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Molecular Modeling and QSAR Analysis of the Interaction of Flavone Derivatives with the Benzodiazepine Binding Site of the $GABA_A$ Receptor Complex

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Abstract—A large number of structurally different classes of ligands, many of them sharing the main characteristics of the benzodiazepine (BDZ) nucleus, are active in the modulation of anxiety, sedation, convulsion, myorelaxation, hypnotic and amnesic states in mammals. These compounds have high affinity for the benzodiazepine binding site (BDZ-bs) of the $GABA_A$ receptor complex. Since 1989 onwards our laboratories established that some natural flavonoids were ligands for the BDZ-bs which exhibit medium to high affinity in vitro and anxiolytic activity in vivo. Further research resulted in the production of synthetic flavonoid derivatives with increased biochemical and pharmacological activities. The currently accepted receptor/pharmacophore model of the BDZ-bs (Zhang, W.; Koeler, K. F.; Zhang, P.; Cook, J. M. *Drug Des. Dev.* **1995**, *12*, 193) accounts for the general requirements that should be met by this receptor for ligand recognition. In this paper we present a model pharmacophore which defines the characteristics for a ligand to be able to interact and bind to a flavone site, in the $GABA_A$ receptor, closely related to the BDZ-bs. A model of a flavone binding site has already been described (Dekermendjian, K.; Kahnberg, P.; Witt, M. R.; Sterner, O.; Nielsen, M.; Liljerfors, T. *J. Med. Chem.* **1999**, *42*, 4343). However, this alternative model is based only on graphic superposition techniques using as template a non-BDZ agonist. In this investigation all the natural and synthetic flavonoids found to be ligands for the BDZ-bs have been compared with the classical BDZ diazepam. A QSAR regression analysis of the parameters that describe the interaction demonstrates the relevance of the electronic effects for the ligand binding, and shows that they are associated with the negatively charged oxygen atom of the carbonyl group of the flavonoids and with the nature of the substituent in position 3′.

Introduction

Several important drugs such as the benzodiazepines (BDZs), barbiturates, neurosteroids, ethanol, some anticonvulsants and general anesthetics interact with γ-aminobutyric acid type A receptors ($GABA_A$ receptors) in order to elicit their pharmacological effects. These receptors are the principal inhibitory system operating in the brain, playing a pivotal role in the regulation of brain excitability.

$GABA_A$ receptors are transmembrane hetero-oligomeric proteins which are expressed in the central and peripheral nervous system. Several subunit classes and isoforms within each class have been cloned from the mammalian brain. The various subunits of the GABA receptors are identified as α1–α6, β1–β3 (plus β4 in chick brain), γ1–γ3 (plus γ4 in chick brain), δ, ϵ, π and ρ1–ρ3.[1] The receptor itself is a pentameric assembly derived from the combination of various subunits.

In addition to $GABA_A$ receptors, which are ligand-gated chloride ion channels,[2,3] GABA activates two other classes of receptors: $GABA_B$ and $GABA_C$. $GABA_B$ receptors are known to be coupled to Ca^{2+} or

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K^+ channels which activate second messenger systems within the cell via G proteins.[2,4] $GABA_C$ receptors are derived from various isoforms of their ρ subunit, and are directly associated with chloride ion channels.

The benzodiazepine binding site (BDZ-bs) is located at the interface of the α and the γ subunits in the $GABA_A$ receptors. The BDZs act by potentiating GABA-induced Cl^- currents.[5,6] The presence of α and γ1 or γ2 subunits is an absolute requirement for fully functional BDZ binding and optimal modulation of Cl^- currents by them.[7,8]

The extensive molecular diversity of $GABA_A$/BDZ-bs which results from the existence of the subunits has been implicated in the multiple pharmacological properties elicited by ligands like the BDZ diazepam, which lacks subtype selectivity. Furthermore, the regional heterogeneity of the $GABA_A$–BDZ-bs complex has been suggested as another reason for the multiplicity of pharmacological properties of BDZ-bs ligands. The enormous amount of structure–activity relationship (SAR) data available for a diversity of ligands has resulted in the formulation of several pharmacophore models for the BDZ-bs.[9–18] The common feature of these models is the attempt to explain ligand efficacy as a function of ligand–receptor interaction at a molecular level. A comprehensive pharmacophore model, developed by Cook and co-workers, unifies the previous models for inverse agonists/antagonists, and agonist activities, including even a recent model for a diazepam insensitive site (DI).[19] This pharmacophore/receptor model which has already been applied to a large number of compounds and shows a high degree of reliability is based on the study of different classes of ligands, such as β-carbolines, diindoles, 1,4-BDZs, imidazobenzodiazepines, triazolopyrimidines, imidazopyridines and pyrazoloquinolin-3-ones. It assumes that agonists, antagonists, and inverse agonists all share the same binding pocket in the BDZ-bs.[19] The general features of this pharmacophore model are shown in Figure 1. It identifies receptor areas of particular physicochemical properties that should be located in a well defined spatial arrangement in order to be counterbalanced by the ligands. H1 and A2 are hydrogen bond donor and acceptor sites, respectively, whereas H2/A3 is a bifunctional hydrogen donor/acceptor site, L1, L2 and L3 are lipophilic pockets, and S1, S2 and S3 denote regions of negative steric interactions.

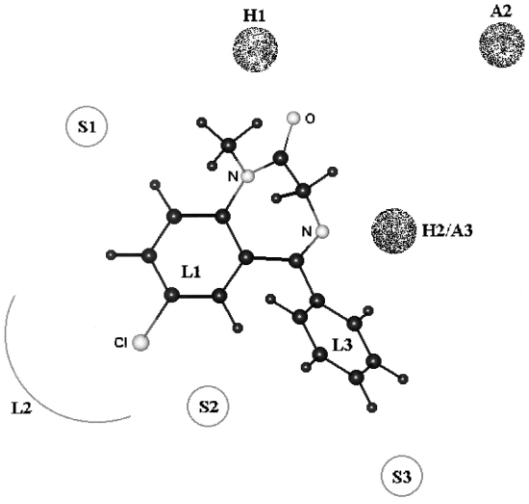


Figure 1. Diazepam (**51**) fitted in the pharmacophore model of the BDZ-bs developed by Cook and co-workers. H1 and A2 are hydrogen bond donor and acceptor sites, respectively, H2/A3 is a bifunctional hydrogen donor/acceptor site, L1, L2 and L3 are lipophilic pockets, and S1, S2 and S3 denote regions of steric interactions.

After extensive studies of BDZ-bs-subtypes[20–26] Cook and co-workers have recently focused their research on BDZ-bs subtype-selective ligands[27] in order to attempt the synthesis of more selective agents which could provide a better understanding of which subtype mediates a particular biological effect. By means of a ligand-mapping approach, they found differences in the size of the lipophilic pocket of five recombinant BDZ-bs subtypes. Their effort resulted in the design and synthesis of a novel ligand, selective for the α5β3γ2 receptor, that enhances memory when injected directly into the hippocampus, but not after systemic administration.[27]

Rudolph and co-workers[28] have recently found that diazepam (a full agonist) failed to show its sedative, amnesic and partly its anticonvulsant action after the introduction of a histidine-to-arginine point mutation at position 101 on the murine α1 subunit of the $GABA_A$ receptors. The anxiolytic-like, myorelaxant and motor impairing effects were retained. It was thus concluded that diazepam responses are mediated by specific $GABA_A$ receptor subtypes. There is an increasing interest in the identification of BDZ-bs subtype-selective ligands, oriented to attain new agents useful for the treatment of anxiety, sleep disorders, convulsions, or memory deficits with low potential for ill side-effects.

