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In Vitro and in Vivo Neuronal Nicotinic Receptor Properties of (+)- and (−)-Pyrido[3,4]homotropane [(+)- and (−)-PHT]: (+)-PHT Is a Potent and Selective Full Agonist at α6β2 Containing Neuronal Nicotinic Acetylcholine Receptors

F. Ivy Carroll,*[,†] Hernán A. Navarro,[†] S. Wayne Mascarella,[†] Ana H. Castro,[‡] Charles W. Luetje,[‡] Charles R. Wageman,[§] Michael J. Marks,[§] Asti Jackson,[∥] and M. Imad Damaj[∥]

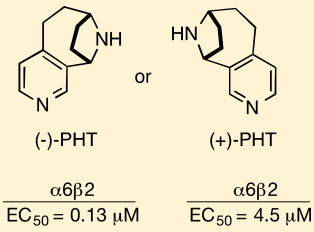
[†]Research Triangle Institute, P.O. Box 12194, Research Triangle Park, North Carolina 27709, United States

[‡]Department of Molecular and Cellular Pharmacology, Miller School of Medicine, University of Miami, Miami, Florida 33101, United States

[§]Institute for Behavioral Genetics, University of Colorado, Boulder, Colorado 80309, United States

[∥]Department of Pharmacology, Virginia Commonwealth University Medical Campus, P.O. Box 980615, Richmond, Virginia 23298-0613, United States

ABSTRACT: Pyrido[3,4]homotropane (PHT) is a conformationally rigid, high affinity analogue of nicotine. (+)-PHT was previously shown to be 266 times more potent than (−)-PHT for inhibition of [^{3}H]epibatidine binding to nAChRs but had no antinociceptive activity in mouse tail-flick or hot-plate tests and was not a nicotinic antagonist even when administered intrathecally. While (−)-PHT had no agonist activity, it was a potent, nicotinic antagonist in the test. Here, electrophysiological studies with rat nAChRs show (+)-PHT to be a low efficacy partial agonist selective for α4β2-nAChRs, relative to α3β4-nAChRs (15-fold) and α7-nAChRs (45-fold). (−)-PHT was an antagonist with selectivity for α3β4, relative to α4β2- (3-fold) and α7- (11-fold) nAChRs. In [^{3}H]DA release studies in mice, (+)-PHT was 10-fold more potent than (−)-PHT at α4β2*-nAChRs and 30-fold more potent at α6β2*-nAChRs. Studies using α5KO mice suggested that much of the activity at α4β2*-nAChRs is mediated by the α4β2α5-nAChR subtype. In conditioned place preference studies, (−)-PHT was more potent than (+)-PHT in blocking nicotine reward. Off-target screens showed (+)- and (−)-PHT to be highly selective for nAChRs. The high potency, full agonism of (+)- and (−)-PHT at α6*-nAChR contrasts with the partial agonism observed for α4*-nAChR, making these ligands intriguing probes for learning more about the pharmacophores for various nAChRs.



KEYWORDS: *(−)- and (+)-Pyrido[3,4]homotropane, (−)- and (+)-PHT, α6β2-nicotine agonist, nicotinic receptors, [^{3}H]dopamine release studies, electrophysiological studies, conditioned place preference studies*

Nicotinic acetylcholine receptor (nAChR) ligands are of interest as potential pharmacotherapies for treating addiction (nicotine, alcohol, methamphetamine, and cocaine), Parkinson's disease, schizophrenia, depression, Alzheimer's disease, attention deficit/hyperactivity disorder, pain, and inflammation (see ref 1 for a recent review). Since the α4β2- and α7-nAChRs are the major nAChR subtypes found in the brain, most of the previous efforts in drug discovery and development have been directed toward these subtypes. However, since the α6 subunit is predominantly expressed in the ventral tegmental area and the mesolimbic system, nAChRs containing this subunit are also of interest as targets for drug development.[2−5] Drug discovery studies using the α6*-nAChR as target have been limited due to difficulties in expressing the α6 subunit in heterologous systems and the lack of suitable small molecule standard compounds with which to characterize the α6*-nAChRs.

In the late 1980s and in a follow-up study in 2005, Kanne et al. reported that racemic pyrido[3,4]homotropane (PHT), a conformationally rigid analogue of nicotine (**1**) or nor-nicotine (**2**) (Figure 1) had good affinity for nAChR labeled by [^{3}H]nicotine and other radioligands and possessed activity similar to nicotine in a prostration assay.[6−8] In 2006 we synthesized (+)- and (−)-PHT (Figure 1) and reported that there was a large difference in the ability of the two enantiomers to compete with [^{3}H]epibatidine binding in rat brain.[9] In addition, their in vivo pharmacological properties in mice were not consistent with their nAChR binding affinities. We speculated that this inconsistency could be due to binding to other nAChR subtypes, differential binding to various conformations of nAChRs (desensitized or inactive state, for example) or simply binding to non-nicotinic receptors.[9] In the present study we investigated the activity and selectivity of (+)- and (−)-PHT using in vitro and in vivo experiments.

