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Laboratory note

Structure and pharmacology of 4,5,6,7-tetrahydroisothiazolo[5,4-*c*]pyridin-3-ol (Thio-THIP), an agonist/antagonist at $GABA_A$ receptors

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Summary — 4,5,6,7-Tetrahydroisothiazolo[5,4-*c*]pyridin-3-ol (Thio-THIP), an analogue of the potent and efficacious partial $GABA_A$ agonist, 4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol (THIP), shows rather potent agonist effects at spinal $GABA_A$ receptors in vivo, but remarkably low affinity for brain $GABA_A$ receptors in vitro. 2-Methyl-4,5,6,7-tetrahydropyrazolo[5,4-*c*]pyridin-3-ol (2-Me-Aza-THIP) does not bind detectably to $GABA_A$ receptors. The conformation of the molecule of Thio-THIP, which has now been determined by an X-ray crystallographic analysis, is very similar to those previously described for THIP and 2-Me-Aza-THIP. At human $GABA_A$ receptors of $\alpha_3\beta_2\gamma_2$ or $\alpha_5\beta_3\gamma_2$ subunit configurations, expressed in *Xenopus* oocytes, at which THIP shows low- (44%) or high-efficacy (99%) $GABA_A$ agonism, respectively, Thio-THIP was shown to be a competitive antagonist. At $GABA_A$ receptors in cultured cerebellar granule cells, Thio-THIP turned out to be a weak low-efficacy (2–9%) partial $GABA_A$ agonist.

$GABA_A$ receptors / recombinant receptors / receptor subunits / molecular pharmacology / cellular electrophysiology / $GABA_A$ partial agonists / $GABA_A$ antagonists / structure–activity studies / heterocyclic carboxyl bioisosteres / X-ray crystallography

Introduction

4-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian central nervous system (CNS) and operates through $GABA_A$, $GABA_B$ and probably also $GABA_C$ receptors [1–3]. Dysfunctions of the GABA system have been associated with certain neurological and psychiatric disorders, and there is an interest in GABA receptors, not least in the $GABA_A$ receptors, as potential therapeutic targets in these diseases [4–6].

A number of heterocyclic $GABA_A$ agonists, bioisosterically derived from GABA, such as muscimol and thiomuscimol [7] as well as isoguvacine and 4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol (THIP) (fig 1) [8], have been extensively used as tools for pharmacological characterization of the $GABA_A$ receptors.

Although there is evidence of impaired function of the central GABA system in epilepsy [9], THIP

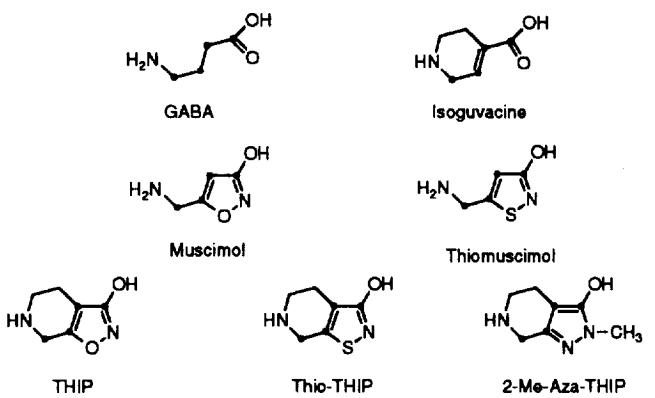

Fig 1. Structures of GABA, a number of mono- and bicyclic $GABA_A$ receptor ligands, and the inactive analogue, 2-Me-Aza-THIP.

Abbreviations: Aza-THIP: 4,5,6,7-tetrahydropyrazolo[5,4-*c*]-pyridin-3-ol; CNS: central nervous system; GABA: 4-aminobutyric acid; 2-Me-Aza-THIP: 2-methyl-4,5,6,7-tetrahydropyrazolo[5,4-*c*]pyridin-3-ol; PET: position emission tomography; Thio-THIP: 4,5,6,7-tetrahydroisothiazolo[5,4-*c*]pyridin-3-ol; THIP: 4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol.

