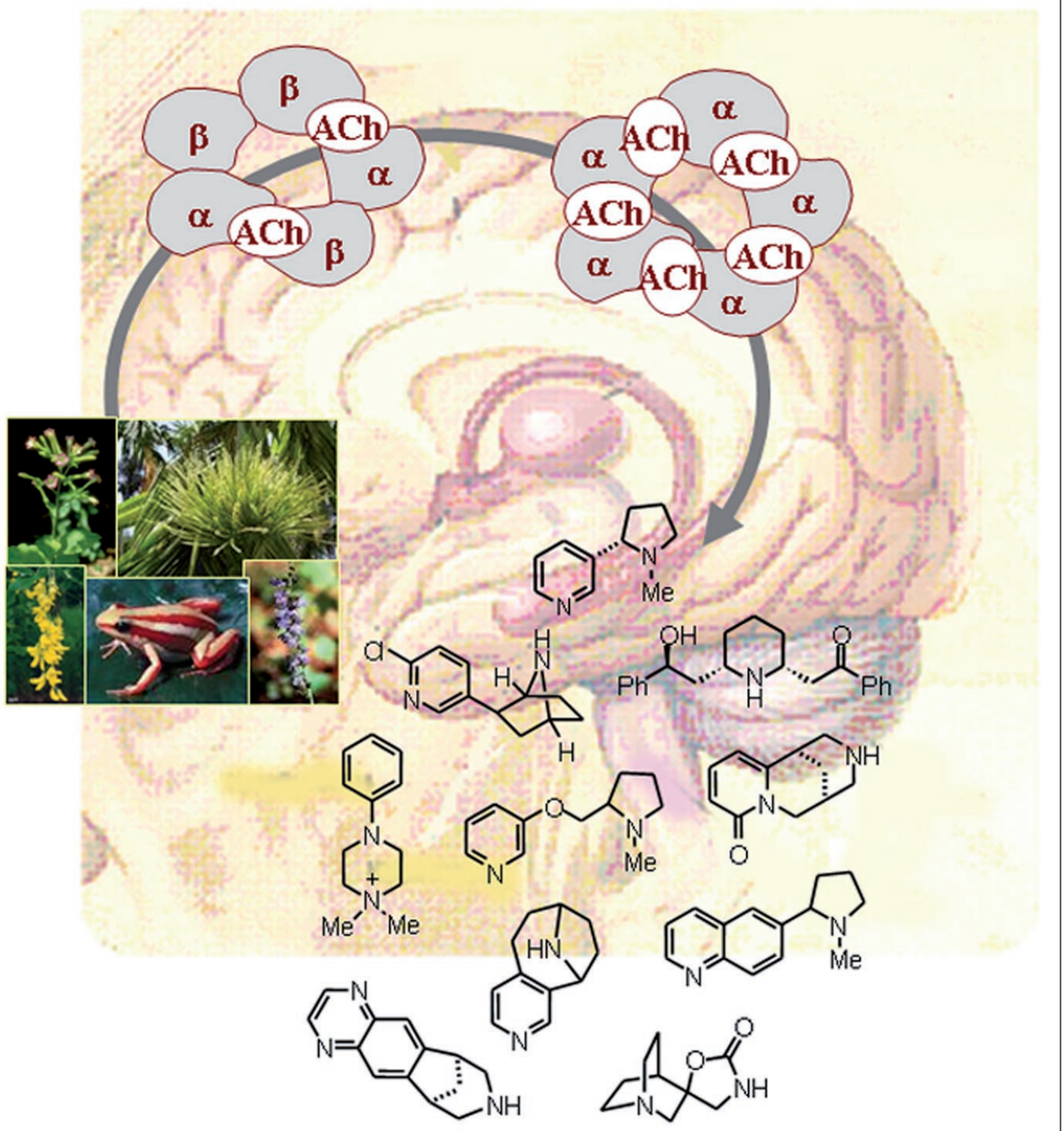

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Central Nicotinic Receptors: Structure, Function, Ligands, and Therapeutic Potential

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The growing interest in nicotinic receptors, because of their wide expression in neuronal and non-neuronal tissues and their involvement in several important CNS pathologies, has stimulated the synthesis of a high number of ligands able to modulate their function. These membrane proteins appear to be highly heterogeneous, and still only incomplete information is available on their structure, subunit composition, and stoichiometry. This is due to the lack of selective ligands to study the role of nAChR under physiological or pathological conditions; so far, only compounds showing selectivity between $\alpha4\beta2$ and $\alpha7$ receptors have been obtained. The nicotinic receptor ligands have been designed starting from lead compounds from natural sources such as nicotine, cytisine, or epibatidine, and, more recently, through the high-throughput screening of chemical libraries. This review focuses on the structure of the new agonists, antagonists, and allosteric ligands of nicotinic receptors, it highlights the current knowledge on the binding site models as a molecular modeling approach to design new compounds, and it discusses the nAChR modulators which have entered clinical trials.

Introduction

The history of cholinergic receptors goes back to the beginning of the 20th century when Sir Henry Dale studied the alkaloids muscarine and nicotine on a variety of cholinergic preparations giving origin to the muscarinic/nicotinic classification of acetylcholine receptors.[1] It was soon discovered that nicotine-activated cholinergic receptors were present in ganglia and on muscle endplates of the neuromuscular junctions.[2,3] In the 1970s, the abundance of nicotinic synapses in the electric organ of *Torpedo*, together with the use of high affinity ligands such as α-bungarotoxin, facilitated the study of the structure and functions of nicotinic receptor protein. The *Torpedo* receptor was purified and resolved into four different subunits designated α, β, γ (or ε), and δ.[4] Shortly after, these subunits were cloned and sequenced, paving the way for the molecular analysis of the nicotinic receptor, in particular that of the neuromuscular junction, a pentameric protein showing a $(\alpha)_2\beta\gamma\delta$ stoichiometry and spanning the membrane, where it functions, like all nicotinic receptors, as a ligand-gated ion channel.

The existence of central nicotinic receptors was discovered and firmly established in the early 1980s,[5,6] almost a decade after the corresponding neuromuscular and ganglionic species had been isolated and characterized. Since then, molecular biology techniques have greatly expanded our understanding of the structure and diversity of peripheral and central nicotinic receptors. The architecture of neuronal nicotinic receptors was found to be similar to that of the peripheral counterparts but, in contrast to the muscle subtype, neuronal receptors contain only subunits of α and β families.[7] Whereas the subunit composition of peripheral nicotinic receptors is quite constant, in the brain the pentameric combination of several α and β subunits makes the existence of a great number of nicotinic receptors possible, whose physiological significance still needs to be completely understood.[8]

The interest in central cholinergic receptors as drug targets is the obvious consequence of the cholinergic hypothesis of Alzheimer's disease, which assumes that the dysfunction of the central cholinergic system is the main determinant of this pathology.[9,10] In the beginning, the search for drugs to restore the impaired central cholinergic tone involved almost exclusively the muscarinic receptors or indirect activators of the cholinergic system such as acetylcholinesterase inhibitors.[11] Nicotinic ligands were practically neglected, mainly because of the lack of information on neural nicotinic receptors, but also because of the negative connotation associated with tobacco smoking. However, in the past few years, thanks to progress in molecular biology, physiology, and pharmacology of neuronal nicotinic receptors,[12–14] the potential of nicotinic ligands for the treatment of neurodegenerative disorders and other pathological states has been recognized, and thus has led to the present high interest in these kinds of drugs.

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Nicotinic receptors organization, localization, and function

The nicotinic acetylcholine receptors (nAChR) belong to the family of ligand-gated ion channels (LGIC), which also include $GABA_A$, $GABA_C$, glycine, and $5HT_3$ receptors; they are composed of five subunits assembled to form a pore which is permeable to cations such as Na^+, K^+, or Ca^{++}. Historically, nAChR were divided into muscle-type receptors, found at the skeletal neuromuscular junction where they mediate neuromuscular transmission, and neuronal receptors, found throughout the central and peripheral nervous system; nowadays this classification does not always hold up as neuronal receptors have been found also in non-neuronal tissues.[15]

The muscle-type nicotinic receptor is the best studied, as it can be found in large amounts in the *Torpedo* electric ray, from which it can be extracted and purified. It is formed by four different subunits with stoichiometry $(\alpha)_2\beta\gamma\delta$ (or $(\alpha)_2\beta\varepsilon\delta$ in the adult), and it carries two nonequivalent binding sites located at the interface between the α/γ and α/δ subunits. All the subunits are formed of a large extracellular N-terminal domain, followed by three hydrophobic transmembrane fragments (M1–M3), a large intracellular loop containing consensus sequences

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of phosphorylation sites, and a fourth transmembrane portion (M4) before the C-terminal part. The α-subunits differ from the others by the presence of two adjacent cysteine residues in the N-terminal domain.[16,17]

The receptor from *Torpedo* has been subjected to several structural studies which culminated in an atomic-scale model at 4 Å resolution;[18] these studies, combined with biochemical studies, in vitro electrophysiology, and molecular genetics, have helped to clarify some structure–function relationships.[19] Much of the structural information extracted from the muscle-type receptor can be extended to the neuronal subtypes, which are more difficult to study for several reasons, the most relevant one being that neuronal nAChR are heterogeneous. Of the seventeen different subunits which have been cloned so far, twelve (α2-10 and β2-4) are found in neuronal receptors; these subunits are differently distributed in the areas of the nervous system, some in low abundance, and the number of possible combinations is not yet known.[17,20]

On the basis of binding studies, the neuronal nAChRs can be divided into two groups: 1) the α-Bungarotoxin (α-Bgtx) sensitive receptors, which can be homomeric, composed of five α (α7–α9) subunits, or heteromeric, made up of different α subunits (α7 and α8, or α9 and α10); 2) the α-Bgtx insensitive receptors, which are composed of different combinations of α (α2–α6) and β (β2–β4) subunits, whose prevalent stoichiometry is believed to be $(\alpha)_2(\beta)_3$. The number of binding sites depends on the number and type of α subunits: for instance in the homomeric receptors such as $(\alpha7)_5$, five identical binding sites are present, whereas in the heteromeric receptors such as $(\alpha)_2(\beta)_3$ there are two binding sites, located at the interface between the α2–4 or α6 and the β2 or β4 subunits, the α5 or the β3 being considered only auxiliary subunits.[21]

The different combination of α and α/β subunits gives receptors which differ in terms of cation permeability, activation and desensitization kinetics, and ligand pharmacology.[20] The stoichiometry between subunits can also affect the functional properties, as it has been reported that different classes of subtypes are formed in heterologous systems when the ratio of the injected cDNA is varied (reviewed in ref. [15]). This is important as many subtypes can be studied only in recombinant systems.

The majority (more than 90%) of the nAChR in the central nervous system (CNS) contain the α4 and β2 subunits, whereas the other major subtype contains α7; the most abundant subunits in the peripheral nervous system (PNS) are α3 and β3. The α4β2*, α7*, and α3β4* are the subtypes which are best characterized in terms of ligand selectivity, as they can be purified from animal tissues and studied by means of binding techniques; [^{3}H]-cytisine, [^{3}H]-nicotine, or [^{3}H]-epibatidine can label α4β2* receptor, [^{3}H]-epibatidine is used for α3β4* receptors, [^{125}I]-Bgtx or [^{3}H]-methyllycaconitine ([^{3}H]-MLA) are used to label α7* receptors. However, selective ligands are still lacking (see next section):[14] for instance α-Bgtx binds to α7*, α8*, and α9* homomeric and heteromeric receptors, MLA also recognizes α3/α6* receptors,[22] epibatidine binds with high affinity to nicotinic receptors containing α2–α4 and β2–β4 subunits expressed in recombinant systems.[23] The asterisk used in the receptor nomenclature means that the receptor complex may contain additional subunits; in this review it is referred to as native receptors.

The nAChR can exist in three different conformations which are in equilibrium: the resting, active, and desensitized states. Agonists bind to the active state, but show higher affinity for the desensitized one owing to a conformational change produced by the agonist, which modifies the geometry of the binding site and increases the affinity of the ligand; the affinity values found in binding studies using an agonist as radioligand should reflect its affinity to the desensitized state.[14] In the desensitized state, the channel does not respond to further stimuli, being functionally deactivated. Therefore, an agonist which induces sustained desensitization behaves as an antagonist. This can explain why in some instances the effect of nicotine can be mimicked by nicotinic antagonists such as mecamylamine.

