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ORIGINAL PAPER

Guanidino Acids Act as ρ1 $GABA_C$ Receptor Antagonists

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Abstract $GABA_C$ receptors play a role in myopia, memory-related disorders and circadian rhythms signifying a need to develop potent and selective agents for this class of receptors. Guanidino analogs related to glycine, β-alanine and taurine were evaluated at human ρ_1GABA_C receptors expressed in *Xenopus* oocytes using 2-electrode voltage clamp methods. Of the 12 analogs tested, 8 analogs were active as antagonists and the remaining were inactive. (*S*)-2-Guanidinopropionic acid ($IC_{50} = 2.2$ μM) and guanidinoacetic acid ($IC_{50} = 5.4$ μM; $K_B = 7.75$ μM [$pK_B = 5.11 \pm 0.06$]) were the most potent being competitive antagonists at this receptor. In contrast, the β-alanine and GABA guanidino analogs showed reduced activity, indicating the distance between the carboxyl carbon and terminal nitrogen of the guanidino group is critical for activity. Substituting the C2-position of guanidinoacetic acid with various alkyl groups reduced activity indicating that steric effects may impact on activity. The results of this study contribute to the structure–activity-relationship profile required in developing novel therapeutic agents.

Keywords $GABA_C$ receptors · Guanidino acids · Taurine · Glycine · β-Alanine

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Abbreviations

TPMPA	(1,2,5,6-Tetrahydropyridin-4-yl) methylphosphinic acid
CACA	(*Z*)-4-Aminobut-2-enoic acid
(+)-CAMP	(+)-*cis*-2-Aminomethylcyclopro panecarboxylic acid
cis- and *trans*-3-ACPBPA	*cis*- and *trans*-(3-aminocyclopentanyl) butylphosphinic acid
ZAPA	(*Z*)-3-[(Aminoiminomethyl)thio] prop-2-enoic acid
(*R*)- and (*S*)-2-Me-GABA	(*R*)- and (*S*)-4-Amino-2-methyl-butyric acid
GAA	Guanidinoacetic acid
5-MeIAA	5-Methyl imidazole-4-acetic acid

Introduction

GABA is an important inhibitory neurotransmitter in the central nervous system (CNS) activating receptors termed $GABA_A$, $GABA_B$ and $GABA_C$ receptors. $GABA_A$, and $GABA_C$ receptors are members of the ligand-gated-ion-channels or cys-loop family that incorporates nicotinic acetylcholine, serotonin type 3 and strychnine-sensitive glycine receptors while $GABA_B$ receptors are members of the family 3 class of G-protein-coupled receptors [1, 2].

$GABA_A$ receptors are hetero-oligomeric pentamers formed from a combination of subunits consisting of α_{1-6}, β_{1-4}, γ_{1-3}, δ, ε, θ and π. In contrast, $GABA_C$ receptors are either homo-oligomeric or pseudo-hetero-oligomeric pentamers comprising only of ρ subunits [1, 2]. The most studied $GABA_C$ receptor is the one consisting of the ρ_1 subunit.

The pharmacology of $GABA_C$ receptors is distinct from $GABA_A$ receptors. $GABA_C$ receptors are not blocked by the alkaloid bicuculline nor modulated by benzodiazepines and steroids that affect $GABA_A$ receptors. Instead $GABA_C$ receptors are selectively activated by (+)-2-(aminomethyl)-cyclopropane-1-carboxylic acid [(+)-CAMP] [3] and 5-methyl imidazole-4-acetic acid [5-MeIAA] [4], and blocked by (1,2,5,6-tetrahydropyridin-4-yl)methylphosphinic acid [TPMPA] [5, 6], the cyclopentane phosphinic acids, *cis*- and *trans*-(3-aminocyclopentanyl)butylphosphinic acid [7–9] and the cyclopentene phosphinic acids, (*R*)- and (*S*)-4-amino-cyclopent-1-enyl phosphinic acids [10].

