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Novel arylaminopyridazine-GABA receptor antagonists examined electrophysiologically in *Ascaris suum*

Richard J. Martin [a,*], Jean-Marie Sitamze [b], Anne H. Duittoz [b], Camille G. Wermuth [b]

[a] *Department of Preclinical Veterinary Sciences R.(D.) S.V.S., Summerhall, University of Edinburgh, Edinburgh EH9-3QT, UK*
[b] *Departement de Pharmacochimie Moleculaire, L'Université Louis Pasteur, 67084 Strasbourg Cedex, France*

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Abstract

The structure-activity relationships of 35 novel derivatives of 2-(carboxypropyl)-3-amino-4-methyl-6-phenyl pyridazine (SR 95103) were examined as γ-aminobutyric acid (GABA) antagonists in the flap preparation of the parasitic nematode, *Ascaris suum*, using a two-microelectrode current-clamp technique. All but one of the potent antagonists displaced GABA dose-response curves to the right without reduction in the maximum response. The dissociation constants of the more potent competitive antagonists were described using a model which assumed that two molecules of GABA were required to open the ion channel but that only one molecule of antagonist acted on each ion channel. By exploring the structure-activity relationship, the potency of the antagonist was increased from a K_B of 64 μM for SR 95103 to a K_B of 4.7 μM for NCS 281-93 (2-(3-carboxypropyl)-3-amino-4-phenylpropyl-6-phenyl pyridazine).

Keywords: Arylaminopyridazine; GABA (γ-aminobutyric acid) derivative; SR 95103; GABA receptor antagonist; (*Ascaris suum*)

1. Introduction

The γ-aminobutyric acid (GABA) receptor of nematode parasites has been identified as a site of action for anthelmintic drugs (Del Castillo et al., 1964a; Martin, 1987). The anthelmintic, piperazine, hyperpolarizes body muscle cells in the porcine nematode, *Ascaris suum*, by mimicking the action of the natural neuromuscular inhibitory transmitter GABA (Del Castillo et al., 1964a,b). Bath application of GABA or piperazine activates extrasynaptic GABA receptors on *Ascaris* muscle cells and produces an increase in membrane Cl^- conductance (Martin, 1980, 1982). The antibiotic anthelmintic, ivermectin, has also been shown to interact with GABA receptors on *Ascaris* muscle (Holden-Dye et al., 1988; Martin and Pennington, 1989) and to act as an antagonist. Thus pharmacological characterization of the *Ascaris* GABA receptor and its differences to vertebrate GABA receptors is of interest for the design of anthelmintic agents. For therapeutic reasons, it is also necessary to compare the *Ascaris* GABA receptor with vertebrate host $GABA_A$ receptors.

Pharmacological examination of the *Ascaris* GABA receptor has shown, to a first approximation, that the agonist profile is like that of a vertebrate $GABA_A$ receptor (Holden-Dye et al., 1989). In contrast, the antagonist profile is unlike that of a $GABA_A$ receptor. The $GABA_A$ antagonists bicuculline, picrotoxin, securinine, pitrazepine, *t*-butylbicyclophosphorothionate (TBPS), RU5135 and dieldrin are either weak or inactive in *Ascaris* (Martin, 1980; Holden-Dye et al., 1989; Martin et al., 1991). In vertebrates the arylaminopyridazine, SR 95331, is about 20 times more potent than SR 95103 as a $GABA_A$ antagonist (Heaulme et al., 1986) but SR 95531 has a lower potency than SR 95103 in *Ascaris* (Duittoz and Martin, 1991a). In *Ascaris* SR 95103 behaves as a competitive antagonist with a dissociation constant, K_B, of 64 μM (Duittoz and Martin, 1991b).

As a result of observations on SR 95103 and SR 95531 it was suggested that the structure-activity relationship of arylaminopyridazine GABA receptor antag-

* Corresponding author. Tel. 00-44-31-650-6094, fax 00-44-31-650-6576, e-mail R.J.Martin@ed.ac.uk.

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onists would be different at the *Ascaris* GABA receptor and be unlike a vertebrate $GABA_A$ receptor (Duittoz and Martin, 1991a). In a study comparing a series of arylaminopyridazine derivatives, this hypothesis was confirmed and the results interpreted to indicate that accessory binding sites required for binding of an antagonist of vertebrate $GABA_A$ receptors were different to the accessory binding sites on *Ascaris* muscle GABA receptors (Duittoz and Martin, 1991b). The study also indicated that the action of novel arylaminopyridazine derivatives should be investigated with a view to examining further the structure-activity relationship of these compounds and to find a more potent antagonist to advance antiparasitic drug development.

This paper then describes results of experiments in which 35 novel arylaminopyridazine-GABA derivatives were synthesized and screened and the potency of the more potent analogues tested to determine their K_B. As a result of these experiments it was possible to identify NCS 281-93 as an antagonist that was an order more potent than SR 95103 at the *Ascaris* muscle receptor.

2. Materials and methods

2.1. Preparation and recording

Ascaris suum were collected from the local abattoir and kept in Locke's solution at 34°C. They were used within 4 days. A muscle flap preparation (Martin, 1980) was pinned, cuticle side down, in an experimental chamber and maintained at 22°C and bathed in a high-Cl^- and low-Ca^{2+} Ringer containing (mM): NaCl, 135; KCl, 3; $MgCl_2$, 15.7; glucose, 3; Tris, 5; pH 7.2 adjusted with maleic acid. For the experiments with the less soluble antagonists, 2% dimethyl sulphoxide (DMSO) was added to all the Ringer solutions to prevent precipitation of the antagonist. 2% DMSO had a small depressant effect on GABA-induced conductance changes so a fixed concentration was used in all solutions throughout the experiments with the less soluble antagonists. The DMSO had no direct effect on resting membrane conductances or potentials in *Ascaris*.

