Allosteric Interactions of Quaternary Strychnine and Brucine Derivatives with Muscarinic Acetylcholine Receptors

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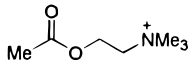
The affinity and allosteric properties of 22 quaternary derivatives of strychnine and brucine at the m1−m4 subtypes of muscarinic receptors have been analyzed and compared. The subtype selectivity, in terms of affinity, was in general m2 > m4 > m1 > m3. The highest affinities were found for *N*-benzyl, *N*-2-naphthylmethyl, and *N*-4-biphenylylmethyl strychnine (**13**, **14**, and **18**, respectively). All the strychnine and brucine derivatives were positively cooperative with the antagonist, *N*-methylscopolamine, at m2 receptors and, in the case of the strychnine analogues, were positively cooperative with *N*-methylscopolamine at least at one other subtype. The strychnine analogues were negatively cooperative with the neurotransmitter, acetylcholine, at all subtypes whereas brucine and five of the six derivatives examined were positively cooperative with acetylcholine at one or more subtypes (m1−m5) and exhibited different patterns of subtype selectivity. The ability to generate subtype-selective allosteric enhancers of acetylcholine binding and function may be of use in the development of drugs for the treatment of Alzheimer's disease.

Introduction

Muscarinic receptors have a widespread distribution in both the central and peripheral nervous systems where they mediate the actions of released acetylcholine (ACh). These actions include the stimulation of smooth muscle contraction, glandular secretion, modulation of cardiac function, motor control, temperature regulation, and memory.[1] There are five subtypes of muscarinic receptors (m1−m5), each with a characteristic regional distribution.[2,3] In addition, different biochemical and electrophysiological responses are associated with the different subtypes.[1−3] Potential targets for subtype-selective agents include disorders of intestinal motility, cardiac and urinary bladder function, asthma, analgesia, and Parkinson's disease.[2]



Acetylcholine (ACh)

Major efforts are also being made to develop agents which act to alleviate the effects on cognition of the cholinergic deficit present in the early stages of Alzheimer's disease. Such agents include cholinesterase inhibitors[4,5] (to prolong the actions of ACh) and muscarinic agonists[6−9] (as a replacement therapy). However, cholinesterase inhibition results in prolonged and non-selective stimulation of all cholinergic receptors, both nicotinic and muscarinic. Equally there are no muscarinic agonists of substantial selectivity,[2,10] at least based on "affinity". We have been investigating an alternative approach, one which aims to increase the efficiency of the binding interaction between ACh and its receptors in a subtype-selective manner.

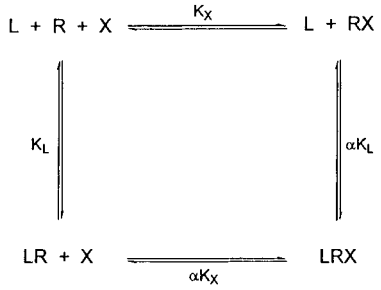


Figure 1. Ternary complex model of allosteric action of a ligand L with an allosteric agent X at a receptor R.

Muscarinic receptors have a second (allosteric) binding site in addition to the site which binds acetylcholine and competitive antagonists.[11−15] Occupancy of the allosteric site modulates but does not abolish the actions of ligands binding to the primary site. The interactions follow the allosteric ternary complex model (Figure 1), where K_X and K_L are the affinity constants for the binding of the allosteric ligand, X, and the primary ligand, L, respectively.[12,13,15] The value of α defines the nature of the cooperative interaction. For $\alpha < 1$ there is negative cooperativity, i.e., X and L inhibit the binding of each other, whereas if $\alpha > 1$ there is positive cooperativity, i.e., X and L increase the binding of each other. In situations where $\alpha = 1$, there is neutral

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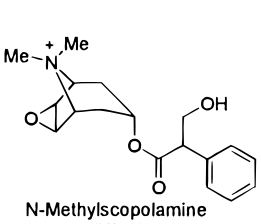
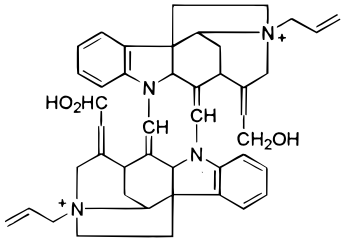
cooperativity, i.e., X and L have no effect on the equilibrium binding of each other to the receptor.

The allosteric interactions with muscarinic receptors were first quantitated using gallamine as the allosteric ligand.[12,13] A number of other ligands were later shown to interact allosterically,[14] but like gallamine, all these ligands were negatively cooperative with agonists and antagonists. More recently the neuromuscular blocker alcuronium **1** has been reported to enhance the binding of the antagonist *N*-methylscopolamine (NMS) to m2 receptors.[16] Subsequently we[15] and others[17] also discovered that the alkaloid strychnine **2** exhibited allosteric properties similar to alcuronium at muscarinic receptors.



N-Methylscopolamine (NMS)



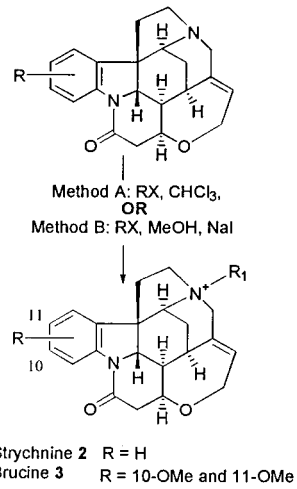
Alcuronium **1**

Strychnine is the prerequisite starting material for the synthesis of alcuronium and its analogues and, in simplest terms, may be viewed as a monomer of the functionalized dimer, alcuronium. Although strychnine is much smaller than alcuronium, it binds with similar affinities to the unliganded and NMS-liganded m1, m3, and m4 receptors.[15] The major difference in the binding of the two agents is at the unliganded and NMS-liganded m2 receptors where alcuronium exhibits a greater than 10-fold higher affinity than strychnine. There are also differences in the cooperativities of the two compounds with an antagonist (NMS) and agonist (ACh). For example, with NMS as antagonist, strychnine manifests neutral cooperativity at m1 receptors and positive cooperativity at m4 receptors,[15] whereas alcuronium inhibits NMS binding to m1 receptors and is neutrally cooperative at m4 receptors.[19] In addition, alcuronium is inhibitory against ACh at all muscarinic subtypes[18,19] whereas strychnine approaches neutral cooperativity with ACh at m1 and m4 receptors.[15]

