

## Article

# A Pharmacological Comparison of Two Isomeric Nicotinic Receptor Agonists: The Marine Toxin Isoanatabine and the Tobacco Alkaloid Anatabine

Hong Xing, Sunil Keshwah, Anne Rouchaud and William R. Kem \*

Department of Pharmacology and Therapeutics, College of Medicine, University of Florida, Gainesville, FL 32610, USA; hong.xing@ufl.edu (H.X.); Sunil.Keshwah@uchospitals.edu (S.K.); arouchau@hotmail.com (A.R.)

\* Correspondence: wrkem@ufl.edu; Tel.: +1-352-392-0669

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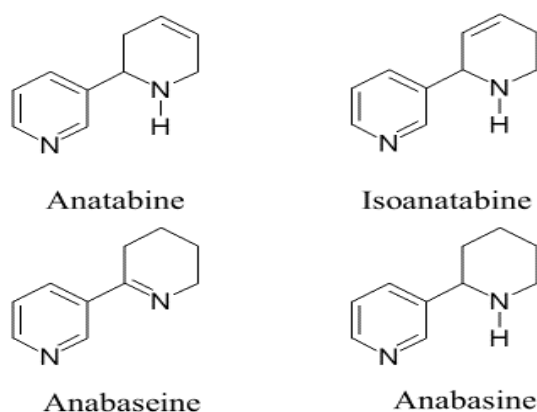
**Abstract:** Many organisms possess “secondary” compounds to avoid consumption or to immobilize prey. While the most abundant or active compounds are initially investigated, more extensive analyses reveal other “minor” compounds with distinctive properties that may also be of biomedical and pharmaceutical significance. Here, we present an initial in vitro investigation of the actions of two isomeric tetrahydropyridyl ring-containing anabasine analogs: isoanatabine, an alkaloid isolated from a marine worm, and anatabine, a relatively abundant minor alkaloid in commercial tobacco plants. Both compounds have a double bond that is distal to the piperidine ring nitrogen of anabasine. Racemic isoanatabine and anatabine were synthesized and their S- and R-enantiomers were isolated by chiral high pressure liquid chromatography (HPLC). Both isoanatabines displayed higher efficacies at  $\alpha 4\beta 2$  nicotinic acetylcholine receptors (nAChRs) relative to the anatabines; R-isoanatabine was most potent. Radioligand binding experiments revealed similar  $\alpha 4\beta 2$  nAChR binding affinities for the isoanatabines, but R-anatabine affinity was twice that of S-anatabine. While the two anatabines and S-isoanatabine were highly efficacious agonists at  $\alpha 7$  nAChRs, R-isoanatabine was only a weak partial agonist. The four compounds share an ability to stimulate both  $\alpha 4\beta 2$  and  $\alpha 7$  nAChRs, a property that may be useful in developing more efficacious drugs to treat neurodegenerative and other medical disorders.

**Keywords:** acetylcholine; alkaloid; Alzheimer’s disease; anabaseine; anabasine; anatabine; inflammation; isoanatabine; nemertine; nicotine; nicotinic acetylcholine receptor; tobacco; toxin

## 1. Introduction

Due to its many effects on the human organism, (S)-nicotine, the most abundant pharmacologically active constituent of the commercial tobacco plant *Nicotiana tabacum*, is one of the most thoroughly investigated natural products. It is also one of the most addictive of drugs and, when administered by smoking, is the major cause of preventable human death. In recent decades, much has been learned about the myriad central nervous system nicotinic acetylcholine receptors (nAChRs) that are activated by nicotine and similar “nicotinoid” compounds [1–5]. It has been demonstrated that nicotine and some other nAChR agonists have pro-cognitive, anxiolytic, analgesic, and anti-inflammatory properties that might be useful in the treatment of certain neurodegenerative, neurodevelopmental, inflammatory, and drug addiction disorders [4–6]. Several drug candidates targeting nAChRs have undergone clinical tests for some of these potential therapeutic indications, but only one, varenicline, has reached the clinic [7–10].

The mammalian organism contains more than fifteen different nAChRs, which are expressed in neurons, skeletal muscles, glia, macrophages, keratinocytes, lung epithelia, and immune cells [1]. Each nAChR is composed of five homologous, protein subunits, which form a barrel-like membrane protein complex. When this membrane protein is naturally activated by the neurotransmitter ACh, endogenous choline, or nicotine, its ion channel opens, causing a membrane potential change that may be sufficient to transiently generate a nerve or muscle impulse in an electrically excitable cell or elevate its intracellular calcium concentration. The nAChRs are conveniently classified according to their subunit compositions. Some are homo-oligomeric, containing five copies of a single subunit. Most receptors containing the  $\alpha 7$  subunit are homo-oligomeric and their ion channel displays selectivity for calcium ions, which activate intracellular signaling cascades [11]. Hetero-oligomeric nAChRs are more common and include several brain, autonomic nervous system, and neuromuscular nAChRs. The  $\alpha 4\beta 2$  receptors are the major heteromeric receptor subtype in the brain and spinal cord. Nicotine has an exceptionally high affinity for  $\beta 2$ -containing nAChRs, which mediate many of its physiological and behavioral effects, including tobacco addiction. Recently, other nAChRs have been found to participate in certain brain circuits involved in nicotine and other drug addictions [12].



