Pyrrolidinyl benzofurans and benzodioxanes: selective α4β2 nicotinic acetylcholine receptor ligands with different activity profiles at the two receptor stoichiometries

Rebecca Appiani,[a] Marco Pallavicini,[a] Ayman K. Hamouda,[b] and Cristiano Bolchi [a,*]

[a] Dipartimento di Scienze Farmaceutiche, Università degli Studi di Milano, via Mangiagalli 25, I-20133 Milano, Italy

[b] Department of Pharmaceutical Sciences and Health Outcomes, Fisch College of Pharmacy, The University of Texas at Tyler, Tyler, TX, USA

rebecca.appiani@unimi.it; marco.pallavicini@unimi.it; Ahamouda@uttyler.edu; Corresponding author: cristiano.bolchi@unimi.it;

Keywords: nicotinic acetylcholine receptors; α4β2 nAChR; α3β4 nAChR; α4β2 nAChR isoforms; pyrrolidinyl benzofuran; pyrrolidinyl benzodioxane.

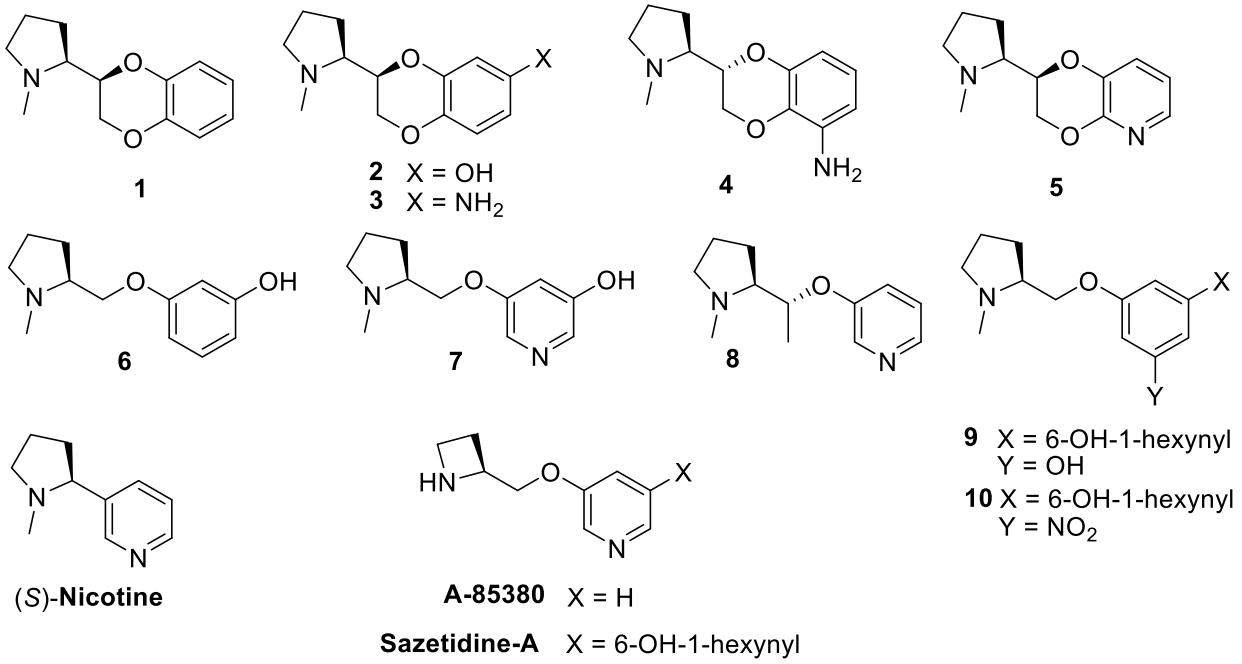
Abstract: A series of racemic benzofurans bearing *N*-methyl-2-pyrrolidinyl residue at C(2) or C(3) has been synthesized and tested for affinity at the α4β2 and α3β4 nicotine acetylcholine receptors (nAChRs). As previously reported for the benzodioxane based analogues, hydroxylation at proper position of benzene ring results in high α4β2 nAChR affinity and α4β2 vs. α3β4 nAChR selectivity. 7-Hydroxy-*N*-methyl-2-pyrrolidinyl-1,4-benzodioxane (**2**) and its 7- and 5-amino benzodioxane analogues **3** and **4**, which are all α4β2 nAChR partial agonists, and 2-(*N*-methyl-2-pyrrolidinyl)-6-hydroxybenzofuran (**12**) were selected for functional characterization at the two α4β2 stoichiometries, the high sensitivity $(\alpha4)_2(\beta2)_3$ and the low sensitivity $(\alpha4)_3(\beta2)_2$. The benzene pattern substitution, which had previously been found to control α4β2 partial agonist activity and α4β2 vs. α3β4 selectivity, proved to be also involved in stoichiometry-selectivity. The 7-hydroxybenzodioxane derivative **2** selectively activates $(\alpha4)_2(\beta2)_3$ nAChR, which cannot be activated by its 5-amino analogue **4**. A marginal structural modification, not altering the base pyrrolidinyl benzodioxane scaffold, resulted in opposite activity profiles at the two α4β2 nAChR isoforms providing an interesting novel case study.