Since 1989 onwards,[29–33] our laboratories established that some naturally occurring flavonoids, as well as the flavone nucleus itself (Scheme 1), were ligands for the BDZ-bs, which exhibit medium to high affinity in vitro and anxiolytic activity in vivo, with minor sedative or myorelaxant effects. Moreover, our research resulted in the production of several synthetic flavone derivatives with electronegative atoms or chemical groups in its nucleus and established that they were high affinity ligands for the BDZ-bs endowed with potent anxiolytic or antagonistic effects.[34–45] As a natural further step in these studies, we have centered our efforts on the study of the main structural features that explain their binding characteristics, analyzing, at the same time, whether they show subtype selectivity. In attempting this work we have considered the results of previous research on related subjects. Major advances in the knowledge of the BDZ-bs pharmacophore are owed to Cook and co-workers (see ref 19 and others therein). Although working with several classes of ligands, they have not included flavonoids in their training set. Dekermendjian and co-workers,[46] who have tested the validity of Cook's model for 21 flavonoids, based their analysis on graphic superposition techniques using as template a non-BDZ agonist, namely CGS-9896 (a pyrazoloquinolinone, Scheme 1). As will be discussed later, this

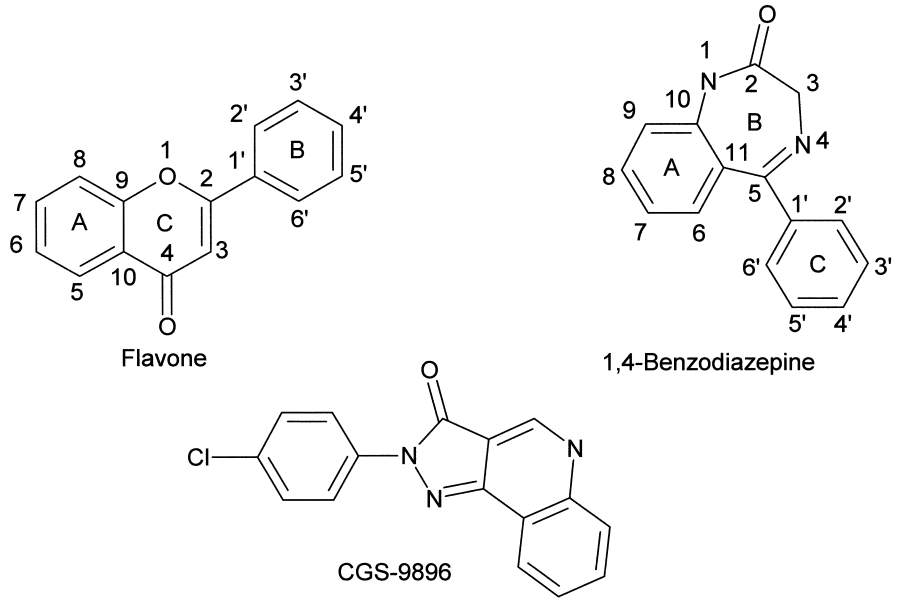
compound differs from diazepines (Scheme 1) in its affinity for certain receptor subtypes. In the present work, instead, we have chosen to work with a training set formed by all the natural and synthetic flavonoids found to be ligands for the BDZ-bs, comparing them with the classical BDZ diazepam. Cinnamic acid, cinnamic acid methyl ester, cinnamic acid ethyl ester and caffeic acid ethyl ester, together with equol, define the validation set (Scheme 2). The compounds in this validation set bind to the BDZ-bs with very low, but measurably different, affinities for the receptor and their structures have many points in common with the flavone nucleus (see Scheme 1).

The model pharmacophore developed on this basis defines the characteristics for a ligand to interact and bind to the flavone site (FS), that is also a site for BDZ binding. The requirements of the model achieved by us are compared with those proposed by Cook and coworkers for the BDZ-bs[19] and Dekermendjian and coworkers for the FS.[46] The effect of substituents in the flavone nucleus (positions 6 and/or 3′) that we have shown to be relevant for high affinity binding[37,39,40] is also quantitatively analyzed. Multiparametric QSAR regression studies show the importance of the electronic interactions in the recognition process and in the modulation of the binding capability by lipophilic and steric effects.

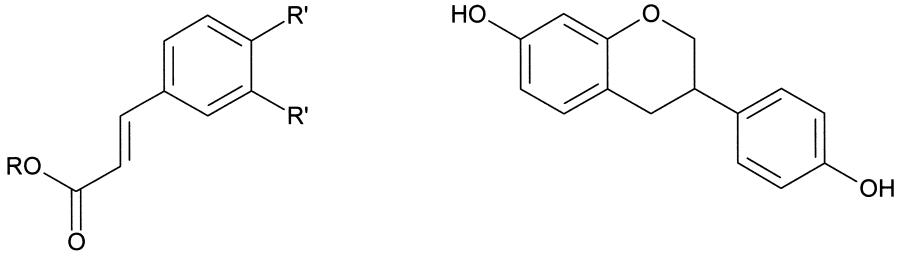
Computational Methods

One hundred and twenty different compounds have been included in the similarity analysis; 36 are natural flavonoids, 67 are flavonoid derivatives synthesized in our laboratory and the rest have been taken from the literature (see ref 43 for review). Table 1 shows the K_i values of the 6,3′-disubstituted flavones. These positions are the most effective to place substituents that give rise to high affinity ligands.

The geometry of the structures has been optimized to minimum energy using an AM1 model Hamiltonian.[47] Because of the lack of flexibility, only the rotation of the



Scheme 1. Chemical structures of flavone derivatives, 1,4-benzodiazepines and CGS-9896.



Scheme 2. Structures of the validation set compounds.

Table 1. Affinity for the BDZ-bs of the compounds mentioned in this work

n	Compound	K_i (μM)[a]
1	6-bromoflavone	0.070[b]
2	6-methylflavone	0.125[c]
3	6-chloroflavone	0.164[d]
4	6-nitroflavone	0.275[d]
5	6-hydroxyflavone	0.580[c]
6	6-methoxyflavone	0.860[c]
7	6-fluoroflavone	4.5[d]
8	flavone	1
9	6-bromo-3′-nitroflavone	0.001[e]
10	6-methyl-3′-nitroflavone	0.0056[f]
11	6-chloro-3′-nitroflavone	0.008[d]
12	6,3′-dinitroflavone	0.026[g]
13	6-fluoro-3′-nitroflavone	0.180[d]
14	3′-nitroflavone	0.285[d]
15	6,3′-dibromoflavone	0.019[d]
16	6-methyl-3′-bromoflavone	0.013[h]
17	6-chloro-3′-bromoflavone	0.023[i]
18	6-nitro-3′-bromoflavone	0.025[d]
19	6-fluoro-3′-bromoflavone	0.236[i]
20	3′-bromoflavone	0.413[d]
21	6-hydroxy-3′-bromoflavone	1
22	6-methoxy-3′-bromoflavone	1
23	6-bromo-3′-chloroflavone	0.017[i]
24	6,3′-dichloroflavone	0.023[i]
25	6-fluoro-3′-chloroflavone	0.199[i]
26	3′-chloroflavone	0.614[d]
27	6-bromo-3′-methylflavone	0.154
28	6,3′-dimethylflavone	0.208
29	3′-methylflavone	10
30	3′-methoxyflavone	2.4[i]
31	3′-fluoroflavone	3.55[i]
32	6-bromo-3′-fluoroflavone	0.042[i]
33	6-bromo-3′-methoxyflavone	0.609[i]
34	6-chloro-3′-fluoroflavone	0.117[i]
35	6-chloro-3′-methoxyflavone	0.848[i]
36	6,3′-difluoroflavone	0.920[i]
37	6-fluoro-3′-methoxyflavone	2.5[i]
38	3-bromoflavone	> 75[b]
39	6,3-dibromoflavone	> 75[b]
40	3-bromo-3′-nitroflavone	> 20[d]
41	5,2′-dihydroxy-6,7,8,6′-tetramethoxyflavone	0.36[j]
42	7-hydroxyflavone	5.3[c]
43	cinnamic acid	> 100
44	cinnamic acid methyl ester	> 100
45	cinnamic acid ethyl ester	100
46	caffeic acid ethyl ester	150[k]
47	caffeic acid 1-butyl ester	> 100
48	caffeic acid isopropyl ester	> 100
49	7,4′-dihydroxyisoflavan (equol)	80[l]
50	II-4′, I-5, II-5, II-7-tetrahydroxy-I-4′, I-7-dimethoxy [I-3′, II-8] biflavone	2–6[m]
51	diazepam	0.007

