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Research paper

Modification of the anabaseine pyridine nucleus allows achieving binding and functional selectivity for the $\alpha 3\beta 4$ nicotinic acetylcholine receptor subtype



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Carlo Matera ^a, Marta Quadri ^a, Miriam Sciaccaluga ^b, Diego Yuri Pomè ^a, Francesca Fasoli ^{d, e}, Marco De Amici ^a, Sergio Fucile ^{b, c}, Cecilia Gotti ^{d, e}, Clelia Dallanoce ^{a, *}, Giovanni Grazioso ^a

^a Dipartimento di Scienze Farmaceutiche, Sezione di Chimica Farmaceutica "Pietro Pratesi", Università degli Studi di Milano, Via L. Mangiagalli 25, 20133 Milano, Italy

^b I.R.C.C.S. Neuromed, Istituto Neurologico Mediterraneo, Via Atinese 18, 86077 Pozzilli Isernia, Italy

^c Dipartimento di Fisiologia e Farmacologia, Università di Roma La Sapienza, Piazzale A. Moro 5, 00185 Roma, Italy

^d Consiglio Nazionale delle Ricerche (CNR), Istituto di Neuroscienze, Via Vanvitelli 32, 20129 Milano, Italy

^e Dipartimento di Biotecnologie Mediche e Medicina Traslazionale, Università degli Studi di Milano, Via Vanvitelli 32, 20129 Milano, Italy

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ABSTRACT

We report the design, synthesis and pharmacological screening of a group of analogues of anabaseine **2**, a naturally occurring unselective nicotinic agonist. The novel nAChR ligands **5-15** were planned following a molecular modeling analysis which suggested the replacement of the pyridine ring of **2** with a 3-substituted benzene ring as a means to gain selectivity for the $\alpha 3\beta 4$ nAChR subtype. Overall, from binding experiments, the synthesized compounds showed high values of $\alpha 3\beta 4$ affinity and $\alpha 3\beta 4$ vs $\alpha 4\beta 2$ selectivity, although they poorly discriminated the homomeric $\alpha 7$ subtype. The three analogues **6**, **12** and **13** were also evaluated in electrophysiological assays, and **12** [6-(3-iodophenyl)-2,3,4,5-tetrahydropyridine] emerged as a rather interesting nicotinic ligand. Indeed, in addition to a noteworthy affinity (K_i = 4.7 nM) for the $\alpha 3\beta 4$ subtype and to an excellent $\alpha 3\beta 4$ vs $\alpha 4\beta 2$ subtype selectivity (806-fold), compound **12** selectively activated the $\alpha 3\beta 4$ nAChR (EC₅₀ = 7.4 µM) while eliciting a negligible response at the $\alpha 7$ subtype and no effect at the $\alpha 4\beta 2$ subtype.

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1. Introduction

Neuronal nicotinic acetylcholine receptors (nAChRs) are a heterogeneous family of ligand-gated cation channels formed by homo- or hetero-pentameric combinations of α and β subunits ubiquitously expressed in the central (CNS) and peripheral (PNS) nervous systems [1–3]. nAChR subtypes share a common basic structure but their pharmacological and functional properties depend on their subunit composition. The $\alpha 4\beta 2$ and $\alpha 7$ nAChR subtypes are widely expressed in the CNS [4,5], while the $\alpha 3\beta 4$ subtype is mainly localized in the PNS [6] and is highly concentrated in a few brain regions, the medial habenula (MHb) and the

* Corresponding author. E-mail address: clelia.dallanoce@unimi.it (C. Dallanoce). interpeduncular nucleus (IPN), and in the pineal gland [7,8].

The $\alpha 4\beta 2$ and $\alpha 7$ nAChR subtypes modulate cognition, anxiety, pain and neuroprotection [9–13], and new selective ligands for these two subtypes are frequently reported in the recent literature [14,15]. On the other hand, the nAChRs containing the $\alpha 3\beta 4^*$ subunits (the asterisk denotes the possible presence of additional subunits) mediate ACh-induced fast excitatory ganglionic transmission and control the autonomic functions [6,16]. Although the $\alpha 3\beta 4$ nAChR is not the predominant subtype in the brain, recent studies have suggested its relevant role in influencing the behavioral effects of nicotine, the nicotine dependence and some manifestations of nicotine withdrawal [17,18]. It has also been shown that injection of $\alpha 3\beta 4$ nAChR antagonists into the MHb decreases self-administration not only of nicotine [19] but also of multiple abused drugs [20].

Recently, a series of linkage analyses indicated that variants in

the human $\alpha 3-\alpha 5-\beta 4$ nAChR subunit gene cluster on chromosome 15q24-25.1 are involved in the risk of nicotine dependence, smoking and lung cancer [21], and two of the clustered nAChR genes ($\alpha 3$ and $\beta 4$) are significantly over-expressed in small-cell lung carcinoma (SCLC) cells, an aggressive form of lung cancer that is closely related to cigarette smoking [22]. The identification of highly potent and selective $\alpha 3\beta 4$ nAChR ligands may thus be very useful not only to treat nicotine dependence, but also in view of their application in the lung cancer therapy.

To date, the crystal structures of the homologue acetylcholinebinding proteins (AChBPs) have contributed to elucidating the molecular determinants of ligand receptor binding [23–25]. AChBP crystal structures in complex with nAChR ligands indicated that agonists may establish water-mediated hydrogen bonds between their hydrogen bond acceptor moieties and residues on the complementary receptor face [26,27]. Experimental evidence by unnatural amino acid mutagenesis has confirmed the existence of these hydrogen bond interactions [28]. Since computational and experimental investigations indicated the presence of several water molecules in the nAChR binding cleft, it has been suggested that a productive interaction between a hydrogen bond acceptor moiety of the ligand and the complementary receptor protein could be mediated by a water molecule [29–31].

In a recent study [31], we computationally analyzed the presence of water molecules as putative hydrogen bond donor/acceptor moieties in the agonist binding site of the $\alpha 4\beta 2$, $\alpha 3\beta 4$ and $\alpha 7$ nicotinic channels. Initially, we evaluated the interactions of the frog toxin epibatidine (–)-**1** (Fig. 1), an exceptionally potent albeit nonselective nicotinic agonist, and of its analogue deschloroepibatidine. Then, our theoretical study was applied to derivatives (±)-**3** and (±)-**4** (Fig. 1), in which the 3-hydroxyl and 3hydroxymethyl groups replacing the nitrogen atom of pyridine should surrogate to some extent the role of a water molecule in the ligand–receptor interaction [31]. The presence of the water molecule in the receptor binding cleft was compatible with our experimental data and both (±)-**3** and (±)-**4** retained a good affinity at $\alpha 3\beta 4$ nAChRs coupled with the appearance, for (±)-**3**, of $\alpha 3\beta 4$ vs



Fig. 1. Structure of the reference compounds $1-4^a$ and of the new derivatives $5-15^b$ investigated in this study. ${}^a(\pm)-3$: $R_1 = H$, $R_2 = OH$; $(\pm)-4$: $R_1 = H$, $R_2 = CH_2OH$; b5 : $R_1 = H$, $R_2 = OH$; 6: $R_1 = H$, $R_2 = CH_2OH$; 7: $R_1 = H$, $R_2 = CH_3$; 8: $R_1 = R_2 = H$; 9: $R_1 = H$, $R_2 = F$; 10: $R_1 = H$, $R_2 = CI$; 11: $R_1 = H$, $R_2 = Br$; 12: $R_1 = H$, $R_2 = I$; 13: $R_1 = Br$, $R_2 = Br$; 14: $R_1 = F$, $R_2 = Br$; 15: $R_1 = F$, $R_2 = CH_2OH$.

 $\alpha 4\beta 2$ functional selectivity [31].

In the present study, we applied a parallel approach to anabaseine 2 (Fig. 1), one among the pyridine-containing nicotinic alkaloids, which is a toxin that some marine worms use as a chemical defense and as a means for capturing prey. Like epibatidine 1, anabaseine 2 rather indiscriminately stimulates all nicotinic cholinergic receptors [32], displaying higher potency on neuromuscular ($\alpha 1\beta 1\gamma \delta$) and neuronal $\alpha 7$ receptor nAChRs and evoking lower detectable responses from $\alpha 4\beta 2$ and $\alpha 3\beta 4$ subtypes. At the time we started this study, the binding affinity of **2** for α 3 β 4 nAChRs was unknown [33]. At variance with epibatidine or nicotine, anabaseine is an achiral molecule bearing a tetrahydropyridine ring, whose imine double bond is conjugated with the 3-pyridyl moiety. Thus, the two rings of **2** are almost coplanar, whereas in **1** the sixmembered ring is part of the 7-azabicyclo[2.2.1]heptane skeleton and the 2-chloro-5-pyridyl moiety adopts an exo orientation. Since we aimed at identifying selective $\alpha 3\beta 4$ nAChR ligands, in analogy to (\pm) -3 and (\pm) -4 we initially designed, synthesized and tested derivatives 5 and 6 (Fig. 1), in which the 3-pyridyl ring of 2 was replaced by the 3-hydroxybenzene or 3-hydroxymethylbenzene moieties, respectively. Then, we enriched the substitution pattern on positions 3 and 3,5 of the aromatic ring by preparing and testing the group of analogues 7-15 (Fig. 1).

Herein we report the synthesis and the pharmacological and computational investigations on target compounds **5-15** at neuronal $\alpha 3\beta 4$, $\alpha 4\beta 2$ and $\alpha 7$ nAChR subtypes. Based on the binding affinity data, we selected derivatives **6**, **12** and **13** to evaluate their functional behavior in electrophysiological experiments performed on the three heterologously expressed human nAChRs under study.

2. Structure-based design

The significance of structural water molecules in ligand protein recognition is a well-known and deeply investigated issue [34–36]. Water molecules may mediate crucial protein—protein and ligand—protein interactions as well as contribute to the favorable orientation of the ligands within the binding crevice. In a previous study, we found that the two hydroxyl-containing analogues (\pm)-**3** and (\pm)-**4** (Fig. 1) perturbed the hydrogen bond network formerly present in the solvated binding cleft hosting deschloroepibatidine [31]. The theoretical binding modes were correlated with the experimental results on (\pm)-**3** and (\pm)-**4**, which, with respect to their parent compound, showed a comparable affinity at the α 3β4 subtype while considerably lost affinity at the α 4β2 subtype. As a consequence, the applied structural modification caused a gain of α 3β4 vs α 4β2 selectivity, which deserved further investigation.