The identification of strychnine as a muscarinic allosteric agent prompted us to search for other closely related structures. This work led to our discovery that another well-known alkaloid, brucine **3** (10,11-dimethoxy strychnine), is an allosteric muscarinic ligand,[20] a result which has now been confirmed.[18,21] Brucine binds with about 2−10-fold lower affinity to the unliganded and NMS-liganded m1−m4 receptors than strychnine.[20,21] Despite its low affinity for muscarinic receptors, brucine is of special interest since it is the only allosteric agent reported so far to increase both the affinity and functional potency of ACh at m1 receptors.[20]

The binding data for strychnine and brucine indicate that small changes in ligand structure (i.e., the two methoxy groups in brucine) result in different allosteric properties and hence different pharmacological properties. This is as one expects, since the effect of chemical modification of an allosteric ligand on its binding to the unliganded and liganded receptors is unlikely to be

Scheme 1. Quaternization Reactions of Strychnine and Brucine



Strychnine **2** R = H
Brucine **3** R = 10-OMe and 11-OMe

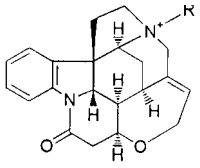
equal. There is therefore the potential for discovering related allosteric agents which exhibit positive, neutral, or negative cooperativities with ligands which occupy the primary binding site (including, and especially, the endogenous agonist). Since the size and nature of the cooperativity of an allosteric ligand depends on its affinity for both the unliganded and liganded state of a receptor, it follows that the analysis of both sets of structure−activity relationships (SARs) is necessary for the design of such agents.

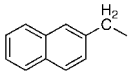
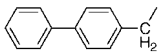
The complex structures of these alkaloids somewhat limit their usefulness as versatile chemical templates for the development of SAR series. Quaternization of the basic tertiary nitrogen atom appeared as the most appropriate synthetic strategy to generate a wide range of analogues or derivatives, taking into consideration that alcuronium itself contains quaternary ammonium centers. In this paper, we examine the effect of quaternization of both alkaloids on the affinity and cooperativity with an antagonist (NMS) and agonist (ACh) at the m1−m4 subtypes. A preliminary report of some of the findings has been published.[22]

Chemistry

The quaternization of strychnine and brucine with a variety of alkyl halides were carried out using either method A or B according to Scheme 1. In general the reactions proceeded in good yields but there were a number of reactions which failed, presumably in part due to the presence of electron-withdrawing groups on the α-carbon atom of the alkyl halide. Strychnine and brucine did not undergo quaternization with the following alkyl halides: bromomethyl methyl ether, 2-bromoethyl methyl ether, 2-iodo-1,1,1-trifluoroethane, 2-iodoethanol, 2-bromomethyl-1,3-dioxolane, and 2-(bromomethyl)tetrahydropyran. Strychnine *N*-oxide **11** was prepared from the reaction of strychnine and hydrogen peroxide.[23] N-Amination of brucine and strychnine to give **12** and **22**, respectively, was achieved with *o*-mesitylenesulphonylhydroxylamine, following a literature procedure described for N-amination of tertiary amines.[24,25] *N*-Chloromethyl brucine[20] was prepared following a procedure similar to that reported for *N*-chloromethyl strychnine.[26]

Table 1. Estimated log Affinities (log M^{-1}) of the Quaternary Derivatives of Strychnine at the Free and NMS Liganded Muscarinic (m1−m4) Receptors[a]



		m1		m2		m3		m4	
compd	R	free	NMS	free	NMS	free	NMS	free	NMS
2	H	4.9	4.9 ± 0.2 (4)	5.0	5.3 ± 0.1 (4)	4.2	4.0 (1)	5.0	5.2 ± 0.1 (4)
4	CH_3	<4.1	4.5 ± 0.1 (2)	4.4	5.1 ± 0.1 (2)	<4.0	4.1 ± 0 (2)	4.1	4.7 ± 0 (2)
5	CH_2CH_3	<4.5	4.6 ± 0.1 (2)	4.7	5.2 ± 0.1 (2)	4.2	4.2 ± 0.2 (2)	4.3	4.9 ± 0.1 (2)
6	$CH_2CH_2CH_3$	5.2	4.8 ± 0.1 (3)	5.2	5.5 ± 0.1 (3)	5.0	4.5 ± 0.1 (3)	5.2	4.9 ± 0.1 (3)
7	$C_6H_{11}CH_2$	<4.6	4.8 ± 0.1 (2)	<4.9	5.5 ± 0.1 (2)	4.0	4.1 ± 0.1 (2)	4.5	4.8 ± 0.1 (2)
8	$CH_2CH{=}CH_2$	4.7	4.7 ± 0.1 (2)	5.3	5.9 ± 0.1 (2)	4.6	4.6 ± 0 (2)	5.0	5.0 ± 0.1 (2)
9	$CH_2C{\equiv}CH$	5.2	4.7 ± 0.1 (2)	5.2	5.4 ± 0.1 (2)	4.7	4.5 ± 0.1 (2)	4.8	5.0 ± 0.1 (2)
10	CH_2CN	<4.0	4.3 ± 0.2 (2)	4.5	5.1 ± 0.1 (2)	<3.9	4.2 ± 0.1 (2)	<4.4	4.8 ± 0.1 (2)
11	O^-	<3.6	3.8 ± 0.1 (4)	<4.1	4.4 ± 0.1 (4)	<3.9	3.9 ± 0.1 (4)	<3.8	4.1 ± 0.1 (4)
12	NH_2	4.3	4.7 ± 0.1 (2)	<4.8	5.4 ± 0.1 (2)	<4.4	4.5 ± 0.2 (2)	<4.4	4.9 ± 0.1 (2)
13	$PhCH_2$	<5.0	5.2 ± 0.2 (2)	5.3	6.3 ± 0.1 (3)	<5.2	5.3 ± 0.1 (3)	<5.3	5.6 ± 0.2 (3)
14	$PhCH_2CH_2$	4.9	4.9 ± 0.1 (2)	5.3	5.7 ± 0.1 (2)	4.8	4.6 ± 0.1 (2)	5.2	5.5 ± 0.1 (2)
15	*m*-NO_2PhCH_2	<4.9	5.1 ± 0.1 (2)	<5.4	6.0 ± 0.1 (2)	<4.7	4.8 ± 0 (2)	<5.2	5.6 ± 0.1 (2)
16	*p*-NO_2PhCH_2	<4.8	5.3 ± 0.2 (2)	4.8	5.6 ± 0.1 (2)	4.6	4.6 ± 0.1 (2)	5.0	5.4 ± 0.1 (2)
17		<5.3	5.5 ± 0.2 (2)	5.5	6.1 ± 0.1 (2)	<5.8	6.0 ± 0.1 (2)	<5.6	5.9 ± 0.1 (2)
18		5.7	5.3 ± 0.2 (2)	5.6	5.9 ± 0.1 (2)	5.6	5.7 ± 0 (2)	<6.0	6.3 ± 0 (2)
1	alcuronium	4.9	4.5 ± 0.1 (2)	6.0	6.5 ± 0.1 (3)	<4	3.5 ± 0 (2)	4.7	4.7 ± 0 (2)

