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The flavone hispidulin, a benzodiazepine receptor ligand with positive allosteric properties, traverses the blood–brain barrier and exhibits anticonvulsive effects

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Dedicated to Prof. Dr S. Ebel on his 70th birthday

1 The functional characterization of hispidulin (4′,5,7-trihydroxy-6-methoxyflavone), a potent benzodiazepine (BZD) receptor ligand, was initiated to determine its potential as a modulator of central nervous system activity.

2 After chemical synthesis, hispidulin was investigated at recombinant $GABA_A$/BZD receptors expressed by *Xenopus laevis* oocytes. Concentrations of 50 nM and higher stimulated the GABA-induced chloride currents at tested receptor subtypes ($\alpha_{1-3,5,6}\beta_2\gamma_2S$) indicating positive allosteric properties. Maximal stimulation at $\alpha_1\beta_2\gamma_2S$ was observed with 10 μM hispidulin. In contrast to diazepam, hispidulin modulated the $\alpha_6\beta_2\gamma_2S$-$GABA_A$ receptor subtype.

3 When fed to seizure-prone Mongolian gerbils (*Meriones unguiculatus*) in a model of epilepsy, hispidulin (10 mg kg^{-1} body weight (BW) per day) and diazepam (2 mg kg^{-1} BW per day) markedly reduced the number of animals suffering from seizures after 7 days of treatment (30 and 25% of animals in the respective treatment groups, vs 80% in the vehicle group).

4 Permeability across the blood–brain barrier for the chemically synthesized, ^{14}C-labelled hispidulin was confirmed by a rat *in situ* perfusion model. With an uptake rate (K_{in}) of 1.14 ml min^{-1} g^{-1}, measurements approached the values obtained with highly penetrating compounds such as diazepam.

5 Experiments with Caco-2 cells predict that orally administered hispidulin enters circulation in its intact form. At a concentration of 30 μM, the flavone crossed the monolayer without degradation as verified by the absence of glucuronidated metabolites.
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Abbreviations: BBB, blood–brain barrier; BW, body weight; BZD, benzodiazepine; CNS, central nervous system; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; GABA, γ-aminobutyric acid; $GABA_A$ receptors, γ-aminobutyric acid type A receptors; HBSS, Hanks' balanced salt solution; HPLC, high-performance liquid chromatography; K_{in}, rate of uptake; P_{oct}, calculated octanol–water partition coefficient; Ro15-1788, flumazenil; RT, retention time; TEER, transendothelial electrical resistance; TMS, tetramethylsilane

Introduction

γ-Aminobutyric acid (GABA) and $GABA_A$ receptors belong to the major inhibitory system in the central nervous system (CNS). GABA opens a chloride ion selective channel in $GABA_A$ receptors, which are equally modulated by a wide range of drugs including barbiturates, steroids and benzodiazepines (BZDs). These receptor modulators interact with distinct allosteric binding sites on the $GABA_A$ receptor complex. The BZD binding site, so-called BZD receptor, can be occupied by a variety of substances classified as positive allosteric modulators, antagonists or negative allosteric modulators according to their intrinsic activity (Sigel & Buhr, 1997). BZDs, the most widely prescribed tranquilizers, are considered classical ligands of this modulatory site. They act as positive allosteric modulators by increasing channel-opening frequency and confer anxiolytic, anticonvulsant, sedative-hypnotic, muscle-relaxant effects (Sieghart & Sperk, 2002). An intensive search has been undertaken over the past decade with the aim to separate these diverse actions and to find new, more selective BZD receptor ligands as drug candidates in the treatment of epilepsy, anxiety and sleep disturbances. Natural flavonoids count among these candidates and hold promise as BZD receptor ligands (Medina *et al*., 1998). They comprise flavones, flavonols, anthocyanins and related ubiquitous constituents of higher plants and represent a significant part of our daily diet. Several biological activities of flavonoids have emerged, for example, antioxidant, antiviral, anticancer and chemopreventive properties (Daniel & Wenzel, 2003).

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Effects of plant flavonoids on the CNS are known since 1990, when the existence of natural anxiolytic flavonoids was first described (Medina *et al*., 1998). Flavones like chrysin, apigenin, wogonin and the recently isolated 6-methylapigenin have been shown to possess anxiolytic effects *in vivo*. In contrast, their sedative, anticonvulsant and myorelaxant effects appeared less prominent indicating a possible more selective action of these substances (Medina *et al*., 1998; Hui *et al*., 2002; Marder *et al*., 2003).

Although the sources of many active flavones are well known, information on their bioavailability and their active forms *in vivo* is limited. In particular, absorption, metabolism and CNS delivery of most flavonoid agents are poorly understood. Recently, hispidulin (4′,5,7-trihydroxy-6-methoxyflavone) was isolated from sage (*Salvia officinalis* L.) and identified as a potent ligand of the central human BZD receptor *in vitro*. With an IC_{50} value of 1.3 μM, hispidulin showed the strongest binding activity to the BZD receptor in comparison to other flavones isolated (Kavvadias *et al*., 2003). Hispidulin is a naturally occurring flavone commonly found in several *Artemisia* and *Salvia* species. Several *in vitro* studies have demonstrated its potent antioxidative, antifungal, anti-inflammatory and antimutagenic activities (Chulasiri *et al*., 1992; Gil *et al*., 1994; Tan *et al*., 1999). The lack of pharmacokinetic data and unresolved issues with regard to the crossing of the blood–brain barrier (BBB) prompted us to investigate its synthesis in a radiolabelled form. In this paper, we describe the conventional synthesis of flavones as applied to generating ^{14}C-labelled hispidulin, and present a comprehensive characterization of this natural ligand, including effects on inhibitory hyperpolarization, BBB permeability and anticonvulsive activity *in vivo*.

Methods

Animals

Seizure-prone, male Mongolian gerbils (*Meriones unguiculatus*) weighing 70–102 g were kindly provided by the Leibniz-Institute for Neurobiology (Magdeburg, Germany) and partly bred at the Institute for Medical Chemistry (Veterinary University, Vienna, Austria). The *in situ* brain perfusion study was carried out on three male Wistar rats (Harlan UK Limited, Oxon, U.K.) aged 6 months with a body weight (BW) of 250 g. The animals were housed in single cages under standard laboratory conditions (20°C, 55% humidity, 12 h light/dark cycle) with free access to food and water.

All experiments were performed in accordance with the UK Home Office, Animal Procedures Act 1986 and in strict compliance with the NIH Guide for the care and use of laboratory animals.

Syntheses

General experimental procedures Column chromatography for compound purification was performed with Merck 60 silica gel (0.040–0.063 mm). ^{1}H-NMR spectra of unlabelled compounds were recorded on Bruker AC-250 MHz instrument. Chemical shifts are reported (δ) in ppm and coupling constants (J) in Hz relative to the internal standard tetramethylsilane (TMS). Chemical and radiochemical purity of ^{14}C-labelled compounds was controlled by high-performance liquid chromatography (HPLC) analysis with the absorbance detector Spectroflow 757 (Kratos, Weiterstadt, Germany) and the flow scintillation analyzer 500 TR (Packard, Groningen, Netherlands) using authentic references from synthesis of unlabelled hispidulin.

