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The proconvulsant effects of the $GABA_A$ α5 subtype-selective compound RY-080 may not be α5-mediated

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Abstract

RY-080 (ethyl 8-ethynyl-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylate) is an imidazobenzodiazepine with 40–50-fold higher affinity for the benzodiazepine binding site of α5- rather than α1-, α2- or α3-containing $GABA_A$ receptors. Previous data describing RY-080 as being convulsant suggests that inverse agonists selective for the α5 subtype may not be suitable for clinical development. In the present study, we show that RY-080 possesses inverse agonism for the α1 and α5 subtypes of human recombinant $GABA_A$ receptors and whilst not convulsant it was proconvulsant. Hence, with pentylenetetrazole alone, the dose predicted to give tonic convulsions in 50% of the mice (ED_{50}) was 108 mg/kg whereas in the presence of 1 and 10 mg/kg RY-080, the ED_{50}s were 93 and 57 mg/kg, respectively. In vivo [^{3}H]L-655,708 and [^{3}H]Ro 15–1788 binding assays showed that the subtype selectivity of RY-080 in vivo was 7–10-fold for α5-relative to α1- and α2/α3-containing receptors (respective ID_{50} values of 0.93, 9.7 and 6.2 mg/kg) and is therefore much lower than seen in vitro. Consequently, it is not possible to define a dose of RY-080 which gives high occupancy of the α5 subtype without binding to other subtypes and accordingly the proconvulsant effects of RY-080 cannot be attributed solely to the α5 subtype.

Keywords: $GABA_A$ receptor; α5 subunit; Benzodiazepine; RY-080; Proconvulsant; In vivo binding

1. Introduction

Mammalian $GABA_A$ receptors are ligand-gated chloride ion channels comprising of pentameric assemblies of subunits from a 19-member gene family (α1–6, β1–3, γ1–3, δ, ε, θ, π and ρ1–3; Simon et al., 2004). The majority of $GABA_A$ receptors contain α, β and γ2 subunits arranged in an αβαβγ sequence in a clockwise direction as viewed from the synapse (Minier and Sigel, 2004).

In addition to GABA bindings sites, which occur at the interface of α and β subunits, most $GABA_A$ receptors also contain a recognition site for prototypic benzodiazepines (exemplified by diazepam) which is found at the interface of the γ2 and either an α1, α2, α3 or α5 (but not α4 or α6) subunit (Sieghart and Sperk, 2002). Of these four subtypes, the α1βγ2 combination predominates in the brain, followed by α2-then α3-containing receptors with the α5 subtype being least abundant (McKernan and Whiting, 1996; Sieghart and Sperk, 2002). Despite its relatively low level expression in the whole brain, the α5 subtype has a highly heterogeneous pattern of expression, most notably high levels of expression within the hippocampus (Wisden et al., 1992; Fritschy and Mohler, 1995; Pirker et al., 2000) where it constitutes around 25% of the total $GABA_A$/benzodiazepine receptor population (Sur et al., 1998, 1999). The highly localized expression of this subtype suggests that it is associated with specific hippocampal functions and indeed these receptors are involved in aspects of learning and memory (Collinson et al., 2002; Crestani et al., 2002). Consequently, this receptor population is an attractive target for potential cognition enhancers (Chambers et al., 2004). More specifically, since non-selective agonists impair and non-selective inverse agonist enhance cognition, it is proposed that an inverse agonist selective for the α5 subtype should enhance cognition (Maubach, 2003) but be devoid of the anxiogenic and proconvulsant liabilities that limit the clinical utility of non-selective inverse agonists such as FG 7142

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(*N*-methyl-β-carboline-3-carboxamide; Horowski and Dorow, 2002).

A number of structurally-related imidazobenzodiazepines with 25–100-fold higher affinity for α5 compared to α1, α2 or α3 subunit-containing $GABA_A$ receptors have been described, including L-655,708 (ethyl (*S*)-[11,12,13,13a-tetrahydro-7-methoxy-9-oxo]-[^{9}H]-imidazo[1,5-*a*]pyrrolo[2,1-c][1,4]benzodiazepine-1-carboxylate; Quirk et al., 1996), RY-010, -023, -024 and -080 (the ethyl 8-ethyl-, *t*-butyl 8-ethynyl-, *t*-butyl 8[(trimethylsilyl)ethynyl]- and ethyl 8-ethynyl-analogues of 5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylate: Liu et al., 1995, 1996; Skolnick et al., 1997). Where measured, these compounds have inverse agonist efficacy at the α5 subtype (Liu et al., 1995; Kelly et al., 2002) and would therefore appear to be ideal compounds for evaluating the hypothesis that an α5-selective inverse agonist might enhance cognition but be devoid of the anxiogenic and convulsant and/or proconvulsant liabilities associated with non-selective inverse agonists (Maubach, 2003). However, RY-023, -024 and -080 have all been reported to be convulsant or proconvulsant in mice (Liu et al., 1996). Moreover, these effects were proportional to the extent of α5 efficacy in so far as RY-024 has full inverse agonist efficacy at this subtype (Liu et al., 1995) and produced convulsions in 80% of mice whereas RY-023, which has partial inverse agonism at the α5 subtype (Liu et al., 1995) was only convulsant in 22% of mice (Liu et al., 1996). In the absence of details of the efficacy of these compounds at the other subtypes as well as the extent of the in vivo receptor occupancy at the α5 subtype (i.e., do doses that produce convulsions selectively occupy only α5-containing receptors?) these data are difficult to interpret but clearly have implications for any attempts to develop α5 subtype selective cognition enhancers. Hence, it is important to establish whether inverse agonism at the α5 subtype is capable of producing convulsant or proconvulsant activity.