8

**Figure 1.** Structures of the two alkaloids, tobacco anatabine and nemertine isoanatabine, compared with anabasine and another tetrahydropyridyl ring isomer, anabaseine. Anatabine and anabasine are known to largely occur as (S)-enantiomers; the possible chirality of natural isoanatabine is not yet known. Both anabaseine and anabasine have been found in marine (nemertine) worms and in ants.

Although nAChRs have been extensively investigated during the past three decades, once they were cloned and expressed in cell lines, much remains to be learned about their *in vivo* functions and their potential as therapeutic targets. In the 1980s, it was reported by many laboratories that the membrane concentrations of some nAChRs, especially the  $\alpha 4\beta 2$  subtypes, are greatly reduced in cognitive circuits of the brain during the progression of neurodegenerative diseases (Alzheimer's and Parkinson's) and are not expressed normally in some other central nervous system disorders [13]. Thus, initial pharmaceutical interest targeting  $\alpha 4\beta 2$  or  $\alpha 7$  nAChRs focused on behavioral effects of nicotinoids, including enhancement of cognition and the treatment of tobacco addiction. Efforts to design and develop drugs that can selectively stimulate or antagonize these central nervous system receptors are still in progress. In addition, recent investigations demonstrating the involvement of nAChRs in acute (sepsis, pancreatitis, etc.) and chronic (rheumatoid arthritis, multiple sclerosis, etc.) inflammatory diseases have opened a new therapeutic frontier that ultimately may lead to better treatments of these medical conditions [14,15].

Nicotine, anabasine, and anabaseine (Figure 1) are potent but unselective agonists at most nAChRs, which limits their therapeutic use. However, many drug candidates, including analogs of nicotine and anabaseine, have been reported to selectively affect a single nAChR subtype, which reduces the possibility of adverse effects. In spite of numerous compounds being developed and the recent appearance of selective allosteric modulators, nAChR-targeted drug design is still an immature field of investigation. It has benefited greatly from the availability of a large number of potent natural products (“molecular probes”) that act upon nAChRs. These include natural agonists such as anatoxin [16], anabaseine [17,18], and epibatidine [19], as well as competitive antagonists such as *Erythrina* [20] and *Delphinium* alkaloids [21].

This paper will describe in vitro studies of two “minor” nicotinoid natural products: (1) isoanatabine, which we isolated from a nemertine (nemertean) worm, *Amphiporus angulatus*, and (2) anatabine, the most abundant minor alkaloid of the commercial tobacco plant (Figure 1). Both compounds are isomers differing only in the position of a single double bond in an otherwise saturated piperidine ring in the “parent” compound anabasine, which is present in wild tobacco [22]. The pharmacological properties of anabasine [23,24] and the related anabaseine [25,26] have been investigated, but not as extensively as those of nicotine.

