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Diazepam and desmethyldiazepam differ in their affinities and efficacies at 'central' and 'peripheral' benzodiazepine receptors

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The in-vitro binding characteristics of three different ligands ([^{3}H]Ro 15-1788, [^{3}H]Ro 5-4864 and [^{3}H]flunitrazepam) and the structural requirements for binding to 'central' and 'peripheral' benzodiazepine receptors have been evaluated in rat cerebral cortex, cerebellum and adrenal glands. [^{3}H]Ro 15-1788 binding was detectable only in the brain. Clonazepam was the most potent inhibitor followed by diazepam and desmethyldiazepam, which showed the same affinity, and by premazepam; Ro 5-4864 did not show appreciable affinity. The same pattern was seen for [^{3}H] flunitrazepam binding in brain areas while in adrenal gland the inhibition pattern was exactly superimposable on that with [^{3}H]Ro 5-4864 in all the areas considered (Ro 5-4864 > diazepam > desmethyldiazepam > clonazepam > premazepam). These data confirm and extend previous reports. A methyl group in position 1 enhances the affinity for peripheral benzodiazepine binding sites which are labelled in the adrenal gland by [^{3}H]Ro 5-4864 and [^{3}H]flunitrazepam; in brain areas, [^{3}H]flunitrazepam, like [^{3}H]Ro 15-1788, selectively labels central binding sites. Methylation in position 1 did not change the affinity for these sites. Desmethyldiazepam is less active than diazepam as an anticonvulsant and in other tests. In-vivo experiments were therefore carried out to assess the 'intrinsic activity' of desmethyldiazepam: it appeared that this compound acts as a partial agonist at central benzodiazepine receptors.

Benzodiazepine (BDZ) binding to central and peripheral binding sites has been studied in detail (Richards et al 1982; Sieghart & Schuster 1984; Wang et al 1984). The two receptors have different structural and steric requirements. However, different ligands and receptor sources were used for those studies, making direct comparison of the two receptor sites difficult. Moreover, most of the experiments were made with [^{3}H]diazepam or [^{3}H]flunitrazepam, two ligands that, because of the presence of an alkyl group in position 1, lack selectivity between the two sites.

The study now reported aimed to verify the structural requirements for binding to these two receptors, and has considered the binding of three different ligands: [^{3}H]Ro 15-1788, reported as a selective ligand of central binding sites (Richards et al 1982; Gee & Yamamura 1983; Bonetti et al 1982); [^{3}H]Ro 5-4864 which has a methyl group in position 1 and a chloro in position 4 (Wang et al 1984) and is therefore considered a selective ligand for peripheral binding sites (Marangos et al 1982; Schoemaker et al 1983), and [^{3}H]flunitrazepam which, with a methyl group in position 1 can label both sites (Richards et al 1982). The in-vitro affinities of diazepam (methyl group in position 1), desmethyldiazepam, clonazepam (both without substituents in position 1), Ro 5-4864, and a pyrrolodiazepine, premazepam (no substituent), have been determined in the cortex and cerebellum and adrenal glands of the rat.

This characterization of the binding of various BDZ compounds to central and peripheral BDZ receptors also helped towards explaining why desmethyldiazepam is less active than diazepam as an anticonvulsant in rats (Caccia & Garattini 1984; Garattini et al 1981) and mice (Frey & Loscher 1982) and also in reducing motor activity in rats and in the anticonflict test (Babbini et al 1979).

As previously described (Mennini & Garattini 1984; Mennini et al 1985) a useful experimental approach to test this hypothesis is to measure [^{3}H]flunitrazepam binding in the hippocampus after in-vivo injection of a tracer dose of the ligand in control rats and in rats treated with equiactive doses (against metrazol convulsions) of different drugs.

Materials and methods

In-vitro binding. Male CD-COBS rats (Charles River, Italy), ca 200 g, were decapitated and their cerebella, cortex and adrenal glands dissected and stored at −80 °C until use. Binding assays were carried out on crude membrane preparations obtained as follows: the

† Correspondence.

regions were homogenized in 50–100 vol of 50 mM Na–K phosphate buffer, pH 7·4 (for [^{3}H]flunitrazepam and [^{3}H]Ro 15-1788 binding assays) or 50 mM phosphate buffer saline (PBS), pH 7·4 ([^{3}H]Ro 5-4864) using an Ultra Turrax TP-1810, and centrifuged at 48 000*g* for 10 min in a Sorvall RC 2B refrigerated centrifuge. The pellets were then washed four ([^{3}H]flunitrazepam and [^{3}H]Ro 15-1788) or five ([^{3}H]Ro 5-4864) times by resuspension in the same fresh buffer and recentrifugation as before. For adrenal glands, an intermediate filtration was made through four gauze layers. The last pellets were resuspended with the same buffer just before the binding assay to give final concentrations of about 6 mg of tissue mL^{-1} and the suspensions divided into aliquots of 1 mL (brain areas) or 0·5 mL (adrenal glands).

