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IN VIVO DETERMINATION OF THE PROFILE OF BENZODIAZEPINE LIGANDS BY COMPARING THE INHIBITION OF ^{3}H-Ro 15-1788 BINDING TO THE MODULATION OF cGMP LEVELS IN MOUSE CEREBELLUM

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Abstract—The *in vivo* effects of various benzodiazepine (BZD) ligands belonging to different chemical families were studied comparatively in mouse cerebellum using displacement of ^{3}H-Ro 15-1788 binding and cGMP content as biochemical tools. It was possible to differentiate four classes of compounds with regard to these biochemical parameters. The first class of compounds such as diazepam and suriclone induced a net effect on *in vivo* ^{3}H-Ro 15-1788 binding and a dose-dependent decrease of cGMP levels. A second class of drugs such as ZK 91296 and CGS 9896 showed *in vivo* activities in displacement studies but relatively small or moderate activities on cGMP levels. A third class was represented by Ro 15-1788 itself which prevented dose-dependently the *in vivo* ^{3}H-Ro 15-1788 binding but was devoid of effect on cGMP levels. Finally, a fourth class of compounds (CGS 8216, FG 7142, β-CCM and DMCM) showed *in vivo* displacement of ^{3}H-Ro 15-1788 with concomitant increase of cGMP levels. The first class of compounds represents full agonists, the second class, partial agonists, the third class, the antagonist Ro-15-1788 itself, and the fourth class corresponds to inverse agonists. Thus it is proposed to use ^{3}H-Ro 15-1788 binding and cGMP levels to differentiate *in vivo* BZD ligands acting on the BZD receptor/GABA receptor/chloride ionophore complex.

There is now compelling evidence supporting the notion initially reported ten years ago [1, 2] that anxiolytic benzodiazepines (BZD) exert their pharmacological actions through the modulation of specific receptors. Anxiolytic, hypnotic, anticonvulsant and muscle relaxant effects of BZD are consecutive to an enhancement of GABAergic transmission [3] and the locus of action of BZD was proposed to be a part of a BZD receptor/GABA receptor/chloride ionophore complex [4]. In cerebellum, the inhibitory effect of Purkinje cells on the cerebellar nuclei and that of basket cells on Purkinje soma are mediated by GABA [5, 6]. Anxiolytic BZD decrease Purkinje cells activity [3] and the 3′,5′-cyclic guanosine monophosphate (cGMP) levels in cerebellum [7]. By measuring the cGMP levels in cerebellum, it was proposed to obtain information of the state of activation of the GABA and BZD receptors in this cerebral structure [8].

Non BZD ligands were found to recognize with a high affinity the BZD receptor: the cyclopyrrolones zopiclone and suriclone [9–11], the triazolopyridazines [12]; the quinolines [13]; the pyrazoloquinolines CGS 8216 and 9896 [14, 15]; the imidazopyridine zolpidem [16], and various derivatives of β-carbolines such as β-carboline-3-carboxylate (β-CCE), methyl 6,7-dimethoxy-4-ethyl-β-carboline (DMCM) and β-carboline-3-carboxylic acid methylamide (FG 7142) [17].

Pharmacological and biochemical studies have clearly demonstrated that new compounds acting on BZD receptors can display actions which differ from those of classical BZD [18–22]. The pharmacological profile of these different drugs shows a continuum from full agonists (diazepam, zopiclone) to full inverse agonist (DMCM). Indeed drugs showing partial agonist (CGS 9896 and ZK 91296), antagonist (Ro 15-1788) and partial inverse agonists (CGS 8216, FG 7142 and β-CCE) activities were also described [14, 17, 21, 23–25]. Braestrup and Nielsen [22] have reported correlations between *in vivo* BZD receptor occupancy and *in vivo* efficacy of BZD ligands as anticonvulsants, inhibitors or facilitators of audiogenic seizures and anticonflict drugs. All these comparisons were effected using ^{3}H-flunitrazepam as an *in vivo* ligand. However, compared to ^{3}H-flunitrazepam, ^{3}H-Ro 15-1788 was found more suitable for *in vivo* labelling of BZD receptors [26].

