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European Journal of Pharmacology 539 (2006) 27–33
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Pharmacological characterisation of strychnine and brucine analogues at glycine and α7 nicotinic acetylcholine receptors

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Received 14 November 2005; received in revised form 9 March 2006; accepted 3 April 2006
Available online 9 May 2006

Abstract

Strychnine and brucine from the plant *Strychnos nux vomica* have been shown to have interesting pharmacological effects on several neurotransmitter receptors, including some members of the superfamily of ligand-gated ion channels. In this study, we have characterised the pharmacological properties of tertiary and quaternary analogues as well as bisquaternary dimers of strychnine and brucine at human α1 and α1β glycine receptors and at a chimera consisting of the amino-terminal domain of the α7 nicotinic receptor (containing the orthosteric ligand binding site) and the ion channel domain of the 5-HT_{3A} serotonin receptor. Although the majority of the analogues displayed significantly increased K_i values at the glycine receptors compared to strychnine and brucine, a few retained the high antagonist potencies of the parent compounds. However, mirroring the pharmacological profiles of strychnine and brucine, none of the analogues displayed significant selectivity between the α1 and α1β subtypes. The structure–activity relationships for the compounds at the α7/5-HT_3 chimera were significantly different from those at the glycine receptors. Most strikingly, quaternization of strychnine and brucine with substituents possessing different steric and electronic properties completely eliminated the activity at the glycine receptors, whereas binding affinity to the α7/5-HT_3 chimera was retained for the majority of the quaternary analogues. This study provides an insight into the structure–activity relationships for strychnine and brucine analogues at these ligand-gated ion channels.

Keywords: Strychnine; Brucine; Glycine receptor; Nicotinic acetylcholine receptor-α7

1. Introduction

The family of ligand-gated ion channels contains receptors for the neurotransmitters acetylcholine, serotonin, γ-aminobutyric acid and glycine (Jensen et al., 2005; Karlin, 2002; Laube et al., 2002; Lynch, 2004; Rajendra et al., 1997; Sieghart and Sperk, 2002). The receptors are homo- or heteromeric assemblies of five subunits, where the orthosteric sites are located in the interfaces of the amino-terminal domains of the subunits, and the transmembrane regions of the subunits constitute the ion channel domain. The nicotinic acetylcholine receptors and the 5-HT_3 serotonin receptors are cationic Na^+/Ca^{2+} channels mediating excitatory transmission through depolarisation of the cell, whereas the glycine and γ-aminobutyric acid receptors are anionic Cl^- channels linked to hyperpolarisation and inhibition of neuronal firing (Jensen et al., 2005; Karlin, 2002; Laube et al., 2002; Lynch, 2004; Rajendra et al., 1997; Sieghart and Sperk, 2002).

Strychnine and brucine are found in the plant *Strychnos nux vomica*, and these alkaloids have pharmacological actions at several neurotransmitter receptors, including a number of ligand-gated ion channels. Strychnine is the prototypic competitive antagonist of glycine receptors (which are often referred to as 'strychnine-sensitive glycine receptors'), displaying nanomolar K_i values at recombinant and native receptors in binding and functional assays (Laube et al.,

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doi:10.1016/j.ejphar.2006.04.010

2002; Lynch, 2004; Rajendra et al., 1997). In addition to its activity at these receptors, strychnine is a moderately potent noncompetitive antagonist of muscle-type and neuronal heteromeric nicotinic acetylcholine receptors (Albuquerque et al., 1998; Garcia-Colunga and Miledi, 1999; Matsubayashi et al., 1998), whereas it has been shown to be a competitive antagonist of the neuronal homomeric α7 nicotinic receptor subtype (Baker et al., 2004; Matsubayashi et al., 1998). Finally, strychnine and brucine are also well known allosteric modulators of muscarinic acetylcholine receptors (Birdsall et al., 1999; Gharagozloo et al., 1999; Lazareno et al., 1998; Zlotos et al., 2003).

In the present study, a series of strychnine and brucine analogues and dimers have been characterised pharmacologically at the human α1 and α1β glycine receptor subtypes and at a α7/5-HT_{3A} receptor chimera. The study provides new insight into the structure–activity relationships for the alkaloids strychnine and brucine at these ligand-gated ion channels.

