The dietary flavonoids apigenin and (−)-epigallocatechin gallate enhance the positive modulation by diazepam of the activation by GABA of recombinant GABA_A receptors

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Abstract

The dietary flavonoids apigenin, genistein and (−)-epigallocatechin gallate (EGCG) inhibited the activation by GABA (40 μM) of recombinant human α1β2γ2L GABA_A receptors expressed in Xenopus laevis oocytes with IC50 values of 8, 30 and 15 μM, respectively. Apigenin and genistein also acted as GABA antagonists at flumazenil-insensitive α1β2 GABA_A receptors, indicating that they were not acting as negative modulators through flumazenil-sensitive benzodiazepine sites on GABA_A receptors. In addition to these GABA_A antagonist effects, a novel second order modulatory action was found for apigenin and EGCG on the first order enhancement of GABA responses by diazepam. Apigenin (1 μM) and EGCG (0.1 μM) enhanced the modulatory action of diazepam (3 μM) on the activation by GABA (5 μM) of recombinant human α1β2γ2L GABA_A receptors by up to 22% and 52%, respectively. This was not found with genistein, nor was it observed with enhancement by allopregnanolone or pentobarbitone.

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1. Introduction

Flavonoids were first linked to GABA_A receptors when three isoflavans isolated from bovine urine were shown to inhibit diazepam binding to brain membranes [1]. They have a range of activities on GABA_A receptors [2], acting as positive and negative modulators of GABA_A receptor function. While many of these actions are sensitive to the benzodiazepine antagonist flumazenil consistent with the ability of flavonoids to inhibit benzodiazepine binding, flumazenil-insensitive actions have also been described indicating that flavonoids can act at sites other than classical benzodiazepine sites on GABA_A receptors. For example, the positive modulatory effects of 6-methylflavone [3] and negative modulatory effects of amentoflavone [4] on recombinant α1β2γ2L GABA_A receptors expressed in oocytes are insensitive to flumazenil. Furthermore, flavonoids are known to inhibit the action of GABA on flumazenil-insensitive ρ1 GABA_C receptors [5]. The present study describes a novel action of two flavonoids, apigenin and (−)-epigallocatechin gallate (EGCG), that enhance the positive modulation of the activation by GABA of recombinant α1β2γ2L GABA_A receptors by diazepam.

Apigenin (5,7,4'-trihydroxyflavone, Fig. 1) is a common flavonoid found in a range of plants, including chamomile. The traditional use of chamomile tea as a treatment for insomnia and anxiety led to investigations of its active constituents. Apigenin was proposed to be a benzodiazepine partial agonist and produced potent anxiolysis (and mild sedation at high doses) without any other benzodiazepine-like effects [6]. However, Avallone et al. [7] found that apigenin fitted the profile of a benzodiazepine inverse agonist and was sedative and mildly pro-convulsant, but not anxiolytic. Dekermendjian et al. [8] reported that apigenin fitted the profile of a benzodiazepine antagonist. Thus, apigenin appears to have a variety of effects at the benzodiazepine site of the GABA_A receptor, but the nature of these effects is unclear.

(+)-Catechin and (−)-epicatechin (Fig. 2) are the most common flavans in nature, appearing in many common
foods [9,10]. (−)-Epigallocatechin gallate (EGCG, Fig. 2) is the most abundant flavan in tea [10] and is unique in that it has not been found elsewhere in the diet [11]. Thus, EGCG is a prime target for pharmacological investigations into the actions of tea. The traditional use of tea as a cognitive stimulant and a digestive aid is consistent with an action as a GABA A antagonist. This is supported by the finding that tea is pro-convulsant [12]. The most likely candidates for the stimulant ingredients of tea are the flavans, especially EGCG. Catechin, epicatechin and (−)-epigallocatechin gallate have previously been reported to be inactive as inhibitors of benzodiazepine binding [13,14]. Based on the structure–activity model developed by Dekermendjian et al. [8] for flavones acting at the benzodiazepine site, catechin, epicatechin and EGCG would be predicted to be inactive on the benzodiazepine site on GABA A receptor, as all three lack the 4-carbonyl group, possess a 7-hydroxy group and are non-planar. In addition, EGCG also possesses a large group at the 3 position (the gallate group), a site of steric hindrance in this model. However, this gallate group contains a carbonyl group, which may be able to substitute for the 4-carbonyl of flavones. EGCG has been shown to inhibit the activation of recombinant α1β1 GABA A receptors expressed in oocytes [15]; classical flumazenil-sensitive sites are not found on these receptors.