As far as we know, there is no biochemical report comparing in the same study the effects of various BZD ligands (full and partial agonists, antagonists, full and partial inverse agonists) on *in vivo* binding of ^{3}H-Ro 15-1788 and cGMP levels in cerebellum. The aim of this study was to compare the receptor occupancy of such different BZD ligands with their activity in a functional model (cGMP) which was proposed as a useful tool to differentiate BZD agonists and antagonists [8].

MATERIALS AND METHODS

Male CD1 COBS mice weighing 20–22 g, Charles River France, were used throughout; food and water were freely available. The colony rooms were illuminated between 7.00 a.m. and 7.00 p.m.

In vivo ^{3}H-*Ro* 15-1788 *binding.* ^{3}H-Ro 15-1788 (83 Ci/mmol, New England Nuclear) was diluted with 0·9% NaCl to 50 μCi/ml and in some cases the specific activity was reduced with non-radioactive Ro 15-1788 to obtain desired doses. Each mouse received 5 μCi/100 μl via the dorsal tail vein. For determination of non-specific binding, mice were pretreated orally with clonazepam (25 mg/kg) 30 min before the ^{3}H-Ro 15-1788 injection. For pharmacological studies, drugs were administered orally 30 min prior to the intravenous (i.v.) injection (4–5 animals per group) of ^{3}H-Ro 15-1788; 20 min later, each mouse was sacrificed by decapitation and the cerebellum was rapidly dissected, weighed, deposited in counting vials and homogenized in 1.5 ml of Tris–HCl (50 mM, pH 7.5). Ten millilitres of liquid scintillation cocktail (HP Beckman) were added to each counting vial and the samples counted using standard counting methods. For determination of chemical identity of radioactivity, the cerebellum was homogenized in 30 vol. of ethanol and centrifuged at 50,000 *g* for 30 min and 50 μl of the supernatant were applied in silica gel plates. Using methylene chloride–methanol (19:1, v/v) as a solvent, more than 90% of the radioactivity migrated with authentic Ro 15-1788.

cGMP assay. Drugs were administered to animals (7–9 per group) 50 min before the sacrifice. The cerebellum was dissected and deposited in 13 vol. of boiling Tris–HCl (50 mM, pH 7.5). The samples were heated for 5 min, sonicated and centrifuged at 14,000 *g* for 15 min. The cGMP levels were measured in the diluted supernatant using a RIA kit (Amersham).

Drugs. Ro 15-1788 was a gift from Hoffmann La Roche & Co. (Basel); ZK 91296 and DMCM were kindly supplied by Schering AG (Berlin). All the other compounds were synthesized in our laboratories. Drugs were suspended in 10% gum arabic.

RESULTS

In agreement with previous data from the literature, all drugs tested showed expected potencies for displacing *in vitro* the specific binding of ^{3}H-flunitrazepam to rat brain BZD receptors (data not shown).

Saturation of ^{3}H-*Ro* 15-1788 *binding in cerebellum*

Estimation of receptor occupancy was determined according to the method described by Goeders and Kuhar [26]. Figure 1 shows that the injection of increasing doses of nonlabelled Ro 15-1788 together with a constant tracer dose of ^{3}H-Ro 15-1788 decreased the quantity of specifically-bound radioactive drug in mouse cerebellum in a dose-dependent manner. The maximal displacement obtained with the highest dose of nonlabelled Ro 15-1788 coincides with the state reflecting the nonspecific binding. This result is in agreement with that reported in mouse cerebral cortex by Goeders and Kuhar [26]. Specific ^{3}H-Ro 15-1788 binding was 158 ± 8 d.p.m./mg of tissue (mean ± SEM of 39 experiments).

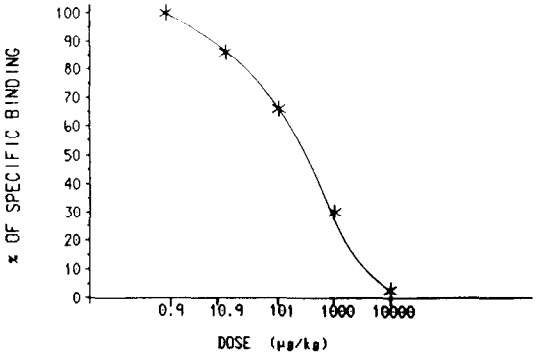

Fig. 1. Saturation of ^{3}H-Ro 15-1788 binding in mouse cerebellum. Mice were intravenously injected with ^{3}H-Ro 15-1788 and increasing doses of unlabelled Ro 15-1788. Animals were sacrificed 20 min after injections. Each point represents the mean of 4–5 determinations.

