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Flavan-3-ol derivatives are positive modulators of $GABA_A$ receptors with higher efficacy for the α_2 subtype and anxiolytic action in mice

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ABSTRACT

Recent genetic and pharmacological studies have demonstrated that α_2-containing $GABA_A$ receptors mediate the anxiolytic effects of benzodiazepines, setting a new strategy in developing novel, non-sedative anxiolytic agents. In this study we show that stereoisomers of 3-acetoxy-4′-methoxyflavan are positive modulators of recombinant $\alpha_{1,2,3,5}\beta_2\gamma_{2L}$ and $\alpha_1\beta_2$ $GABA_A$ receptors expressed in *Xenopus laevis* oocytes. $GABA_C$ receptors are insensitive to modulation by these compounds. In each case, the enhancement was evident at low micromolar concentrations and occurred independently of the classical high affinity benzodiazepine site, as it could not be blocked by the antagonist flumazenil. Importantly, the compound Fa131 was significantly more efficacious at enhancing GABA-induced currents (EC_5) at $\alpha_2\beta_2\gamma_{2L}$ receptors compared to $\alpha_1\beta_2\gamma_{2L}$, $\alpha_3\beta_2\gamma_{2L}$ and $\alpha_5\beta_2\gamma_{2L}$ receptors ($E_{max} = 21.0 \pm 1.7$ times, compared to 8.5 ± 0.7 times at α_1-, 9.5 ± 0.6 times at α_3- and 5.2 ± 0.4 times at α_5-contaning $GABA_A$ receptors), suggesting a potential use as an anxiolytic. In mice, this agent (1–30 mg/kg i.p.) induced anxiolytic-like action in two unconditioned models of anxiety: the elevated plus maze and the light/dark paradigms. No sedative or myorelaxant effects were detected using the hole board, actimeter and horizontal wire tests, and only weak barbiturate-potentiating effects on the loss of righting reflex test. Fa131 demonstrated improved segregation of anxiolytic and sedative doses when compared to the non-selective agonist diazepam. Finally, flavan derivatives highlight the potential of targeting non-benzodiazepine allosteric sites in the search for new anxioselective drugs.

1. Introduction

$GABA_A$ receptors are key therapeutic targets in modern psychiatry. These ligand-gated chloride channels consist of a heteropentameric assembly of proteins derived from a family of 16 genes: α_{1-6}, β_{1-3}, γ_{1-3}, δ, π, ε, and θ (Simon et al., 2004). Despite the great number of possible combinations, it has been shown that less than 20 native subtype combinations occur within the mammalian central nervous system, the most abundant being those containing α_{1-3}:$\beta_{2/3}$:γ_2 subunits in a 2:2:1 stoichiometry (McKernan and Whiting, 1996). $GABA_A$ receptors are a principal component of fast synaptic inhibitory neurotransmission in the mammalian brain, but also participate in the regulation of the resting potential of neurons by activating tonic currents in extrasynaptic locations (Farrant and Nusser, 2005). Therefore, it is not surprising that these receptors are implicated in a series of major psychiatric disorders including anxiety, sleep disorders, cognitive disorders, epilepsies, mood disorders and schizophrenia (Johnston, 2005).

Enhancement of the GABAergic function appears to be the primary mechanism of a range of important therapeutic agents such as benzodiazepines, barbiturates, and some general anesthetics. Indeed, $GABA_A$ positive modulation is translated into a range of desirable, as well as unwanted pharmacological actions, including anxiolysis, sedation, myorelaxation, protection against seizures, cognitive impairment and anesthesia, and development of tolerance and dependence (Johnston, 2005). While the co-occurrence of pharmacological effects might be beneficial in some instances, e.g. sedation and myorelaxation when using an anesthetic, latest research has centered on defining the molecular substrates that mediate these actions (Rudolph and Möhler, 2006; Whiting, 2006). Recent genetic and pharmacological studies have demonstrated that distinct α-containing $GABA_A$ receptors mediate specific benzodiazepine-induced pharmacological effects. The sedative and partly the anticonvulsant actions were attributed to the activation of α_1-containing receptors, suppression of anxiety to the α_2/α_3 subtypes, α_1/α_5 subtypes were associated with anterograde amnesia, whilst myorelaxation and motor impairment

