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Dual-Acting Drugs: an in vitro Study of Nonimidazole Histamine H_3 Receptor Antagonists Combining Anticholinesterase Activity

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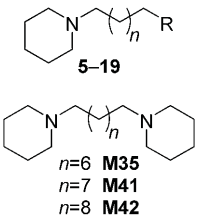
Dual-acting compounds that combine H_3 antagonism with anticholinesterase properties are currently emerging as a novel and promising therapeutic approach in the treatment of multifactorial disorders primarily characterized by cholinergic deficits such as Alzheimer's disease. A series of novel nonimidazole H_3 ligands was developed from the chemical manipulation of 1,1′-octa-, -nona-, and -decamethylene-bis-piperidines—H_3 antagonists that had been the subject of previous investigations. These compounds were evaluated for in vitro binding affinity, antagonistic potency, and selectivity at rodent and human histamine H_3 receptors, inhibitory activity at rat brain cholinesterase, and in vivo CNS access and cholinomimetic effects. Within the present series, the tetrahydroaminoacridine hybrid **18** stands out as one of the most attractive molecules, synergistically combining nanomolar and selective H_3 antagonism with remarkable anticholinesterase activity. From this original starting point, it is hoped that future investigations will lead to dual-acting compounds that can selectively enhance central cholinergic neurotransmission and thus facilitate the treatment of cognitive disorders.

Introduction

The histamine H_3 receptor (H_3R) is a G_i-coupled presynaptic auto- and heteroreceptor that negatively modulates the synthesis and release of histamine and several other neurotransmitters in various brain areas such as the cerebral cortex, hippocampus, basal ganglia, and striatum.[1] It is well known that these regions play a key role in learning and memory processes, in the regulation of the sleep–wake cycle, and in homeostatic functions such as food and water intake. Therefore, H_3 receptor antagonists have long been suggested as potentially effective therapeutics for various central nervous system (CNS) pathologies characterized by neurotransmitter deficits, such as cognitive disorders in Alzheimer's disease and attention-deficit hyperactivity disorder (ADHD), and for the regulation of body weight in obesity.[2] Although generally endowed with good in vitro potency, first-generation imidazole-based H_3 antagonists exhibited a poor pharmacokinetic profile: their inhibitory action on cytochrome P450 activity[3] and their low brain penetration,[4] both of which are due to the presence of the imidazole ring, accounted for their limited in vivo applicability. As a consequence, in the last decades the pharmaceutical industry and academia have undertaken several efforts to synthesize new nonimidazole H_3 antagonists: the most promising compounds, such as GSK189254 and BF2.649, are now being tested at various phases of clinical trials (http://www.clinicaltrials.gov). However, the most recent and attractive approach in the field of the treatment of CNS disorders lies in the development of multitarget molecules that combine H_3 antagonism with inhibition of histamine Nτ-methyltransferase (HTM),[5,6] acetylcholinesterase (AChE),[7] HTM/AChE,[8] M2 antagonism,[9] NO-releasing properties,[10,11] or neuroleptic activity.[12] In particular, the synergic interaction between central H_3 blockade and cholinesterase inhibition could ameliorate cognitive performances in vivo, by improving cholinergic neurotransmission without the undesired peripheral effects typical of anticholinesterase agents. Unfortunately, only in vitro data are available concerning these molecules while no information on their in vivo activity has been provided to date.

Herein we report the preliminary pharmacological investigation of a series of nonimidazole H_3 ligands developed from the flexible structures of 1,1′-octa-, -nona-, and -decamethylene-bis-piperidine derivatives (compounds **M35**, **M41**, and **M42**), already proven to possess moderate affinity and potency toward rodent H_3Rs and good antagonistic properties at human H_3Rs.[13,14] The general formula of the new compounds **5**–**19** and of the previously studied bis-piperidine derivatives are shown.



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The chemical manipulation of one of the terminal piperidine rings and its substitution with secondary or tertiary amines of different steric hindrance and basicity or with the tetrahydroaminoacridine nucleus of the AChE inhibitor (AChEI) tacrine led to the synthesis of a novel class of compounds. They were evaluated for their binding affinity and antagonistic potency at rodent and human H_3Rs and their possible interactions with guinea-pig H_1 and H_2 and human H_4 histamine receptor subtypes. Furthermore, as the molecular structure of these compounds either contains the scaffold of tacrine or shows clear similarities with that of dibasic cyclic amines endowed with anticholinesterase action,[15] their ability to inhibit rat brain cholinesterase activity in vitro was assessed as well. Finally, in vivo experiments were performed to test the CNS access of the most promising compound **18** and to assess the occurrence of peripheral/central cholinomimetic effects. The results obtained for the newly synthesized derivatives, compared with those previously collected for prototypical compounds **M35**, **M41**, and **M42**, will allow a deeper insight into the optimal structural requirements for potent and selective AChEI–H_3 antagonists as dual-acting molecules.