$GABA_C$ antagonists have been shown to prevent experimental myopia development, and inhibited the associated vitreous chamber elongation [9], enhance memory in chicks [11] and rats [9], and affect sleep-waking behavior in rats [12] suggesting that these receptors may also be involved in myopia, memory-related disorders and circadian rhythms [13]. $GABA_C$ receptors have also been found to be present in thyrotropin-secreting cells where they may be involved in the regulation of hormone secretion [14], as well as in the gut where they may have a role in regulating gut motility [15].

To date, the structure–activity-relationship (SAR) profile of $GABA_C$ receptors is not well defined impeding the development of therapeutically useful agents for this receptor. The structural manipulations made in developing GABA analogs for the $GABA_C$ receptor have mainly been confined to the carboxylic acid end of the molecule or conformational restriction of the flexible backbone. Such analogs have led to potent and selective $GABA_C$ receptor ligands [5, 7–10], however few studies have investigated modifications of the amine functional group.

ω-Guanidino acids are known to act at GABA receptors indicating that the guanidino group is equivalent to an amino function with an additional two carbons [16]. Thus, guanidinoacetic acid (GAA) behaves like GABA rather than glycine and indeed guanidinoacetic acid is a partial agonist at $GABA_A$ receptors with no effects on $GABA_B$ receptors [17]. Developments in this field have led to the discovery of the isothiouronium analog of GABA, such as (*Z*)-3-[(aminoiminomethyl)thio] prop-2-enoic acid [ZAPA] [18, 19], a potent agonist at $GABA_A$ receptors but also an antagonist at $GABA_C$ receptors [20], and a substrate for the neuronal GABA transport system [19]. Furthermore, γ-guanidinobaclofen is weak full agonist at ileal $GABA_B$ receptors but almost inactive on rat neocortical slices [21].

Glycine [22–24], taurine [23, 24] and β-alanine [22, 23] have been shown to activate $GABA_C$ receptors at concentrations that may be reached in the synapse indicating that, like GABA, these amino acids can modulate synaptic transmission across GABAergic synapses. The activities of these three amino acids are weak, activating the receptors at millimolar concentrations. This study evaluates a series of guanidino analogs related to glycine, taurine and β-alanine on recombinant ρ_1 $GABA_C$ receptors expressed in *Xenopus laevis* oocytes for SAR analysis to test the hypothesis that by introducing a guanidino moiety the activity of glycine, taurine and β-alanine may improve at $GABA_C$ receptors.

Experimental Procedure

Materials and Methods

Guanidino analogs, guanidinoacetic acid (**1**) 3-guanidinopropionic acid (**2**), 4-guanidinobutanoic acid (**3**), 2-guanidinoethanesulfinic acid (**4**) (*S*)-2-guanidinopropanoic acid (**5**), (±)-2-guanidinopropanoic acid (**6**), (*S*)-2-guanidino butyricacid (**7**), (*S*)-2-guanidino-3-hydroxypropanoic acid (**8**), (*S*)-2-guanidino-3-methylbutyric acid (**9**), (2*S*,3*R*)-2-amino-3-hydroxybutyric acid (**10**), (*S*)-2-guanidino-2-(4-hydroxyphenyl) acetic acid (**11**) and creatine (**12**) were synthesized in house using established methodologies [25–27]. All other chemicals were obtained from Sigma-Aldrich (St Louis, MO, USA) unless otherwise stated. Human ρ1 cDNA encapsulated in the pcDNA1.1 vector (Invitrogen, San Diego, CA, USA) was kindly donated by Dr George Uhl (National Institute for Drug Abuse, Baltimore, MD, USA).

ρ1 cDNA was linearized with *NOT*1 as previously reported and transcribed to mRNA using the T7 "mMESSAGE mMACHINE" kit (Ambion Inc. Austin, Texas, USA) [28]. Electrophysiological methods were performed as previously described [28]. In brief, oocytes were harvested from *Xenopus laevis* (housed in the Department of Veterinary Science at the University of Sydney) and defolliculated. The oocytes were then stored in ND96 solution (in mM) NaCl (96), KCl (2), $MgCl_2$ (1), $CaCl_2$ (1.8), HEPES (hemi-Na salt; 5) supplemented with sodium pyruvate (2.5), theophylline (0.5) and 50 $\mu g/ml^{-1}$ gentamycin.

