Pharmacophore/Receptor Models for $GABA_A$/BzR Subtypes (α1β3γ2, α5β3γ2, and α6β3γ2) via a Comprehensive Ligand-Mapping Approach

Qi Huang,[§] Xiaohui He,[§] Chunrong Ma,[§] Ruiyan Liu,[§] Shu Yu,[§] Charlotte A. Dayer,[‡] Galen R. Wenger,[‡] Ruth McKernan,[†] and James M. Cook*[,§]

Department of Chemistry, University of Wisconsin−Milwaukee, P.O. Box 413, Milwaukee, Wisconsin 53201, Department of Pharmacology & Toxicology, University of Arkansas for Medical Sciences/Mail Slot 611, 4301 West Markham Street, Little Rock, Arkansas 72205, and Department of Biochemistry, Merck Sharpe and Dohme Research Laboratories, Terlings Park, Eastwick Road, Harlow, Essex CM20 2QR, U.K.

Received July 6, 1999

Pharmacophore/receptor models for three recombinant $GABA_A$/BzR subtypes (α1β3γ2, α5β3γ2, and α6β3γ2) have been established via an SAR ligand-mapping approach. This study was based on the affinities of 151 BzR ligands at five distinct (α1−3,5,6β3γ2) recombinant $GABA_A$/BzR receptor subtypes from at least nine different structural families. Examination of the included volumes of the α1-, α5-, and α6-containing subtypes indicated that region L_2 for the α5-containing subtype appeared to be larger in size than the analogous region of the other receptor subtypes. Region L_{Di}, in contrast, appeared to be larger in the α1 subtype than in the other two subtypes. Moreover, region L_3 in the α6 subtype is either very small or nonexistent in this diazepam-insensitive subtype (see Figure 16 for details) as compared to the other subtypes. Use of the pharmacophore/receptor models for these subtypes has resulted in the design of novel BzR ligands (see **27**) selective for the α5β3γ2 receptor subtype. α5-Selective ligand **27** when injected directly into the hippocampus did enhance memory in one paradigm (Bailey et al., unpublished observations); however, systemic administration of either **9** or **27** into animals did not provide an observable enhancement. This result is in complete agreement with the observation of Liu (1996). It has been shown (Liu, 1996; Wisden et al., 1992) that in the central nervous system of the rat (as well as monkeys and pigeons) there are several native subtypes of the $GABA_A$ receptor which exhibit different functions, regional distributions, and neuronal locations. Although **27** binds more potently at α5β3γ2 receptor subtypes and is clearly an inverse agonist (Liu et al., 1996; Liu, 1996), it is possible that this ligand acts as an agonist at one or more subtypes. Liu (1996) clearly showed that a number of imidazobenzodiazepines were negative modulators at one subtype and agonists at another. Therefore, selectivity for a particular subtype at this point is not sufficient to rule out some physiological effect at other $GABA_A$/BzR subtypes. The inability of **27** to potentiate memory when given systemically is again in support of this hypothesis, especially since α1β2γ2 subtypes are distributed throughout the brain (Wisden et al., 1992). A drug delivered systemically is far more likely to interact with all subtypes than one delivered to a specific brain region. This observation (systemic vs intrahippocampal) provides further support for the design of more subtype-specific ligands at the BzR to accurately define their pharmacology, one key to the design of new drugs with fewer side effects.

Introduction

The $GABA_A$/BzR complex contains a chloride ion channel which comprises part of the major inhibitory neurotransmitter system in the central nervous system (CNS).[1−4] This system regulates numerous neurological functions including convulsions, anxiety,[5,6] and sleep activity,[7] as well as memory and learning processes.[8] This membrane-bound heteropentameric protein polymer[9] is composed principally of α, β, and γ subunits. Presently a total of 18 subunits (6α, 4β, 4γ, 1ϵ, 1δ, and 2ρ) of the $GABA_A$ receptor have been cloned and sequenced; moreover, 16 of them have been found in the mammalian CNS.[9,10] Three types of subunits (α, β, and γ) are required for the construction of recombinant $GABA_A$/BzRs which most closely mimic the biochemical, electrophysiological, and pharmacological functions of native $GABA_A$/BzRs obtained from mammalian brain cells.[11,12] Among these, α1β2γ2 mimics many of the pharmacological properties of the classical type-I BzR, while α2β2γ2, α3β2γ2, and α5β2γ2 ion channels are termed type-II BzR.[13,14] Furthermore, recent studies of site-directed mutagenesis by Weiss et al.[15] indicated that a rat recombinant $GABA_A$ receptor α1β2γ2 isoform was composed of 1γ, 2α, and 2β subunits. The α4β2γ2 and α6β2γ2 channels both resemble "diazepam-insensitive" (DI) sites.[6,16−21] It appears now, based on existing evidence from both photoaffinity radiolabeling experiments and site-directed mutagenesis, that the benzodiazepine binding site lies between the α and γ subunits.[15,22−26]

* To whom correspondence should be addressed. Tel: 414-229-5856. Fax: 414-229-5530. E-mail: capncook@csd.uwm.edu.
[§] University of Wisconsin−Milwaukee.
[‡] University of Arkansas for Medical Sciences.
[†] Merck Sharpe and Dohme Research Laboratories.

10.1021/jm990341r CCC: $19.00
Published on Web 12/09/1999

The distribution of various subunits in the brain was found to be distinct and regionally overlapping.[9] The regional heterogeneity of the $GABA_A$/BzR subtypes has also been suggested as a basis for the multiplicity of pharmacological properties of the benzodiazepines.[27] Consequently, determination of the differences among receptor pharmacophores of BzR subtypes may lead to the preparation of more selective agents which would result in a better understanding of which subtype mediates which physiological response(s). This should result in more selective drugs for the treatment of anxiety, sleep disorders, convulsions, and memory deficits with fewer side effects.

A number of structurally diverse compounds have been shown to bind to the benzodiazepine binding site of the $GABA_A$ receptor. There are β-carbolines,[28−31] imidazobenzodiazepines,[18,20,32] triazolopyrimidines such as CL 218872,[33] pyridodiindoles,[34−37] imidazopyridines such as zolpidem,[38] and pyrazoloquinolines represented by CGS 9896,[39] as well as some others.[40−43] Classical benzodiazepines such as diazepam, which bind to the BzR and elicit a wide range of biological activities such as anxiolytic/anticonvulsant, muscle relaxant, sedative/hypnotic, and amnesiac, are termed agonists. On the other hand, inverse agonists such as BCCE[44] and many others related to Ro 15-4513[18,32] exhibit anxiogenic, somnolytic, and/or convulsant/proconvulsant activities.[45−47] In addition, ligands such as flumazenil and BCCT, so-called antagonists, exhibit little or no intrinsic efficacy by themselves but inhibit the effects of both agonists, such as ZK 93423, and inverse agonists, represented by BCCE.[48−51] There are a number of ligands whose pharmacological profiles lie on a continuum at the BzR ranging from full agonist through partial agonist to antagonist, as well as from partial inverse agonist to full inverse agonist.[39,52,53]

Structure−activity relationship (SAR) studies have been carried out by a number of research groups on structurally diverse classes of ligands and have resulted in the formulation of several different pharmacophore/receptor models for the BzR.[54] Models which attempt to explain ligand efficacy as a function of ligand−receptor interaction at the molecular level have been put forth by Loew,[54,55] Crippen,[56,57] Codding,[58,59] Fryer,[60] Gilli and Borea,[61] Wermuth,[62] and Gardner,[63] as well as from our own laboratory.[31,34−36,44,52,53,64−70]

A comprehensive pharmacophore/receptor model for the BzR was developed,[52,53] which unified the previous models for inverse agonist/antagonist and agonist activity as well as included the recent model for the DI site.[20,21,31,34−36,44,64−70] This unified pharmacophore/receptor model was employed to include agonist, antagonist, and inverse agonist ligands, which encompassed 12 families of structurally diverse compounds (Figure 1).[52,53] Four basic anchor points, H_1, H_2, A_2, and L_1, were assigned, and three additional lipophilic regions were defined as L_2, L_3, and L_{Di} (see captions in Figure 1 for details).

Recent efforts have focused on the search for subtype-selective ligands at recombinant $GABA_A$/BzR subtypes ($\alpha x\beta 3\gamma 2$, $x = 1-3$, 5, and 6) via a systematic SAR. Affinities of ligands from a number of different structural classes of ligands (Figure 2) have been evaluated. On the basis of SAR data obtained for these ligands at five recombinant BzR subtypes,[32,51,71−75] an effort has been made to establish different pharmacophore/receptor models for BzR subtypes. This approach is similar to the previous one which resulted in the unified pharmacophore/receptor model.[52,53] Herein are described the results from ligand-mapping experiments at recombinant BzR subtypes of 1,4-benzodiazepines, imidazobenzodiazepines, β-carbolines, diindoles, pyrazoloquinolinones, and others[38,76] (Figure 2), for three of the five recombinant receptor subtypes ($\alpha 1\beta 3\gamma 2$, $\alpha 5\beta 3\gamma 2$, and $\alpha 6\beta 3\gamma 2$). The differences and similarities between these three subtypes comprise much of the present report.

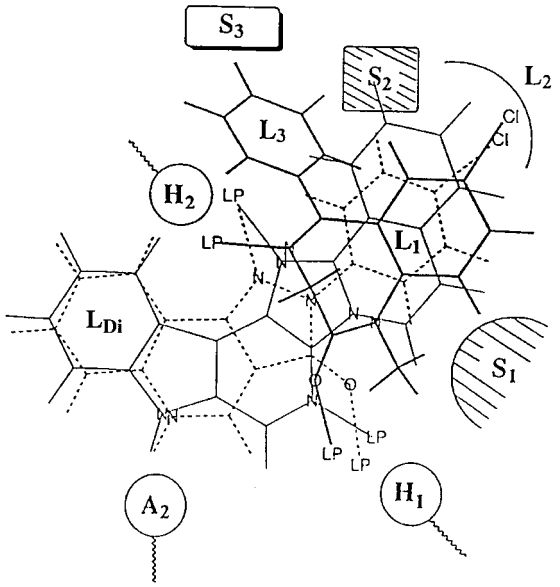


Figure 1. Pyrazolo[3,4-*c*]quinolin-3-one CGS-9896 (**119**) (dotted line), a diazadiindole[36] (**109**) (thin line), and diazepam (**145**) (thick line) fitted to the inclusive pharmacophore model for the BzR. Sites H_1 and H_2 represent hydrogen bond donor sites on the receptor protein complex, while A_2 represents a hydrogen bond acceptor site necessary for potent inverse activity in vivo. L_1, L_2, and L_3 are three lipophilic regions in the binding pharmacophore. Descriptors S_1, S_2, and S_3 are regions of negative steric repulsion.

In brief, analysis of the SAR indicates that region L_2 for the α5-containing BzR subtype appears to be larger in size than the analogous region in the other receptor subtypes.[32,72,77] Region L_{Di}, in contrast, appears to be larger in the α1-containing receptor isoform than at the other subtypes. Moreover, region L_3 in the recombinant receptor which contains the α6 subunit is very small or nonexistent in this DI site; it is believed L_3 in the α4 subtype suffers a similar fate. Efforts continue toward the development of more selective ligands for BzR subtypes. Ultimately, this will permit one to understand the binding mode of BzR ligands at all receptor subtypes as well as to determine the biological significance of each of the $GABA_A$/BzR isoforms. Data from these studies, when correlated with biological activity, may shed a great deal of light on the physiological function of $GABA_A$/BzRs in mammalian brain.

Computer Modeling Methods

The core structures of the ligands were taken from available X-ray crystallographic coordinates or generated using the SYBYL fragment library.[20,58,59,73,78−82]

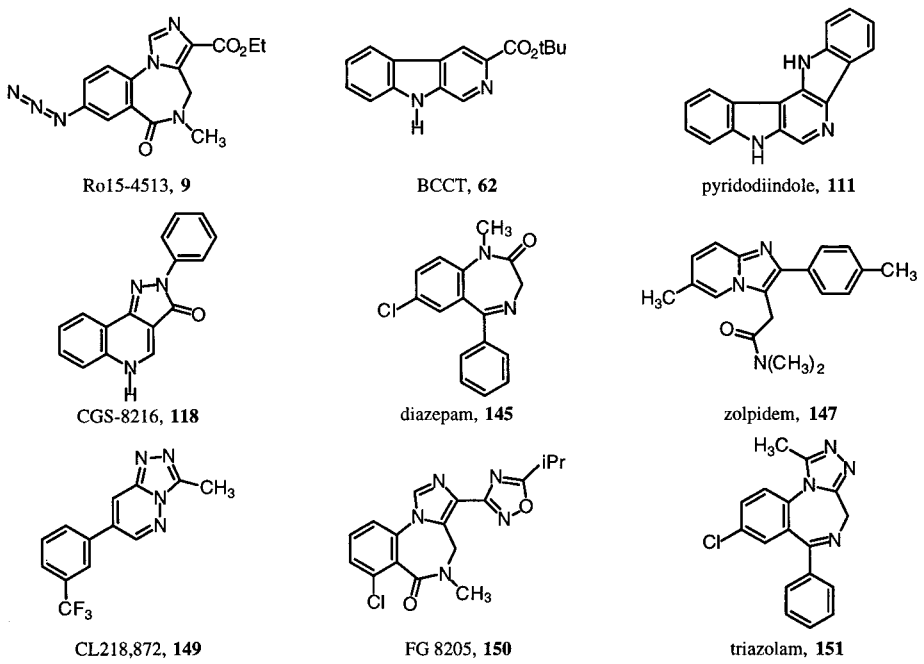


Figure 2. Classes of ligands employed for the study of pharmacophore/receptor models of BzR subtypes.

