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Synergistic interaction between hesperidin, a natural flavonoid, and diazepam

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Abstract

It has been recently reported the presence in Valeriana of the flavone 6-methylapigenin and the flavanone glycoside hesperidin. The apigenin derivative is a ligand for the benzodiazepine binding site in the γ-aminobutyric acid receptor type A ($GABA_A$) and has anxiolytic properties. Hesperidin has sedative and sleep-enhancing properties but is not a ligand for the benzodiazepine binding site. 6-Methylapigenin is able to potentiate the sleep-enhancing effect of hesperidin. In this work we demonstrate that this property is shared with various $GABA_A$ receptor ligands, among them the agonist diazepam, which was used to study the potentiation as measured in the hole board test. Isobolar analysis of the results showed the interaction being synergistic. We discarded pharmacokinetic effects or a direct action of hesperidin on the benzodiazepine binding site.

A possible use of hesperidin properties to decrease the effective therapeutic doses of benzodiazepines is suggested.

Keywords: Flavonoid; Hesperidin; Diazepam; Synergy; Sedation; Sleep-enhancing action

1. Introduction

Enhancement of the inhibitory effect of γ-aminobutyric acid (GABA) acting on its type A receptor in the mammalian brain explains the pharmacological and therapeutic actions of benzodiazepines which bind to a specific site in the $GABA_A$ receptor. However, the usefulness of benzodiazepines as anxiolytics, sedatives, anticonvulsants and muscle relaxants is compromised by the occurrence of several adverse effects such as ataxia, amnesia, alcohol intolerance and residual sedation as well as the related problems of tolerance and dependence after chronic use. Molecular biology studies have established the heterogeneity of $GABA_A$ receptors and the pharmacological and electrophysiological study of the different subtypes has allowed the search of new ligands with improved selectivity which eventually may lead to the development of safer drugs (Möhler et al., 2002, 2004; Vicini and Ortinski, 2004).

Flavonoids have recently attracted interest as new chemical entities with activity on the central nervous system (CNS) (Wang et al., 1999; Marder and Paladini, 2002). We have demonstrated that some naturally occurring flavonoids possess a selective and relatively mild affinity for the central benzodiazepine binding site in the $GABA_A$ receptor, and exert anxiolytic but not depressant effects in rodents (Medina et al., 1990; Wolfman et al., 1994; Viola et al., 1995). Several flavone derivatives with added electronegative groups have been synthesized by our group and found to have enhanced affinities for the benzodiazepine binding site. These compounds are potent anxiolytics in rodents without exerting sedative or myorelaxant effects (Medina et al., 1997; Marder and Paladini, 2002).

The presence of two neuroactive flavonoids in the sedative plants *Valeriana wallichii* and *Valeriana officinalis* has been recently described by us (Wasowski et al., 2002; Marder et al., 2003). They are the flavone 6-methylapigenin and the flavanone 2*S*(−)-hesperidin (Fig. 1). The apigenin

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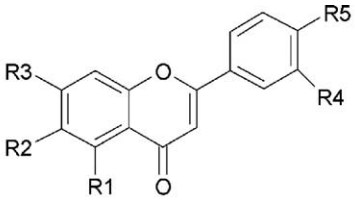

Compound	R1	R2	R3	R4	R5
6-methylapigenin	OH	CH_3	OH	H	OH
Chrysin	OH	H	OH	H	H
Apigenin	OH	H	OH	H	OH
6-methylflavone	H	CH_3	H	H	H
6,3'-dinitroflavone	H	NO_2	H	NO_2	H

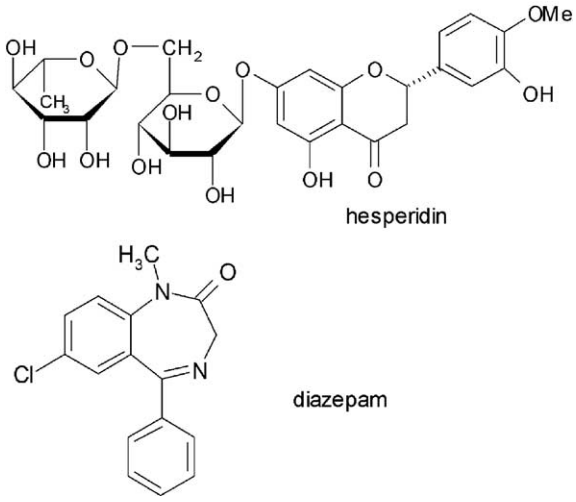

Fig. 1. Molecular structure of the drugs used in the behavioral assays.

derivative exhibits medium–high affinity for the benzodiazepine binding site (K_i=495 nM), and has anxiolytic effects in mice. Hesperidin, in turn, has sedative and sleep-enhancing properties but it is not a ligand for the benzodiazepine binding site. When hesperidin and 6-methylapigenin are jointly injected i.p. in mice during a thiopental-induced sleeping time test, the resultant effect suggests a greater than additive interaction between both drugs (Marder et al., 2003), 6-methylapigenin, however, does not potentiate the sedative action of hesperidin in the hole board test at the doses tested. In this paper we show that a potentiation occurs when hesperidin interacts with other benzodiazepine binding site ligands in the thiopental-induced sleeping time and in the hole board tests. The nature of this interaction was evaluated by an isobolar analysis of the data obtained with hesperidin and the classical agonist diazepam in the hole board assay. The analysis demonstrates that synergism is present when these drugs are co-injected. Although the mechanism of the CNS activity of hesperidin is still unknown, we have discarded a direct action of hesperidin on the plasma levels of diazepam after injection and/or direct effect on the benzodiazepine binding site.