Electrophysiology

Stage V–VI oocytes were injected with 50 µl containing 10 ng of mRNA and then stored at 16°C. Recordings of receptor activity were obtained after 2–8 days by two-electrode voltage clamp by means of a Geneclamp 500 amplifier (Axon Instruments Inc., Foster City, CA), a MacLab 2e recorder (AD Instruments, Sydney, NSW) and Chart version 5.5.6 program. Oocytes were voltage clamped at −60 mV using glass electrodes filled with 3 mM KCl (0.5–1.5 MΩ). The preparation was continually perfused with ND96 solution at room temperature. Known concentrations of ligands dissolved in ND96 were applied

in the absence and presence of GABA, respectively, until maximum current was reached, at which time the oocyte was washed for five to ten minutes to allow complete recovery of response to a known maximal dose of GABA.

The current elicited by each drug on expressed receptors was measured and standardized to GABA (1 μM) on the same cell in the following ratio ($I/I_{GABA(1\ \mu M)}$) unless otherwise stated. The dose response curves were plotted using the current ratios versus concentration. Dose response curves [Sigmoidal dose-response (variable slope)] were obtained using the least squares regression Eq. (1) from Prism (GraphPrism v. 4) where I is the current at a known concentration of agonist; I_M is the maximum current; [A] is the agonist (GABA) concentration; EC_{50}, the concentration of GABA that activates 50% of receptors or IC_{50} the concentration of antagonist that inhibits 50% of receptors at a given agonist concentration; and n_H is the Hill coefficient. This equation is identical to the four parameter logistic equation where "Bottom" refers to the estimated response at zero concentration and "Top" refers to estimated response at infinite concentration.

$$I/I_M = \text{Bottom} + (\text{Top} - \text{Bottom})/(1 + 10\text{^}(\log EC_{50}(\text{or } \log IC_{50}) - \log[A] \times n_H)) \quad (1)$$

The dissociation constant of an antagonist (K_B) is the effective concentration at which an antagonist binds to half the receptor population. Where the EC_{50} of GABA was determined in multiple concentrations of the antagonist, competitive antagonism was tested for via the Gaddum–Schild equation derived from [29].

$$\log(DR - 1) = pA_2 + m \log[Ant] \quad (2)$$

where DR is the EC_{50} of GABA in the presence of inhibitor divided by the EC_{50} of GABA alone where the EC_{50} is the concentration of agonist that gives half maximal response, [Ant] is the concentration of the antagonist and pA_2 is the negative logarithm of the concentration of antagonist needed to shift the dose response curve by a factor of 2. If the SchildSlope is fixed to 1.0 (i.e. assuming competitive antagonism), the pA_2 is the $-\log[K_B]$, the negative log of the equilibrium dissociation constant of inhibitors binding to the receptor.

Where the EC_{50} of GABA in the presence of only a single concentration of antagonist was determined, competitive antagonism was assumed based on the lack of intrinsic efficacy and a linear shift of the GABA does response curve to the right. The pA_2 or $-\log[K_B]$ were determined via Eq. 2 above. The K_B for each antagonist was subsequently determined from the calculated pA_2 or $-\log[K_B]$ values. All compounds were tested on oocytes from at least two harvests.

Results

Oocytes expressing homomeric ρ_1 $GABA_C$ receptors responded to GABA as previously reported [28]. Guanidino analog (**1**)–(**12**) were evaluated for activity alone and in the presence of GABA (1 μM). These compounds had no effect alone when tested at 100 or 300 μM but in the presence of GABA (1 μM), compounds (**1**)–(**8**) inhibited the response in a concentration-dependent manner (Fig. 1; Tables 1, 2).

