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Development and Assessment of a 3D Pharmacophore for Ligand Recognition of BDZR/$GABA_A$ Receptors Initiating the Anxiolytic Response

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Abstract—Benzodiazepine receptor (BDZR) ligands are structurally diverse compounds that bind to specific binding sites on $GABA_A$ receptors and allosterically modulate the effect of GABA on chloride flux. The binding of BDZR ligands to this receptor system results in activity at multiple behavioral end points including anxiolytic, sedative, hyperphagic, anticonvulsant and hyperthermic effects. In the work presented here, 17 structurally diverse BDZR ligands of the receptors initiating the anxiolytic response have been studied using a systematic computational procedure developed in our laboratory. Using this procedure, a five component 3D recognition pharmacophore was obtained consisting of two proton acceptors, a hydrophobic group, an aromatic electron accepting ring and a ring containing polar moieties, all found in a common geometric arrangement in the 15 compounds with an effect at the anxiolytic end point and absent in two control compounds. The 3D pharmacophore developed was validated by searching 3D databases and finding known BDZR ligands active at the anxiolytic end point, including 1,4-BDZ derivatives, imidazo BDZ and β-carboline ligands.

Introduction

Benzodiazepines ligands are structurally diverse compounds that bind to the $GABA_A$ benzodiazepine receptor, a pentameric chloride ion channel. These benzodiazepine receptor (BDZR) ligands bind to a distinct site from gamma-aminobutyric acid (GABA) and allosterically modulate the effect of the endogenous GABA neurotransmitter on chloride flux through the $GABA_A$ benzodiazepine ion channel. BDZR ligands can either enhance, diminish or have no effect on GABA activity and can thus be categorized as agonists, inverse agonists and antagonists respectively. The binding of BDZ ligands to this receptor system also results in activity at multiple behavioral end points including anxiolytic, sedative, hyperphagic, anticonvulsant and hyperthermic effects.

Cloning and sequencing has revealed that the pentameric $GABA_A$ ion channel consists of various combinations of distinct subunit families (α, β, γ, δ, ρ, π and ε) with less than 30% sequence identity between them. Most recently, a novel θ subunit has been determined with high sequence (50%) identity to the β subunits.[1]

The α, β, γ and ρ families themselves exist in various isoforms: α (1–6), β (1–4), γ (1–4), and ρ (1–3).[2–4] While the percent sequence identity between subunit families is typically low (30%), the percent sequence identity between particular members of subunit families is high, ca. 70%. Although, in principle, these subunits can combine to form an essentially infinite number of functional CNS $GABA_A$ receptors, there are believed to be approximately 13 functional combinations in vivo.[5] These complications have thus far prevented the establishment of a robust connection between results obtained in transfected cells and the composition and number of functional GABA receptors in different brain regions. Nevertheless, studies of different combinations of subunits in transfected cell systems have identified important general characteristics of functional $GABA_A$ receptors.

It is now known that a minimum of α, β, and γ subunits must be co-expressed in transfected cell systems to mimic the pharmacological properties of native $GABA_A$ receptors.[6] In particular, recombinant receptors constructed from α (1–6), β2, γ2 subunit combinations seem to most closely match the pharmacological profile of mammalian brain cells. Moreover, given the common occurrence of the γ2 component in many isoforms, α (1–6) subunit variation is emerging as the major discriminant

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of the properties of the reconstituted receptors. Recently, studies of structurally diverse BDZR in transfected cells containing different α subunits in combination with β2, γ3 subunits have indicated some selectivity in ligand binding between receptor subtypes.[7,8] Much work remains, however, in the path to development of ligands which are either highly selective or specific for each of the receptor subtypes.

In the absence of selective ligands for each of the receptor subtypes, it has been difficult to robustly establish the connection between ligand recognition of specific $GABA_A$ /BDZR types and its behavioral profile. A recent study incorporating a mutant α1 subunit, in mice, has however provided direct evidence for a connection between $GABA_A$ receptors containing α1 subunits and the sedation end point.[9] While there are clues to the possible association between the different α subunits of the $GABA_A$ receptors and other behavioral end points most results give an incomplete picture, indicating that a given behavioral activity could be associated with several receptor subtypes.

Recent studies in our laboratory have used an alternative approach, to provide evidence for BDZR/$GABA_A$ receptor heterogeneity. This approach was based on the assessment of the effect of 21 structurally diverse BDZR ligands at five behavioral end points: anxiolysis, sedation, hyperphagia, hyporthermia, and anticonvulsant activity. The results clearly indicated that, among the 21 compounds evaluated, many exhibited behavioral heterogeneity. That is, in many instances, the same compound had qualitatively different effects, i.e. agonist, inverse agonist, or antagonist[10,11] or no effect[12] at different end points. Moreover, the pattern of heterogeneity was not the same for all compounds. For example, the subset of compounds that were agonists at the anxiolytic end point or had no effect at that end point was different from those that were agonists or had no effect at the sedation end point.

These results taken together have two major implications. The first is direct evidence for BDZR/$GABA_A$ receptor heterogeneity in the brain. The second is that not all types of functional BDZR/$GABA_A$ receptors are linked to each behavioral response. Rather a different, but perhaps overlapping, set of functional BDZR/GABA receptor types is responsible for initiating each behavioral effect.

Evidence for the dependence of activity on the composition of the BDZR/GABA receptor types comes from a recent electrophysiological study of the BDZR/GABA ligand, RO15-4513. In that study, it was observed that mutations of a single residue H101 in the rat α1 subunit resulted in a change of RO15-4513, from a partial inverse agonist in WT, to a partial agonist in H101F and H101Y, agonist in H101K and H101E, or antagonist in H101Q, all by different single substitution at one position.[13] This study provides support for the possibility that even minor changes in receptor composition between receptor subtypes can give rise to the pharmacological behavioral heterogeneity described above in our studies.

It is not possible at present to identify the BDZR $GABA_A$ receptor(s) initiating each of the in vivo behavioral responses. However, the heterogeneous behavioral profiles we have observed with structurally diverse BDZR ligands can be used to provide evidence of ligand binding to receptor subtypes which are associated with a given behavioral end point, even in the absence of knowledge of which specific receptor subtype(s) are directly involved. The behavioral data can be used for this purpose because each ligand that has any activity at a given in vivo end point (agonist, inverse agonist, or antagonist) must bind to one or more receptor subtypes associated with that particular BZD behavioral end point. The behavioral heterogeneity of the BDZR ligands is currently being used in our laboratory to develop 3D pharmacophores for recognition of receptors that initiate each of the five behavioral end points.