[a] The values for strychnine analogues were derived from at least two semiquantitative equilibrium assays and the indicated number of off-rate assays (*n*), as described in the Experimental Section, except that the values for strychnine itself (**2**) are from quantitative assays reported earlier.[15]

Pharmacology

The binding assays and analyses have been described previously.[15,21] These provide, where possible, estimates of the affinities of the allosteric compounds for the unliganded and NMS-liganded receptors (Tables 1 and 2) together with the cooperativities of the ligands with NMS and ACh (Tables 3 and 4). Four different types of experimental design were used: semiquantitative and quantitative equilibrium assays, "off-rate" assays to measure the effects of the allosteric ligands on ^{3}H-NMS dissociation, and a nonequilibrium assay. The experimental data were analyzed by nonlinear least squares analysis using equations derived from the ternary allosteric model (Figure 1). Descriptions of the methodology and the analysis and interpretation of the data are provided in the Experimental Section and as Supporting Information.

Results and Discussion

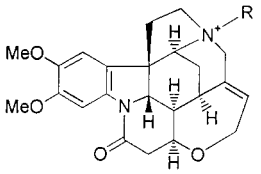
Quaternization of Strychnine. The binding affinities of N-substituted strychnines at the unliganded and NMS-liganded human m1−m4 muscarinic receptors are presented in Table 1.

Simple Alkyl Groups. A number of alkyl substituents of variable chain length and bulk were investigated. At the unliganded m1−m4 receptors, methyl substitution (**4**) decreased affinity (up to 8-fold) relative to strychnine. This decrease was partially reversed by ethyl substitution (**5**) and fully reversed and even increased (up to 6-fold at m3 receptors) by propyl substitution (**6**). A further increase in bulk generated by a cyclohexylmethyl substituent (**7**) decreased affinity. Allyl and propargyl substitution (**8** and **9**, respectively) did not produce any increase in affinity at the unliganded receptor above that produced by the propyl group.

In general, alkylation of strychnine resulted in marginal changes in affinity at the NMS-liganded m1 and m4 receptors. The binding affinity was also largely unaffected at m3 receptors except for propyl, allyl, and propargyl substituents where up to a 4-fold increase in affinity was observed. At m2 receptors, the only notable change in affinity was found with allyl substitution. *N*-Allyl strychnine **8** has a potency of about 1 μM at m2 NMS-liganded receptors, 4 times higher than that of strychnine itself. It is worth pointing out that the allyl group, also present in alcuronium, may be an important substituent for selectively increasing affinity at the NMS-liganded versus unliganded m2 receptors.

Polar Substituents. Studies with simple alkyl groups revealed that the allosteric binding site on muscarinic receptors can tolerate a variety of lipophilic substituents of different bulk without large changes in affinity. Polar or charged substituents can affect the charge density on the nitrogen atom in different ways and offer the potential for a variety of additional electrostatic interactions with the receptor. Quaternization with polar substituents such as CH_2CN and NH_2 (i.e., **10** and **12**) slightly reduced binding at m1, m2, and m4 unliganded and NMS-liganded receptors. However, substitution with *N*-oxide (**11**) causes a more pronounced reduction (up to 10-fold) of binding at m1, m2, and m4 receptors. In contrast, the binding affinity of **10**−**12** at the unliganded and NMS-liganded m3 receptors was not de-

Table 2. log Affinities (log M^{-1}) of the Quaternary Derivatives of Brucine at the Free and NMS Liganded Muscarinic (m1−m5) Receptors[a]



		m1		m2		m3		m4		m5	
compd	R	free	NMS	free	NMS	free	NMS	free	NMS	free	NMS
3	H	4.6 ± 0.1	4.5 ± 0.1 b (5)	4.5 ± 0.1	4.7 ± 0.1 b (4)	3.5 ± 0.1	2.9 ± 0.1 b (4)	4.8 ± 0.2	4.5 ± 0.1 b (4)	4.3 ± 0.1	3.3 ± 0.1 b (2)
19	CH_3	4.0	4.0 ± 0 a (2)	4.0	4.5 ± 0.1 a (2)	3.5	3.6 ± 0.1 a (2)	4.3	4.4 ± 0.1 a (2)	3.3	2.9 ± 1 a (2)
20	CH_2Cl	4.4 ± 0.2	4.1 ± 0.2 b (4)	4.2 ± 0.1	4.4 ± 0.1 b (4)	3.7 ± 0.1	3.3 ± 0.2 b (4)	4.3 ± 0.1	4.3 ± 0.1 b (4)	3.7 ± 0.1	3.3 ± 0.1 b (2)
21	O^-	3.4 ± 0.1	3.6 ± 0.1 b (4)	2.7 ± 0.1	3.3 ± 0.1 b (4)	2.8 ± 0.1	2.8 ± 0.1 c (3)	3.7 ± 0.1	3.7 ± 0.1 c (3)	2.5 ± 0.1	2.7 ± 0.1 b (2)
22	NH_2	3.8	3.8 ± 0.1 a (2)	<4.0	4.2 ± 0.1 a (2)	3.8	3.9 b (1)	<4.0	4.2 ± 0.1 a (2)	3.6	3.3 a (1)
23	$CH_2CH{=}CH_2$	4.4	4.2 c (1)	4.5	4.7 c (1)	3.8	3.5 c (1)	4.5	4.6 c (1)	3.8	3.3 ± 0.1 a (3)
24	$PhCH_2$	<4.6	4.4 ± 0.1 a (3)	4.6	4.8 ± 0.1 a (3)	>4.0	3.8 ± 0.1 a (3)	4.5	4.5 ± 0.1 a (3)	3.7	3.7 ± 0.1 a (2)