Two glass microelectrodes (10–40 MΩ) filled with 2 M K acetate were inserted into the bag region of the muscle cell: one was used for current injection (40 nA, 1 s, 0.25 Hz), the other was used for recording the voltage response. The signals were recorded and amplified with an Axoclamp 2A amplifier, monitored on a Tektronix 2210 oscilloscope and recorded on a Lecromed 216MX two-channel chart recorder. The input conductance of the bag was calculated from the voltage response to the hyperpolarizing current pulses:

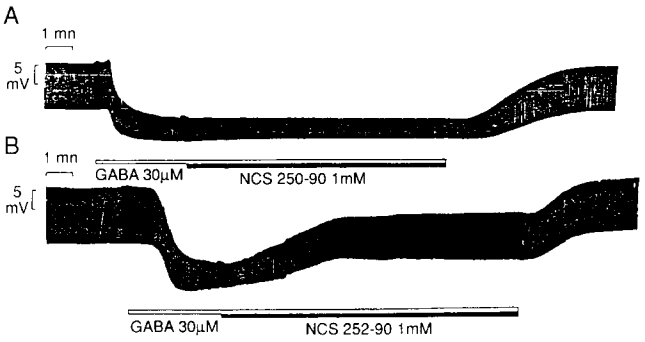


Fig. 1. Effect of 1 mM NCS 250-90 (A) and 1 mM NCS 252-90 (B) on the change in input conductance produced, ΔG_{con} (control) and ΔG_{ant} (with antagonist) by 30 μM GABA. A: NCS 250-90; resting membrane conductance, 3.3 μS; 30 μM GABA induced ΔG_{con}, 1.8 μS; 1 mM NCS 250-90 + 30 μM GABA, ΔG_{ant}, 1.8 μS; 0% antagonism. B: NCS 252-90; resting membrane conductance, 3.1 μS; 30 μM GABA induced ΔG_{con}, 1.4 μS; 1 mM NCS 252-90 + 30 μM GABA, ΔG_{ant}, 0.3 μS; 78.6% antagonism.

the I/V relationship is sufficiently linear during the injection of hyperpolarizing current to permit an accurate assessment of the input conductance.

The preparation was not perfused continuously because of the limited quantities of antagonist available. Initially to detect antagonist activity, 30 μM GABA was applied and when the membrane conductance stabilized to a new value, the bath solution was replaced with 30 μM GABA + 1 mM NCS compound (Fig. 1). In some experiments 30 μM GABA + 1 μM NCS compound or GABA + 0.1 mM NCS compound were also tested. Antagonists found to be more potent at 1 mM than SR 95103, i.e. to have a percent antagonism greater than 93% with this test, were tested at several concentrations against GABA dose-response curves (Fig. 2). GABA dose-response relationships were obtained by cumulative application of increasing GABA concentrations without washing between concentrations. The method used gentle draining of the bath (volume 6 ml) and then flushing the bath with 10 ml of the next GABA concentration. The antagonist was applied before the lowest GABA concentration and remained present when the higher concentrations of GABA were applied. A control GABA dose-response curve without the antagonist was always obtained first from the test cell to permit dose ratios to be calculated. The antagonists that readily dissolved in the aqueous phase and that did not require DMSO for solubility were easily washed off the preparation and repeated application of GABA yielded dose-response curves overlapping the controls; the more lipophilic antagonists requiring DMSO for solubility were only slowly washed from the preparation. Consequently antagonism was assessed by comparing control responses to GABA observed prior to antagonist application with the responses observed in the presence of antagonist.

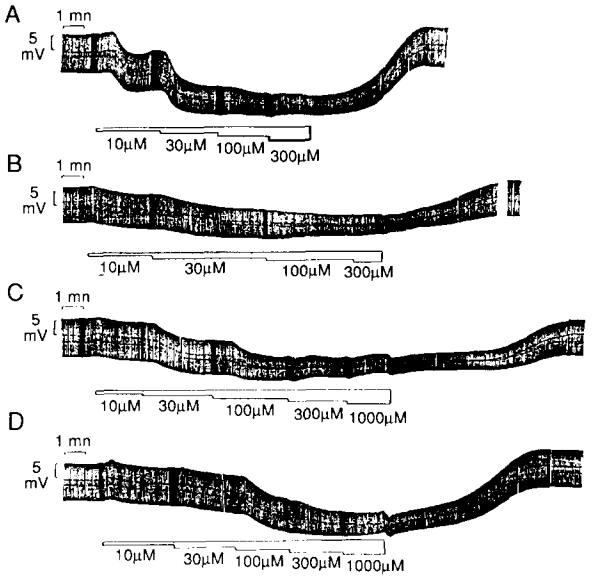


Fig. 2. Effect of 0.1, 0.3, 1 mM NCS 247-90 on GABA dose-response relationship. A: Control responses to cumulative application of GABA. B: In the presence of 0.1 mM NCS 247-90. C: In the presence of 0.3 mM NCS 247-90. D: In the presence of 1 mM NCS 247-90. The corresponding plot is shown in Fig. 3.

2.2. Analysis

The actions of 10 μM, 0.1 mM and/or 1 mM NCS compound on the change in input conductance produced by 30 μM GABA were used to calculate the percentage antagonism thus:

$$\text{percentage antagonism} = 100[1 - (\Delta G_{ant}/\Delta G_{con})],$$

where ΔG_{con} is the change in input conductance produced by the initial control application of 30 μM GABA and ΔG_{ant} is the change in input conductance produced by the application of 30 μM GABA + antagonist, allowing at least 10 min for the antagonist to reach its maximum effect.

Dose ratios determined from the plots of the GABA dose-response curves and the effects of NCS 281-93 were examined using the modified Schild plot (Williams et al., 1988):

$$\log(DR^{n_H} - 1) = M \times \log[X_B] + pK_B,$$

where the Hill coefficient, n_H, is 2 (Duittoz and Martin, 1991b) and M is the number of antagonist molecules interacting with the receptor, and pK_B is the negative logarithm of the dissociation constant of the antagonist receptor complex.