Given the high sequence identity (ca. 70%) between α subunits it is not surprising that a set of ligands eliciting a given behavioral response may be due to binding at several receptor subtypes differing in their α components. The high sequence identity between receptor subtypes associated with a given behavioral end point makes it plausible that the determinants of binding to those receptor subtypes share some similarities. There is also evidence that agonists, inverse agonists and antagonists share some common molecular determinants of receptor recognition. Direct evidence from competitive binding studies indicates that, with the possible exception of low affinity sites, the binding of agonists, inverse agonists and antagonists is mutually exclusive.[13] This observation suggests that the binding pocket of agonists, inverse agonists and antagonists is overlapping. The determination of recognition pharmacophores from such a data set would then result in identification of the molecular determinants shared by these three categories of ligands. In particular, such a pharmacophore contains the minimal recognition elements for the overlapping binding site of agonists, inverse agonists and antagonists.

The work reported here is the development and validation of a 3D pharmacophore for recognition of receptor(s) initiating activity at the anxiolytic end point using a program MOLMOD, which refines on principles in DistComp developed in our laboratory[14] and is similar to the commercially available package DISCO.[15] All of these programs systematically identify potential 3D receptor recognition or activation pharmacophores by employing principles of conformational clustering and distance comparison. The pharmacophores and consequent superpositions of training set ligands deduced by such procedures may then be subsequently used for 3D database searches or QSAR studies. MOLMOD has recently been used to determine both a nonspecific pharmacophore for recognition of $GABA_A$ receptors containing α1, α2, α3 and α5–α6 subunits[16] as well as a pharmacophore for recognition of $GABA_A$ receptor subtypes eliciting hyperphagia effects.[17] This program requires a database of known compounds with the desired recognition or activation end point and as controls some in which this end point is absent.

For the study of the anxiolytic end point, 17 of these compounds from diverse chemical families for which anxiolytic activity has been previously measured in our laboratory[10,11] have been selected. Their structures, together with the type of activity found at this end point, and the minimum effective dose (μmol/kg) at which this activity was observed, are given in Figure 1. As shown in Figure 2, these compounds belong to 8 different chemical families. Since among these compounds are those that had no effect at the other behavioral end points measured previously in our laboratory, the 3D pharmacophore reported here will be different from those to be developed at the other end points. Thus, they can each be used to discover new compounds that could be selective BDZR/$GABA_A$ ligands with enhanced behavioral selectivity.

Methods

Construction of BDZR ligands

Initial structures of the 17 BDZR ligands included in this study were constructed in MSI Quanta.[18] All forcefield parameters used were taken from the Quanta/CHARMM forcefield. The net atomic charges used were based on parameterization in this program, which is based on libraries containing charges based on atom types deduced from ab initio studies of small compounds. The initial structures were then energy minimized using 200 steps of steepest descents followed by 2000–3000 steps of conjugate gradients or until the changes in the gradient were less than 0.01 Å.

Calculation of conformational libraries of the BDZR ligands

The method used for pharmacophore development requires the generation of a conformational library for each compound. These conformational libraries were generated with an assumed dielectric constant of 80 and a long potential function truncation distance at 90 Å in order to minimize truncation effects. The value of $\varepsilon = 80$ chosen is based on an assumption that the accessible BDZR ligand conformational pool is developed in a polar environment prior to binding to the GABA receptor or that the GABA binding sites are themselves partially exposed to a polar environment.

The method chosen for calculation of the conformational libraries depended on the number of rotatable bonds in the molecule. In all cases the same Quanta/CHARMM forcefield was used for the ligands. For BDZR ligands with less than five rotatable bonds, a simple nested rotation (grid search) procedure was used, as embodied in Quanta/CHARMM, employing incremental rotations about each rotatable bond and energy minimization with the torsion angles active in the grid search constrained to their grid values. For one compound in this study, abercanil, a hybrid genetic algorithm/energy minimization method was used, as described below.

The philosophy adopted in preparation of conformational libraries by grid search procedures was based upon a few simple principals: (1) it is desirable to use fine grids for torsion angles affecting the position and orientation of hydrogen bonding moieties in the ligands, (2) the torsion angles affecting the position of large hydrophobic groups may be sampled more coarsely, commensurate with the overall goal of (3) keeping the total number of conformers in each ligand conformational library to less than several thousand. Given that hydrogen-bonding atoms in a ligand will have very specific, distance constrained interactions with a complementary receptor donor or acceptor, a 30-degree increment was chosen in grid searches for torsion angles affecting such moieties. For cases where less than two torsion angles were active in the search, 30-degree increments were used irrespective of the moieties affected by the torsion angle variations. For cases with 3–4 torsion angles, more gross increments of 60 degrees were used for torsion angles affecting the orientation of hydrophobic groups. Because the method used for pharmacophore generation requires a conformational library for each ligand in the training set, even in cases of rigid compounds, such as AHR11797, a single rotational degree of freedom was chosen for exploration.

In the case of abercanil with a greater number of rotatable bonds possibly important in the pharmacophore development, a hybrid genetic algorithm (GA)/minimization method in CCEMD[19] developed by Judson and co-workers[20–23] was employed. Several studies in the literature have pointed out the efficiency of GA exploration[20,22,24–27] and its utility in the exploration of the low energy regions of conformational space of flexible ligands involving many torsional degrees of freedom. The GA/minimization method used employs the usual principles of survival of the fittest, crossover, mutation and niche variation in order to develop a representative low energy pool of energy optimized conformers. In particular, there are three separate steps in each GA run: (1) an initial genetic algorithm step wherein an initial population of low energy (fit) conformers is determined, (2) a subsequent step in which this initial population is clustered into families of unique conformers and (3) energy minimization of the unique conformers. In the case of abercanil, an initial population of low energy conformers was generated using a genetic algorithm search step. Next, this initial population of low energy conformers was screened to find those low energy conformers differing by five degrees rms in their torsion angles. This latter set of conformations was then subjected to energy minimization as described below. This procedure was repeated for six runs until no significant additional low energy conformers were detected.