The structures which resulted were energy-minimized using MM2 (molecular mechanics program 2) or MMFF (Merck molecular force field) force fields,[83−87] and the subsequent Monte Carlo conformational searches were carried out on MacroModel 4.5 or MacroModel 6.0[88] on a Silicon Graphics Personal Iris 4D/35 workstation or a Silicon Graphics Octane SI 2P 175 R10000 workstation, respectively. The low-energy conformations were then fully optimized via molecular orbital calculations at the 3-21G basis set with torsional angles fixed. The structures which resulted were further calibrated with 6-31G* single-point calculations at an "SCF=TIGHT" convergence criteria via Gaussian 92[89] on a Silicon Graphics Indigo^2 R4400 workstation or via Gaussian 94[90] on a Silicon Graphics Octane SI2P175R10000 workstation. With ligands such as **4**, **16**, **17**, **42**, **43**, **48**, **143**, and **144** wherein the molecules contain heavy atoms (bromine or iodine), the necessary 6-31G* basis sets were not included in the commercially available Gaussian 92 and Gaussian 94 programs. These basis sets were taken from splitting the MP4 basis set reported by Andzelm et al. and then addition of d functions.[91−95]

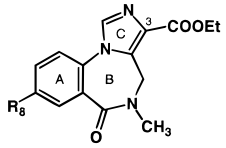
The ab initio 6-31G* geometries of the ligands were used for the included volume analysis. Ligands which possessed BzR affinities of ≤20 nM at one recombinant receptor subtype were considered as "active" and were used for the included volume analysis for that subtype. Zolpidem, CL 218872, and ligands reported to be more selective for one subtype[96,97] were included even though the binding affinities were somewhat higher than 20 nM. Molecular graphics, root-mean-squares (rms) fit, calculations of centroids of substructures, and included volume analyses were carried out by means of SYBYL 5.5 on a Silicon Graphics Personal Iris 4D/35 workstation or SYBYL 6.4[98] on a Silicon Graphics Octane SI 2P 175 R10000 workstation. The lengths of hydrogen bond extension vectors (HBV) were set to 1.84 Å, while the C−N−HBV and C=O−HBV valence angles were chosen to mimic the geometry of an ideal hydrogen bond.[99−103]

Results and Discussion

In vitro affinities (K_i values) for ligands **1**−**151** employed in the study were obtained by competition for [^{3}H]Ro 151788 binding to recombinant receptor subtypes at 4 °C and are depicted in Tables 1−10. Included volumes were obtained for the α1β3γ2, α5β3γ2, and α6β3γ2 recombinant receptor subtypes via the modeling procedure described above and are illustrated below. It is known that in recombinant GABA$_A$ receptors composed of α5β3γ2 subunits, the K_i values of ligands RY 80 (**11**) and RY 24 (**27**) as well as others were entirely consistent with the values obtained on wild-type receptors (α5β2γ2) from rat hippocampal tissue.[32,104]

1. Imidazobenzodiazepines. From the K_i values it has been found that many 8-substituted imidazobenzodiazepines exhibited some degree of selectivity at the α5-containing receptor subtype. Among them, ethyl 8-ethynyl-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylate (RY 80, **11**) exhibited a 44-fold selectivity overall at the α5-containing subtype,[77] while the important α1 to α5 selectivity of *tert*-butyl 8-(trimethylsilyl)ethynyl-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylate (RY 23, **28**) was found to be 75.5-fold. Other ligands with an acetylenic moiety at position-8 of the imidazobenzodiazepine nucleus (**27**, **46**, and **50**) also displayed 45−67-fold selectivity for the α5 isoform over the α1 subtype (Tables 1−5). In comparison to their parent (C8-H) compounds (see **1** vs **11**, Figure 3), RY 80 (**11**) as well as ligand **46** [see **41** (H) vs **46** (C≡CH)] are much more selective for α5-containing subtypes. In fact, the parents **1** and **41** exhibited almost no selectivity. This observation was in agreement with the previous hypothesis that correct occupation of region L_2 can promote α5 selectivity of a ligand.[32,77] Furthermore, studies of a series of framework-constrained 4,5-

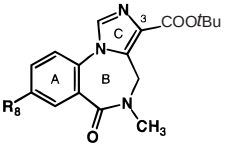
Table 1. Affinities of 5,6-Dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylic Acid Ethyl Esters for α*x*β3γ2 (*x* = 1−3, 5, 6) BzR Isoforms



ligand	R_8	K_i (nM)[a] α1	α2	α3	α5	α6	α1/α5
1	H	1.2	2.0	1.1	0.4	>300	3.0
2 (Ro 15-1788)	F	0.8	0.9	1.1	0.6	148	1.33
3 (Ro 15-1310)	Cl	6.8	16.3	9.2	0.85	54.6	8.00
4	Br	26.0	27.0	13.0	0.7	22.0	3.7
5	CN	10.0	45.0	19.0	6.0	>1000	1.7
6	$CH{=}CH_2$	8.3	10.2	6.9	0.4	NA[b]	20.8
7	Et	20.4	27	26.1	1.5	176	13.6
8	OEt	11.1	36	16.9	1.07	51.5	12.4
9 (Ro 15-4513)	$N{=}N^+{=}N^-$	3.3	2.6	2.5	0.3	3.8	10.5
10	$CH{=}C{=}CH_2$	3.75	7.2	4.1	1.1	44.3	3.4
11 (RY 80)	$C{\equiv}C{-}H$	28.4	21.4	25.8	0.49	28.8	58.0
12	$C{\equiv}C{-}CH_3$	10.1	22.2	16.5	1.68	>100	6.0
13	$C{\equiv}C{-}Si(CH_3)_3$	121	142	198	5.0	114	24.2
14	$C{\equiv}C{-}CH_2Si(CH_3)_3$	>300	>300	>300	>300	>300	NA

[a] Data shown here are the means of two determinations which differed by less than 10%. [b] NA stands for data not available.

Table 2. Affinities of 5,6-Dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylic Acid *tert*-Butyl Esters for α*x*β3γ2 (*x* = 1−3, 5, 6) BzR Isoforms



ligand	R_8	K_i (nM)[a] α1	α2	α3	α5	α6	α1/α5
15	Cl	17.3	21.6	29.1	0.65	4	26.6
16	Br	11.4	10.7	9.2	0.47	9.4	24.3
17	I	9.7	11.2	10.9	0.38	4.6	25.5
18	OH	1.50	NA[d]	0.53	0.14	6.89	10.7
19	OCH_3	6.74	NA	7.42	0.29	8.28	23.0
20	$N(CH_3)_2$	13.1	NA	38.1	0.78	118	16.8
21	X[b]	5.8	NA	169	9.25	325	0.63
22	Y[c]	6.44	NA	148	4.23	247	1.5
23	$N{=}N^+{=}N^-$	7.25	22.1	9.84	0.3	5.25	24.3
24	NCS	17.1	33.7	50	2.5	30.7	6.8
25	NO_2	12.8	49.8	30.2	3.5	22.5	3.7
26	Et	14.8	56	25.3	1.72	22.9	8.6
27 (RY 24)	$C{\equiv}C{-}H$	26.9	26.3	18.7	0.4	5.1	67.3
28 (RY 23)	$C{\equiv}C{-}Si(CH_3)_3$	197	143	255	2.61	58.6	75.5
29	$C{\equiv}CCH_2Si(CH_3)_3$	275	387	337	23	301	12.0

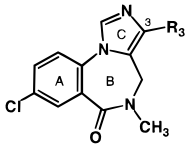
[a] Data shown here are the means of two determinations which differed by less than 10%. [b] X = *N*-tetrahydropyrrole. [c] Y = *N*-hexahydropyridine. [d] NA stands for data not available.

substituted pyrroloimidazobenzodiazepines and azetidinylimidazobenzodiazepines, with both *R* and *S* configurations (Figure 4), demonstrated that only *S* enantiomers of this series of ligands were able to bind to BzR subtypes with high affinity (Tables 4, 5).[71] This was in agreement with the earlier work of Haefely et al.[41] and Fryer et al.[19] on wild-type receptor populations (synaptosomal membranes). The stereopreferences of recombinant receptors for the *S* enantiomers of these ligands suggested the conformational topography at the five recombinant receptor subtypes was well-conserved.[71,105]

Recent results with tritiated RY 80 **(11)** demonstrated that [^{3}H]RY 80 **(11)** could be employed to label specific populations of $GABA_A$ receptor isoforms which contained an α5 subunit.[104] As such, [^{3}H]RY 80 may be used to evaluate the potency and efficacy of ligands at wild-type $GABA_A$ receptors containing α5 subunits, as a radioligand for autoradiographic studies, and as a probe for examining these receptors after physiological and pharmacological manipulation.[104,106,107]

The alignment rule and active conformation of the ligands were determined as required[52,53,108] before the included volume analysis. In general, the imidazobenzodiazepine nucleus is fairly rigid which simplifies the molecular modeling. Moreover, the active conformation of the seven-membered ring is known from both the results on the *S* enantiomers of the framework-constrained ligands[71] as well as from the work of Haefely et al.[41] and Fryer et al.[19] This conformation was employed in the modeling of all benzodiazepine-related ligands in this study. For these reasons the imidazobenzodiazepines were aligned in the pharmacophore model

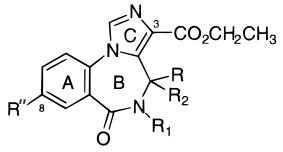
Table 3. Affinities of 3-Substituted 5,6-Dihydro-5-methyl-6-oxo-8-chloro-4*H*-imidazo[1,5-*a*][1,4]benzodiazepines for α*x*β3γ2 (*x* = 1−3, 5, 6) BzR Isoforms



		K_i (nM)[a]					
ligand	R_3	α1	α2	α3	α5	α6	α1/α5
3 (Ro 15-1310)	CO_2Et	6.8	16.3	9.2	0.85	54.6	8.0
15	CO_2t-Bu	17.3	21.6	29.1	0.65	4	26.6
30	CO_2CH_2-*c*-Pro	16.4	48.2	42.5	9.8	168	1.7
31	$C(=O)CH_3$	17500	33800	22100	2610	29500	6.7
32	C(=O)*n*-Bu	1580	2870	2740	166	2930	9.6
33	CH_2OH	>300	>300	>300	>300	>300	−
34	CH_2OCH_3	>300	>300	>300	38.8	>300	>7.7
35	CH_2Cl	>300	>300	>300	28.5	>300	>10.5
36	CH_2OEt	>300	>300	>300	82.7	>300	>3.6
37	$CH_2N(Et)_2$	9480	30000	15400	2580	30200	3.2
38	$CH_2N(i\text{-}Pr)_2$	4200	12600	6270	1350	8600	3.2
39	Et	408	1530	1130	182	3650	2.2
40	*n*-C_5H_{11}	2050	2900	2900	369	960	5.6

[a] Data shown here are the means of two determinations which differed by less than 10%.

Table 4. In Vitro Affinities of Framework-Constrained Imidazobenzodiazepines at Recombinant α*x*β3γ2 GABA$_A$/BzR Subtypes



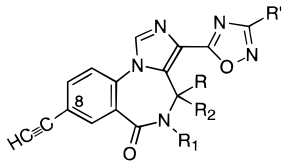
				K_i (nM)[a]					
ligand	R″	R	R_1, R_2	α1	α2	α3	α5	α6	α1/α5
41	H	(*S*)H	$-CH_2CH_2CH_2-$	7.3	NA[b]	7.1	1.6	>300	4.6
42	Br	(*S*)H	$-CH_2CH_2CH_2-$	49	29	15	1	46	**49**
43	Br	(*R*)H	$-CH_2CH_2CH_2-$	>1000	>1000	>1000	>1000	>1000	NA
44	C≡CSi$(CH_3)_3$	(*S*)H	$-CH_2CH_2CH_2-$	200	124	79	4	340	50
45	C≡CSi$(CH_3)_3$	(*R*)H	$-CH_2CH_2CH_2-$	>1000	>1000	>1000	>1000	>1000	NA
46	**C≡CH**	**(*S*)H**	$\mathbf{-CH_2CH_2CH_2-}$	**59**	**44**	**27**	**1.3**	**126**	**45**
47	C≡CH	(*R*)H	$-CH_2CH_2CH_2-$	283	318	102	7.2	61	39
48	Br	(*S*)H	$-CH_2CH_2-$	17	13	6.7	0.3	31	**56**
49	C≡CSi$(CH_3)_3$	(*S*)H	$-CH_2CH_2-$	83	60	48	2.6	180	32
50	C≡CH	(*S*)H	$-CH_2CH_2-$	21	12	10	0.37	42	57
51 (MSD**)**	OMe	(*S*)H	$-CH_2CH_2CH_2-$	48.5	27.4	24.5	0.45	83.2	108
11 (RY 80)	**C≡CH**	**H**	$\mathbf{-CH_3}$	**28.4**	**21.4**	**25.8**	**0.5**	**28.8**	**57**

[a] Data shown here are means of two determinations which differed by less than 10%. [b] NA stands for not applicable.

in agreement with earlier work reported on the unified pharmacophore/receptor model for BzR.[52,53,109] As shown in Figure 3, the centroid of ring-A of RY 80 (**11**), the lone pair of electrons of N2, and the lone pair of electrons of the oxygen atom of the carbonyl group at the C3 position were rms fitted with L_1, H_1, and H_2 of the pharmacophore/receptor model.[52,53,73] Note that the bond lengths between the nitrogen or oxygen atoms and the proton donors at H_1 or H_2 were adjusted to 1.84 Å. The bond angle of C═O−LP was adjusted to 135°.