Synthesis of [^{14}C]hispidulin

*4-Benzyloxy-2,3-dimethoxy-6-hydroxyacetophenone (**5**)* Potassium carbonate (3 g, 21.7 mmol) was added to a solution of 2,4,6-trihydroxyacetophenone-monohydrate (2 g, 10.8 mmol) in acetone (30 ml). The mixture was heated to 65°C. Three portions of dimethyl sulfate (3×0.7 ml, 22.1 mmol) were added to the reaction solution at 3 h intervals. The solution was further heated for 3 h, filtered and the filtrate evaporated to give 2,4-dimethoxy-6-hydroxyacetophenone (**1**) as a yellow solid (92%): ^{1}H-NMR (250 MHz, DMSO-d_6): δ 2.61 (CO-CH_3, s, 3H), 3.88 (2-OCH_3, s, 3H), 3.93 (4-OCH_3, s, 3H), 6.15 (H-3, d, $J = 2.1$ Hz, 1H), 6.18 (H-5, d, $J = 2.1$ Hz, 1H), 13.88 (6-OH, s, 1H). Acetophenone **1** (1.5 g, 7.7 mmol) and dry aluminum chloride (1.5 g, 11.3 mmol) were suspended in chlorobenzene (20 ml) and refluxed for 1 h. The organic solvent was evaporated under reduced pressure and the residue mixed with ice-cold water–HCl (1 : 1) solution (40 ml). The white precipitate was filtered, dissolved in ethyl acetate and extracted with 10% sodium hydroxide solution (3×50 ml). After acidifying the aqueous phase, the 2,4-dihydroxy-6-methoxyacetophenone (**2**) was re-extracted with ethyl acetate (3×100 ml) and crystallized from ethyl acetate (45–60%). Alternatively, column chromatography was performed according to Jain *et al*. (1985) to give **2** as a yellow solid: ^{1}H-NMR (250 MHz, DMSO-d_6): δ 2.58 (CO-CH_3, s, 3H), 3.88 (-OCH_3, s, 3H), 5.94 (H-3, d, 2.1 Hz, 1H), 6.04 (H-5, d, 2.1 Hz, 1H), 13.88 (2-OH, s, 1H). Compound **2** (0.5 g, 2.75 mmol) was dissolved in dry acetone (30 ml) and the solution heated under reflux overnight after adding potassium carbonate (3 g, 21.7 mmol) and benzyl bromide (0.43 ml, 3.6 mmol). After evaporating the solvent, 4-benzyloxy-2-methoxy-6-hydroxyacetophenone (**3**) was obtained as a brown solid (99%): ^{1}H-NMR (250 MHz, DMSO-d_6): δ 2.62 (CO-CH_3, s, 3H), 3.93 (-OCH_3, s, 3H), 5.24 (-CH_2-, s, 2H), 6.25 (H-3, d, 2.5 Hz, 1H), 6.28 (H-5, d, 2.4 Hz, 1H), 7.49 (-Ph, m, 5H). The phenolic compound **3** (0.5 g, 1.8 mmol) and sodium hydroxide (0.4 g) were dissolved in 4 ml water and 0.5 ml pyridine. A solution of potassium persulfate (0.55 g, 2.0 mmol) in water (11 ml) was added over 4 h at a constant temperature of 15°C. After stirring at room temperature for 24 h, the mixture was acidified to pH 5 and filtered. The filtrate was extracted with diethyl ether (2×15 ml). The aqueous phase was acidified with HCl_{conc} (2 ml) and refluxed for 1 h after addition of diethyl ether (15 ml). The diethyl ether phase was dried over sodium sulfate and evaporated to give 4-benzyloxy-2,5-dihydroxy-6-methoxyacetophenone (**4**) as brown syrup (29%): ^{1}H-NMR (250 Hz, DMSO-d_6): δ 2.66 (CO-CH_3, s, 3H), 3.91 (6-OCH_3, s, 3H), 5.28 (-CH_2-, s, 2H), 6.46 (H_{ar}, s, 1H), 7.48 (Ph, m, 5H). For partial methylation of intermediate **4** (0.15 g, 0.5 mmol), 1.5 equivalents of dimethyl sulfate (0.07 ml, 0.7 mmol) and potassium carbonate (0.8 g) were dissolved in dry acetone (8 ml) and refluxed overnight. After filtration and evaporation of the solvent, compound **5** was purified by silica gel column chromatography (2.8×42 cm) using chloroform as eluent. The

product **5** was obtained in fractions with 500–700 ml elution volume (59%): ^{1}H-NMR (250 Hz, $CDCl_3$): δ 2.66 (CO-CH_3, s, 3H), 3.81 (2-OCH_3, s, 3H), 4.01 (3-OCH_3, s, 3H), 5.14 (-CH_2-, s, 2H), 6.30 (H_{ar}, s, 1H), 7.42 (Ph, m, 5H), 13.40 (6-OH, s, 1H).

4-Benzyloxy-^{14}COCl-benzoyl chloride (7) To a solution of radiolabelled 4-hydroxy-^{14}COOH-benzoic acid (55 mCi·mmol^{-1}, 250 μCi) in water–ethanol (1 : 3) mixture (450 μl), unlabelled 4-hydroxybenzoic acid (45 mg, 0.33 mmol), aqueous 4 M potassium hydroxide solution (170 μl) and benzyl bromide (45 μl, 0.38 mmol) were added. The reaction mixture was heated under reflux for 6 h. After addition of 4 M potassium hydroxide solution (0.5 ml), the mixture was further heated for 2 h, cooled down to room temperature and centrifuged at $5000 \times g$. The supernatant was removed and the residue was recrystallized in acetic acid (1 ml) to obtain 4-benzyloxy-^{14}COOH-benzoic acid (**6**) as white solid (chemical yield: 36%; total ^{14}C activity: 78 μCi). The dried acid **6** (27 mg, 0.12 mmol) was suspended in dichloromethane (1 ml), and oxalyl chloride (28 μl, 0.15 mmol) was added. The reaction mixture was stirred at room temperature for 8 h. 4-Benzyloxy-^{14}COCl-benzoyl chloride (**7**) was obtained after evaporation of the solvent under reduced pressure (chemical yield: 100%; total ^{14}C activity: 76 μCi).

