

An Updated Unified Pharmacophore Model of the Benzodiazepine Binding Site on γ -Aminobutyric Acid_A Receptors: Correlation with Comparative Models

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Abstract: A successful unified pharmacophore/receptor model which has guided the synthesis of subtype selective compounds is reviewed in light of recent developments both in ligand synthesis and structural studies of the binding site itself. The evaluation of experimental data in combination with a comparative model of the $\alpha 1\beta 2\gamma 2$ GABA_A receptor leads to an orientation of the pharmacophore model within the Bz BS. Results not only are important for the rational design of selective ligands, but also for the identification and evaluation of possible roles which specific residues may have within the benzodiazepine binding pocket.

Keywords: Aminobutyric acid(A) receptors, gated ion channels, GABA-A, computer-assisted analysis, benzodiazepine.

The GABA_A receptor is the major inhibitory neurotransmitter receptor of the central nervous system (CNS) and the site of action of a variety of pharmacologically and clinically important drugs, such as benzodiazepines, barbiturates, neuroactive steroids, anesthetics and convulsants [1]. It is now clear that these receptors regulate the excitability of the brain, anxiety, muscle tone, circadian rhythms, sleep, vigilance, memory, and learning [1]. There are several disease states associated with the improper functioning of this protein, including anxiety, epilepsy [2], insomnia [3], depression and bipolar disorder [4, 5], schizophrenia [6], as well as mild cognitive impairment and Alzheimer's disease [7]. A role of GABA_A receptors in drug and alcohol abuse has also been reported [8-10].

GABA_A receptors are composed of 5 subunits that form a central chloride channel and can belong to different subunit classes. A total of 19 subunits ($6\alpha, 3\beta, 3\gamma, 1\delta, 1\epsilon, 1\pi, 1\theta, 3\rho$) of the GABA_A receptor have been cloned and sequenced from the mammalian nervous system [11, 12]. All these polypeptides possess an approximate molecular mass of ~ 50 kD and are structurally related. Each subunit consists of a large extracellular region, which contains several potential glycosylation sites and a characteristic "cys-loop" formed by a covalent bond between two conserved cysteines. This extracellular region is also important in contributing to the agonist GABA and modulatory benzodiazepine binding sites. The protein then traverses the lipid bilayer four times and has a large intracellular loop located between transmembrane regions 3 and 4 (M3 and M4). This intracellular region contains possible phosphorylation sites necessary for regulation of the receptor. The homology within each subunit class is about 60 – 80 %, while the homology between subunit classes is about 30 – 40 %. In Fig. (1) the proposed topology of a single GABA_A receptor subunit is shown. The pentameric structure of a ligand-gated ion channel is shown in Fig. (2) [13, 14].

The existence of multiple GABA_A receptor subunits can give rise to a large number of different GABA_A receptor subtypes [15]. The majority of GABA_A receptors, however, is composed of 1γ and 2α and 2β subunits. The presence of a γ subunit within a GABA_A receptor is necessary for the formation of a benzodiazepine binding site that is located at the interface of an α and γ subunit. Whereas the classical benzodiazepines, such as diazepam or flunitrazepam, exhibit a high affinity for receptors composed of $\alpha 1\beta\gamma 2$, $\alpha 2\beta\gamma 2$, $\alpha 3\beta\gamma 2$ or $\alpha 5\beta\gamma 2$ subunits (diazepam sensitive (DS) receptors), as well as for their less intensively investigated analogues containing the $\gamma 3$ subunit, other benzodiazepine binding site ligands are also

able to interact with $\alpha 4\beta\gamma 2$ or $\alpha 6\beta\gamma 2$ receptors (diazepam insensitive (DI) receptors), or with receptors containing $\gamma 1$ subunits [1]. Receptors containing $\gamma 1$ or $\gamma 3$ subunits exhibit a quite low abundance in the brain [16-18] and their contribution to the "in vivo" effects of benzodiazepine binding site (BZ BS) ligands currently is unclear.

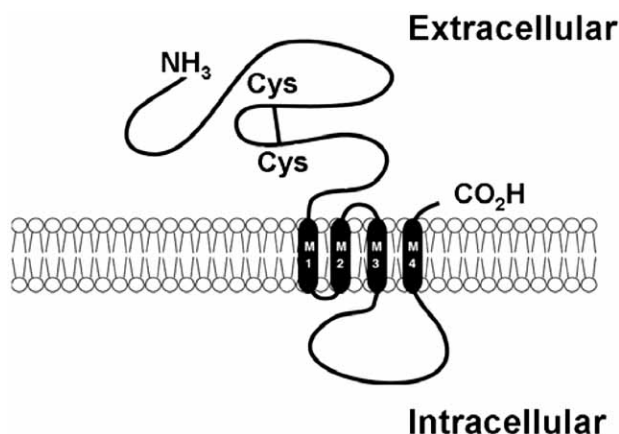


Fig. (1). Proposed topology of a GABA_A receptor subunit. The extracellular domain begins with the N-terminus and M1-M4 represent the four transmembrane domains. Figure reprinted with permission [13].

The concept of receptor multiplicity has been extremely valuable, in that different receptor subtypes reside within anatomically distinct regions of the brain and are responsible for different physiological and pathological processes [15, 19, 20]. For example, the $\alpha 1\beta\gamma 2$ receptor subtype has a prominent role in seizure susceptibility and sedation [21-23], the $\alpha 2\beta\gamma 2$ and possibly also the $\alpha 3\beta\gamma 2$ subtypes are involved in anxiety, whereas the $\alpha 5\beta\gamma 2$ subtype has a prominent role in memory and learning (Table 1) [15, 24-26]. These distinctions have thus become a motivation for the design of subtype selective ligands in order to elicit a single specific response [15, 27-34]. Differences observed in the action of such drugs may be due to subtype-selective affinity and absolute and/or relative subtype-selective efficacy [35].

Agonist binding to the receptor opens an intrinsic chloride ion channel, typically hyperpolarizing the cell membrane or at least opposing depolarization, thereby inhibiting neuronal transmission. Bz BS ligands are allosteric modulators, unable to induce channel openings themselves, but function to vary the frequency and not the channel opening times [37, 38]. Positive allosteric modulators at the benzodiazepine binding site (agonists) increase this frequency,

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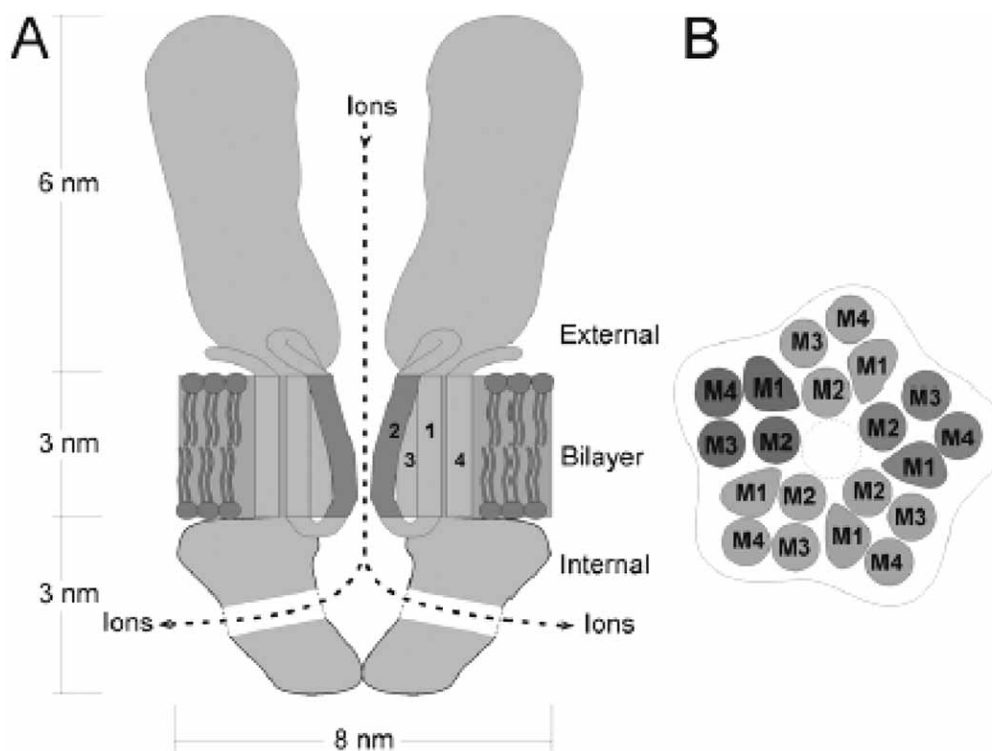


Fig. (2). Longitudinal (A) and cross-sectional (B) schematic representations of a ligand-gated ion channel. The numbers 1-4 refer to the M1-M4 segments. The M2 segment contributes to the majority of the pore lining within the membrane lipid bilayer. Figures reprinted with permission [14].

while negative allosteric modulators (inverse agonists) decrease the frequency. Currently it is not clear whether Bz BS ligands allosterically modulate GABA affinity or channel gating. Recent studies [39] support the view that high-affinity classical benzodiazepines modulate $\alpha 1\beta 2\gamma 2$ GABA_A receptors *via* allosteric coupling to channel gating [40, 41]. Further studies are needed to determine whether the mechanism of modulation varies in different receptor subtypes.

Table 1. Action of Benzodiazepines at GABA_A $\alpha 1-6 \beta 3\gamma 2$ Receptor Subtypes [36]

Subtype	Associated Effect
$\alpha 1$	Sedation, anterograde amnesia, Some anticonvulsant action, ataxia
$\alpha 2$	Anxiolytic, hypnotic (EEG), some muscle relaxation
$\alpha 3$	Some anxiolytic action, some anticonvulsant action, Maybe some muscle relaxation
$\alpha 4$	Diazepam-insensitive site
$\alpha 5$	Cognition, temporal and spatial memory (Maybe memory component of anxiety)
$\alpha 6$	Diazepam-insensitive site

In recent years a unified pharmacophore/receptor model for agonists, antagonists and inverse agonists at the Bz BS was developed, using the techniques of chemical synthesis, radioligand binding and receptor mapping [42, 43]. The overlap of these different modulators within the Bz BS has been supported by experimental data [44-46]. Using this ligand-based pharmacophore/receptor model and our $\alpha 1\beta 2\gamma 2$ GABA_A receptor models [47, 48], the experimental data of recent and past years have been evaluated, and definite trends with regard to the orientation of the regions of the protein relative to the descriptors of the pharmacophore/receptor model have been identified and are presented in this work. The

need to define such an orientation has been established [49], since it permits inspection of ligand docking studies and the identification of possible roles specific residues may have within the Bz BS. These roles may then be explored in future studies involving covalent labeling, site-directed mutagenesis and structure-activity relationships, all of which contribute to the rational design of subtype-specific modulators of the Bz BS of GABA_A receptors.

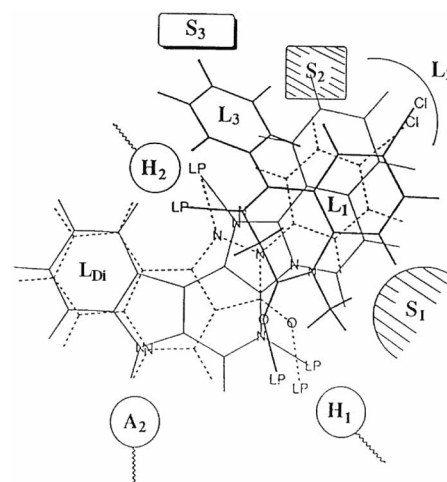


Fig. (3). Relative locations of the descriptors and regions of the unified pharmacophore/receptor model. The pyrazolo[3,4-*c*] quinolin-3-one CGS-9896 (dotted line), a diazadiindole (thin line), and diazepam (thick line) aligned within the unified pharmacophore/receptor model for the Bz BS. H₁ and H₂ represent hydrogen bond donor sites within the Bz BS while A₂ represents a hydrogen bond acceptor site necessary for potent inverse agonist activity *in vivo*. L₁, L₂, L₃ and L_{Di} are four lipophilic regions and S₁, S₂, and S₃ are regions of negative steric repulsion. LP = lone pair of electrons on the ligands.

THE UNIFIED PHARMACOPHORE/RECEPTOR MODEL

More than 150 agonists, antagonists and inverse agonists at the Bz BS [42, 43] which encompassed 15 structural families were used for generating the unified pharmacophore/receptor model. Although the relative affinities, efficacies and functional effects displayed by various ligands from the same structural class at the diazepam-sensitive and diazepam-insensitive benzodiazepine binding sites were taken into account, the approximate locations of descriptors (hydrogen bond donor sites, hydrogen bond acceptor sites, lipophilic regions, and regions of steric repulsion) were based primarily on *in vitro* binding affinities. Ligands from different structural classes were then superposed on each other to satisfy the same descriptors, resulting in the unified pharmacophore model.

Briefly, the pharmacophore/receptor model consists of two hydrogen bond donating descriptors (H_1 and H_2), one hydrogen bond

accepting descriptor (A_2) and one lipophilic descriptor (L_1). In addition to these descriptors, there are lipophilic regions of interaction (L_2 , L_3 and L_{Di}) as well as regions of negative steric repulsion (S_1 , S_2 and S_3). While occupation of L_2 and/or L_3 as well as interactions at H_1 , H_2 , and L_1 are important for positive allosteric modulation, inverse agonists only require interactions with the H_1 , L_1 , and A_2 descriptors of the pharmacophore/receptor model for potent activity *in vivo*. [42, 50-53] The L_{Di} descriptor is a region of lipophilic interaction, for which the difference between the diazepam sensitive (DS) and the diazepam insensitive (DI) sub-pharmacophore models is most pronounced. Depicted in Fig. (3) are the relative locations of the different descriptors and regions of the model.

The structures of ligands frequently referred to in this paper are shown in Fig. (4).

The alignments of several Bz BS ligands within this model are shown in Fig. (5).

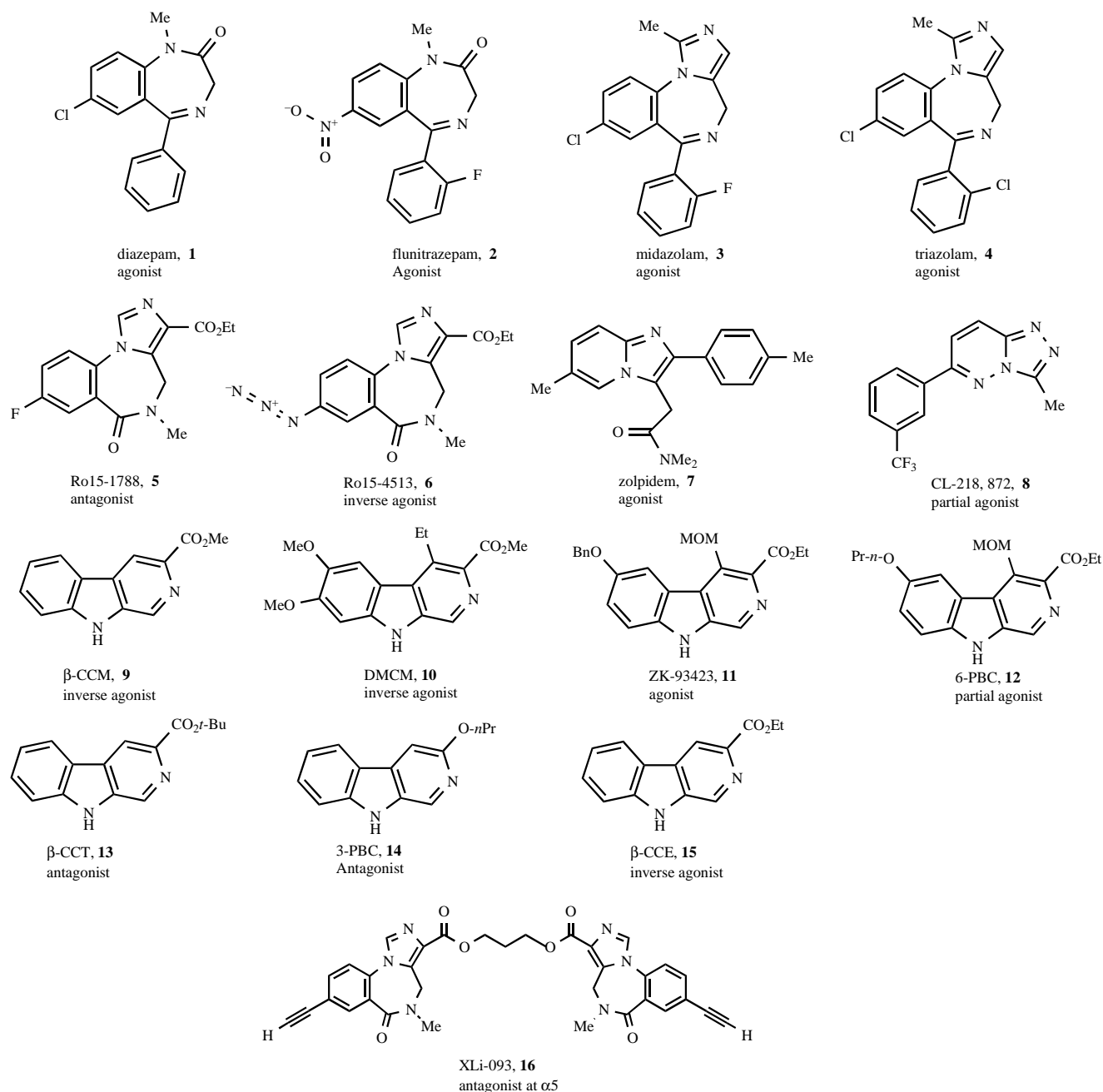
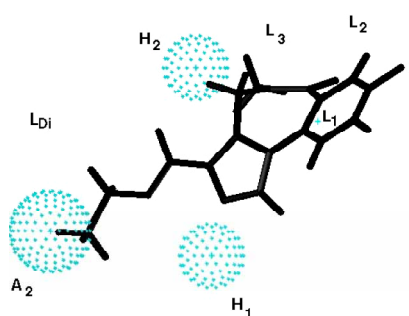
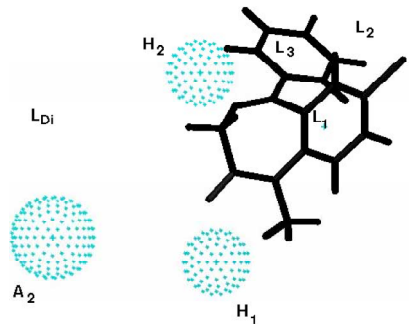


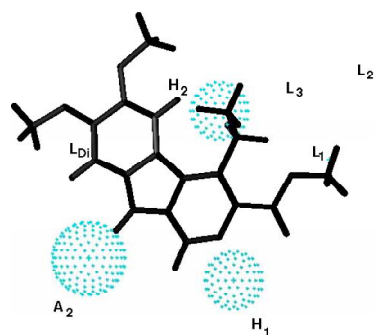
Fig. (4). Structures of ligands and their modulation at the α_1 subtype.