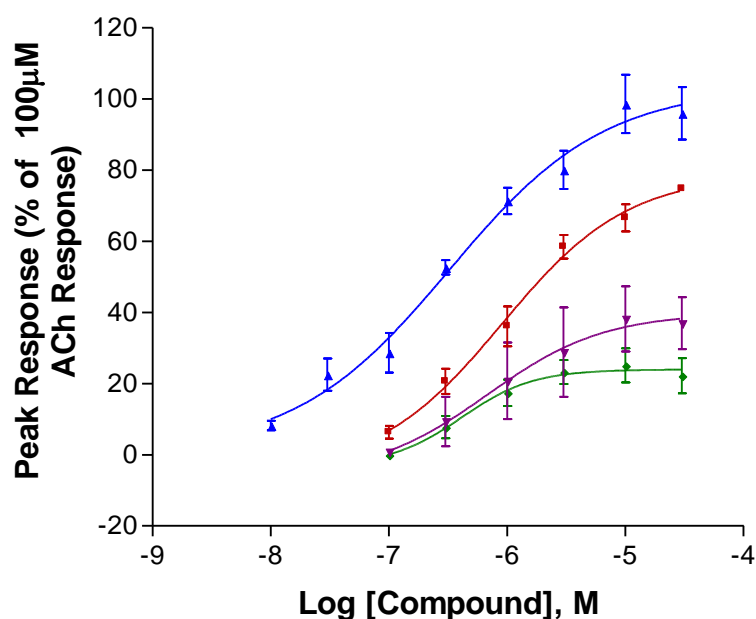
The hoplonemertine *A. angulatus* contains many pyridine alkaloids. We initially isolated two major compounds, 2,3'-bipyridyl and a tetrapyridyl we named nemertelline [27,28]. The worm inhabits the northwestern Atlantic, North American Arctic, and both northern Pacific coasts. It lives under rocks in the lower intertidal and sublittoral zones and preys upon amphipod crustaceans. Its potential predators include crabs and blenny fishes that live under the same rocks. While 2,3'-bipyridyl rapidly produces convulsions and paralysis of crustaceans, nemertelline causes a less rapid, sleep-like paralysis in crustaceans. Many (> 10) “minor” compounds, including isoanatabine, have been isolated from *A. angulatus* by high-pressure liquid chromatography and identified by nuclear magnetic resonance and mass spectrometric methods (Kem et al., in preparation). The presence of 3-methyl-2,3'-bipyridyl and isoanatabine has already been reported [29–31].

Over a century ago, anatabine was isolated from tobacco leaves, and its structure reported by Spath's laboratory [32]. It is the most abundant minor alkaloid in tobacco, representing about 4% of the alkaloids present in tobacco leaves; nicotine represents > 90% of the alkaloids present [33]. Due to its relative resistance to metabolism, it has become a useful biomarker of tobacco use [34]. Several anatabine syntheses and in vivo pharmacological studies have been published, but its in vitro effects on nAChRs have not been published. For that reason, we investigated the effects of anatabine and isoanatabine on the two major central nervous system (CNS) nAChRs.

## 2. Results

### 2.1. Experiments with Human nAChRs Expressed in *Xenopus* Oocytes

Complete concentration–response curves for the stimulation of human  $\alpha 4\beta 2$  nAChRs are shown in Figure 2. Table 1 provides efficacy (current response,  $I_{max}$ ) and potency ( $EC_{50}$ , the concentration producing half the maximal response for the compound) estimates from the curves in Figure 2; these estimates are arranged to facilitate comparisons between different enantiomers of the same compound (top half) and between similar enantiomers of the different compounds (bottom half). The two isoanatabines displayed significantly greater efficacies than the two anatabines (Table 1). R-isoanatabine's potency was greater than that of S-isoanatabine (Student's t-test  $P = 0.020$ ). The efficacy of S-anatabine was larger than that of R-anatabine ( $P = 0.029$ ). Comparing the S-enantiomers of the two compounds, S-isoanatabine's efficacy was significantly greater than that of S-anatabine ( $P = 0.0061$ ). Comparing the R-enantiomers of the two compounds, R-isoanatabine's efficacy was also greater than that of S-anatabine ( $P < 0.001$ ).

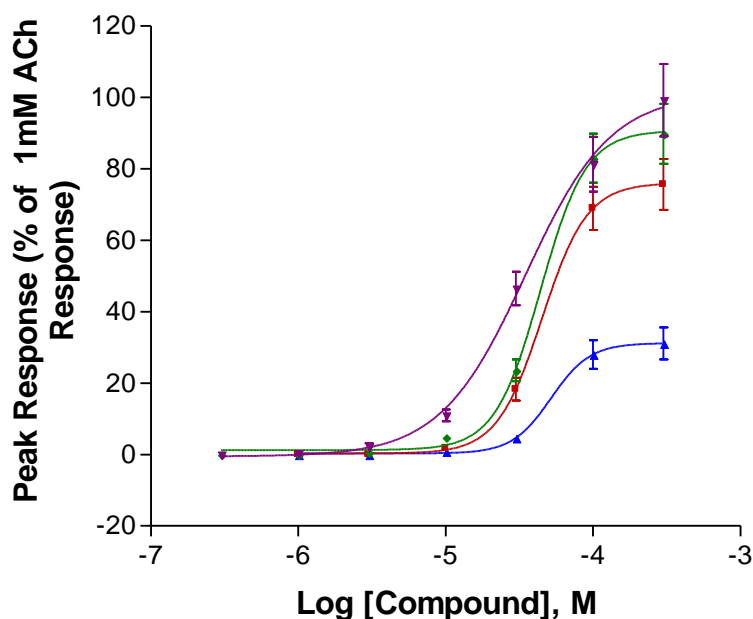


**Figure 2.** Activation of human  $\alpha 4\beta 2$  neuronal nicotinic acetylcholine (nACh) receptors expressed in *Xenopus* oocytes. All peak responses were normalized with respect to the response of the cells to 100  $\mu$ M ACh. R-Isoanatabine (Blue, ▲), S-Isoanatabine (Red, ■), R-Anatabine (Green, ◆), S-Anatabine (Purple, ▼). SEM bars are included.