The binding assays were carried out by incubating at 0 °C for 90 min ([^{3}H]flunitrazepam and [^{3}H]Ro 15-1788, Gee & Yamamura 1983) or for 120 min ([^{3}H]Ro 5-4864, Schoemaker et al 1983) a mixture containing the receptor preparation, the ^{3}H-ligand at a final concentration of 1 nM ([^{3}H]flunitrazepam: 92·3 Ci mmol^{-1}, [^{3}H]Ro 15-1788 [ethyl 8-fluoro-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylate]: 87·0 Ci mmol^{-1}, [^{3}H]Ro 5-4864 [7-chloro-5-(4-chlorophenyl)-1,3-dihydro-1-methyl-2*H*-1,4-benzodiazepin-2-one]: 76·9 Ci mmol^{-1}, all purchased from New England Nuclear), and the different drugs at the desired concentrations. Non-specific binding was obtained in the presence of 100 μM clonazepam for [^{3}H]flunitrazepam and [^{3}H]Ro 15-1788 binding, or of 100 μM Ro 5-4864 for [^{3}H]Ro 5-4864 binding.

Incubations were stopped by rapid filtration under vacuum through Whatman GF/B fibre filters which were then washed with 8–12 mL of ice-cold buffer and counted in 8 mL of Filter Count (Packard) in a Beckman liquid scintillation spectrometer model LS 7500 with a counting efficiency of about 45%.

Each drug was tested at 8–10 concentrations and the resulting inhibition curves were analysed using the 'ALLFIT' non-linear fitting program (De Lean et al 1978), running on a PDP 11/24 computer system, which permits simultaneous curve fitting of a family of sigmoidal dose-response curves according to the logistic equation.

In-vivo binding. Male rats (ca 200 g, food-deprived 12–18 h before experiments) were treated with diazepam or desmethyldiazepam, dissolved in 0·5% carboxymethylcellulose, at doses of 1·9 and 2·5 mg kg^{-1} p.o., respectively (corresponding to the relative anti-metrazol ED50 (Caccia & Garattini 1984). Thirty min later the rats were injected with [^{3}H]flunitrazepam (92·3 Ci mmol^{-1}, New England Nuclear; 50 μCi/0·2 mL saline) into a lateral tail vein, and killed 1 min later. Hippocampi were quickly removed and homogenized in 50 vol of ice-cold 50 mM Tris-HCl buffer, pH 7·4, using an Ultra-Turrax TP-1810 for 20 s. Aliquots of 0·5 mL of the homogenate were either put directly into scintillation vials (total radioactivity) or filtered through Whatman GF/B filters (bound radioactivity). Non-specific binding was determined after incubation of 0·5 mL of the homogenate at 0 °C for 90 min with 1 μM clonazepam, as described by Mennini et al (1985).

[^{3}H]Flunitrazepam specific binding as a percentage of total radioactivity was calculated for each animal (about 50% for the control rats); the effect of drug pretreatment was expressed as percent reduction of this value (% of inhibition).

Drugs were obtained from the following sources; diazepam and clonazepam from Ravizza (Italy); premazepam from Lepetit (Italy); Ro 5-4864 from Hoffman La-Roche (Switzerland) and desmethyldiazepam from Bottu (France).

Results and discussion

[^{3}H]Ro 15-1788, [^{3}H]Ro 5-4864 and [^{3}H]flunitrazepam were chosen as representative of three different ligands for BDZ receptors. The first two are described as selective ligands for the 'central' (Richards et al 1982) and 'peripheral' (Marangos et al 1982; Schoemaker et al 1983) BDZ receptors, respectively, while [^{3}H]flunitrazepam should bind to both classes.

Our results (Table 1) confirm these observations. [^{3}H]Ro 15-1788 showed high affinity binding only in brain areas, cortex and cerebellum, while no specific binding was detectable in the adrenal gland where only peripheral BDZ receptors seem to be present. In both the brain areas, clonazepam and Ro 5-4864, selective ligands for central and peripheral BDZ receptors, respectively, showed the highest (1·3 and 1·7 nM) and lowest (300 μM) affinity for [^{3}H]Ro 15-1788 binding sites while diazepam and desmethyldiazepam had intermediate and identical affinity (30–40 nM).