[a] K_i±SEM values are means of 3 to 5 independent determinations and estimate the inhibition of [^{3}H]flunitrazepam binding to rat cerebral cortical synaptosomal membranes. SEM varies between 6 and 13% of the absolute values listed.
[b] Ref 37.
[c] Ref 54.
[d] Ref 39.
[e] Ref 38.
[f] Ref 46.
[g] Ref 34.
[h] Ref 45.
[i] Ref 40.
[j] Ref 55.
[k] Ref 56.
[l] Ref 57.
[m] Ref 43. The values from literature c and f were obtained in binding tests using [^{3}H]flumazenil.

B ring of the flavone nucleus relative to the bicyclic moiety has to be considered in the conformational analysis of the synthetic flavonoids. Other torsional angles are associated with the study of some natural flavonoids (mainly biflavonoids) and synthetic acids. A systematic procedure, based on modifications of the angles in 30° steps, has been used in all the cases.

The superposition analyses were oriented to find the common features that are shared by all the structures under consideration, which define the pharmacophoric pattern. However, the geometry of a ligand in the interaction site is not associated uniquely with the conformation of lowest energy. Any conformation that can be acquired at a low energy cost can be involved in the interaction, defining the active conformation. For the evaluation of the energy cost associated with the evolution among different conformations, the angles involved in conformational changes have been modified in 4–10° steps, keeping constant its value at each stage of the walk while the other degrees of freedom were fully optimized.

For the QSAR analysis, we have centered our study on the 6,3′-substituted compounds. The 37 flavonoids included in the training set contain eight variable substituent positions in the flavone nucleus. Previous studies[37,39,40] have demonstrated that carbon atoms 6 and 3′ are the most effective positions to place substituents. In order to quantify these effects, each substituent was parameterized by several physicochemical parameters. As a first step we have considered 13 electronic, lipophilic and steric descriptors: molar refractivity (MR), dipole moment (μ), Hammett *meta* and *para* constants (σm, σp), lipophilicity (πm, πp), polar constant (F), resonant constant (R), steric constant (Es), the energy of the highest occupied molecular orbital (HOMO), energy of the lowest unoccupied molecular orbital (LUMO), delta HOMO−LUMO (H−L), charge in carbon 4 (C), charge in oxygen 4 (O). The first nine were taken from ref 48, whereas the others belong to AM1 semiempirical calculations. They were further arranged in subsets on the basis of their lack of mutual dependence. For each subset of descriptors, QSAR regression analysis was performed including compounds **1** to **37** (Table 1), which define the training set. Statistical fittings that correlate K_i, Ln K_i, Ln $(1/K_i)$ versus eight selected physicochemical parameters in multiparametric expressions have been considered. The validation set, defined by compounds **43** to **49**, has been used to confirm the inferences derived from the superposition analysis.

Results and Discussion

Molecular modeling analysis

The conformation of minimum energy for the flavones that do not bear substitution in positions 3, 2′, or 6′ is characterized by a dihedral angle close to 25° between the B ring and the bicyclic system (Scheme 1). In agreement with the results of the MM3 and MM2 calculations of Dekermendjian and co-workers,[46] we found

that no energy is associated with the planarization of the structures at the semiempirical AM1 level. Another point of agreement concerns the effect of substitution in the 3 position (compounds **38**, **39** and **40**, Table 1), which increases the dihedral angle to 63°. A similar effect is found in the 2′ and/or 6′ positions, where substitutions stabilize an angle of 58° (skullcapflavone II, compound **41**).[49] A higher energy is required to decrease this angle in the substituted structures, due to steric repulsion between the more voluminous groups. The calculated energies for planarization are 15 and 6 kcal/mol, for 3-bromoflavone (**38**) and skullcapflavone II (**41**), respectively. Our previous experience demonstrates that 6 kcal/mol, calculated at the AM1 level, defines an energy low enough to allow fitting to a given template,[50] whereas higher values impede the fitting. The present calculations add further weight to the hypothesis that a close-to-planar conformation is required for ligand binding to the BDZ-bs,[19] since the skullcapflavone II (**41**) is capable of binding to the receptor, whereas the 3-substitued structures are not.

Superposition of the planar flavones with the benzodiazepines is very difficult due to the tridimensional conformation of the diazepine ring. Being aware that perfect overlapping between the ligands is not necessary as the ligand interacts with regions of the receptor larger in size than an atom, we have quantified this possibility on a relative basis considering the energy cost for perfect fitting. To this end two conformational changes have been considered for the BDZ structure:

(1) A modification of the angle N1-C10-C11-C5 (see Scheme 1) from its stable value of −43° to a value close to zero. This leads to a structure with positions N1, C3, N4, and C5 (see Scheme 1) almost coplanar and the carbonyl moiety emerging slightly out of the plane.
(2) Rotation of the C ring (see Scheme 1) until coplanarity with the bicyclic moiety is attained.

The first conformational change requires an energy of 6 kcal/mol, which is considerable within the limits of accessibility in the interaction site. The second one implies only 2 kcal/mol, but increases to 9 kcal/mol when performed consecutively to the first one, due to steric repulsion between the hydrogen atoms in positions 6 and 6′ in the final conformation. Hence, a compromise between the consecutive changes, that counterbalance coplanarity and the effect of repulsive interaction, is accepted to define the *active conformation of the BDZs*. This modification attains a planar structure in the A, B fused rings of DZ and a slight distortion from coplanarity in the C ring, keeping the energy associated with the change below 7 kcal. The calculated energies should not be considered as absolute but are useful to compare the possibility of interaction of several ligands according to their flexibilities. The analysis is based, on the other hand, on the planarization of DZ, which does not go strictly to a planar structure, but one closer to the flavone configuration. Perfect overlapping can only be obtained between molecules of the same family (flavones, for example). Comparison of structures of different families, accepting a slight separation between the corresponding centers, on the consideration that the interaction with the receptor is a dynamic process, renders more complete information about the requirements in the receptor site.

The superposition analysis of natural and synthetic flavonoids of the training set with DZ allows two fitting possibilities (Fig. 2a and b).

In both cases, the consideration of the positions that are shared by all the structures (Fig. 3) demonstrates the importance of the carbonyl group of the flavonoids which overlaps with either the keto group at C2 or the N4 atom of the diazepine ring. This center defines a hydrogen bond acceptor group, capable of recognizing H1 or H2 positions in the BDZ-bs model proposed by Cook (see Fig. 1). A hydrogen bond donor group should be present, thence, in the FS of the BDZ-bs.