Drug-induced inhibition of in vivo ^{3}H-*Ro* 15-1788 *binding*

Determination of doses inducing 50% inhibition (ID_{50}) of ^{3}H-Ro 15-1788 binding was performed by a regression-analysis from dose–responses curves represented in Fig. 2 and Fig. 3. We have listed in Table 1 the ID_{50} of the studied drugs. There were marked differences between the potency of the BZD ligands for decreasing the *in vivo* ^{3}H-Ro 15-1788 binding. In the case of proconvulsant or convulsant compounds (FG 7142, β-CCM and DMCM), the ID_{50} were only estimated.

Comparison of the effects of BZD ligands on in vivo ^{3}H-*Ro* 15-1788 *binding and on cGMP levels in mouse cerebellum*

Control cGMP levels were 407 ± 24 pmol/g of wet weight (mean ± SEM of 50 experiments).

Table 1. ID_{50} of various benzodiazepine ligands with respect to *in vivo* ^{3}H-Ro 15-1788 binding in mouse cerebellum

Drug	ID_{50} (mg/kg p.o.)
Diazepam	1.7
Suriclone	12.3
CGS 9896	2.2
ZK 91296	3.4
Ro 15-1788	0.4
CGS 8216	1.9
FG 7142	≥50*
β-CCM	≥100*
DMCM	250*

Animals were treated orally with various benzodiazepine ligands 30 min prior to the i.v. injection of ^{3}H-Ro 15-1788 (5 μCi) and were killed after 20 min. ID_{50} were determined from dose–response curves from Figs 2 and 3. The ID_{50} were only estimated when indicated *.