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failed to protect baboons with photosensitive epilepsy against photocally-induced myoclonic responses [10], and THIP was only marginally effective as a clinical antiepileptic agent [11]. Quite surprisingly, positron emission tomography (PET) studies on epileptic patients and normal volunteers have shown that THIP increases rather than reduces global brain glucose metabolism [12, 13]. Accumulating evidence derived from clinical studies on GABAergic drugs supports the view that activation of $GABA_A$ receptors can provoke psychosis in normal subjects and stimulate psychotic symptoms in schizophrenics [14].

Thus, compounds capable of reducing central $GABA_A$ receptor-mediated neurotransmission may be of therapeutic interest in epilepsy as well as schizophrenia [4]. Since $GABA_A$ antagonists are potential anxiogenics and convulsants, such compounds may be difficult to administer safely to patients, thereby making low-efficacy partial $GABA_A$ agonists therapeutically interesting [15]. Compounds showing prevailing agonist and antagonist effects at spinal and supraspinal $GABA_A$ receptors respectively may, at least theoretically, be of therapeutic interest [4].

These aspects prompted us to re-investigate 4,5,6,7-tetrahydroisothiazolo[5,4-*c*]pyridin-3-ol (Thio-THIP), an analogue of THIP, which shows rather potent agonist effects at $GABA_A$ receptors in the cat spinal cord, though weaker than those of THIP [16] (Curtis et al, unpublished). Using [^{3}H]GABA binding, Thio-THIP (IC_{50} = 42 μM) does, however, show remarkably low affinity for rat brain $GABA_A$ receptors in vitro as compared with THIP (IC_{50} = 0.13 μM) [16].

In order to shed some light on these aspects, we now report on the cellular and molecular pharmacology of Thio-THIP at brain $GABA_A$ receptors. Furthermore, we describe an X-ray crystallographic analysis of Thio-THIP, and a comparative structure–activity analysis of Thio-THIP, THIP and the inactive analogue of THIP, 2-methyl-4,5,6,7-tetrahydropyrazolo[3,4-*c*]-pyridin-3-ol (2-Me-Aza-THIP) [17, 18] (fig 1).

X-ray crystallographic analysis

A perspective drawing [19, 20] of the molecular structure of Thio-THIP hydrochloride as determined by X-ray analysis is shown in figure 2. The atom-labelling scheme, bond distances and angles are given in figure 2 and table I. Bond lengths and angles are in agreement with expected values [21–24]. The bond lengths of the isothiazole ring and the associated bond lengths all indicate some double-bond character. Selected torsion angles are given in table I. The isothiazole ring is planar, and the tetrahydropyridine ring is puckered. The puckering parameters [25–27] (table I) indicate a half-chair conformation for the tetrahydropyridine ring with an approximate two-fold axis running through the C5–N6 and C3A–C7A bonds; the atoms C5 and N6 are displaced –0.344 (1) Å and 0.405(1) Å respectively from the best plane of the remaining four atoms of the ring. The crystal packing is shown in figure 3. The crystal structure is mainly stabilized by O–H···N and N–H···Cl^- hydrogen bonds (table I, figs 2 and 3) [28]. 3-Isothiazolol moieties