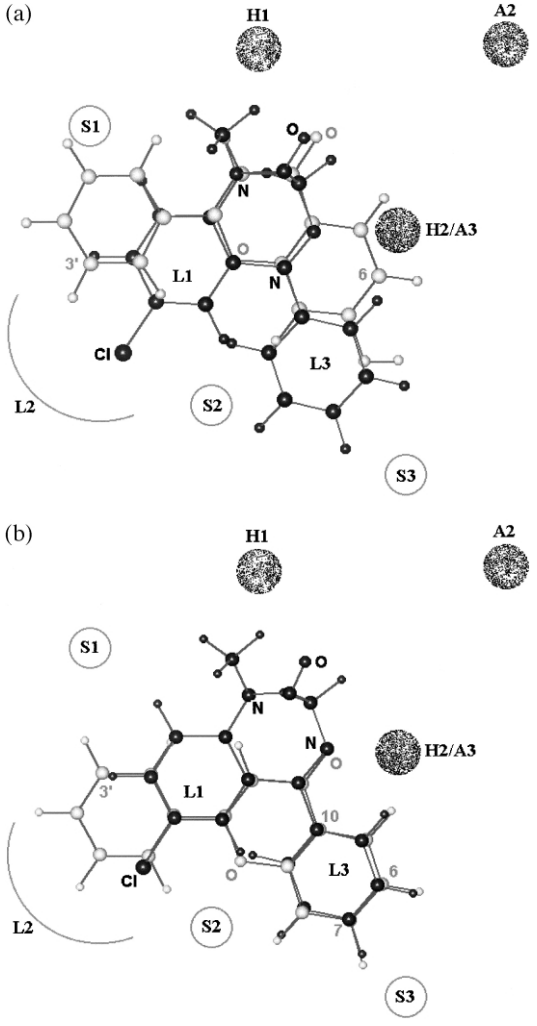


Figure 2. Superposition of flavone (**8**, unfilled atoms) and diazepam (**51**, filled atoms). The carbonyl group of flavone overlaps with the keto group at carbon 2 of diazepam in (a) and with nitrogen 4 of the diazepine ring in (b). H1 and A2 are hydrogen bond donor and acceptor sites, respectively, H2/A3 is a bifunctional hydrogen donor/acceptor site, L1, L2 and L3 are lipophilic pockets, and S1, S2 and S3 denote regions of steric interactions.

The aromatic rings A and B of the flavone structures cannot be related to a requirement for the activity, after the superposition studies, in any of the schemes attempted. Both of them only partially overlap. However, aromaticity of B ring would develop as a requirement after the QSAR analysis.

The superpositions shown in Figure 2a and b extend, to the left, up to position 3′ of the B ring of the flavonoids. The atoms that should be considered in the superposition with the A ring deserve further discussion. Whereas superposition 2a shows that four carbon atoms to the right of the carbonyl are important, superposition 2b shows that positions 10 and 7 match perfectly with atoms 1′ and 4′ of the diazepam template. In the first case, atom 7 will not be perfectly overlapping, due to the lack of planarity of the phenyl ring of the DZ structure. The fact that the size of the ligand should extend up to position 6 or 7 in the A ring of the flavone indicates that a complementary pocket in the binding site (L3) should be filled by the ligand. The same might apply to position 3′ (L2). Position 3′ defines the size of the ligand for efficient binding. Large substituents in this position are not suitable.

When the compounds of the validation set, cinnamic acid and its esters (**43**, **44** and **45**, Scheme 2), are included in the comparison (Fig. 4c), it becomes clear that regardless of the choice of positions 6 or 7 of the flavone ring to define the superposition with BDZs, a minimum size is required that allows reaching position 4′ of the benzodiazepine. Neither the cinnamic acid, nor its methyl ester, which does not comply with this requirement, bind as effectively as the ethyl ester.

The importance of filling the lipophilic pocket L3 is one of the factors that have been considered in supporting the choice of superposition shown in Figure 2b. Further evidence for this choice was provided by the distance between the electron rich centers involved in the interaction with the hydrogen donor group in the receptor, that is the O4 of the carbonyl group of the flavone nucleus with the keto group at C2 of the diazepine ring (2 Å apart in Figure 2a) or with the N4 atom of the diazepine ring (perfect overlapping in Figure 2b).

Moreover, substitution in position 7 does not give a positive contribution to the affinity (compound **42**), presumably due to steric interactions with the receptor

Figure 3. Superimposition of diazepam (**51**, black atoms), 7′-hydroxyflavone (**42**, blue atoms), 3′-methoxyflavone (**29**, green atoms), sciadopitysin (**50**, red atoms) and 6-bromo-3′-nitroflavone (**9**, yellow atoms).

volume that occur when the size of the ligand over-exceeds its optimum value (S3). This model is validated by the fact that larger hydrocarbon chains, like those defined by the 1-butyl and isopropyl esters of caffeic acid (compounds **47** and **48**), inhibit the binding. The compounds of the validation set, together with equol (compound **49**) (Fig. 4c and d), also validate the fact that a second hydrogen bond donor group, located in position 1 (O1) of the flavone derivatives, does not became necessary for binding.

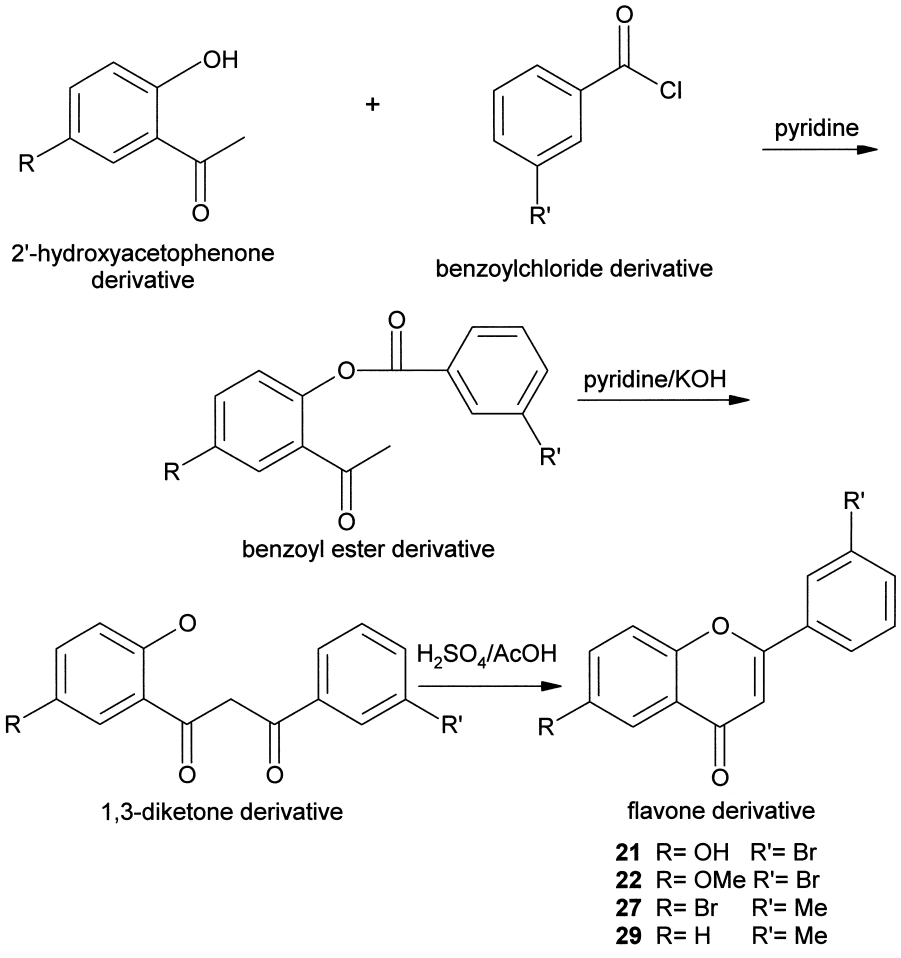
Figure 3 shows the superposition of several natural and synthetic flavonoids representative of the set. According to the previous discussion, important anchor points are defined by H2, L2, and L3 (Fig. 2b). We have not found a second H, nor an H bond acceptor site (A2), as requirements for binding, on the basis of the superposition analysis. We agree, on this point, with the results of Dekermendjian and co-workers.[46] The pharmacophore (Fig. 5), and the associated size of the ligand, should extend up to positions 6 and 3′ to the right and to the left of the carbonyl group of the flavones, respectively.