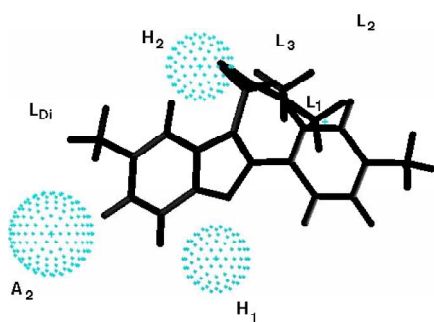
Ro 15-1788, 5



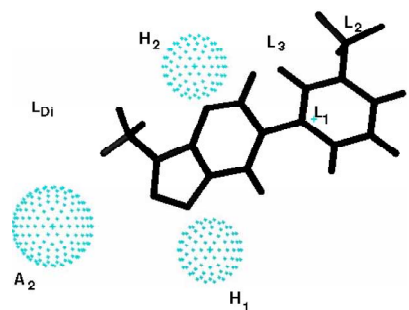
Diazepam, 1



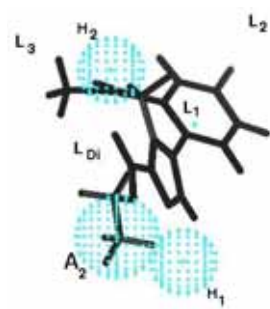
DMCM, 10



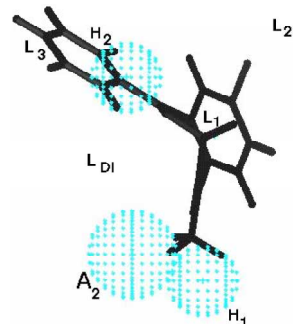
zolpidem, 7



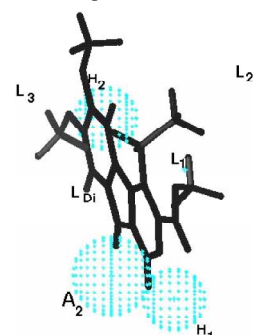
CL-218,872, 8



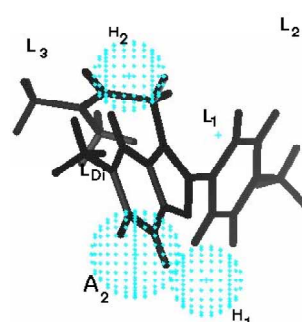
Ro 15-1788 rotated 90°



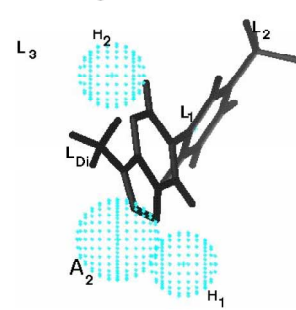
Diazepam rotated 90°



DMCM rotated 90°



zolpidem rotated 90°



CL-218, 872 rotated 90°

Fig. (5). Alignments of several Bz BS ligands within the pharmacophore model.

INCLUDED VOLUME ANALYSIS OF LIGANDS BINDING TO RECEPTORS CONTAINING DIFFERENT ALPHA SUBUNITS

The benzodiazepine binding site of $\alpha\beta\gamma_2$ GABA_A receptors is strongly influenced by the type of α subunit present in these receptors as indicated by the existence of ligands exhibiting certain selectivity for receptors containing the respective α subunits [1, 20, 54]. If subtype selective ligands are then aligned within the pharmacophore model according to the resulting alignment rules [55], their included volumes can be constructed and used to compare the topologies of benzodiazepine binding pockets of different receptor subtypes [55, 56]. Ligands employed in the included volume for each receptor subtype exhibited potent affinity ($K_i \leq 20$ nM) at the respective receptor subtype. CL-218,872 **5** ($K_i = 57$ nM at $\alpha 1$) and zolpidem **4** ($K_i = 26.7$ nM at $\alpha 1$) were added to the included volume of the $\alpha 1\beta 3\gamma 2$ subtype since they are both $\alpha 1$ -subtype selective ligands. The major differences with regard to volume and affinity are shown below and are important for interpreting experimental data.

The included volume requirements show some trends that are useful to explain subtype preferences of ligands and that have already guided substance development:

1. *The included volumes of the $\alpha 1$, $\alpha 2$, and $\alpha 3$ subtypes are similar both in size and topology.* This is consistent with the similar affinity profiles these subtypes displayed for classical benzodiazepine agonists, pyrazoloquinolinones and imidazobenzodiazepines (*i*-BZDs) [57-59]. The included volume of the $\alpha 1$ subtype is slightly different from that of $\alpha 2$ and $\alpha 3$ subtype as indicated by the selective affinities of zolpidem, CL-218,872, 6-substituted β -carbolines (e.g. 6-methylbenzyl amino betacarboline) and pyridodiindoles for this subtype and the space needed for accommodating these structures (Fig. 6a,b) [59]. Results from both the SAR studies and the included volume analysis imply that the $\alpha 2$ and $\alpha 3$ subtypes are very similar in shape, polarity and lipophilicity.
2. *The included volumes of the $\alpha 1$ and $\alpha 4$ or $\alpha 6$ subtypes are very different.* Looking at Fig. (6d), it is evident that the included volume of the $\alpha 6$ subtype is significantly smaller

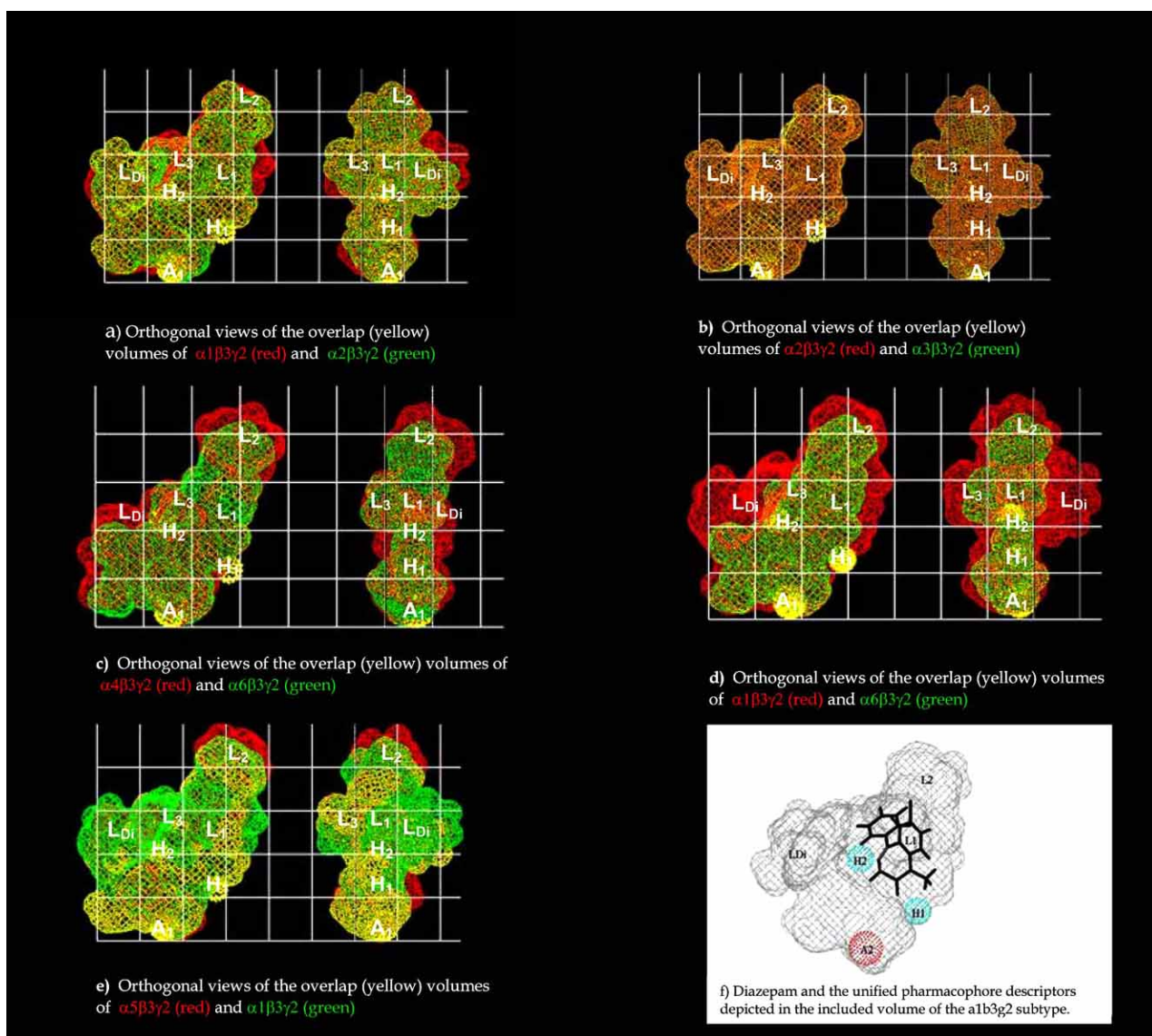


Fig. (6). Overlap between pairs of included volumes derived from receptor subtype selective ligands: a) $\alpha 1$ and $\alpha 2$, b) $\alpha 2$ and $\alpha 3$, c) $\alpha 4$ and $\alpha 6$, d) $\alpha 1$ and $\alpha 6$, e) $\alpha 1$ and $\alpha 5$. Yellow color indicates overlapping regions and each grid measures 4 Å in width and height. In order to provide the connection between this figure and other figures, f) shows diazepam and the descriptors of the unified pharmacophore model depicted in the included volume requirement of the $\alpha 1\beta 3\gamma 2$ subtype.

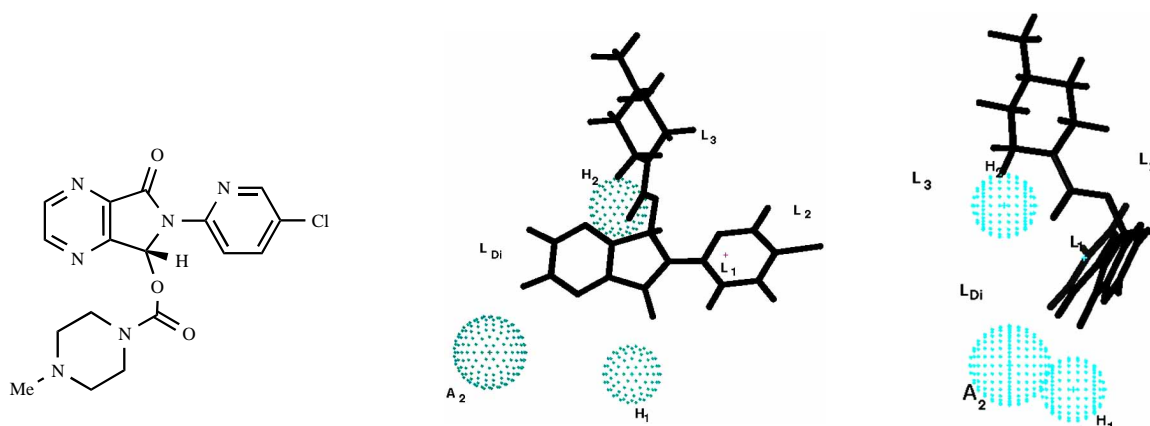


Fig. (7). Structure of zopiclone **17**, its alignment within the pharmacophore/receptor model and rotated 90 degrees.

than that of the $\alpha 1$ subtype. Especially the L_{Di} region is much larger in $\alpha 1$ receptors. Contributions to the L_{Di} region are derived in a large measure from 6-substituted β -carboline and ring-A substituted pyrazoloquinolines, thus implying that occupation of the L_{Di} region may be critical for ligand selectivity at the $\alpha 1$ subtype. This is supported by the finding that the L_{Di} region was also larger for the $\alpha 1\beta 3\gamma 2$ subtype when compared with the $\alpha 5\beta 3\gamma 2$ subtype (Fig. **6e**).

3. *L_3 region is very small or non-existent for the $\alpha 4$ and $\alpha 6$ DI subtypes.* Based on the inability to bind 1,4-benzodiazepines, the lack of region L_3 was believed to be responsible for the diazepam-insensitivity of these receptor subtypes (Fig. **6c, d**) [42, 51]. With a few exceptions, β -carboline also do not bind to the $\alpha 6\beta 3\gamma 2$ receptor subtype, while *i*-BZDs and pyrazoloquinolinones (CGS series) represent the primary ligands that bind to this subtype.
4. *The L_2 and L_{Di} regions are slightly smaller for the $\alpha 6$ versus the $\alpha 4$ subtype.* The sequence of the $\alpha 4$ subunit is most homologous to the $\alpha 6$ subunit and it was determined that the pharmacological profiles of these DI sites toward classical benzodiazepines are very similar [60]. Differences in the included volume of these DI sites are shown in Fig. **(6c)**.
5. *The L_2 region contributes to $\alpha 2$ and $\alpha 5$ selectivity.* It has been observed that ligands with $\alpha 2$ and/or $\alpha 5$ selectivity are generally *i*-BZDs and have a lipophilic C(8)-substituent that

occupies the L_2 pocket. Based on ligands from various studies, examination of data in Fig. **(6)** illustrates that the L_2 region is deeper and larger for the $\alpha 2$ and $\alpha 5$ subtypes, respectively, than for the corresponding $\alpha 1$ or $\alpha 6$ subtypes [42, 43, 51, 61]. This L_2 region seems to account for the selective affinity of ligands at the $\alpha 5$ subtype, as clearly illustrated in Fig. **(6e)**, whereas the same region may account for the selective efficacy observed at the $\alpha 2$ subtype.

RECENT ALIGNMENT OF NON-CLASSICAL BZ BS LIGANDS SUPPORT THE UNIFIED PHARMACOPHORE/RECEPTOR MODEL

Besides the major classes of Bz ligands used to define the model, several non-Bz ligands also fit within the pharmacophore/receptor model very well. Examples include zopiclone (**17**) and its active enantiomer, the flavonoid **25** and the 8-chloropyrazolo[5,1-c][1,2,4]-benzotriazine 5-oxide analog **38**, as illustrated in (Figs. **7** and **8**) and (Figs. **11** and **13**).