2. Materials and methods

2.1. Materials

Culture media, serum, antibiotics and buffers for cell culture were obtained from Invitrogen (Paisley, UK). Glycine was purchased from Sigma (St. Louis, MO), and strychnine and brucine from Tocris Cookson (Bristol, UK). The cDNAs encoding for the human α1 and β glycine receptors subunits, the rat α7 nicotinic acetylcholine receptor and the murine 5-HT_{3A} serotonin receptor were kind gifts from Dr. Peter R. Schofield (Garvan Institute of Medical Research, Sydney, New South Wales, Australia), Dr. James W. Patrick (Baylor College of Medicine, Houston, TX) and Dr. David J. Julius (University of California, San Francisco, CA), respectively. The generation of cell lines stably expressing α1 and α1β glycine receptors and the construction of the α7/5-HT_{3A} chimera consisting of the amino-terminal domain of the α7 nicotinic receptor and the transmembrane and carboxy-terminal domains of the 5-HT_{3A} serotonin receptor have been described previously (Jensen, 2005; Jensen and Kristiansen, 2004; Jensen et al., 2003). The syntheses of the strychnine and brucine analogues examined in this study (Fig. 1) have also been described previously (Gharagozloo et al., 1999; Zlotos et al., 2003, 2004).

2.2. Cell culture

The tsA cells (a modified human embryonic kidney 293 cell line) were maintained at 37 °C in a humidified 5% CO_2 incubator in culture medium [Dulbecco's Modified Eagle Medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% dialyzed fetal bovine serum]. Stable human embryonic kidney 293 cell lines expressing glycine receptors α1 and α1β were cultured in culture medium containing 1 mg/ml G-418 and 30 μM strychnine (α1) and 1 mg/ml G-418, 200 μg/ml hygromycin B and 30 μM strychnine (α1β).

2.3. The FLIPR® membrane potential assay

The functional characteristics of the strychnine and brucine analogues at the α1 and α1β glycine receptor-cell lines were determined in the FLIPR® (Fluorometric Imaging Plate Reader)