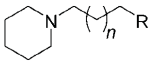
Results and Discussion

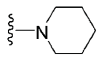
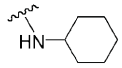
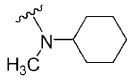
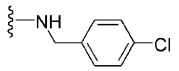
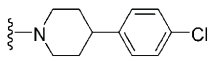
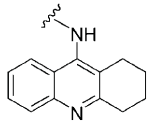
In vitro pharmacology

The binding and functional profile of the novel compounds toward human and rodent H_3 receptors, the data concerning their possible interactions with the other histamine receptor subtypes, and their inhibitory activity against rat brain cholinesterase are reported in Table 1. The new results are compared with those of **M35**, **M41**, and **M42**.[14]

As indicated in Table 1, all the derivatives tested were able to displace the radiolabeled ligand [^{3}H](*R*)-α-methylhistamine from H_3Rs in the rat cerebral cortex membrane assay with similar affinity to **M35**, **M41**, and **M42**; only when considering the decamethylene analogues (compounds **7**, **10**, **13**, **16**), the pK_i values estimated were definitely lower. This negative trend is especially true regarding the cyclohexylamine derivatives **5**–**7**, for which the progressive lengthening of the flexible chain from eight to ten methylene units causes a decrease of 1.5 log units of rat H_3Rs pK_i values (Table 1). Regarding human H_3Rs expressed in cultured SK-N-MC cells, a substantial loss of affinity is common for all the compounds with respect to the bis-piperidine reference molecules, with the exception of tacrine de-

Table 1. Affinity and antagonistic potency of the compounds under study at various histamine receptors[a] and rat brain cholinesterase inhibitory potency.



			Histamine receptors							Cholinesterase	
Compd	*n*	R	rH_3 pK_i[b]	hH_3 pK_i[c]	hH_3 pK_B[d]	gpH_3 pK_B[e]	gpH_1 pK_B[f]	gpH_2 pK_B[g]	hH_4 pK_i[h]	pIC_{50}	I_{max} [%][i]
M35[13,14]	6		7.31 ± 0.01	8.56 ± 0.06	8.28 ± 0.07	8.01 ± 0.06	#	#	#	#	#
M41[13,14]	7		7.80 ± 0.09	8.35 ± 0.09	8.22 ± 0.09	7.65 ± 0.12	#	#	#	5.19 ± 0.05	81 ± 5
M42[13,14]	8		7.53 ± 0.08	8.40 ± 0.07	7.69 ± 0.06	8.12 ± 0.20	#	#	#	5.68 ± 0.17	91 ± 2
5	6		7.79 ± 0.04	7.90 ± 0.02	8.18 ± 0.17	8.65 ± 0.15	#	#	#	#	#
6	7		7.18 ± 0.09	7.18 ± 0.08	7.64 ± 0.08	7.67 ± 0.12	#	#	#	5.03 ± 0.13	85 ± 1
7	8		6.28 ± 0.17	7.00 ± 0.03	7.52 ± 0.09	6.76 ± 0.12	#	§	#	5.46 ± 0.05	93 ± 3
8	6		7.45 ± 0.07	7.53 ± 0.04	7.94 ± 0.07	7.99 ± 0.11	#	§	#	#	#
9	7		7.31 ± 0.04	7.58 ± 0.04	7.47 ± 0.10	7.57 ± 0.21	#	#	#	5.8 ± 0.13	96 ± 4
10	8		7.03 ± 0.05	7.89 ± 0.08	7.77 ± 0.05	6.91 ± 0.24	5.38 ± 0.03	#	#	5.93 ± 0.08	96 ± 1
11	6		7.44 ± 0.09	7.61 ± 0.10	8.07 ± 0.12	7.49 ± 0.17	#	5.26	#	#	#
12	7		7.51 ± 0.10	7.68 ± 0.09	7.72 ± 0.11	7.82 ± 0.17	#	#	#	5.05 ± 0.08	90
13	8		7.15 ± 0.03	8.05 ± 0.12	7.39 ± 0.13	7.21 ± 0.11	4.75 ± 0.05[j]	§	#	5.31 ± 0.02	93 ± 1
14	6		7.56 ± 0.15	7.66 ± 0.12	8.20 ± 0.15	7.86 ± 0.07	5.90 ± 0.05	§	#	5.21 ± 0.03	90 ± 1
15	7		7.74 ± 0.06	7.89 ± 0.07	7.86 ± 0.10	7.36 ± 0.08	6.30 ± 0.10	#	#	5.80	95
16	8		7.04 ± 0.07	7.73 ± 0.10	7.89 ± 0.14	7.27 ± 0.06	4.85 ± 0.22[j]	§	#	5.69 ± 0.11	93 ± 1
17	6		7.54 ± 0.10	7.94 ± 0.06	8.23 ± 0.11	ND	ND	§	#	7.88 ± 0.06	94 ± 3
18	7		7.94 ± 0.03	8.73 ± 0.06	8.93 ± 0.18	ND	ND	§	#	7.57 ± 0.04	95 ± 2
19	8		7.81 ± 0.08	8.66 ± 0.01	8.67 ± 0.21	ND	ND	§	#	7.69 ± 0.05	95 ± 1

