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Binding of 1,4-benzodiazepines to a novel [^{3}H]Ro15-4513 binding site in the rat spinal cord

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Abstract

An alpidem-insensitive benzodiazepine binding site in the rat spinal cord has recently been identified in our laboratory. We report here the binding of 23 1,4-benzodiazepines to this site using [^{3}H]Ro15-4513 (ethyl-8-azido-6-dihydro-5-methyl-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylate) in the presence of 65 μM alpidem (6-chloro-2-(4-chlorophenyl)-*N*,*N*-dipropylimidazo[1,2-*a*]pyridine-3-acetamide). This binding site displays a wide affinity for 1,4-benzodiazepines, most of which show much higher affinity for benzodiazepine receptors in various brain regions and transfected cell systems. The highest affinity ligands are: brotizolam (1-bromo-4-(2-chlorophenyl)-9-methyl-6*H*-thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepine) (4.3 nM), Ro15-4513 (5.0 nM), Ro42-8773 (7-chloro-3-[3-(cyclopropylmethoxy)-1-propynyl]-4,5-dihydro-5-methyl-6*H*-imidazo[1,5-*a*][1,4]benzodiazepin-6-one) (5.7 nM), Ro16-6028 (*t*-butyl (*s*)-8-bromo-11,12,13,13*a*-tetrahydro-9-oxo-9*H*-imidazo[1,5-*a*][1,4]benzodiazepine-1-carboxylate) (5.9 nM) and triazolam (8-chloro-6-(2-chlorophenyl)-1-methyl-4*H*-[1,2,4]triazolo[4,3-*a*][1,4]benzodiazepine) (7.9 nM). The structural feature common to these compounds is an imidazo- or triazolo-ring on the 1- and 2-position of the benzodiazepine. However, the presence of this feature does not guarantee high affinity binding as Ro15-1788 (8-fluoro-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylic acid ethyl ester) (100 nM) and Ro23-0364 (6-[2-chlorophenyl]-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxamide) (360 nM) display much lower affinity for this site. Studies are currently underway to investigate the functional significance of this unusual benzodiazepine binding site.

Keywords: Benzodiazepine; Receptor binding; Spinal cord; Alpidem-insensitive site

1. Introduction

The GABA$_A$/benzodiazepine/chloride channel receptor is a heteromultimeric complex. Six α subunits (α_{1-6}), four β subunits (β_{1-4}), four γ subunits (γ_{1-3} and γ_{2L}) as well as δ and ρ subunits have been cloned and sequenced (see Wisden and Seeburg, 1992 for review). Several combinations have been expressed in transfected cells and receptor binding and channel function studies have been carried out.

Although these expression studies provide much useful information, little is known about the subunit composition, stoichiometry and pharmacological characteristics of native benzodiazepine receptors. Immunoprecipitation studies have shown that there is tremendous diversity in the combinations of subunits within native benzodiazepine receptors (Quirk et al., 1994; Ruano et al., 1994; Endo and Olsen, 1993; Mertens et al., 1993; Duggan et al., 1991; Lüddens et al., 1991; McKernan et al., 1991). In order to study the binding characteristics of native benzodiazepine receptors, we chose to study the binding sites in the rat spinal cord because this tissue was reported to lack specific binding of [^{3}H]zolpidem (*N*,*N*,6-trimethyl-2-(4-methylphenyl)-imidazo[1,2-*a*]pyridine-3-acetamide) to central benzodiazepine receptors, suggesting that this central nervous system (CNS) region lacks type I receptors (Niddam et al., 1987). However, more recent in situ hybridization studies have since demonstrated that there may be extensive heterogeneity within the rat spinal cord (Persohn et al., 1991,1992; Poulter et al., 1992).

