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Molecular Determinants of Recognition and Activation at the Cerebellar Benzodiazepine Receptor Site

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Abstract—Semiempirical quantum mechanical and molecular mechanics calculations were carried out to identify and characterize the steric and electronic properties that modulate ligand recognition and activation of the cerebellar $GABA_A$/benzodiazepine (BDZ) receptor. For this hypothesis development, thirteen compounds belonging to structurally diverse chemical families were selected for study. Among the compounds selected were nine that bind and four that do not bind with appreciable affinity to this receptor and some that are known agonists, antagonists and inverse agonists, as measured by their modulation of GABA (γ-aminobutyric acid) enhanced chloride ion flux in cerebellum. The stereoelectronic requirements for recognition deduced from commonalities among the ligands are the presence of at least two of three hydrogen bonding centers, and a lipophilic aromatic ring, in a specific spatial relationship. The results suggest that the selectivity for the cerebellar or Type I subtype, demonstrated by some of these ligands, could be failure to meet the requirements for binding at other receptors because of the absence of one of the proton accepting centers or the larger surface area and volume of these ligands. The requirement for activation, deduced from comparisons of agonist, antagonist, and inverse agonist properties is the presence of an electron accepting aromatic ring in a specific geometric arrangement with respect to the components of recognition. The validity of the '3D-Pharmacophore' developed was probed by using it for predictions of the behavior of 11 additional compounds not used for its development.

Introduction

The benzodiazepine (BDZ) binding site is known to be localized in the central nervous system (CNS) on the $GABA_A$ (γ-aminobutyric acid) chloride ion channel receptor. BDZ receptor ligands produce their pharmacological effects by modulating the gating action of GABA on the chloride ion channel.[1] A large number of ligands from diverse chemical families have been shown to bind with high affinity to the benzodiazepine binding site(s). The BDZ ligands have been categorized as agonists, antagonists, and inverse agonists, based on their activity in diverse pharmacological endpoints. Ligand labelled agonists are those with similar *in vivo* activities to the prototypical 1,4 BDZ drugs, such as diazepam, exhibiting anxiolytic, anticonvulsant, enhanced feeding, muscle relaxant, and sedative/hypnotic activity. Inverse agonists cause opposite behavioral effects such as anxiogenesis, proconvulsant, anorectic, and procognitive actions. Antagonists are able to inhibit the action of both agonists and inverse agonists.[2]

Based on early receptor binding and thermodynamic stability studies, two distinct BDZ receptor subtypes were the possibility of many functionally distinct receptor subtypes. The techniques of recombinant DNA have led detected that were labeled Type I and Type II.[3] However, at present, the entire $GABA_A$/BDZ receptor complex is known to be composed of five membrane spanning subunits with different subunit combinations resulting in thus far to the identification of six variants of α subunits (α_{1-6}), four β subunits (β_{1-4}) four γ subunits (γ_{1-3} and γ_{21}), as well as others labeled δ and ρ that have been cloned and sequenced.[4a] Several subunit combinations have been expressed in transfected cell systems and receptor binding and Cl^- ion channel function studies have been carried out.[4] Although these expression studies provide much useful information, little is known about the subunit composition and number of functionally distinct central benzodiazepine receptors (BDZR) in different CNS regions.

Recently, our laboratory has reported evidence of at least three central BDZRs in the spinal cord and one in the cerebellum.[5a] Moreover, evidence from both the molecular biology[5b-d] and our own receptor binding studies[5a] indicate that the BDZR central site in cerebellum, is most likely composed of α_1, β_2, γ_2 subunits and are similar to the BDZR originally labeled Type I. While 70 % of cortical BDZR display a 'Type I' BDZ receptor pharmacology, other subtypes are more abundant in the corpus stratum and spinal cord.[6] This lack of regional subtype localization and the lack of selective ligands combine to make the pharmacological characterization of the individual BDZR subtypes and the determination of ligand requirements for recognition and activation of each, very demanding and as yet an unresolved problem. The central cerebellar 'Type I' BDZR is currently the most promising candidate for such in depth characterization, because cerebellum can be used to determine receptor

Abbreviations: BDZ benzodiazepine; CNS central nervous system; GABA γ-aminobutyric acid; BDZR benzodiazepine receptor; Flu flunitrazepam; Ro 15-1788 8-fluoro-3-carboxy-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-*a*]1,4 benzodiazepine; DMCM 3-carbomethoxy-4-ethyl-6,7-dimethoxy-9H-pyrido[4,3-*b*]indole; β-CCE 3-carboethoxy-β-carboline: β-CCP β-carbopropoxy-β-carboline; HOMO highest occupied molecular orbital; LUMO lowest unoccupied molecular orbital; SAR structure–activity relationship.

affinities and activation at this Type I site. There are also Type I selective ligands, namely zolpidem and alpidem, two imidazopyridine ligands,[7] and the less selective ICI 190,622 and 2-oxoquazepam[8] that can be useful in determining molecular requirements for recognition.

Until now, most pharmacophores developed for BDZ/$GABA_A$ receptor ligands were based on the assumption of a single receptor site. In previous work in our laboratory, a model was developed which includes the minimal requirements for recognition and activation of non-selective ligands based on a one-site model.[9] For this purpose, the steric, electronic and environmental properties of fifteen analogs including agonists, inverse agonists, and antagonists classified according to their anticonvulsant profile were computed. The pharmacophore proposed requires as common recognition features, two proton-accepting centers in the ligand separated by ~3.5 Å. In addition, the position of a lipophilic center relative to the proton-accepting sites as defined by the angle between the three centers was found to be an important discriminant of activation.

Proton accepting sites are a common feature of most models of BDZ pharmacophores reported in the literature. In a model by Codding and Muir, one proton accepting site and one moiety involved in a π–π stacking complex with the receptor was invoked.[10] In another study, Fryer *et al.*,[11] proposed that the type of activity elicited by the BDZR ligands could be determined by the distance between a proton-accepting group and a π aromatic ring. We have shown previously[9] that this hypothesis cannot explain all BDZ ligand activities. Tebib and Bourguignon[12] have developed a pharmacophore using crystallographic data and molecular mechanics calculations and found six 'critical zones' of the BDZR ligands: four which modify recognition and two others which modify activation, including two electron rich zones corresponding to proton accepting centers. Cook and co-workers[13] have developed pharmacophores based on the assumption that the inverse agonist/antagonist and agonist bind to different regions of the same domain. There have been several other models proposed for receptor recognition, although few of them address the question of receptor activation and even fewer propose a site-specific receptor model.[14]

In a continued effort to understand the requirements for recognition and activation at the benzodiazepine receptor sites, the goal of the present work is to use the techniques of computational chemistry to identify and characterize the steric and electronic requirements of ligand recognition and activation of the cerebellar (Type I) site of the BDZ receptor sensitive to both agonists and antagonists. To provide a self-consistent database for this hypothesis development, we have used cerebellar receptor binding data obtained in our laboratory for thirteen ligands from diverse families. Four of these had no significant affinity for the receptor and were included as controls. In order to address activation through this receptor, we have measured the effect of three of these ligands on the $GABA_A$ stimulated chloride ion flux in cerebellar microsacs and found one to be an agonist, another an antagonist, and the third an inverse agonist. This assay provides a direct measure of the ability of BDZ receptor ligands to modulate the function of the $GABA_A$ receptor. Such a direct measure of receptor subtype activity is not presently available for any other subtype because of receptor heterogeneity in different brain regions and the lack of selective ligands. The validity of the pharmacophore developed was probed by using it to predict the pharmacological behavior of eleven additional ligands. All the compounds studied are listed in Table 1 and their structures shown in Figure 1. The results obtained allow a refinement of our previous determinants of recognition and activation to those that are specific for 'Type I' receptors.

Computational Methods and Procedures

Initial ligand structures were generated interactively using the ChemNote program as implemented in the Quanta 3.3 package (Polygen Corp., Waltham, MA). The structures were then minimized with the CHARMm force field 2.2[15] using an option that ensures planarity of the amide bond. The steepest descent method was used in minimization with a convergence of 0.01 kcal/Å. CHARMm minimized structures were then reoptimized using the semiempirical quantum mechanical AM1 method included in the MOPAC v. 5.0 package[16] using the PRECISE keyword for minimization. Minima in the conformations of the rotatable aromatic rings (e.g. ring B of the 1,4 BDZs) and other substituents (e.g. ester in β-carbolines) were obtained by nested rotations in 60 ° increments using AM1. The AM1 optimized geometries were used to calculate all thermodynamic and electronic properties reported.

Heats of protonation at competing sites of each analog, labeled 1, 2, 3, 4, and 5 in Table 2, were calculated as the difference between the heat of formation of the protonated and non-protonated form of each ligand. The heat of protonation was used as a measure of the relative proton-accepting ability at competing sites in the ligand. We and others have previously used gas phase proton affinities as an indicator of hydrogen bonding ability of proton acceptors.[17] The smaller the energy, the better the proton accepting ability at that site. Included in this calculation are all sites that have the possibility of acting as proton acceptors including Cl, NO_2, CN, and NH groups as well as the more conventional moieties such as carbonyl, ester and ether oxygens, and imine nitrogens. The proton affinities of all lone pairs of electrons were calculated for each heteroatom as possible proton accepting sites. In each case, it was found that there was no significant difference in proton affinities among the different lone pairs centered on any such moiety.

The energy and electron density of the highest occupied molecular orbital (HOMO) and the energy and virtual electron density in the lowest unoccupied molecular orbital (LUMO) were reported and used as indicators of the ability of a compound to donate and accept electrons respectively, as well as to identify the localized ligand sites of such interactions.