The main goal of pharmacotherapy using a combination of different drugs with similar effects is to increase the efficacy of the treatment while decreasing its toxicity. Multiple-drug therapy has been applied in medical areas such as hypertension, chemotherapy, antibiotic therapy, asthma, etc. (Berenbaum, 1989; Rosow, 1997). The recent findings of Campbell et al. (2004) showing that flavonoids enhance diazepam modulatory action at recombinant $GABA_A$ receptors, plus the synergistic interaction in vivo between hesperidin and diazepam described here, suggest that flavonoids, besides being potentially valuable single drugs, may also be used with advantage in combination with benzodiazepines.

2. Materials and methods

2.1. Drugs and injection procedures

The drugs used to perform the behavioral experiments are shown in Fig. 1 and were obtained as follows: hesperidin isolated by us from *V. wallichii* as described by Marder et al. (2003) was used in the sodium thiopental-induced sleeping time test; genuine hesperidin from SIGMA, USA, was used in the hole board test; diazepam was from Hoffmann-La Roche; 6-methylapigenin was isolated by us from *V. wallichii* as described by Wasowski et al. (2002); apigenin, chrysin and 6-methylflavone were from SIGMA, USA; 6,3′-dinitroflavone was synthesized by us (Marder et al., 1995).

The drugs were dissolved by the sequential addition of: dimethylsulfoxide up to a final concentration of 5%, a solution of 0.25% Tween 80 up to a final concentration of 20%, and saline to complete 100% volume. Sodium thiopental (Fada, Biochemie Gesellschaft m.b.H., Kundl/Tirol, Austria) was dissolved in saline. The rodents were i.p. injected 20 min before performing the pharmacological tests. The volume of i.p. injections was 0.15 ml/30 g of body weight. In each session, a control group receiving only vehicle was tested in parallel with those animals receiving drug treatment.

2.2. Animals

Adult male Swiss mice weighing 25–30 g were used in the pharmacological assays. Animals were housed in a controlled environment (20–23 °C), with free access to food and water and maintained on a 12 h/12 h day/night cycle. Housing, handling, and experimental procedures complied with the recommendations of the European

Community guidelines for the use of experimental animals.

Behavioral experiments were conducted from 10:00 AM to 2:00 PM.

2.3. Behavioral studies

2.3.1. Sodium thiopental-induced sleeping time assay

A sub-hypnotic dose of sodium thiopental (35 mg/kg) was i.p. injected to mice 20 min after a similar injection of vehicle or the drug. Sleeping time was determined as the interval between the loss and the recovery of the righting reflex (Ferrini et al., 1974). For statistical analysis, Dunn's multiple comparison test was used after Kruskal–Wallis test (nonparametric analysis of variance). Significance was reported starting at the 0.05 level.

2.3.2. Hole board assay

This assay was conducted in a walled acrylic arena with a floor of 60 cm×60 cm and 30-cm-high walls, with four equally spaced holes in the floor, 2 cm in diameter each. The holes housed an infrared light emitting diode. The interruption of the light beam by an exploring mouse during, at least 100 ms, triggers a counting device that records, in a computer, the number of head-dips and the time head-dipping. The mice were placed singly at the center of the board, facing away from the observer and the number and time of holes explored as well as the number of rearings, in a 5-min session, were recorded. After each trial, the apparatus was wiped clean to remove traces of the previous assay. A decrease in the number of head-dips, the time spent head-dipping, and/or the number of rearings, reveals a sedative behavior (File and Pellow, 1985).

2.4. Data analysis

2.4.1. Fitting dose–response curves

In order to study the interaction between hesperidin and diazepam in the hole board test, dose–response curves for hesperidin, diazepam and three different fixed-ratio combinations of the drugs (Table 1) were obtained. Dose–response data are presented as the percent of response for each parameter (number of rearings, number of head-dips or time spent head-dipping), calculated as follows:

$$\text{Percent of response} = (\text{compound treatment}/\text{vehicle treatment}) \times 100 \quad (1)$$

All results are expressed as mean ± standard error (S.E.M.). Groups of six or more animals were used for each dose (Table 1). Data were fitted to sigmoidal dose–response (variable slope) curves using nonlinear regression. Maximal effect and ED_{50} were estimated and compared by the extra sum-of-squares F test. F ratio and P value cited in this paper are derived from this test (Prism 4.00, GraphPad Software).