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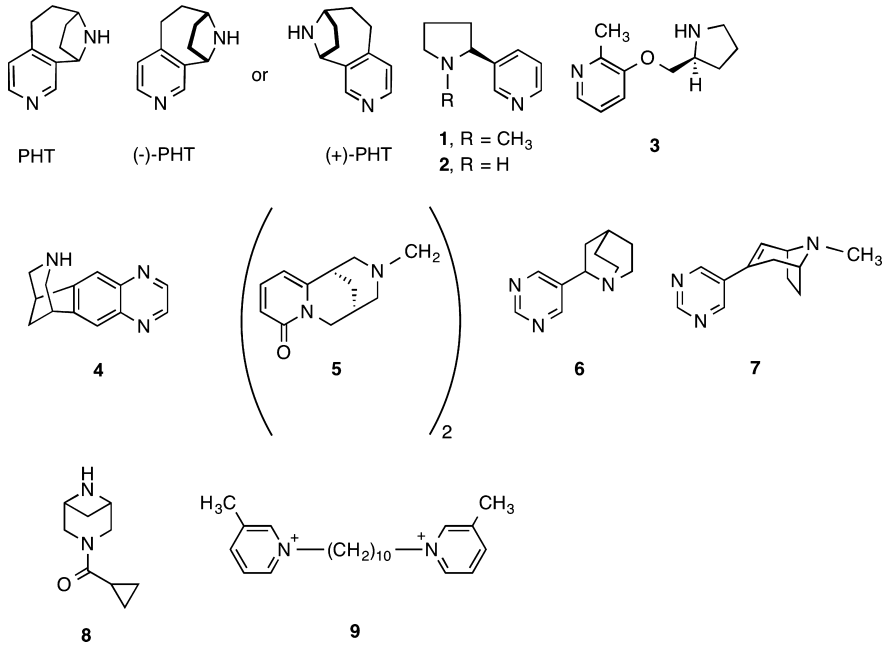

Figure 1. Structures of PHT, (−)-PHT, (+)-PHT, **1** (nicotine), **2** (nor-nicotine), **3** (ABT-089), **4** (varenicline), **5** (CC-4), compounds **6**, **7**, **8** (TC-8831), and **9** (bPiDi).

Table 1. In Vitro Single Concentration Functional Assays for (+)- and (−)-PHT[a]

	agonist activity (% of maximal ACh response)			antagonism (% of EC_{50} ACh remaining)		
cmpd	$\alpha4\beta2$	$\alpha3\beta4$	$\alpha7$	$\alpha4\beta2$	$\alpha3\beta4$	$\alpha7$
(+)-PHT	3.2 ± 0.4	7.7 ± 0.9	0	17 ± 3	93 ± 3	87 ± 1
(−)-PHT	0	0	1.1 ± 0.2	41 ± 4	32 ± 3	84 ± 10

[a]Current responses of rat nAChRs expressed in *Xenopus* oocytes were recorded under a two-electrode voltage clamp. Agonist activity was assessed by comparing the response to 100 μM of each compound to the response to acetylcholine applied at an EC_{20} concentration (20 μM for $\alpha4\beta2$, 110 μM for $\alpha3\beta4$) or an EC_{50} concentration (300 μM for $\alpha7$). Antagonist activities assessed by comparing the current response to an EC_{50} concentration of acetylcholine (70 μM for $\alpha4\beta2$, 200 μM for $\alpha3\beta4$, 300 μM for $\alpha7$) in the presence of 100 μM of each compound are compared to the response to acetylcholine alone.

Table 2. Comparison of Agonist Potency and Efficacy Values, And Antagonist Potency Values, for (+)- and (−)-PHT at $\alpha4\beta2$-, $\alpha3\beta4$-, and $\alpha7$-nAChRs[a]

	agonist activity						
	$\alpha4\beta2$		$\alpha3\beta4$		antagonism, IC_{50} (μM)		
cmpd	EC_{50} (μM)	E_{max} (% ACh)	EC_{50} (μM)	E_{max} (% ACh)	$\alpha4\beta2$	$\alpha3\beta4$	$\alpha7$
(+)-PHT	4 ± 1	4.6 ± 0.4	50 ± 6	8.4 ± 0.5	16 ± 3	242 ± 13	720 ± 290
(−)-PHT					99 ± 13	33 ± 6	353 ± 34

[a]Current responses of rat nAChRs expressed in *Xenopus* oocytes were recorded under a two-electrode voltage clamp. EC_{50} and E_{max} values for (+)-PHT activation of $\alpha4\beta2$- and $\alpha3\beta4$-nAChRs are derived from concentration−response curves. E_{max} values are expressed as a percentage of the E_{max} for acetylcholine. IC_{50} values for (+)-PHT and (−)-PHT inhibition of $\alpha4\beta2$-, $\alpha3\beta4$-, and $\alpha7$-nAChRs are derived from concentration−inhibition curves, in which the current response to an EC_{50} concentration of acetylcholine (70 μM for $\alpha4\beta2$, 200 μM for $\alpha3\beta4$, 300 μM for $\alpha7$) in the presence of various concentrations of each compound are compared to the response to acetylcholine alone.

