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Development of a 3D Pharmacophore for Nonspecific Ligand Recognition of α1, α2, α3, α5, and α6 Containing $GABA_A$/Benzodiazepine Receptors

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Abstract—Transfected cells containing $GABA_A$/benzodiazepine receptors (BDZRs) have been utilized to systematically determine the affinity of ligands at α1, α2, α3, α5 and α6 subtypes in combination with β_2 and γ_2. All but a few of the ligands thus far studied have relatively high affinities for each of these α subtype receptors. Thus, these ligands must contain common stereochemical properties favorable for recognition by each of the subtype combinations. In the present work, such a common three-dimensional (3D) pharmacophore for recognition of α1, α2, α3, α5 and α6 containing $GABA_A$/BDZRs types of receptors has been developed and assessed, using as a database receptor affinities measured in transfected cells for 27 diverse compounds. The 3D-recognition pharmacophore developed consists of three proton accepting groups, a hydrophobic group, and the centroid of an aromatic ring found in a common geometric arrangement in the 19 nonselective ligands used. Three tests were made to assess this pharmacophore: (i) Four low affinity compounds were used as negative controls, (ii) Four high affinity compounds, excluded from the pharmacophore development, were used as compounds for pharmacophore validation, (iii) The 3D pharmacophore was used to search 3D databases. The results of each of these types of assessments provided robust validation of the 3D pharmacophore. This 3D pharmacophore can now be used to discover novel nonselective ligands that could be activation selective at different behavioral end points. Additionally, it may serve as a guide in the design of more selective ligands, by determining if candidate ligands proposed for synthesis conform to this pharmacophore and selecting those that do not for further experimental assessment.

Introduction

The wide spectrum of pharmacological effects that benzodiazepines (BDZs) can produce makes them one of the most versatile classes of drugs used in psychopharmacology. Among the various pharmacological effects are anxiolytic, anticonvulsant, muscle relaxant, and sedative/hypnotic activity. Despite their widespread clinical use, benzodiazepines are also known to produce unwanted side effects, including disruption of learning and memory (amnesia), hyperphagia, hypothermia, and discriminative stimulus actions, which may indicate abuse potential.

Benzodiazepine receptor (BDZR) ligands exert their actions by binding in the central nervous system (CNS)[1,2] to high affinity sites on the $GABA_A$/chloride (Cl-) channel complex.[3] The neurotransmitter γ-aminobutyric acid (GABA) is the principal inhibitory neurotransmitter in the CNS. Inhibition is achieved by regulating Cl- ion flux through the $GABA_A$/BDZRs ion channel. A large number of ligands from diverse chemical families have been shown to bind with high affinity to the $GABA_A$/BDZRs. BDZR ligands have been classified as agonists, antagonists, and inverse agonists, depending on whether they enhance, have no effect, or diminish the effect of GABA on the Cl- ion channel. This categorization has also been used to describe the activity of BDZR ligands in diverse pharmacological endpoints. Specifically, agonists are ligands with similar in vivo activities to the prototypical 1,4 BDZ drugs, exhibiting anxiolytic, anticonvulsant, hyperphagic, hypothermic, muscle relaxant, amnesic and sedative/hypnotic activity. Inverse agonists cause opposite behavioral effects, that is, anxiogenesis, proconvulsant, anorectic[4] and procognitive actions.[5] Antagonists block the action of both agonists and inverse agonists.

$GABA_A$ receptors are heteropentomeric proteins made up of subunits that can be grouped into distinct families, based on homology.[6] Most recently, a novel θ subunit

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with the sequence identity of 50% to the β subunit has been characterized.[7] Thus far, 21 different isoforms of the $GABA_A$ receptor subunit families have been cloned, including α_1–α_6, β_1–β_4, γ_1–γ_4, δ, ρ_1–ρ_3, π, ε, and θ.[7,8] Although, in principle, these subunits can be combined to form an infinite number of functional CNS $GABA_A$/BDZR receptors, there is currently in vivo evidence for approximately 13 functional combinations.[9] However, even this level of complexity has thus far prevented the establishment of a robust connection between transfected cell results, the composition and number of functional GABA receptors in different brain regions, and activity at different behavioral end points. Nevertheless, studies of different combinations of subunits in transfected cell systems have identified important general characteristics of functional $GABA_A$ receptors.