In agreement with this fact, it has been experimentally found, from the analysis of 37 compounds, that carbon atoms 6 and 3′ in the flavone nucleus are the most effective positions to place substituents.[37,39] According to Cook's model,[19] substituents in position 6 efficiently fill the lipophilic pocket L3 (see Fig. 1). In order to gain further insight on the relevance of positions 6 and 3′, we have performed a quantitative QSAR regression analysis including specific descriptors for the effect at these positions.

QSAR analysis

Tables 2 and 3 show the values of the parameters for positions 6 and 3′ of the flavone nucleus that have been included in the regression analysis and lead to more representative results. The descriptors, defined in the computational section, have been grouped to give sets of non-dependent variables.

The descriptors in Table 2 that quantify the electronic effects are derived from quantum chemical calculations. For this set of descriptors the inclusion of all the variables in the correlation renders a good correlation coefficient ($r = 0.83$). However, the coefficients show that the associated equation is not significative. The best correlation is achieved for a multiparametric relation of $\mathrm{Ln}(1/K_i)$ versus H-L, $\pi 3'$, $\pi 6$ and O (eq (1); n = numbers of compounds; r = correlation coefficient; s = standard deviation; F = Fisher value).



Scheme 3. Synthesis of 6-hydroxy-3′-bromoflavone (**21**), 6-methoxy-3′-bromoflavone (**22**), 6-bromo-3′-methylflavone (**27**) and 3′-methylflavone (**29**).

$$\mathrm{Ln}(1/K_i) = -2.066(\pm1.47)\mathrm{H} - \mathrm{L} + 1.408(\pm1.12)\pi6 + 2.657(\pm0.97)\pi3' + 121.741 (\pm64.71)\mathrm{O} + 53.669(\pm18.71)$$
$$(n = 37;\ r = 0.834;\ s = 1.22;\ F = 18.28) \quad (1)$$

The coefficient that precedes the charge on the carboxylic oxygen shows the statistical weight of this variable, and supports the requirement, derived from the superposition analysis, that a negatively charged oxygen should be present in the active structure (Fig. 3). Lipophilic effects on positions 6 and 3′ should also be noticed.

Accepting the primary requirement focused on the negative oxygen, we have analyzed the effect of substituents on positions 6 and 3′, defining a set of independent variables, based on classical descriptors, that accurately describe them (Table 3). Good correlations are attained ($r > 0.85$) when hydrophobic and/or steric descriptors that characterize position 6 are associated with electronic and/or steric descriptors that characterize position 3′ (eqs (2), (3), and (4)). No combination that includes an electronic descriptor for position 6 renders a good correlation.

$$\mathrm{Ln}(1/K_i) = -1.260(\pm0.49)\mathrm{Es6} + 2.417(\pm0.84)\pi6 + 3.912(\pm1.22)\sigma3' - 1.156(\pm0.61) \quad (2)$$
$$(n = 37;\ r = 0.904;\ s = 0.929;\ F = 49.38)$$

$$\mathrm{Ln}(1/K_i) = 2.798(\pm1.39)\mathrm{MR6} - 1.161(\pm0.48)\mathrm{Es3'} + 1.784(\pm1.14)\pi6 - 1.282(\pm0.85) \quad (3)$$
$$(n = 37;\ r = 0.851;\ s = 1.142;\ F = 28.98)$$

Table 2. Physicochemical parameters used in the QSAR analysis that lead to eq (1) and binding affinities for the BDZ-bs of synthetic flavonoids used in the training set

n	Ln(1/K_i)	MR6[a]	MR3′[a]	HOMO[b]	LUMO[b]	H–L[b]	π6[a]	π3′[a]	O[b]
1	2.659	0.890	0.100	−9.381	−0.930	8.449	0.860	0.000	−0.300
2	2.120	0.561	0.100	−9.157	−0.750	8.400	0.560	0.000	−0.307
3	1.808	0.600	0.100	−9.343	−0.910	8.433	0.710	0.000	−0.300
4	1.290	0.740	0.100	−9.760	−1.650	8.109	0.780	0.000	−0.289
5	0.545	0.280	0.100	−9.026	−0.820	8.207	−0.670	0.000	−0.300
6	0.151	0.790	0.100	−8.938	−0.777	8.161	−0.020	0.000	−0.301
7	−1.504	0.090	0.100	−9.430	−0.960	8.470	0.140	0.000	−0.302
8	0.000	0.100	0.100	−8.980	−0.580	8.400	0.000	0.000	−0.301
9	6.901	0.890	0.740	−8.680	−1.530	7.150	0.860	0.010	−0.287
10	5.185	0.561	0.740	−9.430	−1.330	8.100	0.560	0.010	−0.296
11	4.820	0.600	0.740	−8.615	−1.540	7.075	0.710	0.010	−0.292
12	3.644	0.740	0.740	−10.288	−1.780	8.580	0.010	0.010	−0.275
13	1.715	0.090	0.740	−9.619	−1.420	8.199	0.140	0.010	−0.287
14	1.255	0.100	0.740	−9.630	−1.450	8.180	0.000	0.010	−0.298
15	3.963	0.890	0.890	−9.490	−1.080	8.410	0.860	0.860	−0.295
16	4.343	0.561	0.890	−9.250	−0.924	8.326	0.560	0.860	−0.304
17	3.772	0.600	0.890	−9.438	−1.070	8.368	0.710	0.860	−0.297
18	3.689	0.740	0.890	−9.880	−1.736	8.144	0.010	0.860	−0.285
19	1.444	0.090	0.890	9.430	−1.090	8.340	0.140	0.860	−0.296
20	0.884	0.100	0.890	−9.420	−0.939	8.480	0.000	0.860	−0.302
21	0.000	0.280	0.890	−9.113	−0.978	8.135	−0.670	0.860	−0.296
22	0.000	0.790	0.890	−9.020	−0.938	8.081	−0.020	0.860	−0.297
23	4.075	0.890	0.600	−9.480	−1.070	8.410	0.860	0.710	−0.296
24	3.772	0.600	0.600	−9.430	−1.056	8.374	0.710	0.710	−0.297
25	1.614	0.090	0.600	−9.420	−1.080	8.340	0.140	0.710	−0.296
26	0.488	0.100	0.600	−9.410	−0.930	8.480	0.000	0.710	−0.303
27	1.870	0.890	0.561	−9.359	−0.900	8.460	0.860	0.560	−0.300
28	1.570	0.561	0.561	−9.140	−0.720	8.420	0.560	0.560	−0.308
29	−2.302	0.100	0.561	−9.270	−0.740	8.529	0.000	0.560	−0.307
30	−0.875	0.100	0.790	−9.220	−0.825	8.414	0.000	−0.020	−0.307
31	−1.267	0.100	0.090	−9.430	−0.957	8.473	0.000	0.140	−0.302
32	3.170	0.890	0.090	−9.490	−1.100	8.390	0.860	0.140	−0.295
33	0.496	0.890	0.790	−9.314	−0.956	8.358	0.860	−0.020	−0.308
34	2.146	0.600	0.090	−9.449	−1.080	8.369	0.710	0.140	−0.297
35	0.160	0.600	0.790	−9.289	−0.939	8.350	0.710	−0.020	−0.300
36	0.083	0.090	0.090	−9.440	−1.100	8.340	0.140	0.140	−0.295
37	−0.916	0.090	0.790	−9.295	−0.970	8.324	0.140	−0.020	−0.299