RESULTS AND DISCUSSION

The nAChR subtype selectivity of (+)- and (−)-PHT was initially assessed in an electrophysiological assay using rat $\alpha4\beta2$-, $\alpha3\beta4$-, and $\alpha7$-nAChR's expressed in *Xenopus* oocytes and assayed by a two-electrode voltage clamp. To test for agonist activity, currents in response to 100 μM applications of each compound were normalized to the response to ACh. Results from this screen, shown in Table 1, suggested that (+)-PHT is a low efficacy partial agonist of the $\alpha4\beta2$ and $\alpha3\beta4$-nAChRs. No agonist activity was observed for (+)-PHT at $\alpha7$-nAChRs. (−)-PHT displayed little or no agonist activity at the $\alpha4\beta2$, $\alpha3\beta4$, and $\alpha7$-nAChRs.

To test for antagonist properties, the current response to an EC_{50} concentration of ACh in the presence of 100 μM (+)- or (−)-PHT was determined. Results from this screen (Table 1) suggested that (+)-PHT may be selective for $\alpha4\beta2$- relative to the $\alpha3\beta4$ and $\alpha7$-nAChRs and that (−)-PHT appeared to be selective for $\alpha3\beta4$- and $\alpha4\beta2$-, relative to $\alpha7$-nAChRs.

In order to gain more detailed information on the subtype selectivity of the agonist and antagonist activity of (+)- and (−)-PHT, concentration−response and concentration−inhibition curves were generated (Table 2). We found (+)-PHT to

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Table 3. Dopamine Release from Striatal Synaptosomes Stimulated by (+)- and (−)-PHT[a]

	(+) PHT res $\alpha4\beta2$	(+) PHT sens $\alpha6\beta2$	(+) PHT $\alpha4\beta2$ in $\alpha5$KO*	(+) PHT $\alpha6\beta2$ in $\alpha5$KO	(−) PHT res $\alpha4\beta2$	(−) PHT sens $\alpha6\beta2$	(−) PHT $\alpha4\beta2$ in $\alpha5$KO*	(−) PHT $\alpha6\beta2$ in $\alpha5$KO
EC_{50}	0.4 μM	0.13 μM	2 μM	0.20 μM	4 μM	4.5 μM	0.7 μM	2 μM
sem	±0.2 μM	±0.04 μM	±3 μM	±0.06 μM	±3 μM	±0.6 μM	±0.8 μM	±2 μM
max[b] (% nic)	33%	94%	13%	125%	17%	109%	10%	96%
sem	±5%	±18%	±4%	±8%	±3%	±25%	±2%	±12%
binding K_i[c]	1.7 nM	28 nM			35 nM	629 nM		
sem	±0.2 nM	±3.1 nM			±1.8 nM	±19 nM		

[a]EC_{50} from n = 3−4 experiments; DA release from mouse striatal synaptosomes; res = αCtxMII resistant (response); sens = αCtxMII sensitive (response). [b]Max (% nic) from n = 7−8 experiments; comparison of 10 μM nic response to 10 μM (+) PHT or 100 μM (−) PHT. [c]Binding K_i values from inhibition of [^{125}I]epibatidine binding to membranes prepared from mouse cortex ($\alpha4\beta2$ sites only) or membranes prepared from striatum and olfactory tubercle from α4KO mice ($\alpha6\beta2$ sites only).