Dose-response relationships for NCS 281-93 were also fitted to the equation:

$$\Delta G = \Delta G_{max}/\left(1 + [ED_{50}/X_a]^{n_H} + [ED_{50}/X_a]^{n_H} \times [X_b/K_B]^m\right) \quad (1)$$

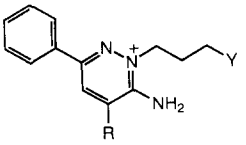
where ΔG is the change in input conductance; ΔG_{max} is the maximum change in input conductance; ED_{50} is the concentration of GABA producing 50% of the ΔG_{max} response; n_H is the Hill coefficient; X_b is the concentration of the NCS antagonist; K_B is the antagonist dissociation constant; M is the degree of cooperativity shown by the antagonist. A non-linear least squares estimate of the parameters of this equation was made using a program written in FORTRAN using the subroutine NAG E04CCF.

Means and standard errors (S.E.) were calculated. Statistical significance was assessed using a two-tailed independent t-test. All compounds cited as having a different potency in the Results section were tested with the t-test and had a significance level of $P < 0.05$.

2.3. Drugs

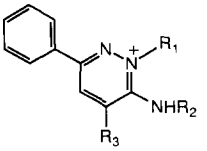
GABA was obtained from Sigma. The NCS compounds, Tables 1–6 and below, were synthesized and their purity confirmed by nuclear magnetic resonance (NMR) (Sitamze et al., 1992; Sitamze, 1993).

Table 1
Chemical structure and percent antagonism of derivatives with modification of the carboxyl function



Compound	Y	R	Benzocyclohepta(e-5,6)	% Antagonism (0.1 mM)		% Antagonism (1 mM)		K_B (μM)
SR 95103	COOH	CH_3	Absent	–		93 ± 3	($n = 5$)	64 ± 13 ($n = 13$)
NCS 248-90	SO_3H	H	Present	–		14 ± 5	($n = 6$)	–
NCS 249-90	SO_3H	CH_3	Absent	–		0, 0	($n = 2$)	–
NCS 250-90	SO_3H	CH_3CH_2	Absent	–		0, 0	($n = 2$)	–
NCS 256-91	COOH	H	Present	0, 16	($n = 2$)	57, 79	($n = 2$)	–
NCS 274-90	Tetrazolyl	$C_6H_5CH_2CH_2$	Absent	0, 10	($n = 2$)	44, 60	($n = 2$)	–
NCS 283-93	$OP(OH)_2$	CH_3	Absent	0 ± 0	($n = 3$)	0 ± 3	($n = 3$)	–

Table 2
Derivatives with modification of the GABA chain



Compound	R1	R2	R3	% Antagonism (0.1 mM)		% Antagonism (1 mM)		K_B (μM)
SR 95103	$(CH_2)_3COOH$	H	CH_3	–		93 ± 3	($n = 5$)	64 ± 13
NCS 194-83	$(CH_2)_4COOH$	H	CH_3	–		46 ± 9	($n = 4$)	–
NCS 259-91	No GABA side-chain	$C_6H_5CH_2CH_2$	CH_3	30 ± 18	($n = 3$)	69 ± 16	($n = 3$)	–
NCS 260-91	No GABA side-chain	$C_6H_5CH_2CH_2$	CH_3CH_2	0, 7	($n = 2$)	23, 29	($n = 2$)	–

2.3.1. Derivatives with modification of the carboxylic function (Table 1)

NCS 248-90, 2-(3-sulphonylpropyl)-3-amino-benzocyclohepta(*e*-5,6) pyridazinium bromide; NCS 249-90, 2-(3-sulphonylpropyl)-3-amino-4-methyl-6-phenyl pyridazine bromide; NCS 250-90, 2-(3-sulphonylpropyl)-3-amino-4-ethyl-6-phenyl pyridazinium bromide; NCS 256-91, 2-(3-carboxypropyl)-benzocyclohepta(*e*-5,6)-3-amino pyridazinium bromide; NCS 274-93, 2-(3-tetrazolylpropyl)-3-amino-4-phenylethyl-6-phenyl pyridazinium chloride; NCS 283-93, 2-(3-phosphonopropyl)-3-amino-4-methyl-6-phenyl pyridazinium bromide.

2.3.2. Derivatives with the length of the GABA chain modified (Table 2)

NCS 194-83, 2-(4-carboxybutyl)-3-amino-4-methyl-6-phenyl pyridazinium bromide; NCS 259-91, 3-phenylethylamine-4-methyl-6-phenyl pyridazinium tartrate; NCS 260-91, 3-phenylethylamine-4-ethyl-6-phenyl pyridazinium tartrate.

2.3.3. Acetoxyethyl derivatives (Table 3)

NCS 276-93, 2-(2′-acetoxyethyl)-3-amino-4-methyl-6-phenyl pyridazinium bromide; NCS 277-93, 2-(2′-acetoxyethyl)-3-amino-6-methoxyphenyl pyridazinium bromide; NCS 278-93, 1-(2′-acetoxyethyl)-2-amino-5-phenyl pyrazinium bromide; NCS 279-93, 2-(2′-acetoxyethyl)-3-amino-5-phenyl pyridazinium bromide.

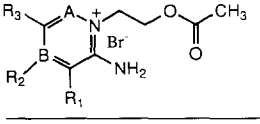
2.3.4. Derivatives with the amine function in position 3 modified (Table 4)

NCS 263-91, 2-(3-carboxypropyl)-3-phenylethylamino-4-ethyl-6-phenyl pyridazinium bromide; NCS 271-92, 2-(3-carboxypropyl)-4-phenylethyl-6-phenyl pyridazinium bromide; NCS 272-92, 2-(3-carboxypropyl)-4-biphenylethyl-6-phenyl pyridazinium bromide; NCS 273-92, 2-(3-carboxypropyl)-4-(2-ethylnaphthyl)-6-phenyl pyridazinium bromide; NCS 280-93, 2-(3-carboxypropyl)-3-(*N*′-acetyl-amino)-4-phenylethyl-6-phenyl pyridazinium bromide.