Performing competitive assays against [^{3}H]Ro15-1788 (8-fluoro-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo-[1,5-*a*][1,4]benzodiazepine-3-carboxylic acid ethyl ester), it became evident that at least three binding sites were

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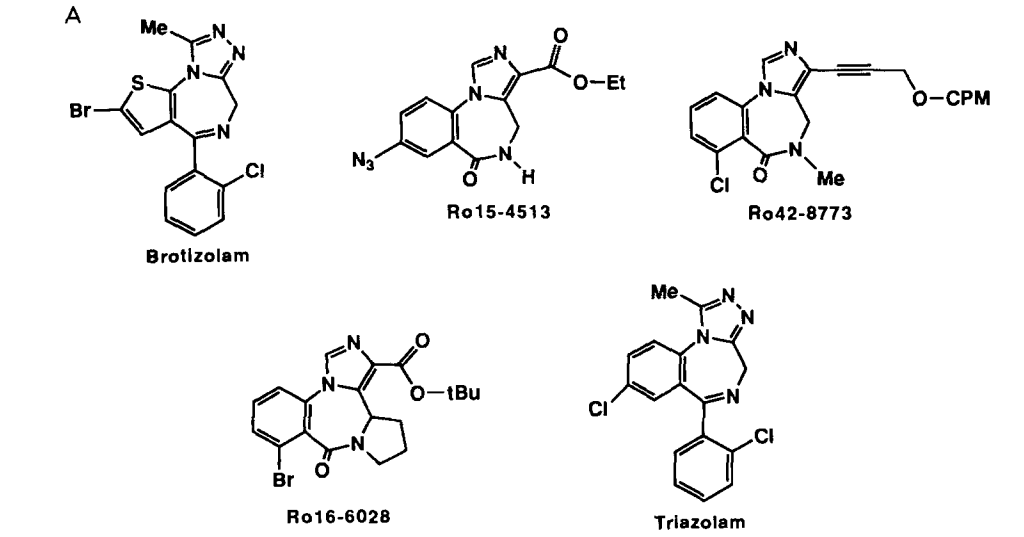
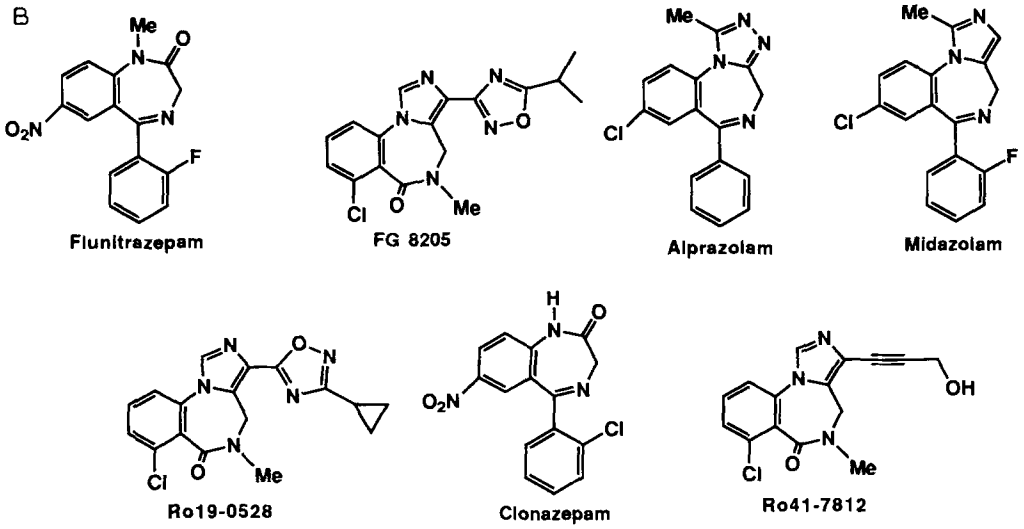
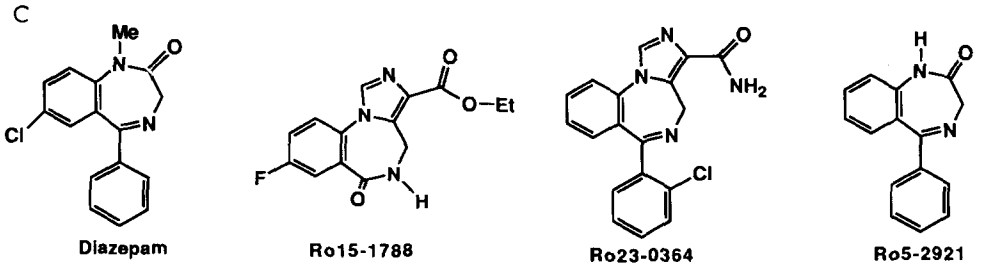


