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HIGH AFFINITY OF THE NATURALLY-OCCURRING BIFLAVONOID, AMENTOFLAVON, TO BRAIN BENZODIAZEPINE RECEPTORS *IN VITRO*

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Abstract—In a search for pharmacologically-active ingredients in the plant extract Karmelitter Geist®, we have isolated and identified a high-affinity benzodiazepine receptor ligand, amentoflavon. Amentoflavon binds in a mix-type competitive and non-competitive manner to brain benzodiazepine receptors with an IC_{50}-value of 6 nM *in vitro*, comparable to the affinity of diazepam. Amentoflavon shows a GABA ratio of 1.4 at 0° and increases ^{35}S-TBPS binding by 12% (60 min incubation at 25°), which indicates a partial agonistic effect on benzodiazepine receptors. The substance does not influence the binding of a variety of other brain receptor ligands. Amentoflavon does not inhibit ^{3}H-flunitrazepam-binding to brain benzodiazepine receptors following i.v. administration in the mouse *in vivo*, and it is therefore not likely that amentoflavon is responsible for any pharmacological effect of Karmelitter Geist®.

The benzodiazepine receptor was identified in 1977 when it was shown that specific binding sites for 1,4-benzodiazepines exist in rat brain. The anxiolytic, anticonvulsive, hypnotic and sedative effects of the clinically-used benzodiazepines are mediated by an interaction with this receptor. Among benzodiazepine receptor ligands several non-benzodiazepine ligands have been discovered (e.g. derivatives of triazolopyridazines, β-carboline-3-carboxylic acid, pyrazoloquinolinone and cyclopyrrolones) [1].

Extracts of plants have been used against a variety of diseases, often without clear rationales. Karmelitter Geist® is an alcoholic tincture of various plants used against anxiety and epilepsy or as sedative hypnotics. It is therefore of interest to investigate this extract for the presence of pharmacologically-active substances, which may interact with the brain benzodiazepine receptor. We now report that the biflavonoid amentoflavon is found in Karmelitter Geist® and has high affinity (IC_{50} = 6 nM) for brain benzodiazepine receptors *in vitro*.

MATERIALS AND METHODS

Isolation of amentoflavon. Batches of Karmelitter Geist® were diluted *ca.* 10 times with distilled water (to obtain a final ethanol concentration of *ca.* 10%), and gently shaked with Chromosorb® for 30 min at room temperature. After washing of the Chromosorb® with water and 20% ethanol, the ^{3}H-diazepam-binding inhibitory activity was eluted with 50% ethanol, which was evaporated at reduced pressure. The dry residue was redissolved in 70% ethanol and chromatographed on a Sephadex LH 20 column (5 × 100 cm). The column was eluted with 70% ethanol, flow 0.6 ml/min. Fractions containing activity were pooled; HPLC analysis showed that one single substance accounted for 80% by weight.

Brain membrane preparations. Tissue was prepared for binding studies as described [2]. In brief, cortex, hippocampus or cerebellum from rat brain were homogenized and washed three times in Tris–citrate (50 mM, pH 7.1) and the tissue was finally resuspended in Tris–citrate buffer 50 mM, pH 7.1 at a concentration of 2 mg original tissue per ml. Aliquots of membranes were used for ^{3}H-diazepam- (0.4 nM, 2.5 ml assay) binding, for ^{3}H-flunitrazepam- or ^{3}H-Ro 15-1788- (1 ml assay) binding. Samples were incubated at 0° for 40 min followed by vacuum filtration through Whatman GF/C glass-fibre filters. Nonspecific binding was obtained in the presence of midazolam (3×10^{-6} M). ^{3}H-muscimol-binding to an Ag^{+}-treated membrane preparation and ^{35}S-TBPS-binding to 3-times-washed membrane preparation were done according to Nielsen *et al.* [2]. All assays were done in duplicate.

Inhibition of ^{3}H-flunitrazepam to mouse brain benzodiazepine receptors was investigated as described [3]. All mice were decapitated 20 min after intravenous administration of 4 μCi of ^{3}H-flunitrazepam.

Materials. Karmelitter Geist®, Vogel, Switzerland, was purchased from a local supplier. The alcoholic tincture is an extract of the following fresh plants: *Folium melissa officinalis* conc.; *Cortex citri.* conc.; *Lignum junipero* conc.; *Fruct. coriandri contusi*; *Folium menthae piperitae* conc.; *Herba echinacea* conc.; *Nuces myristicae* conc.; *Fruc. cardamoni contusi*; *Flores caryophylli* cont. and *Cortex cinnamoni cassiae* conc. ^{3}H-diazepam (78.9 Ci/mmol), ^{3}H-flunitrazepam (methyl-^{3}H, 74.8 Ci/mmol), ^{3}H-Ro 15-1788 ([*N*-methyl-^{3}H], 76 Ci/mmol) and ^{35}S-TBPS (butyl bicyclophosphorothionate, tertiary-^{35}S-, 91.8 Ci/mmol) were from New England Nuclear, Dupont. Amentoflavon was kindly

