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Structural requirements for novel willardiine derivatives acting as AMPA and kainate receptor antagonists

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1 The natural product willardiine is an AMPA receptor agonist. We have examined the structural changes required to convert willardiine into an antagonist at AMPA and kainate receptors. Structure–activity analysis has been carried out to discover the structural features required to increase the potency and/or selectivity of the antagonists at AMPA or kainate receptors.

2 Reduction of the fast component of the dorsal root-evoked ventral root potential (fDR-VRP) has been used to investigate AMPA receptor antagonist activity. To examine antagonist activity at kainate receptors, the ability of compounds to depress kainate-induced depolarisations of dorsal root fibres was assessed.

3 Blocking ionisation of the uracil ring by adding a methyl group to the N^3 position was not sufficient to convert willardiine into an antagonist. However, willardiine derivatives with a side-chain bearing a carboxylic acid group at the N^3-position of the uracil ring could antagonise AMPA and kainate receptors.

4 *S* stereochemistry was optimal for antagonism. When compounds with differing interacidic group chain lengths were compared, a group chain length of two methylene groups was preferable for AMPA receptor antagonism in the series of compounds bearing a carboxyalkyl side chain (UBP275, UBP277 and UBP279 reduced the fDR-VRP with IC_{50} values of 287 ± 41, 23.8 ± 3.9 and $136 \pm 17\,\mu$M, respectively). For kainate receptor antagonism, two or three methylene groups were almost equally acceptable (UBP277 and UBP279 reduced dorsal root kainate responses with apparent K_D values of 73.1 ± 4.5 and $60.5 \pm 4.1\,\mu$M, respectively).

5 Adding an iodo group to the 5-position of UBP277 and UBP282 enhanced activity at kainate receptors (UBP291 and UBP301 antagonised kainate responses on the dorsal root with apparent K_D values of 9.83 ± 1.62 and $5.94 \pm 0.63\,\mu$M, respectively).

6 The most useful antagonist identified in this study was UBP301, which was a potent and ~30-fold selective kainate receptor antagonist. UBP282 may also be of use in isolating a non-GluR5-mediated kainate response.

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Abbreviations: fDR-VRP, fast component of the dorsal root-evoked ventral root potential; (*R*)-AP5, (*R*)-2-amino-5-phosphonopentanoic acid; UBP275, (*S*)-3-carboxymethylwillardiine; UBP276, (*R*)-3-carboxymethylwillardiine; UBP277, (*S*)-3-(2-carboxyethyl)willardiine; UBP278, (*R*)-3-(2-carboxyethyl)willardiine; UBP279, (*S*)-3-(3-Carboxypropyl)willardiine; UBP280, (*R*)-3-(3-carboxypropyl)willardiine; UBP281, (*S*)-1-carboxymethylwillardiine; UBP282, (*S*)-3-(4-carboxybenzyl)willardiine (3-CBW); UBP290, (*S*)-3-(2-carboxyethyl)-5-nitrowillardiine; UBP291, (*S*)-3-(2-carboxyethyl)-5-iodowillardiine; UBP293, (*S*)-1-carboxymethyl-5-methylwillardiine; UBP294, (*S*)-3-methylwillardiine; UBP301, (*S*)-3-(4-carboxybenzyl)-5-iodowillardiine.

Introduction

Ionotropic glutamate receptors in the mammalian central nervous system have been divided into three main types–NMDA, AMPA and kainate receptors–depending on their pharmacology (for comprehensive reviews see Jane *et al*., 2000; Jane, 2002). AMPA receptors are made up from a combination of GluR1–4 subunits, while kainate receptors consist of a combination of GluR5–7, KA1 and KA2 subunits (Bleakman & Lodge, 1998). Although selective AMPA receptor antagonists are available, few discriminate between individual subunits and selective kainate receptor antagonists remain scarce (for reviews see Chittajallu *et al*., 1999; Jane *et al*., 2000). Recently, a number of decahydroisoquinoline analogues have been shown to be GluR5-selective antagonists. These compounds have been used to show that GluR5-selective antagonists may have utility in the treatment of neuropathic pain (Simmons *et al*., 1998), cerebral ischaemia (O'Neill *et al*., 1998) and epilepsy (Smolders *et al*., 2002).

The natural product willardiine acts as an agonist at AMPA receptors and a range of willardiine analogues have been synthesised with selectivity for either AMPA or GluR5-containing kainate receptors depending on the nature of the 5-substituent on the uracil ring (Evans *et al*., 1980; Patneau *et al*., 1992; Wong *et al*., 1994; Jane *et al*., 1997). For example,

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(*S*)-5-iodowillardiine is a highly selective GluR5 agonist whereas (*S*)-5-fluorowillardiine is an AMPA receptor agonist, which binds with higher affinity to GluR1 or GluR2 compared to GluR3 or GluR4 (Jane *et al*., 1997; Thomas *et al*., 1998; Varney *et al*., 1998).