Nicotinic receptors are localized in several areas of the nervous system:[17,20] they are preferentially located presynaptically, regulating the release of other neurotransmitters,[12] but they may be also present on postsynaptic membranes (reviewed in ref. [15]). Neuronal nicotinic receptors may also be present in non-neuronal tissues such as lymphocytes,[24] macrophages,[25] lungs,[26] keratinocytes,[27] vascular endothelium,[28] and others.[29]

Regarding the function of nicotinic receptors, a lot of work has been performed using knock-out and knock-in mice (recently reviewed by Gotti and Clementi[15]). These studies have confirmed the important role of the nicotinic receptors in ganglionic transmission (α3 and β4 subunits), in the neuroprotection of the dopaminergic system and nociception (α4), in learning, memory, and addiction (β2). Moreover, some known diseases result from genetic alterations at the nicotinic receptors: for instance, congenital myasthenic syndromes may be attributable to a genetic defect that occurs in the ligand binding domain or in the channel pore domain of the muscle-type receptor;[19] mutation of the genes for α4 or β2 subunits can lead to autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE);[30] the alpha7 nicotinic acetylcholine receptor gene, CHRNA7, is associated with genetic transmission of schizophrenia.[31]

Therapeutic potential of nicotinic drugs

Nicotine has been used for centuries throughout the world in the form of tobacco products and its pharmacological effects have guided the potential application of nicotinic drugs in therapy. Epidemiological evidence, such as that showing a negative correlation between smoking and the incidence of Alzheimer's diseases (AD) or Parkinson's disease (PD), supported by in vitro and in vivo studies,[32] has identified several pathological states that might benefit from nicotinic drugs. As a matter of fact, the main role of nicotinic receptors in the brain seems to be that of enhancing neurotransmitter release,[33] which can be highly beneficial for several pathological states.[34,35]

Neurodegenerative diseases and cognition

Nicotinic cholinergic systems are involved in several important aspects of cognitive functions, including attention, learning, and memory.[36–38] One of the most consistent observations in relation to normal human brain aging is the widespread decline in nicotinic receptors.[39] It is reasonable that this reduction can be one of the causes of mild cognitive impairment (MCI) and predispose subjects to neurodegenerative disorders such as AD and PD to which cognitive impairment is associated. Targeting presynaptic nicotinic receptors, that enhance the release of neurotransmitters such as acetylcholine and dopamine, may therefore be advantageous for AD and PD, respectively. Among the many subtypes present in the brain, the $\alpha4\beta2^*$ and $\alpha7^*$ subtypes seem particularly involved in cognitive processes.[40] In this respect, it has been recently shown that stimulation of $\alpha7^*$ nicotinic receptors protects neuronal cells from degeneration and neuronal death induced by $A\beta_{42}$ amyloid protein that has a high affinity for this subtype.[41] On this basis, it has been proposed that $\alpha7^*$ receptors, by serving as a gateway for $A\beta_{42}$ entry and accumulation into neurons by endocitosis, may play a key role in pathological accumulation of $A\beta_{42}$ in neurons that express this subtype.[42]

Psychiatric disorders

Central nicotinic receptors are associated with a number of psychiatric disorders.[13] It has been found that nicotinic receptors, in particular the $\alpha7^*$ subtype, are reduced in number in the post mortem brains of schizophrenic patients[43,44] and nicotine is beneficial in normalizing sensory gating and improving cognitive deficit.[45] Several studies have reported an association between smoking and major depression and anxiety.[43] However, the effects of nicotine on these pathological states are complex. For instance nicotine can be either anxiolytic or anxiogenic depending on the model tested.[46] In this respect, it has been shown that a centrally acting nicotinic receptor antagonist such as mecamylamine is able to reduce symptoms of depression and mood instability, suggesting that centrally acting nicotinic antagonists may represent a new class of drugs for treating mood disorders.[47] Very recently, it has been proposed that nicotinic cholinergic antagonists could represent a novel approach for the treatment of autism.[48]

Pain

Control of pain is one of the most promising therapeutic applications of nicotinic drugs, namely agonists.[49,50] The discovery of the antinociceptive activity of nicotine dates back to 1932, but interest in nicotinic receptor mediated analgesia really started only after the discovery of the outstanding analgesic properties of epibatidine and of its high affinity for the nicotinic receptors. However, utilization of epibatidine as an analgesic is compromised by its serious side effects that originate from poor selectivity among the nicotinic receptor subtypes. The $\alpha4\beta2^*$ subtype has been identified as being involved in the antinociceptive activity, but other subtypes could also contribute.[49,51] The properties of epibatidine have stimulated the design and study of a number of new nicotinic agonists, characterized by strong antinociceptive activity but showing better subtype selectivity; some of them have reached clinical development and will be described in a later section.

Tobacco dependence

Nicotine addiction is one of the most prevalent addictive behaviors worldwide, involving almost a billion individuals. Several strategies are available to aid smoking cessation and use of nicotinic drugs is one of the most popular;[52,53] nicotine itself is available in various formulations and delivery systems. Nicotinic antagonists such as mecamylamine can be used but recently it has been proposed that partial agonists could give better results, as has been shown for varenicline.[54]

Epilepsy

The association between a form of genetically transmissible epilepsy, the autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE), and a mutation in CHRNA4, the gene coding for the $\alpha4$ subunit of nicotinic acetylcholine receptors, has started the search for nicotinic drugs to treat this condition. As the mutation increases sensitivity to acetylcholine, antagonists or, better, negative allosteric modulators of the nicotinic receptor could represent a way to control the disease.[55]

Tourette's syndrome

Tourette's syndrome (TS) is a hyperkinetic movement disorder with symptoms of sudden, rapid and brief, recurrent, stereotyped motor movements or sounds. TS represents a disorder related to excess dopamine transmission in the striatum. Transdermal nicotine reduces the symptoms, probably desensitizing the nicotinic receptors that control dopamine release, as confirmed by the effect of an antagonist such as mecamylamine in controlling the pathology.[13]

Ligands

In the last few years several papers have reviewed the high number of nicotinic ligands discussed in the literature;[14,20,56–59] therefore, only the most recent compounds are included in this review.

Agonists

This section reports the molecules which have been shown to activate the nicotinic receptor, or which have been designed starting from an agonist lead compound, but it must be noted that in most of the cases only affinity data have been reported, and no functional effect has been measured.

Nicotine analogues. The prototype of nAChR agonists is nicotine (**1**, Figure 1), which has stimulated the synthesis of several analogues, differing by the pattern of substitution on the pyri-

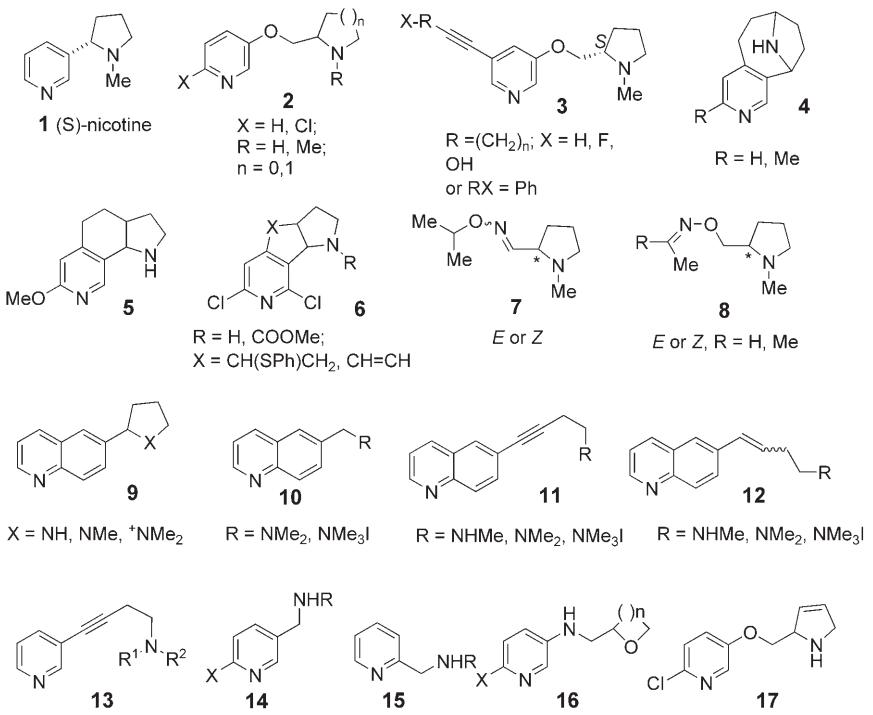


Figure 1. Nicotine analogues.

dine or the pyrrolidine rings, or by the conformational flexibility.

Among the flexible nicotine analogues, the pyridyl ethers **2** synthesized in Abbott laboratories such as **2a** (A-84543: R = Me, $n = 1$, X = H) or **2b** (ABT-594: R = H, $n = 0$, X = Cl) have attracted considerable interest because of their high potency, high selectivity for the $\alpha4\beta2$* subtype, and synthetic accessibility which has made extensive structural modifications possible;[14,20,58] a few of them have also entered clinical trials (see below). Recently, some new analogues have been synthesized (for example **3**, Figure 1), carrying in position 5 of the pyridine ring an unsaturated alkyl chain of variable length. Compounds **3a** ($RX = (CH_2)_4OH$) and **3b** ($RX = (CH_2)_4F$), having an OH or F moiety at the end of the chain, respectively, showed nanomolar affinity for the $\beta2$-containing receptors and higher selectivity compared to the unsubstituted compound.[60] Modeling studies suggested that high affinity and selectivity could be due to a favorable orientation of the ligand in the $\alpha4\beta2$ but not in the $\alpha3\beta4$ nAChR binding site, leading to the formation of a H-bond with a serine residue in the $\beta2$ subunit.

Pyrido[3,4-b]homotropane (PHT, **4a**, R = H) is a rigid nicotine analogue synthesized as racemate in the 1980s.[61] Recently, its methyl derivative **4b** (R = Me) has also been prepared[62] and found to be three times more potent than **4a** in displacing [^{3}H]nicotine from the $\alpha4\beta2$* receptor of mouse fibroblast M10 cells ($K_i = 0.39$ nM and 1.3 nM, respectively). Following the same synthetic pathway used for the racemate, but using an enantiopure reactant, Carroll and co-workers were able to obtain the enantiomers of **4a** and to assess their functional properties.[63] (1*R*,6*R*)-**4a** showed an affinity 260 times higher than (1*S*,6*S*)-**4a** on the rat brain nAChR labeled by [^{3}H]epibatidine. This confirms the previous speculations on the absolute configuration of the eutomer,[64] but such high eudismic ratio is unusual in nicotinic ligands carrying a secondary amine function. However, (1*R*,6*R*)-**4a** and *rac*-**4a** behave as low-efficacy partial agonists in the tail-flick and hot-plate tests for analgesic activity, whereas (1*S*,6*S*)-**4a** is a potent nicotinic antagonist in the same tests. Thus, increasing the volume of the agonists can affect not only efficacy but also affinity, as happens for **5**, another bulky rigid nicotine analogue; compared to nicotine, *rac*-**5** has 500-fold lower affinity for the nAChR of the rat brain and both *rac*-**5** and the eutomer (3a*R*,9b*S*)-**5** are partial agonists on several nAChR subtypes (increase of [Ca^{2+}] influx in HEK cells transfected with human nAChR).[65] Compound **6a** (R = H, $X = CH_2CHSPh$), structurally related to **5**, is also a partial agonist on $\alpha3\beta4$ receptors in the KX$\alpha3\beta4$R2 cell line. Interestingly compound **6b** (R = COOMe, X = CH=CH), in which a carbamate function replaces the potentially cationic pyrrolidine nitrogen, behaved as a full agonist on the same receptors, its potency being similar to nicotine. The affinity of **6a** is however higher than that of **6b**, on both $\alpha4\beta2$ and $\alpha3\beta4$ receptors, when measured by the displacement of epibatidine in liquid chromatography studies utilizing immobilizing nAChR stationary phase.[66,67]

The compounds with general formula **7** and **8** are analogues of nicotine or **2a**, in which the pyridyl ring has been replaced with an immino or oxyimmino group.[68] These compounds showed affinity in the micromolar range for the rat brain nicotinic receptor labeled by [^{3}H]-epibatidine; the activity depends on the configuration at the pyrrolidine stereocenter (the *S* isomer was 4–30 times more active than the *R* form) rather than on the *E*/*Z* isomerism. Among the synthesized compounds, only (*E*)-(*R*)-**7** and (*S*)-**8a** (R = Me) were able to interact with αBtgx-sensitive receptors of rat brain, with an affinity that is 9- and 94-times lower, respectively, than that on the $\alpha4\beta2$* subtype.