Several studies have demonstrated that α, β and γ subunits are needed to create high affinity BDZ-sensitive GABA receptors.[10–12] There is also growing evidence that the nature of the α subunit expressed in each specific pentameric receptor is a key determinant of the properties of the reconstituted receptors.[13–15] However, studies of structurally diverse BDZRs in transfected cells containing different α subunits in combination with β2, γ2 subunits, revealed little or no selective binding. Specifically, in recently reported systematic determination of the affinity of diverse ligands at α1, α2, α3, α5, and α6 containing subtypes of $GABA_A$/BDZRs in transfected cells, all but a few have significant affinity at all these five types of receptors. Thus they must contain common stereochemical properties favorable for recognition of all of these receptor subtypes. In the present work, such a common three-dimensional (3D) pharmacophore for recognition of these subtypes of α_n containing $GABA_A$/BDZRs has been developed and assessed using reported transfected cell binding data for 27 diverse compounds.

To accomplish this goal, our laboratory has recently developed and assessed systematic procedures embedded in software for the determination of 3D pharmacophores for structurally diverse ligands.[16] These procedures can, in principle, be used to develop both 3D recognition and 3D activation pharmacophores. This computer program requires two types of input. The first type consists of a conformational library for each of the compounds to be used in the pharmacophore development. The second type of input required user identification of all chemical moieties, such as hydrophobic groups and proton accepting and donating groups in each molecule to be considered as possible components of the 3D pharmacophores. The underlying hypothesis in the selection of these candidate moieties is that they could be involved in key interactions with complementary amino acid residues in the binding site of the receptor(s) being investigated. The program then systematically searches for a common spatial arrangement of some combinations of chemical moieties selected for each compound that recognize or activate the receptor system under investigation. If successful, the results constitute a 3D pharmacophore defined by the common chemical and geometric determinants identified. An immediate assessment of this 3D pharmacophore is then made by determining if these pharmacophoric components are absent in compounds with little or no receptor affinity or ability to activate them. No a priori assumption of a bioactive conformation of any of the ligands needs be made, no template is required and both structurally diverse and conformationally flexible ligands can be included in the database of compounds used for development of the pharmacophore of interest.

Applying this methodology, in this work a nonspecific 3D pharmacophore for recognition of α1, α2, α3, α5, and α6 containing $GABA_A$ receptors was developed for nineteen nonselective ligands. This pharmacophore was first assessed by determination that its requirements were absent in four ligands which are non-binders. An additional assessment was made by the determination that four nonselective ligands not used in the development of this pharmacophore, complied with it. A third type of assessment was made by using the 3D pharmacophore requirements to search 3D databases in order to provide additional validation. These three assessments taken together provided robust validation of the nonselective 3D pharmacophore developed. It can now be used in a number of potentially fruitful ways, for example as criteria to search 3D databases for novel nonselective $GABA_A$/BDZR ligands.

Methods

Table 1 lists the 27 compounds selected for the present work together with their reported binding affinities from transfected cell studies to the α1, α2, α3, α5 and α6 containing subunits of the $GABA_A$/BDZRs in combination with β_2, γ_2 subunits. As seen in this table, four of the compounds have $K_i > 1$ μM for all the α1, α2, α3, α5 and α6 containing $GABA_A$/BDZRs and were thus considered as negative controls. Since values of receptor binding affinities vary continuously among ligands, rather than discretely, any cut-off chosen to distinguish binders from non-binders to use as controls is necessarily arbitrary. This arbitrary value could represent the limit of detection of the assay. In addition to these four negative controls, four ligands with significant affinity at the five receptors, compounds **2**, **3a**, **6** and **7** were not included in the 3D pharmacophore development and were used as compounds for pharmacophore validation.

The generation of a conformational library for each of these 27 compounds was the first step in this 3D pharmacophore development. In this study, the specific conformational libraries considered in the search for a 3D pharmacophore were the set of energy optimized unique conformations for each compound within 3 kcal/mol of the lowest energy conformer. The selection of this cutoff of 3 kcal/mol could be considered arbitrary. However, the sensitivity of the pharmacophore detection was checked and it was verified that an identical pharmacophore can be obtained using a larger energy window of 5 kcal/mol. In addition, previous studies[16] have demonstrated that many of the ligand binding motifs in enzyme-substrate complexes of known structures are characterized by distances

Table 1. Binding affinities of BZ ligands at the α1, α2, α3, α5, and α6 subunits of $GABA_A$/benzodiazepine cells studies