Figure 1 shows the inhibitory concentration response curves for the active analogs (**1**)–(**8**) against GABA (1 μM). Only guanidino analogs derived from the natural (*S*)-amino acids were active. The potency order of the series was found to be (**1**) ≈ (**5**) > (**2**) > (**6**) ≈ (**3**) > (**7**) ≈ (**8**).

In contrast, guanidino compounds such as (*S*)-2-guanidino-3-methylbutyric acid (**9**), (2*S*,3*R*)-2-amino-3-hydroxybutyric acid (**10**), (*S*)-2-guanidino-2-(4-hydroxyphenyl) acetic acid (**11**) and creatine (**12**) were inactive or had

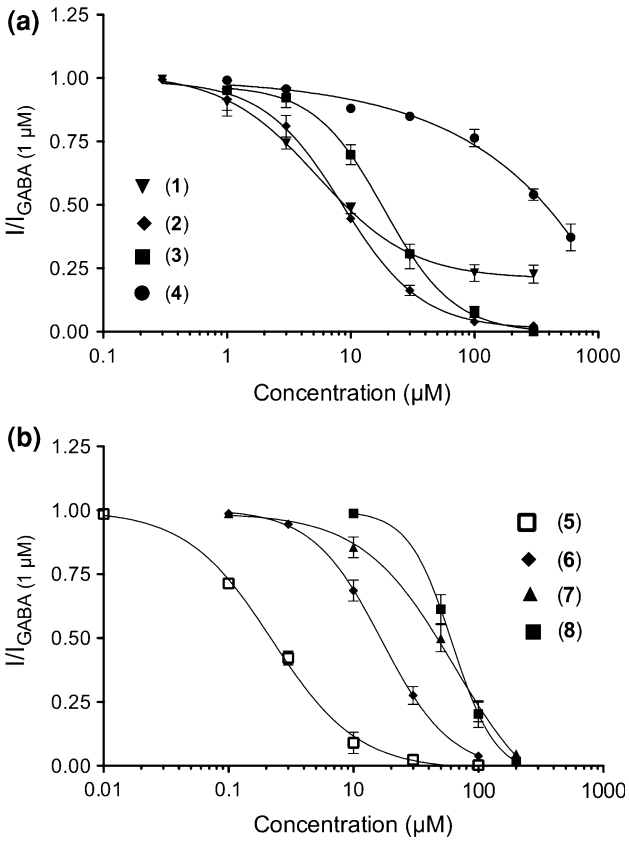
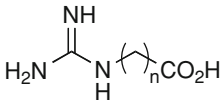
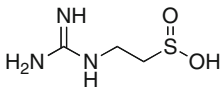
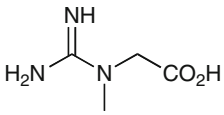


Fig. 1 Inhibitory concentration-response curves for guanidino analogs **a** compounds (1)–(4) and **b** C2-substituted guanidine analogs of guanidinoacetic acid, compounds (5)–(8) against GABA (1 μM). Data are the mean ± SEM ($n \geq 3$ oocytes). The IC_{50} values which is the concentration of the compound that inhibits 50% of the response produced by GABA (1 μM) ranged from 2.2 to 64 μM (Tables 1, 2 summarize the results)

Table 1 Effect of distance between guanidino and acidic moieties on activity at recombinant ρ1 $GABA_C$ receptors expressed in *Xenopus* oocytes

	n	$-pIC_{50} \pm SD$ IC_{50} (95% CI) (μM)
		