The aim of the present study was to examine the effects of apigenin and EGCG, together with some related flavonoids, on the action of GABA on recombinant GABA A receptors and the enhancement of this action by a benzodiazepine (diazepam) and a steroid (allopregnanolone).

2. Materials and methods

2.1. Materials

(−)-Epigallocatechin gallate, catechin, genistein, daidzein, pentobarbitone sodium and GABA were obtained from Sigma (St. Louis, MO, USA), epicatechin was obtained from ICN (Seven Hills, NSW, Australia), apigenin was obtained from Fluka (Castle Hill, NSW, Australia), 5α-pregnan-3α-ol-20-one (allopregnanolone) was provided by Dr. Peter Burden (Department of Pharmacol-
ology. The University of Sydney, NSW, Australia), diazepam was obtained as a solution for injection (5 mg/mL) from David Bull Laboratories (Mulgrave North, Vic., Australia) and dimethyl sulfoxide (DMSO) was obtained from APS Finechem (Seven Hills, NSW, Australia). Flumazenil was a gift from Roche (Basle).

2.2. Methods

Xenopus laevis were anaesthetised with 0.17% ethyl 3-aminobenzoate and a lobe of an ovary was removed and rinsed with oocyte releasing buffer, OR2 (82.5 mM NaCl, 2 mM KCl, 1 mM MgCl2·6H2O, 5 mM HEPES, pH 7.5). It was then treated with Collagenase A (2 mg/ml of OR2, Bohringer Manheim) for 2 h. The released oocytes were rinsed in modified frog Ringer solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl2·6H2O, 1.8 mM CaCl2, 5 mM HEPES, 2.5 mM pyruvate, 0.5 mM theophylline, 50 ng/ml gentamycin, pH 7.5). Stage V–VI oocytes were collected and stored in this buffer.

Human α1, β2 and γ2L cDNA in pcDM8 was provided by Dr. Paul Whiting (Merck, Sharpe and Dohme Research Laboratories, Harlow, UK). Plasmids containing the α1, β2 or γ2L cDNA were linearised using NOT1 enzyme. cRNA was synthesised using the ‘mMessage mMACHINE’ kit from Ambion (Austin, TX, USA). cRNA was mixed in a ratio of 1 α1:1 β2:1 γ2L (or 1 α1:1 β2) and injected into defolliculated oocytes at a concentration of 50 ng/50 nl. Oocytes were stored for 3–9 days at 16 °C, after which receptor activity was measured by two electrode voltage clamp recording using a Geneclamp 500 amplifier (Axon Instruments, Foster City, CA, USA), a MacLab 2e recorder (AD Instruments, Sydney, NSW, Australia) and Chart version 3.5.2 program. Oocytes were voltage clamped at −60 mV and continuously superfused with frog Ringer solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl2·6H2O, 1.8 mM CaCl2, 5 mM HEPES). For receptor activation measurements, the indicated concentrations of drug were dissolved in a total of 200 µL of DMSO and added to 25 mL of the buffer solution.

2.3. Data analysis

The response was taken to be the maximum change in current during drug application. Data were expressed as a proportion of the response to which they were being compared (usually the response to 40 µM GABA). In the case of the effects of flavonoids on modulators, the following formula was used: $Y = \frac{(X - G)/(M - G)}{1 + 10^{(\log EC_{50} - X)/\text{hillslope}}}$, where $X$ is the log of concentration; $Y$ is the response; $Y$ starts at bottom and goes to top with a sigmoid shape. This is identical to the “four parameter logistic equation”.

Logarithmically transformed data for dose response curves were also analysed using a linear fit. $F$ and $P$ values cited in this paper are derived from the results of this fit, except for those comparing two curves, in which case the $F$ and $P$ values are derived from a two-way ANOVA analysis. The percent inhibition and percent enhancement values were calculated using the minimum and maximum values obtained from the sigmoidal fit (variable slope) equation, except where otherwise stated. The effects of flumazenil on apigenin, genistein and diazepam were analysed using a repeated measures ANOVA with Bonferroni’s post hoc test.