During an earlier study of DI BzR ligands, the active conformations of imidazobenzodiazepines employed for CoMFA studies at DS and DI BzR subtypes were determined.[20] In short, the *anti* conformation of the ester functionality at position-3 was more likely to be involved in the active conformation for both the DS and DI subtypes. In addition, in the case of the 4,5-pyrroloimidazobenzodiazepines, the aliphatic ring plays an important role in the active conformation of the ester function. Ab initio calculations at the 6-31G* level revealed that the energy of the *syn* conformer was 2.74 kcal/mol higher than that of the *anti* conformer for the conformation of the 3-ethyl ester moiety of the pyrroloimidazobenzodiazepine **46**.[73] Consequently, the equilibrium constant at the temperature under which the binding study was done (4 °C) between the two conformers of **46** was 144.9 ($\Delta G = -RT \ln K$) in favor of the *anti* conformation (Figure 5). Thus the *anti* conformation was chosen as an active conformation throughout this study. For the 3-oxadiazole analogues (Table 5, ligands **52**−**60**), an *anti* conformation was also chosen, for this led to good overlap with the 3-ester function of **46**, as expected. In the case of 3-substituted imidazobenzodiazepines **31** and **32**, which carried ketone moieties at position-3, the *anti* conformer of the carbonyl function was also chosen; this was a low-energy conformation. For the remainder of the ligands with an imidazobenzodiazepine nucleus which contained a flexible side chain at position-3, a low-energy conformation was chosen which resulted in maximum overlap with the *tert*-butyl ester group of the potent ligand 3-*tert*-butoxycarbonylimidazobenzodiazepine **(15)**.[73]

Table 5. Affinities of 3-Alkyl-1,2,4-oxadiazole 4,5-Substituted Imidazobenzodiazepines at Recombinant $\alpha x\beta 3\gamma 2$ ($x = 1-3, 5, 6$) $GABA_A$/BzR Subtypes



				K_i (nM)[a]					
ligand	R′	R	R_1, R_2	α1	α2	α3	α5	α6	α1/α5
52	CH_3	(*S*)H	$-CH_2CH_2CH_2-$	89	70	91	3.7	301	24
53	CH_3	(*R*)H	$-CH_2CH_2CH_2-$	>1000	>1000	>1000	157	>1000	NA[b]
54	CH_2CH_3	(*S*)H	$-CH_2CH_2CH_2-$	86	40	85	2.4	150	36
55	$CH(CH_3)_2$	(*S*)H	$-CH_2CH_2CH_2-$	73	85	97	4.8	333	15
56	C_6H_5	(*S*)H	$-CH_2CH_2CH_2-$	33%[c]	26%	19%	455	4%	NA
57	CH_3	(*S*)H	$-CH_2CH_2-$	19	56	91	7.2	266	2.6
58	CH_2CH_3	(*S*)H	$-CH_2CH_2-$	220	150	184	12.7	361	17
59	$CH(CH_3)_2$	(*S*)H	$-CH_2CH_2-$	156	88	122	8.5	267	18
60	C_6H_5	(*S*)H	$-CH_2CH_2-$	10%	29%	15%	374	14%	NA

[a] Data shown here are the means of two determinations which differed by less than 10%. [b] NA stands for not applicable. [c] Percentage inhibition at a concentration of 1 μmol.

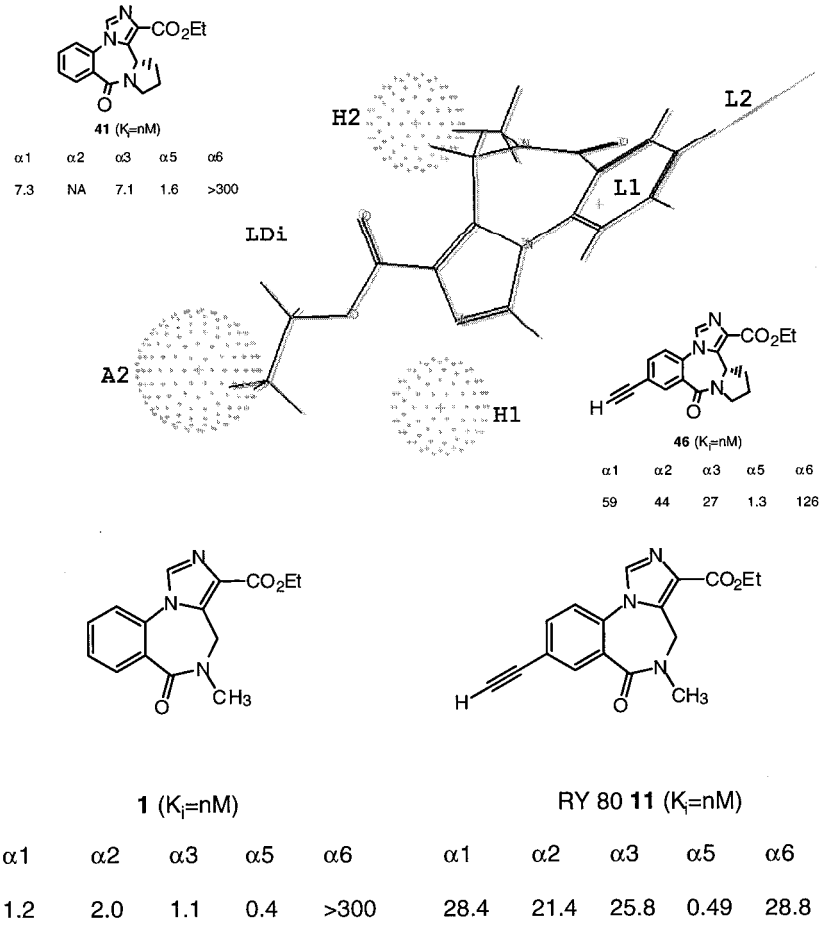


Figure 3. **1** (black) and RY 80 **(11)** (gray) in the pharmacophore/receptor model for BzR. Ligands **41** and **46** are also shown in the background for comparison purposes.

Most of the substituents at position-8 of the imidazobenzodiazepine scaffold were chosen as symmetrical or linear; therefore, conformational concerns were not an issue. In the case of compounds which carried vinyl (**6**), azido (**9** and **23**), and allene (**10**) moieties at C8, *syn* (Figure 6) conformations were chosen for DS sites (α1,5) while *anti* conformations were chosen for the DI site (α6), which is in agreement with the previous study.[20,53,73] For 8-isothiocyanoimidazobenzodiazepine (**24**), examination of ab initio 6-31G* calculations revealed that the bond angle C8−N−C should be close to 180°.[73] To permit conjugation of the π-electrons of the nitrogen atom with the aryl system, the *N*-tetrahydropyrrole (**21**) and *N*-hexahydropyridine (**22**) moieties were located nearly in the same plane as ring-A of the imidazobenzodiazepine nucleus.[73]

In addition, because of the prochiral center at C4, the B-ring of the 5-methylimidazobenzodiazepines may adopt one of the two envelope conformations. Examination of the binding data (Tables 4, 5) for *(R)*- and *(S)*-pyrrolo[2,1-*c*][1,4]imidazobenzodiazepines (see **42** vs **43**)

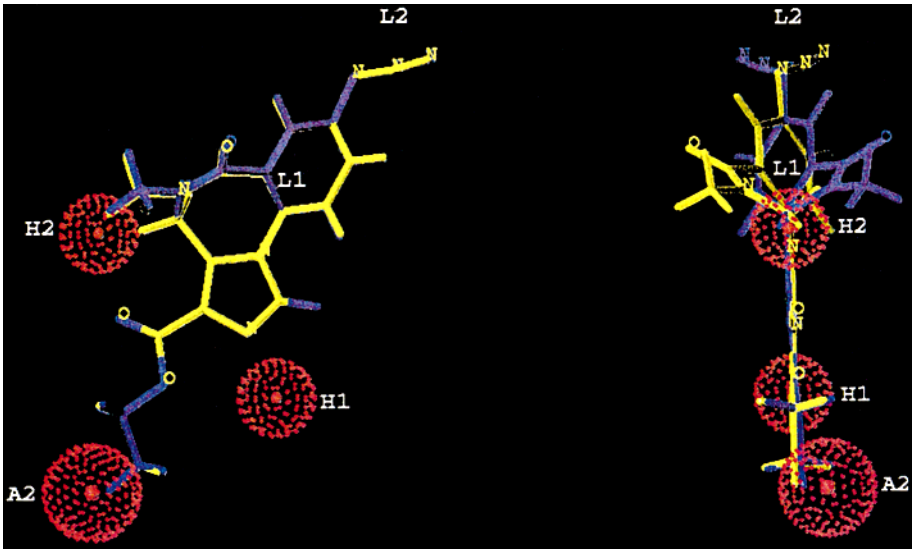


Figure 4. Two conformers (*pro-S* in yellow and *pro-R* in blue) of Ro 15-4513 (**9**) in the benzodiazepine pharmacophore/receptor model.

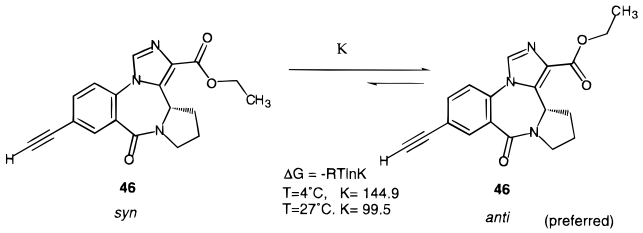


Figure 5. *Syn* and *anti* conformational representations for the C3 ester functionality of the framework-constrained pyrroloimidazobenzodiazepine (**46**).

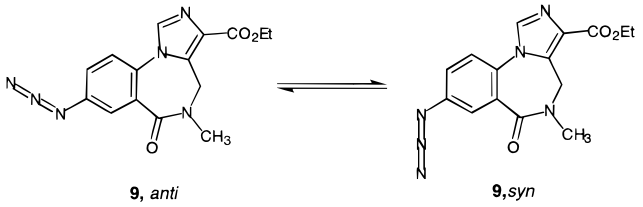


Figure 6. Conformations of the azido moiety of Ro 15-4513 (**9**).

indicated that the *pro-S* conformations are the active conformations for this series[71] (Figure 4).

2. β-Carbolines and Diindoles. β-Carbolines were first discovered to bind to central BzRs by Nielsen et al.[110] A number of active β-carbolines were synthesized and identified as inverse agonists over the years in many laboratories including our own.[31,44,68,111−115] Inverse agonists such as DMCM (**100**), agonists, such as abecarnil (**92**) and ZK 93423 (**91**), and antagonists such as ZK 93426 have been reported by scientists at Schering.[116,117] Furthermore, the discovery of inverse agonist β-carboline-3-carboxylates by Braestrup and Nielsen[45,46,118,119] in the early 1980s resulted in the discovery of the two-way modulation of BzR-mediated GABAergic transmission.[120] β-Carbolines have been found to elicit a continuum of intrinsic activities ranging from agonists [such as ZK 93423 (**91**) and abecarnil (**92**)][116,117,121] through antagonists [such as 3-PBC and BCCT (**62**)][122] to inverse agonists [DMCM (**100**) and BCCE (**63**)].[45,119] There are also partial agonists (6-PBC, **87**) and partial inverse agonists (3-EBC, **64**).[44] SAR studies of β-carbolines at the BzR were also reported during the early 1990s.[31,44,66,69] A predictive inverse agonist/antagonist binding pharmacophore model for β-carbolines was developed via CoMFA/GOLPE approaches, and it was further refined employing ab initio calculations.[31,44]

On the basis of the agonist pharmacophore/receptor model of the BzR, the design and synthesis of the anxiolytic/anticonvulsant 6-(*n*-propyloxy)-4-methoxymethyl-β-carboline-3-carboxylic acid ethyl ester (6-PBC, **87**) was realized.[67,69] This β-carboline inhibited PTZ-induced seizures in a dose-dependent fashion and exhibited anxiolytic activity when evaluated in an elevated plus-maze paradigm.[67] This partial agonist **87** was devoid of muscle-relaxant activity and completely antagonized the myorelaxant actions of diazepam.[67,69] The reason for the partial agonist activity of this ligand was believed to originate from partial occupation of lipophilc region L_3 of the BzR pharmacophore[51,74] in contrast to full occupation of this region by a full agonist, such as ZK 93423 (Figure 7).

