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Identification of Novel α4β2-Nicotinic Acetylcholine Receptor (nAChR) Agonists Based on an Isoxazole Ether Scaffold that Demonstrate Antidepressant-like Activity

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ABSTRACT: There is considerable evidence to support the hypothesis that the blockade of nAChR is responsible for the antidepressant action of nicotinic ligands. The nicotinic acetylcholine receptor (nAChR) antagonist, mecamylamine, has been shown to be an effective add-on in patients that do not respond to selective serotonin reuptake inhibitors. This suggests that nAChR ligands may address an unmet clinical need by providing relief from depressive symptoms in refractory patients. In this study, a new series of nAChR ligands based on an isoxazole-ether scaffold have been designed and synthesized for binding and functional assays. Preliminary structure–activity relationship (SAR) efforts identified a lead compound 43, which possesses potent antidepressant-like activity (1 mg/kg, IP; 5 mg/kg, PO) in the classical mouse forced swim test. Early stage absorption, distribution, metabolism, excretion, and toxicity (ADME-Tox) studies also suggested favorable drug-like properties, and broad screening toward other common neurotransmitter receptors indicated that compound 43 is highly selective for nAChRs over the other 45 neurotransmitter receptors and transporters tested.

INTRODUCTION

Neuronal nicotinic acetylcholine receptors (nAChRs) are pentamers assembled from varying combinations of subunits (α2−α10, β2−β4) and belong to the ligand-gated ion channel superfamily of neurotransmitter receptors.1−3 These receptors are broadly distributed in the central and peripheral nervous systems, where they modulate many processes, such as ganglionic transmission regulated by α3/4*-nAChRs (the * indicates that subunits other than those specified are known or possible partners in the closed assembly), neuroprotection of dopaminergic pathways and nociception mediated by α4*-nAChRs, as well as learning, memory, and addiction by β2*-nAChRs.3−6 Over the past two decades, many compounds targeting nAChRs have been tested in various stages of clinical trials.7 However, only one new chemical entity, varenicline (1), has been launched and marketed as a potent partial agonist at the α4/2-nAChR for smoking cessation (Figure 1).8−10

Given nAChR subtype diversity and their involvement in the modulation of a host of neurotransmitter systems, nicotinic ligands have the potential to treat a multitude of central nervous system (CNS)-related dysfunctions, including chronic depression.8,11 There is considerable evidence to support the hypothesis that the blockade (antagonism or receptor desensitization) of nAChR is responsible for the antidepressant action of nicotinic ligands.12−14 In particular, clinical studies have shown that the cholinesterase inhibitor, physostigmine, produces depressive symptoms in humans,15 whereas mecamylamine16 and the muscarinic antagonist scopolamine17,18 relieve depressive symptoms in humans. Additionally, preclinical studies provide support for the hypothesis that increased cholinergic activity leads to depressed mood states. Flinders sensitive rats, a line selectively bred for increased cholinergic sensitivity, exhibit several depressive-like behaviors.19,20 Moreover, administration of the nicotinic antagonist mecamylamine elicits an antidepressant-like effect in the mouse forced swim test, and this effect is reduced when the β2 subunit gene is knocked out.11 The same effects were also observed in response to the tricyclic antidepressant amitriptyline, strongly suggesting that β2*-nAChRs are involved in the antidepressant efficacy of nicotinic ligands.21 The α4β2-nAChR is the predominant subtype in the vertebrate CNS, and the α4β2 nicotinic agonists...
Selected nicotinic acetylcholine receptor ligands.

Figure 1. Selected nicotinic acetylcholine receptor ligands.

cytisine (2),22 A-85380 (9),23 and compound 124 induce antidepressant-like effects in mice that are similar to the effects of the antagonist mecamylamine. The S-enantiomer of mecamylamine (TC-5214, 5) is an α4β2-nAChR modulator now in Phase II clinical trials for use in the treatment of depression.25 Therefore, the α4β2-nAChR is an attractive target for the development of novel antidepressants, although it is unclear whether nAChR activation, desensitization, or some combination of both is essential. It is also known from clinical studies that α3β4*-nAChRs contribute to adverse side effects in vivo, although roles in mood control also are possible, as exemplified by mecamylamine.26–28 Consequently, we chose to focus on developing potent agonists selective for the α4β2-nAChR, bearing in mind that activity at the α3β4*-nAChR subtype might be an attribute or a detriment.