A number of studies have demonstrated the conversion of glutamate receptor agonists into antagonists by extending the chain length between the α-carboxylic acid and the terminal acidic group (Davies *et al*., 1982; Krogsgaard-Larsen *et al*., 1991; Madsen *et al*., 1996; Jane *et al*., 2000). We recently demonstrated that the agonist willardiine could be converted into an antagonist of AMPA and kainate receptors by increasing the interacidic group chain length when it was shown that 3-CBW ((*S*)-3-(4-carboxybenzyl)willardiine, UBP282) is an antagonist of AMPA receptors on motor neurones and kainate receptors on dorsal root C-fibres (More *et al*., 2002a, b).

The aim of the current study was to investigate the structural changes required to convert willardiine into an antagonist and to find ways of increasing the potency of antagonism and the selectivity towards either AMPA or kainate receptors. To achieve this, several derivatives of willardiine have been assessed for their antagonist activity at AMPA and kainate receptors. In order to investigate whether adding a substituent to the N^3-position is sufficient to convert willardiine into an antagonist, we synthesized the 3-methyl analogue ((*S*)-3-methylwillardiine, UBP294). Other structural changes made to the willardiine structure included changing the stereochemistry at the stereogenic centre, changing the interacidic group chain length and adding substituents to the 5-position of the uracil ring. Thus, the following compounds were investigated for their AMPA and/or kainate receptor antagonist activity (see Figure 1 for structures): (*S*)-3-carboxymethylwillardiine (UBP275), (*R*)-3-carboxymethylwillardiine (UBP276), (*S*)-3-(2-carboxyethyl)willardiine (UBP277), (*R*)-3-(2-carboxyethyl)-willardiine (UBP278), (*S*)-3-(3-carboxypropyl)willardiine (UBP279), (*R*)-3-(3-carboxypropyl)willardiine (UBP280), (*S*)-3-methylwillardiine (UBP294), (*S*)-1-carboxymethyl-5-methylwillardiine (UBP293), (*S*)-1-carboxymethylwillardiine (UBP281), (*S*)-3-(2-carboxyethyl)-5-nitrowillardiine (UBP290), (*S*)-3-(2-carboxyethyl)-5-iodowillardiine (UBP291) and (*S*)-3-(4-carboxybenzyl)-5-iodowillardiine (UBP301).

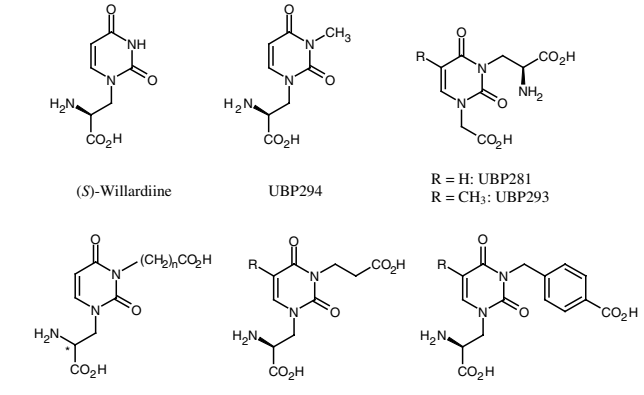


Figure 1 Structure of willardiine and a number of new derivatives.

To compare the antagonists for activity at AMPA receptors, their ability to depress the fast component of the dorsal root-evoked ventral root potential (fDR-VRP) in the neonatal rat hemisected spinal cord preparation was measured. As reported previously, the fDR-VRP has been shown to be evoked by the stimulation of AMPA receptors and can therefore be used as a convenient method to compare AMPA receptor antagonists using native receptors (More *et al*., 2002b).

To investigate the ability of the novel compounds to antagonise kainate receptors, their ability to depress kainate-induced depolarisations of neonatal rat dorsal roots was assessed. Previous studies have shown that this preparation contains predominantly kainate receptors of the GluR5 subtype (Bettler *et al*., 1990; Partin *et al*., 1993), although possibly combined with KA1 or KA2 (Fletcher & Lodge, 1996), making it useful for the examination of selective kainate receptor antagonists using a native receptor population (Agrawal & Evans, 1986; Thomas *et al*., 1998; More *et al*., 2002a, b).

As there are a limited number of selective antagonists available for AMPA and kainate receptors, it is important to discover new families of compounds that have the potential to be used as antagonists. Small changes in substituents can have drastic selectivity effects in willardiine derivatives acting as agonists, as highlighted by (*S*)-5-iodowillardiine and (*S*)-5-fluorowillardiine. It is therefore possible that careful structure–activity analysis of willardiine derivatives may lead to novel selective and potent antagonists for either AMPA or kainate receptors.

Preliminary reports of this work have been published (More *et al*., 2001; More *et al*., 2002a).

Methods

Reduction of the fDR-VRP by AMPA receptor antagonists

Hemisected spinal cords from nonanaesthetised 1- to 5-day-old rats killed by cervical dislocation were prepared and used according to the method of Evans *et al*. (1982). The standard superfusion medium contained (mM): NaCl (118), $NaHCO_3$ (25), KCl (3), $CaCl_2$ (2.5), D-glucose (12), gassed with 95% O_2/5% CO_2, with all solutions being perfused over the preparation at a rate of 1 ml min^{-1}.