1a: K_i < 10 nM



1b: K_i 10 - 100 nM



1c: K_i 100 - 1000 nM

Fig. 1. Structure of the 1,4-benzodiazepines used in this study.

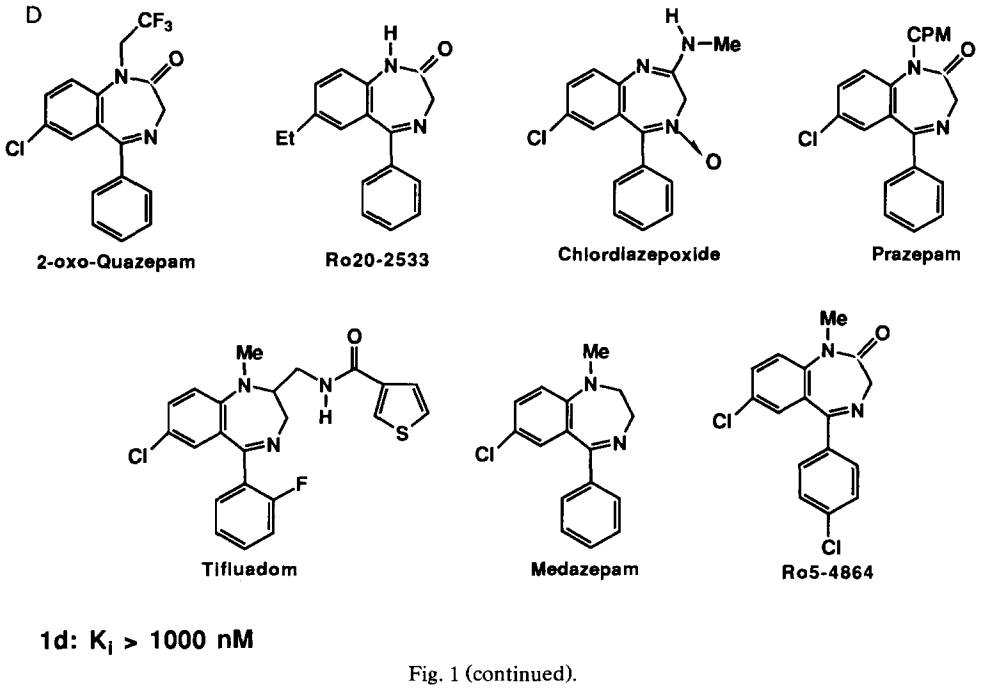


Fig. 1 (continued).

present in the spinal cord membranes, one of which was insensitive to the imidazopyridine, alpidem (6-chloro-2-(4-chlorophenyl)-*N*,*N*-dipropylimidazo[1,2-*a*]-pyridine-3-acetamide) (Maguire et al., 1992a). However, characterization of the alpidem-insensitive site was difficult due to the low affinity of [^{3}H]Ro15-1788 for this binding site (150 nM) (Maguire et al., unpublished observations). We, therefore, investigated the use of [^{3}H]Ro15-4513 (ethyl-8-azido-6-dihydro-5-methyl-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylate) for further characterization of this site.

We report here the binding affinity of 23 benzodiazepine receptor ligands, each with the 1,4-benzodiazepine skeleton (Fig. 1a–d). Assays were performed in rat spinal cord membranes with [^{3}H]Ro15-4513 in the presence of 65 μM alpidem. The results are discussed in terms of binding studies of both cloned and native receptors.