Nicotinic acetylcholine receptors (nAChRs) are widely distributed in the central and peripheral nervous system. In the brain, the α4β2 subpopulation of nAChRs is associated with the majority of nicotine binding and its implication in drug and nicotine addiction and in a number of severe CNS disorders is documented by many pharmacological, clinical, and preclinical studies.[1] Partial α4β2 agonists are currently used for smoking cessation,[2] and recent *in vivo* pharmacological studies revealed their therapeutic potential in the treatment of depressive symptoms in refractory patients,[3] pain modulation,[4] and reduction of ethanol consumption.[5]

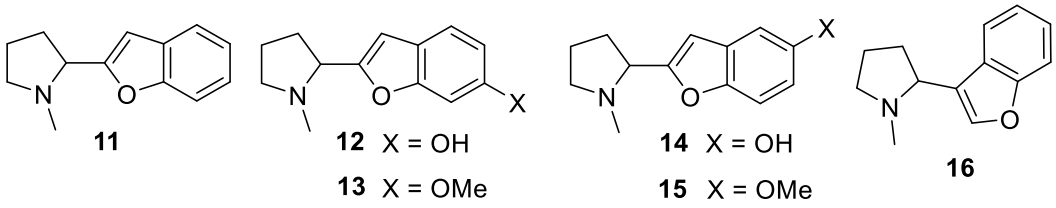
The development of better α4β2 therapeutic ligands became more feasible because of recent advances in the nAChR field including improved knowledge of the 3D structure of nicotinic receptor subtypes [6-11] and of ligand structural requirements for discrimination among α4β2, α3β4 and α7 nAChRs. [12-14] These structural data are especially critical to design α4β2 ligands with selectivity over

the ganglionic α3β4 subtype, because the α4β2 and α3β4 orthosteric binding sites mainly differ for the accommodation capacity of the β minus side.[12] More recently, selective activation of one of the two functional α4β2 isoforms in brain, the low sensitivity $(\alpha4)_3(\beta2)_2$ nAChR (LS) and the high sensitivity $(\alpha4)_2(\beta2)_3$ nAChR (HS),[15,16] and selective potentiation of their response by ligands acting at pseudo-agonist, unorthodox-agonist or allosteric sites [17-22] have been considered as new efficacious strategies to improve the druggability of the α4β2 receptor. The aim is to exploit, on one side, the difference in the effects mediated by the two isoforms and to reduce, on the other, undesired effects resulting from the sustained activation produced by the orthosteric agonists.

Our previous works have demonstrated that *N*-methyl-2-(2'-pyrrolidinyl)-1,4-benzodioxane,[23,24] whose 2*R*,2'*S* diastereomer (**1**) displays a moderate and selective α4β2 affinity and acts as an α4β2 antagonist,[25,26] can be transformed into partial or full agonist by the decoration of the benzodioxane with substituents at C(7) (**2** and **3**) and C(5) (**4**),[27,28] replacement of benzene with pyridine (**5**),[25] deconstructions of the dioxane ring and decoration of the aromatic ring to new phenyl and pyridyl ethers (**6**-**10**).[29,30]