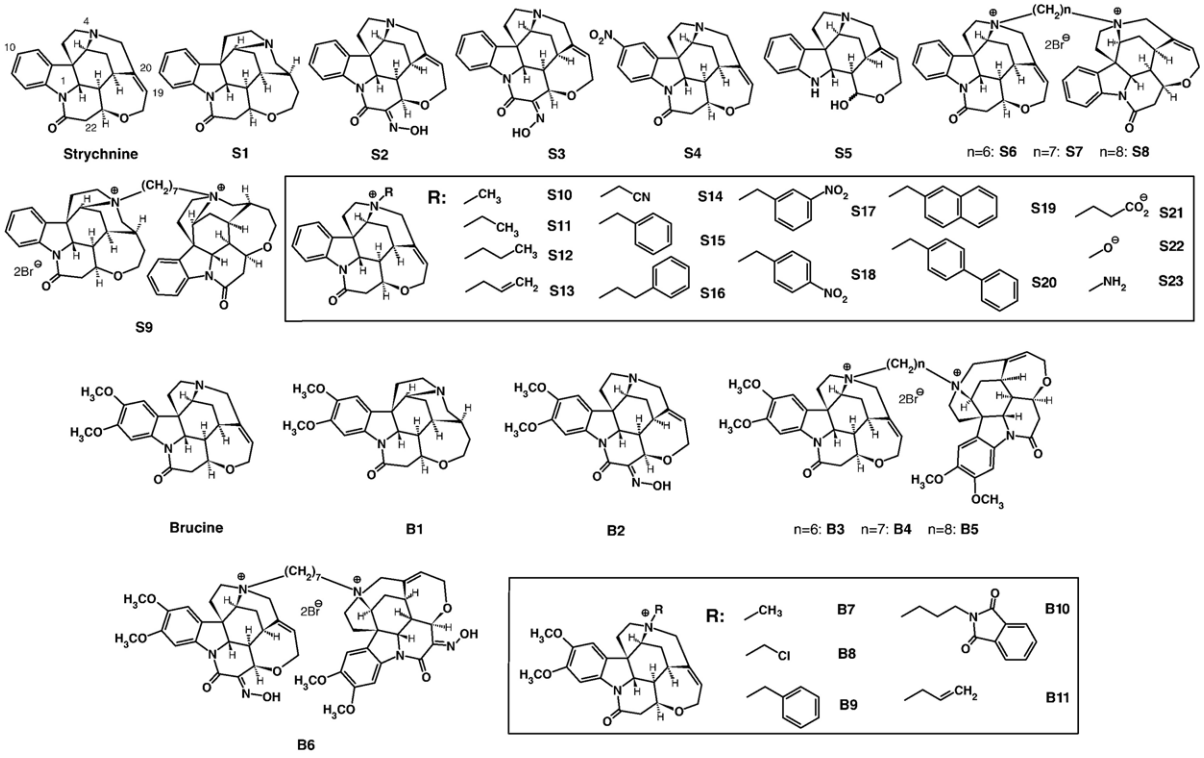

Fig. 1. Chemical structures of the strychnine and brucine analogues characterised pharmacologically in the present study.

Membrane Potential assay (catalogue number R-8042, Molecular Devices, Crawley, UK). The cells were split into poly-D-lysine-coated black 96-well plates with clear bottom (BD Biosciences, Bedford, MA) in culture medium supplemented with the appropriate antibiotics and strychnine. 16–24 h later the medium was aspirated, and washed with 100 μl Krebs buffer [140 mM NaCl/4.7 mM KCl/2.5 mM $CaCl_2$/1.2 mM $MgCl_2$/11 mM HEPES/10 mM D-glucose, pH 7.4]. 50 μl Krebs buffer was added to each well (in the antagonist experiments, different concentrations of the antagonists were dissolved in the buffer). 50 μl of loading buffer (loading dye dissolved in Krebs buffer) was added to each well, and the plate was incubated at 37 °C in a humidified 5% CO_2 incubator for 30 min. The plate was assayed in a NOVOstar™ plate reader (BMG Labtechnologies, Offenburg, Germany) measuring emission [in fluorescence units (FU)] at 560 nm caused by excitation at 530 nm before and up to 1 min after addition of 25 μl agonist solution (agonist was dissolved in Krebs buffer). Glycine was used as agonist at a final assay concentration of 200 μM. The experiments were performed in duplicate at least three times for each compound.

2.4. [^{3}H]Methyllycaconitine binding

For the [^{3}H]methyllycaconitine binding experiments, 1×10^6 tsA cells were split into a 10-cm tissue culture plate and transfected the following day with 10 μg α7/5-HT_3-pCDNA3 using Polyfect as a DNA carrier according to the protocol by the manufacturer (Qiagen, Hilden, Germany). The day after the transfection the medium was changed, and the following day, the [^{3}H]methyllycaconitine binding assay was performed. The cells were scraped into homogenization buffer, homogenized for 10 s in 30 ml assay buffer [50 mM Tris–HCl (pH 7.2)] using a Polytron, and centrifuged for 20 min at 50,000 × *g*. The resulting pellet were homogenized in 30 ml homogenization buffer and centrifuged again. This step was performed twice, after which the pellet was resuspended in assay buffer. The protocol used for the binding assay was slightly modified from that of a previous study (Davies et al., 1999). Membranes of α7/5-HT_3 transfected tsA cells was incubated with 0.5 nM [^{3}H]methyllycaconitine and various concentrations of the test compounds in a total assay volume of 1 ml. Nonspecific binding was determined in assays containing 5 mM (*S*)-nicotine.

The assays were incubated for 2.5 h at room temperature with shaking. Binding was terminated by filtration through GF/C filters, presoaked for 1 h in a 0.2% polyethylenimine solution, using a 48-well cell harvester and washing with 4 × 4 ml ice-cold isotonic NaCl. The filters were dried, 3 ml of Opti-Fluor™ (Perkin-Elmer, Boston, MA) added, and the amount of bound radioactivity was determined in a scintillation counter. The binding experiments were performed in duplicate at least three times for each compound.

2.5. Data analysis

Concentration–response curves for glycine and the strychnine and brucine analogs were constructed based on the maximal responses obtained at different concentrations of the respective ligands in the FMP assay. The curves were generated by nonweighted least-squares fits using the program KaleidaGraph 3.6 (Synergy Software). Data from the [^{3}H]methyllycaconitine competition binding experiments were fitted to the equation: % Bound = 100% Bound/$(1+([L]/IC_{50})^n)$. K_i values were determined using the equation $K_i = IC_{50}/(1+[L]/K_D)$, where [L] is the radioligand concentration, n is the Hill coefficient, and K_D is the dissociation constant of the radioligand.