Table 1
Doses of hesperidin, diazepam and fixed-ratio combinations used in the hole board test, n=number of mice

Hesperidin/diazepam fixed-ratio combinations	Hesperidin dose (mg/kg)	Diazepam dose (mg/kg)	n
Hesperidin alone	0.3	0	6
	1	0	6
	2	0	7
	4	0	11
	9	0	7
	30	0	6
	50	0	6
13:1	1	0.075	6
	2	0.15	6
	4	0.3	8
	8	0.6	7
	26	2	7
4:1	1	0.25	6
	2	0.5	6
	4	1	8
	20	5	7
1:13	0.075	1	7
	0.15	2	7
	0.3	4	7
	0.6	8	6
	2	26	7
Diazepam alone	0	0.3	11
	0	1	8
	0	1.5	6
	0	3	8
	0	6	7
	0	9	8
	0	18	6
	0	30	7

2.4.2. Isobolar analysis

A graphical assessment of synergy was carried out using isobolographic analysis (Berenbaum, 1989; Tallarida et al., 1989; Tallarida, 1992). In an isobologram, the equieffective pairs of doses of two drugs are represented using rectangular coordinates. In the present study the dose of hesperidin needed to reach 50% effect is plotted on the abscissa and the isoeffective dose of diazepam on the ordinate. The straight line connecting these two points represents the theoretical additive combination of the two drugs. If the experimentally determined data points and their confidence interval (CI) lie on this line, then the drug effects are purely additive (no interaction). If the points lie below this line, then there is super-additivity (synergy), and if they lie above this line, then there is sub-additivity (antagonism) (Berenbaum, 1989).

The interaction index is an assessment of the degree of synergism or antagonism. The index, denoted by γ, is defined by the isobolar relation:

$$\gamma = a/A + b/B \quad (2)$$

where A and B are the doses of drug A (alone) and B (alone), respectively, that give the specified effect and (a,b) are the combination doses that cause the same effect. The quantities in Eq. (2) are obtained from the dose response

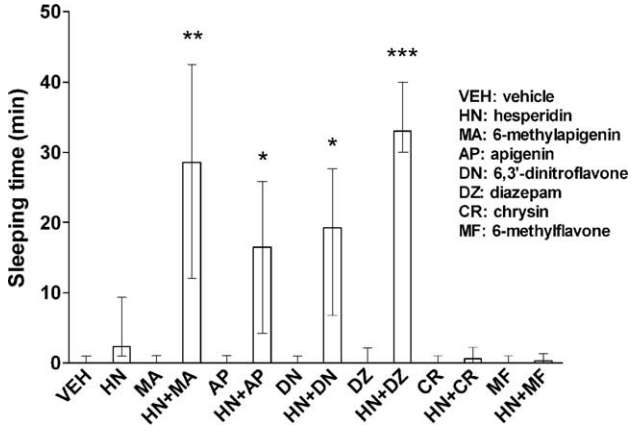

Fig. 2. Effect of the i.p. injection of hesperidin and several ligands for the benzodiazepine binding site, alone or combined with hesperidin, on the sodium thiopental-induced sleeping time in mice. Individual doses are: hesperidin (HN, 2 mg/kg i.p.), 6-methylapigenin (MA, 1 mg/kg), apigenin (AP, 1 mg/kg), 6,3'-dinitroflavone (DN, 0.02 mg/kg), diazepam (DZ, 0.3 mg/kg), chrysin (CR, 1 mg/kg), 6-methylflavone (MF, 1 mg/kg). Bars represent the median (interquartile range) of sleeping time of mice, 20 min after an i.p. injection of vehicle (VEH), or the drugs alone or combined. The sleeping time was measured as the time spent between disappearance and reappearance of the righting reflex (see Materials and methods). Number of mice per group ≥ 6. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, significantly different from vehicle; Dunn's Multiple Comparison test after Kruskal–Wallis non-parametric analysis of variance.

curves of drugs A, B, and their combinations. If $\gamma=1$ the interaction is additive; if $\gamma<1$ it is super-additive (synergy), and if $\gamma>1$ it is sub-additive (antagonism).

2.5. Pharmacokinetic study

2.5.1. Plasma sampling procedure

Mice were housed and treated in groups of five. The animals were anesthetized and sacrificed by heart exsanguination at 15, 30 and 90 min after receiving a single i.p. injection of diazepam (5 mg/kg) or a combination of diazepam (5 mg/kg) plus hesperidin (20 mg/kg). The doses of diazepam and hesperidin were chosen according to the results obtained in the isobolar analysis. Blood samples, collected in tubes containing EDTA, were centrifuged at $3000 \times g$ for 20 min at 3 °C to separate plasma.

2.5.2. Diazepam quantification

Diazepam was extracted from plasma by a liquid–liquid extraction method according to Nakashima et al. (2003) with slight modifications. In brief, acetonitrile (2.5 ml) was added to the plasma sample (1.5 ml) and the resultant mixture was centrifuged. To the supernatant, aqueous ammonia solution (25%, 150 μl) and chloroform (3 ml) were added. The organic layer was separated and evaporated to dryness. The residue was reconstituted with 50 μl of dimethylsulfoxide and 50 μl of a mixture of acetonitrile/water 1:2 (v/v); 50 μl of the resultant solution were injected into the column. Chromatographic quantification of diazepam was performed using a LKB Pharmacia HPLC apparatus with a C18 reversed phase Vydac column (5 μm, 0.46 cm×25 cm, The Separation Group, Hesperia, CA, USA) which was eluted with a lineal