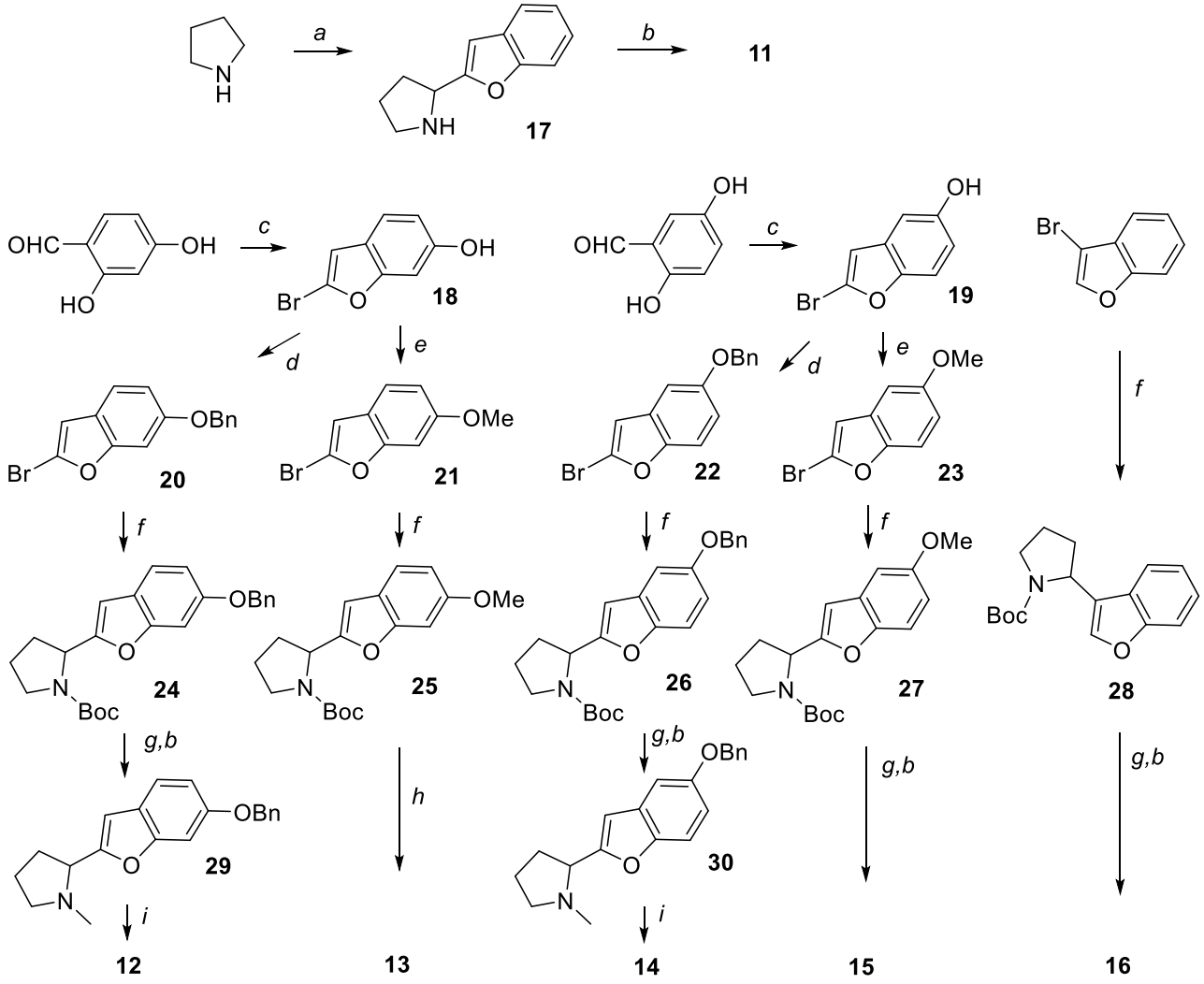
Here, such investigation approaches, based on the replacement of 3-pyridyl residue of 3-(1-methyl-2-pyrrolidinyl)pyridine, i.e. of (*S*)-**nicotine**, with a rigidified and/or decorated (hetero)aryloxymethyl moiety (compounds **1**-**10**), have been extended from pyrrolidinyl-benzodioxanes to a series of pyrrolidinyl-benzofurans (**11**-**16**) and from α4β2/α3β4 selectivity also to selectivity between the two HS and LS α4β2 stoichiometries. In fact, more recent studies suggest that the substitution pattern at the aromatic ring of these nicotine analogues is critical also for the latter type of selectivity, as well exemplified by the 6-hydroxy-1-hexynyl *meta*-substituted analogue of the potent α4β2 agonist 3-pyridyloxymethyl azetidine A-85380,namely **sazetidine-A**, whose high α4β2 vs. α3β4 nAChR and $(\alpha4)_2(\beta2)_3$ (HS) vs. $(\alpha4)_3(\beta2)_2$ (LS) nAChR selectivities are both attributable to the introduction of the hexynyl substituent at the pyridine ring.[16,31]



Previous research indicates that, as in nicotine, *S* configuration is required at the stereogenic pyrrolidine C(2) bearing the (hetero)aryloxymethyl residue, or its rigidified congener, for maximum α4β2 affinity and activity.[23-34] However, at this investigation stage, we decided to preliminarily test the racemates of compounds **11**-**16**, synthetically accessible *via* the addition of 2-benzofuranyllithium to the cyclic imine generated in situ from *N*-lithiated pyrrolidine (compound **11**) and *via* lithiation-transmetalation-Negishi coupling of *N*-Boc-pyrrolidyne with 3-bromobenzofuran (compound **16**) or with 5- or 6-methoxy or benzyloxy substituted 2-bromobenzofuran (compounds **12**-**15**).[35,36] We also planned the synthesis of the 3-benzofuranyl positional isomers of **12**-**15**, but attempts to couple *N*-Boc-pyrrolidyne with methoxy and benzyloxy substituted 3-bromobenzofuran were unsuccessful. The synthetic routes are shown in Scheme 1. Compound **11**, whose *S* enantiomer is reported in the literature but without synthesis and characterization details,[37] was obtained by *N*-methylation of 2-(benzofuran-2-yl)pyrrolidine, prepared from 2-benzofuranyllithium and the imine formed in situ from pyrrolidine according to the procedure by Paul and Seidel.[36] Compounds **12** and **13** were synthesized starting from 2,4-dihydroxybenzaldehyde and compounds **14** and **15** from 2,5-dihydroxybenzaldehyde. Reaction with CBr_4 and PPh_3 converted the aldehydes into *gem*-dibromoalkenes, highly susceptible of decomposition on isolation and therefore directly cyclized to the 2-bromobenzofurans **18** [38] and **19** by treatment with CuI and K_2CO_3 or Cs_2CO_3 and then *O*-alkylated with BnBr or MeI to give the intermediate 2-bromobenzofurans **20**-**23**. Coupling of **20**-**23** and of 3-bromobenzofuran with *N*-Boc-pyrrolidine *via* *s*-BuLi-mediated lithiation-trapping according to the Barker-O'Brien-Campos procedure [35] afforded the intermediate benzofuranyl pyrrolidines **24**-**28**. The *N*-Boc intermediate **25** was directly converted into **13** by treatment with $LiAlH_4$, while the other *N*-Boc intermediates **24**, **26**, **27**, and **28** were *N*-deprotected, *N*-methylated, and, in the case of **24** and **26**, *O*-debenzylated to give the compounds **12**, **14**, **15** and **16**.

