An Orally Bioavailable, Functionally Selective Inverse Agonist at the Benzodiazepine Site of $GABA_A$ α5 Receptors with Cognition Enhancing Properties

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Abstract: (3-*tert*-Butyl-7-(5-methylisoxazol-3-yl)-2-(1-methyl-1*H*-1,2,4-triazol-5-ylmethoxy)pyrazolo[1,5-*d*][1,2,4]triazine (**13**) has been identified as a functionally selective, inverse agonist at the benzodiazepine site of $GABA_A$ α5 receptors. **13** is orally bioavailable, readily penetrates the CNS, and enhances performance in animal models of cognition. It does not exhibit the convulsant, proconvulsant, or anxiogenic activity associated with nonselective $GABA_A$ inverse agonists.

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the central nervous system. $GABA_A$ receptors are GABA-gated chloride ion channels, composed of pentameric assemblies of members of the $GABA_A$ receptor gene family (α1−6, β1−3, γ1−3, δ, ϵ, θ, and π)[1,2] with the majority of $GABA_A$ receptors comprising of α-, β-, and γ-subunits arranged in a 2:2:1 stoichiometry.[3] The binding of GABA to its receptor can be modulated by simultaneous binding of chemical entities to allosteric sites on the ion channel complex, the most studied of which is the benzodiazepine (BZ) binding site. Upon the basis of their modulatory effects on GABA-induced $GABA_A$ receptor activation, BZ site ligands are categorized as either agonists (positive allosteric modulators), inverse agonists (negative allosteric modulators) or antagonists. BZ agonists exert their effect by increasing the frequency of channel opening in the presence of GABA resulting in an increased chloride flux through the ion channel to give a net hyperpolarization of the neuron and a decreased excitability. Conversely, BZ inverse agonists decrease the frequency of channel opening in the presence of GABA and thereby increase neuronal excitability. Spanning these efficacy extremes are partial agonists, antagonists (which bind to the BZ site on the $GABA_A$ receptor but have no intrinsic efficacy), and partial inverse agonists.

$GABA_A$ receptor subtypes which possess a BZ binding site (and which comprise around 75% of all $GABA_A$ receptor subtypes present in the brain)[4] contain β- and γ2-subunits in conjunction with either an α1-, α2-, α3-, or α5-subunit.[4] Diazepam (**1**; Valium) is classified as a nonselective full BZ agonist (i.e. binds to the different $GABA_A$ receptor subtypes with similar affinity and efficacy), and such compounds have found therapeutic use as anxiolytics, hypnotics, and anticonvulsants.[5] However, they also impair learning and memory processes.[6,7] Currently, all clinically effective BZ ligands are full agonists at the BZ binding site on the $GABA_A$ receptor. In contrast, methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM; **2**) is a nonselective full inverse agonist,[8,9] and it has been shown that nonselective BZ inverse agonists enhance cognitive performance in animal models[10] but are anxiogenic,[11] convulsant,[12] and proconvulsant[13] and may alter attentional processing.[14] Using genetically modified, point-mutated mice, it has been demonstrated that $GABA_A$ receptors containing an α1-subunit mediate the sedative/muscle relaxant effects of benzodiazepines, whereas α2- or α3-subunit-containing receptors mediate the anxiolytic and anticonvulsant effects.[15−18] Recently, Collinson et al.[19] and Crestani et al.[20] have used α5 'knock-out' and point-mutated mice, respectively, to demonstrate a role for the $GABA_A$ α5-subtype in cognitive processing. In adult brain, α5-subunit-containing $GABA_A$ receptors have a relatively restricted distribution, being primarily expressed in the hippocampus, a region of the brain associated with learning and memory, and, although α5 receptors account for less than 5% of the total $GABA_A$ receptor population in the brain, in the hippocampus they represent around 20% of all $GABA_A$ receptors.[4,21,22] Given the molecular genetic evidence and the relative abundance of $GABA_A$ α5 receptors in the hippocampus, we proposed that an inverse agonist acting at the BZ site of α5-containing $GABA_A$ receptors (hereafter referred to as the α5-subtype) may have therapeutic utility as a cognition-enhancing agent and, furthermore, that an α5-selective inverse agonist may also lack the unwanted side effects associated with inverse agonist activity at other $GABA_A$ receptor subtypes (i.e., anxiogenesis or convulsant or proconvulsant activity).

