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Structural Requirements for the Binding of Benzodiazepines to Their Peripheral-Type Sites

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SUMMARY

The DS19 Friend erythroleukemia cells possess peripheral-type benzodiazepine recognition sites that are similar to those characterized in other tissues and cells. These sites recognize only specific and well-defined ligand structures. In order for the benzodiazepines to bind to these sites, an alkyl group of 3 carbons or less is required at position 1, together with a carbonyl group at position 2. In addition, the binding affinity is enhanced by 4′-halogen or 4′-methoxy substituents, and by 7- or 2′-halogens. However, the affinity is decreased by substitutions at position 3 or 4. Central-type benzodiazepine binding in brain, on the other hand, is not affected by 1-alkyl groups and is inhibited by 4′-substitutions. Furthermore, unlike the peripheral-type site, the brain receptor is stereoselective. These results demonstrate that the two binding sites are fundamentally different in their recognition of benzodiazepine ligands.

INTRODUCTION

The BZD[3] receptor in brain has been studied extensively since its discovery in 1977 (1–3), and it is thought to mediate at least some of the biological effects of BZDs (for review see refs. 4 and 5). There exists another class of pharmacologically distinct BZD sites in many tissues and cell types, including kidney (6, 7), heart (7, 8), liver (6), lung (6), ileal longitudinal muscle (9), mast cells (10), platelets (11), lymphocytes (12), various cell lines (13), and even brain (14–16). This site has generally been termed "peripheral-type" because initially it was thought to exist only outside of the central nervous system. The peripheral and central-type sites can be differentiated by selective ligands such as Ro 5-4864, which binds preferentially to the former, and clonazepam, which is selective for the latter (6). The two compounds differ only in the presence of a methyl on nitrogen 1 and a chloro at position 4′ of Ro 5-4864 (see Table 1). Structure-activity relationship studies revealed that the behavioral effects of BZDs are decreased by 4′-substitutions and enhanced by 7-substitutions (17). The binding of BZDs to their central nervous system receptor shows similar structural specificity, whereas the 1-methyl substituent is necessary for peripheral-type binding (16). Furthermore, peripheral-type binding is not stereospecific, but central-type binding is (14, 16). This study presents a detailed analysis of the structural requirements for the binding of BZDs to a well-characterized peripheral-type site on intact FEL cells, and the differences between the structure-binding relationships for the peripheral and central-type sites.

MATERIALS AND METHODS

FEL cell line 745A, subclone DS19, was a generous gift from Dr. R. A. Rifkind (Memorial Sloane-Kettering Cancer Center, New York, N. Y.). The cells were maintained in continuous suspension culture in Eagle's minimal essential medium without ribonucleosides or deoxyribonucleosides (MEM-alpha; GIBCO, Grand Island, N. Y.), supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). Cell cultures were maintained in a humidified incubator at 37° in a 95% air/5% CO_2 atmosphere.

The DS19 FEL cells were collected by centrifugation at 100 × *g* for 5 min and washed once by resuspension in a balanced glucose salt buffer as described previously (18). For the binding assay, 0.9 nM [^{3}H] Ro 5-4864 (New England Nuclear Corporation, Boston, Mass., 80.6 Ci/mmole) was incubated with 1×10^6 cells at 0° for 40 min, and the reaction was terminated by filtration as previously described (10). The binding was linear between 0.2 and 2×10^6 cells/assay (data not shown). A crude membrane fraction was prepared from Wistar rat (Charles River Breeding Laboratories, Wilmington, Mass.) brains (19), and the binding of [^{3}H]diazepam (New England Nuclear Corporation, 83.6 Ci/mmole was assayed as described previously (10). The IC_{50} of binding was defined as that concentration of the unlabeled ligand which inhibited specific binding of the labeled ligand by 50%. Nonspecific binding was defined as the amount of binding remaining in the presence of 10 μM unlabeled diazepam. Specific binding was defined as the difference between nonspecific and total binding.

Benzodiazepine compounds were provided by Hoffmann-La Roche Inc. (Nutley, N. J.). All other reagents were obtained through commercial sources.

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[3] The abbreviations used are: BZD, benzodiazepine; FEL, Friend erythroleukemia.

