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REVIEW

Flavonoid modulation of $GABA_A$ receptors

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There has been a resurgence of interest in synthetic and plant-derived flavonoids as modulators of γ-amino butyric acid-A ($GABA_A$) receptor function influencing inhibition mediated by the major inhibitory neurotransmitter GABA in the brain. Areas of interest include (i) flavonoids that show subtype selectivity in recombinant receptor studies *in vitro* consistent with their behavioural effects *in vivo*, (ii) flumazenil-insensitive modulation of $GABA_A$ receptor function by flavonoids, (iii) the ability of some flavonoids to act as second-order modulators of first-order modulation by benzodiazepines and (iv) the identification of the different sites of action of flavonoids on $GABA_A$ receptor complexes. An emerging area of interest is the activation of $GABA_A$ receptors by flavonoids in the absence of GABA. The relatively rigid shape of flavonoids means that they are useful scaffolds for the design of new therapeutic agents. Like steroids, flavonoids have wide-ranging effects on numerous biological targets. The challenge is to understand the structural determinants of flavonoid effects on particular targets and to develop agents specific for these targets.

Abbreviations

BBB, blood–brain barrier; CoMFA, comparative molecular field analysis; CoMSIA, comparative molecular similarity indices analysis; EGCG, epigallocatechin gallate; Fa131, *trans*-(2*R*,3*S*)-*3*-acetoxy-4′-methoxyflavan; GABA, γ-amino butyric acid; $GABA_A$, γ-amino butyric acid-A receptor; HEK, human embryonic kidney; HQSAR, hologram quantitative structure activity relationship; i.p., intraperitoneal injection; TM, transmembrane domain; TPA023, 7-(1,1-Dimethylethyl)-6-(2-ethyl-2H-1,2,4-triazol-3-ylmethoxy)-3-(2-fluorophenyl)-1,2,4-triazolo[4,3-b]pyridazine

Introduction

Flavonoids are ubiquitous in plants and as a result constitute the most common group of polyphenolic compounds in the human diet. It is estimated that human daily consumption of flavonoids ranges from tens of milligrams to over one gram. Dietary flavonoids are predominantly derived from fruits, vegetables, chocolate and beverages such as tea, coffee and red wine as well as herbal preparations. In particular, fruits vegetables, cereals, green tea and coca are rich sources of flavonoids (Manach *et al.*, 2004). Although the absorption and distribution of flavonoids is not well understood, and their bioavailability is a topic of considerable debate (Manach *et al.*, 2004), the lipophilicity of some flavonoids allows them to cross the blood–brain barrier (BBB) (Youdim *et al.*, 2004), and thus, it is likely that diet-derived flavonoids are found in brain.

Flavonoids have been the focus of intense research, displaying many interesting biological activities (Cushnie and Lamb, 2005; Clarke and Wiseman, 2008; Andres *et al.*, 2009; Rathee *et al.*, 2009), and are believed to play a significant role in reducing the risks of age- and lifestyle-related diseases such as cancer (Kale *et al.*, 2008; García-Lafuente *et al.*, 2009), diabetes (Cazarolli *et al.*, 2008), cardiovascular disease (García-Lafuente *et al.*, 2009) and declining neurological function (Spencer, 2009; Spencer *et al.*, 2009). Many flavonoids have well-established antioxidant and free-radical-scavenging activities, and it was initially believed that their protective effects were as a direct result of these actions. However, it is now accepted that in addition to their ability to prevent damage caused by oxidative stress, flavonoids also exert their biological effects through direct actions on enzymes, receptors and signalling pathways (Williams *et al.*, 2004). More recently, the actions of flavonoids on the central nervous system have attracted much attention. Flavonoids are believed to prevent neurodegeneration associated with Parkinson's and Alzheimer's disease and improve cognitive function (Spencer, 2009; Spencer *et al.*, 2009). Additionally, flavonoids have demonstrated anxiolytic, sedative and

anticonvulsant activities. Although their actions in the central nervous system occur through a variety of interactions with different receptors and signalling pathways, it is believed that some of these effects are mediated by ionotropic GABA, in particular $GABA_A$ receptors.

This has led to a resurgence of interest in flavonoids as modulators of $GABA_A$ receptor function influencing inhibition mediated by the major inhibitory neurotransmitter GABA in the brain. Areas of interest include (i) flavonoids that show subtype selectivity in recombinant receptor studies *in vitro* consistent with their behavioural effects *in vivo*, (ii) flumazenil-insensitive modulation of $GABA_A$ receptor function by flavonoids, (iii) the ability of some flavonoids to act as second-order modulators of first-order modulation by benzodiazepines and (iv) the identification of the site(s) of action of flavonoids on $GABA_A$ receptor complexes.

$GABA_A$ receptors

$GABA_A$ receptors are the most important inhibitory receptors in the central nervous system (Chebib and Johnston, 2000; Whiting, 2003; Johnston, 2005; Carter *et al.*, 2010). They are members of the cys-loop superfamily of ligand-gated ion channels (LGICs) that encompasses both cationic [nicotinic acetylcholine and 5-hydroxytryptamine family 3 ($5\text{-}HT_3$)] and anion ($GABA_A$, $GABA_C$ and glycine) receptors. These channels are membrane bound, structurally similar and considered to be composed of pentamers formed from distinct subunit combinations. Each subunit has a large N-terminal domain that encompasses the ligand binding domain and the cys-loop motif. The cys-loop consists of two disulphide bond–forming cysteines separated by 13 amino acid residues, several of which are highly conserved to form a signature sequence. There are four transmembrane domains termed TM1 to TM4 that traverses the membrane, and the TM2 lines the channel lumen. A large intracellular loop exists that contains sites for phosphorylation, anchoring and channel clustering. The GABA agonist binding site (the orthosteric site) is located at the interface between β-α subunits and is formed from loops that occur in the N-terminal region of the subunit (reviewed in Akabas, 2004).

Several classes of subunits with multiple isoforms have been identified by sequence homology (including α1–α6, β1–β3, γ1–γ3, δ) (Alexander *et al.*, 2009). These subunits can 'mix and match' to form receptor subtypes. Given the large number of subunits and possible combinations, the total number of potential receptor subtypes would be huge. However, only 10 distinct subunit combinations have been conclusively identified to be physiologically relevant with the majority of $GABA_A$ receptors in the brain formed from two α-, two β- and one single γ- or δ-subunit (Whiting, 2003).