Multiple modifications to the structure of natural nicotinic ligands, especially nicotine (3), epibatidine (4), and compound 2, have already been explored over the past 20 years. Most of the reported nicotinic ligands bear a substituted pyridine ring as the core scaffold. Some compound classes involving replacement of the pyridine ring by isosteres have been investigated, as exemplified by substituted phenyl derivatives,29 quinolines,30 furopyridines,31 structurally related chomran derivatives,32 and most interestingly the five-membered heteroaromatic rings isoxazole and isothiazole.33–35 The ability to replace the pyridine moiety of S-(-)-nicotine with an isoxazole ring was first investigated by Abbott Laboratories, leading to the clinical study of ABT-418 (6) for the treatment of both Alzheimer’s disease (AD) and attention deficit hyperactivity disorder (ADHD). Compound 6 is a selective, full agonist at the α4β2-nAChR with a Ki value of 7.4 nM.36,37 The complete subtype selectivity profile was not determined. In preclinical studies, compound 6 demonstrated efficacy and potency similar to that of compound 3 in animal models of cognition while exhibiting reduced toxicity.38 Although compound 6 failed in clinical development due to the occurrence of nausea as a side effect, isoxazole-containing nicotinic ligands remain an exciting area of investigation. Substituted isoxazoles were also successfully applied in optimization studies of compound 4. Replacement of the chloropyridyl group in compound 4 with a methylisoxazolyl group led to epiboxidine (7),39,40 which was approximately 10-fold less potent than compound 4 but 17-fold more potent than compound 6 in the displacement of [3H]nicotine at the α4β2-nAChR from rat cerebral cortical membranes. Epiboxidine retained analgesic activity in mice at 25 mg/kg compared to compound 4 at 10 mg/kg, with greatly reduced toxicity. Furthermore, a series of 3-(5-isoxazolyl)-methylene-1-azabicyclic compounds (8) were synthesized as potent nicotinic ligands.41

Pyridyl ethers, in which a CH3O linker is inserted between compound 3’s pyrrolidin (or in analogues, azetidine) ring and its pyridine ring, have attracted considerable interest as α4β2-nAChR agonists because of their high potency.42,43 For example, compound 9 possesses a Ki value of ca. 50 pM and a high efficacy of 163% compared to compound 3 at the human α4β2-nAChR.44 ABT-594 (10), a nAChR agonist, was advanced to a Phase II clinical trial for the treatment of chronic and neuropathic pain with a potency 50 times that of morphine but was discontinued due to an acceptably narrow therapeutic index.45,46 ABT-089 (11) is currently under investigation as a replacement for compound 6 that has an improved preclinical therapeutic index and a better pharmacokinetic profile. It has gone through Phase II clinical studies for cognitive dysfunction.47,48 Upon the basis of the precedent quoted above, we anticipated that an isoxazole moiety could be used as a readily accessible replacement for the pyridine core in the design of new nicotinic ligands. Herein, we report the synthesis and pharmacological evaluation of a novel series of nAChR ligands based on an isoxazole core in combination with the CH3O linker (“isoxazole ethers”; Figure 2). Selected compounds were further assessed in behavioral tests, in a broad screening panel of common CNS neurotransmitter receptors and transporters, as well as in preliminary in vitro ADME-Tox studies.

**RESULTS AND DISCUSSION**

**Chemistry.** First, we designed compounds that could be accessed from readily available starting materials to ascertain whether an isoxazole moiety could replace the pyridine core in the previously published pyridine ether nicotinics developed by Abbott. The 3-alkoxyisoxazoles 18–21 were synthesized in 3–6 steps utilizing the synthetic routes shown in Scheme 1. Intermediate 16 was formed via the Mitsunobu reaction of Boc-protected 2(S)-azetidinylmethanol (15) and 3-hydroxyisoxazole-5-carboxylic acid methyl ester (14), which was in turn prepared as described in the literature from dimethyl 2-butylnedioate (13). The ester 16 was subsequently reduced with LiBH4 to furnish the primary alcohol 17, and this intermediate was carried on to the iodoide. The phenyl ether 19 and the aliphatic ethers 20–21 were obtained through nucleophilic substitution.
substitution following standard methods. After acidic deprotection and subsequent purification by HPLC, compounds 18–21 were obtained as trifluoroacetates. The number of equivalents of trifluoroacetic acid (TFA) in these non-stoichiometric compounds was determined by elemental analysis. The 5-methylated 3-alkoxyisoxazole 24 was synthesized in the same manner from commercially available 3-hydroxy-5-methylisoxazole 22 (Scheme 1).

The preparation of 5-alkoxyisoxazole ligands proceeded through the common intermediates 29 and 30, which were in turn prepared from the commercially available ester 26 (Scheme 2). Compounds 31, 32, 33, and 43 were synthesized by employing the same strategy as described in Scheme 1. The primary alcohol 29 was transformed to an iodide, followed by nucleophilic substitution with aniline or 4-fluoroaniline to afford the precursors of amine derivatives 34 and 35. Carbamate analogues 34–38 were prepared by reaction of 29 with the corresponding isocyanates. The fluoromethyl derivative 39 was obtained by treatment of alcohol 29 with (diethylamino)sulfur trifluoride. Subsequent Boc deprotection of the precursors yielded the desired final compounds 34–39.