[a] Histamine H_3 receptors: rat (rH_3), human (hH_3), guinea pig (gpH_3); guinea pig histamine H_1 (gpH_1) and H_2 (gpH_2) receptors; human histamine H_4 receptors (hH_4). [b] Inhibition of [^{3}H]RAMH binding to rat brain membranes. [c] Inhibition of [^{3}H]RAMH binding to SK-N-MC cells stably expressing the human histamine H_3 receptor. [d] Antagonist potency at human histamine H_3 receptors expressed in SK-N-MC cells. [e] Antagonist potency at H_3 receptors expressed in guinea pig ileum. [f] Antagonist potency at H_1 receptors expressed in guinea pig ileum. [g] Antagonist potency at H_2 receptors expressed in guinea pig atrium. [h] Inhibition of [^{3}H]histamine binding to SK-N-MC cells stably expressing the human histamine H_4 receptor. [i] Maximum percent inhibition of rat brain cholinesterase. [j] pD_2' value. #: Inactive up to 10 μM; §: inactive up to 1 μM; ND: not determined.

rivatives **18** and **19**, which displayed the highest pK_i values in the entire series (Table 1). These data suggest that the chemical manipulation of one of the terminal heterocyclic rings of the bis-piperidine derivatives does not lead, in general, to a significant change in the binding affinity toward rat H_3Rs as long as the flexible bridge contains eight or nine methylene groups. On the other hand, independent of the chain length, the replacement of a piperidine ring with a non-tacrine moiety negatively affects the affinity to human H_3Rs. Therefore, we can speculate that the length of the flexible linker is a critical feature to drive the species preference of this series of dibasic asymmetrical H_3 antagonists.

The results of the functional studies confirm the affinity data: the compounds antagonized (*R*)-α-methylhistamine effects showing surmountable and concentration-dependent antagonism at both human and guinea pig H_3Rs, although with variable potency (Table 1). In detail, the progressive separation of the two basic centers produced a gradual loss of antagonistic potency, analogous to that observed for rat H_3 receptor affinity and especially evident for cyclohexylamine compounds toward guinea pig H_3Rs. Among these derivatives it is worth noting that the passage from the octamethylene analogue **5**, exhibiting the highest pK_B value in the entire series, to the least potent decamethylene **7** brings about a decrease in H_3 antagonistic potency of nearly two log units (Table 1). As regards the tacrine derivatives, the longest molecules **18** and **19** combined the highest affinity with the most potent antagonism toward human H_3 receptors within all the compounds assayed. Their blocking potency on guinea pig H_3 receptors could not be determined because of their ability to enhance basal cholinergic tone and to interfere with electrically induced cholinergic contractions of the guinea pig ileum, probably because of their marked anticholinesterase activity. In fact, as expected, the molecules containing the tetrahydroaminoacridine scaffold displayed the most potent anticholinesterase activity in the entire series, exhibiting pIC_{50} values ranging from 7.57 to 7.88, even higher than that shown by the simple molecule of tacrine on rat brain cholinesterase (p$IC_{50} = 6.54 \pm 0.01$; maximum percent inhibition: 95 ± 2).

It is also interesting to note that, among the remaining compounds, whereas the octamethylene derivatives displayed no or only weak inhibitory activity on brain cholinesterase up to a concentration of 10 μM, the elongation of the flexible core and the presence of a second tertiary nitrogen atom proportionally increased the anticholinesterase activity (Table 1). These results are consistent with our previous findings on the pivotal role played by the length of a rigid linker connecting two basic centers in the anticholinesterase potency of the compound.[15] In particular, as nona- and decamethylene derivatives **M41**, **M42**, **6**–**7**, and **9**–**10** displayed similar activity to their already described corresponding biphenyl analogues,[15] we can speculate that the flexible molecules block the enzyme in their fully extended conformation.

As regards the interactions with the other histamine receptor subtypes, the newly synthesized compounds generally exhibited a good selectivity profile. Weak effects and only at micromolar concentrations were shown on histamine H_1-mediated ileal contractions by the longest *N*-methylcyclohexylamine compound **10**, that is, behaving like its rigid biphenyl analogue,[15] and by *p*-chlorophenyl derivatives **14** and **15**, whereas the homologue **11** slightly affected dimaprit H_2-induced positive chronotropic responses of spontaneously beating atria (Table 1). As concerns tacrine derivatives, the enhancement of the cholinergic tone, due to their potent anticholinesterase activity, prevented the functional evaluation of their selectivity profile on guinea pig tissues at concentrations higher than 1 μM. Moreover, none of the compounds tested displayed any cytotoxic activity in the MTT test on SK-N-MC cell culture up to 10 μM (data not shown).

