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Advances in small molecule selective ligands for heteromeric nicotinic acetylcholine receptors☆

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ABSTRACT

The study of nicotinic acetylcholine receptors (nAChRs) has significantly progressed in the last decade, due to a) the improved techniques available for structural studies; b) the identification of ligands interacting at orthosteric and allosteric recognition sites on the nAChR proteins, able to tune channel conformational states; c) the better functional characterization of receptor subtypes/subunits and their therapeutic potential; d) the availability of novel pharmacological agents able to activate or block nicotinic-mediated cholinergic responses with subtype or stoichiometry selectivity. The copious literature on nAChRs is related to the pharmacological profile of new, promising subtype selective derivatives as well as the encouraging preclinical and early clinical evaluation of known ligands. However, recently approved therapeutic derivatives are still missing, and examples of ligands discontinued in advanced CNS clinical trials include drug candidates acting at both neuronal homomeric and heteromeric receptors. In this review, we have selected heteromeric nAChRs as the target and comment on literature reports of the past five years dealing with the discovery of new small molecule ligands or the advanced pharmacological/preclinical investigation of more promising compounds. The results obtained with bifunctional nicotinic ligands and a light-activated ligand as well as the applications of promising radiopharmaceuticals for heteromeric subtypes are also discussed.

Abbreviations: A85380, 3-(2(*S*)-azetidinylmethoxy)pyridine; A-84543, 3-{[1-Methyl-2(*S*)-pyrrolidinyl]methoxy}pyridine; ACh, acetylcholine; AD, Alzheimer's disease; AMs, Allosteric modulators; AN6001, (2*E*)-3–[3–(4–Chloro-3–fluorophenyl)-1–(3–chloro-4–methoxyphenyl)-1*H*-pyrazol-4–yl]-2–cyano-*N* methanesulfonylprop-2–enamide; AT-1001, *N*-(2-Bromophenyl)– 9-methyl-9-azabicyclo[3.3.1]nonan-3-amine; AT-1012, *N*-(2-Iodophenyl)– 9-methyl-9-azabicyclo [3.3.1]nonan-3-amine; Br-PBTC, (*R*)– 7-Bromo-*N*-(piperidin-3-yl)benzo[*b*]thiophene-2-carboxamide; ^{11}C-LSN3172176, Ethyl 4-(6-(methyl-^{11}C)– 2-oxoindolin-1-yl)-[1,4′-bipiperidine]– 1′-carboxylate; CNS, central nervous system; CMPI, 3-[(2-Chlorophenyl)– 5-(5-methyl-1-(piperidin-4-yl)– 1*H*-pyrazol-4-yl]isoxazole; Cris-104, 1-{2-[5-(4-Fluorophenyl)– 1*H*-pyrazol-4-yl]ethyl}piperidine; cryo-EM, cryogenic electron microscopy; dFBr, *N*-(2-[6-Bromo-2(1,1-dimethyl-2-propyl)– 1*H*-indol-3-yl]ethyl-*N*-methylamine; DMPA, 4-Acetyl-1,1-dimethylpiperazin-1-ium; EPA, 1-(4-Ethylpiperazin-1-yl)ethanone; + -[^{18}F]Flubatine, (+)-(1 *S*,5 *R*,6 *R*)– 6-(6-[^{18}F] Fluoro-pyridine-3-yl)– 8-azabicyclo-[3.2.1]octane; [^{18}F]2-FA, 2-[^{18}F]Fluoro-3-[2(*S*)-azetidinylmethoxy]pyridine; HS receptors, High sensitivity (α4β2)2β2 nAChRs; HTS, High-throughput screening; ITC, isothermal titration calorimetry; LGIC, Ligand-gated ion channels; LS receptors, Low sensitivity (α4β2)2α4 nAChRs; LY2087101, {2-[(4-fluorophenyl)amino]– 4-methylthiazol-5-yl}– 3-thienylmethanone; MB266, 3-Quinuclidinyl-α-methoxydiphenylacetate; MCL-11, (*S*)– 5-[(1-Methylpyrrolidin-2-yl)methoxy]pyridin-3-ol; MCL-28, (*S*)– 3-[(1-Methylpyrrolidin-2-yl)methoxy]phenol; MPA, 1-(4-Methylpiperazin-1-yl)ethanone; nAChRs, Nicotinic acetylcholine receptors; NAMs, Negative allosteric modulators; [^{18}F]Nifene, 2-[^{18}F]Fluoro-3-[2((*S*)– 3-pyrrolinyl)methoxy]pyridine; NS9283, 3-[3-(Pyridin-3-yl)– 1,2,4-oxadiazol-5-yl]benzonitrile; PD, Parkinson's disease; PAMs, Positive allosteric modulators; PET, Positron Emission Tomography; PNS, Peripheral nervous system; RTI-36, 3′-Fluorodeschloroepibatidine; RTI-76, 3′-(3-Dimethylaminophenyl)epibatidine; RTI-102, 3′-(4-Nitrophenyl)epibatidine; SARs, Structure-activity relationships; Soman, 3,3-dimethyl-2-yl methylphosphonofluoridate; SPECT, Single-Photon Emission Computed Tomography; SR14271, (*R*)– 7-Cloro-4-trifluoromethyl-*N*-(1-methylpiperidin-3-yl)benzo[*b*]thiophene-2-carboxamide; SUVN-911 Ropanicant, 3-(6-Chloropyridine-3-yloxymethyl)– 2-azabicyclo[3.1.0] hexane hydrochloride; *S*-T1, (*S*)– 3-[4-(4-fluorophenyl)-1*H*-1,2,3-triazol-1-yl]quinuclidine; TEVC, two-electrode voltage-clamp; UFR2709, (*S*)-1-Methylpyrrolidin-2-yl methyl benzoate hydrochloride.

☆ *In memory of Professor Klaus Mohr*

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1. Introduction

Several natural compounds from various sources that are known to interact with biological targets recognize nicotinic acetylcholine receptors (nAChRs) [1–5]. Historically, advances in the study of cholinergic transmission mediated by nAChRs relied on the investigation of naturally occurring activator compounds, among them *S*-(−)-nicotine, (−)-cytisine, (+)-anatoxin-a, and (+)-epibatidine, whose molecular skeletons inspired the design and synthesis of a number of structurally related analogs, aiming to novel nicotinic ligands provided with subtype selective pharmacological profiles.

The increased application of cryogenic electron microscopy (cryo-EM) to elucidate the high-resolution structures of biological targets allowed the structural determination of human heteromeric α4β2 [6] and α3β4 [7] receptors as well as human homomeric α7 receptor [8]. Acquisition of further structural details, which is essential to achieving higher degrees of subtype selectivity, may also reveal elements that contribute to better understand channel function. In addition to those of desensitized and resting-like states, also the structure of the activated, open-channel conformational state, stabilized by the agonist epibatidine and the positive allosteric modulator PNU-120596, has been reported for the α7 receptor [8]. Accordingly, the main steps of the whole gating cycle [9] may be reconsidered in the light of an enriched structural information.

The number of subtype selective receptor ligands has considerably increased, ranging from synthetic small molecule scaffolds [10–13] to a collection of natural compounds [2,4,14], among them peptides and an array of modified analogs [15–18]. The availability of different binding sites on the nAChR proteins and the variety of functional modulatory effects [19,20] have enhanced the arsenal of pharmacological tools both for in-depth investigation of homomeric or heteromeric receptors and prospective candidate drugs for therapeutic intervention. Among nAChRs formed by different subunits, those with identical subunit composition may differ for their stoichiometry, as in the case of the α4β2* and α3β4* receptors (*means that other subunits may be assembled), the most widely expressed subtypes in the central nervous system (CNS) and peripheral nervous system (PNS), respectively [19,21]. The $(\alpha4\beta2)_2\alpha4$ and $(\alpha4\beta2)_2\beta2$ stoichiometries are different in terms of calcium permeability and agonist or antagonist sensitivity, whereas $(\alpha3\beta4)_2\alpha3$ and $(\alpha3\beta4)_2\beta4$ receptors show differences in single-channel conductance and kinetics as well as in zinc enhancement [21]. For $(\alpha4\beta2)_2\beta2$ and $(\alpha4\beta2)_2\alpha4$ receptors, variations in their properties have been attributed to the presence of a further binding site on the latter at the α4(+)/α4(−) interface, as evidenced in concatamers with controlled subunit composition [22]. In addition to binding at the two conventional (orthodox) orthosteric sites at the α4(+)/β2(−) interfaces, agonist binding at the supplemental (unorthodox) site increases activation from the orthodox sites and accelerates desensitization of the $(\alpha4\beta2)_2\alpha4$ receptor [23]. The selective agonist for the unorthodox α4(+)/α4(−) site NS9283 was initially thought to be a positive allosteric modulator. An in-depth analysis of those subunits able to form unorthodox binding sites has been reviewed by Wang and Lindstrom [19].

Most of investigated ligands for both homomeric and heteromeric nAChRs, and notably those entered clinical trials or on the market, have a pharmacological profile (*i.e.*, full or partial agonism, competitive antagonism) due to their interaction with canonical orthosteric recognition sites. However, over the past fifteen years, positive and negative allosteric modulators (PAMs and NAMs, respectively) of nAChRs have been identified and then explored as alternative therapeutic options for pain, inflammation, smoking cessation as well as cognitive disorders and CNS diseases impacting on cognitive function [19,24–28]. Typically, PAMs do not or barely stimulate receptors themselves but are able to potentiate (or attenuate in the case of NAMs) the response only in the presence of an orthosteric activator ligand. Unlike agonists, AMs do not bind at the conserved ACh binding site but bind at less conserved sites of the nAChR protein structure, and they are in principle provided with higher degrees of subtype selectivity. In terms of structural features, however, small molecule AMs show a variety of molecular skeletons, and structure-activity relationships are more complex to be defined than those of their orthosteric counterparts [11,29,30]. At variance with orthosteric agonists, PAMs do not produce non-physiological continuous activation and desensitization of receptor channels, thus potentially reducing the incidence of undesirable side effects.

Based on the features of allosteric potentiation, ligands have been classified as Type I and Type II PAMs [10,12,28]. Type I refers to compounds that enhance ACh sensitivity and the apparent peak amplitude of agonist-evoked responses, with a negligible effect on channel gating kinetics. Type II PAMs have additional effects on channel gating kinetics (*e.g.*, increase open channel duration, decrease desensitization) and can transiently reactivate desensitized nAChRs. Type I and Type II PAMs have been characterized for both homomeric and heteromeric nAChRs, and their allosteric binding sites are localized in transmembrane as well as extracellular domains [12,19,20]. A third type of PAMs are allosteric agonists, that, besides increasing orthosteric ligand response, activate nAChRs also in the absence of agonists [12]. The investigation on NAMs is relatively less developed, although examples are known for both homomeric and heteromeric receptors, with their related interacting sites [19]. Worth mentioning, NAMs are different from channel blockers, that are noncompetitive antagonists and block the channel pore rather than regulating the channel gating [19]. Interestingly, a variety of relatively new nAChR ligands have been classified as “silent agonists”, and their binding site is partially superimposed with that of typical orthosteric agonists [31]. Silent agonists, which are peculiar of the α7 receptor subtype, engender little channel opening on their own but induce stable desensitization states. These compounds, which have been discovered and intensively investigated by the group of R. L. Papke, will not be discussed in this review, that is focused on ligands for heteromeric nAChRs.

Overall, in the research field of nAChRs the concept of selectivity has evolved from compounds discriminating, in terms of binding affinity and/or functional effect, among different receptor subtypes to ligands able to fine-tune distinct downstream signaling of heteromeric receptors with a closely related subunit composition. For the first group of ligands, translational gaps between preclinical results in animal studies and outcomes from human clinical trials have halted, also in the recent past, the approval of promising candidate drugs meant for CNS pathologies [32]. On the other hand, although attractive therapeutic opportunities could be associated with more subtly selective nicotinic ligands, both identification of new molecular entities with these specific profiles and their eventual optimization towards suitable clinical candidates are far from being effortless tasks.