	K_i (nM)					
Compound	α1	α2	α3	α5	α6	Ref
RY10	20.4	27	26.1	1.5	176	25
RY80	28.4	21.4	25.8	0.5	28.8	25
Rol51788	0.8	0.9	1.0	0.6	148	25
Ro154513	3.3	2.6	*2.5*	0.27	3.8	25
Abercanil	0.2–3.9	4.4	7.1	8.4	348[a]	26–29
DMCM	5.7	8.3	4.0	1.0	134	30
Ro151310	6.8	16.3	9.2	0.8	54.6	25
RY24	197	143	255	2.6	58.6	25
28	275	387	337	23	301	25
42	200	124	79	4	340	25
47	83	60	48	2.6	180	25
49	89	70	91	3.7	301	25
51	86	40	85	2.4	150	25
52	73	85	97	4.8	333	25
55	220	150	184	12.7	361	25
Ro166028 (Bretazenil)	0.3	0.6	0.2	0.5	12.7	30
RY23	26.9	26.3	18.7	0.4	5.1	25
L655,708	48	27	24	0.4	83	31
FG8205	0.7–2	3.7	6	1.5–6.4	227	26, 28, 32
2	11.4	10.7	9.2	0.47	9.4	30
3a	121.1	141.9	198.4	5.0	113.7	30
6	14.8	56	25.3	1.7	22.9	30
7	3.75	7.2	4.14	1.1	44.3	30
30	17,535	33,834	22,125	2612	29,500	25
36	9483	30,000	15,409	2583	30,160	25
37	4201	12,590	6266	1346	8600	25
43	> 1000	> 1000	> 1000	> 1000	> 1000	25

[a]This value corresponds to the abercanil binding affinity at the α6 subtype of $GABA_A$/benzodiazepine receptors in cerebellum.[29] However, although the corresponding K_i value from transfected cells binding studies is not provided, there is evidence from the literature[28] of the abercanil affinity at the α6 subtypes.

between the recognition moieties similar to those belonging to low-energy conformers.

Initial ligand structures of the 27 compounds listed in Table 1 and shown in Figure 1 were constructed using the MSI Quanta package (MSI-Quanta Biosym/MSI, San Diego, CA). The structures were then minimized with the Quanta/CHARMm force field, using 200 steps of steepest descent followed by 2000–3000 steps of conjugate gradient method until the rmsd changes in the gradient were less than 0.01 Å. A dielectric constant of 80 and no-cutoff were used in these calculations.

In previous studies (unpublished results), we have determined that for molecules with 3–4 rotatable bonds, a nested rotation method is sufficient to sample conformational space and generate a library of diverse lowenergy conformers. For more flexible molecules, a hybrid genetic algorithm (GA)/minimization procedure (CCEMD, Sandia, CA) using the same Quanta/CHARMm force field is more effective. Examining the structures shown in Figure 1, the nested rotation method could be applied to all compounds selected for the present study, except abercanil, for which the GA method was used.

Specifically, the nested rotation procedure consisted of employing increments of 30° for each significant rotatable bond and energy minimizing the resulting conformations.

In the case of abercanil, the procedure required three separate steps for each GA run. First, an initial population of low energy conformers was generated using a genetic algorithm step. Subsequently, this initial population was clustered into families of unique conformers, using a 5° rms torsion criterion. Finally, the resulting unique conformers were energy minimized. This three-step procedure was repeated six times until no significant additional low energy conformers were found.

In both computational procedures (nested rotation and GA) used, energy minimization of each conformer was carried out using the same minimization procedure described above.

In addition to these conformational libraries, the procedure for 3D pharmacophore generation used in this work, requires as input user selected candidate chemical moieties common to each ligand with significant affinity for all the α1, α2, α3, α5, and α6 containing $GABA_A$/BDZRs. Specifically, the selection of candidate pharmacophoric points was suggested by identification of the maximal number of chemical moieties common to the most similar ligands within the set of compounds used for the pharmacophore development. Subsequently, corresponding moieties were identified in the remaining more heterogeneous ligands. Shown by different colors in Figure 1 are the maximal number of candidate chemical moieties common to each of the 19 ligands with significant affinity for all the α1, α2, α3, α5, and α6 subtypes of $GABA_A$/benzodiazepine receptor used for pharmacophore development. Specifically, A, B, and C are proton acceptor atoms, D is a hydrophobic group, and E is the centroid of an aromatic ring.