In vivo pharmacology

On the basis of its remarkable in vitro H_3 antagonistic potency and selectivity, and of its anticholinesterase activity, compound **18** was administered in vivo to rats to evaluate its CNS access and side effects profile. As reported in Table 2, when administered intraperitoneally (i.p.) from 5 to 20 mg kg^{-1}, compound **18** showed a very low displacement of the H_3-specific ligand [^{3}H]RAMH from rat cerebral cortex 60 min later, suggesting limited access to the CNS compared with the parent molecule **M41**.[14] However, it dose-dependently induced a pronounced hypothermia ($\Delta T = 1.7 \pm 0.33$ °C at 20 mg kg^{-1} i.p. $^{**}P < 0.01$ vs. vehicle-treated rats) (Figure 1). At the highest dose, tremor was detected in six of seven treated animals, whereas salivation was present in only one animal (Table 2). As regards tacrine, at the dose of 10 mg kg^{-1} i.p., roughly equimolar to 20 mg kg^{-1} of compound **18**, it lowered the body temperature by 1.26 ± 0.25 °C after 60 min ($^{*}P < 0.05$ vs. vehicle-treated rats) (Figure 1), producing a clear tremorogenic effect in all treated rats and sialogogic and chewing effects in three of seven animals (Table 2). Taken together, the data collected herein seem to indicate that, despite the apparently disappointing results of the ex vivo H_3 binding assay, the blood–brain barrier is not impermeable to the tacrine derivative. Indeed, both hypothermia and especially tremor are described as central muscarinic effects,[16] also overtly displayed by the CNS-penetrating anticholinesterase reference compound in accordance with previous findings.[17] It is therefore appealing to hypothesize that, al-

Table 2. Ex vivo H_3 receptor binding, tremor, salivation, and chewing following i.p. administration of compound **18** and tacrine.

Compd	Dose [mg kg^{-1}]	Ex vivo binding [%][a]	Tremor[b]	Salivation[b]	Chewing[b]
18	5	95 ± 19	0/2	0/2	0/2
18	10	85 ± 7	0/2	0/2	0/2
18	20	86 ± 10	6/7	1/7	0/7
Tacrine	10	ND	7/7	3/7	3/7

[a] Percentage of [^{3}H]RAMH-specific binding in rat cerebral cortex 60 min after i.p. administration of compound **18**; ND: not determined. [b] Cholinergic side effects registered 60 min after i.p. administration of compound **18** and tacrine and expressed as a fraction of total animals treated that showed the symptoms.

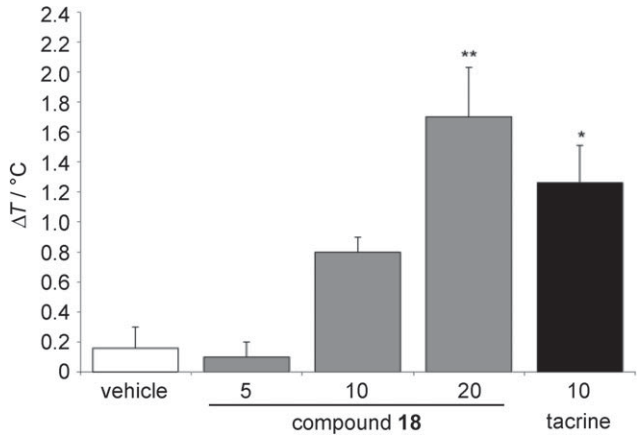


Figure 1. Hypothermic effect induced by vehicle (white column), compound **18** (gray columns), and tacrine (black column), 60 min after intraperitoneal administration (compound concentrations given in mg kg^{-1}); *$P < 0.05$ and **$P < 0.01$ versus vehicle (one-way ANOVA followed by Dunnett's post-test).

though limited, the degree of central H_3 receptor occupancy by compound **18** may be adequate to potentiate its anticholinesterase action in the CNS without evoking unwanted peripheral cholinergic side effects such as sialorrhea. Such a favorable pharmacological profile could be achieved by a molecule that, dually acting as a H_3 blocker/AChEI, selectively boosts CNS cholinergic neurotransmission by blunting the tonic inhibition exerted by histamine, through H_3 receptors, primarily on central ACh release.[18]

Conclusions

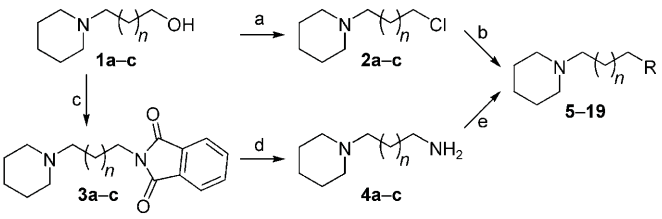
The results collected in the present study widen the conclusions drawn by our previous investigations and allowed further exploration of the steric requirements for an optimal interaction with and inhibition of two distinct molecular targets: the histamine H_3 receptor and cholinesterase enzyme. Furthermore, the original in vivo data reveal that of the novel derivatives, the hybrid molecule **18** was able to combine a potent H_3 antagonism with a remarkable anticholinesterase activity. Thus **18** may represent a promising starting point toward dual-acting compounds specifically enhancing central cholinergic neurotransmission. Future studies in preclinical animal models of learning and/or memory deficits will help to better elucidate its potential impact on the therapy of cognitive disorders.