Zopiclone and its Active Enantiomer

Since the sedative-hypnotic zopiclone **17** was first reviewed in *Drugs* in 1986 [62], a much larger body of clinical data has become available to permit a more detailed comparison of the non-benzodiazepine zopiclone with classical benzodiazepines. Results have shown that, regardless of the duration of action, zopiclone was

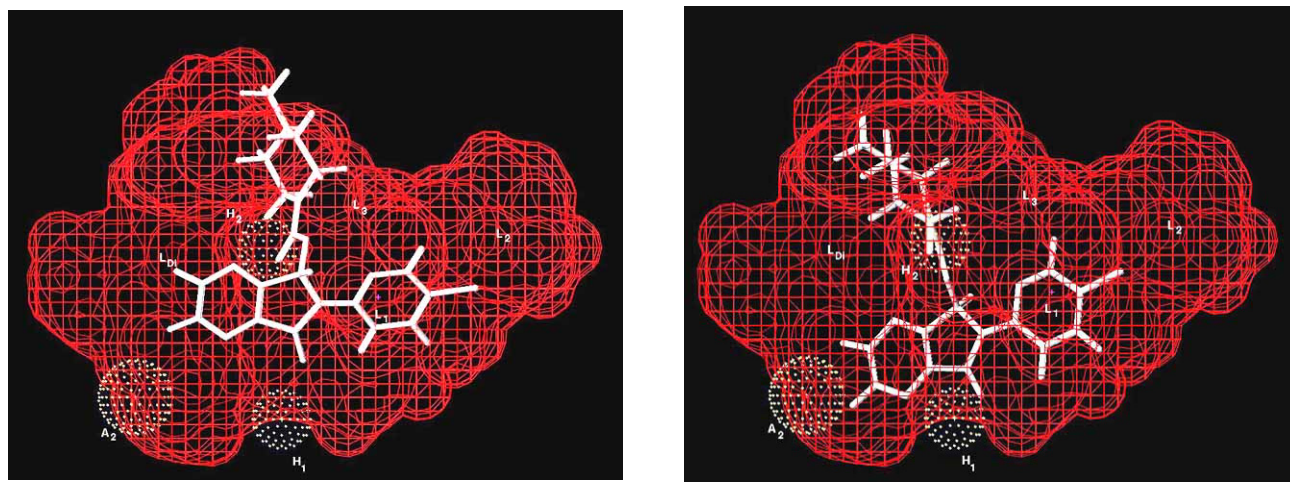


Fig. (8). Alignment of zopiclone **17** (left) and its active enantiomer (right) in the included volume of the pharmacophore/receptor model for the $\alpha 1\beta 3\gamma 2$ subtype.

generally at least as effective as benzodiazepines in the treatment of insomnia, although comparisons between zopiclone and flurazepam have produced inconsistent results. It was observed that zopiclone had a relatively low propensity to elicit residual clinical effects, such as difficulty in waking or reduced morning concentration. While tolerance to the effects of zopiclone was not noticed in short term clinical trials (≥ 4 weeks), the results from longer term studies were conflicting and, therefore, the potential for tolerance during long term administration of zopiclone was unclear. Rebound insomnia to a level above that at baseline can occur after withdrawal of zopiclone. However, on the basis of data from short term studies, this does not appear to be common. Evaluation of prescriptions filled has indicated that zopiclone does not have a high dependence potential, at least in those who are not regular drug abusers/addicts. Zopiclone was well-tolerated in both the elderly and younger patients with insomnia. A bitter after-taste was usually the most common adverse event, but was relatively infrequent at 3.6 % in the largest available post-marketing study. Thus, zopiclone has now been firmly established as an effective and well-tolerated sleep agent [63].

The structure and alignment of zopiclone within the unified pharmacophore/receptor model is shown in Fig. (7). The centroid of the pyridine moiety of zopiclone overlapped with region L₁ of the receptor, while the lone pair of electrons of the carbonyl oxygen (O) atom interacted with H₁ of the receptor to form a hydrogen bond between the ligand and the receptor. A second hydrogen bond was formed between the amide carbonyl oxygen atom (LP) and H₂ of the receptor protein (Fig. 7).

However, a recent study by Sepracor [64] indicated that one of the enantiomers of zopiclone was much more active than zopiclone

itself. This may be the result of receptor subtype-selective efficacy or simply a pharmacokinetic effect, but the pharmacophore/receptor model developed here revealed that the active enantiomer fits better into the included volume of the $\alpha_1\beta_3\gamma_2$ subtype than zopiclone **17** itself (Fig. 8). This enantiomer has just been approved by the FDA for treatment of insomnia.

SH-053BZ Enantiomers

In pursuing this approach using BZ enantiomers, the behavioral activity of three newly-synthesized compounds [65], functionally selective for α_2 , α_3 and α_5 -containing subtypes of GABA_A receptors (SH-053-S-CH₃ and SH-053-S-CH₃-2'F), or essentially selective for α_5 subtypes (SH-053-R-CH₃) were examined. Motor influence was tested in the elevated plus maze, spontaneous locomotor activity and rotarod test, which are considered primarily predictive of the anxiolytic, sedative and ataxic influence of BZs, respectively. There was substantially diminished ataxic potential of BZ site agonists devoid of α_1 subunit-mediated effects, with preserved anti-anxiety effects at 30 mg/kg of SH-053-S-CH₃ and SH-053-S-CH₃-2'F. However, all three ligands, dosed at 30 mg/kg, decreased spontaneous locomotor activity, suggesting that sedation may be partly dependent on activity mediated by α_5 -containing GABA_A receptors. Such an effect could not have been observed previously, because to date, all apparently non-sedating BZ receptor ligands, which are devoid of activity at α_1 -containing GABA_A receptors, engendered essentially antagonist [66] or only partial agonist efficacy at α_5 -containing GABA_A receptors [67]. Therefore, it cannot be ruled out that substantial efficacy at α_5 -containing GABA_A receptors may contribute to sedative effects besides the effects on learning and memory processes [68, 69]. This is supported by two sets of data

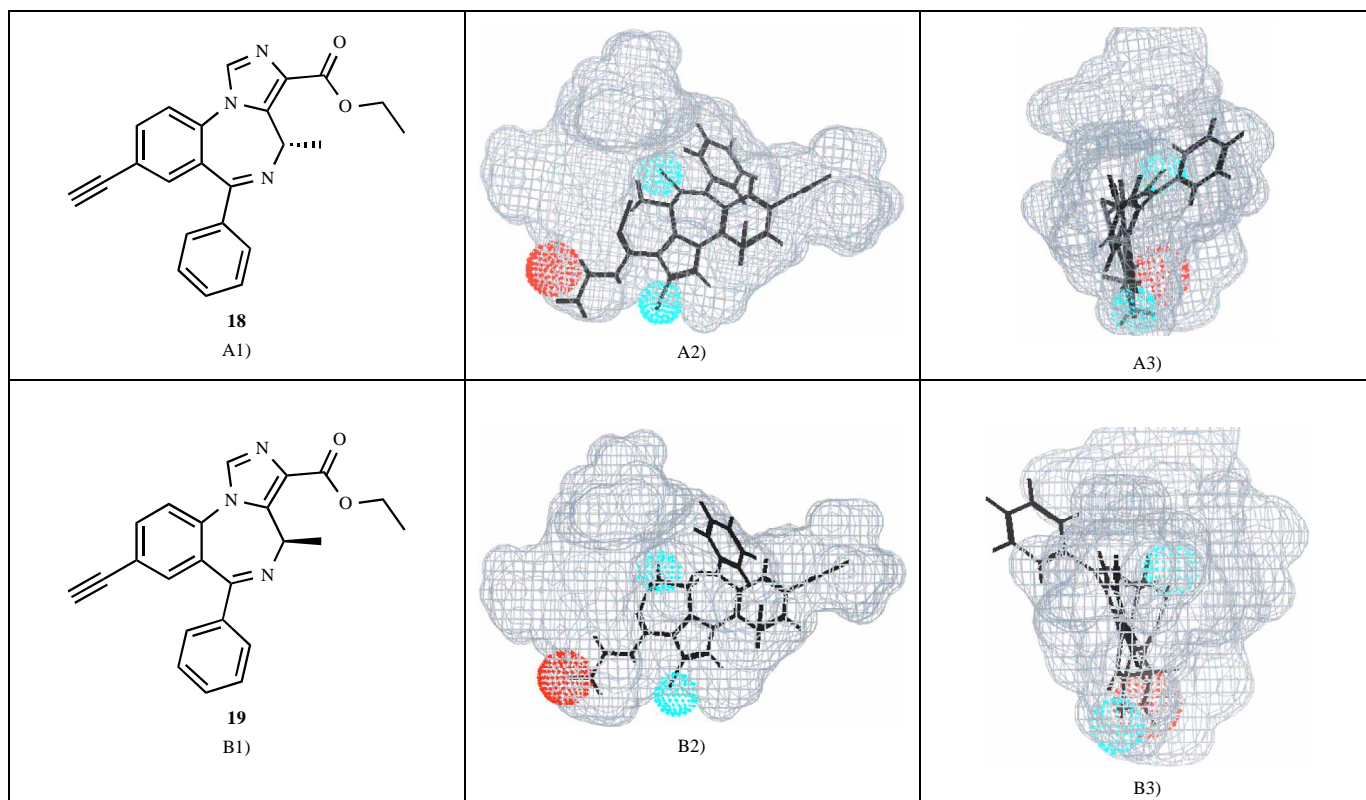


Fig. (9). Structures and conformations of SH-053-S-CH₃ (A1-A3) and SH-053-R-CH₃ (B1-B3). The molecular modeling was carried out as described in Zhang *et al.* 1995 and He *et al.* 2000. SH-053-S-CH₃ fits to the pharmacophore within the included volume of the α_2 subtype (A2); A3) is the same image rotated 90 degrees. It can be clearly seen that this conformer fits within the included volume. SH-053-R-CH₃ fit to the pharmacophore in the included volume of the α_2 subtype (B2); B3) is the same image rotated 90 degrees. The R-CH₃ at the prochiral center C4 changes the conformation of the molecule causing the pendant 6-phenyl to stick outside the included volume, consequently this ligand is not efficacious at the α_2 subtype. It simply does not interact strongly with α_1 , α_2 or α_3 subtypes.

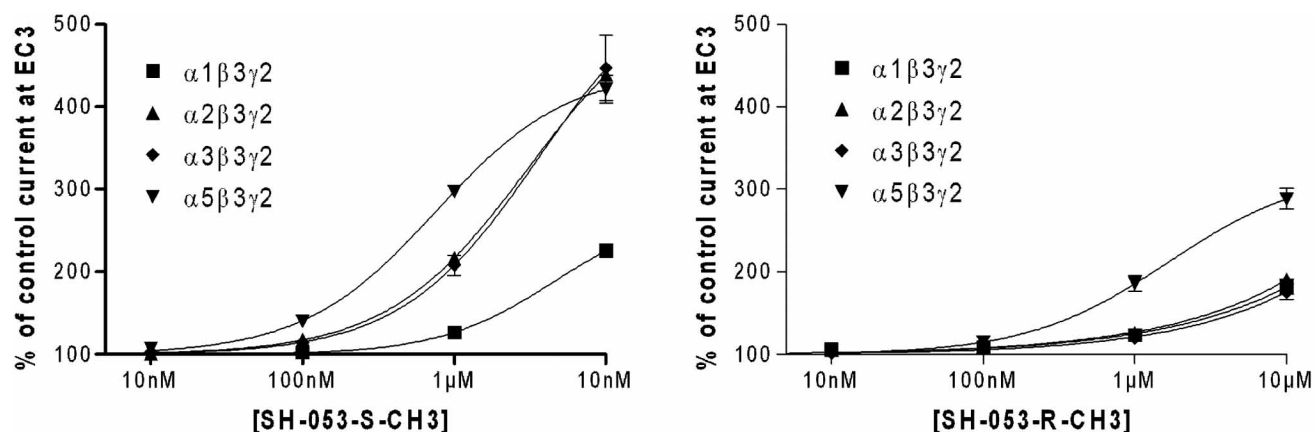


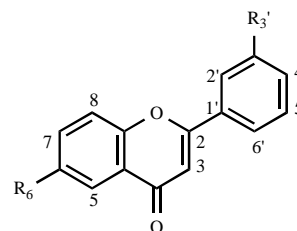
Fig. (10). Concentration-effect curves for SH-053-S-CH3 and SH-053-R-CH3 on $\alpha_1\beta_3\gamma_2$, $\alpha_2\beta_3\gamma_2$, $\alpha_3\beta_3\gamma_2$, and $\alpha_5\beta_3\gamma_2$ GABA_A receptors. Data points represent means \pm SEM from at least 4 oocytes from ≥ 2 batches.

which indicate the possibility of substantial motor influence *via* α_5 -GABA_A receptor modulation: In the spinal cord, somatic and preganglionic motoneurons (lamina IX and lateral cell column) exhibited a moderate to strong staining for the α_5 subunit, suggesting a possible influence of receptors containing these subunits in motor behavior [70]. In addition, the knock-in mice harboring the α_5 subunit insensitive to diazepam are refractory to development of tolerance to the sedative effect of diazepam dosed subchronically. Such a tolerance development might have been caused by a down-regulation of receptors containing α_5 subunits in the appropriate brain regions of wild-type mice [71]. These two sets of evidence indicate that the motor influence of α_5 -GABA_A receptor modulation is not necessarily an indirect consequence of the established effects on learning and memory processes [68, 69]. Hence, it has been hypothesized [65] that locomotor activity changes induced by ligands possessing a substantial α_5 -efficacy may be, at least partly, contributed by modulation at GABA_A receptors containing the α_5 subunit. It therefore could be of importance to avoid substantial agonist potentiation of α_5 -subunits by candidate anxiolytic anxiolytics, if clinical sedation is to be avoided. Nevertheless, as a caveat to all studies examining sedation, it should be remembered that a decrease in automatically measured locomotor activity can be due to a variety of causes other than sedation, including the occurrence of stereotyped behavior, motor impairments or pain [72]. To dissect these overlaps in activity and uncertainties, much is expected from screening of even more selective BZ site ligands in the future.

Flavonoids

Flavonoids represent a class of non-Bz ligands that fit extremely well within the unified pharmacophore/receptor model. These compounds are a class of natural products isolated from a variety of herbal plants and employed as tranquilizers in folk medicine. They exhibit a wide range of biological activity, such as antiviral, anti-inflammatory, antithrombotic, antioxidant and estrogenic effects [73, 74]. Flavonoids also displayed potent anxiolytic effects and appeared devoid of myorelaxant, amnesic or sedative actions. Haberlein *et al.* has shown that the steric orientation of the substituents which lie coplanar to the aromatic ring was crucial for ligand affinity to the Bz BS [75]. This was especially true for the C(5) and C(6) positions. Furthermore, the recent work of Huang *et al.* indicated that 6,3'-dinitroflavone **25** exhibited a K_i value of 12 nM at the Bz BS (Table 2) and was an extremely potent anxiolytic devoid of muscle relaxant effects. The 6-Br-3'-nitroflavone **26** also demonstrated increased binding affinity, however, the anxiolytic effect was lower than the dinitroanalogue **25** [76].

Table 2. Affinities of a Series of Flavonoid Ligands for Benzodiazepine Receptors [76]



Compound	R ₆	R _{3'}	K _i (nM)
20	F	NO ₂	182
21	Cl	NO ₂	8.0
22	Br	Cl	17.0
23	Cl	Br	23.0
24	Br	Br	19.1
25	NO ₂	NO ₂	12.0
26	Br	NO ₂	1.0

The flavonoids **21**, **25** and **26** fit very well within the unified pharmacophore/receptor model. The alignment of 6,3'-dinitroflavone **25** within the $\alpha_2\beta_3\gamma_2$ subtype is shown in Fig. (11). The centroid of the phenyl moiety overlapped with the L₁ region

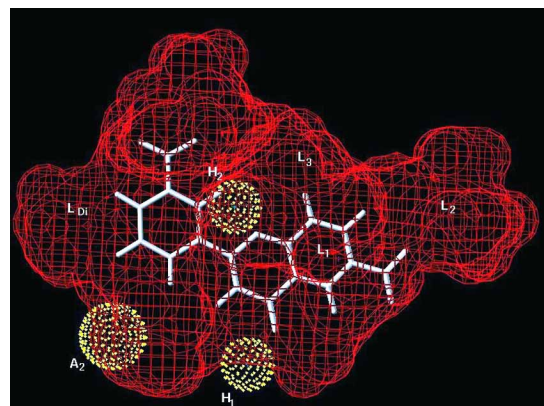


Fig. (11). Alignment of **25** within the included volume of the $\alpha_2\beta_3\gamma_2$ subtype.

and the 3'-nitro group occupied region L₂, while the lone pair of electrons of the carbonyl oxygen (O) atom interacted with H₁ to form a hydrogen bond between the ligand and the receptor. A second hydrogen bond was formed between the oxygen lone pair and H₂ of the receptor protein. This alignment essentially agrees with what has been discussed by Marder [77].

Recently, some flavone-related analogs were prepared (Fig. 12) [78] however, these flexible ligands with additional lipophilic groups did not bind to any GABA_A receptor subtype. It is believed that more planar ligands are required for affinity to these subtypes, as the twist chair of the dihydropyran unit may have prevented the fit required for high affinity at the Bz BS. Together these data, combined with the work of others, indicate the phenyl ring, the carbonyl group and the double bond between C(2) and C(3) of the flavone are the key structural features that contribute to the binding affinity of flavonoids to the Bz BS.

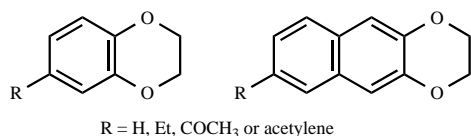
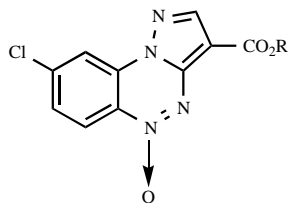


Fig. (12). A series of flavone-related analogs that was inactive at the Bz BS.

Analogues of 8-chloropyrazolo[5,1-c][1,2,4]-benzotriazine 5-oxide

It has been recognized that small structural modifications in the same chemical family could lead to ligands which display different intrinsic activity. Costanzo *et al.* reported that small structural modifications in analogs of 8-chloropyrazolo[5,1-c][1,2,4]-benzotriazine 5-oxide produced ligands that displayed different intrinsic activity (Table 3) [79]. When an aryl ester function occupied position-3, this class of ligands exhibited high affinity at the Bz BS (Table 3, K_i = 11 – 35 nM). It was proposed the methoxy group of ligand **38**, which bound with an affinity of 1.0 nM, acted

Table 3. Affinities of a Series of 8-Chloropyrazolo[5,1-c][1,2,4]-benzotriazine 5-oxide Ligands at the Bz BS of GABA_A Receptors [79]



Ligand	R	K _i (nM)
27	Ph	47.5
28	4-F-Ph	146.0
29	2-F-Ph	14.0
30	4-Cl-Ph	49.2
31	2-Cl-Ph	14.4
32	4-Me-Ph	51.9
33	2-Me-Ph	38.3
34	4-NO ₂ -Ph	41.8
35	2-NO ₂ -Ph	106.0
36	4-MeO-Ph	11.6
37	3-MeO-Ph	41.1
38	2-MeO-Ph	1.0

as an electron donor group to enhance the π - π stacking interactions between the phenyl ring of this ligand and the receptor protein. However, it may also be that the methoxy group enhanced lipophilic interactions within the L₁ region. Costanzo *et al.* proposed that net inductive and resonance effects as well as the electron donating properties (σ = - 0.27) and suitable lipophilic features ($-\pi$) of the ligand facilitated this interaction.