Although there are many structural classes of compound which bind to the BZ site on the $GABA_A$ ion channel complex, relatively few have been reported as being selective ligands for the α5-subtype. The imidazobenzodiazepines[23] such as L-655,708 (**3**)[24] and some diazepam analogues[25] exhibit binding selectivity for the α5-subtype compared to the other receptor subtypes. From our laboratories, a series of 6,7-dihydro-2-benzothiophen-4(5*H*)-ones[26,27] has been described which has binding selectivity for the α5-subtype and from which

Chart 1. $GABA_A$ Benzodiazepine Site Ligands

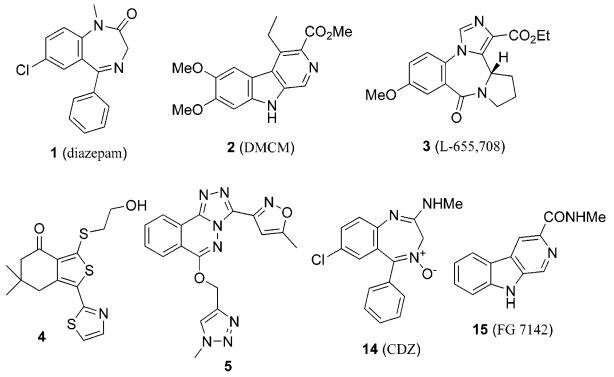

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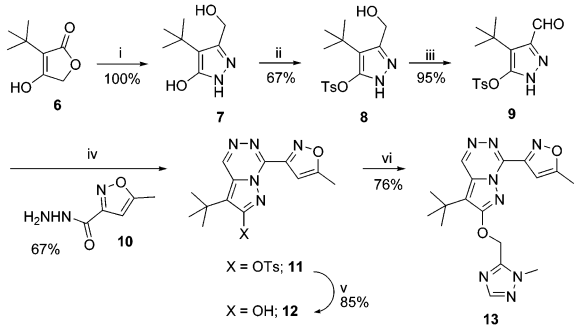
Table 1. Binding Affinity and Efficacy of **13**, **5**, **2**, and **14** at Human Recombinant $GABA_A$ $\alpha x\beta 3\gamma 2$ Receptor Subtypes

	K_i (nM) human $GABA_A$ $\alpha x\beta 3\gamma 2$ receptors[a]				efficacy at human $GABA_A$ $\alpha x\beta 3\gamma 2$ receptors (%)[b]			
compound	α1	α2	α3	α**5**	α1	α2	α3	α**5**
13	0.83 ± 0.08	0.85 ± 0.16	0.77 ± 0.10	**1.4 ± 0.4**	−16 ± 2	+6 ± 5	−9 ± 2	**−55 ± 2**
5	0.88 ± 0.19	0.58 ± 0.17	0.61 ± 0.17	**0.66 ± 0.14**	−15 ± 3	+16 ± 4	−7 ± 4	**−40 ± 1**
2	10 ± 1	13 ± 5	7.5 ± 1.2	**2.2 ± 1.0**	−71 ± 2	−53 ± 3	−62 ± 2	**−57 ± 1**
14[c]	605 ± 136	392 ± 73	471 ± 164	**368 ± 66**	+100 ± 3	+111 ± 4	+113 ± 4	**+99 ± 2**

[a] Displacement of [^{3}H]Ro 15-1788 binding from recombinant human $GABA_A$ receptor subtypes with a $\alpha x\beta 3\gamma 2$ composition (where x = 1, 2, 3, or 5) stably expressed in mouse fibroblast L(tk$^-$) cells. K_i values are the geometric mean ± SEM of four independent determinations. [b] Maximum modulation of the current produced by **13**, **5**, **2**, or **14** relative to a submaximal (EC_{20}) GABA response. Values are the mean maximum modulation ± SEM from at least five individually fitted concentration−response curves for each receptor subtype. [c] Efficacy values are the mean ± SEM from 31 to 37 cells produced using [**14**] = 3 μM.