2. Materials and methods

2.1. Materials

The following compounds were received as generous gifts: alpidem (Synthelabo Recherche, Bagneux, France); Ro15-1788, Ro23-0364 (6-[2-chlorophenyl]-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxamide), clonazepam (5-(2-chlorophenyl)-1,3-dihydro-7-nitro-2*H*-1,4-benzodiazepin-2-one), diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl-2*H*-1,4-benzodiazepin-2-one), midazolam (8-chloro-6-(2-fluorophenyl)-1-methyl-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine), Ro15-4513, Ro16-6028 (*t*-butyl (*s*)-8-bromo-11,12,13,13*a*-tetrahydro-9-oxo-9*H*-imidazo[1,5-*a*][1,4]benzodiazepine-1-carboxylate), Ro19-0528 (7-chloro-5,6-dihydro-5-methyl-6-oxo-3-[3-(cyclopropylmethyl)-1,2,4-oxadiazol-5-yl]-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine), Ro41-7812 (7-chloro-4,5-dihydro-3-(3-hydroxy-1-propynyl)-5-methyl-6*H*-imidazo[1,5-*a*][1,4]benzodiazepin-6-one), Ro42-8773 (7-chloro-3-[3-(cyclopropylmethoxy)-1-propynyl]-4,5-dihydro-5-methyl-6*H*-imidazo[1,5-*a*][1,4]benzodiazepin-6-one), Ro5-2921 (1,3-dihydro-5-phenyl-2*H*-1,-4-benzodiazepin-2-one), Ro20-2533 (7-ethyl-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2-one), (Hoffmann-LaRoche, Nutley, NJ, USA); alprazolam (8-chloro-1-methyl-6-phenyl-4*H*-[1,2,4]triazolo-[4,3-*a*][1,4]benzo-diazepine), triazolam (8-chloro-6-(2-chlorophenyl)-1-methyl-4*H*-[1,2,4]triazolo[4,3-*a*][1,4]benzodiazepine) (Upjohn, Kalamazoo, MI, USA); brotizolam (1-bromo-4-(2-chlorophenyl)-9-methyl-6*H*-thieno[3,2-*f*][1,2,4]-triazolo[4,3-*a*][1,4]-diazepine) (Boehringer Ingelheim, Ridgefield, CT, USA); 2-oxo-quazepam (7-chloro-1-2,2,2-trifluoroethyl)-1,3-dihydro-5-(2-fluorophenyl)-2*H*-1,4-benzodiazepin-2-one) (Schering, Bloomfield, NJ, USA); tifluadom (1-methyl-2-(3-thienylcarbonyl)-aminomethyl-5-(2-fluorophenyl)-*H*-2,3-dihydro-1,4-benzodiazepine) (Sandoz, Basel, Switzerland); FG 8205 (7-chloro-5,6-dihydro-5-methyl-6-oxo-3-(5-isopropyl-1,2,4-oxadiazol-3-yl)-4*H*-imidazo-[1,5-*a*][1,4]benzodiazepine) (Merck Sharp and Dohme, Essex, UK). [^{3}H]Ro15-4513 and [^{3}H]Ro15-1788 were purchased from Dupont NEN (Boston, MA, USA), flunitrazepam (5-(2-fluorophenyl)-1,3-dihydro-1-methyl-7-nitro-2*H*-1,4-benzodiazepin-

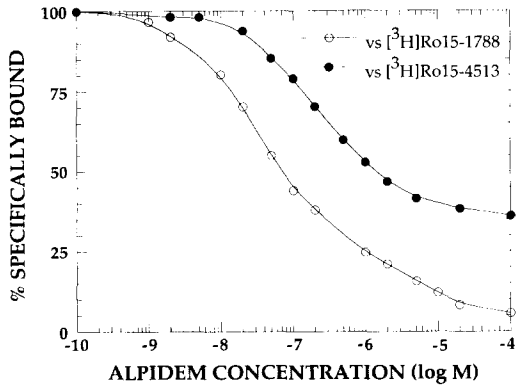


Fig. 2. Inhibition of [^{3}H]Ro15-1788 (open circles) and [^{3}H]Ro15-4513 (closed circles) binding by alpidem in rat spinal cord membranes.