Alignment of **38** overlaid with diazepam is shown in Fig. (13), wherein the N(1) and N(4) functions hydrogen bonded to the H₂ and H₁ donor receptor sites, respectively. It was felt that the 3-ester function fits into a limited dimension lipophilic pocket in the L₁/L₂ region. Moreover, the orientation of the lone pair of electrons of the carbonyl oxygen atom of the ester function reinforced the receptor binding by means of a 3-centered hydrogen bond (N(4)/CO/H₁). This hydrogen bond, which is similar to that described for the C(3) ester substituent in β -carbolines, is also strengthened by the interaction between the 5-oxide group and the nitrogen atom with H₁.

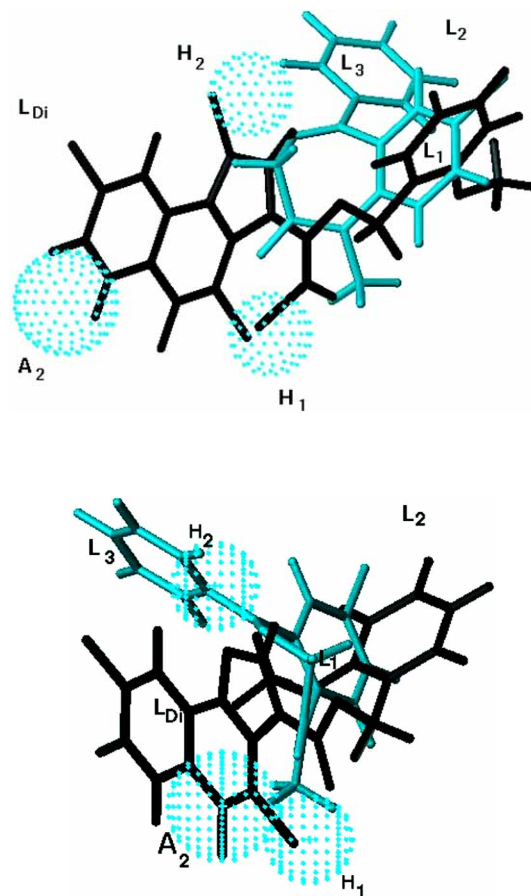
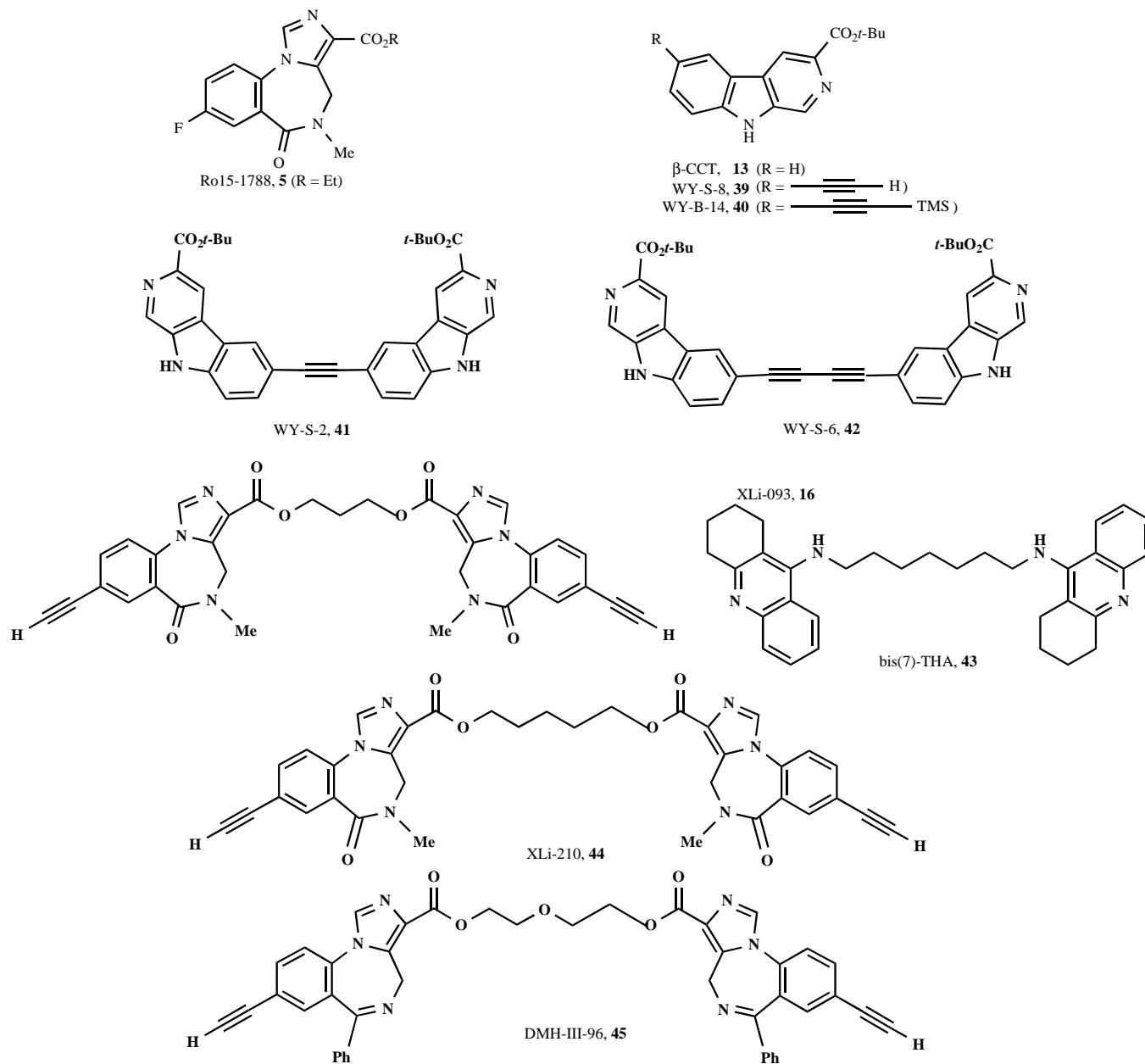


Fig. (13). Alignment of ligand **38** (black) overlaid with diazepam (cyan) within the unified pharmacophore/ receptor model and rotated 90 degrees.

Dimer Affinity for the Binding Site

Some studies which involved monomeric *i*-BZDs indicated that there might be a limit on the size of 3'-imidazo substituents the receptor may accommodate [53]; However, studies that involved bivalent ligands suggested otherwise (Table 4) [80, 81]. It is thus possible that the spacer for *i*-BZDs dimers threaded the second portion of the bivalent ligand through and/or around the L_{Di}/A₂ region that presented steric hindrance for the monomers.

Design of the bivalent ligand XLi-093 **16** was based on the modeling of the α 5-selective monomer RY-80 **52** in the α 5 pharmacophore model. The ability of this ligand to bind (Table 4) and

Table 4. Structures and Affinities at the $\alpha\beta\gamma 2$ Subtype [53]. NR = not reported; ANT = antagonist; IA = inverse agonist; ND = not determined yet. Activity is Defined for Receptor Subtype Listed in Bold

Ligand	K_i (nM)						Activity
	$\alpha 1$	$\alpha 2$	$\alpha 3$	$\alpha 4$	$\alpha 5$	$\alpha 6$	
5, Ro15-1788	0.8	0.9	1.05	NR	0.6	148	ANT
13 , β -CCT	0.72	15	18.9	NR	110.8	5000	IA
39 , WY-S-8	0.97	111	102	NR	208	1980	ND
40 , WY-B-14	6.8	30	36	2000	108	1000	ND
41 , WY-S-2	30	124	100	300	300	4000	ND
42 , WY-S-6	120	1059	3942	NR	5000	5000	ND
16 , XLi-093	1000	1000	858	1550	15	2000	ANT
43 , bis(7)-THA	NR	NR	NR	NR	NR	NR	ANT
44 , XLi-210	231	661	2666	NR	5.4	54.2	ND
45 , DMH-III-96	460	5000	NR	NR	5000	5000	ND

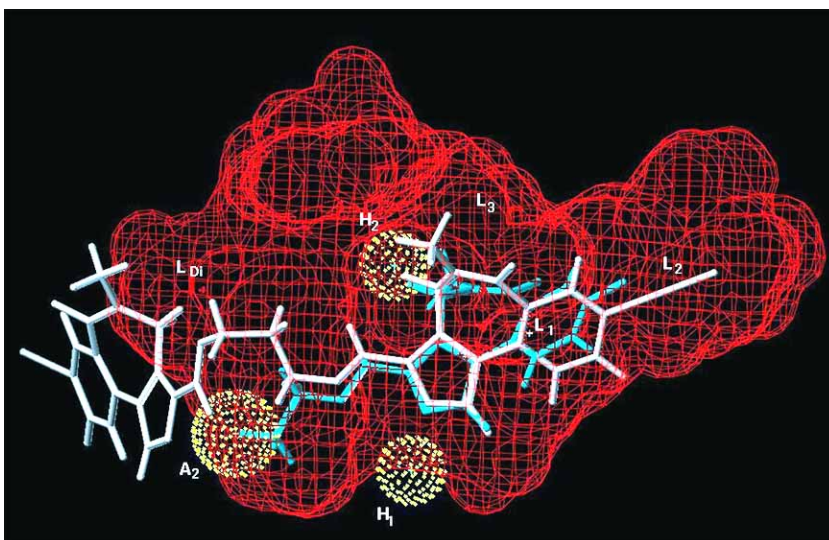


Fig. (14). Alignments of XLi-093 **16** (white) and Ro15-1788 **5** (cyan) within the included volume of the $\alpha 5\beta 3\gamma 2$ subtype.

fit well within the pharmacophore (Fig. 14) requires the 3-carbon linker to be in a linear (*versus* folded) conformation. This linear conformation of XLi-093 has been confirmed both in the solid phase and in solution (Fig. 14) [82]. The J values calculated from the dihedral angles ($J = 5.38$) were in excellent agreement with those determined from the solution NMR spectrum ($J = 6.39$). Because this bivalent ligand is the most $\alpha 5$ -selective ligand reported, the enriched selectivity of this dimer and those similar to it, was presumably entropic in nature, as the loss of affinity at the other subtypes was profound [81, 82].

It has been shown *via* crystallographic and solution NMR studies that modification of the aliphatic spacer to a $-\text{CH}_2\text{OCH}_2-$ or a $-(\text{CH}_2)_2\text{O}(\text{CH}_2)_2-$ group, provided the bivalent ligand with a folded conformation (Fig. 15) [82]. Modeling of this type of ligand (*e.g.* DMH-III-96 **45**, Fig. 16) within the $\alpha 5$ pharmacophore model illustrated the inability of bivalent ligands in the folded conformation to bind presumably because they are too hindered to access the Bz BS. Current data displaying these trends is shown in Table 5, while further studies are underway to evaluate the length the lipophilic spacers may contribute toward the selectivity of both the benzodiazepine and β -carboline bivalent ligands (*e.g.* XLi-210 **44** and WY-S-6 **42**).

Success of XLi-093 and earlier *in vitro* data on WY-S-8 **39** and WY-B-14 **40** (Table 4) indicated the C(6)-substituent of β -

carbolines lies in the L_{Di} region of the pharmacophore model or in the extracellular domain of Bz BS. For this reason, the β -CCT dimer, WY-S-2 **41** was designed and synthesized. Although the $\alpha 1$ subtype selectivity was not amplified with this particular β -carboline dimer, the ligand does bind. It was proposed that the two-carbon linker was not long enough and that crowding between the second β -CCT unit and the receptor protein decreased the binding affinity at $\alpha 1$ subtype, thereby negating potential selectivity [80]. Further evaluation of the β -CCT bivalent ligand with a bis-acetylene linker (WY-S-6 **42**, Fig. 17) should shed light on this hypothesis.

Similar to XLi-093, the dimer bis-(7)-THA **43** of Wang *et al.* was determined to be a competitive antagonist at the Bz BS in both electrophysiological experiments and receptor binding assays (Table 4) [73]. Although additional experimental data is needed, inspection of the alignment of XLi-093 (Fig. 14) and our receptor model (see below) indicated the aliphatic linker of these bivalent ligands would thread the second half of the dimer through the $\text{L}_{\text{Di}}/\text{A}_2$ regions and toward the solvent accessible space outside of the pocket.

Beta-Carbolines

The crystal structures and molecular mechanics simulations of several β -carbolines has recently been reported [83]. In general,

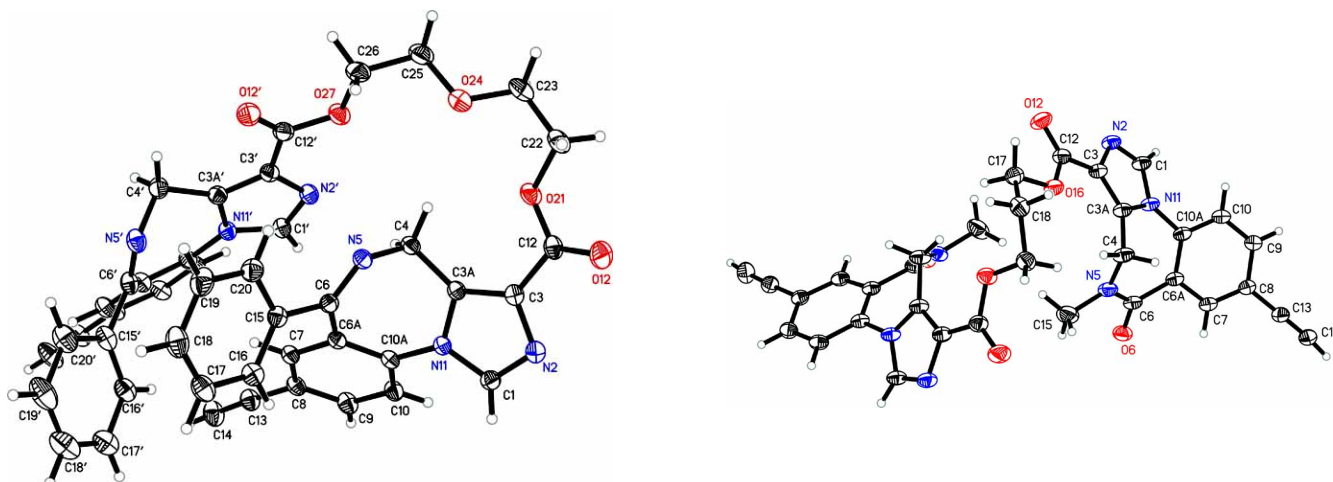
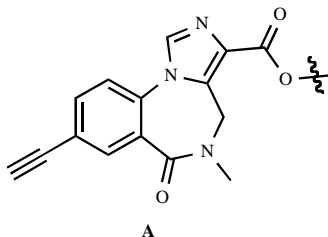


Fig. (15). Crystal structures of the linear XLi-093 **16** (left) and the folded DMH-III-96 **45** (right). Figures reprinted with permission [81, 82].

Table 5. The Molecular Composition and Stable Conformation of Various Bz BS Bivalent Ligands. ND = not determined yet

Ligand	Monounit 1	Monounit 2	Spacer	Conformation in solution	Crystal structure
16 , XLi-093	A	A	(CH ₂) ₃	linear	linear
44 , XLi-210	A	A	(CH ₂) ₅	linear	ND
46 , XLi-347	A	A	(CH ₂) ₂ O(CH ₂) ₂	folded	ND
47 , XLi-374	A	A	CH ₂ OCH ₂	folded	ND
45 , DMH-III-96	-	-	(CH ₂) ₂ O(CH ₂) ₂	folded	folded

substitution at C(3) and C(6) had the greatest effect on affinity. Consistent with the interaction with the H₁ descriptor, high affinity ligands were always associated with groups able to interact as hydrogen bond acceptors at C(3). Furthermore, affinity was much

lower for constrained β -carboline derivatives which contained the carbonyl group in the *anti* conformation, in agreement with the proposed 3-centered hydrogen bond [42, 50, 52, 61] afforded by many β -carboline derivatives in the stable *syn* conformation with the H₁ descriptor.