Additional electronic properties considered as candidate modulators of receptor recognition and activation were

Table 1. Binding affinities in rat cerebellar membrane at 0 ° of the BDZ ligands

LIGAND	K_i (nM)	Pharmacophore Development	Pharmacophore Validation
Flunitrazpeam	1.49 ± 0.09	x	
Prazepam	–		x
2-Oxoquazepam	–		x
Loprazolam	–		x
Ro 05-3305	–		x
Midazolam	–		x
b-CCE	0.35 ± 0.02	x	
b-CCP	0.32 ± 0.02	x	
DMCM	2.50 ± 0.15	x	
Ro 15-1788	0.49 ± 0.04	x	
Ro 14-7437	–		x
Ro 15-3505	–		x
Ro 15-4513	–		x
Alpidem	2.15 ± 0.13	x	
Zolpidem	10.5 ± 0.6	x	
ICI 190,622	–		x
CL 218,872	45.8 ± 2.8	x	
AHR-11797	44.0 ± 2.5	x	
Tifluadom	–		x
PK 11195	> 1000 nM[a]		x
UP 590-1	3.3% @ 1mM	x	
UP 590-3	3.0% @ 1mM	x	
UP 588-1	8.1% @ 1mM	x	
UP 586	8.7% @ 1mM	x	

[a]Preliminary data from our laboratory.

dipole moments, calculated as the expectation value of the corresponding operator, and mean molecular polarizabilities, evaluated by an additive scheme using the parameters proposed by Fraga.[18]

Four geometric descriptors were used to characterize the shape of each ligand.[19] For this purpose a van der Waals sphere was placed around each atom, using the radii reported by Gavezzotti.[20] The parameters evaluated were the surface area, volume, the sterimol parameters L, defined here as the maximum distance between two atoms in the molecule, and B1, the maximum orthogonal distance that constitutes a cross-section perpendicular to L. Each of these four quantities could be important steric/shape descriptors in addition to the geometric requirements that are deduced for the properties found to be important in specific interactions with the receptor sites. For example, the maximum length of the compound, L, and the maximum cross section perpendicular to it, represented by B1, could both be important factors in the ability of the ligand to be accommodated in the binding pocket within the constraints of its maximum dimensions. The total volume and areas, similarly provide general criteria for matching the shape of the binding pocket.

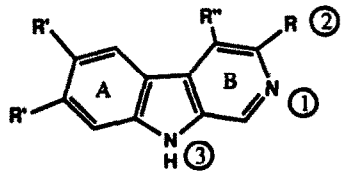
The hydrophobicity of the ligands was estimated by an atom based, conformational-dependent hydrophobic index, computed using a method recently developed in the laboratory.[21] In this procedure, each atom is assigned a hydrophobicity index determined from its contribution to the total van der Waals area and its Mulliken population analysis from AM1 calculations. The logarithm of the partition coefficient is then estimated as the sum of these individual contributions.

The free energies of solvation of the drugs in aqueous solution were calculated using the AM1–SM2 parameter set in AMSOL 3.0.1.[22] In these calculations, the minimized AM1 structure was held rigid and the free energy calculated.

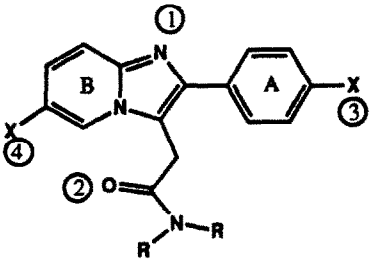
Results and Discussion

Binding studies of the cerebellum site of the BDZ receptor

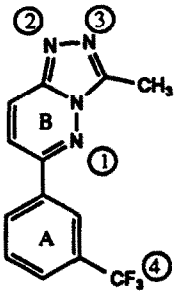
The binding affinities (K_is) for the 13 ligands used for hypothesis development, determined in a previous study in our laboratory[23] are listed in Table 1. K_is were calculated from displacement curves at 0 °C in rat cerebellar membranes. In addition, preliminary unpublished cerebellum binding data were obtained in our laboratory for one of the compounds used for validation (PK 11195). These data were obtained in the same manner as described previously.[23]



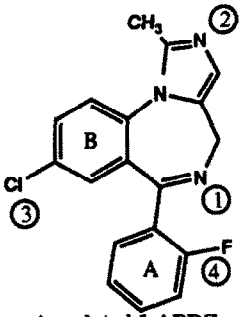
β-Carbolines
β-CCE; R=-$COOC_2H_5$; R'=H
β-CCP; R=-$COOC_3H_7$; R'=H
DMCM; R=-$COOCH_3$; R'=-OCH_3;
R''=-CH_2CH_3



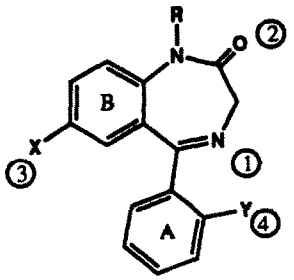
Imidazopyridines
X = R = CH_3 Zolpidem
X = Cl, R = C_3H_7 Alpidem



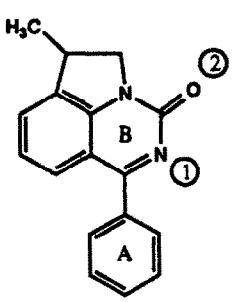
Triazolopyridazines
CL 218,872



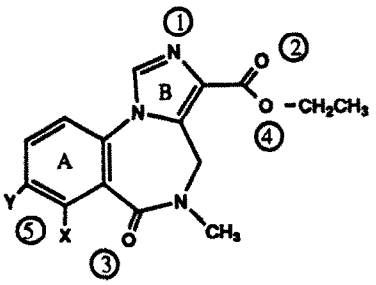
Annelated 1,4 BDZ
Midazolam



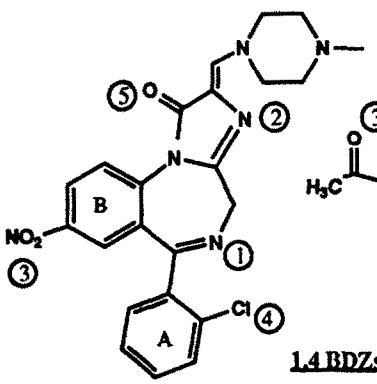
Classical 1,4 BDZs
X= NO_2, Y= F, R= CH_3 Flunitrazepam
X= Cl, Y= F, R= CH_2CF_3 2-Oxo-Quazepam
X= Cl, Y= H, R= CH_2-(cyclopropyl) Prazepam



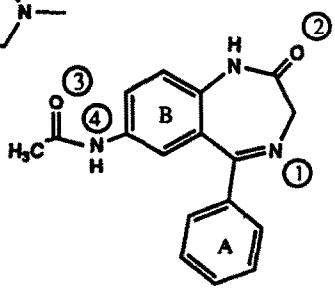
Pyrroloquinazolinones
AHR-11797



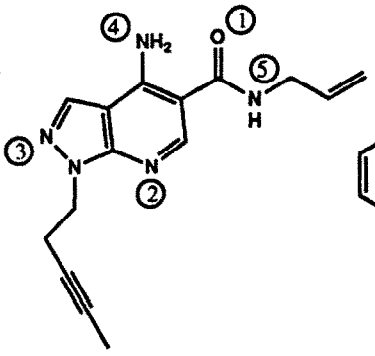
ImidazoBDZs
Y = F, X = H Ro 15-1788
Y = H, X = H Ro 14-7437
Y = H, X = Cl Ro 15-3505
Y = N_3, X = H Ro 15-4513



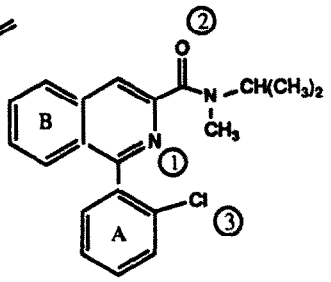
1,4 BDZs
Loprazolam



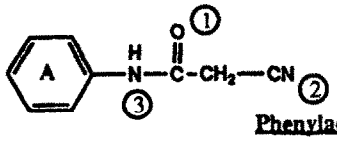
Ro 05, 3308



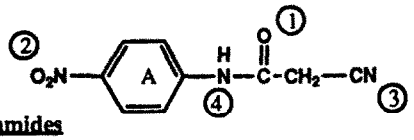
Pyrazolopyridines
ICI 190,622



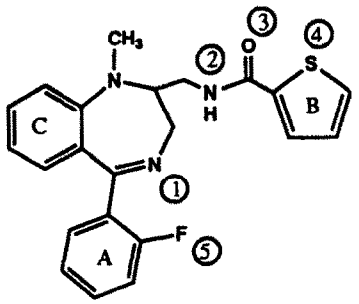
Phenylquinolines
PK 11195



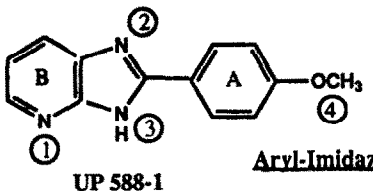
Phenylacetamides
UP 590-1



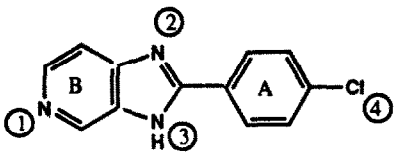
UP 590-3



2-Benzazepines
Tifluadom



Aryl-Imidazopyridines
UP 588-1



UP 586

Figure 1. Chemical formulas and structures of the 24 BDZ Type I receptor ligands contained in the present study. A and B refer to the aromatic rings of the ligands which contain varying degrees of lipophilic and LUMO character. The numbers 1, 2 and 3 correspond to the heat of protonation energies given in Table 2.

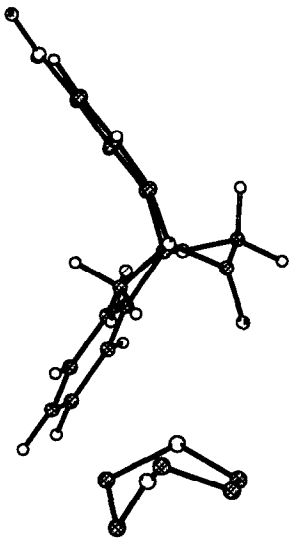
Conformational flexibility of the receptor ligands

Although the compounds included in the present study are fairly rigid, several families have rotational degrees of freedom for aromatic rings and other substituents. In such cases, the conformational profiles of these moieties were evaluated. Energy conformational profiles were calculated in 60 ° increments and the resulting low energy conformations were optimized. In general, the energy differences between the various conformations were ≤ 1.2 kcal/mol. A typical example of the result obtained, shown in Figure 2, indicates the various conformational minima for flunitrazepam (Flu) and their relative energies. Since AM1 is known to underestimate the nitro aromatic torsional energy barrier and although minima were found in which the nitro group was out of plane ($\tau 2$=87 °), these conformers were not included as candidates for recognition. All other conformations given in Figure 2 were considered as candidates for the bioactive conformation.

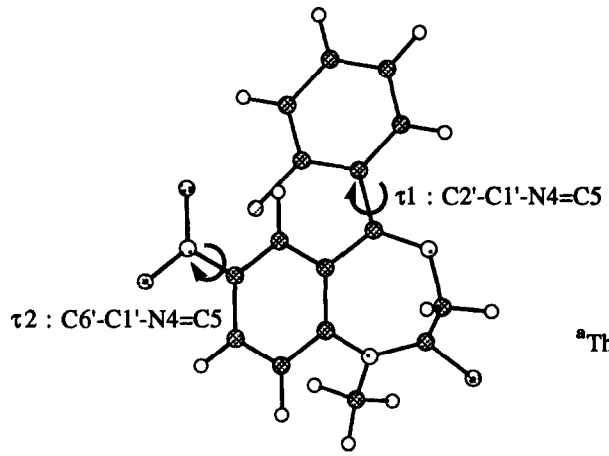
Electronic properties of the BDZ receptor ligands

Various electronic properties and environmental indices were calculated for the BDZR ligands. These properties were evaluated for all conformational minima found in the AM1 calculations. Tables 2 to 7 report these indices for the lowest energy conformer only. The compounds are listed in the tables according to chemical family.