In addition to the above-mentioned ones, other interesting reviews have been recently published that encompass relevant aspects of the nAChR pharmacology [33,34], biochemistry [35,36], and structural biology [37,38]. In this review, we discuss literature reports published in the past five years related to the properties of novel selective small molecules for heteromeric nAChRs, both orthosteric and allosteric ligands. Then, we comment the results on already known nicotinic derivatives in view of their potential clinical investigation. In the last sections of the paper, we report on ligands interacting with nAChRs and additional biological targets, on a light-regulated nicotinic antagonist, and on recent applications of radiopharmaceuticals targeting heteromeric nAChRs.

2. Small molecule orthosteres of heteromeric nAChRs

2.1. Ligands with agonist and partial agonist profiles

The research area of selective orthosteric ligands, mainly agonists, for heteromeric nAChRs is still very active, aiming at a better discrimination among the different subtypes and/or stoichiometries. As far as the α4β2 subtype is the target receptor, Murineddu et al. synthesized and

tested two groups of differently substituted 3,6-diazabicyclo [3.1.1] heptanes (general structures **A** and **B** on top of Fig. 1) [39,40]. In a library of forty-five pyridinyl- and pyridazinyl-3,6-diazabicyclo[3.1.1] heptane anilines (X = CH or X = N in **A**), the 3-(anilino)pyridine derivatives were the most interesting ligands, among them **1** and **2**, showing K_i values of 0.0598 nM and 1.76 nM, respectively, for the α4β2 subtype [39]. Moreover, the studied derivatives showed a significant affinity for the α3β4 subtype (K_i = 2.2 nM for **1** and K_i = 25 nM for **2**), while recognition at the α7 subtype was compromised (K_i = 837 nM for **1** and K_i = 4.1 μM for **2**). The two compounds were selected to assess their agonist activity on human recombinant α4β2, α3β4 and α7 receptors expressed by transient transfection in rat anterior pituitary GH4C1 cell line [39]. Compound **1** acted as a partial agonist at both α4β2 and α3β4 nAChRs, with EC_{50} values of 2.90 ± 0.03 μM and 1.90 ± 0.8 μM, respectively, and maximal evoked currents of 42.3 ± 0.8% and 11 ± 1%, respectively, of the maximal current amplitude elicited by 1 mM ACh. On the other hand, compound **2** was unable to evoke a significant response from the α3β4 expressing cells and showed a reduced potency at the α4β2 subtype, with EC_{50} > 10 μM.

The group of twelve derivatives with the 5-(3,6-diazabicyclo[3.1.1] heptan-3-yl)-*N*-arylnicotinamide structure (general formula **B**, Fig. 1) all displayed a very high affinity for α4β2 nAChRs, bound the α3β4 subtype, and showed a quite remarkable α4β2 over α7 selectivity [40]. Based on the binding data, analogs **3** [K_i (α4β2) = 10 pM, K_i (α3β4) 81 nM], **4** [K_i (α4β2) = 560 pM, K_i (α3β4) 265 nM], and **5** [K_i (α4β2) = 42 pM, K_i (α3β4) 59 nM] were selected for functional analysis. They were found to behave as partial agonists, eliciting inward whole-cell currents in GH4C1 cells transfected with human α4 and β2 subunits with comparable EC_{50} values: 0.52 ± 0.04 μM, 0.6 ± 0.2 μM, 1.0 ± 0.2 μM for **3**, **4**, and **5**, respectively. Similar values of the corresponding maximal evoked currents (41 ± 1%, 38 ± 11%, 37 ± 7% of those elicited by 1 mM ACh) were observed, and the compounds behaved as functionally selective ligands, since their agonist profile at human α3β4 receptors was detected only at high concentrations (> 1 μM) with EC_{50} values > 10 μM. The experimental results have been discussed in the light of a molecular modeling simulation, by docking the most selective derivative **3** and epibatidine to the binding sites of both α4β2 and α7 receptor models.

With the aim of identifying novel chemotypes for the nAChRs, Manetti et al. retrieved LOYMOB from a 3D search of the Cambridge Structural Database as one of the promising hits for the molecular recognition of the nicotinic pharmacophore [41]. The molecular skeleton of LOYMOB was simplified maintaining a bicyclo[2.2.1]heptane moiety as a spacer between the pyridyl ring (H-bond acceptor) and an aliphatic amino group (general formula **C**, Fig. 1), providing a group of *endo*- and *exo*-3-(pyridine-3-yl)bicyclo[2.2.1]heptan-2-amines, which were synthesized as racemates, and tested on α4β2* and α7* nAChRs of rat brain. Based on the affinity profile, some of the new ligands (**6a**-**9a**, Fig. 1) were further investigated for their functional properties on heterologously expressed individual α4β2, α7, and α3β2 subtypes. Notably, primary amines **6a** [K_i (α4β2) = 2.11 nM, K_i (α7) 46 nM] and **7a** [K_i (α4β2) = 1.31 nM, K_i (α7) 10.53 nM] displayed α4β2 antagonistic properties, since 50 μM concentrations blocked (91% for **6a** and 81% for **7a**) nicotine-evoked currents. On the other hand, the two analogs behaved as full agonists on recombinant α7 receptors [EC_{50} = 0.048 ± 0.013 μM (**6a**), EC_{50} = 0.024 ± 0.06 μM (**7a**)], and fully activated nAChRs in human neuroblastoma SH-SY5Y cell line, producing a $[Ca^{2+}]_i$ rise, with EC_{50} = 1.92 ± 0.66 μM and EC_{50} 0.22 ± 0.04 μM, respectively, while nicotine EC_{50} was several times higher (22.47 ± 3.06 μM). Only the chloro-containing compound **7a** evidenced a full agonist activity at the α3β2 subtype with a submicromolar potency (EC_{50} = 0.43 ± 0.10 μM), whereas unsubstituted **6a** behaved as a partial agonist (EC_{50} = 6.32 ± 1.07 μM). Although none of the novel primary amines was selective toward a specific nAChR, their structural skeleton represents a novel chemotype, particularly useful to recognize the α7 and α3* subtypes.

The properties of arecoline and other known *Areka* alkaloids, such as arecaidine, guvacoline, and guvacine, and those of further related analogs (isoarecolone, DMPA, MPA, EPA, Fig. 1) have been re-examined by Papke et al., to achieve an analog endowed with activity/selectivity for α4* nAChRs, not desensitizing the α7 subtype, and displaying reduced mAChR-mediated effects [42]. The goal of this study was that of proposing novel putative drugs to treat both nicotine and betel addiction; in fact, cytisine and varenicline, currently utilized as smoking cessation agents, are partial agonists of α4β2 receptors and, although inactive at muscle-type nAChRs, they behave as efficacious activators of both α3β4 and α7 subtypes. Among the studied derivatives, only isoarecolone and MPA were found to behave as truly α4* nAChR selective partial agonists, with low muscarinic activity. In the case of MPA, further elaboration of the structure, maybe by optimization of the *N*-acyl group or introduction of carbonyl isosteres, might contribute to improve the relatively low potency, that in part could be due to the low pK_a value of 6.9, which limits the charged fraction of MPA at physiological pH, thus reducing nAChR activation [42].

In another recent study, Papke and coworkers critically

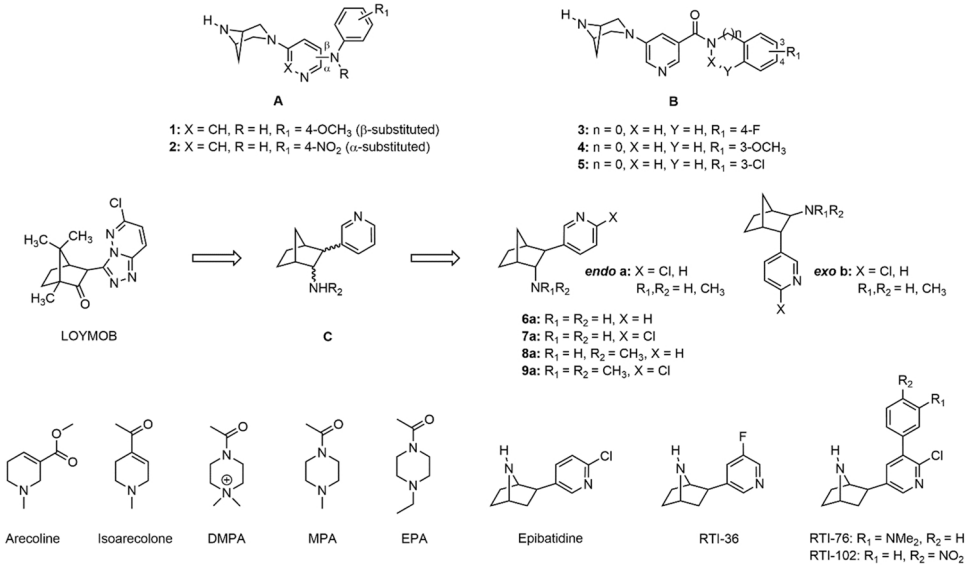

Fig. 1. Selective and nonselective agonists and partial agonists of heteromeric nAChRs.

reinvestigated the activity profile on a range of structurally defined receptor subunits of three known analogs of epibatidine, RTI-36, RTI-76, and RTI-102 (Fig. 1) [43]. The three ligands showed analgesic activity significantly lower than that of epibatidine, though they bound with very high affinity (K_i values from 0.037 to 0.009 nM) to the heteromeric nAChRs of male rat brain cerebral cortices and with very low affinity (K_i values $>$ 1000 nM) to the α7 subtype. Receptors of defined subunit composition and stoichiometry were obtained by injecting RNAs coding for nAChR monomeric subunits and/or concatamers into *Xenopus* oocytes [43]. In brief, RTI-36, the closest analog of epibatidine, was the most efficacious of the three compounds, and effectively activated also α7 and α3β4 receptors. RTI-76 was the most potent desensitizer of α4- and α2-containing receptors, although it was not the most efficacious agonist. On the other hand, RTI-102 behaved as an efficacious agonist for high sensitivity (HS) $(\alpha4\beta2)_2\beta2$ receptors and as an antagonist for low sensitivity (LS) $(\alpha4\beta2)_2\alpha4$ receptors. Overall, these data underline the impact of the presence or absence of specific subunits on brain nAChRs, which are crucial in directing the ligand activity profile.

The paper by Bavo et al. aimed at highlighting the determinants for α4β2 *vs.* α3β4 subtype selectivity in a series of more than forty α4β2 ligands [44], which were docked into the structures of the two human receptors, recently determined by cryo-EM. 1,4-Benzodioxane is known as a suitable scaffold to construct α4β2 ligands endowed with partial agonism when linked to 2-pyrrolidine through the dioxane ring and decorated or isosterically modified to pyridine at the benzene ring [45]. The derivatives gathered in Fig. 2 are selected from a group of flexible, semi-rigid, and rigid analogues of the α4β2 *vs.* α3β4 selective prolinol pyridyl ether A-84543. The binding affinities (K_i, nM) for rat α4β2 nAChRs, those for human α3β4 nAChRs, and α4β2 *vs.* α3β4 binding selectivity of the compounds exhibiting the highest α4β2 affinities [(*S*)-**10**, (*S*)-**11**, (*S*)-**12**, (*S*,*R*)-**13**, (*S*,*R*)-**14**, and (*S*,*S*)-**15**] are reported, together with the data for (*S*)-nicotine and A-84543, with the α3β4 affinity value of the latter being determined at the rat receptor subtype [44]. Worth noting, the α4β2 affinities are similar in rat and in human receptors, whereas the α3β4 affinities are generally much lower in rat than in human subtypes, resulting in much higher α4β2 *vs.* α3β4 selectivity ratio when *r*α3β4 rather than the relatively higher *h*α3β4 affinity values are considered. The authors proposed a structural rationalization of the rat *vs.* human differences of α4β2 *vs.* α3β4 selectivity. Comparative docking into the two human α4β2 and α3β4 nAChR structures of this series of pyrrolidine-based α4β2 ligands evidenced that the α4β2 *vs.* α3β4 subtype selectivity is largely depending on a) the stabilization of the ligand's aromatic ring by the not conserved β2-Phe119 residue, and b) the direct or water-mediated interaction with hydrophilic residues of the β(−) side affected by decoration of the aromatic ring and extensibility of the substructure of the latter [44]. These discrimination factors are mainly controlling the remarkable subtype selectivity achieved by some of the ligands, such as the 5-amino substituted pyrrolidinyl benzodioxane (*S*,*S*)-**15** [46], the semirigid α-methyl prolinol pyridyl ether (*S*,*R*)-**13** [47], and the hydroxyhexinyl phenol derivative (*S*)-**12** [48]. The full agonists prolinol aryl ethers (*S*)-**10** (MCL-28) and (*S*)-**11** (MCL-11) were found to induce a significant cognitive enhancement in behavioral studies in zebrafish, in which the two ligands were very active in increasing spatial learning, memory, and attention [49]. Conversely, the undecorated pyrrolidinyl benzodioxane (*S*,*R*)-**16a** (MCL-117), which was characterized as a relatively potent α4β2 antagonist in electrophysiology experiments [50], in the T-maze test blocked the positive effects of the maximally active nicotine dose on the memory of the zebrafish, with a behavior qualitatively comparable with that of mecamylamine [49].

