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Small molecule ligands for α9 * and α7 nicotinic receptors: A survey and an update, respectively☆

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ABSTRACT

The α9- and α7-containing nicotinic acetylcholine receptors (nAChRs) mediate numerous physiological and pathological processes by complex mechanisms that are currently the subject of intensive study and debate. In this regard, selective ligands serve as invaluable investigative tools and, in many cases, potential therapeutics for the treatment of various CNS disfunctions and diseases, neuropathic pain, inflammation, and cancer. However, the present scenario differs significantly between the two aforementioned nicotinic subtypes. Over the past few decades, a large number of selective α7-nAChR ligands, including full, partial and silent agonists, antagonists, and allosteric modulators, have been described and reviewed. Conversely, reports on selective α9-containing nAChR ligands are relatively scarce, also due to a more recent characterization of this receptor subtype, and hardly any focusing on small molecules. In this review, we focus on the latter, providing a comprehensive overview, while providing only an update over the last five years for α7-nAChR ligands.

1. Introduction

One of the most expressed nAChRs in the mammalian brain is the homomeric α7 subtype. This receptor subtype is composed by five identical subunits arranged around a central pore permeable to cations such as sodium and calcium. Interestingly, otherwise from others nicotinic receptors, the α7 and the α9α10 subtype show very high permeability to calcium. This peculiarity confers to α7 subtypes a very particular behavior; indeed, the influx of calcium ions results in cell depolarization but it may also result in intracellular signaling generation followed by gene transcription [1]. The α7-nAChR contains five identical agonist binding sites, located at subunit interfaces in extracellular domains, but the occupancy of only one site is able to fully activate the receptor [2]. Historically, they are characterized as highly sensitive to α-Bungarotoxin, which acts as an antagonist, and as sensitive to choline, which acts as an agonist [1]. α7-nAChRs are mainly expressed in CNS and, in particular, in the cortex, hippocampus and subcortical limbic regions and, at lower levels, in the thalamus and basal ganglia. In these areas, their main functions are related to the control of neurotransmitter release, mediating effects such as cognitive enhancement, neuroprotection and memory [3]. Reduction of the number and the functional activity of the α7-nAChRs has been correlated with a number of diseases of humans CNS including schizophrenia, Alzheimer's and Parkinson's diseases, bipolar disorder and epilepsy [3]. Therefore, potentiation of such receptors has emerged as a novel therapeutic strategy for these neurological pathologies.

α7-nAChRs are also expressed in non-neuronal tissues such as immune cells, astrocytes, microglia and endothelial cells, where they play a role in immunity, inflammation and neuroprotection [3]. In the last decade, many efforts have been done to elucidate the role of the α7-nAChR in the immune system, discovering that it is an important player of the cholinergic anti-inflammatory pathway [4]. The stimulation of α7-nAChR in macrophages activates several intracellular signal pathways, G proteins-mediated, such as JAK2-STAT3. Therefore, in these cells, the α7-nAChR acts as a metabotropic receptor instead of a "canonical" ionotropic receptor. Indeed, in most of the immune cells, α7-nAChR's ionotropic activity has not been detected. Therefore, also in this context, the potentiation of α7-nAChR activities could be a novel and very promising strategy for the modulation of inflammatory responses [5].

Finally, in the recent past, it has been shown that some tumor types, such as lung cancer and glioblastoma, express α7-containing nAChRs. Their activation promotes cell proliferation and activate the anti-apoptotic AKT and pro-proliferative ERK pathways. These nicotine-

☆ In memory of Professor Luigi Villa

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induced effects are blocked by the α7/α9α10 antagonist 2-triethylamoniumethyl ether of 4-stilbenol (MG624) [6] and by its analogs obtained by elongation of the ethylene linker between oxygen and nitrogen [7]. In this scenario, differently from the therapeutic strategies previously mentioned, the reduction of α7 activity may be beneficial for the treatment of these very aggressive tumors.

The studies on the anti-tumor activity of MG624 and its derivatives [7,8] as well the recent investigations on the α7 silent agonist *p*CF_3-diEPP [9] are very recent striking examples of how the functions and, more in general, the responses mediated by the α7-nAChR are not always clearly distinguishable from those mediated by the α9 * -nAChR (as α9 homomers or, more often, α9α10 heteromers, as indicated by the asterisk), despite many differentiating features of the two subtypes. Until recently, the poor stability and expression of α9 * -nAChRs and the lack of small molecules selectively modulating the α9 * receptor function have hampered investigations intended to sort out the respective roles, for instance, in the cholinergic anti-inflammatory system or in the pro-proliferative pathways. Within this context, the development of α9- and α9α10-nAChR ligands, mainly antagonists, has been pursued only in the last decade and rather sporadically aiming at fully understanding the therapeutic potential of these *sui generis* nicotinic receptors, not conventionally classifiable as neither muscle-type or neuronal. They are composed by five homologous subunits arranging around a central cationic-permeable pore. The α9 subunit, like the α7 subunit, is able to form functional homopentameric nAChR subtypes, when expressed in *Xenopus* oocytes, but it is also able to form a functional heteropentameric receptor combining with the α10 subunit [10,11]. In *Xenopus* oocytes, the expression of α9α10 subtype results, as occurs for α4β2 receptor, in two different stoichiometries, namely $(\alpha9)_2(\alpha10)_3$ [12] and $(\alpha9)_3(\alpha10)_2$ [13], which have different sensitivity to ACh. Otherwise, the α10 subunit is not able to lead functional channels. Hence, it has been proposed to act as a structural subunit, providing both complementary and principal components to the agonist binding site [14]. However, recent research [15] found that mammalian α10 subunits can assemble to form functional homomeric receptor subtypes, when expressed in *Xenopus* oocyte, by exposing the oocytes to some alkaloids (*i.e.*, strychnine, brucine, and methyllycaconitine). This finding could prompt search for α10-nAChR selective ligands to address questions about pharmacology of this potential new receptor subtype.

The α9 * ligand-gated ion channels are mainly expressed in the hair cells of the inner ear, where they represent the primary receptor and are involved in the sound transmission and sound discrimination [16]. The α9 and α10 subunit are also expressed in the cells of the immune system, where they are implicated in the immune response [17]. Interestingly, there are no evidence that the α9-containing nAChRs, expressed by these cells, form functional ion channel receptors suggesting, similarly to α7-nAChRs, that they mediate a metabotropic pathway [18]. The expression of α9 subunits in the dorsal root ganglion neurons has been debated from the first publication in 2002 [19], whose results were not replicated. However, α9-mRNA (and α10-mRNA at low levels) were recently found in 25% of human dorsal root ganglion neurons [20], as reviewed by A. B. Elgoyhen in this special issue [21]. Finally, a number of studies have shown the presence of α9-containig nAChRs in many cancer cells and tissues, where their activation promotes tumor cell growth and, conversely, their inhibition has antiproliferative effects. These results are extensively summarized in a recent review [22]. Therefore, these receptors are mainly expressed in a very few tissues, and most importantly, otherwise than other neuronal nAChRs, they are not present in the human brain [23].

2. α9 * - and α7-nAChR ligands

In this section, we summarize the until now developed ligands of α9-containing receptors (subsection 2.1) and the α7-nAChR ligands reported in the last five years (subsection 2.2).

2.1. α9 * -nAChR ligands

2.1.1. Screening of known nicotinoids and Cys-loop receptors ligands at α9 * -nAChRs

The pharmacological characterization of α9-containing nAChRs started with the pioneering work of Belén Elgoyhen and colleagues, who reported the isolation of the α9-nAChR resulting from injection of α9 cRNA in *Xenopus* oocytes. By screening of previously known cholinergic ligands, the authors proposed mixed nicotinic-muscarinic properties for the newly discovered receptor. Indeed, on one hand, some non-selective agonists for nicotinic or muscarinic receptors (*i.e.*, ACh, and carbachol), some selective nicotinic agonists, such as methylcarbachol and suberyldicholine, and some selective muscarinic agonists (*i.e.*, McN-A-343, and methylfurtrethonium) were able to elicit ion currents in α9-containing nAChR subtypes expressed in *Xenopus laevis* oocytes (Figure and Table 1), while, on the other hand, some nicotinic agonists (*e.g.*, epibatidine, nicotine, and cytisine), some nicotinic antagonists (*i.e.*, mecamylamine, dihydro-β-erythroidine, and methyllycaconitine), and some agonists and antagonist for muscarinic receptors (*e.g.*, muscarine, pilocarpine, bethanechol, atropine, and gallamine) exhibited inhibitory properties [24].(Fig. 1).

The same group investigated other ligands of the Cys-loop receptors superfamily (Fig. 2) and found that nicotinic receptors containing the α9 subunit are blocked by bicuculline, an antagonist of $GABA_A$ receptors, and by strychnine, a glycine receptor antagonist. Bicuculline inhibits ACh evoked currents of α9-nAChRs expressed in *Xenopus laevis* oocytes with IC_{50} value of 0.768 μM, which is similar to the result obtained by inhibition of $GABA_A$ receptors, and strychnine, which inhibits glycine receptors with IC_{50} of 0.05 μM, inhibits ACh evoked currents of α9-nAChRs expressed in *Xenopus laevis* oocytes with IC_{50} value of 0.0178 μM. With lower potency, serotonin, the endogenous agonist at 5-HT_3 receptor, inhibits ACh-evoked currents of α9-nAChRs expressed in *Xenopus laevis* oocytes with IC_{50} of 251 μM (5.4 μM IC_{50} when tested at α9α10-nAChRs). In this scenario, ligands for the 5-HT_3 receptor have been evaluated at α9α10-nAChRs expressed in *Xenopus laevis* oocytes finding a high inhibitory activity for tropisetron (0.0701 μM IC_{50}), ondansetron (0.60 μM IC_{50}), and bemesetron (MDL-72222, 0.70 μM IC_{50}). Such inhibition was proved to be reversible and concentration-dependent [25,26].

As previously mentioned, the α9α10-nAChRs are mainly expressed in the hair cells of the inner ear and their activation modulate the sound transmission. This knowledge triggered the Elgoyhen's group to investigate the possible correlation between the ototoxic activity of some aminoglycosides and their activity at the α9α10-nAChRs [27]. Neomycin (1.2 μM IC_{50}), gentamicin (3.7 μM IC_{50}), streptomycin (6.5 μM IC_{50}), amikacin (160.2 μM IC_{50}), and kanamycin (894.0 μM IC_{50}) were all able to inhibit ACh evoked current in α9-nAChRs expressed on *Xenopus* oocytes. A deeper investigation on gentamicin suggested a non-competitive type of block as a mechanism of action. This inhibition could explain the suppression of the olivocochlear efferent function, one of the initial reversible actions of aminoglycosides at the organ of Corti.

The detection of opioid peptides in the auditory and vestibular efferent neurons prompted Guth and coworkers to the investigation of such peptides as α9α10-nAChR ligands [28]. In this research, the endogenous peptide endomorphin-1 and dynorphin B have been proved to inhibit, in a reversible and concentration-dependent manner, α9α10-nAChRs expressed on *Xenopus laevis* oocytes with 1.7 μM and 1.6 μM IC_{50}, respectively.

2.1.2. Bis-, tris-, and tetrakis-aza-aromatic quaternary ammonium salts

Besides the pharmacological evaluation of already known molecules at the α9α10-nAChR, essential to increase knowledge about the pharmacology of this receptor subtype, and, eventually, to define possible off-target effects possessed by some pharmacological tools or approved therapies, the scientific community has moved the attention to the

Table 1
Functional activity of cholinergic ligands at α9-nAChR subtypes expressed on *Xenopus laevis* oocytes [24].

Activity on α9-nAChR subtypes		Activity on α9-nAChR subtypes	
Compound	**EC_{50}, μM**	**Compound**	**IC_{50}, μM**
ACh	11.4	Epibatidine	1.6
Carbachol	63.7 – partial agonist	Nicotine	31.5
Methylcarbachol	30.4 – full agonist	Cytisine	43.1
Suberyldicholine	44.1 – partial agonist	Mecamylamine	8.5
McN-A-343	Weak partial agonist	Dihydro-β-erythroidine	24.2
Methylfurtrethonium	Weak partial agonist	Methyllycaconitine	0.0011
		Muscarine	84
		Pilocarpine	76
		Bethanechol	105
		Atropine	1.0
		Gallamine	1.5

development of molecules that selectively target this receptor subtype.