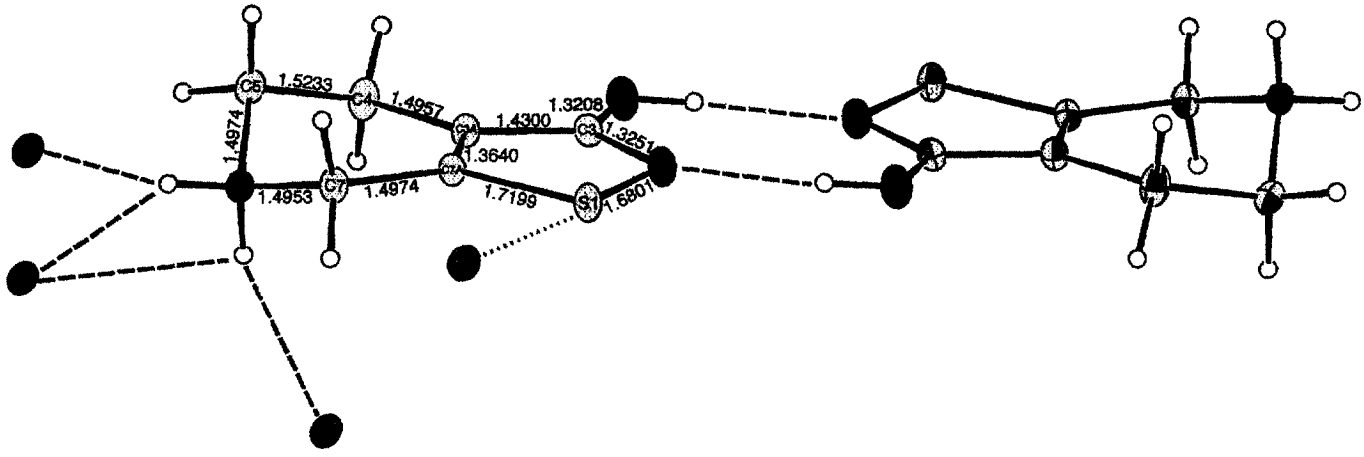

Fig 2. A perspective drawing of the hydrogen bonded dimers of Thio-THIP formed in the crystalline state. The atom-labelling is indicated, and displacement ellipsoids for non-hydrogen atoms are scaled to 50% probability. Hydrogen atoms are represented as spheres of arbitrary radius. Esd's in the bond lengths shown: 0.0006–0.0010 Å. The intramolecular distances from N6 to S1, N2, and O1 are 4.074(1), 4.774(1), and 5.070(1) Å, respectively. Hydrogen bonds are shown by red dashed lines and the close S···Cl^- interatomic distance by a red dotted line.

Table I. Selected geometric parameters for Thio-THIP hydrochloride[a].

Valency angles (°)					
N2-S1-C7A	93.11	(3)	C4-C3A-C7A	123.83	(6)
C3-N2-S1	110.86	(5)	C3A-C4-C5	110.45	(6)
N2-C3-C3A	115.01	(6)	C4-C5-N6	110.01	(5)
N2-C3-O1	123.44	(6)	C5-N6-C7	113.36	(5)
C3A-C3-O1	121.54	(6)	N6-C7-C7A	108.80	(5)
C3-O1-H1	110	(1)	C3A-C7A-C7	123.31	(6)
C3-C3A-C4	125.63	(6)	C3A-C7A-S1	110.54	(5)
C3-C3A-C7A	110.48	(6)	C7-C7A-S1	126.10	(5)
Torsion angles (°)					
C7A-S1-N2-C3	0.27	(6)	C7A-C3A-C4-C5	14.0	(1)
S1-N2-C3-C3A	–0.24	(8)	C3A-C4-C5-N6	–44.06	(8)
N2-C3-C3A-C7A	0.07	(9)	C4-C5-N6-C7	65.33	(7)
C3-C3A-C7A-S1	0.13	(7)	C5-N6-C7-C7A	–48.93	(7)
C3A-C7A-S1-N2	–0.23	(5)	N6-C7-C7A-C3A	16.69	(9)
N2-C3-O1-H1	– 2	(2)	C7-C7A-C3A-C4	– 0.1	(1)

Ring-puckering parameters for the tetrahydropyridine ring [25–27]
(N6 → C5 → C4 → C3A → C7A → C7) $Q = 0.492(1)$ Å; $\Phi = 207.2(1)°$; $\Theta = 130.2(1)°$

Hydrogen bond geometries (Å,°)[b]				
X-H···Y	X-H	H...Y	X...Y	< XHY
O1-H1···N2^{ii}	0.81 (2)	1.87 (2)	2.680 (1)	175 (2)
N6-H6A···Cl^{iii}	0.91 (2)	2.21 (2)	3.088 (1)	162 (1)
N6-H6B···Cl^{i}	0.88 (2)	2.28 (2)	3.109 (1)	156 (1)
N6-H6A···Cl^{iv}	0.91 (2)	3.01 (2)	3.259 (1)	98 (1)
N6-H6B···Cl^{iv}	0.88 (2)	2.91 (2)	3.259 (1)	106 (1)
Some close contacts (Å, °)				
C4-H4A···O1^{v}	0.99 (2)	2.66 (2)	3.162 (1)	111 (1)
C5-H5A···O1^{iii}	0.98 (1)	2.53 (1)	3.465 (1)	159 (1)
C5-H5B···Cl^{iv}	0.93 (2)	2.81 (2)	3.482 (1)	130 (1)
C7-H7B···Cl^{iv}	0.95 (2)	2.83 (2)	3.327 (1)	113 (1)
S1···Cl^{vi}			3.220 (1)	