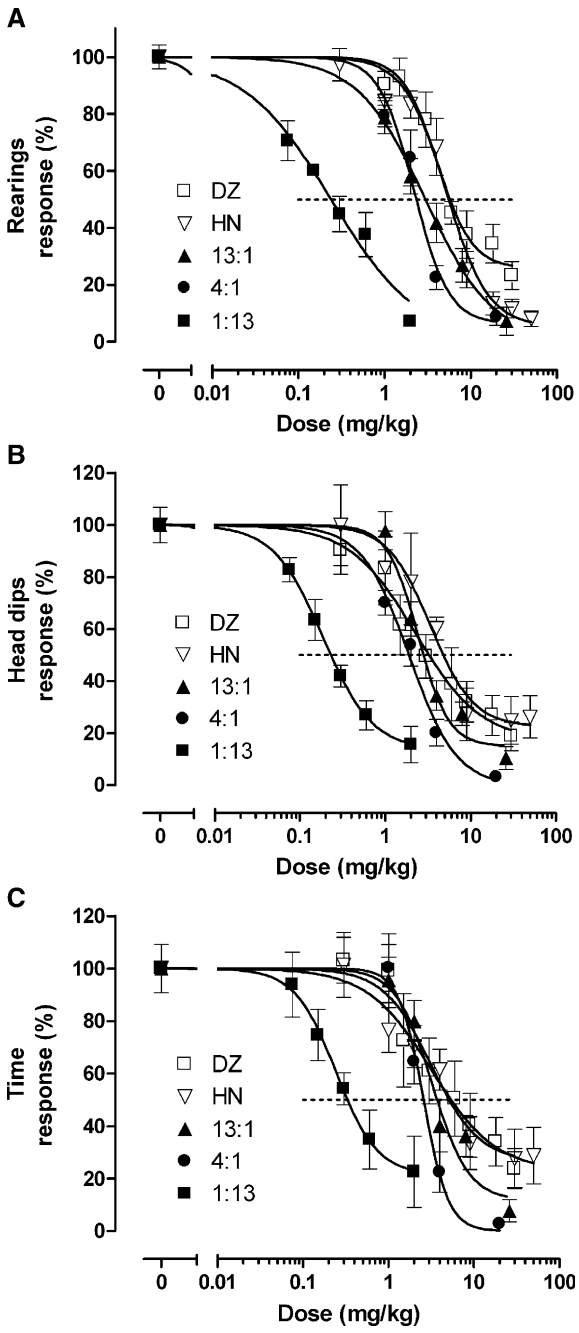

Fig. 3. Dose–response curves in the hole board test of diazepam (□), hesperidin (▽) and hesperidin/diazepam fixed-ratio combinations: 13:1 (▲), 4:1 (●) and 1:13 (■). Doses for hesperidin or diazepam mono injections are indicated; for fixed-ratio combinations the values in the abscissa represent the hesperidin doses. The apparent shift in the curve for combination 1:13 only reflects the low dose of hesperidin in this combination. Data are represented as mean ± S.E.M for the %number of rearings (A), %number of head-dips (B) and %time spent head-dipping (C) registered in a 5-min session in the hole board test performed 20 min after injection of increasing doses of hesperidin, diazepam, or hesperidin/diazepam combinations 13:1, 4:1 and 1:13. Doses and number of mice per dose are as described in Table 1.

gradient of acetonitrile in water rising in 20 min from 30% to 70% with a flow rate of 1 ml/min and monitoring the effluent by UV absorption at 280 nm. The areas of the eluted peaks of diazepam were obtained in a LKB integrator model 2221 connected in series. The concentration of diazepam in each experimental sample was calculated by reference to a calibration curve obtained with standard solutions of diazepam in mice plasma submitted to the same extraction and chromatographic procedure as described. All data are presented as mean ± S.E.M. of three independent assays. Statistical analysis was performed by Student's unpaired *t*-test with $P<0.05$ being considered significant.

2.6. Radioligand binding assay

A radioligand binding assay was used to evaluate a possible action of hesperidin on benzodiazepine binding. Mice, in groups of four, were housed and injected i.p. with hesperidin (10 mg/kg) or vehicle. After 20 min, animals were humanely killed and their cerebral cortices were dissected and pooled. Washed crude synaptosomal membranes from this material were obtained as previously described (Medina et al., 1990). Aliquots of synaptosomal membranes were resuspended in Tris–HCl buffer 25 mM, pH 7.4 (0.2–0.4 mg/ml) and incubated with [^{3}H]flunitrazepam (0.4–12 nM) in a final volume of 200 μl at 4 °C for 60 min. Nonspecific binding measured in the presence of 10 μM flunitrazepam, amounted to less than 15% of total binding. After incubation, the mixtures were filtered onto Whatman GF/B filters using a Brandel 96 channel cell harvester and washed three times with 200 μl of distilled water. Individual filters were incubated overnight with 0.5-ml scintillation cocktail (OptiPhase 'HiSafe' 3) before measurement of radioactivity in a Wallac Rackbeta 1214 liquid scintillation counter.

In each case, data were analyzed by nonlinear regression of specific bound vs. radioligand concentration and one site binding vs. two sites binding models were compared by the extra sum-of-squares *F* test. *F* ratio and *P* value cited in this paper are derived from this test. According to the best fit model, affinity constant (K_d) and number of binding sites (B_{max}) were estimated and compared (Prism 4.00, GraphPad Software).