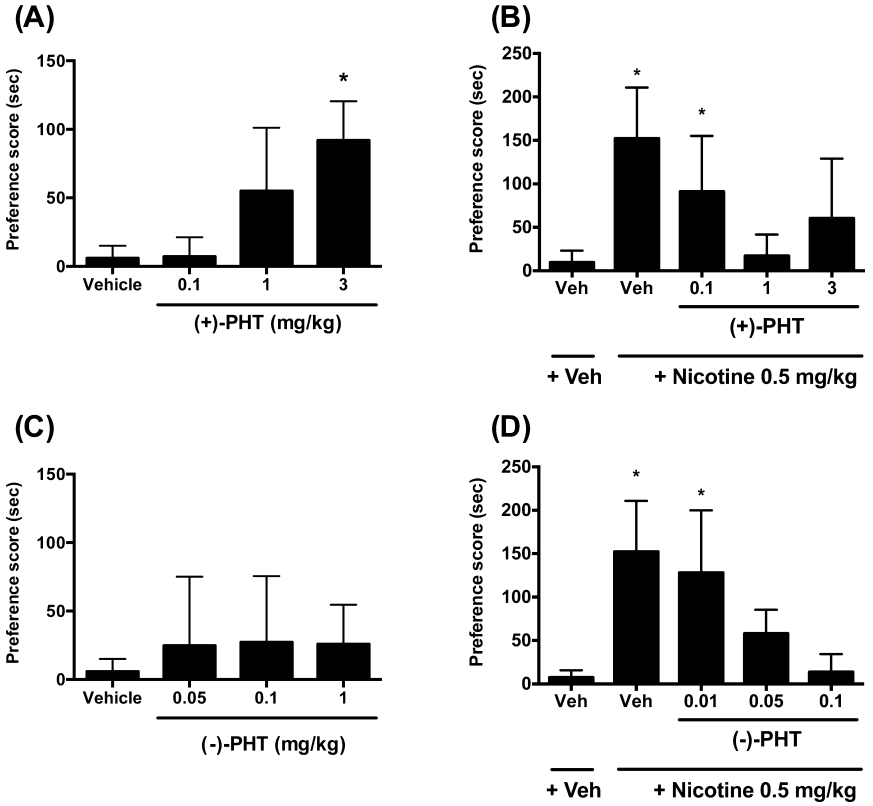

Figure 2. Effects of (+)-PHT and (−)-PHT on development of nicotine-induced CPP in the mouse. (A) Dose−response curve for (+)-PHT in the CPP test given s.c. 15 min before conditioning. (B) Blockade of nicotine-induced CPP by (+)-PHT. The drug was given 15 min before nicotine (0.5 mg/kg, s.c.) during conditioning. (C) Dose−response curve for (−)-PHT in the CPP test given s.c. 15 min before conditioning. (D) Blockade of nicotine-induced CPP by (−)-PHT. The drug was given 15 min before nicotine (0.5 mg/kg, s.c.) during conditioning. Each point represents the mean ± SEM of 8 mice per group. ∗ denotes $p < 0.05$ vs the vehicle control groups. Veh = Vehicle.

be a low efficacy partial agonist at the $\alpha4\beta2$- and $\alpha3\beta4$-nAChRs, yielding maximal responses that were 4.6% and 8.4% of the maximal response to ACh, respectively. A comparison of EC_{50} values showed (+)-PHT to be 12-fold more potent at the $\alpha4\beta2$-nAChR, as compared to the $\alpha3\beta4$-nAChR. A comparison of IC_{50} values showed that as an antagonist (+)-PHT is 15-fold selective for $\alpha4\beta2$- relative to the $\alpha3\beta4$-nAChR and 45-fold selective for $\alpha4\beta2$- relative to the $\alpha7$-nAChR. In contrast, (−)-PHT was a modestly selective antagonist of $\alpha3\beta4$- relative to the $\alpha4\beta2$-nAChR (3-fold) and 11-fold selective for $\alpha3\beta4$- relative to the $\alpha7$-nAChR. We were not able to assess agonist or antagonist activity of (+)- or (−)-PHT at $\alpha6$-containing nAChRs using the *Xenopus* oocyte assay due to the difficulty of expressing wild-type $\alpha6$-containing nAChRs in heterologous systems. Use of chimeric or concatameric constructs is usually required to achieve functional expression of $\alpha6$-containing nAChRs,[10,11] which can be a concern when trying to assess agonist efficacy. Instead, we turned to a striatal synaptosome [^{3}H]dopamine release assay to assess the activity of (+)- and (−)-PHT at $\alpha6$-containing nAChRs.

We performed [^{3}H]dopamine release studies for (+)- and (−)-PHT using wild type and $\alpha5$KO* mice, and the results are provided in Table 3. Maximal responses were normalized to that measured with 10 μM nicotine. In wild type mice (+)-PHT has EC_{50} values of 0.4 and 0.13 μM for the α-CtxMII resistant ($\alpha4\beta2$*-nAChR) and sensitive ($\alpha6\beta2$*-nAChR) response compared to 4 and 4.5 μM for (−)-PHT. Thus, (+)-PHT is

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10-fold more potent than (−)-PHT at the α4β2*-nAChR and 30-fold more potent for the α6β2*-nAChR.

Both isomers are more efficacious at the α6β2*-nAChR and are about as efficacious as nicotine. Both isomers are partial agonists for α4β2*-nAChR with the (+)- and (−)-isomers being $^1/_3$ and $^1/_6$, respectively, as efficacious as nicotine.

In the [^{125}I]epibatidine binding studies, both isomers have higher affinity for the α4β2*- than the α6β2*-nAChR. (+)-PHT has K_i values of 1.7 and 28 nM for the αCtxMII resistant (α4β2*-nAChR) and αCtxMII sensitive (α6β2*-nAChR), respectively, compared to 35 and 629 nM for the (−)-PHT at the α4β2* and α6β2*-nAChRs, respectively.