We evaluated the binding affinity of **11**-**16** at the α4β2 and α3β4 nAChRs using the competitive binding of the high affinity agonist [^{3}H]-epibatidine to membranes isolated from HEK 293 cells stably expressing human α4β2 (HEK- α4β2) and rat α3β4 (HEK- α3β4) nAChRs, respectively. The HEK- α4β2 and HEK- α3β4 membranes were incubated with [^{3}H]-epibatidine in the presence and absence of increasing concentrations of compounds **11**-**16**, and their binding affinities (K_i) were determined from the resulting competition binding curves. The results are listed in Table 1 together with the previously reported affinities of the pyrrolidinyl benzodioxanes **1**-**4**, vice versa determined towards the α4β2 nAChR present on rat cerebral cortex membranes and towards the human α3β4 nAChR transiently transfected on HEK 243 cells.[23,27,28] We have recently evidenced that α4β2 affinities do not significantly different if determined at human or rat α4β2 nAChR, whereas those determined at the rat α3β4 subtype are markedly lower than those at the same human subtype.[12] That said, a comparison of racemic unsubstituted pyrrolidinyl benzofurans **11** and **16** with the eutomer of unsubstituted pyrrolidinyl benzodioxane **1** reveals that replacement of 1,4-benzodioxan-2-yl with 2- or 3-benzofuranyl does not modify the α4β2 affinity, thus suggesting similar interactions for the two oxygenated bicyclic scaffolds. In support of this, hydroxylation of benzene increases the α4β2

affinity if accomplished in the proper position, that is at C(7) of **1** (compound **2**; from 0.26 to 0.012 μM) and at C(6) of **11** (compound **12**; from 0.718 to 0.172 μM), but not at C(6) and C(5) of **1**, as previously reported,[28] and at C(5) of **11** (compound **14**). Unfortunately, we cannot extend such SAR analysis to the hydroxylated analogues of the 3-benzofuranyl derivative **16**, because they are unavailable. The critical role of the hydroxyl substituent in **12** is also highlighted by the drop of α4β2 affinity resulting from its methylation (compound **13**), analogous to that previously observed for the *O*-methylation of **2**.



Scheme 1. Reagents and conditions: (*a*) *n*-BuLi, ether, -78 °C, 10 min, followed by benzophenone, -78 °C, 10 min, 2-benzofuranyllithium in THF and $BF_3{\cdot}Et_2O$ -78 °C and 2 h, rt; (*b*) MeOH, CH_3COOH, 37% HCHO $_{aq}$, pic-BH_3, rt, 4 h, 62% (**11**), 60% (**29**), 41% (**30**), 37% (**15**), 26% (**16**); (*c*) ACN, CBr_4, PPh_3, Zn, rt, 1 h, followed by NH_4Cl, rt, 30 min; Cs_2CO_3 or K_2CO_3, CuI, reflux, 3 h, 43% (**18**), 36% (**19**); (*d*) dry THF, NaH, BnBr, rt, overnight, 84% (**20**), 73% (**22**); (*e*) dry THF, NaH, MeI, rt, overnight, 70% (**21**), 86% (**23**); (*f*) *N*-Boc pyrrolidine, dry THF, *s*-BuLi, $ZnCl_2$, -30 °C to rt, then $Pd(OAc)_2$, tBu_3PHBF_4, rt, 16 h, 16% (**24**), 10% (**25**), 20% (**26**), 17% (**27**), 16% (**28**); (*g*) 1.25 N HCl in MeOH, 50 °C, 3 h; (*h*) dry THF, $LiAlH_4$, rt, 3 h, 14% (**13**); (*i*) H_2, Pd/C 10%, MeOH, rt, 2 h, 78% (**12**), 66% (**14**).

As to α4β2 vs α3β4 nAChR selectivity, we can again observe analogies between 2-(*N*-methyl-2-pyrrolidinyl)benzofuran (**11**) and 2-(*N*-methyl-2-pyrrolidinyl)-1,4-benzodioxane (**1**): the

hydroxylation at C(6) and C(7), to give, respectively, **12** and **2**, increases not only the α4β2 affinity but also the α4β2 selectivity. The higher selectivity ratios of the benzofurans **11** and **16**, compared to benzodioxane **1**, and of benzofuran **12**, compared to the benzodioxane **2**, could be due also to the fact that the α3β4 affinities of **11**, **12**, and **16** were determined at the rat receptor subtype, which is, as previously underlined, more discriminating than the human receptor subtype used to determine the α3β4 affinities of **1** and **2**.