Replacement of the pyridine ring with a quinoline moiety gave compound **9a** (X = NMe); **9a** and the corresponding quaternary ammonium derivative **9b** ($X = NMe_2I$) show affinity for nAChR (displacement of [^{3}H]cytisine from rat brain homogenate) with K_i values 17 and 5 times higher than nicotine, respectively.[69] These compounds behave as nicotinic agonists in the hot-plate test on mice when injected i.c.v.[70] Compounds **9** were obtained by optimization of **10a** ($R = NMe_2$) and the methiodide **10b** ($R = NMe_3I$), which were designed through a 3D-

database search approach. **10a** and **10b** showed affinity in the micromolar range ($K_i = 6$ μM and 0.15 μM, respectively, against [^{3}H]cytisine). Their isomers in position 5 or 7 were completely devoid of affinity.[69]

Extending the distance between the quinoline and the cationic nitrogen atoms through a butynyl chain gave the compounds with general formula **11.** The ammonium derivative **11a** (R = NMe_3I) was shown to interact with the nicotinic receptor, whereas the corresponding tertiary and secondary amines were devoid of affinity. Reduction of the triple bond gave the corresponding *cis* and *trans* alkenes **12**; only the methiodide of the *trans* derivative (**12a**, R = NMe_3I) was able to displace [^{3}H]cytisine from rat cerebral cortex ($K_i = 1.35$ μM). Compound **11a** behaved as an agonist in the hot-plate test after i.c.v. injection, its efficacy being similar to that of **9a** and **9b.**[70] The trend of affinity found in the (6-quinolinyl)butynyl series is different from that reported for their 3-pyridyl analogues: compound **13a** (R^1 = H, R^2 = Me) showed an affinity for rat brain homogenate 4–5 times higher than that of the dimethylamino and trimethylammonium analogues;[71] the agonistic properties of these latter compounds have not been determined.

Compounds **14**–**17** have been designed as nicotinic agonists with the aim of discovering new analgesic drugs, and they may be considered as structural analogues of nicotine or **2b**. However, only analgesic activity is reported, whereas possible interactions with the nicotinic receptor were not determined. Compounds of general formula **14**, but not **15** or **16**, showed appreciable analgesic activity in the writhing test on mice;[72] as far as compound **17** is concerned, the *R* isomer showed analgesic activity in the formalin test on mice.[73]

Epibatidine analogues. Epibatidine **18** (Figure 2) is an alkaloid isolated from the skin of the Ecuadorian frog *Epipedobates tricolor*. The natural isomer has the 1*R*,2*S*,4*S* configuration, but most of the studies have been performed on the racemate as the two enantiomers have the same affinity. Epibatidine is endowed with high potency but also high toxicity due to its low subtype selectivity, which precludes its use as a drug. For this reason, several analogues have been designed and tested to find derivatives which could retain high potency with improved selectivity. This is the case, for instance, of compound **19**, obtained by applying the same rationale used for **3a**, which shows an affinity only two-fold lower for the α4β2 subtype compared to **18**, but much higher selectivity over the α3β4.[60] It would be interesting to know the functional effect of this compound, as it is known that structural modification on the pyridyl ring of **18** may affect efficacy more than affinity. In fact, although small groups in position 2′ are well tolerated,[74] substitution at position 3′ (that is, **20**) gives low efficacy agonists characterized by functional antagonistic properties in the tail-flick and hot-plate tests for analgesia.[75]

Modifications on the basic nitrogen of **18** are not well tolerated: only *N*-methylation gives compounds with comparable affinity, whereas the *N*-ethyl analogue showed an affinity 500 times lower.[76] It is not surprising that compounds **21** showed much lower affinity than **18**; however their K_i values were still in the nanomolar range.[77] It is a pity that these compounds have been tested only as a mixture of isomers, and their functional activity has not been measured, as it is known that *N*-methylation of **18** introduces some enantioselectivity in functional tests on α4β2, α3β4, and α7 receptors.[78] The extrusion of the basic nitrogen out of the bicyclo[2.2.1]heptane ring gave amines **22**: the 7-*endo* and 7-*exo* primary amines **22a** ($R^1 = NH_2$, R^2 = H) and **22b** (R^1 = H, $R^2 = NH_2$) showed affinity at α4β2* receptor and analgesic potency in the same range of nicotine, and no appreciable binding at the α7* subtype. The 7-*endo* and 7-*exo* secondary amines **22c** ($R^1 = NHCH_2Ph$, R^2 = H) and **22d** (R^1 = H, $R^2 = NHCH_2Ph$) bind to neither α4β2* nor α7* receptors.[79]

Homologation of the azanorbornane cycle leads to tropane derivatives. The enantiomers of compounds **23** have been synthesized with high enantiomeric excess to develop ligands for PET studies. The affinity of (+) and (−)-**23a** (R = H) for α4β2 nAChR was found to be in the same range as **18**, whereas that for α3β4 and α7 subtype was lower.[80] Therefore, the selectivity is improved and, as happens for epibatidine, no enantioselectivity is observed. Compounds **24** and **25** derive from ring enlargement on the side carrying the aromatic pendant: the chloropyridyl moiety has been replaced with the methyl-isoxazole group, as in epiboxidine.[81] Compound **24a** (R = H), having the same absolute configuration as the natural epibatidine enantiomer, showed affinity in the nanomolar range for the nicotinic receptor of rat brain, with a K_i 490 times lower than its isomer **25a** (R = H). *N*-methyla-

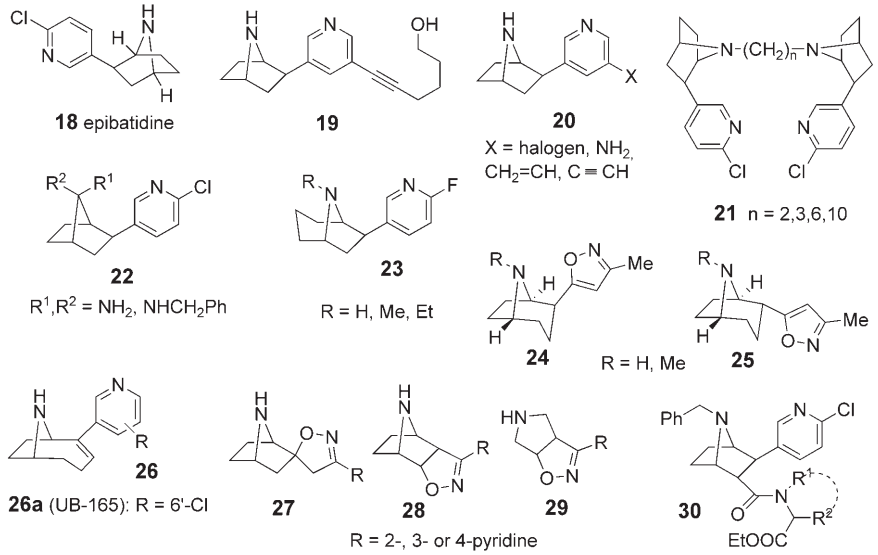


Figure 2. Epibatidine analogues.

tion, or the shift of the isoxazole ring from position 2 to 3 on the tropane nucleus, were detrimental for affinity.[82]

Further expansion of the azabicyclic moiety gives 9-azabicyclo[4.2.1]nonanes, exemplified by UB-165 (**26a**, R = 6′-Cl), which may be considered a hybrid between the structures of anatoxin-a and epibatidine. Karig et al. have synthesized analogues of **26a** with general formula **26** carrying a phenyl ring on the pyridine moiety: the introduction of a phenyl ring in the 2′, 5′, and 6′-position reduced affinity for the α4β2* and the α3β4 subtypes, but the 4′-phenyl analogue showed a three-fold increased affinity for the α7* subtype compared to **26a**, suggesting a possible area of structural modification to improve affinity/selectivity for this subtype.[83] It would be interesting to determine the functional properties of these analogues as it is known that the introduction of a phenyl moiety on the pyridine ring on epibatidine (postiton 3′) or on **2a** (position 5′) gave high affinity compounds endowed with antagonistic properties.[84,85]

Compounds **27** and **28** are deschloro-epibatidine analogues in which the azanorbornane and the pyridine rings are connected through an isoxazolidine moiety. These compounds, and their simplified analogues **29**, show affinity only in the micromolar range for α4β2 and α7 receptors.[86] The epibatidine analogues **30**, incorporating an amino acidic function, and their methiodides have been synthesized and their analgesic properties have been measured in the acetic acid writhing test on mice. These compounds showed analgesic properties, but no comparison has been made with epibatidine, nor has the interaction with the nicotinic receptor been evaluated.[87]

Cytisine analogues. Cytisine (**31**, Figure 3), an alkaloid from *Cytisus* seeds, shows high affinity at the α4β2 nAChR but it behaves as a partial agonist, whereas at the α7 subtype it shows low affinity but behaves as a full agonist. As the total synthesis of this alkaloid is labor intensive, only few analogues have been prepared so far, apart from derivatives carrying different alkyl groups on the nitrogen. A few years ago it was demonstrated that substituents on the pyridone ring can affect both affinity and efficacy: the introduction of a halogen atom in position 9 (compounds **32**, X = Cl, Br, or I) increases affinity and efficacy for α4β2, α7, α3β4, but not for the muscle-type nAChR, whereas a halogen atom in position 11 (compounds **33**, X = Cl, Br, or I) gives different results according to the halogen and the receptor subtype.[88,89] A large decrease in affinity (two to three orders of magnitude) for nAChR containing α2, α3, α4, β2, and β4 subunits is reported after the introduction of an aryl moiety in position 9 (compounds **34**, X = 4-F-phenyl, 4-*n*Bu-phenyl, 5-methyl-2-thienyl) or of a propionyl or a methoxycarbonyl group in position 6 (compounds **35**). On the other hand, a methyl group in position 10 gives a compound (**36a**, R = Me, synthesized as racemate) with similar affinity on human α4β2 receptor, but with higher selectivity over α3β4 with respect to cytisine.[90] The introduction of a vinyl group in position 9 gives a compound (**37**, R = CH=CH$_2$) with an affinity profile similar to that of cytisine; **37** behaves as a partial agonist, its intrinsic activity being higher on the α3β4 than on the α4β2 subtype, as it was able to increase $^{86}Rb^+$ efflux with E_{max} values of 83% and 22%, relative to nicotine, on α3β4 and α4β2 receptors, respectively.[90]

Cytisine has represented the lead compound for the design and development of varenicline, a partial agonist now in clinical trials for smoking cessation (see the last section).[54] In radioligand binding assays this compound is highly selective for α4β2* over α3β4, α7 and muscle-type nAChR: its intrinsic activity on human α4β2 expressed in *Xenopus* oocytes is similar to that of cytisine, whereas its affinity is three-fold higher. In vivo, varenicline displays 30–60% of the efficacy of nicotine (increase in dopamine release from rat nucleus accumbens). Varenicline shares with cytisine the property of being a partial agonist on the α4β2 receptor and a full agonist on the α7 subtype.[91] This compound is the most interesting one among a series of 1,5-methano-2,3,4,5-tetrahydro-*1H*-3-benzazepines (compounds of general formula **38** and **39**) differently substituted on the benzene moiety or carrying nitrogen-containing benzofused heterocycles, showing K_i values from nM to μM on human α4β2, and a wide range of functional activities.[92]

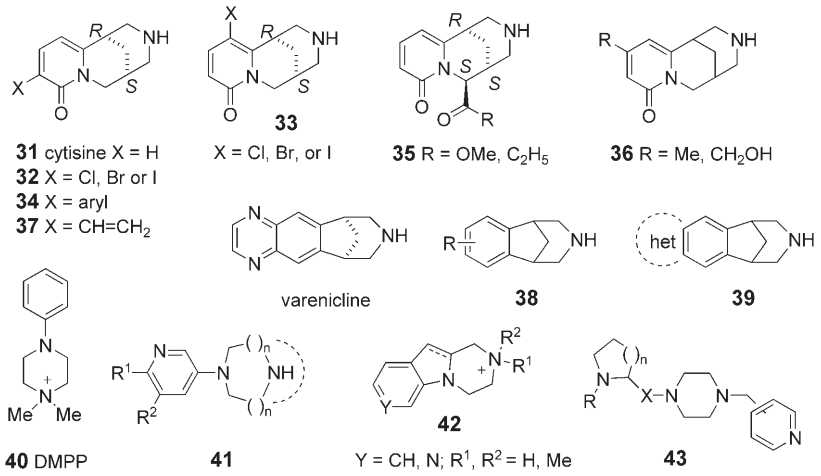


Figure 3. Cytisine and DMPP analogues.