Guanidinoacetic acid (**1**)	1	-0.73 ± 0.08 5.4 (3.75–7.76) $K_B = 7.75$ μM $pA_2 = -\log[K_B] = 5.11 \pm 0.06$
3-Guanidinopropionoic acid (**2**)	2	-0.93 ± 0.04 8.57 (7.16–10.27)
4-Guanidinobutanoic acid (**3**)	3	-1.27 ± 0.04 18.75 (15.29–22.55)
	–	1 mM inhibited GABA (1 μM) by 63%
2-Guanidino-ethanesulfinic acid (**4**)		
	–	Inactive at 300 μM
Creatine (**12**)		
TPMPA	–	$K_B = 2.3 \pm 0.4$ μM [8]

much reduced activity. Figure 2 shows an example of a trace of GABA (30 μM) (duration indicated by filled bar) and GABA (1 μM) (duration indicated by open bar) activating an inward current 50% of the maximal current produced by GABA (30 μM). Compound (**8**) (100 μM, duration indicated by forward hatched bar), compound (10) (100 μM, duration indicated by the vertically hatched bar) and compound (**9**) (100 μM, duration indicated by the cross-hatched bar) guanidino analogs did not activate a current. When co-applied with GABA (1 μM), only compound (**8**) (100 μM, duration indicated by the hatched bar) analog (100 μM) reduced the GABA response by 63%.

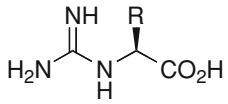
Guanidinoacetic acid (**1**) caused a parallel shift in the concentration-response curve of GABA to the right over 4 antagonist concentrations indicating its competitive nature [Fig. 3; $K_B = 7.75$ μM (pA_2 or $-\log K_B = 5.11 \pm 0.06$; Table 1)]. Furthermore, guanidino analogs (*S*)-2-guanidinopropanoic acid (**5**), (±)-2-guanidinopropanoic acid (**6**) and (*S*)-2-guanidino butyric acid (**7**) showed a parallel shift in the dose-response curve of GABA to the right over one antagonist concentration, indicating possible competitive nature of these compounds (Fig. 4). Table 2 summarizes apparent K_B values for (*S*)-2-guanidinopropanoic acid (**5**), (±)-2-guanidinopropanoic acid (**6**) and (*S*)-2-guanidino butyric acid (**7**).

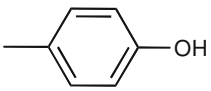
Discussion

ω-Guanidino acids are known to act like GABA at GABA receptors indicating that the guanidino acids behave as though the guanidino group is equivalent to the amino functionality with an additional two carbons [16]. This study expands on this observation and assesses the activity of guanidino compounds related to glycine, taurine and β-alanine on ρ1 $GABA_C$ receptors expressed in *Xenopus* oocytes. Of the guanidino analogs evaluated, compounds (**1**) to (**8**) were active as antagonists at the ρ_1 $GABA_C$ receptor indicating that substituting the amine functionality with a guanidino moiety is tolerated at the ligand-binding site.

It has been shown that the distance between the carboxyl carbon and terminal nitrogen of $GABA_C$ receptor ligands appear to be important for the ligand's affinity at this

Table 2 Effect of C2 substituted guanidinoacetic acid analogs at recombinant ρ1 $GABA_C$ receptors expressed in *Xenopus* oocytes



	R	$-pIC_{50} \pm SEM$ IC_{50} (95% CI) (μM)	Apparent K_B (μM) $pA_2 = -\log[K_B] \pm SEM$
(*S*)-2-Guanidino propanoic acid (**5**)	CH_3	-0.35 ± 0.03	9.0
		2.23 (1.96–2.53)	[5.05 ± 0.03]
2-Guanidino propanoic acid (**6**)	CH_3	-1.22 ± 0.04	20.0
		16.76 (13.85–20.28)	[4.70 ± 0.04]
(*S*)-2-Guanidino butyric acid (**7**)	CH_2CH_3	-1.8 ± 0.13	56.4
		63.8 (33.85–120.7)	[4.25 ± 0.04]
(*S*)-2-Guanidino-3-hydroxy propanoic acid (**8**)	CH_2OH	-1.79 ± 0.04	ND
		61.55 (50.24–75.4)	
(*S*)-2-Guanidino-3-methylbutyric acid (**9**)	$CH(CH_3)_2$	Inactive at 100 μM	–
(2*S*,3*R*)-2-Amino-3-hydroxybutyric acid (**10**)	$(CH)OHCH_3$	Inactive at 100 μM	–
(*S*)-2-Guanidino-2-(4-hydroxyphenyl) acetic acid (**11**)		Inactive at 100 μM	–