Table 1. Affinity of compounds **11**-**16** for heterologously expressed human α4β2-nAChR and rat α3β4-nAChR and of compounds **1**-**4** for native rat α4β2-nAChR and heterologously expressed human α3β4-nAChR, labelled by [^{3}H]epibatidine.

	α4β2-nAChR [^{3}H]Epi K_i (μM)	α3β4-nAChR [^{3}H]Epi K_i (μM)	α4β2/α3β4 selectivity		α4β2-nAChR [^{3}H]Epi K_i (μM)	α3β4-nAChR [^{3}H]Epi K_i (μM)	α4β2/α3β4 selectivity
11	0.718 (0.98)	19.43 (2.7)	27	**16**	0.828 (0.32)	81.55 (69.48)	98
12	0.172 (0.025)	45.31 (13.26)	260	**1**	0.26 (0.08)	1.2 (0.34)	4.6
13	>100	27.10 (11.18)		**2**	0.012 (0.002)	0.31 (0.1)	26
14	>100	>100		**3**	0.022 (0.008)	0.019 (0.006)	0.9
15	181	>100		**4**	0.131 (0.04)	13 (6.5)	100

The K_i values were derived from [^{3}H]epibatidine competition binding experiments using HEK α4β2 and HEK α3β4 membranes. Data from competition binding curves were evaluated by one-site competitive binding curve-fitting procedures using GraphPad Prism version 9. Each compound was tested in triplicated for each subtype and the inhibition constant K_i was estimated using K_d of the radioligand according to Cheng-Prusoff equation. The numbers in brackets represent the standard error. The affinities of compounds **1**-**4** are those previously reported in refs. 23, 27 and 28. For (*S*)-**11**, the literature (ref. 37) reports 0.158 μM K_i derived from [^{3}H]-(–)-cytisine competition binding experiments using a whole rat brain preparation.

The activity at the two α4β2 isoforms, the HS and the LS, were determined for the compounds displaying the highest α4β2 affinities, namely the 6-hydroxylated pyrrolidinyl benzofuran **12** and the 7-hydroxylated pyrrolidinyl benzodioxane **2**. In addition to the α4β2 partial agonist **2**, we thought to test two other benzodioxane based α4β2 partial agonists, namely the 7- and 5-amino analogues of **1** (compounds **3** and **4**). These three benzodioxane derivatives have three different activity profiles. They are all α4β2 partial agonists, but **2** with a moderate α4β2/α3β4 functional selectivity, **3** with no α4β2/α3β4 functional selectivity and **4** with no α3β4 functional activity. The functional effect was assessed using two-electrode voltage-clamp technique measuring the whole cell current elicited in *Xenopus* oocytes expressing wild-type human LS or HS nAChRs in response to application of these ligands alone or in combination with other nAChR ligands as described by Wang et al.[39] and by Deba et al.[40] To circumvent the low current amplitudes observed with these ligands at HS and/or LS receptors, 1 μM of the positive allosteric modulator LY2087101 was coapplied. LY2087101 is expected to enhance maximal responses with little effect on potencies at both stoichiometries based on its effect on the current elicited by ACh under similar experimental conditions.[40] The potencies and the efficacies of **2**, **3**, **4** and **12** at the two receptor isoforms are listed in Table 2. The 6-hydroxybenzofuran-based compound **12** shows similar potencies at the two isoforms, while the three benzodioxane-based compounds **2**, **3** and **4** exhibit three different potency profiles. The 2-

pyrrolidinyl-7-hydroxybenzodioxane **2** is 170 times more potent at the HS isoform, the 2-pyrrolidinyl-7-aminobenzodioxane **3** shows modestly different potencies, while the 2-pyrrolidinyl-5-aminobenzodioxane **4** has no activity at the HS isoform. All the four compounds are partial agonists with markedly higher efficacy at the HS isoform, except for **4**, which has null efficacy at the HS isoform.

Table 2. Potency and efficacy of compounds **2**, **3**, **4** and **12** at the two wild type human α4β2 nAChR isoforms expressed in *Xenopus* oocytes.

Compound	$(\alpha4)_2(\beta2)_3$		$(\alpha4)_3(\beta2)_2$	
	EC_{50} (μM)	E_{max} %	EC_{50} (μM)	E_{max} %
2	0.129 (0.22)	30 (3.7)	21.8 (3.29)	12.1 (1.9)
3	0.799 (0.07)	38 (2.9)	4.8 (4.6)	2.3 (0.2)
4	no currents		18.5 (1.92)	2.1 (0.3)
12	2.34 (0.67)	24 (4.3)	3.0 (0.46)	3.4 (1.5)

Current responses by *Xenopus* oocytes expressing WT $(\alpha4)_2(\beta2)_3$ and $(\alpha4)_3(\beta2)_2$ nAChRs to 5 s applications of increasing concentrations of **2**, **3**, **4** and **12** alone or with 1 μM LY2087101 were recorded and normalized to peak current amplitude elicited by 10 μM ACh alone. Efficacies are expressed as percentage ratio between the maximal effect of the compound, in the absence of LY2087101, and the maximal effect of ACh at the $(\alpha4)_2(\beta2)_3$ (10 μM) or $(\alpha4)_3(\beta2)_2$ (1 mM) isoform.