3. Results

3.1. Apigenin

Apigenin (0.1, 1, 10, 100 µM) produced no effect when tested on sham-injected oocytes or on oocytes expressing functional α1β2γ2L GABA receptors. Nor did apigenin (0.1, 0.3, 1, 3, 10, 100 µM) significantly alter responses to 5 µM GABA at α1β2γ2L GABA receptors. It did significantly inhibit responses to 40 µM GABA at α1β2γ2L GABA receptors ($n = 6; F = 51.9, P < 0.001$). This inhibition reached 49.6%, with an IC50 of 8 µM (95% CI: 2.5–25.1 µM) and Hill coefficient of −1.2 ± 0.5 (Fig. 3).

Apigenin (30 µM) significantly inhibited the GABA dose–response curve at α1β2γ2L GABA receptors ($n = 5; P < 0.0001; \text{Fig. 4}$). There was a significant interaction.
between the effects of GABA and the effects of apigenin \((P < 0.0001)\). This is due to a preferential inhibition of higher doses of GABA. Apigenin reduced the maximal GABA-induced current by 50%.

Low doses of apigenin (Figs. 5 and 6) significantly enhanced the positive modulation of responses to 5 µM GABA by 3 µM diazepam by up to 22.5% \((n = 3–7; \text{Fig. 5})\). Significant differences from control were observed at 1, 3 and 6 µM apigenin \((t\text{-test } P < 0.006, 0.001 \text{ and } 0.12, \text{ respectively})\). Flumazenil (1 µM) significantly inhibited the enhancement of 5 µM GABA responses by 3 µM diazepam at \(\alpha_1\beta_2\gamma_2\) GABA\(_A\) receptors (mean difference in responses; 88.7%, 95% CI: 74.3–103%, \(n = 5, t = 20.6, P < 0.001\)). These conditions represented the maximal flumazenil-sensitive enhancement of 5 µM GABA responses by 3 µM diazepam. Thus, the 22.5% enhancement observed for these low doses of apigenin represented a significant increase on the maximum flumazenil-sensitive enhancement of the GABA responses by diazepam.

At higher doses of apigenin, the inhibitory effects that were seen in the absence of diazepam became apparent (Fig. 5). Inhibition of diazepam enhancement reached a maximum of 55%, with an approximate IC\(_{50}\) of 10 µM. Due to the biphasic response, this IC\(_{50}\) is likely to be an overestimate. Flumazenil (1 µM) failed to significantly alter the effect of 100 µM apigenin on 40 µM GABA responses (mean difference in responses: –4.9%, 95% CI: –19.2 to 9.5%, \(n = 5, t = 1.13, P > 0.05\)).

Apigenin significantly inhibited the response to 5 µM GABA at flumazenil-insensitive \(\alpha_1\beta_2\) GABA\(_A\) receptors \((n = 3; F = 39.58, P < 0.0001)\) with an IC\(_{50}\) of 11.7 µM (95% CI: 2.8–49.6 µM). This inhibition reached 60.3%.

Enhancement of 5 µM GABA responses by 100 nM allopregnanolone was significantly inhibited by 10 µM or higher doses of apigenin (Fig. 7) at \(\alpha_1\beta_2\gamma_2\) GABA\(_A\) receptors \((n = 7; F = 26.8, P < 0.01 \text{ and } n = 5; F = 26.15, P < 0.01, \text{ respectively})\). Inhibition of allopregnanolone
enhancement by apigenin reached 78% with an IC$_{50}$ of 8.4 µM (95% CI: 4.9–14.4 µM) and a Hill coefficient of −1.8 ± 0.5. Enhancement of 5 µM GABA responses by 30 µM pentobarbitone was similarly inhibited by apigenin with over 90% inhibition and an IC$_{50}$ of 22.9 µM (95% CI: 10.5–50 µM) and a Hill coefficient of −2.5 ± 0.5, n = 8. No enhancement of either allopregnanolone or pentobarbitone modulation of GABA responses was seen with apigenin.

3.2. EGCG

EGCG (100 µM) had no effect when tested on sham-injected oocytes. It did significantly inhibit responses to 40 µM GABA at α1β2γ2L GABA$_A$ receptors (Fig. 3; n = 4; F = 59.3, P < 0.0003) and 20 µM (n = 4; F = 57.11; P = 0.0003) GABA. Inhibition reached 90.6% and 97% with IC$_{50}$ values of 14.7 µM (95% CI: 5.03–43.1 µM) and 8.7 µM (95% CI: 3.8–20 µM) and Hill coefficients of −0.95 ± 0.31 and −1.19 ± 0.39, respectively.