[a] The values for brucine analogues were obtained from either (a) semiquantitative and off-rate assays (see caption to Table 1), (b) quantitative equilibrium assays, or (c) quantitative nonequilibrium assays. The number of assays yielding quantitative data is indicated by (*n*). Some affinity values for compounds **3**, **20**, **21**, and **24** have been reported elsewhere.[21] The estimates of affinity at ^{3}H-NMS-occupied receptors derived from quantitative equilibrium and nonequilibrium assays were the product of the affinity estimate at the free receptor and the cooperativity with ^{3}H-NMS.

creased substantially relative to strychnine and in fact was increased 3-fold for *N*-amino strychnine **12** at the NMS-liganded m3 receptor.

Aralkyl Substituents. Several aromatic substituents containing one or two phenyl rings were chosen for this study. At the *unliganded* receptors the substituents in **13**−**17** produced essentially no change in affinity at m1 receptors and only exhibited 2−3-fold increases in affinity at m2 and m4 receptors. Larger increases were found at m3 receptors (at least 10−20-fold in the case of the benzyl and 2-naphthylmethyl analogues, **13** and **17**, respectively). *m*- or *p*-Nitro substitution of the benzyl ring **(15** and **16**, respectively) had little effect on binding to m1 and m4 receptors. The most substantial effect on binding to the unliganded receptor was found for the 4-biphenylylmethyl analogue **18** where 4−25-fold increases in affinity over strychnine at all subtypes was observed. This compound has a higher affinity than alcuronium at the unliganded m1, m3, and m4 receptors and only a slightly lower affinity at m2 receptors.

At the NMS-liganded m1 and m4 receptors, only small increases in affinity (up to 5-fold) were produced by aralkyl substitution. In contrast, larger effects were found at m2 and m3 receptors (10−20-fold increases being found for *N*-benzyl strychnine **13** at m2 and m3 and 100-fold for the 1-naphthylmethyl analogue **17** at m3 receptors). The affinity of *N*-benzyl strychnine **13** for the NMS-liganded m2 receptor approaches that of alcuronium.

In summary, it has been possible to obtain agents which have higher affinities than strychnine at all four muscarinic subtypes examined and which have comparable or higher affinities than alcuronium at m1−m4 subtypes. Substituents such as benzyl, 2-naphthylmethyl, or 4-biphenylmethyl increase muscarinic affinity by 10−100-fold at the different subtypes, an indication that π-electron ring systems may be a requirement for high-affinity interaction at the allosteric site, at least in this series of compounds.

Quaternization of Brucine. A limited number of quaternized brucine analogues were synthesized and their affinities at the unliganded and NMS-liganded m1−m5 receptors determined (Table 2). At the unliganded receptors the substituents produced no significant increases in affinity relative to brucine at any subtype. Substantial decreases in affinity were found for brucine *N*-oxide **21** at all subtypes (up to 60-fold at m2 and m5 receptors) and for *N*-amino brucine at all subtypes except at m3 receptors. At the NMS-liganded m1, m2, m4, and m5 receptors, N-substitution did not increase affinity except for benzyl substitution (**24**) at m5 receptors (∼2-fold increase). However, with the exception of brucine *N*-oxide (where affinity was unchanged), N-substitution increased affinity at the NMS-liganded m3 receptors, with the largest gain (10-fold) residing for *N*-amino substitution (**22**).

Comparison of Affinities of Strychnine and Brucine Analogues. As mentioned in the Introduction, the presence of the two methoxy groups in brucine decreases its affinity by about 2−10-fold at m1−m4 unliganded and NMS-liganded receptors. With the exception of **19** at m4 unliganded receptors, this observation also holds true for all the corresponding analogues of strychnine and brucine (e.g., **4** and **19**, **8** and **23**, **11** and **21**, **12** and **22**, and **13** and **24**). On the other hand, the binding at ACh-liganded receptors is less affected in some cases since these compounds can manifest positive cooperativity with ACh (e.g., brucine at m1, **22** at m3, etc.). The methoxy groups may therefore be regarded as an important structural feature for generating positive cooperativity with ACh in these molecules.

Subtype Selectivity−Affinity. None of the analogues discussed here approached the m2 selectivity of

Table 3. Cooperativity of Quaternary Strychnines with NMS and ACh at m1−m4 Receptors[a]

	cooperativity with NMS				cooperativity with ACh			
compd	m1	m2	m3	m4	m1	m2	m3	m4
alkyl								
2	0	++	-	+	-	--	--	-
4	++	++	+	++	-	--	-	--
5	+	++	0	++	-	--	-	--
6	-	+	-	-	--	--	--	--
7	+	++	+	+	--	--	--	--
8	0	++	0	0	--	--	--	-
9	-	+	-	+	--	--	--	-
polar								
10	+	++	+	++	-	--	-	-
11	+	++	+	+	-	-	-	-
12	+	++	+	++	-	-	-	-
aromatic								
13	+	++	+	+	--	--	-	-
14	0	++	-	+	--	--	--	--
15	+	++	+	++	-	-	-	-
16	++	++	0	++	-	--	-	--
17	+	++	+	+	-	--	-	-
18	-	++	0	+	--	--	--	-
1	-	++	-	0	--	--	-	--

[a] Cooperativity estimates from the data in Tables 1 and 2, and other semiquantitative assays. Key (symbol, cooperativity): --, <0.2; -, 0.2−0.8; 0, 0.81−1.19; +, 1.2−2; ++, >2.