Diamine derivatives. Diamine compounds can be considered to be derived from *N*,*N*-dimethyl-phenylpiperazinium iodide (DMPP **40**, Figure 3) a potent nonselective nicotinic agonist. The ammonium moiety is necessary for activity as the tertiary base is devoid of affinity for central nAChR. However, introducing suitable substituents on the phenyl ring and/or replacing the aromatic moiety with a pyridine ring can considerably improve binding, leading to secondary bases endowed with high affinity for the nicotinic receptor of rat cerebral cortex.[93,94]

Changing the piperazine ring with a seven-membered 1,4-diazepane moiety,[93] with an eight-membered 1,5-diazocane, or with diazabicyclononane or diazabicyclodecane (derivatives with general formula **41**) leads to potent compounds whose structure–affinity relationships have been interpreted by means of a 3D-QSAR model and docking study on a homology model of the α4β2 nAChR.[95]

The reduction of the conformational flexibility of DMPP by freezing it into a 1,2,3,4-tetrahydro-pyrazino[1,2-*a*]indole (**42a**, R^1,R^2 = Me, Y = CH) gave an eight-fold decrease in affinity, whereas the corresponding 1,2,3,4,10,10a-hexahydro analogue **42b**, or the aza derivative1,2,3,4-tetrahydro-pyrido[4′,3′:4,5]pyrrolo[1,2-*a*]pyrazine **42c** (Y = N, R^1 = H, R^2 = Me) did not displace [^{3}H]cytisine from rat cerebral cortex.[96]

Using the piperazine moiety as a spacer between the pyridine and the pyrrolidine rings of nicotine, Crooks and co-workers have synthesized a series of compounds of general formula **43**.[97] These molecules show affinity for the rat striatal nAChR in the micromolar range (against [^{3}H]nicotine) but do not displace [^{3}H]MLA from the α7* receptor, thus showing some selectivity for the α4β2* subtype.

α7 *selective ligands.* Compounds showing selectivity for the α7 receptor can be divided into two different groups: those containing the pyridyl piperidine scaffold, typical of the marine worm toxin anabaseine (for instance GTS21, Figure 4), and those containing an azabicyclic moiety such as the 5-HT_3 antagonist tropisetron or the quinuclidine compound AR-R17779.

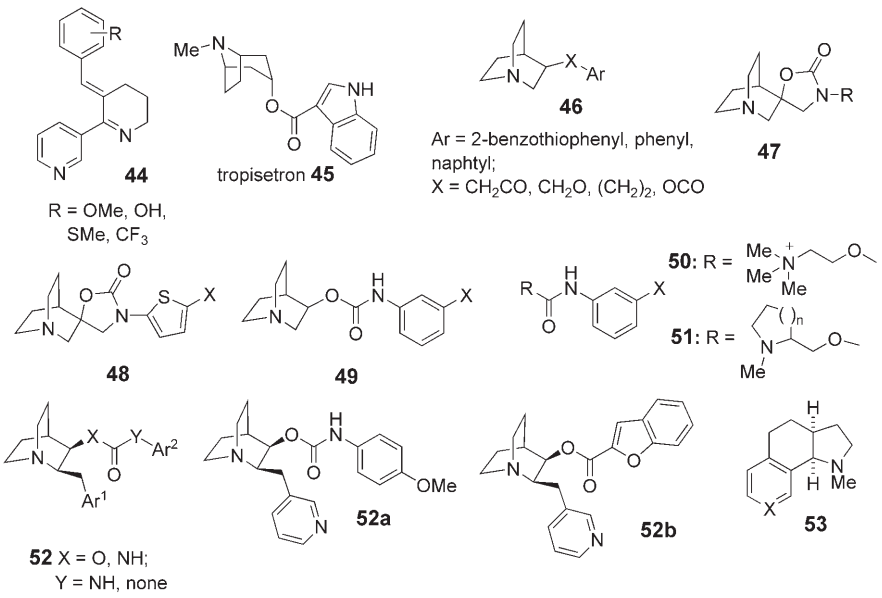


Figure 4. Selective α7 agonists.

GTS-21 (**44a**, R = 2,4-$(OMe)_2$) binds to α4β2* receptors with higher affinity than to the α7* subtype, but it is able to activate only the α7 receptor, behaving as a partial agonist.[98] Some new analogues of **44a**, carrying different substituents on the phenyl ring (compounds with general formula **44**), have been prepared and tested for their affinity and functional properties on the nAChR subtypes. It was found that the benzylidene moiety is important for selective activation at α7 receptors, and that the pattern of substitution can influence both affinity and efficacy at different subtypes: in fact, whereas the 4-OH derivative is able to activate the α7 receptor but does not show antagonistic properties at the α4β2 or α3β4 subtypes, the 4-SMe and 4-CF_3 analogues are poor α7 agonists but displayed α4β2 and α3β4 antagonistic activity.[99]

Tropisetron (**45**), developed as a 5-HT_3 antagonist, behaves as a partial agonist at the α7 receptor (EC_{50} = 0.6 μM), and as an antagonist at the non-α7 subtypes.[100] The simple bicyclic amine tropane (8-methyl-8-azabicyclo[3.2.1]octane) showed a similar profile but displayed lower potency. Thus, the introduction of a 3-indolecarbonyl moiety in position 3 on the tropane ring improves the interaction with the α7 nAChR but it does not affect activation as the two compounds show the same intrinsic activity.[101] It seems, therefore, that a rigid scaffold, containing the basic nitrogen, and an aryl moiety are required for good interaction with the α7* receptor; these two groups can be connected through spacers characterized by different length and/or functional groups as in the compounds with general formula **46**. As an example, the (+)-(2-benzothienyl)-2-oxoethyl derivative **46a** (X = CH_2CO, Ar = 2-benzothiophene) showed affinity in the nanomolar range and agonistic properties in PC12 cells higher than **44a**.[102]

When the azabicyclic moiety is a quinuclidine, the spacer can be an oxazolidinone ring as in AR-R17779 (**47a**, R = H), a full agonist at the α7* receptor. Whereas minor structural changes, such as *N*-alkylation, are reported to reduce affinity and/or selectivity,[103] the introduction of an aryl moiety on the carbamate nitrogen, to give **48**, considerably improved activity: the 5′chlorothiophenyl derivative **48a** (X = Cl) shows a binding affinity 38 times higher than **47a** for α7* receptor, and high selectivity for this subtype with respect to α4β2* or the muscle-type nAChR. Compound **48a** behaves as partial agonist in electrophysiological studies, and it was able to reduce the MK-801-induced auditory gating deficit in rats, suggesting a possible use of this compound in schizophrenia.[104] Compound **48b** (X = ^{125}I) has been synthesized as a radiotracer, but later it appeared not suitable for this use because of its high nonspecific binding.[105]

The spacer between the azabicycle and the aromatic ring can also be a carbamate function, as in compounds with general formula **49**: as expected, these derivatives show selectivity for the α7* over the α4β2*, the α3β4*, and the muscle-type nAChR subtypes. The replacement of the quinuclidine moiety with choline gives compounds (**50**) with a similar affinity pro-

file, whereas a pyrrolidine or a piperidine ring (compounds **51**) decrease affinity for the α7* subtype and selectivity. No functional data are reported for these compounds.[106]

The introduction of a arylmethyl moiety in position 2 on the quinuclidine ring gave compounds with general formula **52**, where the spacer can be a carbamate (X = O, Y = NH), an urea (X ,Y = NH), or an amide (X = NH, Y = none) function. In general, these molecules show high affinity for α7* receptor, and selectivity over the α4β2*, the α3β4*, and the muscle-type nAChR subtypes. The compounds selected for further evaluation, that is, the (+)-enantiomers of **52a** and **52b**, displayed agonistic properties (measure of current in *Xenopus* oocytes expressing the rat α7 subtype), but whereas (+)-**52a** is a partial agonist, with EC_{50} 300 nM, (+)-**52b** is a full agonist endowed with higher potency with respect to **52a** and AR-R17779.[107]

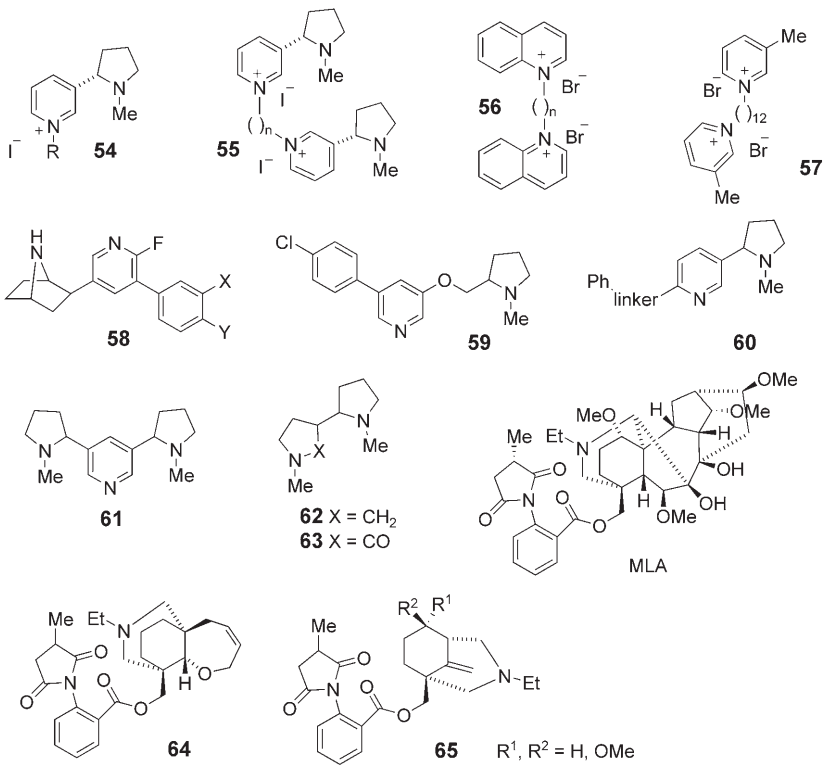


Figure 5. Antagonists.

Compound **53a** (X = N), in which the *syn* conformation of nicotine has been frozen into a tricyclic structure, may seem out of place in this section, as nicotine and its analogues display selectivity for the α4β2* subtype, yet this compound and its boron-inclusion derivative **53b** (X = N·BH_2CN) have been previously shown to bind with similar affinity to both α4β2* and α7* receptors.[108] However, **53a** and **53b** did not activate α4β2 and α3β4 receptors, and behaved as partial agonist on the α7 subtype, therefore showing functional selectivity; **53b** is more potent than **53a**, as the compounds elicited approximately 26% and 10% of the maximal effect of ACh, respectively. The *N*-conjugation of (*S*)-nicotine with cyanoborane decreased efficacy for α3β4 and α4β2 receptors, as well as for α7 nAChR.[109]

Antagonists

Whereas considerable efforts have been directed toward the development of nicotinic agonists because of their interesting therapeutic potential, the search for nicotinic antagonists has so far attracted less attention. It was shown in the previous section that structural changes on the lead compound can shift the activity from agonist to antagonist, but only in few instances have the structural requirements for such transformation been elucidated.