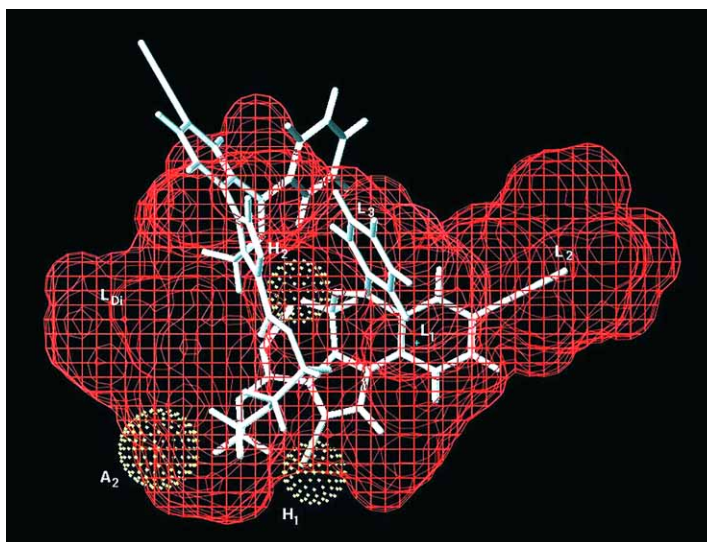
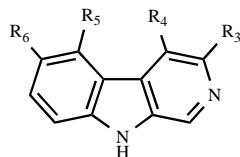


Table 6. Structures and Affinities at α 5 β 2 γ 2 Recombinant Receptors and Modulation by β -Carbolines. pAg = partial agonist; Ag = agonist [84]

Ligand	K _i (nM)										Activity
	R ₆	R ₅	R ₄	R ₃	α 1	α 2	α 3	α 4	α 5	α 6	
12, 6-PBC	On-Pr	H	MOM	CO ₂ Et	0.49	1.21	2.2	NR	2.39	1343	pAg
11, ZK-93423	OCH ₂ Ph	H	MOM	CO ₂ Et	4.1	4.2	6	NR	4.5	1000	Ag
48, ZK-91296	H	OCH ₂ Ph	MOM	CO ₂ Et	NR	NR	NR	NR	NR	NR	pAg
49, Abecarnil	OCH ₂ Ph	H	MOM	CO ₂ -i-Pr	12.4	15.3	7.5	NR	6	1000	pAg

As previously stated, the C(6)-substituent of β -carbolines lies in the L_{Di} region of the unified pharmacophore/ receptor model for inverse agonists and antagonists. However, based on the CoMFA studies of Huang *et al.* [78] β -carboline agonists have been proposed to take on a vertical alignment with respect to the horizontal alignment of inverse agonists and antagonists. While only four β -carbolines have been reported to display agonistic activity, each of these compounds had a 4-methylmethoxy group that permitted interaction with the H₂ descriptor and partial interaction with the region near the entrance to the L₃ pocket (Table 6). It was postulated that 6-PBC **12** should display agonistic activity, and indeed, since partial occupation of a lipophilic region near the C(6)-substituent is probable (relative to ZK-93423 **11**), 6-PBC is a partial agonist [42, 84]. However, it is likely that the type of activity displayed by 6-PBC may be receptor subtype-dependent, because when evaluated *in vivo*, it inhibited PTZ-induced seizures in a dose-dependent fashion and exhibited anxiolytic activity when evaluated in the elevated plus-maze paradigm. Yet, unlike typical 1,4-benzodiazepines, 6-PBC **12** was devoid of muscle relaxant activity and even antagonized the muscle relaxant/ataxic activity of diazepam [85, 86]. Further studies are needed to fully address this issue.

LIGANDS THAT OCCUPY THE L₂ REGION AND ARE SELECTIVE FOR α 5 CONTAINING RECEPTORS

RY-24 and Related Analogs

Continued interest in the development of α 5 selective ligands goes forward in the CNS area for many reasons. One of these involves the localization of these receptors and their presumed importance in developmental biology. Over 30 % of GABA_A receptors in neonatal rat pups are comprised of α 5 β 3 γ 2 subtypes, whereas they only comprise about 5 % in adult rats [87]. In addition, these α 5 subtypes are primarily found in the hippocampus [88], which prompted interest in memory and learning [25]. From the evaluation of K_i values, it was found that many 8-substituted *i*-BZDs, such as RY-24 **50** and RY-80 **52** and their trimethylsilyl precursors **51** and **53** (Table 7), exhibited a significant degree of binding selectivity at the α 5 subtype *in vitro*. This observation was in agreement with the previous hypothesis that correct occupation of the L₂ region can promote α 5 selectivity of a ligand [29, 51]. Therefore, the efficacy of the α 5-subtype selective ligand RY-24 **50** was evaluated *in vitro*. Recently, it was determined that this ligand was a potent inverse agonist at the α 5 subtype with a much weaker efficacy at the other subtypes (Fig. 18). These results confirmed previous binding data, which indicated that this ligand was α 5-selective due to the lipophilic C(8)-substituent which fully occupied the L₂ pocket [28, 29]. The data were also in agreement with previous studies *in*

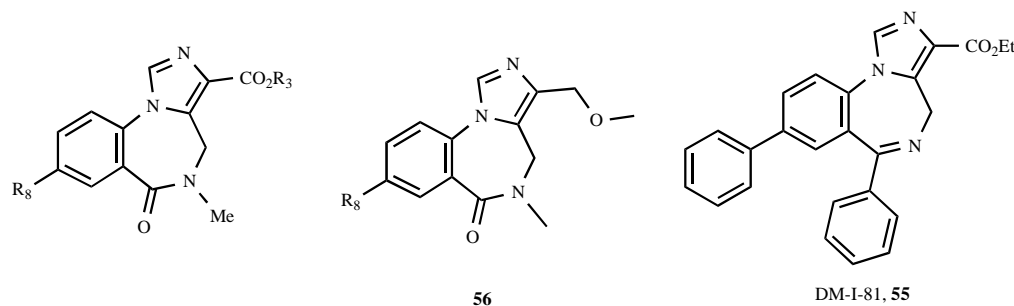
vivo, which indicated that some of these ligands enhanced cognition while other Bz BS ligands were not as effective [25, 89]. Furthermore, ligands devoid of a lipophilic substituent at the C(8) position showed no selectivity for the α 5 subtype (Table 7).

Inspection of Table 7 revealed some observations worth noting. While the lipophilic substituent at R₈ of RY-24 **50** and RY-80 **52** decreased the affinity for α 1, α 2 and α 3, it retained affinity for α 5 and actually increased affinity for the α 6 subtype. Furthermore, selective affinity of *i*-BZDs at the α 5 subtype was independent of the occupation of the L₃ pocket, as illustrated by the *in vitro* data of DM-I-81 **55** (Table 7). This data again supports the importance of the occupation of the lipophilic pocket L₂ for potent selectivity at α 5 subtype.

June *et al.* recently reported the neurobehavioral results of RY-23 and RY-24 in rats. In agreement with previous studies [28], these α 5 selective ligands were highly selective in suppressing ethanol-maintained responding (Fig. 19) [91]. As previously stated, the hippocampus contains the greatest concentration of α 5-containing receptors in the CNS [88, 92], and it is possible that these hippocampal α 5 receptors may regulate alcohol-motivated responding following systemic administration of an α 5-selective agent. Furthermore, RY-24 also antagonized the motor-impairing and sedative effects of ethanol in Long-Evans rats. Combined with additional studies within the ventral pallidum (VP), it has been proposed that the GABAergic systems within the VP and hippocampal pathways may represent new extensions of the mesolimbic ethanol reward circuitry. Although these data do not strongly support a direct role for the modulatory influences of intrinsic efficacy in the behaviors examined, the synthesis of α 5 subtype selective ligands provides researchers a unique opportunity to explore the role of this subtype in the neurobehavioral effects of alcohol [28, 93].

In studies involving the α 1 subtype, β -CCT **13** and 3-PBC **14** were observed to selectively reduce alcohol-motivated behaviors for a variety of experiments [94, 95]. However, unlike the α 5 selective inverse agonist RY-23, both the β -carboline antagonists β -CCT and 3-PBC displayed mixed agonist-antagonist profiles *in vivo* in alcohol P and HAD rats. Therefore, in addition to being able to study the molecular basis of alcohol reinforcement, α 1 Bz BS ligands which display mixed agonist-antagonist pharmacology in alcohol P and HAD rats may be capable of reducing alcohol intake while eliminating or greatly reducing the anxiety associated with habitual alcohol, abstinence or detoxification. Thus, these types of ligands may be ideal clinical agents for the treatment of alcohol-dependent individuals [94, 95].

Additional behavioral studies of RY-24 were performed by Helmstetter *et al.* and provided further support for the role of the

Table 7. Structures and Affinities of Some $\alpha 5\beta 3\gamma 2$ -Subtype Selective Ligands

Ligand	R_8	R_3	K_i (nM)				
			$\alpha 1$	$\alpha 2$	$\alpha 3$	$\alpha 5$	$\alpha 6$
50, RY-24	— H	<i>t</i> -Bu	26.9	26.3	18.7	0.4	5.1
51, RY-23	— TMS	<i>t</i> -Bu	197	143	255	2.61	58.6
52, RY-80	— H	Et	28.4	21.4	25.8	0.49	28.8
53, RY-79	— TMS	Et	121	142	198	5.0	114
54	H	Et	1.2	2.0	1.1	0.4	> 300
55, DM-I-81	Ph	NA	> 2000	> 2000	> 2000	176	> 2000
56, PWZ-029 ⁹⁰	Cl	NA	>300	>300	>300	38.8	>300

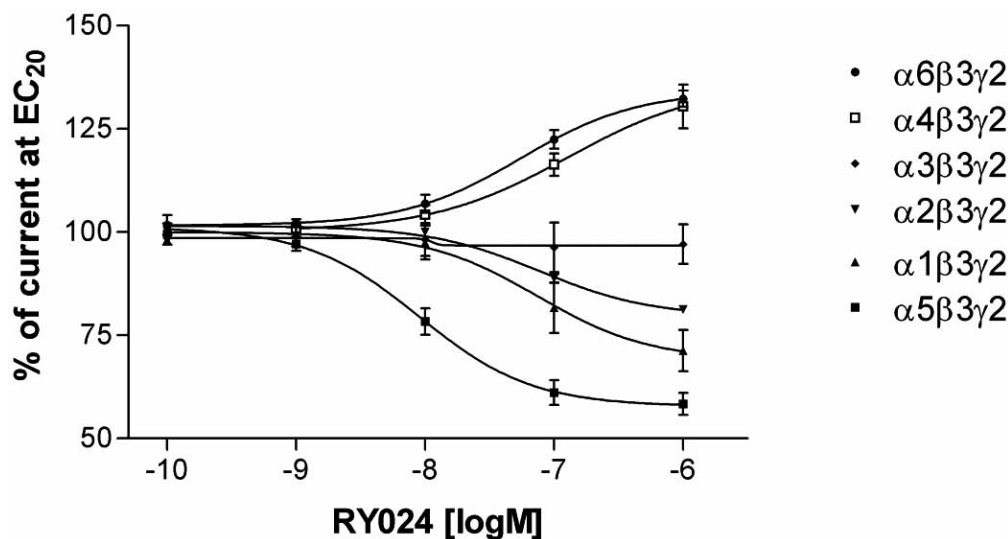


Fig. (18). Subtype efficacy of RY-24 50. Dose response curves for RY-24 in oocytes expressing different subunit combinations of GABA_A receptors. Subtype combinations are indicated in legends. cRNA-injected *Xenopus* oocytes were held at -60 mV under two-electrode voltage clamp. Increasing concentrations of RY-24 were superfused together with a GABA concentration eliciting $\sim 20\%$ of the maximal current amplitude. RY-24 was pre-applied for 30 sec before the addition of GABA, which was co-applied with the drugs until a peak response was observed. Data were normalized for each curve assuming 100% for the response in the absence of RY-24. RY-24 was made up and diluted as stock solution in DMSO. Final DMSO concentrations perfusing the oocyte were 0.1%. Values are presented as mean \pm SD of at least 4 oocytes from at least 2 batches.

hippocampus in anxiety and learning [25]. Moreover, the data suggested that Bz BSs within the hippocampus are important for the acquisition of fear conditioning. Although this subtype selective ligand has been shown to be an inverse agonist at the $\alpha 5$ subtype [29, 96], this study suggested that RY-24 may act as an agonist at other alpha subtypes because larger doses of RY-24 were not as anxiogenic as the smaller doses and resulted in decreased learning. Consistent with the studies of Stephens *et al.* using $\alpha 5$ knock-out mice [97] and the efficacy studies of Lüddens, June and Cook *et al.* [98] these findings support the concept that the pharmacology observed depends upon the dose, behavioral paradigm employed and subunit composition activated. Ligands such as RY-24 have proven to be valuable in the study of the biochemical and pharmacological

properties of GABA_A receptors and have permitted insight into the role this protein plays in anxiety and learning.

QH-ii-066

Due to the pharmacological profile RY-24 exhibited *in vivo*, the development of additional $\alpha 5$ subtype selective ligands was pursued. Thus, the 7-acetyleno analog of diazepam, QH-ii-066 **57**, was synthesized and was determined to also exhibit a binding and functional selectivity at the $\alpha 5$ subtype over the $\alpha 1$ subtype (Table 8) [27]. This was due to the full occupation of the L_2 pocket, relative to diazepam (Fig. 20). To our knowledge, this was the first agonist ligand to display some $\alpha 5$ selectivity from the 1,4-benzodiazepine

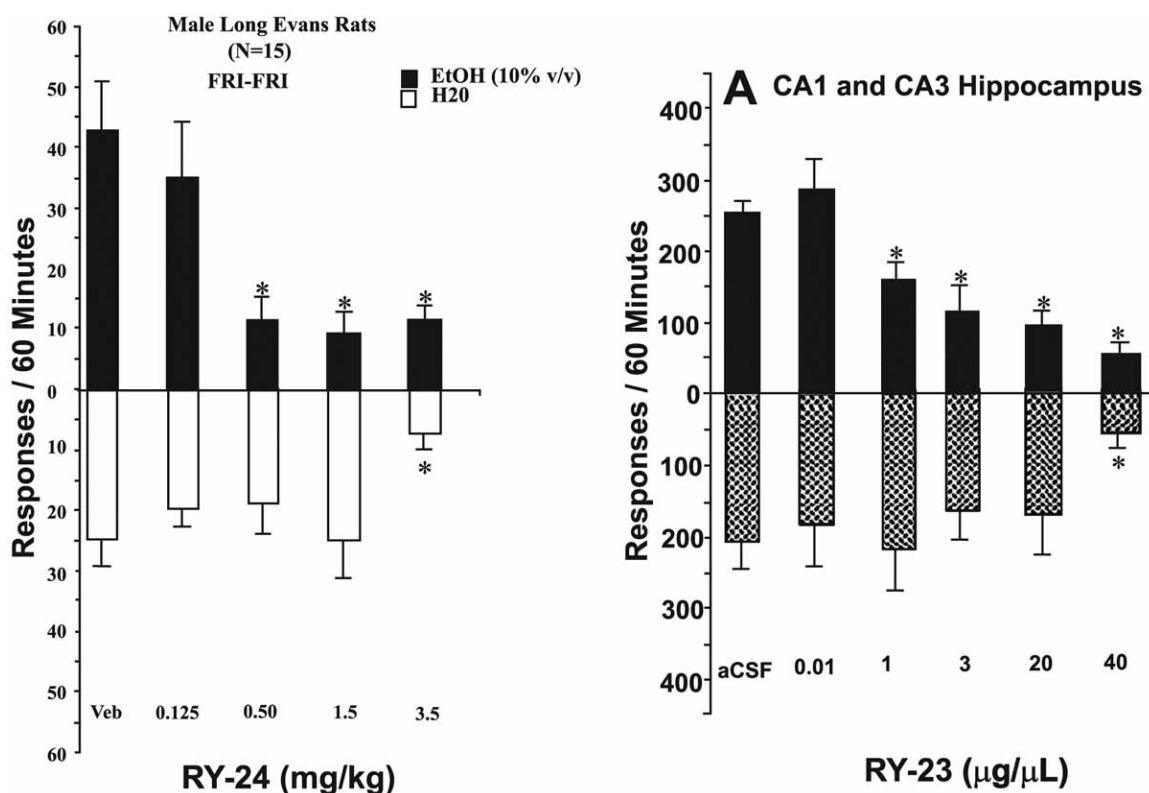


Fig. (19). Suppression of alcohol-motivated responding by RY-24 **50** and RY-23 **51** [93, 96]. Left: Dose-response of IP RY-24 (0.0–3.5 mg/kg) and vehicle on responding maintained by ethanol (10 % v/v) (*top panel*) and water (*bottom panel*) in male Long-Evans rats. Right: Dose-response of unilateral infusions of RY-23 (0.0–40 µg) in the hippocampus on a concurrent fixed-ratio (FR4) schedule for ethanol (10 % v/v) (*top panel*) and saccharin-maintained (0.025 % v/v) (*bottom panel*). For both studies, $p \leq 0.05$ versus control condition values was determined using ANOVA and *post hoc* Newman-Keuls test. Each bar represents the mean (\pm SEM) ($n = 15$ for RY-24 and $n = 7$ for RY-23). Figures reprinted with permission of the authors [93, 96].

family. Importantly, the 7-cyano congener **58** (Table 8) did not potently bind to recombinant receptors of the $\alpha 5$ subtype, which is in agreement with earlier work of Haefely and Fryer *et al.* on the SAR of 1,4-benzodiazepines [99, 100]. This cyano ligand also did not exhibit any subtype selectivity, re-emphasizing that occupation of the L_2 region with lipophilic groups is important for $\alpha 5$ selectivity as well as for high affinity. The selective efficacy of this QH-ii-066 ligand over the $\alpha 1$ subtype was demonstrated by reversing the convulsant actions of RY-24 **50**, an $\alpha 5$ -selective inverse agonist, in NIH mice [87]. This ability was not observed at comparable doses for the $\alpha 1$ -selective agonist zolpidem **7**.