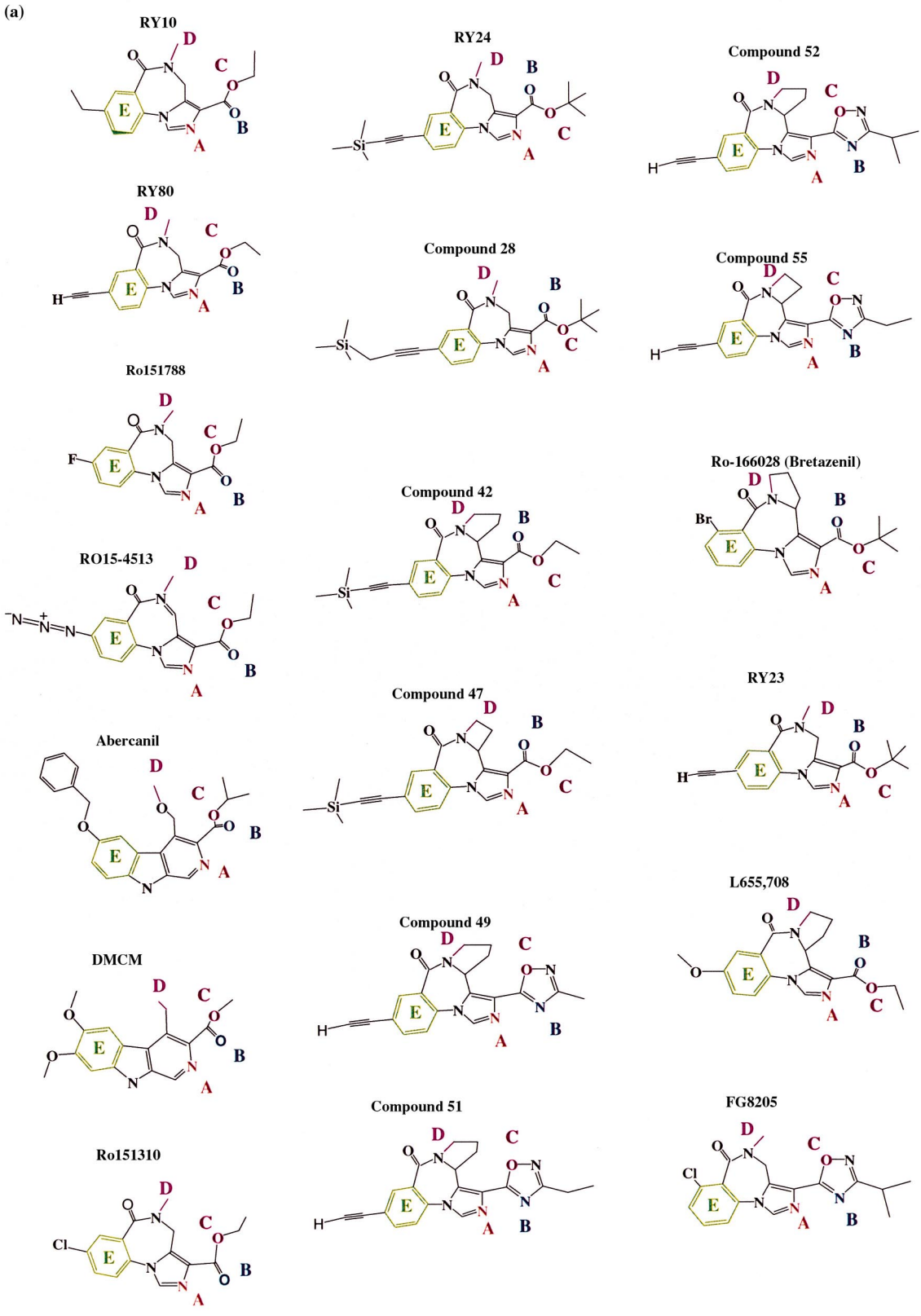


Figure 1a. Chemical structures of the 27 compounds included in the present work: (a) The 19 nonselective ligands chosen for 3D pharmacophore development.