The neonatal rat spinal cord preparation is a convenient source of a number of glutamate receptor subtypes (Watkins & Evans, 1981; Tölle *et al*., 1993; Schoepp *et al*., 1999; Stegenga & Kalb, 2001). Stimulation of the dorsal root allows the dorsal root-evoked ventral root potential (DR-VRP) to be recorded from the corresponding ventral root. The fast component of the DR-VRP (fDR-VRP) is mediated chiefly by activation of postsynaptically expressed AMPA receptors (More *et al*., 2002b), while the slow component is mediated mainly by NMDA receptors (Evans *et al*., 1982).

AMPA receptor antagonists were tested for their ability to depress the fDR-VRP in the neonatal rat spinal cord preparation (More *et al*., 2002b). A dorsal root in the lumbar region of the spinal cord was stimulated supramaximally (16 × threshold, 2 pulses min^{-1}, pulse width 0.2 ms) and recordings were made from the corresponding ventral root. To allow isolation of the non-NMDA receptor-mediated component of

the DR-VRP, which includes the fast component mediated *via* AMPA receptors (Long *et al*., 1990; More *et al*., 2002b), 2 mM $MgSO_4$ and 50 μM (*R*)-AP5 were included in the standard medium (30 min preincubation) to block any NMDA receptor-mediated component. The test antagonists were superfused over the preparation for 5 min at a range of concentrations, and the percentage depression of the fDR-VRP was calculated to generate noncumulative concentration–response curves for each antagonist (More *et al*., 2002b). Averages of the peak amplitudes of two consecutive responses in the absence of the antagonist and when the minimum response size was achieved after antagonist application were used to calculate the percentage depression of the fDR-VRP. Throughout the experiments used to measure the fDR-VRP, a slow trace was also recorded which showed d.c. shifts in ventral root potential. Depolarisations observed on this trace indicated that the test compound had agonist activity.

Antagonism of kainate responses on dorsal root C-fibres by novel willardiine derivatives

Experiments to test the antagonistic effect of the novel compounds on GluR5-containing kainate receptors were conveniently carried out on kainate-induced responses on isolated dorsal roots (More *et al*., 2002b). The dorsal root (L3–L5) was dissected from the point of exit from the spinal cord to just proximal to the dorsal root ganglion of 1- to 5-day-old rats, as reported previously (Agrawal & Evans, 1986). In order to record the shift in d.c. potential, the peripheral end of the dorsal root was electrically insulated from the rest of the preparation by a grease seal. To prevent desensitisation of kainate receptors, the dorsal root was superfused with 1 mg ml^{-1} concanavalin A for 20 min after a 20 min exposure to glucose-free superfusion medium. Standard superfusion medium was then applied throughout the experiments. This allowed measurement of depolarisations evoked by the exogenously applied agonist kainate (1 min applications). Noncumulative, nonsequential concentration–response curves were constructed for kainate in the absence and presence of 100 μM UBP277, UBP279, UBP291 or 50 μM UBP301 (30 min preincubation). In preliminary studies, it was found that 30 μM kainate was the maximum concentration that could be used in the absence of antagonist to maintain repeatable results; therefore, this concentration was used as the maximum kainate dose in the absence of antagonist (More *et al*., 2002b). Using this methodology EC_{50} values for kainate were obtained that were similar to previously reported values (Agrawal & Evans, 1986; Pook *et al*., 1993; Thomas *et al*., 1998; More *et al*., 2002b).

Data analysis

Concentration–response curves were analysed by iterative nonlinear regression (GraphPAD Prism). IC_{50} values for the antagonists were measured as the concentration required to obtain a 50% reduction of the fDR-VRP. Apparent K_D values were determined using the Gaddum–Schild equation: $K_D = [\text{ANTAGONIST}]/DR - 1$ where the dose ratio (DR) is determined by the EC_{50} in the presence of the antagonist/EC_{50} in the absence of the antagonist.

The K_D and IC_{50} values obtained are from at least three independent experiments and are given as means $\pm$ s.e.m.

Materials

The novel willardiine derivatives were synthesised in our own laboratory by methods that will be reported elsewhere. Stock solutions were made up in one equivalent of 100 mM aqueous sodium hydroxide with the exception of UBP301, which was made up to 50 mM in two equivalents of aqueous sodium hydroxide. Concanavalin A (type VI) was obtained from Sigma (UK). Kainate and (*R*)-AP5 were obtained from Tocris Cookson (Bristol, UK). All other chemicals were of analytical grade or above.

Results

Depression of the fDR-VRP by novel AMPA receptor antagonists

To investigate whether blocking the ionisation of the uracil ring was sufficient to convert willardiine into an AMPA receptor antagonist, the 3-methyl analogue UBP294 was tested for its ability to depress the fDR-VRP. When tested at a concentration of 1 mM UBP294 was found to be a weak agonist (see Table 1 for a summary of the test results). Next, the 3-carboxymethyl analogue UBP275 was tested to investigate whether increasing the interacidic group chain length would confer AMPA receptor antagonist activity. UBP275 reduced the fDR-VRP with an IC_{50} value of 287 ± 41 μM ($n = 3$; mean $\pm$ s.e.m.). To investigate the effect of positional isomerism, two analogues of UBP275 in which the alanine and carboxymethyl side-chains were swapped were tested. Neither UBP281 nor UBP293 had potent antagonist activity, as when they were tested at a concentration of 1 mM they depressed the fDR-VRP by 2.9 ± 0.7 and 3.8 ± 0.7% ($n = 3$, mean $\pm$ s.e.m.), respectively.