Functional antagonist potencies were calculated from the inhibition curves using the equation $K_i = IC_{50}/[(2+([A]/EC_{50})^b)^{1/b}-1]$, where [A] is the glycine concentration used and b is the slope of the glycine concentration–response curve (Leff and Dougall, 1993; Lazareno and Birdsall, 1993). The slopes of the inhibition curves varied from 0.9 to 1.5 for all the strychnine and brucine analogues in this study where complete concentration–inhibition curves could be obtained. The equation assumes that the Hill slope is 1, and the use of the equation can be justified considering that other studies where full Schild analysis with glycine receptor antagonists have been performed have found Schild slopes of 1 (Lewis et al., 1998; Han et al., 2004) or close to 1 (Kumamoto and Murata, 1996).

3. Results

3.1. Characterisation of compounds at α1 and α1β glycine receptors in the FMP assay

The pharmacological characteristics for a range of standard ligands have previously been determined in the stable α1 and α1β glycine receptor-human embryonic kidney 293 cell lines using the FLIPR Membrane Potential assay (Jensen, 2005; Jensen and Kristiansen, 2004). Analogous to the pharmacological properties recently obtained for glycine receptors using another fluorescence-based high throughput screening assay (Kruger et al., 2005), the potencies of agonists and antagonists determined in the FLIPR Membrane Potential assay are lower than those found in conventional electrophysiological set-ups. However, the rank orders of EC_{50}, K_i and IC_{50} values obtained from the assay are in concordance with those obtained in previous electrophysiology studies (Jensen, 2005; Jensen and Kristiansen, 2004).

In the present study glycine exhibited EC_{50} values of 110 μM (pEC_{50} ± S.E.M: 3.95 ± 0.02, n = 6) and 92 μM (pEC_{50} ± S.E.M: 4.04 ± 0.03, n = 5) at α1 and α1β, respectively. The Hill slopes of the concentration–response curves for glycine at the two receptors varied between 1.9 and 2.7 between the individual experiments (data not shown). Furthermore, picrotoxin displayed an IC_{50} value of 7.2 μM (pIC_{50} ± S.E.M: 5.14 ± 0.04, n = 3) at α1, whereas 100 μM picrotoxin resulted in only ~40–70% inhibition (n = 3) of the response to 200 μM glycine at α1β. These properties are in agreement with previous reported values for these cell lines (Jensen, 2005; Jensen and Kristiansen, 2004).

Table 1
Binding characteristics of the strychnine and brucine analogues at the α1 and α1β glycine receptor-cell lines in the FLIPR® Membrane Potential assay

Compounds	α1	Ratio	α1β	Ratio
	K_i [pK_i]	$K_i/K_i^{strychnine}$	K_i [pK_i]	$K_i/K_i^{strychnine}$
Strychnine	0.16 [6.8]	1	0.21 [6.7]	1
S1	5.2 [5.3]	33	2.1 [5.7]	10
S2	0.092 [7.0]	0.58	0.11 [7.0]	0.52
S3	2.1 [5.7]	13	1.8 [5.7]	8.5
S5	9.2 [5.0]	58	2.1 [5.7]	10
	K_i [pK_i]	$K_i/K_i^{brucine}$	K_i [pK_i]	$K_i/K_i^{brucine}$
Brucine	1.7 [5.8]	1	1.4 [5.9]	1
B1	42 [4.4]	25	9.6 [5.0]	6.9
B2	2.7 [5.6]	1.6	1.9 [5.4]	1.4

The K_i values (in μM) and pK_i values (in brackets) of the compounds are listed, together with the ratios of the K_i values of the strychnine and brucine analogues compared to strychnine and brucine, respectively. SEM values in the estimates of pK_i values were 0.02–0.05. In neither of the two cell lines, were compounds **S4**, **S6**–**S23** and **B3**–**B11** able to inhibit the responses to levels below 50% at 100 μM concentrations. The data are the means of 3–6 individual experiments performed in duplicate as described in Materials and methods.

The antagonist potencies of the strychnine and brucine analogues at the two glycine receptors in this assay are given in Table 1, and concentration–response curves for selected analogues at the α1 subtype are depicted in Fig. 2. As mentioned above, the slopes of the inhibition curves varied from 0.9 to 1.5 for all the strychnine and brucine analogues, where complete concentration–inhibition curves could be obtained (data not shown).