The conformational libraries and candidate chemical moieties selected for each compound were then used as input to a 3D pharmacophore generating program. This in-house computer program searches for a common 3D arrangement of the candidate chemical moieties by performing systematic pairwise comparisons using every low energy conformer of every analogue. The current version of this program, called MOLMOD, builds on concepts embodied in the original program DISTCOMP[17] for 3D pharmacophore generation developed in our laboratory. However, MOLMOD clusters the conformers into families based on a distance criterion such that each

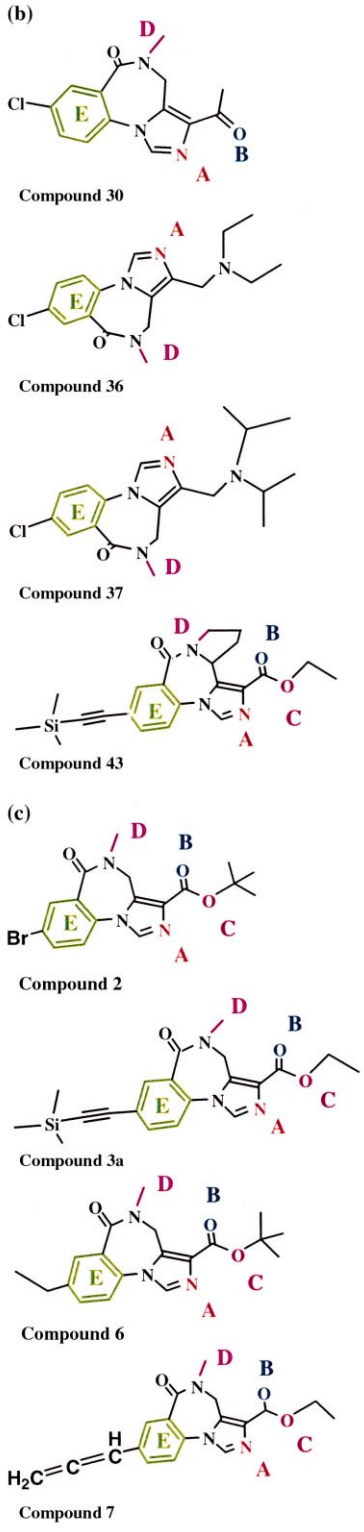


Figure 1b,c. Chemical structures of the 27 compounds included in the present work: (b) The four compounds chosen as negative controls. (c) The four compounds chosen as compounds for pharmacophore validation. Also shown in color in these figures are the five candidate chemical moieties (A, B, C, D, and E) selected as possible components of the 3D recognition pharmacophore. A, B, and C are proton acceptor atoms, D is a hydrophobic group, and E is the centroid of an aromatic ring.

conformation appears only in one family; allows the user to employ independent distance criteria for the conformer clustering; tests for extent of compliance with pharmacophoric distance as part of the algorithm; and allows for development of pharmacophores that include points which are receptor based. Finally, a measure of the uncertainties in the geometric descriptors of the 3D pharmacophore identified using this program is calculated as sum of the variances of each inter-pharmacophore distance.

In the current study, use of this procedure resulted in a 3D pharmacophore for nonspecific ligand recognition of α1, α2, α3, α5, and α6 containing subunits of $GABA_A$/BDZRs.

This 3D pharmacophore was then initially assessed by use of both the positive and negative controls. It was then further assessed by using it as input to the Tripos Inc. SYBYL/UNITY package (SYBYL/UNITY, Tripos Associates, Inc., St. Louis, MO, 1999) to search 3D databases. The 3D databases searched were the Cambridge Structural Database, the Chapmann and Hall Chemical Database, and the NCI and Maybridge Databases.

Results and Discussion

The chemical structure of each compound is given in Figure 1. Specifically, Figure 1a shows the 19 ligands used to develop the 3D pharmacophore, Figure 1b show the four low affinity compounds used as negative controls and Figure 1c shows the four compounds excluded from the pharmacophore used as compounds for pharmacophore validation. As can be seen in these figures, 25 compounds belong to the imidazobenzodiazepine family while two ligands (DMCM and abercanil) are members of the β-carboline family. These appear to be the only compounds for which affinities at α1, α2, α3, α5, and α6 containing GABAA/BDZRs from transfected cell studies have been reported. Also shown in Figure 1 by different colors are the maximal number of candidate chemical moieties common to each of the 19 ligands with significant affinity for all the α1, α2, α3, α5, and α6 subtypes of $GABA_A$/benzodiazepine receptor used for pharmacophore development. The identification of these candidate chemical moieties is an important input information required by the pharmacophore generating program. In this figure, A, B, and C are proton acceptor atoms, D is a hydrophobic group, and E is the centroid of an aromatic ring.