**In Vitro Characterization—Radioligand Binding Studies.** In vitro binding affinities of the five 3-alkoxyisoxazoles (18–21, 24) were determined by the standard [3H]epibatidine binding assay at seven rat nAChR subtypes (Table 1). While this initial set of compounds showed weak binding to all seven nAChR subtypes tested, compound 18 exhibited a moderate affinity for α4β2- and α4β2*-nAChRs.
It is commonly accepted that the essential pharmacophore of nicotinic ligands consists of a cationic center (e.g., quaternized or protonated nitrogen) and a hydrogen-bond acceptor (e.g., a nitrogen atom in the case of pyridine-containing ligands). The inactivity of our first batch of isoxazole-ether compounds is possibly a result of misalignment of these two key elements. Therefore, to align these pharmacophoric elements differently, and hopefully more appropriately, isoxazoles with a reverse position of their N and O ring atoms were synthesized. As the nitrogen atom in the case of pyridine-containing ligands). The inactivity of our first batch of isoxazole-ether compounds is possibly a result of misalignment of these two key elements.

Table 1. Binding Affinities of 3-Alkoxyisoxazole Ligands at Seven Rat nAChR Subtypes

<table>
<thead>
<tr>
<th>ID</th>
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<th>$K_i$ (nM) $^a$</th>
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<td>H</td>
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</table>

$^a$See Experimental Section. $^b$ $\alpha_4\beta_2\mu^b$, endogenous receptors prepared from rat forebrain. Besides $\alpha_4$ and $\beta_2$, other unidentified subunits may also be present. Details are provided in the Experimental Section. $^c$The $K_i$ values for compound 3 are taken from the PDSP Assay Protocol Book. $^d$Not active, defined as <50% binding in the primary assay at 10 $\mu$M.

Table 2. Binding Affinities of 5-Alkoxyisoxazole Ligands at Seven Rat nAChR Subtypes

<table>
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<th>$K_i$ (nM) $^a$</th>
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$^a$See Experimental Section. $^b$ $\alpha_4\beta_2\mu$ or $\alpha_7\mu$, endogenous receptors prepared from rat forebrain. Besides $\alpha_4$, $\beta_2$, or $\alpha_7$, other unidentified subunits may also be present. Details are provided in the Experimental Section. $^c$The $K_i$ values for compound 3 are taken from the PDSP Assay Protocol Book. $^d$ND: not detected. $^e$Not active, defined as <50% binding in the primary assay at 10 $\mu$M. $^f$The $K_i$ values for compound 1 are from ref 51.
In Vitro Functional Characterization. The most potent α4/β2-nAChR ligands based on binding assays, 39 and 43, as well as pyrrolidine analogue 44 were selected for evaluation of functional activity using the 86Rb+ ion flux assay in SH-EP1-h4β2, SH-SYSY (α3/β4+) and TE671/RD (α1/β1γδ) cells (Figure 3; Tables 3 and 4). Consistent with the binding data, the azetidines 39 and 43 were found to be more potent than the pyrrolidine 44, both in agonism and functional inactivation.

Table 3. Potencies and Efficacies of Ligand Agonism and Inactivation of Human α4/β2-nAChRs

<table>
<thead>
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<th>Compound</th>
<th>EC50 (nM)</th>
<th>Efficacy (%)</th>
<th>IC50 (nM)</th>
<th>Efficacy (%)</th>
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<td>169</td>
<td>78</td>
<td>4.6</td>
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<tr>
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<td>&gt;3000</td>
<td>&gt;50</td>
<td>1100</td>
<td>69</td>
<td>129</td>
</tr>
<tr>
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<td>290</td>
<td>88</td>
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<td>92</td>
<td>4.9</td>
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<td>53</td>
<td>~1100</td>
<td>~85</td>
<td>0.05</td>
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</table>

“See Experimental Section for details. The term “inactivation” is used because compounds may be acting to desensitize receptors or as competitive or non-competitive antagonists, and further work is needed to make such a distinction. SEM values were determined for each parameter and, although not presented here, typically are less than 3% and frequently less than 1% of the maximal carbamylcholine response for efficacy measures for ligands potent enough to reach maximal efficacy at 10 μM. SEM values for EC50 and IC50 values were no more than a factor of 2. See Table 2 for structures. For compounds that were not potent enough to cause maximal inhibition at the highest concentration tested, inactivation efficacy was fixed at 100% to allow IC50 values to be fit during graphical analysis.

at the α4/β2-nAChR (Figure 3, Table 3). Compounds 39 and 43 had agonist efficacies at the α4/β2-nAChR comparable to compound 3 and higher than that of compound 1. Compounds 39 and 43 have functional inactivation efficacies lower than those of compound 3 or compound 1. They were both full agonists at the α3/β4*-nAChR, with potencies similar to those seen at the α4/β2-nAChR, though they were less potent in the functional inactivation of the α3/β4*-nAChR (Table 4, Figure 3). Whereas compounds 39 and 43 have high selectivity for α4β2- over α3β4*-nAChRs (174- and 150-fold) in the binding affinity assays (Table 2), this selectivity does not translate to the functional assay (Tables 3 and 4, Figure 3). None of the compounds displayed significant activity at the α1β1γδ-nAChR. Compounds 31–38 were not potent in our preliminary functional screening.