Experimental Section

Chemistry

The synthesis of the studied compounds was accomplished as shown in Scheme 1. The target compounds **5**–**16** were prepared from ω-hydroxyalkylpiperidine derivatives **a**–**c**, previously synthesized in our laboratory,[5,13] by treatment with $SOCl_2$ followed by reaction with the appropriate amine.

For the synthesis of **17**–**19** a different route was used. The starting products **1a**–**c** were treated with phthalimide followed by reduction with $NH_2NH_2{\cdot}H_2O$ of **3a**–**c** to afford the intermediates **4a**–**c**. The amines **4a**–**c** were then condensed with 9-chloro-1,2,3,4-tetrahydroacridine to give the final compounds.



Scheme 1. Reagents and conditions: a) $SOCl_2$, reflux, 4 h; b) primary or secondary amine, DMF, K_2CO_3, KI, 80 °C, 8 h; c) phthalimide, PPh_3, DIAD, THF, 0 °C, 1 h, then room temperature, overnight; d) $NH_2NH_2{\cdot}H_2O$, reflux, 1 h; e) 9-chloro-1,2,3,4-tetrahydroacridine, pentanol, DIPEA, reflux, 3 days.

General methods

Melting points were not corrected and were determined with a Gallenkamp melting point apparatus. The final compounds were analyzed on a ThermoQuest Flash EA 1112 elemental analyzer for C, H, and N. The percentages we found were within ±0.4% of the theoretical values. The ^{1}H NMR spectra were recorded on a Bruker 300 spectrometer (300 MHz); chemical shifts (δ) are reported in parts per million (ppm). ^{1}H NMR spectra are reported in the following order: multiplicity, approximate coupling constants (J value) in hertz (Hz) and number of protons; signals were characterized as s (singlet), d (doublet), dd (doublet of doublets), t (triplet), m (multiplet), br s (broad signal). Mass spectra were recorded using an API-150 EX instrument with APCI interface (Applied Biosystems, MDS Sciex, Toronto, Canada). Reactions were monitored by TLC on Kieselgel 60 F_{254} (DC-Alufolien, Merck). Final compounds and intermediates were purified by preparative flash chromatography on Büchi Sepacore®, using SiO_2 column (Prepacked Cartridges, SiO_2 60, 40–63 μm, Büchi); the eluents were mixtures of CH_2Cl_2/CH_3OH at various volume ratios. When indicated, gaseous NH_3 was added to the methanolic phase to obtain a 5% *w/w* solution. Abbreviations are the following: THF: tetrahydrofuran, DMSO: dimethyl sulfoxide, DMF: *N*,*N*-dimethylformamide, DIAD: diisopropylazodicarboxylate, DIPEA: *N*,*N*-diisopropylethylamine.

General procedure for the synthesis of piperidinoalkyl derivatives (5–16): We synthesized the derivatives **1a**–**c** and **2a**–**c** using the condition described in the literature[5] by condensation of the appropriate commercially available α,ω-hydroxyhalogenoalkane with piperidine followed by treatment with $SOCl_2$.

A stirred suspension of ω-chloroalkyl intermediate **2a**–**c** (1 mmol), the appropriate amine (1 mmol), K_2CO_3 (0.138 g, 1 mmol), and KI (33.2 mg, 0.2 mmol) in DMF (4 mL) was heated at 80 °C for 8 h. The solvent was removed in vacuo to give the crude products, which were purified by flash chromatography [SiO_2, CH_2Cl_2/CH_3OH (NH_3) 9:1].

***N*-(8-(Piperidin-1-yl)octyl)cyclohexylamine (5·2 HCl·0.5 H_2O).** White solid (244 mg, 65% yield); mp: 245–247 °C (EtOH/Et_2O); ^{1}H NMR (D_2O): $\delta = 1.11$–1.57 (m, 14H, $(CH_2)_2$-$(CH_2)_4$-$(CH_2)_2$, *c*Hex, pip), 1.57–1.88 (m, 10H, *c*Hex, pip), 1.88–2.02 (m, 2H, CH_2-CH_2-pip), 2.02–2.15 (m, 2H, CH_2-CH_2-NH), 2.85–2.97 (m, 2H, CH_2-pip), 2.97–3.16 (m, 5H, *c*Hex, pip, CH_2-NH), 3.44–3.58 (m, 2H, pip); MS (APCI) *m/z*: 294 $[M+1]^+$; Anal. calcd for $C_{19}H_{38}N_2{\cdot}2HCl{\cdot}0.5H_2O$: C 60.62, H 10.98, N 7.44, found: C 60.49, H 10.96, N 7.50.