*2-^{14}C-hispidulin (2-^{14}C-4′,5,7-trihydroxy-6-methoxyflavone) (**11**)* Acetophenone derivative **5** (36 mg, 0.12 mmol) was added to a solution of ^{14}C-labelled 4-benzyloxybenzoyl chloride (**7**) (29 mg, 0.12 mmol, 76 μCi) in dry pyridine (0.7 ml) and the mixture was stirred at room temperature for 3 h. The reaction solution was poured on ice-cold 3% HCl (11 ml) and extracted with ethyl acetate (25 ml). The organic phase was washed twice with aqueous saturated sodium carbonate, once with water and then evaporated under reduced pressure to give ester **8** (chemical yield: 81%; total ^{14}C activity: 55 μCi). Ester **8** was dried in vacuum and redissolved in dry pyridine (0.5 ml). After addition of freshly powdered potassium hydroxide (175 mg), the mixture was stirred at 60°C for 4 h. The reaction was stopped by pouring on ice-cold 3% HCl (11 ml). The diketone compound **9** was extracted with ethyl acetate (25 ml), washed with aqueous saturated sodium carbonate and then evaporated under reduced pressure to give the diketone **9** (chemical yield: 55%; total ^{14}C activity: 26 μCi). Diketone **9** was dissolved in acetic acid (1.7 ml), and sulfuric acid (42 μl) was carefully added. The solution was stirred at 60°C for 90 min and then poured on ice (10 g). The resulting flavone **10** was extracted with ethyl acetate (25 ml) and dried under reduced pressure after solvent evaporation (chemical yield: 60%; total ^{14}C activity: 14 μCi). For selective demethylation and deprotection of the hydroxy groups, flavone **10** was dissolved in dichloromethane (1 ml) and cooled down to about −65°C. Boron trichloride solution (1 M in dichloromethane, 0.25 ml) was then added and the mixture was stirred at –65°C for 90 min. The reaction was stopped by addition of saturated sodium bicarbonate (1 ml), and the aqueous solution was extracted with diethyl ether (3×5 ml). The crude 2-^{14}C-hispidulin (**11**) was purified by silica gel column chromatography (1.9×18 cm) with methanol : chloroform (3 : 97) as eluent. The major activity of pure flavone **11** (3 mg, 0.01 mmol) was found in fractions eluted with 55–70 ml of the MeOH/$CHCl_3$ mixture (chemical yield: 44%; total ^{14}C activity: 9 μCi). Identity was confirmed by HPLC analysis using unlabelled hispidulin as reference.

Synthesis of hispidulin

Unlabelled hispidulin was synthesized from 4-benzyloxy-2,3-dimethoxy-6-hydroxyacetophenone (**5**) and unlabelled 4-benzyloxybenzoic acid chloride (**7**), both prepared in separate routes from 2,4,6-trihydroxyacetophenone and 4-hydroxybenzoic acid, respectively. Synthesis steps were carried out on a large gram-scale as described above for **11**. The product was purified by silica gel column chromatography (diameter 2.2 cm, length 42 cm) and was eluted by methanol : chloroform (3 : 97) with an elution volume of 250–370 ml. Identity was confirmed on the basis of published spectral data using isolated natural hispidulin as reference (UV, NMR, MS) (Kavvadias *et al*., 2003).

Electrophysiological studies

Xenopus laevis oocytes were prepared, injected and defolliculated and currents were recorded as previously described (Sigel, 1987; Sigel *et al*., 1990). Briefly, oocytes were injected with 50 nl of capped, polyadenylated cRNA dissolved in 5 mM K-HEPES (pH 6.8). For the triple subunit combinations, 10 nM of the cRNA coding for the different α subunits (α1, α2, α3, α5 and α6) and the β2 subunit was used, whereas 50 nM of the γ2S subunit cRNA was used (Boileau *et al*., 2002). RNA transcripts were synthesized from linearized plasmids encoding the desired protein using the mMESSAGE mMACHINE® kit according to the manufacturers' recommendations. A poly(A) tail of about 300 residues was added to the transcripts by using yeast poly(A) polymerase. The cRNA combinations were co-precipitated in EtOH and stored at −20°C. Transcripts were quantified on agarose gels after staining with Radiant Red Fluorescent RNA Stain by comparing staining intensities with various amounts of molecular weight markers. Electrophysiological experiments were performed by the two-electrode voltage clamp method at a holding potential of −80 mV. The medium (pH 7.4) contained 90 mM NaCl, 1 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM HEPES–NaOH (pH 7.4). GABA, diazepam and hispidulin were applied for 20 s and a washout period of 4–15 min was allowed to ensure full recovery from desensitization. Hispidulin was dissolved every day freshly in dimethyl sulfoxide (DMSO) at a concentration of 10 mM. The stock solution of Apigenin was 10 mM in DMSO. The final concentration of DMSO in the medium was always adjusted to 0.5%, except when hispidulin was assayed at 100 μM. In this case, final DMSO concentration in the medium was 1%. These concentrations of DMSO did not by themselves affect significantly GABA-elicited currents. Both flavone compounds were tested for their effect on GABA concentrations that elicited 2–5% of the maximal current amplitude.

Gerbil model

Only seizure-sensitive animals were used in the experiments. They were assigned to treatment groups of 10 animals each (with the exception of eight animals in the diazepam group). Drugs (hispidulin and diazepam) were dissolved in ethanol, diluted with water and added to a pastry manufactured with

ground chow (Altromin® Pellets). Pellets were formed to give final concentrations of 1.08 mg hispidulin or 0.21 mg diazepam and max. 1.4% ethanol per 5 g (daily portion). For the control group, pellets from ground chow, ethanol and water were manufactured in the same manner and used as vehicle. Test substances were administered orally for 7 days at a dosage of 10 mg kg^{-1} hispidulin and 2 mg kg^{-1} diazepam per day. During this period, the animals were left undisturbed to avoid any unintended provocation of seizures. Epileptic seizures were induced by a standardized handling procedure before and after 7 days of treatment. For this purpose, each animal was stroked on the back and was separately placed outside its home cage while its seizure activity was monitored and graded in terms of severity as follows: no visible seizure or only minimal seizure correlates (no noticeable changes of gross behavior, possibly twitching of vibrissae and pinnae for a few seconds) and full seizure (clonic–tonic seizure, loss of righting reflexes and body rollover). Only seizures occurring within 3 min of provocation tests were counted.

In situ *rat brain perfusion model*

Hemiperfusions were carried out using a modified short-duration technique previously described by Takasato *et al.* (1984). Briefly, [^{14}C]hispidulin (1 μCi, specific activity 67.5 μCi $mmol^{-1}$) was dissolved in ethanol (1 ml), diluted with perfusion buffer (according to Egleton *et al.*, 1998) to give a final ethanol concentration of 5% and subsequently perfused *via* the right carotid artery of rats for 30, 45 and 60 s ($n=3$ animals for each time point). The right hemisphere of the brain was dissected and the ^{14}C content was determined with a scintillation counter. Results were expressed as volume of distribution V_d (μl g^{-1}) and uptake rate K_{in} (ml min^{-1} g^{-1}). To ensure that ethanol concentration did not disrupt the BBB and affect permeability data, a control experiment was performed perfusing [^{14}C]sucrose (as a vascular marker) in perfusate containing ethanol (5% final concentration) for 60 s.