Despite great understanding of how GABA binds to these receptors, the complex pharmacology of $GABA_A$ receptors is not well characterized due to the diversity of subunit combinations and a lack of subtype selective agents. In general, $GABA_A$ receptors are activated by GABA and selectively blocked by the alkaloid bicuculline. Of the agonists known, most are not selective for any particular subtype of the $GABA_A$ receptor. However, $GABA_A$ receptors incorporate a large number of allosteric modulatory sites, agents known to modulate $GABA_A$ receptors, including benzodiazepines, barbiturates, neurosteroids, general anaesthetics such as etomidate, and propofol, ethanol, the sedative and anticonvulsant loreclezole and flavonoids, amongst others.

Modulation of $GABA_A$ receptors

Since their discovery in the mid-1970s, benzodiazepines have been some of the most widely prescribed drugs. They have a range of wanted (including anticonvulsant, sedative–hypnotic and anxiolytic) and unwanted (including sedative, memory impairment and physical dependence) effects. These agents have no direct action on mammalian $GABA_A$ receptors but modulate (either positively or negatively) the action of GABA via an allosteric site.

The effects of benzodiazepines on $GABA_A$ receptors are complex and dependent on receptor subunit composition. In general, the γ2-subunit is required for the most widely observed effects of benzodiazepines on native $GABA_A$ receptors (Puia *et al.*, 1991; Wafford *et al.*, 1993), with the α-subunit determining benzodiazepine sensitivity. Benzodiazepine agonists such as diazepam and flunitrazepam enhance the action of GABA with high affinity (nM activity) for $GABA_A$ receptors consisting of α1-3,5 such as α1βγ2, α2/3βγ2 and α5βγ2 receptors. In contrast, receptors containing α4- or α6-subunits are insensitive to diazepam (Wieland *et al.*, 1992).

Walters *et al.* showed that benzodiazepines can act on $GABA_A$ receptors via 'two distinct and separable mechanisms' (Walters *et al.*, 2000). At nanomolar concentrations, benzodiazepines act in a classic flumazenil-sensitive manner to enhance the action of GABA, while at micromolar concentrations, benzodiazepines act in a flumazenil-insensitive manner. They showed that specific mutations in the second membrane spanning domains of α-, β- and γ-subunits abolished the micromolar but not the nanomolar component. Furthermore, the biphasic action of benzodiazepines apparent in recombinant α1β2γ2S $GABA_A$ receptors was absent in receptors consisting of α1β2 subunits, with these receptors exhibit only the micromolar component. Given that plasma levels of benzodiazepines can reach micromolar concentrations (Bond *et al.*, 1977) and there is evidence for the occurrence of $GABA_A$ receptors consisting of only α- and β-subunits (Olsen and Sieghart, 2009), especially during epilepsy, hormone treatment, ethanol intake, or during development, the flumazenil-insensitive, low-affinity benzodiazepine site may have pharmacological relevance.

Recently the use of transgenic mice (knock-out and knock-in) has provided new knowledge towards our understanding of the physiological role of the various α-subunit containing receptors (Rudolph and Mohler, 2004; Atack, 2005; Whiting, 2006). Comparison of drug-induced behavioural responses in the mutated and wild-type mice has allowed the identification of diazepam effects that were missing, or reduced, in the mutant mice. With this approach, it was demonstrated that the α1-subunits mediate sedation and serve as targets for sedative–hypnotics, while α2- and/or α3-containing receptors mediate anxiolysis and α5-containing receptors are related to memory (Rudolph and Mohler, 2006).

Many intravenous and volatile anaesthetics potentiate the action of GABA at clinically relevant concentrations. At least two binding sites exist: a high-affinity site whereby the action of GABA is potentiated by low micromolar concentrations of anaesthetic and a low-affinity site whereby high micromolar concentrations of anaesthetics directly activate the receptor. Recombinant $GABA_A$ receptor studies, chimeric and mutagenesis studies evaluating anaesthetic activity along with transgenic mice studies have identified sites within the β- and α-subunits to be the target for a number of anaesthetics (reviewed in Rudolph and Antkowiak, 2004).

The site of action for general anaesthetics is complex and differs for volatile versus intravenous anaesthetics (reviewed in Franks, 2008). Irrespective of the type of anaesthetic, the general consensus for the binding site is within the TM2 and TM3 domains of the receptor. Specifically, mutation of sites on the α-subunit reduce or eliminate the effects of volatile anaesthetics (Krasowski *et al.*, 1997; Mihic *et al.*, 1997; Jenkins *et al.*, 2001) but not the effects of intravenous agents (Krasowski *et al.*, 1998; Schofield and Harrison, 2005). In contrast, sites on the β-subunit (Belelli *et al.*, 1997; Mihic *et al.*, 1997) are critical for allosteric modulation of $GABA_A$ receptors by intravenous anaesthetics such as etomidate, propofol, enflurane, isoflurane and other modulators such as loreclezole and ethanol (reviewed in Franks, 2008). Studies using knock-in transgenic mice (Drexler *et al.*, 2009) and a photoreactive analogue of etomidate, [^{3}H]azietomidate (Li *et al.*, 2010) suggest that general anaesthetics do not necessarily bind to a common binding site but may bind to an overlapping binding site or bind to separate sites but use a common transduction mechanism.

Barbiturates exert anxiolytic and hypnotic actions, and although most are no longer used clinically, pentobarbitone is still used as an anaesthetic and phenobarbitone as an anticonvulsant. Like general anaesthetics, the pharmacology of barbiturates is complex. Pentobarbitone for example has several actions: low micromolar concentrations potentiate GABA, high micromolar concentrations directly activate $GABA_A$ receptors while millimolar concentrations inhibit the receptor (Thompson *et al.*, 1996; Drafts and Fisher, 2006). The various effects seen by pentobarbitone indicate several sites may be involved in mediating the various such effects. Both the α- and β-subunits are important in determining the affinity and efficacy of barbiturates, with the α-subunit having more pronounced effects (Hadingham *et al.*, 1993; Thompson *et al.*, 1996).