Energy minimizations for both the GA and nested rotation conformational searches were carried out using a combination of steepest descent and conjugate gradient methods until a gradient of less than 0.01 kcal/Å was obtained. For the grid search optimizations were performed with harmonically constraining active torsion angles. For the GA procedure, used for abercanil, unconstrained optimizations were performed. The library of conformers obtained for each compound was the

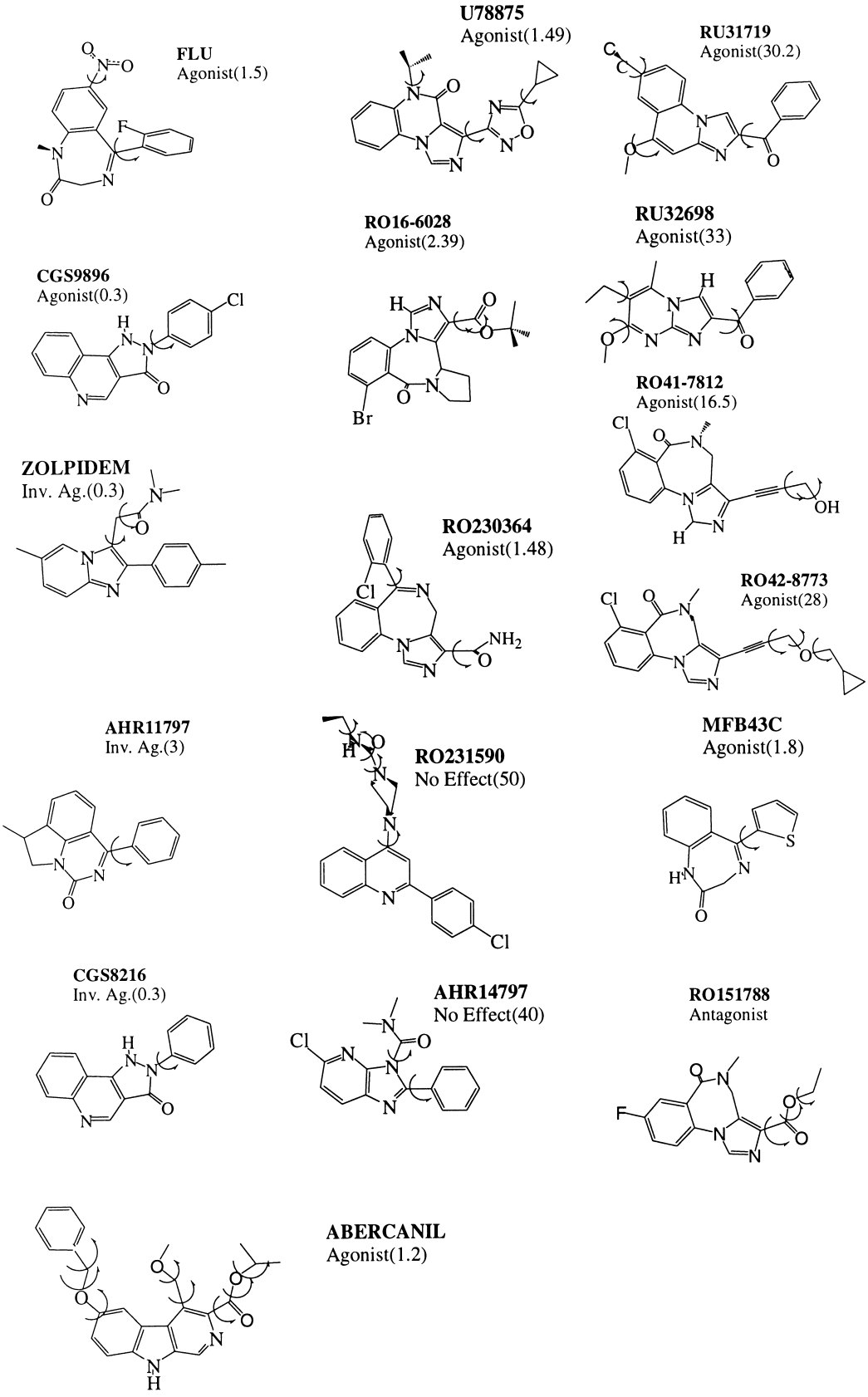

Figure 1. The structure of the 17 ligands included in this study. Also indicated is whether they were found to be an agonist, inverse agonist, antagonist or have no effect at the anxiolytic end point, together with the minimum effective dose in μmoles/kg in parentheses. The torsion angles explored in the conformational libraries for each ligand are indicated by arrows.

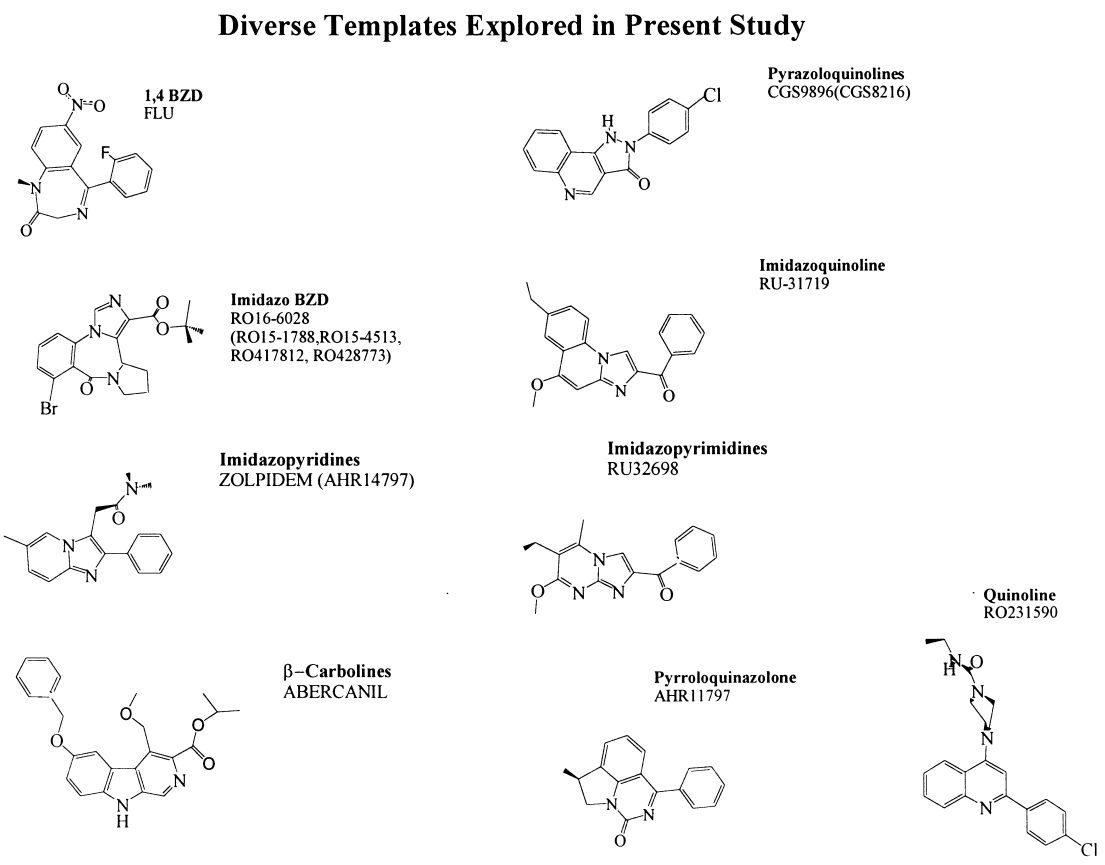

Figure 2. The 8 different templates examined with a single representative structure for each.

one used as input to the pharmacophore development program.