[a]Estimated standard deviations are given in parentheses; [b]symmetry code: i) x, y, z; ii) 1 – x, 2 – y, 1 – z; iii) x, – 1+y, z; iv) 1 – x, 1 – y, – z; v) 1/2 – x, y – 1/2, 1/2 – z; vi) 1 1/2 – x, y – 1/2, 1/2 – z.

of two molecules related by a centre of symmetry are linked in a dimeric manner, resembling the common H-bonding motif found for carboxylic acids [29]. This feature is also found in the crystal structure of 3-hydroxy-5-(methylsulphonyl)-4-phenylisothiazole [24], the only 3-isothiazolol found in a search of the Cambridge Structural Database (version April 1996) [30], and for molecules containing a 3-isoxazolol moiety [31–33]. The hydrogen atoms of the ammonium group are hydrogen bonded to chloride ions piled up in columns along the **b** axis and a close S···Cl$^-$ interaction [3.220(1) Å] along the **a** axis is observed. Futhermore, a few close C–H···O and C–H···Cl$^-$ contacts are listed in table I.

Molecular and cellular pharmacology

Thio-THIP has previously been shown to be a very weak inhibitor of the binding of [^{3}H]-GABA to rat brain membranes (IC_{50} = 42 μM) [16]. This low affinity of Thio-THIP for brain $GABA_A$ receptors was confirmed in the present study, the IC_{50} values for inhibition of [^{3}H]-GABA ($GABA_A$) and [^{3}H]THIP binding being 43 ± 8 and 36 ± 12 μM, respectively.

In *Xenopus* oocytes with human $\alpha_3\beta_2\gamma_2$ or $\alpha_5\beta_3\gamma_2$ $GABA_A$ receptor subunits co-injected, Thio-THIP did not show significant agonist activity at concentrations up to 1 mM, which was the maximum concentration used. At these recombinant human $GABA_A$ receptors

