Chapter 15

Flavonoid Actions on Receptors for the Inhibitory Neurotransmitter GABA

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Abstract

Flavonoids, both naturally occurring and synthetic, are known to have multiple effects on the activation of ionotropic receptors for γ-aminobutyric acid (GABA), the major inhibitory neurotransmitter in our brains. They can act as positive or negative allosteric modulators, enhancing or reducing the effect of GABA. They can elicit a direct activation of the receptors. They can also act to modulate the action of other modulators. This ability to influence function via their actions on GABA receptors permits a range of effects of flavonoids, including relief of anxiety, anticonvulsant, analgesic and sedative actions.

Keywords: apigenin, hispidulin, luteolin, EGCG, synthetic flavonoids, synergism

1. Introduction

Flavonoids have shown a range of effects, such as anxiolytic, sedative, anticonvulsant and analgesic properties, via their actions on the central nervous system (CNS). These effects occur through a variety of interactions with different receptors and signalling systems, including γ-aminobutyric acid (GABA) receptors. γ-Aminobutyric acid (GABA) is the primary inhibitory neurotransmitter in the mammalian brain, released by up to 40% of neurons [1]. GABA acts on two classes of receptors—ionotropic and metabotropic [2]. Ionotropic receptors for GABA are ligand-gated chloride channels located in the neuronal membrane. When activated by GABA, these channels permit the passage of chloride ions down their electrochemical gradient. This usually results in the inward flow of chloride ions and the inhibition of neuronal firing. Metabotropic receptors for GABA are G-protein-coupled receptors that modulate neuronal activity via a variety of second messengers. While an extensive literature on the

interactions of flavonoids with ionotropic GABA receptors exists [3], there are no examples of flavonoids acting on metabotropic GABA receptors, though they are known to act on other G-protein-coupled receptors such as adrenergic receptors [4].

This overview highlights the effects of some representative flavonoids on ionotropic GABA receptors acting as positive or negative allosteric modulators, increasing or decreasing the effect of GABA, as directly acting allosteric agonists, and as second-order modulators influencing the action of other modulators. Of particular interest are flavonoids that show subtype selectivity on GABA receptors. This overview also highlights the pre-clinical evidence for these representative flavonoids as anxiolytics, sedatives and anticonvulsants through their interactions with the GABAergic system. Further, the synergistic actions of flavonoids are reviewed.

2. Ionotropic GABA receptors

There are two classes of ionotropic GABA receptors: $GABA_A$ and $GABA_C$ receptors. $GABA_A$ receptors are relatively complex proteins, while $GABA_C$ receptors are relatively simple [2]. These receptors are pharmacologically distinguished by selective antagonists—$GABA_A$ receptors are antagonized by the convulsant alkaloid bicuculline and are insensitive to (1,2,5,6-tetrahydropyridin-4-yl)methylphosphinic acid (TPMPA), whereas $GABA_C$ receptors are insensitive to bicuculline and are selectively antagonized by TPMPA. $GABA_B$ receptors are metabotropic receptors selectively activated by the GABA analogue baclofen, and insensitive to bicuculline and TPMPA. $GABA_A$, $GABA_B$ and $GABA_C$ receptors differ in their physiology, pharmacology and molecular biology.

Ionotropic GABA receptors are part of a superfamily of ligand-gated ion channels comprising excitatory, cation-selective channels such as nicotinic acetylcholine receptors, $5\text{-}HT_3$ receptors and zinc-activated channels, as well as inhibitory, anion-selective channels such as $GABA_A$ and $GABA_C$ receptors, strychnine-sensitive glycine receptors and invertebrate glutamate-gated chloride channels [5]. Receptors of this superfamily require five subunits to assemble a single ion channel. The ion channel may be homomeric formed by five identical subunits as is the case of $GABA_C$ receptors, or heteromeric, consisting of a combination of at least two different subunits, for example, the $GABA_A$ receptors [6]. This superfamily of ligand-gated ion channels is referred to as the cys-loop receptors due to a conserved characteristic cys-cys disulphide bond forming a loop of 13 amino acids in the N-terminal extracellular domain that contains the orthosteric agonist-binding site for the transmitter. The cys-loop is believed to be important for both cell surface expression of the receptor and cooperative interaction between agonist-binding sites and the channel gate.