**Table 1.** Comparison of *Xenopus* oocyte-expressed human  $\alpha 4\beta 2$  nAChR efficacy ( $I_{\max}$ ) and potency ( $EC_{50}$ ) estimates for the four compounds. These efficacy and potency estimates were obtained by Prism analysis of the data used to construct the curves in Figure 2  $n$  = number of oocytes tested. Tables 1–3 are organized to facilitate comparison of the two enantiomers (above) of each isomer and the same enantiomers of the two isomers (below). SEM values are included.

Compound	$I_{\max}$ (%ACh)	P	$EC_{50}$ ( $\mu$ M)	P	N
S-isoanatabine	$78.7 \pm 1.9$	0.02*	$1.01 \pm 0.33$	0.12	4
R-isoanatabine	$102 \pm 5.7$		$0.31 \pm 0.09$		4
S-anatabine	$43.2 \pm 4.3$	0.029*	$2.65 \pm 1.4$	0.3	3
R-anatabine	$25.0 \pm 5.2$		$0.74 \pm 0.21$		8
S-isoanatabine	$78.7 \pm 1.9$	0.0061 <sup>††</sup>	$1.01 \pm 0.33$	0.36	4
S-anatabine	$43.2 \pm 4.3$		$2.65 \pm 1.4$		3
R-isoanatabine	$102 \pm 5.7$	<0.001 <sup>†††</sup>	$0.31 \pm 0.09$	0.099	4
R-anatabine	$25.0 \pm 5.2$		$0.74 \pm 0.21$		8

\* P value from  $t$ -test between S- and R-enantiomers of the same compound (S-/R-Isoanatabine and S-/R-Anatabine); \* < 0.05. <sup>†</sup> Value from  $t$ -test comparing the same (S- or R-) enantiomers of different compounds; <sup>††</sup>  $p$  < 0.01, <sup>†††</sup>  $p$  < 0.001.



**Figure 3.** Activation of human  $\alpha 7$  (right) neuronal nACh receptors expressed in *Xenopus* oocytes. R-Isoanatabine (Blue, ▲), S-Isoanatabine (Red, ■), R-Anatabine (Green, ◆), S-Anatabine (Purple, ▼). All peak responses were normalized with respect to the response of the cells to 1 mM ( $\alpha 7$ ) ACh. SEM values are included.

**Table 2.** Comparison of *Xenopus* oocyte human  $\alpha 7$  nAChR efficacy ( $I_{max}$ ) and potency ( $EC_{50}$ ) estimates for the four compounds. These efficacy and potency estimates were obtained by Prism analysis of the data used to construct the curves in Figure 2. n = numbers of oocytes tested. SEM values are included.

Compound	$I_{max}$ (% ACh)	P	$EC_{50}$ ( $\mu M$ )	P	n
S-Isoanatabine	81.9 $\pm$ 9.1	0.0007***	52.3 $\pm$ 13	0.61	9
R-Isoanatabine	35.4 $\pm$ 5.3		61.3 $\pm$ 12		9
S-Anatabine	113 $\pm$ 20	0.788	69.7 $\pm$ 30	0.569	9
R-Anatabine	105 $\pm$ 16		51.8 $\pm$ 6.5		8
S-Isoanatabine	81.9 $\pm$ 9.1	0.193	52.3 $\pm$ 13	0.599	9
S-Anatabine	113 $\pm$ 20		69.7 $\pm$ 30		9
R-Isoanatabine	35.4 $\pm$ 5.3	0.003 <sup>††</sup>	61.3 $\pm$ 12	0.50	9
R-Anatabine	105 $\pm$ 16		51.8 $\pm$ 6.5		8

\* P value from t-test for S- and R-enantiomers of the same compound (S-/R-Isoanatabine and S-/R-Anatabine): \*\*\*  $p < 0.001$ . <sup>†</sup> Value from t-test comparing the same (S- or R-) enantiomers of different compounds: <sup>††</sup>  $p < 0.01$ .