Table 2 lists the calculated proton affinities at competing sites labeled in circles in Figure 1 for each analog. For sites with multiple lone pairs, proton affinities were calculated for each lone pair. The differences in AM1 gas phase energies among the proton affinities of lone pairs of electrons centered on the same atom or associated with a given group, for example the four lone pairs of electrons of the NO_2 group, were found to be < 0.75 kcal/mol, and thus all possible protonation directions for each moiety were considered in development of the pharmacophore. The value of the lowest energy proton affinity at each site is



BENZODIAZEPINE BOAT CONFORMATION



τ1	τ2	ΔE (kcal/mol)
62°	0°	0.0[a]
2°	0°	0.5
172°	0°	0.1

[a]This is the conformation used for calculated properties.

Figure 2. Geometries and relative AM1 optimized energies of stable conformers of flunitrazepam.

Table 2. Heat of protonation (in kcal/mol) at different nucleophilic sites for various BDZ ligands

COMPOUND	SITE 1	SITE 2	SITE 3	SITE 4	SITE 5
Flunitraz	149.5 (N4)	167.3 (O2)	174.2 (NO_2)	201.2 (F)	–
Prazepam	142.6 (N4)	154.4 (O2)	175.7 (Cl)	–	–
2-Oxoquaz	146.6 (N4)	167.3 (O2)	171.0 (Cl)	202.2 (F)	202.3 (CF_3)
Loprazolam	142.2 (N7)	140.7 (N4)	167.9 (NO_2)	198.1 (Cl)	172.3 (O1)
Ro 05-3305	139.1 (N4)	154.1 (O2)	159.4 ($C=O_7$)	177.3 (NH)	–
Midazolam	144.5 (N7)	139.5 (N3)	178.5 (Cl)	204.3 (F)	–
b-CCE	145.3 (N2)	160.9 ($C=O_3$)	165.8 (NH)	173.9 (OEt)	–
b-CCP	145.2 (N2)	165.8 ($C=O_3$)	160.6 (NH)	174.3 (OEt)	–
DMCM	144.4 (N2)	162.6 ($C=O_3$)	162.5 (NH)	174.1 (OMe)	–
Ro 15-1788	129.7 (N2)	138.9 (O3)	145.2 (O2)	172.1 (OEt)	201.2 (F)
Ro 14-7437	147.0 (N2)	155.4 (O3)	163.1 (O2)	170.6 (OEt)	–
Ro 15-3505	100.9 (N2)	107.0 (O3)	116.8 (O2)	171.9 (OEt)	197.5 (Cl)
Ro 15-4513	146.2 (N2)	155.9 (O3)	163.1 (O2)	172.2 (OEt)	189.3 (N_3)
Alpidem	137.0 (N1)	152.7 (O9)	194.3 (Cl_5)	194.8 (Cl_4)	–
Zolpidem	132.3 (N1)	154.1 (O9)	–	–	–
ICI 190,622	149.4 (O9)	143.0 (N7)	160.1 (N2)	163.8 (NH_2)	165.8 (NH)
CL 218, 872	158.2 (N5)	159.6 (N1)	162.1 (N2)	204.6 (CF_3)	–
AHR-11797	139.3 (N1)	143.5 (O2)	–	–	–
Tifluadom	144.4 (N4)	150.2 (N2)	158.4 (O2)	200.8 (S2)	203.3 (F2')
PK 11195	140.0 (N1)	148.7 (O2)	158.9 (Cl2')	–	–
UP 590-1	165.7 (O1)	179.7 (NH)	182.4 (CN2)	–	–
UP 590-3	171.5 (CN2)	176.6 ($NO_2$4)	177.5 (O1)	182.9 (NH)	–
UP 588-1	139.4 (N1)	147.7 (N6)	182.6 (NH)	192.5 (O4')	–
UP 586	144.1 (N2)	146.7 (N6)	183.3 (NH)	184.3 (Cl4')	–

reported in Table 2. As seen in this table, the imine N site, common to all analogs, shows the strongest proton accepting ability, while the nature of the remaining proton accepting groups vary among all classes of compounds. Since both high and low affinity analogs have at least 2 or 3 such sites, their presence alone is not a determinant of recognition. However, a specific geometric relationship among them could be such a factor. Since there is no preference in protonation energies of the lone pairs in probing the geometric relationships of different atoms, all were considered as possible interaction sites and those common to high affinity analogs and absent in low affinity ligands determined the lone pair direction to interact with a complementary proton donating receptor moiety. Of all moieties studied, only one (the NH group) has the possibility of acting as both a proton acceptor and a proton donor. This duality has been addressed by this laboratory before in a study of the β-carbolines in which this hydrogen was replaced with a methyl group without significant loss of affinity.[24] Alternatively, it is possible that torsional rotation (e.g. serine side chain) or tautomeric equilibria (e.g. histidine residue) in the receptor may allow interconversion of accepting and donating sites thus allowing interaction with both proton donating and accepting regions of the ligand. Thus, the interaction of the NH moiety is not definitive, without a complete understanding of the receptor point with which it is proposed to interact.

Table 3 lists the dipole moment, total hydrophobic index, and hydrophobic index for various rings for the BDZR ligands. The dipole moment has considerable variation, with the compounds zolpidem and alpidem having unusually low dipole moments for the high affinity ligands. Comparing compounds which do and do not bind to the receptor, it is evident that there is no relationship between dipole moment values and recognition.

The hydrophobic index also shows variation among the ligands. Most members of the 1,4 benzodiazepine family have rather large hydrophobic indices, although flunitrazepam and loprazolam are notable exceptions. Since the non-binding compounds show no marked difference in lipophilicity from the high affinity ligands, no relationship of this property to recognition is obtained. However, all compounds with known high affinity to the receptor are more soluble in a hydrophobic phase than in an aqueous environment. Examining the local contribution to the hydrophobicity, the most lipophilic moiety for each ligand except the ICI compound was found to be an aromatic ring, labeled ring A in Figure 1. This ring, and in the case of the ICI compound the unsaturated hydrocarbon

Table 3. Dipole (in D) and hydrophobic index for various BDZ ligands

COMPOUND	DIPOLE	Hydophobic Index		
			Ring A	Ring B
Flunitraz	3.1	0.5	1.9	1.6
Prazepam	3.1	4.1	2.0	1.6
2-Oxoquaz	4.3	4.0	2.0	1.5
Loprazolam	6.9	0.3	2.0	1.5
Ro 05-3305	7.5	1.5	1.8	1.5
Midazolam	3.7	3.9	1.8	1.6
β-CCE	3.9	1.4	1.9	1.7
β-CCP	3.9	1.7	1.9	1.7
DMCM	4.9	0.7	1.6	0.2
Ro 15-1788	2.6	0.8	1.8	-0.1
Ro 14-7437	3.4	0.6	1.8	0.0
Ro 15-3505	3.8	0.9	1.8	0.1
Ro 15-4513	2.3	-2.3	1.6	0.1
Alpidem	1.9	4.3	1.7	0.8
Zolpidem	1.8	3.8	1.7	0.8
ICI 190,622	3.2	1.1	1.4[a]	1.7[b]
CL 218, 872	3.9	0.8	1.7	-0.5
AHR-11797	5.6	3.0	1.9	1.7
Tifluadom	4.5	5.2	2.0	-
PK 11195	3.7	4.4	1.8	1.8
UP 590-1	1.6	0.7	2.2	-
UP 590-3	6.2	-1.6	2.0	-
UP 588-1	2.0	0.9	1.9	-
UP 586	2.8	1.7	1.8	-

[a]This is the value for the vinyl group (B1 in Figure 1).
[b]This is the value for the alkyne (B2 in Figure 1).

chain, could participate either in $\pi-\pi$ stacking or a hydrophobic interaction with the receptor.

The calculated polarizabilities are shown in Table 4. This quantity can be considered as a general measure of the dispersion contribution to the binding energy. Table 4 shows no direct relation of either the total polarizability or the local contributions of different rings with observed binding affinities, although compounds with unusually low polarizabilities show low affinity for the Type I BDZ receptor or do not bind at all. This conclusion is in agreement with our previous studies.[9,14c]

In Table 5, we report the general steric and shape descriptors for all compounds studied. The UP compounds have very low molecular areas and volumes and show no binding to Type I or any other known receptor site. This result suggests a minimum size requirement for recognition of any BDZR. At the other extreme, alpidem and zolpidem have the largest areas and volumes indicating that, while they can be accommodated at the cerebellar Type I receptor, they could exceed a size requirement at the other BDZR subtypes that prevents these compounds from binding to them, thus contributing to their Type I selectivity. The other descriptors show no systematic pattern among the compounds.

Table 6 lists the solvation free energies of the ligands. This parameter gives an indication of the importance of desolvation in binding to the receptor. There is no clear distinction in this value between high and low affinity analogs and consequently, this property is not, by itself, a discriminant of recognition.

Table 7 gives the energies of the HOMO and LUMO and the virtual electron density distribution on each aromatic ring in LUMO for the BDZR ligands. Energies of the LUMOs are small negative quantities. Although negative energies for unoccupied orbitals are not physically realistic and a consequence of using semi-empirical methods, comparisons of the relative order of the LUMO energies for zolpidem and alpidem from *ab initio* calculations[25] with the results here, indicated the relative magnitudes of these values can be adequately used as a measure of electron accepting abilities. Thus, these ligands can function as efficient electron acceptors with the electron

density localized on one of the aromatic rings. In most ligands, the aromatic ring with the localized electron density in LUMO is distinct from the most lipophilic ring. In other compounds, however, such as the Ro compounds, the electron accepting and most lipophilic ring are the same.