$GABA_A$ receptors are heteromeric pentamers, composed of a variety of protein subunits. In humans, there are 19 isoforms of $GABA_A$ subunits, that is, six α, three β, three γ and one of δ, ε, π, θ, known to form heteromeric $GABA_A$ receptors. The most widely distributed complex in the brain is composed of two α, two β and one γ subunit, but many other combinations are known to be found in specific brain areas. Theoretically, many thousands of $GABA_A$ receptors

could exist, made up of different combinations of subunits. Specific subunit combinations are thought to be associated with selective actions [7]. Using transgenic mice, it was demonstrated that receptors containing α1-subunits serve as targets for sedative-hypnotics-mediating sedation, while α2- and/or α3-containing receptors mediate anxiolysis, and α5-containing receptors are involved in memory [8].

The action of GABA on $GABA_A$ receptors can be modulated by many well-known agents. These include benzodiazepines, such as diazepam, barbiturates, anaesthetic agents, ethanol, neurosteroids and flavonoids. Consequently, modulators of $GABA_A$ receptors are important targets for drug development, particularly modulators that are selective for $GABA_A$ receptors made up of specific subunit combinations [7].

$GABA_C$ receptors are relatively simple compared to $GABA_A$ receptors. Three subunits, ρ1, ρ2 and ρ3, have been cloned from retina and restricted brain regions [9]. These subunits usually express as pentameric homomeric $GABA_C$ receptors that are activated by lower concentrations of GABA than $GABA_A$ receptors. The amino acid sequence similarity between $GABA_A$ and $GABA_C$ subunits is 35–45% and is as high as 75% in the transmembrane region. The genes coding for the three $GABA_C$ subunits are found on different chromosomes to those coding for $GABA_A$ subunits. Modulators of $GABA_C$ receptors are rare (zinc, lanthanides and some neurosteroids), although some flavonoids have been show to inhibit $GABA_C$ receptors, for example, apigenin and quercetin [10, 11]. $GABA_A$ and $GABA_C$ receptors have distinct pharmacological profiles, with some agents having opposite effects (agonist/antagonist) on the two classes of receptors. As $GABA_C$ receptors are much less widely distributed in the brain than $GABA_A$ receptors, they are considered to be important targets for drug development [9].

2.1. Flavonoids and benzodiazepines

Flavonoids were first linked to benzodiazepines when *S*-(−)-equol, 4′-hydroxy-7-methoxyisoflavone and 3′,7-dihydroxyisoflavone, isolated from bovine urine, were shown to inhibit benzodiazepine binding to brain membranes [12]. These flavonoids most likely originated from plant sources in the bovine diet. The pioneering studies on naturally occurring and synthetic flavonoids carried out by the research groups of Marder, Medina, Paladini in Argentina during the 1990s drew attention to flavonoids as initially as 'a new family of benzodiazepine receptor ligands [13, 14].

At the time of initial research into flavonoids at GABA receptors, benzodiazepines were amongst the most widely prescribed pharmaceuticals, and numerous flavonoids of the various classes were investigated both *in vitro* and *in vivo* as potential leads for novel benzodiazepine site ligands. The major therapeutic actions of benzodiazepines are now known to result from their action as positive allosteric modulators of GABA at $GABA_A$ receptors, that is, they enhance the action of GABA on these receptors by acting at another site on $GABA_A$ receptors distinct from site that interacts with GABA. This positive modulatory action of benzodiazepines can be antagonized by flumazenil, a neutralizing modulator used therapeutically to reverse benzodiazepine effects. Benzodiazepines have also been shown to act at high concentrations via a flumazenil-insensitive low-affinity-binding site, separate to the high-affinity,

flumazenil-sensitive-binding site [15]. Thus, benzodiazepines act via 'two distinct and separable mechanisms' on $GABA_A$ receptors.

Flavonoids have been shown to modulate $GABA_A$ receptors at low concentrations in either a flumazenil-sensitive or flumazenil-insensitive manner [3]. Thus, flavonoids can influence $GABA_A$ receptors via the classical benzodiazepine-binding site, as well as independently of the classical benzodiazepine-binding site [3, 16]. Many flavonoids elicit biphasic responses, enhancing GABA actions at low concentrations and inhibiting at high concentrations. Additionally, some flavonoids act as agonists particularly at high concentrations and directly gate the receptor in the absence of GABA [17]. Clearly then, flavonoids interact with at least two, and possibly more specific active sites on $GABA_A$ receptors.

2.2. Types of flavonoids

Flavonoids form a class of molecules that consist of a benzopyran moiety (A and C rings) with a phenyl substituent (B ring), as shown in **Figure 1**. The degree of oxidation of the C ring, the hydroxylation pattern of the C ring structure and the substitution in the 3-position demarcate the different subgroups of flavonoids [18]. The predominant subgroups of naturally occurring flavonoids include flavonols (e.g. quercetin [10]), flavones (e.g. apigenin and luteolin [19, 20]), isoflavones (e.g. genistein [21]), flavanones (e.g. naringenin [22]) and flavanols (e.g. epigallocatechin gallate (EGCG) [11]). Each of the flavonoids listed is known to influence $GABA_A$ receptors and to produce CNS effects.