General procedure for the synthesis of piperidinoalkylphthalimides 3a–3c.[19] DIAD (0.202 g, 1 mmol) was added to a mixture of ω-piperidinoalkylalcohol (0.87 mmol), phthalimide (0.147 g, 1 mmol), and PPh_3 (0.262 g, 1 mmol) in dry THF (2.5 mL), stirred at 0 °C and kept under N_2 atmosphere. The reaction mixture was then allowed to warm to room temperature and stirred overnight. Solvent was evaporated under reduced pressure, and the residue was purified by flash chromatography [SiO_2, CH_2Cl_2/CH_3OH (NH_3) 95:5] to give the piperidinoalkylphthalimide derivative.

2-(8-(Piperidin-1-yl)octyl)isoindoline-1,3-dione (3a). White solid (179 mg, 60% yield); mp: 56–57 °C (PE); ^{1}H NMR HCl (DMSO): $\delta =$ 1.25–1.29 (m,(CH_2)$_6$-, pip), 1.55–1.88 (m, 6H, pip), 2.73–2.95 (m, 4H, pip), 3.32–3.44 (m, 2H, CH_2-phth), 3.52–3.57 (m, 2H, CH_2-NH), 7.68–7.88 (m, 4H, Ph), 10.65 (s, 1H, NH^+-pip); MS (APCI) *m/z*: 343 [*M*+1]$^+$.

General procedure for the synthesis of piperidinoalkylamines 4a–4c.[5] The phthalimide derivative (0.47 mmol) was dissolved in CH_3OH (6 mL) and $NH_2NH_2{\cdot}H_2O$ (0.070 g, 1.4 mmol) was added to this solution. The mixture was held at reflux for 1 h. The suspension was cooled, acidified with concentrated HCl, and filtered. The filtrate was evaporated under reduced pressure, and the residue was purified by flash chromatography [SiO_2, CH_2Cl_2/CH_3OH (NH_3) 95:5] to give the piperidinoalkylamine derivative.

8-(Piperidin-1-yl)octan-1-amine·2 HCl (4a). Oil (74.8 mg, 75% yield); ^{1}H NMR 2 HCl (DMSO): $\delta =$ 1.22–1.55 (m, 12H, (CH_2)$_6$-, pip), 1.67–1.88 (m, 6H, pip), 2.67–2.85 (m, 4H, pip), 2.69–2.88 (m, 2H, CH_2-pip), 3.37–3.55 (m, 2H, CH_2-NH_3^+), 8.11 (s, 3H, NH_3^+), 10.63 (s, 1H, NH^+-pip); MS (APCI) *m/z*: 213 [*M*+1]$^+$.

General procedure for the synthesis of piperidinoalkyl derivatives (17–19). A solution of 9-chloro-1,2,3,4-tetrahydroacridine (0.218 g, 1 mmol) in *n*-pentanol (6 mL) was treated with the appropriate amine **4a**–**c** (1 mmol) and DIPEA (0.517 g, 4 mmol) and held at reflux for 3 days. The solvent was evaporated under reduced pressure; the residue was purified by flash chromatography [SiO_2, CH_2Cl_2/CH_3OH (NH_3) 95:5].

1,2,3,4-Tetrahydro-*N*-(8-(piperidin-1-yl)octyl)acridin-9-amine (17·$C_2H_2O_4$·2 H_2O). White solid (234 mg, 45% yield); mp: 128–129 °C (EtOH/Et_2O); ^{1}H NMR 2 HCl (DMSO): $\delta =$ 1.14–1.38 (m, 10H, (CH_2)$_2$-(CH_2)$_4$-(CH_2)$_2$, pip), 1.57–1.86 (m, 12H, pip, CH_2-CH_2-pip, CH_2-CH_2-NH, acr), 2.61–2.79 (m, 2H, acr), 2.72–2.96 (m, 4H, CH_2-pip, pip), 2.96–3.08 (m, 2H, acr), 3.28–3.38 (m, 2H, pip), 3.80–3.84 (m, 2H, CH_2-NH), 7.55 (t, $J =$ 7.8 Hz, 1H, Ph), 7.83 (t, $J =$ 7.8 Hz, 1H, Ph), 7.95 (br s, 1H, NH), 8.05 (d, $J =$ 8.4 Hz, 1H, Ph), 8.43 (d, $J =$ 8.4 Hz, 1H, Ph), 10.77 (s, 1H, NH^+-pip), 14.31 (s, 1H, NH^+-acr); MS (APCI) *m/z*: 394 [*M*+1]$^+$; Anal. calcd for $C_{26}H_{39}N_3{\cdot}C_2H_2O_4{\cdot}2H_2O$: C 64.71, H 8.73, N 8.06, found: C 64.95, H 8.68, N 7.99.