Caco-2 system model

Caco-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 1% MEM non-essential amino-acid solution, GLUTAMAX-1 (2 mM) and penicillin–streptomycin (100 U ml^{-1}; 100 μg ml^{-1}), and maintained at 37°C in a humidified atmosphere (5% CO_2). For transport studies, Caco-2 cells (passage 30–50) were seeded on Transwell inserts (polycarbonate membrane, 12 mm diameter and 0.4 μm pore size, Corning Costar Co.) at a density of 1×10^5 cm^{-2}. The transendothelial electrical resistance (TEER) across the Caco-2 monolayers was measured daily using an epithelial volt-ohmmeter (EVOM, World Precision Instruments Inc., Hertfordshire, U.K.) with a planar electrode chamber (Endohm-12, World Precision Instruments Inc.). Monolayers used in transport studies (20–25 days postseeding on Transwells) exhibited TEER $>500\,\Omega\,cm^{-2}$. Inserts were washed with Hanks' balanced salt solution (HBSS) pH 7.4 and were incubated at 37°C for 30 min. Hispidulin and apigenin (30 mM in DMSO) were diluted to 30 μM in transport buffer (HBSS containing 25 mM HEPES, 0.1% BSA (w v^{-1})). BSA was included to stabilize membrane and tight junctions. ^{3}H-Mannitol (0.1 μCi ml^{-1}, specific activity 20 Ci $mmol^{-1}$) was used as a marker for the paracellular (tight junctional) pathway to ensure that integrity was maintained during the permeability studies. Aliquots (0.5 ml) of the transport buffer containing 30 μM hispidulin or apigenin were added to the donor (apical) side of the inserts. The receiver (basolateral) chamber contained 1 ml of transport buffer (HBSS, 25 mM HEPES and 0.1% BSA). Plates were then incubated at 37°C in a Wesbart shaking plate set at 200 rpm (Wesbart Ltd, U.K.). After 30 min, the inserts were transferred into new wells containing fresh transport buffer (1 ml). After 60 min, 50 μl aliquots from receiver ($t=30$ and 60 min) and donor wells ($t=60$ min) were collected, added to scintillation cocktail and the [^{3}H] radioactivity was measured using a Beckman scintillation counter. Flavonoid content in donor and receiver wells was analyzed by HPLC with photodiode array detection. To identify glucuronide conjugates, samples (200 μl) were incubated with β-glucuronidase (20 μl, 10,000 U ml^{-1}) for 2 h at 37°C and assessed for a decrease in the suspected glucuronide peak and concomitant increase in the respective aglycone peak. Stability of both flavonoids was verified by measurements following incubation of samples in transport buffer at 37°C for 60 min.

HPLC analysis

HPLC was performed on a Waters HPLC system using a Nova-Pak C18 column (4.6×250 mm, 4 μm) and a guard column (4.6×15 mm, 4 μm) as previously described (Youdim *et al.*, 2003). Flavonoids were identified by comparing retention times (RTs), photodiode array spectra and sample spiking with reference substances.

Chemicals

The following chemicals and reagents of analytical purity were used for the synthesis and experiments: acetic acid, acetone, aluminum chloride, apigenin, benzyl bromide, boron trichloride solution (1 M in dichloromethane), chlorobenzene, chloroform, diazepam, dichloromethane, diethyl ether, dimethyl sulfate, DMSO, DMSO-d_6, ethanol, ethyl acetate, GABA, 4-hydroxybenzoic acid, methanol, potassium carbonate, potassium hydroxide, potassium persulfate, pyridine, flumazenil (Ro15-1788), sodium hydroxide, sodium sulfate, sulfuric acid, TMS and 2,4,6-trihydroxyacetophenone-monohydrate from Sigma-Aldrich (Deisenhofen, Germany). 4-Hydroxy-^{14}COOH-benzoic acid was obtained from American Radiolabelled Chemicals Inc. (St Louis, MO, U.S.A.). Ultima-FloTM AF LSC-Cocktail (Packard Bioscience Company, Groningen, Germany) was used for scintillation counting.

The following were used for electrophysiological studies: mMESSAGE mMACHINE® kit (Ambion Inc.), yeast poly(A) polymerase (Amersham Biosciences, U.K.), Radiant Red Fluorescent RNA Stain (Bio-Rad, U.S.A.) and RNA Ladder (Gibco BRL, Crewe-Cheshire, U.K.). Caco-2 cells were obtained from the European Collection of Animal Cell Cultures (ECACC, Wiltshire, U.K.). Further on, fetal bovine serum, DMEM, MEM solution, GLUTAMAX-1, penicillin–streptomycin and HBSS were from Invitrogen (U.K.), and HEPES, BSA, β-glucuronidase (type L-II from Limpets) and ^{3}H-Mannitol were from Sigma (Dorset, U.K.).

Data analysis

Electrophysiological studies Currents were measured using a modified OC-725 amplifier (Warner Instruments Corp.) in combination with an XY-recorder or digitized using a MacLab200 (AD Instruments). Current responses have been fitted to the Hill equation [$I = I_{max}(1 + (IC_{50}[A]^{-1})^n)^{-1}$], where I is the peak current at a given concentration of GABA, I_{max} the maximum current, IC_{50} the concentration of agonist eliciting half-maximal current and n the Hill coefficient. The resulting data are shown as mean ± s.e.m.

Gerbil model Paired proportion tests (http://www.ubmail.ubalt.edu/~harsham/Business-stat/otherapplets/PairedProp.htm) were used to examine the null hypothesis that 7 days' pharmacological treatment had no modulatory effect on seizure propensity. P-value of less than 0.05 was considered as significant.

In situ *rat brain perfusion model* Results were expressed as volume of distribution V_d ($\mu l\,g^{-1}$) using the following equation: $V_d = (dpm\,g^{-1})_{tissue}$ $(\mu l\,dpm^{-1})_{perfusate}$, where dpm expresses disintegrations per minute of the sample. The uptake rate K_{in} ($ml\,min^{-1}\,g^{-1}$) was obtained as slope of the plot of V_d ($ml\,g^{-1}$) against time (min). Log P_{oct} was calculated using the KowWin (LogKow) software at http://www.esc.syrres.com/interkow/logkow.htm.