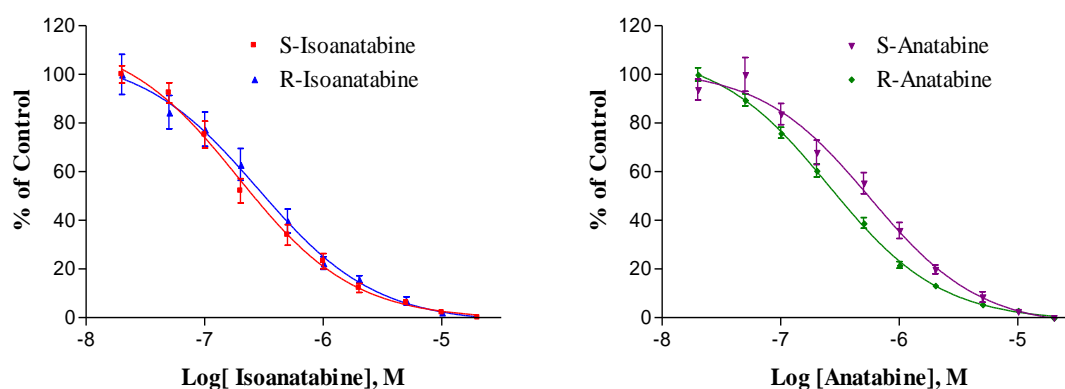
The two isoanatabines were more efficacious and the two anatabines were less efficacious at human  $\alpha 4\beta 2$  nAChRs compared with S-anabasine ( $I_{max} = 78\%$ , Kem et al., in preparation). However, all compounds except R-isoanatabine still had efficacies at human  $\alpha 4\beta 2$  nAChRs that were inferior to that of nicotine (Xing et al., submitted). All four compounds displayed potencies at human  $\alpha 4\beta 2$  nAChRs that were greater than that for S-anabasine ( $EC_{50} = 4 \mu M$ , Kem et al., in preparation).

The two anatabines displayed the highest efficacies at  $\alpha 7$  receptors (Figure 3 and Table 2). However, the  $\alpha 7$  receptor potencies of all four compounds were rather similar, the  $EC_{50}$ s ranging from 52 to 70  $\mu M$ . All four compounds possessed potencies that were similar to that of nicotine ( $EC_{50} = 65 \mu M$ , Xing et al., submitted).

## 2.2. Radioligand Binding Experiments with Rat Brain nAChRs

We utilized [ $^3\text{H}$ ]-cytisine displacement to measure the relative affinities of the isoanatabines and anatabines for rat brain high-nicotine-affinity receptors, which have been shown to be >90% of the  $\alpha 4\beta 2$  subtype. At the low [ $^3\text{H}$ ]-cytisine concentration (0.3 nM) employed in these experiments, one can be confident that binding to the high-sensitivity  $\alpha 4\beta 2_3$  stoichiometry receptors is being measured, since the low-sensitivity  $\alpha 4\beta 2$  receptors have a much lower affinity (~50X) for cytisine [35].

Compound affinities for  $\alpha 4\beta 2$  nAChRs, measured by radioligand binding methods, are shown in Figure 4, and the binding constants are presented and compared in Table 3. The affinities of the four compounds were rather similar, being in the 100–250 nM range. The binding affinity of S-isoanatabine was slightly higher than that of the S-anatabine ( $P = 0.005$ ). Although the affinities of the two isoanatabines were not statistically different, the R-anatabine  $K_i$  was half of the S-anatabine  $K_i$  ( $P = 0.001$ ). By comparison, the nicotine  $K_i$  for rat brain receptors is approximately 2 nM (Xing et al., submitted). The affinities measured by displacement of cytisine binding were approximately 100× higher than would be predicted from the  $\text{EC}_{50}$  values of the compounds. This is not surprising since other binding studies using cytisine and related radioligands (nicotine and epibatidine) have found much higher affinities than would be inferred from functional estimates. Radioligand binding experiments measure ligand interaction under steady-state conditions, unlike functional experiments and, thus, are greatly affected by high affinity for the desensitized state of the  $\alpha 4\beta 2$  receptors. Thus, one cannot expect a strict proportionality between  $\text{EC}_{50}$  and  $K_i$  estimates.



**Figure 4.** Competition binding assay of (S and R)-Isoanatabines (left) and (S and R)-Anatabines (right). Displacement of [ $^3\text{H}$ ]-cytisine from rat brain membranes. R-Isoanatabine (Blue,  $\blacktriangle$ ), S-Isoanatabine (Red,  $\blacksquare$ ), R-Anatabine (Green,  $\blacklozenge$ ), S-Anatabine (Purple,  $\blacktriangledown$ ). Each point represents the mean of 12 replicates (three experiments, identical concentrations tested in quadruplicate); SEM bars are shown.

Our binding data for rat brain  $\alpha 7$  receptors were limited to single [ $^{125}\text{I}$ ]- $\alpha$ -bungarotoxin displacement experiments (ten compound concentrations, each with four identical samples) for S-anatabine ( $K_i = 236 \pm 20$  nM) and R-anatabine ( $K_i = 680 \pm 120$  nM).