Based on the results obtained with some benzodioxane derivatives, where introduction of a hydroxyl group in a suitable position of the benzene ring yielded high α4β2 affinity as well as α4β2 *vs.* α3β4 selectivity, Appiani et al. synthesized racemic benzofurans **17**-**19** (Fig. 2),

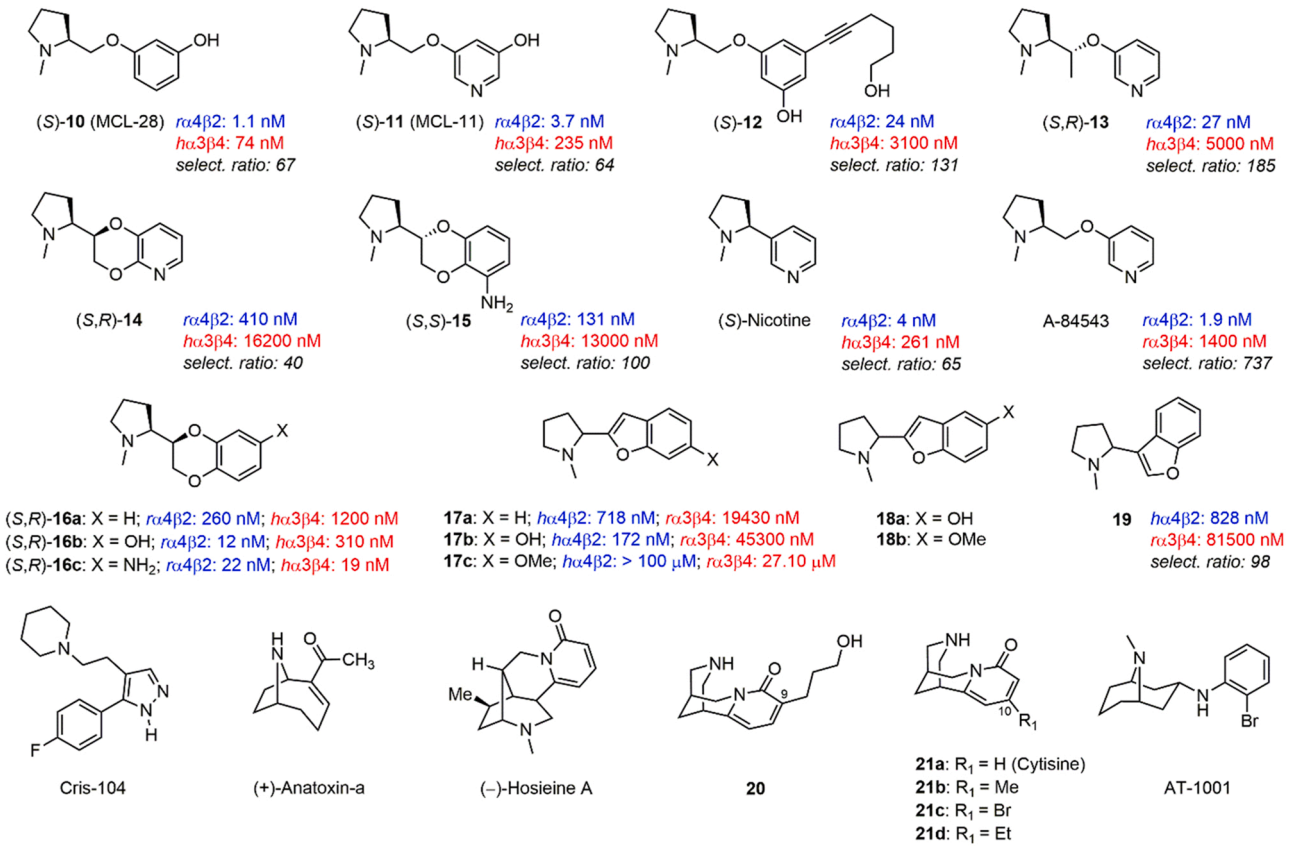

Fig. 2. Selective agonists and partial agonists of α4β2 nAChRs.

bearing the *N*-methyl-2-pyrrolidinyl residue at C-2 or C-3, that were tested for affinity at heterologously expressed human (α4β2) and rat (α3β4) receptor subtypes labelled by [^{3}H]epibatidine [51]. The most interesting ligands in this set of new derivatives were **17a** [K_i (α4β2) = 0.718 μM, K_i (α3β4) = 19.43 μM, α4β2/α3β4 selectivity = 27] and **17b** [K_i (α4β2) = 0.172 μM, K_i (α3β4) 45.31 μM, α4β2/α3β4 selectivity = 260]; these data evidenced the role of the OH group at (C-6) on both α4β2 affinity and selectivity, that were absent in the MeO-containing analog **17c**. Derivative **17a** was chosen for functional tests, which involved also the 7-hydroxylated pyrrolidinyl benzodioxane (*S*,*R*)-**16b**, the related 7-amino (*S*,*R*)— **16c** and 5-amino (*S*,*S*)— **15** analogs (Fig. 2), which had been previously characterized as α4β2 partial agonists. Their potencies and efficacies were evaluated against ACh in *Xenopus* oocytes expressing the wild-type human LS and HS α4β2 nAChR isoforms, using the two-electrode voltage-clamp (TEVC) technique. The 6-hydroxybenzofuran **17b** showed similar potencies at the two isoforms, while benzodioxanes **16b**, **16c**, and **15** exhibited different potency profiles. The 2-pyrrolidinyl-7-hydroxybenzodioxane **16b** was 170 times more potent at the HS isoform, the 2-pyrrolidinyl-7-aminobenzodioxane **16c** showed comparable potencies, while the 2-pyrrolidinyl-5-aminobenzodioxane **15** was inactive at the HS isoform. The four compounds behaved all as partial agonists with markedly higher efficacy at the HS isoform, except for **15**. Therefore, for these structurally related nicotinic ligands, the benzene pattern substitution is affecting selectivity between α4β2 and α3β4 subtypes as well as the degree of discrimination between α4β2 stoichiometries [51].

As far as putative therapeutic applications for heteromeric nAChR agonists are concerned, Cris-104 (Fig. 2), that had been characterized as an α4β2* selective ligand, showed efficacy in rats with diabetic neuropathy coupled to an encouraging physicochemical profile [52]. More recently, Cris-104 was found to induce dose-dependent antinociception in hot-plate and formalin tests in mice, and these effects were blocked by the nAChR antagonist mecamylamine, the selective α4β2* nAChR antagonist dihydro-beta-erythroidine, and the α2-adrenoceptor antagonist yohimbine, but not by the α1-adrenoceptor antagonist prazosin [53]. The analgesic potential of Cris-104 was assayed by evaluating its effects in rats on systemic and local administration on noradrenaline (NA) release in the spinal dorsal horn cord, and neuronal activity in the locus coeruleus (LC) with spinal nerve ligation, using behavioral, microdialysis and extracellular recording methods. Systemic Cris-104 increased neuronal activity in the LC of normal rats without affecting locomotion, and mecamylamine blocked its effects on spinal NA release and LC neuronal activity [53]. Thus, this selective α4β2* agonist is characterized by a promising analgesic profile, due to the interference with the descending noradrenergic pathways by stimulating noradrenergic neurons in the LC and their terminals in the spinal cord.

(−)-Hosieine-A (Fig. 2) is the most abundant among the alkaloids isolated from *Ormosia hosiei*, an ingredient of Chinese herbal medicines. A recent, convenient total synthesis of (−)-hosieine-A [54] allowed a reinvestigation of its molecular recognition pattern by nAChRs, in a study involving also (+)-anatoxin-a (Fig. 2), a natural neurotoxin targeting nAChRs [55]. The affinity and thermodynamic parameters were evaluated for the interactions of the two alkaloids with the acetylcholine-binding protein (AChBP), a soluble, highly conserved homolog of the nAChR extracellular domain in mollusks such as *Aplysia californica* (hence *Ac*AChBP), that has been used as a suitable surrogate for crystallographic and binding studies. The results obtained using a fluorescence-quenching assay and isothermal titration calorimetry (ITC) were compared with those available for (−)-nicotine and (−)-cytisine (**21a**, Fig. 2) [55]. Both (+)-anatoxin-a and (−)-hosieine-A exploit interactions with residues common to those of archetypal orthosteric agonists, but with higher affinity. The ITC data revealed that the binding of the four ligands to *Ac*AChBP is an exothermic event dominated by a favorable enthalpic component. Binding of (−)-hosieine-A shows the lowest contribution from enthalpic terms, but the favorable entropic term engenders a greater affinity for the target than those observed for the other agonists. The K_d values determined for (−)-hosieine-A are 0.025 ± 0.005 μM (ITC) and 0.040 ± 0.001 μM (fluorescence), whereas for (−)-cytisine ITC-derived K_d values of 1.6 μm and 0.60 ± 0.03 μM had been reported [55]. The binding profile was complemented by X-ray crystallographic data, that were compared with those of other relevant ligands such as (−)-cytisine and varenicline within *Ac*AChBP and with known structural parameters available for the α4β2 nAChR subtype. Partial agonists with high binding affinities based on the hosieine-A chemical scaffold could be developed that target the (−) face, *i.e.*, the complementary side of the orthosteric site, thus paralleling the approach leading to varenicline as an optimized analog of (−)-cytisine.

In a similar study, Davis et al. explored the binding of the C(9)-substituted cytisine derivative carrying a 3-(hydroxypropyl) moiety on the pyridine ring (compound **20**, Fig. 2), to exploit additional interactions in this region of the cytisine scaffold that has been associated with enhanced discrimination among receptor subtypes [56]. The authors characterized the binding of cytisine and its analog to *Ac*AChBP using ITC and reported a high-resolution crystal structure of the *Ac*AChBP-**20** complex. ITC evidenced that the favorable binding of **21a** and **20** to *Ac*AChBP is driven by the enthalpic component, which prevails on an unfavorable entropic contribution**.** Although **20** had a less unfavorable entropic contribution compared to **21a**, the affinity for *Ac*AChBP was considerably decreased due to reduction of the enthalpic component. Based on the crystal structure data, incorporation of this C(9)-substituent in the skeleton of cytisine causes a perturbation of the side chain of a Met133 residue on the protein structure, where a steric clash gives a major contribution to the reduced affinity of **20**.