2.3. Apigenin, hispidulin and luteolin

The flavones apigenin, hispidulin and luteolin are closely related structurally, as shown in **Figure 2**. Compared with apigenin, hispidulin has an extra methoxy group in the 6-position and luteolin has an extra hydroxyl group in the 4′-position (**Figure 2**). These small structural differences significantly impact the effects of these flavones on experimental animal behaviours and on $GABA_A$ receptors.

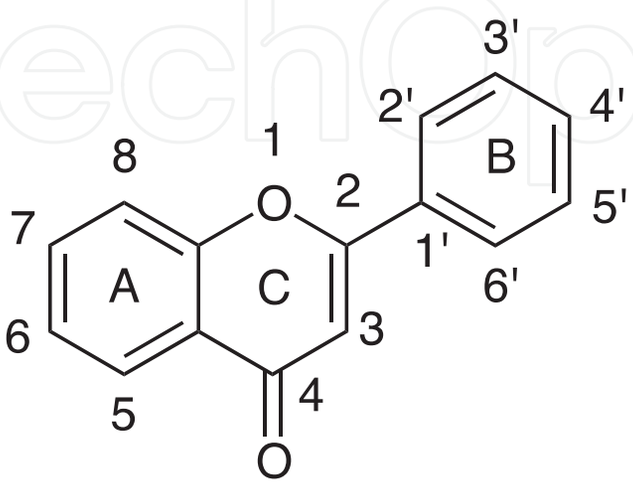

Figure 1. Structure of flavone, showing the generic structure of flavonoids with numbering system and ring designation.

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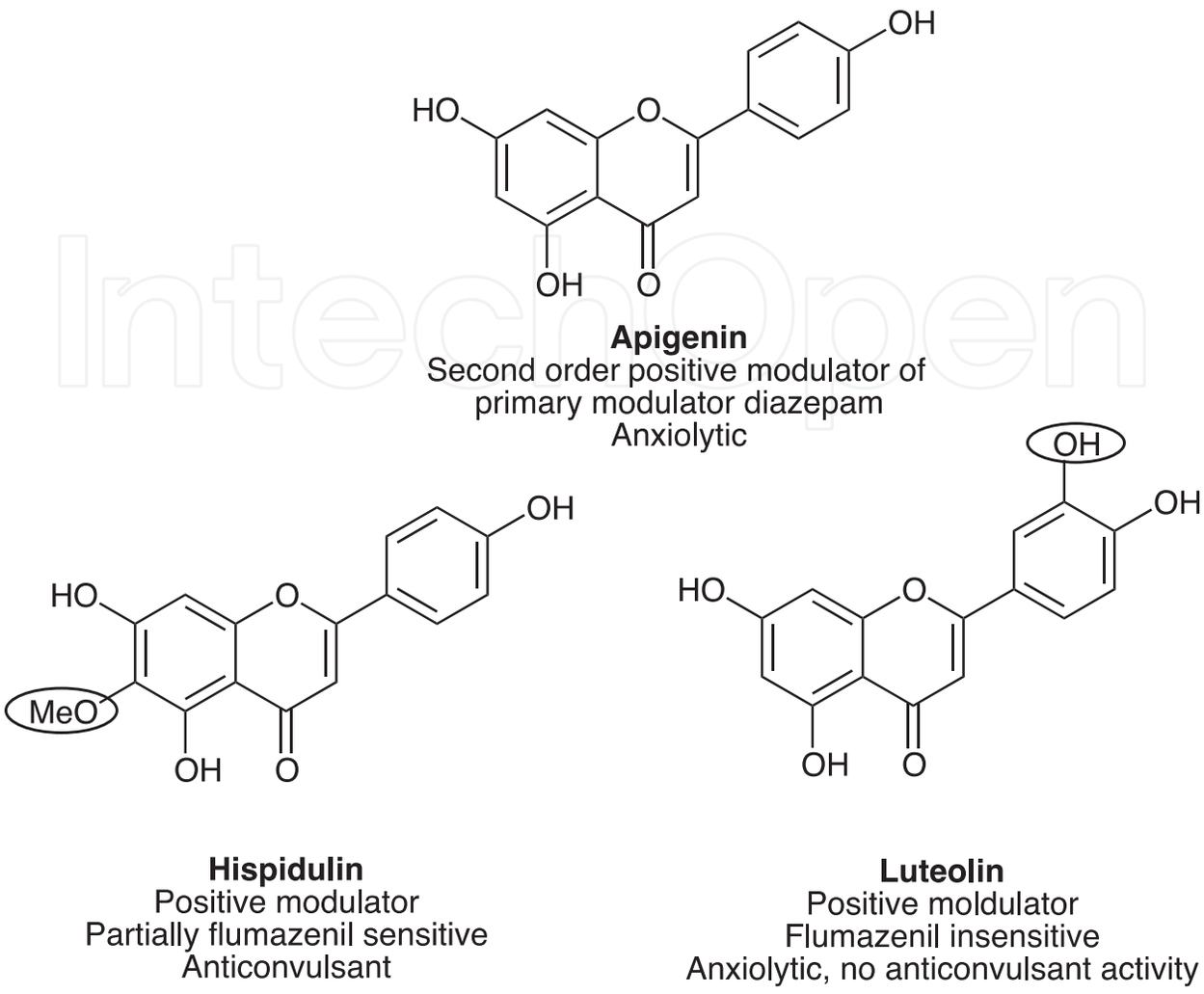