In addition, compounds 39 and 43 are similar in potency at the α4/β2-nAChR (EC50 values of 1090 nM and 1180 nM, respectively) compared to compound 1 (EC50 value of 1400 nM). Both are inactive at the α1β1γδ-nAChR and have activity similar to compound 1 at the α3β4*-nAChR. Whereas in vitro binding and functional data are intriguing, a more proximal measure of therapeutic value is likely to be behavioral pharmacological activity in an animal model of the indication of interest. Therefore, we decided to further test these compounds for their antidepressant profile in vivo.

In Vivo Behavioral Studies—Mouse Forced Swim Test. Antidepressant efficacy was assessed with the mouse forced swim test, an assay in which a mouse is placed into a beaker of water, and the time it spends passively floating in the water (immobility) is recorded (Figure 4). Most traditional antidepressants decrease the amount of time the mouse spends immobile. Mice were administered compounds 43 (1 and 5 mg/kg ip) or the selective serotonin reuptake inhibitor (SSRI) antidepressant, sertraline, as a positive control (10 mg/kg). Drug administration produced a reduction in immobility. Fisher’s post hoc tests showed that compound 43 reduced immobility at both doses (1 and 5 mg/kg), suggestive of a
potent antidepressant-like effect. Moreover, 43 was also active at an orally administered dose of 5 mg/kg.

**Broad Screening at Other Neurotransmitter Receptors and Transporters.** A broad-range screening study was carried out for compound 43 to further determine its effects at 10 μM on about 45 other CNS neurotransmitter receptors and transporters, including serotonin receptors, dopamine receptors, GABA receptors, biogenic amine transporters, adrenergic receptors, muscarinic receptors, opioid receptors, sigma receptors, and histamine receptors (NIMH-PDSP, University of North Carolina, Chapel Hill). No inhibition caused by 10 μM on about 45 other CNS neurotransmitter receptors and transporters was reduced to less than 70% of the control, suggesting that 43 will not alter the metabolism of other xenobiotics or endogenous compounds that are substrates for the CYP isoforms tested.

**CYP Inhibition.** The inhibitory effect of 43 on in vitro CYP activity in human liver microsomes was screened using a high-throughput multiple CYP assay for CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, and CYP3A4. In the presence of 1 μM and 10 μM of 43, none of these CYP isoforms’ activity was reduced to less than 70% of the control, suggesting that 43 will not alter the metabolism of other xenobiotics or endogenous compounds that are substrates for the CYP isoforms tested.

**Metabolic Stability.** The metabolic stability of 43 was studied using human and mouse liver microsomes. The test article was incubated at two concentrations (1 μM and 10 μM), and aliquots (100 μL) were removed at various time points (0, 15, 30, and 60 min) for analysis by LC-MS/MS. This study showed that liver microsomes from both species caused a time-dependent decrease in parent drug at both concentrations. Human liver microsomes metabolized 43 to a greater extent in 60 min than mouse liver microsomes; 53.4% and 67.2% of the parent drug remained unchanged at 1 μM and 10 μM, respectively. Incubation with mouse liver microsomes resulted in 67.5% and 73.7% of 43 remaining unchanged after 60 min incubation at 1 μM and 10 μM, respectively.

**PPB (Plasma Protein Binding).** The binding of 43 to proteins in human and mouse plasma was determined using equilibrium dialysis. Binding of 43 was evaluated at concentrations of 0, 0.1, 1, and 10 μM. The mean percentage of binding of this compound to human plasma ranged from 8.2% to 17.7%. Its mean percentage of binding to mouse plasma ranged from 12.9% to 19.3%.

**hERG Inhibition.** HEK293 cells stably expressing recombinant human hERG were used in a fluorescence-based membrane potential assay. The observed hERG inhibition of 43 at 10 μM was similar to that of the negative control, giving 0% hERG blockade.

**CONCLUSIONS**

In the present study, a new series of isoxazole ether nAChR ligands have been identified, and their preliminary SAR has been explored. In the PDSP binding study, most of the 5-alkoxyisoxazole ligands were found to bind to the rat α4/β2-nAChR with a significantly higher affinity than to the α3/β4*-nAChR. Compound 43 was identified as the lead compound from this series as it displayed favorable in vitro nAChR binding affinities. The functional potency of isoxazole 43 at the α4/β2-nAChR is similar to that of compound 1, but it has higher efficacy, helping to distinguish it from compound 1. When tested in vivo, compound 43 demonstrated a potent antidepressant-like activity at both 5 mg/kg and 1 mg/kg ip, and was orally active at 5 mg/kg in the mouse forced swim test. Preliminary in vitro ADME-Tox characterization further suggested promising drug-like properties of 43. The PDSP broad range screening indicated that compound 43 was highly selective for nAChRs and did not have significant binding affinity to the other 45 neurotransmitter receptors and transporters tested.