During the course of the search for subtype-selective ligands, β-carbolines have been found to exhibit higher affinity at α1-containing receptor subtypes than at other isoforms.[51,74] In this regard, the antagonist BCCT (**62**) was among the most selective ligands at the α1-containing receptor reported at the time (1995)[122] and displayed subnanomolar affinity at the α1 subtype. This *tert*-butyl ester **62** exhibited 20-fold selectivity at the α1 subtype in comparison to other subtypes and was more selective in vitro at the α1 subtype than the classical Bz_I-selective ligands, CL 218,872 **(149)** (about 10-fold overall more selective at α1) and zolpidem **(147)** (about 6-fold overall more selective at α1). Since α1-containing receptor subtypes are the most abundant GABA$_A$/BzR isoforms and are located throughout the brain, an α1-selective ligand such as BCCT should be an important tool to define the exact location of α1 subtypes in the brain with pharmaceutical potential. Recently, June et al. has shown that BCCT reversed the self-administration of ethanol in alcohol-preferring (P) rats.[123] This GABA$_A$-related phenomenon may provide a significant new pathway for treatment of alcohol abuse. More work remains in this area to exploit this observation.

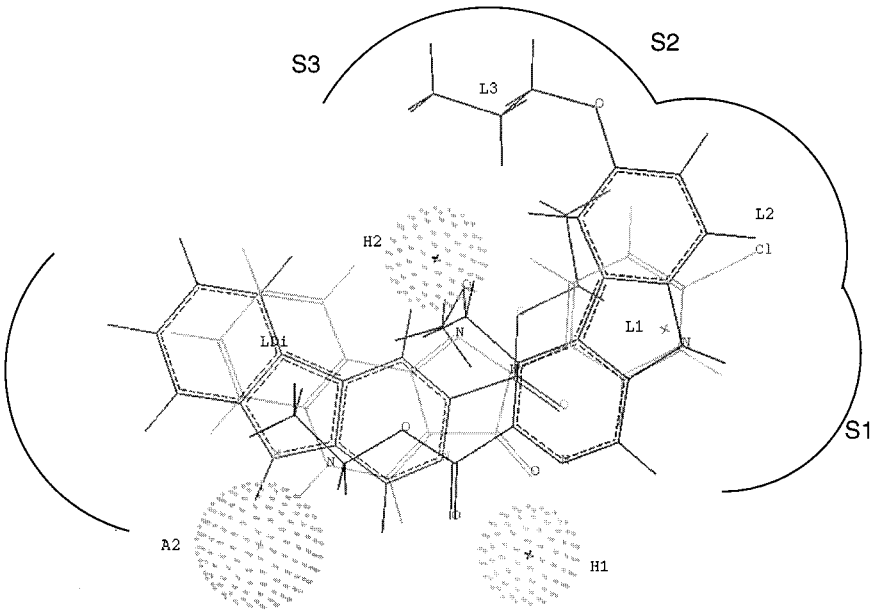


Figure 7. Partial agonist 6-PBC (**87**; black, aligned vertically), inverse agonist BCCE (**63**; gray, aligned horizontally), and CGS-9896 (**119**; light gray) in the pharmacophore/receptor model.

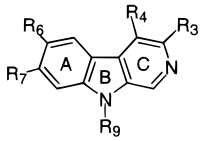
Most β-carbolines do not bind to the α6-containing BzR subtype. DMCM (**100**) and its ethyl ester congener **99**, however, are among the few β-carbolines which exhibit moderate affinity at this receptor isoform (see Table 6 for details). β-Carbolines generally displayed some selectivity at α1-containing receptors, as described previously.[51,74] A detailed 3-D QSAR examination of these ligands at five receptor subtypes via a CoMFA approach was reported recently.[74] The alignment for agonists vs inverse agonists depicted in Figure 7 was based on this CoMFA.[74] This alignment was employed in the present study to define the included volumes of BzR subtypes.

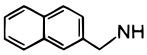
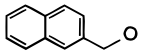
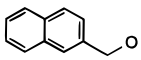
The rigid pyridodiindole **113** was employed as a template to define the alignment rule and the active conformation of flexible BzR ligands including β-carbolines.[52,74] As previously reported, diindole **113** was rms fitted into the pharmacophore/recepor model in such a way that the proton of N7 interacted with A_2 with a bond length of 1.84 Å and a bond angle (N7−H−A_2) of 180°. The lone pair of electrons of N5 were paired with H_1 with a bond length of 1.84 Å and an angle (C6−N5−H_1) of 120°. The centroid of the E-ring was paired with the lipophilic region L_1 of the pharmacophore/receptor model. The 2-chloropyridodiindole template **113** was especially useful since it was one of the largest completely rigid ligands known to bind with potent affinity to three of the five receptor subtypes (see Table 7 for in vitro data). Thus, it could be employed immediately to map out a significant portion of the sterically allowed regions of the receptor. Furthermore, the region of the BzR in the vicinity of the 2-chloro group of bound **113** defined a sterically tolerated region in the receptor. The longer side chains of the 3-substituted ether and ester functions of β-carbolines were threaded through this sterically allowed region (see Figure 8) requiring that the torsional angle of N2−C3−C═O be in the *syn* conformation (τ = 0°). The remainder of the torsional angles were arbitrarily fixed in the extended *trans* conformation (Figure 8). The *syn* conformation of the 3-ester group permitted the interaction via a three-centered hydrogen bond between the carbonyl oxygen group and N2 nitrogen atom of the β-carbolines with the proton donor site H_1 of the receptor protein (Figure 9). The three-centered hydrogen bond interaction was predicted to be a very stable arrangement based on analysis of the X-ray crystal structure of BCCM.[59] In principle, the *anti* conformation (N2−C3−C═O torsional angle = 180°) would also permit the formation of a three-centered hydrogn bond with H_1; however, this would involve an interaction between H_1 and the less electronegative ether oxygen atom of the ester group and therefore would be less favorable.

In a previous study, an *anti* conformer of the 3-alkoxy function was proposed with a torsional angle of 180° for N2−C3−O−C which resulted in good alignment with the active 3-alkoxycarbonyl group.[31,44] A study of active conformations also suggested that the *syn* conformer would sterically hinder the formation of a hydrogen bond between the N2 lone pair of electrons and the H_1 site on the receptor which is essential for tight binding of the ligand at the BzR. The low affinity of DMCM analogues **97** and **98** (see Table 6) was consistent with the above assumption (Figure 10). In the case of **97** the energy difference between the *anti* conformer (the proposed active conformer in a near-planar conformation to fit in the pharmacophore receptor model) and the global optimal low-energy conformer in the gas phase was over 18 kcal/mol by calculations using an MMFF force field. Because of the steric repulsion between the 3-methoxy and 4-ethyl functions, formation of the active conformation was disfavored (see Figure 10). In the case of the monosubstituted 3-alkoxy-β-carbolines, the energy differences between the *anti* "active" conformation and the global optimal low-energy conformation of these ligands were calculated to be considerably less and could be compensated for by the free energy released upon the binding of the ligand to the receptor.[52,53,74]

3. Pyrazoloquinolin-3-ones (CGS Series). The pyrazoloquinolines, discovered in the laboratories of Ciba-Geigy (the so-called CGS series),[39] were found to bind to both DS and DI BzR sites (α1−6 isoforms).[39,80,124,125]

Table 6. Affinities of β-Carbolines at $\alpha x\beta 3\gamma 2$ ($x = 1-3$, 5, 6) Receptor Subtypes



						K_i (nM)[a]				
ligand	R_9	R_7	R_6	R_4	R_3	α1	α2	α3	α5	α6
61	H	H	H	H	CO*n*-Pr	2.1	16.0	21.0	995	>3000
62 (BCCT)	H	H	H	H	CO_2*t*-Bu	0.72	15	18.9	111	>5000
63 (BCCE)	H	H	H	H	CO_2Et	1.2	4.9	5.7	26.8	2700
64 (3-EBC)	H	H	H	H	OEt	6.43	25.1	28.2	826	>1000
65	H	H	H	H	NCS	67.2	120	141	>3000	>10000
66	H	H	H	H	Cl	60.4	125	126	~1000	>10000
67	H	H	H	H	O*n*-Pr	5.3	52.3	68.8	591	>1000
68	H	H	H	H	O*n*-Bu	36.9	194	245	~1000	>1000
69	H	H	H	H	OBn	830	>3000	>3000	>10000	>10000
70	H	H	H	H	O*i*-Bu	24.9	124	139	~1000	>10000
71	H	H	H	H	O*i*-Pent	350	>3000	>3000	>3000	>10000
72	H	H	H	H	O*i*-Pr	283	>3000	>3000	>10000	>10000
73	H	H	H	H	O*i*-Bu	245	818	869	>10000	>10000
74	H	H	NO_2	H	NO_2	12.9	83.1	NT[b]	314	>5000
75	H	H	SCN	H	SCN	13.6	423	NT	2910	>5000
76	H	H	SCN	H	CO_2CH_3	3.41	NT	30.0	141	>10000
77	H	H	BnO	H	CO_2Et	7.2	169	284	271	>10000
78	H	H		H	CO_2CH_3	2.39	17.4	14.4	316	>10000
79	H	H	BnNH	H	CO_2CH_3	5.42	30.2	48.9	475	>10000
80	H	H		H	CO_2CH_3	30.7	205	271	814	>10000
81	H	H	H	$MeOCH_2$	CO_2Et	0.63	2.79	4.85	10.4	1150
82	H	H	BnO	Et	CO_2Et	20.8	78.3	58.7	67.3	>10000
83	H	H		Et	CO_2Et	183	291	300	270	>10000
84	H	H	MeO	Et	CO_2Et	1.6	2.9	2.8	1.0	>1000
85	H	H	$CH_3(CH_2)_7O$	Et	CO_2Et	3650	29700	35000	12700	>30000
86	H	H	MeO	$MeOCH_2$	CO_2Et	0.14	1.19	1.72	4.0	479
87 (6-PBC)	H	H	PrO	$MeOCH_2$	CO_2Et	0.49	1.21	2.2	2.39	1340
88	H	H	PrO	$MeOCH_2$	OPr	24.6	214	270.4	332	>1000
89	H	H	PrO	$MeOCH_2$	$CONHNH_2$	46	422	400	108	>300
90	H	H	PrO	$MeOCH_2$	NH_2	45	540	700	380	>3000
91 (ZK93423)	H	H	BnO	$MeOCH_2$	CO_2Et	4.1	4.2	6	4.5	>1000
92 (abecarnil)	H	H	BnO	$MeOCH_2$	CO_2*i*-Pr	12.4	15.3	7.5	6	>1000
93	H	H	BnO	$MeOCH_2$	SCN	25	82.6	58.8	159	>10000
94	H	H		$MeOCH_2$	CO_2Et	15.4	NT	293	323	>1000
95	H	H	OH	$MeOCH_2$	CO_2Et	2.0	5.4	10.8	18.5	~3000
96	H	H	$CH_3(CH_2)_7O$	$MeOCH_2$	CO_2Et	>1000	>3000	>3000	>3000	>3000
97	H	OMe	MeO	Et	OMe	>300	>300	>300	>300	>300
98	H	OMe	MeO	Et	OEt	>300	>300	>300	>300	>300
99	H	OMe	MeO	Et	CO_2Et	6.2	18.7	4.0	3.3	74.9
100 (DMCM)	H	OMe	MeO	Et	CO_2Me	5.7	8.3	4.0	1.04	134
101	H	OMe	MeO	Et	$CONHNH_2$	896	>1000	570	91.5	>3000
102	Me	H	H	Et	CO_2Et	>1000	>1000	>1000	>1000	>10000
103	Me	H	PrO	$MeOCH_2$	CO_2Et	25.6	105	164	161	>10000
104	Me	H	$CH_3(CH_2)_7O$	$MeOCH_2$	CO_2Et	>3000	>3000	>3000	>3000	>3000
105	Me	H	$CH_3(CH_2)_7O$	Et	CO_2Et	>1000	>1000	>1000	>1000	>1000

[a] Data shown here are the means of two determinations which differed by less than 10%. [b] NT stands for not tested.