**Table 3.** Isoanatabine and anatabine binding to rat brain  $\alpha 4\beta 2$  nAChRs measured by displacement of [ $^3\text{H}$ ]-cytisine-specific binding. There were no statistically significant differences ( $P > 0.05$ ) between isoanatabine and anatabine or for S-anatabine versus R-anatabine. SEM values are shown. By comparison, S- and R-anabasine  $K_i$  values for rat brain  $\alpha 4\beta 2$  nAChRs measured under similar conditions were 1100 and 910 nM, respectively [24]. n = total number of replicates.

Compound	$K_i$ (nM)	P	Hill Slope	P	n
S-Isoanatabine	$108 \pm 14$	0.13	$1.08 \pm 0.15$	0.40	12
R-Isoanatabine	$136 \pm 11$		$0.94 \pm 0.05$		12
S-Anatabine	$249 \pm 32$	0.001**	$0.98 \pm 0.09$	0.70	12
R-Anatabine	$119 \pm 16$		$0.94 \pm 0.06$		12
S-Isoanatabine	$108 \pm 14$	0.005**	$1.08 \pm 0.15$	0.59	12
S-Anatabine	$249 \pm 32$		$0.98 \pm 0.09$		12

R-Isoanatabine	136 ± 11	0.39	0.94 ± 0.05	0.99	12
R-Anatabine	119 ± 16		0.94 ± 0.06		12

\* P value from *t*-test for S- and R-enantiomers of the same compound (S-/R-Isoanatabine and S-/R-Anatabine): \* \* < 0.01. † † Value from *t*-test comparing the same (S- or R-) enantiomers of different compounds: ††*p* < 0.01.

### 3. Discussion

Insertion of the double bond in an otherwise piperidine ring flattens this part of the ring, preventing the 3,4 (isoanatabine) or 4,5 position (anatabine) carbons from taking a half-chair or half-boat conformation. Our main observations are that the presence of a double bond in either position increases agonist potency at  $\alpha 4\beta 2$  nAChRs but decreases potency at  $\alpha 7$  nAChRs, relative to S-anabasine. Anabasine displays about a 10-fold lower potency than nicotine for  $\alpha 4\beta 2$  nAChRs, but an approximately threefold higher potency at  $\alpha 7$  nAChRs (Xing et al., in preparation). Anabasine binds about fivefold less tightly to rat  $\alpha 4\beta 2$  nAChRs than to  $\alpha 7$  nAChRs [25]. The larger ring size of anabasine seems less favorable for binding and activation of  $\alpha 4\beta 2$  nAChRs [35]. Addition of the double bond appears to partially compensate for the enlargement of the ring. Crystal structures of nicotine bound to *Lymnaea* acetylcholine binding protein (AChBP, Protein Data Bank IUW6 [36]) and anabaseine bound to *Aplysia* AChBP (Protein Data Bank 2WNL [37]) indicate that these nicotinoid agonists occupy most of the volume of the binding site. Introduction of the 3,4-double bond in isoanatabine enhances efficacy at  $\alpha 4\beta 2$  receptors relative to anabasine, but 4,5-double bond introduction in anatabine reduces its efficacy at these receptors relative to anabasine. Perhaps this is due to the proximity of the 3,4-double bond to the 2-position carbon, to which the pyridyl ring is connected; the lone sp<sup>2</sup> hydrogen at the 3-position may improve the relative orientations of the two rings for isoanatabine binding at the  $\alpha 4\beta 2$  binding site. Crystal structures of anabasine, isoanatabine, and anatabine bound to nAChRs or to AChBPs could provide a firmer basis for interpreting the pharmacological effects of an added double bond in the two isomeric compounds.

The S- and R-enantiomers for each compound did not display large differences in their interactions with the two nAChRs, as was also observed for the anabasine enantiomers [23, and (Kem et al., in preparation)]. An exception was the much lower efficacy of R-isoanatabine at  $\alpha 7$  nAChRs (Figure 3 and Table 2) relative to S-isoanatabine. S-anatabine accounts for approximately 85% of tobacco anatabine [33]. Anabasine also occurs predominantly as the S-form, as does nicotine [33].

Biologically, the presence of a distal double bond in isoanatabines and anatabines provides the organism (nemertine and tobacco plant) with a toxin capable of interacting more potently and efficaciously with  $\alpha 4\beta 2$  nAChRs than either anabasine or anabaseine [25]. Thus, the organism probably acquires an enhanced ability to neutralize predators and prey (nemertine) or herbivores (tobacco) by targeting an additional subtype of nAChR more effectively than anabasine or anabaseine.