Scheme 1[a]



[a] Reagents: (i) $NH_2NH_2{\cdot}H_2O$, EtOH, reflux; (ii) *p*-toluenesulfonyl chloride, Et_3N, DCM; (iii) MnO_2, $CHCl_3$, reflux; (iv) xylene, reflux; (v) 4 N NaOH, MeOH, 65 °C; (vi) K_2CO_3, DMF, 5-chloromethyl-1-methyl-1*H*-1,2,4-triazole.

thiophene **4** was identified as a *functionally* selective α5-subtype inverse agonist.[27] Recently, we have also described work leading to the identification of the triazolophthalazine **5**, which was chosen for clinical evaluation as a potential treatment for cognitive disorders.[28]

In this manuscript, we describe the synthesis and biological data of a structurally novel, orally bioavailable, and functionally selective α5-subtype inverse agonist which has also been selected for clinical evaluation as a potential cognition-enhancing agent.

In the search for such a compound, our strategy was to modify the triazolophthalazine core of **5**. Various benzofused and nonbenzofused heterocyclic alternatives were investigated. In the course of these studies, a series of pyrazolo[1,5-*d*][1,2,4]triazines was identified as providing high affinity α5-subtype inverse agonists. Within this class of compounds, a *tert*-butyl group and a 5′-methyl-3′-isoxazolyl substituent at the C3 and C7 positions, respectively, of the pyrazolotriazine core were the optimum groups for providing high α5 inverse agonism. As was the case in the triazolophthalazine series,[28] small changes in the heteroarylmethyloxy substituent at C3 could have a profound effect on efficacy at one or more of the $GABA_A$ receptor subtypes, and, while the pyrazolotriazines did not exhibit significant *binding* selectivity for any particular subtype, varying degrees of *functional* (i.e., efficacy) selectivity for the $GABA_A$ α5-subtype could be attained. Following an extensive study of the series, the optimum compound was identified as (3-*tert*-butyl-7-(5-methylisoxazol-3-yl)-2-(1-methyl-1*H*-1,2,4-triazol-5-ylmethoxy)pyrazolo[1,5-*d*][1,2,4]triazine (**13**).

As shown in Scheme 1, **13** was synthesized in six steps from 3-*tert*-butyl-4-hydroxy-2-furanone (**6**).[29] Heating **6** with hydrazine hydrate afforded the pyrazole **7** in quantitative yield. The C3-hydroxyl was selectively protected in 67% yield as the tosylate **8**. Protection of this hydroxyl functionality was necessary in order to ensure efficient oxidation of the primary alcohol, to give aldehyde **9**. Condensing **9** with 5-methyl-3-isoxazolecarbohydrazide (**10**)[30] produced, after concomitant cyclization, the pyrazolotriazine **11**. Removal of the tosyl group, under basic conditions, gave the hydroxypyrazolotriazine **12** in 85% yield, and subsequent alkylation with 5-chloromethyl-1-methyl-1*H*-1,2,4-triazole[31] produced **13**.