Table 4. Cooperativity of Quaternary Brucines with NMS and ACh at m1−m5 Receptors

	cooperativity with NMS					cooperativity with ACh				
compd	m1	m2	m3	m4	m5	m1	m2	m3	m4	m5
3	0	+	-	-	--	+	-	-	-	--
19	0	++	0	0	-	-	-	+	-	--
20	-	+	-	0	-	-	--	++	0	--
21	+	++	0	0	+	0	-	++	+	-
22	0	+	0	+	-	-	-	++	-	-
23	-	+	-	+	-	--	--	+	-	--
24	-	++	-	0	0	-	-	+	-	-

[a] Cooperativity estimates from the data in Tables 1 and 2, and other semiquantitative assays. Key (symbol, cooperativity): --, <0.2; -, 0.2−0.8; 0, 0.81−1.19; +, 1.2−2; ++, >2.

alcuronium. However, the pattern of affinity found for alcuronium and strychnine at the unliganded and NMS-liganded receptors (m2 > m4 > m1 > m3) was, in general, found in an attenuated form for all the strychnine and brucine analogues. The only marginal exceptions to the rank order were **17**, **18**, and **21** where some differences from the expected sequence were found.

Subtype Selectivity−Cooperativity with NMS. All strychnine analogues examined were positively cooperative with NMS at m2 receptors and, except for alcuronium and **6**, were positive with at least one other subtype (Table 3).

Strychnines with polar N-substituents were positively cooperative with NMS at all subtypes, and the *N*-aralkyl substituted strychnines also supported positive cooperativity more than strychnine itself or its *N*-alkyl derivatives. It is evident therefore that the ability of a muscarinic receptor to support positive cooperativity with NMS is dependent on both the receptor subtype as well as the general characteristics of the N-substituent.

The brucine analogues examined were all positively cooperative with NMS at m2 receptors, as found for the strychnine analogues (Table 4). However, they showed a much lower incidence of positive cooperativity with NMS at the other subtypes than did the strychnines (17% versus 72% for comparable N-substituents and subtypes).

Subtype Selectivity−Cooperativity with Acetylcholine. No strychnine analogue exhibited positive cooperativity with ACh at m1−m4 receptors (Table 3) as determined by the semiquantitative equilibrium binding assay in conjunction with the off-rate assays,[15,21] although for several derivatives, this cooperativity appeared to be low and approached neutrality for *N*-methyl strychnine **4** at m1 receptors. Low negative cooperativities with ACh were observed least often at m2 receptors (17% incidence versus 47−59% at the other subtypes) and most often for the strychnine derivatives with polar substituents (92% incidence) relative to the other analogues (37% incidence).

In contrast, brucine and its analogues show a propensity for positive cooperativity with ACh (Table 4). The data were derived from the assays described in this paper together with more detailed binding assays on **18**, **20**, and **21**[21] and include data from m5 as well as m1−m4 receptors. Positive cooperativity was manifest in a subtype-selective fashion with brucine enhancing acetylcholine binding at m1 receptors, **19**−**24** enhancing at m3 receptors, and brucine *N*-oxide **21** enhancing acetylcholine binding at m4 receptors. No brucine analogue exhibited positive cooperativity at m2 or m5 receptors. This tendency to enhance acetylcholine binding at m3 receptors and to inhibit acetylcholine binding at m2 receptors was the reverse of the allosteric interactions of the brucine and strychnine analogues with NMS at these subtypes. Neutral cooperativity with ACh was observed for **20** at m4 receptors and **21** at m1 receptors.

The ability of allosteric agents to exhibit neutral cooperativity with a neurotransmitter (in this case acetylcholine) at a receptor subtype means that it will bind to that subtype but not change the binding (and function) of that subtype at any concentration while having the ability to modulate receptor function at another subtype at which it has non-neutral, i.e., positive or negative, cooperativity. This property is unique for allosteric agents and represents an additional level of receptor subtype selectivity over competitive agents.

Conclusions

In this paper we have been able to demonstrate that modification of the structure of strychnine and brucine has generated a series of compounds in which the affinities and cooperativities with NMS and acetylcholine have been altered substantially in a subtype-dependent manner. Quaternization of strychnine has led to a number of agents with greater affinities and cooperativities with NMS than strychnine itself, although none of the compounds increased the affinity of ACh. Notable compounds are *N*-benzyl strychnine (m2), *N*-naphthylmethyl strychnine (m1−m4), and *N*-biphenylmethyl strychnine (m1−m4). In contrast, quaternization of brucine has led to derivatives which increase ACh affinity at m3 and m4 receptors. These are the first examples of allosteric enhancers of ACh at m3 and m4 receptors and may serve as useful pharmacological tools in this field. Detailed SAR analyses are precluded because of the relatively low-affinity interac-

tions observed in this series of compounds and the complexities associated with cooperativity being a comparison of two very closely related SAR series. Even with more potent ligands the effect of substituents on cooperativity will be difficult to predict and may only be possible after a thorough examination of quantitative SAR at both the unliganded and liganded receptors.

This is the first time that the properties of a substantial series of muscarinic allosteric agents which demonstrate positive cooperativity with both an antagonist and acetylcholine has been described. Similar studies with more potent allosteric templates may furnish agents which could be useful for the treatment of the cognitive deficits in the early stages of Alzheimer's disease.

Experimental Section

General Methods. All starting materials and brucine N-oxide **21** are available commercially and were used without purification except for liquid halides which were distilled before use. Melting points (mp) were carried out in open capillaries on an electrothermal digital melting point apparatus (IA9100) and are uncorrected. Mass spectra were run by fast atom bombardment on a VG Analytical ZAB-SE double-focusing magnetic sector mass spectrometer and were consistent with the assigned structures. Elemental analyses were performed by the microanalytical section of the Chemistry Department, University College London, and such analyses reported by the symbols of the elements indicate observed values that were within ±0.4% of the calculated values. Analytical high-pressure liquid chromatography (HPLC) was performed on a Beckman System Gold with UV detection at 254 nm and a (4 × 4 + 250 × 4 mm) Lichrosorb RP Select B 5 mm column with a flow rate of 1 mL/min. HPLC retention times (rt) were obtained using the following conditions: a gradient run from 25% MeOH in H_2O to 75% MeOH in H_2O over 17.5 min and then to 100% MeOH over the next 7.5 min. Strychnine **2** and brucine **3** had retention times of 9.5 and 10.1 min, respectively.