ND not determined

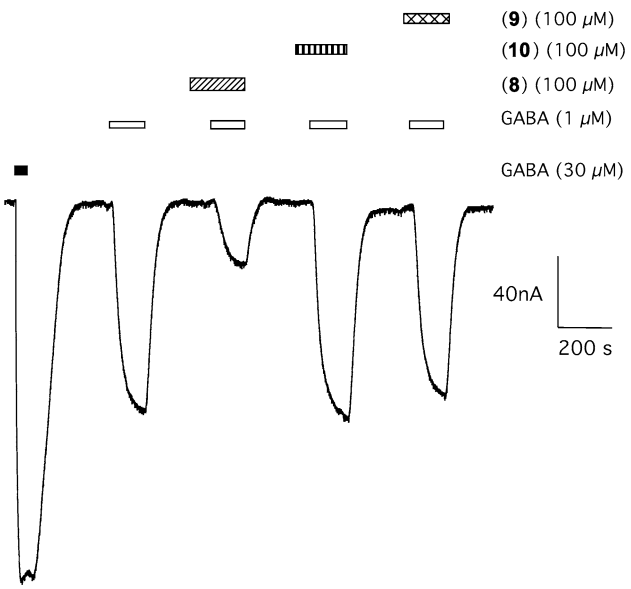


Fig. 2 GABA (30 μM) (duration indicated by *filled bar*) activated a maximal inward current in oocytes expressing ρ1 $GABA_C$ receptors and clamped at −60 mV. GABA (1 μM) (duration indicated by *open bar*) activated an inward current 50% of the maximal current produced by GABA (30 μM). Compound (8) (100 μM, duration indicated by *forward hatched bar*), compound (10) (100 μM, duration indicated by the *vertically hatched bar*), and compound (9) (100 μM, duration indicated by the *cross-hatched bar*), guanidino analogs did not activate a current. When co-applied with GABA (1 μM), only compound (8) (100 μM, duration indicated by the *forward hatched bar*) reduced the GABA response by 63%

receptor. As the activity of the guanidino analogs of glycine and β-alanine are more potent than the original amino acids, and these in turn are more potent than either the β-alanine or GABA guanidino analogs indicates that the distance between the carboxyl carbon and terminal nitrogen of the guanidino group is critical for activity at the ρ_1 $GABA_C$ receptor.

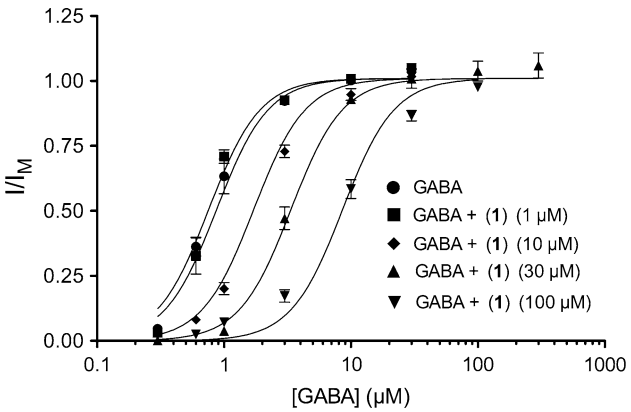


Fig. 3 Concentration response curves for GABA alone (●, $n = 6$) and GABA in the presence of 1 μM (■, $n = 3$), 10 μM (◆, $n = 6$), 30 μM (▲, $n = 6$) and 100 μM (▼, $n = 3$) guanidinoacetic acid (1) at human ρ1 $GABA_C$ receptors expressed in *Xenopus* oocytes; Data are the mean ± SEM. Table 1 summarizes the results. A pA_2 or $-\log K_B$ of 5.11 ± 0.06 was determined using the Gaddum–Schild equation

