RESEARCH PAPER

Flavan-3-ol esters: new agents for exploring modulatory sites on GABA_A receptors

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BACKGROUND AND PURPOSE
Enhancement of GABAergic function is the primary mechanism of important therapeutic agents such as benzodiazepines, barbiturates, neurosteroids, general anaesthetics and some anticonvulsants. Despite their chemical diversity, many studies have postulated that these agents may bind at a common or overlapping binding site, or share an activation domain. Similarly, we found that flavan-3-ol esters act as positive modulators of GABA_A receptors, and noted that this action resembled the in vitro profile of general anaesthetics. In this study we further investigated the interactions between these agents.

EXPERIMENTAL APPROACH
Using two-electrode voltage clamp electrophysiological recordings on receptors of known subunit composition expressed in Xenopus oocytes, we evaluated positive modulation by etomidate, loreclezole, diazepam, thiopentone, 5α-pregnan-3α-ol-20-one (THP) and the flavan-3-ol ester 2S,3R-trans 3-acetoxy-4′-methoxyflavan (Fa131) on wild-type and mutated GABA_A receptors.

KEY RESULTS
The newly identified flavan, 2S,3S-cis 3-acetoxy-3′,4′-dimethoxyflavan (Fa173), antagonized the potentiating actions of Fa131, etomidate and loreclezole at α1β2 and α1β2γ2L GABA_A receptors. Furthermore, Fa173 blocked the potentiation of GABA responses by high, but not low, concentrations of diazepam, but did not block the potentiation induced by propofol, the neurosteroid THP or the barbiturate thiopental. Mutational studies on ‘anaesthetic-influencing’ residues showed that, compared with wild-type GABA_A receptors, α1M236Wβ2 and α1β2N265Sγ2L receptors are resistant to potentiation by etomidate, loreclezole and Fa131.

CONCLUSIONS AND IMPLICATIONS
Fa173 is a selective antagonist that can be used for allosteric modulation of GABA_A receptors. Flavan-3-ol derivatives are potential ligands for etomidate/lorepcelezole-related binding sites at GABA_A receptors and the low-affinity effects of diazepam are mediated via the same site.

Abbreviations
DMSO, dimethyl sulphoxide; Fa131, 2S,3R-trans 3-acetoxy-4′-methoxyflavan; Fa173, 2S,3S-cis 3-acetoxy-3′,4′-dimethoxyflavan; LGIC, ligand-gated ion channel; THP, 5α-pregnan-3α-ol-20-one; TM, transmembrane
Introduction

GABA type A (GABA<sub>A</sub>) receptors are ligand-gated chloride channels consisting of a heteropentameric assembly of proteins derived from a family of 19 genes, namely α1–6, β1–3, γ1–3, δ, π, ε, β and ρ1–3 (Simon et al., 2004). Common topography of these subunits is a large extracellular-amino-terminal domain, four transmembrane segments (TM1–TM4) and a long intracellular domain between TM3 and TM4. The chloride channel is delimited by the second transmembrane domain of each of the five subunits, with the large extracellular domain constituting the agonist/antagonist orthosteric binding sites (Mckernan and Whiting, 1996; Miller and Smart, 2010). In addition, the receptor contains a variety of allosteric sites that can modulate its function by altering the energy barrier required for conformational changes in the chloride channel (Johnston, 2005). Many important therapeutically agents such as benzodiazepines, barbiturates, loreclezole, and general anaesthetics like etomidate and propofol bind to these allosteric sites to produce an enhancement of GABA<sub>A</sub> receptor function.

Functional GABA<sub>A</sub> channels require the presence of α and β subunits, but the vast majority of naturally expressed receptors also contain a γ subunit (Mckernan and Whiting, 1996). Interestingly, agents such as barbiturates, neurosteroids, loreclezole and etomidate can positively modulate the receptor type receptors, suggesting that their corresponding binding sites lie within one of these subunits, or their interface (Malherbe et al., 1990; Thompson et al., 1996; Rudolph and Antkowiak, 2004; Belelli and Lambert, 2005). Furthermore, while the presence of a γ subunit is necessary for high-affinity modulation by benzodiazepine ligands, later studies proved that these drugs can also exert low-affinity (µM) modulation of αβ GABA<sub>A</sub> receptors (Stevenson et al., 1995; Walters et al., 2000). Although much evidence suggests the presence of discrete binding sites for these agents, many reports also suggested a common mechanism or overlapping sites. For example, mutational studies have identified a glycine at position 219 on the second transmembrane domain of the β2 subunit as fundamental for anaesthetic modulation of GABA currents (Chang et al., 2003). These authors found a linear correlation between the volume of the amino acid at this position and the loss of modulation by etomidate, propofol, pentobarbital and alphaxalone. Similarly, modulation by etomidate, propofol and loreclezole can be greatly reduced when the N265 residue at β-TM2 is replaced by serine, indicating a common mechanism. This position, however, seems to have little influence on the modulation by barbiturates and neurosteroids (Wingrove et al., 1994; Belelli et al., 1997; 1999; Siegwart et al., 2003; Desai et al., 2009). Also, several mutational studies at M286 on β-TM3 demonstrated its relevance for both propofol and etomidate modulatory activity, although modulation by loreclezole, neurosteroids and barbiturates remained unaffected (Krasowski et al., 1998; Siegwart et al., 2003).