It remains unclear whether activity at the α3/β4*-nAChR subtype is an attribute or a detriment for treatment of depression. Further studies are required to fully understand the importance of α3/β4*-nAChR involvement in depression. Our previously reported nicotinic ligands such as sazetidine-A and 3-alkoxy-S-aminoypyridine derivatives, that contain appropriate substituents in the S-position of their pyridine rings, exhibited improved selectivity for β2- over β4-containing nAChRs.6,52–55 Considering that the present isoxazole ligands are easily accessible through substitution reactions performed upon a preassembled isoxazole building block, the S-alkoxyisoxazole scaffold should be a useful starting point for a broader optimization campaign to discover more α4/β2-selective nAChR ligands, perhaps importantly with higher efficacy than...
compound I, that may in due course lead to a novel treatment option for depression.

## EXPERIMENTAL SECTION

### General Methods.

Starting materials, reagents, and solvents were purchased from commercial suppliers and used without further purification, unless otherwise stated. Anhydrous THF and CH₂Cl₂ were obtained by distillation over sodium wire or CaH₂, respectively. All nonaqueous reactions were run under an argon atmosphere with exclusion of moisture from reagents, and all reaction vessels were oven-dried. The progress of reactions was monitored by TLC on SiO₂. Spots were visualized by their quenching of the fluorescence of an indicator admixed to the SiO₂ layer, or by dipping into KMnO₄ solution followed by heating. SiO₂ for column chromatography (CC) was of 230–400 mesh particle size, and an EtOAc/hexane mixture or gradient was used unless stated otherwise. ¹H NMR spectra were recorded at a spectrometer frequency of 300 or 400 MHz. ¹³C NMR spectra were at 75 or 100 MHz. Chemical shifts are reported in δ (ppm) using the δ 7.26 signal of CDCl₃, the δ 4.80 signal of D₂O, or the δ 3.31 signal of CD₃OD, or the δ 2.50 signal of DMSO-d₆ as internal standards. ¹H Chemical shifts are reported in δ (ppm) using the δ 77.23 signal of CDCl₃, the δ 49.15 signal of CD₃OD, or the δ 39.51 signal of DMSO-d₆ as internal standards. ¹³C NMR spectra in D₂O were not adjusted. Purities of final compounds (>98%) were established by analytical HPLC, which was carried out on an Agilent 1100 HPLC system with a Synergy 4 µ Hydro-RP 80Å column, with detection at 220 or 254 nm on a variable wavelength detector. The column was a Sunfire 4 µ, 150 × 25 mm, with purity >95% and retention time 6.3 min. Hydrogenation reactions were carried out in a Parr 4720 autoclave (10 mL) under 1 atm H₂ pressure at room temperature (80 °C) for 100% yield employing Method A and Gradient I. 

### General Procedure for the Deprotection of N-Boc-Amines to Afford TFA Salts (Method A).

To a solution of an (iodomethyl)isoxazole (1 mmol) in anhydrous THF (20 mL) was added LiBH₄ (4 mmol) with ice cooling under Ar. After stirring overnight at rt, the solvent was evaporated. The residue was purified by CC on SiO₂ to give the product.

### General Procedure for the Preparation of Amines from Iodides from Alcohols (Method B).

To a solution of an (iodomethyl)isoxazole (1 mmol) in anhydrous DMF (2 mL) was added I₂ (1.5 mmol) with ice cooling under Ar. After stirring overnight at rt, saturated aqueous NH₄Cl solution was added. The mixture was extracted with EtOAc (2 × 15 mL), and the combined organic phases were washed with water (3 × 10 mL), dried over Na₂SO₄, and evaporated. The residue was purified by CC on SiO₂ to give the product.

### General Procedure for the Preparation of Carbamates from Alcohols (Method F).

To a solution of an (iodomethyl)isoxazole (1 mmol) in anhydrous DMF (2 mL) was added NaH (60% dispersion in oil, 1.9 mmol) with ice cooling under Ar. After stirring for 30 min at rt, alkyl halide (1 mmol) was added. Stirring continued for 2 h at rt, then the reaction was quenched with saturated aqueous NH₄Cl solution with ice cooling. The mixture was extracted with EtOAc (2 × 15 mL), and the combined organic phases were washed with water (3 × 10 mL), dried over Na₂SO₄, and evaporated. The residue was purified by CC on SiO₂ to give the product.