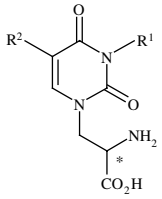
The effect of increasing the interacidic group chain length was then investigated. The next two higher homologues of the lead compound UBP275, the 3-(2-carboxyethyl) (UBP277) and the 3-(3-carboxypropyl) (UBP279) derivatives depressed the fDR-VRP (Figure 2) with IC_{50} values of 23.8 ± 3.9 and 136 ± 17 μM ($n = 3$, mean $\pm$ s.e.m.), respectively.

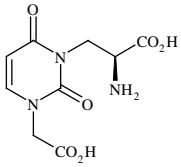
To examine the effect of changing the stereochemistry at the stereogenic centre of the willardiine derivatives, the *S*-enantiomers UBP275, UBP277 and UBP279 were compared to the corresponding *R*-enantiomers UBP276, UBP278 and UBP280. At a concentration of 1 mM, UBP276, UBP278 and UBP280 depressed the fDR-VRP by 44.4 ± 3.0, 50.3 ± 1.4 and 61.3 ± 4.9%, respectively (Table 1) ($n = 3$; mean $\pm$ s.e.m.). The effect of adding a substituent to the 5-position of the 3-(2-carboxyethyl) derivative UBP277 was also investigated. The 5-nitro (UBP 290) and 5-iodo (UBP291) derivatives depressed the fDR-VRP with IC_{50} values of 79.9 ± 15.9 and 13.7 ± 1.7 μM, respectively (Figure 3a) ($n = 3$; mean $\pm$ s.e.m.). To investigate the effect of adding an iodo group to the 5-position of the previously reported compound 3-CBW (UBP282), the ability of UBP301 to depress the fDR-VRP was assessed. UBP301 depressed the fDR-VRP with an IC_{50} value of 164 ± 25 μM. This is compared to UBP282, which gave an IC_{50} value of 10.3 ± 2.4 μM (More *et al*., 2002), in Figure 3b.

With the exception of UBP294, none of the compounds tested caused concentration–dependent depolarisations of

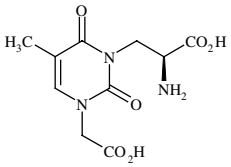
Table 1 Summary of the depressant activity of willardiine analogues on the fDR-VRP or the kainate response on isolated dorsal root (all values $n=3$, mean$\pm$s.e.m.)

General Formula





UBP281



UBP293

Compound	*R^1*	*R^2*	*	*IC_{50} (μM) vs fDR-VRP*	*% depression of fDR-VRP at 1 mM*	*K_D (μM) vs kainate*	*% reduction of kainate response at 200 μM*
UBP275	CH_2CO_2H	H	S	287$\pm$41		ND	
UBP276	CH_2CO_2H	H	R		44.4$\pm$3.0%	ND	
UBP277	$(CH_2)_2CO_2H$	H	S	23.8$\pm$3.9		73.1$\pm$4.5	
UBP278	$(CH_2)_2CO_2H$	H	R		50.3$\pm$1.4%		13.2$\pm$2.1%
UBP279	$(CH_2)_3CO_2H$	H	S	136$\pm$17		60.5$\pm$4.1	
UBP280	$(CH_2)_3CO_2H$	H	R		61.3$\pm$4.9%		22.8$\pm$9.6%
UBP281					2.9$\pm$0.7%	ND	
UBP282	CH_2-Ph-4-CO_2H	H	S	10.3$\pm$2.4[a]		4.96[b]	
UBP290	$(CH_2)_2CO_2H$	NO_2	S	79.9$\pm$15.9		ND	
UBP291	$(CH_2)_2CO_2H$	I	S	13.7$\pm$1.7		9.83$\pm$1.62	
UBP293					3.8$\pm$0.7%	ND	
UBP294	CH_3	H	S	Agonist		ND	
UBP301	CH_2-Ph-4-CO_2H	I	S	164$\pm$25		5.94$\pm$0.63	

All values are from three independent experiments and are expressed as the mean$\pm$s.e.m. ND: not determined: [a]values taken from More *et al.* (2002b). [b]pA_2 value given for antagonism of kainate-induced responses.

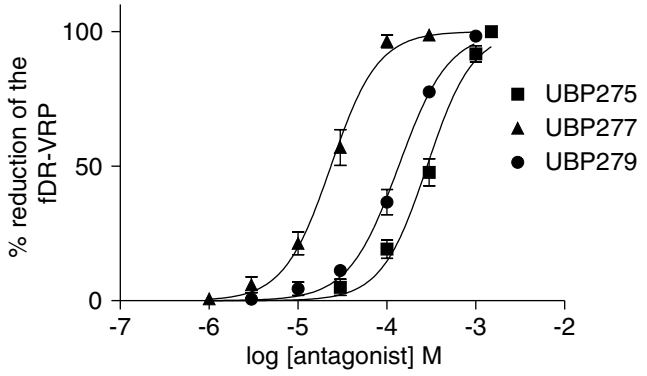


Figure 2 Concentration–response curves for the reduction of the fDR-VRP by UBP275, UBP277 and UBP279. The IC_{50} values were 287$\pm$41, 23.8$\pm$3.9 and 136$\pm$17 μM, respectively ($n=3$; mean$\pm$s.e.m.).

motor neurones, indicating that UBP294 was the only agent with agonist activity.