Similarly, the contribution of the α subunit to anaesthetic action has been examined by site-directed mutagenesis. For example, Drafts and Fisher (2006) found that the mutation T69K in α6 subunit reduced both pentobarbital and etomidate agonist action, and mutations in α-TM1 alter sensitivity to neurosteroids (Hosie et al., 2006; Akk et al., 2008). Li and colleagues (2006) utilized a radiolabelled photoreactive etomidate analogue to identify residues involved in the binding of etomidate. Their results pointed not only at the previously mentioned M286 in β2-TM3, but also at M236 in α1-TM1 (and the homologous methionines in α2,3,5), an amino acid not previously implicated in the binding of this anaesthetic. Later functional studies revealed that substitution of this residue by a bulky amino acid such as tryptophan significantly reduces the modulation elicited by etomidate, but not by alphaxalone or pentobarbital (Stewart et al., 2008).

Studies involving site-directed mutagenesis intrinsically carry the paradox of whether the mutated residue being studied is directly involved in ligand–protein interactions, or is involved in the allosteric changes taking place as a consequence of the binding process, making the results difficult to interpret. Whereas the use of techniques such as photoaffinity labelling, radioligand binding competition or the simple use of specific antagonists may provide a deeper insight into unravelling the mysteries of these potentially overlapping binding sites. We have previously described a series of flavan-3-ol derivatives as positive modulators of GABA<sub>A</sub> receptors (Fernandez et al., 2008; Mewett et al., 2009). The derivative 2S,3R-trans 3-acetoxy-4′-methoxyflavann (Fa131) demonstrated positive allosteric properties over a range of GABA<sub>A</sub> subunit combinations, and in particular had a higher efficacy at α2-containing GABA<sub>A</sub> receptors. We noted at the time that the mode of action of Fa131 resembled that of other GABA<sub>A</sub> modulators such as neurosteroids, barbiturates and anaesthetics such as etomidate. In this present study, we carried out mutational studies on ‘anaesthetic-influencing’ residues to test their role in flavan-elicited modulation of GABA<sub>A</sub> receptors. Furthermore, we discovered a new flavan derivative, namely 2S,3S-cis 3-acetoxy-3′,4′-dimethoxyflavan (Fa173), featuring antagonist properties. Fa173 is the first reported ligand to inhibit the modulation of GABA by etomidate, loreclezole, diazepam and flavans, but not thiopentone or 5o-pregn-an-3a-ol-20-one (THP), indicating that these drugs may share a common binding pocket or activation domain.

Methods

DNA constructs and ligands
cDNA for human α1, β2 and γ2L GABA<sub>A</sub> receptor subunits subcloned into pCDM8 were provided by Dr Paul Whiting (Merck, Sharpe and Dohme Research Labs, Harlow, UK). The protocol for in vitro transcription of cRNA has been described previously (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, USA). cRNA was diluted and stored in diethylpyrocarbonate-treated water at ~80°C until use. Site-specific mutations were introduced into the cDNAs of the GABA<sub>A</sub> receptor subunits using QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). Mutagenic oligonucleotides used were (S’ to 3’): β2N265S, ACAATCACCCACTTCCAGGGAAAAC; α1M236A, CCTGCCTGGTCATA GCCAGCTTATCTC; α1M236W, CCTGCCTGGCATA TGCC. Mutant clones were submitted to complete
Flavans as novel GABA<sub>a</sub> modulatory agents

from connective tissue and follicular cells. Released oocytes were rinsed in ND96 wash solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl<sub>2</sub>, 1.8 mM CaCl<sub>2</sub>, 5 mM HEPES, pH 7.5). Stage V-VI oocytes were injected (Nanoject, Drummond Scientific Co., Broomall, PA, USA) with a total of 3–5 ng of cRNA. When expressing receptors containing a γ subunit, a 1:1:2 ratio of α : β : γ subunits was used. After the injection, oocytes were incubated at 16°C in ND96 storage solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl<sub>2</sub>, 1.8 mM CaCl<sub>2</sub>, 5 mM HEPES, pH 7.5, supplemented with 2.5 mM pyruvate, 0.5 mM theophylline and 50 µg·mL<sup>-1</sup> gentamicin) for 2 days before use in electrophysiological studies.

Currents were recorded using the two-electrode voltage-clamp technique as previously described (Walters et al., 2000; Hall et al., 2005). Oocytes were individually placed in a 100 μL chamber connected to a reservoir bottle containing ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl<sub>2</sub>, 1.8 mM CaCl<sub>2</sub>, 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<sup>-1</sup>). Stock solutions of the drugs were prepared in dimethyl sulphoxide (DMSO), except for GABA and sodium thiopental 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. Voltage clamp experiments were conducted using a Geneclamp 500 amplifier (Axon Instruments Inc., Foster City, CA, USA). Current amplitudes were calculated off-line using Chart software v3.6 (ADInstruments, NSW, Australia).