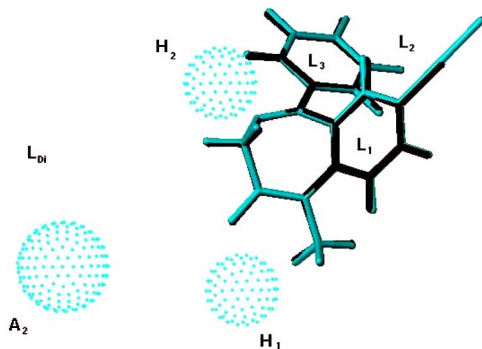
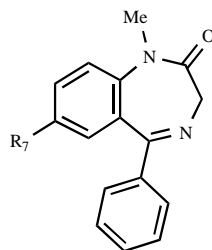


Fig. (20). Comparison of non-selective diazepam (black) with the $\alpha 5$ -selective QH-ii-066 (cyan) when aligned within the unified pharmacophore/receptor model. The acetylene group of QH-ii-066 increased the occupation of the L_2 region relative to that of diazepam.

Furthermore, Lelas and Cook *et al.* have recently determined that although QH-ii-066 had similar affinity for the DS subtypes in rats, it displayed functional selectivity *in vivo*, with diazepam-like efficacy at the $\alpha 5$ subtype and partial efficacy at the $\alpha 1$ subtype [27]. The study also indicated that this 7-acetyleno substituted diazepam analog exhibited less potency in protection against ECS-induced seizures relative to diazepam than against PTZ-induced seizures. Hence, the $\alpha 1$ subtype may play a more prominent role in ECS-induced seizures than in PTZ-induced seizures [27].

COMPARATIVE MODEL OF THE BENZODIAZEPINE BINDING SITE

Crystallization of GABA_A receptors thus far has not been accomplished, but the successful structure determination of the water-soluble acetylcholine binding protein (AChBP) [101] has generated much interest in the GABA_A receptor community. Although this protein shares only ~ 18 % sequence homology with the extracellular domain of the GABA_A receptor [101], the structural resemblance has been estimated to be relatively high (60 – 75 %) [48]. Several comparative modeling studies have used the AChBP structure to derive models of the extracellular domain of GABA_A receptors [47, 48]. Following the cryo-EM determination of the extracellular and transmembrane domain structure of the nACh receptors, these structures also were used as templates for modeling GABA_A receptors [102]. Sequence homology is so low however, that detailed features of the models are highly uncertain, and the proposed dockings of Bz BS ligands [103–105] have a qualitative character and do not sufficiently explain the observed differential effects of

Table 8. Structures and Affinities of 1,4-Benzodiazepines

Ligand	R ₇	K _i (nM)				
		α1	α2	α3	α5	α6
1, diazepam	Cl	14	20	15	11	> 3000
57, QH-ii-066		76.3	42.1	47.4	6.8	> 3000
58	C≡N	320	310	350	265	> 3000

ligands interacting with different receptor subtypes. The experimental structure of the nAChR [106] has provided first data on how much the fold can vary between members of a family [106]. From this data the extent to which GABA_A receptor subunits differ from each other in structure can be qualitatively extrapolated, but not predicted in detail.

As mentioned before, the majority of GABA_A receptors are composed of 1γ, 2α and 2β subunits. Each subunit has per convention a “plus” and a “minus” side (Fig. 21). The subunit interfaces consequently consist of the plus and minus sides of neighboring subunits. The modulatory Bz BS is located at the α*γ subunit interface and is larger than, but homologous to the two agonist (GABA) binding sites, which are located at the β*α subunit interfaces [49, 107, 108]. The absolute subunit configuration for the α1β2γ2 GABA_A receptor appears to be γβαα, when viewed counter clockwise (from + to -, Fig. 21) [48, 109-111].

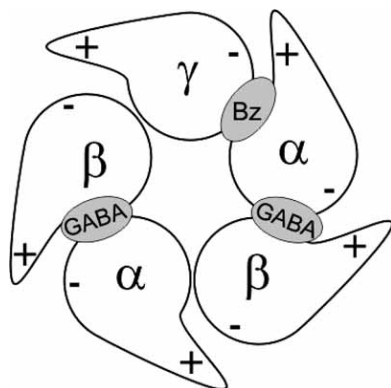


Fig. (21). Absolute subunit arrangement of the α1β2γ2 GABA_A receptor when viewed from the synaptic cleft. The GABA binding sites are located at the β*α subunit interfaces and the modulatory Bz binding site is located at the α*γ subunit interface. The part of the schematically drawn subunits marked by the + indicates loop C of the respective subunits.

While the γ2 subunit is required for recognition and binding of benzodiazepines as well as many other substance classes that act *via* the Bz BS [112, 113], it is now clear that sequence variations between different α and γ subunits determine subtype selectivity and efficacy of Bz BS ligands [20, 112-114]. The Bz BS has been proposed to consist of three segments provided by the α* side, the so-called “loops A, B and C” and by three segments of the γ*, the so-called “loops D, E and F” [112, 113]. These segments were then confirmed by X-ray crystallographic and EM-structures of AChBP [101] and the nAChR [106] to form a groove-like pocket at

the interface boundary between subunits that appears to be conserved in the entire superfamily.

Even later than the nAChR structure, a series of AChBP crystal structures with co-crystallized ligands appeared [115]. These structures revealed how ligand binding can alter the local conformation of the binding site. Particularly loop C has been found to be a highly mobile subdomain, additional more subtle changes are seen along the entire subunit boundary [115]. These findings are consistent with the hypothesis that many receptor conformations exist, that are separated by low energy barriers, and can be stabilized by different ligands. Unfortunately it cannot be decided a priori which of the experimental structures is the “best” template to model a particular receptor/ligand complex. Depending on template and alignment choice, model Bz pockets differ in total volume by as much as 40% and can vary by several Angstrom in the distances between key residues in the binding site loops.

Although changes in protein conformation may be minor, it has been demonstrated that they can profoundly affect the efficacy of Bz BS ligands [116]. Furthermore, efficacy can vary for the same ligand at different GABA_A receptor subtypes [27, 31, 35]. As proteins are inherently dynamic and able to sample many conformations, and the stabilization of the active state relative to the inactive state has been calculated to be less than 1 kcal/mol [117], it is impossible to provide absolute assignments of specific side chains to specific descriptors for any particular conformation. This conformational flexibility may imply that residues which satisfy certain pharmacophoric descriptors can vary, resulting in a “soft” orientation of the pharmacophore in the receptor. Thus, a unified view of a pharmacophore model and a homology derived receptor model will assign large areas of lipophilic interaction to specific regions in the protein, but allow a flexible assignment of specific interactions such as H-bridges or π-π stacking.

RELATIVE ORIENTATION OF THE PHARMACOPHORE WITHIN THE COMPARATIVE MODEL

Prior to structure determination of the AChBP, we published a review which evaluated results of site-directed mutagenesis and provided insights as to where certain side chains in the Bz BS might be located relative to the pharmacophoric descriptors [118]. However, with the knowledge gained from recent experimental data and with the aid of our GABA_A receptor models, built by homology to lymnea AChBP [47], aplysia AChBP and the nAChR [102] an update of the orientation is provided here. We now propose an orientation which is favored by experimental evidence, allows some degree of conformational flexibility which can lead to variable as-

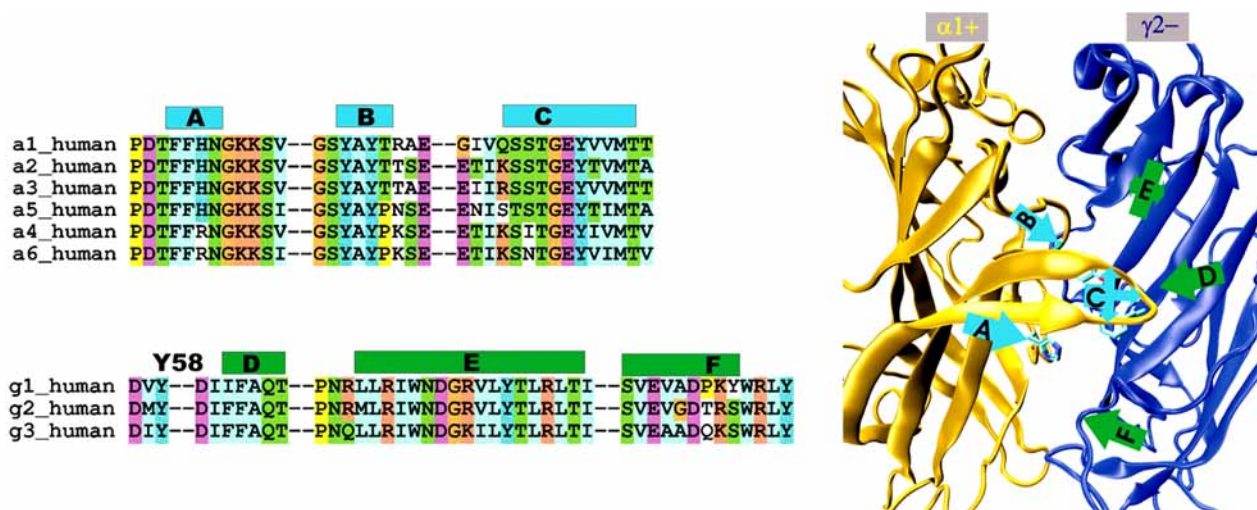


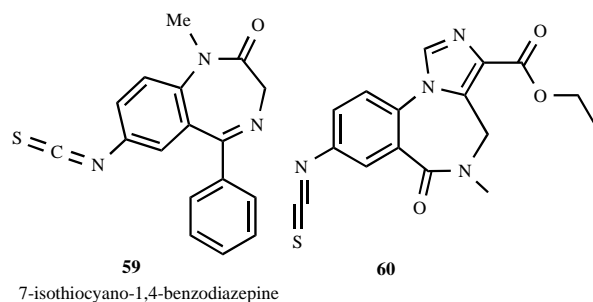
Fig. (22). Alignment and homology model depiction of the so called “loops A-F” and flanking regions of the human sequences of the Bz recognition site in different subunits. The homology model is as seen from “outside” of the channel mouth, the membrane would be parallel to the lower edge of the image. Key residues are shown in stick representation: Loop A His102, loop B Tyr 160 and loop D Phe77.

signments for H-bridge interactions, but is based on specific areas of lipophilic interactions that are determined by binding site geometry.

Evidence from Covalently Reactive Ligands Allows to Position the L2 Lipophilic Pocket

Covalent labeling studies contributed significantly to the determination of residues that are located within the Bz BS [104, 119-124].

Table 9. Ligands Used for Affinity Labeling Studies [104, 121-124]



7-isothiocyano-1,4-benzodiazepine

Ligand	Activity	Site of Interaction
2, [³ H]flunitrazepam	Agonist	α 1H102
6, [³ H]Ro15-4513	inverse agonist	α 1Y210
59	Agonist	α 1H101C (rat)
60	part. Agonist	α 1H101C, α 1G157C α 1V202, α 1V211C (rat)

Photoincorporation studies with the agonist [³H]flunitrazepam identified α 1H102 of the human sequence as the primary site of incorporation [123]. Although it is possible that flunitrazepam is coupling *via* the nitro group at the 7-position, the coupling group of flunitrazepam is currently not known. Studies with the inverse agonist [³H]Ro15-4513 indicated α 1Y210 as the primary site of incorporation [104]. Thus, the azido group at the 7-position of Ro15-4513 should be in close apposition to α 1Y210, assuming no rearrangement of the photo-activated intermediate. Further information comes from recent studies reporting the covalent coupling of 7-isothiocyano- derivatives of a 1,4-benzodiazepine, (substance 59

[122]) and of Ro15-1788 (substance 60 [124]) to GABA_A receptors in which individual amino acid residues had been mutated to cysteines. Primary site of reaction of both substances is the rat α 1H101C mutant that is homologous to α 1H102 of the human subunit. Thus, the 7-substituent both of 1,4-benzodiazepines and of imidazobenzodiazepines appears to be in apposition to α 1H102. The imidazobenzodiazepine 60 reacts with additional cysteines in positions corresponding in the human sequence to positions α 1V203C and α 1V212C in the loop C stem, and with α 1G158C in loop B (see alignment in Fig. 22). Thus, the 7-substituent of this compound, in agreement with the data from photolabeling H102 with flunitrazepam and Y210 with Ro15-4513, is in apposition to loop A, the loop C base, and additionally loop B.

Thus, all the findings from covalently incorporated ligands given above can be reconciled by placing the 7-substituent, and thus the L2 descriptor of the unified pharmacophore model, between the loop C base near α 1V203/ α 1V212/ α 1Y210 and loop A α 1H102. The homology models reveal that this is not only topologically possible, but also provides the additional apposition to loop B that is suggested by the reaction of 60 with rat α 1G157C. Thus, the L2 lipophilic pocket is most likely formed by the base of loop C, together with parts of loop A.

THE A2 DESCRIPTOR AND THE L_{DI} REGION

Experimental data yields some very convincing evidence for assigning the A2 descriptor and the L_{DI} region of lipophilic interaction next to A2 to protein segments close to residues γ 2A79 and γ 2T81 at loop D. Certain imidazobenzodiazepines, which occupy the pharmacophore volume close to A2 (see Fig. 5), are more sensitive to γ 2A79 mutations than classical benzodiazepines or benzodiazepines that lack a 3'-imidazo substituent. This was shown in a study where mutations of γ 2A79 and γ 2T81 had little effect on the binding of the agonists diazepam and flunitrazepam, but in a roughly size dependent fashion decreased the affinities of antagonist Ro15-1788 and inverse agonists Ro15-4513 [125, 126]. In agreement with other studies, midazolam, which lacks a 3' substituent, behaved more closely to classical benzodiazepines than *i*-BZDs [44, 125, 127, 128]. This trend is enhanced by rigid and bulky 3'-imidazo substituents, altogether suggesting that γ 2A79 and γ 2T81 are located close to the region of the Bz BS surrounding the 3' substituent of *i*-BZDs [125]. The localization of the 3'-imidazo substituents in the unified pharmacophore model, see Fig. (5), thus positions the A₂ and L_{DI} descriptors near residues γ 2A79 and

γ 2T81. Further support for this localization comes from the observation that DMCM also loses affinity upon mutagenesis in positions γ 2A79 and γ 2T81, [125, 126] consistent with the alignment of this substance in the pharmacophore. Fig. (5) shows that changes to the protein near the A₂ descriptor would in all likelihood not be tolerated by DMCM.

COMBINING HOMOLOGY MODEL AND EXPERIMENTAL EVIDENCE

If the two assignments discussed above are correct, they should be reflected in results of computational ligand docking to homology models of the binding site. Thus, computational docking of diazepam and flumazenil was performed in multiple different models based on different templates [101, 106, 115] and the best 100 docking poses per model were collected in a database. A database query was then formulated to search among the (unrefined) docking poses for those with the following properties: The seven membered ring of diazepam or flumazenil is in the conformation that is assumed to

be the active one; [99] the 7-substituents are near residues α 1H102/ α 1V203/ and α 1Y210/ α 1V212 in order to be consistent with the covalent labeling data; the 3' ester group of flumazenil is near residues γ 2A79 and γ 2T81. Such a query yields docking poses that are consistent with all covalent labeling studies on one hand, and with the steric requirements that were found for 3' substituted imidazobenzodiazepines [125] on the other hand. Such poses were indeed present in our database, and one representative diazepam pose is shown in Fig. (23): To be consistent with the presentation of the pharmacophore and its descriptors so far, the orientation of the benzodiazepine binding pocket had to be turned upside down and slightly tilted.