In the α5KO* mice (+)-PHT has EC_{50} values of 2 and 0.2 μM, respectively, at the α4β2*- and α6β2*-nAChR, respectively, compared to EC_{50} values of 0.7 and 2 μM at the α4β2*- and α6β2*-nAChR, respectively, for (−)-PHT. These results show that deletion of the α5 subunit greatly reduces α-CtxMII-resistant activity of both isomers suggesting that much of the activity at the α4β2*-nAChR is mediated by the α4β2α5-nAChR subtype. These results are similar to those found for nicotine in the striatum, but the effect of the α5 gene deletion on (+)- and (−)-PHT is even greater than it is for nicotine.[12]

Since the in vitro data indicate that both (+)- and (−)-PHT modulate nicotinic receptors function and DA release in the striatum, we evaluated the effects of (+)- and (−)-PHT in vivo in the conditioned place preference (CPP) test. As shown in Figure 2A, (+)-PHT induced a dose-related preference when given s.c. to mice. Post hoc analysis showed that (+)-PHT significantly increased CPP scores at a dose of 3 mg/kg, whereas it did not at doses of 0.1 and 1 mg/kg. In contrast, all doses of (−)-PHT tested were ineffective in inducing significant preference in the CPP test (Figure 2C). In the antagonism studies, in mice given (+)-PHT before each drug pairing with nicotine (0.5 mg/kg, s.c.), preference scores in comparison to nicotine alone (Figure 2B) were significantly reduced after pretreatment with 1 mg/kg of the drug. In addition, nicotine-induced CPP was blocked by pretreatment with (−)-PHT in a dose-dependent manner with the dose of 0.05 mg/kg completely preventing nicotine preference (Figure 2D).

(+)-PHT (10 μM) was inactive in radioligand binding assays for the 5HT1A, 5HT2A, 5HT2C, DAD1, DAD2, DAD3, DAD4, and μ, δ, and κ opioid receptors, and the DAT and SERT. (+)-PHT (10 μM) inhibited 61% of specific [^{125}I]RTI-55 binding to the NET. (+)-PHT (10 μM) was inactive at off site targets in a 62 receptor/enzyme screen (NovaScreen).

(−)-PHT (10 μM) was inactive in radioligand binding assays for the 5HT1A, 5HT2A, 5HT2C, DAD1, DAD2, DAD3, DAD4, and μ, δ, and κ opioid receptors, and the DAT, NET, and SERT. (−)-PHT (10 μM) was inactive at off site targets in a 62 receptor/enzyme screen (NovaScreen).

CHRNA6, the gene encoding for the α6 subunit, is located on chromosome 8b11.21 and is predominantly expressed in visual pathways, the ventral tegmental area, and the mesolimbic system.[2−5] These anatomical distributions identified by in situ hybridization studies and physiological roles of α6*-nAChRs, using subunit-null transgenic animals and peptide ligands, suggest the relevance of these nicotinic subtypes in drug addiction (nicotine, alcohol, and others) and other CNS disorders, making them potential targets for pharmacotherapy development.

The paucity of suitable pharmacological tools has made it difficult to conduct studies with small molecule α6*-nAChR ligands. Most of the tools available are peptides. The discovery of α-Ctx MII allowed positive identification of the α3β2*- and α6β2*-nAChRs[13] and the development of the only radioligand currently available: [^{125}I]α-Ctx MII.[14,15] A subtraction approach was used to identify an α-CtxMII-selective nAChR population that mediates dopamine release. By measuring the functional response in the presence and absence of α-Ctx MII, the activity of α-Ctx MII-sensitivity can be distinguished from that of α4β2*-nAChR.[10,12,15,16] Even though α-Ctx MII has been highly useful in the characterization of α6*-nAChR, its pharmacological properties have some limitations. In addition to lack of CNS availability via systemic administration and stability, α-Ctx MII interacts with α3β2*-nAChR with similar potency with α6β2*-nAChRs. The lack of selectivity was overcome by the development of the more α6β2*-nAChR selective α-conotoxin PIA (α-CtxPIA).[16] However, α-CtxPIA is also a peptide and lacks CNS availability after systemic administration. In addition, expression of α6 with α4 and β2 and/or β3 subunits in oocytes yielded functional receptors with different pharmacological properties than α4β2-nAChRs, such as enhanced acetylcholine (ACh) sensitivity, slower desensitization, and different desensitization rate and potency for nicotine.[17] Clearly small molecule-based α6*-nAChR ligands and their radioligand analogues are needed to reliably distinguish between α6*-nAChR and other nAChR subtypes as well as the various α6*-nAChR subtypes.