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involve $\alpha_2/\alpha_3/\alpha_5$ subtypes (Rudolph et al., 1999; Löw et al., 2000; McKernan et al., 2000; Rudolph and Möhler, 2006). The subtype dependence of specific benzodiazepine actions is a reflection of the differential expression of $GABA_A$ receptors subtypes across brain regions (Möhler et al., 2002). Given this evidence, the search for new non-sedative anxiolytics has been focused on developing α_2 selective agonists for the benzodiazepine binding site. In 2000, McKernan and collaborators reported the in vivo and in vitro profile of the experimental drug L-838417, a benzodiazepine partial agonist selective for $\alpha_2/\alpha_3/\alpha_5$ subtypes. As expected, this agent exerted non-sedative anxiolytic properties in a range of animal models of anxiety. Since then, other benzodiazepine agonists have been described that essentially show little or no efficacy in activating α_1-containing $GABA_A$ receptors (Möhler et al., 2002; Atack et al., 2006; Rudolph and Möhler, 2006; Whiting, 2006).

Conversely, less attention has been directed towards identifying α-selective positive modulators for non-benzodiazepine allosteric sites, since classical GABAergic modulators such as barbiturates, neurosteroids and anesthetics, like etomidate and propofol, show little or no α-subtype specificity (Thompson et al., 1996; Belelli and Lambert, 2005; Rudolph and Antkowiak, 2004). Notwithstanding, some non-benzodiazepine selective ligands have been identified. In 2004, Johnstone and collaborators reported the potentiation of GABA-elicited currents by the dihydroquinolone "compound 4" at α_2-containing, but not α_1, $GABA_A$ receptors. Although the specific binding sites could not be defined, the compound was shown to exert anxiolytic-like properties in rodents without signs of sedation. In a later paper, Hall et al. (2005) described the positive modulatory action of the flavonoid 6-methylflavanone on $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_2\beta_2\gamma_{2L}$ $GABA_A$ receptors. Although the potency for these subtypes was essentially the same, the drug was more efficacious in modulating α_2-containing receptors. Importantly, this potentiation of GABA currents occurred independently of the benzodiazepine binding site, although the exact mechanism was not elucidated and the in vivo actions of this drug were not explored. This evidence suggests that the targeting of non-benzodiazepine allosteric sites is a viable alternative in the search for subtype selective $GABA_A$ positive modulators with specific pharmacological action.

In the present study we examined the actions of the four stereoisomers of 3-acetoxy-4′-methoxyflavan on human $\alpha_{1,2,3\&5}$-containing $GABA_A$ and ρ_1 $GABA_C$ receptors expressed in *Xenopus laevis* oocytes, and showed that these compounds are positive modulators of $GABA_A$ receptors. The isomer Fa131 showed greater efficacy at $GABA_A$ receptors containing the α_2 subunit compared to α_1, α_3 and α_5, with a mechanism independent of the benzodiazepine binding site. The systemic actions of the stereoisomer Fa131 were evaluated in mice using a battery of behavioral tests. At the doses tested, this drug exerted anxiolytic but not sedative or myorelaxant action, and only weak barbiturate-potentiating effects, suggesting that these agents are lead compounds in the search for new anxioselective drugs.

2. Materials and methods

2.1. Drugs

All four stereoisomers of 3-acetoxy-4′-methoxyflavan, namely Fa131, Fa132, Fa144 and Fa145 (Fig. 1) were synthesized in our laboratories following a modified version of the procedure of van Rensburg et al. (1997). Diazepam was purchased from Apin Chemicals LTD (Oxon, UK) and sodium thiopental from Jurox (Rutherford, NSW, Australia). [^{3}H]Flunitrazepam (84.5 Ci/mmol) was obtained from PerkinElmer (Boston, MA).

2.2. Animal use

All procedures involving animals were in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and were approved by the Animal Ethics Committee of the University of Sydney.

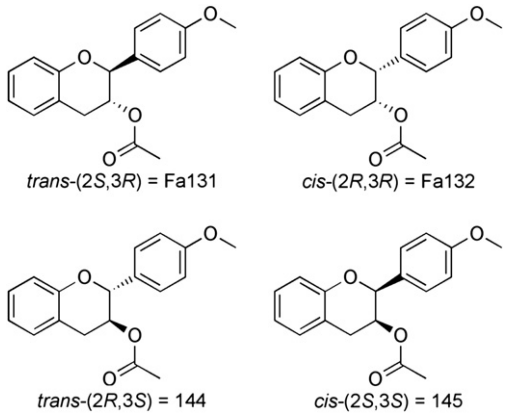

Fig. 1. Molecular structure of 3-acetoxy-4′-methoxyflavan stereoisomers. Chiral centers at positions C2 and C3 allow four possible configurations of this molecule, denoted here as *trans*-(2*R*,3*S*), *trans*-(2*S*,3*R*), *cis*-(2*R*,3*R*) and *cis*-(2*S*,3*S*).