Investigation of N^3*-substituted willardiine analogues as antagonists of kainate receptors expressed on isolated dorsal root fibres*

To determine the antagonist activity at GluR5-containing kainate receptors, some of the compounds described above were compared for their ability to antagonise kainate-induced depolarisations of dorsal roots (see Table 1 for a summary of the results). The shorter chain compounds UBP275, UBP281 and UBP293 and *R* enantiomers were not tested as kainate

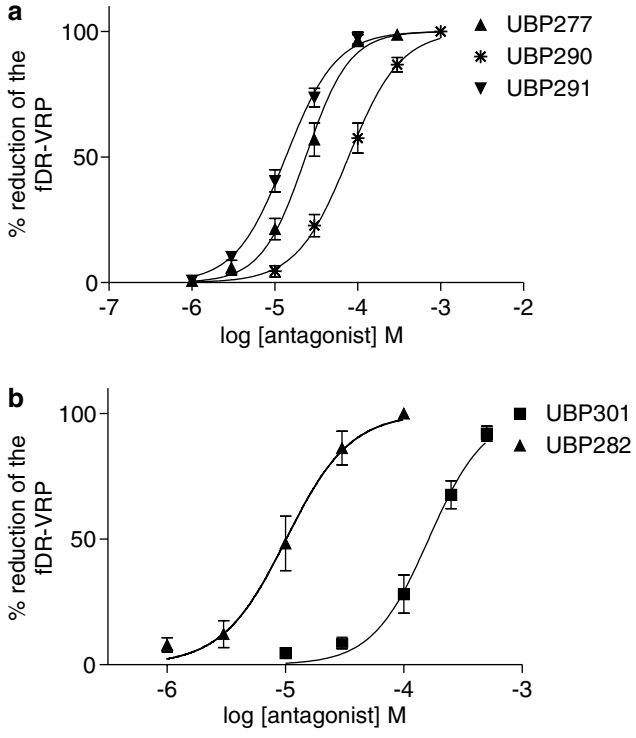


Figure 3 (a) Concentration–response curves for the reduction of the fDR-VRP by UBP290, UBP291 and UBP277. The IC_{50} values were 79.9$\pm$15.9, 13.7$\pm$1.7 and 23.8$\pm$3.9 μM, respectively ($n=3$; mean$\pm$s.e.m.). (b) Concentration-response curves for the reduction of the fDR-VRP by UBP282 and UBP301. The IC_{50} values were 10.3$\pm$2.4 and 164$\pm$25 μM, respectively ($n=3$; mean$\pm$s.e.m.).

receptor antagonists as preliminary studies suggested that longer chain lengths and *S* stereochemistry were requirements for potent antagonist activity. Two analogues with extended interacidic group chain lengths, UBP 277 and UBP279, depressed kainate-induced depolarisations of dorsal roots with apparent K_D values of 73.1 ± 4.5 and $60.5 \pm 4.1\ \mu$M, respectively (Figure 4) ($n = 3$; mean $\pm$ s.e.m.). In agreement with the findings on AMPA receptors, the *R*-enantiomers were relatively inactive on kainate receptors. At a concentration of 200 μM, UBP278 and UBP280 depressed responses to 10 μM kainate on the dorsal root by 13.2 ± 2.1 and $22.8 \pm 9.6\%$, respectively ($n = 3$; mean $\pm$ s.e.m.).

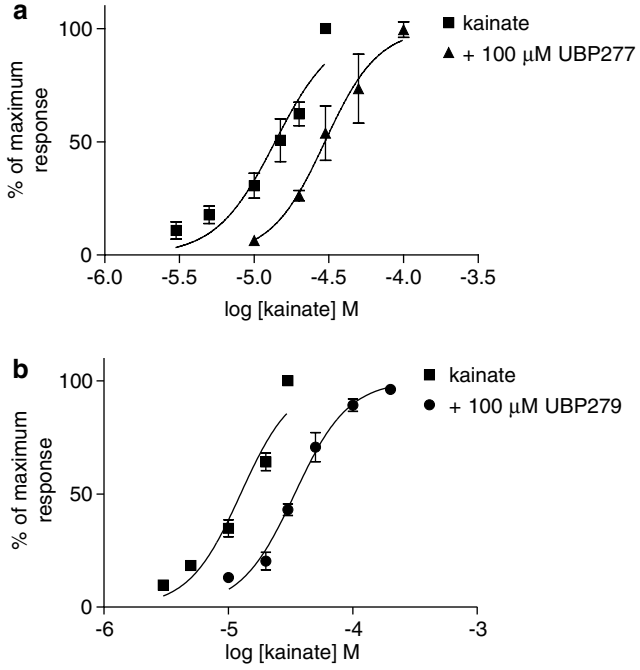


Figure 4 (a) Concentration–response curves for kainate on the dorsal root in the absence and presence of 100 μM UBP277. The apparent K_D value was $73.1 \pm 4.5\ \mu$M ($n = 3$; mean $\pm$ s.e.m.). (b) Concentration–response curves for kainate in the absence and presence of 100 μM UBP279. The apparent K_D value was $60.5 \pm 4.1\ \mu$M ($n = 3$; mean $\pm$ s.e.m.). To normalise the data, responses were analysed as the percentage of the response to 30 μM kainate in the absence of antagonist.