Rego Campello et al. reported the successful C(10)-substitution of (−)-cytisine, aimed to improve its overall profile at nAChRs by a) preserving the α4β2 partial agonism profile; b) increasing α4β2 *vs.* α3β4 and, in particular, α4β2 *vs.* α7 subtype selectivity; c) suppressing α7 agonistic properties [57]. From a synthetic viewpoint, a group of C(10)-substituted cytisine variants was prepared *via* a site-selective Ir-catalyzed borylation reaction on the parent natural compound [57]. This late-stage diversification approach allows isolation of target derivatives in enantiomerically pure form and could offer the opportunity to easily achieve cytisine-based analogs for, *e.g.*, smoking cessation, that are more prone to cross the blood-brain barrier, and thus with an improved therapeutic profile. The studied compounds were assayed on heterologously expressed human receptors, and the most relevant ligands (Fig. 2) were **21b** [R_1 = Me, K_i (α4β2) = 2.60 nM; α4β2 *vs.* α3β4 selectivity = 864; α4β2 *vs.* α7 selectivity = 1911], **21c** [R_1 = Br, K_i (α4β2) = 1.77 nM; α4β2 *vs.* α3β4 selectivity = 303; α4β2 *vs.* α7 selectivity = 182], and **21d** [R_1 = Et, K_i (α4β2) = 3.01 nM; α4β2 *vs.* α3β4 selectivity = 1901; α4β2 *vs.* α7 selectivity = 2301]. Overall, when functionally tested, the C(10)-substituted cytisine analogs retained the partial agonism of (—)-cytisine at α4β2 nAChRs and displayed a preference for the HS $(\alpha4\beta2)_2\beta2$ receptor stoichiometry, a discrimination that is attenuated on passing, for example, from methyl (**21b**) to ethyl (**21d**). The new compounds did not activate (or inhibit) α7 Rs at therapeutically meaningful concentrations and behaved as weak partial agonists at α3β4 Rs, although observed efficacies at this subtype were consistently lower than those of their model compound. An accurate computational section, performed on the three investigated receptor subtypes and including docking analysis and molecular dynamics simulations, allowed to correlate the observed selectivity profile to key interactions (or loss of key interactions) with essential protein residues associated with, as well as beyond, the primary ligand binding site [57].

More recently, Knox et al. utilized this panel of C(10)-substituted cytisine analogues to evaluate the effects of slight structural changes on the ligand binding profile, and identified pivotal features of both the receptor and agonist structure that may increase selectivity for either α3β4 or α4β2 subtype [58]. While α4β2 selectivity is enhanced with smaller ligands that minimize steric clashes, increased hydrophobic interactions and a larger ligand size are those factors affecting selectivity for α3β4 receptor, as evidenced by analyzing relevant binding

interactions of the known α3β4 selective partial agonist AT-1001 (Fig. 2). The results of this study will help design nAChR-targeted ligands with an improved selectivity profile for putative therapeutic approaches with reduced off-target effects.

2.2. Competitive antagonists

In the framework of the study of nicotinic ligands characterized by an antagonist profile at heteromeric nAChRs, Kachel et al. applied a solid-phase synthetic procedure to prepare and test a group of 17 derivatives of PhTX-343 (Fig. 3), a synthetic analog of philanthotoxin-433 (PhTX-433, Fig. 3), a polyamine-containing toxin isolated from the Egyptian digger wasp *Philanthus triangulum*, behaving as a potent inhibitor of both, ionotropic glutamate receptors (iGluRs) and nAChRs [59]. Conversely, PhTX-343 is strongly selective for neuronal over muscle-type nAChRs and its analogs potentiated the trend of model compound, since they exhibited high antagonistic potency, particularly at the α3β4 subtype. TEVC current responses of α3β4 or α4β2 nAChRs, expressed in *Xenopus* oocytes, to 100 or 10 μM ACh were evaluated. IC_{50} values for PhTX-343 inhibition of α3β4 and α4β2 receptors were 7.65 and 80.2 nM, respectively (α3β4 *vs.* α4β2 selectivity = 10.5). Among the novel ligands, **22**, which incorporates a bulky hydrophobic cyclohexyl moiety, showed an increased potency at both studied subtypes [IC_{50} = 0.46 nM (α3β4) and 36.6 (α4β2) nM], with an enhanced α3β4 *vs.* α4β2, equal to 80. On the other hand, for the two doubly substituted antagonists **23** [IC_{50} = 2.62 nM (α3β4) and 2.58 (α4β2) nM] and **24** [IC_{50} = 0.16 nM (α3β4) and 14.6 nM (α4β2); α3β4 *vs.* α4β2 selectivity = 91] (Fig. 3), the loss of selectivity caused by exchanging the tyrosine moiety of PhTX-343 for a cyclohexylalanine in compound **23** was more than counterbalanced by introducing an aromatic, sterically demanding naphthyl moiety, that elevated potency of **24** at the β4-containing receptor subtype [59]. Compounds with a selective profile of this kind could be investigated for smoking cessation therapies and/or represent valuable probes in the study of nicotine addiction.

In another application of *Ac*AChBP with nAChRs, Bueno et al. characterized the 2′-fluoro-(carbamoylpyridinyl)deschloroepibatidine derivatives of epibatidine **25–28** (Fig. 3) for their binding affinity [60]. These compounds, already synthesized and tested as racemates in *in vitro* and *in vivo* experiments, are high affinity ligands (K_i < 1 nM) for the α4β2 nAChRs, but, at variance with their model full agonist, behave as antagonists at α3β4 and α4β2 subtypes and show a varied profile at the α7 subtype [61]. As an example, derivative **27b** displays a stimulating combination of properties, among them a subnanomolar (K_i = 0.07 nM) binding affinity at α/β nAChRs coupled with a submicromolar (IC_{50} = 0.46 μM) inhibition of the α4β2 subtype in functional assays, with a high degree of selectivity over α3β4 and α7 subtypes (54- and 348-fold, respectively) [61]. The authors assessed the affinity of *Ac*AChBP for the six ligands by bio-layer interferometry, all compounds displaying K_d values in the low nanomolar range, comparable to that of epibatidine, with a higher affinity than that evaluated for nicotine [60]. Three (**25**, **26a**, and **26b**) among the studied analogs were successfully crystallized in complex with *Ac*AChBP, with resolutions of 2.2, 2.4 and 2.5 Å, respectively. Given the limitations due to the use of this surrogate protein, the authors carefully compared the acquired data on the *Ac*AChBP-ligand crystals with the cryo-EM structure of nAChR in complex with nicotine and with the sequences of human α4, α7, and β2 subunits. The results of this study may be valuable for designing new nAChR-targeting ligands with defined pharmacological properties.

The two competitive nAChR antagonists UFR2709 and SUVN-911 (Ropanicant), whose structures are reported in Fig. 3, have been investigated over the last few years in view of potential therapeutic applications. UFR2709, endowed with markedly higher potency at displacing radioligand binding to human α4β2 than to α7 nAChRs [62], was evaluated for its ability to reduce the alcohol intake of high-alcohol drinking University of Chile bibulous (UChB) rats [63,64]. These animals have been bred for over 90 generations to ingest 10% ethanol solution in preference to water and represent a suitable model to screen medications to treat alcoholism. In a first report, UChB rats were given free access to ethanol for 24 h periods in a two-bottle free choice paradigm and their ethanol and water intake were measured. The animals were i.p. injected daily for 17 days with a 10, 5, 2.5, or 1 mg/kg dose of UFR2709, which reduced voluntary ethanol intake in a dose-dependent manner without affecting body weight or locomotor activity [63]. The 2.5 mg/kg dose of UFR2709 was the most effective and elicited a long-lasting effect that induced a 56% reduction in alcohol consumption, without affecting the weight or locomotor activity of the rats. In a more recent report [64], the same research group assessed the effect of UFR2709 administration in the acquisition and maintenance of ethanol intake in UChB rats that were chronically exposed to ethanol consumption. Treatment with UFR2709 decreased alcohol consumption at both stages even after ceasing its administration to the animals. At 2.5 mg/kg i.p., the compound reduced the seek behavior and ethanol intake, even when the drug administration was stopped, and induced a reduction in the overall ethanol intake by around 55% [64]. Therefore,

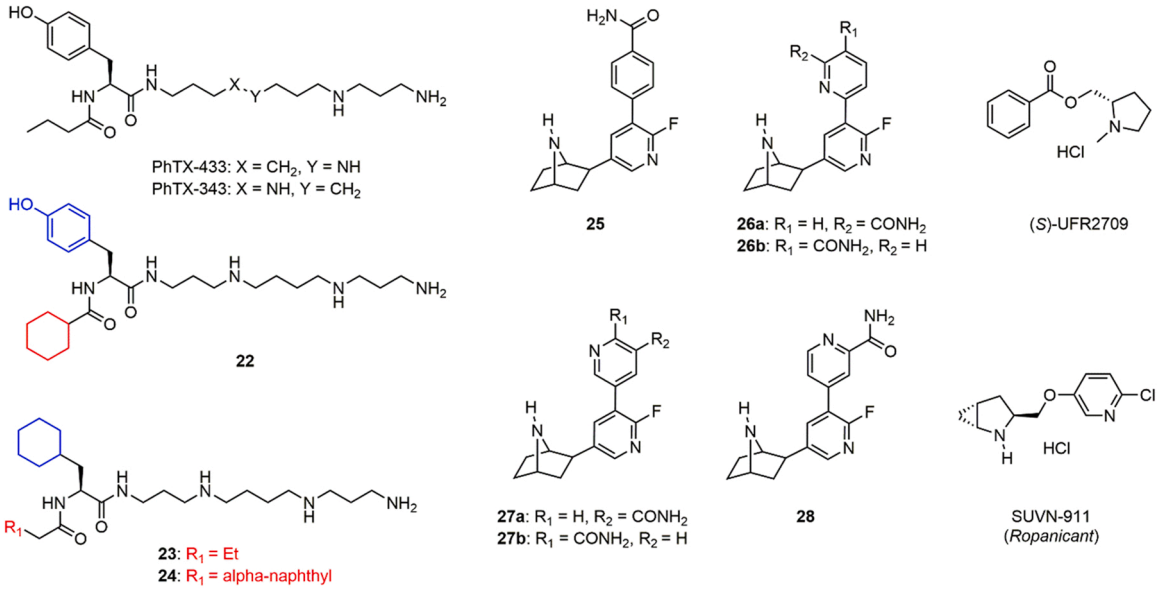

Fig. 3. Selective competitive antagonists of α3β4 or α4β2 nAChR subtypes.

α4β2-preferring nicotinic antagonists delay the acquisition and reduce the ethanol intake even in long-term experiments, thus suggesting their potential to treat or prevent alcohol abuse.

In a third report on UFR2709, its anxiolytic and anti-addictive properties were estimated in adult zebrafish, utilizing two behavioral paradigms to test for addiction, the novel tank diving test (NTT) to assess anxiety and the conditioned place preference (CPP), which resembles the models used for rodents [65]. The studied antagonist showed anxiolytic properties in the NTT and blocked the effect on the CCP evoked by nicotine. Moreover, UFR2709 caused a marked decrease in the expression of α4 nicotinic receptor subunit, without affecting, at variance with nicotine, the α7 subunit expression [65], a behavior that is in line with the α4β2 selective affinity profile shown by this ligand. Overall, the *in vivo* data at present available for UFR2709 suggest its potential for the treatment of nicotine addiction and/or anxiety, further supporting zebrafish as a suitable neuropharmacological protocol.

Nirogi et al. performed an in-depth structural optimization of the *in vitro* affinity profile in a series of constrained methanopyrrolidinol and methanopiperidinol compounds, focused on high affinity ligands for α4β2 nAChRs with marked selectivity against α3β4 nAChRs [66]. The structure-activity investigation was then combined with microsomal metabolic stability studies, pharmacokinetic evaluation, efficacy in a forced swim test (FST), target engagement, and safety assessments on the most promising derivative in the series, *i.e.*, SUVN-911, which was characterized as a safe, potent, and selective α4β2 nAChR clinical candidate. In fact, SUVN-911 is a potent receptor antagonist with a K_i value of 1.5 nM for the α4β2 subtype, showing > 10 μM binding affinity toward the ganglionic α3β4 receptor and selectivity over 70 other biological targets. Marked antidepressant activity and dose-dependent receptor occupancy in rats supported a potential therapeutic utility in the treatment of depression. The compound is devoid of cardiovascular and gastrointestinal side effects, and it does not affect the locomotor activity at doses several folds higher than its efficacy dose [66]. In addition to the effects in the rat FST, Ropanicant exhibited antidepressant-like properties in the differential reinforcement of low rate-72 s (DRL-72 s) [67]. A significant reduction in anhedonia was observed in the sucrose preference test. Oral administration of Ropanicant produced a meaningful increase in serotonin and brain-derived neurotrophic factor (BDNF) levels, with a reduction in the ionized calcium-binding adaptor molecule 1 (Iba1) activity. The onset of antidepressant-like effect with Ropanicant was within a week of treatment and was devoid of cognitive dulling and sexual dysfunction. A first Phase 1 study has been conducted on Ropanicant, *i.e.*, a randomized, double-blind, placebo-controlled, first-in-human study to evaluate the safety, tolerability, and pharmacokinetics of single ascending doses (0.5, 6, 15, 30, and 60 mg) and multiple ascending doses (15, 30, and 45 mg) of the drug administered orally for 14 days to healthy male subjects [68]. In a second Phase 1 study, the effect of food, sex, and age on drug pharmacokinetics was evaluated following a single 30 mg oral dose [68]. Ropanicant was found to be a safe and well tolerated drug following single and multiple oral administrations in healthy subjects and is currently being advanced into a Phase 2 clinical trial for depression.