2.3.5. Derivatives with the substituants in position 4 modified (Table 5)

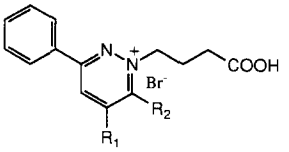
NCS 247-90, 2-(3-carboxypropyl)-3-amino-4-ethyl-6-phenyl pyridazinium bromide; NCS 252-90, 2-(3-carboxypropyl)-3-amino-4-isopropyl-6-phenyl pyridazinium bromide; NCS 251-90, 2-(3-carboxypropyl)-3-amino-4-benzyl-6-phenyl pyridazinium bromide; NCS 253-90, 2-(3-carboxypropyl)-3-amino-4-paramethoxybenzyl-6-phenyl pyridazine bromide; NCS 254-90, 2-(3-carboxypropyl)-3-amino-4-phenylethyl-6-phenyl pyridazinium bromide; NCS 258-91 2-(3-carboxypropyl)-3-amino-4-paramethylbenzyl-6-phenyl pyridazinium bro-

Table 3
Acetoxyethyl derivatives



Compound	R1	R2	R3	A	B	% Antagonism (1 mM)	
NCS 276-93	CH_3	H	C_6H_5	N	C	0, 10	($n = 2$)
NCS 277-93	$CH_3OC_6H_5$	H	H	N	C	0, 22	($n = 2$)
NCS 278-93	H	H	C_6H_5	C	N	37, 40	($n = 2$)
NCS 279-93	H	C_6H_5	H	N	C	50, 25	($n = 2$)

Table 4
Derivatives with the amine function in position 3 modified



Compound	R1	R2	% Antagonism (10 μM)		% Antagonism (0.1 mM)		% Antagonism (1 mM)		K_B (μM)	
SR 95103	CH_3	NH_2	–		–		93 ± 3	(n = 5)	64 ± 13	(n = 13)
NCS 263-91	CH_3CH_2	$NH(CH_2)_2C_6H_5$	–		24.3 ± 9	(n = 4)	68 ± 13	(n = 3)	–	
NCS 271-92	$C_6H_5CH_2CH_2$	H	0	(n = 1)	6, 0	(n = 2)	49, 38	(n = 2)	–	
NCS 272-92	Biphenyl$(CH_2)_2$	H	0	(n = 1)	12	(n = 1)	88 ± 12	(n = 3)	–	
NCS 273-92	Naphthyl$(CH_2)_2$	H	0, 0	(n = 2)	14, 21	(n = 2)	100, 100	(n = 2)	120, 140	(n = 2)
NCS 280-93	$C_6H_5CH_2CH_2$	$NHCOCH_3$	7, 0	(n = 2)	26, 31	(n = 2)	59, 100	(n = 2)	–	

mide; NCS 266-92, 2-(3-carboxypropyl)-3-amino-4-paramethoxyphenethyl-6-phenyl pyridazinium bromide; NCS 267-92, 2-(3-carboxypropyl)-3-amino-4-meta-methoxyphenethyl-6-phenyl pyridazinium bromide; NCS 268-92, 2-(3-carboxypropyl)-3-amino-4-[(4,3-methylenedioxy)]phenethyl-6-phenyl pyridazinium bromide; NCS 281-93, 2-(3-carboxypropyl)-3-amino-4-phenylpropyl-6-phenyl pyridazinium bromide; NCS 282-93, 2-(3-carboxypropyl)-3-amino-4-diphenylethyl-6-phenyl pyridazinium bromide.

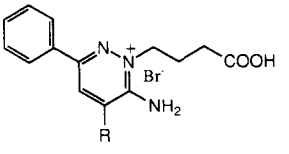
2.3.6. Derivatives with modification to the pyridazine ring (Table 6)

NCS 255-90, 3-(3-carboxypropyl)-2-amino-5-phenyl thiadiazolium; NCS 257-91, 1-(3-carboxypropyl)-2-amino 5-phenyl pyridazinium bromide; NCS 261-91, 3-phenethylamine-8-phenyl phthalazine; NCS 262-91, 3-(*n*-(2-aminoethylmorpholine))-8-phenyl phthalazine chlorhydrate; NCS 264-91, 2-(3-carboxypropyl)-3-*N*-(2-(aminoethylmorpholine))-8-phenyl phthalazinium bromide; and NCS 265-91, 2-(3-carboxypropyl)-3-phenethylamine-8-phenyl bromide.

3. Results

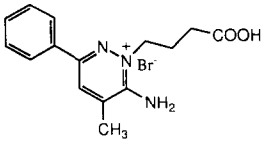
The results reported here are based on the analysis of experiments performed on 123 cells from more than 80 preparations. The results of similar tests on SR compounds (Duittoz and Martin, 1991b) are included for comparative purposes. Cells selected for recording and analysis had a resting membrane potential more

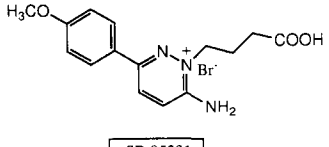
Table 5
Derivatives modified in position 4



Compound	R	% Antagonism (10 μM)		% Antagonism (0.1 mM)		% Inhibition (1 mM)		K_B (μM)	
SR 95103	CH_3	–		–		93 ± 3	(n = 5)	64 ± 13	(n = 13)
SR 95132	C_6H_5	–		–		89 ± 3	(n = 5)	65 ± 20	(n = 9)
NCS 247-90	CH_3CH_2	–		–		95 ± 3	(n = 5)	55 ± 16	(n = 13)
NCS 251-90	$C_6H_5CH_2$	–		24, 48	(n = 2)	99 ± 1	(n = 3)	31 ± 14	(n = 3)
NCS 252-90	$(CH_3)_2CH$	–		–		81 ± 3	(n = 3)	–	
NCS 253-90	p-HO-$C_6H_5CH_2$	–		30,	(n = 1)	91 ± 5	(n = 3)	–	
NCS 254-90	$C_6H_5CH_2CH_2$	–		56 ± 4	(n = 4)	97, 100	(n = 2)	11 ± 3	(n = 4)
NCS 258-91	p-CH_3-$C_6H_4CH_2$	5, 11	(n = 2)	55, 42	(n = 2)	93, 86	(n = 2)	–	
NCS 266-92	p-MeO-$C_6H_4(CH_2)_2$	0	(n = 1)	15, 38	(n = 2)	88, 97	(n = 2)	–	
NCS 267-92	m-MeO-$C_6H_4(CH_2)_2$	0, 0	(n = 2)	18, 35	(n = 2)	73, 93	(n = 2)	–	
NCS 268-92	4,3-Methylenedioxy	0, 0	(n = 2)	0, 0	(n = 2)	74, 58	(n = 2)	–	
NCS 281-93	$C_6H_5CH_2CH_2CH_2$	24, 10	(n = 2)	57, 50	(n = 2)	94, 96	(n = 2)	4.7 ± 0.7	(n = 11)
NCS 282-93	$(C_6H_5)_2CHCH_2$	11, 27	(n = 2)	75, 42	(n = 2)	73, 100	(n = 2)	Non-competitive	