Results

Chemical synthesis

A route for the chemical synthesis of hispidulin, including its ^{14}C-labelled form, was developed in our laboratory utilizing the Baker Venkataraman rearrangement starting with 4-benzyloxy-2,3-dimethoxy-6-hydroxyacetophenone (**5**) and 4-benzyloxybenzoic acid chloride (**7**) (Figure 1c). Both educts were prepared by separate routes from 2,4,6-trihydroxyacetophenone and 4-hydroxybenzoic acid. The acetophenone **5** was synthesized from commercially available 2,4,6-trihydroxyacetophenone in five steps as shown in Figure 1a. Transformation into 2,4-dimethoxy-6-hydroxyacetophenone (**1**) was achieved by reaction with dimethyl sulfate (Srivastava & Srivastava, 1987). Subsequent selective demethylation with aluminum chloride in chlorobenzene was performed to obtain 2,4-dihydroxy-6-methoxyacetophenone (**2**) (Jain *et al*., 1985). After protection of the 4-hydroxy group with a benzyl group, compound **3** was applied to Elbs persulfate oxidation (Baker *et al*., 1939). In the last step of the acetophenone preparation, the newly introduced hydroxy group at position C-5 was methylated using dimethyl sulfate. The benzoyl chloride **7** was prepared in two steps according to Figure 1b. Starting from 4-hydroxybenzoic acid, we protected the hydroxy group with a benzyl group and prepared the acid chloride by reaction of compound **6** with oxalyl chloride (Cherpeck, 1998; Bracon *et al*., 1999). For hispidulin synthesis, compounds **5** and **7** (76 μCi) were converted into dry pyridine to the corresponding benzoyl ester (Figure 1c). The crude benzoate was treated with potassium hydroxide to induce an intramolecular Claisen condensation resulting in formation of diketone **9**. The diketone **9** cyclized to the corresponding flavone by heating in acetic acid with small amounts of sulfuric acid (Horie *et al*., 1997). The protecting groups and the labile methoxy group at C-5 were selectively cleaved by reaction with boron trichloride in dichloromethane at about −65°C to obtain hispidulin, which was identified on the basis of published spectral data (UV, NMR, MS) (Kavvadias *et al*., 2003). For synthesis of ^{14}C-labelled hispidulin (**11**), radiolabelled 4-hydroxy benzoic acid with a total activity of 250 μCi was used. Radiolabelled flavone **11** (total activity: 9 μCi) was obtained with 12% isotopic incorporation and a specific activity of 67.5 $\mu Ci\,mmol^{-1}$ as determined by liquid scintillation counting after silica gel column purification.

Electrophysiological studies

In electrophysiological studies at recombinant $GABA_A$ receptors expressed in *X. laevis* oocytes, hispidulin exhibited a positive allosteric modulatory effect. The current elicited by GABA in $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors was enhanced in a concentration-dependent way (Figure 2b). Maximum relative stimulation at $\alpha_1\beta_2\gamma_2$ was achieved with 10 μM hispidulin. Interestingly, at higher concentrations, a second inhibitory phase was observed. At 10 μM, stimulation by hispidulin amounted to about 24% of the stimulation by 1 μM diazepam. The potentiation of 10 μM hispidulin amounted to 47 ± 5% ($n = 3$, s.e.m.) and was largely blocked by co-application of the BZD receptor antagonist Ro15-1788 with a residual stimulation of 17 ± 2% ($n = 3$, s.e.m.) (Figure 2c).

Substance concentrations of about 50 nM and higher stimulated the GABA-induced chloride current at all five receptor subtypes ($\alpha_{1-3,5,6}\beta_2\gamma_2$) investigated (not shown). In contrast to diazepam, hispidulin also enhanced the GABA-activated current at $\alpha_6\beta_2\gamma_2$ $GABA_A$ receptors (Figure 2d).

In order to relate hispidulin's effects to the effects of a familiar flavone, apigenin was equally investigated (Figure 1d). This compound reduced the GABA-induced chloride ion current and therefore acted as negative allosteric modulator of GABA (Figure 2e).

Gerbil model

For the investigation of hispidulin effects on ictal events, gerbils were selected for their susceptibility to the triggering of seizures by typical environmental stressors. A visible confirmatory seizure was inducible at baseline in 28 animals immediately prior to treatment. Drug effects of hispidulin and diazepam on seizure incidence are presented in Figure 3. No ictal phenomena were observed during the period of treatment. When re-exposed to the same stimuli on day 7, gerbils experienced milder/fewer seizures in both the hispidulin (10 $mg\,kg^{-1}$ BW per day) and the diazepam (2 $mg\,kg^{-1}$ BW per day) groups (full seizures in 30% ($P < 0.04$) and 25% ($P < 0.04$) of animals, respectively), when compared to gerbils in the vehicle group (full seizures in 80% of animals).

Blood–brain barrier study

In accordance with our behavioral findings, perfusion of ^{14}C-labelled hispidulin into the right carotid artery of the rat resulted in a linear flux of the flavone to the brain. [^{14}C]hispidulin in artificial saline was perfused into the right carotid artery of the rat for 30, 45 and 60 s. Figure 4 shows the

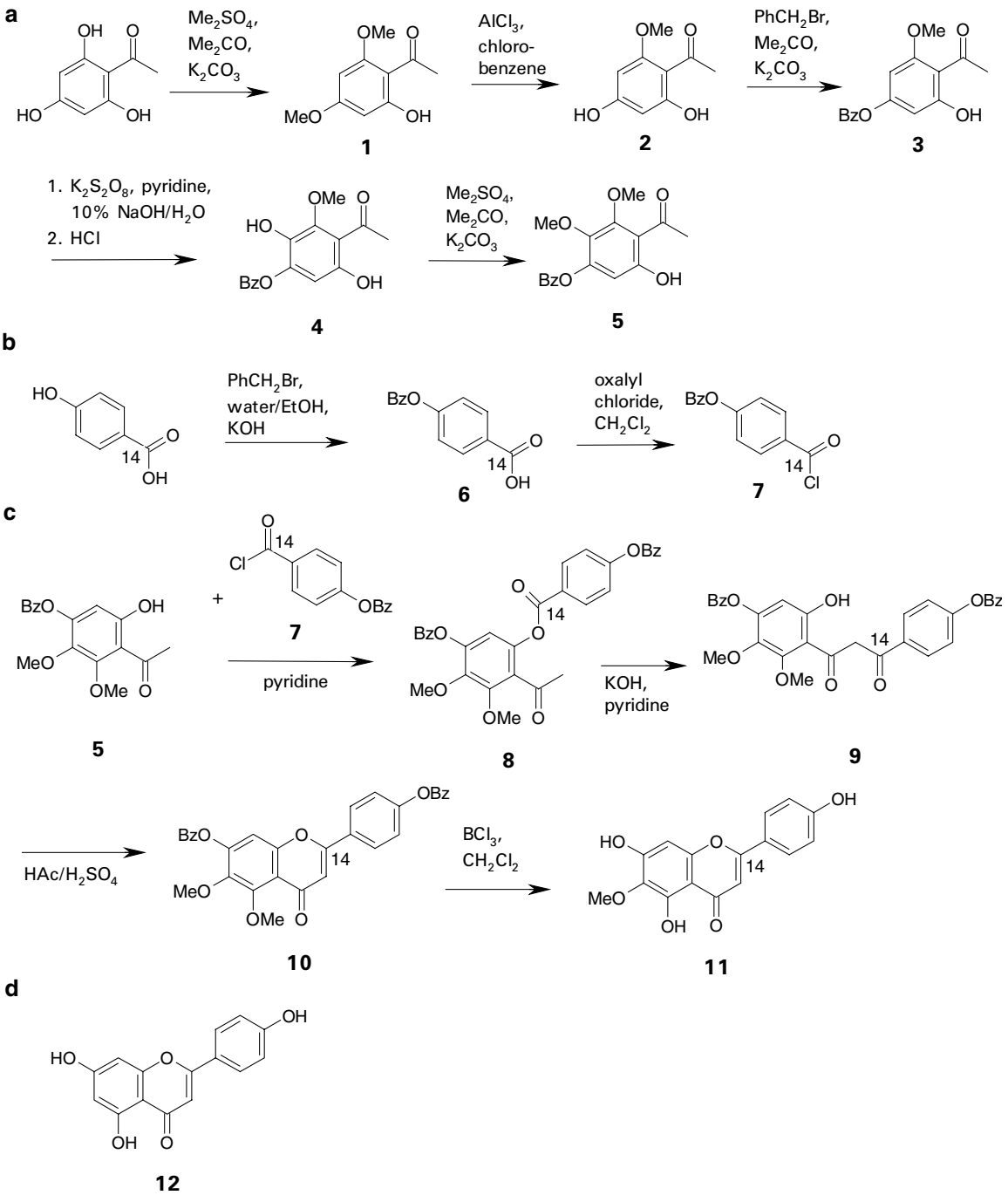

Figure 1 Chemical syntheses of ^{14}C-labelled hispidulin **11** (c) and its building blocks **5** (a) and **7** (b) and the structure of apigenin (d).