Relying on previous series of bis-, tris-, and tetrakis-aza-aromatic quaternary ammonium salts [29–32], Zheng and coworkers, in 2011 [33], reported the α9α10-nAChRs antagonist activity of forty compounds belonging to these structural classes. All the compounds were initially screened at a 100 nM concentration at cloned α9α10-nAChR, heterologously expressed in *Xenopus* oocytes, for their ability to block ACh-induced currents. A selection of these derivatives was further evaluated in full concentration-response studies for inhibition of ACh-induced currents at both α9α10- and α7-nAChR. The structure activity relationship analysis of the synthesized compounds suggested that: a) analogs containing three or four quaternary ammonium head-groups were more effective in inhibiting α9α10 receptors; b) the presence of a rigid linker unit (*i.e.*, molecules containing triple bond linker) confers great inhibitory activity at α9α10-nAChRs and high selectivity over α7-nAChRs; c) the cationic head-group appears to be important in conferring selectivity while it has less impact on the inhibitory potency at α9α10-nAChRs. Within the reported series, compounds **1**, **2**, and **3** (Fig. 3) were the most interesting molecules with peculiar pharmacological properties. Compounds **1** and **2** block

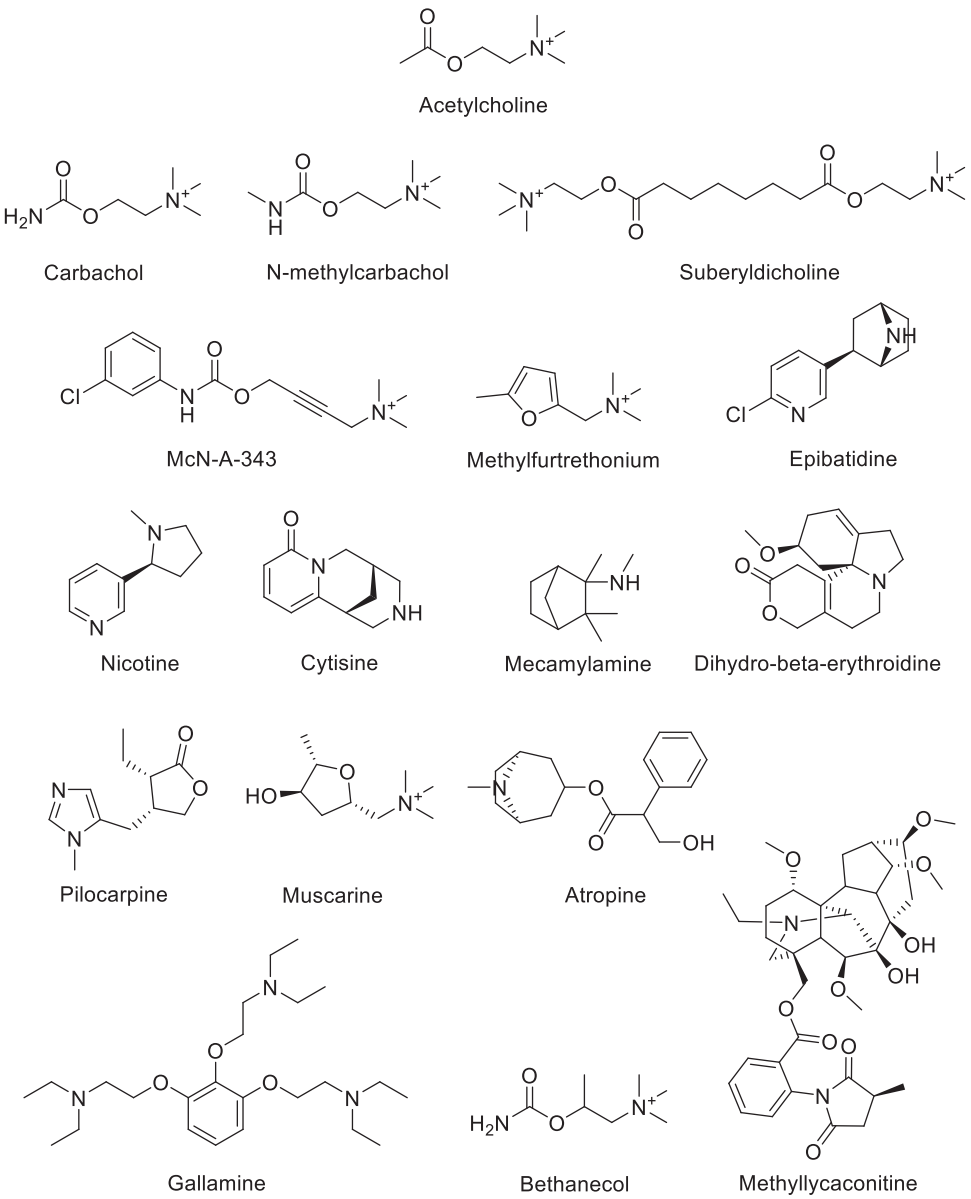


Fig. 1. Structure of cholinergic ligands screened at α9-nAChRs.

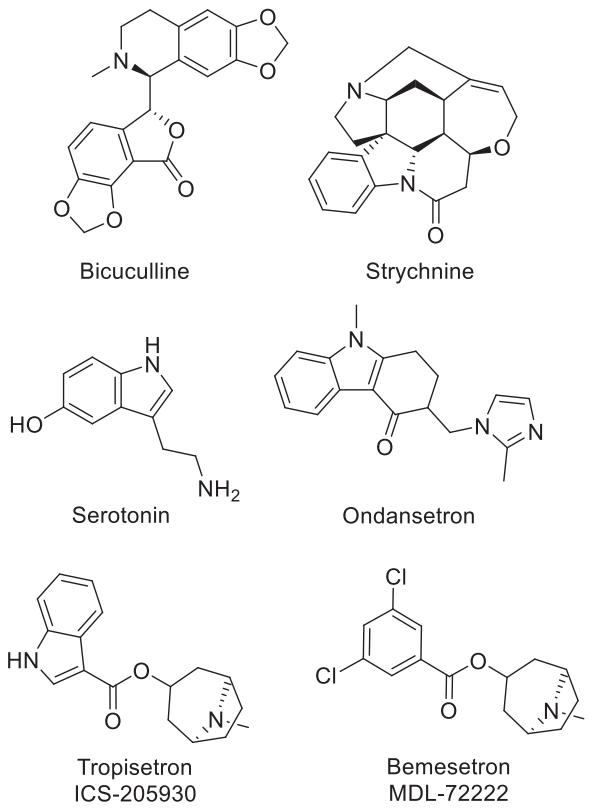


Fig. 2. Structure of agonists and antagonists of the Cys-loop receptors superfamily.

α9α10-nAChR subtype with 16 nM and 4.2 nM IC_{50}, respectively, with a remarkably high selectivity for α9α10- *vs* α7-nAChR subtype (75 and 360-fold respectively). On the other hand, **3** was the most potent antagonist of the series capable of inhibiting α9α10-nAChRs with 0.56 nM IC_{50} but endowed with lower selectivity toward α7-nAChRs compared to other analogs. Compounds **1**, **2**, and **3** were also tested *in vivo* to evaluate potential analgesic effects and they proved to be efficacious in two animal pain models, namely the rat formalin model of tonic inflammatory pain and the rat chronic constriction nerve injury, while they were ineffective in the rat tail-flick test [33,34].

The inhibitory activity of compound **3**, was also evaluated against α6/α3β2β3- (0.1 μM IC_{50}), α6/α3β4- (6.1 μM IC_{50}), α4β2- (3.8 μM IC_{50}), α3β4- (0.8 μM IC_{50}), and muscle- (0.12 μM IC_{50}) nAChR [33,34]. Moreover, **3** had not affinity for $GABA_A$ and $GABA_B$ receptors and several orders of magnitude lower affinity for $5HT_3$ and κ-opioid receptors compared to α9α10-nAChRs.

2.1.3. 1,1-Diethyl-4-phenylpiperazinium salts

Grasping from a pool of previously synthesized derivatives of 1,1-diethyl-4-phenylpiperazinium (diEPP), Papke and coworkers have recently reported some selective agonist and antagonist of α9- and α9α10-nAChR subtypes by conducting an extensive pharmacological characterization of more than 25 compounds [35,36]. Some molecules of this subset (Fig. 4), that have been previously characterized as partial (*e.g.*, *o*-Cl-diEPP) and silent (*e.g.*, *p*-F-diEPP) agonists for the human α7-nAChR subtype, when tested on human α9-nAChR subtype, expressed in *Xenopus* oocyte, showed inhibitory activity with 4.45 μM IC_{50} (*o*-Cl-diEPP) and 5.67 μM IC_{50} (*p*-F-diEPP). On the other hand, *p*-CN-diEPP and *p*-CF_3-diEPP, a partial and silent agonist at α7-nAChR subtype respectively, both elicit ion currents in human α9-nAChR subtype expressed in *Xenopus* oocyte with 0.368 μM EC_{50} (I_{max} = 0.76) and 7.04 μM EC_{50} (I_{max} = 0.36) respectively. In a parallel study, *p*-CF_3-diEPP was also evaluated as an anti-inflammatory agent in human whole blood cultures. The compound was able to inhibit the LPS-induced release of pro-inflammatory cytokines and the authors concluded that these effects are probably mediated by nAChR subunits α7, α9 and α10 [9]. Noteworthy, *p*-F-diEPP was tested in competition experiment demonstrating that diEPP analogs compete with ACh for the same binding site at the α9-nAChR subtype. *In vivo,* α9 is assembled with α10, therefore the authors tested the key compounds also on α9α10-nAChR and found no significant differences from the results obtained at α9-nAChR. Subsequently, using the reported EM structure for the homologous α7 receptor as a template, the authors built a homology model of the α9 receptor and docked a series of these agonists and antagonists. The identification of a peculiar binding pocket subsite, located on the (-) side, that is involved in the agonist's interaction led to the design of a novel compound with a modified phenyl core. The new derivative, namely APA-diEPP, showed a potent full agonist activity at the α9-nAChR subtype (0.67 μM EC_{50} and 1.11 I_{max}) with 10-fold preference over the α7-nAChR subtype (6.4 μM EC_{50}), where it behaves as a partial agonist (0.42 I_{max}).

2.1.4. Analogs of the 2-triethylammonium ethyl ether of 4-stilbenol (MG624)

The 2-triethylammonium ethyl ether of 4-stilbenol (MG624) was described, in the past century, as a ganglioplegic agent, devoid of activity on neuromuscular junction and with low activity at muscarinic ACh receptors [37,38]. In 1998, it was first characterized as an antagonist at the α7-nAChRs, with moderate and high selectivity over the β4- and β2-containing nAChRs respectively, and, more recently, its antagonist activity at the α9α10-nAChRs was highlighted (6.68 μM IC_{50}) [6, 39]. Starting from the ability of nicotine to promote cancer progression

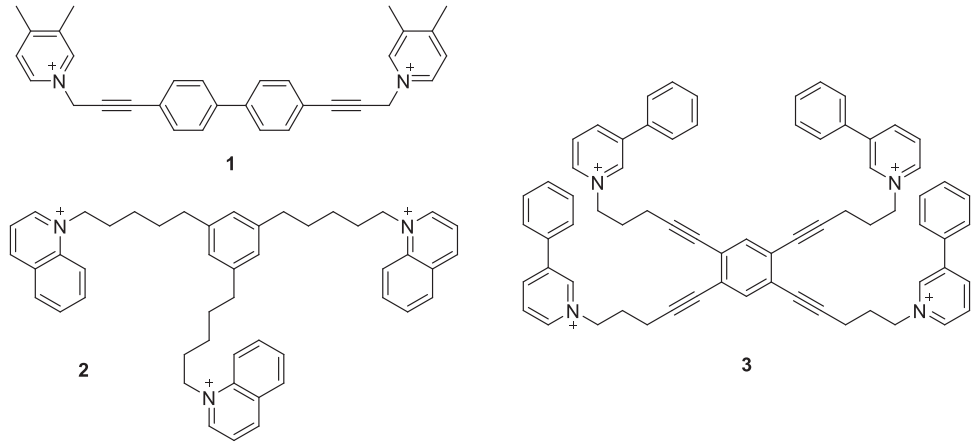


Fig. 3. Structure of representative bis-, tris-, and tetrakis-aza-aromatic quaternary ammonium salts as selective antagonists of α9α10-nAChRs.

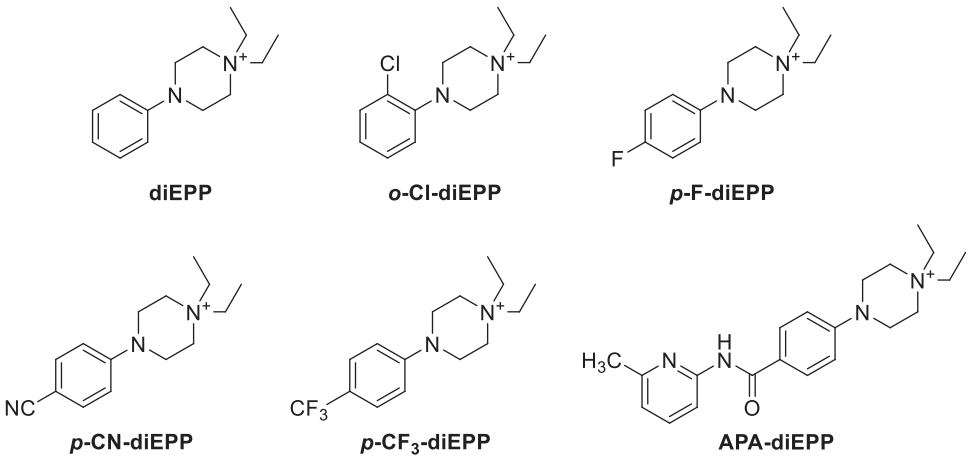


Fig. 4. Representative compounds from a series of diEPP derivatives with selective agonist and antagonist activities at α9- *versus* α7-nAChRs.

in tumor type, overexpressing α7- and α9α10-nAChRs, Bavo et al. reported the antiproliferative properties, over adenocarcinoma and glioblastoma cells, of MG624 and of some its derivatives with a lengthened alkylene linker between the charged nitrogen and the ethereal oxygen. The lengthening of ethylene bridge of MG624 results in potent antiproliferative effect paralleled by increased α7- and α9α10-nAChRs antagonism [7]. A very recent pharmacological investigation of these compounds highlighted some additional non-nicotinic mechanisms that could contribute to their antitumor activity [6,8]. A structure-activity relationship study around the structure of MG624, conducted by the same research group, led to the discovery of two interesting small-molecules with selective antagonism activity for either the α9α10- or the α7-nAChRs (please refer to Section 2.2.2 for compounds with selective antagonism activity at the α7-nAChRs) [40,41]. Overall, approximately sixty derivatives of MG624 were synthesized and characterized for binding affinity at the α7-nAChRs and, some of them, at the α3β4-, and α4β2-nAChRs. *In vitro* functional characterization has been performed for a selection of these compounds to evaluate their inhibitory activity, using *Xenopus laevis* oocytes expressing human α7- or α9α10-nAChRs. The biological results of the modifications of MG624 concerning the cationic head, the ethylene linker and the styryl portion allow to define the following structure activity relationships (Fig. 5): (a) the increase or decrease of the ammonium head volume (*e.g.*, compound **4** and **5**, with 5.74 μM and 34.3 μM IC_{50} at α9α10-nAChR, respectively), and the inclusion of the ethylene bridge between *O*- and triethylammonium group into a piperidine or quinuclidine ring (*e.g.*, compound (±)-**6**, 10.4 μM IC_{50} at α9α10-nAChR) lead to selective antagonists at α9α10-nAChRs, devoid of any antagonist activity at α7-nAChR; (b) the derigidification of styryl moiety (*i.e.*, compound **7**, 9.12 μM IC_{50} at α9α10-nAChR) leads to a selective antagonist at α9α10-nAChR, devoid of any antagonist activity at α7-nAChR.