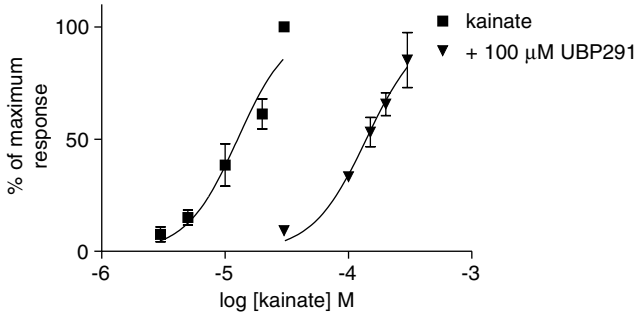


Figure 5 Concentration–response curves for kainate on the dorsal root in the absence and presence of 100 μM UBP291. The apparent K_D value was $9.83 \pm 1.62\ \mu$M ($n = 3$; mean $\pm$ s.e.m.). To normalise the data, responses were analysed as the % of the response to 30 μM kainate in the absence of antagonist.

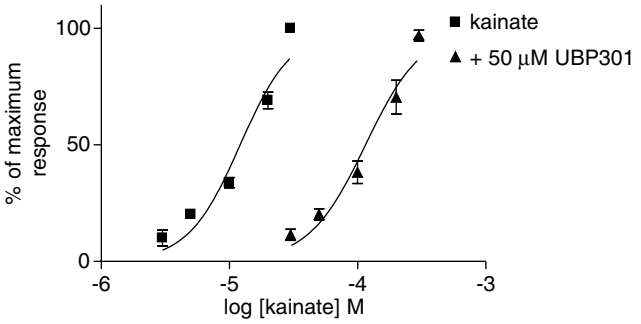


Figure 6 Concentration–response curves for kainate on the dorsal root in the absence and presence of 50 μM UBP301. The apparent K_D value was $5.94 \pm 0.63\ \mu$M ($n = 3$; mean $\pm$ s.e.m.). To normalise the data, responses were analysed as the percentage of the response to 30 μM kainate in the absence of antagonist.

The 5-iodo derivatives UBP291 and UBP301 were found to antagonise kainate responses on isolated dorsal root fibres with apparent K_D values of $9.83 \pm 1.62\ \mu$M (Figure 5) and $5.94 \pm 0.63\ \mu$M (Figure 6), respectively ($n = 3$; mean $\pm$ s.e.m.). No indication of kainate receptor agonist activity was observed upon application of any of the novel compounds to the dorsal root at concentrations of 50–100 μM.

Discussion

The main aim of this study was to determine the structural requirements necessary to convert the AMPA receptor agonist willardiine into an antagonist. Structure–activity analysis was also carried out to find ways of improving the antagonism of novel willardiine derivatives at both AMPA receptors and the GluR5-containing kainate receptors present on neonatal rat dorsal root fibres. The use of these recording methods allowed us to compare the activity of the novel compounds at native AMPA and kainate receptors *in situ*. This is important, as the subunit composition of these receptors in native tissue is not always known. Thus, by testing antagonists on native receptors we can get an idea of their ability to discriminate between AMPA and kainate receptor populations expressed in different tissues.

Structural changes required to convert willardiine into an AMPA receptor antagonist

The 3-methyl analogue of willardiine, UBP294, was found to be a weak agonist, indicating that merely blocking ionisation of the uracil ring of willardiine is not sufficient to convert it into an antagonist, although agonist activity is markedly reduced. Increasing the interacidic group chain length of willardiine by introducing a carboxymethyl substituent to the N^3 position, to give UBP275, did result in weak AMPA receptor antagonist activity. However, two positional isomers of UBP275 in which the alanine and carboxymethyl side-chains were swapped (UBP281 and UBP293) were both much less potent as AMPA receptor antagonists. This led to the conclusion that the best way to convert willardiine into an antagonist would be to add substituents to the N^3 position of the uracil ring of willardiine.

When three N^3-carboxyalkyl analogues UBP275, UBP277 and UBP279 were compared, it was evident that a chain length

for the N^3 substituent equivalent to two methylene groups was preferable to those of one or three methylene groups for AMPA receptor antagonism (Figure 2). The compounds represented in this study were also compared to the previously reported compound UBP282 (3-CBW), which was more potent as an AMPA receptor antagonist than the N^3-carboxyalkyl-substituted derivatives (see Table 1 and More *et al*., 2001, 2002b). This observation suggests that the use of a benzyl moiety as an interacidic group spacer unit enhances antagonism at AMPA receptors, possibly because of conformational restriction of the carboxyalkyl side-chain. Alternatively, the benzene ring may be involved in either hydrophobic or π–π interactions with amino acids at the receptor binding site. The benzyl group is likely to be equivalent to a chain of three to four methylene groups, and as the carboxyethyl analogue (UBP277) with a chain of two methylene groups had optimal antagonist activity among the straight chain carboxyalkyl-substituted derivatives, the terminal carboxylic acid groups of UBP277 and UBP282 may interact with different binding sites in the receptor. It is unlikely that the terminal carboxyl group of the shorter chain derivative UBP277 could reach the same binding site as that of the carboxybenzyl analogue UBP282.