Until now, the in vitro agonist properties of anatabine have not been investigated. However, anatabine has been shown to have anti-inflammatory actions, both in vivo and in vitro [38,39]. Racemic anatabine was marketed as an herbal supplement called Anatabloc by Rock Creek Pharmaceuticals. Several studies supported by this company have shown that anatabine, in addition to its anti-inflammatory actions, has procognitive [40–43], neuro-protective [44], immune disease modulating [45,46], and smoking cessation actions. Anatabine partially substitutes for nicotine in drug discrimination behavioral assays but does not have the addictive potency of nicotine [47,48]. Racemic anatabine released dopamine from striatal slices obtained from adult mice [49] but failed to reduce the threshold for intracranial electrical stimulation, a useful measure of its reinforcing ability [50]. A recent investigation also found that anatabine has anti-depressant effects in mice [51]. Most (if not all) of these studies apparently used synthesized, racemic anatabine. Ours is the first study where both enantiomers were tested separately.

While similar in vivo studies have yet to be carried out with an isoanatabine, the similarity of its in vitro properties with those of anatabine suggest that isoanatabine will also have similar in vivo actions. Both isomeric analogs of anabasine—isoanatabine and anatabine—have therapeutic potential and merit further investigation. It was recently reported that agonists that co-stimulate both

of the major brain nAChRs have greater efficacy in protecting brain cells from  $\beta$ -amyloid [52]. Similarly, the precognitive effects of varenicline seem to depend on stimulation of both  $\alpha 4\beta 2$  and  $\alpha 7$  nAChRs [53]. These interesting findings will stimulate further investigations of agonists that have this co-stimulatory ability at a common concentration. Isoanatabine and anatabine are compounds which should be capable of co-stimulating these two receptors, particularly since it has been shown that  $\alpha 7$  nAChRs are maximally stimulated at concentrations that are as much as  $10 \times$  lower than are found to be effective when measured by peak current measurements such as the ones we used in this study. It will also be important to evaluate the effects of isoanatabine and anatabine on autonomic ganglion and neuromuscular nAChRs, which are potential sites for their adverse effects.

#### 4. Materials and Methods

##### 4.1. Isoanatabine and Anatabine Syntheses

Isoanatabine synthesis was achieved via a procedure using simple and inexpensive starting materials [31]. The key step of our synthesis was introduction of the pyridyl group via a Bruylants reaction, reacting 1-methyl-2-cyano-3-piperidine [54] with 3-pyridyl magnesium chloride. N-Demethylation of the N-oxide of the N-Me isoanatabine by  $\text{FeSO}_4$  in MeOH at  $10^\circ\text{C}$  (non-classical Polonovski reaction) led to isoanatabine. The  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of racemic isoanatabine were identical to those of natural isoanatabine (Kem et al., in preparation). Racemic anatabine analytical data were also in agreement with published data. Enantio-separation was achieved by chiral-phase HPLC (Chiralcel OJ-H column,  $1\text{ cm} \times 25\text{ cm}$ , Daicel Chemical Industries Ltd.; Affiliate: Chiral Technologies, Inc., West Chester, PA, USA). For anatabine, an initial 10 min development with hexane-2.5% ethanol-0.1% trifluoroacetic acid-0.1% diethylamine was followed by a 40 min linear gradient attaining hexane-10% ethanol-0.1% TFA-0.1% DEA after 40 min. The S-anatabine peak occurred at 43 min and the R-anatabine peak at 47 min. For isoanatabine, an initial 10 min development with 98% (v/v) hexane-2.0% ethanol-0.15% trifluoroacetic acid-0.1% diethylamine was followed by a linear gradient attaining 80% hexane-10% ethanol-0.1% TFA-0.1% DEA after 40 min. The S-isoanatabine peak occurred at 33 min and the R-anatabine peak at 40 min. See Tang et al. [55] for more details regarding chiral separations of nicotine alkaloids with this column.

##### 4.2. Two-Electrode Voltage-Clamp Analysis of Human Brain nAChRs

Because the two nAChRs of interest often occur on the cell membranes of the same neurons, we chose to separately investigate the functional effects of the two alkaloids using the frog (*Xenopus*) oocyte expression system, which has been widely used to investigate the agonistic or antagonistic effects of many compounds acting on nAChRs. It has been especially useful for investigating  $\alpha 7$  nAChRs, due to the difficulties that were initially encountered in functional expression of this particular homomeric nAChR in mammalian cells. We measured the peak current response of an oocyte to a given concentration of the compound and expressed its amplitude with respect to the mean response to ACh before and after the compound test concentration. These  $\text{EC}_{50}$  values are minimal estimates of  $\alpha 7$  receptor potency, as it has been shown that the peak current is not a direct measure of receptor activation by the concentration being tested, since the rate of desensitization of this receptor exceeds the rate of equilibration of the compound with the receptors on the oocyte surface [56]. The actual  $\text{EC}_{50}$  values of the compounds may be as much as 10-fold lower than our peak current estimates due to this difference in the method of estimating the  $\alpha 7$  receptor responses [56]. However, since we were interested in comparing the relative efficacies and potencies of the four isomeric compounds and their rates of diffusion to the receptors are expected to be almost identical, our peak current measurements should allow quantitative comparisons.