Using the tools available, the α6*-nAChR properties of nicotine and several other nicotine ligands have been studied. This information is summarized below in order to show the lack of suitable small molecules selective α6β2*-nAChR agonists. Nicotine is reported to have 70% partial agonist activity at α4α6β2-nAChRs and could desensitize the α4β2-nAChR and activate the α4α6β2-nAChR.[17] 2-Methyl-3-[(2*S*)-pyrrolidin-2-yl]methoxypyridine (pozanicline, ABT-089, **3**) (Figure 1), which is a potent partial agonist at α4β2*-nAChR, increases in vitro striatal DA release via activation of α6β2*- and α4β2*-nAChRs.[18] 6,7,8,9,10-Tetrahydro-6,10-methano-6H-pyyrazino[2,3-h][3]benzazepine (varenicline, **4**) (Figure 1) has EC_{50} values of 0.007 and 0.086 μM at α6β2*- and α4β2*-nAChR-mediated [^{3}H]DA release from rat striatal synaptosomes, respectively, and thus, was functionally more potent at the α6β2-nAChR.[19] Varenicline acted as a partial agonist in these studies with maximal efficacies of 49 and 24% at the α6β2- and α4β2-nAChRs, respectively.[19] In monkeys, varenicline potently inhibited striatal α6β2*- and α4β2*-nAChR with K_i values of 0.13 and 0.19 nM.[19] In functional studies it stimulated α6β2*- and α4β2*-nAChR-mediated [^{3}H]DA-release from monkey striatal synaptosomes with EC_{50} values of 0.14 and 0.026 μM, respectively. The E_{max} was 13 and 42% for the α6β2*- and α4β2*-nAChRs, respectively. 1,2-*bis*-*N*-Cystinylethane (**5**, CC4) is another selective partial agonist at α4β2/α6β2-nAChR. CC4-induced CPP and its self-administration show an inverted U dose response curve. Pretreatment with nonreinforcing doses of CC4 significantly reduced nicotine-induced self-administration and CPP without affecting motor function.[14] The receptor binding affinity and functional efficacy and selectivity of **6** and **7** at α6β2*- relative to α4β2- and α3β4-nAChR were reported.[13] Compound **6** had K_i values of 1.8, 1100, and 8 nM at α4β2-, α7-, and α6β2*-nAChRs while **7** had K_i values of 1.2, 9100, and 17.3 nm. Using DA release studies, compound **6** had an EC_{50} = 90 nM (E_{max} = 104%) and **7** had an EC_{50} = 100 nM (E_{max} = 77%). The α3β4*/α6β2* EC_{50} ratio for **6** and **7** were 26.7 and 410,

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respectively, and the $\alpha3\beta4^*/\alpha4\beta2^*$ EC_{50} ratio for **6** and **7** were 5.3 and 22.7, respectively. Very recently, 3-cyclopropylcarbonyl-3,6-dazabicyclo[3.1.1]heptane (**8**, TC-8831) was reported to have K_i = 3.0 and 21 nM at $\alpha4\beta2$- and $\alpha6/\alpha3\beta2\beta3$-nAChRs, respectively.[20] Using calcium-flux functional studies, **8** had EC_{50} = 80 nM (E_{max} = 100%) at the $\alpha6/\alpha3\beta2\beta3$-nAChR. Previous studies had shown that compound **8** ameliorated L-dopa induced dyskinesia (LID) without reducing antiparkinsonian benefits in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-lesioned nonhuman primates with established LID.[21] Compound **8** also reduced abnormal involuntary movements (AIMs) in rats.[22] In another study, *N,N′*-decane-diyl-*bis*-3-picolinium **9** (bPiDI) with selectivity for $\alpha6\beta2^*$-nAChRs over $\alpha4\beta2^*$-nAChRs has been shown to reduce nicotine SA in rats.[23] Even though studies from these compounds and a few others have provided useful information about the pharmacological properties of compounds interacting at the $\alpha6^*$-nAChR, few of the compounds were a full agonist at the $\alpha6^*$-nAChR with any degree of selectivity. In this study, we report that both (+)- and (−)-PHT are $\alpha6\beta2$-nAChR agonists. (+)-PHT is more potent and more selective for $\alpha6\beta2^*$-nAChR than (−)-PHT.

PHT is a conformationally rigid analogue of nicotine that showed high affinity for nAChRs using various radiolabeled nicotinic ligands.[6−8] In 2006, we reported the synthesis and pharmacological characterization of (+)- and (−)-PHT.[9] We found that PHT and (+)- and (−)-PHT had K_i values of 6.2, 1.29, and 346 nM, respectively, for inhibition of [^{3}H]epibatidine binding to nAChRs. Thus, (+)-PHT has a 266-times higher affinity than (−)-PHT for inhibition of [^{3}H]epibatidine binding to nAChRs. Even though (+)-PHT has a K_i = 1.29 nM at nAChRs, it has no antinociceptive activity in mouse tail-flick or hot-plate tests even when given intrathecally (10 μg/mouse). (+)-PHT did show weak activity in mouse hypothermia and locomotor-activity tests, but the effects were not reversed by the nicotinic antagonist mecamylamine. Not surprisingly, (−)-PHT with a K_i = 346 nM for inhibition of [^{3}H]epibatidine binding was inactive as an agonist in the mouse tail-flick, hot-plate, hypothermia, and locomotor-activity behavioral tests. Like (+)-PHT, it was also inactive when given intrathecally (10 μg/mouse) in the tail-flick test. Surprisingly, (−)-PHT was highly potent in antagonizing nicotine-induced antinociception in the tail-flick test (AD_{50} = 0.07 μg/kg) and also antagonized the antinociceptive effects of nicotine in the hot-plate test (AD_{50} = 0.8 μg/kg). In an in vitro functional test, (+)-PHT behaved as a weak partial agonist at $\alpha4\beta2$-nAChR with an intrinsic activity of 17% versus a full agonist acetylcholine (1 mM) and EC_{50} = 5.5 μm. (−)-PHT did not activate currents at $\alpha4\beta2$-nAChR. Neither compound activated or inhibited $\alpha3\beta4$-nAChR expressing cells. In nicotine drug discrimination studies, PHT and its enantiomers failed to fully generalize nicotine at doses that did not affect the rate of responding in rat (0.3−56 μg/kg). In addition, they failed to antagonize nicotine discrimination. Our in vivo results with the CPP test show that (+)- but not (−)-PHT possesses reward-like effects at the range of doses tested, reflecting perhaps the difference of potency of these compounds at $\alpha6\beta2^*$-nAChR and their ability to release DA in the striatum. Pretreatment with (+)- and (−)-PHT significantly reduced nicotine preference, suggesting antagonistic properties of these compounds on nicotine reward and motivation. These results are in line with the partial agonism and antagonistic properties of (+)- and (−)-PHT at $\alpha4\beta2$ expressed nAChRs, respectively. The higher potency of (−)-PHT in blocking nicotine CPP than (−)-PHT was also observed in their ability of blocking the acute effects of nicotine.[9]