Table 2 gives the total number of unique conformers and the number of low energy conformers within 3.0 kcal/mol of the lowest energy conformation found for all of the 27 compounds included in the present study. Specifically, the subset of conformers within 3.0 kcal/mol was used as input for the generation of the 3D pharmacophore.

Shown in Figure 2, is the five component 3D pharmacophore obtained for nonspecific ligand recognition of

Table 2. Total number of conformers and number of the low energy conformers within a 3.0 kcal/mol threshold in respect to each lowest energy conformation of the 27 compounds studied

Compound	Total number of conformers	Number of conformers within 3.0 kcal/mol threshold
RY10	44	31
RY80	144	12
Ro151788	864	50
Ro154513	71	11
Abercanil	1331[a]	1128
DMCM	144	20
Ro151310	144	12
RY24	144	13
28	432	12
42	432	7
47	432	12
49	12	6
51	144	24
52	144	24
55	144	114
Ro166028 (Bretazenil)	144	11
RY23	144	12
L655,708	144	7
FG8205	144	12
2	432	51
3a	864	637
6	1728	79
7	1728	47
30	12	2
36	144	45
37	144	41
43	432	34

[a]Total number of unique conformers.

the α1, α2, α3, α5, and α6 containing of $GABA_A$/BDZRs. In this figure are given the pairwise distances between the five components of the pharmacophore common to the 19 ligands that bind with significant affinity to the five different α*n* containing of $GABA_A$/BDZR, used as the database to generate this pharmacophore. These distances describe the common geometric arrangement of the five pharmacophore components. In addition, the standard deviation of the mean values of these distances are also reported as a measure of the uncertainties in the geometric descriptors of the 3D pharmacophore. Also shown in this figure are all the 19 ligands used superimposed with spatial overlap of the five common recognition moieties, A, B, C, D, and E. For this superposition the lowest energy conformer of each ligand that conformed to the 3D pharmacophore was used. As can be seen from this figure, a good superposition of the five recognition moieties A, B, C, D, and E is obtained for all these compounds with significant affinity for all α1, α2, α3, α5, and α6 containing $GABA_A$/BDZRs.

Four compounds shown in Figure 1b with little or no affinity ($K_i > 1$ μM) at all α1, α2, α3, α5, and α6 containing $GABA_A$/ BDZRs (Table 1) were used as negative controls. As seen in this figure, three of these, compound **30**, **36** and **37** do not fit the pharmacophore because of the lack of at least one proton acceptor atom moiety (B or C for compound **30** and both of them for compounds **36** and **37**). The absence of one or two possible polar interactions with the receptor could explain the lack of affinity of these compounds.