A wide range of pyrazoloquinolinones have been synthesized by a number of research groups to correlate the substitution pattern and structure with the intrinsic activity of a ligand.[39,124−127] Although many of the ligands are active in vivo as anxiolytic/anticonvulsant agents or as inverse agonists, the future for the original CGS series as clinical agents was limited due to the poor solubility of these ligands. However, the unique topology of their structure along with the potent affinity in vitro establishes them as excellent templates for the development of pharmacophore/receptor models.[52,125] The fit of the CGS series at the H_1, H_2, and L_1 descriptors of the pharmacophore/receptor models (α1−6 isoforms) for BzR is nearly perfect. A number of new pyrazoloquinolinones with linear side chains (such as acetylenes) have been synthesized to explore regions L_2 and/or L_{Di} of the pharmacophore/receptor model.[128] In general, this series of CGS-like ligands exhibited potent affinities at all five receptor subtypes (Table 8). Ligands substituted with the trimethylsilylacetylene moiety at position C4′ (**123**) displayed affinities which were 20−30 times less potent than the protio analogue (see **118**). This ligand (**123**) also bound poorly to α6-containing receptor isoforms. This suggested that the size of the

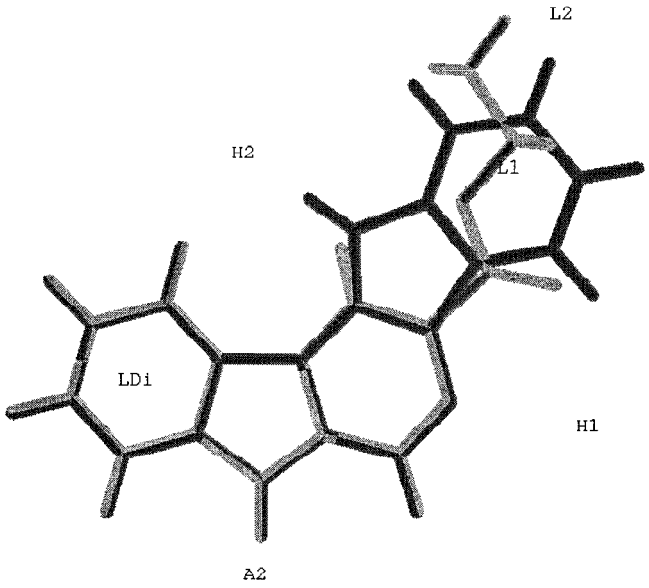


Figure 8. BCCE (**63**) (gray) and diindole (**111**) (black) aligned in the pharmacophore/receptor model for BzR.

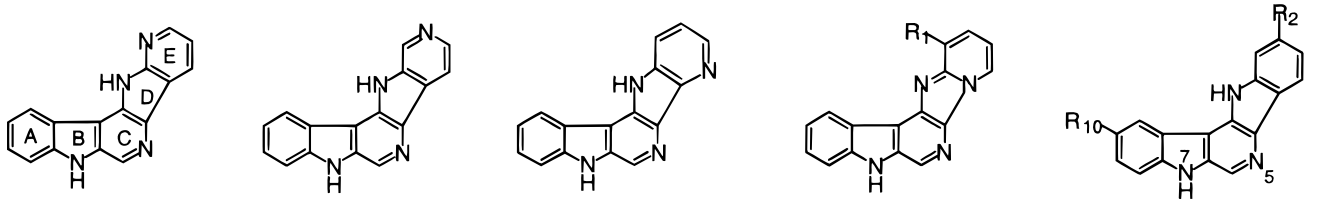
binding pharmacophore in the α6 receptor subtype was smaller in region L_2 than the other receptor subtypes, in agreement with earlier work on the unified pharmacophore/receptor model.[21,52] Most of these new pyrazoloquinolinone ligands (see Table 8) exhibited less potent affinity at the α5 subtype than at the α1 subtype including those substituted with acetylene and trimethylsilylacetylene substituents. Examination of the overlap of 4′-acetylenopyrazoloquinolinone **(124)** with RY 80 (**11**) can be employed to illustrate the differences in occupation of regions L_{Di} and L_2 of the pharmacophore/receptor model exhibited by the two compounds (see Figure 11). It is proposed that potent interaction of ligand **124** in region L_{Di} in the pharmacophore/receptor model may have compromised the key interaction at L_2 required for α5 selectivity, accounting for the α1 selectivity as reported elsewhere.[129]

In addition to pyridodiindoles, phenylpyrazoloquinolinones were also employed as templates for the construction of the unified pharmacophore/receptor model of BzR due to the high potency of this series at BzRs.[52] In brief, in the study the pyrazoloquinolinone **124** was rms fitted into the pharmacophore/receptor model in such a manner that the lone pair of electrons of N1 interacted with the hydrogen bond donor site H_2 of the model with a bond length of 1.84 Å and a bond angle (N2−N1−H_2) of 126°. The lone pair of electrons of the C3 carbonyl oxygen atom was aligned with the hydrogen bond donor site H_1 with a bond length of 1.84 Å and an angle (C═O−H_1) of 135°. The proton on the N5 nitrogen atom interacted with hydrogen bond acceptor site A_2 to form a hydrogen bond with a bond length of 1.84 Å and a bond angle (N6−H−A_2) of 180°. Finally, the centroid of the phenyl moiety at position-2 was aligned in the lipophilic region L_1 of the pharmacophore/receptor model (Figure 11). This alignment has been employed for ligands **118**−**129** in the present work and is in complete agreement with earlier modeling reported in this series.[52,53,127]

4. 1,4-Benzodiazepines. Since the introduction of Librium in the 1960s and the subsequent discovery of its mode of action via $GABA_A$ receptors by Squires, Braestrup, Haefely, Möhler, and Okada,[1,130−132] the benzodiazepines have enjoyed widespread use rivaled by few other classes of compounds. In general, 1,4-benzodiazepines are nonselective ligands[133] which bind to all BzR isoforms which are DS (see Table 9). As a result, most of the 1,4-benzodiazepines display a wide range of pharmacological activities, such as anxiolytic/anticonvulsant, sedative/hypnotic, ataxic/myorelaxant, and amnesiac. It is now believed that agents selective for specific BzR subtypes may permit one to separate out the pharmacological activities of these isoforms. Furthermore, the actual intrinsic activity of BzR ligands plays an important role. Compounds with low intrinsic activity (so-called partial agonists) do not cause motor deficits even at doses at which ligands occupy a high percentage of BzRs in the CNS. However, full agonists, such as flunitrazepam, interrupt the rotarod performence of rodents even on occupation of only 13% and 19% of BzRs in cerebellum and the spinal cord, respectively. In contrast, the ED_{50} of a partial agonist (clonazepam) was found to occupy 80% and 78% of the BzRs in the same regions, respectively, of the CNS in order to interrupt the same test.[134] The mechanism of intrinsic activity of a ligand is still not clear. Attempts to define this activity at the pharmacophore/receptor level have been made.[52,53]

Studies of the pharmacophore/receptor model via the synthesis of 1,4-benzodiazepines[70,135] and a series of β-carboline ligands[30,51] suggested that occupation of region L_3 of the receptor by a BzR ligand would lead to agonist activity.[52,67] From the CGS series it was assumed that full occupation of regions L_2 and L_3 would lead to a full agonist, while partial occupation of region L_3 would provide a partial agonist. The synthesis of the partial agonist 6-PBC (**87**) was based on this hypothesis.[51] Many 1,4-benzodiazepines are found to be full agonists, since the 5-aryl moiety fully occupies region L_3 of the receptor site. Benzofused 1,4-benzodiazepines have been employed to further probe the pharmacophore/receptor models of BzR subtypes.[72] It was shown that molecular descriptors (repulsive regions) S_1 and S_2 were valid for all four DS sites, since the 7,8-benzofused 1,4-benzodiazepines bound to all four BzR subtypes (DS sites) better than the 6,7-benzofused and 8,9-benzofused systems (see Figure 12).[72] A few of these benzofused 1,4-benzodiazepines were found to be moderately selective at α5 isoforms. More importantly, differences in the ability to accommodate the lipophilic substituent of a ligand at region L_2 were observed in comparison to different receptor subtypes. Examination of the affinities of these benzofused 1,4-benzodiazepines at five recombinant $GABA_A$/BzR subtypes revealed that region L_2 in the α5-containing receptor isoform was larger than the others, which was in agreement with findings from the imidazobenzodiazepine series.[32,73,77] On the basis of this work and the α5 selectivity of RY 80 (**11**), the synthesis of a 7-acetyleno-substituted 1,4-benzodiazepine, a diazepam congener, was executed and resulted in a ligand (**139**) with 7-fold overall selectivity in vitro for the α5 receptor subtype.[72] The ligand also displayed an 11-fold better affinity at the α5-containing receptor isoform than at the α1 subtype (Figure 13). To our knowledge this was the first agonist ligand to exhibit

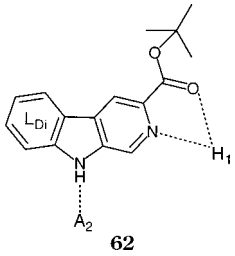
Table 7. Affinities of Pyridodiindoles at $\alpha x\beta 3\gamma 2$ GABA$_A$/BzR Subtypes



106 **107** **108** **109-110** **111-117**

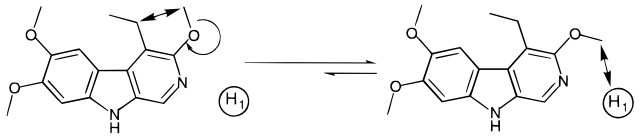
				K_i (nM)[a]				
ligands	R_1	R_2	R_{10}	α1	α2	α3	α5	α6
106				2.2	11.5	16.3	200	>10000
107				3.0	23.8	30.5	240	>10000
108				28	118	156	~1000	>10000
109	H			105	228	284	>3000	>10000
110	Cl			>3000	>3000	>3000	>10000	>10000
111		H	H	1.1	1.2	1.1	40.3	>1000
112		H	NO_2	244	500	532	1560	>10000
113		Cl	H	3.9	12.2	24.4	210	>10000
114		F	H	5.1	10.4	18.4	260	>10000
115		OCH_3	H	3.4	11.7	11.0	225	>10000
116		BnO	H	432	~3000	~3000	>10000	>10000
117		OC(O)*t*-Bu	H	156	NAb	NA	>3000	>10000

[a] Data shown here are the means of two determinations which differed by less than 10%. [b] NA stands for data not available.



62

Figure 9. BCCT **(62)** in the pharmacophore/receptor model.



97, *anti* **97**, *syn*

Figure 10. Energy difference between the two conformers of **97** is over 16 kcal/mol. The *anti* conformer (the active conformer) is a high-energy conformer. However, the low-energy *syn* conformer is not favored for high-affinity binding at BzRs due to the steric repulsion between the 3-methoxy moiety of the β-carboline nucleus and the H_1 site on the receptor protein.

some α5 selectivity reported from the 1,4-benzodiazepine family (see Table 9).

Recently this ligand **(139)** was shown to reverse the convulsant activity of RY 24 (**27**), an α5-selective inverse agonist, in NIH mice.[104] Under similar conditions, zolpidem, an α1-selective agonist, was not able to effectively block these convulsions in mice at comparable doses.[104] Importantly, a 7-cyano congener **(140)** did not bind to this series of recombinant receptor subtypes in agreement with earlier work of Haefely and Fryer on the SAR of 1,4-benzodiazepines.[41,60] This ligand **(140)** also did not exhibit any subtype selectivity. This again supports the hypothesis that occupation of region L_2 with lipophilic groups is important for α5 selectivity as well as for high affinity.

The 1,4-benzodiazepines in general are nonselective at all four of the DS subtypes (α1−3 and α5), as was expected. Moreover, 5-phenyl-substituted 1,4-benzodiazepines did not bind to the α6-containing receptor isoform, which is one of the DI subtypes. A study of this DI subtype via molecular modeling[52,53,73] again revealed that this receptor subtype may be devoid of region L_3 which accommodates the aryl moiety at position-5 of the 1,4-benzodiazepine nucleus of DS sites, according to the pharmacophore/receptor model. Based on a limited amount of data, it appears the DI α4 site shares this characteristic with the α6 isoform.[105]

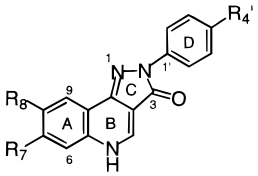
For the present modeling study the 1,4-benzodiazepines were aligned into the pharmacophore/receptor model in such a manner that the centroid of ring-A interacts with region L_1. The lone pair of electrons of the C2 oxygen atom interact with H_1, while the lone pair of electrons on N4 interact with H_2 of the receptor protein (Figure 13).[52,53,105] This alignment is in agreement with previous reports on the unified (average) pharmacophore/receptor model.[52,53]

5. Triazolopyrimidines (CL 218872) and Imidazopyridines (Zolpidem). a. CL 218872. The triazolopyrimidine, CL 218872 (**149**), was found by Beer and Lippa to be a partial agonist at BzRs.[33,136] This ligand was also reported to be selective for the classical type-I BzR and has been used to identify and characterize the type-I BzR in the brain.[137,138] In addition to CL 218872 (**149**), zolpidem (**147**) and alpidem (**152**) (two type-I-selective ligands) have been employed in the laboratory to identify receptors which contain an α1 subunit. [14,96,139]

For the present study, compound **149** was aligned in the pharmacophore/receptor model to permit the lone pair of electrons of N1 to interact with H_1 while the lone pair of electrons on N4 interacted with the H_2 descriptor of the receptor protein. The centroid of the phenyl moiety at position-6 of the triazolopyrimidine nucleus overlapped with region L_1 of the pharmacophore/receptor model (Figure 15a).