As shown in Table 1, **13** has high in vitro binding affinity[32] at all four BZ-sensitive $GABA_A$ receptor subtypes, ranging from 0.8 to 1.4 nM. It has very weak affinity at the $GABA_A$ $\alpha 4\beta 3\gamma 2$-subtype (K_i 395 ± 173 nM) and is essentially inactive at the $GABA_A$ $\alpha 6\beta 3\gamma 2$ receptor (K_i > 4000 nM). Furthermore, when examined in 147 radioligand binding and enzyme assays,[33] **13** showed no significant off-target activity (IC_{50} values > 10 μM). The efficacy values shown in Table 1 were determined using whole cell patch clamp recordings[34] from mouse fibroblast cells stably expressing the human $GABA_A$ receptor subtypes.[32] The in vitro efficacy is measured as the percentage maximum modulation of the GABA-evoked current using a submaximal (EC_{20}) GABA concentration. Positive values represent a potentiation of the GABA-induced current (agonist) whereas negative values represent an attenuation (inverse agonist). The values for **13** are compared to the clinical compound **5**, the nonselective full inverse agonist **2**,[9] and the nonselective full agonist chlordiazepoxide (CDZ; **14**). **13** is a full inverse agonist at the α5-subtype and is functionally selective over the α1-, α2-, and α3-subtypes. In mouse fibroblast L(tk$^-$) cells expressing the $\alpha 5\beta 3\gamma 2$-subtype, **13** attenuates the GABA-evoked current by 55% (efficacy = −55%), which is essentially identical to that of the full inverse agonist **2** (efficacy = −57%) and greater than that produced by **5** (efficacy = −40%). In contrast, efficacy at the α1-, α2-, and α3-subtypes is much lower, with respective efficacy values of −16%, +6%, and −9% being in the range of weak partial inverse agonists or antagonists. The efficacy of **13** at the α1- and α3-subtypes is comparable with the recordings obtained for **5**; whereas at the α2-subtype **13** is essentially an antagonist (efficacy = +6%), **5** exhibits partial agonism (efficacy = +16%). Indeed, in our quest for a compound with full inverse agonism at the α5-subtype and little functional activity at the other $GABA_A$ receptor subtypes, pyrazolotriazine **13** has a more impressive efficacy profile compared to the clinical

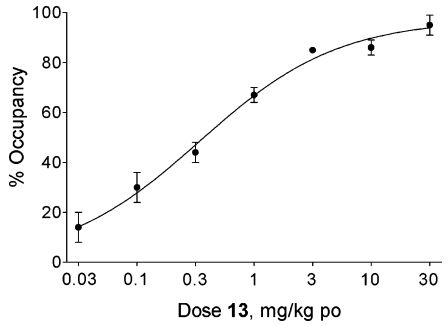


Figure 1. Occupancy of $GABA_A$ receptor benzodiazepine binding site by **13** as a function of dose 0.75 h after oral administration in a 0.5% Methocel vehicle as measured using an in vivo [^{3}H]Ro 15-1788 binding assay. Values shown are mean ± SEM (n = 4−8/group).

compound **5**. The EC_{50} value of **13** at the α5-subtype is 3.0 nM, which complements its in vitro binding affinity of 1.4 nM.

The inhibition of in vivo binding of [^{3}H]Ro 15-1788 by **13** was used to determine the occupancy of this compound at the BZ site of rat brain $GABA_A$ receptors. [^{3}H]Ro 15-1788 binds with equal affinity to the BZ site of $GABA_A$ receptors containing α1-, α2-, α3-, and α5-subunits, and, therefore, in vivo binding of [^{3}H]Ro 15-1788 reflects binding to this combined receptor population. However, since **13** also binds with equal affinity to $GABA_A$ receptors containing α1-, α2-, α3-, and α5-subtypes, the dose of **13** required to occupy the α5-subtype is the same as that required to occupy the combined α1-, α2-, α3-, and α5-subtypes. As shown in Figure 1, the occupancy of **13** in rats was dose-dependent after oral dosing, with the mean dose of **13** required to occupy 50% of $GABA_A$ receptor subtypes (0.75 h postdose) being 0.35 mg/kg.

The oral bioavailability of **13** in rats and dogs is 52% and 8%, respectively.

The proconvulsant potential of **13** was determined in mice.[36] At doses up to 10 mg/kg ip, where 99% of $GABA_A$ receptor subtypes are occupied, **13** did not potentiate pentylenetetrazole-induced convulsions in mice. This contrasts with the significant *convulsant* effects reported in mice for the nonselective BZ full inverse agonist **2**[12] and the proconvulsant effects reported for the nonselective BZ partial inverse agonist **15** (FG 7142).[13] While nonselective BZ partial inverse agonists are without direct convulsant effects in mice when given acutely, they can induce seizures if given daily over a number of days, a process known as 'kindling'.[13] Repeated dosing of **13** (30 mg/kg po for 20 days) did not induce seizures, although seizures were induced with **15** (40 mg/kg ip for 20 days) which occupied fewer receptors than **13** and had a shorter duration. The rat elevated plus maze test[37] is sensitive to the anxiogenic effects of nonselective BZ inverse agonists such as **15**.[11] In this test, **13** was without significant anxiogenic-like effects in rats at doses that occupy >95% of BZ binding sites. In contrast, **15** produced a robust anxiogenic effect.