One such case regards the alkylation of the pyridyl nitrogen of nicotine: *N*-alkyl-nicotinium salts (**54**, Figure 5) are reported to bind to the nicotinic receptors with affinities ranging from mid-micromolar to high-nanomolar for the rat brain α4β2* receptor, displaying selectivity over the α7* subtype. A correlation between the length of the alkyl group and affinity at α4β2* receptors has been found,[110] and there is also a quantitative relationship between structure and antagonism at the neuronal nAChR mediating dopamine (DA) release.[111] However, the compound with the highest affinity, NDNI (**54a** R = *n*-dodecyl), does not antagonize nicotine-evoked dopamine release, and the most potent functional antagonist NONI (**54b** R = *n*-octyl) has low affinity for the α4β2* nAChR, thus suggesting that they interact with different subtypes.[112] As a matter of fact, **54a** is a potent inhibitor of nicotine-evoked $^{86}Rb^+$ efflux from rat synaptosomes.[113] Both affinity and functional activity are sensitive to structural modifications on the conformational flexibility of the alkyl chain.[114] Indeed, introduction of double or triple bonds into the octyl chain of **54b** increases the affinity for [^{3}H]nicotine binding sites and the potency in inhibiting nicotine-evoked [^{3}H]DA overflow with respect to the parent compound. The same modification in **54a** reduced affinity for α4β2* but introduced some activity on the subtype mediating DA release.[115] In a series of bis-ammonium derivatives (**55**–**57**), variation of *N*-*n*-alkyl chain length, together with structural modification of the azaaromatic quaternary ammonium moiety, afforded selective antagonists for the α4β2* nAChR subtype such as, for instance, **55a** (bNDI, *n* = 10), and ligands with selectivity at α7* nAChRs such as compound **56a** (bQDDB, *n* = 12).[116] Compound **57** (bPiDDB) was found to specifically decrease nicotine self-administration, and for this reason it has been proposed as a new lead compound for the

development of a clinically useful treatment for tobacco dependence.[117]

Structural changes on the pyridyl ring of epibatidine can also lead to antagonists. For instance, the introduction of a phenyl ring in position 3′ on the agonist 2′-fluoro-deschloroepibatidine gave high affinity antagonists **58**.[84] The antagonistic potency can be improved by aromatic substitution with electron-withdrawing groups:[118] the 4-NO_2 derivative **58a** (Y = NO_2, X = H) blocked ACh-induced current on human $\alpha4\beta2$ expressed in HEK 293 cells with an IC_{50} value of 0.1 μM, but it was much less active on rat $\alpha3\beta4$ nAChR (SH-EP1 cells, IC_{50} ~64 μM), showing selectivity in both binding and functional tests.[119] This trend of affinity/selectivity is confirmed by the work of other researchers[120] who have reported that compounds **58** show selectivity for the β2-containing receptors in binding studies; interestingly, the 4-CN derivative **58b** (X = H, Y = CN) possesses some agonistic properties on rat $\alpha3\beta4$ nAChR (measurement of $^{86}Rb^+$ efflux on KX$\alpha3\beta4$R2 cells) although at a high dose (100 μM).

Similar to what happens for epibatidine, the introduction of an aromatic moiety on the 5 position of the pyridine ring of A-84543 gives antagonists (such as A-186253, **59**) except when the aryl group is a pyrimidine ring.[85] On the other hand, a phenyl ring in position 5 of nicotine gives compounds endowed with agonistic properties whose activity, unfortunately, is described only in the patent literature.[121] Meanwhile, the introduction of a phenylethyl moiety in the 2 position of nicotine gives a compound, **60a** (X = CH_2CH_2), showing affinity in the nanomolar range for the rat brain nAChR, which did not produce nicotinic-like action in several tests after s.c. administration, but was able to antagonize nicotine-induced analgesia in the tail-flick test when injected via the intrathecal route. Modification of the linker (that is, introduction of a β-OH, an insaturation, or an additional CH_2 unit) decreased affinity, whereas substitution on the phenyl ring had little effect.[122]

New alkaloids (**61**–**63**) have been extracted from the root of *Nicotiana tabacum*: **61** and **62** were shown to interact with the $\alpha4\beta2$* nAChR (K_i = 1.18 μM and 15.8 μM, respectively, against [^{3}H]nicotine from rat brain homogenate) but not with the $\alpha7$ subtype; **62**, which did not show interaction with either $\alpha4\beta2$ or $\alpha7$ subtypes, was 30-fold more potent than **61** in blocking nicotine-evoked dopamine release from rat striatal slices, indicating an antagonistic behavior.[123]

MLA is a competitive nicotinic antagonist possessing much higher affinity for the $\alpha7$* than for the $\alpha3\beta4$*, $\alpha4\beta2$*, and the muscle-type nAChR, but which may antagonize, at concentrations frequently used to selectively block $\alpha7$* receptors, other nAChR subtypes located at rat striatal dopaminergic nerve terminals.[22] Simplification of the structure of MLA into the structures **64** and **65** gives compounds endowed with antagonistic properties: in fact they reduced ACh-evoked current on $\alpha7$, $\alpha3\beta4$, and $\alpha4\beta2$ nAChR expressed in *Xenopus* oocytes. Compound **64**, the most potent derivative, behaved as a competitive antagonist on $\alpha7$, noncompetitive on $\alpha4\beta2$, and with mixed effect on $\alpha3\beta4$ nAChR. This is in contrast with the parent compound MLA, which shows competitive antagonism on both $\alpha7$* and $\alpha3\beta4$* subtypes.[124,125]

The binding affinity of a series of strychnine and brucine derivatives at the $\alpha7$/5-HT_3 chimera were reported by Jensen et al.; the compounds bound with K_i in the micromolar range and with structure–affinity relationships different from those relative to the binding at the glycine receptor.[126] Although the functional activity of these analogues has not been measured, the antagonism by strychnine is reported to be competitive at the $\alpha7$ receptor, but noncompetitive at the muscle-type and neuronal heteromeric nAChR; in addition, strychnine and brucine are allosteric modulators of muscarinic acetylcholine receptors.[127,128] Surprisingly, atropine, the prototype of muscarinic antagonists, has also been reported to competitively block $\alpha3\beta4$* receptors in bovine chromaffin cells, and bovine $\alpha7$ and $\alpha3\beta4$ receptors expressed in oocytes; the IC_{50} values were in the nanomolar range, the $\alpha7$ receptor being more sensitive than the $\alpha3\beta4$ subtype (IC_{50} = 11.2 nM and 46.8 nM, respectively).[129]

Peptidic modulators.

Several peptides are known to modulate nicotinic receptors. The best known are toxins from snakes, such as α-bungarotoxin (α-Btgx) and cobratoxin, and from the *Conus* snail (conotoxins). These peptides are released by the animal to paralyze the prey, and therefore behave as antagonists for the muscle-type receptors; nevertheless, they are active on, and often selective for, other nAChR subtypes. Some recent papers have reviewed the current knowledge about such peptides and, therefore, are not treated in this review.[20,130,131] Meanwhile, several other congeners have been discovered or synthesized.[132–138]

In recent years, two peptides called SLURP (secreted mammalian Ly-6/uPAR-related protein) -1 and -2 have been described which modulate nicotinic receptors in keratinocytes. SLURP-1 has higher affinity for the nicotinic receptors labeled by [^{3}H]nicotine while SLURP-2 has higher affinity for the subtypes labeled by [^{3}H]epibatidine. The experimental evidence suggests that these peptides activate different nAChR subtypes mediating opposite effects on keratinocytes.[139,140] The protein $A\beta_{42}$ is another modulator of nicotinic receptors: this peptide is reported to bind with high affinity to nicotinic receptors,[41,141] but its functional effects are controversial as in some instances activation has been reported[142,143] but in other cases only inhibition has been found.[144–147] These findings suggest that the nicotinic receptors may be the target of the toxic effects of $A\beta_{42}$.

Allosteric modulators

Like other LGIC, nAChRs are modulated by structurally diverse compounds, acting through allosteric mechanisms, which can be divided into potentiators and inhibitors. Inhibition can be achieved by different mechanisms: by blocking the open channel, either by binding to and stabilizing the resting or desensitized state of the receptor, or by increasing the desensitization rate.[148]

Compounds such as local, dissociative or general anesthetics, barbiturates, and other molecules bind to the lumen of the

channel, at different positions; local anesthetics can also bind to another site at the protein–lipid interface, although with lower affinity. The location of the binding site depends on receptor conformational state and on receptor subtype.[149]

Steroids are reported to modulate nAChRs, with both activation or inhibition. Again, the mode of action depends on the receptor subtype. In fact, corticosteroids and progesterone block the muscle-type and ganglionic nAChRs, and estradiol, which however is able to activate the human $\alpha4\beta2$ subtype. Alkanols can also modulate nAChR with a dual mode of action: whereas long-chain alkanols behave as blockers, ethanol and other short-chain alkanols are reported to activate muscle-type and neuronal subtypes.[150–152]

Among the activators, the most important compounds are the so-called allosteric potentiating ligands (APL) such as codeine, galantamine, and physostigmine. The nAChR activation by these compounds is dependent on the presence of acetylcholine, thus providing a physiological stimulation of the receptor. Galanthamine combines nAChR activation and AChE inhibiting properties, and it has been approved for the treatment of AD. Docking studies using homology models of $\alpha4\beta2$, $\alpha7$, and $\alpha3\beta4$ nAChRs have identified the possible binding site(s) of these modulators, located not far from the orthosteric site.[153]

Pfizer researchers have recently reported a novel APL, **66** (PNU-120596, Figure 6), which is able to increase agonist-evoked currents in $\alpha7$ receptor but not in the $\alpha4\beta2$, $\alpha3\beta4$, or $\alpha9\alpha10$ subtypes.[154] Interestingly, this compound was discovered by testing a library of newly-synthesized substances on an $\alpha7/5\text{-}HT_3$ chimeric receptor, an approach that allowed the same researchers to discover PNU-282987 (see last section and Figure 13), a structural analogue endowed with direct agonistic properties which is now in clinical trials for schizophrenia (see below).[155] Employing high-throughput screening of an in-house chemical library, researchers at Lilly have discovered compound **67** (Figure 6) which is able to allosterically activate $\alpha2\beta4$, $\alpha4\beta4$, $\alpha4\beta2$, and $\alpha7$ receptors but not the muscle-type, $\alpha3\beta2$ or $\alpha3\beta4$ subtypes.[156]

There is a long list of natural and synthetic products that inhibit nicotinic receptors which has been the topic of recent reviews[56, 58, 148, 149, 157] and is continuously increasing; some of the new entries are shown in Figure 6. It has been reported that aminoglycoside antibiotics, such as neomicine, block ACh-evoked currents at neuronal receptors at concentrations below those used in therapy, their effects being more pronounced at $\alpha7$ than at $\alpha4\beta2$ receptors. This finding can explain some of the side effects on the auditory system of these chemotherapeutics.[158]

Some frog-poison alkaloids have been isolated and several analogues have been synthesized and tested on recombinant nAChRs expressed in *Xenopus laevis* oocytes.[159] These alkaloids were found to be noncompetitive blockers, with the most potent being **68**. Compound **68** behaved as an open channel blocker, and it showed some selectivity for $\alpha4\beta2$ ($IC_{50}=$ 0.07 μM) compared to $\alpha7$ and $\alpha3\beta4$ subtypes ($IC_{50}=0.4$ and 3.5 μM, respectively). The quinolizidine **69** and the tricyclic derivative **70** were less potent, but they displayed some selectivity for $\alpha7$ over $\alpha4\beta2$ and $\alpha3\beta4$ subtypes.[160] (−) Pictamine (**71**), another quinolizidine alkaloid from *Clavelina picta*, and (−) lepadin B (**72**), its decahydroisoquinoline analog from *Clavelina lepadiformis*, noncompetitively blocked the $\alpha4\beta2$ and $\alpha7$ nAChR with IC_{50} in the micromolar range; the blockade of the $\alpha4\beta2$ but not the $\alpha7$ subtype by **71** was irreversible.[161]

