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Synthesis and Pharmacological Evaluation of Novel 9- and 10-Substituted Cytisine Derivatives - Nicotinic Ligands of Enhanced Subtype Selectivity

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Abstract

We report the synthesis and pharmacological properties of several cytosine derivatives. Among them, two 10-substituted derivatives showed much higher selectivities for the $\alpha 4\beta 2$ nAChR subtype in binding assays than cytosine. The 9-vinyl derivative was found to have a very similar agonist activity profile to that of cytosine.

Neuronal nicotinic acetylcholine receptors (nAChRs) belong to a heterogeneous family of pentameric ligand-gated ion channels which are differently expressed in many regions of the central nervous system (CNS) and peripheral nervous system.^{1,2} In the CNS, nAChRs regulate transmitter release, cell excitability, and neuronal integration. Neuronal nicotinic receptors constitute therapeutically relevant targets for the treatment of neurodegenerative disorders and other CNS disorders including inter alia, Alzheimer's and Parkinson's disease, Tourette's syndrome, schizophrenia, attention deficit disorder, anxiety, and pain. Moreover, as the addictive properties of tobacco products are due to the nicotine contained therein, nAChRs also become important targets for the discovery of medications for use in smoking cessation.³

The nAChRs are comprised of various combinations of different subunits, of which seventeen ($\alpha 1$ - $\alpha 10$, $\beta 1$ - $\beta 4$, γ , δ and ϵ) are known at present. Different subunit combinations define the various nAChR subtypes, and different receptor subtypes have characteristic pharmacological and biophysical properties, as well as different locations within the nervous system.⁴ Therefore, subtype selectivity is an important issue for the effectiveness and safety of nicotinic therapeutics.

While a host of nAChR ligands have been identified, there is still a substantial need to discover subtype-selective ligands that can be used to establish the physiological and pathophysiological significance of each of the receptor subtypes. As is now apparent from clinical results obtained with a variety of drugs, both the safety and efficacy of therapeutic agents often depend upon their subtype selectivity. While, as noted, a large number of nicotinic agonists and non-competitive antagonists exist, very few of these are subtype-selective.^{1,6} Exquisite subtype selectivity is difficult to achieve because of the large number of possible subtypes together

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with their relatively subtle structural differences, but is of great societal value in terms of possible disease treatment.

The $\alpha 4\beta 2$ nAChR is the most abundant subtype in the brain.⁷ Several findings suggest that $\alpha 4\beta 2$ receptors are involved in behavioral activity such as nicotine dependence, avoidance learning, and antinociception.⁸ Nicotine (**1**) and epibatidine (**2**) are both naturally occurring nAChR agonists that have attracted interest as lead candidates for analog synthesis aimed at identifying structures with improved pharmacological properties.^{1,9,10} For example, we recently reported that introduction of a hydrophobic or hydrogen-bonding alkynyl group into the C-5 position of the pyridine ring of epibatidine and A-84543 (**3**) significantly increased the selectivity for nAChRs containing $\beta 2$ subunits.¹¹ As part of our continuing interest in this project, we focused on chemically modifying (-)-cytisine **4**, a natural chiral quinolizidine alkaloid with a unique tricyclic structure.¹²

(-)-Cytisine (**4**) is reported to behave as a partial agonist at the $\alpha 4\beta 2$ nAChR with $EC_{50} \approx 1$ μ M and possess a low nanomolar binding affinity ($K_i \approx 1$ nM).¹³⁻¹⁶ [³H]Cytisine is frequently used as a radioligand in the study of nAChRs.^{13,17} Three total syntheses of cytisine¹⁸ were achieved in the 1950's. Recently, further interest in this alkaloid was stimulated by the two alternative approaches to cytisine reported by Coe¹⁹ and O'Neill et al.²⁰ Their efforts eventually resulted in the discovery of varenicline (**5**), a substantially re-engineered version of cytisine which has become a clinical candidate for use in smoking cessation.²¹ Following these reports, several other reports, including two enantioselective routes to this alkaloid as well the synthesis of other novel cytisine analogs have been published.²²⁻²⁴

Our own work in the nicotine area has been ongoing for approximately four years. Herein we report the synthesis and pharmacological evaluation of novel 9- and 10-substituted cytisine derivatives. To the best of our knowledge, the present work provides the first example of cytisine derivatives with 10-substitution. We modified O'Neill's synthetic strategy so as to allow implementation of the desired structural changes.²⁰

At the onset of our work in this area, we chose to synthesize some simplified cytisine analogs. Deletion of the C-1/C-13 bond in cytisine yields structure **6** (Figure 2). This compound and its isomers **7a** and **7b** can be prepared from piperidin-3-yl- and -4-ylmethanol by N-protection, iodide installation, and then reaction with α -pyridone, followed by deprotection (for the synthetic scheme, see Supporting Information).