Figure 2. Structures of the flavanones apigenin, hispidulin and luteolin with the differences from apigenin circled.

Apigenin (**Figure 2**), the major flavonoid found in chamomile (*Matricaria chamomilla*), has complex modulatory actions on $GABA_A$ receptors. In cultured cerebellar granule cells, apigenin is described as a negative modulator of GABA action, and it is a weak inhibitor of flumazenil binding to cerebellar membranes [19]. The inhibition of the GABA response at $\alpha 1\beta 2\gamma 2S$ $GABA_A$ receptors expressed in oocytes in the presence of flumazenil (0.1–1 mM) was the first definitive report of the flumazenil-insensitive actions of flavonoids on recombinant $GABA_A$ receptors [10]. Similar flumazenil-insensitive negative modulatory actions of apigenin on recombinant $GABA_A$ receptors were subsequently reported by other investigators [11, 23].

Functional electrophysiological studies have also demonstrated that apigenin can act as a second-order modulator of the first-order modulation of $GABA_A$ receptors by benzodiazepines [11]. This effect of apigenin was observed at concentrations where apigenin alone had no detectable modulatory effects on GABA responses. The second-order positive modulation of the diazepam-enhanced response was observed at the maximal flumazenil-sensitive concentration of diazepam. 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 and was not observed for pentobarbitone or allopregnanolone enhancement. In order for this to be a mechanism for the inhibition of locomotor activity by apigenin, there would have to be primary modulation by endogenous benzodiazepines, the so-called endozepines [24].

The second-order modulation (or metamodulation) has also been noted in other systems [25, 26] and may represent an obscure novel mechanism of drug action deserving further investigation, with the potential to lead to decreased therapeutic doses. It is not easy to study as it involves the dose-dependent interactions between three ligands, requiring the study of a number of dose combinations, and thus may be difficult to observe. Synergistic interactions have been described between other flavonoids on GABA receptor-related behaviours [27–29] and at glycine receptors between strychnine and flavonoids [30]. Complex tertiary interactions between flavonoids and other substances may be a subtle feature of cys-loop ligand-gated ion channels.

Clear anxiolytic effects for apigenin have been shown using the elevated plus maze model of anxiety in mice at doses that did not cause myorelaxation or sedation [29, 31], and in rats (5 mg/kg) [32], as did apigenin 7-glucoside (2.5 and 5 mg/kg) [32]. One study using rats was unable to demonstrate an anxiolytic effect of apigenin on the light-dark avoidance model of anxiety at doses of 1–25 mg/kg [33]. On the other hand, 25 mg/kg apigenin was shown to inhibit locomotor activity in a flumazenil-insensitive manner, since flumazenil pre-treatment did not inhibit this effect [33]. It was concluded that the sedative action of apigenin seen in this study 'cannot be ascribed to an interaction with GABA-benzodiazepine receptor, since it is not counteracted by the benzodiazepine antagonist flumazenil' [33]. Nevertheless, this study was undertaken prior to the discovery of the flumazenil-insensitive action of flavonoids on $GABA_A$ receptors. Other possible mechanisms for the action of apigenin on locomotor activity include reduced activation by L-glutamate of NMDA receptors [34] and inhibition of L-glutamate release via reduction of calcium ion entry [35].