Coclaurine (**73a**) is a tetrahydroisoquinoline alkaloid extracted from the leaves of *Nelumbo nucifera*.[162] It can be considered half of the more complex alkaloids *d*-tubocurarine, the prototype of the nAChR competitive antagonists, and tetrandrine, which has been found to be a noncompetitive inhibitor of both muscle and neuronal nAChR.[163] Coclaurine analogues (general formula **73**) have been synthesized as racemate[164] and tested for their nAChR modulating properties on recombinant human $\alpha4\beta2$, $\alpha4\beta4$, and $\alpha7$ subtype. They were found to be

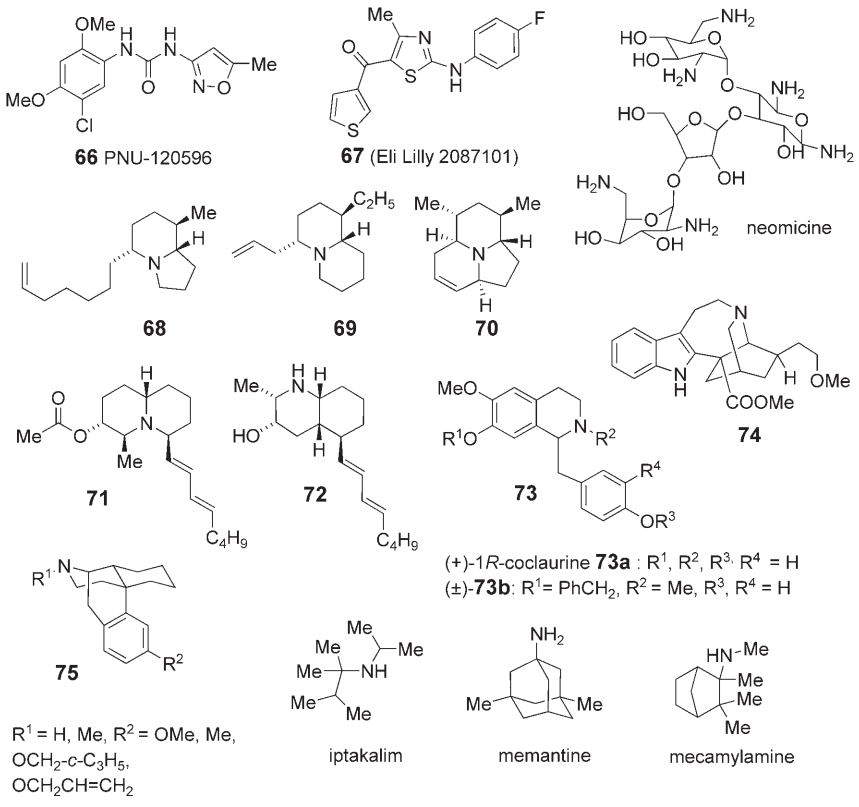


Figure 6. Allosteric modulators.

noncompetitive antagonists, with IC_{50} values in the micromolar range, the most potent of the three subtypes being compound **73b**.[165]

In a search for anti-addictive agents, some indole alkaloids, such as 18-methoxycoronaridine **74** and its synthetic analogues,[166] were found to block the $\alpha3\beta4$ nAChR subtype.[167] Interestingly, contrary to what happens regarding their interaction with opioid receptors,[168] the enantiomers of **74**, the most potent antagonist, display the same potency on recombinant $\alpha3\beta4$ nAChR expressed in HEK 293 cells.[169] Dextromethorphan (**75a**: $R^1 = Me$, $R^2 = OMe$) is another noncompetitive nicotinic antagonist, potentially useful as anti-addictive agent.[170] Some analogues (general formula **75**) were tested on recombinant $\alpha3\beta4$ receptor in *Xenopus* oocytes by means of voltage-clamp, showing a potency similar to that of the parent compound, with an IC_{50} value in the micromolar range.[171]

Iptakalim, a K_{ATP}-channel blocker endowed with neuroprotective properties,[172] has shown antagonistic activity on human $\alpha4\beta2$, $\alpha4\beta4$, and $\alpha7$ nAChRs in SH-EP1 cell lines, the $\alpha7$ receptor being much less sensitive than the $\alpha4\beta2$ subtype.[173] Interestingly, iptakalim is a bulky aliphatic alkyl amine showing structural similarity with mecamylamine and memantine. Mecamylamine (see the last section) is a nonselective, noncompetitive antagonist, widely used as pharmacological tool to study nAChR, whereas memantine, classified as an NMDA antagonist, has been recently reported to also be a noncompetitive blocker at $\alpha7$[174,175,176] and $\alpha4\beta2$ receptors.[177] There is evidence that both memantine and iptakalim may interact with more than one binding site within the receptor and one of these may be located within the channel lumen.

Pharmacophore, 3D QSAR, and structural models

Excellent and comprehensive reviews have been written over the years which comprise the general structure–activity relationships and molecular modeling studies on the nicotinic receptor nAChR.[178–180] The focus of this section is to go over the steps that led to present-day knowledge on the pharmacophore 3D QSAR and especially structural nAChR models.

Over the years, conformational preferences of nicotinic ligands have been the focus of several studies. An interesting report by Beer and Reich in the 1970s, describes one of the first attempts to define the nicotinic pharmacophore. Starting from the analysis of the structure of two agonists and three antagonists, the paper proposes a two point pharmacophore constituted of a positively charged nitrogen ($N+$) and an H-bond acceptor group (HBA) separated by a distance of 5.9 Å between the onium group and the Van der Waals (VdW) surface of the HBA.[181]

Using a distance geometry approach and only four nicotinic ligands (that is, (*S*)-nicotine, muscarone, ferruginine methiodide, cytisine), Sheridan et al.[182] refined this model deriving an improved nicotinic "triangle" pharmacophore with a distance of 4.8 Å from the quaternary N to the VdW surface of the HBA, and a third point located 1.2 Å from the pyridine centroid of nicotine-like compounds.

Since then, also taking advantage of the development of three-dimensional QSAR analysis, numerous studies have dealt with the pharmacophore and ligand demands for the molecular recognition in nAChRs.[183–190]

Barlow et al.[183] introduced the "point plus a flat area" theory, pointing out the importance of a flat, hydrophobic area for activity.

Further refinements to the nAChR pharmacophore came with the so called "four points" and "vector" hypothesis of Mayer et al.[191] and Tønder et al.,[192] respectively. The models shifted attention to points *a* and *b* on nAChRs 2.9 Å from the basic amine and the HBA moiety of the ligands, and to these intrareceptor features (distance *a*-*b*, 7-8 Å), rather than to the intraligand distances (for example, the internitrogen distance), thus introducing the concept of directions (vectors) in the receptor as determining features for the activity. The theory was further modified[193] by the inclusion of an "aromatic centroid point *c*" and the definition of the *b*-*a*-*c* angle, although the addition of the *c*-point has brought up criticisms based on the possible forcing in the alignment of aryl rings in the ligands, regardless of different microenvironments in the receptor which could accommodate them in a different manner.[178]

These models were subsequently "revisited" and combined by Glennon and Dukat[194] in an interesting paper where the new theory of the "water-extension" concept was introduced for the first time. As presented by the authors, this concept emerged from the attempt to clarify why the vector models failed in explaining the biological data for certain compounds. The water-extension theory suggests that a water molecule can mediate the binding of ligands to the receptor; a short ligand, such as nicotine, (N–N distance = 4.8 Å) can be converted into a longer ligand through a water bridge between the ligand and the receptor, thus providing a possible explanation to the wide range of the proposed nAChR pharmacophoric intraligand distances.

These above mentioned studies were carried out using a number of ligands not sufficient for the model validation, with mixed functional properties (agonist and antagonist) and in some cases considering biological data (not necessarily binding data) from different laboratories, and when binding data were considered they were often obtained with different radioligands.

3D QSAR and QSAR studies on nAChR paralleled the efforts in the development of the pharmacophore models,[95,189,190,193,195–198] supporting the rational design and synthesis of novel nicotinic ligands.[60,71,95] The pharmacophoric patterns of nAChR, both from SAR and computational techniques such as DISCO and Catalyst HipHop, were widely used in the conformer selection and in the alignment of the ligands involved in the development of the nicotinic 3D QSAR models, that is, in the construction of mathematical models aimed at uncovering relationships between chemical properties, the 3D molecular features of the compounds, and biological activity of the set of nicotinic ligands. Such models can be regarded as an empirical picture of the biomacromolecular target and their determination has been accomplished by procedures such as CoMFA, CoMSIA, GRID/GOLPE, or related-field approaches.

Actually, these studies agreed in indicating some essential features guiding the interaction in the nAChRs: 1) a protonated nitrogen N^+ for π-cation or hydrogen bond interactions; 2) a hydrogen bond acceptor and/or a π-electron rich moiety in the ligands; 3) the presence of a π-system in the ligands able to give π-π interactions; 4) steric interactions involving a hydrophobic area of the ligands.[179]

To be suitable for developing a 3D QSAR model, a set of compounds should 1) belong to defined drug classes which essentially bind to the same site on the receptor; 2) represent a large variety in chemical structures; 3) have the same pharmacological profile (agonists, antagonists, etc.); 4) present biological data that cover a wide range, determined homogeneously. However, none of the studies discussed above fulfils all these requirements. Thus, caution is necessary when using the models for the rational design of new compounds, drawing attention to the importance of structural data on the nAChR for the design of new and selective modulators.

Thanks to the progress made in experimental techniques, the structural architecture of the nicotinic receptor has been increasingly better defined over the years. Initial studies were aimed at purification[199–201] and characterization[202,203] of the protein and by the end of the 1970s it was common knowledge that *Torpedo* receptor was a pentameric protein carrying two binding sites for ACh.[204,205] The first structural studies with X-Ray diffraction and electron microscopy led to the hypothesis of the transmembrane nature of nAChR[206] which was subsequently demonstrated by the use of specific antibodies[207] and photoreactive phospholipids.[208]

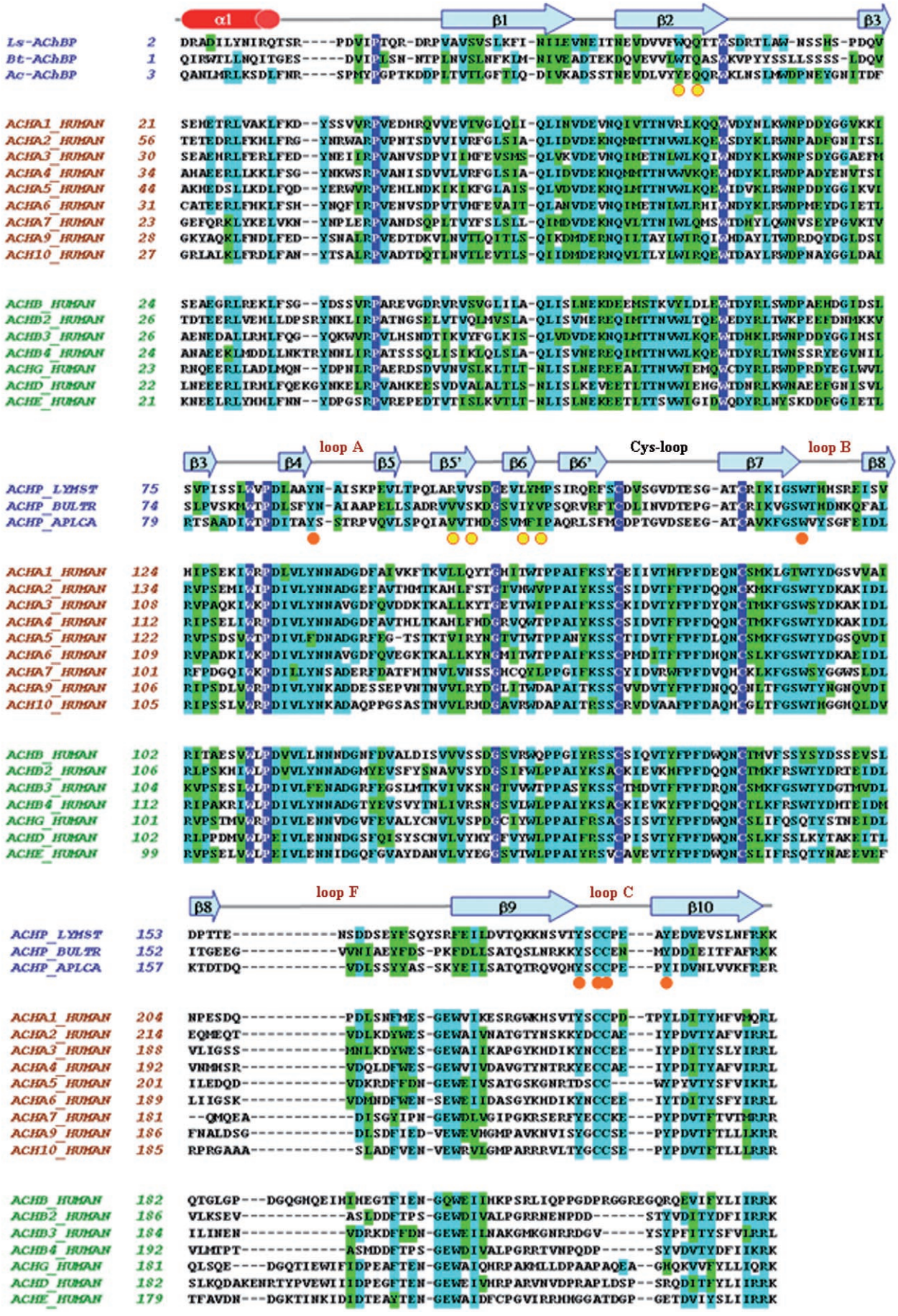
Thanks to advances in sequencing and cloning techniques, at the beginning of the 1980s it was possible to obtain the primary sequences of the various *Torpedo* receptor subunits and to compare them, highlighting their homology and the possible evolution from a common ancestor.[4,209,210] Extending the sequence comparison to receptor subunits from different species,[211] it was possible to identify conserved residues critical for ligand binding or receptor function which, together with hydropathy analysis, guided the first attempts to derive secondary structure predictions and topographical models of the receptor channel and of the ligand binding site.[212–216] Site-directed mutagenesis experiments on *Torpedo* receptor confirmed the importance of several residues for protein–ligand interactions and delineated two components of the binding site: a "principal component" located on the α subunit and a "complementary component" on the γ or δ subunit.