Responses to GABA applications were normalized as P<sub>%</sub> = (I/I<sub>max</sub>), where I is the peak amplitude of current response and I<sub>max</sub> is the maximal current produced by GABA measured in each individual cell. Modulation of GABA-elicted currents was tested by co-applying increasing concentrations of the drugs with a concentration of GABA that produced 3% of maximal activation (EC<sub>3</sub>, determined for each cell). Current responses were recorded and normalized as: fractional potentiation = (I<sub>drug</sub> − I<sub>GABA</sub>) / I<sub>GABA</sub>, where I<sub>drug</sub> is the current in the presence of a given concentration of drug, and I<sub>GABA</sub> is the amplitude of the control GABA current. Finally, experiments involving the antagonist Fa173 were conducted by co-applying GABA EC<sub>50</sub>, the positive modulator at a concentration that produced 50% of its maximal effect (EC<sub>50</sub>, determined for each cell) and increasing concentrations of Fa173. Data were normalized according to the equation: % inhibition: 100 × (I<sub>control</sub> − I<sub>drug</sub>) / (I<sub>control</sub> − I<sub>GABA</sub>), where I<sub>control</sub> is the response in the presence of Fa173, I<sub>GABA</sub> is the response to GABA alone and I<sub>drug</sub> is the response to GABA plus positive modulator. In all cases, a 3–5 min washout period was allowed between drug applications to avoid receptor desensitization. Normalized responses were pooled and graphed as mean ± SEM from at least two different batches of injected oocytes. Responses were fitted to the four-parameter logistic equation: I = I<sub>max</sub> / (1 + (EC<sub>50</sub> / [A])<sup>H</sup>), where I is the peak amplitude of the current elicited by a given concentration of agonist [A], I<sub>max</sub> is the maximum amplitude of the current, EC<sub>50</sub> is the concentration required for half-maximal response, and n<sub>H</sub> is the Hill coefficient (Prism 5 GraphPad Software, San Diego, CA).
USA). Best-fit parameters were first compared by extra-sum-of-squares F-test to detect whether an estimated parameter differs among data sets. Individual differences between best-fit values were detected by non-overlapping confidence intervals. All data analyses were conducted using Prism v5 and a P value lower than 0.05 was considered of statistical significance.

Results

Mutational analysis of β2-N265 and α1-M236

The co-injection of mRNA encoding point-mutated subunits of the GABA receptor type A resulted in functional GABA-activated channels. The potency of GABA to activate α1β2N265Sα2L receptors was slightly lower compared with wild-type, but this change was not statistically significant (Table 1). Conversely, receptors containing a tryptophan point mutation on methionine 236 (α1M236Wβ2α2L) were more sensitive to GABA activation as revealed by a significantly lower EC₅₀ (Table 1). Interestingly, when the same residue was substituted by an alanine, which is much smaller in size, no significant changes in GABA activation were observed (Table 1). To test for spontaneous channel opening, we applied picrotoxin alone at 1 mM concentration to all receptors expressed in *Xenopus* oocytes. Similarly, the anaesthetic etomidate and the anticonvulsant loreclezole elicited a positive modulatory action on these receptor types in a way resembling the effect of Fa131. Corresponding EC₅₀ values for the modulation of GABA currents at both receptor subtypes were in the low micromolar range for all three drugs, and corresponding concentration-response curves presented high Hill coefficient values.

### Table 1

Estimated parameters from best-fit to four-parameter logistic equation for GABA activation and positive modulation by Fa131, etomidate, and loreclezole to wild-type and mutated GABAₐ receptors

<table>
<thead>
<tr>
<th>Drug/receptor type</th>
<th>Maximal fractional effect*</th>
<th>EC₅₀</th>
<th>Hill coefficient</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>GABA</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>α1β2α2L</td>
<td>1.02 ± 0.06</td>
<td>36.2 ± 4.8</td>
<td>1.0 ± 0.2</td>
<td>10</td>
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<tr>
<td>α1β2</td>
<td>0.95 ± 0.04</td>
<td>5.9 ± 1.1</td>
<td>1.0 ± 0.1</td>
<td>7</td>
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<tr>
<td>α1β2N265Sα2L</td>
<td>1.01 ± 0.04</td>
<td>63.6 ± 5.5</td>
<td>1.1 ± 0.2</td>
<td>7</td>
</tr>
<tr>
<td>α1M236Wβ2α2L</td>
<td>1.04 ± 0.05</td>
<td>8.5 ± 0.9*</td>
<td>0.73 ± 0.07</td>
<td>4</td>
</tr>
<tr>
<td>α1M236α2β2α2L</td>
<td>1.02 ± 0.02</td>
<td>46.7 ± 3.2</td>
<td>0.80 ± 0.04</td>
<td>4</td>
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<tr>
<td>Fa131</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α1β2α2L</td>
<td>14.7 ± 0.6</td>
<td>9.7 ± 1.7</td>
<td>1.9 ± 0.6</td>
<td>5</td>
</tr>
<tr>
<td>α1β2</td>
<td>10.2 ± 1.2</td>
<td>7.5 ± 1.2</td>
<td>2.1 ± 0.9</td>
<td>6</td>
</tr>
<tr>
<td>α1β2N265Sα2L</td>
<td>6.0 ± 0.2***</td>
<td>18.1 ± 1.7***</td>
<td>1.6 ± 0.2</td>
<td>5</td>
</tr>
<tr>
<td>α1M236Wβ2α2L</td>
<td>7.0 ± 0.6***</td>
<td>4.6 ± 1.3</td>
<td>1.3 ± 0.5</td>
<td>6</td>
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<tr>
<td>α1M236α2β2α2L</td>
<td>13.3 ± 0.9</td>
<td>12.5 ± 3.7</td>
<td>2.3 ± 0.9</td>
<td>4</td>
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<tr>
<td>Etomidate</td>
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<tr>
<td>α1β2α2L</td>
<td>28.9 ± 1.9</td>
<td>3.2 ± 1.0</td>
<td>1.5 ± 0.5</td>
<td>6</td>
</tr>
<tr>
<td>α1β2</td>
<td>33.6 ± 2.1</td>
<td>7.5 ± 1.2</td>
<td>2.1 ± 0.7</td>
<td>4</td>
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<tr>
<td>α1β2N265Sα2L</td>
<td>18.1 ± 1.1*</td>
<td>22.0 ± 5.0***</td>
<td>1.6 ± 0.4</td>
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<tr>
<td>α1M236Wβ2α2L</td>
<td>18.0 ± 0.9*</td>
<td>14.2 ± 2.1***</td>
<td>1.4 ± 0.2</td>
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<tr>
<td>α1M236α2β2α2L</td>
<td>32.8 ± 1.2</td>
<td>5.7 ± 0.9</td>
<td>1.3 ± 0.2</td>
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<tr>
<td>Loreclezole</td>
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<tr>
<td>α1β2α2L</td>
<td>9.5 ± 0.9</td>
<td>9.8 ± 2.1</td>
<td>2.2 ± 1.1</td>
<td>5</td>
</tr>
<tr>
<td>α1β2</td>
<td>7.4 ± 0.8</td>
<td>11.3 ± 1.1</td>
<td>1.8 ± 0.6</td>
<td>4</td>
</tr>
<tr>
<td>α1β2N265Sα2L</td>
<td>3.0 ± 0.5**</td>
<td>24.0 ± 3.8*</td>
<td>1.6 ± 0.6</td>
<td>5</td>
</tr>
<tr>
<td>α1M236Wβ2α2L</td>
<td>6.1 ± 0.6*</td>
<td>9.3 ± 1.9</td>
<td>1.4 ± 0.4</td>
<td>4</td>
</tr>
<tr>
<td>α1M236α2β2α2L</td>
<td>8.7 ± 0.7</td>
<td>12.0 ± 3.5</td>
<td>2.1 ± 0.7</td>
<td>4</td>
</tr>
</tbody>
</table>