The strongest evidence for flavonoid modulation of $GABA_A$ receptors in the brain comes from the anticonvulsant hispidulin, found in the sage plant (*Salvia officinalis*) (**Figure 2**) [36]. Structurally, hispidulin differs from apigenin only by the addition of a 6-methoxy substituent. In functional studies on recombinant $\alpha1\beta2\gamma2$ $GABA_A$ receptors expressed in *Xenopus* oocytes, hispidulin was inactive when applied alone, and at concentrations up to 10 μM was found to positively modulate the effect of 4 μM GABA [23]. This positive modulation was partially blocked by flumazenil. However, hispidulin at 10 μM was inactive at $\alpha1\beta2$ receptors, which lack the flumazenil-sensitive benzodiazepine site. Hispidulin was further shown to exhibit a biphasic action and to be approximately equipotent at each of six different α-subunit containing $GABA_A$ receptors—$\alpha1,2,3,5,6\beta2\gamma2S$, enhancing at low concentrations (EC_{50} 0.8–5 mM) and inhibiting at higher concentrations (>30 mM). The fact that hispidulin is inactive at $\alpha_1\beta_2$ receptors but is active at $\alpha6$-containing receptors that are insensitive to benzodiazepines suggests that hispidulin may act via more than one binding site on $GABA_A$ receptors, at least one of which may represent a novel site. Interestingly, previous studies indicated that a range of

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natural and synthetic flavones had no affinity for recombinant $\alpha 6\beta 3\gamma$ receptors [37]. Thus, hispidulin appears to show a different profile of activity to apigenin at $GABA_A$ receptor subtypes.

Hispidulin was also shown to displace ^{3}H-flumazenil binding in human frontal cortex crude synaptic membrane preparations [38]. Using ^{14}C-hispidulin, the flavone was found to pass the blood-brain barrier using a rat perfusion model [23]. Further, hispidulin exhibited a flumazenil-sensitive anticonvulsant action, similar to diazepam, in seizure-prone Mongolian gerbils used as an animal model of epilepsy [23].

Luteolin (**Figure 2**), found in many plants including celery and green pepper, differs from apigenin by an additional 3′-hydroxyl group. Following acute administration in mice, luteolin at doses of 1–50 mg/kg failed to demonstrate anticonvulsant or myorelaxation effects, or to have any impact on locomotor activity [39]. On the other hand, 5 mg/kg luteolin increased open-arm entries in the elevated plus maze, suggesting an anxiolytic effect, and reduced haloperidol-induced catalepsy [39]. Both of these effects, however, disappeared at higher doses tested. Another study testing 50 mg/kg luteolin in rats in the elevated plus maze also failed to demonstrate any anxiolytic action [40], though this may be partly explained by the finding that the same dose reduced locomotor activity, suggesting a sedative effect that may have masked any anxiolytic action. Although it has not been tested *in vitro*, it may also be possible that, like some other flavonoids, luteolin possesses positive allosteric modulatory actions at low doses and negative allosteric modulation at higher doses. A combination of flumazenil with luteolin also failed to show any significant difference to the control group, and the researchers concluded that 'luteolin does not produce anxiolysis by modulation of the $GABA_A$ receptor' [40]. Given that we now know of flumazenil-insensitive actions of flavonoids on $GABA_A$ receptors, this may be an incorrect conclusion.

Following chronic administration (14 days), luteolin showed antidepressant activity in the forced swim test, significantly reducing latency to immobilization and increasing total immobilization to the same extent as diazepam, interpreted as increased adaptation (rather than increased helplessness) in the model used [39]. Further evidence of luteolin's antidepressant activity was shown using the forced swim test [20]. A dose-dependent reduction in the duration of immobility was observed in mice following acute administration of doses (5 and 10 mg/kg) that did not alter locomotor activity when tested using the open field.

In a rat neuropathic pain model, luteolin produced analgesia in a bicuculline-sensitive, flumazenil-insensitive manner [23]. Luteolin (10 mg/kg) also improved spatial memory in rats in a scopolamine-induced amnesia model in the Y maze [41], although the proposed mechanisms relate to brain-derived neurotrophic factor, acetylcholine and lipid peroxidation. Further studies are required to demonstrate any involvement of the GABAergic system in this observed memory enhancement. Finally, a study of luteolin in four mouse seizure models showed no anticonvulsant activity [42].

Evidence that some of these behavioural effects of luteolin may be mediated by binding to $GABA_A$ receptors exists. Luteolin displaced ^{3}H-flunitrazepam (1.5 nM) binding on rat cortical crude synaptic membrane preparations, with a K_i of 60 μM, suggesting weak binding to the

benzodiazepine-binding site on $GABA_A$ receptors [39]. Further, luteolin was shown to promote GABA-mediated chloride influx in human neuroblastoma cells, which was attenuated by the $GABA_A$ receptor antagonist bicuculline [20].