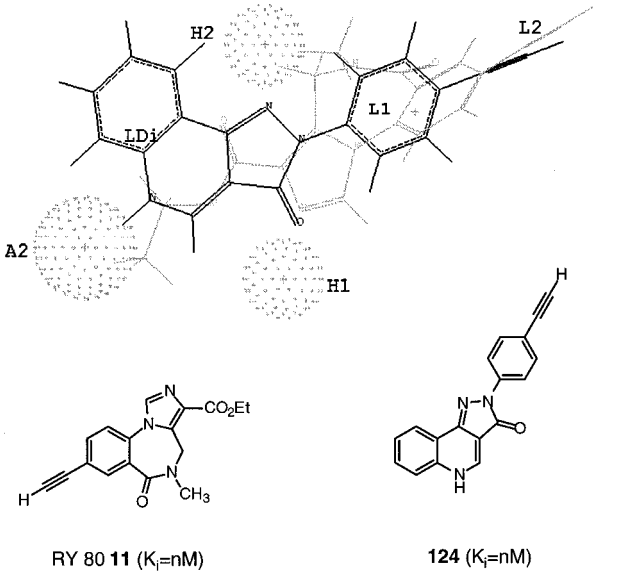
b. Zolpidem. The alignment of zolpidem (**147**) in the pharmacophore/receptor model is illustrated in Figure

Table 8. Affinities of Linearly Substituted Pyrazoloquinolinones at $\alpha x\beta 3\gamma 2$ $GABA_A$/BzR Subtypes



				K_i (nM)[a]				
ligand	R_8	$R_{4'}$	R_7	α1	α2	α3	α5	α6
118 (CGS-8216)	H	H	H	0.05	0.08	0.12	0.25	17
119 (CGS-9896)	H	Cl	H	NA[b]	NA	NA	NA	NA
120	Br	H	H	0.06	0.08	0.05	0.12	4
121	C≡CH	H	H	0.08	0.06	0.02	0.08	NA
122	H	Br	H	0.18	0.21	0.25	1.3	40
123	H	$C{\equiv}CSiMe_3$	H	3.0	3.7	4.7	24	>1000
124	H	C≡CH	H	0.07	0.03	0.04	0.17	NA
125	OMe	H	H	0.11	0.10	0.09	0.20	>10
126	Cl	OMe	H	0.20	NA	0.20	0.32	1.90
127	H	OMe	OMe	0.34	NA	0.79	0.52	10
128	H	H	OMe	1.34	1.31	1.26	0.84	2.03
129	Cl	H	H	0.23	0.17	0.12	0.44	17.3

[a] Data shown here are the means of two determinations which differed by less than 10%. [b] NA stands for data not available.



RY 80 **11** (K_i=nM)

α1	α2	α3	α5	α6
28.4	21.4	25.8	0.49	28.8

124 (K_i=nM)

α1	α2	α3	α5	α6
0.07	0.03	0.04	0.17	NA

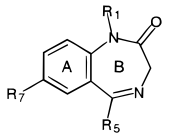
Figure 11. RY 80 (**11**; gray) and Ligand **124** (black) in the pharmacophore/receptor model.

15c. The centroid of the phenyl moiety at position-2 of zolpidem overlapped with region L_1 of the receptor, while the lone pair of electrons of N1 interacted with H_1 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 H_2 of the receptor protein (Figure 15c).

c. α1 Selectivity and L_{Di}. Examination of the alignment of CL 218872 (**149**), zolpidem (**147**), and RY 80 (**11**) revealed some of the differences among these ligands in regard to the occupation of the pharmacophore/receptor model of the BzR (Figure 15). As clearly illustrated in Figure 15, the α1-selective ligands, CL 218872 (**149**) and zolpidem (**147**), had better occupation of region L_{Di}, in the receptor model, while the α5-selective ligand RY 80 (**11**) demonstrated more complete occupation of region L_2. The α1 subtype selectivity of many β-carbolines, such as BCCT (**62**), further supports the observation that occupation of region L_{Di} should result in a ligand with enhanced α1 selectivity. The further exploration of this region is expected to lead to more selective ligands at α1-containing $GABA_A$/BzR isoforms.

6. Pharmacophore/Receptor Models for $GABA_A$/BzR Subtypes. Depicted in Figure 16 are the included volumes of the pharmacophore receptor models for α1-, α5-, and α6 (DI)-containing receptor subtypes. Ligands employed in the included volume for each receptor subtype are those which exhibited potent affinity ($K_i \leq$ 20 nM) at the receptor subtype. However, CL 218872 (**149**, $K_i\alpha 1$ = 57 nM) and zolpidem (**147**, $K_i\alpha 1$ = 26.7 nM), as mentioned, have been added to the included volume of the $\alpha 1\beta 3\gamma 2$ receptor subtype since both ligands are more selective for the α1 subtype.[33,38,138] Depicted in each figure on the right-hand side is the orthogonal view of the same object illustrated at the left. When the included volume of the α5 pharmacophore/receptor model (yellow) was overlapped with that of the volume of the α1 isoform (blue), it was clear that the left (west) side of the pharmacophore of the α1-containing receptor subtype was larger than that of the α5-containing receptor isoform (see Figure 17a). Contributions to this L_{Di} area are derived in large measure from 7-substituted β-carbolines as well as ring-A-substituted pyrazoloquinolinones (CGS series) (see figure legend for details). Most β-carbolines demonstrated some α1 selectivity even though it may be moderate in level. The small pyrazoloquinolinones exhibited tight affinity at all subtypes with very little selectivity for the α1 subtype. More work in this area is underway at present. Both series of ligands occupy lipophilic region L_{Di} in the pharmacophore/receptor model with ring-A of their molecular structure. This result implies that occupation of L_{Di} may be critical for the selectivity of a ligand at receptors which contain α1 subunits. Results from other laboratories also suggest that occupation of region L_{Di} is highly recommended for selective binding at receptors which contain an α1 subunit.[140]

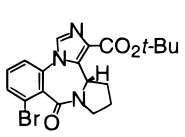
Table 9. In Vitro Affinities of Substituted 1,4-Benzodiazepines at Recombinant α*x*β3γ2 GABA$_A$/BzR Subtypes



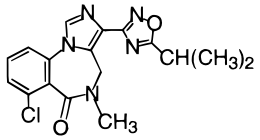
				K_i (nM)[a]					
ligand	R_1	R_5	R_7	α1	α2	α3	α5	α6	α1/α5
130	Me	2-furyl	Cl	>300	>300	>300	>300	>300	NA[b]
131	Me	2-thienyl	Cl	19.2	13.2	13.4	11.5	>300	1.7
132	Me	3-thienyl	Cl	>300	>300	>300	>300	>300	NA
133	Me	*c*-butyl	F	>300	>300	>300	>300	>300	NA
134	H	2-thienyl	F	175	335	405	150	>300	1.17
135	Me	2-thienyl	F	141	215	205	65.5	>300	2.15
136	H	2-azidophenyl	N_3	2160	NA	4620	1640	>1000	1.32
137	Me	2-nitrophenyl	NO_2	0.49	NA	0.76	7.7	>10000	0.06
138	Me	phenyl	C≡CSiMe$_3$	94	73	203	63	>3000	1.49
139 (QH-II-66)	**Me**	**phenyl**	**C≡CH**	**76.3**	**42.1**	**47.4**	**6.8**	**>3000**	**11.2**
140	Me	phenyl	CN	320	310	350	265	>3000	1.2
141	H	phenyl	H	286	233	350	140	>3000	2.04
142	Me	phenyl	H	81	138	318	96	>3000	0.84
143	H	2-nitrophenyl	Br	16	31	52	199	>3000	0.08
144	Me	phenyl	Br	9.4	9.3	31	7.7	>3000	1.22
145 (diazepam)[c]	**Me**	**phenyl**	**Cl**	**14**	**20**	**15**	**11**	**>3000**	**1.3**
146 (flunitrazepam)	Me	2-nitrophenyl	F	2.2	2.5	4.5	2.1	>2000	1.05

[a] Data shown here are means of two determinations which differed by less than 10%. [b] NA stands for not applicable. [c] Data obtained from ref 133 for comparison purposes.

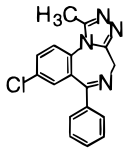
Table 10. Affinities of Miscellaneous Ligands at α*x*β3γ2 (*x* = 1−3, 5, 6) Receptor Subtypes



148



150



151

	K_i (nM)[a]				
ligand	α1	α2	α3	α5	α6
147 (zolpidem)[b]	26.7	156	383	>10000	>10000
148 (bretazenil)[b]	0.35	0.64	0.2	0.5	12.7
149 (CL218,872)[b]	57	1960	1160	561	>10000
150 (FG8205)[b]	0.4	2.08	1.16	1.54	227
151 (triazolam)[b]	0.8	0.59	1.43	1.54	>10000

[a] Data shown here are the means of two determinations which differed by less than 10%. [b] These ligands were employed for comparison purposes in these sets of assays and the reported values obtained (see ref 137 for details).

In contrast, as illustrated in Figure 17a, region L_2 is believed to be deeper in the pharmacophore of the α5 receptor subtype than in the α1 isoform. Examination of the illustrations in Figure 17a demonstrate that region L_2 for the α5-containing receptors is deeper in size than in the corresponding α1 or α6 receptor subtypes, based on the ligands from this study (**1**−**151**) as well as others.[52,73,74] It has been observed that ligands with α5 selectivity are generally imidazobenzodiazepines. The C8 substituent of this series of ligands occupies region L_2 of the pharmacophore. For example, the acetyleno function of RY 80 (**11**), one of the most potent α5-selective ligands, occupied this region L_2 and displayed α5 selectivity.[77] The selectivity of QH-II-66 (**139**) at α5-containing receptor subtypes further supported this hypothesis.[72] Furthermore, the chloro analogue of this 1,4-benzodiazepine, diazepam, was nonselective (Figure 13). Occupation of region L_{Di} of ligands in the imidazobenzodiazepine series was poor, as illustrated in Figure 11, in comparison to both β-carbolines and the CGS series. In a fashion similar to imidazobenzodiazepines, 1,4-benzodiazepines do not occupy region L_{Di} to the degree that β-carbolines and pyrazoloquinolinones (CGS series) occupy this area.

Illustrated in Figure 17b is the included volume of the pharmacophore/receptor model of the α6 subtype (in green) overlapped with the volume of the α5 receptor subtype (purple), respectively. It was found this α6 (DI) receptor subsite was devoid of lipophilic pocket L_3. This was in agreement with the previous finding that the BzR DI site was devoid of lipophilic pocket L_3.[21,52] This was based on the activity of 1,4-benzodiazepines which do not bind to this α6-containing receptor subtype. Lack of region L_3 was believed to be responsible for the insensitivity of this receptor subtype to diazepam and its congeners. With a few exceptions, β-carbolines also do not bind to the α6β3γ2 receptor subtype. Ligands which bind to this DI site are small and are represented mostly by imidazobenzodiazepines and pyrazoloquinolinones (CGS series). It was previously claimed that Ro 15-4513, an 8-azidoimidazobenzodiazepine, was a DI-selective ligand.[20] On the basis of the affinity of Ro 15-4513 (**9**) at the five recombinant receptor subtypes, this series of ligands (in large part) exhibited some selectivity toward receptors which contain α5 subunits, rather than those at α6.[32,71,77] In general, pyrazoloquinolinones bind tightly to this receptor subtype. However, the affinity diminished when the size of a substituent at ring-D increased (**123**), similar to the finding observed at the classical DI site.[124] Comparison of the pharmacophore/receptor included volume of this receptor subtype with the volumes of the α1 and α5 receptor subtypes revealed that the pharmacophore/receptor model for the α6-containing receptor was much smaller in size than the receptor subtypes which contain α1 or α5 subunits.

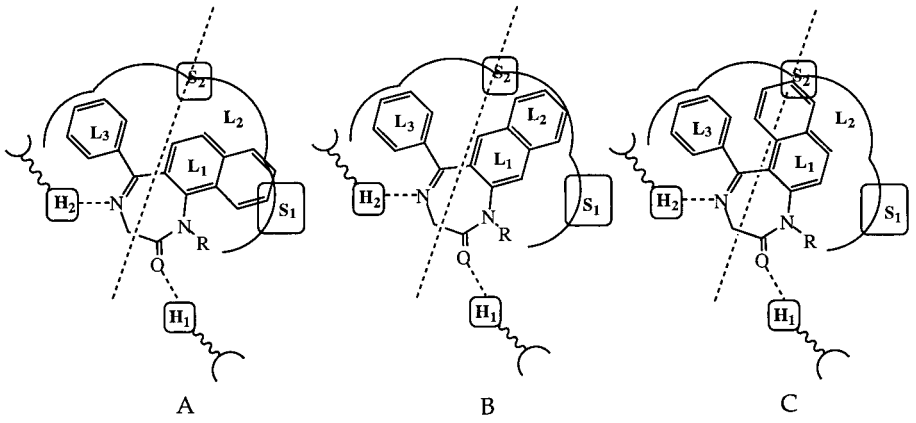


Figure 12. Benzofused 1,4-benzodiazepines as molecular yardsticks to probe the size and dimensions of region L_2 of the BzR pharmacophore model. H_1 and H_2 are the hydrogen bond donor sites on the receptor. L_1, L_2, and L_3 are three lipophilic pockets in the receptor pharmacophore. S_1 and S_2 are two areas of steric repulsion in the receptor binding domain.