Pharmacology

Drugs used were purchased from Sigma (St. Louis, MO, USA). Cultured SK-N-MC cells stably expressing human histamine H_3 or H_4 receptors and the reporter gene β-galactosidase (Johnson & Johnson R&D, San Diego, CA, USA) were used for binding and functional studies. Functional experiments were also performed on isolated organs excised from guinea pigs (250–350 g) whereas in vitro and ex vivo binding, colorimetric, and behavioral assays were carried out in male Wistar rats (150–200 g) (Charles River, Italy). Animals were housed, handled, and cared for according to the European Community Council Directive 86 (609) EEC, and the experimental protocols were carried out in compliance with Italian regulations (DL 116/92) and with the local Ethical Committee Guidelines for Animal Research.

Rat histamine H_3 receptor binding assay. Rat brain membranes, prepared according to the method of Kilpatrick and Michel,[20] were incubated for 30 min with [^{3}H](*R*)-α-methylhistamine (RAMH) 0.5 nM and the compounds under study (1 nM–10 μM), in Tris-HCl 50 mM, pH 7.4, NaCl 50 mM, EDTA 0.5 mM, then rapidly filtered (AAWP Millipore filters 0.8 mm) under vacuum and rinsed twice with ice-cold buffer (50 mM Tris-HCl/5 mM EDTA). Nonspecific binding was defined with 10 μM thioperamide as competing ligand.

Human histamine H_3 and H_4 receptor binding assay. Homogenates of SK-N-MC cells, a human neuroblastoma cell line stably expressing the human histamine H_3 or H_4 receptors, were used in radioligand displacement studies according to the method of Lovenberg et al. for H_3 receptors[21] or Liu et al. for H_4 receptors.[22] Membranes were incubated for 60 min at room temperature with 0.5 nM [^{3}H]RAMH (30.0 Ci mmol^{-1}, Amersham Bioscience) or with 10 nM [^{3}H] histamine (18.1 Ci mmol^{-1}, PerkinElmer) in the absence or presence of competing ligands (0.01 nM–10 μM). Incubation was terminated by rapid filtration over Millipore AAWPO2500 filters followed by two washes with ice-cold buffer (50 mM Tris-HCl/5 mM EDTA). Nonspecific binding was defined by 10 or 100 μM histamine as competing ligand for H_3 and H_4 receptors, respectively.

Human histamine H_3 receptor functional assay. Compounds were added directly to the media containing SK-N-MC cells expressing the human histamine H_3 receptor and the construct gene (β-galactosidase), followed 5 min later by addition of forskolin (5 μM). The compounds (1 nM–10 μM) were added 10 min prior to RAMH (0.1–100 nM). After a 6 h incubation at 37 °C, the medium was aspirated and the cells were lysed with 25 mL 0.1× assay buffer (mM composition: NaH_2PO_4 10; Na_2HPO_4 10; pH 8, $MgSO_4$ 0.2; $MnCl_2$ 0.01) and after 10 min with 100 mL 1× assay buffer (NaH_2PO_4 100; Na_2HPO_4 100; pH 8; $MgSO_4$ 2; $MnCl_2$ 0.1) containing 0.5% Triton and 40 mM β-mercaptoethanol. Color was developed using 25 μL 1 mg mL^{-1} substrate solution (chlorophenol red β-D-galactopyranoside; Roche Molecular Biochemicals, Indianapolis, IN, USA) and quantified with a microplate reader by measuring the absorbance at λ 570 nm (Bio-Rad microplate reader 550, Segrate, MI, Italy).[23]

Cell viability. Cell viability was determined through colorimetric quantification of formazan derived from 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) metabolic reduction.[24] SK-N-MC cells were incubated with the compounds under study (1–10 μM) or with the vehicle for 6 h. At the end of the period of incubation, 10 μL 5 mg mL^{-1} MTT solution were added to each well. After 3 h, the culture medium was removed, the cells washed with phosphate-buffered saline (PBS), and 200 μL formazan solubilization solution (0.1 N HCl in anhydrous *i*PrOH) was added. Culture medium absorbance was spectrophotometrically read at λ 570 nm (Bio-Rad microplate reader 550, Segrate, MI, Italy). Cell viability was expressed as viability relative to control.

Functional studies on isolated tissues

Field stimulated guinea pig ileum. Portions of guinea pig ileum were longitudinally mounted (1 g load) in organ chambers, filled with Krebs–Henseleit solution (mM composition: NaCl 118.9; KCl 4.6; $CaCl_2$ 2.5; KH_2PO_4 1.2; $NaHCO_3$ 25; $MgSO_4{\cdot}7H_2O$ 1.2; glucose 11.1) and gassed with 95:5 O_2/CO_2 at 37 °C. The tissues were electrically stimulated (0.1 Hz, 1 ms, submax voltage) (LACE, Ospedaletto, PI, Italy). The H_3 antagonistic activity of the tested compounds (1 nM–1 μM) was functionally determined on twitch contraction in-

hibition induced by RAMH cumulatively administered (1 nM–1 μM) in the presence of 1 μM mepyramine.[25]