3. Results

3.1. Sleep-enhancing action of hesperidin co-administered with different ligands for the benzodiazepine binding site

The effects of the single administration of hesperidin, 6-methylapigenin, apigenin, 6,3′-dinitroflavone, diazepam, chrysin and 6-methylflavone or of the co-administration of each of these compounds with hesperidin on the sleeping time of mice are shown in Fig. 2. The administration of the single drugs caused no significant increase on the sleeping time, at the doses tested. Most of these compounds, such as 6-methylapigenin, apigenin, 6,3′-dinitroflavone and chrysin, only possess depressant effects at high doses (Wolfman et al., 1994, 1996; Viola et al., 1995; Marder et al., 2003). The effect of each drug administered alone was compared to the response obtained when they were co-administered with hesperidin. The responses given by the combination of hesperidin with 6-methylapigenin, apigenin, 6,3′-dinitroflavone or diazepam showed a striking sleep-enhancing effect. Neither chrysin nor 6-methylflavone in combination with hesperidin had any effect on the sleeping time, at the doses assayed.

3.2. Sedative action of hesperidin and diazepam as measured in the hole board test

Hesperidin and diazepam reduced all three exploratory parameters in a dose-dependent manner (Fig. 3). Both drugs administered alone displayed similar efficacy, as their maximal effects were not significantly different ($F_{1,9}=4.38$, $P>0.05$ for rearings; $F_{1,9}=0.46$, $P>0.5$ for head-dipping and $F_{1,9}=0.07$, $P>0.5$ for time head-dipping). Also ED_{50} of single-drug treatments were similar (Table 2), however since the hesperidin used was a partially racemic drug (Marder et al., 2003), with app. 60% of the active 2S-diastereomer, it is clear that the sedative potency of hesperidin is higher than that of diazepam.

3.3. Synergistic effects of hesperidin plus diazepam in the hole board test

Three different fixed-ratio combinations of hesperidin and diazepam were tested (Table 1) and dose–response

Table 2
ED_{50} of hesperidin (HN), diazepam (DZ) and their fixed-ratio combinations in the hole board test

HN/DZ combinations	ED_{50}[a] (mg/kg) (95% CI)					
	Number of rearings		Number of head-dips		Time head-dipping (s)	
	HN	DZ	HN	DZ	HN	DZ
Hesperidin alone	5.52 (5.27, 5.79)	–	4.80 (4.49, 5.14)	–	5.38 (4.86, 5.98)	–
13:1	2.98 (2.92, 3.05)	0.23 (0.22, 0.24)	2.81 (2.69, 2.94)	0.22 (0.21, 0.23)	3.71 (3.47, 3.97)	0.29 (0.27, 0.31)
4:1	2.33 (2.22, 2.45)	0.58 (0.56, 0.61)	1.90 (1.82, 1.97)	0.48 (0.46, 0.49)	2.58 (2.51, 2.65)	0.65 (0.63, 0.66)
1:13	0.22 (0.16, 0.29)	2.86 (2.08, 3.77)	0.24 (0.23, 0.25)	3.12 (2.99, 3.25)	0.34 (0.33, 0.35)	4.42 (4.29, 4.55)
Diazepam alone	–	5.74 (5.45, 6.06)	–	3.24 (3.05, 3.45)	–	5.14 (4.66, 5.70)

[a] Effective dose resulting in a 50% reduction on control response, expressed with the 95% CI.

curves are shown in Fig. 3. No significant differences were observed in the efficacy elicited by these treatments ($F_{2,5}=1.82$, $P>0.2$ for rearings; $F_{2,5}=1.66$, $P>0.2$ for head-dipping and $F_{2,5}=0.83$, $P>0.4$ for time head-dipping).

The doses of the combinations needed to reach 50% effect and their CI (Table 2) are plotted in Fig. 4. The experimentally determined data indicates that the combinations hesperidin/diazepam 13:1 and 4:1 were below the theoretical additive line for the three parameters tested in the assay (Fig. 4A–C), indicating a synergistic interaction between the two drugs. Instead, combination hesperidin/diazepam 1:13 showed a synergistic interaction only in the number of rearings measured in the assay.