Neurosteroids, for example allopregnanolone and tetrahydrocorticosterone, are physiologically important allosteric modulators of $GABA_A$ receptors, acting with highest potencies on α5- and δ-subunit containing receptors (Hosie *et al.*, 2007). Neurosteroids have two distinct actions on $GABA_A$ receptors, at nanomolar concentrations such as those experienced during stress they act as positive modulators and at higher (μM) concentrations such as those that occur during pregnancy that directly activate $GABA_A$ receptor. These actions are mediated by two distinct binding sites in the transmembrane region of the receptor. The modulatory site is located in a lipophillic pocket between the highly conserved M1 domain and M4 domain of the α-subunit (Hosie *et al.*, 2009). The binding site for direct activation is at the α–β interface between the M1 domain of the α-subunit and the M2 domain of the β-subunit (Hosie *et al.*, 2006; 2009).

Flavonoids

Flavonoids are characterized by a phenylbenzopyran chemical structure. The basic structure includes a C15 (C6-C3-C6) skeleton consisting of two aromatic rings and an oxygen containing heterocyclic benzopyran ring. The fused aromatic ring is known as the A ring, phenyl constituent as the B ring and the benzopyran ring as the C ring. Flavonoids are subdivided in to eight different classes, defined according to the oxidative status and the number and type of substituents on the heterocyclic ring. These are flavones, flavanones, flavonols, flavanonals, flavan-3-ols (catechins), flavan-3,4-diol, isoflavones and anthocyanins (Figure 1).

The presence of the double bond in the C ring of flavones, flavonols and isoflavones results in the A ring and C ring being planar. The absence of this double bond in flavanones, flavan-3-ols and flavandiols means that the two rings are not planar, the C ring existing in a puckered conformation with the C2 and C3 carbons lying on opposite sides of the plane of the A ring. In addition, the absence of the double bond means these non-planar compounds are chiral at the C2 and/or C3 centres, existing as stereoisomers.

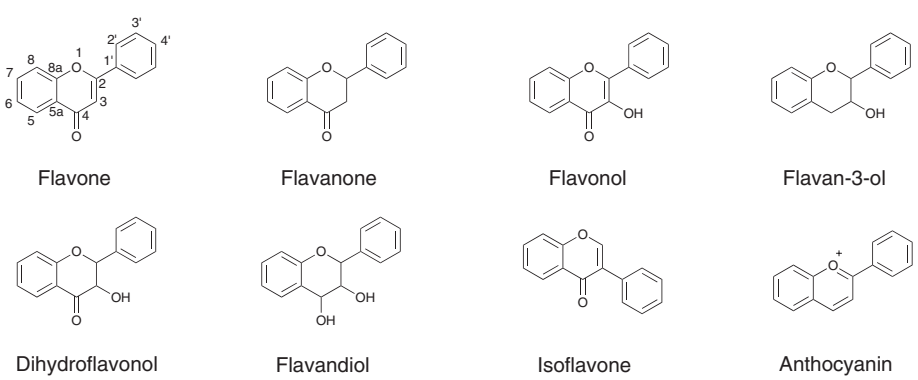

Figure 1
Different classes of flavonoids.

There is an abundance of natural flavonoids, with over 6000 different members of the flavonoid family reported. Extensive modification of the parent structure through hydroxylation accounts for many of these compounds. However flavonoids may also be *C*-prenylated or *O*-methylated. In nature, many flavonoids are also found as *O*- or *C*-glycosides (Bohm, 1998). Flavonoids may also exist in polymeric form with dimers being the most common, through a variety of different C–C and C–O–C linkages. For example, apigenin (4′,5,7-trihydroxyflavone) forms the natural products 6,8″-biapigenin (agathisflavone), 3′8″-biapigenin (amentoflavone), 8,8″-biapigenin (cupressiflavone), 3′6″-biapigenin (robustaflavone) through C–C linkages and 4′,6″-biapigenin (hinokiflavone) through a C–O–C linkage.

Flavonoids as modulators of $GABA_A$ receptors

The search for the elusive endogenous benzodiazepine receptor ligand led to the first report of flavonoids interacting with benzodiazepine receptors. The isoflavones *S*-(–)-equol, 4′-hydroxy-7-methoxyisoflavone and 3′,7-dihydroxyisoflavone (Figure 2) isolated from bovine urine were found to displaced ^{3}[H]-diazepam binding in rat brain. *S*-(–)-Equol was found to be most potent with an IC_{50} of 80 μM but reported to have no significant pharmacological effects *in vivo* (Luk *et al.*, 1983). However, it was the pioneering work of Marder, Medina, Paladini and their coworkers in Argentina during the 1990s drew attention to flavonoids as 'a new family of benzodiazepine receptor ligands' (Medina *et al.*, 1997; Paladini *et al.*, 1999; Marder and Paladini, 2002).

As benzodiazepines were at that time amongst the most widely prescribed pharmaceuticals, a wide range of natural and synthetic flavonoids were investigated *in vitro* and *in vivo* as potential leads for new benzodiazepine ligands. Structure activity studies led to development of synthetic flavonoid ligands with the high affinity for the classical benzodiazepine binding site, including 6-bromoflavone, 6,3′-dinitroflavone, 6-bromo-3′-nitroflavone and 6-chloro-3′-nitroflavone (Figure 3). The relatively rigid shape of flavonoids means that they are useful scaffolds for the design of new therapeutic agents. Flavonoids have been used as scaffolds in the design of a variety of combinatorial libraries for drug discovery (Yao *et al.*, 2007). The use of a combinatorial library of synthetic flavones provided further evidence that substitution at the 6- and/or 3′-positions with electronegative moieties were important determinants of high affinity at benzodiazepine receptors (Marder *et al.*, 1988).

Using GABA ratios determined by the impact that ligand binding has on the GABA binding, it was established that the

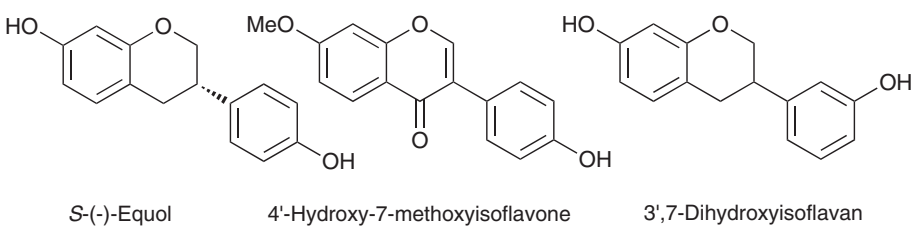

Figure 2
Naturally occurring flavonoids shown to displace diazepam binding.