Studies showing that $\alpha6^*$-nAChR knockout mice did not self-administer nicotine which could be restored by re-expression of the $\alpha6$ subunit in the mesolimbic dopamine system provided additional evidence that the $\alpha6^*$-nAChR was involved in nicotine-induced reward.[24] In addition, intracerebroventricular injection of α-Ctx MII in mice dose-dependently attenuated the rewarding effect of nicotine in the CPP model without reducing locomotor behavior.[25] α-Ctx MII also attenuated the affective signs of nicotine withdrawal but not the physical signs and attenuated mecamylamine-precipitated CPA.[25] In addition, α-Ctx MII produced anxiolytic effects in mice withdrawn from nicotine after chronic exposure. The microinjection of α-Ctx MII directly into the VTA or nucleus accumbens shell attenuated established nicotine self-administration in rats[26] without nonspecific changes in food response.[27] These studies indicate that blockade of $\alpha6\beta2^*$-nAChRs reduces the rewarding effects of nicotine in CPP, attenuates nicotine intake in an SA paradigm, reduces anxiety associated with nicotine withdrawal, and underscores the role of $\alpha6\beta2^*$-nAChRs in nicotine abuse and, thus, suggests the $\alpha6\beta2^*$-nAChR ligands may have potential as pharmacotherapies for smoking cessation.[28]

In summary, the high potency and full agonist activity of (+)- and (−)-PHT at $\alpha6^*$-nAChR contrasts with the partial agonist activity observed for $\alpha4^*$-nAChR. Our in vitro studies suggest that (+)- and (−)-PHT are more efficacious than varenicline in releasing striatal DA. Similar to varenicline, (+)- and (−)-PHT are partial agonists at $\alpha4\beta2^*$-nAChRs. The partial activity at $\alpha4^*$-nAChR is further reduced following deletion of the $\alpha5$ nAChR subunit, suggesting both compounds may be more active, although still partial agonists, at the $\alpha4\beta2\alpha5$-nAChR subtype. (+)-PHT, in particular, may be a useful tool for studying $\alpha6$-nAChR systems. Our in vivo studies using CPP as a test of nicotine reward showed that (+)- but not (−)-PHT is rewarding, albeit to a lesser extent than nicotine. This difference in CPP response probably reflects their difference in potency at $\alpha6^*$-nAChR subtypes. The ability of (+)- and (−)-PHT to block nicotine-induced CPP is reflective of their partial agonistic properties at $\alpha4\beta2^*$-nAChRs. Importantly, (+)- and (−)-PHT are novel lead structures for the development of selective small molecule-based agonists and antagonists and radioligands for studying the various receptors in the $\alpha6^*$-nAChR subfamily. In addition, the rigid structure of PHT and its enantiomers combined with their unexpected in vitro and in vivo pharmacological properties make them intriguing probes for learning more about the pharmacophores for various nAChRs. Finally, our in vivo and in vitro findings show that (+)- and (−)-PHT are highly useful pharmacological probes for studying various nAChR subtypes and support the possible development of PHT enantiomers or future analogues as possible smoking cessation agents.

METHODS

Materials. (+)- and (−)-PHT were synthesized using previously reported methods.[9]

Binding Studies. Binding K_i values were determined from inhibition of [^{125}I]epibatidine binding to membranes prepared from mouse cortex ($\alpha4\beta2$ sites only) or membranes prepared from striatum and olfactory tubercle from α4KO mice ($\alpha6\beta2$ sites only).[29] The production, care, and use of mice used for the binding assays and the functional assays described below was approved by the Institutional

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Animal Care and Use Committee of the University of Colorado Boulder and meet the guidelines of the National Institutes of Health.

In Vitro Efficacy Studies. (+)- and (−)-PHT were tested for agonist and antagonist activity at rat $\alpha4\beta2$-, $\alpha3\beta4$, and $\alpha7$-nAChR expressed in *Xenopus laevis* oocytes and assayed in an electrophysiology assay as previously described.[30] The care and use of *X. laevis* frogs in this study was approved by the University of Miami Animal Research Committee and meet the guidelines of the National Institutes of Health.