Further pharmacological characterization of compound **4** and other α9α10-nAChR antagonists of the same series for intrinsic activity allowed the authors to discover the partial agonism behavior at supramicromolar concentration at α9α10-nAChR and to hypothesize a mechanism of their selective α9α10-nAChR antagonism, which could consist of opening, engaging the channel, and then blocking the receptor into a non-conducting and open state. Compound **4** is the most interesting compound of the series being a potent and selective antagonist at α9α10-nAChR (5.74 μM IC_{50}), with very low affinity at α7- and α3β4-nAChR, and completely devoid of activity at the α7-nAChR subtype at the maximum tested concentration (100 μM). For these features, compound **4** represents a candidate to investigate the debated multifaceted aspects of α9α10-nAChRs druggability.

*2.1.5. α-Conotoxins targeting the α9 * -nAChR*

An exhaustive report about the pharmacology and therapeutic potential of conotoxins antagonizing nicotinic acetylcholine receptors is already presented in this issue by Adams et al. [42] However, an examination of the structural evolution of conotoxins through medicinal chemistry techniques together with pharmacological properties of native and modified α-conotoxins targeting α9α10-nAChRs deserves a chapter in this review. Scientific interest has recently emerged to modify and functionalize conopeptides aiming to enhance knowledge about structure activity relationships and to develop new pharmacological tools with improved potency and different subtype selectivity.

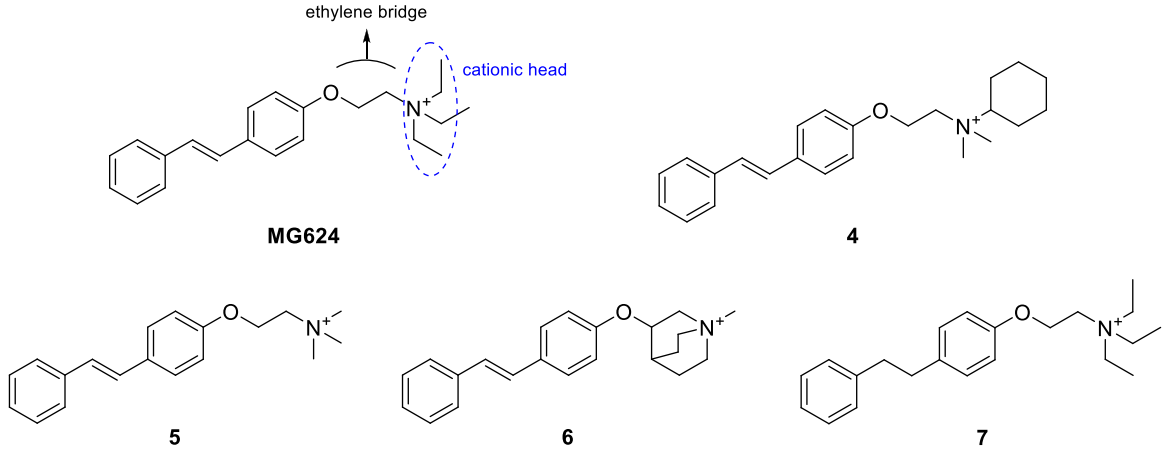


Fig. 5. Analogs of MG624 endowed with selective antagonist activity toward α9α10- over α7-nAChR subtypes.

Conotoxins can be divided into subfamilies based on different criteria: the signal peptide sequence (*e.g.*, A-, D-, I- *etc.*), the pharmacological targets (*e.g.*, α-, γ-, δ-, κ- *etc.*), and the cysteine framework pattern. α-Conotoxins are generally composed of 12–40 amino acid residues, with four cysteine residues that are important to determine the secondary structure of this peptides and are essential for the biological activity. Indeed, cysteine residues form disulfide bridges that confer different secondary structure (Fig. 6A) based on the bridge pattern, namely globular (C1–C3, C2–C4), ribbon (C1–C4, C2–C3), and bead (C1–C2, C3–C4) [43,44].

In 2005, McIntosh et al. reported the first toxin able to distinguish between α7- and α9α10-nAChRs, namely the α-conotoxin PeIA identified from *Conus pergrandis* [45]. The α-conotoxin PeIA inhibits ACh evoked current in α9α10-nAChRs with 6.9 nM IC_{50} and displays a 260-fold higher selectivity for α9α10- respect to α7-nAChRs. α-Conotoxin PeIA has a high sequence similarity with toxins MII and GIC, isolated by other Conus species, but neither of these two α-conotoxins inhibit responses elicited by ACh at α9α10-nAChRs (Table 2).

A novel α-conotoxin from the species *Conus regius*, namely the α-RgIA, was identified and characterized as a subtype specific blocker of the α9α10-nAChR. α-RgIA was able to inhibit ACh evoked current at α9α10-nAChR with 5.2 nM IC_{50} and a remarkable selectivity for α9α10-nAChR respect to α7-nAChR (approximately 1000 fold), α3β2-nAChR (>2000 fold) and other heteromeric nicotinic subtypes [51]. The critical residues conferring the activity toward α9α10-nAChR of this α-conotoxin were determined by Huyn et al. using mutational approach [62]. Both side chain length and charge of Arg7 were proved to be critical for pharmacological activity, together with the side chain length, but not charge of Arg9. On the contrary, substitution of Tyr10 (*e.g.*, with 3-iodo-L-tyrosine) and Arg11 (*e.g.*, with L-homoarginine) allowed to increase potency for human α9α10-nAChR subtype.

Another important α-conotoxin is Vc1.1 from the venom ducts of *Conus victoriae*, a molecule that entered in clinical trial for the treatment of neuropathic pain. This conotoxin can selectively block α9α10-nAChR, with 19 nM IC_{50}, compared to > 3,0000 nM IC_{50} at α7-nAChR. Vc1.1 also blocks α6-containing nAChRs with lower potency and it is a potent antagonist of *N*-type calcium channel function through the agonist activation of $GABA_B$ receptor [47,63]. A structure activity relationship study conducted by scanning mutagenesis of Vc1.1 revealed that residues Asp5–Arg7 and Asp11–Ile15 are fundamental for activity at α9α10-nAChR. However, introduction of N9G mutation or S4R mutation resulted in more potent antagonist at this receptor subtype, with IC_{50} of 6 nM and 17 nM respectively (please note that in this article, Vc1.1 activity was reported to be 109 nM at rat α9α10-nAChR). The new analogs maintained selectivity for α9α10-nAChR *versus* α7, α3β2 and α3β4 [48]. Similarly, a structure activity relationship study of Vc1.1 at human α9α10-nAChR conducted by minimal side chain replacement revealed that single mutations S4 Dab (Dab = diaminobutyric acid), N9W and N9A all increased antagonistic properties at α9α10-nAChR. These findings prompted the authors to synthesize analogs Vc1.1[S4 Dab, N9W] and Vc1.1[S4 Dab, N9A] which possessed a 20-fold increased potency at human α9α10-nAChR (IC_{50} of 38.7 nM and 52.5 nM, respectively) compared to Vc1.1 (IC_{50} of 1000 nM) [49].

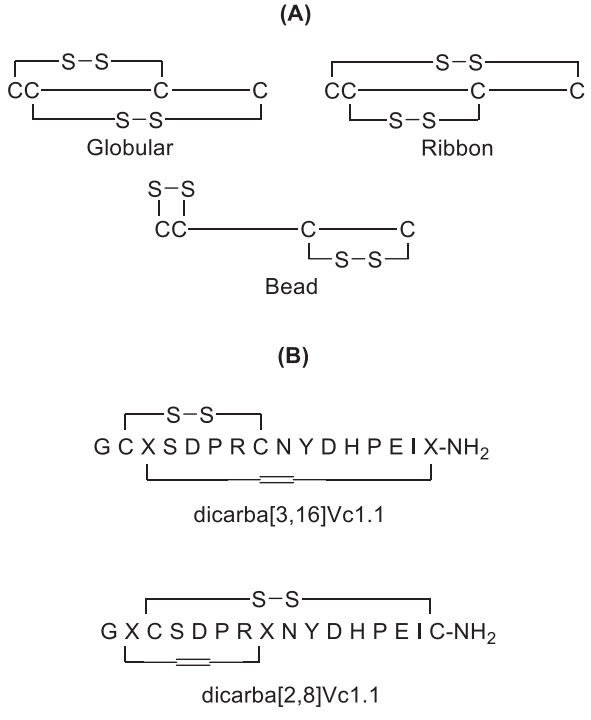


Fig. 6. (A) Possible arrangements of disulfide bridges of α-conotoxins: globular (the most common), ribbon and bead structures. (B) Dicarba bridges modifications of Vc1.1.

Vc1.1, as well as all natural conopeptides, contains disulfide bridges that are essential for the biological activity but hamper stability and large-scale production. For these reasons, Vc1.1 has been modified with dicarba moiety (*i.e.*, the disulfide moiety was substituted with *cis* or *trans* HC=CH moiety) to address the stability issue and to study possible influence on pharmacological properties. Interestingly, the introduction of dicarba bridge in position [3,16] (Fig. 6B) allowed selective inhibition of α9α10-nAChR with 2.4 μM IC_{50} for the *trans* isomer, and 12.0 μM for the *cis* isomer, and null activity at $GABA_B$ receptors. On the other hand, the introduction of dicarba bridge in position [2,8] afforded analogs with selective activity toward $GABA_B$ receptors and null activity at the α9α10-nAChR [50].

A similar study was conducted by structural modification of α-RgIA. In this case, the introduction of dicarba bridge in position [3,12] allowed selective inhibition of α9α10-nAChR with 1.15 μM IC_{50} for the *trans* isomer, and 1.47 μM for the *cis* isomer. The introduction of the dicarba bridge also allowed to obtain improved α9α10- *vs* α7-nAChR selectivity for the *cis* isomer (inactive at the α7-nAChR subtype), whereas the *trans* isomer was unselective for the α9α10-nAChR subtype, exhibiting a 3.95 μM IC_{50} at the α7- nAChR. [53] Subsequently, the structure of α-RgIA was modified with the development of a synthetic peptide, namely RgIA4. This molecule antagonizes selectively α9α10-nAChRs, with 1.2 nM IC_{50}, compared to α7-nAChR (4500 nM IC_{50}), other nAChRs subtypes and other targets (*i.e.*, opioid and $GABA_B$ receptors). RgIA4 was demonstrated to prevent chemotherapy-induced cold allodynia *in vivo* in rat and mice after oxaliplatin administration and to produce a sustained analgesic effect lasting 21 days after oxaliplatin administration in mice [54,64]. RgIA4 was also successful in the treatment of mechanical allodynia caused by paclitaxel, thus supporting its efficacy in multiple chemotherapeutics models [65]. The prevention of cold allodynia after oxaliplatin administration mediated by RgIA4 was proved to not occur in α9-nAChR-encoding gene (*i.e.*, CHRNA9) knock out mice and in mice lacking CD3 + T-cells indicating that both CHRNA9 and CD3 + T-cells are required to mediate this effect [66].

Recently, Liang et al. reported the synthesis and pharmacological characterization at nAChR subtypes of α-conotoxin dimers. Using click chemistry reactions between an azide group of a modified α-conotoxin and an alkyne-lysine dendron with two alkyne moieties, dimers of Vc1.1, RgIA# (an analog of α-RgIA truncated and amidated at C-terminal), and PeIA were synthesized. The dimerization generally induced enhanced potency at the α9α10-nAChR expressed on *Xenopus* oocyte, with 266 nM IC_{50} for the Vc1.1 dimer (1000 nM IC_{50} for Vc1.1), 38.5 nM for the RgIA# dimer (248 nM IC_{50} for RgIA#), and 1.9 nM for the PeIA dimer (21.9 nM IC_{50} for PeIA). The human α9α10- *vs* α7-nAChR selectivity compared to parent α-conotoxin was preserved for Vc1.1 dimer, reduced for PeIA dimer and abolished for RgIA# dimer (Table 2). Therefore, RgIA# dimer is an important pharmacological tool that would be useful to prove dual inhibition targeting these two nAChR

Table 2
Conotoxins sequence and activity at α9α10- and α7-nAChRs expressed on *Xenopus* oocytes. # = amidated at C terminus; * = citrulline; ‡ = 3-Iodo-L-Tyrosine; β^3hY = β^3-homo tyrosine; bhY = L-β-homotyrosine; [] = side chain cyclization; X = methylene thioacetal replacement; Pen = L-penicillamine.