3. Small molecule allosteres and noncompetitive antagonists of heteromeric nAChRs

3.1. Positive allosteric modulators

Given the limited knowledge on the SARs available from patent and scientific literature, AMs with sufficient potency and selectivity are often discovered by applying complementary drug discovery approaches, among them scaffold hopping, HTS and virtual screening, as commented in a report focused on PAM derivatives binding to the α7 nAChR subtype [29]. Owing to their commercial availability, some AMs of heteromeric nAChRs have been extensively investigated and compared in the last years, among them desformylflustrabromine (dFBr, Fig. 4), a naturally occurring compound that was identified more than fifteen years ago as a selective α4β2* Type II PAM. In a recent study, Bagdas et al. evaluated whether dFBr could mitigate mouse chronic constriction injury (CCI)-induced neuropathic pain by increasing the endogenous cholinergic tone or potentiating the nicotine-evoked antiallodynic response [69]. Although subcutaneous administration of dFBr did not reduce pain behavior, this PAM potentiated dose- and time-dependently the nicotine-evoked antinociception without causing motor impairment. Such an effect was blocked by dihydro-β-erythroidine (DHβE), thus confirming that it is primarily mediated by β2*-nAChR subtypes [69].

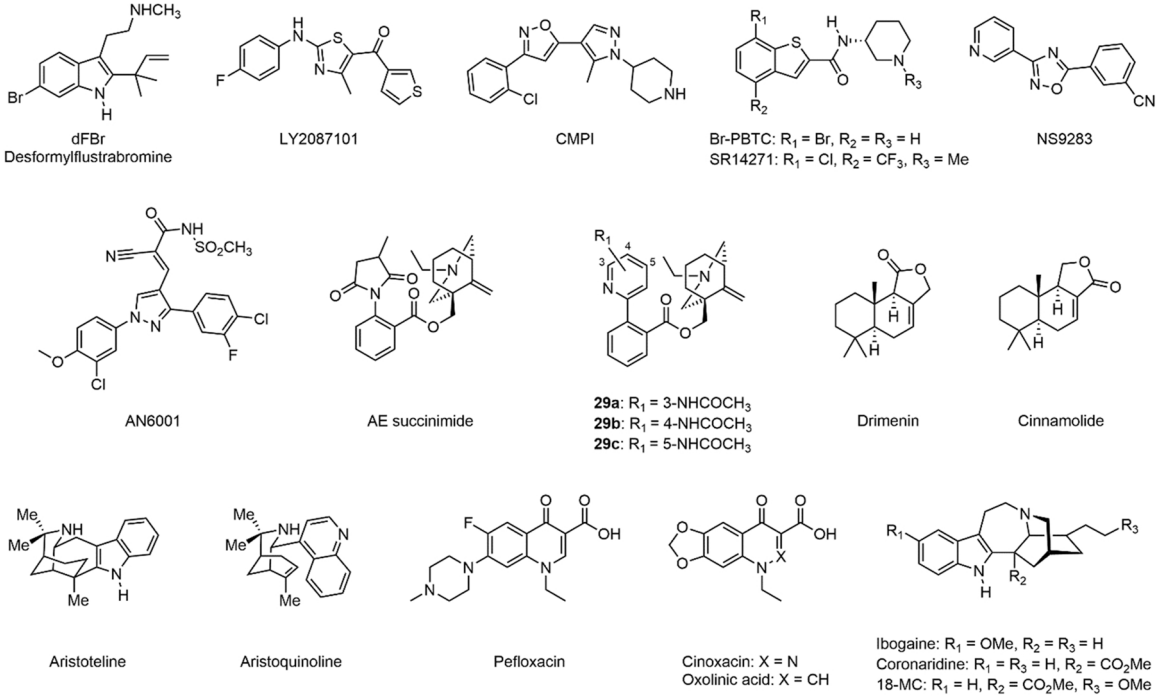

Fig. 4. PAMs and noncompetitive antagonists of heteromeric nAChRs.

In a study by DeCristofano et al., ethanol-induced loss of righting reflex (LORR) duration in rats was measured in the presence and absence of dFBr. Pretreatment with 6 mg/kg dFBr reduced ethanol-induced LORR duration as compared to rats treated with ethanol alone, and LORR experiments with DhβE suggested the involvement of the β2 subunit [70]. Pretreatment with the same dose of dFBr significantly reduced also the ethanol-induced α4 thalamic nAChR subunit protein levels, that were assessed by crosslinking-based western analyses. In TEVC recordings in recombinant human α4β2 nAChR, ethanol potentiated ACh-induced currents and slightly reduced dFBr potentiation of maximal ACh currents [70]. These overall results indicate dFBr as an option to reverse ethanol intoxication.

In an additional study by Nikiforuk et al., the cognitive effects of dfBr were investigated in the novel object recognition task (NORT) and in the attentional set-shifting task (ASST) in rats [71]. The compound attenuated the delay-induced impairment in NORT performance (3 mg/Kg) and facilitated cognitive flexibility in the ASST (0.1–0,3 mg/kg). The pro-cognitive activities elicited by dFBr were inhibited by DHβE, indicating the involvement of α4β2 nAChRs in cognitive processes. The tested α4β2R PAM was also effective against ketamine- and scopolamine-induced deficits of object recognition memory. Moreover, precognitive effects were observed after combined treatment with inactive doses of dFBr and TC-2403, a selective α4β2 nAChR agonist, a result in line with previous data in which dFBr was found to enhance nicotine-induced antinociception in a mouse model of neuropathic pain [71].

In an interesting report by Deba et al., the previously characterized α4 *vs.* α3/5 selective nAChR PAM LY2087101 (Fig. 4) was further studied at the α4β2 subtype, also by means of mutational and computational analyses [72]. LY2087101 behaved as a potentiator of the ACh-induced currents of LS and HS human nAChRs expressed in *Xenopus* oocytes, with comparable potency (EC_{50} = 1.4 ± 0.03 μM and 1.9 ± 0.04 μM, respectively) albeit to a different maximum potentiation (I_{max} about 840% and 460%, respectively). Amino acid replacements within the α4 subunit transmembrane domain [*e.g.*, α4Leu256 and α4Leu260 within the transmembrane helix 1 (TM1); α4Phe316 within the TM3; and α4Gly613 within TM4] markedly reduced LY2087101 potentiation of LS nAChR response [72]. Homology modeling and docking analyses allowed identification of the binding sites of LY2087101 in the transmembrane domain of the α4β2 nAChR protein: an intra-subunit site within the α4 subunit helix bundle and an inter-subunit site at the α4(+)/α4(−) subunit interface. The two recognition sites identified for LY2087101 are those involved also in the interaction of dFBr with α4β2 nAChRs, although with non-superimposing amino acid contacts.

The two nAChR PAMs dFBr, which does not distinguish between the LS and HS α4β2 nAChRs, and CMPI (Fig. 4), which is selective for the LS nAChR, have been evaluated in adult male mice in intravenous nicotine self-administration and withdrawal studies [73]. Only dFBr was found to decrease intravenous nicotine self-administration in a dose-dependent manner, and to fully reverse somatic and affective symptoms of nicotine withdrawal. Conversely, CMPI, at doses up to 15 mg/Kg, was only positively linked to nicotine-induced hypothermia, and negatively linked to nicotine-induced antinociception. These results indicate that potentiation of the HS isoform is necessary to modulate nicotine's reinforcing properties that underlie nicotine intake and to reverse nicotine withdrawal symptoms that influence nicotine abstinence. On the contrary, the LS isoform has a limited role in mediating body temperature and nociceptive responses [73].

The effects of dFBr and CMPI were further compared in the hot plate and tail flick tests, in which the latency to acute thermal nociceptive responses in rats was determined [74]. Intraperitoneal injection of dFBr, but not of CMPI, dose-dependently increased latency in the hot plate test. In the tail flick test, the effect at the highest dFBr or CMPI dose tested was only < 20% of the maximum possible effects reported for nicotine and other nicotinic agonists. Therefore, the direct acute effects of dFBr were superior to those found for CMPI, thus suggesting that selectivity for the LS is not beneficial in treating acute pain conditions [74]. However, these results do not preclude the suitability of PAMs like CMPI in chronic pain models.

The same research group recently aimed at better characterizing the binding and gating properties at the α4(+)/α4(−) binding site. To this end, whole-cell currents from *Xenopus* oocytes expressing LS nAChRs were recorded in response to applications of nicotinic ligands, such as ACh, cytisine, and nicotine (which bind at both α4(+)/α4(−) and α4(+)/β2(−) interfaces) and CMPI (which binds at the α4(+)/α4(−) but not at the α4(+)/β2(−) interface) [75]. CMPI enhanced channel-gating activation triggered by ACh occupancy at the α4(+)/β2(−) agonist-binding sites by binding to the α4(+)/α4(−) subunit interface, which becomes occupied by ACh only at high concentrations. Therefore, exposure to nicotinic ligands specifically recognizing the low-affinity ACh binding site located at the α4(+)/α4(−) subunit interface is expected to increase the efficacy of the neurotransmitter ACh, an effect that may become therapeutically useful in conditions associated with decline in nAChR-mediated activity in the CNS [75].

Br-PBTC (Fig. 4) was characterized some years ago as a subtype-selective type II PAM, able to selectively increase ACh-evoked responses at α2*- and α4*-containing subtypes and to reactivate desensitized α4*-containing subtypes in nAChRs expressed in HEK cells [76]. The same research group combined functional and mutagenesis studies with computational protocols to identify the Br-PBTC binding site in the transmembrane domains [77]. The amino acids Glu-282, Phe-286, and Ile-601 near the extracellular domain of the third transmembrane helix of the α4 subunit were found to be relevant to the Br-PBTC's allosteric effect. The located Br-PBTC binding site overlapped well with that of other known nAChR PAMs that have been docked within the transmembrane domains; some of them, like dFBr, shared with Br-PBTC common intra-subunit cavities. Although a refinement of the Br-PBTC-binding site is crucial, these results appear promising in view of designing new subtype-selective nAChR PAMs. Worth mentioning, some of the analogs of Br-PBTC, particularly SR14271 (Fig. 4), behaved as the first PAMs able to promote activation of α5-containing heteromeric receptors [77].