Table 6
Derivatives with a modified ring structure


SR 95103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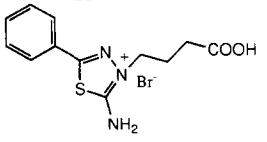
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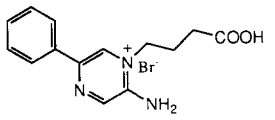
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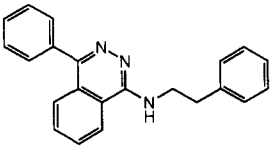
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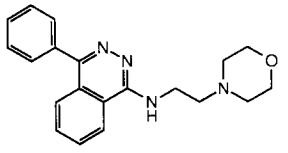
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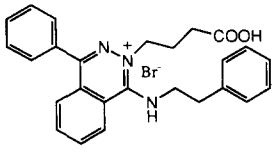
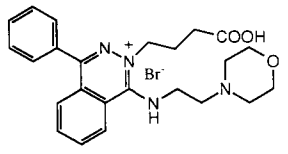

NCS 265-91


NCS 264-91

Compound	Ring modification	% Inhibition (0.1 mM)		% Inhibition (1 mM)	
SR 95103	None	–		93 ± 3	(n = 5)
SR 95531	None	–		44 ± 6	(n = 6)
NCS 255-90	Thiazolium			49 ± 9	(n = 4)
NCS 257-91	Pyrazinium	0, 6	(n = 2)	20, 65	(n = 2)
NCS 261-91	Phthalazine	0, 0	(n = 2)	14, 0	(n = 2)
NCS 262-91	Phthalazine	0, 8	(n = 2)	0, 25	(n = 2)
NCS 264-91	Phthalazine	2.7 ± 3		8 ± 5	(n = 4)
NCS 265-91	Phthalazine	43 ± 18	(n = 4)	75 ± 24	(n = 3)

negative than −20 mV and a resting input conductance between 2.0 and 3.5 μS. Recordings were rejected if the resting input conductance failed to return to less than 120% of the control after the final wash.

All of the antagonists were applied at a concentration up to 1 mM in the absence of GABA and found to have no effect on membrane potential or input conductance. The lack of effect of the compounds on input conductance showed that the compounds do not act as GABA agonists despite the presence of the GABA side-chain in the 2-[3-carboxypropyl] derivatives or as nicotinic agonists despite the acetylcholine side-chain in the 2-[2′-acetoxyethyl] derivatives.

Fig. 1A and B illustrates the first test for activity that was carried out on all of the antagonists: 30 μM GABA was applied initially and the conductance increase produced by the GABA allowed to come to a steady state; the antagonist was then applied at a concentration of 1 mM, on top of the GABA and again the input conductance permitted to come to a steady state. Fig. 1A shows the effect of a low potency compound, NCS 250-90: GABA increased the resting input conductance from 3.2 μS to 5.0 μS but this remained unchanged in the presence of the antagonist so that the percent antagonism was 0%. Fig. 1B shows the effect of a more potent antagonist, NCS 252-90: GABA increased the resting input conductance from 3.1 μS to 4.5 μS so that the input conductance change, ΔG, was 1.4 μS. In the presence of the antagonist, ΔG was 0.3 μS and the percent antagonism was 79%. The effects of 10 μM and 0.1 mM antagonist were also examined in a similar manner. Tables 1, 2, 3, 4, 5 and 6 summarize the percentage antagonisms observed and illustrate the structures of the compounds.

3.1. Derivatives with modification of the carboxylic function

Table 1 shows the list of compounds examined where the carboxylic group on the GABA chain has been replaced with sulphonic (NCS 248-90, NCS 249-90 and NCS 250-91), phosphonic (NCS 283-93) and tetrazole (NCS 274-93) groups. Comparison of the mean percentage antagonism for NCS 249-90 and NCS 250-90 with the mean percentage antagonism shown by SR 95103, and the percent antagonism shown by NCS 248-90 with the percent antagonism shown by NCS 256-91, demonstrates that the introduction of the sulphonic group significantly (all values at 1 mM $P < 0.01$) reduces potency and renders compounds inactive. The same is true when the phosphonic group of NCS 283-93 replaces the carboxylic group of SR 95103. Comparison of the antagonism shown by 0.1 mM concentrations of NCS 274-93 with that of NCS 254-90 (Table 5) shows that replacing a carboxylic group with a tetrazole group significantly ($P < 0.01$) reduces the potency of the antagonist.

3.2. Derivatives with the GABA side-chain modified

The significant ($P < 0.001$) reduction in the mean percent antagonism of 1 mM NCS 194-83 when compared to SR 95103 (Table 2) shows the effect of increasing the length of the acid side-chain from 4 to 5 atoms. Comparison of the structure and activity of NCS 260-91 (Table 2) with the structure and activity of NCS 263-91 (Table 4) shows that omission of the GABA side-chain leads to a significant ($P < 0.02$) reduction in activity. Similarly a comparison of the structure and activity of NCS 276-93 (Table 3) with that of SR 95103 (Table 1) shows that replacing the GABA side-chain with an acetylcholine side-chain leads to a large (significant $P < 0.01$) reduction in potency. None of the acetoxyethyl derivatives were potent. It appears then that a 4-carbon carboxylic side-chain on the 2-position of the pyridazine ring is important for the potency of the antagonist.