As initial biological assays revealed that the binding affinities of compounds **6a-c** and **7a-b** at the nAChR subtypes were much lower (supporting information, Table A) than that of cytisine,¹⁶ we subsequently retained the core tricyclic structure while placing substituents on the pyridone ring. Our first objective was to introduce a hydroxymethyl group at the 10-position. The modified starting material required for this purpose, compound **10**, was prepared by borane reduction of commercially available 2-chloro-6-methoxyisonicotinic acid (**8**),²⁵ followed by hydroxyl protection (Scheme 1).

Pd-catalyzed Stille coupling of the preformed stannane²⁰ **11** with **10** under the optimized conditions proceeded smoothly to afford compound **12** in 79% isolated yield (Scheme 2). With *trans*-benzyl(chloro)bis(triphenylphosphine)palladium(II) as the catalyst, the reaction proceeded much faster, but gave low yields especially on scale-up. The in situ Stille coupling reaction gave inferior results.²⁰ Our attempts to repeat O'Neill's ester reduction at room temperature gave only the over-reduced methyl derivative. The required alcohol was obtained when the reduction was carried out using 1M LiAlH₄ solution in THF at -20 °C for 3.5 h. Following the strategy of O'Neill with some modifications, and after deprotection of the methoxymethyl (MOM) group with trifluoroacetic acid (TFA) at room temperature, we obtained *N*-benzyl-10-(hydroxymethyl) cytisine (**14**).

Attempted N-debenzylation of this compound using a variety of methods failed. Several catalytic hydrogenation protocols as well as the use of allyloxycarbonyl chloride²⁶ resulted in either no reaction or the formation of complex mixtures. Prolonged hydrogenation over 10% Pd-C at room temperature and 1 atm in MeOH gave the over-reduced product **15**. Our difficulty in carrying out this step was finally resolved by hydrogenation over Pd-C in the presence of Boc₂O.²⁷ A mixture of two products was formed, separation of which by semi-preparative HPLC gave the less polar over-reduced compound **16** and the more polar 10-hydroxymethyl derivative **17**. Final N-Boc deprotection with TFA²⁸ gave the 10-substituted racemic cytosine derivatives **15** and **17a**. Later we found that, under optimized conditions (20% Pd(OH)₂-C, (0.01 eq), H₂ (1 atm), (Boc)₂O, MeOH, 5 min, reflux), N-debenzylation proceeded smoothly to afford the 10-hydroxymethyl derivative **17** in 97% yield.

We further expanded the SAR of cytosine by preparing some additional analogs starting from (-)-cytosine itself. Most of the previously reported SAR of this molecule has focused on modifications at the alicyclic nitrogen (position 3) and also on the 9- and 11-positions of the pyridone ring.^{24,29,30} Moreover, a recent report showed that substitution at the 6-position could be brought about *via* a novel N-acyl migration reaction.³¹ As the biological activity of some of these known compounds has not been described in full, we selected four of the reported compounds together with new analogs in order to more fully flesh out our SAR studies. Following a literature procedure we synthesized the N-Boc-protected 9-bromocytosine **18** and its Stille coupling product with tri-*n*-butylvinylstannane.²⁹ Final deprotection with TFA afforded the derivative (-)-**19** (Scheme 3).^{29b}

We succeeded in carrying out a Suzuki coupling reaction of **18** with various boronic acids **20** to afford **21a-c** (Scheme 3). The synthesis of **21a** using the Stille coupling procedure²⁹ has already been reported. The 6-substituted derivatives **22** and **23** were also prepared following known procedures (Figure 3).³¹

In vitro binding affinities (*K_i* values) of the eight cytosine analogs **15**, **17a**, **19**, **21a**, **21b**, **21c**, **22** and **23** were measured at six defined nAChR subtypes expressed in stably transfected cell lines, using competition binding assays as previously reported (Table 1).^{10,16,32} In comparison to cytosine, the 10-methyl derivative **15** showed a similar binding affinity to α4β2 nAChRs, the major subtype in brain, but its affinities to the other subtypes were lower than those of cytosine. Thus, the selectivity of **15** for the α4β2 subtype over the other subtypes was improved significantly. This is especially true for selectivity between the α4β2 subtype and α3β4 subtype, the main subtype of ganglionic nAChRs. The affinity ratio of α3β4/α4β2 is larger than 3000-fold, which puts compound **15** among the most selective cytosine analogs that have been reported to date. The racemic nature of **15** probably will not have a substantial impact on this selectivity ratio. Interestingly, compound **17a** with a 10-hydroxymethyl group showed reduced activity at all subtypes, but again displays a larger α3β4/α4β2 affinity ratio. The 9-vinyl compound **19** was slightly more potent than cytosine at some of the nAChRs, but overall it behaves much like cytosine. All of the other six cytosine analogs showed considerably reduced binding affinities at all nAChRs. Compound **21a** did, however, retain its selectivity for the α4β2 nAChR.