Table 4. Polarization volumes, total volumes (α), and continuation of different rings, a and b

COMPOUND	α Total	α (A)	α (B)
Flunitrazpeam	42.8	13.8	13.9
Prazepam	46.9	13.5	13.7
2-Oxoquazepam	44.0	13.8	13.7
Loprazolam	57.3	13.8	13.9
Ro 05-3305	43.3	13.7	13.8
Midazolam	48.5	13.8	13.7
β-CCE	34.2	13.2	–
β-CCP	37.8	13.3	–
DMCM	43.5	13.5	–
Ro 15-1788	40.3	12.8	10.2
Ro 14-7437	39.9	12.8	10.1
Ro 15-3505	41.1	12.9	10.0
Ro 15-4513	42.2	13.3	10.0
Alpidem	47.5	14.3	14.3
Zolpidem	43.7	14.2	14.2
ICI 190,622	45.2	11.3	–
CL 218, 872	34.7	16.4	–
AHR-11797	35.8	13.7	–
Tifluadom	45.6	13.9	13.7
PK 11195	43.7	13.9	13.7
UP 590-1	17.6	13.2	–
UP 590-3	18.8	13.8	–
UP 588-1	32.6	14.0	–
UP 586	31.2	13.7	–

Table 5. Sterimol parameters for various BDZ ligands

Ligand	Area	Volume	L	B1
Flunitrazep	296.7	259.1	12.2	7.3
Prazepam	334.0	290.8	14.5	7.5
2-Oxoquazep	324.9	276.5	12.1	7.7
Loprazolam	447.0	391.0	17.8	8.2
Ro 05-33	304.6	267.0	13.3	7.6
Midazolam	313.5	274.5	12.8	6.5
β-CCE	256.8	217.0	13.9	5.5
β-CCP	277.9	233.2	14.9	6.8
DMCM	329.7	281.9	16.1	5.7
Ro 15-1788	301.2	256.1	15.7	5.5
Ro 14-7437	296.7	253.0	15.1	5.0
Ro 15-3505	311.7	266.8	14.9	6.1
Ro 15-4513	327.9	278.1	17.0	5.5
Alpidem	420.6	366.3	15.6	9.2
Zolpidem	379.4	332.1	15.6	8.1
ICI 190, 622	329.1	268.5	19.4	4.7

Table 5. *Continued.*

Ligand	Area	Volume	L	B1
CL 218, 872	259.0	219.2	13.7	5.9
AHR 11797	274.4	245.6	14.1	5.0
Tifluadom	396.9	343.6	15.9	6.3
PK 11195	360.9	314.3	15.0	8.5
UP 590-1	184.2	151.2	11.3	4.7
UP 590-3	209.1	170.3	12.7	4.4
UP 588-1	241.2	206.9	15.2	4.7
UP 586	228.6	196.5	14.4	4.5

Table 6. Free energy of solvation

COMPOUND	ΔG_{SOL} (kcal/mol)
Flunitrazepam	-11.3
Prazepam	-4.2
2-Oxoquazepam	-9.2
Loprazolam	-12.1
Ro 05-3305	-16.0
Midazolam	-7.5
β-CCE	-10.8
β-CCP	-10.6
DMCM	-12.4
Ro 15-1788	-15.3
Ro 14-7437	-14.9
Ro 15-3505	-16.0
Ro 15-4513	-18.8
Alpidem	-8.3
Zolpidem	-10.3
ICI 190,622	-9.8
CL 218, 872	-6.9
AHR-11797	-9.7
Tifluadom	-17.2
PK 11195	-9.0
UP 590-1	-8.5
UP 590-3	-12.5
UP 588-1	-11.6
UP 586	-11.4

Model of ligand-receptor recognition

The large diversity of chemical families that bind with relatively high affinity to the Type I cerebellar BDZ site suggest a versatile binding region. In determining the requirements for recognition of this receptor, in order to compare the role of proton donating sites in compounds of diverse structure, complementary proton donor receptor points RP_1, RP_2, RP_3, etc. were defined. These were placed 3.0 Å from the nucleophilic ligand atoms with which they are proposed to interact, with the possible direction(s) of these receptor points determined by the direction of the lone pair(s) of electrons on the ligand heteroatoms. There are six different types of candidate proton accepting centers in these compounds with different degrees of flexibility in the placement of complementary receptor points for interaction with them. For an imine nitrogen and an amine nitrogen, there is only one lone pair of electrons and its direction is well determined in each case. The carbonyl oxygen atom has two lone pairs of electrons whose positions can be defined by two different values of torsion angles with respect to a specific atom to which the C=O moiety is bonded (i.e. τ C–C=O–RP).[26] Values of $\tau = 0°$ or 180 °, *syn* and *anti*, were obtained only for those compounds in which the three atoms and the lone pair are in the same plane. Although one or the other lone pairs of electrons are shown to be favored in crystal structures,[27] protonation energies are comparable and the preferred direction of interaction in the binding site will be largely determined by the position of the corresponding proton donor in the binding site of the receptor. Thus both possible sites have been examined as candidate receptor sites in our calculations of distances and angles. For the Cl substituent, there are three non-bonded pairs of electrons that form a nearly spherical electron density distribution around it and the complementary interacting receptor point can be placed at a 3 Å radius in many directions from the Cl atom. The position of the complementary site with the Cl atom was chosen considering an sp^3 hybridization for the halogen and placing three receptor points in all sp^3 directions, any of which could be the receptor point. For the NO_2 group, there are two oxygen atoms each with two lone pairs of electrons, allowing four positions of the complementary proton donating moiety in the receptor. Again, protonation energies *in vacuo* are similar for the different sites and the preferred lone pair sites of intermolecular interactions from crystal structures are not helpful in choosing those in the receptor binding site since the 3D architecture of this binding site is very different from the crystal packing geometries. Thus, all four possible directions of the receptor point interacting with the nitro group were considered as receptor point candidates.

In addition to the proton acceptors, both aromatic rings, labeled A and B in Figure 1, were also considered possible moieties involved in receptor recognition. Consequently, the corresponding receptor points (RP_A and RP_B) were also defined. Since the interaction expected (van der Waals) is not directional, these receptor points were placed

Table 7. Energy (in eV) of HOMO and LUMO and virtual electron density in LUMO on the rings for the BDZ type I ligands

LIGAND	E_{HOMO}	E_{LUMO}	Ring A	Ring B	Ring C
Flunitraz	-9.856	-1.435	0.010	0.737	–
Prazepam	-9.200	-0.607	0.072	0.686	–
2-Oxoquaz	-9.655	-0.840	0.012	0.801	
Loprazol	-8.603	-1.101	0.007	0.769	–
Ro 05-3305	-8.635	-1.250	0.110	0.766	–
Midazolam	-8.954	-0.781	0.002	0.717	–
β-CCE	-8.814	-0.594	0.269	0.610	–
β-CCP	-8.824	-0.593	0.266	0.612	–
DMCM	-8.619	-0.578	0.464	0.584	–
Ro 15-1788	-9.683	-1.110	0.692	0.0	–
Ro 14-7437	-9.634	-0.882	0.689	0.0	–
Ro 15-3505	-9.728	-0.986	0.731	0.1	–
Ro 15-4513	-9.608	-1.248	0.655	0.1	–
Alpidem	-8.749	-0.671	0.239	0.539	–
Zolpidem	-8.468	-0.307	0.224	0.544	–
ICI 190,622	-8.938	-0.256	0.677	–	–
CL 218, 872	-9.368	-1.242	0.361	0.576	–
AHR-11797	-8.909	-0.839	0.118	0.525	0.361
Tifluadom	-8.585	-0.347	0.000	0.927	0.215
PK 11195	-9.104	-0.803	0.029	0.403	–
UP 590-1	-9.018	+0.078	0.701	–	–
UP 590-3	-9.832	-1.206	0.691	–	–
UP 588-1	-8.667	-0.557	0.411	0.307	–
UP 586	-9.001	-0.861	0.556	0.199	–

in the center of each ring, representing the centroid of the interaction.

Once these candidate receptor points were identified, flunitrazepam, a non-selective high affinity ligand, was used as a template for pairwise comparisons of geometric commonalties of them with all the other ligands studied. All distances between possible receptor proton donor sites and the angles made by these sites with the aromatic rings were evaluated. As an illustration of this procedure, Table 8 gives such a comparison between flunitrazepam and two of these ligands. As illustrated in Table 8 all possible lone pair directions of the nitro, carbonyl oxygen and fluorine substituents of flunitrazepam were considered in determining the position of possible complementary proton donor receptor points. These included two possible receptor points for the carbonyl oxygen, four for the nitro group, and three for the fluorine atom. As shown in Table 8, the distance between the receptor point interacting with the imine nitrogen and all the other candidate receptor donor sites were then calculated. In addition, the corresponding angles to the lipophilic rings (RP_A and RP_B), which could be involved in either recognition or activation, were also calculated. These distances and angles in flunitrazepam were then compared with AHR 11797, which has only an imine nitrogen and a carbonyl oxygen as possible proton accepting sites. We see from Table 8 that only for receptor points corresponding to interactions with :N and the lone pair direction of the carbonyl labeled O_2 are the distances and angles the same in these two compounds. These receptor points labeled RP_1 and RP_2 in Figure 1 and Tables 9–11 were thus chosen as common recognition sites between the template and this analog. Similarly, comparing flunitrazepam and zolpidem in Table 8, we see that the common receptor proton donor site in addition to that interacting with the imine nitrogen (RP_1) is a specific one of the nitro group of the flunitrazepam, (labeled RP_3 in Figure 1 and Tables 9–11) and one direction of the carbonyl oxygen of zolpidem (also labeled RP_3 in Figure 1 and Table 9–11). Neither the high affinity Type I selective zolpidem nor the AHR 11797 compound, each with only two proton accepting moieties, had a lone pair in a similar position as any of those of the fluorine atom in flunitrazepam. Therefore, this fourth site was eliminated as a determinant of Type I recognition. While the pairwise comparison between flunitrazepam and zolpidem illustrated in Figure 8 alone did not totally determine which of the lone pairs of the nitro group of flunitrazepam and of the carbonyl group of zolpidem are involved in receptor interactions, further pairwise comparisons between flunitrazepam and all other compounds did allow a unique choice of all three proton

donating receptor points (RP_1, RP_2, and RP_3) in all compounds. These unique lone pair directions chosen for each proton accepting center in each ligand to determine the direction of the three corresponding receptor points (RP_1, RP_2, and RP_3) are given in Table 9 in terms of their specific torsion angles. These proposed receptor points were evaluated for steric tolerance for all ligands and were found to be acceptable. Figure 1 indicates for each compound the proton accepting moiety used for the labeling of these receptor points.

The specific receptor points, RP_1 and RP_2, can be regarded in every case as proton accepting sites, as shown in Table 2. RP_3, however, cannot be considered as a proton accepting site for all ligands studied. Two alternative modes of interaction of this site with the ligand are presented here. First, this receptor site may function as a hydrogen bond donor, a role that is supported by a negative region in the MEP at complementary ligand sites in the majority of the ligands included in this study. Second, the receptor point 3, RP3, might function as a hydrogen bond acceptor as in the β-carboline and phenylacetamide series of benzodiazepine ligands. Receptor site RP_3 could, for example, be a freely rotating serine or a partially protonated histidine side chain which could explain how substituents such as the carbonyl oxygen atom of zolpidem and the amine NH moiety in the β-carbolines can both interact with the same receptor site. Therefore, in order to develop a pharmacophore model which will accommodate all classes of ligands included in this study, RP_3 is considered as a nucleophilic center located 3.0 Å from the corresponding nucleophilic atom located in the ligand, realizing that the hydrogen atom that forms the hydrogen bond between these two nucleophilic centers may be covalently bound to either the receptor or the ligand, depending on the nature of the ligand.