3.3. Derivatives with modification of the 3-amino moiety

Acylation of the 3-amino group leads to a reduction in activity: this is illustrated by comparing the percent antagonism produced by a 0.1 mM concentration of the acylated compound NCS 280-93 (Table 4) which is significantly ($P < 0.01$) less than the non-acylated analogue, NCS 254-90 (Table 5). Alkylation of the 3-amino group also leads to significant reduction ($P < 0.05$ at 1 mM) in activity and is seen by comparing NCS 263-91 (Table 4) with NCS 247-90 (Table 5). The requirement for the 3-amino group for activity is illustrated by comparing the poor activity of a derivative that lacks the amine moiety, NCS 271-92 (Table 4) with the derivative which possesses the amine group and a greater inhibitory potency, NCS 254-93 (Table 4); the significance level at 0.1 mM was $P < 0.01$. Thus it appears that a free amine group in position 3 is required for the potency of the antagonist.

3.4. Modification of groups in position 4

The effect of substitution in the 4-position of the pyridazine ring has already been pointed out (Duittoz and Martin, 1991b). Substitution in this position appears to be detrimental to antagonist potency at the vertebrate $GABA_A$ receptor but it is a prerequisite for antagonist potency at the *Ascaris* muscle GABA receptor. The series of compounds shown in Table 5 were designed to investigate further the structure-activity relationships of substituents in this position.

The high percentage antagonisms shown by the compounds: SR 95103, SR 95132, NCS 252-90, NCS 247-90, NCS 251-90, NCS 254-90, NCS 258-91, NCS 281-93, NCS 282-93 (Table 5) are consistent with the view that introduction of lipophilic groups in position 4 facilitates the potency of the antagonist. The effect of introduction of the methoxy group on the phenyl ring of the 4-position chain in NCS 266-92 and NCS 267-92 is to reduce the lipophilicity of the side-chain and to reduce the mean activity of the antagonists when compared to NCS 254-90 that lacks the methoxy group; the differences in potency, however, did not reach statistical significance. Comparison of NCS 268-92 (a 4,3-methylene dioxy derivative of NCS 251-90) did reveal a statistically significant reduction ($P < 0.05$ at 1 mM) following substitution of more lipophilic moieties.

3.5. Mode of action of more potent antagonists

It order to examine further the mode of action of the more potent antagonist, GABA dose-response curves were obtained in the absence and then in the presence of different concentrations of antagonist: Fig. 2 shows an example of an experiment carried out with the antagonist NCS 247-90. Representative dose-response curves are shown in Fig. 3 for NCS 273-92, NCS 247-90, NCS 251-90, and NCS 254-90, in Fig. 4 for NCS 282-93, and in Fig. 5 for NCS 281-93.

Although there may be a small shift to the right with NCS 282-93 (Fig. 4) it was clear that the antagonism shown by NCS 282-93 was non-competitive (two experiments) because of the reduction of the slope of the dose-response curves: the other antagonists produced a parallel shift to the right without an obvious reduction in the maximum response, suggesting a competitive mechanism.

Dose ratios were determined from these plots as the ratio of the concentrations producing nearly half the

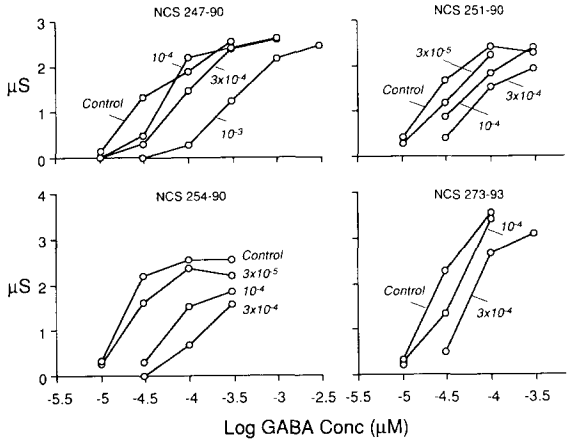


Fig. 3. Plots of GABA log-dose conductance-response relationships in single cells and the effect of antagonists: NCS 247-90, NCS 251-90, NCS 254-90 and NCS 273-90. Note that the shift to the right and the lack of depression in the maximum response suggest competitive antagonism.

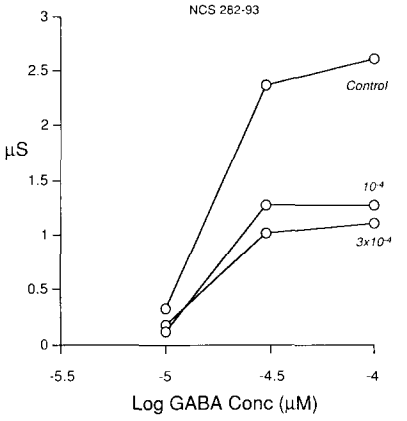


Fig. 4. Plot of GABA log-dose conductance-response relationship in a representative cell and the effect of the antagonist NCS 282-93. Note the small shift to the right but the reduction in the maximum response showing non-competitive antagonism.

maximum response observed. The dissociation constant, K_B, of the antagonists were then estimated for the antagonists with the equation:

$$K_B = (DR^2 - 1)/X_B,$$

where X_B is the antagonist concentration and DR is the dose ratio. The equation was used to take account of the cooperativity of the *Ascaris* GABA receptor which may be explained by two molecules of GABA being required to open the GABA channel but only one molecule of antagonist may interact with the receptor to prevent opening (Duittoz and Martin, 1991b).

The values of K_B determined are shown in Table 5. It can be seen that the potency of the antagonists increased from: SR 95103 with a K_B 64 μM (Duittoz and Martin, 1991b); to NCS 247-90 with a K_B of 55 μM; to NCS 251-90 with a K_B of 31 μM; to NCS 254-90 with a K_B of 11 μM; to NCS 281-93 with a K_B of 4.7 μM. Thus substituting the methyl group at position 4 in SR 95103 with a phenyl, phenylmethyl, phenylethyl, phenylpropyl leads to a progressive increase in the potency of the antagonist.