EGCG (100 µM) significantly inhibited the GABA dose–response curve at α1β2γ2L receptors (n = 4; F = 307.7; P < 0.0001), increasing the GABA EC$_{50}$ by 573% and decreasing the maximum by 70% (Fig. 8). A two-way ANOVA also found a significant interaction effect (F = 39.69; P < 0.0001). This interaction is due to a preferential inhibition of higher doses of GABA.

EGCG significantly inhibited both diazepam (3 µM) and allopregnanolone (100 nM) enhancement of GABA (5 µM) currents at α1β2γ2L GABA$_A$ receptors (n = 7; F = 48.4; P < 0.0001; Fig. 6 and n = 4; F = 69.3; P < 0.0002; Fig. 7, respectively). Inhibition of steroid reached 167% (which is below the response due to 5 µM GABA alone) with an IC$_{50}$ of 5.7 µM (95% CI: 4.3–7.5 µM) and inhibition of diazepam reached 182% (which is also below the response due to 5 µM GABA alone) with an IC$_{50}$ of 7 µM (95% CI: 2.3–21.6 µM).

As with apigenin, lower concentrations of EGCG enhanced the effects of diazepam (Fig. 6). This enhancement reached 52%. At 0.1 µM EGCG the response was significantly different to control at P < 0.019 (t-test). Due to the biphasic response, the calculated IC$_{50}$ of 7 µM for the inhibitory phase is probably an overestimate.

Enhancement of 5 µM GABA responses by 30 µM pentobarbitone was inhibited by EGCG with over 160% inhibition and an IC$_{50}$ of 22.1 µM (95% CI: 4.8–101 µM) and a Hill coefficient of −0.86 ± 0.5, n = 8. No enhancement of either allopregnanolone or pentobarbitone modulation of GABA responses was seen with EGCG.

3.3. Catechin and epicatechin

Neither catechin (n = 4; F = 3.24; P = 0.13) nor epicatechin (n = 5; 1.49; P = 0.28) were able to significantly inhibit currents due to 40 µM GABA at α1β2γ2L GABA$_A$ receptors at concentrations ranging from 1 to 100 µM (Fig. 3) and thus were not tested further.

3.4. Genistein

Genistein (100 µM) had no effect when tested on sham-injected oocytes. It did significantly inhibit responses to 40 µM GABA at α1β2γ2L GABA$_A$ receptors (n = 4; F = 12.3; P = 0.017). This inhibition reached 51% with an IC$_{50}$ of 29.2 µM (95% CI: 23.4–36.4 µM) and Hill coefficient of −5.3 ± 6.2 (Fig. 3) and was not altered by 1 µM flumazenil (mean difference in responses: −3.44%, n = 4, t = 0.15, P > 0.05). Furthermore, genistein significantly inhibited the response to 5 µM GABA at α1β2 receptors (n = 4; F = 6.9, P < 0.016) with an IC$_{50}$ of 11.7 µM. This inhibition reached 40%.

Genistein (100 µM) significantly inhibited the GABA dose–response curve at α1β2γ2L GABA$_A$ receptors (Fig. 9, n = 5; P < 0.003). Neither the EC$_{50}$ nor the Hill coefficient of GABA was significantly affected, while the maximal GABA-induced current was reduced by 28.3%.

Genistein significantly inhibited diazepam (3 µM) and allopregnanolone (100 nM) enhancement of GABA
(5 μM) currents at α1β2γ2L GABA<sub>A</sub> receptors (n = 6, F = 31.28, P < 0.0001, Fig. 6; n = 5, F = 9.845, P < 0.026, Fig. 7, respectively). Inhibition of diazepam by genistein showed an IC<sub>50</sub> of 52 μM (95% CI: 37.7–72.4 μM) and Hill coefficient of −1.83 ± 0.58. Inhibition of allopregnanolone reached 97% with an IC<sub>50</sub> of 83 μM (95% CI: 45.6–152.2 μM) and Hill coefficient of −1.7 ± 0.35 (Fig. 7). Unlike apigenin and EGCG, low doses of genistein did not enhance the effects of diazepam (Fig. 6).