2.3. Electrophysiological studies

cDNA for human α_1, α_2, β_2 and γ_{2L} $GABA_A$ receptor subunits subcloned into pCDM8 were provided by Dr Paul Whiting (Merck, Sharpe and Dohme Research Labs, Harlow, UK). Human α_3 and α_5 DNA in pGEMHE and pCDNA3, respectively, were a gift from Dr Bjarke Ebert (H. Lundbeck A/S, Valby, Denmark). Human ρ_1 DNA in pCDNA1.1 was provided by Dr George Uhl (National Institute for Drug Abuse, Baltimore, MD). The protocol for in vitro transcription of cRNA has been previously described (Walters et al., 2000; Hall et al., 2005). Briefly, cDNA vectors were linearized with the appropriate restriction endonucleases and capped transcripts were produced from linearized plasmids using the mMessage mMachine T7 transcription kit (Ambion, Austin, TX). cRNA was diluted and stored in diethylpyrocarbonate-treated water at −80 °C. The procedure involving the extraction, separation, and enzymatic treatment of *Xenopus laevis* oocytes was identical to that described previously (Huang et al., 2003; Hall et al., 2005). Stage V–VI oocytes were selected and injected (Nanoject, Drummond Scientific Co., Broomali, PA) with 2–3 ng of cRNA in a 1:1:2 ratio of α:β:γ subunits to facilitate the incorporation of the γ subunit and to achieve the desirable level of expression (maximal response to GABA less than 1 μA). High-expressing oocytes were obtained by injection of 10 ng of RNA (maximal GABA activation 1.5–2.5 μA). After injection, oocytes were incubated at 16 °C in ND96 storage solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2 \cdot 6H_2O$, 1.8 mM $CaCl_2$, 5 mM HEPES, pH 7.5, supplemented with 2.5 mM pyruvate, 0.5 mM theophylline and 50 μg/ml gentamycin), for 3–4 days before use in electrophysiological studies.

Currents were recorded using the two-electrode voltage clamp technique as described elsewhere (Walters et al., 2000; Hall et al., 2005). Oocytes were placed in a 100 μl chamber connected to a reservoir bottle containing ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2 \cdot 6H_2O$, 1.8 mM $CaCl_2$, 5 mM HEPES, pH 7.5). Glass microelectrodes were made using a micropipette puller (Narishige Scientific Instrument Laboratory, Tokyo, Japan) and filled with 3 M KCl (0.5–2 MΩ). The oocytes were impaled and the membrane potential was clamped at −60 mV while continuously superfused with ND96 solution (10 ml/min). Stock solutions of the drug were prepared in DMSO, except for GABA where distilled water was used, and applied into the perfusate until a peak response was reached. DMSO concentration in the perfusate was 0.6% and did not produce any alteration in the recording. Current measurements were obtained using a Geneclamp 500 voltage clamp amplifier (Axon Instruments INC, Foster City, CA), a MacLab/8 recorder (ADInstruments, Sydney, NSW, Australia) and Chart program v3.6. Responses were graphed as mean ± SEM from at least six oocytes from at least two different batches.

Modulation of GABA-elicited currents was tested by applying increasing concentrations of the sample drugs with a concentration of GABA that produced 5% of maximal activation (EC_5). A 3–5 min washout period was allowed between drug applications to avoid receptor desensitization. Responses were recorded and normalized as: fractional potentiation $= (I_{drug} - I_{GABA})/I_{GABA}$, where I_{drug} is the current in the presence of a given concentration of the drug, and I_{GABA} is amplitude of the control GABA current. EC_{50}, Hill coefficient (n) and maximal potentiation (E_{max}) values were estimated by fitting the concentration–response curves to the Hill equation (Prism v4, GraphPad software, San Diego, CA), according to the formula: $E = E_{max}/(1 + [EC_{50}/(X)]^n)$.

To study direct channel activation by flavans, oocytes expressing high levels of $GABA_A$ receptors were used. The drugs were applied to the perfusate alone and current responses were normalized as $I\% = (I_{drug}/I_{max}) \times 100$, where I_{drug} is the peak amplitude of current in response to drug and I_{max} is the maximal current produced by GABA measured in each individual cell. An isobolografic analysis (Berenbaum, 1989) was applied to evaluate synergistic interaction between GABA and drugs with direct action. For that purpose, increasing concentrations of the sample drug were