### General Procedure for the Mitsunobu Reaction of 1-(tert-Butyloxycarbonyl)-2-(5)-azetidinyl)methanol-5-isoxazolylmethyl (17). This compound was obtained from 1-(tert-butyloxycarbonyl)-2-(5)-azetidinyl)methanol-3-hydroxyisoxazole-5-carboxylic acid methyl ester in two steps as a colorless oil in 84% yield employing Method B and Method C. "H NMR (400 MHz, CDCl₃) δ 6.57 (s, 1H), 4.58–4.54 (m, 1H), 4.48 (m, 1H), 4.35 (d, 2H, J = 10.8 Hz), 3.92 (s, 1H), 3.84 (t, 2H, J = 7.6 Hz), 2.33–2.28 (m, 2H), 2.22–2.18 (m, 1H), 1.39 (s, 9H).

### General Procedure for the Preparation of Iodides from Alcohols (Method G).

To a solution of an (iodomethyl)-azetidinyl)methoxy]-5-isoxazolylmethanol-11, that may in due course lead to a novel treatment option for depression.

### General Procedure for the Preparation of Amides from Iodides (Method H). A solution of 29 (1 mmol), isocyanate (2 mmol), and 4-(dimethylamino)pyridine (0.1 mmol) in anhydrous tolune (5 mL) was stirred at 80 °C under Ar for 5 h. The solvent was removed under reduced pressure, and the residue was purified by CC on SiO₂ (acetone/hexane) to obtain the product.

### General Procedure for the Preparation of Carbamates from Alcohols (Method I).

To a solution of an (iodomethyl)isoxazole (1 mmol) in anhydrous CH₂CN (10 mL) were added at rt K₂CO₃ (6 mmol) and amine (4 mmol). After stirring overnight, the reaction mixture was concentrated. The residue was purified by CC on SiO₂ (CH₂Cl₂/Methanol) to obtain the product.

### General Procedure for the Mitsunobu Reaction of 1-(tert-Butyloxycarbonyl)-2-(5)-azetidinyl)methanol-15 or 1-(tert-Butyloxycarbonyl)-2-(5)-pyrrolidinylmethanol (25) with Hydroxysio-azoles to Afford Alkoxyisoxazoles (Method B). To a stirred solution of an (iodomethyl)isoxazole (1 mmol), alcohol 15 or 25 (1.2 mmol), and PPh₃ (1.5 mmol) in anhydrous THF (20 mL) was added diethyl azodicarboxylate or diisopropyl azodicarboxylate (1.5 mmol) dropwise. After stirring overnight at rt, the solvent was evaporated, and the residue was dissolved in EtOAc. The solution was washed with water (20 mL) and brine (15 mL), dried over Na₂SO₄, and concentrated under a vacuum. The residue was purified by CC on SiO₂ to give the alkoxysiozole.

### General Procedure for the Reduction of Isoxazolacarboxylic Acid Esters to Alcohols (Method C).

To a stirred solution of an isoxazolacarboxylic acid ester (1 mmol) in anhydrous THF (20 mL) was added LiBH₄ (4 mmol) with ice cooling under Ar. After stirring overnight at rt, saturated aqueous NH₄Cl solution was added with ice cooling. Extraction with EtOAc, drying over Na₂SO₄, and CC on SiO₂ gave the alcohol.

### General Procedure for the Preparation of Iodides from Alcohols (Method D).

To a stirred solution of an isoxazolylmethanol (1 mmol), imidazole (1.5 mmol), and PPh₃ (1.5 mmol) in anhydrous PhMe (8 mL) was added I₂ (1.5 mmol) with ice cooling under Ar. After stirring overnight at rt, the solvent was evaporated. The residue was purified by CC on SiO₂ to give the iodide.

### General Procedure for the Preparation of Phenyl Ethers from Iodides (Method E).

To a stirred solution of an (iodomethyl)-isoxazole (1 mmol) and phenol (2 mmol) in anhydrous DMF (4 mL) was added K₂CO₃ (6 mmol) under Ar. After stirring overnight at rt, saturated aqueous NH₄Cl solution was added. The mixture was extracted with EtOAc (2 × 15 mL), and the combined organic phases were washed with water (3 × 10 mL), dried over Na₂SO₄, and evaporated. The residue was purified by CC on SiO₂ to give the product.

### General Procedure for the Preparation of Alkyl Ethers from Alcohols (Method F).

To a stirred solution of an isoxazolylmethanol (1 mmol) in anhydrous DMF (2 mL) was added NaH (60% dispersion in oil, 1.9 mmol) with ice cooling under Ar. After stirring for 30 min at rt, alkyl halide (1 mmol) was added. Stirring continued for 2 h at rt, then the reaction was quenched with saturated aqueous NH₄Cl solution with ice cooling. The mixture was extracted with EtOAc (2 × 15 mL), and the combined organic phases were washed with water (3 × 10 mL), dried over Na₂SO₄, and evaporated. The residue was purified by CC on SiO₂ to give the product.