To investigate the effect that **13** has on learning and memory we assessed the performance of rats dosed orally with **13** in the delayed 'matching-to-place' variant of the Morris water maze, a hippocampal-dependent cognitive test.[38,39] In this assay, the difference in time taken to find a hidden platform (the position of which

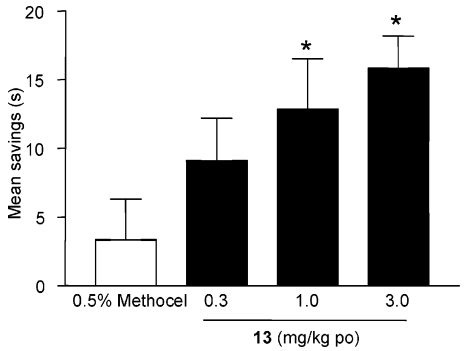


Figure 2. Enhanced performance of rats dosed with pyrazolotriazine **13** in the delayed 'matching-to-place' water maze test.[39] **13** was dosed po at 0.3, 1, and 3 mg/kg in 0.5% Methocel. Vehicle-treated animals were dosed po with 0.5% Methocel. The difference in time taken between trial 1 and trial 2 (savings) to find the hidden platform over a five-day test period is shown. Rats dosed orally at 1 and 3 mg/kg with **13** made significantly higher savings (*) compared with vehicle-treated animals. Values shown are mean ± SEM (n = 9−10/group).

varies day-to-day but is fixed on any given day) on the first and second trial is used as an index of how well the rat has remembered the position of the platform. Spatial cues around the pool assist the rat in navigating its way to the platform.[39] As can be seen in Figure 2, **13** showed a dose-dependent (0.3−3 mg/kg po) improvement in performance between trial 1 and trial 2 compared with vehicle-treated animals ($p < 0.05$). The occupancy of BZ sites on $GABA_A$ receptors in a satellite group of rats dosed at 0.3, 1, and 3 mg/kg po was 61%, 72%, and 85%, respectively. Since **13** was without effect on swim speed, these data indicates that the functionally selective $GABA_A$ α5 inverse agonist **13** significantly enhanced performance in a dose-dependent manner in this hippocampal-dependent memory task. These data complements the findings obtained with the structurally diverse thiophene **4**[27] and triazolophthalazine **5**[28] and supports the hypothesis that a selective α5-subtype inverse agonist may have therapeutic utility for the treatment of cognitive disorders.

In conclusion, the structurally novel, functionally selective $GABA_A$ α5-subtype inverse agonist **13** enhances cognitive performance in a hippocampal-dependent memory task, without the anxiogenic, convulsant, or proconvulsant side effects associated with nonselective BZ $GABA_A$ inverse agonists. **13** has greater inverse agonism at the α5-subtype and reduced α2 agonism compared with the clinical compound **5**, and, on the basis of its overall in vitro and in vivo profile, **13** was selected for clinical evaluation as a potential cognition-enhancing agent.

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Supporting Information Available: Details of the synthesis and characterization of **13** and intermediates (chemistry, NMR, MS, microanalytical data and melting points), as well as a detailed description of the biological test methods used. Data showing the lack of proconvulsant effect produced by **13** on pentylenetetrazole-induced seizures in mice. Data showing

kindling potential of **13** compared to **15**. Data demonstrating the anxiogenic potential of **13** and **15** in the elevated plus maze and associated occupancy of BZ sites on $GABA_A$ receptors. This material is available free of charge via the Internet at http://pubs.acs.org.

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