The effect of the most potent antagonist, NCS 281-93, on the GABA dose-response relationship was examined in three experiments by using a modified Schild plot, Fig. 4B, and by estimating the parameters of Eq. 1. The gradient of the modified Schild plot of Fig. 4B was 0.74. The non-linear least squares estimates for the constants of Eq. 1 were: n_H was 1.55; M was 0.95; G_{max} was 6.3 μS; the ED_{50} for GABA was 21 μM; and the K_B for NCS 281-83 was 8.6 μM. In two other

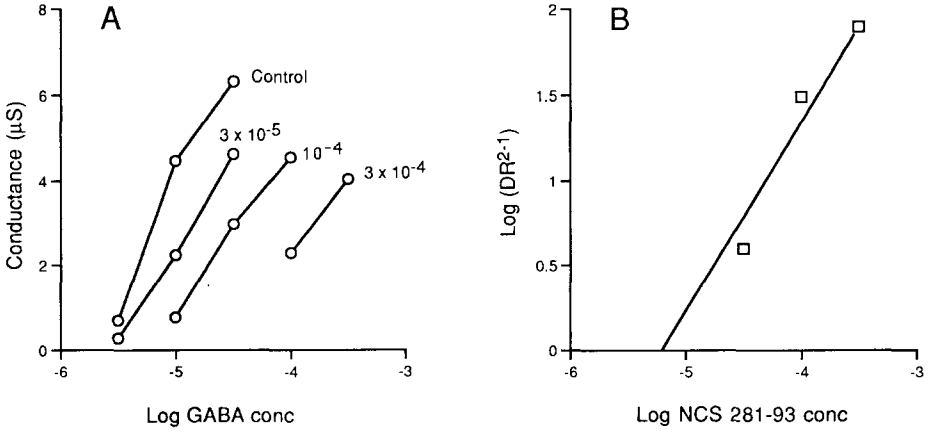


Fig. 5. A: Plot of the GABA log-dose-conductance response relationship in a representative cell and the effect of the antagonist NCS 281-93. Note the shift to the right and the absence of a reduced maximum response associated with the application of the antagonist. B: Modified Schild plot derived from the plot in (A); slope, 0.74; intercept on abscissa, 8 μM.

experiments similar results were obtained. These observations are consistent with the proposed mechanism whereby two molecules of GABA are required to produce opening but only one molecule of antagonist is required to produce blocking (Duittoz and Martin, 1991b). Similar values were estimated in single experiments for n_H and M with the antagonists: NCS 273-92, NCS 247-90, NCS 251-90, NCS 254-90 and NCS 282-93.

3.6. Modification of the pyridazine ring structure

Table 6 shows that NCS 255-90, a compound in which the pyridazine ring is replaced by a thiadiazolium ring, and NCS 257-91, a compound in which the pyridazine ring is replaced by a pyrazine ring, have approximately the same inhibitory potency as SR 95531, a pyridazine derivative not substituted in the 4-position. However NCS 255-90, NCS 257-91 and SR 95531 are all significantly ($P < 0.01$) less potent than SR 95103 (a pyridazine derivative with a 4-methyl substituent). These observations suggest that either the thiadiazolium or pyrazine ring could replace the pyridazine ring without a large change in potency. However, without substitution in a position equivalent to the 4-position on the pyridazine ring, there would be no increase in potency of the analogue.

Table 6 also shows the percent inhibition results obtained with the phthalazine derivatives: NCS 261-93, NCS 262-93, NCS 264-93, NCS 265-93. These compounds, except for NCS 265-93, are significantly ($P < 0.01$) less potent than SR 95103. Comparison of the structure activities of NCS 247-90, NCS 263-91 and NCS 265-91 suggests that the phthalazine ring does not reverse the loss in potency produced by acylation of the 3-amino moiety.

4. Discussion

We have measured the percent antagonism of the conductance response to 30 μM GABA as an initial measure of antagonist potency. Since this method used single concentrations of agonist and antagonist, it does not provide information about the type of antagonism involved. However, within a group of chemically related compounds such as the arylaminopyridazine derivatives, the percent antagonism can be used to indicate relative potencies. The method is a sensitive technique for detecting antagonism at the GABA receptor because the concentration of GABA selected, 30 μM, is near the EC_{50} for the GABA dose-response curve (Martin, 1980) and therefore in the linear section of the log dose-response curve. The method also permits a larger number of compounds to be screened for activity. The compounds that had a higher percent antagonism than the starting compound, SR 95103, were also examined by estimating their K_B values using a range of GABA as well as antagonist concentrations. The modified Schild analysis (Williams et al., 1988; Duittoz and Martin, 1991b) was used to determine K_B, since it allows for the cooperativety produced by two molecules of GABA being required for channel opening but only one molecule of antagonist being required to block the effect of GABA: this model has previously been shown to be appropriate for describing the action of the arylaminopyridazine derivatives at the *Ascaris* GABA receptor.

The estimated dissociation constant, K_B, or the mean antagonism obtained when applied at a concentration of 0.1 mM or 1 mM in the presence of 30 μM GABA gave the rank order of potency at the *Ascaris* muscle GABA receptor as: NCS 281-92 > NCS 254-90 > NCS 251-90 > NCS 247-90 > SR 95103 > NCS 258-91 > SR 95132 > NCS 272-92 ≈ NCS 282-93 > NCS 267-92 > NCS 252-90 > NCS 280-93 > SR 42666 ≈ NCS 273-92 > NCS 259-91 ≈ NCS 268-91 > SR 95133 > NCS 255-90 > NCS 194-83 ≈ SR 95531 ≈ NCS 271-93 ≈ NCS 257-91 > SR 42627 > NCS 260-90 > NCS 248-90 > SR 42640 ≈ NCS 264-90 ≈ NCS 261-91 ≈ NCS 283-90 > NCS 249-90 ≈ NCS 250-90. The potencies of the arylaminopyridazine derivatives obtained previously (Duittoz and Martin, 1991b) are also included to permit a fuller description of the antagonist profile. It is pointed out that because of the large number of compounds used in this list that the first of adjacent compounds is not necessarily significantly more potent than the second of the adjacent compounds but the list gives an indication of the relative potencies of the series of arylaminopyridazine derivatives tested. At a mammalian $GABA_A$ receptor the potency order of the compounds tested is: SR 95531 > SR 42666 > SR 42627 > SR 95103 > SR 95133 > SR 42640 > SR 95132 and it has already been pointed out that the different order in *Ascaris* (SR 95103 ≈ SR 95132 > SR 42666 > SR 95133 > SR 95531 > SR 42627 > SR 42640) distinguishes the receptors. This difference may be explained by differences in the accessory binding sites required by competitive antagonists rather than differences in the agonist binding site (Duittoz and Martin, 1991a,b).