Thus, we extended our study to anabaseine 2 by performing docking calculations and molecular dynamics (MD) simulations in the fully solvated $\alpha 3\beta 4$ nAChR model previously developed by us [37]. Using the computational protocol applied to epibatidine **1** and deschloroepibatidine [31], we identified a plausible binding mode of 2 and then analyzed its interaction with a putative water molecule bound in the receptor area surrounded by the residues α 3-Thr106, α 3-Trp149 and β 4-Leu119 (Fig. 2A). During the MD simulations, we observed that the water molecule was firmly bound to the backbone of these residues and only occasionally interacted with the pyridine nitrogen of 2 (Fig. 2B). As predicted for epibatidine **1**, in the $\alpha 3\beta 4$ binding site the solvent molecules interact with the above cited receptor residues and create a hydrophilic environment which dictates the ligand orientation [31]. In addition, to confirm the presence of the solvent molecules in the binding site, we calculated the water occupancy by the Volmap tool of VMD [38], over 50 ns of MD simulations of a solvated α3β4 model in the apo state. The results of this calculation suggested that the binding cleft is frequently visited by solvent molecules, as displayed



Fig. 2. (A) Hypothetical binding mode of anabaseine **2** within the α 3 β 4 binding site. In this frame of MD simulation, WAT molecules engaged H-bond interactions with β 4-Leu119 and β 4-Thr106. In the equilibrated system, water molecules created H-bond network also with the α 3-Trp149 side chain. (B) Distance fluctuation between the oxygen atom of WAT and the protein residue involved in the H-bond network in the binding site of the α 3 β 4 model in complex with **2**.

by the yellow area in Fig. 3.

Taking into account the results of our computational analysis, we designed new analogues of **2** with the aim to access the space occupied by solvent molecules. From our modeling studies (Fig. 2B), we envisioned that the introduction of a benzene ring with suitable substituents at the 3-position would provide more productive interactions with the α 3β4 receptor residues in the binding cleft than those afforded by the nitrogen atom of the anabaseine pyridine ring, which are presumably mediated by the water molecules bound to the backbone. By analogy with compounds (\pm)-**3**, (\pm)-**4** (Fig. 1), to improve the affinity for the target subtype we initially designed and prepared the 3-hydroxybenzene **5** and 3-hydroxymethylbenzene **6** derivatives, which incorporate the hydroxyl group of the water molecule in the ligand molecular skeleton [39]. Then we synthesized the group of analogues **7-15**, to further probe the effect on the pharmacological profile of the

electronic and spatial features of the 3-substituent on the aromatic ring. With the *m*-tolyl and phenyl derivatives, **7** and **8** respectively, we aimed at exploring the competitive influence of van der Waals contacts or hydrogen bonding in the interaction with the binding site. Instead, to engender stronger polar interactions within the receptor crevice, we synthesized a subgroup of 3-substituted mono-halogenated derivatives, i.e., 9 (3-F), 10 (3-Cl), 11 (3-Br), 12 (3-I), in which the progressive increase of the halogen size paralleled its electron-withdrawing properties. It is well known that the halogen atoms can form halogen bonds with nucleophiles, e.g. electronegative atoms like oxygen, displaying a roughly linear arrangement, but also give rise to hydrogen bonds with electrophiles (H-bond donors), occurring laterally [40,41]. Halogen bonds may stabilize ligand-receptor interactions and thus positively affect the process of mutual molecular recognition. Finally, we planned the synthesis and the pharmacological evaluation of the

Fig. 3. Average water occupancy (dark yellow areas) calculated by the VolMap tool of VMD over 50 ns of MD simulations. The surface was plotted considering an isovalue of 0.6. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

doubly substituted congeners 13-15.

3. Chemistry

Target compounds 5, 6, 10, 12 and 13 were prepared along with a rapid synthesis of 2-substituted cyclic imines from lactams, with a reaction sequence involving the in situ introduction of the N-Boc group, an organometallic ring-opening reaction, and the nitrogen deprotection with concomitant ring-closure and dehydration [42]. As illustrated in Scheme 1, commercially available δ -valerolactam was sequentially reacted with butyllithium in THF at -78 °C, ditert-butyldicarbonate and the appropriate Grignard reagent (3methoxyphenylmagnesium bromide 16. 3chlorophenylmagnesium bromide 17, 3-iodophenylmagnesium bromide 18 and 3,5-dibromophenylmagnesium bromide 19), in turn obtained from the corresponding aryl bromide with magnesium turnings [43]. The intermediates *N*-Boc-ω-amino ketones 20-**23** were treated with trifluoroacetic acid, then with 30% aqueous sodium hydroxide, providing the cyclic imines 24, 10, 12 and 13, respectively [42].

Demethylation of derivative **24** with concentrated hydrobromic acid afforded the desired final compound **5**. On the other hand, the synthesis of **6** took advantage of a microwave-mediated fast carbonylation of aryl iodide **12**, using molybdenum hexacarbonyl as a solid carbon monoxide source [44] to provide the corresponding methyl ester **25**. Red-Al[®] reduction [45] of the latter produced the related hydroxymethylbenzene cyclic imine **6**.

In a parallel way, cyclic imines **7-9**, **11**, **14** and **15** were synthesized utilizing the commercially available 5-bromovaleronitrile as the key intermediate (Scheme 2). The *m*-tolyl derivative **7** was prepared transforming 3-bromotoluene into the corresponding *m*tolylmagnesium bromide **26** [43], which was reacted with 5bromovaleronitrile in a tandem addition–cyclization reaction at room temperature in tetrahydrofuran to give the desired compound [46]. Alternatively, 5-bromovaleronitrile was coupled and cyclized with organolithiums [47]. Indeed, compounds **8**, **9**, **11** and **14** were synthesized by reaction of 5-bromovaleronitrile, respectively, with commercially available phenyllithium **27** or (3fluorophenyl)lithium **28**, (3-bromophenyl)lithium **29** and (3bromo-5-fluorophenyl)lithium **30**, which were in turn prepared from precursor bromophenyl derivatives by treatment with 1.6 M *n*-butyllithium in hexane [48].

According to the protocol applied to **12** (Scheme 1), the cyclic 3bromo-5-fluorophenyl imine **14** was converted into the corresponding methyl ester **31**, which was reduced to the desired hydroxymethyl derivative **15**. At last, the target compounds **5-15** were converted into their corresponding hydrochlorides by treatment with a 4.0 M hydrochloric acid solution in 1,4-dioxane.

4. Pharmacology

4.1. Binding studies

As detailed in Table 1, the new compounds as well as the reference ligand **2** were tested for their ability to compete with [³H] epibatidine for binding to heterologously expressed human $\alpha 3\beta 4$ and rat $\alpha 4\beta 2$ nAChRs. The target derivatives were also assayed at the rat hippocampal $\alpha 7$ nAChR subtype, using [¹²⁵I] α -bungarotoxin as radioligand.

It is worth remarking that replacement of the pyridine nucleus of anabaseine **2** with the 3-hydroxybenzene and 3-hydroxymethylbenzene moieties to give **5** and **6** caused, in terms of binding affinity, a 500-fold reduction at the $\alpha 4\beta 2$ subtype, and, at least for **5**, a 300-fold decrease at the $\alpha 7$ subtype. On the other hand, the new compounds exhibited a moderately lower (K_i = 680 nM for **5**) or a slightly higher (K_i = 80 nM for **6**) affinity than **2** (K_i = 107 nM) at the $\alpha 3\beta 4$ nAChR. Moreover, the affinity of the anabaseine-related ligand **6** at the latter subtype matched that of the two epibatidine-related analogues (\pm)-**3** and (\pm)-**4**, with an additional, remarkable gain (438-fold) of $\alpha 3\beta 4$ over $\alpha 4\beta 2$ subtype selectivity.

As shown in Table 1, when the *m*-hydroxymethyl group of **6** was replaced by a lipophilic moiety (CH₃, compound **7**) or a hydrogen atom (compound **8**), an almost complete loss of $\alpha 3\beta 4$ vs $\alpha 4\beta 2$ selectivity was observed, mainly due to a partial increase of affinity for the $\alpha 4\beta 2$ subtype. The 3-halogen derivatives **9-12**, synthesized to ascertain the importance of the polar halogen bond interactions in the binding site, indeed exhibited from high to low nanomolar binding affinities at α 3 β 4 and α 7 nAChRs, and the related K_i values gradually decreased at both receptor subtypes with increasing the size of the halogen substituent. Compound 12, carrying the largest 3-iodo atom, showed the highest affinity for α 3 β 4 and α 7 nAChRs, with K_i values of 4.7 nM and 11.3 nM, respectively. In addition, this derivative had the highest value of $\alpha 3\beta 4$ vs $\alpha 4\beta 2$ selectivity (806fold) in the series. The introduction of a second bromine atom on the 5 position of the phenyl ring in compound **11** led to the dibromo analogue 13, which showed a moderate decrease of binding affinity at α 3 β 4 and a more significant one at α 4 β 2 and α 7 nAChRs, thereby increasing subtype selectivity respect to the monobromo congener. Conversely, the presence of the additional 5-F (compounds 14 and 15) had only a negligible or modest effect on the affinity at $\alpha 3\beta 4$ and α 7 subtypes relative to the corresponding mono-substituted derivatives 11 and 6, respectively. However, compound 15 exhibited a 47-fold lower K_i value than that found for its analogue **6** at the heteromeric $\alpha 4\beta 2$ nAChRs, suggesting a different binding mode of the two ligands with a concomitant drop in subtype selectivity.

Together, the modified anabaseine derivatives **5-15** bearing different *meta* substituents on the benzene ring behaved as good to excellent $\alpha 3\beta 4$ ligands with a poor $\alpha 3\beta 4$ vs $\alpha 7$ selectivity. In this set of compounds, the introduction of heavier halogen atoms on the same 3-position, on one hand caused a clear, comparable increase of both $\alpha 3\beta 4$ and $\alpha 7$ affinity, on the other amplified the ability of the ligands to distinguish the $\alpha 3\beta 4$ from the $\alpha 4\beta 2$ heteromeric nAChRs.

5, 6, 10, 12, 13 ^f 5, 6, 10, 12, 13 × HCI

Scheme 1. Synthesis of target compounds 5, 6, 10, 12 and 13. Reagents and conditions: (a) 2.5 M BuLi/hexane (1 equiv), (Boc)₂O (1 equiv), THF, -78 °C, 7 h; (b) CF₃COOH (16 equiv), rt, 4 h; (c) 48% HBr, reflux 6 h, then rt, 12 h; (d) Mo(CO)₆ (1.5 equiv), 10% Pd/C, DMAP (2 equiv), DIPEA (2 equiv), MeOH (10 equiv), 1,4-dioxane, MW, 50 W, 130 °C, 1 h; (e) 65% Red-Al/ toluene (4.2 equiv), THF, rt, 1 h; (f) 4N HCl (5 equiv), 1,4-dioxane, rt, 30 min.