3.2. Characterisation of compounds at the α7/5-HT$_{3A}$ chimera in a [^{3}H]methyllycaconitine binding assay

We and others have previously demonstrated that the binding affinities of orthosteric nicotinic ligands at an α7/5-HT$_{3A}$ chimera consisting of the amino-terminal domain of the α7 receptor and the transmembrane and carboxy terminal domains of 5-HT$_{3A}$ obtained in [^{3}H]methyllycaconitine binding assays are very similar to those obtained using native α7* receptors (Baker et al., 2004; Jensen et al., 2003). Since strychnine has been shown to be a competitive antagonist of α7 targeting the orthosteric site located in the amino-terminal domain of the receptor (Baker et al., 2004; Matsubayashi et al., 1998), the α7/5-HT$_{3A}$ chimera could be used to determine the binding affinities of the strychnine and brucine analogues to this nicotinic receptor subtype.

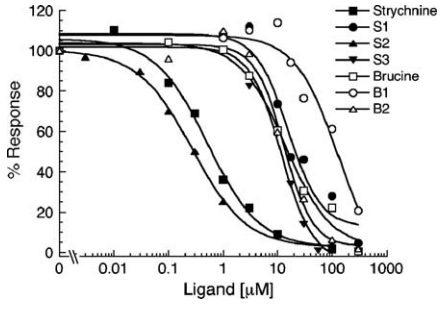

Fig. 2. Concentration–inhibition curves of strychnine and brucine and selected analogues of these at the human α1 glycine receptor in the FLIPR® Membrane Potential assay. The data are expressed as percentage of the response of 200 μM glycine in the absence of antagonist, and the figure depicts data from a single experiment. For reasons of clarity, error bars are omitted from the figure. The size of the errors (SD) on each data point were generally in the range 2–7% and did not exceed 12%.

Table 2
Binding characteristics of the strychnine and brucine analogues at the α7/5-HT$_{3A}$ chimera in the [^{3}H]methyllycaconitine binding assay

Compounds	K_i [pK_i]	$K_i/K_i^{strychnine}$
Strychnine	2.7 [5.6]	1
S1	>100	–
S2	1.8 [5.7]	0.67
S3	1.1 [6.0]	0.41
S4	3.8 [5.4]	1.4
S5	22 [4.7]	8.1
S6	3.9 [5.4]	1.4
S7	5.6 [5.3]	2.1
S8	2.4 [5.6]	0.89
S9	11 [5.0]	3.9
S10	18 [4.7]	6.8
S11	14 [4.9]	5.2
S12	19 [4.7]	7.1
S13	14 [4.8]	1.7
S14	9.8 [5.0]	3.6
S15	0.52 [6.3]	0.19
S16	13 [4.9]	4.8
S17	1.1 [6.0]	0.41
S18	1.4 [5.9]	0.52
S19	0.52 [6.3]	0.19
S20	2.0 [5.7]	0.74
S21	36 [4.4]	13
S22	>300 [<3.5]	–
S23	20 [4.7]	7.4
	K_i[pK_i]	$K_i/K_i^{brucine}$
Brucine	6.2 [5.2]	1
B1	>100 [<4]	–
B2	3.4 [5.5]	0.55
B3	2.5 [5.6]	0.40
B4	3.3 [5.5]	0.53
B5	8.9 [5.1]	1.4
B6	4.4 [5.4]	0.71
B7	~100 [~4]	~16
B8	29 [4.5]	4.6
B9	0.98 [6.0]	0.15
B10	5.2 [5.3]	0.84
B11	~100 [~4]	~16

The K_i values (in μM) and pK_i values (in brackets) of the compounds are given together with the ratios of the K_i values of the strychnine and brucine analogues compared to strychnine and brucine, respectively. SEM values in the estimates of pK_i values were 0.02–0.06. The data are the means of 3–5 individual experiments performed in duplicate as described in Materials and methods.