Schild analysis of the antagonist activity of UBP282 (3-CBW) on (*S*)-5-fluorowillardiine-induced depolarisations suggested a competitive mode of antagonism (More *et al*., 2002b). As the willardiine derivatives described in this study have a marked structural similarity to UBP282, it is likely that they are also acting as competitive antagonists.

It is apparent from the lack of activity of the *R*-isomers UBP276, UBP278 and UBP280 that *S*-stereochemistry is optimal for willardiine derivatives acting as AMPA receptor antagonists (see Table 1).

Substitution of the 5-position of willardiine has a marked effect on agonist potency and selectivity for AMPA *vs* kainate receptors (Wong *et al*., 1994; Jane *et al*., 1997). An investigation into the effect of adding substituents to the 5-position of N^3-substituted willardiine analogues was carried out to determine whether antagonist potency and selectivity would be affected by this substitution. As UBP277 was found to be the most potent of the carboxyalkyl-substituted analogues, this was chosen for further elaboration by adding either an iodo (UBP291) or a nitro group (UBP290). These two substitutions had differential effects, with the 5-nitro derivative decreasing and the 5-iodo derivative slightly increasing AMPA receptor antagonist potency compared to the parent compound (see Figure 3a).

Interestingly, when an iodo group was added to the 5-position of the carboxybenzyl derivative UBP282 to give UBP301, AMPA receptor antagonist activity was decreased, as highlighted in Figure 3b.

Several structure–activity conclusions can therefore be made about the requirements for AMPA receptor antagonism from the results of these studies. It appears that adding substituents bearing an acidic group to the N^3 position of willardiine is a means of converting willardiine into an antagonist. *R*-enantiomers are relatively inactive; therefore *S*-stereochemistry is optimal for these antagonists. A carboxyalkyl chain length equivalent to two methylene groups in the N^3 substituent or a benzyl group spacer is optimal for antagonism at AMPA receptors. Also, the addition of an iodo group at the 5-position of UBP277 enhances AMPA receptor antagonism whereas the addition of a nitro group to this position is detrimental to antagonist activity. However, 5-iodo substitution of the longer chain analogue UBP282 led to a decrease in antagonist potency at AMPA receptors, strengthening the theory that UBP277 and UBP282 are not interacting with all of the same amino-acid residues in the AMPA receptor binding site.

Structural changes required to convert willardiine into a kainate receptor antagonist

As some willardiine analogues were potent GluR5 agonists (Wong *et al*., 1994; Jane *et al*., 1997), it was important to investigate whether any of the N^3-substituted analogues had antagonist activity on GluR5-containing kainate receptors. Structure–activity studies were carried out to investigate the structural changes required to increase the potency or selectivity of antagonism at the GluR5-containing kainate receptors present on the dorsal root. In contrast to the results found for AMPA receptor antagonism, when the N^3-carboxyalkyl analogues UBP277 and UBP279 were investigated for activity at kainate receptors on dorsal root fibres there was little difference in their antagonist activity (Figure 4a and b; Table 1). It therefore appears that for kainate receptor antagonism, a longer interacidic chain length equivalent to three methylene groups is equally as well accommodated as the shorter chain length. The previously reported compound UBP282 (3-CBW) had a pA_2 value of 4.96 on the dorsal root, which is equivalent to a K_D value of $\sim 11\,\mu$M (More *et al*., 2002b). It therefore appears that, as with AMPA receptor antagonism, use of a benzyl group as the interacidic spacer unit increases the potency of antagonism at GluR5-containing kainate receptors. Schild analysis of the antagonist activity of UBP282 (3-CBW) on kainate-induced depolarisations suggested a competitive mode of antagonism (More *et al*., 2002b). As the willardiine derivatives described in this study have a marked structural similarity to UBP282, it is likely that they are also acting as competitive antagonists.

In agreement with the studies investigating AMPA receptor antagonist activity, the *R*-enantiomers UBP278 and UBP280 were relatively inactive as kainate receptor antagonists when compared to UBP277 and UBP279. It was therefore concluded that *S*-stereochemistry is optimal for antagonism of GluR5-containing kainate receptors. It has been reported that 5-iodo substitution of willardiine results in an agonist with an $\sim$100-fold selectivity for kainate *vs* AMPA receptors (Wong *et al*., 1994; Thomas *et al*., 1998). For antagonists this switch in selectivity was less apparent, as when a 5-iodo substituent was added to the uracil ring of UBP277, the resultant compound UBP291, although nearly nine times as potent as a kainate receptor antagonist than the parent compound, had a similar potency on AMPA receptors (Table 1). The 5-iodo-substituted analogue of UBP282 (3-CBW), UBP301, was also more potent than the parent compound as a kainate receptor antagonist and indeed was the most potent antagonist tested in the dorsal root assay. UBP301 was also more selective, showing $\sim$16-fold weaker antagonist potency at AMPA receptors than the parent compound UBP282. Previous studies have shown that the dorsal root preparation contains predominantly kainate receptors of the GluR5 subtype (Bettler *et al*., 1990; Partin *et al*., 1993), although possibly combined with KA1 or KA2 (Fletcher & Lodge, 1996), making it useful for the examination of kainate receptor responses. The presence of GluR5 subunits in kainate receptors expressed on DRG cells has been