V_d (ml g^{-1}) for hispidulin into the right hemisphere. The rate of uptake (K_{in}) 1.14 ml min^{-1} g^{-1} was obtained from the slope of a plot of V_d (μl g^{-1}) against time (min). Figure 5 shows the relationship between log K_{in} (experimental) and lipophilicity of hispidulin (log P_{oct} 2.67, calculated octanol–water partition coefficient) compared with the behavior of known passively permeating compounds and efflux transport substrates.

Caco-2 cell layer permeability study

The permeation of hispidulin and apigenin aglycones across the Caco-2 monolayers was investigated in a model of intestinal absorption to test for any compound modification secondary to uptake by epithelial cells. Figure 6a shows the HPLC profile of the receiver medium following hispidulin (30 μM) exposure at the donor side of the Caco-2 cell monolayer for 60 min. The peak with RT at 50.93 min represents the hispidulin aglycone, in parallel with RT and diode array spectra of a standard solution. Apigenin (RT 43.40 min) also crossed the Caco-2 cell monolayer in its intact form as shown in Figure 6b. Following incubation with β-glucuronidase, no change in either peak was noted, suggesting the absence of glucuronidated metabolites of hispidulin or apigenin. Identification of the unknown peaks

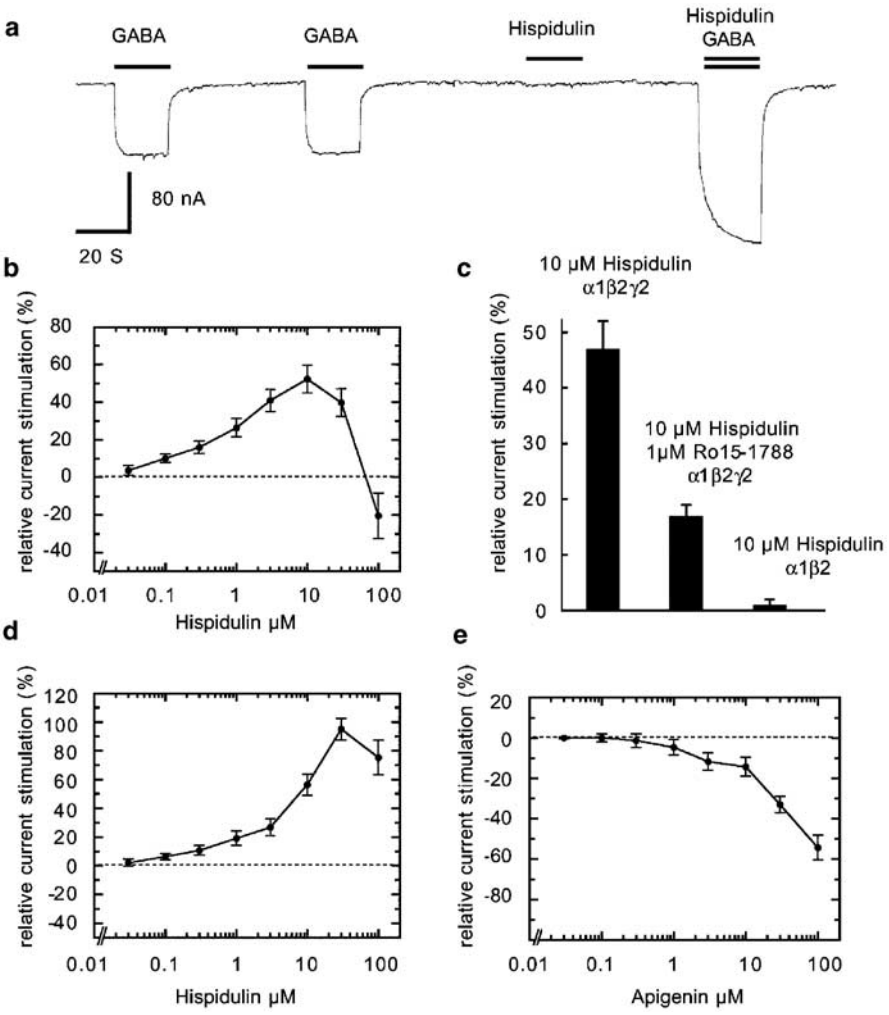

Figure 2 Functional properties of hispidulin and apigenin. (a) Hispidulin at 10 μM by itself did not elicit any current but stimulated currents elicited by 4 μM GABA at recombinant $\alpha1\beta2\gamma2$S $GABA_A$ receptors expressed by *X. leavis* oocytes. (b) Concentration dependence of allosteric stimulation by hispidulin. (c) Stimulation by hispidulin (10 μM) at $\alpha1\beta2\gamma2$S $GABA_A$ receptors is partially inhibited by co-application of the BZD receptor antagonist Ro15-1788 (1 μM). $\alpha1\beta2$ $GABA_A$ receptors are not stimulated by 10 μM hispidulin. (d) Allosteric stimulation by hispidulin at $\alpha6\beta2\gamma2$S $GABA_A$ receptors. (e) Inhibition by apigenin of GABA-induced currents at recombinant $\alpha1\beta2\gamma2$S $GABA_A$ receptors. The points indicate the mean $\pm$ s.e.m. ($n = 3$–5).

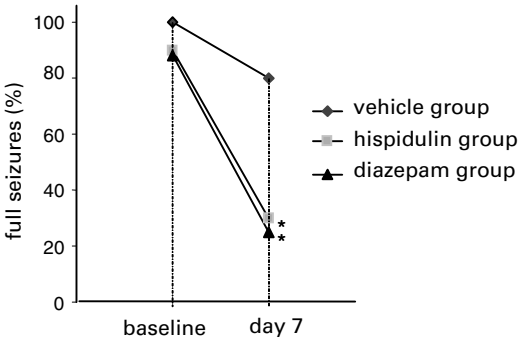

Figure 3 Effects of vehicle, hispidulin and diazepam on the occurrence of epileptic seizures in the Mongolian gerbil model (*M. unguiculatus*) before and after the application of test substances. Paired proportion tests (http://www.ubmail.ubalt.edu/~harsham/Business-stat/otherapplets/PairedProp.htm) were used to examine the null hypothesis that 7 days' pharmacological treatment had no modulatory effect on seizure propensity. $^*P < 0.04$.

