8), 1.20 [2 d, 6H, $J = 6.2$ Hz, $CO_2CH(CH_3)_2$]; ^{13}C NMR ($CDCl_3$, 75.4 MHz) δ 166.3 (C=O), 159.4 (C2), 154.2 (C10a), 153.9 (C9a), 150.3 (C5), 143.9 (C1′), 128.6 (C5′), 128.5 (C6′), 127.0 (C4′), 113.4 (C5a),(C2′), 106.8 (C3), 99.7 (C4a), 67.8 [$CO_2CH(CH_3)_2$], 38.6 (C4), 32.4 (C9), 22.8, 22.5, 22.3 (C6, C7, C8), 22.0, 21.7 [$CO_2CH(CH_3)_2$], 19.6 [C(2)–CH_3]; MS (APCI+) *m*/*z* 379 [$(M+H)^+$, 100], 337 (3), 256 (2). Anal. calcd for $C_{23}H_{26}N_2O_3$: C, 72.98; H, 6.93; N, 7.41. Found: C, 73.43; H, 6.24; N, 7.08.

Biological Methods

Cell isolation and culture of bovine chromaffin cells

Bovine adrenomedullary chromaffin cells were isolated following standard methods[44] with some modifications.[45] Cells were suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum, 10 μM cytosine arabinoside, 10 μM fluorodeoxyuridine, 50 IU mL^{-1} penicillin and 50 μg mL^{-1} streptomycin. Cells were plated at a density of 2×10^5 cells/well in 96-multiwell Costar plates and were used 1–5 days after plating. Medium was replaced after 24 h and then after 2–3 days.

Measurements of $^{45}Ca^{2+}$ uptake

$^{45}Ca^{2+}$ uptake studies were carried out in cells after 2–3 days in culture. Before the experiment, cells were washed twice with 0.1 mL Krebs-HEPES solution of the following composition (in mM): NaCl 140, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 1, glucose 11, HEPES 10, at pH 7.2 and 37 °C. $^{45}Ca^{2+}$ uptake in chromaffin cells was studied by incubating the cells at 37 °C with $^{45}CaCl_2$ at a final concentration of 5 μCi mL^{-1} in Krebs-HEPES (basal uptake), high K^+ concentration solution (Krebs-HEPES containing 70 mM KCl with isosmotic reduction of NaCl) or 100 μM dimethylphenylpiperazinium (DMPP) in Krebs-HEPES. This incubation was carried out during 5 s in the absence (control) or in the presence of the drugs; at the end of the incubation period the test medium was rapidly aspirated and the uptake reaction was ended by adding 0.1 mL of a cold Ca^{2+}-free Krebs-HEPES containing 10 mM $LaCl_3$. Finally, cells were washed five times more with 0.1 mL of Ca^{2+}-free Krebs-HEPES containing 10 mM $LaCl_3$ and 2 mM EGTA, at 15 s intervals.

To measure radioactivity retained by chromaffin cells, the cells were scraped with a plastic pipette tip while adding 0.1 mL of 10% trichloroacetic acid. 3.5 mL of scintillation fluid (Optiphase Hisafe II, EGG Instruments) was added and the samples counted in a Packard beta counter. Results are expressed as cpm 2×10^5 cells, as fmol single $cell^{-1}$, or as % of Ca^{2+} taken up by control cells.

Experiments with rat thoracic aorta

Male Sprague–Dawley rats weighting 225–250 g were used in all experiments. The animals were killed by asphyxiation. The thoracic aorta as close as possible to the heart was quickly removed and placed in a Petri dish containing Krebs bicarbonate solution (NaCl 119 mM, KCl 4.7 mM, $MgSO_4$ 1.2 mM, KH_2PO_4 1.2 mM, $CaCl_2$ 1.5 mM, $NaHCO_3$ 24.9 mM, glucose 10.9 mM), and the excess fat and connective tissue were removed. The aorta was cut spirally into a strip approximately 2–3 mm wide. In each experiment segments 1.5–2 cm long were used; the strips were kept in Krebs solution gassed with 95% oxygen and 5% CO_2 throughout the experiment.

The segments of aorta were mounted in an organ bath (muscle chamber with a capacity of 40 mL, kept at 37 °C) so that one end was fixed to an isometric transducer connected with an amplifier and recorder. In all the experiments the muscles were loaded with 1 g tension. Results are expressed as maximum relaxation attained or IC_{50} value.

Statistical analysis of the results

Data are expressed as means ± SEM. IC_{50}'s for each drug were estimated through non-linear regression analysis using ISI software for a PC computer. Differences between non-paired groups were compared by Student's *t*-test with the statistical program Statworks TM; a value of p equal or smaller than 0.05 was taken as the limit of statistical significance.

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