In a report by Wang et al., activation of the $(\alpha4\beta2)_2\alpha4$ stoichiometric form of α4β2 nAChRs by NS9283 (Fig. 4), another PAM selective for the LS receptors, may represent a new strategy to reduce alcohol consumption [78]. The alcohol intake is reduced by the smoking cessation agent varenicline, a partial agonist of α4β2 nAChRs, whose use is limited by side effects at high therapeutic doses. In this study, NS9283 was found to increase *in vitro* the potency of varenicline by 235-fold in activating LS nAChRs, and to a lesser extent (tenfold), in desensitizing them. This PAM also slightly increased varenicline efficacy, with no effect on other human nAChRs [$(\alpha4\beta2)_2\beta2$, α3β4 and α7 subtypes, all stably expressed in HEK cells] or $5HT_{3A}$ receptors. In male and female mice, NS9283 (10 mg/kg) reduced ethanol intake in a two-bottle choice, intermittent drinking procedure without affecting saccharin intake, ethanol-induced incoordination or ethanol-induced LORR [78]. To further shed light on the recognition site of the stoichiometry-selective PAM NS9283, Mazzaferro et al. generated concatemeric receptors with mutations at specific subunit interfaces, then assessed the ability NS9283 to potentiate $(\alpha4\beta2)_2\alpha4$ single-channel currents elicited by ACh [79]. The authors proved that a mutation at the principal face of the β2 subunit at either β2(+)/α4(−) pseudo-agonist site inhibited potentiation by NS9283, whereas a mutation at the complementary face of the α4 subunit at the α4(+)/α4(−) agonist site resulted in a pronounced potentiation. Since orthosteric agonists do not bind to the β2(+)/α4(−) interface, as shown by the cryo-EM structures of the α4β2 nAChR in complex with nicotine [6], NS9283 could bind to this interface and exert its potentiating effect, with a mechanism reminiscent of that of benzodiazepines at the $GABA_A$ receptor [79].

It is known that α6β2* nAChRs are predominantly expressed in midbrain dopaminergic neurons, including substantia nigra pars

compacta (SNc) neurons and their projections to striatal regions, where they regulate dopamine release and nigrostriatal activity. van Hout et al. identified and characterized the novel PAM of α6β2* -containing nAChRs AN6001 (Fig. 4), whose selectivity profile and functional properties were assessed in functional *in vitro* studies using recombinant nAChRs expressed in HEK293 cells and *Xenopus* oocytes and in dopaminergic neurons with a combination of imaging and electrophysiological techniques [80]. Moreover, AN6001 augmented agonist-induced dopamine release from striatal synaptosomes and potentiated the neuroprotective effect of nicotine by increasing the survival of cultured MMP^+-treated dopaminergic neurons. Hence, AN6001 is a relevant tool for further exploration of α6β2* nAChR expression and function, although the potential of allosteric modulation of this subtype in PD still needs a more detailed investigation.

3.2. Noncompetitive antagonists

In the framework of ligands displaying antagonistic effects at heteromeric nAChRs, a recent study by Qudah et al. focused on some simplified analogs of methyllycaconitine (MLA), a potent nicotinic antagonist at both α7 and α4β2 receptors, with higher selectivity for the α7 subtype. The anthranilate-succinimide ester side chain, that was found to be relevant to the activity and selectivity profile of MLA, has been preserved in the investigated compounds, which included AE succinimide (Fig. 4) [81], designed to identify ligands with a higher potency for the α4β2 subtype. Remarkably, AE succinimide inhibited ACh-activated currents at both α4β2 stoichiometries of nAChRs, generated by injecting *Xenopus* oocytes with different ratios of α4 and β2 mRNA. Two different mechanisms were identified: a direct competitive antagonism with ACh when the compound was not preincubated, and an apparent unsurmountable effect when the compound was preincubated. The authors found that upon binding and in the presence of ACh, a conformational change occurred in the channel membrane that was evidenced in the mutated $(\alpha4V13'C)_3(\beta2)_2$ nAChR. With this 3:2 stoichiometry, the two close α4 subunits containing 13′ cysteine mutations formed a disulfide bridge and obstructed ion conductance, an effect that was reversed with the reducing agent dithiothreitol. Thus, at variance with MLA and other investigated AE analogs, AE succinimide, upon binding to an unidentified site and in the presence of ACh, engenders a ligand-bound nonconducting state of the α4β2 receptor [81].

In a similar study by the same research group, a set of twelve newly synthesized MLA-related analogs (exemplified by compounds **29a-c**, Fig. 4) were studied at α7 and α4β2 nAChRs [82]. Electrophysiology experiments using *Xenopus* oocytes expressing nAChR subtypes revealed a general profile of noncompetitive antagonists, endowed with a preference for the α4β2 over α7 nAChR subtypes. Selected derivatives in the series were compared on both α4β2 stoichiometries, showing a more pronounced inhibitory effect at LS over HS receptors. The IC_{50} values of regioisomers **29a-c** at $(\alpha4\beta2)_2\alpha4$ receptors ranged from 3.9 to 4.9 μM, and those at $(\alpha4\beta2)_2\beta2$ receptors from 15.0 to 29.1 μM, suggesting that the position of the amide group has marginal influence on the activity profile of these compounds. Although MLA was 50–200-fold more potent at both receptor stoichiometries than its analogs **29a-c**, the latter showed a degree of selectivity for the LS receptor that is absent in their model compound [82].

In the field of natural compounds, H. R. Arias et al. studied two groups of noncompetitive nicotinic antagonists with a different profile. In the first report, the four drimane sesquiterpenoids drimenin, cinnamolide, polygodial, and dendocarbin A were purified from the Canelo tree *Drimys winteri* and structurally characterized by spectroscopic techniques. These natural compounds were evaluated on human receptors by Ca^{2+} influx measurements, displaying inhibitory activity of nAChR subtypes following the order: *h*α4β2 > *h*α3β4 > *h*α7 [83]. In the case of the *h*α4β2 subtype, drimenin and cinnamolide (Fig. 4) were by far the most potent ligands ($IC_{50} = 0.97 \pm 0.35$ μM and 1.57 ± 0.36 μM, respectively). In the second report, the three alkaloids aristoteline, aristoquinoline and aristone were purified from the leaves of the Maqui tree *Aristotelia chilensis* and chemically characterized by NMR spectroscopy. Following the previously applied protocol, the compounds were evaluated on nAChR subtypes, and their inhibitory potency was in the order *h*α3β4 > *h*α4β2 ≫ *h*α7. In the case of *h*α3β4 receptors, aristoteline and aristoquinoline (Fig. 4) showed interesting potency values ($IC_{50} = 0.40 \pm 0.20$ μM and 0.96 ± 0.38 μM, respectively) 50 [84]. Overall, drimenin and aristoteline represent two novel molecular scaffolds for the development of more potent noncompetitive antagonists with higher selectivity for α4β2 and α3β4 nAChRs, respectively.

Sanders et al. investigated a group of quinolone antibiotics, belonging to a therapeutic class which is known to inhibit both ionotropic $GABA_A$ receptors and human α7 nAChRs; the compounds were assayed on both stoichiometries of the human α4β2 nAChR, expressed in *Xenopus* oocytes, by TEVC recordings [85]. The seven fluoroquinolone antibiotics (ciprofloxacin, enoxacin, enrofloxacin, difloxacin, norfloxacin, pefloxacin, and sparfloxacin) more potently inhibited $(\alpha4\beta2)_2\beta2$ than $(\alpha4\beta2)_2\alpha4$ nAChRs, with a particularly relevant effect shown by pefloxacin (Fig. 4), which inhibited $(\alpha4\beta2)_2\beta2$ receptors with an IC_{50} value of 26.4 ± 3.4 μM, and a four-fold selectivity over the $(\alpha4\beta2)_2\alpha4$ isoform. On the other hand, the two tested nonfluorinated quinolone antibiotics cinoxacin and oxolinic acid (Fig. 4) were about twofold less potent than pefloxacin on the $(\alpha4\beta2)_2\beta2$ isoform and lacked stoichiometry selectivity. Computational docking analyses, performed with three-dimensional atomic models of the $(\alpha4\beta2)_2\beta2$ and $(\alpha4\beta2)_2\alpha4$ nAChRs from cryo-EM structures, allowed to rationalize the preference shown by pefloxacin by identifying its selective interaction with an allosteric transmembrane site at the β2(+)/β2(−) subunit interface [85].

In a recent perspective article, Straub et al. critically discussed the biological results available on all the known α3β4 nAChR inhibitory ligands, including iboga alkaloids and related analogs (Fig. 4) [86]. Ibogaine, the psychoactive alkaloid isolated from *Tabernanthe iboga*, is characterized by complex pharmacological properties, and the antagonist profile at α3β4 nAChRs may represent the main mechanism of action for the antiaddictive activity of this naturally occurring substance. In fact, anatomical and genetic outcomes suggest that α3β4 nAChRs expressed in the Medial habenula (MHb)-Interpeduncular nucleus (IPN) axis exert a crucial role in addiction and their inhibition could be one of the approaches against disorders from use of substances like nicotine, morphine, methamphetamine, and alcohol [86]. Experimental data on ibogaine and its synthetic analog 18-Methoxycoronaridine (18-MC) indicate that they do not bind the orthosteric recognition site of human α3β4 nAChRs. Docking and molecular dynamics analyses put in evidence that, besides blocking the ion channel's lumen, 18-MC and its derivatives create a series of negative allosteric interactions with various non-luminal sites [87]. Despite its moderate potency and the limited degree of selectivity for α3β4 receptors, 18-MC was found to be efficacious against different drugs of abuse, and in 2022 the biopharmaceutical company MindMed published the results of a Phase 1 clinical trial, dealing with the potential of Zolunicant (18-MC HCl) for the treatment of opioid use disorder [88].

4. Dual-acting nAChR ligands and an example of photoswitchable nicotinic probe

In the last two decades, an increasingly pursued drug discovery approach in the medicinal chemistry research has been the development of bifunctional/multitarget compounds, that rationally incorporate in one structure pharmacophoric moieties able to recognize different biological targets. Although examples of this strategy are mainly related to G protein-coupled receptors, some recent reports focused on pentameric LGICs, among them nAChRs [89]. Interestingly, a systematic study collected the results of an *in silico* analysis contributing to a better understanding of polypharmacology across LGICs [90].

As far as the nAChRs are concerned, Matera et al. synthesized and tested the new compounds **30a-c** (Fig. 5) potentially targeting the D3R-

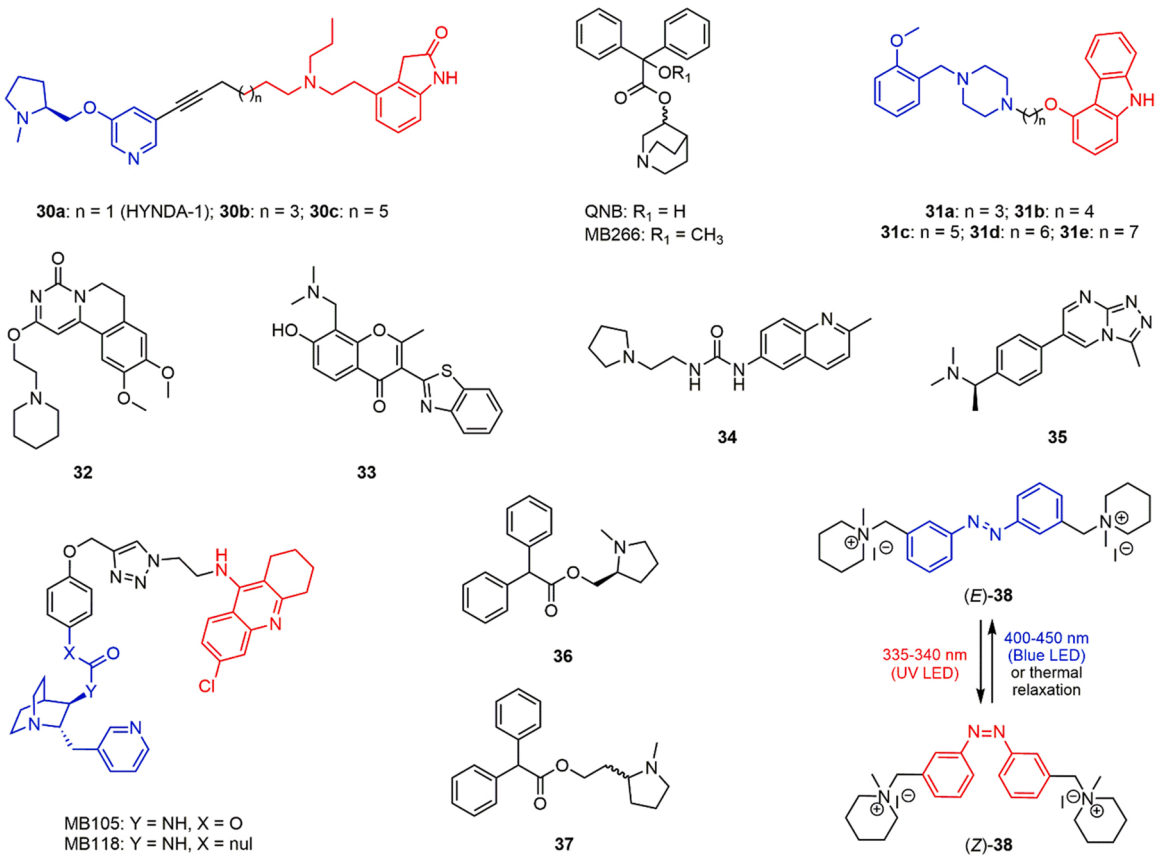

Fig. 5. Hybrid, dual-acting nAChR ligands and the isomers of a photoswitchable neuromuscular blocker.