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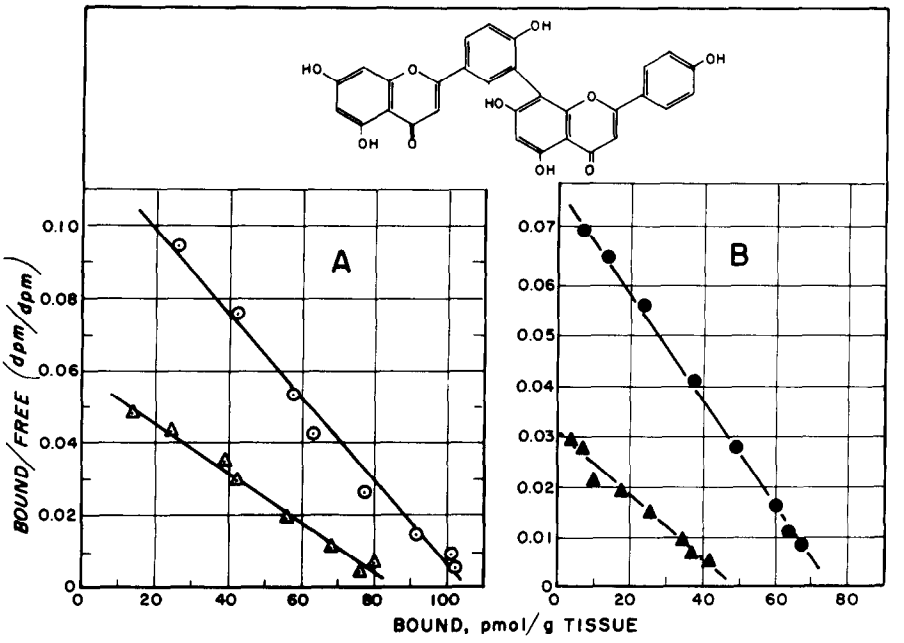

Fig. 1. Chemical structure of amentoflavon. Scatchard plot of (A) ^{3}H-flunitrazepam (74.8 Ci/mmol, NEN) binding (0.7–46.3 nM) to hippocampus membranes (○), and (B) ^{3}H-Ro 15-1788 (76 Ci/mmol, NEN) binding (0.12–7.90 nM) to cerebellum membranes (●). Inhibition by amentoflavon (3.7 nM) (△ and ▲).

donated by Professor Dr. Hans Geiger, Institut für Chemie, Universität Hohenhein, Stuttgart, F.R.G. ZK 93423 (6-benzyloxy-4-methoxy-methyl-β-carboline-3-carboxylic acid ethyl ester) and DMCM (4-ethyl-6,7-dimethoxy-β-carboline-3-carboxylic acid methyl ester) were from Ferrosan (Soeborg, Denmark). Amentoflavon dissolved in DMSO and diluted in 50% ethanol was added to assays just prior to radioligands.

RESULTS

Elution of Sephadex LH20 column with 70% ethanol yielded one single peak of ^{3}H-diazepam-inhibitory activity. HPLC analysis suggests that 80% of the absorbing material represented one single substance, which was identified by NMR/mass spectrometry as amentoflavon. The substance showed similar potency and displacement curves as authentic amentoflavon on ^{3}H-diazepam- and ^{3}H-flunitrazepam-specific binding *in vitro* (data not shown).

Amentoflavon inhibits ^{3}H-flunitrazepam- and ^{3}H-Ro 15-1788-specific binding to hippocampal and cerebellar membranes in a mix-type competitive–non-competitive manner (Fig. 1). The GABA ratio at 0° [4] of amentoflavon inhibiting ^{3}H-diazepam binding was 1.50 ± 0.34 (7) and 1.35 ± 0.18 (6) (mean ± SD of N values) in membranes from hippocampus and cerebellum, respectively [The GABA ratio is the IC_{50} of amentoflavon inhibiting ^{3}H-diazepam-binding without GABA added to the IC_{50} with GABA (10^{-5} M) added to assays]. The IC_{50}-value in hippocampus [5.0 ± 1.5 (7) nM] was not different from the IC_{50}-value in cerebellum [6.5 ± 2.2 (6) nM] (mean ± SD of N values). The Hill coefficient was not different from the one in hippocampus and cerebellum (1.09 ± 0.14, mean ± SD of 13 values). Amentoflavon (1.85 μM) has no effect on ^{3}H-muscimol-binding to high-affinity GABA receptors but showed a small increase in ^{35}S-TBPS-binding (Fig. 2).