[^{3}H]Ro 5-4864 binding was higher in adrenal gland where the most effective inhibitor was unlabelled Ro 5-4864 (IC50 = 75 nM). Clonazepam had no affinity for these sites (IC50 = 100 μM).

Another important piece of evidence, suggesting that these peripheral binding sites are different from the central sites labelled by [^{3}H]Ro 15-1788, was the different pattern shown by diazepam and desmethyldiazepam. [^{3}H]Ro 5-4864 was preferentially inhibited by diazepam (IC50 = 1·6 μM) while desmethyldiazepam had 75 times lower affinity (statistically significant difference). Specific [^{3}H]Ro 5-4864 binding was also seen in brain areas; the pattern of inhibition obtained with the drugs tested was similar to that in adrenal glands, on account of the presence of 'peripheral' type BDZ receptors in the brain. [^{3}H]Flunitrazepam binding in the brain is represented by the 'central' type as indicated by the pattern of affinities of the tested drugs which was exactly superimposable on that with [^{3}H]Ro 15-1788 (clonazepam > diazepam = desmethyldiazepam > premazepam > Ro 5-4864). In the adrenal gland, however, the specific [^{3}H]flunitrazepam binding

Table 1. In-vitro affinities of different drugs for benzodiazepine binding sites. Inhibition curves, 8–10 drug concentrations, were analysed by non-linear fitting using the logistic function. Cx/Cb is the ratio between the IC50's in cortex and cerebellum.

	IC50 ± s.e. [M]			
	Cortex	Cerebellum	Adrenal gland	Cx/Cb
[^{3}H]Flunitrazepam (1 nM)				
Clonazepam	1·08 ± 0·13 E-9	0·51 ± 0·06 E-9	2·37 ± 0·32 E-5	2·128†
Diazepam	4·19 ± 0·36 E-8	4·35 ± 0·39 E-8	9·06 ± 1·48 E-7	0·963
Desmethyldiazepam	4·06 ± 0·38 E-8	4·07 ± 0·39 E-8	5·01 ± 1·38 E-5**	0·997
Ro 5-4864	4·57 ± 0·95 E-4	5·55 ± 1·30 E-4	8·37 ± 1·15 E-8	0·823
Premazepam	2·90 ± 0·30 E-7	4·00 ± 0·40 E-7	4·00 ± 0·40 E-4	0·725
[^{3}H]Ro 15-1788 (1 nM)				
Clonazepam	1·69 ± 0·12 E-9	1·30 ± 0·11 E-9	—	1·300
Diazepam	3·44 ± 0·20 E-8	3·94 ± 0·25 E-8	—	0·873
Desmethyldiazepam	3·34 ± 0·21 E-8	4·05 ± 0·27 E-8	—	0·825
Ro 5-4864	3·37 ± 0·35 E-4	3·71 ± 0·48 E-4	—	0·908
Premazepam	3·20 ± 0·30 E-7	5·20 ± 0·50 E-7	—	0·615†
[^{3}H]Ro 5-4864 (1 nM)				
Clonazepam	3·25 ± 1·43 E-6	8·80 ± 2·63 E-6	9·07 ± 0·86 E-5	0·369
Diazepam	8·39 ± 3·32 E-7	3·29 ± 0·69 E-7	1·58 ± 0·11 E-6	2·550
Desmethyldiazepam	1·54 ± 1·07 E-5**	2·02 ± 1·18 E-5**	1·18 ± 2·54 E-4*	0·762
Ro 5-4864	1·84 ± 0·58 E-8	1·53 ± 0·32 E-8	7·52 ± 0·51 E-8	1·203
Premazepam	>1 E-4	>1 E-4	>1 E-4	—

* $P < 0·05$, ** $P < 0·01$ } Desmethyldiazepam vs diazepam.

† $P < 0·05$ Cortex vs cerebellum (Student's t-test).

detected was clearly different, the pattern of inhibition being superimposable on that of [^{3}H]Ro 5-4864 (Ro 5-4864 > diazepam > clonazepam > desmethyldiazepam > premazepam). Thus in this area [^{3}H]flunitrazepam appears to label the 'peripheral' type of BDZ receptors.

Premazepam was considered in this study because it was reported to interact differently with BDZ receptors in rat hippocampus and cerebellum after in-vivo administration (Mennini et al 1985). However, Sieghart & Schuster (1984), considering BDZ receptors in the same areas, did not find premazepam to have any selectivity, although in the same paper they showed that some BDZs, including clonazepam, had higher affinity for BDZ receptors in the cerebellum than in the hippocampus.