The studies reviewed here reveal that apigenin, hispidulin and luteolin appear to show differing profiles of activity at $GABA_A$ receptor subtypes and differing effects *in vivo*, demonstrating that small differences in chemical structure have profound effects on the biological properties of these flavonoids.

2.4. (–)-Epigallocatechin gallate (EGCG)

EGCG (**Figure 3**) is a flavanol, and the major polyphenol in green tea (*Camellia sinensis*) [43]. At low concentrations, EGCG (0.1 mM) has a potent second-order modulatory action on the first-order modulation by diazepam at $\alpha 1\beta 2\gamma 2L$ $GABA_A$ receptors but inhibits the action of GABA at higher concentrations (>1 mM) [11]. As a second-order modulator, EGCG is an order of magnitude more potent than apigenin [11]. In addition, it has been found that EGCG, at concentrations that have no influence on the activation of $GABA_A$ receptors by GABA, was able to reverse β-carboline (a negative modulator of $GABA_A$ receptors)-mediated inhibition of GABA currents in cultured hippocampal neurons [44]. Also, up to 100 μM EGCG significantly increased chloride influx in primary cultured cerebellar cells [45]. This indicates that EGCG may act as a second-order modulator with respect to the first-order modulation by both positive and negative modulators that act via benzodiazepine-binding sites on $GABA_A$ receptors.

EGCG demonstrates dose-dependent stress-reducing, anxiolytic, sedative and hypnotic properties in a number of animal models [44–46], with evidence that these activities are mediated at least in part by $GABA_A$ receptors [44, 46]. EGCG has effects on learning and memory [47] that may be useful in the treatment of Alzheimer's disease [48]. It has also been suggested for Parkinson's disease therapy [49] while some novel EGCG derivatives may be useful for neuropathic pain [50]. It is possible that activity at $GABA_A$ receptors underlies many of the reported actions of EGCG.

2.5. Synthetic flavonoids

Classical structure activity-based design led to the development of synthetic flavonoid ligands with high affinity for the classical flumazenil-sensitive benzodiazepine-binding site on $GABA_A$ receptors [51]. Numerous synthetic flavonoids have been shown to influence $GABA_A$ receptors. Of particular interest are a series of 6-substituted flavones that show the full repertoire of effects on $GABA_A$ receptors: positive, neutralizing and negative modulation and direct activation [3, 52], at both the flumazenil-sensitive benzodiazepine site and flumazenil-insensitive site(s).

6-Bromoflavone was shown to be a flumazenil-sensitive positive allosteric modulator at $GABA_A$ receptors, whereas 6-fluoro- and 6-chloroflavone were demonstrated to act as neutralizing modulators [52]. On the other hand, 6,2′-dihydroxyflavone was found to be a negative modulator. By contrast, 6-methylflavone has been shown to act as a flumazenil-insensitive modulator of $GABA_A$ receptors [53].

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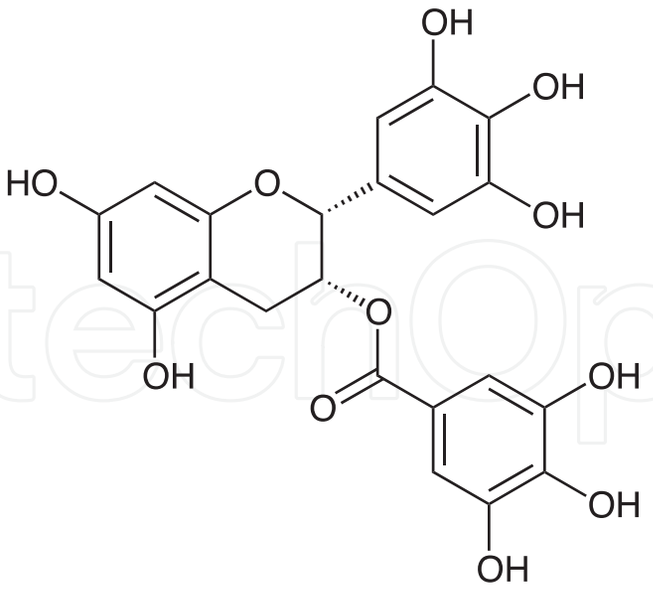
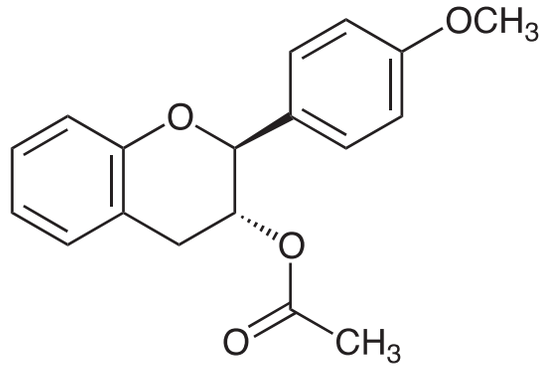
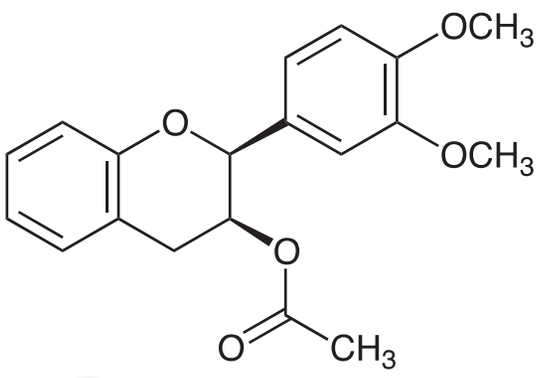