Quantum chemical assessments of ligands

In addition to conformational libraries, the second type of input required by MOLMOD for each compound is the selection of appropriate chemical moieties for consideration as pharmacophoric elements. To aid in this selection, quantum chemical calculation of selected chemical properties such as relative proton donating and accepting abilities of candidate ligand moieties, group hydrophobicities,[28] electron distribution in the highest occupied (HOMO) and lowest empty (LUMO) molecular orbital distributions were calculated. Three distinct types of quantum chemical methods were used for these calculations. The AM1 semiempirical method present in MOPAC7[29] was the method used to characterize all the ligands in the training set. As a check on the robustness of geometries and properties calculated with AM1, several of the ligands were chosen for complementary calculations using density functional theory (DFT) present in Oxford Molecular DGauss/Unichem, and standard Hartree Fock methods in Gaussian 98.[30] No significant differences in results, relevant to the goals of the present study, were noted.

Pharmacophore development

Pharmacophores were determined using a stand-alone program built on an extensible C++ molecule class library using dynamic memory allocation to allow for working with large numbers of compounds (> 25) with extensive conformational variability (2000 conformations per compound). The program searches for commonalities in the 3D-display of moieties in training sets and superimposes ligands at user defined pharmacophore points. The program employed, MOLMOD, builds on the previously validated principles of clustering and distance matrix comparisons present in DISTCOMP which we have previously reported.[14] MOLMOD augments the procedure described for DISTCOMP[14] by: (1) using exclusive conformational clustering (ensuring that each conformer appears in at most one conformational cluster), (2) permitting the use of receptor-based pharmacophore points including donor receptor point geometries based upon atomic chemical hybridization (sp^2, sp^3 etc) of the ligand acceptor atom, and (3) permits use of distinct distance parameters for the purpose of conformational clustering and pharmacophore deduction.

MOLMOD requires user selection of candidate moieties in each compound that could be considered as possible components of the 3D pharmacophores such as hydrophobic groups and proton accepting and donating groups. The program then systematically searches for the combination of chemical moieties selected for each compound that have common spatial arrangements in the compounds that affect this end point and that are absent in the compounds with no effect. MOLMOD adds to the capabilities of a previously developed program DISTCOMP by exhaustively searching for all possible

pharmacophores, based on the set of user selected moieties. The 3D pharmacophores found by this procedure are hence defined as a set of specific chemical moieties in a specific geometric arrangement required for the pharmacological end point under consideration. These pharmacophores can then be assessed by using them to search 3D databases to identify compounds that satisfy their requirements. Initial validation consists of finding known BDZR ligands by this procedure. The validated 3D pharmacophores can then be used to discover novel BDZR ligands based on unique scaffolds.

Database searches for validation

The 3D pharmacophores selected can then be used to search 3D databases for compounds that fulfill the steric and chemical requirements of that pharmacophore. Such a search was used to find known BDZR ligands eliciting an anxiolytic response to validate the 3D pharmacophore. In addition, this procedure can also be used to discover compounds with novel molecular scaffolds that could recognize or activate the receptors under investigation and give rise to anxiolytic activity.

In particular the databases searched in this work were the Chapmann and Hall Chemical Database, NCI and Cambridge Structural Databases. The pharmacophore query was defined in Tripos Inc. SYBYL/UNITY,[31] which permits definitions of such queries in terms of ligand based donors and acceptors, as well as donors and acceptors complementary to them on receptors, centroids and hydrophobic groups.

Results and Discussion

Table 1 gives the total number of conformations found for each of the 17 ligands. Also given in this Table are the subset of conformers within 3 kcal/mol of the lowest energy conformation found for each of the 17 compounds studied. It is this subset of energy accessible conformers of each ligand that was chosen as input to identify a 3D pharmacophore. Even with these cut-off criteria it is impossible to a priori select a bioactive conformation for each of these structurally diverse compounds. The identification of the bioactive conformation is made only after testing each conformer for the possibility of conforming to a common 3D pharmacophore with all the other ligands in the test set.

Table 1. Total number of conformers and number of conformers within 3 kcal/mol of the minimum for each of the 17 BDZR ligands studied

Compound	Total no. of conformers[a]	No. of conformers (3 kcal)
Flunitrazepam	144 (2)	36
U78875	1728 (3)	46
RU31719	864 (3)	110
CGS9896	12 (1)	8
RO16-6028	144 (2)	11
RU32698	1296 (4)	14
Zolpidem	144 (2)	36
RO23-0364	144 (2)	6
RO41-7812	144 (2)	12
RO42-8773	864 (3)	16
AHR11797	8 (1)	6
RO23-1590	1728 (3)	12
MFB43C	8 (1)	2
CGS 8216	12 (1)	12
AHR14797	144 (2)	14
RO15-1788	864 (3)	50
Abecarnil	1331	1128

[a]The total number of torsion angles explored is shown in parentheses. The torsion angles explored are indicated in Figure 1.

In addition to the conformational libraries, all potential moieties which can be candidate recognition or activation pharmacophore elements must be identified in each of the molecules. To help identify the essential physical properties of these moieties quantum chemical calculations were performed on each of the ligands.

Figure 3 shows the choices of potential pharmacophore points in each of the molecules in this study. The polar groups are labeled by numbers (1–5) and the various classes of hydrophobic groups by letters (A–F). Examining this particular set of molecules, the presence of proton donors can be excluded as a common recognition or activation element because only a few molecules in this set have such moieties. In each compound, there are, however, a number of heteroatoms that could be proton acceptors. However, as shown in Figure 3, two of these compounds, AHR11797 and CGS8216, contain only two such proton acceptors, limiting the inclusion of this type of moiety to two. Since all the other compounds contain a greater number of such sites, to aid in selection of two among them, the relative proton affinities of each of these candidate atoms in each of the 17 BDZRs were calculated using the semiempirical quantum chemical method AM1. These results are shown in Table 2. For each compound, this Table gives the relative value of the enthalpies of protonation at each site compared to the site with the smallest value. The numbers used in this table to identify each site for each compound are consistent with the numbering scheme used in Figure 3. In selected cases, molecular electrostatic potential surfaces at ab initio levels were computed to verify particular sites in the ligands as potential hydrogen bonding/proton accepting sites determined initially from the AM1 studies. In general, the two sites selected for each compound were the most favorable proton acceptors.