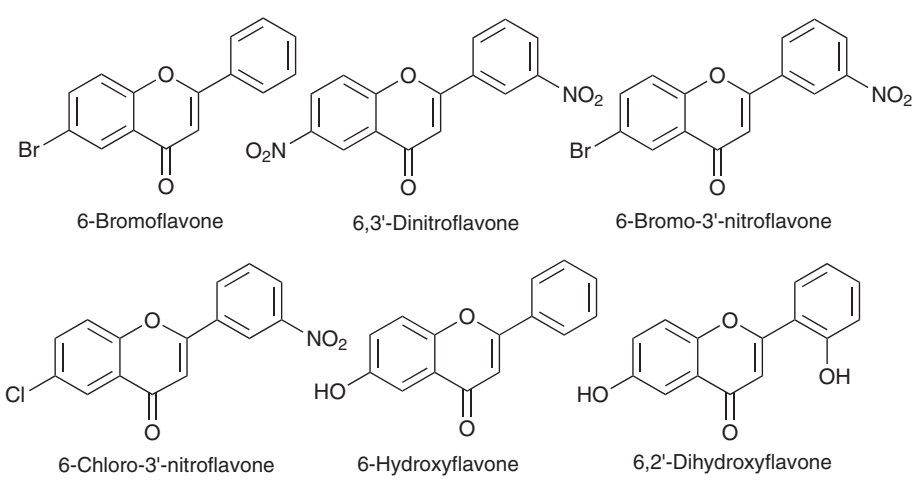

Figure 3
Some synthetic flavonoids that displace benzodiazepine binding.

flavones exhibited the full spectrum of biological activities at benzodiazepine receptors. 6-Bromoflavone with a GABA ratio of 1.6–2.0 was identified as a full agonist (Marder *et al.*, 1996); 6-bromo-3′-nitroflavone with a GABA ratio of 1.38 as a partial agonist (Wolfman *et al.*, 1998); and 6-chloro-3′-nitroflavone with a GABA ratio of 2.0 as an antagonist (Viola *et al.*, 2000). One study found 6-bromo-3′-nitroflavone was a potent anxiolytic in the elevated plus maze (Marder *et al.*, 2001), and that this effect could be antagonized by flumazenil (Griebel *et al.*, 1999). However, anticonflict activity was not antagonized by flumazenil, and 6-bromo-3′-nitroflavone was found to lack anticonvulsant activity but failed to substitute of these compounds for the stimulus produced by chlordiazepoxide (Griebel *et al.*, 1999). Although these results suggest that 6-bromo-3′-nitroflavone may affect anxiolysis via a mechanism other than through the benzodiazepine receptor, it is possible that the effects of 6-bromo-3′-nitroflavone are subtype selective.

Recent studies have identified 6-hydroxyflavone (Figure 3) as a subtype selective partial positive allosteric modulator at the flumazenil-sensitive benzodiazepine site. 6-Hydroxyflavone displayed significant preference for α2- and α3- compared with α1- and α5-containing receptors expressed in HEK 293T cells. *In vivo* 6-hydroxyflavone exhibited anxiolytic effects in the elevated plus-maze test, with no sedation, cognitive impairment, myorelaxation, motor incoordination or anticonvulsant effects at anxiolytic doses (Ren *et al.*, 2010).

In functional electrophysiological studies, 6,2′-dihydroxyflavone (Figure 3) was characterized as a flumazenil-sensitive partial inverse agonist or negative modulator at α1β3γ2, α2β3γ2, and α5β3γ2, but not α3β3γ2 receptors, demonstrating subtype selectivity. As expected for an inverse agonist 6,2′-dihydroxyflavone elicited anxiogenic effects in the elevated plus-maze test and enhanced cognitive performance in the step-through passive avoidance test. Surprisingly, no proconvulsant effects were noted, and this was attributed to the fact that it may be α3β3γ2 receptors that are responsible for convulsant/anticonvulsant activity (Wang *et al.*, 2007).

Wogonin (Figure 4), a flavone found in *Scutellaria baicalensis* Georgi, an important medicinal herb used in traditional Chinese medicine, has been reported to elicit anxiolysis in mice when administered orally (7.5 to 30 mg·kg^{-1}) (Hui *et al.*, 2002) and to significantly block convulsions induced by pentylenetetrazole and electroshock but not strychnine when administered i.p. (10 mg·kg^{-1}) (Park *et al.*, 2007). No sedative or myorelaxation effects were observed, and both the anxiolytic and anticonvulsant actions could be blocked by co-administration of flumazenil. Another flavonoid isolated from *S. baicalensis* Georgi, oroxylin A, identified as an antagonist at the benzodiazepine site, selectively abolished the anxiolytic, myorelaxant and motor incoordination, but not the sedative and anticonvulsant effects of diazepam (Huen *et al.*, 2003b). These reports suggest that both compounds act with some subtype selectivity; however, further studies using specific recombinant receptors are needed to verify this.

Using the abundant biological data afforded by the radioligand binding studies, a number of quantitative structure activity studies have been carried out to define the structural

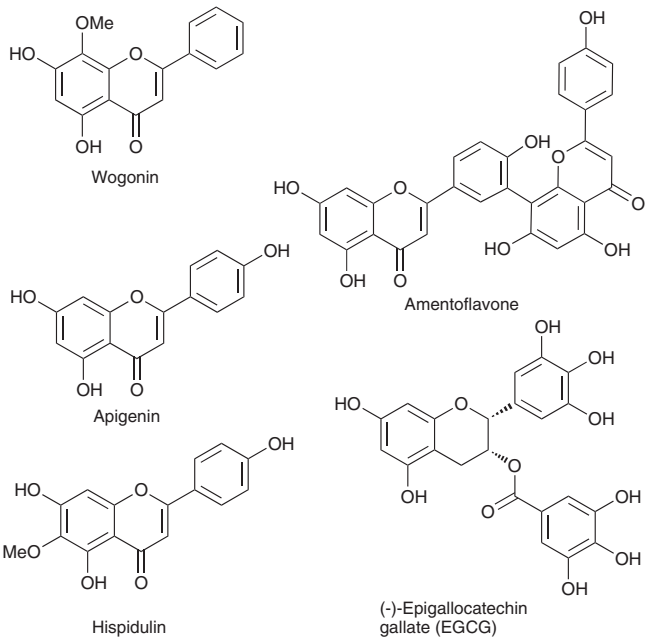

Figure 4

Naturally occurring flavonoids that influence the activation of $GABA_A$ receptors. $GABA_A$, γ-amino butyric acid-A.