[^{3}H]Dopamine Release. The [^{3}H]dopamine release studies were conducted using previously reported methods.[10,18] Concentration−effect curves were constructed for (+)- and (−)-PHT in the absence (total release) and the presence of 50 nM α-conotoxin MII (α-Ctx MII) to inhibit $\alpha6\beta2$*-nAChR subtypes. [^{3}H]dopamine release in synatosomes prepared from $\alpha5$ null mutant mice was measured to evaluate the role of the $\alpha5$ subunit (as a component of $\alpha4\beta2\alpha5$-nAChR subtype). These mice were originally generated at and obtained from Baylor College of Medicine, Houston, TX, and null mutants were obtained from mating of $\alpha5$ heterozygotes.[31] The difference in activity between [^{3}H]dopamine release measured in the absence and presence of α-Ctx MII provides the measure of the response mediated by $\alpha6\beta2$*nAChRs and release measured in the presence of α-Ctx MII provides the measure of the response mediated by $\alpha4\beta2$*-nAChRs. Maximal release and ED_{50} values were calculated by nonlinear least-squares fits of the data to the Michaelis−Menten equation. Relative efficacy was determined by comparison to the release mediated by stimulation with 10 μM nicotine.

In Vitro Off-Target Binding. Both (+)- and (−)-PHT were evaluated for their in vitro receptor binding and functional properties at the following receptors: 5HT1A, 5HT2A, 5HT2C, DAD1, DAD2, DAD3, and DAD4, μ, δ, and κ opioid receptors and DAT, SERT, and NET as well as their profiles in a 62 radioligand/enzyme assay (NovaScreen) at 100 nM and 10 000 nM concentrations in duplication. The studies and data were provided through the National Institute on Drug Abuse (NIDA) Addiction Treatment Discovery Program.

Nicotine Conditioned Place Preference (CPP) Studies. Naive male 8−10 week-old ICR mice (Harlan Laboratories; Indianapolis, IN) served as subjects. They were housed five per cage in a 21 °C humidity controlled facility with ad libitum access to food and water. The animal facility was approved by the Association for Assessment and Accreditation of Laboratory Animal Care. Experiments were performed during the light cycle and were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University. (−)-Nicotine hydrogen tartrate salt was purchased from Sigma Chemical Company (Milwaukee, WI), and it was dissolved in physiological saline (0.9% sodium chloride). Nicotine was injected s.c. at a volume of 10 mL/kg body weight. The pH of the nicotine solution was checked and neutralized if necessary. All doses are expressed as the free base of the drug. Nicotine CPP was conducted using an unbiased design as previously described.[32] In brief, separate groups of male ICR adult mice (n = 8 per group) were handled for 3 days prior to initiation of CPP testing. The CPP apparatus consisted of a three-chambered box with a white compartment, a black compartment, and a center gray compartment. The black and white compartments also have different floor textures to help the mice further differentiate between the two environments. On day 1, mice were placed in the gray center compartment for a 5 min habituation period, followed by a 15 min test period to determine baseline responses. A prepreference score was recorded and used to randomly pair each mouse with either the black or white compartment. Drug-paired sides were randomized so that an even number of mice received drug on the black and white side. Over the next 3 days, mice were conditioned for 20 min twice a day with conditioning sessions no less than 5 h apart. The saline group received saline on both sides of the boxes and drug groups received nicotine (0.5 mg/kg, s.c.) on one side of the box and saline on the opposite side. Animals in the drug group received drug each day. On test day (day 5), mice were exposed to the chambers, and day 1 procedure was repeated. In addition, mice were pretreated with vehicle (s.c.), (+)- and (−)-PHT at different doses 15 min prior nicotine. Data were expressed as time spent on the drug-paired side postconditioning minus time spent on the drug-paired side preconditioning. A positive number indicated a preference for the drug-paired side, whereas a negative number indicated an aversion to the drug-paired side. A number at or near zero indicated no preference for either side.

AUTHOR INFORMATION

Corresponding Author

*Phone: 919-541-6679. E-mail: fic@rti.org.

Author Contributions

F.I.C., M.I.D., C.W.L., and M.J.M. participated in research design. A.H.C., C.R.W., and A.J. conducted experiments. F.I.C., H.A.N., S.W.M., A.H.C., C.W.L., C.R.W., M.J.M., A.J., and M.I.D. performed data analysis. F.I.C., H.A.N., S.W.M., C.W.L., M.J.M., and M.I.D. wrote or contributed to the writing of the manuscript.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

ACh, acetylcholine; nAChR, nicotinic acetylcholine receptor; α-CtXMII, α-conotoxin MII; CC4, 1,2-*bis*-*N*-cystinylethane; CPP, conditional place preference; CPA, conditional place adversion; SA, self-administration; α4KO, alpha 4 knockout; 5HT1A, 5HT2A, and 5HT2C, (serotonin-1A, 2A, and 2C receptors, respectively); DAD1, DAD2, DAD3, and DAD4, (dopamine D1, D2, D3, and D4 receptors, respectively); DAT, dopamine transporter; SERT, serotonin transporter; NET, norepinephrine transporter

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