Ring systems and other pendent aliphatic groups may be hydrophobic recognition sites. To help select the hydrophobic groups, group hydrophobicities of rings and substituents were calculated by a method developed in our laboratory.[28] The results shown in Table 3 guided the choice of two moieties as hydrophobic recognition elements. These are indicated with capital letters A and B in Table 3 and Figure 3.

Aromatic rings are most often the regions in these molecules that can act as electron donors or acceptors to the receptor. Electron donating rings can be identified as those with the greatest electron density from the highest energy occupied molecular orbitals (HOMO). Electron accepting regions are those with the greatest

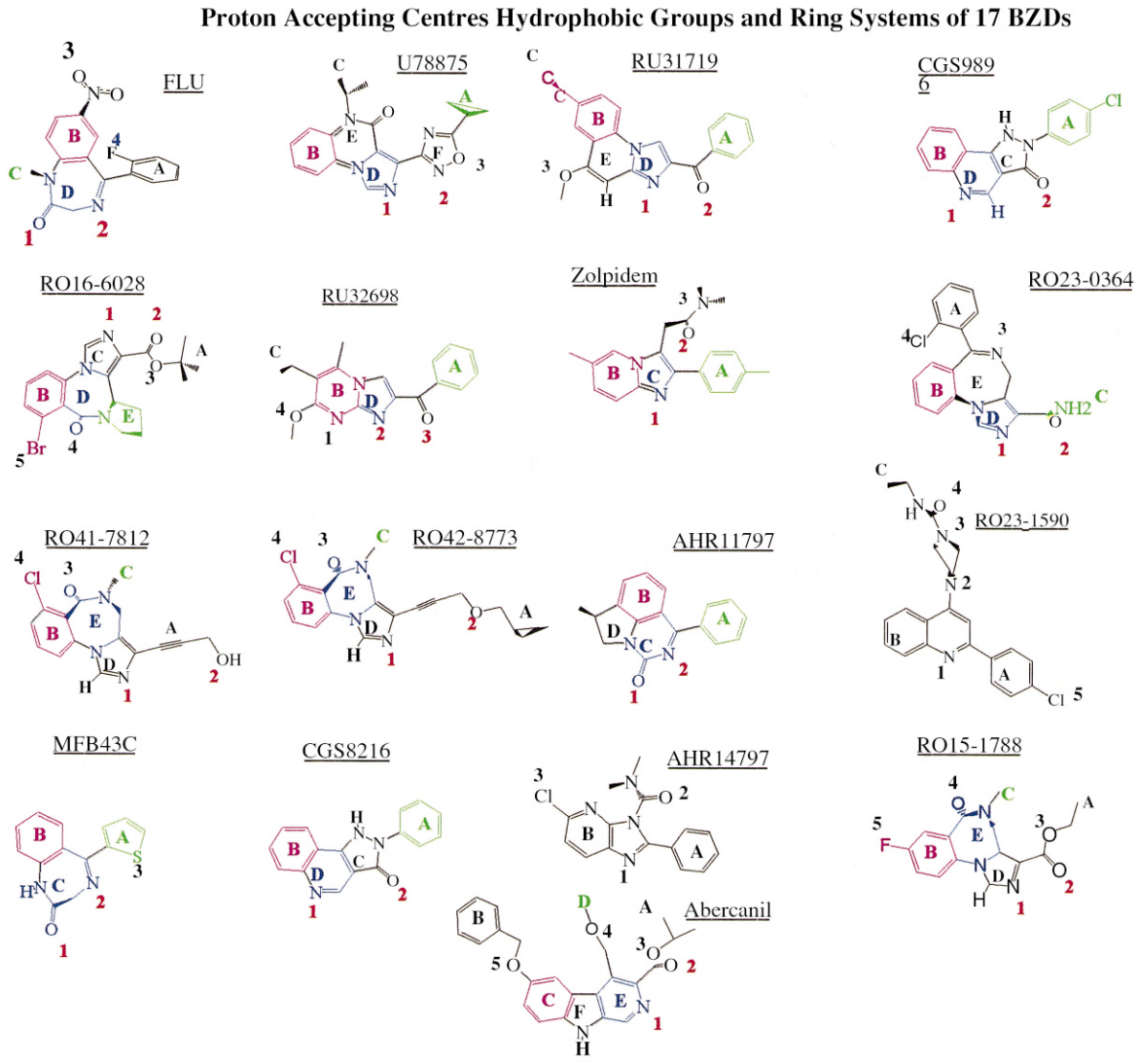

Figure 3. Identification of the candidate pharmacophoric components selected for each compound. (Color-coding of the elements determined to be pharmacophoric elements as described in the Results and Discussion.)

potential for accepting electron density into their lowest energy unoccupied molecular orbitals (LUMO). These rings were identified by quantum chemical calculations. Previous work has suggested that such a LUMO center could be an important component in activation of BDZR/$GABA_A$ receptors in rat cerebellum.[32] Table 3 indicates the LUMO centered rings which were found and included as potential pharmacophore components in Figure 3.

Two of the compounds included in this study, CGS9896, an agonist, and CGS8216, an inverse agonist at the anxiolytic end point, can exist either in two keto or an enol tautomeric forms. The relative energies of each tautomer were calculated with the effect of solvent using the ab initio density functional theory or the semiempirical AM1 method. These results suggest that all three tautomeric forms might play a role in receptor recognition. However, as shown in Figure 4, both keto I and the enol form contain proton donors that have already been excluded as potential pharmacophore entities since they are absent in most of the compounds in this training set. At least one study has alluded to the possible role of N5H proton donors in the keto I form as partially responsible for the high affinities of pyrazoloquinolines.[33] Yet, the compounds in this training set have substantial binding affinities[34,35] despite lacking such a proton donor. Moreover, both the keto I and enol forms lack the number of proton accepting moieties with the spatial disposition present in the other compounds in the present training set that are present in the keto II forms of these CGS compounds. On this basis, the keto II form of these two compounds was selected as the candidate form relevant to determination of the core recognition features of agonists, inverse agonists and antagonists binding to the BDZR/$GABA_A$ receptor(s) associated with anxiolytic activity.