4.1. Modification of the 2-position moiety

The structure-activity relationship showed that substitution of the carboxylic acid of the 2-(3-carboxypropyl) derivatives with sulphonic (NCS 249-90, NCS 250-90) or phosphonic acid (NCS 283-93) reduced potency. This effect appears comparable to the effect of substitution of the carboxylic group of GABA to produce 3-APS and P4S (Holden-Dye et al., 1989) which produces low potency agonists at the *Ascaris* GABA receptor. Lengthening the side chain of the 2-position

moiety (NCS 194-83, 5C like δ-aminovaleric acid) was also detrimental to the potency as it is for the agonist δ-aminovaleric acid. The observation that an intact GABA sequence is optimal for receptor recognition by the antagonist suggests that it is the 2-position moiety of the antagonist that binds to the *Ascaris* GABA agonist binding site. A distance of 5 A° between the acidic and basic function is likely to pertain since extended agonists (*trans*-aminocrotonic acid) are more potent than folded agonists (*cis*-crotonic acid), Holden-Dye et al., 1989). Complete removal of the 2-position moiety (NCS 259-91, NCS 260-91) did not lead to abolition of GABA receptor antagonist activity suggesting that the remaining molecule can still recognize part of the GABA receptor site. Similar conclusions have been obtained with other arylaminopyridazine GABA receptor antagonists for a mammalian $GABA_A$ receptor (Wermuth et al., 1987).

4.2. Modification of the 3-position

It has been concluded from agonist and antagonist structure-activity studies for a vertebrate $GABA_A$ receptor that: a GABA moiety bearing a positive charge is necessary for optimal receptor recognition by arylaminopyridazine derivatives and that additional binding sites by *N* substitution are only tolerated if they are part of a charge-delocalized amidinic or guanidinic system; replacement of the endo-exo amidinic system leads to total inactivation of the compound (Wermuth et al., 1987). This is supported by the fact that isosteric replacement of the 3-amino group by a neutral group leads to total inactivation. In *Ascaris*, by contrast, the compounds NCS 270-92, NCS 271-92, and NCS 273-92 which lack the 3-amino group still show antagonist activity at the GABA receptor, although they are reduced in potency compared to the 3-amino derivatives. The anthelmintic piperazine, which is a selective GABA agonist in *Ascaris* (Martin, 1982), also lacks an amidinic or guanidinic system delocalizing charge, again suggesting that delocalization is not an absolute requirement in *Ascaris*.

4.3. Substitution in the 4-position

In vertebrates, substitution in the 4-methyl group of SR 95103 with hydrogen (SR 95531), leads to an increase in potency but replacement with a phenyl group is detrimental to antagonist potency (Wermuth et al., 1987). By contrast, 4-position substitution appears to be a prerequisite for antagonist potency in *Ascaris* (Duittoz and Martin, 1991b). The difference between the vertebrate GABA receptor and the *Ascaris* receptor may be explained by obstruction of a free access zone between the anionic and cationic binding site (Wermuth and Rognan, 1987). The free access zone is required for receptor recognition in vertebrates but not *Ascaris* (Duittoz and Martin, 1991b). In the novel compounds studied in this paper, the introduction of more lipophilic groups increased the potency of the antagonist compound. Starting from a 4-methyl substitution (SR 95103), the potency of the antagonists increased from: a K_B of 64 μM, Duittoz and Martin (1991b); to NCS 247-90 with 4-phenyl substitution and a K_B of 55 μM; to NCS 251-90 with 4-phenylmethyl substitution and a K_B of 31 μM; to NCS 254-90 with 4-phenylethyl substitution and a K_B of 12 μM; to NCS 281-93 with a 4-phenylpropyl and K_B of 4 μM. Thus, substituting the methyl group at position 4 in SR 95103 leads to a progressive increase in the potency of the antagonists.

4.4. The GABA receptor as target site for anthelmintics

GABA receptors mediating an increase in the Cl^- conductance of neuronal membranes are found in vertebrates as well as in invertebrate muscle and nerve membranes (Nistri and Constanti, 1979) including nematode parasite membranes (Martin, 1980,1982). It has already been pointed out in the Introduction that the agonist profile of the *Ascaris* muscle GABA receptor and a vertebrate $GABA_A$ receptor are similar but that the antagonist profile is very different (Holden-Dye et al., 1989; Martin et al., 1991). These observations may be explained by vertebrate $GABA_A$ receptors and *Ascaris* muscle GABA receptors having similar agonist binding sites. The differences in the accessory binding sites required for antagonists may explain differences in the antagonist profiles (Ariëns et al., 1979; Wermuth et al., 1987; Duittoz and Martin, 1991b). It is therefore suggested that a selective GABA receptor antagonist for nematodes and *Ascaris* may be easier than an agonist to find.

Selective cholinergic agonist like levamisole, pyrantel and morantel are used as anthelmintics and appear to work by acting as agonists on nematode muscle acetylcholine receptors (Harrow and Gration, 1985; Martin et al., 1991). Stimulation of the acetylcholine receptors produces depolarization and spastic paralysis of the nematode leading to its expulsion. Unfortunately resistance to therapy with these compounds is increasing requiring higher concentrations of anthelmintic to be used (Sangster et al., 1991) or the change to a drug that has another mode of action. One way of increasing the effectiveness of a therapeutic regimen would be to add a selective GABA receptor antagonist along with the selective cholinergic agonist so that the two compounds could act in concert producing depolarization, contraction and spastic paralysis. Further study of the structure-activity relationships of arylaminopyridazines may permit the development of therapeutic approaches that can control nematode parasites resistant to other anthelmintic agents.

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