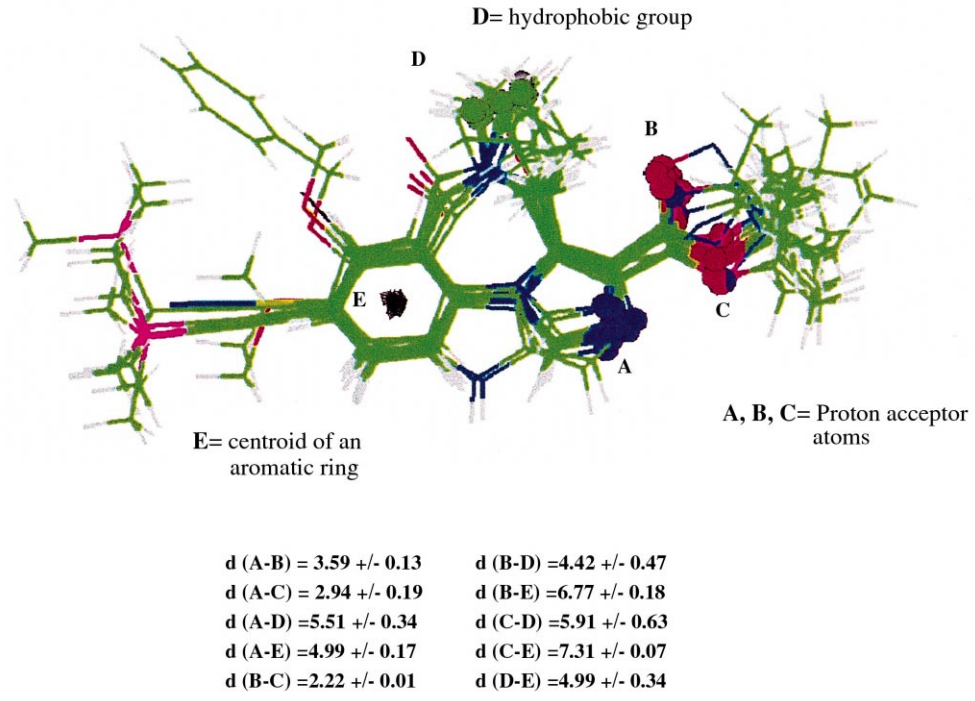


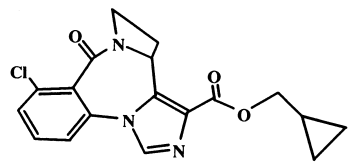
Figure 2. The 5-component 3D pharmacophore developed for nonselective recognition of the α1, α2, α3, α5, and α6 containing $GABA_A$/benzodiazepine receptors. This pharmacophore consists of the pairwise distances given in this figure between the five common chemical moieties (A, B, C, D, and E) where A, B, and C are proton acceptor atoms, D is a hydrophobic group, and E is the centroid of an aromatic ring. Also shown in this figure is the spatial overlap of these five common recognition moieties in the 19 nonselective ligands used for the pharmacophore development. In this superposition, the lowest energy conformer of each ligand that complied with the pharmacophore was used.

In contrast, compound **43**, does satisfy the requirements of the 3D pharmacophore. However, this compound is the least robust of the four negative controls selected. The other three compounds that clearly do not satisfy the pharmacophore have no significant affinity even at concentrations 4 to 17 times greater than 1 μM. By contrast, the K_is of compound **43** for binding to all the α1, α2, α3, α5, and α6 are reported only as >1 μM (Table 1). These values are right at the border of the cutoff used to identify controls (i.e., a K_i value of >1 μM for all α1, α2, α3, α5, and α6 containing receptors). Because compound **43** is a borderline control, its compliance with the 3D recognition pharmacophore does not necessarily detract from its validity.

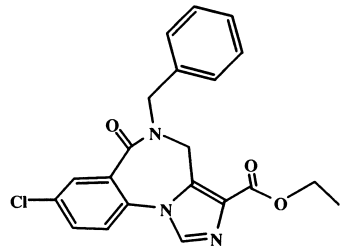
Compounds **42** and **43** are optical isomers, differing only in the configuration of the five member ring containing the proposed recognition moiety D. Specifically, in compound **42** this five member ring contains a chiral carbon atom in an *S* configuration while in compound **43** it has an *R* configuration. In a continued search for more subtle modulators of the differences in affinity between these two compounds, the solvent accessible surface of both compound **42** and compound **43** were obtained. These solvent exposed surfaces can be used as an indicator of ligand accessibility for receptor interactions in the binding pocket. Comparison between them indicates that, in the *R* configuration of the five member ring of compound **43**, the acceptor atom B is less solvent exposed than in compound **42** and hence would be less favorably disposed for H-bonding interactions with a proton donating receptor group than in compound **42**. The weakening of the one hydrogen bond could explain the lower receptor affinities of compound **43** compared to compound **42**. Although these differences are subtle, it should be remembered that every order of magnitude difference in measured K_i corresponds to only 1.4 kcal/mole in free energy of binding.

Continued assessment of the 3D pharmacophore developed was made by use of the four compounds for pharmacophore validation shown in Figure 1c, the high affinity compounds **2**, **3a**, **6** and **7** excluded from the pharmacophore development. All four compounds were found to satisfy this pharmacophore. The five components that satisfied these requirements for each compounds are shown in Figure 1c. They are labeled A, B, C, D, and E consistent with the definitions used in Figure 1a.

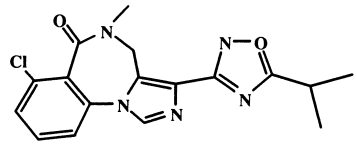
Another type of assessment of the 3D pharmacophore made was to use it to search 3D databases. In this search, the same criterion for compliance, 1.25 Å, of distance similarities between the selected recognition moieties was used as in the development of the pharmacophore. Using this criterion, a rigid 3D search was made of the Cambridge Structural Database. Among the compounds found were two imidazobenzodiazepines Ro166028[18] and Ro151788[19] and the β-carboline DMCM[20] known non selective ligands included in this study. In addition, three known BDZR ligands shown in Figure 3, were identified for which binding profiles are incomplete but consistent with non selective affinities. As shown in this figure, for two of these, Ro171812[18] and compound



Ro171812
DS (nM) = 0.1
DI (nM) =55



Compound 11i
DS (nM) = 13.7
DI (nM) =10.9



Compound 10h
IC_{50} (nM) = 3.6

Figure 3. Structures and binding data for the known $GABA_A$/BDZR ligands found in 3D database searching using the criteria of the 3D pharmacophore developed here.