Table 10 gives the distances between the proton donor receptor points chosen for each compound. Examining this table we see that all the high affinity ligands, except AHR 11797 and CL 218,872, have sites 1 and 3 in common with the high affinity Type I selective ligands zolpidem and alpidem, with a very similar distance of 9.5 ± 1 Å between complementary receptor points RP_1 and RP_3. AHR 11797 and CL 218,872 have sites 1 and 2 in common with flunitrazepam, a classical high affinity non-selective 1,4 BDZ. The distance between these two proton donating receptor points, RP_1–RP_2 is about 6.5 ± 1 Å, also very similar for all the high affinity analogs that contain them. For the high affinity compounds that have both RP_2 and RP_3, this distance between these two proton accepting sites is also very similar, 7.5 ± 0.51 Å.

Table 8. Comparison of all possible receptor points (distances and angles) for flunitrazepam, zolpidem, and AHR 11797

Flunitrazepam	Zolpidem	AHR 11797
:N - O^1= 3.603Å	:N - O^1= 10.760Å	:N - O^1= 2.718Å
:N - O^1= - RP_A 18.1°	:N - O^1= - RP_A 29.5°	:N - O^1= - RP_A 146.2°
:N - O^1= - RP_B 85.9°	:N - O^1= - RP_B 27.1°	:N - O^1= - RP_B 61.9°
:N - O^2= 5.616Å	*:N - O^2= 9.272Å*	**:N - O^2= 6.702Å**
:N - O^2= - RP_A 116.5°	*:N - O^2= - RP_A 58.6°*	**:N - O^2= - RP_A 107.8°**
:N - O^2= - RP_B 67.3°	*:N - O^2= - RP_B 19.9°*	**:N - O^2= - RP_B 58.3°**
:N - NO^1 10.007Å	:N - NR 6.300Å	
:N - NO^1 - RP_A 59.4°	:N - NR - RP_A 63.9°	
:N - NO^1 - RP_B 48.9°	:N - NR - RP_B 34.6°	
:N - NO^2 11.824Å		
:N - NO^2 - RP_A 33.8°		
:N - NO^2 - RP_B 16.7°		
:N - NO^3 11.892Å		
:N - NO^3 - RP_A 30.0°		
:N - NO^3 - RP_B 23.5°		
:N - NO^4 8.576Å		
:N - NO^4 - RP_A 31.9°		
:N - NO^4 - RP_B 33.5°		
:N - F^1 4.079Å		
:N - F^1 - RP_A 169.6°		
:N - F^1 - RP_B 65.0°		
:N - F^2 7.165Å		
:N - F^2 - RP_A 38.8°		
:N - F^2 - RP_B 27.5°		
:N - F^3 7.610Å		
:N - F^3 - RP_A 34.8°		
:N - F^3 - RP_B 64.3°		

Table 9. Torsional values for the specific receptor points chosen as candidate proton donor sites in recognition

COMPOUND	RP1 TORSION	RP2 TORSION	RP3 TORSION
Flunitrazepam	$C\text{-}C_5\text{=}N_4\text{-}LP = 179.4$	$C_3\text{-}C_2\text{=}O\text{-}LP = 0.7$	$C_7\text{-}N\text{-}O\text{-}LP = 0.3$
Prazepam	$C\text{-}C_5\text{=}N_4\text{-}LP = 178.8$	$C_3\text{-}C_2\text{=}O\text{-}LP = 1.2$	$C_6\text{-}C_7\text{-}Cl\text{-}LP = 0.8$
2-Oxoquazepam	$C\text{-}C_5\text{=}N_4\text{-}LP = 179.0$	$C_3\text{-}C_2\text{=}O\text{-}LP = 1.6$	$C_6\text{-}C_7\text{-}Cl\text{-}LP = 1.9$
Loprazolam	$C\text{-}C_5\text{=}N_4\text{-}LP = 177.4$	$C_6\text{-}C_5\text{=}N_4\text{-}LP = 0.4$	$C_{10}\text{-}N\text{-}O\text{-}LP = 1.1$
Ro 05-3305	$C\text{-}C_5\text{=}N_4\text{-}LP = 177.3$	$C_3\text{-}C_2\text{=}O\text{-}LP = 2.2$	$N\text{-}C\text{=}O\text{-}LP = 0.5$
Midazolam	$C\text{-}C_5\text{=}N_4\text{-}LP = 179.9$	$C_5\text{=}C_4\text{-}N_3\text{-}LP = 181.3$	$C_9\text{-}C_{10}\text{-}Cl\text{-}LP = 0.8$
β-CCE	$C_3\text{-}C\text{=}O\text{-}LP = 94.2$	$C\text{-}C_3\text{-}N_2\text{-}LP = 179.8$	$C\text{-}C\text{-}N_9\text{-}LP = 180.3$
β-CCP	$C_3\text{-}C\text{=}O\text{-}LP = 97.8$	$C\text{-}C_3\text{-}N_2\text{-}LP = 178.3$	$C\text{-}C\text{-}N_9\text{-}LP = 182.7$
DMCM	$C_3\text{-}C\text{=}O\text{-}LP = 89.6$	$C\text{-}C_3\text{-}N_2\text{-}LP = 180.6$	$C\text{-}C\text{-}N_9\text{-}LP = 179.8$
Ro 15-1788	$C_5\text{-}C_4\text{-}N_3\text{-}LP = 180.2$	$C_4\text{-}C\text{=}O\text{-}LP = 180.3$	$N_7\text{-}C_8\text{=}O\text{-}LP = 0.3$
Ro 14-7437	$C_5\text{-}C_4\text{-}N_3\text{-}LP = 180.7$	$C_4\text{-}C\text{=}O\text{-}LP = 179.5$	$N_7\text{-}C_8\text{=}O\text{-}LP = 0.9$
Ro 15-3505	$C_5\text{-}C_4\text{-}N_3\text{-}LP = 179.8$	$C_4\text{-}C\text{=}O\text{-}LP = 181.1$	$N_7\text{-}C_8\text{=}O\text{-}LP = 0.5$
Ro 15-4513	$C_5\text{-}C_4\text{-}N_3\text{-}LP = 181.3$	$C_4\text{-}C\text{=}O\text{-}LP = 182.4$	$N_7\text{-}C_8\text{=}O\text{-}LP = -0.8$
Alpidem	$C\text{=}C_2\text{-}N_1\text{-}LP = 179.9$	–	$C_8\text{-}C_9\text{=}O\text{-}LP = 0.4$
Zolpidem	$C\text{=}C_2\text{-}N_1\text{-}LP = 178.7$	–	$C_8\text{-}C_9\text{=}O\text{-}LP = -0.7$
ICI 190,622	$C_5\text{-}C_9\text{=}O\text{-}LP = 0.7$	$C_5\text{-}C_6\text{=}N_7\text{-}LP = 179.6$	$C\text{-}N_1\text{-}N_2\text{-}LP = 179.5$
CL 218, 872	$C_7\text{-}C_6\text{=}N_5\text{-}LP = 181.3$	$C_3\text{=}N_2\text{-}N_1\text{-}LP = 181.8$	–
AHR-11797	$C\text{-}C\text{=}N_1\text{-}LP = 182.0$	$N_1\text{-}C_2\text{=}O\text{-}LP = 180.3$	–
Tifluadom	$C\text{-}C_5\text{=}N_4\text{-}LP = 178.7$	$C_2\text{-}C\text{-}N\text{-}LP = 178.4$	$N\text{-}C\text{=}O\text{-}LP = 180.3$
PK 11195	$C\text{-}C\text{=}N_1\text{-}LP = 179.5$	$C_8\text{-}C\text{=}O\text{-}LP = 179.9$	$C\text{=}C_{2'}\text{-}Cl\text{-}LP = 182.7$
UP 590-1	$N\text{-}C\text{=}O\text{-}LP = 183.2$	$C\text{-}C\text{=}N\text{-}LP = 180.3$	$C\text{=}C\text{-}N\text{-}LP = 175.3$
UP 590-3	$N\text{-}C\text{=}O\text{-}LP = 179.1$	$C\text{-}N\text{-}O\text{-}LP = 183.4$	$C\text{-}C\text{=}N\text{-}LP = 177.9$
UP 588-1	$C\text{-}C\text{=}N\text{-}LP = 179.7$	$N\text{-}C\text{=}N\text{-}LP = 179.3$	$C\text{=}C\text{-}O\text{-}LP = 0.5$
UP 586	$C\text{=}C\text{-}N\text{-}LP = 180.6$	$N\text{-}C\text{=}N\text{-}LP = 179.5$	$C\text{=}C\text{-}Cl\text{-}LP = 180.5$

While the distances between proton accepting receptor sites RP_1, RP_2 and RP_3 are very similar for all the high affinity ligands, these distances alone are not adequate determinants of recognition since at least one of the four compounds with no significant receptor affinity also satisfies two of the three distance criteria. The geometric relationship of the most lipophilic ring A to the three proton donating receptor points identified as possible determinants of recognition was hence explored as a possible additional determinant of a 3D pharmacophore for recognition. Table 11 gives the corresponding angles between pairs of these points and the center of the lipophilic ring. This table clearly demonstrates that the angle between this ring and all three pairs of proton donor receptor points i.e. the value of the RP_1–RP_2–Ring A angle the RP_2–RP_3–Ring A angle and the RP_1–RP_3–Ring A angle is very similar for all high affinity analogs, namely, ~80–115 ° for the first, ~60 °–64 for the second and ~52 °–60 ° for the third. Moreover, these angles have a different value for the non-binding compounds. Thus, the relative position of the lipophilic ring, with respect to the three proton accepting sites is an additional determinant of recognition, possibly involving π–π stacking with a complementary aromatic ring in the receptor.