With all of the input requirements thus satisfied, the systematic search for 3D pharmacophores performed by

MOLMOD resulted in two distinct 5-component pharmacophores. Both pharmacophores consisted of 2-proton donating receptor points, a hydrophobic region represented by a centroid, a ring containing polar moieties and a fifth moiety. In one, by design, the fifth moiety was both hydrophobic and an electron accepting LUMO center. In the other it was any general hydrophobic center. Statistically, the pharmacophore that included LUMO was found to be the more robust. It had about half the total variance of the other candidate pharmacophore ($\sigma^2 = 15$ versus $\sigma^2 = 24$). This 3D pharmacophore for the recognition of BDZR/GABA$_A$ receptors initiating the anxiolytic activity was thus selected as the most reliable since it (i) contained the maximal number of pharmacophore component moieties, and (ii) had the smallest total variances in the distance matrix comprising the pharmacophore.

Table 2. Calculated relative heats of protonation for each compound using the AM1 method[a]

Compound	Protonation site (cf. Fig. 3)				
	1	2	3	4	5
Flunitrazepam	13.9	0	21.8		
U78875	0	8.3	1	3.9	11
RU31719	0	17.8	61.7		
CGS9896	0	14.3			
RO16-6028	0	21.2	28.1	6.7	3.877
RU32698	31	0	20.1	62.1	
Zolpidem	0	26.1	37		
RO23-0364	5.8	41.9	0	47.6	31.7
RO41-7812	0	37.7	9.4	180.0	
RO42-8773	27.93	21.14	0	55.53	
AHR11797	10.79	0			
RO23-1590	0	23.1	28.9	32.1	73.8
MFB43C	15	0	123.5		
CGS8216	19.1	0			
AHR14797	0	17.1	58.7	9.3	
RO15-1788	0	6.5	28.4	16.8	36.3
Abercanil	0	15.8	22.8	25.7	27.5

[a]The heats of protonation of all sites have been expressed relative to the site with the smallest heat of protonation.

Table 3. Table of group hydrophobicities corresponding to labels in Figure 3. The ring system containing the lowest unoccupied molecular orbital of the molecule is also shown in parentheses

Compound	Group hydrophobicities		
	A	B	C
Flunitrazapam	2.14	1.4 (LUMO)	0.4
U78875	1.2	1.8 (LUMO)	1.2
RU31719	1.9	2.4 (LUMO)	
CGS9896	2.5	1.9 (LUMO)	
RO16-6028	1.5	1.6[a] (LUMO)	
RU32698	1.9	0.01 (LUMO)	
Zolpidem	2.3	1.0 (LUMO)	
RO23-0364	2.3	1.7 (LUMO)	−0.63
RO41-7812	1.4	2.2 (LUMO)	
RO42-8773	1.3	2.1 (LUMO)	
AHR11797	1.9	1.6 (LUMO)	
RO23-1590	2.5	1.7 (LUMO)	
MFB43C	2.4	1.8 (LUMO)	
CGS8216	2.0	1.8 (LUMO)	
AHR14749	3.1	0.8 (LUMO)	
RO15-1788	0.95	2.1 (LUMO)	
Abercanil	1.3	2.0	1.6 (LUMO)

[a]The hydrophobicity contribution of an atom [Br(RO16-6028); S(MFB43C)] has not been included in this case but would significantly alter the hydrophobicity of this ring system.

The 3D pharmacophore selected is shown schematically in Figure 5. Given in this figure are the identity of the five pharmacophoric components and the geometric relationship among them. In addition, the calculated distances between the pharmacophoric elements common to all the active ligands, 11 agonists, three inverse agonists and one antagonist, are given. The pharmacophoric elements are two donor receptor points on one side of the long axis of the molecule. This axis is defined by a hydrophobic moiety at one end and the aromatic ring containing the dominant electron density in the lowest unoccupied molecular orbital (LUMO) at the other. The polar ring pharmacophore element lies between the hydrophobic and LUMO elements.

To supplement this description, Table 4 specifically identifies the moieties in each of these 15 compounds that comprise the five point pharmacophore. These pharmacophoric moieties are color coded in Figure 3. For each compound, proton acceptor moieties 1 and 2 in Table 4 are indicated in red numerals in Figure 3. These acceptors are the partners of the donor receptor points. Hydrophobic moieties identified in Table 4 are

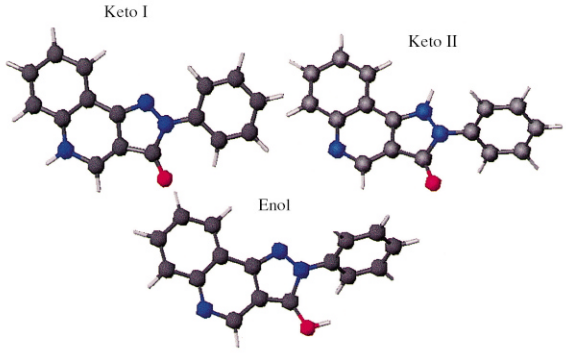

Figure 4. Three possible tautomeric forms of the CGS compounds (CGS9896 and CGS8216; shown for CGS8216).

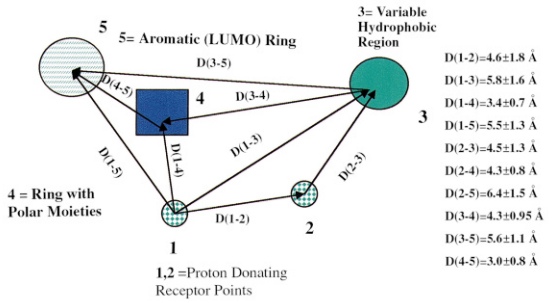

Figure 5. The best five point pharmacophore for recognition of BDZR/GABA receptors initiating the anxiolytic end point determined from the conformational libraries of 11 agonists, 3 inverse agonists and 1 antagonist at the anxiolytic end point.

Table 4. Summary of moieties[a] in each molecule comprising the best five point pharmacophore

Molecule	Acceptor 1 (receptor donor Pt. 1)	Acceptor 2 (receptor donor Pt. 2)	Hydrophobic moiety	Polar ring system	LUMO
Flunitrazepam	2	1	C	D	B
U78875	1	2	A	D	B
RU31719	1	2	A	D	B
CGS9896	1	2	A	D	B
RO16-6028	1	2	E	D	B
RU32698	2	3	A	D	B
Zolpidem	1	2	A	C	B
RO23-0364[b]	1	2	C	D	B
RO41-7812	1	2	C	E	B
RO42-8773	1	2	C	E	B
AHR11797	1	2	A	C	B
MFB43C	1	2	A	C	B
CGS8216	1	2	A	D	B
RO15-1788	1	2	C	E	B
Abercanil	1	2	D	E	C

[a]These moieties have been color coded in Figure 3 (red = acceptor points complementary to the receptor donor points; green = hydrophobic moiety; blue = polar ring system; magenta = ring with dominant LUMO coefficients).
[b]The compound RO23-0364 is a single exception to this general pattern.

marked in green, the polar ring elements in blue and the LUMO ring in magenta in Figure 3.