requirements for high-affinity binding of flavonoid ligands at the benzodiazepine binding site. Incorporation of flavonoids (flavones) into the unified pharmacophore model of Cook (Zhang *et al.*, 1995) using the non-benzodiazepine high-affinity partial agonist CGS-9896 as a template gave rise to a model in which the flavones lie between the two hydrogen bond-donating sites and with the carbonyl groups of the flavones and benzodiazepines overlap, interacting with a hydrogen bond–donating site (H1) in essentially the same manner. The ether oxygen of the flavones making similar H-bond interactions to the benzodiazepine N1 with a second hydrogen bond–donating site (H2), and the fused aromatic ring of benzodiazepines and the B ring of flavones superimpose. The A-ring of the flavones occupies the lipophilic region (L1) occupied by 5-phenyl substituent of benzodiazepines. However, this model does not explain the fact that 6-methyl-3′-nitroflavone acts as an inverse agonist, but does not interact with a H-bond acceptor site (A2) required in the Cook model for benzodiazepine inverse agonist activity (Dekermendjian *et al.*, 1999).

Further three-dimensional studies using comparative molecular field analysis (CoMFA), comparative molecular similarity indices (CoMSIA) and hologram quantitative structure activity relationship (HQSAR) approaches with flavone as a structural template afforded a similar pharmacophore to Dekermendjian's and proposed specific S1 and S2 subsites relating to electrostatic interaction of substituents in 6-position of flavone (Huang *et al.*, 2001). Refinement of their model by the Danish group using a larger dataset of compounds led to the identification of two addition regions of steric repulsive interactions around the 4′- and 5′-regions of the B-ring (Kahnberg *et al.*, 2002). Using benzodiazepine itself as a template, Marder *et al.* (2001) proposed an

alternative model for the interaction of flavones with the benzodiazepine-binding site. In this model, the carbonyl group of the flavones interacts only with a bifunctional H2/A3 site, and the B-ring is positioned close to the lipophilic region L2. The A-ring in the flavones superimposes the 5-phenyl ring in diazepam; there are no interactions between the flavones and the H1 site and no possibility for interactions with A2. This model does not account for the increased affinity of compounds with a 2′-OH group (Huen *et al.*, 2003a) or the strong decrease in affinity due to a 4′-methyl substituent.

Although these models can predict the binding affinity of the synthetic flavones, none of these studies have accounted for the different pharmacological activities reported of flavones. Unlike the Cook model, which qualitatively accounts for the relative affinities, efficacies and functional effects displayed by various ligands at the benzodiazepine receptor (Zhang *et al.*, 1995), the pharmacophores proposed for flavones have only accounted for relative binding affinities of flavones. In addition, none of the studies include the biflavone, amentoflavone (Figure 4), that binds to the benzodiazepine site with an affinity similar to that of diazepam. Kahnberg *et al.* suggest that as it is a biflavone it must bind to other receptor sites and not be compared directly with the flavones used in the pharmacophore studies (Kahnberg *et al.*, 2002). However, given its high affinity for the benzodiazepine binding site, it is likely that it does interact with this site, and a subsequent study postulated that one monomer of the biflavone binds in a manner similar to apigenin (Figure 4), and the increased affinity of amentoflavone is due to the second monomer forming additional binding interactions in the interface between the α- and γ-subunits (Svenningsen *et al.*, 2006).

Concurrent with the elegant structure–activity studies relating the interactions of flavonoids with the high-affinity benzodiazepine binding site of $GABA_A$ receptors, many actions of flavonoids *in vivo* and *in vitro* were found to be insensitive to the classical benzodiazepine antagonist flumazenil. In particular, functional electrophysiological studies indicated that flavonoids act on $GABA_A$ receptors via two separate mechanisms involving a flumazenil-sensitive high-affinity site and an alternative site that may be the flumazenil-insensitive low-affinity benzodiazepine site.

In addition, certain flavonoids were found to modulate the flumazenil-sensitive modulatory effects of benzodiazepine ligands on $GABA_A$ receptors under conditions where the flavonoids alone had no detectable modulatory effects on GABA responses. This gave rise to the concept of second-order modulation by flavonoids of first-order modulation by benzodiazepines (Campbell *et al.*, 2004; Fernandez *et al.*, 2005; Vignes *et al.*, 2006). The second-order positive modulation of diazepam enhancement of GABA responses by apigenin and EGCG was observed under conditions of the maximal flumazenil-sensitive enhancement of the action of low doses of GABA. It is unlikely that apigenin acts by enhancing diazepam binding as it is known to inhibit such binding. Furthermore, apigenin does not influence the binding of muscimol, a potent $GABA_A$ agonist. The observed second-order modulation may result from alterations in the coupling of the flumazenil-sensitive benzodiazepine allosteric sites with the orthosteric GABA sites on $GABA_A$ receptors. This action was selective for diazepam modulation as it was not observed for enhancement by pentobarbitone or allopregnanolone. Second-order modulation (or metamodulation) of receptor activation has been noted in other systems (Mesce, 2002; Ribeiro and Sebastiao, 2010) and may represent a new, albeit obscure, mechanism of drug action deserving of further investigation. It is not easy to study as it involves the dose-dependent interactions between three ligands and thus may be difficult to observe. Apigenin and EGCG may serve as lead compounds for the development of more selective agents for the study of second-order modulation of $GABA_A$ receptors.

Flumazenil-insensitive effects of flavonoids on $GABA_A$ receptors

Avallone and colleagues found that the inhibitory action of apigenin on locomotor activity in rats was not influenced by pretreatment with flumazenil (Avallone *et al.*, 2000; Zanoli *et al.*, 2000). *In vitro* studies showed that apigenin was a relatively weak inhibitor (IC_{50} 250 μM) of the binding of labelled flumazenil to rat cerebellar membranes. Furthermore, they showed that flumazenil (1 μM) was able to antagonise the inhibitory effect of apigenin (1–10 μM) on the action of GABA on cultured cerebellar granule cells. In contrast to other studies (Viola *et al.*, 1995; Salgueiro *et al.*, 1997; Viola *et al.*, 1998), Avallone and colleagues were unable to observe any anxiolytic action of apigenin (Avallone *et al.*, 2000). They concluded that 'the sedative action of apigenin cannot be ascribed to an activation of $GABA_A$ receptors' (Zanoli *et al.*, 2000).