Name	Sequence	IC_{50} α9α10 (nM) [species]	IC_{50} α7 (nM) [species]
MII	GCCSNPVCHLEHSNLC#	> 10,000 [rat][a]	
GIC	GCCSHPACAGNNQHIC	> 10,000 [rat][a]	
PeIA	GCCSHPACSVNHPELC#	6.9 [rat][a]	1800 [rat][a]
		21.9 [human][b]	> 10,000 [human][b]
PeIA dimer		1.9 [human][b]	353.6 [human][b]
ImI	GCCSDPRCAWRC#		
Vc1.1	GCCSDPRCNYDHPEIC#	19 [rat][c]	≥ 30,000 [rat][c]
		109 [rat][d]	7123 [rat][d]
		1000 [human][b]	> 10,000 [human][b]
Vc1.1[N9G]		6 [rat][d]	≥ 3000 [rat][d]
Vc1.1[S4R]		17 [rat][d]	≥ 3000 [rat][d]
Vc1.1[S4 Dab, N9W]		38.7 [human][e]	
Vc1.1[S4 Dab, N9A]		52.5 [human][e]	
Vc1.1-dicarba[3,16]trans		2400 [rat][f]	
Vc1.1-dicarba[3,16]cis		12,000 [rat][f]	
Vc1.1 dimer		266 [human][b]	> 10,000 [human][b]
α-RgIA	GCCSDPRCRYRCR	5.2 [rat][g]	4660 [rat][g]
		510 [human][h]	
α-RgIA-dicarba[3,12]trans		1150 [rat][i]	3950 [human][i]
α-RgIA-dicarba[3,12]cis		1470 [rat][i]	> 3000 [human][i]
α-RgIA#	GCCSDPRCRYRC#	248.7 [human][b]	> 1000 [human][b]
α-RgIA# dimer		38.5 [human][b]	63.1 [human][b]
RgIA4	GCCTDPRC* ‡QCY	1.2 [mouse][j]	4500 [mouse][j]
		1.5 [human][k]	
Cyclic-RgIA4	[GCCTDPRCR‡QCY]	3.4 [human][k]	504 [human][k]
RgIA-5524	GCXTDPRCR‡QX(bhY)R	0.9 [human][h]	186 [human][h]
RgIA-5474	GC(Pen)TDPRCR‡QC(β^3hY)R	0.0504 [human][l]	115 [human][l]
αB-VxXXIVA	VRCLEKSGAQPNKLFRPPCCQKG PSFARHSRCVYYTQSRE	1200 [rat][m]	30,000 [rat][m]
αS-GVIIIB	SGSTCTCFTSTNCQGSCECLSP PGCYCSNNGIRQRGCSCTCPGT#	9.79 [rat][n]	> 1000 [rat][n]
αO-GeXIVA	TCRSSGRYCRSPYDRRRRYCRRITDACV	4.61 [rat][o]	415 [rat][o]
[C2A,C9A,C20S,C27S]GeXIVA		6.1 [rat][p]	1270 [rat][p]
		33 [human][p]	
Bt14.12	GDCKPCMHPDCRFNPGRCR#	62.3 [rat][q]	> 1000 [rat][q]
Bt14.12[△2D,+ 19RRR]	GCKPCMHPDCRFNPGRCRRRR#	12.7 [rat][q]	-

[a] Data from ref. [45]
[b] Data from ref. [46]
[c] Data from ref. [47]
[d] Data from ref. [48]
[e] Data from ref. [49]
[f] Data from ref. [50]
[g] Data from ref. [51]
[h] Data from ref. [52]
[i] Data from ref. [53]
[j] Data from ref. [54]
[k] Data from ref. [55]
[l] Data from ref. [56]
[m] Data from ref. [57]
[n] Data from ref. [58]
[o] Data from ref. [59]
[p] Data from ref. [60]
[q] Data from ref. [61]

subtypes as a promising therapy of various type of cancer [46], similar to previously reported analogs of MG624, dual inhibitors useful to investigate the involvement of these receptors in tumor progression [7].

In 2020, a cyclic derivative of RgIA4 was developed aiming to increase stability and to maintain the good pharmacological properties of the native α-conotoxin. The followed approach was the side-chain cyclization, meaning that a third cyclization bridge is introduced at the *C*- and *N*-termini in order to not perturb the backbone and, thus, to maintain high potency. A screening of four different side chains was conducted, followed by two single amino acid modification that allowed to select the best cyclic RgIA4 derivative (Cyclic-RgIA4, Table 2). The potency (3.4 nM IC_{50}) on human α9α10-nAChR expressed on *Xenopus* oocyte indicated a comparable activity to RgIA4 (1.5 nM IC_{50}). Selectivity was maintained *versus* all nAChR subtypes (*i.e.*, >1000 fold selectivity for α9α10 *versus* α2β4, α3β2, α3β4, α4β2, α4β4, α6/α3β2β3, α6/α3β4, β4β4, and muscle type) while for α7-nAChR subtype a 148-fold selectivity for α9α10- *versus* α7-nAChR (504 nM IC_{50}) was found. Moreover, cyclic RgIA4 derivative showed improved serum stability compared to RgIA4 and it was able to prevent oxaliplatin induced cold allodynia [55].

The disulfide replacement with methylene thioacetal bridge has been evaluated by Zheng et al. who synthesized RgIA-5524, a potent (0.9 nM IC_{50}) and selective antagonist at the α9α10-nAChR with improved serum stability. The RgIA analog showed a good selectivity for α9α10 *vs* α7 subtype (206-fold), other nAChRs (more than 11,000-fold over α2β2, α2β4, α3β2, α3β4, α4β2, α4β4, α6/α3β2β3, and α6/α3β4) and exerted low or negligible activity on pain-associated receptors or ion channels (*e.g.*, opioid receptors, NMDAR, BZD, OCT receptors and various voltage

gated ion channels). RgIA-5524 relieved neuropathic pain induced by oxaliplatin administration through inhibition of α9-containing nAChRs, as demonstrated by *in vivo* experiments [52].

Another series of RgIA4 derivatives were developed by introduction of penicillamine, a cysteine surrogate, which allowed the discovery of RgIA4–5474, a potent inhibitor of α9α10-nAChR (0.0504 nM IC_{50}) with 2000-fold greater potency compared to α7-nAChR (115 nM IC_{50}). The new conotoxin expresses high selectivity also *vs* all other nAChRs assayed and exhibits low or negligible activity at opioid receptor subtypes, N- and L- Ca^{2+} channels and a large panel of other receptors and ion channels. This substitution also allowed to increase metabolic stability in serum and the obtained peptide, RgIA4–5474, reversed cold allodynia and painful cold sensation in oxaliplatin-induced pain model in mouse [56].

Interestingly, introduction of a fluorescent dye (*i.e.*, Cy3, a cyanine dye) to the *N*-terminus of α-conopeptide RgIA-5474 *via* click chemistry reaction, allowed the preparation of a novel biomarker, namely RgIA-5727, capable of potently inhibiting α9α10-nAChR (23 pM IC_{50}), selectively *versus* α7 (290-fold) and other nAChR subtypes (40,000-fold). The compound can serve as a pharmacological tool to study α9α10-nAChRs in various cells and tissues [67].

Another conotoxin with selective antagonist activity for the α9α10-nAChR has been reported in 2015, when McIntosh et al. described the isolation and identification of αS-GVIIIB. This peptide blocks the α9α10-nAChR with a 9.79 nM IC_{50}. Moreover, αS-GVIIIB is selective for α9α10-nAChR compared to other nAChR subtypes, and it is also selective compared to 5-HT_3 receptor, another member of the Cys-loop receptor superfamily [58]. In the same year, the authors also reported the isolation of the αO-conotoxin GeXIVA [1,2], a peptide able to antagonize the α9α10-nAChR subtype with 4.61 nM IC_{50} and with a certain selectivity *vs* other nAChRs (*e.g.*, 90-fold selectivity over α7-nAChR subtype). Kinetic analysis of toxin dissociation indicated that the binding site of GeXIVA and that of conotoxin RgIA do not overlap. When tested in the rat chronic constriction injury model of neuropathic pain, GeXIVA reduced mechanical hyperalgesia [59]. Furthermore, *in vivo* administration of GeXIVA [1,2] in rats relieved mechanical and cold allodynia caused by oxaliplatin, and promoted recovery from neuropathic pain after repeated treatments [68].

GeXIVA was also tested to evaluate its antitumor activity; in particular, *in vitro* assays on breast cancer MDA-MB-157 cells highlighted antiproliferative and pro-apoptotic activity of the conotoxin. The antiproliferative activity was associated to the inhibition of α9α10-nAChR [69]. Interestingly, Yu et al. reported an assessment of the stability of GeXIVA [1,2] in serum, enzymes, thiols and in forced stress conditions. GeXIVA [1,2] was unstable in human serum, similarly to other previously assayed conopeptides (*i.e.*, RgIA). GeXIVA [1,2] was also unstable when tested in enzymatic stability studies (*i.e.*, in the presence of simulated gastric fluid and simulated intestinal fluid) and in buffers containing reducing thiol (*i.e.*, GSH and HSA) [70]. A recent article investigated the structure activity relationships of this peptide by single modification of the amino acid sequence and showed that disulfide bridges are not essential for the biological activity of GeXIVA. This finding suggests that the structure can be simplified and prompted the researchers to synthetize some derivatives leading to a new peptide (*i.e.*, [C2A,C9A,C20S,C27S]GeXIVA) able to inhibit human α9α10-nAChR expressed on *Xenopus* oocyte with 33 nM IC_{50} and endowed with good selectivity *vs* other nAChRs [60].

More recently, a new conotoxin member of the A-superfamily, Bt14.12, was cloned from *Conus betulinus* and was characterized in pharmacological assays and in SAR studies. Bt14.12 exhibited high potency for inhibition of α9α10-nAChR expressed on *Xenopus* oocyte with 62.3 nM IC_{50} and weaker inhibition of other nAChR subtypes (*e.g.*, α4β2, IC_{50} = 4117 nM; α3β2, IC_{50} = 1797 nM; other subtypes tested α2β2, α2β4, α3β4, α4β4, α7, IC_{50} > 1000 nM). The SAR study allowed the authors to understand that all amino acid residues significantly contribute to its potency. A further improvement of the functional activity was achieved by deletion of Asp2 combined with the addition of three Arg residues at *N*-terminus that resulted in 4-fold increase of potency [61].

2.1.6. α9 * -nAChR allosteric ligands

L-Ascorbic acid is a vitamin capable of modulating the activity of the Cys-loop receptor superfamily (*e.g.*, $GABA_A$Rs, $GABA_C$Rs *etc.*). For this reason, Boffi et al. evaluated the modulation, mediated by L-ascorbic acid, of α4β2-, α7-, and α9α10-nAChRs. The compound did not have any effect on the α4β2- and α7-nAChR subtypes, while was able to potentiate the ACh-induced current at the α9α10-nAChR expressed on *Xenopus* oocyte. The potentiation occurs in a concentration dependent manner with an effective concentration ranging from 1 to 30 mM, and it is stereo-specifically induced by L-ascorbic acid, but not by D-ascorbic acid. The authors concluded that the current modulation induced by L-ascorbic acid could be explained by an allosteric mechanism [71].

2.2. α7-nAChR ligands

In this subsection, we review the most recent α7-nAChR agonists and antagonists. The agonists are more numerous, and we present them grouped by molecular structures, while the antagonists are fewer and structurally heterogeneous and thus reported in a single subsection. Furthermore, a small collection of allosteric modulators, radioligands and an update on pharmacological characterization of previously known ligands for the α7-nAChR are described in separate sections.

2.2.1. α7-nAChR agonists

2.2.1.1. Tetrahydroisoquinolines, and tetrahydropyridyl pyridines. Kristensen and coworkers have previously described derivatives of aromatic *Erythrina* alkaloids as nAChR antagonists, with compound *O*-methylcorypalline being characterized as a strong and selective binder at α4β2-nAChR (0.87 μM K_i) *versus* other nAChR subtypes (*i.e.*, α4β4, α3β4, and α7) [72]. Starting from this study, a series of O-methylcorypalline and tetrahydroisoquinoline derivatives were reported by the same research group allowing the discovery of ligands for the α7- and α4β2-nAChRs (Fig. 7). The functional characterization of the compounds was determined at *m*α4β2-HEK293T and *r*α3β4- HEK293 in the FLIPR Membrane Potential Blue assay, and at *h*$\alpha7^{Ric\text{-}3/NACHO}$-HEK293 cell line in the Ca2 + /Fluo-4 assay in the presence of the α7-nAChR PAM PNU-120596. Within the series, compounds **8** and **9** displayed potent α7-nAChR agonist activity (*i.e.*, 0.99 μM and 1.2 μM EC_{50}, respectively), α4β2-nAChR antagonist activity (*i.e.*, 1.2 μM and 1.8 μM IC_{50}, respectively), and negligible or low activity at α3β4-nAChR. Furthermore, compound (±)-**10** stands out for the best activity at α4β2-nAChRs with half-maximal inhibitory concentration of 0.52 μM, null activity at α3β4, and agonist activity at α7-nAChR with 2.6 μM EC_{50}. Enantiomeric resolution of (±)-**10** and subsequent pharmacological evaluation demonstrated that its α4β2 antagonist activity resides on the *R*-enantiomer, (***R***)-**10** (0.22 μM IC_{50}), while the *S*-enantiomer has negligible activity at this subtype and maintained agonist activity at α7-nAChR with 5.4 μM EC_{50}. However, considering the agonist activity of (***R***)-**10** at α7-nAChRs (*i.e.*, 1.6 μM EC_{50}), the compound is considered a dual α4β2-antagonist and α7-nAChRs agonist. In vivo administration of (***R***)-**10** in the mouse forced swim test allowed to discover antidepressant-like effects. The SAR study also led to the identification of compound **11**, a *N*-benzyl substituted analog of O-methylcorypalline, endowed with α7-nAChR antagonist activity with 2.0 μM IC_{50} value and negligible activity at α4β2- and α3β4-nAChRs [73].