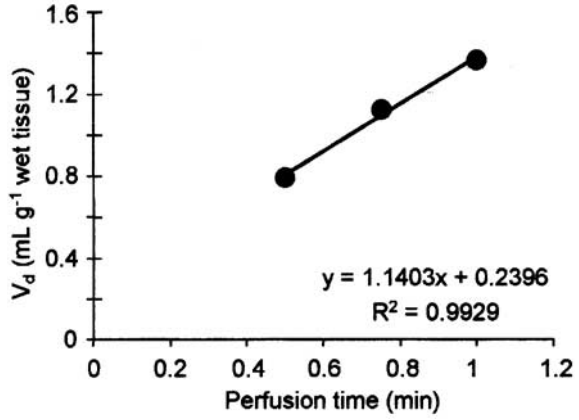

Figure 4 Volume of distribution (V_d) in the rat brain (right hemisphere) for [^{14}C]hispidulin plotted against time ($n = 3$), as assessed using the Takasato *in situ* perfusion method. $R^2 = 0.9929$.

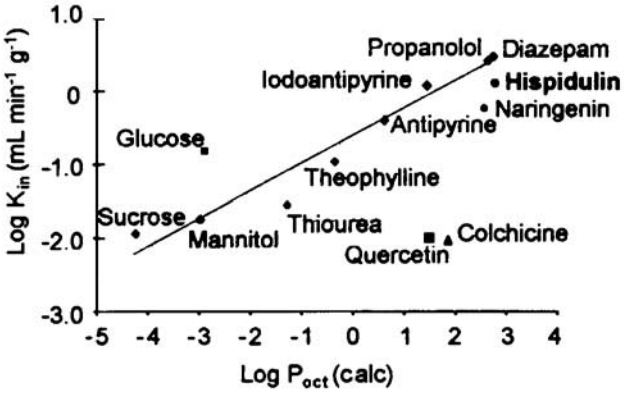

Figure 5 Relationship between log K_{in} (experimental) and log P_{oct} (calculated) of passively permeating compounds (diamonds), other flavonoids (naringenin, quercetin), substrates for known uptake (glucose) and efflux (colchicine) transport. Log K_{in} values with the exception of hispidulin are taken from Qaiser *et al*. (in preparation; see also Youdim *et al*., 2004). Log P_{oct} was calculated using the KowWin (LogKow) software at http://www.esc.syrres.com/interkow/logkow.htm. This program uses fragmental analysis of the compound structure for its prediction (values show high correlation with quoted experimental values, $P = 0.98$).

at RT 43.88 min (Figure 6a) and 36.81 min (Figure 6b) fell outside the scope of the current study.

Discussion

In a previous study on novel natural ligands of the BZD receptor from *S. officinalis*, we isolated the flavone hispidulin and provided details on its affinity to the BZD binding site *in vitro* (Kavvadias *et al*., 2003). The subsequent successful synthesis of hispidulin including its ^{14}C-labelled form allowed us to perform additional *in vitro* and *in vivo* investigations.

Electrophysiological studies

Functional effects of hispidulin were investigated in electrophysiological studies at recombinant $GABA_A$ receptors expressed in *X. laevis* oocytes. GABA was always used at concentrations eliciting 2–5% of the maximal current amplitude in the corresponding $GABA_A$ receptor. Hispidulin at 10 μM did not by itself elicit current but exhibited a positive allosteric modulatory effect by enhancing the GABA-stimulated current at $\alpha_1\beta_2\gamma_2$S (Figure 2a). This stimulation was concentration dependent (Figure 2b). Maximum relative stimulation at $\alpha_1\beta_2\gamma_2$S was achieved with 10 μM hispidulin. Interestingly, at higher concentrations, a second inhibitory phase was observed. The fact that action of hispidulin is two-phasic, going through a maximum, predicts that an overdose will result in a submaximal effect. At 10 μM, stimulation by hispidulin amounted to about 24% of the stimulation by 1 μM diazepam. Assuming that the optimum stimulation observed corresponds closely to the maximum stimulation, hispidulin is a partial positive allosteric modulator. The potentiation of 10 μM hispidulin at $\alpha_1\beta_2\gamma_2$S amounted to $47 \pm 5\%$ ($n = 3$, s.e.m.) and was largely blocked by co-application of the BZD receptor antagonist Ro15-1788 with a residual stimulation of $17 \pm 2\%$ ($n = 3$, s.e.m.) (Figure 2c). Potentiation of 10 μM hispidulin at $\alpha_1\beta_2$ amounted to $1 \pm 1\%$ ($n = 3$, s.e.m.). We conclude that hispidulin acts predominantly through the BZD binding site.

Substance concentrations of about 50 nM and higher stimulated the GABA-induced chloride current at all five receptor subtypes ($\alpha_{1-3,5,6}\beta_2\gamma_2$S) investigated (not shown). In this study, we could not detect any receptor subtype selectivity

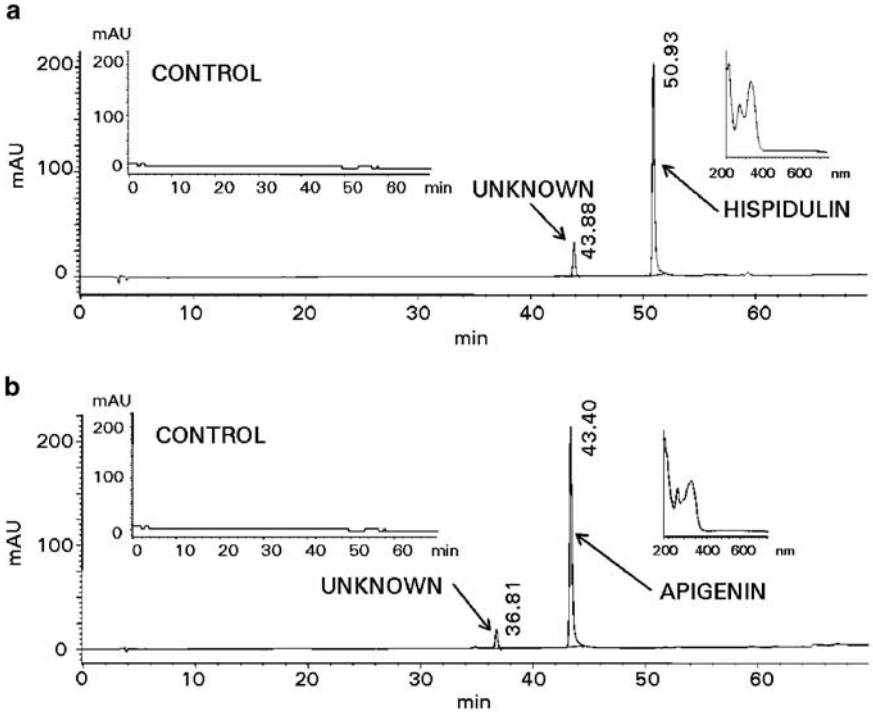

Figure 6 HPLC traces at 320 nm obtained from receiver wells of the Caco-2 cells showing permeability of hispidulin (a) and apigenin (b) in their intact aglycone forms.