*P < 0.05; **P < 0.01; ***P < 0.001; significantly different compared to value at corresponding wild-type receptor.

*Note that the normalization method differs between GABA-alone dose-response curves and potentiation by positive modulators.
Point mutation of the 265 serine residue on β2-TM2 proved to form functional channels that exhibited a substantial resistance to the modulatory action of Fa131. Representative current traces depicting this effect are shown in Figure 2A, where the potentiating action of Fa131 (10 μM) is shown at different receptor subunit combinations. Fa131 can potentiate the GABA EC₃ 10 times at this concentration at α1β2γ2L wild-type GABAₐ receptors; however, this enhancement was reduced to three times at α1β2N265Sγ2L. Full concentration-response curves are shown in Figure 2A right panel. The Fa131 EC₅₀ was shifted from 9.7 to 18.1 μM, while the maximal potentiation was reduced to half, compared with wild-type, both effects reaching statistical significance (Table 1). Similarly, the methionine-to-tryptophan substitution at position 236 in α1-TM1 had drastic, but unequal effects on the potentiation induced by this drug. A concentration of 10 μM Fa131 only potentiated GABA currents five times in this case (Figure 2A), and while the EC₅₀ was slightly.
shifted to the left (4.6 μM), the maximal enhancement was significantly lowered from 14.7 to 7 times the GABA EC₃₀ (Figure 2A and Table 1). Finally, replacing this same residue by an alanine induced no significant changes in the modulatory action of Fa131 over GABA-elicted currents (Table 1).

In oocytes expressing wild-type α1β2γ2L, GABAₐ receptors, etomidate induced a significant potentiating effect, enhancing GABA EC₃₀ more than 20 times at 3 μM (Figure 2B). Both N266S in β2-TM2 and M236W in α1-TM1 mutations substantially impaired the etomidate modulatory action, with 3 μM etomidate having virtually no effect on α1β2N266Sγ2L receptors, and only enhancing GABA currents two times at α1M236Wβ2γ2L (Figure 2B). Concentration-response curves for etomidate at both mutated receptors were significantly shifted to the right (Figure 2B right panel). Changes in potency were characterized by a significant increase in etomidate EC₃₀ from 3.2 μM at wild-type receptors to 22.0 and 14.2 μM at α1β2N266Sγ2L and α1M236Wβ2γ2L, receptors, respectively (Table 1). Similarly, maximal potentiation induced by this drug was significantly lowered from 28.9 times the GABA EC₃₀ response at wild-type receptors, to approximately 18 times at each of the mutated receptors (Table 1). However, mutation M236A at α1-TM1 presented no significant changes on etomidate-induced potentiation (Figure 2B, Table 1).