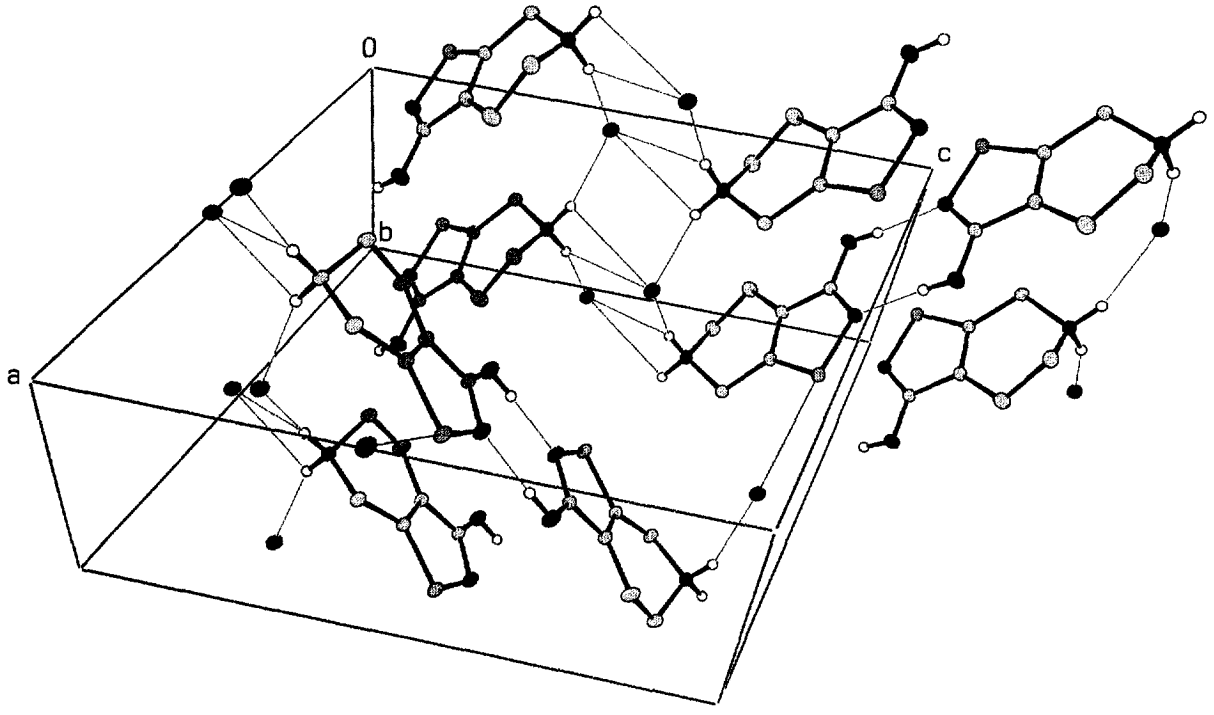

Fig 3. An illustration of the molecular packing of Thio-THIP hydrochloride. For clarity only hydrogen atoms bonded to oxygen and nitrogen atoms are shown. Displacement ellipsoids for non-hydrogen atoms are scaled to 50% probability. Hydrogen bonds are indicated by thin red lines and the close S···Cl⁻ interatomic distance by a thin black line.

of the indicated subunit composition, Thio-THIP was, however, shown to antagonize the responses to GABA (30 μM) in a concentration-dependent manner with an IC_{50} value of 200 μM, as illustrated for the $\alpha_3\beta_2\gamma_2$ receptor configuration in figure 4A. In oocytes expressing $GABA_A$ receptors of this subunit composition, Thio-THIP induced a rightward shift of the dose–response curve for GABA in an apparently parallel fashion, with a pK_i value of 3.71 ± 0.03 (K_i = 196 ± 33 μM) (fig 4B). In oocytes expressing recombinant $GABA_A$ receptors of the $\alpha_5\beta_3\gamma_2$ configuration, Thio-THIP also antagonized GABA-induced currents in an apparently competitive fashion, with a pK_i value of 3.43 ± 0.37 (not shown).

In cultured rat cerebellar granule cells, Thio-THIP showed the characteristics of a very low-efficacy partial $GABA_A$ agonist. At a concentration of 1 mM, Thio-THIP produced an agonist response which was approximately 26% of that of 20 μM of the full agonist, isoguvacine [34] (95% confidence limits [19–33%], n = 9 cells). This response was completely blocked by the competitive $GABA_A$ antagonist, bicuculline methobromide (BMB, 100 μM, n = 5 cells) (not shown). At 1 mM concentration, Thio-THIP was capable of reducing the currents produced by 20 μM of the full $GABA_A$ agonist isoguvacine to about 74% (95% confidence limits [66–81%], n = 8 cells) of that of isoguvacine alone. In this test system, the response to 20 μM isoguvacine is approximately 11% of the maximum isoguvacine response (Kristiansen and Schousboe, unpublished). Thus, it can be concluded that the efficacy of Thio-THIP is between 2 and 9% relative to isoguvacine.

Discussion

On the basis of quite comprehensive clinical studies on the specific $GABA_A$ agonist/partial agonist THIP, it has been concluded that in principle, $GABA_A$ antagonists may constitute therapeutically useful drugs in brain disorders such as epilepsy, schizophrenia and Alzheimer's disease [4, 35]. Due to a high risk of severe side-effects such as anxiety and seizures, such compounds would, however, be difficult to administer safely to patients [4, 35]. These aspects prompted us to develop a series of low-efficacy partial $GABA_A$ agonists showing different levels of efficacy [15].