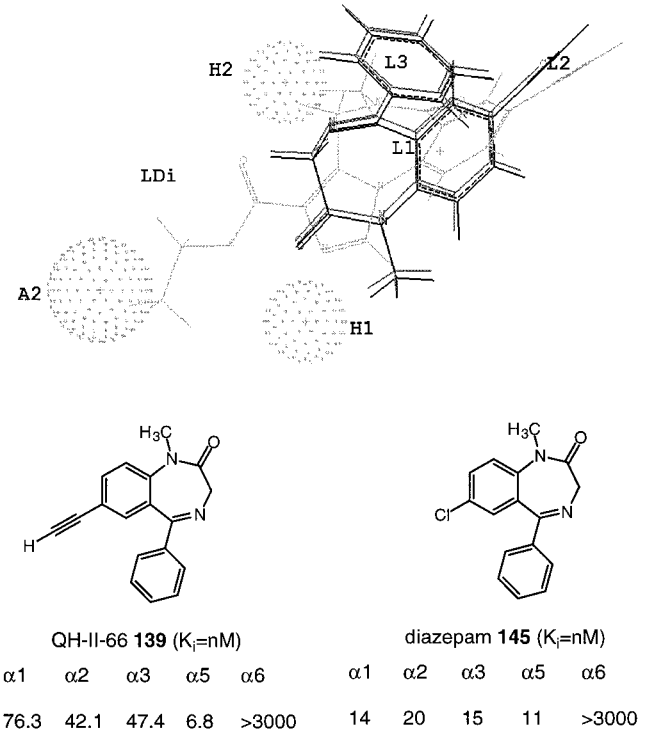


Figure 13. Overlap of RY 80 (**11**; light gray with an acetylene moiety at position-8 of the imidazobenzodiazepine nucleus) with diazepam (**145**; gray with a chlorine at position-7 of the 1,4-benzodiazepine) illustrates the difference in the occupation of region L_2 in comparison to RY 80 (**11**). The ligand QH-II-66 (**139**, a 7-acetylenyl-1,4-benzodiazepine; black) was designed to offer better occupation of this region and exhibit α5 selectivity. Note that diazepam shown in the figure was intentionally offset from its original position, which had overlapped completely with QH-II-66, solely for the sake of visualization.

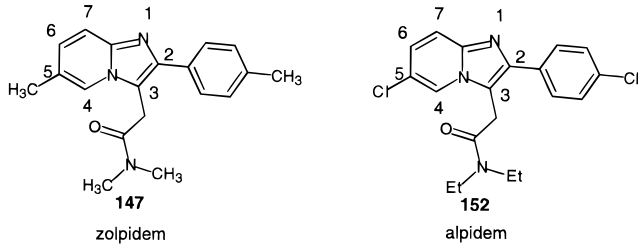


Figure 14. Zolpidem (**147**) and alpidem (**152**).

In summary, pharmacophore receptor models for three $GABA_A$/BzR subtypes have been established via an SAR study based on the affinities of 151 BzR ligands at five distinct recombinant $GABA_A$/BzR receptor subtypes. Ligands have been chosen for this study from at least nine different structural families. The pharmacophore receptor models for these receptor subtypes which resulted revealed differences among receptor subtypes which have directed the design of novel selective BzR ligands at the α5-containing receptor subtype. Further investigation in the area is expected to result in even more selective ligands for a specific receptor subtype. This will provide agents to probe which receptor subtype mediates a specific physiological response as well as potential agents for clinical use.

Biology

Imidazobenzodiazepines have become important in the design of the pharmacophore/receptor model for the α5β3γ2, subtype and modification of the structure of Ro 15-4513 (**9**) has provided some insight into α5-selective ligands.[32,104] The inverse agonist Ro 15-4513 (**9**) had formerly been described as a DI ligand; however, much work indicates **9** binds with high affinity to the α5 subtype as well.[32,73] Ligands such as **11** and **27** have been shown to be some of the most selective ligands at BzR subtypes reported to date and are being employed to decipher some of the pharmacology regulated by α5 subtypes in the hippocampus.[32,104,141]

Recent results with the allene **10** [a geometric bioisostere of Ro 15-4513 (**9**)] are of interest in this regard. The 8-allenylimidazobenzodiazepine **(10)** (Table 1) antagonized the sedation produced by a 0.75 g/kg dose of EtOH when given intrastriatally; however, **10** did not alter the sedative effect of the same dose of EtOH when given systemically.[142] The allene **10** did, however, attenuate the sedation produced by a higher dose (1.25 g/kg) of EtOH.[142] The ligand **10** when given alone was without effect. Intrastriatal modulation of the moderate dose of EtOH was site-specific; no antagonism by allene **10** was observed after infusion into the nucleus accumbens. For this reason two other imidazobenzodiazepines, Ro 15-4513 (**9**) and Ro 15-1788 (**2**), were compared with **10** for their capacity to decrease GABAergic function in the *Xenopus* oocyte expression system and their capacity to decrease EtOH sedation. Examination of data reported recently[142] indicates that reversal of the

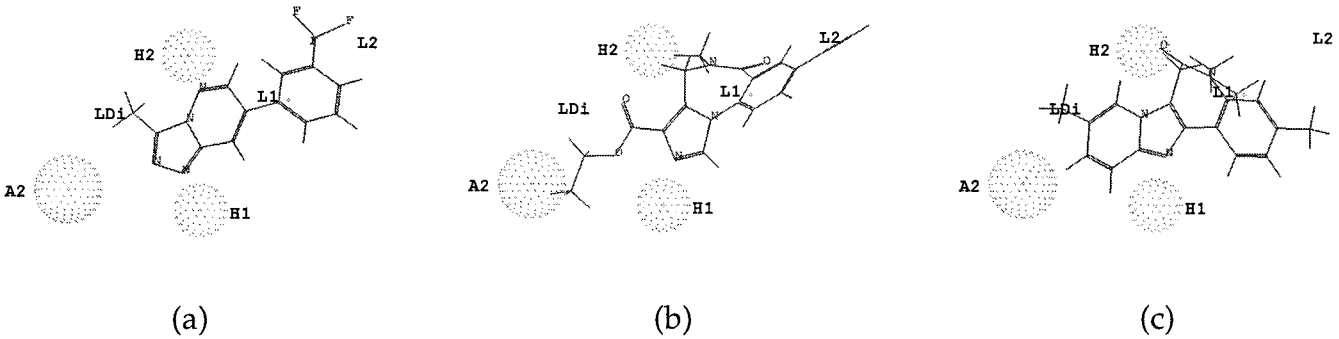


Figure 15. (a) CL 218872 (**149**), (b) RY 80 (**11**), and (c) zolpidem (**147**) in the pharmacophore/receptor model.

sedation produced by EtOH by **10** was not due to a decrease of GABAergic function at α1 or α6 receptor subtypes. At α1 and α6 BzR, **10** exhibited a neutral−partial agonist pharmacological profile, despite maintaining the ability to reverse the effects of EtOH.[142] The lead ligand Ro 15-4513 (**9**)[143−145] exhibited a slightly negative pharmacological profile at α1β2γ2 subtypes which correlated with its classification as a "partial inverse agonist" capable of reversing the action of EtOH across a number of behavioral paradigms.[144,145] This ligand **(9)** also displayed a partial agonist profile at α6β2γ2 receptors in *Xenopus* oocytes.[142] Although flumazenil (**2**) was not injected directly into the striatum, June et al. previously reported[146] that systemic administration of **2** did not alter EtOH-induced sedation (0.5−1.5 g/kg).[146] In that study, Ro 15-1788 (**2**) did not alter GABAergic function at α1 subtypes (i.e. zero efficacy in oocytes), in agreement with the classification as a neutral BzR ligand. However at the α6β2γ2 subtype, **2** exhibited a partial agonist profile similar to allene **10** and Ro 15-4513 (**9**).[142] Analysis of these findings indicates that allene **10** exhibited a behavioral profile similar to that of **9** but that its pharmacological profile (efficacy in oocytes) at the α1 and α6 subunits is more similar to that of flumazenil (**2**).[142,147] Consequently, it is unlikely that negative intrinsic efficacy (i.e. reduced GABAergic function) is a sufficient explanation of the ability of **10** to antagonize the sedative action of EtOH. However, despite the inability of allene **10** to decrease GABAergic function, accompanied by its high affinity at several recombinant receptors (see Table 1), it is possible that these data may not extrapolate to whole animal pharmacology.

Importantly, the results of the study by June et al.[142] demonstrated that the novel ligand, allene **10**, was capable of attenuating the EtOH-induced sedation produced by both a 0.75 and 1.25 g/kg dose of EtOH, depending on whether **10** was administered systemically or directly infused into the dorsal striatum.[142] The finding with **10** provided further evidence that the GABA$_A$/BzR complex plays an important role in mediation of the sedative properties of EtOH. Moreover, analysis of the data of June et al.[142] provide new evidence that specific GABA$_A$/BzR receptors in the dorsal striatum, but not in the NA (a neuro-anatomical substrate proposed to play a role in the rewarding properties of EtOH[148]), mediate the sedative effects of EtOH.[142]

Helmstetter et al.[141] have recently reported a study with the α5-selective imidazobenzodiazepine RY 24 (**27**, see Table 2) in regard to learning and memory. In a fashion similar to the work of June et al.,[142] Helmstetter injected **27** into known structures in the brain. Since RY 24 (**27**) and RY 80 (**11**) demonstrated selectivity for α5 receptor subtypes,[32] Helmstettler administered **27** into the dorsal hippocampus, a structure known to be involved in learning and memory, prior to training in a Pavlovian fear-conditioning paradigm (shock presentation).[141] Hippocampal injections of **27** (1−5 μg/μL) produced fear-related behavioral responses prior to presentation of aversive stimuli during training. At higher concentrations (5−10 μg/μL), **27** decreased the strength of the conditioning observed in a retention test 24 h after training when animals were returned to the environment in which they received a shock. Analysis of the reported data[141] provided further evidence of the involvement of hippocampal GABA$_A$ receptors in anxiety and the acquisition of fear conditioning.

The behavioral effects of hippocampal injections of **27** in this paradigm were clearly dependent on the concentration employed. Ligand **27** was anxiogenic at the 1 and 5 μg/μL doses during the baseline period in the training session. However, the largest dose (10 μg/μL) did not elicit significant amounts of freezing behavior in the rats.[141] At 1 μg/μL, pretraining injection of **27** significantly increased the strength of conditional fear responses observed 24 h after training in the absence of **27**. However, contrary to previous studies using different measures with similar inverse agonists (see **11**),[32] as the dose of **27** delivered into the hippocampus during training was increased, the strength of conditioning decreased. Consequently, pretraining injections had a dose-related, although biphasic, effect on learning. Since no seizures[104] were observed in any of the animals at any of the doses employed, **27** does not appear to be proconvulsant at these dose levels.

Helmstetter reported that the shape of the dose−effect relationship on conditioning measured 24 h after training may be related to the fact that although **27** was highly selective for α5-containing GABA$_A$/BzRs, it does possess some affinity for other subtypes as well (see Table 2).[147] Higher doses of **27** are not as anxiogenic as the smaller doses and appear to elicit amnesiac effects, which suggested that at higher concentrations **27** behaved as an agonist at some of the other α-subtypes.[142,147] At low concentrations **27** may retain the properties of an inverse agonist.[32] However, as the concentration increases, it is possible that the α5β2γ2 subtypes become saturated and the remainder of **27** was available to bind at other subtypes and exhibited a response more characteristic of BzR agonists. Certainly ligands which bind to BzR have shown opposite effica-