confirmed by the observation that the previously reported AMPA receptor antagonist (3*S*, 4a*R*, 6*R*, 8a*R*)-6-[2-(1(2*H*)-tetrazole-5-yl)ethyl]decahydro-isoquinoline-3-carboxylic acid (LY293558) (Schoepp *et al*., 1995) is an antagonist at homomeric GluR5 but not GluR6 and also antagonises the effects of kainate on DRG neurones (Bleakman *et al*., 1996). The antagonism of kainate-induced depolarisations of dorsal root fibres by the novel willardiine derivatives described in this study suggests that they are antagonists of the GluR5-containing kainate receptors expressed on these fibres. As UBP282 (3-CBW) was found to be a much less potent antagonist of the kainate receptors present on neonatal rat motor neurones (More *et al*., 2002b), it is possible that the antagonists described here show diverse levels of antagonist activity at populations of kainate receptors with different subunit compositions. Previous work has shown that UBP282 (3-CBW) is selective for AMPA/kainate receptors over NMDA and group I, II and III metabotropic glutamate receptors expressed in the spinal cord (More *et al*., 2002b). Preliminary studies have suggested that none of the willardiine derivatives described here have significant activity at NMDA receptors (More, Thomas and Jane, unpublished observations).

Although the IC_{50} values for depression of the fDR-VRP cannot be directly compared with the apparent K_D values found for antagonism of kainate responses on the dorsal root, relative effects of the structural changes can be approximated. For example, the addition of an iodo group to the 5-position of UBP277 to yield UBP291 increased AMPA receptor antagonism (measured as the depression of the fDR-VRP) by a factor of $\sim$2 times, whereas the same addition increased kainate receptor antagonism on the dorsal root by $\sim$7 times. It can therefore be suggested that the addition of an iodo group to the 5-position of the 2-carboxyethyl willardiine derivative UBP277 is one way of increasing its selectivity towards the kainate receptors present on the dorsal root over AMPA receptors. Additionally, it was found that the longer chain length compound (UBP279) had a similar potency to UBP277 at kainate receptors but was much less potent than UBP277 at depressing the fDR-VRP. This may suggest, therefore, that longer chain analogues may be more selective kainate receptor antagonists with less activity at AMPA receptors. Also, 5-iodo substitution of the longer chain analogue UBP282 to yield UBP301 increased selectivity for kainate *vs* AMPA receptors. It is of interest to compare the properties of the present willardiine-based antagonists to previously described AMPA and kainate receptors. Although the willardiine derivatives are less potent and selective than the AMPA receptor antagonists NBQX and GYKI53655 and the GluR5-selective antagonist LY382884 (see Jane *et al*., 2000 for a review), they do have some advantages over these compounds. The quinoxalinedione- and decahydroisoquinoline-based antagonists such as NBQX and LY382884, respectively, are not very soluble in water and aqueous solutions need to be made up by the addition of DMSO. In contrast, solutions of the willardiine derivatives described here can be prepared up to concentrations of 50–100 mM in the form of either the mono- or di-sodium salt. The bioavailability of the willardiine derivatives described here is not yet known but it is likely that they would have similar properties to the decahydroisoquinolines (which are known to cross the blood–brain barrier, O'Neill *et al*., 1998) as they have a similar ratio of polar to lipophilic groups.

One of the most useful antagonists to be identified in this study would appear to be UBP282 (3-CBW) as this compound has been found to be selective for AMPA- and GluR5-containing kainate receptors *vs* NMDA, mGlu and kainate receptors expressed on motor neurones (More *et al*., 2002b). Thus, UBP282 can be used to isolate a non-GluR5-mediated kainate response in native tissue without the need to add an antagonist to block the AMPA-receptor-mediated component of the kainate response (More *et al*., 2002b).

UBP301 was found to be the most potent and selective kainate receptor antagonist tested and, although not as selective for kainate receptors *vs* AMPA receptors as LY382884, it nonetheless may be useful for selectively antagonising GluR5-containing kainate receptors.

Conclusion

A range of novel antagonists acting at AMPA and kainate receptors has been synthesised. The main structural conclusions that can be made from this study are that, for willardiine derivatives to act as AMPA and/or kainate receptor antagonists, an N^3-substituent bearing a carboxylic acid side-chain should be present, *S*-stereochemistry is optimal and addition of an iodo moiety to the 5-position of the uracil ring enhances antagonism at kainate receptors.

New analogues based on the structure of willardiine are likely to prove useful tools for the characterisation of the physiological roles of AMPA and kainate receptors and it is envisaged that further structure–activity analysis may lead to antagonists with greater selectivity for individual AMPA or kainate receptor subunits.

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