In our study we were not able to support this concept of heterogeneity of central BDZ receptors. Clonazepam was found to be slightly more active (×2) in inhibiting [^{3}H]flunitrazepam binding in cerebellum than in cortex but no significant difference was found for [^{3}H]Ro 15-1788 binding. Premazepam, on the other hand, showed higher affinity (×2) in the cortex only with [^{3}H]Ro 15-1788 and not with [^{3}H]flunitrazepam.

In conclusion, our data indicate that the 'central' and 'peripheral' types of BDZ receptors have different structural characteristics. This is supported by the lack of selectivity of [^{3}H]flunitrazepam, which labels both the sites (even if it can be successfully used as a selective ligand in relation to the region considered), and by the central selectivity shown by clonazepam and premazepam which have no substituents in position 1. [^{3}H]Ro 15-1788 and [^{3}H]Ro 5-4864 were confirmed as selective ligands for 'central' and 'peripheral' binding sites, respectively.

Diazepam and desmethyldiazepam showed the same affinity for the central type but there was a significant difference for the peripheral sites, confirming that methylation of position 1 is required to enhance the affinity for peripheral binding sites. Thus desmethyldiazepam behaves like clonazepam, showing a higher selectivity for the central type of BDZ receptors.

Desmethyldiazepam is less active than diazepam as an anticonvulsant in rats (Garattini et al 1981; Caccia & Garattini 1984) and mice (Frey & Loscher 1982). It has also been reported to be less active than diazepam in Geller's test and in reducing motor activity in rats (Babbini et al 1979). Table 2 shows that desmethyldiazepam caused significantly higher inhibition (twice) than in diazepam-treated rats. As previously described (Mennini & Garattini 1984), measurement of inhibition of [^{3}H]flunitrazepam binding with equiactive doses of different drugs can be considered an index of the receptors occupied by these drugs (RD), and thus the relative intrinsic activity (α) can be calculated. From the relation, effect = $\alpha \cdot$ RD, we calculated the intrinsic activity of desmethyldiazepam relative to that of diazepam (taken as 1) from the ratio:

$$\text{RD (diazepam)/RD (desmethyldiazepam)}.$$

Desmethyldiazepam proved to be a 'partial agonist', having an intrinsic activity about half that of diazepam.

In conclusion, the present in-vivo experiment shows that the same effect (anti-metrazol ED50) may be

Table 2. Effect of diazepam and desmethyldiazepam on hippocampal [^{3}H]flunitrazepam binding in-vivo.

Drug	% inhibition of [^{3}H]flunitrazepam specific binding	Relative intrinsic activity
Diazepam	7·83 ± 3·90	1·00
Desmethyldiazepam	18·03 ± 4·76*	0·43

Diazepam and desmethyldiazepam were given, p.o., 30 min before the i.v. injection of [^{3}H]flunitrazepam; the doses used (1·9 and 2·5 mg kg^{-1}, respectively), corresponded to those producing the same pharmacological effect (ED50 anti-metrazol), giving the possibility of estimating the intrinsic activity of desmethyldiazepam in relation to the intrinsic activity of diazepam, taken as 1.

Each value represents the mean ± s.d. of three different experiments (3–4 animals per group in each of them).

* $P < 0·05$ different from diazepam (Student's t-test).

obtained with desmethyldiazepam when the occupation of central BDZ receptors is double that with diazepam. If we assign an intrinsic affinity of 1·0 to diazepam, the calculated value for desmethyldiazepam is 0·47, suggesting that it could act as a partial agonist at central BDZ receptors.

Similar results have been reported in mice, where a potency ratio of 0·37 has been calculated for desmethyldiazepam relative to diazepam (Frey & Loscher 1982).

Since the ratio between diazepam and desmethyldiazepam in individual patients may vary widely (Garattini et al 1973), it might to some extent explain the variability in the degree of sedation exerted by diazepam and other benzodiazepines which are metabolized with the formation of desmethyldiazepam.

Partial agonists at BDZ receptors may be of clinical relevance because they may retain only the pharmacological effects needing a low degree of BDZ receptor stimulation, with lower sedative and ataxic properties (Mennini et al 1985).

Moreover, 'peripheral' type benzodiazepine receptors have been recently related to calcium-dependent phenomena (Cantor et al 1984; Mestre et al 1985). Therefore, the possibility that desmethyldiazepam has less cardiovascular and hypotensive effects than diazepam (Daniell 1975) merits further investigation.

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