Furthermore, in 1984 Brisson and Unwin reached a turning point in the structural determination of the receptor, obtaining for the first time tubular crystals from *Torpedo marmorata* receptor-rich vesicles suspension[217] which were successfully used in structural studies.[218,219] The initial low resolution images obtained by cryoelectron-microscopy showed the cylindrical shape of the receptor and its protruding into the synapse to form the ligand binding domain (LBD).[220,221] The topology of the ion channel was extensively investigated, as was the mechanism of ion selectivity and channel gating. Computational approaches were used to predict the arrangement of secondary structure elements[222] and affinity labeling experiments led to the hypothesis of a four transmembrane helices model for each subunit, where the bundle of the second transmembrane segments (M2) forms the wall of the channel and defines its biological properties.[223] Further electron microscopy studies conducted by Unwin produced images of the receptor in the closed[224] and open states[225] at 9 Å of resolution, revealing details about the secondary structure and in particular the presence of a kink in the middle of the central pore, which has been postulated to be the channel gate. Furthermore, comparison of the two forms of the receptor showed that, after exposure to acetylcholine, a rotation at the level of the extracellular domain takes place which causes a variation in the transmembrane domain conformation, probably at the base of the mechanism of channel gating. Recently a more detailed structure of the pore domain of *Torpedo marmorata* receptor has been obtained by electron microscopy which provides further insight into structural and functional aspects of channel opening.[226]

Even though many efforts were also devoted to investigating the extracellular ligand binding domain (LBD), its folding was more difficult to model: through the use of spectroscopy studies the presence of beta structure was observed mainly in this domain, with some strands that could be present also in the transmembrane domain.[227–230] Starting from this observation, several hypotheses regarding potential template scaffolds to be used in comparative modeling of LBD have been formulated, which comprise pyrophosphatase,[231] Cu-binding protein such as plastocyanin and pseudoazurin,[232] and SH2 and SH3 domains of the biotin repressor structure.[233] Certainly the major breakthrough in nAChR research came in 2001 with the discovery and atomic structure determination of a soluble acetylcholine binding protein from *Lymnaea stagnalis* (Ls-AChBP) with pharmacological properties analogous to those of nicotinic receptor.[234,235] The atomic structure of another protein which binds acetylcholine, that is, acetylcholinesterase, was already known,[236] but the major importance of AChBP has been its potential use as representative structure for studying the ligand binding domain of nicotinic receptors.

AChBP is a 120 kDa homopentameric protein secreted by snail glial cells in the cholinergic synapses, where it acts as a modulator of acetylcholine transmission. Ls-AChBP binds nicotinic ligands with an affinity similar to homomeric neuronal nAChR, the closest being $(\alpha7)_5$, and possesses many structural features typical of this class of receptors, although it lacks the transmembrane domain. Each subunit of Ls-AChBP is 210 amino acids long with 20–24% of sequence identity with respect to the extracellular domain of nAChR, most closely related to α subunits. The secondary structure of Ls-AChBP consists of ten β-strands arranged as an immunoglobulin-like fold, with a α-helix at the amino-terminal end. The signature cys-loop characteristic of the LGIC class to which AChRs belong is located at the bottom of each subunit and links $\beta6$ and $\beta7$ strands (Figure 7).

The ligand-binding site was confirmed to be located at the interface between two subunits, as emerged by photoaffinity labeling experiments. Therefore, given the identity of the five AChBP-forming subunits, five binding sites were found in



Ls-AChBP. As one HEPES molecule from the crystallization buffer was present within each binding site, it was possible to observe the details of the binding, in particular the π-cation interaction made by Trp 143 of Ls-AChBP (corresponding to α-Trp 149 in *Torpedo* receptor) and the quaternary ammonium of the ligand: this kind of interaction was previously observed in the modeled complex acetylcholinesterase and acetylcholine[236] and hypothesized to have a role in nAChR ligand recognition.[237,238]

The availability of the structure of AChBP opened the way to build realistic structural models of nicotinic receptor LBD domain through homology modeling, a computational technique which is based on the alignment between the sequence of a target protein and that of a homologous protein of known structure. To obtain models with significant reliability, lysine scanning mutagenesis experiments have been performed on AChR subunits which determined the orientation of residue side chains toward the hydrophobic core or the hydrophilic surface, thus allowing assignment of correct correspondence between residues in the alignment with AChBP.[239]

Using the AChBP structure as template, nicotinic receptor structural studies underwent great improvement, to the point that in 2005 Unwin, using AChBP for modeling parts of the extracellular domain, succeeded in refining electron-microscopy images of the entire *Torpedo* receptor to 4 Å resolution.[18]

Homology models of different types of nicotinic receptors based on HEPES-bound AChBP structure were used in docking experiments[240–242] or molecular dynamics simulations[243,244] for studying ligand–protein interactions and conformational variations upon binding. The results of these studies have been confirmed by the more recently solved crystal structures of Ls-AChBP bound with agonists,[245] or those of complexes of *Aplysia californica*[246–248] and *Bulinus truncatus*[247,249] proteins with different agonists and antagonists.

In all these complexes a tryptophan residue (Trp 143 in Ls-AChBP) lies at the centre of the binding site and is involved in π-cation interaction with positively charged groups of the agonist; in nicotinic receptors, a tryptophan residue in this position is conserved in all AChR α subunits (but not always in non-α, see Figure 7). Several conserved aromatic residues form the rest of the cavity (Ls-AChBP Tyr 89, Tyr 185, and Tyr 192 from the principal side and Trp 53 and Tyr 164 from the complementary) and interact with ligands both through π systems or hydroxyl groups (Figure 8).

Charged residues do not directly contribute to ligand binding but can have a role in polarizing main-chain atoms for hydrogen bonding. Agonists such as nicotine are completely buried in the binding site, trapped by the loop C that caps the cavity. Molecular dynamics (MD) simulations on AChBP structure and nicotinic receptor homology models highlighted the possibility of loop C to pass from capped to uncapped conformation depending on the presence/absence of the agonist.

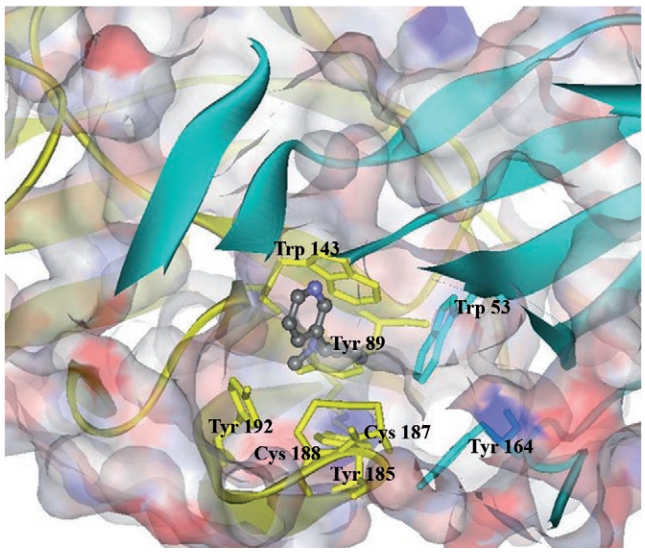


Figure 8. The nicotinic binding site of Ls-AChBP bound to the agonist nicotine (pdb code 1UW6). Aromatic residues interacting with the ligand and the disulfide-bridged cysteines of loop C are shown, colored in yellow if given from the principal side or in cyan if given from the complementary side.

The extended uncapped conformation has been observed in the agonist-free structure of nicotinic receptor and AChBP, and in complexes of AChBP with bulky ligands such as toxins, suggesting an important role in the diffusion and binding of agonists and antagonists (Figure 9).

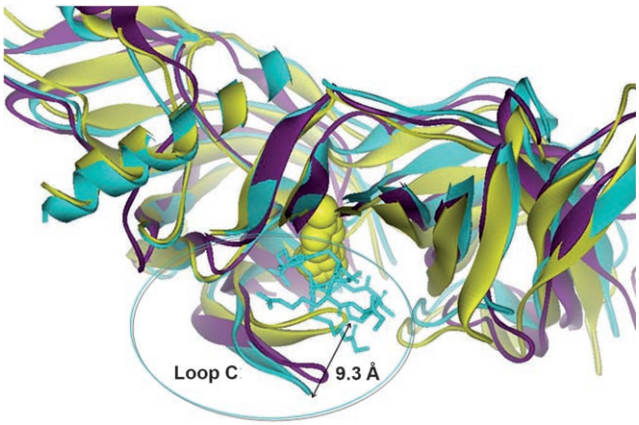


Figure 9. The nicotinic binding site of Torpedo receptor (pdb code 2BG9, purple), Ac-AChBP in complex with the agonist epibatidine (pdb code 2BYQ, yellow), and Ac-AChBP in complex with the peptide antagonist Imi (pdb code 2BYP, cyan); it can be observed the movement which loop C is subjected to from its capped agonist-bound structure to the uncapped one in the free state or after the binding of toxin-like ligands.

Figure 7. Structural superposition of the subunit sequences of AChBP from *Lymnaea stagnalis* (Ls-AChBP), *Bulinus truncatus* (Bt-AChBP), and *Aplysia californica* (Ac-AChBP) and sequence alignment of human nicotinic receptor α1-10 (ACHA), β1-4 (ACHB), γ (ACHG), δ (ACHD), and ε (ACHE) subunits. Secondary structure elements from AChBP structures are indicated. Residues are highlighted in blue if identical in all the sequences, in cyan if identical in more than 50% of sequences, and in green if conserved in more than 50% of sequences. Solid circles under AChBP sequences indicate amino acids principally involved in ligand binding both in the principal (orange) and in the complementary face (yellow).