4. Discussion

Apigenin is not a GABA, benzodiazepine or steroid agonist at α1β2γ2L GABA<sub>A</sub> receptors expressed in Xenopus laevis oocytes as it did not enhance the effects of a low dose of GABA or produce a response when applied by itself. Nor did it have any effect on sham-injected oocytes and, thus, apigenin is not an agonist at any of the receptors native to the oocyte. However, apigenin at high doses did inhibit the effects of a higher dose of GABA. This effect is most clearly seen by the effects of 30 μM apigenin on the GABA dose–response curve. The blunting of the response to supramaximal doses of GABA (1000 μM) and the change in the Hill coefficient both indicate that apigenin is a non-competitive antagonist of GABA<sub>A</sub> receptors. The inhibitory effect of apigenin was not blocked by the benzodiazepine antagonist flumazenil; furthermore, apigenin was a GABA antagonist at flumazenil-insensitive α1β2 receptors. These findings are in agreement with those of Goutman et al. [5] who have shown that apigenin is a flumazenil-insensitive GABA antagonist at α1β1γ2S GABA<sub>A</sub> receptors and at p1 GABA<sub>C</sub> receptors. This GABA antagonist action is thus independent of classical benzodiazepine modulatory sites.

Low doses of apigenin enhanced the modulatory action of diazepam. This action was observed under conditions of the maximal flumazenil-sensitive enhancement of the action of 5 μM GABA by diazepam. This action was not seen in any other test, including the apigenin dose–response curves against GABA or against the modulatory action of allopregnanolone. It is unlikely that apigenin mediates the enhancement of the actions of diazepam by enhancing diazepam binding as it is known to inhibit the binding of benzodiazepines to GABA<sub>A</sub> receptors [6–8]. Diazepam itself has a biphasic effect at α1β2γ2L GABA<sub>A</sub> receptors [16] that suggests two sites of action. However, at GABA<sub>A</sub> receptors containing only α1 and β2 subunits, only a high dose effect of diazepam is seen and this effect is not blocked by flumazenil. Thus, it appears that there are at least two sites of action for diazepam on α1β2γ2L GABA<sub>A</sub> receptors—one high affinity site which is sensitive to flumazenil and another low affinity site which is flumazenil-insensitive. Apigenin showed no enhancement of the actions of GABA alone or in the presence of pregnanolone or pentobarbitone in the present study, nor did it effect the binding of the GABA<sub>A</sub> agonist muscimol [6]; hence, it is unlikely that the enhancement is due to an enhancement of either the binding of GABA or of its effects independent of diazepam.

Apigenin has been characterised as a centrally acting benzodiazepine ligand with anxiolytic effects [6]. Apigenin was described as having ‘a clear anxiolytic effect in mice in the elevated plus maze without evidencing sedation or muscle relaxation effects at doses similar to those used for classical benzodiazepines’ and it was devoid of anticonvulsant effects [6]. These findings are in contrast to a later study in rats where apigenin was shown to reduce the latency of onset of picrotoxin-induced convulsions and to reduce locomotor activity but was devoid of anxiolytic or muscle relaxant activities [7]. This later study showed that apigenin could reduce GABA-activated chloride currents in cultured cerebellar granule cells, an action that could be blocked by flumazenil and thus likely to involve a classical negative modulatory (‘inverse agonist’) action on GABA<sub>A</sub> receptors; this was not observed in the present study. The inhibitory action of apigenin on locomotor behaviour, however, could not be blocked by flumazenil and thus could not ‘be ascribed to an interaction with GABA<sub>A</sub>-benzodiazepine receptors’ [7]. Another study from the same group reported that apigenin exerted sedative effects on locomotor activity in rats in a flumazenil-insensitive manner, whereas chrysin, a structurally related flavonoid lacking the 4'-hydroxy substituent of apigenin, showed a clear flumazenil-sensitive anxiolytic effect in addition to the flumazenil-insensitive sedation [17]. The apparent discrepancy between the behavioural effects of apigenin on mice [6] and rats [7] may be due to mice having higher baseline levels of anxiety.