[a]Physicochemical parameters taken from ref 48.
[b]Descriptors derived from AM1 semiempirical calculations. Both sets are defined in the text in Computational Methods.

$$\mathrm{Ln}(1/K_i) = 2.770(\pm 1.18)\mathrm{MR6} + 1.999(\pm 0.95)\pi 6 + 4.291(\pm 1.27)\sigma 3' - 1.331(\pm 0.69) \quad (4)$$

$$(n = 37;\ r = 0.896;\ s = 0.965;\ F = 45.03)$$

The good correlations of hydrophobic and steric descriptors for positions 6 and 3′ against the binding capability validates the inference, derived from the superposition analysis, of the existence of an optimum size to allow an efficient binding, which is accurately defined by these centers. The negative sign of the coefficients of Es in eqs (2) and (3) shows the negative effect that an increase of the size exerts on the binding.

The fact that an electronic descriptor for position 3′ also renders a good correlation (eqs (2) and (4)) points to this position as the most effective for the introduction of substituents that modify the electronic density in the flavonoid system. Higher binding is associated with electronic deactivation induced by a substituent in this position (NO_2, Table 1), indicating that an electron rich site (ER) is likely to be present in the receptor site of the flavone derivatives (FS). The interaction of the carbonyl with a hydrogen donor group of the receptor fits in a direct correlation, indicative of an increase in the binding as the negative charge on the oxygen increases. Both electronic effects should be balanced for a most efficient binding.

A more thorough analysis of the effect of substitution in position 3′ indicates that because σ3′ describes deactivation due to inductive effects, it is restricted to the B ring of the flavone structure. This fact leads us to postulate the presence of an ER center close to the lipophilic pocket L2, capable of interacting with position 5′ in the B ring (see Fig. 2b). As has been previously mentioned, aromaticity of the B ring of the flavone structure is defined as a requirement for the activity after the QSAR analysis, which is necessary to achieve efficient deactivation by means of substitution in the 3′ position. This fact shows SAR and QSAR as complementary methodologies, as they are used in the present research.

The importance of this ER center in relation to the strength of the binding led us to consider the superposition shown in Figure 6, which is attained from the one shown in Figure 2a by means of a 180° rotation of the flavone about the axis defined by O1-C4-O4. This superposition allows the association of ER with A3. As for the other superpositions previously considered, only one H center is also necessary, together with L3, S3, and S2. However, this model does not justify the importance of position 6, neither in relation to a maximum size to the right end of the flavone derivative, nor in the necessity of filling the lipophilic pocket L2.

It has been found experimentally that substitution in position 4′ has a negative effect on the binding affinity.[43] This position is capable of interacting with L2, but this would result in a positive effect on the binding, which is not found. The low affinity of the 4′-substituted flavones can be explained through the interaction with the ER site (close to L2), giving, on the other hand, further support to the existence of this center.

On the basis of this analysis we suggest the fitting to the receptor shown in Figure 2b, which introduces the described lipophilic, steric and electronic requirements. However, regardless of the model pharmacophore chosen (Figs 2a, b and 6) the requirements of the FS are fully defined by one H, three L, three S centers and the proposed ER center (Fig. 7).

Table 3. Physicochemical parameters used in the QSAR analysis that lead to eqs (2), (3), and (4) and binding affinities for the BDZ-bs of synthetic flavonoids used in the training set

n	Ln(1/K_i)	MR6[a]	MR3′[a]	Es6[a]	Es3′[a]	π6[a]	π3′[a]	σ6[a]	σ3′[a]
1	2.659	0.890	0.100	−1.340	0.000	0.860	0.000	0.270	0.000
2	2.120	0.561	0.100	−1.120	0.000	0.560	0.000	−0.160	0.000
3	1.808	0.600	0.100	−1.140	0.000	0.710	0.000	0.230	0.000
4	1.290	0.740	0.100	−2.520	0.000	0.780	0.000	0.780	0.000
5	0.545	0.280	0.100	−0.550	0.000	−0.670	0.000	−0.380	0.000
6	0.151	0.790	0.100	−0.550	0.000	−0.020	0.000	−0.210	0.000
7	−1.504	0.090	0.100	−0.550	0.000	0.140	0.000	0.210	0.000
8	0.000	0.100	0.100	0.000	0.000	0.000	0.000	0.000	0.000
9	6.901	0.890	0.740	−1.340	−2.520	0.860	0.010	0.270	0.710
10	5.185	0.561	0.740	−1.120	−2.520	0.560	0.010	−0.160	0.710
11	4.820	0.600	0.740	−1.140	−2.520	0.710	0.010	0.230	0.710
12	3.644	0.740	0.740	−2.520	−2.520	0.010	0.010	0.780	0.710
13	1.715	0.090	0.740	−0.550	−2.520	0.140	0.010	0.210	0.710
14	1.255	0.100	0.740	0.000	−2.520	0.000	0.010	0.000	0.710
15	3.963	0.890	0.890	−1.340	−1.340	0.860	0.860	0.270	0.390
16	4.343	0.561	0.890	−1.120	−1.340	0.560	0.860	−0.160	0.390
17	3.772	0.600	0.890	−1.140	−1.340	0.710	0.860	0.230	0.390
18	3.689	0.740	0.890	−2.520	−1.340	0.010	0.860	0.780	0.390
19	1.444	0.090	0.890	−0.550	−1.340	0.140	0.860	0.210	0.390
20	0.884	0.100	0.890	0.000	−1.340	0.000	0.860	0.000	0.390
21	0.000	0.280	0.890	−0.550	−1.340	−0.670	0.860	−0.380	0.390
22	0.000	0.790	0.890	−0.550	−1.340	−0.020	0.860	−0.210	0.390
23	4.075	0.890	0.600	−1.340	−1.140	0.860	0.710	0.270	0.370
24	3.772	0.600	0.600	−1.140	−1.140	0.710	0.710	0.230	0.370
25	1.614	0.090	0.600	−0.550	−1.140	0.140	0.710	0.210	0.370
26	0.488	0.100	0.600	0.000	−1.140	0.000	0.710	0.000	0.370
27	1.870	0.890	0.561	−1.340	−1.120	0.860	0.560	0.270	−0.100
28	1.570	0.561	0.561	−1.120	−1.120	0.560	0.560	−0.160	−0.100
29	−2.302	0.100	0.561	0.000	−1.120	0.000	0.560	0.000	−0.100
30	−0.875	0.100	0.790	0.000	−0.550	0.000	−0.020	0.000	0.120
31	−1.267	0.100	0.090	0.000	−0.550	0.000	0.140	0.000	0.340
32	3.170	0.890	0.090	−1.340	−0.550	0.860	0.140	0.270	0.340
33	0.496	0.890	0.790	−1.340	−0.550	0.860	−0.020	0.270	0.120
34	2.146	0.600	0.090	−1.140	−0.550	0.710	0.140	0.230	0.340
35	0.160	0.600	0.790	−1.140	−0.550	0.710	−0.020	0.230	0.120
36	0.083	0.090	0.090	−0.550	−0.550	0.140	0.140	−0.210	0.340
37	−0.916	0.090	0.790	−0.550	−0.550	0.140	−0.020	−0.210	0.120

[a]Physicochemical parameters were obtained from ref 48 and are defined in Computational Methods.