Xing et al. recently reported the pharmacological characterization of two isomeric nAChR agonists, the natural compounds isoanatabine from nemertine worm, and anatabine from the commercial tobacco plant. The racemic compounds were synthesized, and pure enantiomers were isolated by chiral preparative HPLC (compounds **12**–**15**, Fig. 7). The

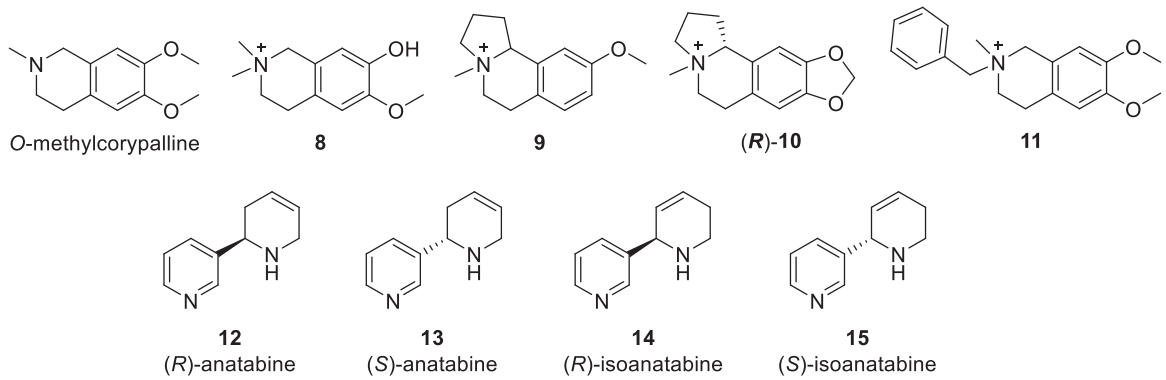


Fig. 7. Tetrahydroisoquinolines, and tetrahydropyridyl pyridines derivatives with agonist activity at α7-nAChR subtype.

pharmacological characterization of all 4 compounds showed a dual agonist activity at α4β2- and α7-nAChR. [74].

2.2.1.2. Quinuclidines. Xue et al. developed a series of derivatives of Br-IQ17B (Fig. 8), previously identified as a potent agonist for α7-nAChRs with a 1.8 μM EC_{50} (64 % E_{max}) [75], replacing the indolizine moiety with benzoindolizines (*e.g.*, compound **16**, Fig. 8) and with reduced aromatic pyrrole derivatives (*i.e.*, dihydropyrrolizine, and 5,6,7,8-tetrahydroindolizine). Overall, more than thirty derivatives were synthesized and almost all compounds showed agonist activity at human α7-nAChRs expressed on *Xenopus* oocytes. Compound **16** showed 1.88 μM EC_{50} (72.4 % E_{max}), and moderate selectivity over other nAChR subtypes (*e.g.*, α4β2, and α3β4) [76].

Another series of α7-nAChR ligands (Fig. 8) containing the quinuclidine scaffold was recently reported by Mazurov et al. Thirteen compounds were synthesized and assayed for binding affinity at α7-, α4β2-, and α3β4-nAChRs. It is worth noting that the configuration of quinuclidine ether influences the selectivity at α7- *versus* α4β2-nAChR subtype, being ***trans*-17** more selective for α7-nAChRs (1.4 nM α7 K_i, 73 nM α4β2 K_i) compared to ***cis*-17** (59 nM α7 K_i, 17.3 nM α4β2 K_i). Compound ***trans*-18** displayed the best profile of the series in term of binding selectivity for α7- *versus* α4β2-nAChRs (11.1 nM α7 K_i, 220 nM α4β2 K_i). Moreover, *in vivo* antitussive activity was evaluated in a guinea pig model. Compound ***trans*-18** showed a significant reduction of cumulative number of coughs, meaning that α7-nAChR modulators may be a novel option to develop antitussive drugs [77].

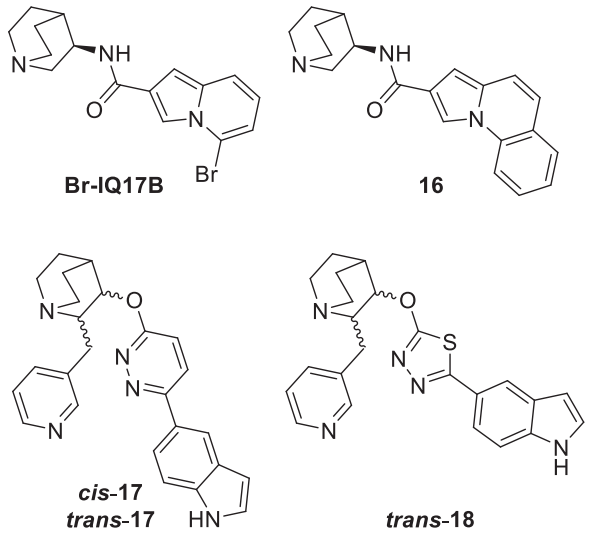


Fig. 8. Quinuclidine containing small molecules as agonists of α7-nAChR subtype.

Dallanoce et al. previously reported the discovery of spirocyclic quinuclidines as potent agonists (*e.g.*, compound **19**, namely (***R*)-ICH3**, Fig. 9) or silent agonists at α7-nAChRs (*e.g.*, **20**, and **21** Fig. 9) [78,79]. Recently, the ADMET properties of the racemate of **ICH3** have been characterized as well as the anti-inflammatory properties and the anti-proliferative effects on adipose-derived stem cells of (***R*)-ICH3** resulting from selective activation of α7-nAChRs [80–83]. Based on the results obtained with spirocyclic quinuclidines, two novel series of compounds were designed maintaining the quinuclidine nucleus of **20** and **21**, by introducing a methylene spacer between quinuclidine and the hydrogen bond acceptor moiety. 1,2,4-oxadiazoles bearing different (hetero)aryl moieties in 3-position were selected for isoxazoline replacement with the aim of exploring the α7-nAChRs binding pocket and possibly finding additional beneficial interactions (*e.g.*, general structures **a** and **b**, Fig. 9). More than twenty-five compounds were synthesized and assayed in electrophysiological experiments on human α7-nAChRs expressed in *Xenopus laevis* oocytes. In general, the previously mentioned structural changes (*i.e.*, introduction of a methylene spacer and 3-aryl-1,2,4-oxadiazole moiety) shifted the activity toward partial agonisms at α7-nAChRs (*e.g.*, compounds **32a**, and **32b**). In brief, SAR studies showed that: (a) partial agonism diminished by increasing size of the halogen in the tertiary amine series **22a**–**25a**, while the opposite trend was found within the quaternary ammonium series **22b**–**25b**; (b) polar methoxy group conferred partial agonism activity in tertiary amine series (compound **26a**–**27a**); (c) the introduction of heteroaryl substituent generally enhanced partial agonism activity for tertiary amines (*i.e.*, compounds **28a**–**31a**) and resulted in silent agonism for quaternary analogs (*i.e.*, **28b**–**30b**) [84].

2.2.1.3. 3-Substituted (3-pyridyloxymethyl)piperidines and 4,6-disubstituted 2-aminopyrimidines. Shen and coworkers have recently conducted a research campaign to discover novel acetylcholine-binding protein (AChBP) ligands. Starting from a mini-library screening (70 compounds), a piperidine derivative with high AChBP affinity was selected as a promising candidate for further development (*i.e.*, compound **34**, Fig. 10) [85]. At this stage, the authors have continued the SAR study through the synthesis and biological evaluation of 27 analogs after X-ray analysis of compound **33** co-crystallized with *Ls*-AChBP. A selection of compounds based on affinity studies have been evaluated for functional activity at α7-nAChR. Compounds (***S*)-34** and **35** behaved as the best partial agonist efficacy at α7-nAChR (33.3 % and 33.8 % agonist activity at 100 μM), while compound **36** was the most potent antagonist at α7-nAChRs with IC_{50} around 1 μM. Interestingly, compound (***S*)-34** showed good pharmacokinetic properties after intravenous and oral administration when tested in mice [86].

The research group led by Taylor reported a series of substituted 2-aminopyrimidines with unique cooperative binding behavior at *Lymnaea* AChBP [87]. Recently, the same research group extended the search of 2,4,6-trisubstituted pyrimidine analogs targeting α7-nAChRs.

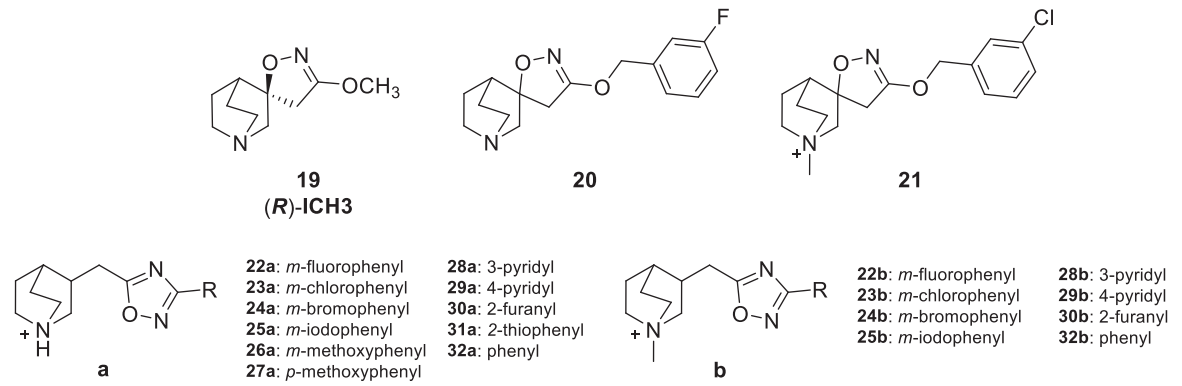


Fig. 9. Quinuclidine containing analogs of (*R*)-ICH3, a potent agonist of the α7-nAChRs.

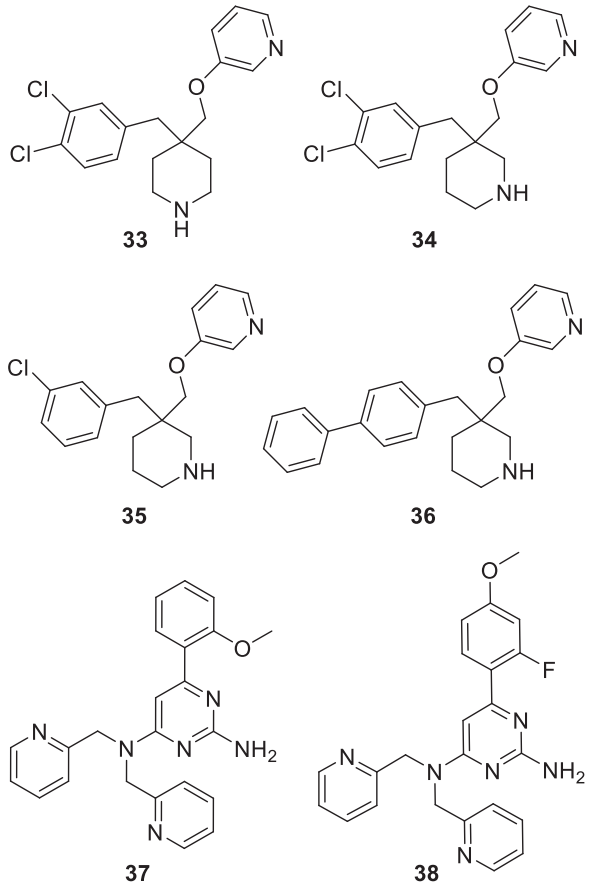


Fig. 10. 3-Substituted (3-pyridyloxymethyl)piperidines and 4,6-disubstituted 2-aminopyrimidines as α7-nAChR agonists.

Starting from compound **37** (Fig. 10), 22 derivatives were synthesized and pharmacologically characterized in a PNU-120596 dependent, cell-based calcium influx assay. The data analysis allowed to understand that: (a) the amino moiety in 2-position is important for the activity at α7-nAChRs; (b) its removal or substitution changes activity and selectivity without eliminating submicromolar agonist responses; (c) the di-(2-picolyl)amino group in 4-position is required for α7-nAChR agonist activity; (d) the functionalization of the 6-position is critical for activity and selectivity at α7-nAChR; (e) the presence of 2-fluoro-4-methoxyphenyl at 6-position (compound **38**) is a winning strategy to increase α7-nAChR EC_{50}. Electrophysiological recordings performed for selected compounds allowed to determine half maximal effective concentrations (0.11 μM EC_{50}, 0.84 I_{max} of the ACh maximum for compound **38**; 0.35 μM EC_{50}, 0.98 I_{max} for compound **37**) and to understand that the new series of compounds differ from silent agonists for the ability of activating the α7 nAChR without the presence of PNU-120596. However, the compounds stabilize the PAM-sensitive desensitized state. These data prompted the authors to consider a possible dual activity at orthosteric and potential allosteric binding sites, a hypothesis that will need further pharmacological evidence to explain the activity of these noncanonical agonists [88].

2.2.2. *α7-nAChR antagonists*

As mentioned in the Section 2.1.4, a structure-activity relationship study around the structure of MG624 was conducted by Bolchi and coworkers, which enabled the discovery of selective small-molecules targeting the α7- or the α9α10-nAChRs [40,41]. Within this study, binding affinity at α7-, α3β4-, and α4β2-nAChRs guided the *in vitro* functional characterization on *Xenopus* oocytes expressing human α7- or α9α10-nAChRs. The biological results of analogs modified at the cationic head, the ethylene linker and the styryl portion allowed to define the following structure activity relationships (Fig. 11): (a) the inclusion of the ethylene bridge between O- and triethylammonium group into (*R*)−3-pyrrolidiniumoxy substructure, namely compound **39**, (23 nM K_i and 1.49 μM IC_{50} at α7-nAChR and 36.5 μM IC_{50} at α9α10-nAChR), produces a 4-fold improvement in term of α7-nAChR binding affinity compared to MG624 and a 7-fold improvement in term of α7 *versus* α9α10-nAChR selective antagonist activity compared to MG624 (104 nM K_i and 1.99 μM IC_{50} at α7-nAChR and 6.68 μM IC_{50} at α9α10-nAChR); (b) the rigidification of the styryl portion of MG624 into an aromatic bicycle including a H-bond donor NH, such as 2-benzoimidazolyl (*i.e.*, compound **40**), 5-indolyl (*i.e.*, compound **41**) leads to increased α7-nAChR binding affinities (33.6 nM and 18.7 nM K_i, respectively) and α7- over α3β4-nAChR selectivity compared to the parent compound.