This and similar docking poses not only satisfy the two assignments for L2 and A2 that are discussed in the sections above, but at the same time position the remaining descriptors as follows: H1 can be satisfied by appropriate side chains near the tip of loop C, the L_{DI} region is near the subunit interface, essentially between α 1 loop B (Y160) and the γ 2 region spanned by the interface forming sheet involving M130, T142 and F77; A2 can be satisfied by H-bridge

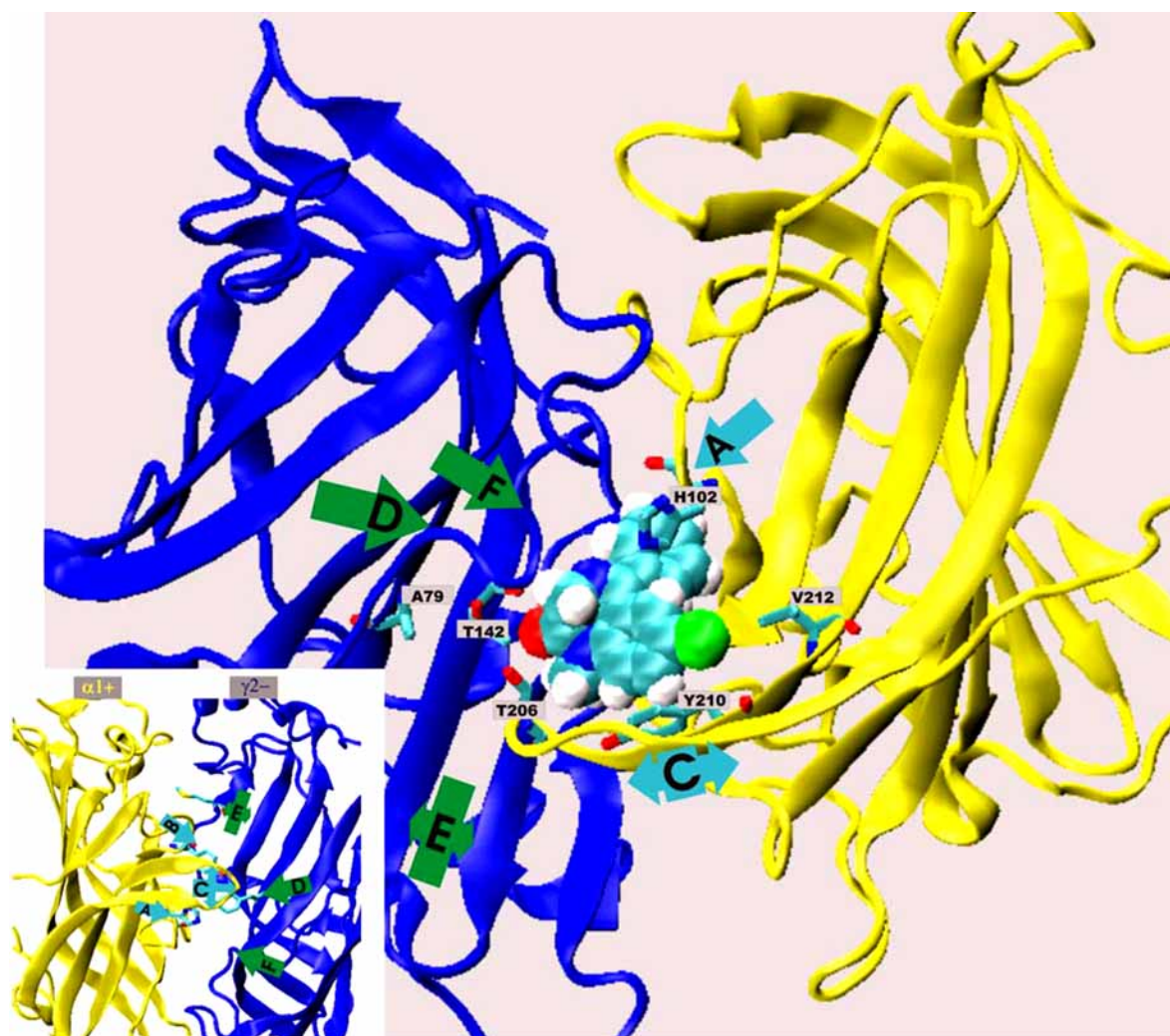


Fig. (23). The main figure shows a diazepam docking pose in an α 1 γ 2 pocket matching the orientation proposed in the text. Diazepam is rendered space filling, (turquoise: carbon, white: hydrogen, green: halogen, red: oxygen, blue: nitrogen), the protein as ribbon, and key amino acids in stick representation. The insert figure shows the empty pocket in the “upright” position to facilitate orientation in the main figure, where the model has been turned and tilted to bring diazepam approximately into the orientation depicted in the unified pharmacophore model, Fig. (3). In this particular docking pose, His102 and Thr206 could satisfy H2 and H1, respectively, and His 102, Val 212 and Tyr 210 would be part of the L2 hydrophobic pocket. This positions 7-substituted reactive analogues such as compound **59** into a position where the reactive groups find free access to His102, Tyr 210, Val 212, Val 203. It should be noted that due to high uncertainties in model backbone position and side chain rotamers, high accuracy docking that correctly reflects all ligand-protein interactions is not feasible, this image is one representative of a big cluster of similar docking poses.

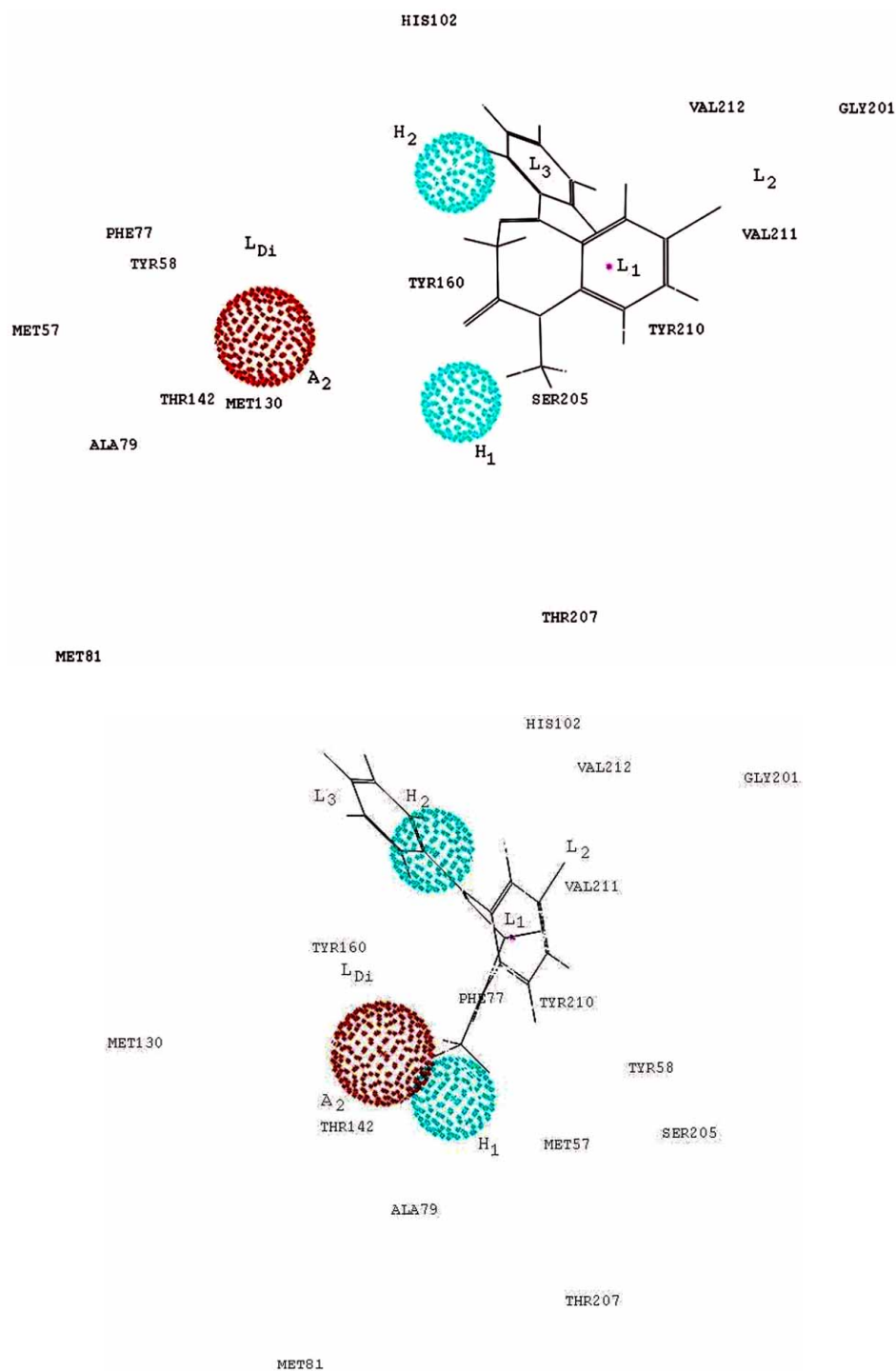


Fig. (24). Location of important Bz BS residues relative to the pharmacophoric descriptors in two views that correspond to a 90 rotation about L_1 , with A_2 in front, as determined by evaluation of experimental data and the comparative model of the $\alpha 1\beta 2\gamma 2$ GABA_A receptor. The ligand shown is diazepam. Since inverse agonists stabilize protein conformations that vary from the conformations preferred by agonists, it seems plausible that more than one side chain could satisfy the same descriptor.

accepting sites near $\alpha 1Y160$, $\gamma 2A79$, $\gamma 2T81$, $\gamma 2M130$ and $\gamma 2T142$. If these proximity relations that are found in the docking poses are transferred to the unified pharmacophore model, the following picture emerges:

Within the different poses that exhibit this orientation, there is still some degree of variation in structural details. There are several sources for this variability in the computationally derived structures: template and alignment choice, model construction and re-

finement, as well as docking algorithms contribute to the variability of the poses that are found.

This orientation is also strongly supported by the observation that XLi-093, as aligned in Fig. (14) with the docked diazepam shown above, will be able to thread the portion of the molecule not inside the included volume towards the solvent accessible space through the subunit boundary.

Agreement between this orientation of the pharmacophore model inside the structural model of the protein with single ligand dockings that have been proposed in the past [103-105] will have to be examined in detail. The overall picture appears to be pointing towards a satisfactory orientation of the pharmacophore model in the pocket, and for those instances where there could be a discrepancy, further experiments will clarify.

Refinement of the predicted protein-ligand complexes with the aid of the unified pharmacophore model appears to be a very promising approach to arrive at 3D structures that are of sufficient accuracy to be used for structure guided drug design. Studies that combine advanced protein modeling techniques with the classical pharmacophore approach are currently underway.

CONCLUSION

A unified pharmacophore model incorporating many substance classes that act at the DS and DI benzodiazepine binding sites of GABA_A receptors has been updated to include new substance classes. Compound development guided by this pharmacophore model has led to new agents with interesting pharmacological profiles, particularly enhanced preference for $\alpha 2$ or $\alpha 5$ containing GABA_A receptor subtype. Based on the evaluation of experimental data and comparative models of the $\alpha 1\beta 2\gamma 2$ GABA_A receptor, the location of several residues relative to the descriptors of the pharmacophore/receptor model has been proposed. Although no absolute assignments were made regarding which amino acids satisfy the pharmacophoric descriptors, experimental data strongly indicated definite trends with regard to how ligands of varying pharmacological activity are oriented within the receptor. Because the unified pharmacophore/receptor model accounts for the binding and activity profiles at the six GABA_A receptor subtypes containing any one of the different alpha subunits, the proposed orientation should also be similar within the different models [110] of the various receptor subtypes. Information to be immediately gained from this proposed orientation can have far reaching benefits, not only for the rational design of selective ligands and the interpretation of ligand docking results, but also for the identification and evaluation of possible roles certain residues may have within the pocket. As structure determination of the GABA_A receptor is eagerly awaited, it is hoped that this proposed orientation may be used by others to gain additional insight into the potential mechanisms underlying binding and modulation at the Bz site, all of which will lead to a better understanding of the structure and function of GABA_A receptors.

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REFERENCES

- [1] Sieghart, W.; Ernst, M. *Curr. Med. Chem. Central Nervous Syst. Agents* **2005**, *5*, 217-242.
- [2] Bowser, D. N.; Wagner, D. A.; Czajkowski, C.; Cromer, B. A.; Parker, M. W.; Wallace, R. H.; Harkin, L. A.; Mulley, J. C.; Marini, C.; Berkovic, S. F.; Williams, D. A.; Jones, M. V.; Petrou, S. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 15170-15175.
- [3] Bateson, A. N. *Sleep Med.* **2004**, *5*, S9-S15.
- [4] Otani, K.; Ujike, H.; Tanaka, Y.; Morita, Y.; Katsu, T.; Nomura, A.; Uchida, N.; Hamamura, T.; Fujiwara, Y.; Kuroda, S. *Neurosci. Lett.* **2005**, *381*, 108-113.
- [5] Dean, B.; Scarr, E.; McLeod, M. *Brain Res. Mol. Brain Res.* **2005**, *138*, 145-155.
- [6] Guidotti, A.; Auta, J.; Davis, J. M.; Dong, E. B.; Grayson, D. R.; Veldic, M.; Zhang, X. Q.; Costa, E. *Psychopharmacology* **2005**, *180*, 191-205.
- [7] Maubach, K. *Drug Targets-CNS Neuro Disorders* **2003**, *2*, 233-239.
- [8] Barrett, A. C.; Negus, S. S.; Mello, N. K.; Caine, S. B. *J. Pharmacol. Exp. Ther.* **2005**, *315*, 858-871.
- [9] Anthenelli, R.; Schuckit, M. A. *Int. J. Addict.* **1990/1991**, *25*, 81-94.
- [10] Schuckit, M. A. *Alcohol Clin. Exp. Res.* **1988**, *12*, 465-470.
- [11] Barnard, E. A.; Skolnick, P.; Olsen, R. W.; Möhler, H.; Sieghart, W.; Biggio, G.; Braestrup, C.; Bateson, A. N.; Langer, S. Z. *Pharmacol. Rev.* **1998**, *50*, 291-313.
- [12] Simon, J.; Wakimoto, H.; Fujita, N.; Lalande, M.; Barnard, E. A. *J. Biol. Chem.* **2004**, *279*, 41422-41435.
- [13] Burt, D. R.; Kamatchi, G. L. *FASEB J.* **1991**, *5*, 2916-2923.
- [14] Keramidas, A.; Moorhouse, A.; Schofield, P. C.; Barry, P. *Prog. Biophys. Mol. Biol.* **2004**, *86*, 161-204.
- [15] Sieghart, W.; Sperk, G. *Curr. Top. Med. Chem.* **2002**, *2*, 795-816.
- [16] Mossier, B.; Togel, M.; Fuchs, K.; Sieghart, W. *J. Biol. Chem.* **1994**, *269*, 25777-25782.
- [17] Togel, M.; Mossier, B.; Fuchs, K.; Sieghart, W. *J. Biol. Chem.* **1994**, *269*, 12993-12998.
- [18] Pirker, S.; Schwarzer, C.; Wieselthaler, A.; Sieghart, W.; Sperk, G. *Neuroscience* **2000**, *101*, 815-850.
- [19] Doble, A.; Martin, I. L. *Trends Pharmacol. Sci.* **1992**, *13*, 76-81.
- [20] Sieghart, W. *Pharmacol. Rev.* **1995**, *47*, 181-234.
- [21] Kralic, J. E.; Korpi, E. R.; O'Buckley, T. K.; Homanics, G. E.; Morrow, A. L. *J. Pharmacol. Exp. Ther.* **2002**, *302*, 1037-1045.
- [22] Crestani, F.; Assandri, R.; Tauber, M.; Martin, J.; Rudolph, U. *Neuropharmacology* **2002**, *43*, 679-684.
- [23] Rudolph, U.; Crestani, F.; Benke, D.; Brunig, I.; Benson, J. A.; Fritschy, J. M.; Martin, J. R.; Bluethmann, H.; Möhler, H. *Nature* **1999**, *401*, 796-800.
- [24] Collinson, N.; Cotliff, R.; Rosahl, T.; Sur, C.; Kuenzi, F. M.; Howell, O.; Seabrook, G.; Attack, J.; McKernan, R.; Dawson, G. R.; Whiting, P. *Eur. J. Neurosci.* **2000**, *12*, 171.
- [25] Bailey, D. J.; Tetzlaff, J. E.; Cook, J. M.; He, X. H.; Helmstetter, F. J. *Neurobiol. Learn. Mem.* **2002**, *78*, 1-10.
- [26] DeLorey, T. M.; Lin, R. C.; McBrad, B.; He, X. H.; Cook, J. M.; Lameh, J.; Loew, G. H. *Eur. J. Pharmacol.* **2001**, *426*, 45-54.
- [27] Lelas, S.; Rohrbach, K.; Glick, S. D.; Zeller, K.; Bourin, C.; Sieracki, K.; Bertekap, R.; Chen, J.; Cook, C. M.; Helmstetter, F. J.; Li, X. H.; Westphal, R.; Lindner, M.; Tertyshnikova, S.; Cook, J.; McElroy, J. F. *Manuscript in preparation*.
- [28] Liu, R. Y.; Zhang, P. W.; McKernan, R. M.; Wafford, K.; Cook, J. M. *Med. Chem. Res.* **1995**, *5*, 700-709.
- [29] Liu, R. Y.; Hu, R. J.; Zhang, P. W.; Skolnick, P.; Cook, J. M. *J. Med. Chem.* **1996**, *39*, 1928-1934.
- [30] Griebel, G.; Perrault, G.; Simiand, J.; Cohen, C.; Granger, P.; Decobert, M.; Francon, D.; Avenet, P.; Depoortere, H.; Tan, S.; Oblin, A.; Schoemaker, H.; Evanno, Y.; Sevrin, M.; George, P.; Scatton, B. *J. Pharmacol. Exp. Ther.* **2001**, *298*, 753-768.
- [31] Minier, F.; Sigel, E. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 7769-7774.
- [32] Wafford, K. A.; Macaulay, A. J.; Fradley, R.; O'Meara, G. F.; Reynolds, D. S.; Rosahl, T. W. *Biochem. Soc. Trans.* **2004**, *32*, 553-556.
- [33] Casula, M. A.; Bromidge, F. A.; Pillai, G. V.; Wingrove, P. B.; Martin, K.; Maubach, K.; Seabrook, G. R.; Whiting, P. J.; Hadingham, K. L. *J. Neurochem.* **2001**, *77*, 445-451.
- [34] Wafford, K. A.; Whiting, P. J.; Kemp, J. A. *Mol. Pharmacol.* **1993**, *43*, 240-244.
- [35] Attack, J. R. *Expert Opin. Invest. Drugs* **2005**, *14*, 601-618.
- [36] Möhler, H.; Rudolph, U.; McKernan, R. In *ACNP*, December 20, **2004**.
- [37] Study, R. E. B. J. L. *Proc. Natl. Acad. Sci. USA* **1981**, *78*, 7180-7184.
- [38] Rogers, C.; Twyman, R. E.; MacDonald, R. L. *J. Physiol. (London)* **1994**, *475*, 69-82.
- [39] Kash, T. L.; Jenkins, A.; Kelley, J. C.; Trudell, J.; Harrison, N. L. *Nature* **2003**, *421*, 272-275.
- [40] Rüsch, D.; Forman, S. A. *Anesthesiology* **2005**, *102*, 783-792.
- [41] Baur, R.; Sigel, E. *Mol. Pharmacol.* **2005**, *67*, 1005-1008.
- [42] Zhang, W.; Koehler, K. F.; Zhang, P.; Cook, J. M. *Drug Des. Discov.* **1995**, *12*, 193-248.
- [43] Zhang, W.; Diaz-Araujo, H.; Allen, M. S.; Koehler, K. F.; Cook, J. M. *Chemical and Computer Assisted Development of the Inclusive Pharmacophore of Benzodiazepine Receptors, in Studies in Medicinal Chemistry, M.I. Choudhary, Editor. Harwood Academic Publishers: 1996; pp. 303.*
- [44] Sigel, E.; Schaerer, M. T.; Buhr, A.; Baur, R. *Mol. Pharmacol.* **1998**, *54*, 1097-1105.
- [45] Dunn, S. M. J.; Davies, M.; Muntoni, A. L.; Lambert, J. J. *Mol. Pharmacol.* **1999**, *56*, 768-774.
- [46] Davies, M.; Bateson, A. N.; Dunn, S. M. J. *J. Neurochem.* **1998**, *70*, 2188-2194.
- [47] Cromer, B.; Morton, C.; Parker, M. W. *Trends Biochem. Sci.* **2002**, *27*, 280-287.
- [48] Ernst, M.; Brauchart, D.; Boresch, S.; Sieghart, W. *Neuroscience* **2003**, *119*, 933-943.
- [49] Sigel, E. *Curr. Top. Med. Chem.* **2002**, *2*, 833-840.
- [50] Allen, M. S.; Tan, Y. C.; Trudell, M. L.; Narayanan, K.; Schindler, L.; Martin, M. J.; Schultz, C. A.; Hagen, T. J.; Koehler, K. F.; Coddington, P.; Skolnick, P.; Cook, J. J. *Med. Chem.* **1990**, *33*, 2343-2357.
- [51] Zhang, P. W.; Zhang, W. J.; Liu, R. Y.; Harris, B.; Skolnick, P.; Cook, J. M. *J. Med. Chem.* **1995**, *38*, 1679-1688.