(-)-Epigallocatechin gallate (EGCG)
Second order positive modulator of primary modulator diazepam
Counteracts negative modulation by methyl β-carboline-3-caroxylate
Anxiolytic, sedative hypnotic



Fa131
Positive modulator
Flumazenil-insensitive
Selective for α2 over α1, α3, α5
Non-sedating anxiolytic



Fa173
Blocks Fa131, etomidate, loreclezole and low but not high affinity diazepam.
Inactive against propofol, thiopental and allopregnanolone

Figure 3. Structures of the flavanols EGCG, Fa131 and Fa173.

2′-Methoxy-6-methylflavone has demonstrated anxiolytic effects in mice at 1 and 10 mg/kg using the elevated plus maze model of anxiety [54]. It was also found to act as a positive modulator at $\alpha 2\beta 1\gamma 2L$ and all $\alpha 1$-containing $GABA_A$ receptor subtypes, demonstrated via recombinant $GABA_A$ receptors expressed in *Xenopus* oocytes [54]. By contrast, it directly activated $\alpha 2\beta 2/3\gamma 2L$ receptors without potentiating GABA [54]. Activation by 2′-methoxy-6-methylflavone was attenuated by bicuculline and gabazine but not flumazenil, indicating

a novel site of action. This suggests that there is a further flavonoid site on $GABA_A$ receptors that mediate opening of the chloride channel in the absence of GABA.

Quantitative structure-efficacy relationships have shown that flavone analogues differing only at position 6 show significantly different pharmacological properties at $GABA_A$ receptors [52]. This study clearly shows the importance of the 6-position as a determination of activity. However, further studies on 6-substitued flavones are needed to study the complex nature of the activation and modulation of $GABA_A$ receptor subtypes and to explore the unique therapeutic potential of these synthetic flavones.

Another interesting series of synthetic flavonoids are the flavan-3-ol esters, analogues of EGCG, a naturally occurring flavanol-3-ester. Fa131 (trans-(2S,3R)-3-acetoxy-4′-methoxyflavan, **Figure 3**) is a non-sedating anxiolytic and a selective positive modulator of α2-containing $GABA_A$ receptors, shown on the basis of efficacy [55, 56]. The efficacy of 2100% enhancement exceeds the highest efficacy previously recorded, which was 1250% by (+)-borneol at these receptors [57].

Fa173 (cis-(2S,3S)-3-acetoxy-3′,4′-dimethoxyflavan, **Figure 3**), a diastereo-isomeric flavan-3-ol ester with additional methoxy in the 3′ position, was shown to block the modulatory actions of Fa131 [58]. Additionally, Fa173 also blocked the enhancement of the GABA response by the anaesthetic etomidate, the sedative anticonvulsant loreclezole, and selectively blocked the low-affinity effect of diazepam (100 μM) at $\alpha1\beta2\gamma2L$ and $\alpha1\beta2$ $GABA_A$ receptors, but not the high-affinity effect of diazepam (100 nM). Fa173 was found not to inhibit the positive modulation of GABA by the anaesthetic propofol, barbiturate thiopental, or neuroactive steroid allopregnanolone. This suggests that Fa131, etomidate, loreclezole and high (non-flumazenil-sensitive) doses of benzodiazepine all exert their positive modulatory effects via a common or overlapping binding site that can be blocked by the neutralizing modulator Fa173. Of these agents, Fa131 alone shows selectivity for α2-containing $GABA_A$ recombinant receptors. Fa131 is the first positive modulator to distinguish between α2- and α3-containing $GABA_A$ receptors, highlighting the potential of targeting flumazenil-insensitive allosteric sites in the search for new anxio-selective drugs.