The anticonvulsant loreclezole also potentiates the GABA response at wild-type α1β2γ2L. GABAₐ receptors expressed in Xenopus oocytes; 10 μM loreclezole induced a clear enhancement of GABA currents (six times the GABA EC₃₀) as depicted in current traces shown in Figure 2C. The point-mutation N266S at β2 completely abolished the potentiation by 10 μM loreclezole (Figure 2C), and produced a significant shift-to-the-right in its concentration-response curve (Figure 2C right panel). Consequently, the EC₅₀ for this drug increased from 9.8 μM at wild-type receptors to 24.0 μM at α1β2N266Sγ2L receptors, while the maximal effect achieved decreased from 9.5- to 3-fold potentiation (Table 1). In contrast, the mutation M236W at α1-TM1 had less pronounced effects on loreclezole potentiation; 10 μM loreclezole potentiated the corresponding GABA EC₃₀ by three times at α1M236Wβ2γ2L receptors, compared with the sixfold potentiation in wild-type receptors (Figure 2C). The concentration-response curves for loreclezole presented a similar EC₅₀ value at both receptor subtypes, while the maximal potentiation significantly decreased from 9.5 times to 6.1 times at α1M236Wβ2γ2L receptors (Table 1). As seen with Fa131 and etomidate, mutation of the methionine 236 at α1-TM1 to an alanine did not affect the modulatory action of loreclezole (Figure 2C, Table 1).

Fa173 is a neutral modulator and antagonizes the modulatory action of flavans

The flavan analogue Fa173 (100 μM) was inactive when applied in the absence of GABA and showed no significant modulatory effects on GABA-elicted (EC₅₀ and EC₉₀) currents at α1β2 or α1β2γ2L receptors expressed in Xenopus oocytes. Current traces shown in Figure 3A illustrate the lack of significant effects of Fa173 (1–300 μM) over corresponding GABA EC₃₀ concentrations at these two receptor subunit combinations. Only at very high concentrations, 100 and 300 μM, some weak potentiation of GABA EC₃₀ was evident. This effect reached approximately 20% enhancement over the corresponding GABA-elicted current response (Figure 3B).

In turn, Fa173 was able to dose-dependently reduce the potentiation of GABA currents induced by Fa131 at both α1β2γ2L and α1β2 GABAₐ receptors (Figure 4A,B respectively). For these experiments, we selected a low concentration of GABA (EC₅₀), and combined it with a concentration of Fa131 that produces half its maximal potentiation response (EC₉₀). We then co-applied these two drugs with increasing concentrations of Fa173 and recorded the final current peak amplitude. Representative current traces of these experiments are depicted in Figure 4A,B. Concentration-response curves for Fa173 inhibitory action over Fa131 potentiation at both receptor subtypes are shown in Figure 4C. Fa173 was equipotent at both receptor subunit combinations (IC₅₀ = 11.4 ± 0.9 μM at α1β2γ2L and 7.8 ± 1.2 μM at α1β2), and similarly, its neutralizing efficacy was comparable (maximal inhibition = 100 ± 5% and 93 ± 5%, respectively, Figure 4C).
**Fa173 antagonizes the potentiation induced by etomidate and loreclezole, but not by propofol, THP or thiopental**

Considering the ability of Fa173 to antagonize the potentiating effects of Fa131, we tested its antagonistic profile over a series of well-known GABA$_A$ positive modulators. Thus, etomidate, propofol, loreclezole, the neurosteroid THP and the barbiturate thiopental were selected to conduct antagonism experiments. Assays were carried out by co-applying GABA (EC$_3$) plus modulator (EC$_{50}$) and Fa173 (increasing concentrations), in order to estimate an inhibitory potency for the antagonist.

Fa173 similarly neutralized the potentiating effects of etomidate at α₁β₂ and α₁β₂γ₂L GABA$_A$ receptors (Figure 5A,B). Representative current traces shown in Figure 5A,B depict the potentiation induced by etomidate of the GABA EC$_3$ response, and its subsequent blockade by increasing concentrations of Fa173. The potency of Fa173 to antagonize etomidate was comparable at both receptor subunit combinations (IC$_{50}$ = 12.0 ± 1.1 μM and 6.1 ± 1.3 μM, at α₁β₂ and α₁β₂γ₂L receptors, respectively), as was its maximal inhibitory effect (maximal inhibition = 86 ± 8% and 81 ± 5%, respectively) (Figure 5C).

Loreclezole can also induce large potentiation of GABA-elicited currents at both at α₁β₂ and α₁β₂γ₂L GABA$_A$ receptors (Figure 6). This enhancement was antagonized by co-applying increasing concentrations of the antagonist Fa173, as depicted in the current traces shown in Figure 6A,B. At α₁β₂γ₂L receptors, the IC$_{50}$ was 6.6 ± 0.2 μM with a maximal inhibition of 99 ± 11%, while at α₁β₂ the IC$_{50}$ was 3.8 ± 0.9 μM with a maximal inhibition of 94 ± 4% (Figure 6C).

The antagonizing properties of Fa173 were tested against the positive modulatory action of THP, thiopental and propofol at α₁β₂ and α₁β₂γ₂L GABA$_A$ receptors (n = 4) (Figure 7). Fa173 (1–100 μM) failed to alter the enhancement of the GABA response induced by THP (Figure 7A) or by thiopental (Figure 7B), at both receptor subtypes. Finally, Fa173 (60 μM) also failed to alter the enhancement of the GABA response induced by propofol (10 μM) at α₁β₂ and α₁β₂γ₂L receptors (n = 3) (Figure 7C).