7, 8, 9, 11, 14, 15 d 7, 8, 9, 11, 14, 15 × HCl

Scheme 2. Synthesis of target compounds 7, 8, 9, 11, 14 and 15. Reagents and conditions: (a) anhydrous THF, rt, 1–12 h (27, 75 °C, 3 h); (b) Mo(CO)₆ (3 equiv), 10% Pd/C, DMAP (2 equiv), DIPEA (2 equiv), MeOH (10 equiv), 1,4-dioxane, MW, 50 W, 130 °C, 2 h; (c) 65% Red-Al/toluene (8.4 equiv), THF, rt, 12 h; (d) 4N HCl (5 equiv), 1,4-dioxane, rt, 30 min.

4.2. Electrophysiological experiments

Based on the above discussed binding data, derivatives **6**, **12** and **13** are among the analogues with the highest affinity for the α 3 β 4 nAChR subtype ($K_i = 80, 4.7$ and 70.8 nM, respectively) and possess the highest values (438, 806 and 266, respectively) of α 3 β 4 vs α 4 β 2 selectivity. Thus, these ligands were selected for evaluation of their functional activity at the human α 3 β 4, α 4 β 2 and α 7 nAChR subtypes

heterologously expressed in the rat anterior pituitary GH4C1 cell line. The functional expression of the different nAChRs was evaluated by measuring the whole-cell inward current elicited by ACh 1 mM, with mean current amplitudes of 3.5 ± 0.5 nA, 1.1 ± 0.2 nA and 0.7 ± 0.1 nA for $\alpha 3\beta 4$ (n = 36), $\alpha 4\beta 2$ (n = 27) and $\alpha 7$ (n = 22), respectively.

As depicted in Fig. 4A, the 3-hydroxymethylbenzene derivative **6** elicited significant inward currents when applied on GH4C1 cells

Table 1

Binding affinities of reference compounds **1-4** and of new derivatives **5-15** at $\alpha 3\beta 4$, $\alpha 4\beta 2$ and $\alpha 7$ nAChR subtypes.

5-15

Compound	$K_{i} (n_{M})^{a}$					α3β4 vs α4β2 selectivity	α3β4 vs α7 selectivity
	R ₁	R ₂	$\alpha 3\beta 4^{b}$	$\alpha 4\beta 2^{c}$	α7 ^d		
Epibatidine (-)-1			0.3 ^e	0.030 ^e	16/22 ^f	0.1	53
Anabaseine 2 ^g			107 (33)	67 (15)	32 (43)	0.6	0.3
(±)- 3	Н	OH	80 (26)	127 (11)	609 (36)	1.6	7.6
(±)- 4	Н	CH ₂ OH	92 (24)	429 (14)	1800 (35)	4.7	20
5	Н	OH	680 (23)	32000 (8)	9300 (42)	47	14
6	Н	CH ₂ OH	80 (35)	35000 (13)	158 (35)	438	2.0
7	Н	CH ₃	155 (20)	1450 (37)	384 (53)	9.4	2.5
8	Н	Н	194 (35)	2080 (20)	2400 (53)	11	12
9	Н	F	108 (20)	2040 (23)	2370 (57)	19	22
10	Н	Cl	77.2 (33)	11500 (62)	394 (38)	149	5.1
11	Н	Br	29.8 (32)	5020 (45)	43.2 (37)	168	1.5
12	Н	I	4.7 (37)	3790 (44)	11.3 (29)	806	2.4
13	Br	Br	70.8 (30)	18800 (41)	343 (47)	266	4.8
14	F	Br	48 (17)	1770 (21)	204 (44)	37	4.3
15	F	CH ₂ OH	80 (24)	744 (38)	821 (43)	9.3	10

^a *K*_i values are derived from three competition-binding experiments. The numbers in brackets refer to the % coefficients of variation.

^b α3β4 nAChRs heterologously expressed in HEK 293 cells.

^c Native $\alpha 4\beta 2$ nAChRs present in rat cortical membranes.

^d Native α 7 nAChRs present in rat hippocampal membranes.

^e *K*_d value from Ref. [69].

^f Binding affinity data for the (-) and (+) enantiomers of **1** are from Ref. [70].

^g The dihydrochloride of **2** was assayed.

expressing $\alpha 3\beta 4$ and $\alpha 7$ nAChRs, while its action on $\alpha 4\beta 2$ nAChRs was completely absent. In particular, the analysis of the dose-response relationships disclosed EC_{50} values of 20.0 μM and 59.7 μ M at α 3 β 4 and α 7 nAChRs, respectively. These results are comparable to those found for the epibatidine-related derivative (\pm) -3, which showed a similar activation pattern [31], and indicate that **6** behaves as a partial agonist at α 3 β 4 and as a full agonist at α 7 nAChRs. Replacement of the CH₂OH group of **6** with iodine gave **12**, which exhibited the highest binding affinity at $\alpha 3\beta 4$ nAChRs and the most pronounced $\alpha 3\beta 4$ over $\alpha 4\beta 2$ selectivity. In addition, compound **12** was a partial agonist at $\alpha 3\beta 4$ nAChRs displaying higher potency and efficacy than its analogue 6, with an EC₅₀ value of 7.4 µM and a maximal current reaching 60% of that evoked by ACh (Fig. 4B). Furthermore, **12** had significantly lower efficacy and potency at α 7 nAChRs and no effect at α 4 β 2 nAChRs (Fig. 4B), thus evidencing a good functional selectivity at the investigated subtypes. The functional profiles of compounds 6 and 12 showed a trend consistent with the outcome of the binding assays (Table 1). However, for the 3,5-dibromo derivative 13, characterized by rather different binding affinity values [K_i : 70.8 nM (α 3 β 4), 343 nM (α 7), 18.8 μ M (α 4 β 2)], an almost complete lack of response was observed in electrophysiological experiments at the three studied subtypes (Fig. 4C). This result strongly recalls the complex relationships between the gating properties of a ligand and its binding affinity for a given receptor channel [49]. Furthermore, both 12 and 13 exhibited negligible effects when assayed as antagonists of heteromeric nAChRs (data not shown). Thus, considering the above discussed electrophysiological data, **12** behaves as an interesting $\alpha 3\beta 4$ partial agonist with a very high binding/functional selectivity over the $\alpha 4\beta 2$ subtype as well as a pronounced functional selectivity over the α 7 subtype.

5. Docking and molecular dynamics analysis

It is worth noting that, in the subset of derivatives 8-12, the overall poor $\alpha 4\beta 2$ binding affinity was slightly affected by the nature of the substituent on the 3-position of the benzene ring. Conversely, as previously discussed, a gradual enhancement of the affinity was observed on passing from the unsubstituted derivative **8** ($K_i = 194 \text{ nM}$) to the 3-iodo analogue **12** ($K_i = 4.7 \text{ nM}$) at the $\alpha 3\beta 4$ subtype. A parallel trend characterized the affinity of the same compounds at the α 7 subtype (from $K_i = 2400$ nM for **8** to $K_i = 11.3$ nM for **12**). This may be due to the presence of a highly conserved sub-area in the two nAChR proteins involved in the ligand-receptor interactions. To shed light on this speculation, we performed docking and MD simulations on the most interesting compound **12** within the $\alpha 3\beta 4$ nAChR binding site. As shown in Fig. 5, the docking studies revealed that the protonated nitrogen of the cyclic imine ring of 12 is ideally positioned to generate a stabilizing H-bonding interaction with the carbonyl oxygen of α 3-Trp149 and a cation- π interaction with the side chain of the same residue. Moreover, the ligand aryl moiety was projected under loop-C toward the same zone previously occupied by the pyridine nitrogen of anabaseine and, noteworthy, the iodine atom was able to interact with the protein area in which solvent molecules are expected to be present, close to β 4-Leu119 and α 3-Ser150 residues (Leu119 and Ser149 in the α 7 sequence, respectively). Most striking is the observation that the side chain of β 4-Arg79 was in close proximity to the halogen atom, creating a good environment for the acceptance of heavy and electron-rich atoms like iodine. This hypothetical binding mode likely accounts for the enhanced affinity associated with the increased size and volume of the halogen atom in compounds 9-12.

Fig. 4. Dose-current response relationships for derivatives **6**, **12** and **13** administered to GH4C1 cells expressing human $\alpha 3\beta 4$, $\alpha 4\beta 2$ or $\alpha 7$ nAChRs. (A) Current amplitudes elicited by compound **6**, and mediated by $\alpha 3\beta 4$ (\odot ; n = 7), $\alpha 4\beta 2$ (\blacksquare ; n = 5) or $\alpha 7$ (\triangle ; n = 5) nAChRs, were normalized to the current amplitude elicited on the same cell by ACh 1 mM. Solid lines represent best fit curves (see Experimental Section) yielding the following parameters: EC₅₀ values of $20 \pm 1 \ \mu$ M and $60 \pm 2 \ \mu$ M, nH values of 1.4 ± 0.3 and 3.3 ± 0.4 , for $\alpha 3\beta 4$ and $\alpha 7$ nAChRs, respectively. (B) Normalized current amplitudes elicited by compound **12**, and mediated by $\alpha 3\beta 4$ (\odot ; n = 8), $\alpha 4\beta 2$ (\blacksquare ; n = 21) or $\alpha 7$ (\triangle ; n = 12) nAChRs. Solid line represents the best fit curve for $\alpha 3\beta 4$ nAChR, yielding the following parameters: EC₅₀ = 7.4 $\pm 2.9 \ \mu$ M and nH = 0.97 ± 0.3 . (C) Normalized current amplitudes elicited by compound **13**, and mediated by $\alpha 3\beta 4$ (\odot ; n = 22), $\alpha 4\beta 2$ (\blacksquare ; n = 5) or $\alpha 7$ (\triangle ; n = 5) nAChRs.

The same Arg79 was found in the $\alpha 7$ and $\beta 2$ sequences, but in

Fig. 5. Hypothetical binding mode of 12 within the α3β4 nAChR binding site.

our $\alpha 4\beta 2$ model it was differently oriented at the end of the MD equilibration. Indeed, being involved in a salt bridge with the side chain of $\alpha 4$ -Glu193, this residue was unavailable for interacting with ligand molecules in the orthosteric site. Moreover, the $\alpha 3$ -Ser150 residue corresponds to a threonine in the $\alpha 4$ sequence ($\alpha 4$ -Thr148), and the presence of an additional methyl group in the side chain should significantly hamper the acceptance of large atoms. This indicates a restrictive size limitation for the substituents on the phenyl ring and justifies the overall low affinity of the compounds **8-12** at the $\alpha 4\beta 2$ nAChR subtype.