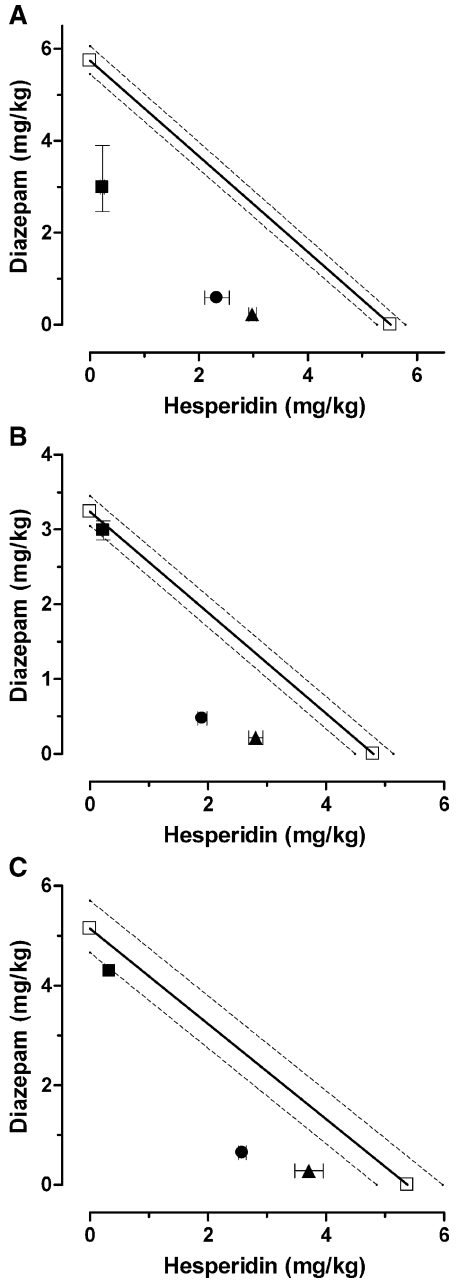

Fig. 4. Isobolograms for % of rearings (A), % of head-dips (B) and % of time spent head-dipping (C), qualifying the nature of the interaction between hesperidin and diazepam in the hole board test. The open symbols (□) on the axes represent the ED_{50} values of the pure compounds, defining the isobole. Closed symbols represent ED_{50} observed for hesperidin/diazepam fixed-ratio combinations 13:1 (▲), 4:1 (●) and 1:13 (■). Dotted lines indicate the 95% CI. Points corresponding to combinations 13:1 and 4:1 lie below the additive line for the three measured parameters demonstrating synergism. In contrast, combination 1:13 only showed synergistic interaction in the reduction of rearings.

The interaction index values (γ) are shown in Table 3. The analysis of these values indicate that a 1.3- to 2-fold ($0.52<\gamma<0.75$) increase in potency was achieved with the combinations hesperidin/diazepam 13:1 and 4:1, in the three parameters tested. When the combination 1:13 was used purely additive interaction is present in the number of head-dips and the time of dipping, however, the rearings are synergistically reduced.

3.4. Effect of hesperidin on diazepam plasma levels

The variation in diazepam plasma concentration after a single i.p. injection of 5 mg/kg was not modified by the concomitant injection of 20 mg/kg of hesperidin (Fig. 5). This experiment was designed considering (a) that both the hole board and the sleeping time tests were carried out 20 min after drug injections and (b) that the ratio 4/1 of hesperidin to diazepam chosen exerts a major synergistic interaction, according to the isobolar analysis.

3.5. Effect of hesperidin on flunitrazepam binding

Saturation radioligand binding studies were performed with synaptosomal membranes prepared from cerebral cortices of mice previously injected i.p. with vehicle or hesperidin (10 mg/kg) (see Materials and methods). In both cases, data obtained were best fitted to one site binding hyperbola ($F_{2,4}=0.30$, $P>0.5$) (Fig. 6). No significant differences in [^{3}H]flunitrazepam binding affinity (K_d) and maximal binding (B_{max}) were observed between vehicle- or hesperidin-treated membrane preparations ($F_{1,12}=0.03$, $P>0.5$ and $F_{1,12}=1.17$, $P>0.3$, respectively). K_d was 2–4 nM and the number of sites was app. 1.3–1.9 pmol/mg. Both values were similar to those obtained by other investigators (Stephenson et al., 1982; Sigel and Barnard, 1984; Churn et al., 2002).

4. Discussion

It is a well-known fact in phytochemistry that crude plant extracts are usually more powerful medicines than pure isolated compounds (Williamson, 2001). The rationale behind this empirical finding may be based on the multiple actions of complex mixtures but also on the synergistic interactions between their components. This is apparently the case with *V. wallichii* where we found that its components, the flavanone glycoside hesperidin and 6-methylapigenin, in combination, produce enhanced effects

Table 3
Interaction index (γ) values for fixed-ratio combinations of hesperidin and diazepam

Hesperidin/diazepam combinations	γ[a] (95% CI)		
	Number of rearings	Number of head-dips	Time head-dipping (s)
Hesperidin alone	1 (0.95, 1.05)	1 (0.92, 1.07)	1 (0.89, 1.11)
13:1	0.58 (0.57, 0.59)	0.65 (0.63, 0.68)	0.75 (0.70, 0.80)
4:1	0.52 (0.50, 0.55)	0.54 (0.52, 0.56)	0.61 (0.59, 0.62)
1:13	0.54 (0.39, 0.71)	1.01 (0.97, 1.05)	0.92 (0.90, 0.95)
Diazepam alone	1 (0.95, 1.06)	1 (0.94, 1.06)	1 (0.91, 1.11)

[a] Interaction index as defined in Materials and methods expressed with its 95% CI.

in mice submitted to the thiopental-induced sleeping assay (Marder et al., 2003; present paper). Recent experiments with *V. officinalis* similarly demonstrated that the sedative and sleep-enhancing properties of its component linarin, a flavone glycoside, were potentiated by the simultaneous administration of valerenic acid, another component in this valeriana species (Fernández et al., 2004).