regarding maximal stimulation. The IC_{50} was 0.8–1.8 μM for $\alpha1\beta2\gamma2S$, $\alpha2\beta2\gamma2S$ and $\alpha5\beta2\gamma2S$, and about 5 μM for $\alpha3\beta2\gamma2S$ and $\alpha6\beta2\gamma2S$. In contrast to diazepam, hispidulin also enhanced the GABA-activated current at $\alpha6\beta2\gamma2S$ $GABA_A$ receptors (Figure 2d). At these receptors, the stimulation by 10 μM hispidulin was $65\pm17\%$ ($n=3$, s.e.m.) and in the presence of 1 μM Ro15-1788 $37\pm7\%$ ($n=3$, s.e.m.). Recent studies of Marder *et al*. (2001) showed absence of binding activity of several flavonoids for $GABA_A$ receptors containing α_6-subunits. Sterical reasons owing to the small lipophilic pocket L3 at receptors containing α_6-unit were hypothesized to explain this phenomenon. In our studies, hispidulin interacted also with this receptor subtype. As with other ligands of the BZD binding site, action at $\alpha6\beta2\gamma2S$ receptors seems to depend on ligand structure.

Apigenin was also investigated. Despite its strong structural similarity to hispidulin (Figure 1d), apigenin reduced the GABA-induced chloride ion current and therefore acted as negative allosteric modulator of GABA in $\alpha1\beta2\gamma2S$ (Figure 2e) in the present electrophysiological study. Most probably it lacks the stimulatory phase observed at low concentrations of hispidulin and only has the inhibitory properties observed at higher concentrations. A similar behavior was observed in $\alpha2\beta2\gamma2S$, $\alpha3\beta2\gamma2S$ and $\alpha5\beta2\gamma2S$. Interestingly, in $\alpha6\beta2\gamma2S$, the transient stimulatory phase was also observed. A negative allosteric effect by apigenin has previously been noted by Avallone *et al*. (2000) in primary cultures of cerebellar granule cells. Thus, its modulatory activity at the BZD receptor site as suggested by behavioral data (Viola *et al*., 1995) has been difficult to ascertain both in primary cell cultures and in oocytes expressing recombinant receptors (Avallone *et al*., 2000; Goutman *et al*., 2003). Sedative or other BZD-like effects observed *in vivo* are therefore presumed to result from apigenin affinity to an alternative site.

Epileptic seizures are a correlate of hypersynchronous electrical discharges in the brain resulting from neuronal overexcitation or deficient inhibition in a given brain area. The GABA-mediated inhibition is readily enhanced by a large number of BZD receptor ligands. Consequently, classical BZD ligands such as diazepam act as effective anticonvulsants (Treiman, 2001). We therefore hypothesized that other BZD receptor ligands that possess an agonistic pharmacological profile similar to that of hispidulin could also have these effects. In this study, the diazepam-like activity of hispidulin *in vitro* led us to hypothesize anticonvulsant effects of the flavone *in vivo*. The Mongolian gerbil (*M. unguiculatus*) is well characterized as an animal model for studying epileptiform seizures, in particular, human myoclonic and grand mal seizures (Bertorelli *et al*., 1995). A variable proportion of Mongolian gerbils are known to suffer from clonic–tonic seizures, which may be elicited by exposure to minor environmental stimuli, for example, by manually stroking the animal or by placing it in a novel environment. Seizure sensitivity is enhanced in certain inbred strains well suited for the study of antiepileptic drugs (Rausch *et al*., 1988). Owing to their innate vulnerability, affected gerbils need not undergo chemical or electroshock challenges to induce seizures (Rausch *et al*., 1988). Intravenous or intraperitoneal injections are mostly avoided so as not to cause immediate seizure induction. In the present study, efficacy of hispidulin was tested in comparison to diazepam on the basis of earlier observations (Rausch *et al*., 1988). As we confirmed, antiepileptic treatment with either hispidulin or the classic tranquilizer diazepam aided in the treatment of spontaneous seizures.

Few studies have addressed BBB permeability for flavone derivatives. It was recently shown in an *in vitro* model by members of our group that a number of flavonoids, for example, naringenin and hesperetin, are able to enter the brain endothelium and cross the BBB as are several flavonoid metabolites (Youdim *et al*., 2003). Other related compounds, for example, epicatechin, possess little or no potential for permeation. The permeability of hispidulin across the BBB has not been investigated in cell culture or *in vivo*. The anticonvulsant effects of this flavone, illustrated in seizure-prone Mongolian gerbils, strongly suggested its delivery to the CNS. To further confirm this important mode of distribution, we investigated the penetration of hispidulin into the brain in an *in situ* rat model. When compared to other highly BBB-penetrating agents, hispidulin approached both diazepam and propanolol in terms of uptake by the total right cerebral hemisphere. When rate of uptake (K_{in}) by the total right cerebral hemisphere was plotted against compound lipophilicity ($\log P_{oct}$ 2.67), the behavior of hispidulin was similar to that of known highly penetrating agents such as diazepam and propanolol. These findings suggest that hispidulin permeates the BBB by simple passive diffusion. However, a control perfusion with [^{14}C]sucrose indicated that 5% ethanol did not disrupt BBB integrity, so the high penetration of [^{14}C]hispidulin was not due to paracellular (junctional) flux, but likely to be transcellular, *via* passive diffusion across the endothelial cell membranes. As recently observed by Youdim *et al*. (2003) in an *in vitro* BBB model, the potential for permeation by other flavonoids is strongly correlated with lipophilicity. Here we show that the lipophilicity of hispidulin appears to facilitate its permeation through the BBB.

Many flavones and their glucoside derivatives are glucuronidated during the absorption process (Spencer *et al*., 1999). Glucuronidation during transfer across the jejunum and ileum is possible without the need for gut microflora. The more highly reducing phenolics like quercetin or luteolin are absorbed, more than 90% as glucuronidated metabolites. To establish whether orally administered hispidulin enters the blood circulation in its intact form, permeation across the Caco-2 monolayer was investigated. Hispidulin and apigenin were predominantly absorbed without any structural modification. These studies provide evidence that the active aglycone form of hispidulin is present after absorption.

The present study illustrates the chemical synthesis of the flavone hispidulin. As we point out, this compound probably acts as a partial positive allosteric modulator at $GABA_A$ receptors, penetrates the BBB and possesses anticonvulsant activity in the CNS. Electrophysiological data and data on BBB permeation provide important insights into the role of hispidulin as a potent anticonvulsant compound and are supported by our *in vivo* observations, with the limitations of a single-dose design. Further experiments will establish whether hipidulin displays other effects typical of BZDs, such as sedative, anxiolytic and muscle relaxant activity. Further research is warranted on the bioavailability, distribution parameters and the putative, active metabolites plus the effects in other epileptic phenotypes, to put into perspective the flavone's impact on inhibitory brain activity.

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