Subtype selectivity of flavonoids

1,4-BDZs are non-selective ligands[51] that bind to all BDZ-bs isoforms which are diazepam sensitive (DS). As a result, most of the BDZs display a wide range of pharmacological activities, such as anxiolytic/anticonvulsant, sedative/hypnotic, ataxic/myorelaxant, and amnesiac. Nevertheless, recent experiments suggest that

agents selective for specific BDZ-bs subtypes may permit us to discriminate between the various pharmacological activities controlled by these isoforms.[28]

Several studies[19,22,23,27] have shown that region L2 is larger in size in the pharmacophore of the α5-containing subtypes than the analogous region in other receptor subtypes and that the occupation of region L2 with lipophilic groups is important for α5 subtype selectivity as well as for high affinity.

It was also found that the lipophilic pocket L3 of the α6 subtype diazepam insensitive (DI) is very small or non-existent.[19] This was based on the inactivity of 1,4-BDZs which do not bind to this α6-containing receptor subtype. Ligands which bind to this DI site are small and are represented mostly by imidazobenzodiazepines and pyrazoloquinolinones. The CGS series of compounds bind to both diazepam sensitive and insensitive BDZ-bs (α1 to α6 isoforms).[27] For example, for CGS-8216 binding to α(1–5)β3γ2 receptor subtype has K_i values of 0.05–0.25 nM and for the α6β3γ2 a K_i of 17 nM.[27]

Our modeling studies and the QSAR analysis, that is based exclusively on flavonoid structures and DZ, lead to a pharmacophore model for the FS (Fig. 7) that fits well with the model currently accepted for the BDZ-bs (Fig. 1).[19]

Figure 4. Superimposition of flavone (**8**, blue atoms) with diazepam (**51**, unfilled atoms) in (a) and (b), cinnamic acid (**43**, red atoms) in (c), and equol (**49**, yellow atoms) in (d).

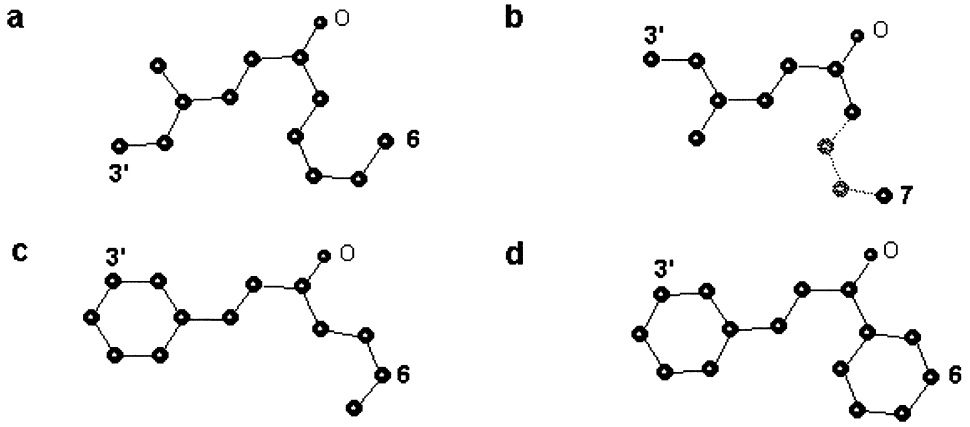


Figure 5. Pharmacophores derived from the superpositions shown in Figure 4. The letters refer to the same systems as in Figure 4. In Figure 4b the dashed line indicates a distance inferior to 1 Å between superimposed centers.

Preliminary experiments with recombinant human $GABA_A$ receptor subtypes revealed that among nine natural flavonoids (flavone, chrysin, apigenin, amentoflavone, kaempferol, flavanone, naringenin, genistein and phloretin) and three synthetic flavone derivatives (6-bromoflavone, 6,8-dibromochrysin and 2′-chlorochrysin) none had affinity for the recombinant human $\alpha6\beta3\gamma2$-$GABA_A$ receptor. For example, the K_i values for inhibition of [^{3}H]Ro 15-1788 binding in membranes from cells expressing $\alpha(1–5)\beta3\gamma2$ receptor subtypes and for inhibition of [^{3}H]Ro 15-4513 in membranes from cells expressing $\alpha6\beta3\gamma2$ receptor subtypes are 3.5–8 nM and $>10\,\mu M$ for amentoflavone, respectively; and 0.53–2.56 μM and $>10\,\mu M$ for 6,8-dibromochrysin, respectively. Parallel experiments realized with flunitrazepam arrived at K_i values of 2.1–4.5 nM and $>2\,\mu M$, respectively (unpublished results). Competition binding curves were carried out at recombinant human $GABA_A$ receptor of composition $\alpha(1–6)\beta3\gamma2$ according to the methods described by Hadingham and co-workers.[52]

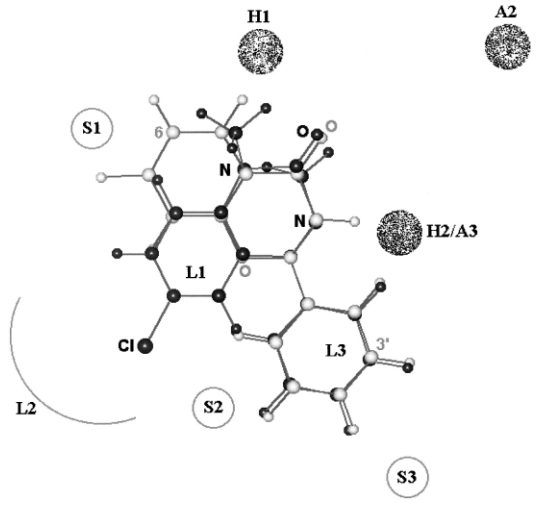


Figure 6. Superimposition of flavone (**8**, unfilled atoms) with diazepam (**51**, filled atoms). The flavone structure is rotated 180° relative to the orientation considered in Figure 2a.

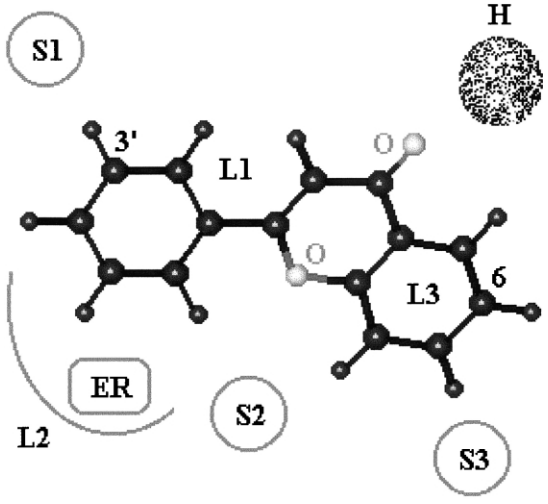


Figure 7. The flavone molecule fitted in the FS pharmacophore model, as established in the present work.