- [52] Allen, M. S.; Laloggia, A. J.; Dom, L. J.; Martin, M. J.; Constantino, G.; Hagen, T. J.; Koehler, K. F.; Skolnick, P.; Cook, J. *J. Med. Chem.* **1992**, *35*, 4001-4010.
- [53] Wong, G.; Koehler, K. F.; Skolnick, P.; Gu, Z. Q.; Ananthan, S.; Schönholzer, P.; Hunkeler, W.; Zhang, W. J.; Cook, J. *M. J. Med. Chem.* **1993**, *36*, 1820-1830.
- [54] Hevers, W.; Luddens, H. *Mol. Neurobiol.* **1998**, *18*, 35-86.
- [55] He, X. B.; Huang, Q.; Ma, C. R.; Yu, S.; McKernan, R.; Cook, J. *Drug Des. Discov.* **2000**, *17*, 131-171.
- [56] Huang, Q.; He, X. H.; Ma, C. R.; Liu, R. Y.; Yu, S.; Dayer, C. A.; Wenger, G. R.; McKernan, R.; Cook, J. *M. J. Med. Chem.* **2000**, *43*, 71-95.
- [57] Hadingham, K. L.; Wingrove, P. B.; Wafford, K. A.; Bain, C.; Kemp, J. A.; Palmer, K. J.; Wilson, A. W.; Wilcox, A. S.; Sikela, J. M.; Ragan, C. I.; Whiting, P. *J. Mol. Pharmacol.* **1993**, *44*, 1211-1218.
- [58] Barnard, E. A. *Trends Biochem. Sci.* **1992**, *17*, 368-374.
- [59] Huang, Q.; Liu, R. Y.; Zhang, P. W.; He, X. H.; McKernan, R.; Gan, T.; Bennett, D. W.; Cook, J. *M. J. Med. Chem.* **1998**, *41*, 4130-4142.
- [60] Wafford, K. A.; Thompson, S. A.; Thomas, D.; Sikela, J.; Wilcox, A. S.; Whiting, P. *J. Mol. Pharmacol.* **1996**, *50*, 670-678.
- [61] Huang, Q.; Cox, E.; Gan, T.; Ma, C. R.; Bennett, D. A.; McKernan, R.; Cook, J. *Drug Des. Discov.* **1999**, *16*, 55-76.
- [62] Drover, D. R. *Clin. Pharmacokinet.* **2004**, *43*, 227-238.
- [63] Noble, S.; Langtry, H. D.; Lamb, H. M. *Drugs* **1998**, *55*, 277-302.
- [64] Jerussic, T. **2006**, unpublished results.
- [65] Savic, M. M.; Huang, S.; Furtmuller, R.; Clayton, T.; Huck, S.; Obradovic, D. I.; Ugresic, N. D.; Sieghart, W.; Bokonic, D. R.; Cook, J. *M. Neuropharmacology* **2007**.
- [66] Dias, R.; Sheppard, W. F. A.; Fradley, R. L.; Garrett, E. M.; Stanley, J. L.; Tye, S. J.; Goodacre, S.; Lincoln, R. J.; Cook, S. M.; Conley, R.; Hallett, D.; Humphries, A. C.; Thompson, S. A.; Wafford, K. A.; Street, L. J.; Castro, J. L.; Whiting, P. J.; Rosahl, T. W.; Attack, J. R.; McKernan, R. M.; Dawson, G. R.; Reynolds, D. S. *J. Neurosci.* **2005**, *25*, 10682-10688.
- [67] McKernan, R. M.; Rosahl, T. W.; Reynolds, D. S.; Sur, C.; Wafford, K. A.; Attack, J. R.; Farrar, S.; Myers, J.; Cook, G.; Ferris, P.; Garrett, L.; Bristow, L.; Marshall, G.; Macaulay, A.; Brown, N.; Howell, O.; Moore, K. W.; Carling, R. W.; Street, L. J.; Castro, J. L.; Ragan, C. I.; Dawson, G. R.; Whiting, P. *J. Nat. Neurosci.* **2000**, *3*, 587-592.
- [68] Collinson, N.; Kuenzi, F. M.; Jarolimek, W.; Maubach, K. A.; Cothliff, R.; Sur, C.; Smith, A.; Otu, F. M.; Howell, O.; Attack, J. R.; McKernan, R. M.; Seabrook, G. R.; Dawson, G. R.; Whiting, P. J.; Rosahl, T. W. *J. Neurosci.* **2002**, *22*, 5572-5580.
- [69] Crestani, F.; Keist, R.; Fritsch, J.; Benke, D.; Vogt, K. E.; Prut, L. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 8980-8985.
- [70] Bohlhalter, S.; Weinmann, O.; Möhler, H.; Fritschy, J. M. *J. Neurosci.* **1996**, *16*, 283-297.
- [71] van Rijnsoever, C.; Tauber, M.; Choulli, M. K.; Keist, R.; Rudolph, U.; Möhler, H.; Fritschy, J. M.; Crestani, F. *J. Neurosci.* **2004**, *24*, 6785-6790.
- [72] Porsolt, R. D.; McArthur, R. A.; Lenegre, A. *Psychotropic Screening Procedures*. Elsevier: **1993**; pp. 23-51.
- [73] Wang, Q.; Han, Y.; Xue, H. *CNS Drug Rev.* **1999**, *5*, 125-144.
- [74] Miksiek, R. *Mol. Pharmacol.* **1993**, *44*, 37-43.
- [75] Häberlein, H.; Tschiersch, K. P.; Schäfer, H. L. *Pharmazie* **1994**, *49*, 912-922.
- [76] Huang, X. Q.; Liu, T.; Gu, J. D.; Luo, X. M.; Ji, R. Y.; Cao, Y.; Xue, H.; Wong, J. T. F.; Wong, B. L.; Pei, G.; Jiang, H. L.; Chen, K. X. *J. Med. Chem.* **2001**, *44*, 1883-1891.
- [77] Marder, M.; Estiu, G.; Blanch, L. B.; Viola, H.; Wasowski, C.; Medina, J. H.; Paladini, A. C. *Bioorg. Med. Chem.* **2001**, *9*, 323-335.
- [78] Yu, J.; Cook, J. **1999**, unpublished results.
- [79] Costanzo, A.; Guerrini, G.; Ciciani, G.; Bruni, F.; Costagli, C.; Selleri, S.; Besnard, F.; Costa, B.; Martini, C.; Malmberg-Aiello, P. *J. Med. Chem.* **2002**, *45*, 5710-5720.
- [80] Yin, W. Y.; Sarma, P.; Han, J.; Chen, J. L.; Cook, J. M. *Tetrahedron Lett.* **2005**, *46*, 6363-6368.
- [81] Li, X. Y.; Cao, H.; Zhang, C. C.; Furtmueller, R.; Fuchs, K.; Huck, S.; Sieghart, W.; Deschamps, J.; Cook, J. M. *J. Med. Chem.* **2003**, *46*, 5567-5570.
- [82] Han, D. M.; Forsterling, F. H.; Li, X. Y.; Deschamps, J. R.; Cao, H.; Cook, J. M. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 1465-1469.
- [83] Ferretti, V.; Gilli, P.; Borea, P. A. *Acta Crystallogr. Sect. B Struct. Sci.* **2004**, *60*, 481-489.
- [84] Cox, E. D.; Diaz-Araujo, H.; Huang, Q.; Reddy, M. S.; Ma, C. R.; Harris, B.; McKernan, R.; Skolnick, P.; Cook, J. M. *J. Med. Chem.* **1998**, *41*, 2537-2552.
- [85] Diaz-Araujo, H.; Evoniuk, G.; Skolnick, P.; Cook, J. *J. Med. Chem.* **1991**, *34*, 1754-1756.
- [86] Diaz-Araujo, H.; Koehler, K. F.; Hagen, T. J.; Cook, J. M. *Life Sci.* **1991**, *49*, 207-216.
- [87] Skolnick, P.; Hu, R. J.; Cook, C. M.; Hurt, S. D.; Trometer, J. D.; Lu, R. Y.; Huang, Q.; Cook, J. M. *J. Pharmacol. Exp. Ther.* **1997**, *283*, 488-493.
- [88] Wisden, W.; Laurie, D. J.; Monyer, H.; Seeburg, P. H. *J. Neurosci.* **1992**, *12*, 1040-1062.
- [89] Harris, D. L.; DeLorey, T. M.; He, X. H.; Cook, J. M.; Loew, G. H. *Eur. J. Pharmacol.* **2000**, *401*, 271-287.
- [90] Roth, B. **2006**, unpublished results.
- [91] Kelly, M. D.; Smith, A.; Banks, G.; Wingrove, P.; Whiting, P. W.; Attack, J.; Seabrook, G. R.; Maubach, K. A. *Br. J. Pharmacol.* **2002**, *135*, 248-256.
- [92] Turner, D.; Sapp, D.; Olsen, R. W. *J. Pharmacol. Exp. Ther.* **1991**, *257*, 1236-1242.
- [93] McKay, P. F.; Foster, K. L.; Mason, D.; Cummings, R.; Garcia, M.; Williams, L. S.; Grey, C.; McCane, S.; He, X. H.; Cook, J. M.; June, H. L. *Psychopharmacology* **2004**, *172*, 455-462.
- [94] Harvey, S. C.; Foster, K. L.; McKay, P. F.; Carroll, M. R.; Seyoum, R.; Woods, J. E.; Grey, C.; Jones, C. M.; McCane, S.; Cummings, R.; Mason, D.; Ma, C. R.; Cook, J. M.; June, H. L. *J. Neurosci.* **2002**, *22*, 3765-3775.
- [95] Rowlett, J. K.; Spealman, R. D.; Lelas, S.; Cook, J. M.; Yin, W. Y. *Psychopharmacology* **2003**, *165*, 209-215.
- [96] June, H. L.; Harvey, S. C.; Foster, K. L.; McKay, P. F.; Cummings, R.; Garcia, M.; Mason, D.; Grey, C.; McCane, S.; Williams, L. S.; Johnson, T. B.; He, X. H.; Rock, S.; Cook, J. M. *J. Neurosci.* **2001**, *21*, 2166-2177.
- [97] Stephens, D. N.; Pistovcakova, J.; Worthing, L.; Attack, J. R.; Dawson, G. R. *Eur. J. Pharmacol.* **2005**, *526*, 240-250.
- [98] Luddens, H.; June, H. L.; Cook, J. (In preparation).
- [99] Haefely, W.; Kyburz, E.; Gerecke, M.; and Mohler, H. *Recent Advances in the Molecular Pharmacology of Benzodiazepine Receptors and in the Structure-Activity Relationships of their Agonists and Antagonists*. Academic Press: **1985**; pp. 165-322.
- [100] Fryer, R. I. *Comprehensive Medicinal Chemistry*. Pergamon Press: **1989**; pp. 539-566.
- [101] Brejc, K.; van Dijk, W.; Klassen, R.; Schuurmans, M.; van Der Oost, J.; Smit, A. B.; Sixma, T. *Nature* **2001**, *411*, 269-276.
- [102] Ernst, M.; Bruckner, S.; Boresch, S.; Sieghart, W. *Mol. Pharmacol.* **2005**, *68*, 1291-1300.
- [103] Kucken, A. M.; Wagner, D. A.; Ward, P. R.; Teissere, J. A.; Boileau, A. J.; Czajkowski, C. *Mol. Pharmacol.* **2000**, *57*, 932-939.
- [104] Sawyer, G. W.; Chiara, D. C.; Olsen, R. W.; Cohen, J. B. *J. Biol. Chem.* **2002**, *277*, 50036-50045.
- [105] Sancar, F.; Ericksen, S. S.; Kucken, A. M.; Teissere, J. A.; Czajkowski, C. *Mol. Pharmacol.* **2007**, *71*, 38-46.
- [106] Unwin, N. *J. Mol. Biol.* **2005**, *346*, 967-89.
- [107] Sigel, E.; Buhr, A. *Trends Pharmacol. Sci.* **1997**, *18*, 425-429.
- [108] Baumann, S. W.; Baur, R.; Sigel, E. *J. Biol. Chem.* **2002**, *277*, 46020-46025.
- [109] Neish, C. S.; Martin, I. L.; Davies, M.; Henderson, R. M.; Edwardson, J. M. *Nanotechnol.* **2003**, *14*, 864-872.
- [110] Chou, K. C. *Biochem. Biophys. Res. Commun.* **2004**, *316*, 636-642.
- [111] Trudell, J. R. *Biochim. Biophys. Acta* **2002**, *1565*, 91-96.
- [112] Buhr, A.; Baur, R.; Malherbe, P.; Sigel, E. *Mol. Pharmacol.* **1996**, *49*, 1080-1084.
- [113] Sigel, E.; Baur, R.; Trube, G.; Möhler, H.; Malherbe, P. *Neuron* **1990**, *5*, 703-711.
- [114] Khom, S.; Baburin, I.; Timin, E. N.; Hohaus, A.; Sieghart, W.; Hering, S. *Mol. Pharmacol.* **2006**, *69*, 640-649.
- [115] Hansen, S. B.; Sulzenbacher, G.; Huxford, T.; Marchot, P.; Taylor, P.; Bourne, Y. *EMBO J.* **2005**, *24*, 3635-46.
- [116] Mihic, S. J.; Whiting, P.; Klein, R. L.; Wafford, K.; Harris, R. A. *J. Biol. Chem.* **1994**, *269*, 32768-32773.
- [117] Downing, S.; Lee, Y. F.; Farb, D. H.; Gibbs, T. T. *Br. J. Pharmacol.* **2005**, *145*, 894-906.
- [118] He, X. H.; Zhang, C. C.; Cook, J. M. *Med. Chem. Res.* **2001**, *10*, 269-308.
- [119] Colquhoun, D. *Br. J. Pharmacol.* **1998**, *125*, 923-947.
- [120] Smith, G. B.; Olsen, R. W. *Neuropharmacology* **2000**, *39*, 55-64.
- [121] McKernan, R.; Farrar, S.; Collins, I.; Emms, F.; Asuni, A.; Quirk, K.; Broughton, H. B. *Mol. Pharmacol.* **1998**, *54*, 33-43.
- [122] Berezhnoy, D.; Nyfeler, Y.; Gonthier, A.; Schwob, H.; Goeldner, M.; Sigel, E. *J. Biol. Chem.* **2004**, *279*, 3160-3168.
- [123] Duncalf, L.; Carpenter, M.; Smillie, L.; Martin, I. L.; Dunn, S. L. *J. Biol. Chem.* **1996**, *271*, 9209-9214.
- [124] Tan, K. R.; Baur, R.; Gonthier, A.; Goeldner, M.; Sigel, E. Structure of the benzodiazepine binding pocket in GABAA receptors: proximity-accelerated chemical reaction. In Abstract D32-527.7 SFN **2006**.
- [125] Kucken, A. M.; Teissere, J. A.; Seffinga-Clark, J.; Wagner, D. A.; Czajkowski, C. *Mol. Pharmacol.* **2003**, *63*, 289-296.
- [126] Teissere, J. A.; Czajkowski, C. *J. Neurosci.* **2001**, *21*, 4977-4986.
- [127] Buhr, A.; Baur, R.; Sigel, E. *J. Biol. Chem.* **1997**, *272*, 11799-11804.
- [128] Ogris, W.; Polt, A.; Hauer, B.; Ernst, M.; Oberto, A.; Wulff, P.; Hoyer, H.; Wisden, W.; Sieghart, W. *Biochem. Pharmacol.* **2004**, *68*, 1621-1629.