Table 10. Distances in Å between the proton accepting centers in various BDZ ligands

COMPOUND	RP1-RP2	RP2-RP3	RP1-RP3
Flunitrazpeam	5.616	8.556	10.007
Prazepam	7.443	9.823	10.957
2-Oxoquazepam	7.049	9.298	10.527
Loprazolam	5.954	11.259	10.998
Ro 05-3305	6.669	12.766	9.916
Midazolam	9.108	11.018	10.479
β-CCE	5.424	7.392	8.606
β-CCP	5.426	7.393	8.608
DMCM	6.158	7.348	8.609
Ro 15-1788	6.522	8.307	9.595
Ro 14-7437	6.488	8.325	9.861
Ro 15-3505	6.799	8.297	9.463
Ro 15-4513	6.491	8.110	9.645
Alpidem	–	–	9.296
Zolpidem	–	–	9.272
ICI 190,622	7.459	7.577	12.615
CL 218, 872	7.663	–	–
AHR-11797	6.702	–	–
Tifluadom	5.367	10.130	6.719
PK 11195	5.581	4.400	2.664
UP 590-1	4.823	7.308	3.726
UP 590-3	7.430	9.866	15.404
UP 588-1	9.166	8.174	10.902
UP 586	9.608	6.046	7.100

Table 11. Angles (in degrees) between the ring with the highest lipophilic character and the proton accepting centers in various BDZ ligands

COMPOUND	RP1-RP2-RING	RP2-RP3-RING	RP1-RP3-RING
Flunitrazepam	116.5	60.4	59.4
Prazepam	86.3	49.1	33.1
2-Oxoquazepam	89.9	53.6	40.2
Loprazolam	114.3	49.0	43.7
Ro 05-3305	133.9	38.6	32.3
Midazolam	83.9	39.9	28.5
β-CCE	82.5	82.2	57.3
β-CCP	82.4	82.5	57.5
DMCM	80.5	83.6	54.2
Ro 15-1788	85.3	81.3	52.1
Ro 15-3505	85.0	81.9	54.5
Ro 15-4513	85.3	82.8	52.3
Alpidem	–	–	57.6
Zolpidem	–	–	58.6
ICI 190,622	88.2[a]	–	–
CL 218, 872	112.8	–	–
AHR-11797	107.8	–	–

Table 11. *Continued.*

COMPOUND	RP1-RP2-RING	RP2-RP3-RING	RP1-RP3-RING
Tifluadom	71.8	24.6	38.1
PK 11195	89.6	91.6	93.7
UP 590-1	87.9	81.3	80.0
UP 590-3	59.9	67.6	10.3
UP 588-1	31.7	12.9	34.4
UP 586	28.0	51.7	110.2

*This is the angle to the center of the vinyl group.

In summary, as indicated in Table 12, using a small number of structurally diverse compounds that do and do not bind to the cerebellar Type I $GABA_A$/BDZ receptor, we have determined the minimum requirements for recognition of this receptor. This receptor appears to have three proton donating moieties, which we have labelled RP_1, RP_2 and RP_3. However, interactions of complementary ligand moieties with all three of these points do not seem to be a requirement for high affinity binding to this receptor subtype. Rather ligand recognition of this receptor appears to require complementary nucleophilic moieties to interact with RP_1 and either RP_2 or RP_3. Thus ligands can bind to this receptor if they can interact with two proton donating receptor sites (RP_1, RP_3) in common with zolpidem and alpidem, separated by about 9–11 Å and if they have an aromatic lipophilic ring making an angle of about 60 ° with these sites. Alternatively, recognition of this receptor can occur by ligands that share one of these sites (site 1) with zolpidem and alpidem and one with high affinity non-selective ligands such as flunitrazepam (site 2). Sites 1 and 2 are separated by a shorter distance of about 5–7 Å with these two sites making an angle in the range of 80–120 ° with a lipophilic aromatic ring. The absence of proton accepting site 2 in Type I selective ligands zolpidem and alpidem, in addition to a size restriction already noted, might contribute to their lack of binding to other receptor subtypes that require this site. The implications of the absence of proton accepting site 3, as in the AHR and CL compounds are less apparent. Compounds that have all three nucleophilic sites, appear to be non-selective and have similar distances between RP_2 and RP_3 of 7.5–8.5 Å and similar angles of 60–85 ° with the lipophilic ring A.

Mode of ligand–receptor activation

Until evidence is provided that specific BDZR subtypes mediate different behavioral endpoints, modulation of GABA effects on chloride ion flux is one of the only properties that can be directly related to activation of the $GABA_A$/BDZ receptor through a specific receptor subtype. Such studies are underway in our laboratory and we currently have reliable experimental results for the effect on the chloride ion flux in cerebellum for three of the compounds included in the present study.[28] These results are consistent with the observation based on *in vivo* endpoints that flunitrazepam is an agonist, since it was found to enhance GABA stimulated chloride ion flux, that DMCM is an inverse agonist, since it diminishes this flux, and that Ro15-1788 is an antagonist, since it has no effect on the flux and antagonizes the effect of both flunitrazepam and DMCM. These three compounds can then be used as templates to identify possible discriminants of activation of the cerebellar $GABA_A$/BDZ receptor.

Table 12. Minimium requirements for recognition of the type I BDZ receptor

RECEPTOR POINTS	RANGE (Å)
RP_1-RP_2	5.4-7.7
RP_1-RP_2-RING	81-117
RP_1-RP_3	8.6-11.0
RP_1-RP_3-RING	52-60
RP_2-RP_3	7.4-8.5
RP_2-RP_3-RING	60-85

As discussed previously, the energy of the LUMO for all the compounds studied is small enough to implicate a charge transfer mechanism of activation. In each of the 13 compounds considered the electron density in LUMO is centered mainly on an aromatic ring that would then be the main moiety involved in such a charge transfer mechanism. We have thus examined whether the aromatic ring with the greatest electron density in LUMO is a determinant of activation.

In order to investigate this possibility, we superimposed the three compounds flunitrazepam, Ro 15-1788, and DMCM of differing activity, as shown in Figure 3, such that they could all interact with the common receptor points RP_1, RP_2, RP3 and the lipophilic region already identified as determinants of recognition. With the

recognition conditions satisfied, we then examined the relative positions of the aromatic ring with maximum electron density in LUMO that would act as an electron acceptor in each of the three ligands. We see in this figure that a second aromatic ring (ring B) on which the electron density in LUMO is primarily located (Table 7) is found in a different location for the agonist and inverse agonist. In the antagonist, the most lipophilic ring involved in recognition is also the one with the most electron density in LUMO. The discriminant position of this electron accepting ring in the agonist and inverse agonist and antagonist (Figure 3) and its already identified role in recognition rather than activation in the antagonist, strongly implicate ring B in a mechanism of activation of the cerebellar BDZ receptor by charge transfer to it from a corresponding aromatic donor ring such as histidine, in the receptor.

This mechanism of activation, namely electron transfer, is similar to that originally proposed by us in our previous studies of non-selective BDZR ligands[9] and has been proposed as a possible mode of interaction by others.[29] In our previous study, electron transfer was implicated in activation since relative activity of the six agonists studied varied as their electron accepting ability as measured by E_{LUMO}. However, in reference 9, it was the position of the most lipophilic ring in each compound (ring A) that was found to have a different position in agonists, antagonists, and inverse agonists. The results of this study differ in that we have determined that the most lipophilic ring is the most electron accepting ring only for the antagonists and that it is ring B for agonists and for inverse agonists. In addition, in this work we define criteria for recognition in terms of common receptor points, rather than moieties of the ligands themselves. Therefore, the moiety responsible for electron transfer and its geometric relationship to the determinants of recognition differ significantly from our previous work.

Since receptor points RP_1 and the lipophilic ring are proposed as universal requirements for recognition of the cerebellar BDZ receptor by agonists, antagonists and inverse agonists, the discriminant for activation can be defined as the angle between the aromatic ring implicated as an electron acceptor site in LUMO, RP_1 and the lipophilic aromatic ring. This angle was measured from the center of the electron accepting ring to RP_1 to a point in the center of the lipophilic ring. The value of this angle calculated for the compounds studied is reported in Table 13. It is 47 ° in flunitrazepam, a value that should be predictive of agonist activity and 77 ° in DMCM, a value that should be predictive of inverse agonist activity. This angle is not defined in the antagonist Ro15-1788, since the electron accepting and lipophilic ring are the same ring.

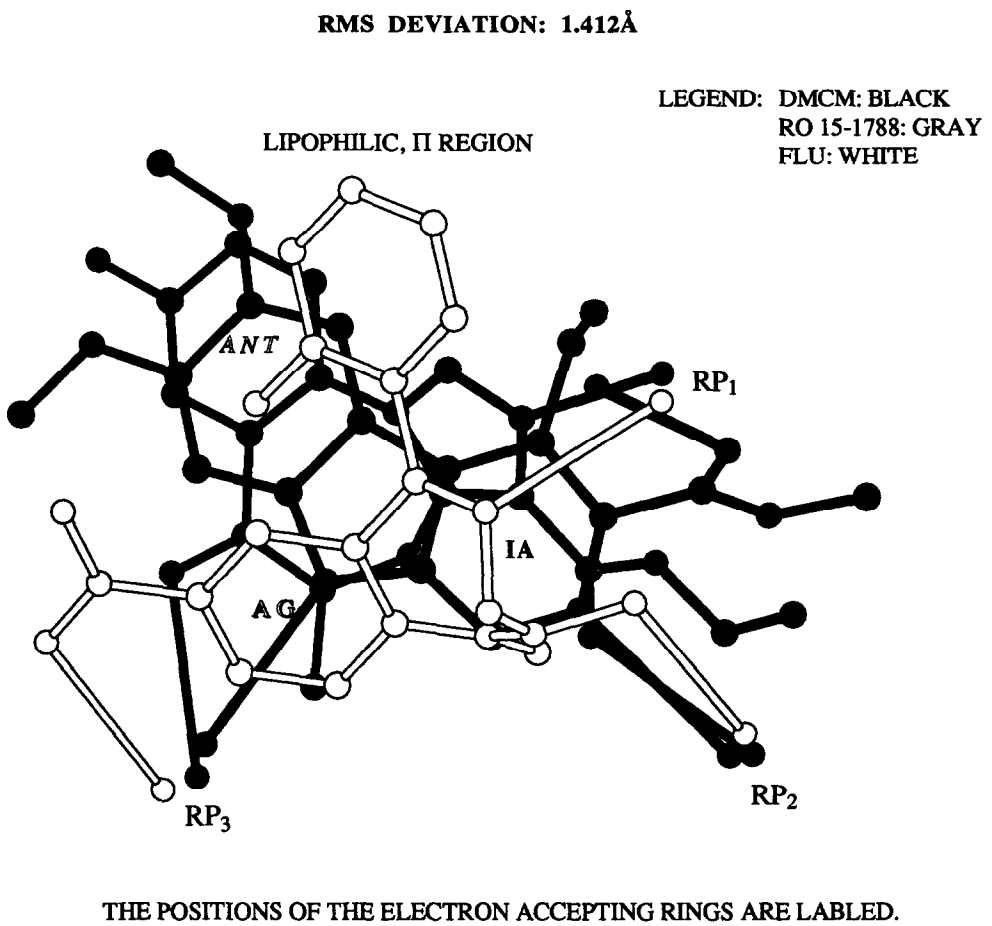


Figure 3. Superimposition of flunitrazepam (agonist), DMCM (inverse agonist), and Ro15-1788 (antagonist) with overlap of the three common proton donating recognition sites (RP_1, RP_2, and RP_3, repectively) and the aromatic, lipophilic ring. Positions of the electron accepting rings are labeled.