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RESULTS

The BZD binding site in DS19 FEL cells is similar to those already characterized in other tissues and cells (7–11, 13–16). The binding was specific, saturable, reversible, inversely dependent on incubation temperature, and of the peripheral-type (data not shown). Scatchard analysis of [^{3}H]Ro 5-4864 binding showed a K_d of 6.7 ± 0.6 nM and a B_{max} of 331 ± 32 fmoles/10^6 cells (mean ± SE, $N = 5$). The binding equilibrium was achieved after 40 min of incubation at 0° and was reversible in a monophasic manner, with a $t_{1/2}$ of dissociation of 23.9 min.

Structural analogues of diazepam were tested for binding activity in rat brain and in DS19 FEL cells. The results are shown in Table 1. A methyl substituent at position 1 of the BZD molecule was critical for the binding to peripheral-type sites. Ro 5-2180, for instance, is similar to diazepam except for its lack of a 1-methyl group. It did not bind to the cells, whereas diazepam did. The same was true for 5-3027 (des-methyl 5-3448) and 5-2752 (des-methyl 5-4864). On the other hand, the brain binding affinity wa not affected by the 1-methyl group (compare 5-2180 and 5-3027 with their 1-methyl analogues). An ethyl (5-6993) or propenyl group (5-6945) at position 1 slightly increased the peripheral-type binding (compared with 4-4864), but bulkier substitutions abolished it (compare 12-5400 with 5-4864, and 13-3050 with 5-6669).

Halogens at position 4′ enhanced the affinity for the peripheral-type site, with chloro substituents superior to fluoro. Ro 5-4864 and 5-5115, as the 4′-chloro analogues of diazepam and 5-3464, respectively, were 10 times more potent in binding, whereas 5-5122, the 4′-fluoro derivative of 5-3464, was only twice as potent. Moreover, the central-type binding was abolished by 4′-halogens (compare 5-2752 with 5-2180, and 5-4864 with diazepam). A 4′-methoxy likewise eliminated the brain affinity and enhanced the peripheral binding (5-6669 versus diazepam). However, a 4′-hydroxy group diminished but did not totally eliminate the former and failed to affect the latter (compare 7-3351 with diazepam).

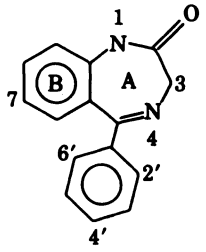
Another important position for enhancing the peripheral binding was position 7, where chloro and fluoro were better than nitro substituents. Diazepam and 5-4864, the 7-chloro analogues of 5-3464 and 5-5115, respectively, were 10 times more potent. Ro 5-6531, a 7-fluoro analogue of 5-5115, was about 4 times more potent, and a 7-nitro substitution increased the binding by less than 2-fold (compare 5-5120 with 5-5115). The 7-chloro substitution also increased the central nervous system affinity (diazepam versus 5-3464). Substitution at position 2′ similarly enhanced the binding to both sites. Ro 5-4608, for example, is a 2′-chloro analogue of 5-3464 and had much higher central and peripheral affinities.

The peripheral-type binding was decreased by substitutions at position 3 or 4. Thus, a 3-hydroxy caused a 3- to 4-fold reduction (5-5345 versus diazepam, and 7-9277 versus 5-4864), and a hydrogen or methyl substituent at position 4 (hence saturating the nitrogen double bond) also caused a 4- to 10-fold decrease (compare 5-5888 with 5-4864, 5-6524 and 5-6528 with 5-6531, and 5-5119 with 5-5115). Eliminating the carbonyl group at position 2 also greatly diminished the binding (22-6070 versus 5-4864).

TABLE 1

Structural requirements of BZD binding

[^{3}H]Diazepam used for brain, and [^{3}H]Ro 5-4864 for FEL binding. Values are means from at least three experiments and are nanomolar unless otherwise noted.