Hispidulin (Figure 4), which differs from apigenin only by the addition of a 6-methoxy substituent, is a ligand at benzodiazepine binding sites with an IC_{50} value of 1.3 μM and has demonstrated anticonvulsant activity in a model of epilepsy in seizure-prone Mongolian gerbils. In functional studies on recombinant receptors, expressed oocytes hispidulin is inactive when applied alone (Figure 5A) and found to be approximately equipotent and exhibit a biphasic activity (Figure 5B) at α1,2,3,5,6β2γ2S receptors, enhancing at low concentrations (EC_{50} 0.8–5 μM) and inhibiting at higher concentrations (>30 μM), was only partially blocked by flumazenil but was inactive at α1β2 receptors at a concentration of 10 μM (Figure 5C) (Kavvadias *et al.*, 2004). This is biphasic action, and the fact that hispidulin is active at α6-containing receptors that are benzodiazepine insensitive suggests that hispidulin may act via more than one binding site on $GABA_A$ receptors. Interestingly, previous studies indicated that a range of natural and synthetic flavones had no affinity for recombinant α6β3γ receptors (Marder *et al.*, 2001).

The first definitive description of flumazenil-insensitive actions of flavonoids on recombinant $GABA_A$ receptors reported that micromolar concentrations of apigenin and quercetin inhibited the action of GABA on $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptors expressed in oocytes in the presence of 0.1–1 μM flumazenil (Goutman *et al.*, 2003). Other investigators reported similar flumazenil-insensitive negative modulatory actions of apigenin and genistein on recombinant $GABA_A$ receptors (Campbell *et al.*, 2004). These flavonoids, however, had similar negative modulatory actions on a variety of other transmitter-gated ion channels, and such actions were thus not specific to $GABA_A$ receptors.

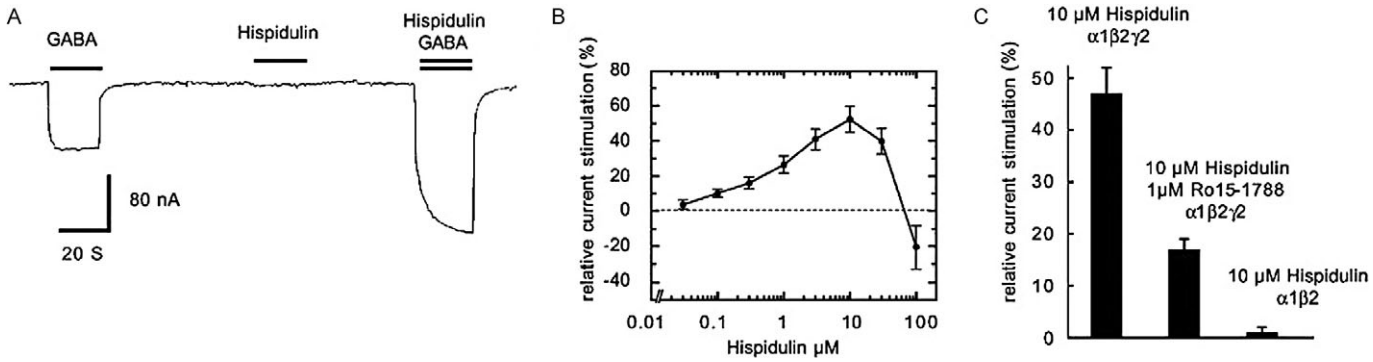

Figure 5

(A) Hispidulin at 10 mM by itself did not elicit any current but potentiated currents elicited by 4 μM GABA at recombinant α1β2γ2S $GABA_A$ receptors expressed in *X. leavis* oocytes. (B) Concentration dependence of allosteric potentiation by hispidulin demonstrating a biphasic response with potentiation at lower concentrations and inhibition at higher concentrations (>10 μM) of hispidulin. (C) The potentiation of currents elicited by GABA $EC_{2\text{-}5}$ by hispidulin (10 μM) at α1β2γ2S GABAA receptors is partially inhibited by co-application of the BZD receptor antagonist Ro15-1788 (flumazenil, 1 μM). The GABA $EC_{2\text{-}5}$ response at benzodiazepine insensitive α1β2 $GABA_A$ receptors is not potentiated by 10 μM hispidulin. From Kavvadias *et al.* 2004. $GABA_A$, γ-amino butyric acid-A.

The biflavone amentoflavone was initially identified as a high-affinity benzodiazepine receptor ligand through the investigation of the pharmacologically active constituents of the plant extract Karmelitter Geist® (Nielsen *et al.*, 1988) and later *Hypericum* species (Baureithel *et al.*, 1997). Displaying an affinity similar to that of diazepam, amentoflavone is the most potent non-nitrogen containing benzodiazepine ligand known; in contrast, I3, II8-biapigenin has no effect at concentrations of 1 μM. Despite later being reported to cross the BBB by passive diffusion in the porcine brain endothelial capillary cells model of BBB (Gutmann *et al.*, 2002), amentoflavone displayed only poor inhibition of [^{3}H]-flunitrazepam binding *in vivo* following i.v. administration (Nielsen *et al.*, 1988). Later studies demonstrated that amentoflavone in fact displays a biphasic activity acting as a benzodiazepine antagonist at nanomolar concentrations (Hansen *et al.*, 2005) and flumazenil-insensitive negative modulator at higher concentrations (Hanrahan *et al.*, 2003; Hansen *et al.*, 2005).

In 2004, Hall *et al.* showed that 6-methylflavone acted as a flumazenil-insensitive positive modulator of recombinant α1β2γ2L and α1β2 $GABA_A$ receptors expressed in oocytes (Hall *et al.*, 2004). Subsequently, these authors found that 6-methylflavanone (Figure 6) was also a fluamazenil-insensitive positive modulator of recombinant $GABA_A$ receptors that, unlike 6-methylflavone, was subtype selective being a more efficacious positive modulator at α2β2γ2L receptors that at α1β2γ2L and α1β2 receptors (Hall *et al.*, 2005). 6-Methylflavanone acted at micromolar concentrations, producing a maximal enhancement of 417% of the action of GABA on $\alpha_2\beta_2\gamma_{2L}$ compared with 120% at α1β2γ2L receptors. This subtype-selective efficacy, together with the finding that neither flavonoid influenced ρ_1 $GABA_C$ receptors, indicates that these actions are relatively selective for $GABA_A$ receptors. Targeting specific $GABA_A$ receptors through subtype-selective efficacy as distinct from potency has been validated for other modulators of $GABA_A$ receptors, including the non-sedating anxiolytic TPA023 and the cognitive enhancer alpha 5IA (Atack, 2008).