nAChR heteromer [91]. This receptor complex has been identified and characterized as the molecular entity that, in dopaminergic neurons, mediates the neurotrophic effects of nicotine, and ligands with a dual agonist profile at this heteromeric receptor should exert a synergistic neurotrophic effect, since the neurotrophic properties of nicotinic agonists and of D3R agonists have been documented [92]. The planned derivatives were designed by linking with a partially rigidified spacer A-84543 (see structure in Fig. 2), a selective α4β2 nAChR agonist (blue fragment), to ropinirole (red fragment), a D3R preferential agonist. The analogs all bound with high affinity to both β2-subunit-containing nAChRs (from native rat cortical membranes) and D3Rs (expressed in HEK cells), but only **30a** (named HyNDA-1, K_i (rα4β2) = 4.50 nM, K_i (D3R) = 3.80 nM), characterized by the shortest linker, significantly promoted neurotrophic remodeling of both mouse and human DA neurons, an effect prevented by either nAChR or D3R antagonists [91]. Moreover, disrupting the D3R-nAChR heteromer with specific interfering peptides counteracted the neurotrophic effects of HyNDA-1. By using the BRET assay, HyNDA-1 was also found to increase the affinity of interaction between D3R and nAChR in the HEK-293 transfected cell system, reflecting its property of bridging the two individual receptors [91]. These results suggest that this bivalent compound may represent the framework to develop new drug candidates with disease-modifying features, such as the capability to block neurodegeneration or to promote neuroregeneration/neurogenesis.

Racemic MB266, the *O*-methylated analog of the mAChR antagonist 3-quinuclidinyl benzilate QNB (see structures in Fig. 5), was synthesized by Timperley et al. and tested in a functional assay based on agonist-induced elevation of intracellular calcium ion concentration in three different cell lines [93]. MB266 behaved as an antagonist at multiple subtypes of mAChRs, displaying an overall 18-fold selectivity for mAChRs *versus* nAChRs (compared to the 15,200-fold selectivity known for QNB). Thus, *O*-methylation of QNB significantly reduced the affinity for mAChR and increased the relative potency at both muscle and neuronal nAChRs. This overall cholinergic antagonist profile may indicate MB266 as a useful ligand for treatment of neuromuscular dysfunction following anticholinesterase poisoning; however, its administration did not improve the neuromuscular function in a soman-poisoned guinea-pig diaphragm preparation pretreated with the organophosphorus nerve agent soman [93].

An example of the multitarget drug-design strategy combined potentially synergistic pharmacophores into hybrid compounds **31a-e** (Fig. 5), by linking a 1-(2-methoxybenzyl)-piperazine function (blue moiety, designed for the recognition of both, AChE catalytic site and α7 nAChR) to a carbazole fragment (red moiety, exerting anti-amyloidogenic activity) through spacers of different length [94]. AChE inhibition was strictly dependent on the chain length separating the pharmacophoric functions, compounds **31d** (IC_{50} = 0.773 μM, n = 6) and **31c** (IC_{50} = 2.39 μM, n = 5) being the most effective. The studied derivatives displayed a micromolar affinity profile (K_i values ranging from 15 to 120 μM) at nAChRs, without discriminating the α7 from α4β2 subtypes from rat hippocampal and cortical membranes, respectively. The compounds reached a low but significant activation only of the α4β2 subtype (15–22% of the response, reference 100 μM ACh). Notably, homologs **31c** and **31d** showed an inhibition ability (34% and 28%, respectively) of Aβ(1−42) self-aggregation at 10 μM, as determined with a thioflavin T fluorescence assay. Overall, these results, obtained for some of the studied analogs on three altered pathways in the AD pathogenesis, represent the basis for further optimization steps.

Balle et al. published two interesting reports on the search for bimodal compounds through computational chemistry protocols, among them homology modeling and high throughput virtual screening [95, 96]. The purpose was again to identify derivatives with AChE inhibitory activity as well as selective agonism at α7 nAChR subtypes, thus gaining a dual modulation of cholinergic signaling potentially useful for the

palliative treatment of AD and related disorders. In the first study, the authors analyzed a ZINC database of 87,250 natural products and their derivatives, purchased the identified hit compounds, and assayed them by TEVC electrophysiology *in vitro* at the human α7 Rs expressed in *Xenopus* oocytes and against *Electrophorus electricus* AChE with the Ellman's colorimetric test. This screening put in evidence the two ligands **32** (IC_{50} = 5.04 μM for AChE, and IC_{50} = 14.5 μM at *h*α7 Rs) and **33** (IC_{50} = 10.6 μM for AChE, and IC_{50} = 34.3 μM at *h*α7 Rs) (Fig. 5), characterized by comparable and balanced activities at the two targets, but behaving as antagonists at the α7 subtype [95]. In the second study, the parallel and independent screening of a much larger virtual compound library of 3848,234 drug-like and commercially available molecules from the ZINC15 database allowed identification of about 60 compounds potentially active at the two selected targets [96]. Based on ligand efficiency as well as scaffold and molecular diversity, sixteen of these compounds were purchased for *in vitro* validation, and two of them, **34** and **35** (Fig. 5), were found to behave as dual-acting compounds with the expected profile. In particular, derivative **35**, inhibited AChE (IC_{50} = 2.58 ± 0.96 μM) and slightly activated α7 nAChRs (7.0 ± 0.9% at 200 μM), thus representing a starting structure to be optimized in terms of both activity/efficacy profile and physicochemical properties [96].

The racemic carbamate MB105 and benzamide MB118 represented in Fig. 5 were rationally synthesized using a "click-chemistry" approach to link the chlorotacrine moiety (in red) to a 2,3-disubstituted quinuclidine scaffold (in blue), which are known for their anticholinesterase and α7 nAChR agonist activities, respectively [97]. The two analogs inhibited human AChE and BChE in the nanomolar range (for MB105, the IC_{50} values were 15.2 nM and 131 nM, respectively). Electrophysiological recordings on *Xenopus* oocytes expressing human α7 nAChRs showed that they behaved as partial agonists at the studied subtype, with MB105 having about 340-fold higher potency than MB118 (for MB105, IC_{50} = 3.98 μM, B_{max} = 53%, compared to the maximal efficacious 100 μM dose of ACh), and the effects were counteracted by α-bungarotoxin. Thus, MB105 acted as a partial agonist at the α7 receptor in a concentration range at which it completely inhibited human AChE activity [97], an encouraging result in view of further optimization steps.

González-Gutiérrez et al. designed and synthesized a set of twenty-one arylpyrrolidine ester derivatives as novel ligands for nAChRs. With the aim to discover potential multitarget interactions with serotonin and dopamine transporters, the studied compounds were assayed in binding experiments on whole rat brain synaptosomes for α4β2 affinity (using [^{3}H]cytisine as the radioligand), and on homogenized membranes prepared from human clonal cell lines (HEK293 for h-SERT and CHO-K1 for h-DAT) [98]. Only derivative **36** (Fig. 5) recognized the three targets, exhibiting high affinity for α4β2 nAChRs (K_i = 0.023 ± 0.006 μM), moderate affinity for h-DAT (K_i = 1.208 ± 0.230 μM), and producing a marked increase in the percent of [^{3}H]-paroxetine binding at h-SERT (192%). The related racemic homologue **37** (Fig. 5) displayed very high affinity for h-DAT (K_i = 0.075 ± 0.009 μM) and α4β2 nAChRs (K_i = 0.113 ± 0.037 μM), behaving as a dual-acting ligand. Molecular docking analyses on homology models of α4β2 nAChR, h-DAT and h-SERT allowed to identify the interaction domains with the orthosteric binding site of α4β2 nAChR, the central binding site of h-DAT, and the allosteric modulatory site of h-SERT [98].

We devote a brief comment on the utilization of light-dependent compounds to control nAChR activity, a subject that has been discussed in the framework of a mini-review focused on LGICs [99]. In a recent study performed by Herrera-Arozamena et al., two *N*-methyl--*N*-carbocyclic quaternary ammonium groups were linked to an azobenzene scaffold in *meta*- or *para*-positions, to give rise to a set of photoswitchable neuromuscular ligands, which the authors named "azocuroniums" [100]. Albeit they are uncommon for LGICs, the advantage associated to this type of light-activated tools in controlling cellular processes is their ease of use, since no genetic manipulation of the related biological targets is required, and the compounds can in principle be applied like conventional drugs. In this example, the derivatives were easily photoisomerized between the (*E*)- and (*Z*)-forms by irradiation at 335–340 nm and 400–450 nm, respectively, as indicated for the *meta*-disubstituted derivative **38** in Fig. 5. The latter behaved as a potent nicotinic ligand on human muscular nAChRs (K_i = 35 ± 3 nM), with selectivity over human neuronal-type α7 (K_i = 910 ± 90 nM) and α4β2 (K_i = > 10,000 nM) subtypes. Moreover, it showed good solubility in physiologic media and negligible cell toxicity. Electrophysiological studies in muscle-type nAChRs expressed in *Xenopus* oocytes showed that (*E*)-isomers were more potent than (*Z*)-forms. These *meta*--azocuroniums may be considered as photo-modulated neuromuscular blockers, representing novel pharmacological tools potentially useful as muscle relaxants for clinical applications [100].

5. Selective radiopharmaceuticals for heteromeric nAChRs

As documented in a review article by Tiepolt et al., *in vivo* exploration on the integrity of cholinergic transmission in the diverse compartments of this complex neurotransmitter system by means of Positron Emission Tomography (PET) imaging is of major relevance in the study of the pathophysiology of neurodegenerative syndromes such as AD and Lewy body disorder [101]. Various PET ligands for the two major nAChRs, *i.e.*, the α4β2 and α7 subtypes, were developed in the last two decades, and some of them have been successfully translated to application in humans. Among the most utilized radioligands for α7 nAChR imaging in humans, substituted diazabicyclononanes with sufficient brain uptake ([^{11}C]NS14492, [^{18}F]NS10743, and [^{18}F]NS14490) were developed, as well as the dibenzothiophene sulfone derivatives [^{18}F]ASEM and its isomer [^{18}F]DBT10 [101]. The relatively new α4β2 nAChR PET radiotracers for application in humans belong to three chemical classes, *i.e.*, 3-pyridyl ethers ([^{18}F]Nifene and [^{18}F]2-FA-85380), epibatidine-related ([^{18}F]AZAN and [^{18}F]XTRA) and homo-epibatidine-related {(+)-[^{18}F]Flubatine and (−)-[^{18}F]Flubatine} derivatives [101]. In this paragraph, the results obtained on already known or more recent, promising radiopharmaceuticals for heteromeric nAChR subtypes are summarized.