Quaternization of Strychnine and Brucine. The quaternization of strychnine and brucine were carried out according to methods A or B, and to our best of knowledge, only their novel quaternary derivatives are reported here. The target compounds for which no synthetic procedure is described were either available commercially or synthesized according to method A.

Method A. To a solution of the alkaloid (1 g) in chloroform (50 mL) was added a solution of the respective halide (3-fold excess) in chloroform (25 mL). This was stirred at room temperature or elevated temperatures until crystallization occurred. The product was collected by filtration, washed with chloroform, and dried at room temperature in vacuum. The following compounds were prepared in quantitative yields and no further purification was necessary.

N-Propargyl Strychnine Bromide (9): Obtained as a white solid; mp > 300 °C (dec); HPLC rt = 9.5 min; MS *m*/*z* 373 (M^+, 100). Anal. ($C_{24}H_{25}N_2O_2Br$): C, H, N.

***N*-Phenylethyl Strychnine Bromide (14):** Obtained as a white solid; mp 258−261 °C; HPLC rt = 16.1 min; MS *m*/*z* 439 (M^+, 100). Anal. ($C_{29}H_{31}N_2O_2Br\cdot1.5H_2O$): C, H, N.

***N*-3-Nitrobenzyl Strychnine Bromide (15):** Obtained as a white solid; mp > 280 °C (dec); HPLC rt = 13.8 min; MS *m*/*z* 470 (M^+, 27). Anal. ($C_{28}H_{28}N_3O_4Br\cdot1.5H_2O$): C, H, N.

***N*-4-Nitrobenzyl Strychnine Bromide (16):** Obtained as a white solid; mp 269−270 °C; HPLC rt = 13.8 min; MS *m*/*z* 470 (M^+, 100). Anal. ($C_{28}H_{28}N_3O_4Br\cdot1H_2O$): C, H, N.

***N*-Allyl Brucine Iodide (23):** Obtained as a white solid; mp > 230 °C (foamed); HPLC rt = 10.4 min; MS *m*/*z* 435 (M^+, 100). Anal. ($C_{26}H_{31}N_2O_4Br\cdot1.5H_2O$): C, H, N.

***N*-Benzyl Brucine Iodide (24):** Obtained as a white solid; mp > 230 °C (foamed); HPLC rt = 13.2 min; MS *m*/*z* 485 (M^+, 100). Anal. ($C_{30}H_{33}N_2O_4Br\cdot1H_2O$): C, H, N.

Method B. To a solution of strychnine (1 g) in MeOH (50 mL) were added sodium iodide (0.5 g) and a solution of the respective halide (3-fold excess) in MeOH (25 mL). The solution was stirred at room temperature until precipitation occurred. The product was collected by filtration, washed with small amounts of MeOH and H_2O, and dried in vacuum.

***N*-Cyclohexylmethyl Strychnine Iodide (7):** Obtained as a white solid; mp 305−307 °C; HPLC rt = 14.7 min; MS *m*/*z* 431 (M^+, 100). HRMS ($C_{28}H_{35}N_2O_2I$) found, *m*/*z* 431.2730; calcd, 431.2717.

***N*-2-Methylnaphthyl Strychnine Iodide (17):** Obtained as a white solid; mp 276−277 °C; HPLC rt = 17.9 min; MS *m*/*z* 475 (M^+, 17). Anal. ($C_{32}H_{31}N_2O_2I$): C, H, N.

***N*-4-Methylbiphenyl Strychnine Iodide (18):** Obtained as an off-white solid; mp 250−251 °C; HPLC rt = 19.4 min; MS *m*/*z* 501 (M^+, 90). Anal. ($C_{34}H_{33}N_2O_2I\cdot0.5H_2O$): C, H, N.

***N*-Chloromethyl Brucine Chloride (20).** A solution of brucine (0.2 g, 0.5 mmol) in CH_2Cl_2 (20 mL) was allowed to stand at room temperature for 2 days. The crystalline product was collected by filtration, washed with CH_2Cl_2, and dried in vacuo to give **20** in 80% yield; mp = 210 °C (dec); HPLC rt = 10.1 min; MS *m*/*z* 394 (M^+, 66). Anal. ($C_{24}H_{28}Cl_2N_2O_4\cdot H_2O$): C, H, N.

***N*-Amino Brucine Mesitylenesulfonate (22).** A solution of *o*-mesitylenehydroxylamine (0.24 g, 1.1 mmol) in CH_2Cl_2 (2 mL) was added dropwise to a cold solution of brucine (0.44 g, 1.1 mmol) in CH_2Cl_2 (2 mL). The solution was allowed to stand at room temperature for 15 min, and ether was then added until precipitation occurred. The white crystalline product was collected by filtration and recrystallized from a mixture of 2-propanol/ether (1:1, v/v) to give **22** in 51% yield: mp = 242−243 °C (dec); HPLC rt = 10.2 min; MS *m*/*z* 410 (M^+, 100). Anal. ($C_{32}H_{39}N_3O_7S\cdot0.9H_2O$): C, H, N.

Radioligand Binding. CHO cells stably expressing cDNA encoding human muscarinic m1−m5 receptors were grown to confluence in α-MEM medium (GIBCO) containing 10% (v/v) newborn calf serum, 50 U/mL penicillin, 50 mg/mL streptomycin, and 2 mM glutamine at 37 °C under 5% CO_2 and were harvested by scraping in a hypotonic medium (20 mM Hepes + 10 mM EDTA, pH 7.4). Membranes were prepared at 0 °C by homogenization with a Polytron followed by centrifugation (40000*g*, 15 min), were washed once in 20 mM Hepes + 0.1 mM EDTA, pH 7.4, and were stored at −70 °C in the same buffer at protein concentrations of 2−5 mg/mL. The yields of receptor were approximately 5, 1, 7, 2, and 1 pmol/mg of total membrane protein for the m1−m5 subtypes, respectively.