The above eight cytosine analogs were next tested for their agonist activities at the two major neuronal nAChR subtypes, α3β4 and α4β2, using ⁸⁶Rb⁺ efflux assays previously reported.^{15,32} They were tested at 4 concentrations, 0.1, 1, 10 and 100 μM. Compound **19** stimulated ⁸⁶Rb⁺ efflux from cells expressing either α3β4 or α4β2 nAChR subtypes. The other seven compounds failed to show agonist activity at any of the concentrations used at these two nAChR subtypes. Compound **19** was further evaluated for its agonist potency and efficacy (Table 2). Consistent with its higher binding affinity at α4β2 than at α3β4 nAChRs, the compound was 20-fold more potent at the α4β2 subtype (EC₅₀ = 1.3 μM) than at the α3β4

subtype ($EC_{50} = 30 \mu M$). Compared to the efficacy of (-)-nicotine, the maximal efficacies of **19** were 83% and 22% of those of nicotine at the $\alpha 3\beta 4$ receptors and $\alpha 4\beta 2$ receptors, respectively. The overall agonist activity profile of **19** is very similar to that of cytosine.^{15, 33} Compounds **15** and **17a** didn't show agonist activity at $\alpha 4\beta 2$ nAChRs despite their high selectivity for this nAChR subtype in the binding assays. We tested these two compounds at concentrations from 0.1 μM to 100 μM for their antagonist activities at the $\alpha 4\beta 2$ and $\alpha 3\beta 4$ receptors. At concentrations up to 10 μM , the compounds did not significantly block nicotine stimulated responses. However, at 100 μM , both compounds inhibited more than 50% of the function of the $\alpha 4\beta 2$ nAChR subtype but only slightly inhibited the function of the $\alpha 3\beta 4$ nAChR subtype. These results indicate that the two 10-substituted cytosine analogs, compound **15** and **17a**, are weak antagonists of the $\alpha 4\beta 2$ nAChR subtype. These two compounds appear to have high affinity for the $\alpha 4\beta 2$ nAChR subtype in its desensitized conformation (i.e., in the receptor binding assays), but low affinity for the receptors in their resting conformation, as shown by their low potency in functional assays. This is typical of mostmost classical nicotinic ligands.³⁴

While (-)-cytosine is a potent, partial $\alpha 4\beta 2$ nAChR agonist, it does not show strong efficacy as a smoking cessation aid.³⁵ This lack of efficacy may result at least in part from its poor penetration of the blood-brain barrier (BBB).³⁶ Lipophilicity is one of the important indicators for predicting absorption and BBB penetration.³⁷ Compound **19** has a higher calculated ClogP value than that of cytosine (Table 1).³⁸ Its ClogP value is between those of nicotine (1.00) and epibatidine (1.80), both of which penetrate the BBB easily. Though far from conclusive, these data indicate that compound **19** may have an improved BBB penetration in comparison to cytosine. In light of the similarity of **19** and cytosine in relation to their binding affinity profiles and agonist activity profiles, if **19** is confirmed to have a better absorption and BBB penetration profile, it may be a better candidate to use in targeting CNS receptors *in vivo*, in particular for use in smoking cessation.

In conclusion, we have synthesized several new cytosine derivatives. The 10-substituted analogs **15** and **17a** are more than 3000-fold and 900-fold selective for the $\alpha 4\beta 2$ subtype over the $\alpha 3\beta 4$ nAChR subtype in binding assays, respectively, making them among the most discriminating nicotinic ligands that have been derived from cytosine. The 9-vinyl derivative **19** showed very similar functional features to those of cytosine. The fact that compound **19** may have an improved ability to penetrate the BBB adds to the value of this compound as a research tool. Therefore, compared to cytosine, ligand **19** may be a better compound for targeting CNS nicotinic receptors. Further efforts to characterize the pharmacological properties of these three compounds both *in vitro* as well as *in vivo* (drug discrimination) are underway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Figure 2.

Figure 3.

Scheme 1.

^aReagents and conditions: (a) $\text{BH}_3\text{-THF}$, THF, 0 °C, 5 h, 85%; (b) $\text{CH}_2(\text{OMe})_2$, $\text{BF}_3\cdot\text{OEt}_2$, CH_2Cl_2 , 0 °C, 3 h, 85%.

Scheme 2.