The crucial role of the halogen atom in the ligand-receptor interaction can be evaluated by comparing the binding affinities of the newly synthesized compounds 7 and 12. Indeed, the anabaseine-related derivative 12 (3-I) showed a 33-fold higher affinity for the α 3 β 4 subtype than its analog **7** (3-CH₃). As a matter of fact, the two compounds differ only for the substituent in position 3 of the phenyl ring, from iodine to methyl, which have similar van der Waals radii (close to 2 Å) but show relevant differences in terms of electronegativity, electron density and capability to accept hydrogen bonds. This clearly suggests that the most suitable interaction of the ligands in the $\alpha 3\beta 4$ receptor binding pocket should be lipophilic as well as polar in nature. The same conclusions can be drawn if the binding data on the α 7 nAChR are taken into account, since, quite similarly, the iodinated derivative 12 displayed a 34-fold higher affinity than the methyl-substituted derivative **7** at this subtype.

In an attempt to rationalize the low biological activity of the 3,5dibromo derivative **13** at the α 3 β 4 nAChR subtype, further docking and MD simulations were performed. The results highlighted that the binding mode of **13** was not dissimilar from that predicted for the 3-iodo analogue **12**, but the presence of an additional halogen atom in 5 led to a different induced-fit effect in the receptor binding cleft upon MD simulations. Indeed, whereas **12** did not essentially alter the distance between loop C and the α 3-Ser150, at the end of simulations the 3,5-dibromo compound **13** increased the same distance by 3 Å. This computational evidence could account for the different electrophysiological profile of **13**, which lost the agonist activity shown by its analogue **12** at the α 3 β 4 nAChR subtype.

The role exerted by loop C in modulating the permeability of nicotinic channels is still debated. Recently, X-ray crystal structures of nicotine analogues, endowed with functional profiles spanning from agonists to antagonists within the LBD of *Limnea S.*, were reported in the PDB [50]. The authors concluded that the loop C conformation is not directly involved in the functional profile shown by the ligands. Nevertheless, a direct connection between

the bulkiness of orthosteric ligands and the capacity to limit the opening of the channel exists. In this respect, Hansen et al. [25] suggested that the loop C extension and closure might be the distinguishing feature between complexes of the nAChR with agonists and antagonists. On the other hand, Mukhtasimova et al. [51] proposed that some key interactions between residues belonging to loop C are required to efficiently modulate the channel-gating equilibrium. Our data suggest that a combined ligand interaction with the loop C and the alpha subunit counterpart may control the functional response of the nicotinic channel.

6. Conclusion

The $\alpha 3\beta 4$ nAChR, mainly expressed in the in sensory and autonomic ganglia and in the adrenal gland, is frequently referred to as the "ganglionic nAChR". Recent studies have provided compelling evidence that the $\alpha 3\beta 4^*$ nAChRs present in the MHb-IPN brain pathway regulate nicotine reinforcement, dependence and withdrawal [52,19,53]. Injection of $\alpha 3\beta 4$ nicotinic receptor antagonists into the MHb decreases self-administration of multiple abused drugs, including nicotine, morphine, cocaine and alcohol [54]. Due to desensitization of $\alpha 3\beta 4$ nAChRs, even the high affinity $\alpha 3\beta 4$ selective partial agonist AT-1001 was found to block nicotine self-administration and relapse-like behavior in rats [55], thus making this compound a potentially safer and clinically useful agent for smoking cessation.

The goal of the present study was to identify and characterize new $\alpha 3\beta 4$ selective ligands aiming at new compounds for smoking cessation and/or to treat lung cancers expressing a high level of the mRNAs for the $\alpha 3$ and $\beta 4$ subunits. To this end, we initially performed an MD theoretical investigation on the natural nicotinic agonist anabaseine **2** within the $\alpha 3\beta 4$ binding cleft. This analysis highlighted that water molecules may generate H-bond interactions with complementary residues of the surrounding receptor protein, thus favoring a proper orientation of the ligand. To learn more about the structural determinants which bring about a gain in $\alpha 3\beta 4$ nAChR subtype selectivity, at first we chose to modify the structure of **2** by replacing its 3-pyridinyl moiety with the 3-OH or the 3-CH₂OH benzene ring (compounds **5** and **6**, respectively), then the set of investigated derivatives was extended to the 3mono- or 3,5-disubstituted analogs **7-15**.

The novel compounds behaved as good to high affinity $\alpha 3\beta 4$ subtype ligands [K_i values from 680 nM for **5** to 4.7 nM for **12**] and, in some instances, a noteworthy $\alpha 3\beta 4$ vs $\alpha 4\beta 2$ selectivity was observed (i.e., 438-fold for **6** and 806-fold for **12**). Remarkably, in the subgroup of 3-halogen-phenyl analogues **9-12**, the $\alpha 3\beta 4$ affinity progressively increased with the size of the substituent, highlighting a critical contribution of halogen bonding in the process of recognition by the $\alpha 3\beta 4$ subtype. Computational studies confirmed that, in the binding crevice of the $\alpha 3\beta 4$ receptor, a favorable subarea is available for the acceptance of heavy and electron-rich atoms like halogens. We hypothesized the presence of a similar conserved sub-area also within the binding site of the $\alpha 7$ receptor, since the same derivatives **9-12** showed a quite comparable affinity trend, which precluded their ability to discriminate between the heteromeric $\alpha 3\beta 4$ and the homomeric $\alpha 7$ subtype.

Finally, three relevant $\alpha 3\beta 4$ ligands were further assessed in electrophysiological experiments. This analysis indicated that the 3-iodo-substituted derivative **12** stands out as a high affinity $\alpha 3\beta 4$ nAChR partial agonist, which couples a very high $\alpha 3\beta 4$ vs $\alpha 4\beta 2$ selectivity with a selective activation of the $\alpha 3\beta 4$ -mediated functional response. In summary, inspired by the structural skeleton of anabaseine, we successfully identified a position on the benzene ring of a small set of new analogs which is crucial in triggering a gain in both $\alpha 3\beta 4$ affinity and binding/functional selectivity.

7. Experimental protocols

7.1. Chemistry

7.1.1. General methods

¹H NMR and ¹³C NMR spectra were recorded with a Varian Mercurv 300 (¹H, 300.063; ¹³C, 75.451 MHz) spectrometer in CDCl₃ solutions (unless otherwise indicated) at 20 °C. Chemical shifts (δ) are expressed in ppm and coupling constants (1) in Hz. TLC analyses were performed on commercial silica gel 60 F₂₅₄ aluminum sheets; spots were further evidenced by spraying with a dilute alkaline potassium permanganate solution or a phosphomolybdic acid solution and, for tertiary amines, with the Dragendorff reagent. All microwave irradiation experiments were carried out in a CEM Discover SP microwave apparatus, operating at a frequency of 2.45 GHz with continuous irradiation power from zero to 300W, utilizing the standard absorbance level of 50W maximum power. The reactions were performed in a standard pressurized 10 mL reaction vessel, sealed with Teflon septum and placed in the microwave cavity. The reaction was irradiated at a required ceiling temperature using maximum power for the stipulated time. Then it was cooled to 40 °C with gas jet cooling. Melting points were determined on a model B 540 Büchi apparatus and are uncorrected. ESI mass spectra were obtained on a Varian 320 LC-MS/MS instrument. Data are reported as mass-to-charge ratio (m/z) of the corresponding positively charged molecular ions. Microanalyses (C, H, N) agreed with the theoretical value within $\pm 0.4\%$. Compound 27 and all other reagents and solvents were purchased from Sigma--Aldrich Srl (Milan, Italy) and used without further purification. Anabaseine dihydrochloride was prepared according to a known procedure [56].

7.1.2. Synthesis of target compounds 5, 6, 10, 12 and 13

7.1.2.1. Tert-Butyl 5-(3-methoxyphenyl)-5-oxopentylcarbamate (20). To a solution of δ -valerolactam (1 g, 10.10 mmol) in anhydrous THF (27 mL) at $-78 \degree$ C under an argon atmosphere was added dropwise 2.5 M butyllithium in hexanes (4.04 mL, 10.09 mmol) and after 30 min of stirring, a solution of di-tert-butyldicarbonate (2.2 g, 10.10 mmol) in anhydrous THF (3.5 mL) was added and temperature was maintained at -78 °C for 3 h. Then a solution of 3methoxyphenylmagnesium bromide 16, prepared from 2.45 g of 3-bromoanisole and 368 mg of magnesium turnings in anhydrous THF (14 mL), was added and stirring was continued at the same temperature for further 3 h. The reaction was allowed to warm to room temperature and quenched with 2.0 M HCl (10 mL). The mixture was extracted with diethyl ether (3 \times 15 mL) and the combined organic layers were washed with a 5% NaHCO₃ aqueous solution (1 \times 20 mL) and brine (1 \times 20 mL), dried over anhydrous sodium sulphate and concentrated in vacuo to obtain 20 as a yellow oil (2.84 g, 92% yield). $R_f = 0.21$ (cyclohexane/ethyl acetate 9:1). ¹H NMR: δ 1.43 (s, 9H), 1.51–1.61 (m, 2H), 1.70–1.81 (m, 2H), 2.97 (t, *J* = 7.2 Hz, 2H), 3.14 (t, *J* = 6.4 Hz, 2H), 3.84 (s, 3H), 4.63 (br s, 1H), 7.09 (ddd, J = 8.3, 2.8, 1.1 Hz, 1H), 7.35 (t, J = 8.3 Hz, 1H), 7.46–7.47 (m, 1H), 7.50–7.53 (m, 1H). ¹³C NMR: δ 21.2, 28.4 (3C), 29.4, 38.4, 40.0, 55.8, 79.5, 115.9, 118.7, 122.1, 129.3, 138.9, 155.9, 160.5, 185.1. MS (ESI) m/z [M+H]⁺ Calcd for C₁₇H₂₆NO₄⁺: 308.19. Found: 308.2.