The high affinity at α7-nAChR of **41** and **39** prompted the authors to hybridize the two compounds leading to **42**, which stands out for the very high α7-nAChR affinity (0.82 nM K_i) and for the potent and selective antagonism at α7-nAChR (1.07 μM IC_{50}) producing a full inhibition of ACh induced function at this receptor subtype with remarkable selectivity *vs* α9α10-nAChR (15.9 μM IC_{50}). The mechanism of action of this compound was compatible with open-channel type of block and further experiments will be required to confirm this hypothesis. The SAR analysis showed that, starting from MG624, it was possible to obtain selective antagonist at α7-nAChR by making modifications of both the styryl moiety and cationic head.

López et al. have previously reported compound **43** as a selective antagonist for α7-nAChRs [89]. The same research group has recently elaborated on the structure of this compound by modification of the cationic head (Fig. 12). In general, compounds **45a–c** inhibited ion currents elicited by choline in interneurons from the stratum radiatum hippocampal CA1 area (*i.e.*, endogenous rat α7-nAChRs) more potently than **44a–c**. **45a** was the most potent antagonist of the series at

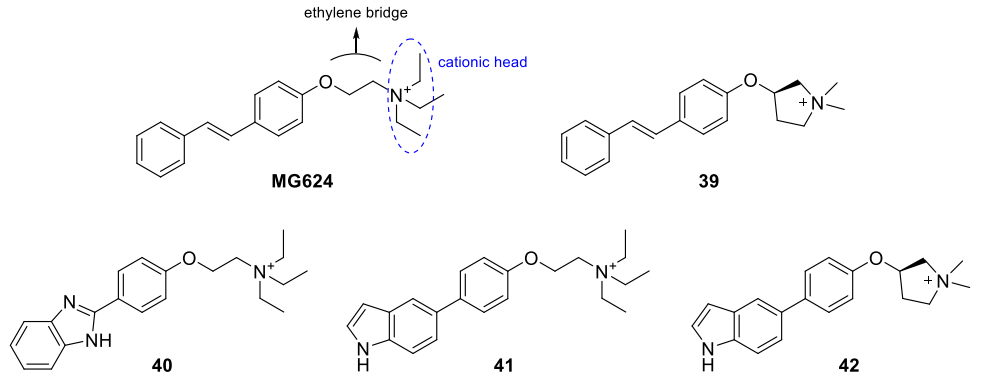


Fig. 11. Structural modifications of MG624 leading to compound 42, a small molecule with high α7-nAChR affinity, and potent and selective antagonism at α7-nAChR expressed in *Xenopus* oocyte.

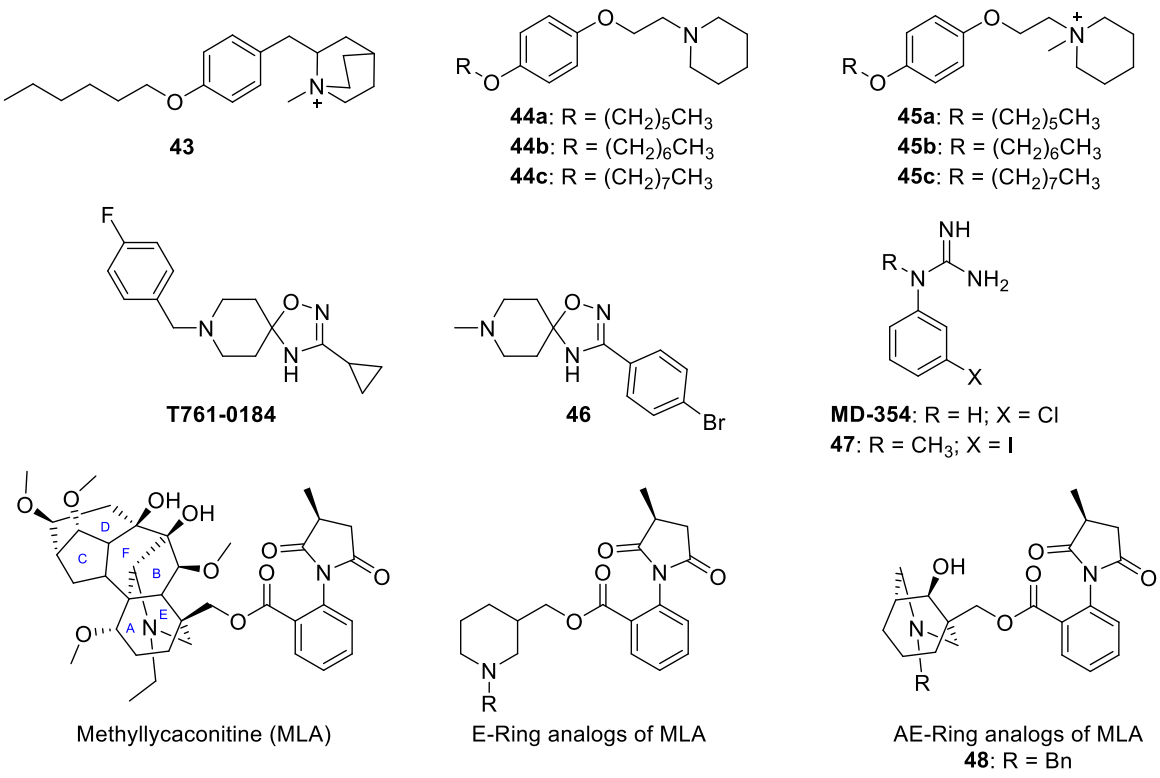


Fig. 12. Miscellaneous structures of antagonists of the α7-nAChR subtype.

α7-nAChRs, with 10 μM concentration capable to induce complete inhibition of the ion currents elicited by choline [90].

With the aim of finding novel chemotypes targeting the α7-nAChRs, Zhang et al. have performed a pharmacophore-based virtual screening of the commercial small-molecule database ChemDiv. Based on virtual screening results, a pool of 13 compounds was than assayed using two-electrode voltage clamp (TEVC) technique in *Xenopus* oocytes expressing human α7-nAChRs allowing to select **T761–0184** (Fig. 12) as a candidate for further development. A SAR study guided by molecular docking was performed by exploring different aromatic substituents in 3-position and, then, combined modifications at 3- and 8- positions, providing the synthesis and pharmacological characterization of fifty-one compounds. Selected analogs of the series exhibited antagonist activity with IC_{50} values ranging from 3.3 μM to 13.7 μM. The most interesting compound, **46**, exhibited selective inhibition of α7-nAChR subtype (5.4 μM IC_{50}) *versus* other nAChR subtypes (*i.e.*, α4β2, and α3β4) [91].

Dukat et al. have previously identified **MD-354** (Fig. 12) as a new chemotype capable of inhibiting ACh elicited currents on α7-nAChRs. This compound (at 10 or 100 μM concentration) was ineffective at displacing [^{125}I]iodo-MLA in ligand binding assay using rat cerebral cortex homogenate and was consequently postulated to act as a negative allosteric modulator [92]. The same research group has recently published a SAR study, consisting of twenty analogs of **MD-354**, by modulating the 3-position of phenyl moiety and/or by introducing alkyl substituents on *N*-aryl. Within the presented series of compounds and with exemption of three cases, the inhibitory potency at α7-nAChRs expressed in *Xenopus* oocytes using two-electrode voltage-clamp technique was generally maintained with IC_{50} ranging from 13 μM to 170 μM. On one hand, introduction of bulkier halogen in 3-position (*i.e.*, Br or I) was beneficial, and introduction of -CF_3 or -OCH_3 moieties was tolerated. On the other hand, substitution on N_1 with -CH_3, -CH_2CH_3, and -$CH(CH_3)_2$ was beneficial for inhibitory activity, while introduction of a bulkier cyclopentyl in the same position abolished inhibitory activity. The most potent compound of the series, **47**, exhibited inhibition of α7-nAChR with 13 μM IC_{50}, with a 3-fold potency increment compared to parent compound **MD-354** (42 μM IC_{50}). The compound

also showed negligible affinity for other nAChR subtypes and 5-HT_3 receptor. Compound **47** completely inhibited binding of [^{125}I]Tyr54--monoiodo-α-bungarotoxin to the native α7-nAChR in autoradiography experiment conducted in rat brain sections at 100 μM, a result that suggested that the inhibitory action would not be only imputable to competitive antagonism because the required concentration to obtain substantial displacement of radioligand was greater than IC_{50} [93].

Methyllicaconitine (MLA) is a potent and selective α7-nAChR competitive antagonist (2 nM IC_{50}). The structure activity relationships have been examined in previous reports, proving the importance of nitrogen atom, side chain of nitrogen atom, and ester side chain [94–97]. The structure of MLA has also been previously simplified synthesizing ring E analogs (Fig. 12) [98], an approach very recently recalled by Blagbrough and coworkers who synthesized AE-bicyclic analogs of MLA, bearing different ester moieties and *N*-side chain substituents. All compounds displayed antagonist activity at α7-nAChR expressed on *Xenopus* oocyte in electrophysiological experiments, despite decreased if compared to parent compound MLA (1 nM of MLA reduces the ACh [100 μM] evoked currents at α7-nAChR to 3.4 %). Introduction of benzyl substituent on nitrogen atom allowed to obtain the most potent compound of the series, namely compound **48**, capable of reducing the ACh (100 μM) evoked currents at α7-nAChR to 53%, when applied at 1 nM concentration. This finding sets the basis for a new series of α7-nAChR antagonists that will need further development and pharmacological characterizations [99].

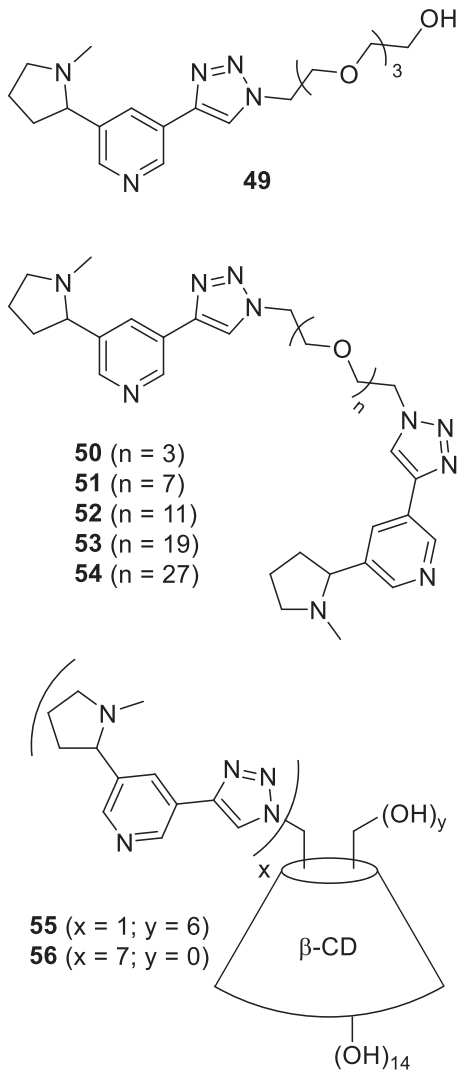


Fig. 13. Structures of a series of di- and hepta-valent nicotine derivatives as antagonists of the α7-nAChR subtype.

The research group of Gouin et al. recently reported the synthesis and pharmacological characterization of di- (*i.e.*, compounds **50**–**54**) and hepta-valent (*i.e.*, compound **56**) nicotine derivatives (Fig. 13), which were constructed using ethylene glycol chains of different length or cyclodextrin cores, respectively. All compounds showed inhibition of ACh evoked currents on α7-nAChR subtype expressed in *Xenopus laevis* oocytes. Compound **50** was the most potent compound of the two series showing a 12 μM IC_{50}, namely 16-fold greater potency than parent compound **49** (195 μM IC_{50}). For the other analogs, increasing the ethylene glycol spacer length produced a decrease of IC_{50} values (*i.e.*, greater potency), with compound **51** and **54** exhibiting 611 μM and 21 μM IC_{50}, respectively. The heptavalent compound **56** showed a 196 μM IC_{50}, with 4-fold decrement compared to parent compound **55** (56 μM IC_{50}) [100].

2.2.3. Allosteric ligands

A very recent review by Romanelli and coworkers, focusing on allosteric modulators of α4β2- and α7-nAChRs, describes in deep different types of modulators, mechanism of actions and the most important α7 positive and negative allosteric modulators (PAM and NAM, respectively) [101]. This review will cover only allosteric modulators of α7-nAChR published from 2018 that were not analyzed in the previously mentioned work.

With the aim of finding new chemotype targeting α7-nAChRs as allosteric modulators, Qi Sun and coworkers have synthesized a series of fused pyrimidin-ones (general structure **A**, Fig. 14) and performed pharmacological characterization at α7-nAChRs expressed in *Xenopus* oocytes using two-electrode voltage clamp technique. Compound **57** was characterized as type I PAM with EC_{50} of 3.20 μM and a maximum effect of 320% in the presence of ACh (100 μM). A SAR study was then conducted to improve efficacy of **57** by modification of all moieties and highlighted the key pharmacophore moiety being the *N*6-(2-chloro-6-methyl)-phenyl. The most promising compound of the series **58** exhibited an EC_{50} of 1.26 μM and a maximum effect of 1633% in the presence of 100 μM ACh. The compound is selective for α7- over other nAChR subtypes (*i.e.*, α4β2, α3β4), and *versus* other receptors 5-HT_{3A}, NMDA, and $GABA_A$. Upon assessment of excellent pharmacokinetic profile and good brain tissue distributions, compound **58** was evaluated *in vivo* in mouse schizophrenia model where it was able to reverse the prepulse inhibition deficit induced by MK-801 [102]. Another analog of **57**, JWX-A0108 (**59**), behaved as α7-nAChR type I PAM capable of selectively enhance currents in the presence of the agonist ACh with EC_{50} value of 4.35 μM. This compound was also proved to be effective in mice models of schizophrenia as it was able to reverse the prepulse inhibition deficit and impaired spatial working memory, both induced by MK-801 [103].