2-one), prazepam (7-chloro-1-(cyclopropylmethyl)-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2-one), medazepam (7-chloro-2,3-dihydro-1-methyl-5-phenyl-1*H*-1,4-benzodiazepine) and chlordiazepoxide (7-chloro-*N*-methyl-5-phenyl-3*H*-1,4-benzodiazepin-2-amide-4-oxide) from Sigma (St. Louis, MO, USA) and Ro5-4864 (7-chloro-5-(4-chlorophenyl)-1,3-dihydro-1-methyl-2*H*-1,4-benzodiazepin-2-one) from Research Biochemicals (Natick, MA, USA). All other chemicals were from standard commercial sources.

2.2. Preparation of membranes

Frozen rat spinal cords (Pel Freeze, Rogers, AR, USA) were homogenized with a Polytron homogenizer in 40 volumes of 50 mM Tris HCl, pH 7.7 at 0° C (assay buffer) and centrifuged at 20000 × *g* for 10 min. The pellet was rehomogenized and centrifuged twice, frozen, thawed and washed 2 additional times. For all binding assays, membranes were suspended to a tissue concentration of 10 mg wet weight/ml in assay buffer.

2.3. Receptor binding assay

The binding assay method was performed using a modification of our previously reported procedure (Maguire et al., 1992b). Briefly, membranes (1 ml) were incubated at 0° C with 5 nM [^{3}H]Ro15-4513 in the presence of 65 μM alpidem and 8 concentrations of unlabeled drug in a total volume of 2 ml for 2 h. Nonspecific binding was determined with 100 μM Ro15-1788. The reaction was terminated by filtration (Brandel cell harvester) through glass fiber filters (Whatman GF/B) followed by three 5 ml washes with ice-cold buffer. Radioactivity retained on the filters was determined by liquid scintillation with EcoLite(+) (ICN) after 6 h at room temperature.

2.4. Data analysis

Data obtained from competitive binding assays were analyzed by a modified version (Toll et al., 1984) of the program LIGAND (Munson and Rodbard, 1980), which calculates the receptor binding affinities (K_d for the radioligand and K_i for the competing ligands) and capacities using weighted nonlinear, least-squares regression analysis.

3. Results

Competitive binding assays were performed in rat spinal cord membranes to determine the feasibility of using [^{3}H]Ro15-4513 as a radioligand for characterization of the spinal cord alpidem-insensitive site. Fig. 2 shows the inhibition of [^{3}H]Ro15-4513 and [^{3}H]Ro15-1788 binding by alpidem. As we have previously reported (Maguire et al., 1992a), alpidem was unable to displace ~8% of the specifically bound [^{3}H]Ro15-1788. By contrast, ~36% of the specifically bound [^{3}H]Ro15-4513 was insensitive to inhibition by alpidem. This suggested that the alpidem-insensitive site had a higher affinity for [^{3}H]Ro15-4513, making this ligand a better choice for the characterization of the binding properties of this site.

Table 1
Affinities of 1,4-benzodiazepines for the alpidem-insensitive site in rat spinal cord

	K_i (nM)
Brotizolam	4.3 ± 0.4
Ro15-4513	5.0 ± 0.9
Ro42-8773	5.7 ± 1.0
Ro16-6028	5.9 ± 0.9
Triazolam	7.9 ± 1.3
Flunitrazepam	15 ± 3
FG 8205	21 ± 3
Alprazolam	24 ± 2
Midazolam	28 ± 4
Ro19-0528	29 ± 3
Clonazepam	41 ± 6
Ro41-7812	45 ± 8
Diazepam	100 ± 20
Ro15-1788	130 ± 50
Ro23-0364	360 ± 30
Ro5-2921	890 ± 250
2-oxo-Quazepam	3200 ± 400
Ro20-2533	4400 ± 800
Chlordiazepoxide	9000 ± 1400
Prazepam	10000 ± 6000
Tifluadom	14000 ± 2000
Medazepam	51000 ± 8000
Ro5-4864	> 100000