7.1.2.2. Tert-Butyl 5-(3-Chlorophenyl)-5-oxopentylcarbamate (**21**). The title compound was obtained according to the method described for compound **20** by employing δ -valerolactam (992 mg, 10.0 mmol) and 3-chlorophenylmagnesium bromide **17** prepared by treatment of 1-bromo-3-chlorobenzene (1.2 mL, 10.00 mmol) with magnesium turnings (243 mg) to afford **21** as a dark yellow oil (1.44 g, 46% yield). $R_{\rm f} = 0.28$ (cyclohexane/ethyl acetate 4:1). ¹H NMR: δ 1.41 (s, 9H), 1.49–1.62 (m, 2H), 1.69–1.79 (m, 2H), 2.95 (t,

 $J = 7.2 \text{ Hz}, 2\text{H}, 3.13 \text{ (t, } J = 6.9 \text{ Hz}, 2\text{H}), 4.64 \text{ (br s, 1H)}, 7.37 \text{ (t, } J = 7.8 \text{ Hz}, 1\text{H}), 7.48-7.52 \text{ (m, 1H)}, 7.78-7.82 \text{ (m, 1H)}, 7.89 \text{ (t, } J = 1.7 \text{ Hz}, 1\text{H}). ^{13}\text{C} \text{ NMR: } \delta 21.3, 28.6 \text{ (3C)}, 29.8, 38.3, 40.4, 79.4, 126.3, 128.4, 130.2, 133.2, 135.2, 138.6, 156.2, 198.9. \text{ MS} \text{ (ESI) } m/z \text{ [M+H]}^+ \text{ Calcd for } C_{16}H_{23}\text{CINO}_3^+: 312.14. \text{ Found: } 312.1.$

7.1.2.3. Tert-Butyl 5-(3-iodophenyl)-5-oxopentylcarbamate (22). The title compound was obtained according to the method described for compound 20 by employing δ -valerolactam (1 g, 10.10 mmol) and 3-iodophenylmagnesium bromide 18 prepared by treatment of 3-bromoiodobenzene (1.67 mL, 13.11 mmol) with magnesium turnings (368 mg) to afford 22 as a yellow oil (2.72 g, 67% yield). $R_{\rm f}$ = 0.32 (cyclohexane/ethyl acetate 4:1). ¹H NMR: δ 1.44 (s, 9H), 1.52–1.65 (m, 2H), 1.72–1.83 (m, 2H), 2.98 (t, J = 7.2 Hz, 2H), 3.16 (t, J = 6.3 Hz, 2H), 4.58 (br s, 1H), 7.23–7.29 (m, 1H), 7.44 (dt, J = 8.0, 5.8 Hz, 1H), 7.61–7.65 (m, 1H), 7.71–7.75 (m, 1H). ¹³C NMR: δ 21.2, 28.6 (3C), 29.5, 38.4, 40.6, 79.5, 124.5, 127.7, 130.1, 133.8, 136.1, 138.7, 155.1, 199.2. MS (ESI) m/z [M+H]⁺ Calcd for C₁₆H₂₃INO⁺₃: 404.07. Found: 404.1.

7.1.2.4. *Tert-Butyl* 5-(3,5-*dibromophenyl*)-5-*oxopentylcarbamate* (**23**). The title compound **20** by employing δ -valerolactam (1.24 g, 12.50 mmol) and 3,5-*dibromophenylmagnesium bromide* **19** prepared by treatment of 1,3,5-tribromobenzene (3.94 g, 12.50 mmol) with magnesium turnings (304 mg) to afford **23** as a yellow oil (2.01 g, 37% yield). *R*_f = 0.41 (cyclohexane/ethyl acetate 4:1). ¹H NMR: δ 1.42 (s, 9H), 1.47–1.62 (m, 2H), 1.71–1.78 (m, 2H), 2.93 (t, *J* = 7.2 Hz, 2H), 3.05–3.20 (m, 2H), 4.62 (br s, 1H), 7.81 (t, *J* = 1.6 Hz, 1H), 7.97 (d, *J* = 1.6 Hz, 2H). ¹³C NMR: δ 21.4, 28.7 (3C), 29.6, 38.2, 40.3, 79.4, 123.3 (2C), 128.6 (2C), 133.7, 139.5, 156.1, 198.7. MS (ESI) *m/z* [M+H]⁺ Calcd for C₁₆H₂₂Br₂NO⁺₃: 434.00. Found: 434.0.

7.1.2.5. 6-(3-Methoxyphenyl)-2,3,4,5-tetrahydropyridine (24). Trifluoroacetic acid (7 mL) was added dropwise at 0 °C to compound **20** (2.74 g, 8.90 mmol) and then stirring was continued at room temperature for 3 h. After completion of the reaction, the mixture was cooled to 0 °C and quenched by dropwise addition of 30% NaOH aqueous solution until pH 10-11 and then extracted with diethyl ether (5 \times 20 mL). The combined organic layers were washed with brine (1 \times 60 mL), dried over anhydrous sodium sulphate and concentrated in vacuo to obtain 24 as a yellow oil (1.20 g, 71% yield). $R_{\rm f} = 0.22$ (cyclohexane/ethyl acetate 9:1). ¹H NMR: δ 1.64–1.71 (m, 2H), 1.79–1.88 (m, 2H), 2.60–2.66 (m, 2H), 3.81–3.86 (m, 2H), 3.84 (s, 3H), 6.94 (ddd, J = 7.7, 2.8, 1.7 Hz, 1H), 7.25–7.31 (m, 2H), 7.37–7.38 (m, 1H). ¹³C NMR: δ 19.9, 22.0, 27.4, 50.0, 55.6, 111.0, 116.2, 118.7, 129.4, 141.7, 159.9, 166.1. MS (ESI) m/z [M+H]⁺ Calcd for C₁₂H₁₆NO⁺: 190.12. Found: 190.1.

7.1.2.6. 6-(3-Chlorophenyl)-2,3,4,5-tetrahydropyridine (10). Reaction of compound **21** (1.10 g, 3.53 mmol) with trifluoroacetic acid as described for the synthesis of **24** gave compound **10** as a yellow oil (612 mg, 90% yield). $R_{\rm f} = 0.34$ (cyclohexane/ethyl acetate 4:1). ¹H NMR: δ 1.58–1.65 (m, 2H), 1.73–1.82 (m, 2H), 2.49–2.55 (m, 2H), 3.78–3.82 (m, 2H), 7.22–7.31 (m, 2H), 7.56–7.59 (m, 1H), 7.74–7.75 (m, 1H). ¹³C NMR: δ 19.8, 22.0, 27.1, 50.2, 124.2, 126.4, 129.6 (2C), 134.6, 142.1, 164.4. MS (ESI) *m/z* [M+H]⁺ Calcd for C₁₁H₁₃ClN⁺: 194.07. Found: 194.1.

7.1.2.7. 6-(3-Iodophenyl)-2,3,4,5-tetrahydropyridine (12). Reaction of compound **22** (2.60 g, 6.45 mmol) with trifluoroacetic acid as described for the synthesis of **24** gave compound **12** as a yellow oil (1.62 g, 88% yield). $R_{\rm f} = 0.38$ (cyclohexane/ethyl acetate 4:1). ¹H NMR: δ 1.63–1.71 (m, 2H), 1.80–1.88 (m, 2H), 2.55–2.61 (m, 2H), 3.82–3.86 (m, 2H), 7.10 (t, J = 7.8 Hz, 1H), 7.21–7.26 (m, 1H), 7.48–7.52 (m, 1H), 7.66–7.62 (m, 1H). ¹³C NMR: δ 19.8, 22.0, 27.2, 50.2, 122.9, 124.7, 129.4, 132.6, 138.6, 142.4, 164.2. MS (ESI) *m/z* [M+H]⁺ Calcd for C₁₁H₁₃IN⁺: 286.01. Found: 286.0.

7.1.2.8. 6-(3,5-Dibromophenyl)-2,3,4,5-tetrahydropyridine (13). Reaction of compound 23 (1.80 g, 4.14 mmol) with trifluoroacetic acid as described for the synthesis of 24 gave compound 13 as a yellow oil (591 mg, 45% yield). $R_{\rm f} = 0.66$ (cyclohexane/ethyl acetate 4:1). ¹H NMR: δ 1.57–1.63 (m, 2H), 1.73–1.78 (m, 2H), 2.44–2.49 (m, 2H), 3.75–3.79 (m, 2H), 7.58 (t, J = 1.8 Hz, 1H), 7.79 (d, J = 1.8 Hz, 2H). ¹³C NMR: δ 19.7, 21.9, 27.2, 50.3, 123.2 (2C), 128.1 (2C), 134.9, 143.6, 163.3. MS (ESI) m/z [M+H]⁺ Calcd for C₁₁H₁₂Br₂N⁺: 315.93. Found: 316.0.

7.1.2.9. 3-(3,4,5,6-Tetrahydropyridin-2-yl)phenol (5). A solution of compound 24 (201 mg, 1.06 mmol) in 48% aqueous HBr (3 mL) was stirred under refluxing conditions for 6 h and then was cooled to room temperature and stirring was continued overnight. The reaction mixture was concentrated under vacuum, the residue was adjusted to pH 11 with a saturated aqueous solution of sodium hydrogen carbonate and then extracted with ethyl acetate $(5 \times 20 \text{ mL})$. The combined organic layers were washed with brine $(1 \times 50 \text{ mL})$, dried over anhydrous sodium sulphate, filtered and concentrated under reduced pressure to obtain compound 5 as a yellow oil (147 mg, 79% yield). $R_f = 0.33$ (dichloromethane/methanol 9:1). ¹H NMR: δ 1.66–1.73 (m, 2H), 1.78–1.86 (m, 2H), 2.59-2.63 (m, 2H), 3.77-3.81 (m, 2H), 6.81-6.85 (m, 1H), 7.09-7.19 (m, 2H), 7.25–7.26 (m, 1H). ¹³C NMR: δ 19.6, 21.7, 28.0, 49.1, 113.9, 117.9, 118.2, 129.7, 140.8, 157.3, 169.4. MS (ESI) m/z [M+H]⁺ Calcd for C₁₁H₁₄NO⁺: 176.11. Found: 176.2.

7.1.2.10. Methyl-3-(3,4,5,6-tetrahydropyridin-2-yl)benzoate (25)To a solution of 12 (1.2 g, 4.21 mmol) in dioxane (4.5 mL), molybdenum hexacarbonyl (1.67 g, 6.32 mmol), 10% palladium on activated charcoal (200 mg), 4-(dimethylamino)pyridine (1.03 g, 8.42 mmol), N,N-diisopropylethylamine (1.09 g, 8.42 mmol) and methanol (1.7 mL, 42.10 mmol) were added and the mixture was sealed and heated in a microwave reactor at 130 °C using 50 W for 35 min (2 cycles). The reaction mixture was filtered over a Celite[®] pad and the filtrate was concentrated under reduced pressure. The residue was then taken up with a saturated aqueous solution of NaHCO₃ (15 mL) and extracted with ethyl acetate (3 \times 10 mL). The combined organic layers were washed with brine (1 \times 15 mL), dried over anhydrous sodium sulphate, filtered and concentrated under reduced pressure to afford a crude mixture which was purified by silica gel column chromatography (cyclohexane/ethyl acetate 85:15). The methyl ester intermediate 25 was obtained as a yellow oil (400 mg, 44% yield). $R_f = 0.3$ (cyclohexane/ethyl acetate 7:3). ¹H NMR: δ 1.64–1.72 (m, 2H), 1.81–1.89 (m, 2H), 2.62–2.69 (m, 2H), 3.81–3.88 (m, 2H), 3.91 (s, 3H), 7.44 (t, J = 7.7 Hz, 1H), 7.99 (dd, I = 7.7, 1.9 Hz, 1H), 8.04 (d, I = 7.7 Hz, 1H), 8.38 (d, I = 1.9 Hz, 1H). ¹³C NMR: *δ* 19.8, 22.0, 27.3, 50.2, 53.4, 126.2, 127.3, 128.6, 130.6, 130.8, 140.7, 165.2, 166.2. MS (ESI) m/z [M+H]⁺ Calcd for C₁₃H₁₆NO₂⁺: 218.12. Found: 218.1.