Starting from compound **57**–**59**, the authors replaced the central core with nicotinamide structure and synthesized more than 30 analogs. Among them, compound **60** stands out as a potent (*i.e.*, EC_{50} = 3.34 μM and a maximum effect of 1474 % in the presence of 100 μM ACh) and selective α7-nAChR PAM *versus* other nAChR subtypes and 5-HT_{3A} receptor [104]. The same research group continued to develop compounds as PAM of α7-nAChRs and identified a novel key pharmacophore, namely 1,3,5-triazin-2-amine. Compound **61** (Fig. 14) is the representative compound of the new series characterized as type I PAM, with an EC_{50} of 3.0 μM and a maximum effect of 3860 % in the presence of 100 μM ACh. The compound is characterized by high selectivity, good pharmacokinetic profile in mice and effective to reverse the prepulse inhibition deficit induced by MK-801 in mouse schizophrenia model [105].

Smelt et al. described a virtual screening of the DrugBank database using a pharmacophore model as template for the search of α7-nAChRs allosteric modulators. 81 compounds were selected in this first phase and the compounds were the divided into four classes: carbonic anhydrase inhibitors, cyclin-dependent kinase inhibitors, diuretics acting at

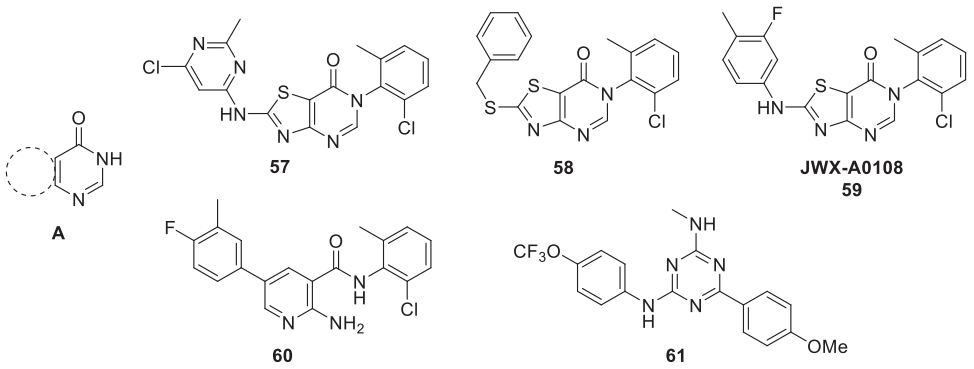


Fig. 14. Fused pyrimidin-one, nicotinamide, and 1,3,5-triazin-2-amine containing PAM of the α7-nAChR.

the Na^+-K^+-Cl^- cotransporter, and fluoroquinolone antibiotics. The best ranked compounds of each class (*i.e.*, DB04763, DB08122, furosemide and pefloxacin, Fig. 15) were selected for the following pharmacological characterization performed at α7-nAChRs expressed on *Xenopus* oocyte using two-electrode voltage-clamp technique. Furosemide was found to be an α7-nAChR PAM with EC_{50} value of 0.2 mM in the presence of 50 μM ACh (maximum level of potentiation of 1.6-fold at 1 mM concentration of furosemide). DB04763, DB08122, and pefloxacin were characterized as α7-nAChR NAM with IC_{50} of 46.4 μM, 1.7 mM, and 388 μM respectively when co-applied with 100 μM of ACh [106].

Being inspired by structures of other important α7-nAChR PAMs, Nielsen et al. designed a new series of functionalized 1,4-disubstituted 1,2,3-triazoles by applying cycloaddition reactions using supported copper nanoparticles as catalyst (Fig. 15). The new series of compounds were assayed at α7-nAChRs expressed on BOSC23 cells by single-channel and whole-cell recordings. **62**, the most interesting and efficacious compound of the series, was characterized as type 1 PAM. The SAR study summarized in Fig. 15 allowed to uncover phosphonate-functionalized 1,2,3-triazoles as novel building block to reach α7-nAChR PAM activity [107].

Despite the discovery of new chemotype as allosteric modulators, known compounds with other pharmacological target have also been recently studied to assess the activity toward α7- and α9α10-nAChRs as allosteric modulators.

Cannabidiol (CBD, Fig. 16) is the second most abundant cannabinoid compound contained in marijuana. The increasing interest in this therapeutic agent has intensified investigations on molecular targets, also comprising α7-nAChRs. The research group of Cecilia Bouzat recently published an extensive pharmacological characterization of CBD by high-resolution single-channel recordings and confocal microscopy. A concentration-dependent decrease of single-channel activity on α7-nAChR expressed in BOSC-23 cell line was revealed with IC_{50} estimated value of 0.5 μM. The mechanism of inhibition of CBD was investigated in deep and postulated to be compatible with the stabilization of the closed or desensitized conformational states of α7-nAChR subtype [108].

Tricyclic antidepressants (TCAs) have been proved to inhibit different nAChR subtypes, thus, García-Colunga et al. studied the α7- and α9α10-nAChRs activity of this class of compounds by using a combination of Ca^{2+} influx and voltage clamp recordings. Imipramine, amitriptyline, and doxepin (Fig. 16) have inhibitory activity at α7 (IC_{50}s of 6.6 μM, 2.7 μM, and 5.9 μM respectively, inhibition of Ca^{2+} influx in GH3-α7 cells), α9α10 (IC_{50} of imipramine = 0.53 μM, voltage-clamp), and hippocampal α7 * -nAChRs (IC_{50} of imipramine = 42.2 μM, voltage-clamp). A different mechanism of action was postulated for α7- and α9α10-nAChRs inhibition by functional and molecular modeling studies, for instance a noncompetitive inhibition of α7-nAChRs through interaction with two overlapping luminal sites, and a competitive inhibition of α9α10-AChRs through interaction with the orthosteric sites [109]. The same research group continued pharmacological investigations at α7-nAChRs of antidepressants with different structure and different pharmacological profile. The effects of norfluoxetine, fluoxetine, escitalopram, imipramine, mirtazapine, bupropion, and venlafaxine (Fig. 16) on ion current elicited by choline in rat CA1 hippocampal interneurons were measured by electrophysiological recordings. At the 20 μM concentration, all tested compounds were capable to inhibit ion current of α7-nAChRs with the following rank, being escitalopram the most potent (42.3% I_{Ch} control inhibition), and norfluoxetine the weakest (17.7% I_{Ch} control inhibition): escitalopram ~ venlafaxine ~ fluoxetine ~ bupropion > imipramine ~ mirtazapine ~ norfluoxetine [110].

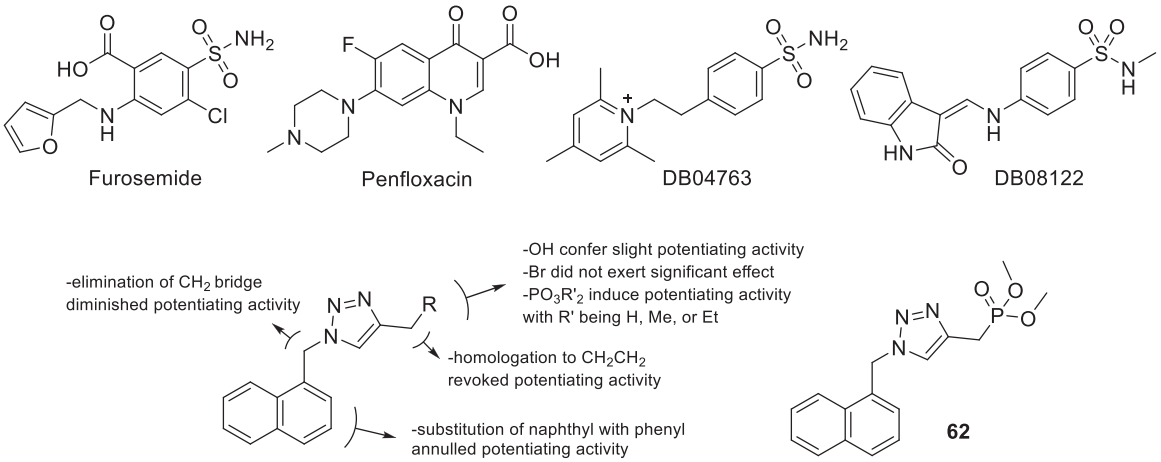


Fig. 15. Miscellaneous structures of novel PAM and NAM of the α7-nAChR subtype.

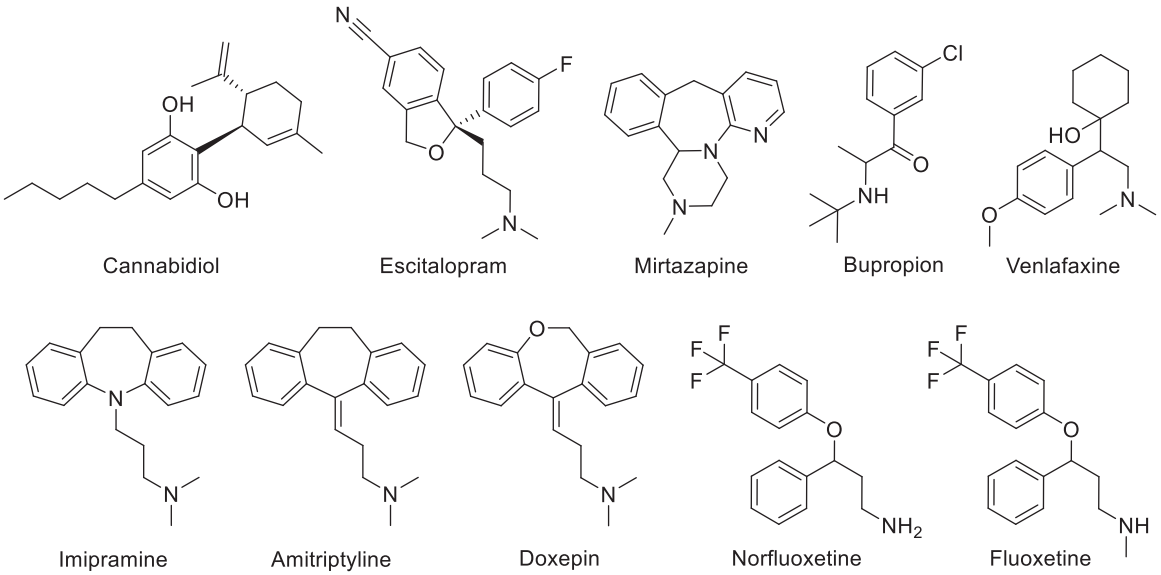


Fig. 16. Structure of cannabidiol e various antidepressants studied for allosteric modulatory activity at α7- and α9α10-nAChR subtypes.

2.2.4. α7-nAChR radioligands

As far as the α7-nAChR radioligands are concerned, the recent literature is mainly focused on PET tracers of the two selective α7-nAChR agonists **63** (K_i = 0.023 nM) and **64** (K_i = 0.28 nM) (Fig. 17). In addition to the most utilized derivatives of **63**, *i.e.*, [^{18}F]DBT10 and especially [^{18}F]ASEM, a group of fluorinated derivatives and one iodinated analog of **64** were identified and preliminarily investigated (Fig. 17). Among them, the radiosynthesis of compounds **65** and **66**, that emerged from newly prepared fluorene-9-one analogs, was translated to a rapid and fully automated process that should in principle promote their preclinical study [111].

In a set of new derivatives of 1,4-diazobicyclo[3.2.2]nonane synthesized and evaluated as nicotinic ligands by Wang et al., five compounds displayed high binding affinity (K_i = 0.001–25 nM) for α7-nAChRs. Among them, primary amine **67** (K_i = 0.0069 nM, Fig. 17) bound α7-nAChRs with exceptionally high affinity coupled with a > 10,000-fold selectivity over the α4β2 subtype, exhibited no significant *h*ERG (human ether-a-go-go-related gene) inhibition, and showed agonistic activities in patch clamp electrophysiology assays. The corresponding fluoro-containing derivative **68** maintained a good affinity and selectivity profile (K_i = 2.98 nM at α7 *vs* K_i = 3.69 μM at α4β2) and was radiolabeled with ^{18}F to afford [^{18}F]**68**. The latter showed high *in vitro* stability and permeated the BBB to specially label α7 receptors in the brain. Moreover, micro-PET/CT experiments in normal rats confirmed accumulation of the compound in the brain, thus indicating in [^{18}F]**68** a promising PET radiotracer for α7-nAChR imaging [112].

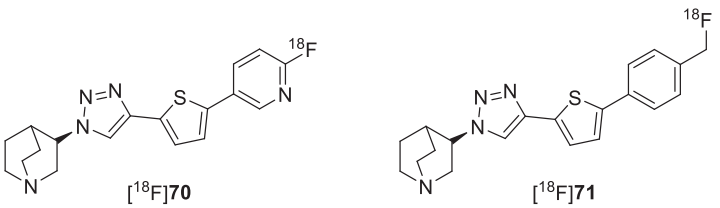
The same research group further utilized the 9 H-fluoren-9-one scaffold to design novel potential radiotracers for imaging cerebral α7-nAChRs. The meta-iodine substituted 9-fluorenone **69**, that retained a high binding affinity (K_i = 9.3 nM) and > 500-fold α7 *vs* α4β2 selectivity, was chosen for radiolabeling with ^{125}I. [^{125}I]**69** (Fig. 17) showed good *in vitro* stability and BBE permeation. In addition, cerebral biodistribution analyses, self-blockade studies with nonlabelled **69**, ex vivo autoradiography, and micro-SPECT/CT in mice put in evidence an overall encouraging *in vivo* pharmacokinetic profile [113].