Based on the favorable neuroimaging results obtained with [^{18}F]Nifene (Fig. 6), Betthauser et al. utilized whole body (WB) PET/computerized tomography (CT) scans in human subjects to characterize and confirm the WB biodistribution and estimate, for the first time, the radiation burden of [^{18}F]Nifene in humans [102]. Dosimetry data indicated that the bladder was the dose limiting organ, and hourly bladder voiding allowed for a maximum injected dose of 278 MBq (7.5 mCi) [^{18}F]Nifene, or up to four 185 MBq (5.0 mCi) [^{18}F]Nifene scans annually. These findings point to the compound as a safe PET radioligand in humans, thus supporting the development of further [^{18}F]Nifene studies to image the α4β2* nAChR system. [^{18}F]Nifene binding to α4β2* nAChRs in PD was analyzed by Campoy et al., using the transgenic Hualpha-Syn(A53T) PD mouse model of α-synucleinopathy for PET/CT studies *in vivo* and autoradiography *in vitro* [103]. Moreover, postmortem human PD brain sections of anterior cingulate were employed in *in vitro* assays to assess potential alterations in α4β2* nAChRs. Transgenic Hualpha-Syn(A53T) mice brain slices exhibited 20–35% reduction in [^{18}F]Nifene binding, while an *in vivo* 20–30% decrease was observed. Lewy bodies and α-synuclein aggregates were confirmed in human PD brain sections which lowered the [^{18}F]Nifene binding by more than 50% in anterior cingulate, suggesting that this PET ligand is a promising option for translation to human studies.

Both (+)-[^{18}F]Flubatine and its enantiomer (−)-[^{18}F]Flubatine (represented in Fig. 6) are homoepibatidine-related radioligands for the neuroimaging of α4β2 nAChRs in brain by PET. To support the evaluation of (+)-[^{18}F]Flubatine during the first clinical PET comparing the status of α4β2 nAChRs in healthy controls and patients with AD, the metabolic fate of the radioligand was investigated by Ludwig et al. to

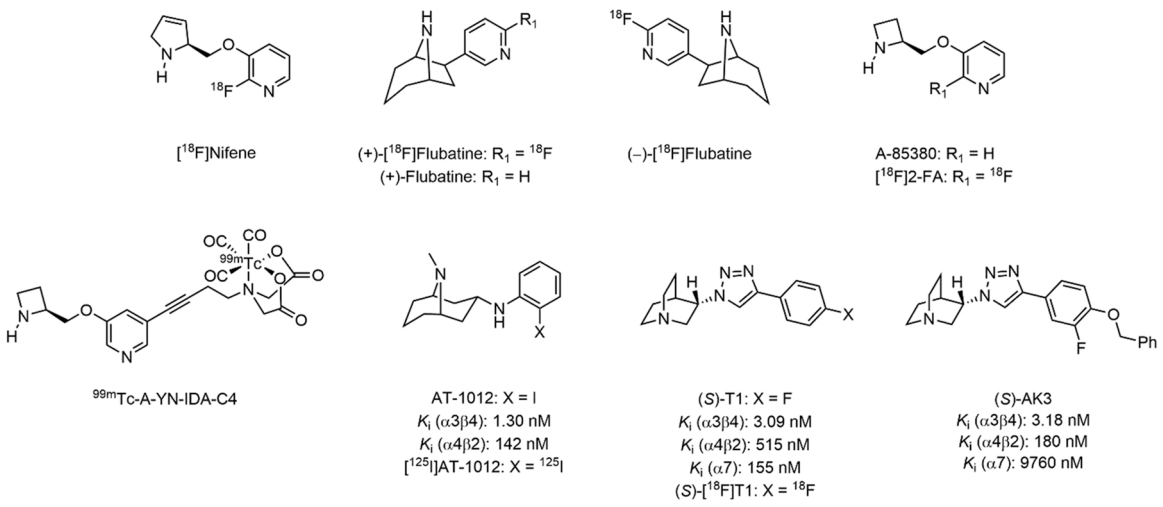

Fig. 6. Selective radiopharmaceuticals for heteromeric nAChR subtypes.

identify the degradation products detected in plasma and urine [104]. The study included an *in vivo* investigation of (+)-Flubatine in pigs, and incubations of (+)-Flubatine and (+)-[^{18}F]Flubatine with human liver microsomes, to generate *in vitro* metabolites, as well as radiometabolites, whose structures were assigned by comparing LC-MS/MS and radio-HPLC data. (+)-[^{18}F]Flubatine behaved as an appropriate tracer with exceptionally high metabolic stability in humans, whose radiometabolites were however characterized as a *C*-hydroxylation product at the azabicyclic ring system and a glucuronide conjugate of the previously formed *N*-8-hydroxylated derivative. In a more recent report, the same research group: a) developed a kinetic modeling-based approach to quantify the dynamic (+)-[^{18}F]Flubatine data, and compared the data of 11 healthy controls (HCs) with those of 9 patients with mild AD; b) investigated the partial volume effect (PVE) on regional (+)-[^{18}F]Flubatine binding; c) correlated (+)-[^{18}F]Flubatine binding with cognitive test data and with β-amyloid (Aβ) plaques [105]. In comparison with HCs, patients with mild to moderate AD showed a reduced availability of α4β2 nAChRs in the bilateral mesial temporal cortex. Worth noting, correlation between white matter β-amyloid PET uptake and (+)-[^{18}F]Flubatine binding denoted an association between white matter integrity and availability of α4β2 nAChRs. No adverse event related to (+)-[^{18}F]Flubatine occurred and, although a limitation of this study is the small sample size, this α4β2-targeting PET radiotracer has the potential of being studied in further clinical trials involving neurological or psychiatric diseases such as dementia, parkinsonian syndromes or depression [105].

ACh exerts distinct functional roles in striatum compared with cortex and imbalance between these systems may affect neuropsychiatric diseases. Smart et al. proposed PET imaging with (−)-[^{18}F]Flubatine, in the presence of nicotine, to evaluate regional differences in occupancy at nAChRs and quantify relative ACh concentration across the human brain [106]. A parallel analysis was performed in the same study with the M_1 selective muscarinic receptor radioligand [^{11}C]LSN3172176 in the presence of scopolamine. Occupancy estimates within striatal regions were found to be considerably lower in both healthy human volunteers and nonhuman primates with both radiotracers, thus indicating markedly higher striatal ACh concentration. This novel approach may allow to assess alterations in distribution of endogenous ACh across brain regions of living people, which may thus correlate with the occurrence of a neuropsychiatric disorder [106].

Changes in neuronal nAChRs have been identified in a form of familial focal epilepsy, the autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE), characterized by hypermotor seizures during sleep. A study by Garibotto et al. [107] aimed at evaluating potential changes in nAChR availability also in patients with idiopathic generalized epilepsy (IGE); 34 male participants, belonging to three clinical groups, were included in the study and PET/CT analyses were conducted with [^{18}F]2-FA-85380 (or [^{18}F]2-FA, Fig. 6), a well-known radiotracer targeting α4β2* nAChRs. In short, the group of patients with IGE showed a meaningful increase of [^{18}F]2-FA ratio index of binding potential (BP_{RI}, corresponding to the receptor availability) in the anterior cingulate cortex (ACC), without structural changes on magnetic resonance imaging. At an individual level, this BP_{RI} increase in the ACC allowed to clearly discriminate IGE subjects from controls or patients with focal epilepsy. Although the sample size was small, data from this study suggest that the modulation of α4β2 nAChR density is crucial not only in ADNFLE but could serve as a biomarker of other genetic epilepsy syndromes such as IGE [107].

Another recent application of PET imaging involved a comparative study on [^{18}F]2-FA and [^{18}F]Nifene in *in vivo* imaging, in male and female mouse brain, to provide further evidence that differences in PET ligand trapping in acidic vesicles affect differences in ligand kinetics and subcellular distribution [108]. Moreover, a fluorescent labeled high-affinity nicotinic ligand allowed to clarify that these intracellular vesicles are α4β2R-containing Golgi satellites (GSats) [108]. Of the PET ^{18}F-labelled imaging ligands, [^{18}F]2-FA has varenicline-like pK_a and affinity whereas [^{18}F]Nifene has nicotine-like pK_a and affinity. [^{18}F]2-FA PET-imaging kinetics were found to be very slow, consistent with trapping of this radiotracer in α4β2R-containing GSats. In contrast, [^{18}F]Nifene showed rapid kinetics, consistent with its binding to α4β2Rs but without trapping. The combined *in vitro* and *in vivo* imaging outcomes of this study add further information to the kinetics of weak base nAChR ligands and to the subcellular mechanisms underlying nicotine addiction.

Technetium-99 m (^{99m}Tc) is a versatile radionuclide, clinically used as a tracer in Single-Photon Emission Computed Tomography (SPECT)/CT imaging. A chelating moiety stably binding ^{99m}Tc was incorporated by Mori et al. in the molecular skeleton of the high affinity α4β2 nAChR agonist A85380 (see Fig. 6), the model ligand for the PET radiotracer [^{18}F]2-FA [109]. For an efficient design of the probe, a computational protocol was applied and, initially, nonradioactive rhenium (Re) as a ^{99m}Tc surrogate was utilized in synthetic as well as theoretical approaches. Among the prepared Re-containing ligands, Re-A-YN-IDA-C4 exhibited high affinity for α4β2 nAChR in docking simulation predictions (−19.3 kcal/mol) as well as binding assays (K_i = 0.4 ± 0.04 nM, expressed as the extent of inhibition of [^{3}H]cytisine binding to the cerebral cortex's crude synaptic membrane fraction). Thus, the related ^{99m}Tc-A-YN-IDA-C4 (Fig. 6) derivative was synthesized in 31% radiochemical yield and was found to maintain affinity and selectivity for α4β2 nAChR *in vitro*. A high correlation was found between the

nAChR densities in the rat brain sections (cerebral cortex, striatum, hippocampus, thalamus, and cerebellum) *in vitro* and the accumulation amount of ^{99m}Tc-A-YN-IDA-C4 (R^2 = 0.93) in each area of interest, suggesting that this compound is a promising molecular imaging probe in nuclear medicine [109].

The development of selective α3β4 nAChR radiotracers as promising tools for studying psychostimulant and drug-seeking behaviors has produced some results. The selectivity profile shown by AT-1012 (Fig. 6), the iodine-containing analog of the previously mentioned α3β4 partial agonist AT-1001 (see Fig. 2), inspired some years ago the synthesis of [^{125}I]AT-1012, a SPECT tracer that selectively labels α3β4 nAChRs in the presence of other nAChR subtypes [110]. On the other hand, the first developed PET α3β4 tracer (*S*)-[^{18}F]T1 took advantage of the affinity profile at nAChRs shown by "cold" (*S*)-T1, illustrated in Fig. 6 [111]. The same research group has recently prepared and tested a small set of closely related analogs, in which the quinuclidine ring, the triazole spacer, and the (*S*)-configuration of (*S*)-T1 have been preserved. When assayed on cell lines stably transfected with human nAChRs, (*S*)-AK3 (Fig. 6) emerged as the most relevant among the new α3β4 ligands, and its extended hydrophobic moiety triggered a significant gain of selectivity towards the α7 subtype [112]. The presence of a fluorine atom in (*S*)-AK3 gives the opportunity to develop a novel selective PET tracer to further investigate the role of α3β4 nAChRs in drug addiction and related disorders.

6. Concluding remarks

The past few years have witnessed the increased availability of high-resolution structures of druggable biological targets, among them nicotinic acetylcholine receptors. These achievements represent a relevant step forward in our understanding of the function of this tightly regulated biological channels and should pave the way to the design of efficacious nicotinic drugs for the treatment of neurological disorders, psychiatric diseases, substance abuse, food intake behavior, pain, inflammation, and cancer. Indeed, if the behavior of conventional orthosteric ligands reached a satisfactory level of knowledge, *e.g.*, the agonist-mediated transient ion channel activation, the complexity of conformations favoring non-conducting states, the silent desensitization and G protein-signaling pathways independent of channel opening, and the responses triggered by allosteric agonists and/or modulators deserve further in-depth studies, to understand if their still latent therapeutic potential may be favorably exploited. In view of putative therapeutic options, additional challenges for heteromeric nAChRs are to improve the characterization and expression levels in native tissues of different stoichiometries of the same subtypes, to better define the stoichiometry-dependent properties of the channels and to clarify to which extent disease-related mutations of a receptor subtype involve its stoichiometric composition.

CRediT authorship contribution statement

MDA conceptualized the review. **CM**, **CP**, **CD** and **MDA** equally contributed to writing, reviewing, and editing the manuscript. All authors approved the submitted final version.

Declaration of Competing Interest

The Authors declare that they have no competing interests.

Data Availability

No data were used for the research described in this article.

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