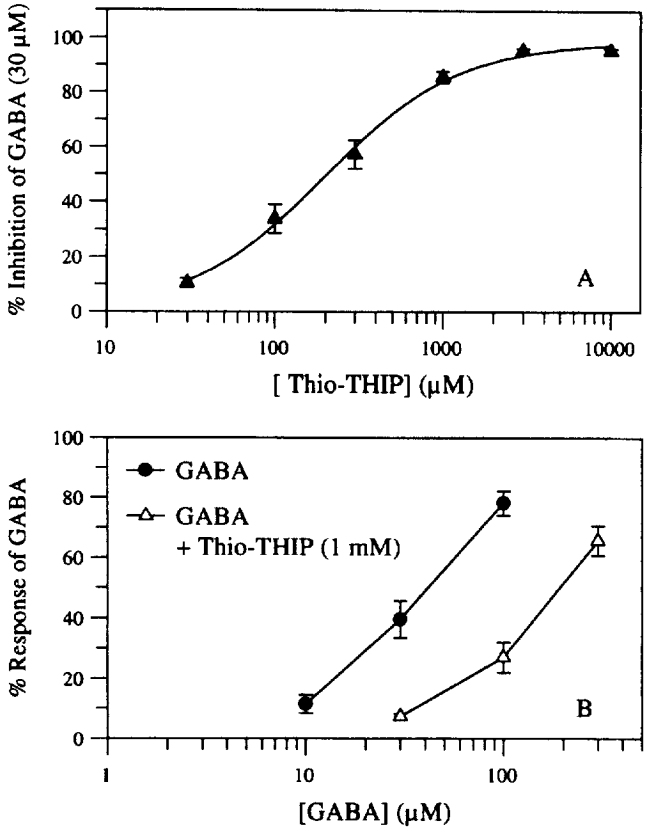

Fig 4. Effects of Thio-THIP in $\alpha_3\beta_2\gamma_2$ injected *Xenopus* oocytes. **A**: concentration-dependent inhibition of responses to 30 µM GABA by Thio-THIP; **B**: effects of 1 mM Thio-THIP on the concentration-response curve for GABA.

Previous attempts to synthesize active $GABA_A$ receptor ligands structurally related to THIP have been largely negative [36, 37]. With the notable exception of Thio-THIP [16], such compounds, including 4,5,6,7-tetrahydropyrazolo[5,4-*c*]pyridin-3-ol (Aza-THIP) and 2-Me-Aza-THIP [17], are totally devoid of affinity for $GABA_A$ receptors. Thio-THIP actually shows an unusual pharmacological profile, being a rather potent agonist at spinal $GABA_A$ receptors in vivo but showing very low affinity for brain $GABA_A$ receptors [16] (Curtis et al, unpublished). In contrast, THIP potently activates spinal $GABA_A$ receptors in vivo and binds tightly to brain $GABA_A$ receptors [8, 16], and THIP is capable of activating, with somewhat different potencies and efficacies, $GABA_A$ receptors of a broad range of brain subunit configurations [38]. In the present study, we selected two such $GABA_A$ receptors of $\alpha_3\beta_2\gamma_2$ and $\alpha_5\beta_3\gamma_2$ configurations at which THIP shows its lowest (44%) and highest (99%) efficacy, respectively [38], to investigate the molecular pharmacology of Thio-THIP at the $GABA_A$ receptors. Recent immunohistochemical studies have shown that $GABA_A$ receptors of these subunit configurations are present in the spinal cord [39]. At both of these recombinant $GABA_A$ receptors, expressed in *Xenopus* oocytes, Thio-THIP showed competitive antagonist effects, with pK_i values of 3.71 ± 0.03 and 3.43 ± 0.37, respectively (fig 4A, B). In cultured cerebellar granule cells containing native $GABA_A$ receptors of subunit configuration(s) which are not known in detail, Thio-THIP showed weak and low efficacy (2–9%) partial $GABA_A$ agonist effects.

This pronounced difference between the pharmacology of THIP (pK_a values: 4.4, 8.5 [17]) and Thio-THIP (pK_a values: 6.1, 8.5 [16]) can hardly be explained by the differences in the pK_aI values of these compounds, since muscimol (pK_a values: 4.8, 8.4 [7, 40]) and thiomuscimol (pK_a values: 6.1, 8.9 [7, 41]) (fig 1) are both very potent $GABA_A$ agonists [7]. Furthermore, Aza-THIP (pK_a values: 6.3, 9.9) and 2-Me-Aza-THIP (pK_a values: 5.8, 9.8 [17]), which are completely devoid of $GABA_A$ receptor affinity [17], show protolytic properties comparable with those of Thio-THIP.