Compounds in development

Despite the large number of nicotinic ligands synthesized and studied after the relevance in CNS of nicotinic receptors was discovered,[14,57,58,179,180] only a few molecules have been examined in detail in preclinical studies, and even fewer have progressed to clinical trials. Cognition impairment and pain are the most frequent indications but also a number of neurological and neuropsychiatric conditions and addiction disorders are possible targets.[250]

For a long time the Abbott group (http://www.abbott.com) has been engaged in the development of drugs acting at nicotinic receptors, so it is not a surprise that several of the molecules that have been studied in detail and entered clinical trials were discovered at Abbott (Figure 10). ABT-089 (**76**) seems the most advanced compound as it recently entered phase II for treatment of cognitive problems. Compound **76** is a weak partial agonist at $\alpha4\beta2$ receptors, equipotent with nicotine in stimulating ACh release but less efficacious in stimulating DA release. In preclinical studies **76** showed to be effective in improving cognitive functions and in phase I was reported to show a good pharmacokinetic profile with markedly limited adverse cardiovascular and gastrointestinal side effects.[251] Recently, **76** has been shown to be effective in treating adult ADHD whilst being well tolerated.[252] ABT-418 (**77**), an $\alpha4\beta2$ receptor full agonist, is another compound that reached phase I for the treatment of cognitive problems but its development was stopped when, as a transdermal patch, it failed to show a differentiation from placebo in a six-month trial.[250] As far as pain control is concerned, the most promising compound seems to be ABT-894 which is reported to be in phase I for neuropathic pain. Its structure has not yet been disclosed nor is there any information available on its pharmacology. ABT-894 is a second-generation agonist, a follow-on to ABT-594 (**2b**, tebanicline) that has been evaluated rather extensively[253,254] in preclinical studies, where it displayed the antinociceptive properties of epibatidine but with an improved safety profile. However, its development was discontinued after phase II because of adverse side effects in initial clinical trials.[255] Two other compounds, ABT-202 (**78**) and A-366833 (**79**)[256] are reported to be in phase I for pain treatment,[255] but details on their pharmacology are not available.

Two compounds of the SIBIA group are in development for Parkinson disease (SIB-1508Y, **80**) and cognition dysfunction (SIB-1553A, **81**), respectively (Figure 11). Compound **80** (altinicline) is an $\alpha4\beta2$ agonist that is more potent and selective than nicotine, stimulates striatal DA and cortex ACh release in rodents and is also active on primates. It has entered phase II for Parkinson treatment.[257] Compound **81** was shown to be equi-efficacious as nicotine in improving working memory performance in mice and aged mice. Side effects were observed at doses much higher than those required to increase cognitive performance;[258] the compound is reported to be in phase I.

Another company heavily involved in nicotinic ligand development is Targacept (http://www.targacept.com) with several compounds in preclinical and clinical phases (Figure 12). Ispronicline (**82**, TC-1734) is a highly selective $\alpha4\beta2$ agonist that has shown cognition enhancement properties in several animal

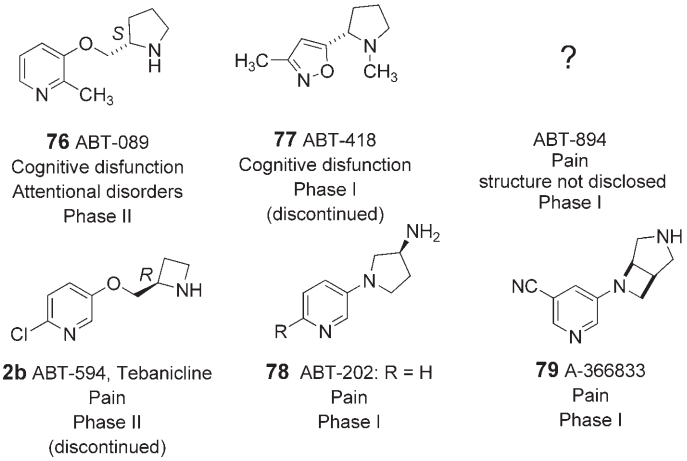


Figure 10. Abbott's nicotinic agonists in development.

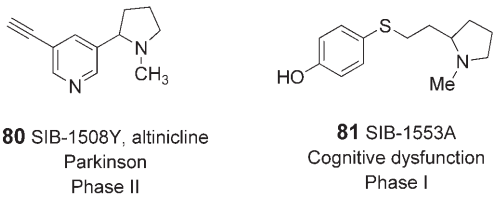


Figure 11. SIBIA's nicotinic agonists in development.

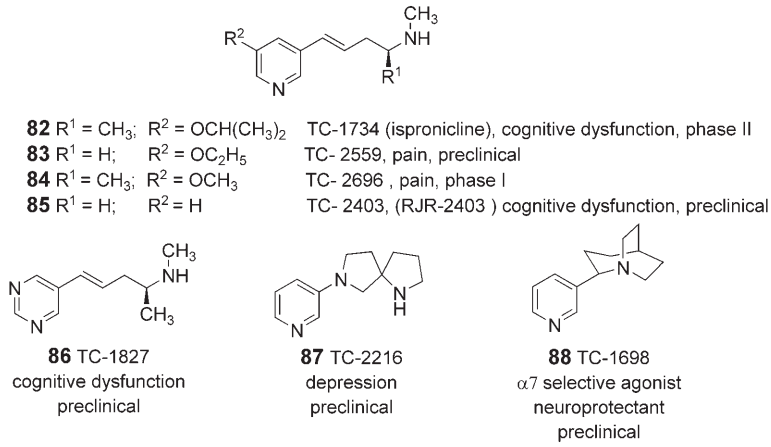


Figure 12. Targacept's nicotinic ligands in development.

models and is now in phase II for treatment of age-associated memory impairment and mild cognitive impairment, after it was shown in phase I that doses up to 320 mg were well tolerated.[259,260] Two close analogues, **83** (TC-2559)[260] and **84** (TC-2696), are in preclinical and phase I trials for pain treatment, respectively, whereas a third one, **85** (TC-2403), is in the preclinical phase for cognitive dysfunction. TC-1827 (**86**), an isoster of **82** which is also an $\alpha4\beta2$ selective agonist, is still in the preclinical phase where it has shown cognition enhancing properties on rodents and primates.[57] TC-2216 (**87**), also a pyridine derivative but with quite a different structure, showed properties in preclinical studies that suggested its development for depression and anxiety disorders. The company is currently conducting additional preclinical safety studies to support its progression to clinical trials. Compound **88** (TC-1698) is an $\alpha7$ selective agonist that in preclinical studies has shown promising neuroprotective properties.[262] Finally, the company is developing an old nonsubtype selective nicotinic antagonist used in hypertension, mecamylamine (Figure 6), for the treatment of depression.[255] The compound is now in phase II and, if successful, the company will accelerate the development of its pure enantiomer TC-5214.

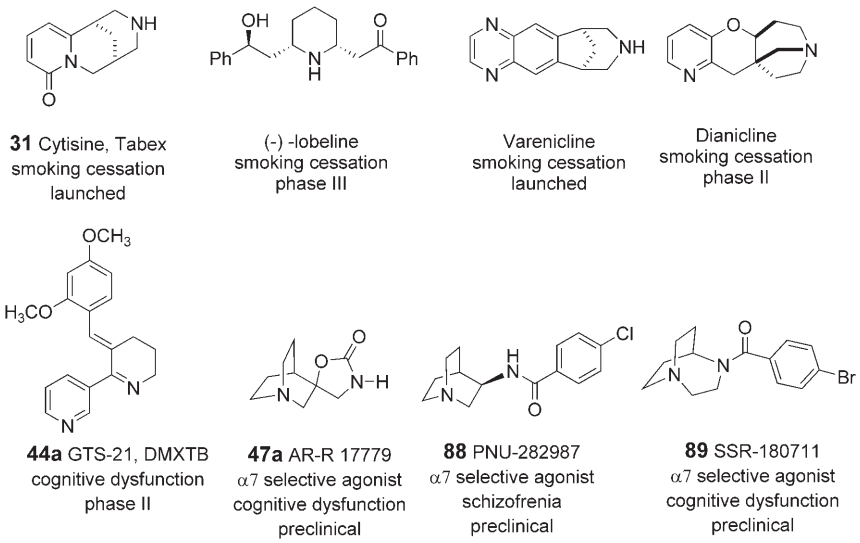


Figure 13. Other nicotinic ligands in development.

Other compounds in development are shown in Figure 13; the most advanced are those that promise to be useful for the treatment of smoking addiction. The alkaloid cytisine (**31**), an $\alpha4\beta2$ partial agonist obtained from *Cytisus laborinum* is already used in Europe (Tabex: tablets containing 1.5 mg of the drug) and (−)-lobeline, an alkaloid from *Lobelia inflata*, which has mixed nicotinic actions depending on the assay used, is in phase III as a treatment for smoking dependency.[250] Among synthetic compounds, varenicline, a partial agonist at the $\alpha4\beta2$ nicotinic receptor subtype, has been developed by Pfizer (http://www.pfizer.com) for the same purpose[263] and appears to be a more effective antismoking agent than bupropion, an antidepressant drug with nicotinic receptor antagonistic properties currently used to treat smoking addition.[264] Pfizer has recently presented regulatory submissions in the US and Europe and intends to market varenicline under the brand name of Chantix and Champix, respectively; the drug has been recently approved.[265] A similar tetracyclic compound, also an $\alpha4\beta2$ nicotinic receptor partial agonist, dianicline, from Sanofi-Aventis is reported to be in phase II clinical trials.[264] GTS-21 (**44a**) was the first $\alpha7$ selective agonist to be developed as a drug candidate for cognitive dysfunction, reaching phase II. However, it was found to present negligible agonist activity at human $\alpha7$ and $\alpha4\beta2$ receptors.[250] Compounds AR-R-17779 (**47a**), PNU-282987 (**88**), and SSR180711 (**89**) are selective $\alpha7$ agonists that are reported to be in advanced preclinical studies for cognitive dysfunction. Compound **47a** is a full nicotinic agonist that is more potent than nicotine and has shown anti-anxiety and memory improving properties.[266] Compound **88**, by selective interaction with $\alpha7$ nicotinic receptors and via GABA ergic neurotransmission activation, was efficacious in improving auditory gating in rats, showing potential for the treatment of schizophrenia;[267] its ring homologous **89** has shown similar properties, being active in animal models of cognitive deficits related to schizophrenia.[57] Two other $\alpha7$ selective agonists, whose structure so far have not been disclosed (PH-399733, from Pfizer and MEM 3454 from Memory), are reported to be in phase I development.[268]

Finally, it is necessary to mention that many of the most interesting molecules synthesized, such as the pyridyl ethers **2a** (A-84543) or **90** (A-85380) or the nicotine analogue **77** (ABT-418), have been labeled with suitable radioisotopes to give compounds (**91**–**96**) that can be used for a variety of studies (Figure 14).[58]

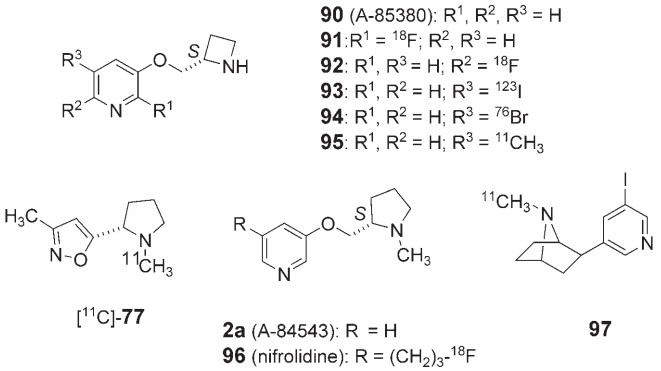


Figure 14. Labeled nicotinic ligands in development.

Of particular interest is the introduction into the molecule of radioisotopes such as ^{11}C, ^{18}F, ^{76}Br, ^{123}I that give compounds useful for in vivo imaging with PET (Positron Emission Tomography) and SPECT (Single Photon Emission Computerized Tomography), two noninvasive techniques that can be used to visualize nicotinic receptors in living subjects.[269,270] Compounds of this kind must present several critical properties such as adequate penetration into the brain, low nonspecific binding, low toxicity, slow metabolism, low incidence on cerebral blood flow and proper half-life of the complex with receptors. Very recently, a new potent and selective nicotinic antagonist **97** entered development as a radioligand for PET and SPECT studies.[271]

Keywords: neurodegenerative disorders · nicotinic ligands · nicotinic receptors · pain

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