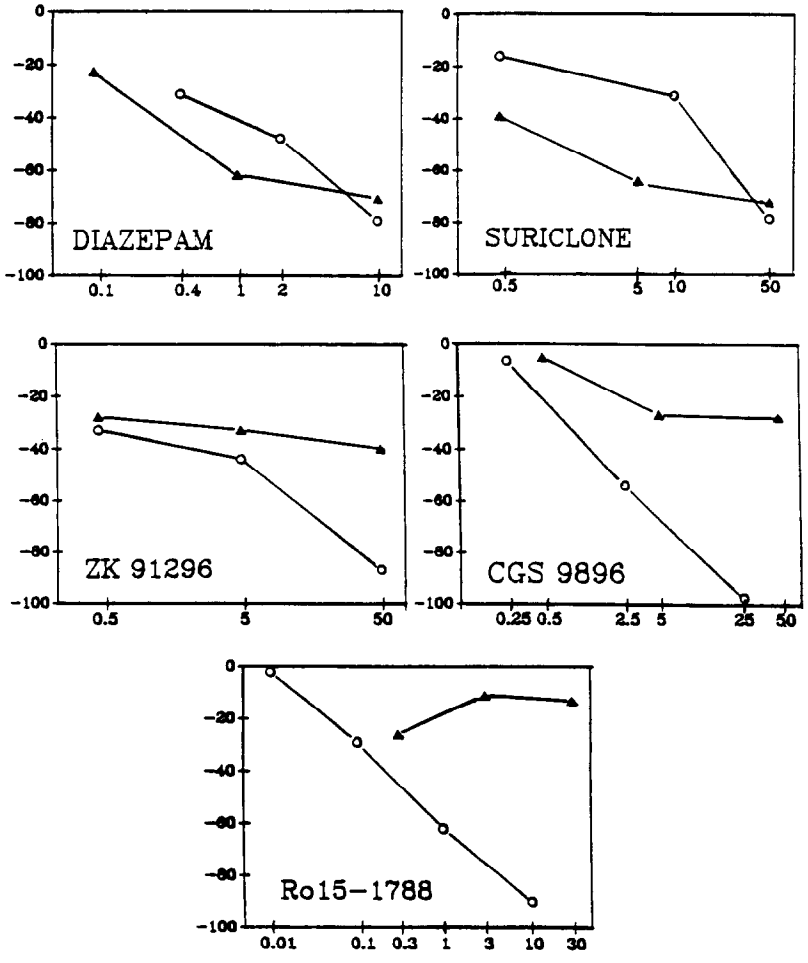

Fig. 2. Effects of diazepam, suriclone, ZK 91296, CGS 9896 and Ro 15-1788 on *in vivo* ^{3}H-Ro 15-1788 binding (○) and cGMP levels (▲) in mouse cerebellum. Animals were treated with indicated doses of studied compounds. For displacement experiments, mice (4–5 per group) received 5 μCi (i.v. route) 30 min after the oral administration and were sacrificed after 20 min. For cGMP experiments, mice (7–9 per group) were sacrificed 50 min after the oral administration. Abscissa: log drug concentration (mg/kg p.o.). Ordinate: per cent decrease in *in vivo* ^{3}H-Ro 15-1788 binding or cGMP levels.

The efficacies of the tested compounds (two BZDs: diazepam and Ro 15-1788; a cyclopyrrolone: suriclone (R.P. 31264), two pyrazoloquinolines: CGS 8216 and CGS 9896 and four β-carboline derivatives: β-CCM, DMCM, FG 7142 and ZK 91296) on *in vivo* ^{3}H-Ro 15-1788 binding and cGMP levels are given in Figs 2 and 3. There is a clear relationship between the displacement of ^{3}H-Ro 15-1788 and the decrease of cGMP levels in cerebellum for diazepam and suriclone (Fig. 2). Interestingly, the two curves do not exactly overlap since there is a displacement to the left of cGMP curves. CGS 9896 and ZK 91296 displaced ^{3}H-Ro 15-1788 from the cerebellar BZD receptors (Fig. 2). However, with these two compounds, the decrease in cGMP levels hardly reached a 40% decrease. The shift to the right of the cGMP curves (compared with the binding displacement curves) and the moderate decrease in cGMP levels could indicate that CGS 9896 and ZK 91296 are partial agonists in this paradigm. Ro 15-1788 inhibited the *in vivo* ^{3}H-Ro 15-1788 binding but did not modify significantly cGMP levels (Fig. 2).

As shown in Fig. 3, CGS 8216, FG 7142, β-CCM and DMCM induced an opposite effect on *in vivo* binding and cGMP levels: the ^{3}H-Ro 15-1788 binding is decreased whereas the cGMP levels are increased.

DISCUSSION

In this study we report the effects of different compounds possessing *in vitro* affinity for BZD receptors on *in vivo* ^{3}H-Ro 15-1788 binding and cGMP levels in mice cerebellum. Goeders and Kuhar [26] proposed the use of ^{3}H-Ro 15-1788 to label *in vivo* BZD receptors. In agreement with the results reported by these workers in cerebral cortex, we observed that the injection of increasing doses of unlabelled Ro 15-1788 with a constant tracer dose of

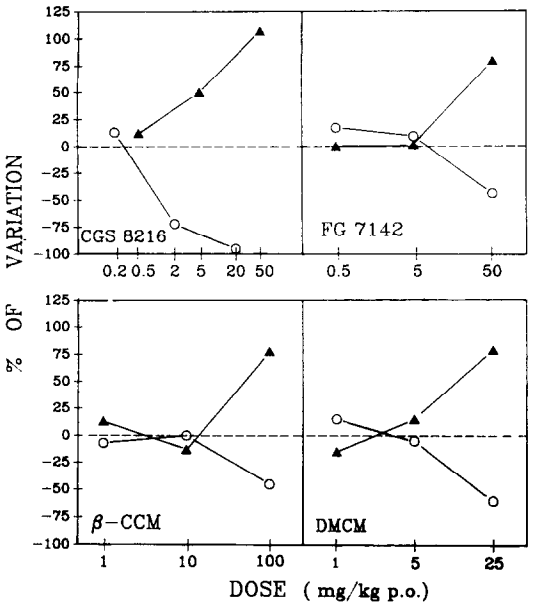

Fig. 3. Effects of CGS 8216, FG 7142, β-CCM and DMCM on *in vivo* ^{3}H-Ro 15-1788 binding (○) and cGMP levels (▲) in mouse cerebellum. Experiments were effected as described in the legend of Fig. 2.