Since 6-methylapigenin is a ligand for the benzodiazepine binding site, the efficacy of other ligands for the same receptor to potentiate hesperidin action was explored. The results in Fig. 2 show that compounds with agonistic properties viz.: diazepam, apigenin and 6,3′-dinitroflavone gave responses clearly greater than the sum of their independent actions. As the doses of single drugs used in this experiment were far apart from the ones that produced a significant effect when administered alone, the potentiation responses could not be explained as the consequence of very steep dose–response curves of these compounds. For example, for 6,3′-dinitroflavone the dose used in the potentiation assay was of 0.02 mg/kg while the reported dose of this drug that evidences a significant sleep-enhancing effect is 3 mg/kg (Wolfman et al., 1996). 6-Methylflavone, which was reported as an antagonistic ligand (Ai et al., 1997), is apparently ineffective. A negative result was also obtained with chrysin, in spite of being a natural flavonoid with quite similar properties to apigenin. Further research is evidently necessary in these two instances.

Proof of synergy between hesperidin and benzodiazepine binding site ligands requires a more thorough study than simply recording the effects of single-dose combination injections shown in Fig. 2. Hence the isobole method (Berenbaum, 1989; Tallarida et al., 1989) was applied, using diazepam as a benzodiazepine binding site agonist in the hole board assay, which was preferred to the sleeping time test because it does not need the use of a third drug in the experiments. Hesperidin and diazepam both showed a dose-dependent response in the hole board test with similar efficacy (see Fig. 3). However, since the hesperidin used in this experiment was a partially racemic compound, these results indicate that hesperidin has a higher sedative potency than diazepam (see Results). The isobologram was constructed for the ED_{50} values since these points lie on the steep part of all the measured dose–effect curves. Isobolar

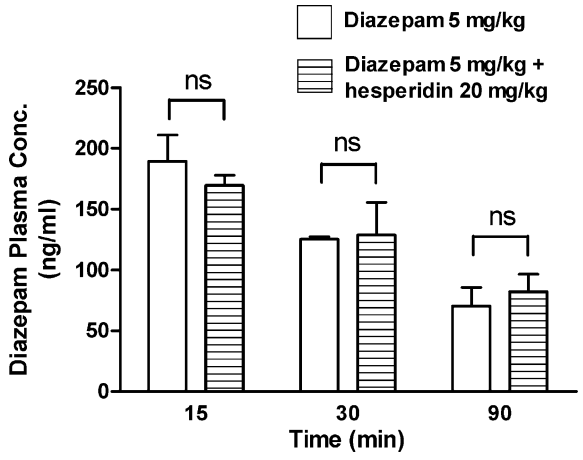

Fig. 5. Effect of hesperidin on diazepam plasma levels. Mean ± S.E.M. of diazepam concentration in mice plasma after 15, 30 and 90 min of an i.p. injection of diazepam 5 mg/kg alone (white bars), or co-injected with hesperidin 20 mg/kg (hatched bars). ns = not significantly different, Student's unpaired *t*-test, $n = 3$.

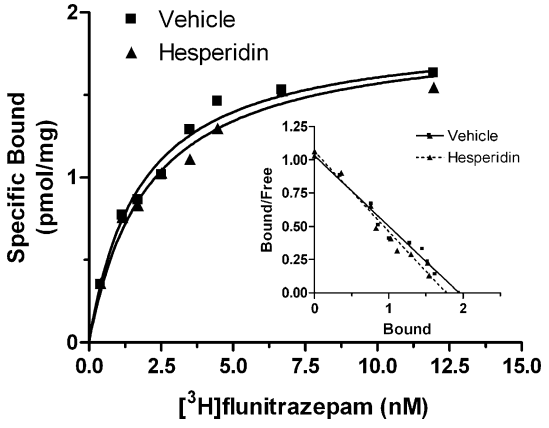

Fig. 6. Effect of hesperidin on flunitrazepam binding to mouse cerebral cortex benzodiazepine receptor in vitro. Saturation isotherms and Scatchard plots of [^{3}H]flunitrazepam (0.4–12 nM) binding are shown. Mice were i.p. injected with hesperidin (HN, 10 mg/kg) or vehicle (VEH) and after 20 min, cerebral cortex was dissected and used to prepare synaptosomal membranes to carry out the experiment (see Materials and methods). In each case data were fitted to one site binding hyperbolas and the affinity constant (K_d) and the number of binding sites (B_{max}) were estimated. The figure shows a representative experiment replicated six times. No significant differences were found in both K_d and B_{max} parameters (extra sum-of-squares *F* test). K_d and B_{max} ranged between 2–4 nM and 1.3–1.9 pmol/mg, respectively.

analysis of the results showed the interaction between hesperidin and diazepam to be synergistic (Fig. 4). The hesperidin/diazepam fixed-ratio combination 4:1 was better than all the others tested, as can be seen in the isobolograms and also considering the γ obtained with the three parameters measured (Table 3). The combination 1:13 showed no synergy in two of these parameters, which suggest that synergistic effects only occur when the amount of hesperidin in the dose combination is higher than that of diazepam.