The purpose of the present study, therefore, was to characterise the intrinsic efficacy and assess the proconvulsant effects of RY-080, a compound with higher affinity for the α5 compared to α1, α2 or α3 subtypes (respective K_i values = 0.5, 28, 21 and 26 nM; Liu et al., 1995, 1996; Skolnick et al., 1997) and convulsant activity (Liu et al., 1996). The efficacy of this compound at the α5 subtype has not previously been reported but given its structural similarity to RY-023 and RY-024 is assumed to possess inverse agonist efficacy at this subtype. Finally, the in vivo effects of RY-080 were related to occupancy at α5 versus α1, α2 and α3-containing $GABA_A$ receptors using [^{3}H]L-655708 and [^{3}H]Ro 15–1788 ([^{3}H] 8-fluoro 5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylic acid ethyl ester) in vivo binding assays, respectively.

2. Methods

2.1. Drugs

[^{3}H]L-655708 was synthesised in-house as described elsewhere (Quirk et al., 1996). This compound is also now commercially available from American Radiolabelled Chemicals, Inc. [^{3}H]Ro 15–1788 (70–87 Ci/mmol) was purchased from PerkinElmer Life Sciences, Boston, MA). Diazepam, flunitrazepam and zolpidem were obtained from RBI (Sigma-Aldrich, Gillingham, UK) and bretazenil was a gift from Roche Labs. RY-080 was prepared in a similar manner to that described previously (Liu et al., 1996).

2.2. Intrinsic efficacy of RY-080

Ovary tissue was removed from female adult *Xenopus laevis* anaesthetized by immersion in 0.4% 3-aminobenzoic acid ethylester. Stage V and VI oocytes were isolated using fine forceps and treated with collagenase to remove follicle cells. cDNAs encoding different human $GABA_A$ receptor subunit expressed in either a pCDM8 or pcDNAI/Amp expression vector were directly injected into the nuclei of individual oocytes in 10–20 nl of injection buffer (88 mM NaCl, 1 mM KCl, 15 mM HEPES buffer, pH 7.0) at a concentration of 20 ng/μl. After 24–72 h., oocytes were placed in a 50 μl bath perfused with modified Barth's medium, impaled with two 1–3 MΩ electrodes containing 2 M KCl and voltage clamped at −30 to −80 mV. RY-080 was dissolved in dimethylsulphoxide (DMSO) and tested at a concentration of around 500-fold the K_i (i.e., 10 and 0.25 μM for α1- and α5-containing oocytes, respectively), with a DMSO concentration in the perfusate of 0.1%. The drug was preapplied for 30 s prior to the addition of a concentration of GABA that elicited a current 20% of the maximal response (EC_{20}) produced at a GABA concentration of 3 mM. The modulation of the EC_{20} was calculated as:

$$((\text{Current}_{\text{GABA EC}_{20}+\text{RY}-080} - \text{Current}_{\text{GABA EC}_{20}}) / \text{Current}_{\text{GABA EC}_{20}}) * 100$$

2.3. In vivo studies

Male Swiss–Webster mice (23–30 g; B&K International, Hull, UK) were used in the convulsant, proconvulsant and in vivo binding experiments. Dose volumes for i.p. and s.c. injections were 10 μl/g and for i.v. injections were 5 μl/g. All procedures were carried out in accordance with the Animals (Scientific Procedures) Act 1986 and associated guidelines.

2.3.1. Convulsant and proconvulsant activity of RY-080

2.3.1.1. Convulsant activity. Mice (n = 10/group) received injections of either RY-080 (40 and 100 mg/kg i.p. in 0.5% methyl cellulose vehicle) or pentylenetetrazole (PTZ; 40 or 100 mg/kg s.c. in isotonic saline). Immediately afterwards, mice were placed in perspex observation boxes and observed for 30 min during which time the number of animals undergoing tonic convulsions was noted.

2.3.1.2. Proconvulsant activity. Mice (25–28 g) were pretreated for 15 min with vehicle (0.5% carboxy methylcellulose) or RY-080 (10, 20 and 40 mg/kg i.p.) following which a subthreshold dose of PTZ was injected (40 mg/kg. s.c.). Animals were then observed for 30 min as described above. In a