The activation profile of **2** resembles that of sazetidine-A, which selectively activates the HS over the LS receptor,[16] while compound **4** has the reverse activation profile, in that it cannot activate the HS receptor. Such results indicate that substituents at benzodioxane C(7) and C(5), which are determinant for the α4β2/α3β4 selectivity, as we have previously rationalized, are involved also in the stoichiometry selectivity. This is intriguing and not surprising. In fact, the substituents at the benzodioxane aromatic ring interact mostly with the complementary β2(−), and β4(−) faces controlling the α4β2/α3β4 selectivity and it is reasonable that these substituents also interact with the α4(−) face of the third "low" affinity ACh site identified at the α4/α4 interface, unique in the LS receptor, thus being critical also for the HS/LS selectivity. Docking into the α4/α4 binding site, site-directed mutagenesis at its minus side and combination with PAMs or unorthodox agonists will aid the understanding of subunit- and stoichiometry selectivity of these α4β2 partial agonists, which are an interesting novel case study, in that marginal structural modifications, not altering the base pyrrolidinyl benzodioxane scaffold, can sharply direct the preference for one of the two α4β2 isoforms. As recently reported, delineation of the contributions and of the roles of the two stoichiometries in the response resulting from α4β2 activation and selectivity for one over the other are central issues that must be addressed to facilitate the development of efficacious and safe nAChR-based therapeutics with more targeted indications.[5,20,22]

Declaration of Competing Interest

All authors have given approval to the final version of the manuscript.

The authors declare no competing financial interest.

Acknowledgements

Funding was provided by Università degli Studi di Milano and by University of Texas System Faculty Science and Technology Acquisition and Retention (STARs) Program.

References

1. Hurst R, Rollema H, Bertrand D. Nicotinic acetylcholine receptors: from basic science to therapeutics. *Pharmacol Ther* 2013;137;22-54.

2. Rollema H, Hurst R S. The contribution of agonist and antagonist activities of α4β2* nAChR ligands to smoking cessation efficacy: a quantitative analysis of literature data. *Psychopharmacol* 2018;235;2479-2505.

3. Yu L F, Zhang H K, Caldarone B J, Eaton J B, Lukas R J, Kozikowski A P. Recent developments in novel antidepressants targeting α4β2-nicotinic acetylcholine receptors. *J Med Chem.* 2014;57;8204-8223.

4. Toma W, Ulker E, Alqasem M, AlSharari S D, McIntosh J M, Damaj M I. Behavioral and molecular basis of cholinergic modulation of pain: focus on nicotinic acetylcholine receptors. *Curr Topics Behav Neurosci.* 2020; 45;153-166.

5. Wang J, Blasio A, Chapman H L, Doebelin C, Liaw V, Kuryatov A, Giovanetti S M, Lindstrom J, Lin L, Cameron M D, Kamenecka T M, Pomrenze M B, Messing R O. Promoting activity of $(\alpha4)_3(\beta2)_2$ nicotinic cholinergic receptors reduces ethanol consumption. *Neuropsychopharmacology.* 2020;45:301-308.

6. Walsh R M, Roh S H, Gharpure A, Morales-Perez C L, Teng J, Hibbs R E. Structural principles of distinct assemblies of the human α4β2 nicotinic receptor. *Nature*. 2018;557;261-265.

7. Gharpure A, Teng J, Zhuang Y, Noviello C M, Walsh R M Jr, Cabuco R, Howard R J, Zaveri N T, Lidahl E, Hibbs R E. Agonist selectivity and ion permeation in the α3β4 ganglionic nicotinic receptor. *Neuron.* 2019;104;501-511.

8. Saladino A C, Xu Y, Tang P. Homology modelling and molecular dynamics simulations of transmembrane domain structure of human neuronal nicotinic acetylcholine receptor. *Biophys*. 2005;88;1009-1017.

9. Pedretti A, Marconi C, Bolchi C, Fumagalli L, Ferrara R, Pallavicini M, Valoti E, Vistoli G. Modelling of full-length human α4β2 nicotinic receptor by fragmental approach and analysis of its binding modes. *Biochem Biophys Res Commun*. 2008;369;648-653.

10. Morales-Perez C L, Noviello C M, Hibbs R E. X-ray structure of the human α4β2 nicotinic receptor. *Nature*. 2016;538-411-415.