**Fa173 blocks the GABA-potentiating effects induced by large, but not low, concentrations of diazepam**

It has been well documented that diazepam can enhance GABA-induced currents in a biphasic mode when acting on α₁β₂γ₂L GABA$_A$ receptors (Walters et al., 2000), an observation that has been reproduced in our laboratories (Hall et al., 2005). We selected two concentrations of diazepam to study the high-affinity (100 nM) and low-affinity (100 μM) effects of this drug at α₁β₂γ₂L receptors (Figure 8A,B). At a concentration of 100 nM, diazepam induced a twofold potentiation of the GABA response, and this enhancement was completely blocked by the co-application of flumazenil 10 μM (93% inhibition), but not by Fa173 100 μM (2% inhibition, n = 3) (Figure 8A). However, when diazepam was applied at a high concentration (100 μM), flumazenil produced a mild neutralizing effect (about 10%), while the flavan Fa173 (100 μM) almost completely abolished the potentiation (87% inhibition, n = 3) (Figure 8B).

**Figure 4**

The flavan Fa173 acts as an antagonist of the GABA$_A$ positive modulator Fa131 at both α₁β₂γ₂L and α₁β₂ L recombinant receptors expressed in Xenopus oocytes. Current traces (nA vs. min) illustrating the concentration-dependent blockade that Fa173 exerts over the potentiating action of Fa131 at (A) α₁β₂γ₂L and (B) α₁β₂ receptors. Drug concentrations were 5 μM GABA and 15 μM Fa131 at α₁β₂γ₂L and 1 μM GABA and 10 μM Fa131 at α₁β₂, equivalent to their corresponding EC$_3$ and EC$_{50}$, respectively. Horizontal bars represent drug application. (C) Concentration-response curve for Fa173 antagonistic action over Fa131-induced potentiation of GABA currents at both receptor subtypes. Data points represent mean ± SEM peak current response normalized as described in Methods. Data were fitted using four-parameter logistic equation and the best-estimated values for each parameter are: IC$_{50}$ = 11.4 ± 0.9 μM, maximal inhibition = 100 ± 5%, n = 7 at α₁β₂γ₂L; IC$_{50}$ = 7.8 ± 1.2 μM, maximal inhibition = 93 ± 5%, n = 6 at α₁β₂. Hill coefficient values were not different from unity and are not reported.
**Figure 5**
The flavan Fa173 acts as an antagonist of the etomidate-induced enhancement of GABA currents. (A) Current traces (nA vs. min) illustrating the blockade that increasing concentrations of Fa173 exert over the potentiating action of etomidate at α1β2γ2L receptors. (B) Current traces (nA vs. min) illustrating the blockade that increasing concentrations of Fa173 exert over the potentiating action of etomidate at α1β2 receptors. Drug concentrations were 3 μM GABA and 10 μM etomidate at α1β2γ2L and 1 μM GABA and 10 μM etomidate at α1β2, equivalent to their corresponding EC30 and EC50, respectively. Horizontal bars represent drug application. (C) Concentration-response curves for Fa173 blockade of etomidate-induced potentiation (IC50 = 12.0 ± 1.1 μM and 6.1 ± 1.3 μM, maximal inhibition = 86 ± 8% and 81 ± 5%, at α1β2γ2L and α1β2 receptors, n = 6 and 5, respectively).

**Figure 6**
The flavan Fa173 acts as an antagonist of the loreclezole-induced enhancement of GABA currents. (A) Current traces (nA vs. min) illustrating the blockade that increasing concentrations of Fa173 exert over the potentiating action of loreclezole at α1β2γ2L receptors. (B) Current traces (nA vs. min) illustrating the blockade that increasing concentrations of Fa173 exert over the potentiating action of loreclezole at α1β2 receptors. Drug concentrations were 3 μM GABA and 10 μM loreclezole at α1β2γ2L and 1 μM GABA and 10 μM loreclezole at α1β2, equivalent to their corresponding EC30 and EC50, respectively. Horizontal bars represent drug application. (C) Concentration-response curves for Fa173 blockade of loreclezole-induced potentiation (IC50 = 6.6 ± 0.2 μM and 3.8 ± 0.9 μM, maximal inhibition = 99 ± 11% and 94 ± 4%, n = 7 and 5, at α1β2γ2L and α1β2 receptors, respectively).
Conversely, at GABAA receptors composed of α1β2 subunits, diazepam potentiates GABA-induced currents in a monophasic manner, consistent with a single ‘low-affinity’ site. Diazepam (50 μM) induced a fivefold increase in GABA-elicited currents at this subunit combination, and this effect was unaffected by flumazenil (10 μM). However, when this dose of diazepam was co-applied with increasing concentrations of Fa173, the potentiation was completely abolished. Fa173 antagonized diazepam with an IC50 of 2.7 ± 0.8 μM and maximal inhibition of 94 ± 5% (n = 4) (Figure 8C).