The binding affinities of strychnine and brucine analogues to the chimera in this assay are given in Table 2, and concentration–response curves for selected analogues are depicted in Fig. 3. In good agreement with previously reported values, the reference compounds methyllycaconitine and (*S*)-nicotine exhibited K_i values of 1.2 nM and 35 μM, respectively, at the chimera (Jensen et al., 2003). Furthermore, the low micromolar K_i value displayed by strychnine at the chimera was in agreement with previously published binding affinities to

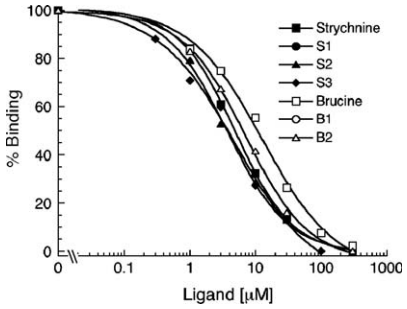

Fig. 3. Concentration–inhibition curves of strychnine and brucine and selected analogues of these at the α7/5-HT_{3A} chimera in a [^{3}H]methyllycaconitine binding assay. The data are expressed as percentage of the specific binding of the radioligand, and the figure depicts data from a single experiment. For reasons of clarity, error bars are omitted from the figure. The size of the errors (SD) on each data point were generally in the range 3–6% and did not exceed 10%.

another α7/5-HT_{3A} chimera and to native α7* receptors (Table 2) (Baker et al., 2004).

4. Discussion

At present only a few ligands capable of differentiating between glycine receptor subtypes have been identified, the most well known being picrotoxin, which is a more potent antagonist of homomeric glycine receptors consisting exclusively of α subunits (α1, α2, α3 or α4) than of heteromeric αβ receptors (α1β, α2β, α3β or α4β) (Jensen, 2005; Jensen and Kristiansen, 2004; Laube et al., 2002; Lynch, 2004; Rajendra et al., 1997). In the search for a competitive antagonist with selectivity for a subset of glycine receptor subtypes, we have in this study characterised the functional properties of a series of strychnine and brucine analogues and dimers at recombinant α1 and α1β glycine receptors (Fig. 1). These two subtypes were chosen because the α1β subtype is the predominant physiological receptor combination, and α1 is the prototypic subtype used in in vitro studies of glycine receptors. In addition we predicted that the greatest chance of initially identifying subtype selective compounds was by comparing a homomeric with a heteromeric glycine receptor, because the protein sequences of the α subunits are highly homologous, whereas the sequence of the β subunit is considerably more different from those of the α subunits.

However, none of the analogues exhibited significant selectivity for either of the two subtypes (Table 1). At best, **S1**, **S5** and **B1** exhibited a 3–5 fold selectivity for the α1 subtype. Furthermore, the glycine receptor activity of the strychnine/brucine molecule appeared to be quite sensitive to structural modifications, since the only modification that did not impair the antagonist potencies of the parent compounds was an *E*-configured hydroxyimino group at the C22-position of strychnine and brucine (**S2** and **B2**, respectively). Interestingly, the *Z*-stereoisomer, **S3**, exhibited 23-fold and 16-fold lower antagonist activities than the corresponding *E*-stereoisomer **S2** at the α1 and α1β subtypes, respectively. Saturation of the C19–C20 double bond of strychnine and brucine, yielding compounds **S1** and **B1**, respectively, resulted in significant reductions in antagonist potencies (33-fold and 10-fold for **S1** compared to strychnine at α1 and α1β, and 25-fold and 7-fold for **B1** compared to brucine at α1 and α1β, respectively). A similar impairment of glycine receptor inhibitory potency between **S1** and strychnine and between **B1** and brucine in [^{3}H]strychnine binding studies of native glycine receptors in membranes from rat brain stem and spinal cord has been reported by Mackerer et al. (1977). In the case of compound **S5**, degradation of the lactam ring of strychnine to give the Wieland-Gumlich aldehyde resulted in a 58-fold and 10-fold decrease in antagonist potency at the α1 and α1β subtypes, respectively. 10-Nitrostrychnine (**S4**) only slightly inhibited glycine receptor signalling at a concentration of 100 μM (Table 1). Although the sizes of the nitro group in **S4** and the methoxy group in the same position of brucine are similar, the electronic properties of the two groups are significantly different, and this probably accounts for the inactivity of this analogue at the glycine receptors. Finally, the most striking structure–activity relationship feature is the fact that all mono- and bis-quaternary compounds were inactive at the glycine receptors (at concentrations up to 100 μM) regardless of the steric and electronic properties of their respective N4-substituents. The orthosteric sites of the two glycine receptor subtypes either appear to be sterically restricted, not tolerating introduction of even small N4-substituents, or the tertiary amines bind in the unprotonated state. The lack of glycine receptor activity of the strychnine and brucine dimers, originally designed to target muscarinic acetylcholine receptors (Zlotos et al., 2003) and having 6–8 carbon-linkers, is compatible with an inability to bind simultaneously to two orthosteric sites in the glycine receptor which would be predicted from the dimensions of the acetylcholine-binding protein (Brejc et al., 2001; Celie et al., 2004).