### General Procedure for the Preparation of Amines from Iodides (Method G).

To a solution of an (iodomethyl)-isoxazole (1 mmol) in anhydrous CH₂CN (10 mL) were added at rt K₂CO₃ (6 mmol) and amine (4 mmol). After stirring overnight, the reaction mixture was concentrated. The residue was purified by CC on SiO₂ (CH₂Cl₂/Methanol) to obtain the product.
in 65% yield employing Method B. 1H NMR (400 MHz, CDCl₃) of 5-hydroxyisoxazole-3-carboxylic acid ethyl ester as a pale-yellow solid.

C₁₄H₁₆FN₃O₂·0.5H₂O (FW 385): C, 50.12; H, 4.64; N, 9.06; F, 21.01. Found: C, 49.88; H, 4.43; N, 9.11; F, 21.41.

3-(2-(2-(tert-Butyloxycarbonyl)-2-(4-fluoroanilino)methyl)isoxazol-3-yl)carboxylic acid Ethyl Ester (27). This compound was obtained from 1-(tert-butyloxycarbonyl)-2-(2-fluoroanilino)methyl)isoxazole-3-carboxylic acid Ethyl Ester.

C₁₄H₁₄FN₄O₂·0.5H₂O (FW 335): C, 50.65; H, 4.31; N, 11.31; F, 21.58. Anal. Calcd for C₁₄H₁₄FN₄O₂·0.5H₂O (FW 335): C, 50.69; H, 4.36; N, 11.35; F, 21.61. Found: C, 50.69; H, 4.34; N, 11.33; F, 21.60.

3-(2-(2-(tert-Butyloxycarbonyl)-2-(4-fluoroanilino)methyl)isoxazol-3-yl)carboxylic acid Ethyl Ester (28). This compound was obtained from 1-(tert-butyloxycarbonyl)-2-(2-fluoroanilino)methyl)isoxazole-3-carboxylic acid Ethyl Ester.

C₁₄H₁₄FN₄O₂·0.5H₂O (FW 335): C, 49.68; H, 4.18; N, 9.06; F, 21.01. Found: C, 49.88; H, 4.43; N, 9.11; F, 21.41.

5-(1-(tert-Butyloxycarbonyl)-1-(tert-butyloxycarbonyl)-3-fluoroisoxazol-4-yl)-2-(1-tert-butyloxycarbonyl)-3-isoxazolylmethyl)isoxazole (50). This compound was obtained from 3-(2-(2-(tert-butyloxycarbonyl)-2-(2-fluoroanilino)methyl)isoxazol-3-yl)carboxylic acid ethyl ester as a pale-yellow solid in 94% yield employing Method C.

C₁₄H₁₄FN₄O₂·0.5H₂O (FW 335): C, 50.13; H, 4.97; N, 9.46; F, 21.28. Found: C, 50.18; H, 4.97; N, 9.49; F, 21.14.

5-(5-(tert-Butyloxycarbonyl)-2-fluorophenyl)isoxazolylmethyl)isoxazole (30). This compound was obtained from 3-(2-(2-(tert-butyloxycarbonyl)-2-(2-fluoroanilino)methyl)isoxazol-3-yl)carboxylic acid ethyl ester as a pale-yellow solid.

C₁₄H₁₄FN₄O₂·0.5H₂O (FW 335): C, 50.65; H, 4.31; N, 11.31; F, 21.58. Anal. Calcd for C₁₄H₁₄FN₄O₂·0.5H₂O (FW 335): C, 50.69; H, 4.36; N, 11.35; F, 21.61. Found: C, 50.69; H, 4.34; N, 11.33; F, 21.60.

5-(2-(2-(tert-Butyloxycarbonyl)-3-isoxazolylmethyl)isoxazole (31). 5-(1-(tert-Butyloxycarbonyl)-2-(1-tert-butyloxycarbonyl)-3-isoxazolylmethyl)isoxazole (29) was obtained from 27 as a pale-yellow solid in quantitative yield employing Method C. The title compound was obtained from 29 employing Method A and Method B. Colorless oil; yield 77%; purity 99.6%.

C₁₄H₁₄FN₄O₂·0.5H₂O (FW 335): C, 50.12; H, 4.64; N, 9.06; F, 21.01. Found: C, 50.18; H, 4.67; N, 9.09; F, 21.01.
null
plates and the second efflux/drug plates defined the amount of intracellular $^{86}$Rb$^+$ available at the start of the second, 5 min assay and were used to normalize nAChR function assessed.