Discussion

GABAa receptors expressing β2-N265 subunit

Etomidate and loreclezole present enhanced modulatory action at GABAa receptors that contain a β2/3 subunit, and their activity is comparatively weaker at β1 (Wafford et al., 1994; Hill-Venning et al., 1997). Later studies found that this selectivity was conferred by a single asparagine residue located at position 265 on the second transmembrane domain of the β2 subunit (and equivalent position at β3). When this asparagine is mutated to a serine (homologous amino acid in the β1 subunit), the ability of etomidate and loreclezole to potentiate GABA currents is diminished (Wafford et al., 1994; Wingrove et al., 1994; Belelli et al., 1997), and, accordingly, knock-in mice carrying either β3N265M or β2N265S present impaired sensitivity to the in vivo actions of these drugs (Jurd et al., 2003; Reynolds et al., 2003; Rudolph and Antkowiak, 2004; Groves et al., 2006). Notably, mutations at this point do not interfere with the potentiation induced by the neurosteroid alphaxalone and the barbiturate pentobarbitone (Belelli et al., 1999; Siegwart et al., 2003). In this study, the β2N265S mutant was
co-expressed with α1 and γ2L subunits. As expected, etomidate and loreclezole were less capable of enhancing currents at this mutant receptor, but more interestingly, the modulatory action of Fa131 was also affected by this mutation, suggesting that these agents share a common binding site domain or signal transduction mechanism. Some studies utilizing computational modelling have suggested that this residue could contribute to the binding pocket for etomidate (Campagna-Slater and Weaver, 2007). However, more recent studies have suggested that N265 is not involved in the binding of etomidate (Li et al., 2006). As there is no high resolution crystal structure available for the GABA_A receptor

Figure 8

The flavan 173 selectively antagonizes the current enhancement induced by diazepam acting on its low-affinity site. (A) Representative current traces showing that Fa173 does not antagonize the potentiating effects of a low concentration of diazepam acting on α1β2γ2L receptors, while this effect is completely blocked by flumazenil (left panel). The quantification of the inhibitory effect of both drugs is shown on the right panel (n = 3). (B) Conversely, the response elicited by diazepam at a high concentration is blocked up to 87% by the flavan Fa173, while the co-application of flumazenil only inhibited about 10% of this response (n = 3). (C) At α1β2 receptors, diazepam induces a potentiation of GABA-elicited currents that can be almost completely blocked by the co-application of Fa173, but not flumazenil (left panel) (n = 4). The panel on the right shows the concentration-response curve for this effect (IC50 = 2.7 ± 0.8 μM, maximal inhibition = 94 ± 5%).
complex, the exact role of N265 remains unclear, dampening further speculations on our hypothesis.

**GABA<sub>A</sub> receptors expressing α1-M236 subunit**

In 2006, Li and collaborators, utilizing a radiolabelled photoreactive etomidate analogue, identified M236 at α-TM1 and M286 at β-TM3, to be directly involved in the binding of this anaesthetic (Li et al., 2006). In later work, Stewart et al. (2008) conducted a tryptophan mutation on M236 and characterized the properties of α1M236Wβ2γ2L receptors, showing that these channels present increased sensitivity to GABA transduction, and reduced etomidate modulation. Our findings suggest that these channels present increased sensitivity to GABA and reduced etomidate modulation. Our α1M236Wβ2γ2L GABA<sub>A</sub> mutant receptors presented very similar characteristics to those described by Stewart et al., including augmented GABA gating, mild spontaneous activity and reduced potentiation by etomidate. Importantly, we found that this mutant was also less sensitive to allosteric modulation by loreclezole, and the flavan-3-ol Fa131, further reinforcing the idea that these three modulators share their binding pocket or activation domain. Interestingly, the mutation M236A did not affect the modulatory properties of etomidate, loreclezole or Fa131. Methionine, the original amino acid, tryptophan and alanine, the chosen mutations, all share the characteristics of non-polar hydrophobic amino acids; however, they differ largely in their molecular size. Thus, it is tempting to speculate that tryptophan impairs drug activity by sterically impeding the accommodation of large aromatic rings, or alternatively by offering nearby residues an intramolecular aromatic interaction, thus weakening the binding of a drug with similar needs. In this line, Stewart et al. (2008) proposed that a α1-M236W mutation mimicked the effects of etomidate on wild-type GABA<sub>A</sub> receptors. Conversely, alanine has a much smaller side chain that would allow the binding process to remain unaltered.

**Fa173 is an antagonist of positive modulators such as Fa131, etomidate and loreclezole**

Despite the worldwide use of GABA<sub>A</sub> receptor positive modulators in human therapy, the development of site-specific antagonists, best defined as neutral modulators, has been scarce. This may be due to the large number of sites for allosteric regulation present in these channels (Johnston, 2005). For example, positive or negative modulation by benzodiazepine ligands at the high-affinity site can be blocked by the antagonist flumazenil. Similarly, bemegride and 3α,5α,17-phenylandrosten-16-en-3-ol have been proposed to be antagonists of the barbiturate and neurosteroid sites, respectively, but these drugs seem to present intrinsic limitations (Schechter, 1984; Mennerick et al., 2004). In our study, the flavan derivative Fa173 showed characteristics of a specific flavan-site modulator with extremely low efficacy to potentiate GABA-induced currents. Importantly, the main structural requirement for this change in intrinsic activity seems to be a cis conformation at C2 and C3. At recombinant α1β2 and α1β2γ2L receptors, Fa173 antagonized the potentiation induced by the positive modulator Fa131. Importantly, Fa173 also antagonized the enhancing effects of etomidate and loreclezole at α1β2 and α1β2γ2L receptors, suggesting that these drugs bind to a single binding site. However, it failed to neutralize the potentiating action of the neurosteroid THP, the barbiturate thiopental and the anaesthetic propofol.