Also given in Table 13 is the angle between the two alternate pairs of receptor points RP_1 and RP_2 and RP_1 and RP_3 required for recognition and the electron accepting aromatic ring. These values can serve as additional discriminants of activation. Finally, the predicted type of activities of the compounds on $GABA_A$ stimulated Cl^- ion flux in cerebellar tissue is also given in Table 13. These activities are predicted from the known effect of Cl^- ion flux on flunitrazepam, DMCM, and Ro15-1788 and on the LU-RP_1-LIPO angle found for these analogs.

The predicted qualitative effect on $GABA_A$ stimulated chloride ion flux for these ligands parallels their known anticonvulsant behavior.[1b,2a] We have previously reported the convulsant, proconvulsant, and anticonvulsant properties of some of the ligands studied here.[9] We reported compounds such as flunitrazepam and CL 218,872 as agonists (ED_{50} = 0.007 nM and 12.0 nM, respectively), Ro15-1788 as an antagonist, and β-CCE and DMCM as inverse agonists. These results match our predicted Cl^- ion flux activity. Although no direct correlation between Type I receptors and the anticonvulsant endpoint has been shown to date, this relationship implies that binding to Type I receptors plays a role in producing this physiological effect.

In summary, based on the results of this study, a model for ligand binding and activation of the BDZ Type I receptor has been developed and is shown schematically in Figure 4. The model incorporates three hydrogen bonding receptor

Table 13. Angles (in °) between the various rings in the ligands and the proton accepting centers and the predicted activity based on experimental data

LIGAND	RP_1-RP_2-LU (°)	RP_1-RP_3-LU (°)	LU-RP_1-LIPO (°)	Activity[a]
Flunitrazep	67.3	48.9	47.3	AG[b]
Prazepam	52.3	22.8	47.2	AG
2-Oxoquaz	55.4	31.7	46.2	AG
Loprazolam	55.3	48.5	48.6	AG
Midazolam	56.6	49.9	51.1	AG
β-CCE	54.9	44.6	74.4	IA
β-CCP	44.7	54.7	74.5	IA
DMCM	48.4	44.2	76.9	IA[b]
Ro 15-1788	85.2	52.1	–	ANT[b]
Ro 14-7437	85.3	52.2	–	ANT
Ro 15-3505	85.2	54.5	–	ANT
Ro 15-4513	85.0	52.3	–	ANT
Alpidem	–	19.5	58.7	AG
Zolpidem	–	19.9	60.1	AG
ICI 190,622	58.2	8.9	58.6	AG
CL 218, 872	54.8	–	37.4	AG
AHR-11797	58.3	–	55.0	AG

[a]IA = Inverse Agonist; ANT = Antagonist; AG = Agonist; predicted from the Cl ion flux data.

[b]Known activity from experimental Cl^- Ion Flux.

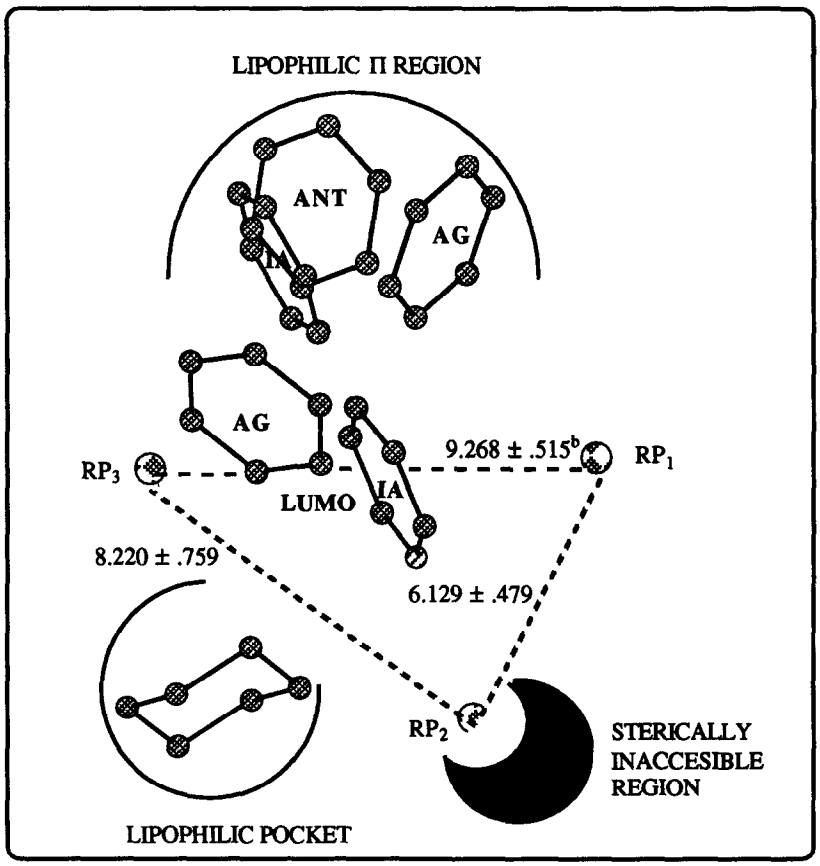


Figure 4. Proposed model for recognition and activation at the Type I BDZ receptor. Coordinates of four ligands studied (flunitrazepam, DMCM, Ro15-1788, and loprazolam) were used for construction of the model and the average distances between receptor points for all compounds with known affinity are given.

points and a lipophilic ring in a specific geometry arrangement as criteria for recognition. It also indicates that activation occurs through an electron transfer mechanism, with the position of the electron accepting aromatic ring acting as a discriminant for agonism, inverse agonism, and antagonism.

Validation of the proposed recognition pharmacophore

A large number of structure–activity relationship (SAR) studies has been reported in the literature for diverse compounds.[30] Among these, a number of specific examples of various classes of ligands were chosen to validate our current pharmacophore.

Tifluadom and Ro05-3305 were included as additional ligands which show no affinity for the BDZ receptor(s). These ligands were added to our control compounds because of their similarity in structure to other, higher affinity, ligands. Although as shown in Tables 10–11, Ro05-3305 fulfills the distance requirements for both RP_1–RP_3 and RP_1–RP_2, its lipophilic ring does not meet the angle criteria (Table 11). Tifluadom meets the distance requirements for RP_1–RP_2 (Table 10) only but also does not have the appropriate angle (Table 11). Therefore, in agreement with experiments, using our criteria, we would classify these two compounds as non-binders at the Type I BDZ receptor, although assuming greater flexibility, a low affinity might be exhibited by these compounds, especially tifluadom.

Seven high affinity ligands which are thought to be non-selective were also included for validation, assuming that measured high affinities in whole brain would parallel those in cerebellum.[2a] These ligands are: Ro15-3505, Ro15-4513, Ro14-7437, 2-oxoquazepam, loprazolam, midazolam, and ICI 190,622. As shown in Tables 10 and 11, all seven of these compounds fulfill the requirements for recognition, based on our current pharmacophore.

Another validation of the criteria for recognition is that they provide an explanation for a puzzling aspect of substituent modulation of 1,4 BDZ receptor affinities, again, assuming that measured affinities in whole brain of these non-selective compounds parallel those in cerebellum. It has generally been observed that electron-withdrawing groups (NO_2, Cl, CF_3) at either the 7 or 2' positions increases the binding affinity whereas having such substituents at both positions lead to only a small additional increase in binding.[31] This trend can be satisfactorily explained using the present recognition pharmacophore. Figure 5 shows the positions of the 2' and 7 substituent in flunitrazepam. As can be seen, either of these substituents can interact with the proton donor receptor point RP_3, assuming there is free rotation of the aromatic ring and nitro substituent. Thus, both sites are not needed for recognition at RP_3, since either substituent at the 2' or the 7 position will allow recognition.

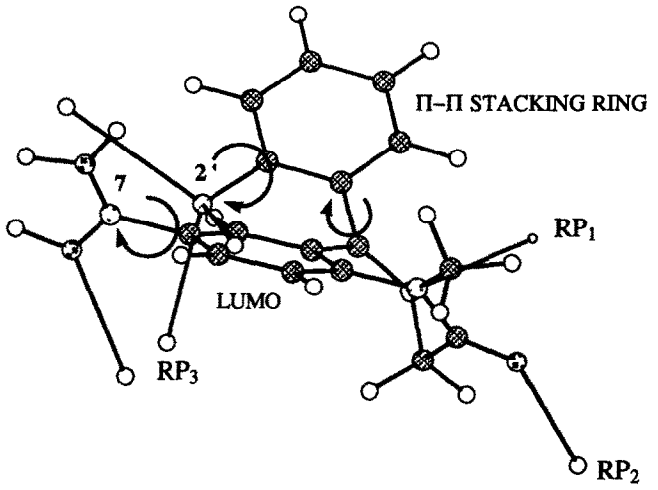


Figure 5. Illustration of the interaction of a classical 1,4 BDZ ligand, flunitrazepam, with postulated recognition sites of the receptor. This figure shows that substituents at either the 2' or 7 positions of the ligand can interact with one of the receptor sites, RP_n, and that both are not needed.

Refinement of recognition pharmacophore: addition of steric factors

Examination of the results for PK 11195 and prazepam have led to the proposal of an additional steric requirement for recognition. PK 11195 is known to bind to the peripheral BDZ binding site but shows no significant binding to the CNS site. Preliminary cerebellum data for PK 11195 ($K_i > 1000$), is consistent with the observation that this ligand has virtually no binding in whole brain. Examining the requirements for recognition (Tables 10, 11 and 12), PK 11195 is found to fulfill the requirements for receptor points 1, 2, and the lipophilic ring, similar to AHR 11797. Thus, based on these criteria alone, we would predict PK 11195 to bind with some affinity to the Type I BDZ receptor.

To probe the lack of binding of this ligand, it was superimposed on flunitrazepam in such a manner that the three common recognition sites, RP_1, RP_2, and the lipophilic rings, overlapped (Figure 6). We see from this figure that the bulky isopropyl group ($CH(CH_3)_2$) would sterically interfere with the proton donating function of receptor point two (RP_2).