11. Harpsøe K, Hald H, Timmermann D B, Jensen M L, Dyhiring T, Nielsen E Ø, Peters D, Balle T, Gajhede M, Kastrup J S, Ahring P K. Molecular determinants of subtype-selective efficacies of cytisine and the novel compound NS3861 at heteromeric nicotinic acetylcholine receptors. *J Biol Chem*. 2013;288,2559-2570.

12. Bavo F, Pallavicini M, Appiani R, Bolchi C. Determinants for α4β2 vs α3β4 subtype selectivity of pyrrolidine-based nAChRs ligands: a computational perspective with focus on recent cryo-EM receptor structures. *Molecules*. 2021;26;3603.

13. Rego Campello H, Del Villar S G, Honraedt A, Minguez T, Oliveira A S F, Ranaghan K E, Shoemark D K, Bermudez I, Gotti C, Sessions R B, Mulholland A J, Wonnacott S, Gallagher T. Unlocking nicotinic selectivity via direct C-H functionalization of (–)-cytisine. *Chem*. 2018;4;1710-1725.

14. Bavo F, Pucci S, Fasoli F, Lammi C, Moretti M, Mucchietto V, Lattuada D, Viani P, De Palma C, Budriesi R, Corradini I, Dowell Cheryl, McIntosh J M, Clementi F, Bolchi C, Gotti C, Pallavicini M. Potent antiglioblastoma agents by hybridizing the onium-alkyloxy-stilbene based structures of an α7-nAChR, α9-nAChR antagonist and of a pro-oxidant mitocan. *J Med Chem*. 2018;61;10531-10544.

15. Da Siva Tavares X, Blum A P, Nakamura D T, Puskar N L, Shanata J A P, Lester H A, Dougherty D A. Variations in binding among several agonists at two stoichiometries of the neuronal, α4β2 nicotinic receptor, *J Am Chem Soc.* 2012;134;1474-11480.

16. Marotta C B, Rreza I, Lester HA, Dougherty D A. Selective ligand behaviors provide new insights into agonist activation of nicotinic acethylcholine receptors. *ACS Chem Biol.* 2014;9;1153-1159.

17. Grupe M, Grunnet M, Bastlund J F, Jensen A A. Targeting α4β2 nicotinic acetylcholine receptors in central nervous system disorders: perspectives on positive allosteric modulation as a therapeutic approach. *Basic Clin Pharmacol Toxicol.* 2015;116;187-200.

18. Maurer J J, Sandager-Nielsen K, Schmidt H D. Attenuation of nicotine taking and seeking in rats by the stoichiometry-selective alpha4beta2 nicotinic acetylcholine receptor positive allosteric modulator NS9283. *Psychopharmacology*. 2017;234;475-484.

19. Wang J, Lindstrom J. Orthosteric and allosteric potentiation of heteromeric neuronal nicotinic acetylcholine receptors. *Br J Pharmacol.* 2018; 175;1805-1821.

20. Mazzaferro S, Bermudez I, Sine S M. Potentiation of a neuronal nicotinic receptor via pseudo-agonist site. *Cell Mol Life Sci.* 2019;76;1151-1167.

21. Wilkerson J L, Deba F, Crowley M L, Hamouda A K, McMahon L R. Advances in the *in vitro* and *in vivo* pharmacology of alpha4beta2 nicotinic receptor positive allosteric modulators. *Neuropharmacol*. 2020;168;108008.

22. Hamouda A K, Bautista M R, Akinola L S, Alkhlaif Y, Jackson A, Carper M, Toma W B, Garai S, Chen Y C, Thakur G A, Fowler C D, Damaj M I. Potentiation of (α4)2(β2)3, but not (α4)3(β2)2

nicotinic acetylcholine receptors reduces nicotine self-administration and withdrawal symptoms. *Neuropharmacol*. 2021;190;108568.

23. Pallavicini M, Bolchi C, Binda M, Cilia A, Clementi F, Ferrara R, Fumagalli L, Gotti C, Moretti M, Pedretti A, Vistoli G, Valoti E. 5-(2-Pyrrolidinyl)oxazolidinones and 2-(2-pyrrolidinyl)benzodioxanes: Synthesis of all the stereoisomers and α4β2 nicotinic affinity. *Bioorg Med Chem Lett.* 2009;19;854-859.

24. Bolchi C, Bavo F, Appiani R, Roda G, Pallavicini M. 1,4-Benzodioxane, an evergreen, versatile scaffold in medicinal chemistry: A review of its recent applications in drug design. *Eur J Med Chem*. 2020;200;112419.

25. Bolchi C, Bavo F, Gotti C, Fumagalli L, Fasoli F, Binda M, Mucchietto M, Sciaccaluga M, Plutino S, Fucile S, Pallavicini M. From pyrrolidinyl-benzodioxane to pyrrolidinyl-pyridodioxanes, or from unselective antagonism to selective partial agonism at α4β2 nicotinic acetylcholine receptor. *Eur J Med Chem.* 2017;125;1132-1144.