The conformations of the THIP cation [42], Thio-THIP cation, and 2-Me-Aza-THIP zwitterion [18] in the solid state are very similar (fig 5), and in light of the low degree of conformational mobility of these annulated bicyclic compounds, the receptor–active conformations of THIP and Thio-THIP must be very similar to those illustrated in figure 5.

It can be concluded that the molecular structures, including the electron distributions of the negatively charged carboxyl bioisosteres of this class of $GABA_A$ receptor ligands, are factors of importance for their pharmacological profiles. It is noteworthy that substitution of sulfur for the isoxazole oxygen atom of

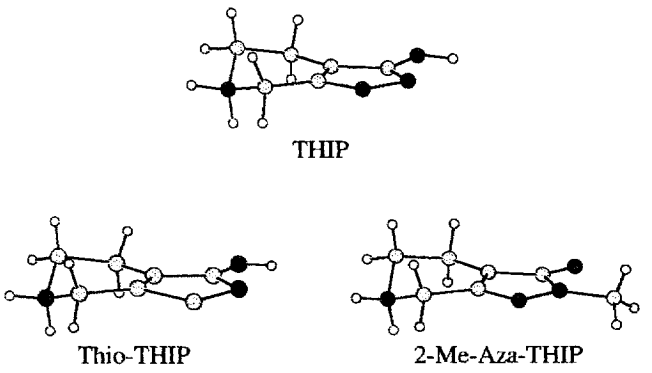

Fig 5. Perspective drawings of the molecular structures determined by X-ray analyses of THIP cation, Thio-THIP cation and 2-Me-Aza-THIP zwitterion.

THIP converts an agonist into an antagonist at recombinant $GABA_A$ receptors. It is at the present time essentially unknown which amino-acid residues are involved in the binding of ligands to the $GABA_A$ receptor recognition site(s) [43]. However, when such information becomes available in the future, the present structure–activity studies may contribute to an understanding of the mechanisms underlying activation of the $GABA_A$ receptors.

Experimental protocols

Crystal data

Thio-THIP hydrochloride ($C_6H_8N_2OS$•HCl), M_r = 192.67, mp = 230 °C (decomp) [16], monoclinic, space group $P2_1/n$, a = 11.736(2), b = 5.471(1), c = 12.952(2) Å, β = 103.83(1)°, V = 807.5(2) Å^3, Z = 4, D_c = 1.585 Mg m^{-3}, $F(000)$ = 400, μ(Mo K_α) = 0.67 mm^{-1}, T ~ 122 K. Crystal dimensions: 0.16 x 0.31 x 0.10 mm.

Crystal data collection and processing

Diffraction data were collected on an Enraf–Nonius CAD-4 diffractometer using graphite monochromated Mo K_α radiation (λ = 0.71073 Å). The crystal was cooled to 122 ± 0.5 K in a stream of N_2 gas. Unit-cell dimensions were determined by least-squares refinement of 22 reflections with θ values in the range of 18–23°. The intensities of the reflections [$2 < \theta < 45°$, $hk \pm l$; $2 < \theta < 30°$, $h - k \pm l$ (partly)] were measured by use of the ω–2θ scan technique. Three standard reflections monitored every 10^4 s showed no significant variation in intensities. Data were reduced using the programs of Blessing (DREADD) [45, 46]. Absorption corrections were applied using the numerical program ABSORB [47] (T_{min} = 0.869, T_{max} = 0.944). The equivalent reflections were averaged to give 6641 independent reflections ($R_{int} = \Sigma |F_0^2 - F_0^2(\text{mean})| / \Sigma |F_0^2| = 0.026$).