Prazepam, a 'classical' 1,4 BDZ with low affinity and agonist activity, has an unusual substituent at its one position, a cyclopropyl group. Although the binding in cerebellum has not yet been reported, this compound has been shown to bind significantly weaker in whole brain that other 1,4 BDZs. As seen in Figure 7, prazepam also has a significant steric hindrance to interaction with receptor point two. Unlike PK 11195, however, the presence of RP_3 in prazepam still allow it to meet the minimum requirements for recognition and it would be predicted to have some affinity for the Type I BDZ receptor. Thus consideration of both PK 11195 and prazepam have allowed the identification of a sterically inaccessible region in the binding site around RP_2.

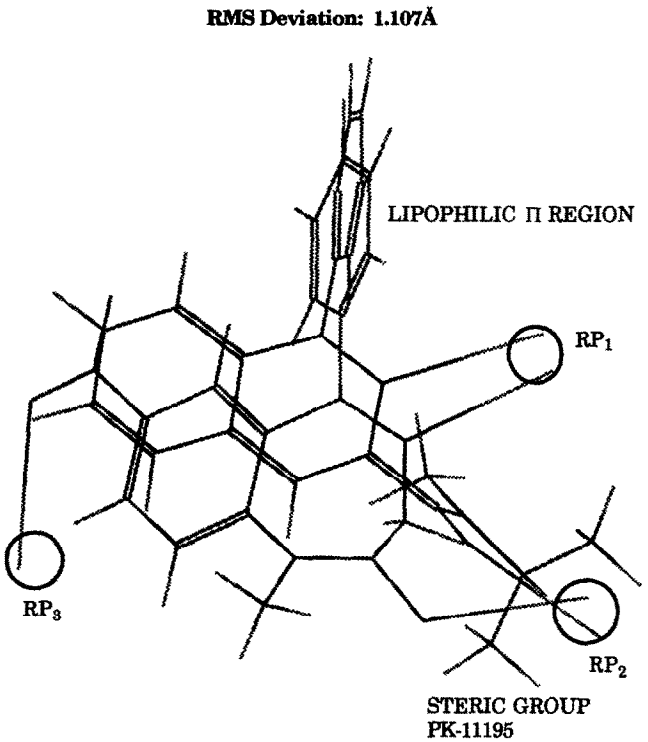


Figure 6. Comparison of PK 11195 and flunitrazepam with common recognition sites overlapped. This comparison allows the identification of the position of the alkyl group in PK 11195 as a steric hindrance to binding.

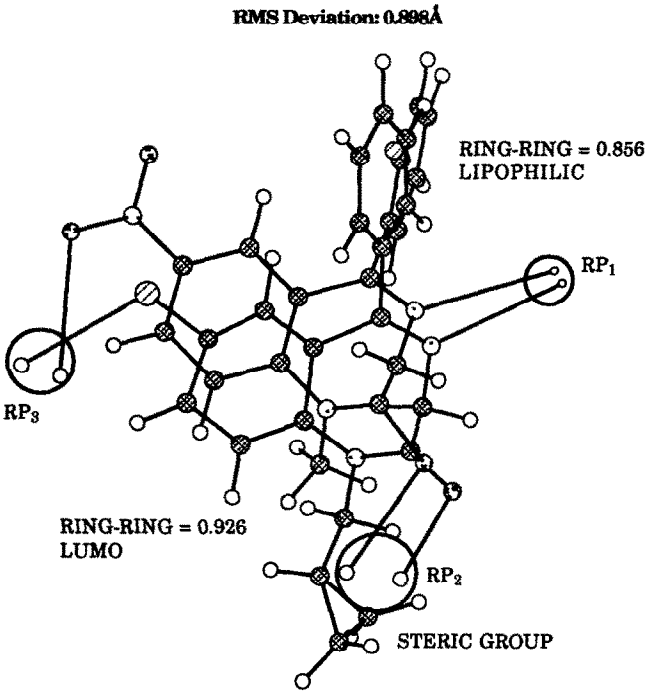


Figure 7. Overlay of flunitrazepam and prazepam with common receptor recognition sites providing additional evidence for a sterically forbidden region near RP_2.

By contrast, a sterically accessible region, a lipophilic receptor pocket between receptor points 2 and 3, has been identified. Figure 8 shows a superimposition of flunitrazepam and loprazolam so that their common

recognition sites overlap. The large, lipophilic group of loprazolam fits nicely between RP_2 and RP_3, without causing the receptor points to move from the ideal overlap with flunitrazepam. This result indicates that loprazolam should bind to the Type I BDZR with high affinity, and since it contains all three receptor points, it should show no appreciable selectivity. Although binding in cerebellum has not been reported, this prediction is consistent with the high affinity of loprazolam in whole brain.[2a] Both this sterically accessible region and the inaccessible area found with PK 11195 are shown in the pharmacophore in Figure 4.

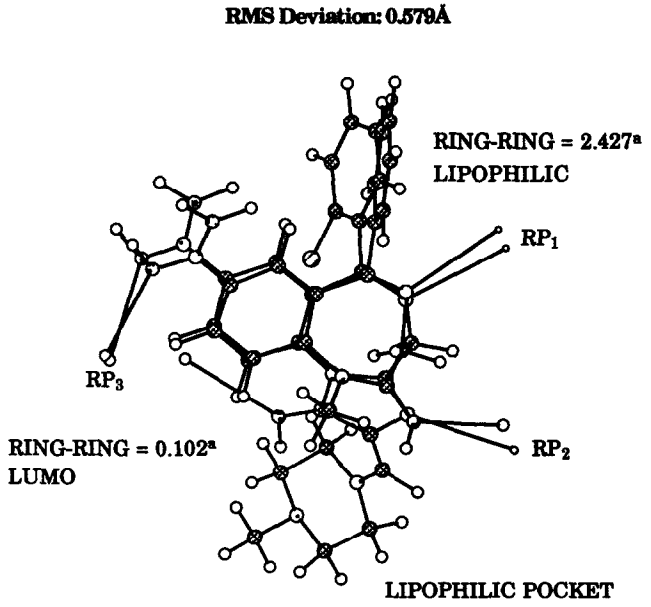


Figure 8. Superimposition of flunitrazepam and loprazolam with overlap of common receptor recognition sites illustrating the lipophilic receptor pocket available to loprazolam between RP_2 and RP_3.

Validation of activation criteria

While there are no additional compounds as yet with robust and reproducible effects on $GABA_A$ stimulated Cl^- ion flux in cerebellum the observation that this behavior parallels that of *in vivo* endpoints allows some comparison to be made between our predicted effect on Cl^- ion flux and known behavioral endpoints. The high affinity Ro15-3505 and Ro14-7437 have antagonist properties at behavioral endpoints thus far reported. Midazolam and loprazolam, two high affinity 'classical' BDZ ligands have agonist activity at most behavioral endpoints as do the more novel ligands 2-oxoquazepam and ICI 190,622. Consistent with these observations, Ro15-3505 and Ro14-7437 are predicted to be antagonists and the other four to be agonists (Table 13) based on criteria developed for Cl^- ion flux activity.

Conclusions

Comparing the Type I BDZR pharmacophores developed here with previously proposed models reported in the literature for a single generic BDZ receptor, the following similarities and differences emerge. In comparison with our previously proposed single receptor pharmacophore,[9] a third recognition component, a lipophilic ring possibly involved in π–π stacking with the receptor, is proposed in addition to two proton accepting sites. Cook and co-workers[13] have proposed a recognition pharmacophore for a single generic BDZ receptor, assuming that the inverse agonist/antagonist and the agonist binding sites are each different regions of the same domain and using a series of β-carbolines, benzodiazepines, and pyrazoloquinoline compounds. By contrast, in this study, we have demonstrated that all compounds characterized that bind to the Type I BDZ receptor possess similar recognition properties, independent of their activity. Therefore, there is no apparent reason to impose different binding sites for compounds of differing activities. The agonist recognition criteria proposed by Cook includes two proton donating receptor points H1 and H2 and an aromatic, lipophilic ring and in the inverse agonist model, he includes one accepting site A2, with the distances between these four receptor points comparable to those between RP_1, RP_2, RP_3 and ring A in our model. However, he includes a second lipophilic region in recognition criteria, that occupied by the alkyl ester group of the β-carbolines. In our pharmacophore, this region is a site of limited lipophilic tolerance, as shown by PK 11195 and prazepam while the region that can accommodate a larger lipophilic moiety lies between RP_2 and RP_3, as seen, for example, with loprazolam.

With respect to requirements for activation, Cook and co-workers proposed that agonism was the result of the combined effect of a lipophilic π-ring and the small lipophilic moiety between RP_1 and RP_3 along with three receptor points. By contrast, in the pharmacophore proposed here, these are determinants of recognition and not activation since all ligands, regardless of their activity, fulfill these requirements. Instead, activation in our model is proposed to occur through a charge transfer interaction between an electron accepting aromatic ring of the ligand and an electron donating ring such as histidine or tryptophan, in the receptor.

In summary, characterization of the conformational electronic and physical properties of 13 BDZR ligands, together with receptor binding activation data have allowed a self-consistent model for ligand recognition and activation of the BDZ cerebellum Type I receptor to be formulated (Figure 4). The model incorporates three hydrogen bonding receptor points and a lipophilic ring in a particular geometric arrangement, a sterically excluded region near RP_2 and an additional lipophilic receptor pocket, not required for recognition but which can accommodate a lipophilic region of the ligand between RP_2 and RP_3. The major determinant of activation is proposed to be the position of an electron accepting aromatic ring that could be involved in a charge transfer complex with the receptor. This moiety is located in the center of the binding pocket but in different positions for agonists and inverse agonists. This feature of the proposed pharmacophore is consistent with data reported by Pritchett[1c] which suggest that the binding cleft is composed of residues from more than one subunit rather

than being localized in a binding site composed of a single polypeptide chain. It is conceivable that the inverse agonists could activate a different subunit than the agonists, since their electron accepting rings are in different regions of the pocket. Agonist activity could then be further modulated by differences in the energy of the LUMO (Table 7). Thus, the difference in the agonist activity of compounds such as flunitrazepam and zolpidem would be a result of the decrease in energy of their LUMOs, from –1.4 to –0.3 kcal/mol, respectively. Experimental data for 11 additional compounds, not included in hypothesis development, were used to successfully evaluate the predictive ability of the current pharmacophore and to add prohibited and allowed steric regions to it. The resulting pharmacophore developed here for recognition and activation at the Type I benzodiazepine receptor explains disparate binding capability and type of activity for 24 ligands from a large variety of chemical families. All high affinity compounds studied fulfill the requirements of the pharmacophore and, by complementarity, allows a description of many features of the benzodiazepine Type I binding site.

Acknowledgments

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