The structure–activity relationships of the strychnine and brucine analogues at the α7/5-HT_{3A} chimera were significantly different from those at the glycine receptors. Introduction of a hydroxyimino group in the 22-position (**S2**, **S3** and **B2**) or a nitro group in the 10-position (**S4**) had little effect on the binding affinity to the chimera, whereas a reduction of the C19–C20 double bond (**S1** and **B1**) had detrimental effects on the binding (Table 2). Since the 3D-structures of strychnine and dihydrostrychnine are very similar, the double bond in the strychnine ring system must be important for binding. Notably, several of the quaternary N4-substituted analogues **S10**–**S23** and **B7**–**B11**, which all were virtually inactive at the glycine receptors, exhibited binding affinities to the chimera similar to those of strychnine and brucine, respectively. Interestingly, quaternization of strychnine and brucine with small aliphatic groups (**S10**–**S13**, **B7**, **B8** and **B11**) or small charged groups (**S21**–**S23**) resulted in increased K_i values compared to the parent compounds, whereas aromatic substituents in this position mostly gave rise to similar or slightly increased affinities for the chimera (**S15**, **S17**–**S20**, **B9** and **B10**) (Table 2). Strychnine-*N*-oxide (**S22**), the only compound in this series with the N^+-charge fully compensated by the negatively charged oxygen atom, was inactive, indicating the importance of cationic centres for this ligand–receptor interaction.

The binding affinities of the bisquaternary strychnine and brucine dimers **S6**–**S9** and **B3**–**B6** to the α7/5-HT_{3A} chimera did not differ significantly from those of the parent compounds (Table 2). This finding is in accord with the linkers in the dimers being too short to allow binding of the two moieties to two different orthosteric sites of α7/5-HT_{3A} receptors, as hypothesised for their interaction with glycine receptors. Furthermore, the similar binding affinities of the monomers and the dimers suggest that the "second" strychnine/brucine moiety of the dimer does not make any significant contacts with the receptor, which would increase the affinity compared to the monomer. Instead, the 'additional' strychnine/brucine molecule in the dimer probably protrudes from the orthosteric binding sites of the chimera.

In conclusion, the structure–activity relationships of the tertiary strychnine- and brucine-derived compounds at the α1 and α1β glycine receptors suggest that there may be limited room for improvement of the parent molecules, both in terms of antagonist potency and subtype selectivity. On the other hand, compounds inactive at glycine receptors, such as **S4** and several quaternary strychnine and brucine salts, display moderate binding affinities at the α7/5-HT_{3A} chimera and thus have clear selectivities as antagonists of the nicotinic α7 receptors over the glycine receptors. However it remains to be investigated whether the reported noncompetitive antagonism of strychnine at other nicotinic receptors is retained in these analogues (Albuquerque et al., 1998; Garcia-Colunga and Miledi, 1999; Matsubayashi et al., 1998), and furthermore several of the compounds are potent allosteric modulators of muscarinic acetylcholine receptors (Birdsall et al., 1999; Gharagozloo et al., 1999; Lazareno et al., 1998; Zlotos et al., 2003). Nevertheless we have shown distinct structure–activity relationships displayed by these compounds at the glycine and α7 nicotinic acetylcholine receptors which could be exploitable in the design of more selective agents.

Acknowledgements

Dr. Peter R. Schofield, Dr. James W. Patrick and Dr. David J. Julius are thanked for their kind gifts of the cDNAs. Dr. Sebastian Lazareno is thanked for supplying some of the compounds examined in this study, and he and Dr. Hans Bräuner-Osborne are thanked for commenting on the manuscript. This work was supported by the Lundbeck Foundation, the Director Ib Henriksen's Foundation, the Augustinus Foundation and the Medical Research Council.

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