The binding assays and analyses have been described previously.[15,21] In general, binding of ^{3}H-NMS was conducted in a buffer containing 20 mM Hepes + 100 mM NaCl + 10 mM $MgCl_2$ (pH 7.4) at 30 °C in a volume of 1 mL. Membranes were collected by filtration over glass fiber filters (Whatman GF/B) presoaked in 0.1% polyethylenimine, using a Brandel cell harvester (Semat, Herts, UK), extracted overnight in scintillation fluid (ReadySafe, Beckman), and counted for radioactivity in Beckman LS6000 scintillation counters. Membrane protein concentrations (5−50 μg/mL) were adjusted so that not more than about 15% of added radioligand was bound. Nonspecific binding was measured in the presence of 10^{-6} M QNB (an antagonist with picomolar potency) and accounted for 1−5% of total binding. Most brucine analogues were dissolved in DMSO which, at the highest final concentration of 1%, had no effect on binding. *N*-Chloromethyl brucine **20** was dissolved in water and brucine *N*-oxide **21** was dissolved in buffer.

The semiquantitative and quantitative equilibrium assays utilized an incubation time of at least 2 h in the presence of GTP (2×10^{-4} M) and ^{3}H-NMS concentrations of 0.1−0.7 nM, depending on receptor subtype (the K_d values at m1−m5 receptors from at least six assays were 111 ± 12, 337 ± 34, 211 ± 16, 78 ± 7, and 603 ± 35 pM, respectively). All strychnine and brucine analogues were assessed with a semiquantitative assay in which binding of ^{3}H-NMS in the absence and presence of an IC_{50} concentration of ACh was measured alone and in the presence of three concentrations

of test agent. These data were transformed into "affinity ratios"—the apparent affinity of the primary ligand (^{3}H-NMS or ACh) in the presence of test agent divided by the "true" affinity in the absence of test agent. According to the ternary complex allosteric model, this novel measure follows the occupancy of the allosteric agent at the unliganded receptor and the asymptotic value corresponds to the cooperativity factor with the primary ligand. Visual inspection of affinity ratio plots allows an estimate of the direction and magnitude of cooperativity of the test agent with each primary ligand, and if the agent is negatively cooperative with either ^{3}H-NMS or ACh, then its IC_{50} corresponds approximately to its K_d at the unliganded receptor. Selected compounds were studied further with a quantitative equilibrium assay which measured inhibition curves with ACh alone and in the presence of three concentrations of test agent. The data were fitted to the allosteric model using nonlinear regression analysis (SigmaPlot, SPSS, Erkrath, Germany), to provide quantitative estimates of the affinity of the agent at the unliganded receptor and its cooperativity with ^{3}H-NMS and ACh.

In the off-rate assay a high concentration of membranes (2−4 mg of protein/mL) was incubated with a high concentration of ^{3}H-NMS (5 nM) for about 15 min. Then 10 μL aliquots were distributed into tubes which were empty or contained 1 mL of 10^{-6} M QNB alone and in the presence of four concentrations of allosteric agent. Nonspecific binding was measured in separately prepared tubes containing 10 μL of membrane and 2 mL of ^{3}H-NMS + QNB. Some time later, about 2.5 dissociation half-lives, the samples were filtered (^{3}H-NMS dissociation rate constants at m1−m5 receptors from at least seven assays were 0.075 ± 0.006, 0.361 ± 0.018, 0.064 ± 0.003, 0.066 ± 0.003, and 0.033 ± 0.001 min^{-1}, respectively, corresponding to half times of 9.2, 1.9, 10.8, 10.5, and 21 min, respectively). The data were transformed to dissociation rate constants, expressed as the percentage inhibition of the "true" ^{3}H-NMS dissociation rate constant measured in the absence of allosteric agent, and fitted to a hyperbolic function using nonlinear regression analysis. This curve corresponds theoretically to the occupancy curve of the allosteric agent at the ^{3}H-NMS-occupied site, and the regression analysis provides a quantitative estimate of the affinity of the agent for the ^{3}H-NMS-occupied receptor. This value is used with the results from the semiquantitative equilibrium assay to obtain an estimate of the affinity of the agent at the free receptor and its cooperative effects. Examples of the logic applied to the analyses of the binding data to obtain affinities and cooperativities are given as Supporting Information.

The nonequilibrium assay utilizes the fact that inhibition of ^{3}H-NMS dissociation by an agent will also result in inhibition of ^{3}H-NMS association, so that at high concentrations of agent the assay will not attain binding equilibrium. There were two sets of tubes, initially containing 1 mL of test agent at its final concentration. One set of tubes then received 10 μL of receptors, followed by 10 μL of ^{3}H-NMS; so in this set of tubes binding equilibrium was attained by ^{3}H-NMS association. The other set of tubes received 20 μL of a mixture of receptors and ^{3}H-NMS, preincubated for a few minutes to allow all the receptors to bind ^{3}H-NMS; so in this set of tubes binding equilibrium was attained by ^{3}H-NMS dissociation. The results of this assay allowed a clear distinction between inhibitory effects on ^{3}H-NMS binding caused by negative cooperativity or by incomplete equilibration and, when fitted to the appropriate model,[15] provided quantitative estimates of the affinity of the agent for the free receptor and its cooperativity with ^{3}H-NMS.

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Supporting Information Available: A detailed description of the methodology and the analysis and interpretation of the data are provided for some the compounds (9 pages). Ordering information is given on any current masthead page.