Amentoflavon (1.85 μM, pure or isolated from Karmelitter Geist®) showed less than 5% inhibition of specific binding *in vitro* of the following ligands: ^{3}H-spiroperidol, ^{3}H-SCH 23390, ^{3}H-ketanserin, ^{3}H-8OH-PAT, ^{3}H-dihydroalprenolol, ^{3}H-prazosin, ^{3}H-QNB (data not shown). As compared to authentic amentoflavon, the purified substance showed similar potency in inhibiting ^{3}H-diazepam binding as also mix-type competitive–non-competitive inhibition of ^{3}H-flunitrazepam binding *in vitro*.

Administration of amentoflavon 3 mg/kg, 10 mg/kg or 30 mg/kg i.p. 30 min before decapitation resulted in no inhibition, 15% and 20% inhibition, respectively, of specific ^{3}H-flunitrazepam binding in the mouse brain *in vivo*.

DISCUSSION

Several natural products which act on the brain GABA receptor–benzodiazepine receptor–chloride ion channel complex have been discovered: muscimol, a potent GABA receptor agonist, is synthesized in the mushroom *Amanita muscaria* by decarboxylation of ibotenic acid; the convulsant alkaloid, bicuculline, which blocks actions of GABA, was extracted from a species of *Cordyalis*; picrotoxinin, a convulsant drug, which inhibits the function of the chloride channel complex, was obtained from seeds of *Anamirta cocculus*; avermectin B_{1a}, an anthel-

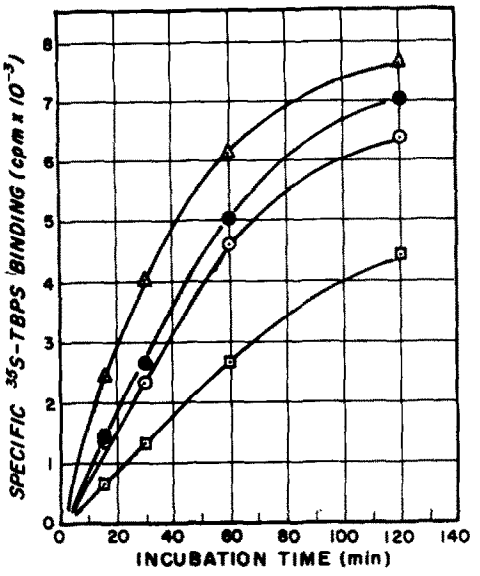

Fig. 2. ^{35}S-TBPS binding (0.9 nM, 1 M NaCl, 25°) to membranes from rat cortex: control binding (○——○); amentoflavon (56 nM) (●——●); ZK 93423 (100 nM) (△——△) and DMCM (100 nM) (□——□). The results shown are representative of four independent experiments. Amentoflavon increased ^{35}S-TBPS specific binding by 112 ± 3% (N = 4); $P < 0.01$ at 60 min of incubation. ZK 93423 and DMCM are benzodiazepine receptor full agonist and inverse agonist, respectively.

minthic product, which modulates the binding of GABA and benzodiazepines to their receptors, was isolated from *Streptomyces avermitilis*, and anisation, a toxic substance isolated from the seeds of *Illicum anisatum*, acts as a picrotoxin-like non-competitive GABA antagonist (for reference, see [5]).

To our knowledge amentoflavon is the first substance, described with high affinity for the brain benzodiazepine receptor, which does not contain nitrogen in its chemical structure. The affinity is comparable to the affinity of diazepam. Amentoflavon does not differentiate between BZ_1 and BZ_2 subtypes of benzodiazepine receptors, since amentoflavon shows similar IC_{50}-values on inhibition of ^{3}H-diazepam-binding to cerebellar and hippocampal membranes. The receptor interactions showed a GABA ratio of *ca.* 1.4 and a small increase in ^{35}S-TBPS binding. The GABA ratio for benzodiazepine receptor antagonists is about one and for agonists about two. Therefore, a GABA ratio of about 1.4 for amentoflavon indicates that the substance should show partial agonistic activity *in vivo*; this is consonant with the increase in ^{35}S-TBPS binding. However, amentoflavon (10 or 30 mg/kg i.p.) showed only a small inhibition of ^{3}H-flunitrazepam binding in mouse brain *in vivo*, indicating that the substance is either rapidly metabolized or does not penetrate the blood–brain barrier. This finding suggests that interaction with benzodiazepine receptors cannot explain pharmacological or clinical effects of amentoflavon.

We have not determined which of the ten plants constituents in Karmelitter Geist® contain high contents of amentoflavon. Biflavonoids are widely distributed in plant species [6]; amentoflavon has been found in eight genera of the subfamily Arpressoideae [7]. It is of interest to learn if other biflavonoid structures show affinity to benzodiazepine receptors; we have found that one such substance, hinokiflavon, has low affinity for brain benzodiazepine receptors.

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