For each experiment, in one set of control samples, total $^{86}$Rb$^+$ efflux was assessed in the presence of a fully efficacious concentration of carbamylcholine alone (1 mM for SH-EP-I-ho4662 and TE671/RD cells, or 3 mM for SH-SY5Y cells). Nonspecific $^{86}$Rb$^+$ efflux in another set of control samples was measured either in the presence of the fully efficacious concentration of carbamylcholine plus 100 μM mecamylamine, which gave full block of agonist-induced and spontaneous nAChR-mediated ion flux, or in the presence of efflux buffer alone. Both determinations of nonspecific efflux were equivalent. Specific efflux was then taken as the difference in control samples between total and nonspecific $^{86}$Rb$^+$-efflux. The same approaches were used to define total, nonspecific, and specific ion flux responses in samples subjected to the second, 5 min, exposure to test drug with or without carbamylcholine at its $\sim EC_{50}$ concentration.

Intrinsic agonist activity of test drugs was ascertained during the first 9.5 min of the initial 10 min exposure period using samples containing test drug only at different concentrations and was normalized, after subtraction of nonspecific efflux, to specific efflux in carbamylcholine control samples. Specific $^{86}$Rb$^+$ efflux elicited by test drug as a percentage of specific efflux in carbamylcholine controls was the same in these samples whether measured in absolute terms or as a percentage of loaded $^{86}$Rb$^+$. Even in samples previously giving an efflux response during the initial 10 min exposure to a partial or full agonist, residual intracellular $^{86}$Rb$^+$ was adequate to allow assessment of nAChR function in the secondary, 5 min assay. However, care was needed to ensure that data were normalized to the amount of intracellular $^{86}$Rb$^+$ available at the time of the assay, as absolute levels of total, nonspecific, or specific efflux varied in cells partially depleted of intracellular $^{86}$Rb$^+$ due to action of any agonist present during the 10 min drug exposure period. That is, calculations of specific efflux as a percentage of loaded $^{86}$Rb$^+$ typically were corrected for any variation in the electrochemical gradient of $^{86}$Rb$^+$ created by intracellular ion depletion after the first (agonism/pretreatment) drug treatment. Ion flux assays $(n \geq 3$ separate studies for each drug and cell line combination) were fit to the Hill equation, $F = F_{max}/(1 + (X/EC_{50})^n)$, where $F$ is the percentage of control, $F_{max}$ for $EC_{50}$ $(n > 0$ for agonists) or IC$\_50$ $(n < 0$ for antagonists) values using Prism 4 (GraphPad, San Diego, USA). Most ion flux data were fit allowing maximum and minimum ion flux values to be determined by curve fitting but in some cases, where agonists or antagonists had weak functional potency, minimum ion flux was set at 0% of control or maximum ion flux was set at 100% of control, respectively.

**GENERAL PROCEDURES FOR BEHAVIORAL STUDIES**

**Animals.** BALB/cj male mice (8–10 weeks old at testing) were obtained from Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed four to a cage in a colony room maintained at 22 ± 2 °C on a 12 h light–dark cycle. All animal experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the PsychoGenics Animal Care and Use Committee.

**Drugs.** Compounds 43 were synthesized as described above, and sertraline was purchased from Toronto Research Chemicals (Ontario, Canada). All compounds were dissolved in injectable water and administered by intraperitoneal (IP) injection or oral gavage (PO) in a volume of 10 mL/kg.

**Mouse Forced Swim Test.** Procedures were based on those previously described. Mice were individually placed into clear glass cylinders (15 cm tall ×10 cm wide, 1 L beakers) containing 23 ± 1 °C water 12 cm deep (approximately 800 mL). Mice were administered vehicle, the SSRI sertraline (10 or 20 mg/kg) as a positive control, or compound 43 (1 or 5 mg/kg). Thirty minutes following IP or PO administration, mice were placed in the water, and the time the animal spent immobile was recorded over a 6 min trial. Immobility was defined as the postural position of floating in the water.

**Statistical Analysis.** Data were analyzed with analysis of variance (ANOVA) with treatment group (vehicle, sertraline, or compound 43 (1 and 5 mg/kg)) as the between group variable and total time immobile (seconds over the 6 min trial) as the dependent variable. Significant main effects were followed up with the post hoc Fisher’s test.

**ASSOCIATED CONTENT**

**Supporting Information**

Broad screening data and detailed preliminary in vitro ADME-Tox profile. This material is available free of charge via the Internet at http://pubs.acs.org.

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**ABBREVIATIONS**

CNS, central nervous system; AD, Alzheimer’s disease; ADHD, attention deficit hyperactivity disorder; NIMH-PDSP, National Institute of Mental Health Psychoactive Drug Screening Program; nAChR(s), nicotinic acetylcholine receptor(s); SAR, structure–activity relationship; ADME-Tox, absorption, distribution, metabolism, excretion, and toxicity; SSRI, selective serotonin reuptake inhibitor; CYP, cytochrome P450; PBP, plasma protein binding; hERG, human ether-a-go-go-related gene; CC, column chromatography; rt, room temperature; TFA, trifluoroacetic acid

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