We found in the molecular modeling and in the QSAR analysis that the filling of L3 in the FS is influenced by negative steric effects. This is interpreted as showing that L3 is small in size, which is in agreement with the fact that flavonoids do not bind to the $\alpha6\beta3\gamma2$-$GABA_A$ receptor subtype. L2, on the other hand, accepts bulky substituents, as is the case of the second flavonoid unit in the biflavone shown in Figure 3, a fact that might reflect an interaction with α5 BDZ-bs subtype.

Comparison with the previous model for the FS

As has been previously mentioned, Dekermendjian and co-workers[46] have described how Cook's receptor/pharmacophore model can be shown to accept flavonoid derivatives, on the basis of a similarity analysis where the partial agonist CGS-9896 was used as template. The comparison of their data with our present results is not simple because the reference compounds used by both groups (CGS-9896 and DZ, respectively) have different structures. In addition, the flavonoid structure in the Dekermendjian model is placed in such a position within the pharmacophore/receptor which does not allow structural superposition with DZ.

Models, however, agree on the importance of the interaction of the flavonoids with an H bond donor group. Dekermendjian and co-workers, on the other hand, give more weight to the lipophilic pocket L2 than to L3. It should be noticed that L2 in the Dekermendjian model and L3 in ours are both related to the interaction of position 6 of the flavone nucleus with the receptor site. Both studies agree, thence, on the importance of position 6 of the ligand. Also, A2 is not considered necessary for flavone binding in any of the models.

In relation to the B ring of the flavone structure, Dekermendjian and co-workers have not discussed the importance of substitution in position 3′, which we associate with the existence of an ER center in the FS.

When the flavones are superimposed with DZ (Fig. 2b), the O1 center does not became necessary for binding. Further support for this statement is given by the consideration of the compounds of the validation set (Fig. 4c and d). At variance with this finding, Dekermendjian and co-workers,[46] after superimposing the flavones with CGS-9896, locate O1 as interacting with H2 which renders necessary two H centers in their postulated model.

As discussed before, DZ and flavonoids do not bind to the $\alpha6\beta3\gamma2$-$GABA_A$ receptor subtype, where L3 does not exist or is very small. CGS-9896 binds to this receptor subtype, a fact that casts doubts on the success of the superposition studies when it is used as template.[46] The choice of using DZ, as in the present research, seems to define a safer decision, since flavonoids and DZ bind to the same receptor subtypes.

Conclusions

We have shown that the pharmacophore for the flavonoid-ligands fits in the model proposed for the BDZ-bs by Cook and co-workers. The superposition analysis of the flavonoids and the 1,4-BDZs shows the importance of steric and electronic effects for the binding of both types of ligands. The superposition shown in Figure 2b defines the best choice, according to the results obtained in this work. QSAR studies point to a steric effect associated with substitution in position 6 together with an electronic effect for 3′ substitution of the flavone derivatives. A hydrogen acceptor group in the carbonyl site appears as a requirement for binding capability.

The requirements in the FS are defined by one H, one ER, three L and three S centers (Fig. 7).

Experimental

Chemistry

The syntheses of 6-hydroxy-3′-bromoflavone (**21**), 6-methoxy-3′-bromoflavone (**22**), 6-bromo-3′-methylflavone (**27**) and 3′-methylflavone (**29**) were carried out according to procedures that have previously been described for other flavones.[39,40] The synthesis involved the reaction of 2′,5′-dihydroxy-acetophenone, 2′-dihydroxy-5′-methoxy-acetophenone, 2′-dihydroxy-5′-bromo-acetophenone or 2′-dihydroxy-acetophenone with 3-bromo-benzoylchloride or 3-methyl-benzoylchloride, respectively. The products obtained were recrystallized from ethanol/water and their identification was achieved on the basis of ^{1}H NMR and mass spectra.[53]

Radioreceptor binding assays

Displacement curves were performed using [^{3}H]flunitrazepam (84 Ci/mmol, NEN) as radioligand in washed crude synaptosomal membranes from rat cerebral cortex. Membranes were prepared according to Medina and co-workers (1990).[30] Briefly, the brains were rapidly dissected out on ice and the different structures were homogenized in 10 volumes of 0.32 M sucrose and centrifuged at $900\times g$ for 10 min. The resulting supernatant was centrifuged at $100{,}000\times g$ for 30 min and the pellet washed twice in 25 mM Tris–HCl buffer (pH 7.4) at $100{,}000\times g$ for 30 min, and stored at -20 °C until used.

For the displacement curves, various concentrations of the compounds were added to 0.3 mg membrane protein suspended in 1 mL of 25 mM Tris–HCl buffer in presence of 0.6 nM of the radioligand. Non-specific binding ($<5\%$) was determined in parallel incubations with 10 μM flunitrazepam (Hoffmann-La Roche). The incubation was carried out at 4 °C for 1 h. The assays were terminated by filtration under vacuum through Whatman GF/A glass fiber filters, and two washes with 3 mL each of incubation medium. The filters were dried and counted after the addition of 5 mL 2,5-diphenyl-oxazole-xylene as scintillation fluid.

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53. The reagents for the investigation and syntheses were of the highest purity commercially available and were used without further purification. EIMS were measured in a Shimadzu QP-1000 quadrupole mass spectrometer. NMR spectra were recorded in a Bruker AMX 400 spectrometer, at room temperature; the chemical shifts are presented in ppm and the solvent signals were used as reference. **6-Hydroxy-3′-bromoflavone** (**21**): EIMS M^+ 316 and 318 ($C_{15}H_9O_3Br$); ^1H NMR ($CDCl_3$, 400 MHz) δ 8.25 (1H, t, $J=1.9$ Hz), 8.04 (1H, dt, $J=1.1$ and 7.8 Hz), 7.75 (2H, m), 7.56 (1H, d, $J=9.0$ Hz), 7.37 (2H, m), 6.93 (1H, s). **6-Methoxy-3′-bromoflavone** (**22**): EIMS M^+ 330 and 332 ($C_{15}H_9O_3Br$); ^1H NMR ($CDCl_3$, 400 MHz) δ 8.25 (1H, t, $J=1.8$ Hz), 8.05 (1H, dt, $J=1.4$ and 7.8 Hz), 7.75 (1H, ddd, $J=8.0$, 1.1 and 1.1 Hz), 7.61 (H, d, $J=3.1$ Hz), 7.53 (1H, d, $J=9.1$ Hz), 7.37 (2H, m), 6.93 (1H, s), 3.93 (3H, s). **6-Bromo-3′-methylflavone** (**27**): EIMS M^+ 314 and 316 ($C_{16}H_{11}O_2Br$); ^1H NMR ($CDCl_3$, 400 MHz) δ 8.34 (1H, d, $J=2.4$ Hz), 7.77 (1H, dd, $J=2.4$ and 8.8 Hz), 7.48 (1H, d, $J=8.8$ Hz), 7.39 (2H, m), 6.82 (1H, s), 2.46 (3H, s). **3′-Methylflavone** (**29**): EIMS M^+ 236 ($C_{16}H_{12}O_2$); ^1H NMR ($CDCl_3$, 400 MHz) δ 8.23 (1H, dd, $J=1.5$ and 7.9 Hz), 7.69 (1H, dd, $J=1.5$ and 8.6 Hz), 7.70 (2H, m), 7.58 (1H, dd, $J=1.1$ and 8.3 Hz), 7.40 (3H, m), 6.83 (1H, s), 2.46 (3H, s).
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