11i,[21] affinities were reported at two types of receptors called diazepam insensitive (DI) and diazepam sensitive (DS). Subsequent studies now link DI sites with α6 and perhaps α4 containing receptors and DS with the other α containing receptors. Therefore the non-selectivity reported for these two compounds is indicative of nonselective high affinity binding to all the α containing types of $GABA_A$/BDZRs. For compound **10h**,[22] only one site with an apparent IC_{50} of 3.6 nM in rat brain homogenate was reported using [^{3}H] Ro151788. Again, while incomplete, this observation is consistent with the identification of this compound as a nonselective ligand. Finally, although present in the 3D database searched, classical 1,4-benzodiazepine agonists, such as flunitrazepam and diazepam, were not identified, consistent with their known lack of binding at α6 containing $GABA_A$/BDZ receptors. These results taken together provide additional validation for the reliability of this 3D pharmacophore in describing the stereochemical properties of BDZR ligands that are favorable for recognition of the α1, α2, α3, α5, and α6 containing $GABA_A$/BDZRs.

The validated 3D pharmacophore has also been used as criterion to search 3D databases for novel nonselective ligands. It is possible that ligands that bind with high affinity to all the different α*n* containing receptors can

exhibit activation selectivity (i.e., be agonists at some, inverse agonists at other and antagonists at still other $GABA_A$/BDZRs). Although none of the nonselective ligands studied here have been assessed for such activation selectivity in transfected cell studies, our laboratory has obtained indirect evidence for activation selectivity from in vivo studies of diverse BDZR ligands at different behavioral end points.[23,24] For example, Bretazenil, included in this study, has been found to be an agonist at the anxiolytic endpoint and an antagonist at the sedation endpoint. These results provide some support for activation selectivity and hence potential therapeutic usefulness of novel nonselective ligands. In particular, experimental studies of the new molecules identified from the 3D searches performed in the present study are currently under investigation in our laboratory.

Conclusions

A self-consistent database of transfected cell binding data for 27 different compounds has been used to develop a nonspecific 3D pharmacophore for recognition of α1, α2, α3, α5, and α6 containing $GABA_A$/benzodiazepine receptors. Among them: (i) the 19 ligands shown in Figure 1a, with $K_i < 1\,\mu M$ at these five $GABA_A$/benzodiazepine receptors were used for 3D pharmacophore development, (ii) the four compounds shown in Figure 1b with $K_i > 1\,\mu M$ at these five $GABA_A$/benzodiazepine receptors were used as negative controls, and (iii) the four high affinity non selective compounds shown in Figure 1c were excluded from pharmacophore development and were used as compounds for pharmacophore validation.

Conformational libraries were obtained for all compounds and a set of candidate chemical moieties that are common to each of the 19 compounds in Figure 1a with significant affinity at the five receptors was selected. This information was used as input to an in-house computer program that systematically identifies 3D recognition pharmacophores.

The nonspecific 3D pharmacophore for recognition of α1, α2, α3, α5 and α6 containing $GABA_A$ receptors found by this procedure consisted of three proton-acceptor sites, a hydrophobic moiety and the centroid of an aromatic ring found in a specific geometric arrangement in the 19 nonselective ligands. These stereochemical properties were absent in the three robust non-binders used as negative controls and present in the four high affinity nonselective ligands used as compounds for pharmacophore validation. This 3D pharmacophore was then further assessed by using it to search 3D databases. Both positive and negative results of this search provided validation of the pharmacophore. Specifically: (i) compounds known to be nonselective BDZR ligands were identified, (ii) known BDZR ligands shown in Figure 3, were identified for which binding profiles were incomplete but consistent with non selective affinities, and (iii) Although present in the 3D database searched, classical 1,4 benzodiazepine agonists, such as flunitrazepam and diazepam, were not identified, consistent with their known lack of binding at α6 containing $GABA_A$/BDZ receptors.

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