26. Braida D, Ponzoni L, Moretti M, Viani P, Pallavicini M, Bolcgi C, Appiani R, Bavo F, Gotti C, Sala M. Behavioural and pharmacological profiles of zebrafish administrated pyrrolidinyl benzodioxanes and prolinol aryl ethers with high affinity for heteromeric nicotinic acetylcholine receptors. *Psychopharmacol.* 2020;237;2317-2326.

27. Bolchi C, Gotti C, Binda M, Fumagalli L, Pucci L, Pistillo F, Vistoli G, Valoti E, Pallavicini M. Unichiral 2-(2'-pyrrolidinyl)-1,4-benzodioxanes: the 2*R*,2'*S* diastereomer of the *N*-methyl-7-hydroxy analogue is a potent α4β2- and α6β2-nicotinic acetylcholine receptor partial agonist. *J Med Chem.* 2011;54;7588-7601.

28. Bavo F, Pallavicini M, Gotti C, Appiani R, Moretti M, Colombo S F, Pucci S, Viani P, Budriesi R, Renzi M, Fucile S, Bolchi C. Modifications at C(5) of 2-(2-pyrrolidinyl)-substituted 1,4-benzodioxane elicit potent α4β2 nicotinic acetylcholine receptor partial agonism with high selectivity over the α3β4 subtype. *J Med Chem.* 2020;63;15668-15692.

29. Bolchi C, Valoti E, Gotti C, Fasoli F, Ruggeri P, Fumagalli L, Binda M, Mucchietto V, Sciaccaluga M, Budriesi R, Fucile S, Pallavicini M. Chemistry and pharmacology of a series of unichiral analogues of 2-(2pPyrrolidinyl)-1,4-benzodioxane, prolinol phenyl ether, and prolinol 3-pyridyl ether designed as α4β2-nicotinic acetylcholine receptor agonists. *J Med Chem*. 2015;58;6665-6677.

30. Bolchi C, Bavo F, Fumagalli L, Gotti C, Fasoli F, Moretti M, Pallavicini M. Novel 5-substituted 3-hydroxyphenyl and 3-nitrophenyl ethers of S-prolinol as α4β2-nicotinic acetylcholine receptor ligands. *Bioorg Med Chem Lett.* 2016;26;5613-5617.

31. Wei Z L, Xiao Y, Yuan H, Baydyuk M, Pethukov P A, Musachio J L, Kellar K J, Kozikowski A P. Novel pyridyl ring C5 substituted analogues of epibatidine and 3-(1-methyl-2(S)-pyrrolidinylmethoxy)pyridine (A-84543) as highly selective agents for neuronal nicotinic acetylcholine receptors containing α2 subunits. *J Med Chem.* 2005;48;1721-1724.

32. Bunnelle W H, Dart M J, Schrimpf M R. Design of ligands for Nicotinic acetylcholine receptors: the quest for selectivity. *Curr Top Med Chem.* 2004;4;299-334.

33. Elliot R L, Ryther K B, Anderson D J, Piattoni-Kaplan M, Kuntzweiler T A, Donnelly-Roberts D, Arneric S P, Holladay M W. Novel 2-(2'furo[3,2-*b*]pyridinyl)pyrrolidines: potent neuronal nicotinic acethylcholine receptor ligands. *Bioorg Med Chem Lett. 1997*;7;2703-2708.

34. Dart M J, Wasicak J T, Ryther K B, Schrimpf M R, Kim K H, Anderson D J, Sullivan J P,Meyer M D. Structural aspects of high affinity ligands for the α4β2 neuronal nicotinic receptor. *Pharm Acta Helv.* 2000;74;115-123.

35. Barker G, O'Brien P, Campos K R. Diamine-free lithiation-trapping of *N*-Boc heterocycles using *s*-BuLi in THF. *Org Lett*. 2010;12;4176-4179.

36. Paul A, Seidel D. α-Functionalization of cyclic secondary amines: Lewis acid promoted addition of organometallics to transient imines. *J Am Chem Soc*. 2019; 141;8778-8782.

37. Dart M J, Wasicak J T, Ryther K B, Schrimpf M R, Kim K H, Anderson D J, Sullivan J P, Meyer M D. Structural aspects of high affinity ligands for the α4β2 neuronal nicotinic receptor. *Pharm Acta Helv*. 2000;74;115-123.

38. Sivaraman A, Harmalkar D S, Kang J, Choi Y, Lee K. A protecting group-free divergent synthesis of natural benzofurans *via* one-pot synthesis of 2-bromo-6-hydroxybenzofurans. *Org Biomol Chem* 2019;17;2153-2161.

39. Wang Z J, Deba F, Mohamed T S, Chiara D C, Ramos K, Hamouda A K. Unraveling amino acid residues critical for allosteric potentiation of (α4)3(β)2-type nicotinic acetylcholine receptor responses. *J Biol Chem* 2017;292;9988-10001.

40. Deba F, Ali H L, Tairu A, Ramos K, Ali J, Hamouda A K. LY2087101 and dFBr share transmembrane binding sites in the (α4)3(β2)2 nicotinic acetylcholine receptor. *Sci Rep*. 2018;8:1249.