Structure solution and refinements

All non-H atoms were found by direct methods using the programs MULTAN11/82 [48] and DIRDIF [49] in the Enraf–Nonius Structure Determination Package (SDP) [19]. All H-atoms were clearly located in a difference electron density map. Final full-matrix least-squares calculations (SHELXL-93 [50]) included an overall scale factor, atomic coordinates for all atoms, anisotropic displacement parameters for the non-hydrogen atoms and isotropic displacement parameters for the hydrogen atoms. Refinements were performed on F^2, minimizing $\Sigma w(F_0^2 - kF_c^2)^2$. All reflections (6641), except three with negative intensities [ie, $F_0^2 < -3\delta(F_0^2)$] were used. The refinement (136 parameters, 6638 reflections) converged at R_F = 0.0332, wR_{F^2} = 0.0799 [5518 reflections with $F_0 > 4\delta(F_0)$; $w^{-1} = (\delta^2(F_0^2) + (0.0418P)^2 + 0.1581P)$ where $P = (F_0^2 + 2F_c^2)/3$, S = 1.12]. The largest shift/esd in the final least-squares cycle was 0.002, and the residual electron density varied between –0.72 and 0.73 e Å^{-3}, the two largest peaks being close to Cl^- and S, respectively. Complex scattering factors (Cl^-, S, O, N, C, H) were taken from International Tables for X-ray Crystallography [51].

Inhibition of $GABA_A$ receptor binding

Dissection of rat brain regions was performed as described by Glowinski and Iversen [52]. Tissue preparation was performed as described by Ransom and Stec [53]. The [^{3}H]GABA binding assay was performed as previously described [54] with the following modifications: the samples were incubated for 45 min instead of 15 min and bound [^{3}H]GABA was determined by filtration through Whatmann GF/B filters using a Brandell 48R cell harvester instead of centrifugation. Nonspecific binding was determined in the presence of 1 mM THIP.

The [^{3}H]THIP binding assay [55] was performed as described above for [^{3}H]GABA binding using 5 nM [^{3}H]THIP as radioligand. Nonspecific binding was determined in the presence of 1 mM GABA.

Electrophysiology on cDNA injected oocytes

Electrophysiological experiments on cDNA-injected *Xenopus* oocytes were performed as described previously [56–58]. After mild collagenase treatment to remove follicle cells (Type IA [0.5 mg/mL] for 10 min) the oocyte nuclei were then directly injected with 10–20 nL of injection buffer (88 mM NaCl, 1 mM KCl, 15 mM HEPES, at pH 7.0 [nitrocellulose filtered]) containing different combinations of human α_3, α_5, β_2, β_3 and γ_2 $GABA_A$ subunit cDNAs (6 ng/mL) engineered into the expression vector pCDM8 or pcDNAAmp. GABA or $GABA_A$ agonists were applied until the peak of the response was observed, usually 30 s or less. At least 3 min wash time was allowed between each agonist application to prevent desensitization. The computer programme GraFit 3.0 (Erithacus Software, Staines, UK) was used to analyze and plot data.

Electrophysiology on cultured rat cerebellar granule cells

Granule cells were cultured from cerebella of 7-day-old rats after dissociation of the tissue as described by Messer [59] and Schousboe et al [60, 61].

Whole-cell patch-clamp recordings were made at 20–22 °C from cells cultured for 13–14 d. The culture medium in the 35-mm Petri dish was replaced by about 4 mL of artificial balanced salt solution (ABSS) of the following composition: NaCl 140 mM, KCl 3.5 mM, Na_2HPO_4 1.25 mM, $MgSO_4$ 2 mM, $CaCl_2$ 2 mM, glucose 10 mM, and HEPES 10 mM (pH 7.35, 22 °C) which was continuously renewed by constant perfusion at 0.5 mL/min. Standard patch-clamp techniques [62] were used to record from the neurones in the whole-cell configuration using an EPC-9 (HEKA, Germany) amplifier. Drugs used were premixed at the required concentrations in ABSS. The solutions were applied in the vicinity (≈ 100 μm) of the recorded neurone from a multi-barrelled perfusion pipette, the multiple barrels ending in a single glass cap with an opening of ≈ 100 μm [34, 63]. Drugs were applied until peak response was reached, and for at least 5 s with 1 min intervals. Responses were quantified by measuring the peak current during application of drugs.

Acknowledgments

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