BZD	1	7	4′	2′	6′	3	4	Brain	FEL
5-2180	H	Cl	H	H	H	H	—	2	>10 μM
5-2752	H	Cl	Cl	H	H	H	—	>10 μM	>10 μM
5-3027	H	Cl	H	Cl	H	H	—	1	5 μM
5-3448	CH_3	Cl	H	Cl	H	H	—	1	11
5-3464	CH_3	H	H	H	H	H	—	600	704
5-4608	CH_3	H	H	Cl	H	H	—	10	175
5-4864	CH_3	Cl	Cl	H	H	H	—	5 μM	6
Clonazepam	H	NO_2	H	Cl	H	H	—	3	>10 μM
Diazepam	CH_3	Cl	H	H	H	H	—	6	79
5-5115	CH_3	H	Cl	H	H	H	—	>10 μM	54
5-5119	CH_3	H	Cl	H	H	H	CH_3	>10 μM	180
5-5120	CH_3	NO_2	Cl	H	H	H	—	>10 μM	34
5-5122	CH_3	H	F	H	H	H	—	>10 μM	327
5-5345	CH_3	Cl	H	H	H	OH	—	13	236
5-5888	CH_3	Cl	Cl	H	H	H	H	>10 μM	37
5-6524	CH_3	F	Cl	H	H	H	CH_3	2 μM	152
5-6528	CH_3	F	Cl	H	H	H	H	5 μM	205
5-6531	CH_3	F	Cl	H	H	H	—	>10 μM	13
5-6669	CH_3	Cl	OCH_3	H	H	H	—	>10 μM	15
5-6945	+[a]	Cl	Cl	H	H	H	—	>10 μM	3
5-6993	++[b]	Cl	Cl	H	H	H	—	>10 μM	4
7-3351	CH_3	Cl	OH	H	H	H	—	350	100
7-9277	CH_3	Cl	Cl	H	H	OH	—	>10 μM	20
11-6893	CH_3	NO_2	H	H	F	CH_3	—	3	1037
11-6896	CH_3	NO_2	H	H	F	CH_3	—	673	1080
12-5400	+++[c]	Cl	Cl	H	H	H	—	>10 μM	>10 μM
13-3050	+++[c]	Cl	OCH_3	H	H	H	—	>10 μM	>10 μM
22-6070[d]	CH_3	Cl	Cl	H	H	H	—	8 μM	1 μM

[a] $CH_2CH{=}CH_2$.

[b] CH_2CH_3.

[c] $CH_2CH_2N(CH_2CH_3)2$.

[d] Two hydrogens substituted for the oxygen at position 2.

Consistent with previous reports (14, 16), we observed that peripheral-type binding did not exhibit stereospecificity. Both Ro 11-6896 (S) and its enantiomer 11-6893 (R) had essentially identical affinities, which were relatively low (about 1 μM). This was in contrast to their binding to brain BZD receptors, where 11-6896 was the active stereoisomer with an affinity of 3 nM, compared with 673 nM for 11-6893.

DISCUSSION

The binding of BZDs to peripheral-type sites in FEL cells has specific structural requirements; even slight

changes in the molecule can have profound consequences for its binding activity. Similar results have been observed in rat kidney membranes (19). The need for 1-alkyl substituents suggests that the peripheral-type binding may be dependent on the ability of these groups to donate electrons into the A-ring of the BZD molecule, thereby increasing the nucleophilicity in the adjacent B-ring. This increased negative charge in the B-ring may enhance its ability to interact with electrophilic reagents such as positive charges on binding site proteins. Ethyl and propenyl groups are better electron donors than methyl; hence these substituents are better than methyl in enhancing peripheral-type binding. Loss of peripheral binding when the 2-carbonyl group is missing further supports this hypothesis. Without the 2-carbonyl, nitrogen 1 becomes an amine instead of an amide linkage and can be protonated by any nearby proton source. The protonated nitrogen localizes the electron-donating capacity of the 1-alkyl groups and prevents delocalization of electrons into the A-ring. The net result is a decrease of nucleophilicity in the B-ring, and hence a loss of peripheral-type binding.

The structural requirements for peripheral binding, although not yet fully understood in molecular terms, do imply that there exists a very specific 3-dimensional conformation that the ligand must possess before it can be recognized by the peripheral-type binding site. On the other hand, the lack of stereospecificity suggests that this site is more flexible in this regard than is the stereospecific central BZD receptor. As is the case with the latter, there are non-benzodiazepine compounds that bind to peripheral-type sites, presumably because they happen to possess the correct 3-dimensional conformation. For example, dipyridamole competes against [^{3}H]diazepam in binding to heart membranes with a K_i of 100 nM (8), and PK11,195, a quinoline derivative, competes against [^{3}H]Ro 5-4864 in binding to kidney membranes with a K_i of 1 nM (20). The structural relationship between the non-benzodiazepines and the BZDs is unclear. The many differences in the structure-binding relationship between central- and peripheral-type sites constitute further evidence that the two sites are fundamentally different at the molecular level.

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