One may argue if the potentiation effect is simply reflecting pharmacokinetic interactions whereby hesperidin alters the absorption, distribution, metabolism or elimination of diazepam. No such interactions have been as yet described with the pure drugs but it is known that hesperidin is the major flavanone in sweet oranges (*Citrus sinensis*) and it is also present in grapefruit (*Citrus paradisi*), and it has been found that the ingestion of juices from both fruits can markedly elevate the bioavailability of benzodiazepines (Ozdemir et al., 1998; Dresser et al., 2000; Dahan and Altman, 2004). However oral intake of grapefruit juice did not alter kinetic parameters of drugs intravenously injected (Ducharme et al., 1995; Lundahl et al., 1997; Uno et al., 2000). In the present paper we clearly show that hesperidin does not alter diazepam plasma concentration levels when both drugs are co-injected intraperitoneally (Fig. 5).

Concerning transport across the blood–brain barrier, Mitsugana et al. (2000) investigated the effects of some bioflavonoids on vincristine passage. They found that hesperetin (the aglicone of hesperidin), but not hesperidin, increased the uptake of [^{3}H]vincristine by cultured mouse brain capillary endothelial cells. We examined the effect of hesperidin on [^{3}H]flunitrazepam brain disposition in mice: hesperidin (4 mg/kg i.p.) did not modify [^{3}H]flunitrazepam brain concentration when it was co-injected with the radiolabelled benzodiazepine (data not shown). In the light of all this evidence, it seems likely that the supra-additive effects described in this work cannot be explained by pharmacokinetic interactions between diazepam and hesperidin.

Since the discovery of the CNS activities of flavonoids, many investigations have been carried out to correlate their affinity for the benzodiazepine binding site located in the $GABA_A$ receptor to their pharmacological profile in vivo (Marder et al., 1995; Viola et al., 1995; Huen et al., 2003). Also, thanks to the increased knowledge of the diversity of $GABA_A$ receptor subtypes, the number of studies with cloned receptors of defined subunit composition has risen in recent years. For example, apigenin was found to decrease GABA-evoked currents mediated by $\alpha_1\beta_1\gamma_{2s}$ $GABA_A$ and ρ_1 $GABA_C$ receptors expressed in *Xenopus laevis* oocytes (Goutman et al., 2003) and also by $\alpha_1\beta_1\gamma_2$ $GABA_A$ receptors expressed in HEK293 cells (Losi et al., 2004). However, Campbell et al. (2004) found that apigenin did not modify the effects of a low concentration of GABA and did inhibit the effects of a higher dose at $\alpha_1\beta_1\gamma_{2L}$ $GABA_A$ receptors expressed in *X. laevis* oocytes. These findings, however, do not explain the anxiolityc activity of apigenin reported by Viola et al. (1995), the sedative and antidepressant activities of apigenin described by Salgueiro et al. (1997) and Nakazawa et al. (2003) and the results shown here (Fig. 2). We also found that benzodiazepine binding parameters of a synaptosomal cortex preparation from mice are not modified by previous treatment of mice with hesperidin. Hesperidin is not a ligand for the benzodiazepine-binding site, AMPA glutamatergic, adenosine$_1$ and 5-hydroxytryptamine type 1A and type 2 receptors (Marder et al., 2003). Recently, it has been described the effects of (−)-epigallocatechin gallate on GABA responses at $\alpha_1\beta_1\gamma_{2L}$ $GABA_A$ receptors expressed in *X. laevis* oocytes. This flavonoid is abundant in tea and it has been reported to be inactive as inhibitor of benzodiazepine binding. However it has, like apigenin, inhibitory effects on GABA responses at $\alpha_1\beta_1\gamma_{2L}$ receptors. Apigenin and (−)-epigallocatechin gallate possess a bimodal effect on diazepam modulatory action. Enhancement of the positive modulation of responses to GABA by diazepam was produced by both flavonoids at low doses (Campbell et al., 2004). Overall then, evidences for synergy between diazepam and flavonoids are appearing.

Concerning the effect of these drugs as expressing an increase of sleeping time of mice, we are aware that the test used in this investigation may be only detecting changes in motor behavior hence its results are not a measure of real sleep as determined by polysomnography.

The isobole method describes interactions only in empirical terms and is not appropriate to assign them a mechanistic significance (Berenbaum, 1989; Tallarida et al., 1989; Tallarida, 1992) but the results presented here suggest plausible approaches to further investigate the nature of this effect. The synergy detected and described in this work could be explained, for example, by the probable existence of a new binding site specific for hesperidin in a particular $GABA_A$ receptor subtype and by resulting allosteric effects of hesperidin and diazepam acting as modulators. Also, a possible interaction of hesperidin with enzymes that regulate $GABA_A$ receptor function may not be discarded since flavonoids are well-known enzyme inhibitors (Middleton et al., 2000).

Aside from the mechanism of the observed synergy, this effect may possibly be put to use to improve present human therapy with benzodiazepines. It has been proved that a chronic benzodiazepine administration is associated with benzodiazepine binding site down regulation and behavioral tolerance to these drugs (Rosenberg and Chiu, 1981; Miller et al., 1988). Our results suggest that it may be possible to obtain the same therapeutic effects with a substantial decrease in the diazepam dose when used in synergistic combination with hesperidin. In addition, the chemistry and pharmacology of this flavonoid, which is also a part of the human diet, is well known and supports the safety of its therapeutic use (Garg et al., 2001).

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