^aReagents and conditions: (a) Pd(PPh₃)₄, DMF, 130 °C, 15 h, 79%; (b) LiAlH₄, THF, -20 °C, 3.5 h, 59%; (c) BnBr, CH₃CN, reflux, 2 h; (d) H₂ (1 atm), PtO₂, Et₃N, MeOH, rt, 15 h, *cis* : *trans* = 5:1; *cis*, 67%; (e) MsCl, Et₃N, DCM, 0 °C, 30 min, 84%; (f) Toluene, reflux, 3 h, 83%; (g) TFA, rt, 3 h, 91%; (h) H₂ (1 atm), 10% Pd-C (1eq w/w), MeOH, rt, 15 h; (i) H₂ (1 atm), 20% Pd(OH)₂-C (0.1 eq), (Boc)₂O, MeOH, reflux, 30 min, 92%; (j) TFA, CH₂Cl₂, rt, 1 h, 87-93%.

Scheme 3.

^aReagents and conditions: (a) $\text{PdCl}_2(\text{PPh}_3)_2$, Dioxane, 120 °C, 1 h, 73%; (b) TFA, CH_2Cl_2 , 30 min, 81%. (c) $\text{Pd}(\text{PPh}_3)_4$, K_2CO_3 , DME/ H_2O , 85 °C, 15 h, 87%; (d) TFA, CH_2Cl_2 , 30 min, 85%.

Table 1

Binding affinities and calculated lipophilicities of (±)-Epibatidine (2), (-)-Cytisine (4) and Cytisine analogs at nAChR subtypes^a

Ligand	K_i (nM) ^b					Affinity ratio ($\alpha 3\beta 4/\alpha 4\beta 2$)		Calculated ClogP ^c
	$\alpha 2\beta 2$	$\alpha 2\beta 4$	$\alpha 3\beta 2$	$\alpha 3\beta 4$	Forebrain			
<i>rac</i> -15	7.5	180	540	6700	1.9	38	3526	1.15
<i>rac</i> -17a	32	300	467	10000	11	68	909	-0.32
(-)-19	0.7	9.0	28	95	0.73	2.3	130	1.50
(-)-21a	820	8100	12000	66000	420	3300	157	2.56
(-)-21b	8000	28000	36000	140000	8200	13000	17	4.38
(-)-21c	500	1700	6000	23000	390	590	59	2.73
(-)-22	17000	75000	250000	320000	24000	33000	13	0.57
(-)-23	14000	50000	300000	280000	16000	20000	18	0.71
(±)-Epibatidine (2)	0.02	0.09	0.04	0.57	0.06	0.16	10	1.81
(-)-Cytisine (4)	1.07	5.41	37.20	217.00	1.51	2.10	144	0.60

^a Competition binding assays were carried out in membrane homogenates of stably transfected cells or rat forebrain tissue as described previously¹⁶. The nAChRs were labeled with [³H]epibatidine. The K_d values for [³H]epibatidine used for calculating K_i values were 0.02 for $\alpha 2\beta 2$, 0.08 for $\alpha 2\beta 4$, 0.03 for $\alpha 3\beta 2$, 0.04 for $\alpha 4\beta 2$, 0.09 for $\alpha 3\beta 4$ and 0.05 for rat forebrain.

^b K_i values of the cytosine analogs shown are the mean of three to five independent measurements. For clarity and to save space, the SEM for the K_i values shown are omitted, but in all cases were less than 45% of the mean values. The K_i values of epibatidine (2) and cytosine (4) were published previously and are shown here for comparison.¹⁶

^c The ClogP values are calculated using the online version of Syracuse Research Corporation's LogKow/KOWWIN program; J. Pharm. Sci. **1995**, 84, 83-92.

Table 2Comparison of agonist activities of (-)-nicotine and (-)-**19** at two major nAChR subtypes, $\alpha 3\beta 4$ and $\alpha 4\beta 2$

Compound	$\alpha 3\beta 4$ nAChR's ^a		$\alpha 4\beta 2$ nAChR's ^b	
	EC ₅₀ (μ M)	Relative E _{max} (% of nicotine E _{max})	EC ₅₀ (μ M)	Relative E _{max} (% of nicotine E _{max})
(-)-Nicotine	35 $\pm$ 8	100	10 $\pm$ 1	100
(-)- 19	30 $\pm$ 7	83 $\pm$ 3	1.3 $\pm$ 0.4	22 $\pm$ 2

^a Agonist activities were measured using ⁸⁶Rb⁺ efflux assays. Values shown are the mean $\pm$ standard error of three independent experiments performed in quadruplicate.

^b KX $\alpha 3\beta 4$ R2 cells stably expressing rat $\alpha 3\beta 4$ nAChRs were used as described previously.^{16,32,33}

^c A procedure for measuring ⁸⁶Rb⁺ efflux from SH-EP1-h $\alpha 4\beta 2$ cells was adopted¹⁵ with minor modifications.