Figure 6(A) shows three structurally diverse compounds with these moieties clearly indicated. In addition, as shown in Figure 6(B) and (C), despite their structural diversity, all the pharmacophore elements can be superimposed. As shown, the greatest variability in this overlap is the hydrophobic and LUMO ring regions.

As a further test of the reliability of this pharmacophore, the two compounds that have no effect at the anxiolytic end point, AHR14947 and RO23-1590, were used as controls. Both of these ligands have been shown in our laboratory to have some activity at one or more of the remaining five end points tested. Specifically, AHR14797 is an agonist at both the sedation and hypothermic end points and RO23-1590 is an agonist at both the sedation and hyperphagic end points. These results then provide clear evidence that lack of bioavailability is not the reason for the lack of effect of these two compounds. It is thus reasonable to deduce that they do not bind with appreciable affinity to the receptors that initiate anxiolytic activity.

Consistent with this deduction is the finding that neither of these compounds fulfilled the requirements for recognition of these receptors as embodied in the 3D recognition pharmacophore shown in Figure 5. Figure 7 shows the disparate geometric relationship of these pharmacophore moieties for one of these compounds, AHR14947, compared to a typical active compound. As illustrated in this figure, in compounds with no effect, the proton accepting centers are on opposite sides of the long axis of the molecule, resulting in a completely different juxtaposition of polar and hydrophobic groups from all of the compounds that bind to the receptor subtypes associated with anxiolysis.

The development of a pharmacophore which simultaneously explains the properties of the compounds that bind and those that have no effect provides support for 3D pharmacophore development based on in vivo behavioral data. In addition to these internal criteria for reliability and robustness, the pharmacophore developed here was validated by using the requirements to search 3D databases. Representative ligands found in three different families including 1,4-BDZ derivatives, imidazoBDZs and β-carbolines are shown in Figure 8. These ligands can be divided into three categories: the first includes all the compounds with known BDZR ligand scaffolds. The second are BDZR ligands found that were among the training set studied which had anxiolytic effects. These are flunitrazepam, RO16-6028 and abercanil. The third are BDZR ligands with known activity at the anxiolytic end point that were not included in the 17 compound training set studied. These are prazepam and DMCM. These combined results provide additional convincing support for the validity of the 3D pharmacophore developed here from a very diverse ligand training set.

There have been a number of prior studies addressing the molecular requirements for receptor recognition or activity at behavioral end points. A number of these have included the involvement of proton acceptor groups.[36–40] In addition, moieties with lipophilic groups had, from the early literature, been invoked as being important in determining binding.[41–43] Moreover, in previous studies in our laboratory, molecular requirements for activation at the anticonvulsant end point[43] and of recognition and activation of the BDZR α1 containing receptors in cerebellum have also been reported[32] that included proton acceptors and hydrophobic moieties, including the LUMO. A 'comprehensive' pharmacophore has been proposed, based upon extensive COMFA studies using 150 compounds from 15 distinct families with activity at many different behavioral end points.[44] While this comprehensive pharmacophore encompassing binding to receptors eliciting several behavioral end points cannot be directly compared with the one developed here for recognition of receptors eliciting specifically an anxiolytic response, there are several commonalities in the presence of proton donor

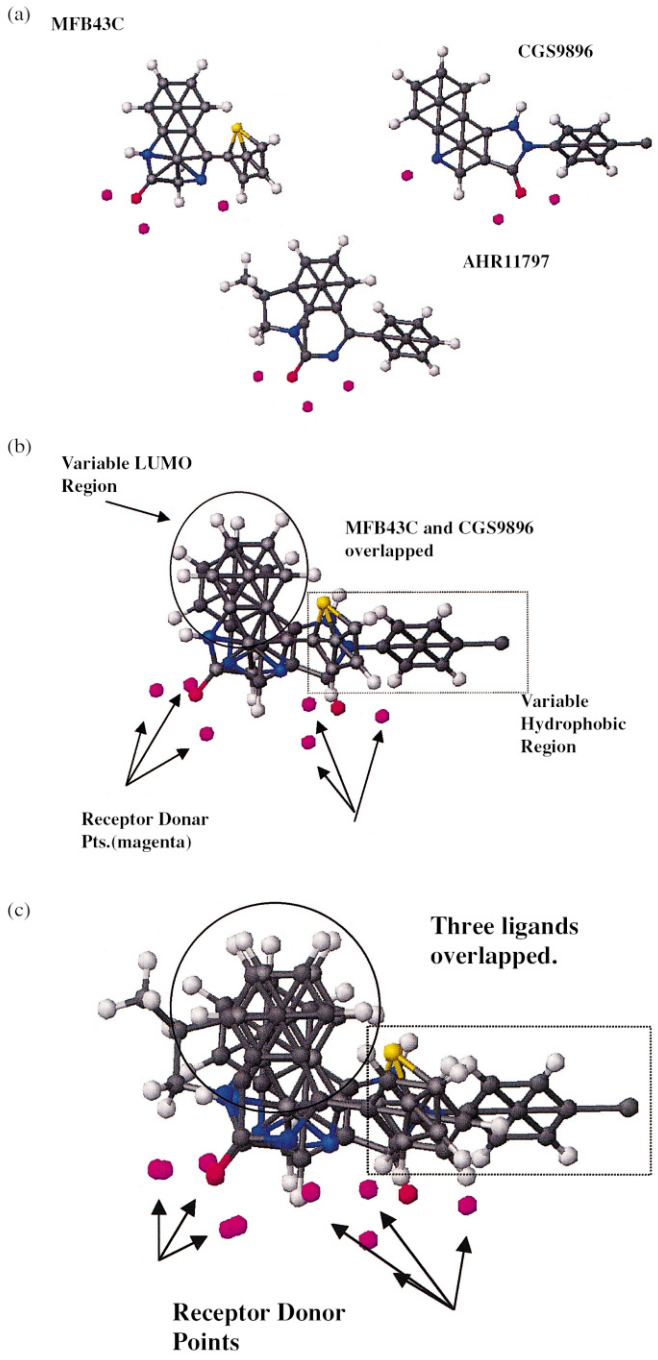

Figure 6. Superposition of 3 diverse ligands binding to receptor subtypes giving rise to an anxiolytic response.