7.1.2.11. (3-(3,4,5,6-Tetrahydropyridin-2-yl)phenyl)methanol (6). To a solution of **25** (400 mg, 1.84 mmol) in anhydrous THF (37 mL) under inert atmosphere was added Red-Al 65% in toluene (2.4 mL, 7.73 mmol) and the mixture was stirred for 1 h at room temperature. Then, a saturated aqueous solution of NaHCO₃ (10 mL) was added to the reaction and the mixture was extracted with ethyl acetate (3×10 mL). The combined organic layers were dried over anhydrous sodium sulphate, filtered and concentrated under reduced pressure. The crude residue was purified by silica gel column chromatography (ethyl acetate) to provide the alcohol

derivative **6** as a yellow oil (178 mg, 51% yield). $R_f = 0.41$ (dichloromethane/methanol 85:15). ¹H NMR: δ 1.64–1.73 (m, 2H), 1.75 (br s, 1H), 1.77–1.88 (m, 2H), 2.60–2.65 (m, 2H), 3.81–3.86 (m, 2H), 4.71 (s, 2H), 7.34–7.38 (m, 2H), 7.63–7.66 (m, 1H), 7.76–7.77 (m, 1H). ¹³C NMR: δ 19.6, 22.6, 27.1, 49.8, 64.6, 126.8, 127.1, 128.8, 129.1, 140.8, 142.8, 168.2. MS (ESI) m/z [M+H]⁺ Calcd for C₁₂H₁₆NO⁺: 190.12. Found: 190.1.

7.1.3. Synthesis of target compounds 7, 8, 9, 11, 14 and 15

7.1.3.1. 6-m-Tolyl-2,3,4,5-tetrahydropyridine (7). A solution of mtolylmagnesium bromide 26, prepared from 416 µL of 3bromotoluene and 83 mg of magnesium turnings in anhydrous THF (3 mL), was added dropwise under inert atmosphere to a solution of 5-bromovaleronitrile (400 µL, 3.43 mmol) in anhydrous THF (3 mL). The mixture was stirred at room temperature for 1 h, guenched with a saturated agueous solution of NaHCO₃ (3 mL) and extracted with dichloromethane (3 \times 10 mL). The combined organic layers were dried over anhydrous sodium sulphate, filtered and concentrated under reduced pressure. The crude residue was purified by silica gel column chromatography (dichloromethane/ methanol 95:5) to provide the compound 7 as a yellow oil (225 mg, 38% yield). $R_f = 0.40$ (dichloromethane/methanol 95:5). ¹H NMR: δ 1.63–1.71 (m, 2H), 1.79–1.87 (m, 2H), 2.37 (s, 3H), 2.60–2.66 (m, 2H), 3.80-3.86 (m, 2H), 7.18-7.29 (m, 2H), 7.51-7.54 (m, 1H), 7.61–7.62 (m, 1H). ¹³C NMR: δ 20.0, 21.7, 22.1, 27.4, 50.1, 123.3, 126.8, 128.3, 130.5, 138.1, 140.4, 166.2. MS (ESI) m/z [M+H]⁺ Calcd for C₁₂H₁₆N⁺: 174.13. Found: 174.2.

7.1.3.2. 6-Phenyl-2,3,4,5-tetrahydropyridine (**8**). The title compound was obtained according to the method described for compound **7** by dropwise addition of a 1.8 M phenyllithium solution of **27** (914 μL, 1.65 mmol) in dibutyl ether to 5-bromovaleronitrile (170 μL, 1.46 mmol) in anhydrous THF (2.5 mL) under inert atmosphere. The mixture was refluxed for 3 h and after the usual workup the crude residue was purified by silica gel column chromatography (dichloromethane/methanol 95:5) to provide **8** as a yellow oil (197 mg, 85% yield). $R_{\rm f} = 0.32$ (dichloromethane/methanol 95:5). ¹H NMR: δ 1.63–1.71 (m, 2H), 1.79–1.88 (m, 2H), 2.60–2.66 (m, 2H), 3.81–3.86 (m, 2H), 7.36–7.39 (m, 3H), 7.75–7.78 (m, 2H). ¹³C NMR: δ 20.0, 22.1, 27.3, 50.2, 126.1 (2C), 128.4 (2C), 129.7, 140.5, 165.8. MS (ESI) m/z [M+H]⁺ Calcd for C₁₁H₁₄N⁺: 160.11. Found: 160.2.

7.1.3.3. 6-(3-Fluorophenyl)-2,3,4,5-tetrahydropyridine (9). The title compound was obtained according to the method described for compound 7. (3-Fluorophenyl)lithium 28 was prepared by dropwise addition of 1.6 M n-butyllithium (1.40 mL) in hexanes to 1bromo-3-fluorobenzene (259 µL, 2.32 mmol) in anhydrous diethyl ether (2.5 mL) at -78 °C. After stirring for 1 h at the same temperature, the reaction mixture was slowly cannulated into a flask containing a solution of 5-bromovaleronitrile (200 µL, 1.72 mmol) in anhydrous THF (2.5 mL) and stirred at room temperature for 12 h. After the usual work-up, the crude residue was purified by silica gel column chromatography (dichloromethane/methanol 95:5) to provide **9** as a light yellow oil (82 mg, 27% yield). $R_f = 0.31$ (dichloromethane/methanol 95:5). ¹H NMR: δ 1.62–1.70 (m, 2H), 1.78-1.86 (m, 2H), 2.55-2.60 (m, 2H), 3.80-3.85 (m, 2H), 7.02-7.09 (m, 1H), 7.26–7.35 (m, 1H), 7.45–7.52 (m, 2H). ¹³C NMR: δ 19.9, 22.0, 27.3, 50.1, 113.1 (d, J = 22.5 Hz), 116.5 (d, J = 21.3 Hz), 121.7 (d, *J* = 2.6 Hz), 129.8 (d, *J* = 8.0 Hz), 142.8, 163.1 (d, *J* = 244.0 Hz), 164.8. MS (ESI) *m*/*z* [M+H]⁺ Calcd for C₁₁H₁₃FN⁺: 178.10. Found: 178.3.

7.1.3.4. 6-(3-Bromophenyl)-2,3,4,5-tetrahydropyridine (**11**). The title compound was obtained according to the method described for compound **7**. (3-Bromophenyl)lithium **29** was prepared by addition of 1.6 M *n*-butyllithium (1.40 mL) in hexanes to 1,3dibromobenzene (280 μ L, 2.32 mmol) in anhydrous diethyl ether (2.5 mL) at -78 °C. After stirring for 1 h at the same temperature, the reaction mixture was slowly cannulated into a flask containing a solution of 5-bromovaleronitrile (200 μ L, 1.72 mmol) in anhydrous THF (2.5 mL) and stirred at room temperature for 12 h. After the usual work-up, the crude residue was purified by silica gel column chromatography (dichloromethane/methanol 98:2) to provide **11** as a yellow oil (129 mg, 32% yield). *R*_f = 0.30 (dichloromethane/methanol 98:2). ¹H NMR: δ 1.61–1.69 (m, 2H), 1.77–1.86 (m, 2H), 2.53–2.59 (m, 2H), 3.79–3.85 (m, 2H), 7.22 (t, *J* = 8.0 Hz, 1H), 7.48 (ddd, *J* = 8.0, 1.9, 0.8 Hz, 1H), 7.64–7.67 (m, 1H), 7.92 (t, *J* = 1.9 Hz, 1H). ¹³C NMR: δ 19.8, 22.0, 27.2, 50.2, 122.9, 124.7, 129.3, 130.0, 132.6, 142.3, 164.6. MS (ESI) *m/z* [M+H]⁺ Calcd for C₁₁H₁₃BrN⁺: 238.02. Found: 238.2.

7.1.3.5. 6-(3-Bromo-5-fluorophenyl)-2,3,4,5-tetrahydropyridine (14). The title compound was obtained according to the method described for compound 7. (3-Bromo-5-fluorophenyl)lithium 30 was prepared by addition of 1.6 M *n*-butyllithium (7 mL) in hexanes to 1,3-dibromo-5-fluorobenzene (1.5 mL, 11.57 mmol) in anhydrous diethyl ether (2.5 mL) at -78 °C. After stirring for 1 h at the same temperature, the reaction mixture was slowly cannulated into a flask containing a solution of 5-bromovaleronitrile (1 mL, 8.57 mmol) in anhydrous THF (12 mL) and stirred at room temperature for 12 h. After the usual work-up, the crude residue was purified by silica gel column chromatography (dichloromethane/ methanol 97:3) to provide **14** as a vellow oil (352 mg, 16% vield). $R_{\rm f} = 0.30$ (dichloromethane/methanol 99:1). ¹H NMR: δ 1.61–1.69 (m, 2H), 1.78–1.86 (m, 2H), 2.50–2.56 (m, 2H), 3.80–3.85 (m, 2H), 7.21–7.25 (m, 1H), 7.38–7.25 (m, 1H), 7.68–7.71 (m, 1H). ¹³C NMR: δ 19.7, 21.9, 27.2, 50.2, 112.2 (d, I = 22.4 Hz), 120.0 (d, I = 24.5 Hz), 122.7 (d, J = 9.4 Hz), 125.3 (d, J = 2.9 Hz), 143.8 (d, J = 7.1 Hz), 162.8 (d, J = 248.8 Hz), 163.5. MS (ESI) m/z [M+H]⁺ Calcd for C₁₁H₁₂BrFN⁺: 256.01. Found: 256.1.