Oocytes were harvested from *Xenopus laevis* frogs obtained from NASCO Scientific Co. (Ft. Atkinson, WI, USA) according to an accepted Kem laboratory Institutional Animal Care Committee (IACUC) approved protocol. We have already described the methods of oocyte injection, perfusion, rapid solution changes, and recording in considerable detail [57]. Oocytes were injected with identical amounts (usually 12 ng) of human  $\alpha 4$  and  $\beta 2$  mRNAs for  $\alpha 4\beta 2$  nAChR expression, or 20 ng of human

$\alpha 7$  mRNAs for  $\alpha 7$  nAChR expression. The two-electrode voltage-clamp method was used for recording peak responses (holding  $E_m$ -50 mV for  $\alpha 4\beta 2$  and -60 mV for  $\alpha 7$  receptors). For convenience, we only measured peak current responses to various concentrations of the two compounds and normalized the data relative to ACh responses before and after the test pulse; the standard ACh concentrations were 100  $\mu$ M for  $\alpha 4\beta 2$  nAChRs and 1000  $\mu$ M for  $\alpha 7$  nAChRs, since the latter receptor is less sensitive to ACh (Xing et al., submitted). The oocyte was no longer used if the current response to ACh decreased more than 20% during the experiment. Compounds dissolved in Barth's saline (pH 7.3) were rapidly administered by an AutoMate perfusion system. Compound applications were separated by a 5 min wash period. We utilized GraphPad Prism software to analyze the data, always assuming a single homogeneous receptor population.

#### 4.3. Radioligand Binding

[ $^3$ H]-Cytisine was purchased from New England Nuclear and iodinated  $\alpha$ -bungarotoxin [ $^{125}$ I]-BTX from Amersham. Unstripped whole male Sprague-Dawley rat brains obtained from Pel-Freez Biologicals (Rogers, AZ, USA) were prepared in Tris binding saline (pH = 7.4) containing 2 mg/mL BSA. [ $^3$ H]-Cytisine binding experiments were performed according to [57] with several modifications [58]. Rat brain membranes were incubated with [ $^3$ H]-cytisine at 4  $^{\circ}$ C for 4 h. Then, 200  $\mu$ g of membrane protein was incubated in a final volume of 500  $\mu$ L binding saline at 4  $^{\circ}$ C for 4 h, while binding assays with [ $^{125}$ I]-bungarotoxin were incubated at 37  $^{\circ}$ C for 3 h to assure that equilibrium was reached. Nonspecific binding was measured in the presence of 1 mM (S)-nicotine hydrogen tartrate salt. Reactions were terminated by vacuum filtrations through Whatman GF/C filters (presoaked in PEI) using a Brandel cell harvester. Bound [ $^3$ H]-cytisine and [ $^{125}$ I]-bungarotoxin were measured with liquid scintillation and gamma counters, respectively. Data were analyzed using GraphPad Prism software (San Diego, CA, USA). The data were fit by nonlinear regression analyses to a sigmoidal dose-response with variable slope.  $K_i$  values were calculated using the Cheng-Prusoff equation ( $K_i = IC_{50}/(1 + [RL]/K_d)$ ) with a  $K_d$  value for each radioligand that had been previously determined.  $IC_{50}$  is the median inhibition concentration of the experimental compound and [RL] is the concentration of the radioligand.

## 5. Conclusions

Both isoanatabine enantiomers were more potent  $\alpha 4\beta 2$  agonists than the two anatabines; R-isoanatabine was the most efficacious and potent agonist. Both anatabine enantiomers and S-isoanatabine were strong partial agonists at the human  $\alpha 7$  receptor relative to R-isoanatabine. Further pharmacological analysis of these two isomeric piperidine analogs of anabasine may provide structural explanations for the effects of the added double bond and differences between isoanatabines and anatabines. The ability of these isomeric natural products to co-stimulate the two major brain nAChRs may provide a means of increasing therapeutic efficacy beyond what would be achieved by solely stimulating one of these nAChRs.

**Patents:** No patents have resulted from the present investigation.

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