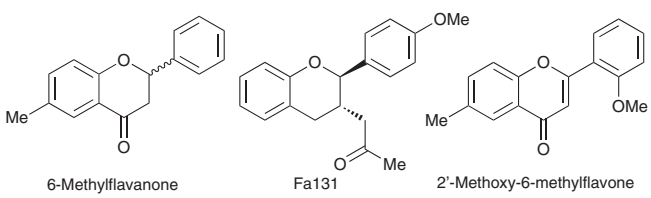

Figure 6

Some synthetic flavonoids with subtype-selective actions on the activation of $GABA_A$ receptors. $GABA_A$, γ-amino butyric acid-A.

6-Methylflavanone differs from 6-methylflavone in having a single rather than a double bond at C2–C3. This results in changes in the delocalization of π-electrons throughout the structure such that 6-methylflavanone is relatively non-planar compared to 6-methylflavone where the phenyl and quinone rings are effectively planar (Hall *et al.*, 2001). The C2–C3 single bond is crucial to the observed subtype-selective efficacy of 6-methylflavanone.

In general, flavanones have shown little or no activity in benzodiazepine-binding studies (Haberlein *et al.*, 1994; Paladini *et al.*, 1999; Marder and Paladini, 2002) and have not been considered in molecular modelling of benzodiazepine binding sites on $GABA_A$ receptors (Dekermendjian *et al.*, 1999; Huang *et al.*, 2001; Clayton *et al.*, 2007). 6-Bromoflavanone and 5-methoxy-6,8-dibromoflavanone have been show to act as anxiolytics in mice, but no details are available as to their possible interactions with $GABA_A$ receptors (Ognibene *et al.*, 2008). (2*S*)-5,7,8,4′-Tetrahydroxyflavanone and 2,5-dihydroxy-7-methoxy-6,8-dimethylflavan-3-one have been shown to inhibit flunitrazepam binding (IC_{50}s 21 and 1 μM respectively), but no *in vivo* data on these compounds have been reported (Wang *et al.*, 2002; Ognibene *et al.*, 2008). Narigenin, the flavanone analogue of apigenin, has been shown to only weakly inhibit (IC_{50} 2.6 mM) flumazenil binding (Jager *et al.*, 2007).