7.1.3.6. *Methyl* 3-fluoro-5-(3,4,5,6-tetrahydropyridin-2-yl)benzoate (**31**). This compound was prepared according to the method described for compound **25** by employing **14** (200 mg, 0.78 mmol) and MeOH (316 μ L) to afford **31** as a yellow oil (57 mg, 31% yield) in 2 h. $R_f = 0.38$ (cyclohexane/ethyl acetate 7:3). ¹H NMR: δ 1.56–1.72 (m, 2H), 1.80–1.89 (m, 2H), 2.57–2.65 (m, 2H), 3.82–3.87 (m, 2H), 3.93 (s, 3H), 7.71–7.76 (m, 2H), 8.18 (s, 1H). ¹³C NMR: δ 19.6, 22.4, 26.3, 50.1, 53.5, 125.8 (d, *J* = 22.5 Hz), 126.1 (d, *J* = 24.7 Hz), 127.4 (d, *J* = 9.9 Hz), 131.8 (d, *J* = 2.7 Hz), 141.8 (d, *J* = 7.2 Hz), 162.7 (d, *J* = 246.3 Hz), 165.2, 166.3. MS (ESI) *m*/*z* [M+H]⁺ Calcd for C₁₃H₁₅FNO[±]₂: 236.11. Found: 236.2.

7.1.3.7. (3-Fluoro-5-(3,4,5,6-tetrahydropyridin-2-yl)phenyl)methanol (**15**). This compound was prepared according to the method described for compound **6** by employing compound **31** (57 mg, 0.24 mmol) and Red-Al 65% in toluene (308 μL, 1.01 mmol) to afford **15** as a yellow oil (17 mg, 34% yield) in 12h. $R_f = 0.20$ (cyclohexane/ ethyl acetate 2:8). ¹H NMR: δ 1.64–1.72 (m, 2H), 1.80–1.88 (m, 2H), 1.92 (br s, 1H), 2.57–2.62 (m, 2H), 3.81–3.85 (m, 2H), 4.69 (s, 2H), 7.06–7.14 (m, 1H), 7.31–7.37 (m, 1H), 7.51–7.53 (m, 1H). ¹³C NMR: δ 19.8, 21.9, 27.4, 50.0, 64.7, 112.3 (d, J = 22.5 Hz), 114.9 (d, J = 21.7 Hz), 120.0, 142.5 (d, J = 7.4 Hz), 143.7 (d, J = 7.1 Hz), 163.3 (d, J = 244.5 Hz), 165.4. MS (ESI) m/z [M+H]⁺ Calcd for C₁₂H₁₅FNO⁺: 208.11. Found: 208.2.

7.1.4. Preparation of hydrochlorides of derivatives **5-15**

7.1.4.1. 3-(3,4,5,6-Tetrahydropyridin-2-yl)phenol hydrochloride (**5** × **HCl**). A solution of the free base **5** (140 mg, 0.80 mmol) in dioxane (1 mL) was treated with a 4.0 M solution of HCl in dioxane.

After stirring at room temperature for 30 min, the solvent was removed in vacuo to provide the corresponding salt, which was crystallized from 2-propanol (135 mg, 80%).

5 × **HCI**: Light brown solid, mp 192–193 °C. ¹H NMR (CD₃OD): δ 1.99–2.03 (m, 4H), 3.26–3.29 (m, 2H), 3.82 (m, 2H), 7.18 (dd, J = 8.0, 2.2 Hz, 1H), 7.26 (t, J = 2.2 Hz, 1H), 7.33 (dd, J = 8.0, 1.1 Hz, 1H), 7.45 (dt, J = 8.0, 1.1 Hz, 1H). ¹³C NMR (CD₃OD): δ 17.0, 17.1, 19.2, 45.2, 114.2, 118.6, 121.9, 130.6, 133.1, 158.6, 183.8. MS (ESI) *m/z* [M]⁺ Calcd for C₁₁H₁₄NO⁺: 176.11. Found: 176.4. Anal. Calcd for C₁₁H₁₄CINO (211.69): C, 62.41; H, 6.67; N, 6.62. Found: C, 62.31; H, 6.89; N, 6.38.

7.1.4.2. (3-(3,4,5,6-*Tetrahydropyridin-2-yl*)*phenyl*)*methanol* hydrochloride (**6** × **HCl**). Crystallized from 2-propanol as light yellow prisms (yield 65%), mp 141–142 °C. ¹H NMR (CD₃OD): δ 2.03–2.04 (m, 4H), 3.30–3.32 (m, 2H), 3.84 (m, 2H), 4.72 (s, 2H), 7.62 (t, *J* = 7.7 Hz, 1H), 7.75–7.80 (m, 2H), 7.87 (m, 1H). ¹³C NMR: δ 17.0, 17.1, 19.1, 45.3, 62.9, 125.8, 126.5, 129.5, 132.0, 133.2, 143.9, 184.0. MS (ESI) *m/z* [M+H]⁺ Calcd for C₁₂H₁₆NO⁺: 190.12. Found: 190.2. Anal. Calcd for C₁₂H₁₆CINO (225.71): C, 63.85; H, 7.14; N, 6.21. Found: C, 63.75; H, 7.41; N, 6.03.

7.1.4.3. 6-*m*-Tolyl-2,3,4,5-tetrahydropyridine hydrochloride (**7** × **HCl**). Hygroscopic light brown solid, yield 88%. ¹H NMR (CD₃OD): δ 2.01–2.05 (m, 4H), 2.47 (s, 3H), 3.30–3.34 (m, 2H), 3.83–3.87 (m, 2H), 7.50–7.55 (m, 1H), 7.59–7.62 (m, 1H), 7.70–7.75 (m, 2H). ¹³C NMR (CD₃OD): δ 17.2, 17.3, 19.2, 20.2, 45.3, 125.1, 128.3, 129.4, 132.0, 135.7, 139.9, 183.9. MS (ESI) *m*/*z* [M+H]⁺ Calcd for C₁₂H₁₆N⁺: 174.13. Found: 174.2. Anal. Calcd for C₁₂H₁₆ClN (209.72): C, 68.73; H, 7.69; N, 6.68. Found: C, 68.41; H, 8.02; N, 6.40.

7.1.4.4. 6-Phenyl-2,3,4,5-tetrahydropyridine hydrochloride (**8** × **HCl**). Crystallized from ethanol/diethyl ether (4:1) as a beige solid (yield 91%), mp 88–90 °C. ¹H NMR (CD₃OD): δ 2.03 (m, 4H), 3.31 (m, 2H), 3.85 (m, 2H), 7.61–7.66 (m, 2H), 7.74–7.79 (m, 1H), 7.89–7.92 (m, 2H). ¹³C NMR (CD₃OD): δ 17.2, 17.3, 19.3, 45.5, 128.0 (2C), 129.6 (2C), 132.0, 135.0, 183.8. MS (ESI) *m/z* [M+H]⁺ Calcd for C₁₁H₁₄N⁺: 160.11. Found: 160.2. Anal. Calcd for C₁₁H₁₄ClN (195.69): C, 67.51; H, 7.21; N, 7.16. Found: C, 67.41; H, 7.43; N, 7.01.

7.1.4.5. 6-(3-*Fluorophenyl*)-2,3,4,5-*tetrahydropyridine hydrochloride* (**9** × **HCl**). Crystallized from 2-propanol as a light brown solid (yield 86%), mp 155 °C dec. ¹H NMR (CD₃OD): δ 2.04 (m, 4H), 3.31 (m, 2H), 3.87 (m, 2H), 7.52–7.57 (m, 1H), 7.68–7.76 (m, 3H). ¹³C NMR (CD₃OD): δ 17.0 (2C), 19.1, 45.7, 115.0 (d, *J* = 24.5 Hz), 121.6 (d, *J* = 21.4 Hz), 124.2 (d, *J* = 2.9 Hz), 131.8 (d, *J* = 8.0 Hz), 134.0 (d, *J* = 7.7 Hz), 163.0 (d, *J* = 246.5 Hz), 183.1. MS (ESI) *m*/*z* [M+H]⁺ Calcd for C₁₁H₁₃FN⁺: 178.10. Found: 178.3. Anal. Calcd for C₁₁H₁₃CIFN (213.68): C, 61.83; H, 6.13; N, 6.56. Found: C, 61.64; H, 6.27; N, 6.31.

7.1.4.6. 6-(3-*Chlorophenyl*)-2,3,4,5-*tetrahydropyridine hydrochloride* (**10** × **HCl**). Crystallized from 2-propanol as a beige solid (yield 85%), mp 144 °C dec. ¹H NMR (CD₃OD): δ 2.01–2.05 (m, 4H), 3.29–3.32 (m, 2H), 3.86 (m, 2H), 7.62–7.67 (m, 1H), 7.78–7.85 (m, 2H), 7.95–7.97 (m, 1H). ¹³C NMR (CD₃OD): δ 17.2 (2C), 19.0, 45.5, 126.6, 127.9, 131.1, 133.9, 134.5, 135.4, 183.0. MS (ESI) *m*/*z* [M]⁺ Calcd for C₁₁H₁₃ClN⁺: 194.07. Found: 194.1. Anal. Calcd for (230.13): C, 57.41; H, 5.69; N, 6.09. Found: C, 57.16; H, 5.97; N, 5.88.

7.1.4.7. 6-(3-Bromophenyl)-2,3,4,5-tetrahydropyridine hydrochloride (**11** × **HCl**). Crystallized from 2-propanol/diethyl ether (7:3) as a dark yellow solid (yield 78%), mp 161–164 °C. ¹H NMR (CD₃OD): δ 1.99–2.04 (m, 4H), 3.29–3.32 (m, 2H), 3.84 (m, 2H), 7.57 (t, *J* = 7.9 Hz, 1H), 7.83–7.86 (m, 1H), 7.93–7.97 (m, 1H), 8.07–8.08 (m, 1H). ¹³C NMR (CD₃OD): δ 16.9 (2C), 19.0, 45.5, 123.2, 126.8, 130.7,

131.2, 134.1, 137.5, 183.1. MS (ESI) *m/z* [M+H]⁺ Calcd for C₁₁H₁₃BrN⁺: 238.02. Found: 238.1. Anal. Calcd for C₁₁H₁₃BrClN (274.59): C, 48.12; H, 4.77; N, 5.10. Found: C, 47.85; H, 5.12; N, 4.84.