Membranes were incubated at 0° C with [^{3}H]Ro15-4513 (5 nM) and competing ligand in the presence of 65 μM alpidem. Values are mean ± S.E. as calculated using LIGAND.

With the radioligand chosen, we proceeded to perform competitive binding assays against a group of 23 1,4-benzodiazepines. The assays were performed in the presence of 65 μM alpidem, a concentration that we have shown blocks binding to other benzodiazepine receptors in spinal cord membranes (Maguire et al., 1995). The data was best fit to a one-site model using LIGAND. The calculated K_i values are shown in Table 1. The compounds have been presented in order of decreasing affinity and can be grouped into four categories based on their affinity: < 10 nM, 10 nM to 100 nM, 100 nM to 1 μM, and over 1 μM. Fig. 1a–d shows the structures of the ligands in each of these categories.

4. Discussion

The results presented here demonstrate that the alpidem-insensitive binding site in the rat spinal cord is indeed a novel site, never before described in the literature. As can be clearly seen in Table 1, the range of affinities, even among structurally related compounds, is very wide, ranging from 4 nM to > 100 μM.

Although the compounds studied vary widely in their substituents on the 1,4-benzodiazepine skeleton, there are pairs of ligands differing in one substituent that may give some insight into the structure-activity relationship of this binding site. One of the changes that makes the largest difference in binding affinity is the nature of the substituent in the 1-position. Diazepam, prazepam and 2-oxo-quazepam differ only in this substituent, diazepam with methyl, prazepam with cyclopropylmethyl and 2-oxo-quazepam with trifluoroethyl. The change in this substituent from a methyl to a larger group, as seen from diazepam to prazepam or 2-oxo-quazepam, resulted in a large decrease in affinity (100-fold and 32-fold, respectively). Comparing this to binding in rat whole brain (vs. [^{3}H]diazepam), the same structural modifications result in 12-fold and 4-fold decreases in affinity, respectively. In addition, these ligands displayed 10-fold to 100-fold higher affinity in whole brain than in the alpidem-insensitive site. (Haefely et al., 1985).

The effect of changes in the 1-position might suggest that bulkier groups in this position would, in general, reduce affinity. However, adding an imidazo- or triazolo-group linking the 1- and 2-positions, as in Ro15-4513 and brotizolam (among others) has the opposite effect, leading to some of the highest affinity analogs at this site. This fused ring is in a somewhat different and fixed position compared to the 1-substituents and also contains a proton-accepting center, the imine nitrogen, which may enhance the binding interaction. However, the addition of this nitrogen-containing ring does not assure high affinity, as Ro15-1788 and Ro23-0364, both imidazobenzodiazepines, have only moderate affinity for the alpidem-insensitive site.

Other modifications produce varying changes in affinity. For example, substituting a Cl for a H in the 2′-position of alprazolam, producing triazolam, induces a small, 3-fold, increase in affinity. This is consistent with the classical 1,4-benzodiazepine structure/activity relationship of the rank order of affinities within different 7-substituted ligands and was also observed in [^{3}H]diazepam binding in rat cortex (Haefely et al., 1985).

Changes in the 7-position can produce a variety of different affinity changes. Substituting an ethyl group for the H in Ro5-2921 to give Ro20-2533 leads to a 5-fold decrease in affinity. An opposite effect is seen in binding to rat whole brain membranes, Ro20-2533 displaying a 10-fold higher affinity than Ro5-2921 (Haefely et al., 1985). Alternately, substituting an azide for F in Ro15-1788 to give Ro15-4513 leads to a 25-fold increase in affinity at the alpidem-insensitive site. However, these two ligands have similar high affinities in whole brain (Haefely et al., 1985).