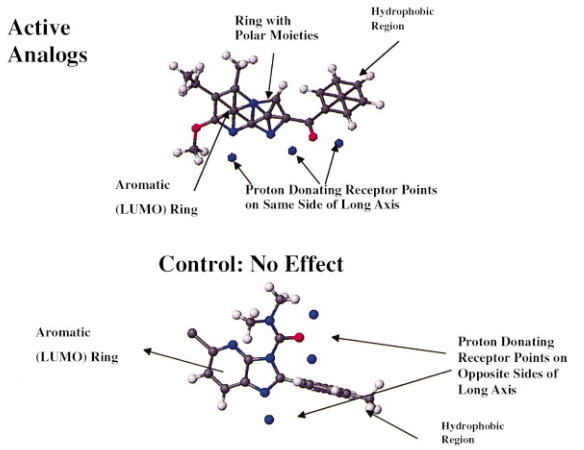

Figure 7. Comparison of the spatial patterns of pharmacophore points in ligands that (a) bind to the anxiolytic receptor subtypes and (b) those which do not (no effects).

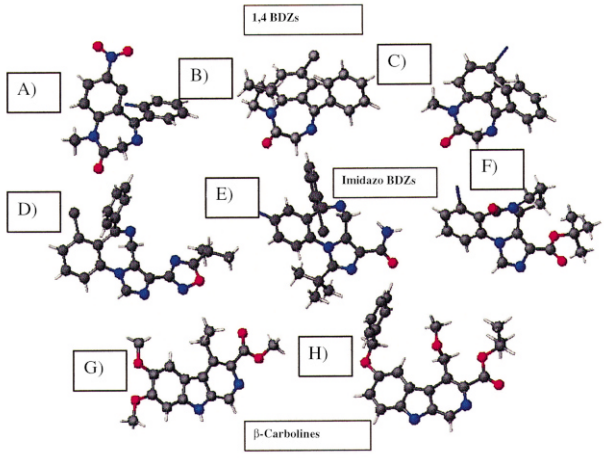

Figure 8. Examples of compounds found by performing 3D database searching using the 3D pharmacophore shown in Figure 5 as criteria. These compounds are divided into three different classes, all corresponding to known anxiolytics. These are 1,4-BDZs. (A) Flunitrazepam, (B) prazepam, (C) 7-bromo-2,3-dihydro-1-methyl-5-phenyl-1,4-benzodiazepin-2-one chloral hydrate. (Two of these compounds are known anxiolytics.) The imidazoBDZs are (D) 7-chloro-3-(5-isopropyl-1,2,4-oxadiazol-3-yl)-6-phenyl 4H-imidazo(1,5-a)(1,4)benzodiazepine, (E) (*S*)-(+)-6-(2-chlorophenyl)-1-t-butyl-8-fluoro-4H-imidazo (1,5-a)(1,4)benzodiazepine-3-carboxamide, (F) RO16-6028: *t*-butyl 11,12,13,13a-tetrahydro-8-bromo-9-oxo-9H-imidazo(1,5-a)pyrrolo (2,1-c)(1,4)benzodiazepine-1-carboxylate, a known anxiolytic. The β-carbolines are (G) DMCM, (H) abercanil, both of which have activity at the anxiolytic end point.

and hydrophobic features. The examination of the subset of ligands in that study that elicited an anxiolytic response indicates two receptor donor points on the same side of the long axis of the anxiolytic ligands, consistent with that component of the pharmacophore developed here. No pharmacophore has been reported, to date, for explicit recognition of receptors that initiate the anxiolytic end point by the systematic examination of a significant set of compounds with this property determined in the same laboratory and that have been characterized with the capabilities of the systematic protocols used here.

Conclusions

A 3D pharmacophore for recognition of BDZR/$GABA_A$ receptors eliciting an anxiolytic response has been developed from a training set consisting of 17 diverse BDZR ligands using an in-house computer program, MOLMOD. This program allows the identification of a common spatial arrangement of chemical moieties common to all agonists, inverse agonists and antagonists of $GABA_A$ receptor subtypes initiating an anxiolytic response. The criteria used to select the most robust and reliable pharmacophore among those generated

were (i) the maximum number of chemical moieties and (ii) the minimum variance in the pairwise distances between them common to all active compounds.

To achieve this goal, conformational libraries and quantum chemical characterization were completed for 17 structurally diverse BDZR ligands. The 3D pharmacophore selected, shown in Figure 5, was the one with the smallest statistical uncertainty and maximum number of components. It has two essential proton acceptors complementary to receptor proton donor groups, on one side of the long axis of the ligands, a hydrophobic group at one end of the long axis, a LUMO ring at the other end and an intervening ring center containing polar moieties.

The validity of this pharmacophore was successfully assessed in two ways. First, it was determined that the two compounds known not to have any effect at the anxiolytic end point did not comply with the requirements of this pharmacophore. Second, using this pharmacophore to search 3D databases a number of compounds, as shown in Figure 8, were identified with known activity at this end point.

The working hypothesis underlying this effort is that the ligands giving rise to agonism, inverse agonism or antagonism at a given end point have similar determinants of binding at the receptor subtypes associated with that end point. Given that the structure of these receptors is unknown, it is also unknown to what degree agonists, inverse agonists and antagonists share common elements permitting them to bind with some affinity to a common region of the BZD binding pocket of $GABA_A$ receptors. While this assumption is in part supported by experimental findings, the finding of a pharmacophore for these compounds including agonists, inverse agonists and antagonists at a given behavioral end point which has predictive ability in retrieval of known anxiolytic compounds in chemical databases provides additional support for it. The development of a pharmacophore that identifies properties common to the compounds that have any one of these three types of activities and demonstrates that these are absent in those that have no effects provides support for 3D recognition pharmacophore development based on in vivo behavioral data.

This validated pharmacophore can now be used to identify novel compounds, not based on any known BDZR ligand template, that could be promising new anxiolytic agents. Moreover, such compounds could be more selective for this end point. This prognosis is based on prior assessment in our laboratory of the 17 compounds used to develop the current 3D pharmacophore, at other end points such as sedation, hyperphagia, convulsions, and hyperthermia.[10,11] Most were found to exhibit qualitatively different types of responses at each of these end points, ranging from agonism, inverse agonism, antagonism or no effect. On this basis, it is clear that the 3D pharmacophore developed here for recognition of receptors initiating anxiolytic activity will be different from the subsequent ones developed for the other end points, leading to identification of compounds more specific for this activity.

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