As Fa173 had little direct effect on GABA-elicited currents, it is unlikely that this compound acts via binding to the GABA<sub>A</sub> site. Rather, the antagonistic properties of Fa173 are highly selective, and may occur through competition for the etomidate/flavan site; however, further experimentation is needed to clarify the specific mechanism. Despite these reservations, this is, to our knowledge, the first report of a specific neutral modulator for the etomidate/loreclezole site and Fa173 represents a lead compound in the development of novel antagonists.

**Diazepam binds to the ‘etomidate’ site with μM affinity**

Both benzodiazepines and β-carbolines have been shown to possess two distinct components when interacting with GABA<sub>A</sub> receptors. The first component, via the classical benzodiazepine site, possesses high-affinity (nM), is flumazenil-sensitive and requires the presence of α1,2,3,5 and γ subunits. The second site presents low ligand affinity (µM), is insensitive to blockade by flumazenil and is not dependent on the presence of a γ subunit (Malherbe et al., 1990). It has been hypothesized that this second binding component could respond to an interaction with the etomidate/loreclezole site, as it could be eliminated by mutations at N265 in β2-TM2 (Stevenson et al., 1995; Walters et al., 2000). Our results corroborate this hypothesis; the flavan Fa173 blocked the effects of etomidate, loreclezole and high concentrations of diazepam at α1β2 and α1β2γ2L receptors. Importantly, we demonstrated that the two binding sites to which diazepam interacts with can be selectively blocked by the addition of flumazenil (high-affinity site) and Fa173 (low-affinity site).

**Implications for anaesthetic action**

Several mutational studies at M286 on β-TM3 led to the proposal that propofol may bind in the M3 domain near β2M286 (Krasowski et al., 1998; Siegwart et al., 2003). However, the finding that propofol only partially inhibits the affinity labelling of both α1M236 and β2M286 by [3H]-azetomidate suggests that the effect of propofol on the reaction of [3H]-azetomidate with these residues is allosteric rather than direct (Li et al., 2010). Importantly, the finding that Fa173 blocks potentiation by etomidate but not propofol supports the notion that propofol does not have a direct interaction with the etomidate binding site α1β2γ2L in GABA<sub>A</sub> receptors. Recently, the crystal structure of the bacterial LGIC with propofol bound was published, showing the location to be an inter-subunit cavity between the TM3 and TM4 domains (Nury et al., 2011). However, it is not yet known whether propofol binds in a similar location at GABA<sub>A</sub> receptors.

We previously observed that while the intrinsic activity of the flavan Fa131 on GABA<sub>A</sub> receptors resembled general anaesthetics, its in vivo profile was quite distinct. Unlike drugs such as etomidate, loreclezole and diazepam, Fa131 failed to induce strong sedative and hypnotic effects in mice. Rather, it exerted a robust anxiolytic action, as measured by the elevated plus maze and the light/dark paradigm. This discrepancy can be explained by the higher efficacy of Fa131 to
activate GABA$_A$ receptors containing an $\alpha_2$ subunit (Fernandez et al., 2008), as this GABA$_A$ receptor subtype is believed to mediate the anxiolytic activity of benzodiazepines and barbiturates (Möhler et al., 2002; Dixon et al., 2008). In contrast, etomidate and loreclezole present limited $\alpha$-subunit selectivity and their ability to induce sedation, anaesthesia and seizure protection is markedly subjugated by the activation of all $\beta_2/3$-containing GABA$_A$ receptors, which represent more than 90% of all receptors expressed in the mammalian brain (Rudolph and Antkowiak, 2004; Groves et al., 2006). Benzodiazepines, such as diazepam, are much used as therapy for anxiety, but this group of substances has also proved useful as anticonvulsants, hypnotics and muscle relaxants. However, this spectrum of pharmacological activities does not all occur in the same dose range, and extensive studies have suggested that the incremental CNS effects of benzodiazepines may be the consequence of gradual nM receptor occupancy (Gardner, 1988; Ito et al., 1993; 1997). The identification of a second $\mu$M potentiation component, present in any $\alpha X \beta 2/3$ subunit combination, thus less specific, indicates the possibility that the deep CNS-depressant actions of benzodiazepines could be the result of their effect, at $\mu$M concentrations, on GABA$_A$ receptor channels (Walters et al., 2000). Whether flumazenil does (Hoffman and Warren, 1993), or does not (Little and Richard, 1984), antagonize the anesthetic effects of benzodiazepines, might reveal whether a double nM/$\mu$M potentiation is an essential requirement. However, despite these speculations, the physiological and pharmacological relevance of the low-affinity benzodiazepine site is currently unknown, and, consequently, the development of a site-specific antagonist such as Fa173 may assist in addressing this question.

**Conclusions**

In summary, our studies have demonstrated that Fa173 is a selective antagonist that can be used for allosteric modulation of GABA$_A$ receptors. Using a combination of mutational studies and this novel ligand, we showed that flavan-3-ol derivatives are potential ligands for etomidate/loreclezole-related binding sites at GABA$_A$ receptors. Furthermore, the low-affinity potentiation induced by benzodiazepines, perhaps related to their high-dose anesthetic-like effects, can also be explained by a second binding component to this same site.

**Acknowledgements**

This research was supported by a grant from the National Health and Medical Research Council of Australia. NK acknowledges funding from The University of Malakand, Pakistan (Faculty Development Programme Scholarship) and a John Lambert Scholarship The authors are grateful to Dr Paul Whiting for providing GABA$_A$ receptor subunit DNAs.

**Conflict of interest**

The authors state no conflict of interest.

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