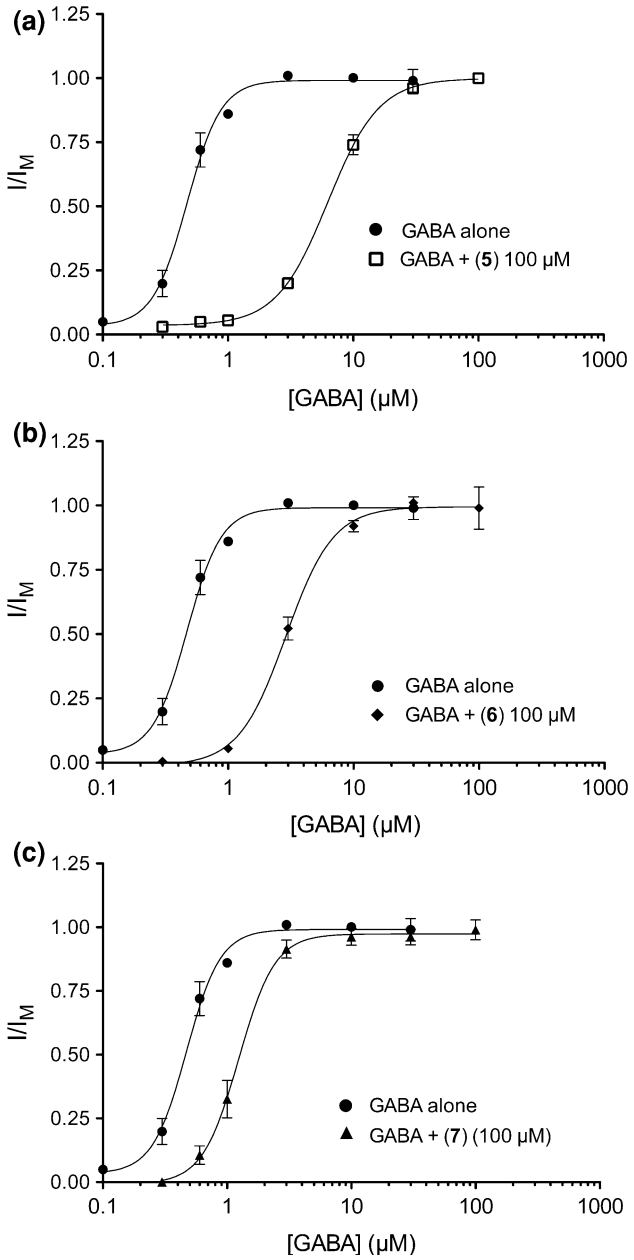


Fig. 4 Concentration response curves for GABA alone (●, $n = 6$) and GABA in the presence of **a** compound (**5**) 100 μM (□, $n = 3$); **b** compound (**6**) 100 μM (◆, $n = 3$) and **c** compound (**7**) 100 μM (▲, $n = 3$) at human $\rho 1$ $GABA_C$ receptors expressed in *Xenopus* oocytes. Data are the mean ± SEM. Table 2 summarizes the results

The most potent analog of this series at the ρ_1 $GABA_C$ receptor was guanidinoacetic acid (**1**), indicating that a carbon linker between the guanidino and the carboxylic acid is optimal for activity. Thus guanidinoacetic acid (**1**) behaves like GABA rather than glycine reflecting similar length molecules. Using guanidinoacetic acid (**1**), we evaluated the effect of substituents at the C2 position because substituents in this position are generally tolerated at the ligand-binding domain of the $GABA_C$ [30]. It was found that the guanidino analog of alanine, compound (**5**) was the most potent. Increasing the size of the substitutions in the C2 position of guanidinoacetic acid (**1**) to an ethyl, isopropyl or tyrosine moiety resulted in analogs with much reduced activity as antagonists at the ρ_1 $GABA_C$ receptor, indicating that the pocket proposed to accommodate such a group is small and steric effects may play a major effect on activity.