Perhaps the greatest disparity in affinity is between diazepam and medazepam, in which the carbonyl oxygen has been removed. This change leads to a 500-fold decrease in affinity for the alpidem-insensitive site. This is consistent with the effect of this change on whole brain binding (Haefely et al., 1985) and suggests the importance of this proton-accepting moiety for recognition of the alpidem-insensitive and other benzodiazepine binding sites.

Several of the ligands in Table 1 display similar affinities for other binding sites in the CNS. These include: Ro15-4513 (Kyburz, 1989; Wong and Skolnick, 1992), Ro42-8773 (Moreau et al., 1991), alprazolam (Haefely et al., 1985), Ro5-2921 (Haefely et al., 1985), tifluadom (Hill et al., 1984) and Ro5-4864 (Santi et al., 1988; Kyburz, 1989). The highest affinity ligands at the alpidem-insensitive site also display high affinity at other central benzodiazepine receptors. However, their affinity is, in general, 5- to 10-fold lower for the alpidem-insensitive site (Wong and Skolnick, 1992; Hirouchi et al., 1992; Tricklebank et al., 1990; Richelson et al., 1991; Haefely et al., 1985; Moreau et al., 1991).

Several of these 1,4-benzodiazepines have been studied in transfected cell systems constructed with various combinations of α, β and γ subunits. However, comparisons of the binding affinities at the alpidem-insensitive site and in the transfected cell systems yields no patterns that would give insight into the identification of the subunit composition of this unusual site.

The affinity of Ro15-4513 (5 nM) is characteristic for this ligand in all reported systems except those containing an α_5 subunit (Lüddens et al., 1994; Hadingham et al., 1993a,b). The low affinity of Ro15-1788 (130 nM) is characteristic of the α_4 and α_6 containing

receptors (Lüddens et al., 1990; Wisden et al., 1991). However, the affinity of the alpidem-insensitive site for flunitrazepam (15 nM) and diazepam (100 nM) are significantly higher than has been reported for the α_4 and α_6 subunit containing receptors (Wisden et al., 1991; Lüddens et al., 1990).

Flunitrazepam has been shown to bind to various constructs in transfected cell systems with similar affinities to the alpidem-insensitive site (Hadingham et al., 1993a,b; Lüddens et al., 1994). However, other groups, studying the same subunit combinations, found higher affinity binding for flunitrazepam (Faure-Halley et al., 1993; Lüddens et al., 1990, 1994; Wisden et al., 1991; Ymer et al., 1990; Herb et al., 1992). The exception is binding to the $\alpha_5\beta_3\gamma_3$ complex, which displayed a lower affinity for flunitrazepam than other subunit combinations within the same study (Lüddens et al., 1994). This subunit combination binds Ro15-1788 with high affinity (Lüddens et al., 1994), eliminating it as a candidate for the alpidem-insensitive site.

In summary, the alpidem-insensitive site identified in the rat spinal cord is a unique benzodiazepine binding site. Its binding characteristics are unlike any receptor thus far reported, both from whole brain and brain regional binding and transfected cell studies with a number of different $GABA_A$ receptor subunit combinations. This binding site displays a wide affinity for 1,4-benzodiazepine ligands, and in general, these ligands have a lower affinity for this site than for the previously characterized native and cloned receptors. The highest affinity ligands are: brotizolam, Ro15-4513, Ro42-8773, Ro16-6028 and triazolam. The structural feature in common among these compounds is the presence of an imidazo- or triazolo-ring on the 1 and 2 position of the 7-membered ring of the benzodiazepines. However, the presence of this feature does not guarantee high affinity binding, as Ro15-1788 and Ro23-0364 display a much lower affinity for the alpidem-insensitive site. Studies are currently underway in our laboratory to investigate the possible functional significance of this unusual benzodiazepine binding site.

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