The flumazenil-sensitive effects of apigenin following systemic administration to rodents could be interpreted on the basis of apigenin potentiating the action of endogenous benzodiazepine-like agents, endozepines [18,19]. Evidence for physiologically relevant endozepines has come from the discovery of a mutant GABA<sub>A</sub> receptor in childhood absence epilepsy and febrile seizures that has diminished sensitivity to benzodiazepines with no other apparent alteration in functioning [20]. Overall, it seems that the effects of apigenin on GABA<sub>A</sub> receptors are complex and that the behavioural effects of apigenin may involve both flumazenil-sensitive and flumazenil-insensitive components.

EGCG also shares the bimodal action of apigenin against diazepam, enhancing at low flavonoid concentrations and inhibiting at high concentrations. In both the enhancement and inhibition phases, EGCG was more potent than apigenin. EGCG at low doses inhibited the response to 40 μM GABA and the enhancement of responses to 5 μM GABA by 100 nM allopregnanolone, 30 μM pentobarbitone and by 3 μM diazepam. In all these tests, EGCG was more potent than apigenin, producing a greater degree of inhibition. This supports the hypothesis
that apigenin and EGCG are acting via the same site(s), at which EGCG has either greater affinity or efficacy.

Neither catechin nor its isomer, epicatechin, inhibited the response to 40 μM GABA. These differ from EGCG in two key features—they lack an hydroxy group at the 5’ position and they have an hydroxy group at the 3 position rather than the 3-O-gallate group. Assuming that apigenin and EGCG are acting via the same mechanism, the lack of an hydroxy group at the 5’ position can be ruled out as the cause of the loss of effect as apigenin also lacks this group. Thus, it is most probably the different group at the 3 position that renders catechin and epicatechin inactive. The 3-O-gallate group of EGCG contains a carbonyl group which may substitute for the 4-carbonyl group of apigenin.

Genistein, the isoflavone equivalent of apigenin, is a phytoestrogen with a wide variety of pharmacological effects on animal cells [21]. Its action as a GABA<sub>A</sub> receptor antagonist has been observed by other workers. While it is widely used as a tyrosine kinase inhibitor, its action as a negative modulator of the action of GABA on recombinant GABA<sub>A</sub> receptors is the result of a direct action on the receptors and is independent of tyrosine kinase [22,23]. Genistein did not enhance the actions of diazepam at any of the concentrations tested.

The second order modulation by apigenin and EGCG of the maximum first order modulatory action of diazepam of the activation by GABA of GABA<sub>A</sub> receptors observed in the present studies may result from these flavonoids altering the coupling of the benzodiazepine allosteric sites with the orthosteric GABA sites on GABA<sub>A</sub> receptors. There is evidence from binding studies that the nexus between the benzodiazepine and GABA sites on GABA<sub>A</sub> receptors is complex and involves other factors, such as phospholipids, that can be removed from brain membranes by detergent extraction [24]. Apigenin and EGCG may influence the interactions of these other factors with the benzodiazepine and GABA sites.

There have been extensive structure–activity studies aimed at developing models of flavonoid pharmacophores for their interaction with GABA<sub>A</sub> receptors [8,25–28]. A problem common to most of these studies is that the activity data is based on ligand binding studies to what is now known to be a mixture of benzodiazepine binding sites and GABA receptor subtypes. Given our increased knowledge of the diversity of benzodiazepine and flavonoid actions on cloned receptors of defined subunit composition, future structure–activity studies need to be based on data from functional studies on GABA<sub>A</sub> receptors of known subunit composition. It would be surprising if the novel second order modulatory action of apigenin and EGCG did not show some GABA<sub>A</sub> receptor subtype selectivity.

Fruits, vegetables, and beverages such as tea and red wine are major sources of flavonoids in our diet [29]. It has been estimated that the average daily intake of flavonoids from a healthy diet is 1–2 g [30]. They have a wide variety of biological activities and are being studied especially as anticancer agents [31]. As both apigenin and EGCG have been widely consumed in the diet for generations, it is unlikely that they would have side effects severe enough to prevent their possible use as therapeutic agents alone or in combination with other agents, e.g. in reducing the therapeutic dose of diazepam. Apigenin clearly has CNS effects on systemic administration [6,7] and it is known that EGCG accumulates in the brain following systemic administration [32]. Furthermore, apigenin and EGCG serve as lead compounds for the development of more selective agents for the second order modulation of benzodiazepine enhancement of the action of GABA on GABA<sub>A</sub> receptors. The second order modulation of a primary modulator is unlikely to be restricted to the modulation of GABA<sub>A</sub> receptors.

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