Stereoisomers have been shown to exhibit opposite pharmacological effects at $GABA_C$ receptors [3, 31]. In the case of (*R*)- and (*S*)-4-amino-2-methyl-butyric acid ((*R*)- and (*S*)-2-Me-GABA) [32], the (*S*)-isomer is an agonist while the (*R*)-isomer was an antagonist. Docking of these molecules in the model developed by Abdel-Halim et al. [33] showed that the methyl group of the (*S*)-isomer is well accommodated within the binding site cavity of the 'agonist' bound conformation while the methyl group of the (*R*)-isomer is sterically interacting with loop C, which may interfere with loop C closure resulting in antagonist activity. Accordingly, the ease of loop C closure and opening may be the characteristic feature differentiating agonist and antagonist effects of the stereoisomers. However, this hypothesis does not explain the effects observed with C2 substituted guanidinoacetic acid analogs. With C2 substituted guanidinoacetic acid analogs, only the (*S*)- or natural stereoisomer was active at the $\rho 1$ $GABA_C$ receptor. One explanation may be that in general antagonists are larger than agonists and by incorporating the guanidino function produces larger molecules that interfere with loop C closure resulting in antagonist activity and having C2 substituents with a D-configuration may hinder the binding of the compound to the pocket.

Both taurine [23, 24] and homohypotaurine [30], the sulfonic acid and sulfinic acid analogs of glycine, respectively, have been shown to be agonists at $GABA_C$ receptors. In contrast, the guanidino analog of homohypotaurine, 2-guanidino-ethanesulfinic acid (**4**) acted as an antagonist. This may be because of the increased steric bulk of the guanidino group compared to the amine or due to delocalised charge of the guanidino group binding to a different amino acid residue on the receptor, preventing the conformational change that is required for activation of the receptor.

The levels of guanidinoacetic acid (**1**) produced in mammalian brain (2.2–6.5 nmol/g tissue; approximately 2–6 μM) [34] are of the order that can antagonize $GABA_C$ receptors ($K_B = 5.4$ μM). When levels of guanidinoacetic acid (**1**) increase due to metabolic disorders, such as in guanidinoacetate methyltransferase (GAMT) deficiency, an autosomal recessive inherited disorder of creatine biosynthesis, non-specific effects start to occur resulting in neurological symptoms, such as seizures and dyskinesia. It has been proposed that the partial agonist effect of

guanidinoacetic acid (**1**) with neuronal $GABA_A$ receptors in cortical neurons represents a candidate mechanism explaining the neurological symptoms associated with GAMT deficiency [17]. However, agonist activity at GABA receptors by guanidinoacetic acid (**1**; 167 μM) is unlikely to lead to seizures. Other mechanisms may be involved such as inhibition of glutamate transporters [35] that can potentially cause siezures. Furthermore, in cerebellar granule cells, guanidinoacetic acid (**1**) has been shown to activate two populations of $GABA_A$ receptors that mediate tonic inhibition. One population involves α6-containing $GABA_A$ receptors, while the other does not. Guanidinoacetic acid (**1**) could not distinguish between these receptor populations and the concentrations required in cerebellar granule cells (mM concentrations) may not be reached in GAMT deficiency [36] compared to concentrations required in cortical neurons. Interestingly, creatine had no effect as an agonist, antagonist or modulator despite suggestions that it interacts with GABA-benzodiazepine receptor complexes in chicks [37].

In conclusion, ω-guanidino acids inhibit $GABA_C$ receptors composed of ρ1 subunits however they are not selective having been shown to act at $GABA_A$ receptors and GABA transporters [17, 19, 36, 38]. Despite the non-selective nature of amino acids at GABAergic systems, differences in the pharmacology have been reported with glycine, β-alanine and taurine at $GABA_C$ subunit composed of perch ρ-subunits [23]. As a result, it would be interesting to evaluate the guanidino compounds reported here against other $GABA_C$ receptor subunits such as the human ρ2 to determine if there are differences in their activity and thus further contribute to the structure–activity profile of these receptors.

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