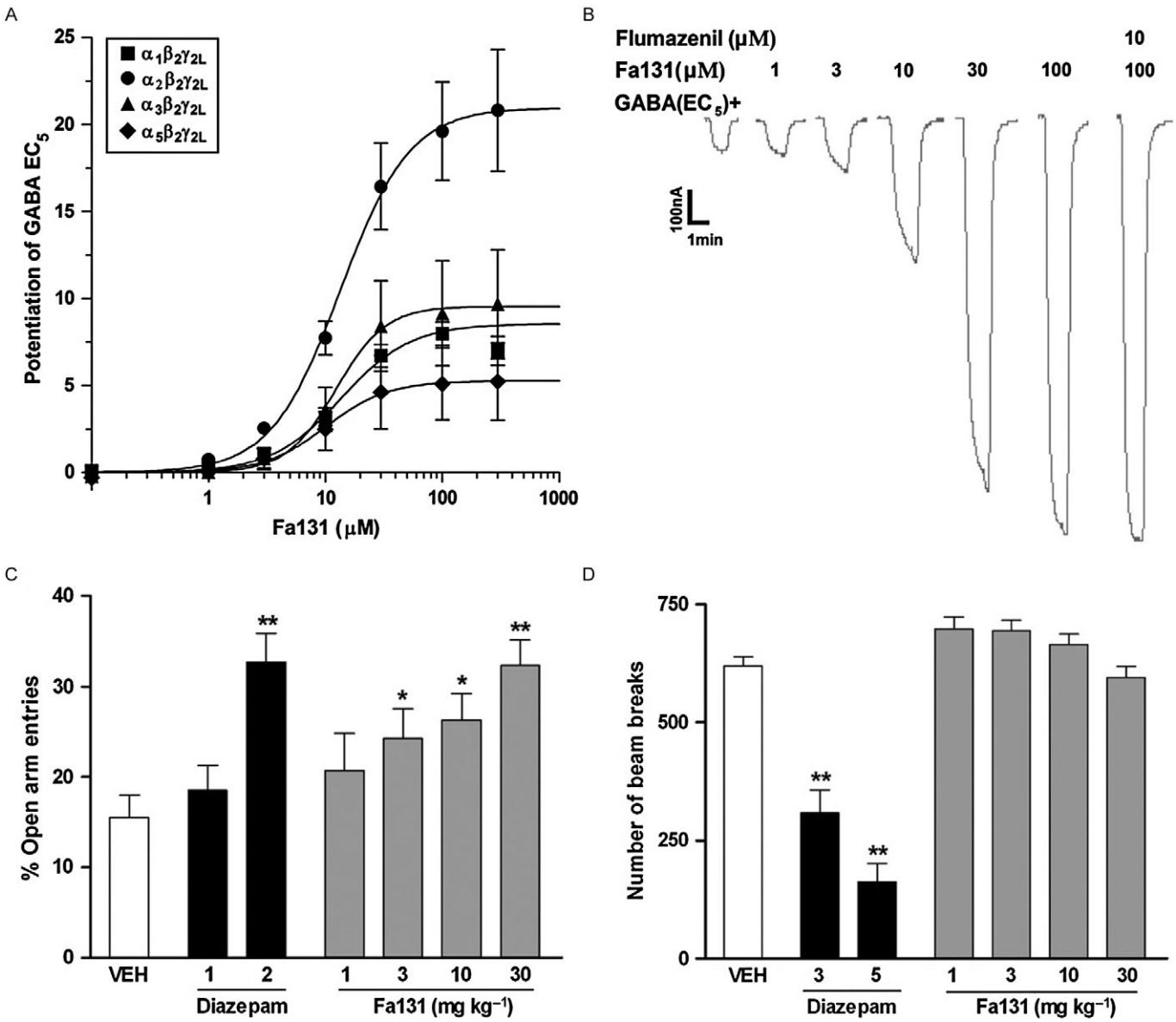

Figure 7

(A) Fractional potentiation of GABA EC_5 current response by Fa131 at human recombinant $GABA_A$ receptors expressed in *Xenopus* oocytes. Concentration-response curves on α1β2γ2L, α2β2γ2L, α3β2γ2L, α5β2γ2L receptors. (B) Potentiation of GABA EC_5 by Fa131 at GABAA α1β2γ2L could not be blocked by coapplication of the benzodiazepine antagonist Ro15-1788 (flumazenil, 10 μM). (C) Effect of diazepam (1 and 2 mg·kg^{-1}) and Fa131 (1–30 mg·kg^{-1}) on the behaviour of mice in the elevated plus maze. Bars represent mean ± SEM of the % open arm entries % time spent on the open arms recorded over a 5 min session, 20 min after an i.p. injection of drugs or vehicle. *$P < 0.05$ or **$P < 0.01$ compared with vehicle group, ANOVA followed by Dunnett's multiple comparison test. (d) Effect of diazepam (3 and 5 mg·kg^{-1}) and Fa131 (1–30 mg·kg^{-1}) on the behaviour of mice in the actimeter test. Bars represent mean ± SEM of number of beam breaks recorded over a 5 min session, 20 min after an i.p. injection of drugs or vehicle, showing a significant decrease in the number of beam breaks induced by diazepam at doses of 3 and 5 mg·kg^{-1} ($P < 0.01$). Fa131 did not produce any changes in this parameter at doses up to 30 mg·kg^{-1}. *$P < 0.05$ or **$P < 0.01$ compared with vehicle group, ANOVA followed by Dunnett's multiple comparison test. From Fernandez *et al.* 2008. GABA, γ-amino butyric acid.

Epigallocatechin gallate (EGCG; Figure 4), a flavanol constituent of green tea, demonstrates dose-dependent anxiolytic, sedative–hypnotic and amnesiac activity, with evidence that these activities are mediated at least in part by $GABA_A$ receptors (Adachi *et al.*, 2006; Vignes *et al.*, 2006). Using 6-methylflavanone and EGCG as lead compounds, structure–activity studies led to the discovery of Fa131 (2*S*,3*R*-3-acetoxy-4′-methoxyflavan) (Figure 6) as the most efficacious compound in a series of flavanol esters with positive modulatory activity of $GABA_A$ receptors (Fernandez *et al.*, 2008; Mewett *et al.*, 2009). Interestingly, similar to barbiturates, Fa131 also acts as weak partial agonist α1β2γ2L receptors and at higher doses exerts a negative modulation (Fernandez *et al.*, 2008).

Fa131 was found to show subtype selectivity for receptors containing α_2-subunits on the basis of efficacy (Fernandez *et al.*, 2008) being more than twice as efficacious on $\alpha2\beta2\gamma2L$ receptors (EC_{50} 14 μM; 2100% enhancement) as on $\alpha1\beta2\gamma2L$ receptors (EC_{50} 14 μM; 850% enhancement) and still less efficacious on $\alpha3\beta2\gamma2L$ and $\alpha5\beta2\gamma2L$ receptors (Figure 7A). The efficacy of 2100% exceeds the highest efficacy yet recorded of 1250% by (+)-borneol at these receptors (Granger *et al.*, 2005). The actions of Fa131 were flumazenil insensitive (Figure 7B) and could be observed on $\alpha1\beta2$ receptors that lack high-affinity benzodiazepine receptors. It was inactive on $\rho1$ $GABA_C$ receptors (Fernandez *et al.*, 2008).

In mice, Fa131 (1–30 $mg{\cdot}kg^{-1}$ i.p.) induced anxiolytic-like action in two unconditioned models of anxiety: the elevated plus maze (Figure 7C) and the light/dark paradigms. No sedative or myorelaxant effects were detected using the hole board, actimeter (Figure 7D) and horizontal wire tests, and only weak barbiturate-potentiating effects on the loss of righting reflex test. Fa131 thus demonstrated improved segregation of anxiolytic and sedative doses when compared with the nonselective agonist diazepam (2 $mg{\cdot}kg^{-1}$ i.p.) consistent with a preferential enhancement at α_2 containing receptors (Fernandez *et al.*, 2008). Fa131 is the first positive modulator to distinguish between α2- and α3-subunit containing $GABA_A$ receptors highlighting the potential of targeting flumazenil-insensitive allosteric sites in the search for new anxioselective drugs.

An emerging area of interest is the activation of $GABA_A$ receptors by flavonoids in the absence of GABA, similar to such activation produced by barbiturates and steroids. There is preliminary evidence for 2′-methoxy-6-methylflavone (Figure 6) activating recombinant $GABA_A$ receptors as micromolar concentrations in the absence of GABA in a flumazenil-insensitive manner (Karim *et al.*, 2010). This activation is blocked by the $GABA_A$ receptor antagonists bicuculline and gabazine and is subtype selective being observed with $\alpha2\beta2/3\gamma2L$ and $\alpha4\beta\delta$ receptors. This suggests that there is a further flavone site on certain $GABA_A$ receptors that mediates opening of the chloride channel in the absence of GABA.

Conclusions

It is now over 25 years since binding studies first demonstrated that flavones are ligands for benzodiazepine binding sites on $GABA_A$ receptors. Since that time, it has become apparent that the actions of flavonoids on these receptors are far more complex than a single action at this one site. It addition to the relatively well-characterized flumazenil-sensitive benzodiazepine-binding sites, there is significant interest in flumazenil-insensitive binding sites for flavonoids. Then there is the question of the site(s) involved in the second-order modulation of benzodiazepine first-order modulation by certain flavonoids and the activation of some $GABA_A$ receptor subtypes by flavonoids in the absence of GABA.

Areas for further study include:

- Much more work needs to be done on the subtype specificity of flavonoid actions on $GABA_A$ receptors.
- Single channel studies may shed light on the different mechanisms of action of flavonoids on $GABA_A$ receptors.
- Identification of the various flavonoid binding sites and their possible overlap with binding sites for other agents acting on $GABA_A$ receptors, for example etomidate and loreclezole.
- Flavonoids that bind to $GABA_A$ receptors but show no intrinsic activity in functional studies should be investigated as possible antagonists.

As significant constituents of our diet, it is important that we understand how natural flavonoids might influence brain function. Understanding the structural determinants of flavonoid effects on $GABA_A$ receptors and developing agents specific for $GABA_A$ receptor subtypes is a key challenge. Synthetic flavonoids are attractive leads for not only drugs to treat brain dysfunction but also for the development of molecules that can be used to study and investigate the role of the modulatory sites at $GABA_A$ receptors and further the development of $GABA_A$ subtype-selective agents.

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Conflict of interest

The authors state no conflict of interest.

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