References

(1) For a review, see: Caulfield, M. P. Muscarinic receptors-characterization, coupling and function. *Pharmacol. Ther.* **1993**, *58*, 319−379.
(2) Caulfield, M. P.; Birdsall, N. J. M. International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. *Pharmacol. Rev.* **1998**, *50*, 279−290.
(3) Hulme, E. C.; Birdsall, N. J. M.; Buckley, N. J. Muscarinic receptor subtypes. *Annu. Rev. Pharmacol. Toxicol.* **1990**, *30*, 633−673.
(4) Kawakami, Y.; Inoue, A.; Kawai, T.; Wakita, M.; Sugimoto, H.; Hopfinger, A. J. The rationale for E2020 as a potent acetylcholinesterase inhibitor. *Bioorg. Med. Chem.* **1996**, *4*, 1429−1446.
(5) Wagstaff, A. J.; McTavish, D. Tacrine. A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic efficacy in Alzheimer's disease. *Drugs Aging.* **1994**, *4*, 510−540.
(6) Lambrecht, G.; Moser, U.; Grimm, U.; Hermann, U.; Hildebrandt, C.; Waelbroeck, M.; Christophe, J.; Mutschler, E. New functionally selective muscarinic agonists. *Life Sci.* **1993**, *52*, 481−486.
(7) Ensinger, H. A.; Doods, H. N.; Immel-Sehr, A. R.; Kuhn, F. J.; Lambrecht, G.; Mendla, K. D.; Muller, R. E.; Mutschler, E.; Sagrada, A.; Walther, G.; Hammer, R. WAL 2014 - a muscarinic agonist with preferential neuron-stimulating properties. *Life Sci.* **1993**, *52*, 473−480.
(8) Freedman, S. B.; Dawson, G. R.; Iversen, L. L.; Baker, R.; Hargreaves, R. J. The design of novel partial muscarinic agonists that have functional selectivity in pharmacological preparations in vitro and reduced side-effect profile in vivo. *Life Sci.* **1993**, *52*, 489−495.
(9) Jaen, J.; Barrett, S.; Brann, M.; Callahan, M.; Davis, R.; Doyle, P.; Eubanks, D.; Lauffer, L.; Lipinski, W.; Moreland, D.; Nelson, C.; Raby, C.; Schwarz, R.; Spencer, C.; Tecle, H. In vitro and in vivo evaluation of the subtype-selective agonist PD 151832. *Life Sci.* **1995**, *56*, 845−852.
(10) Caulfield, M. P. Muscarinic receptor classification. In *Muscarinic receptor subtypes in smooth muscle*; Eglen, R. M., Ed.; CRC Press: New York, 1997; pp 1−37.
(11) Clark, A. L.; Mitchelson, F. The inhibitory effect of gallamine on muscarinic receptors. *Br. J. Pharmacol.* **1976**, *58*, 323−331.
(12) Stockton, J. M.; Birdsall, N. J. M.; Burgen, A. S.; Hulme, E. C. Modification of the binding properties of muscarinic receptors by gallamine. *Mol. Pharmacol.* **1983**, *23*, 551−557.
(13) Ehlert, F. J. Gallamine allosterically antagonizes muscarinic receptor-mediated inhibition of adenylate cyclase activity in the rat myocardium. *J. Pharmacol. Exp. Ther.* **1988**, *247*, 596−602.
(14) Lee, N. H.; El-Fakahany, E. E. Allosteric antagonists of the muscarinic acetylcholine receptor. *Biochem. Pharmacol.* **1991**, *42*, 199−205.
(15) Lazareno, S.; Birdsall, N. J. M. Detection, quantitation and verification of allosteric interactions of agents with labelled and unlabelled ligands at G protein-coupled receptors: interactions of strychnine and acetylcholine at muscarinic receptors. *Mol. Pharmacol.* **1995**, *48*, 362−378.
(16) Tucek, S.; Musilkova, J.; Nedoma, J.; Proska, J.; Shelkovnikov, S.; Vorlicek, J. Positive cooperativity in the binding of alcuronium and N-methylscopolamine to muscarinic acetylcholine receptors. *Mol. Pharmacol.* **1990**, *38*, 674−680.
(17) Proska, J.; Tucek, S. Competition between positive and negative allosteric effectors on muscarinic receptors. *Mol. Pharmacol.* **1995**, *48*, 696−702.
(18) Jakubik, J.; Bakakova, L.; El-Fakahany, E. E.; Tucek, S. Positive cooperativity of acetylcholine and other agonists with allosteric ligands on muscarinic acetylcholine receptors. *Mol. Pharmacol.* **1997**, *52*, 172−179.
(19) Lazareno, S.; Gharagozloo, P.; Popham, A.; Birdsall, N. J. M. Unpublished work.
(20) (a) Birdsall, N. J. M; Farries, T.; Gharagozloo, P.; Kobayashi, S.; Kuonen, D., Lazareno, S.; Popham, A.; Sugimoto, M. Selective allosteric enhancement of the binding and actions of acetylcholine at muscarinic receptor subtypes. *Life Sci.* **1997**, *60*, 1047−1052. (b) Birdsall, N. J. M.; Farries, T.; Gharagozloo, P.; Kobayashi, S.; Lazareno, S.; Sugimoto, M. Subtype-selective positive cooperative interactions between brucine analogues and acetylcholine at muscarinic receptors: functional studies. *Mol. Pharmacol.*, in press.
(21) Lazareno, S.; Gharagozloo, P.; Kuonen, D.; Popham, A.; Birdsall, N. J. M. Subtype-selective positive cooperative interactions between brucine analogues and acetylcholine at muscarinic receptors: radioligand binding studies. *Mol. Pharmacol.* **1998**, *53*, 573−589.

(22) Hiraoka, T.; Birdsall, N. J. M.; Gharagozloo, P.; Lazareno, S.; Strychnine and Brucine derivs., indole derivs. and pentacyclic tetrahydrocarbazoles as allosteric effectors at muscarinic receptors. Brit. UK Pat. Appl. 2292685 A, 1996; *Chem. Abstr.* **1996**, *125*, 86966d.
(23) Oesterlin, M. Investigations and Reactions with Strychnine Oxide. *Chem. Ber.* **1943**, *76B*, 224−229.
(24) Tamura, Y.; Minamikawa, J.; Ikeda, M. *o*-Mesitylenesulphonylhydroxylamine and Related Compounds - Powerful Aminating Reagents. *Synthesis* **1977**, 1−17.
(25) Tamura, Y. Tertiary amines, Novel Method for 1,1,1-Trisubstituted Hydrazinium Salts. *Tetrahedron* **1973**, *29*, 1063−1068.
(26) Caws, A. C.; Foster, G. E. The purity of chloroform B.P. *J. Pharm. Pharmacol.* **1957**, *9*, 824−832.

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