7.1.4.8. 6-(3-Iodophenyl)-2,3,4,5-tetrahydropyridine hydrochloride (**12** × **HCl**). Crystallized from 2-propanol as a colorless solid (yield 83%), mp 202–204 °C dec. ¹H NMR (CD₃OD): δ 2.00–2.04 (m, 4H), 3.27–3.32 (m, 2H), 3.85 (m, 2H), 7.41 (t, *J* = 7.9 Hz, 1H), 7.88–7.91 (m, 1H), 8.10–8.14 (m, 1H), 8.25 (t, *J* = 1.8 Hz, 1H). ¹³C NMR (CD₃OD): δ 16.9, 17.0, 19.0, 45.4, 94.3, 127.3, 131.0, 133.9, 136.5, 143.5, 183.0. MS (ESI) *m*/*z* [M]⁺ Calcd for C₁₁H₁₃IN⁺: 286.01. Found: 286.0. Anal. Calcd for C₁₁H₁₃CIIN (321.59): C, 41.08; H, 4.07; N, 4.36. Found: C, 40.99; H, 4.32; N, 4.28.

7.1.4.9. 6-(3,5-Dibromophenyl)-2,3,4,5-tetrahydropyridine hydrochloride (13 × HCl). Crystallized from diethyl ether as a colorless solid (yield 44%), mp 95–97 °C. ¹H NMR (CD₃OD): δ 2.00–2.03 (m, 4H), 3.26 (m, 2H), 3.86 (m, 2H), 8.07 (d, *J* = 1.5 Hz, 2H), 8.17 (t, *J* = 1.5 Hz, 1H). ¹³C NMR (CD₃OD): δ 16.8 (2C), 18.9, 45.6, 123.8 (2C), 129.8 (2C), 135.5, 139.4, 182.3. MS (ESI) *m*/z [M]⁺ Calcd for C₁₁H₁₂Br₂N⁺: 315.93. Found: 316.0. Anal. Calcd for C₁₁H₁₂Br₂ClN (353.48): C, 37.38; H, 3.42; N, 3.96. Found: C, 37.21; H, 3.72; N, 3.88.

7.1.4.10. 6-(3-Bromo-5-fluorophenyl)-2,3,4,5-tetrahydropyridine hydrochloride (**14** × **HCl**). Crystallized from 2-propanol/diethyl ether (7:3) as a dark yellow solid (yield 51%), mp 65 °C dec. ¹H NMR (CD₃OD): δ 2.03 (m, 4H), 3.27 (m, 2H), 3.86 (m, 2H), 7.70 (d, J = 8.0 Hz, 1H), 7.82 (d, J = 8.0 Hz, 1H), 7.92 (s, 1H). ¹³C NMR (CD₃OD): δ 16.9 (2C), 18.9, 45.7, 114.4 (d, J = 24.5 Hz), 123.8 (d, J = 9.7 Hz), 124.7 (d, J = 24.5 Hz), 127.1 (d, J = 3.1 Hz), 135.4 (d, J = 8.6 Hz), 162.8 (d, J = 251.1 Hz), 182.7. MS (ESI) *m/z* [M]⁺ Calcd for C₁₁H₁₂BrFN⁺: 256.01. Found: 256.0. Anal. Calcd for C₁₁H₁₂BrCIFN (292.58): C, 45.16; H, 4.13; N, 4.79. Found: C, 44.91; H, 4.39; N, 4.52.

7.1.4.11. (3-Fluoro-5-(3,4,5,6-tetrahydropyridin-2-yl)phenyl)methanol hydrochloride (**15** × **HCl**). Hygroscopic dark yellow solid, yield 75%. ¹H NMR (CD₃OD): δ 2.01–2.05 (m, 4H), 3.30–3.32 (m, 2H), 3.84–3.86 (m, 2H), 4.72 (s, 2H), 7.52–7.58 (m, 2H), 7.69 (m, 1H). ¹³C NMR: δ 17.1 (2C), 19.1, 45.3, 62.8, 115.3 (d, *J* = 18.3 Hz), 125.2 (d, *J* = 2.9 Hz), 132.7 (d, *J* = 7.4 Hz), 133.2 (d, *J* = 24.2 Hz), 143.8 (d, *J* = 7.1 Hz), 163.0 (d, *J* = 247.9 Hz), 183.8. MS (ESI) *m/z* [M+H]⁺ Calcd for C₁₂H₁₅FNO⁺: 208.11. Found: 208.2. Anal. Calcd for C₁₂H₁₅ClFNO (243.71): C, 59.14; H, 6.20; N, 5.75. Found: C, 58.79; H, 6.54; N, 5.43.

7.2. Pharmacology

7.2.1. Receptor binding assays

Details of the binding experiments to the nicotinic receptor subtypes have been recently reported for the $\alpha 4\beta 2$ and $\alpha 7$ subtypes [57] as well as for the $\alpha 3\beta 4$ subtype [58]. The K_i values of the novel compounds **5-15** were determined by pre-incubating cortex or hippocampus homogenates with increasing doses (10 pM–10 mM) of the reference nicotinic agonists, epibatidine or nicotine, and the drug to be tested for 30 min at room temperature, followed by overnight incubation with a final concentration of 0.100 nM [³H]-epibatidine (cortex) or 1 nM [¹²⁵I]- α -bungarotoxin (hippocampus), at the same temperatures as those used for the saturation experiments.

In the case of HEK 293 transfected $\alpha 3\beta 4$ receptors, the inhibition of [³H]epibatidine binding by the studied derivatives was measured by incubating increasing concentrations of the compounds for 5 min followed by overnight incubation with 0.25 nM [³H]epibatidine. For each subtype, the experimental data were analyzed using the LIGAND program as described by Munson and Rodbard [59]. The binding parameters were calculated by simultaneously fitting three independent saturation experiments and the K_i values were determined by fitting the data of three independent competition experiments. The errors in the K_D and K_i values of the simultaneous fits were calculated using the LIGAND software, and were expressed as percentage coefficients of variation (% CV). When final compound concentrations up to 100 μ M did not inhibit radioligand binding, the K_i value was defined as being >100 μ M based on the Cheng and Prusoff's equation [60].

7.2.2. Electrophysiological recordings

The human α 7, α 4 β 2 and α 3 β 4 nAChRs were expressed by transient transfection in the rat anterior pituitary GH4C1 cell line [61], grown in Ham's F10 medium supplemented with 10% heatinactivated FBS and 1% penicillin/streptomycin, at 37 °C in a 5% CO₂ humidified atmosphere. Cells were plated on poly-L-lysinecoated coverslides (1 \times 10⁵ cells/mL) and transiently transfected 24 h later using MagnetofectionTM: NeuroMag (OZ Biosciences, France) according to manufacturer's protocol, adding 0.5 µg of each cDNA subtype per well. All culture media were purchased from Invitrogen (San Giuliano Milanese, Italy). Whole-cell current recordings were performed 2-3 days after plating. Recordings and data analysis were performed by using borosilicate glass patch pipette (3- to 6-M Ω tip resistance) connected to an Axopatch 200A amplifier (Axon Instruments, Foster City, CA). Data were stored on a PC computer by using PCLAMP10 software (Molecular Devices). During the recording period, the cells were bathed in the following solution (mM): 140 NaCl, 2 CaCl₂, 2.8 KCl, 2 MgCl₂, 10 Hepes/NaOH and 10 glucose; pH 7.3. The patch pipettes were filled with a solution containing (mM): 140 CsCl, 2 MgATP, 10 Hepes/CsOH and 5 BAPTA; pH 7.3. Whole-cell capacitance and patch series resistance $(3-5 M\Omega)$ were estimated from slow transient compensations. A series resistance compensation of 85-90% was obtained in all cases. The cells were voltage-clamped at a holding potential of -70 mV and continuously perfused with a gravity-driven system using independent external tubes for the control and agonistcontaining solutions. These tubes were positioned 50-100 µm from the patched cell and connected to a fast exchanger system (RSC-160, BioLogic, France). Dose-response relationships were constructed by sequentially applying different concentrations of agonists, and normalizing the obtained current amplitudes to the value obtained by applying 1 mM ACh on the same cell. For guantitative estimations of agonist actions, dose-response relationship were fitted to the Equation (1):

$$I = I_{\max} \left\{ \left[C \right]^{nH} / \left(E C_{50}^{nH} + \left[C \right]^{nH} \right) \right\}$$

$$\tag{1}$$

where I is the current amplitude induced by the agonist at concentration [*C*], I_{max} is the maximum response of the cell, nH is the Hill coefficient and EC₅₀ is the concentration for which a half maximum response is induced.

7.3. Docking and MD simulations

The $\alpha 4\beta 2$, $\alpha 3\beta 4$ and $\alpha 7$ nAChR models [62,63,37], obtained by homology modeling on the crystal structure of *A*-AChBP/**1** (PDB accession code 2BYQ), were utilized to design and to rationalize the results obtained for the compounds under investigation. The receptor models were previously geometrically refined following the molecular modeling standard procedures: minimization, equilibration and molecular dynamics simulation. Ligands utilized for docking were preliminarily minimized by Gaussian09 [64] at the DFT/B3LYP/6-31G(d) level. The net charge of all ligands was +1. Docking calculations were then performed with the GOLD 5.2 program [65] into the binding cleft depicted by α 4-Trp147 and the homologue residues in the α 3 and α 7 subtypes. The cavity was detected with an active site radius of 12.0 Å from the carbonyl oxygen atom of the Trp residue. The PLP fitness function was used to assign the score to the different docking poses and the genetic algorithm parameters were kept at the default value. Cluster analysis was performed by means of the GOLD internal algorithm, based on the RMSD of each pose compared with the one previously obtained. The solutions with the highest score, belonging to the first cluster, were visually inspected and then chosen for further analysis. Then, the resulting complexes were submitted to molecular dynamics (MD) simulations with the sander and pmemd.cuda modules of the AMBER12 [66] package. ff12 [64] and GAFF [67] force fields were applied for the protein and the ligands, respectively. The complexes were immersed in a box containing about 30000 water molecules and the TIP3P model was employed to explicitly represent the solvent [68]. Counter ions were added to neutralize the total charge of the simulating systems.

At first, the energy of the water molecules was minimized, keeping the atoms of the protein frozen. Then, a minimization of the whole system was performed by setting a convergence criterion on the gradient of 10^{-4} kcal mol⁻¹ Å⁻¹. Prior to starting the MD simulations, the system was equilibrated for 40 ps at 300 K in isocore conditions (NVT). Subsequently, 50 ns of MD simulations in isothermal-isobaric ensemble were carried out at 300 K with a 2 fs time-step (NPT). In the production runs, the systems were performed in periodic boundary conditions. Van der Waals and shortrange electrostatic interactions were estimated within a 8 Å cutoff. SHAKE algorithm was applied to all bonds involving hydrogen atoms. When the geometrical stabilization of the complexes was reached, a new minimization of the whole system was performed. The water occupancy over a 50 ns MD simulations was calculated by VolMap tool of VMD [38]. Figures were acquired by the PyMOL software (The PyMOL Molecular Graphics System).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2015.11.045.

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