By applying an efficient reaction sequence that involved a Suzuki-Miyaura cross coupling reaction, Ouach et al. described the synthesis of a library of bis(het)aryl-1,2,3-triazole quinuclidine ligands targeting α7-nAChR. The exploration of SAR indicated that nine of the new compounds exhibited below nanomolar K_i values for the α7 subtype, the best scores being invariably obtained when the triazole was substituted with the 5-phenyl-2-thiophenyl moiety [114]. Once the agonist α7 profile and the selectivity over α4β2-nAChRs and serotoninergic 5-HT$_3$ receptors for the most interesting ligands was assessed, the two

63: $R_1 = R_2 = H$
[^{18}F]DBT10: $R_1 = H$, $R_2 = {}^{18}F$
[^{18}F]ASEM: $R_1 = {}^{18}F$, $R_2 = H$
[^{125}I]ASEM: $R_1 = {}^{125}I$, $R_2 = H$

64: $R_1 = R_2 = R_3 = H$
65: $R_1 = {}^{18}F$, $R_2 = R_3 = H$
66: $R_1 = R_2 = H$, $R_3 = {}^{18}F$
67: $R_1 = NH_2$, $R_2 = R_3 = H$

68: $R_1 = F$, $R_2 = R_3 = H$;
[^{18}F]**68**: $R_1 = {}^{18}F$, $R_2 = R_3 = H$;
69: $R_1 = R_3 = H$, $R_2 = I$;
[^{125}I]**69**: $R_1 = R_3 = H$, $R_2 = {}^{125}I$



[^{18}F]**70** [^{18}F]**71**

Fig. 17. Molecular structures of α7-nAChR radioligands using ^{18}F and ^{125}I.

fluorine-containing compounds **70** (K_i = 13 nM) and **71** (K_i = 5 nM) were radiolabeled with ^{18}F for preliminary *in vivo* evaluation and CNS compatibility. Although the two compounds crossed the BBB after i.v. injection, neither [^{18}F]**70** nor [^{18}F]**71** (Fig. 17) specifically accumulated in brain regions rich in α7-nAChR and were not significantly displaced by the α7-nAChR antagonist MLA [114]. These data further demonstrate that the previously cited [^{18}F]ASEM, due to its very high affinity for the α7-nAChR (K_i = 0.4 nM), is at present the reference PET-tracer for *in vivo* binding of the α7-nAChR. The outcomes from a study using [^{18}F]ASEM in patients with schizophrenia indicated the feasibility of investigating this receptor subtype as a potential target [115], and the same radiotracer entered a study by Coughlin et al., which compared the binding of [^{18}F]ASEM in the hippocampus of individuals who had recent-onset psychosis with that in healthy controls [116]. Although further studies are needed, the results are consistent with lower hippocampal availability of the α7-nAChR subtype in nonsmoking individuals (11) with recent-onset psychosis, particularly those with nonaffective psychosis, than in healthy volunteers (15).

The same research group performed a cross-sectional study, in which 14 patients with mild cognitive impairment (MCI), a prodromal stage to dementia, and 17 cognitively intact, elderly controls completed [^{18}F]ASEM PET. The overall data evidenced higher availability of α7-nAChRs in MCI patients than in healthy controls across all brain regions, in accordance with both postmortem studies, reporting higher α7-nAChR levels in the early stages of AD, and animal models of AD [117]. A potential validation of the high availability of α7-nAChRs as a biomarker of MCI may therefore prove suitable for earlier detection of AD.

Among the recent PET exploration of α7-nAChRs with [^{18}F]ASEM, Vetel et al. investigated the evolution of these receptor subtype *in vivo* with PET imaging in a rat model mimicking early stages of PD, a study carried out at 3, 7, and 14 days following a partial striatal unilateral lesion with 6-hydroxydopamine in adult rats [118]. After collecting the last imaging data, the status of nigrostriatal dopamine neurons as well as different markers of neuroinflammation was evaluated on brain sections by autoradiography and immunofluorescence experiments. An over-expression of α7-nAChRs at early stages after lesion was observed, that could reflect a biphasic M2 (anti-inflammatory)/M1 (inflammatory) phenotype of the activated microglia. These findings indicate α7-nAChR agonists as a putative therapeutic treatment in PD, highlighting their use at the early stages of the disease.

Another report is related to the application of [^{18}F]ASEM as radiotracer for the imaging of α7-nAChR expression in the vasculature. Yang et al. established atherosclerotic plaques models of carotid arteries in ApoE− /− mice and abdominal aorta in New Zealand rabbits and reported a PET/CT imaging conducted with [^{18}F]ASEM, that made it feasible to image atherosclerotic plaques and to evaluate the vulnerability of plaques toward rupture [119]. Overall, [^{18}F]ASEM as a molecular probe showed a good sensitivity in detecting inflammation and may display a stronger signal intensity of inflamed atherosclerotic plaques compared to the standard ^{18}F-fluorodeoxyglucose ([^{18}F]FDG)-based nuclear medicine imaging protocol, thus being a promising radiotracer for the early identification of atherosclerotic plaques and other chronic inflammation diseases.

Since α7-nAChRs are involved in several cognitive and physiologic processes and their expression levels and patterns change in neurologic and psychiatric diseases, Donat et al. explored new selective radioligands for the α7-nAChR in view of investigating its distribution and occupancy profile in the mammalian brain. To such an end, the *in vitro* binding properties of [^{125}I]ASEM (Fig. 17) in the mouse, rat and pig brain using autoradiography were analyzed, together with the *in vivo* binding profile of [^{18}F]ASEM in the pig brain by PET/CT [120]. [^{125}I]ASEM allowed sensitive and selective imaging of α7-nAChR *in vitro*, with better signal-to-noise ratio and other advantages over a tracer like [^{125}I]α-bungarotoxin. Data also suggested that [^{125}I]ASEM may potentially bind heteromeric α7β2-nAChRs. On the other hand, [^{18}F]ASEM was characterized by high brain uptake and suitable kinetic properties for *in vivo* quantification of α7-nAChR in the pig, in line with previously published data.

2.2.5. *Recent advancements in the characterization of previously discovered α7-nAChR ligands*

The recent literature also offers pharmacological advancements of previously discovered compounds targeting the α7-nAChRs. Among others, GTS-21, PNU-282987, PHA-543613, and TC-5619 (*i.e.*, Bradanicline) have been extensively used as pharmacological tools *in vitro*, *ex vivo*, and *in vivo* studies (Fig. 18).

GTS-21 is an α7-nAChR agonist [121,122] that was previously studied in clinical trials for schizophrenia, Alzheimer disease, attention deficit hyperactivity disorder, obesity, and for anti-inflammatory effect in human endotoxemia model [123]. Recently, GTS-21 was studied in animal models of several pathologies that, on one hand, prove the involvement of α7-nAChRs in such pathological conditions and, on the other, highlight potential therapeutic usefulness of GTS-21 and other α7-nAChR agonists.

The anti-inflammatory and neuroprotective activity of GTS-21 was evaluated in neuroinflammation and Parkinson's disease (PD) mouse models with positive outcome of effectiveness and with a proof of the α7-nAChRs mediation for the anti-inflammatory activity [124]. Cardioprotective effect *via* the cholinergic anti-inflammatory pathway of GTS-21 was proved with two interesting reports investigating the effects of GTS-21 in LPS-induced sepsis myocardial injury in mice and in streptozotocin-induced diabetic cardiomyopathy in rats. In the latter case, the authors mentioned a possible independent mechanism in addition to α7-nAChR activation due to only a partial reverse of GTS-21 action after pretreatment with α7-nAChR antagonist (*i.e.*, MLA) [125,126]. In this regard, B. K. Garg and R. H. Loring previously investigated the activity of GTS-21 in two cell models and proved that a part of its anti-inflammatory activity is mediated by α7-nAChRs in macrophages, but other cell-specific independent mechanisms are also involved [127].

Some other reports recently examined and proved the anti-inflammatory activity of GTS-21 to abate diabetes-induced kidney injury [128], to attenuate acute lung injury after renal ischemia-reperfusion injury in mice model [129], to suppress the production of IL-6 and NO in peripheral blood mononuclear cells of chronic obstructive pulmonary disease patients [130], to improve intestinal barrier function in mice DSS-induced intestinal colitis model [131], and to reduce radiation-induced lung injury in mouse model [132]. Furthermore, GTS-21 administered in mice after intracerebroventricular administration of streptozotocin proved to protect from oxidative stress, neuroinflammation, and to improve insulin signaling, thus indicating potential usefulness of α7-nAChR agonists for insulin resistance induced

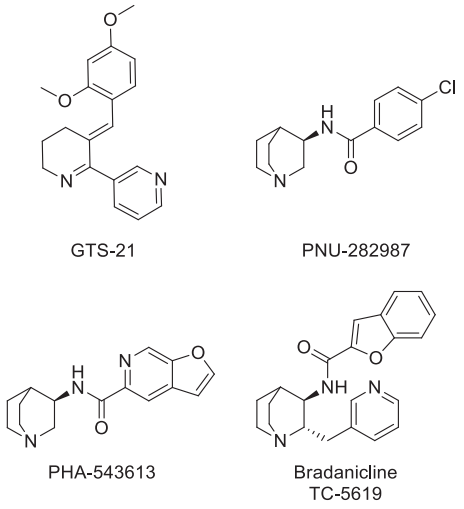


Fig. 18. Structure of GTS-21, PNU-282987, PHA-543613, and TC-5619.

by Alzheimer disease [133].

PNU-282987 is an α7-nAChR agonist characterized by high binding affinity (K_i = 27 nM) and high potency (EC_{50} at α7–$5HT_3$ chimera = 154 nM) at this receptor subtype. PNU-282987 showed selectivity over neuromuscular junction receptor subtype (*i.e.*, α1β1γδ) and the α3β4-nAChR subtype, and a 62-fold preference for α7-nAChR *versus* $5\text{-}HT_3$ receptor [134]. This compound was used to prove the importance of cholinergic anti-inflammatory system in a model of chronic allergic airway inflammation in mice [135], and was proved to suppress the secretion of pro-inflammatory factors in DSS-induced colitis mice model, thus inducing a protective effect in this model [136]. Recent advancements also allowed to deepen mechanistic knowledge on the neurogenic and regenerative effect that was observed previously in adult murine retina and could be useful in the future to develop novel therapeutic opportunities for functional vision loss [137].

PHA-543613 is an α7-nAChR agonist characterized by high binding affinity (K_i = 8.8 nM), and high potency (EC_{50} at α7–$5HT_3$ chimera = 65 nM). This compound was discovered as potential therapeutic for cognitive deficits associated to schizophrenia [138]. Recently, PHA-543613 was used as pharmacological tool in Alzheimer disease models allowing to understand that (a) the administration of PHA-543613 improved impaired cognitive function in presenilin 1 (PS1) and presenilin 2 (PS2) conditional double knockout mice model [139], and (b) the co-administration subeffective doses of PHA-543613 and memantine enhance cognitive effect greater then respective mono treatments in a rats scopolamine-induced amnesia model [140].

Bradanicline (*i.e.*, TC-5619) is a selective agonist of the α7-nAChRs with high binding affinity (K_i = 1.4 nM) and high potency (EC_{50} = 17 nM, E_{max} = 76%) at this receptor subtype. This compound is characterized by a 1000-fold greater selectivity for α7- *versus* α4β2-nAChR subtype and has negligible activity for muscle or ganglionic nAChR subtypes. TC-5619 entered in multiple clinical trials to treat cognitive impairment associated to neurological disorders as schizophrenia, Alzheimer and attention deficit hyperactivity disorder [141]. More recently, the compound also entered in clinical trials for refractory chronic cough treatment. In this respect, TC-5619 has been proved to have antitussive effect in guinea pigs. In addition, a selective agonist of α4β2-nAChR subtypes (*i.e.*, TC-6683) did not have effect on evoked cough responses in this animal model, while another α7 selective agonist (*i.e.*, PHA-543613) inhibited evoked cough responses [142].

3. Concluding remarks

α9- and α10- were the last nAChR subunits to be identified and successful expression of α9α10-receptors in mammalian cells is relatively recent as well as the possibility of extended screening for α9α10-nAChR selective lead compounds. Consequences of this are the here reviewed shortage of α9α10 selective small molecules ligands and the far from complete characterization of the pharmacology of this distinct non-neuronal and non-muscle nicotinic heteromeric receptor composed only of α subunits. The combination of the two α9 and α10 subunits in the pentamer giving different stoichiometries and the contribution of each subunit to the principal and complementary components of the orthosteric binding site are additional issues to be addressed in the study of this receptor and the action modes of its agonists and antagonists. Moreover, the development PAMs targeting α9α10-nAChRs is an attractive, but still unexplored approach.

Quite different is, instead, the scenario for α7-nAChR ligands. Thanks to deeper structural and pharmacological characterization of this receptor subtype, several selective agonists, partial agonists, silent agonists and positive allosteric modulators have been identified and their action elucidated in the last decades. Although none of them has reached the goal of pharmacotherapeutical application to date, their therapeutic potentialities are undisputable. Ligand- and target-based design of selective α7 ligands has been successfully pursued with the synthesis and the pharmacological characterization of a large number of compounds that have allowed relatively sound SARs to be drawn. For these compounds and those of new generation, an additional spectrum of investigation is opened by the relatively recent discovery of the α7β2-nAChR, whose functional and pharmacological profile just begins to be deciphered and needs to be distinguished from the homomeric α7-nAChR.

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CB conceptualized the review. **CB, AG, and MP** equally contributed to writing, reviewing, and editing the manuscript. All authors approved the submitted final version.

Declaration of Competing Interest

The authors declare no conflicts of interest.

Data availability

No data was used for the research described in the article.

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