2.6. Synergism between flavonoids

As flavonoids are significant constituents of our diet, it is important that we understand how natural flavonoids might influence brain function. Except when consumed as dietary supplements, flavonoids are generally consumed as a mixture of different flavonoids from one or more foodstuffs [59]. The effects of mixtures of flavonoids and other modulators on $GABA_A$ receptors need to be more thoroughly investigated. Synergies have been noted between individual flavonoids [29, 60, 61], and between flavonoids and benzodiazepines [27, 28].

Hesperidin, a glycosylated flavonone isolated from Valerian species, has shown synergistic effects in mice. The combination of hesperidin (2 mg/kg) with apigenin (1 mg/kg), 6,3′-dinitroflavone (0.02 mg/kg) or diazepam (0.3 mg/kg) enhanced the barbiturate-induced sleeping time in mice [27, 29]. Both hesperidin and diazepam administered separately showed a dose-dependent reduction in exploratory parameters (number of head dips,

time spent head-dipping and rearing behaviour), indicative of increased sedation, in mice on the holeboard assay [27]. Isobolar analysis revealed synergism between diazepam and hesperidin when administered together. For all exploratory parameters measured, a 1.3 to 2-fold increase in potency was observed compared to the administration of either drug alone. Further, these synergistic actions could not be explained by any influence of either drug on plasma concentrations of the other [27]. Loscalzo et al. [28] further demonstrated a potentiation of sedation in mice when hesperidin was administered together with either bromazepam, alprazolam, flunitrazepam or midazolam, through a reduction in exploratory parameters and locomotor activity using the holeboard assay and open-field test, respectively.

Using human mammary epithelial carcinoma cells (MCF-7), Choi and colleagues [60] showed that isoflavones daidzein (derived from soybean) and baicalein (from *Scutellaria baicalensis*) stimulated oestrogen receptor phosphorylation and transcriptional activation of oestrogen-responsive element (ERE). When tested together, the observed effects on oestrogen receptor phosphorylation and transcription of the ERE were further increased in comparison to when the drugs were tested alone. A combination of baicalein and daidzein was shown to produce a synergistic effect in stimulating oestrogenic activity in MCF-7 cells, calculated using the median-effect principle. Further, using an Aβ-aggregation assay to model cellular pathology of Alzheimer's disease, daidzein and baicalein were demonstrated to reduce Aβ-aggregation. As oestrogen is neuroprotective, this synergistic action of the isoflavones on oestrogen receptors, as well as in reducing Aβ-aggregation, suggests that the combination of flavonoids could provide valuable neuroprotective effects and prevention of neurodegenerative disease [60].

Another study examining the synergistic effects of flavonoids found that the flavonol EGCG and the flavone luteolin synergistically inhibited TGF-β-induced myofibroblast phenotypes in prostate fibroblast cell lines [61]. Myofibroblasts are converted from fibroblasts by TGF-β and IL-6 in the tumour microenvironment. These cells play a role in cell proliferation, migration and invasion. TGF-β-induced fibronectin expression was used as a marker of myofibroblast expression. Both EGCG (20–40 μM) and luteolin (20 μM) were shown to reduce TGF-β-induced fibronectin expression alone. In combination, these compounds showed greater efficacy in reducing fibronectin expression at concentrations that were less effective when administered alone. The authors concluded that a combination of EGCG and luteolin could prove effective in reducing the toxic effects of either compound by requiring lower doses, and in preventing advancement of tumour growth by reducing the myofibroblast phenotype.

3. Conclusion

Since flavonoids were first linked to benzodiazepine-binding sites on $GABA_A$ receptors many years ago, recent studies have clearly demonstrated that the actions of flavonoids on these receptors are far more complex than a single action at a single site. In addition to the now relatively well-characterized flumazenil-sensitive benzodiazepine-binding sites, there is significant interest in flumazenil-insensitive, non-benzodiazepine-binding sites for flavonoids. This

overview has sought to highlight the action of representative flavonoids on $GABA_A$ receptors to illustrate the range of activities.

Recent studies have identified the presence of multiple sites on ionotropic GABA receptors at which flavonoids can act, modulating the effect of GABA. The sites may include ones that are insensitive to the classical benzodiazepine-binding site antagonist (neutralizing modulator) flumazenil and described as low-affinity benzodiazepine sites [15]. Perhaps, these would be more appropriately described as flavonoid sites as they appear to be activated by many naturally occurring and synthetic flavonoids.

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