^{3}H-Ro 15-1788 decreased the quantity of specifically bound ^{3}H-Ro 15-1788 radioactive drug in a dose dependent manner in cerebellum. The specific binding decreased to reach non-specific binding levels, indicating that the available binding sites were saturated.

All drugs studied were able to displace *in vivo* ^{3}H-Ro 15-1788 binding in cerebellum in a dose dependent manner. Concerning the cGMP levels, our results confirm and extend previous reports on compounds acting on BZD receptors [7, 28–30]. Interestingly, marked differences were observed when studying the effects of these different compounds on cGMP levels. Consequently, it was possible to differentiate four classes of drugs with regard to their effects on this second messenger.

A first class of compounds such as diazepam and suriclone induced a net effect on *in vivo* ^{3}H-Ro 15-1788 binding and a dose-dependent decrease of cGMP levels. Interestingly, when comparing the dose–response curves, a shift to the left was observed with the two compounds concerning the cGMP levels. The use of ^{3}H-Ro 15-1788 initially reported by Goeders and Kuhar [26] to label *in vivo* the BZD receptors was recently proposed to measure BZD receptor occupancy [27]. Thus, assuming that inhibition of ^{3}H-Ro 15-1788 binding will correspond to an estimation of *in vivo* occupancy of BZD receptors, we can observe that diazepam and suriclone induce a decrease of cGMP levels at doses corresponding to a small receptor occupancy. This proposal is of interest since full agonists produce an antipentylenetetrazol efficacy (ED_{50}) at an occupancy of 25–50% [21, 27, 28]. Thus, cGMP decrease and anticonvulsant activities elicited by full BZD receptor agonists may occur without complete receptor saturation.

A second class of compounds (CGS 9896 and ZK 91296) showed *in vivo* displacement of ^{3}H-Ro 15-1788 with moderate decrease in cGMP levels. Effectively, the displacement of nearly 90% of ^{3}H-Ro 15-1788 binding led to a decrease of 30–40% of cGMP levels. The shift to the right of cGMP curves suggests that these two compounds behave like partial agonists in this model.

A third class showed *in vivo* activity in displacement studies but with no activity on cGMP levels. The displacement of 90% or more of ^{3}H-Ro 15-1788 binding did not modify the cerebellar cGMP levels. Ro 15-1788 represents the only compound we have studied to be assimilated to an antagonist.

Finally, a fourth class of drugs showed *in vivo* displacement of ^{3}H-Ro 15-1788 with concomitant increase of cGMP levels; CGS 8216, FG 7142, β-CCM and DMCM were found in this group of drugs.

Interestingly, the first class of compounds represents full agonists, the second class is represented by drugs known to act as partial agonists, the third class represents an antagonist, in this study, Ro 15-1788 itself, and the fourth class corresponds to inverse agonists. Our results are in agreement with those of the literature (for reviews, see Refs 20–22).

One cannot totally exclude that some BZD ligands could interfere with cGMP metabolism in a way unrelated to the interaction with specific BZD receptors. However, it is well-known that the antagonistic effect of Ro 15-1788 is highly specific for agents acting through specific BZD receptors [29]. Moreover, recent studies have clearly demonstrated that Ro 15-1788 antagonizes BZD ligand-induced variations of cGMP levels [30–32], indicating that these modifications are consecutive to the interaction with specific BZD receptors.

In conclusion, using ^{3}H-Ro 15-1788 *in vivo* binding and measurement of cGMP levels, we have confirmed and largely extended the initial proposal of Serra *et al.* [8] and demonstrated that these biochemical techniques can be used to differentiate *in vivo* different classes of drugs acting on the GABA receptor/BZD receptor/chloride channel complex. Indeed, our study shows that it is possible to differentiate *in vivo* BZD receptor agonists, partial agonists, antagonists and inverse agonists by comparing the ^{3}H-Ro 15-1788 displacement curves to the cGMP curves in the same cerebral structure, the cerebellum.

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