Guinea pig isolated ileum and atria. Guinea pig terminal ileum portions (H_1 receptors) and atria (H_2 receptors) were isometrically suspended in organ baths filled with Krebs–Henseleit (37 °C) and Ringer–Locke solution (31 °C) (mM composition: NaCl 154.0; KCl 5.6; $CaCl_2$ 1.08; $NaHCO_3$ 5.95; glucose 11.1), respectively, and aerated with 95:5 O_2/CO_2. Atrial responses were determined as changes in the rate of spontaneous beating by means of a connected cardiotachograph. Cumulative dose–response curves of histamine in ileum (1 nM–1 μM) or of H_2 agonist dimaprit in atria (0.1–100 μM) were obtained in the absence and in the presence of the compounds tested up to a 10 μM concentration.[25]

Rat brain cholinesterase inhibition. The inhibition of brain cholinesterase was determined spectrophotometrically using acetylthiocholine as substrate according to the method of Ellman et al.[26] Aliquots of rat brain homogenates were incubated in phosphate buffer 0.1 M (pH 8.0) with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (5 mM) and test compounds or tacrine at appropriate concentrations (10 nM–100 μM). The reaction was started at 37 °C by adding 20 μL acetylthiocholine (75 mM). The reaction was stopped after 15 min by adding formalin (4%). The hydrolysis of acetylthiocholine catalyzed by the enzyme was determined by monitoring the formation of the yellow 5-thio-2-nitrobenzoate anion at a λ 412 nm (Bio-Rad microplate reader 550, Segrate, MI, Italy). The percent inhibition of cholinesterase was calculated as: $[(A_{control}-A_{sample})/A_{control}]\times 100$. Results are provided as pIC_{50} ($-\log IC_{50}$, where IC_{50} is the concentration causing half-maximal inhibition of cholinesterase activity) and maximum percent inhibition of rat brain cholinesterase.

Ex vivo binding study. Ex vivo binding studies were performed by measuring the displacement of [^{3}H]RAMH from cerebral cortical membranes of rats intraperitoneally (i.p.) treated with compound **18** (doses ranging from 5 to 20 mg kg^{-1}) or vehicle saline 60 min before the binding assay. Animals were killed by CO_2 asphyxiation. Cerebral cortical membranes were prepared according to Taylor's method,[27] by homogenizing cerebral tissues isolated after rat transcardial perfusion with ice-cold buffer at pH 7.4 (Tris-HCl 50 mM, pH 7.6, NaCl 50 mM, EDTA 0.5 mM). Cerebral cortex homogenates were incubated with [^{3}H]RAMH (0.5 nM) for 45 min at room temperature, then rapidly washed twice with ice-cold buffer and separated by centrifugation (12800 *g* at 4 °C for 5 min). Pellets were resuspended in buffer solution, mixed with liquid scintillation (Ultima Gold, PerkinElmer), and bound radiolabel was determined by liquid scintillation counting. Thioperamide 10 μM was used to determine nonspecific binding. Specific [^{3}H]RAMH binding in drug-treated animals (2–7 rats for each dose) was expressed as percentage of the specific [^{3}H]RAMH binding measured in vehicle-treated animals.

Cholinergic side effects. Cholinergic side effects produced by compound **18** were monitored in rats used for ex vivo binding studies and compared with those evoked by tacrine 10 mg kg^{-1} administered i.p. to a group of age-matched male rats. Body temperature was rectally measured within 0.1 °C accuracy using a digital thermometer (Delta Ohm HD8704, Padova, Italy) before i.p. injection of compound **18**, tacrine, or vehicle (basal temperature) and 60 min later (final temperature). Decrease in body temperature was evaluated as difference between basal and final temperature (ΔT). At the same time, tremor, salivation, and chewing were registered as present or not present. Salivation was scored as present if the areas surrounding the mouth were wet, while tremor was scored as present on the basis of the intensity of tremor provoked by handling during the temperature measurement.[16]

Data analysis

Data are presented as mean ± SEM of 4–6 independent experiments. The antagonistic potencies were estimated by determining pK_B ("apparent pA_2") as described by Furchgott's equation.[28] When insurmountable antagonism was detected, the antagonistic potency of the drugs was expressed by pD_2' values, determined according to Van Rossum's equation.[29]

In the in vitro binding assays, pIC_{50} values, estimated from the displacement curves of the tested compounds versus [^{3}H](*R*)-α-methylhistamine or [^{3}H]histamine, were converted into pK_i values according to the Cheng–Prusoff equation.[30]

Temperature differences were measured and compared in the drug-treated and vehicle-treated animals. One-way ANOVA followed by Dunnett's post-test using Prism 5.0 (GraphPad Software, San Diego, CA, USA) was performed. Tremor, salivation, and chewing were reported in terms of the fraction of total treated animals that showed the symptoms.

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