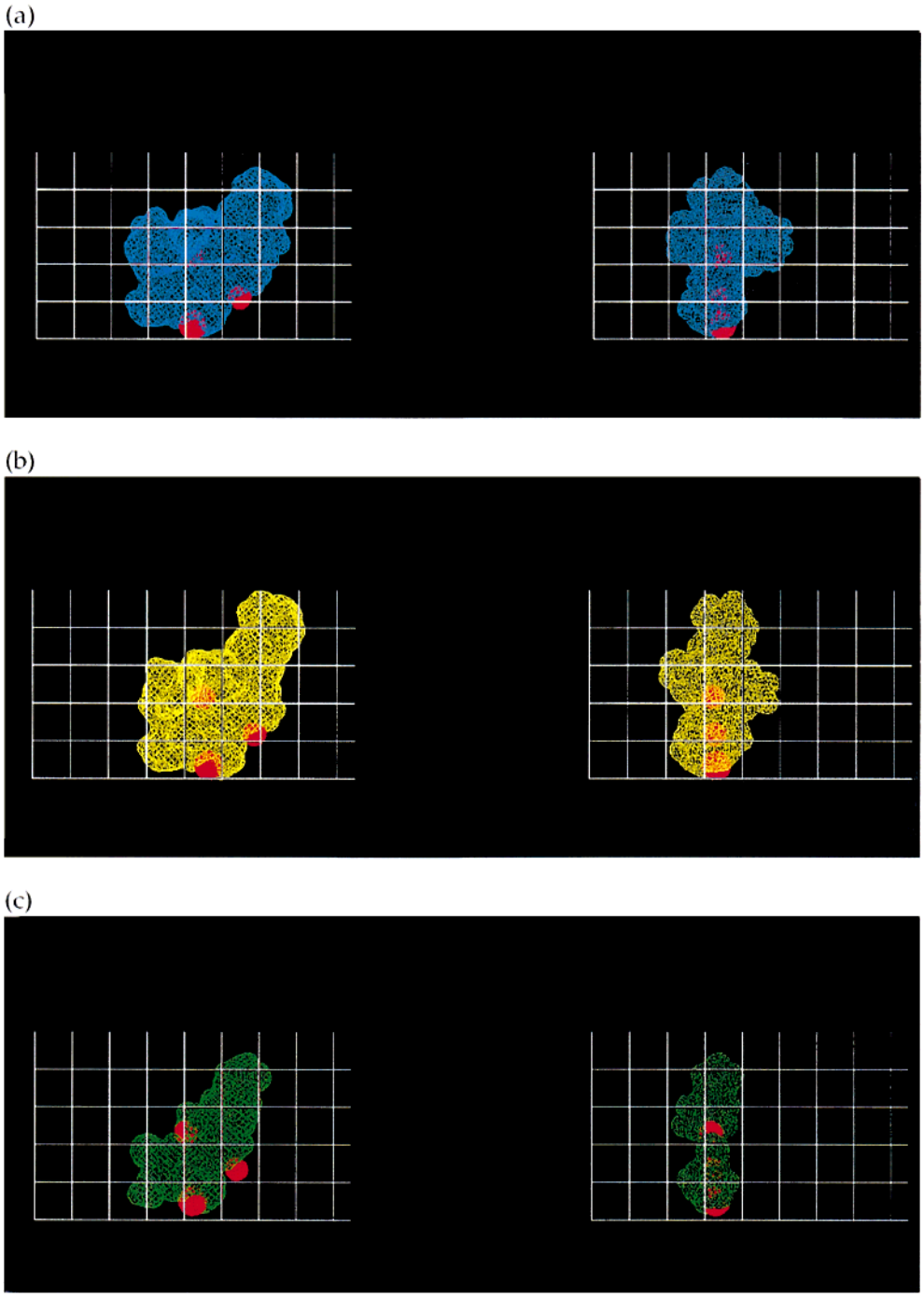


Figure 16. (a) Orthogonal views of the included volume of the pharmacophore/receptor model for the α1β3γ2 subtype. Ligands **1**−**3**, **5, 6**, **8**−**10**, **12**, **15**−**26**, **30**, **41**, **48**, **57**, **61**−**64**, **67**, **74**−**79**, **81**, **84**, **86, 87**, **91, 92**, **94, 95**, **99, 100**, **106, 107**, **111**, **113**−**115**, **118**, **120**−**129**, **131, 137**, and **143**−**151** have been included in this illustration. (b) Orthogonal views of the included volume of the pharmacophore/receptor model for the α5β3γ2 receptor subtype. Ligands **1**−**13**, **15**−**28**, **30**, **41, 42**, **44**, **46**−**51**, **52**, **54, 55**, **57**−**59**, **81**, **84**, **86, 87**, **91, 92**, **95**, **99, 100**, **118**, **120**−**122**, **124**−**129**, **131**, **137**, **139**, **144**−**146**, **148, 150,** and **151** have been included in this illustration. (c) Orthogonal views of the included volume of the pharmacophore/receptor model for the α6β3γ2 receptor subtype. Ligands **9**, **15**−**19**, **23**, **27**, **15**, **118**, **120**, **126**−**129,** and **148** have been included in this illustration. (Each grid measures 4 Å in width and height.)

cies at different receptor subtypes expressed in oocytes.[142,147] This may be the reason the smaller increase in fear-related behavior was observed in the 10 μg/μL group relative to controls during the pretraining baseline as well as the apparent memory-impairing effects on acquisition observed 24 h following training.

Analysis of the data from the previous report[141] lends additional support to the role of the hippocampus in anxiety and learning. Moreover, the data suggest that BzRs within the hippocampus are important for the acquisition of fear conditioning and that this form of learning may be modulated by compounds with specificity for the α5 subunit. It must be remembered that nearly all of the α5β2γ2 subtypes are located in the hippocampus while α1 subtypes are found throughout the brain.[149] It was pointed out that disruption of acquisition observed 24 h after training probably does not reflect state-dependent learning[150] since injection

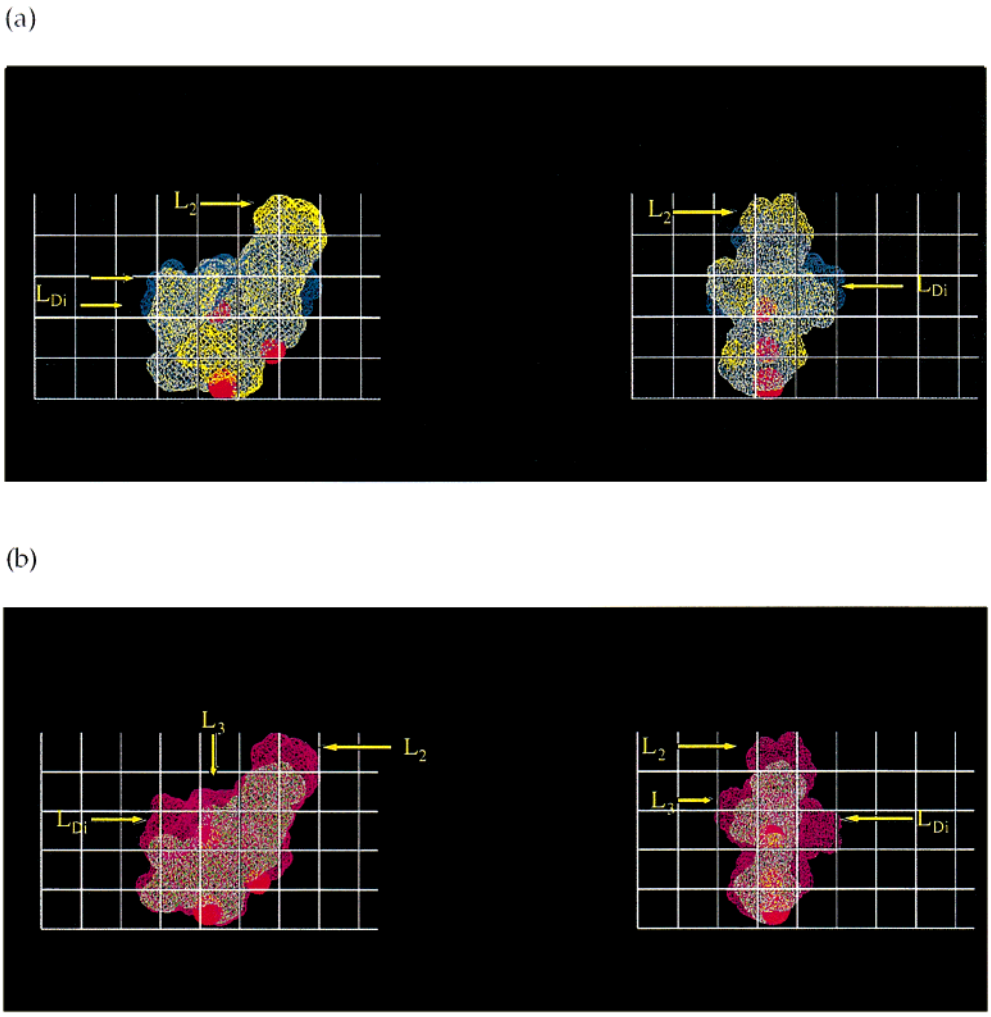


Figure 17. (a) Orthogonal views of the overlap of the included volumes of the pharmacophore/receptor models for α1β3γ2 (blue) and α5β3γ2 (yellow) receptor subtypes. (b) Orthogonal views of the overlap of the included volumes of the pharmacophore/receptor models for α5β3γ2 (purple) and α6β3γ2 (green) receptor subtypes. (Each grid measures 4 Å in width and height.)

of the drug before training and testing revealed no significant change in behavior when compared to pretraining injections alone.

Since **27** was shown to enhance memory and learning when injected into the hippocampus, it was of interest here to determine if either Ro 15-4513 (**9**) or **27** would enhance memory when given systemically. Previous research which involved stress- or fear-producing paradigms points to BzR inverse agonists as possessing "cognitive-enhancing properties". For example, FG 7142, but not all inverse agonists, improve short-term memory function in laboratory animals,[151] and **9** is reported to decrease punished responding.[152] Ligand Ro 15-3505 injected before retention tests in aged mice performing a T-maze resulted in marked improvement of response accuracy.[153] Another inverse agonist, BCCM (Table 6), enhanced acquisition of a passive avoidance task in rats but had no effect after posttraining administration.[154] The inverse agonists BCCE (**63**), CGS-8216 (**118**), and S-135 all augmented the magnitude of long-term potentiation in hippocampal slices induced by tetanic stimulation of input fibers in both the CA1 and CA3 regions.[155]

Although **27** had been shown to be an inverse agonist previously,[32] the effects of systemic administration of **27** on memory had not been evaluated. The importance of this assessment is amplified by the work of Helmstetter (hippocampal injections of **27**)[141] described above. Since **27** behaves as an inverse agonist in some paradigms,[32] a test of short-term memory and a test widely utilized to predict clinical antianxiety activity were utilized to further investigate the pharmacological activity of RY 24 (**27**). In addition, the activity of the prototypical inverse agonist Ro 15-4513 (**9**) was evaluated for comparison purposes. The effect of systemic administration of **27** on short-term memory was determined and compared to that of **9** in both pigeons and squirrel monkeys responding under a titrating matching-to-sample schedule of reinforcement. Matching-to-sample is widely used as a measure of short-term memory[156−159] and has been shown to be sensitive to the effects of agents acting at the GABA$_A$ chloride channel complex in laboratory animals and humans.[160−163] In addition, the possible anxiogenic effects of **27** and **9** were determined in pigeons responding under a schedule of punished and unpunished responding.

Three groups of animals were used for this study. One group of animals consisted of 3 adult male squirrel monkeys (*Saimiri sciureus*). The second and third groups consisted of a total of 15 white Carneau pigeons (8 in group 2 and 7 in group 3). A 12-h light:dark cycle

(lights were on from 7:00 am − 7:00 pm) was utilized for both species, and all experimental sessions occurred during the light cycle. The pigeons had received acute administration of a variety of drugs prior to the start of this experiment, but the monkeys were drug-naive prior to these studies. The apparatus employed for the squirrel monkeys has been described elsewhere (see Experimental Section for details).[164] Pigeons in groups 2 (n = 8) and 3 (n = 7) were trained and tested in standard pigeon chambers containing three response keys, each of which could be transilluminated with red or green light (group 2) or blue and yellow light (group 3). The titrating matching-to-sample procedure for monkeys was initiated by illumination of the houselight and the center key being transilluminated with a white light or a blinking blue light. After 20 responses on the center key (FR 20), the center key was extinguished (observation phase). Following a delay of at least 3 s, during which responses had no scheduled consequences, two of the three keys, randomly selected, were transilluminated. A correct response was defined as a single depression on the key that matched the sample stimulus presented on the center key during the observation phase (before the delay). Following a correct response the lights on the stimulus panel were turned off, a food pellet was delivered to the food-well, and the food-well light was turned on for 3 s. If a response was made on the incorrect key (nonmatch) all illumination in the chamber was turned off for a period of 10 s. A response on the nonilluminated key was counted but had no consequence. The next trial was initiated immediately following the 3-s food presentation cycle or following the 10-s time out period following an incorrect response. Auditory feedback upon a response was provided by a relay mounted in the chamber.

The titrating matching-to-sample procedure used for testing the pigeons in group 2 was very similar to that already described for the squirrel monkey. Briefly, each trial began with the illumination of the house light and center key in a standard three-key operant chamber, which was randomly assigned either a red or green color (observation phase). The 15th response on this center key (FR 15) turned off the center key and initiated a delay period during which all stimulus key lights were extinguished. After the delay of at least 3 s, two of the three keys were transilluminated, one with red light and one with green light. Which two of the three keys were illuminated on a given trial varied randomly among the left, center, and right response keys. A single response on the key transilluminated with the same color as presented on the center response key during the observation phase was defined as a correct response (matching response) and resulted in a 5-s access period to Purina pigeon checkers. A response on the key transilluminated with the stimulus color that was not presented during the observation phase was considered an incorrect response and resulted in a 5-s time out period during which all lights in the experimental chamber were extinguished. A response on a darkened key was counted but had no consequences.

The pigeons in group 3 responded under a mult VI90 VI90 (pun) [multiple variable interval 90 s, variable interval 90 s (punishment)] schedule of food presentation. At the start of the session, the houselight was illuminated and the center key was transilluminated with a blue light indicating the start of the VI90 component of the multiple schedule. Under this component the first response after a variable interval of time resulted in 5 s access to mixed grain. The variable interval of time averaged 90 s in length but could be as short as 10 s or as long as 180 s. This component remained in effect for a 5-min period. At the conclusion of the 5-min period, the key light color changed from blue to yellow indicating the start of the VI90 (pun) component presentation. Under the VI90 (pun) component the first response after a variable interval of time (average = 90 s, range = 10−180 s) resulted in 5-s access to mixed grain. In addition, under the VI90 (pun) component every fifth response resulted in the presentation of a brief (300-ms) mild 110-V electric shock. The shock was delivered through permanently placed electrodes according to the method of Azrin.[165] The intensity of the electric shock was adjusted for each pigeon such that the rate of responding under the VI90 (pun) component was approximately 50% of the response rate under the VI90 component. The shock intensities ranged across pigeons from 0.5 to 2.5 mA. The VI90 (pun) remained in effect for a 5-min period after which the key color changed back to blue indicating the presentation of the original VI90 component. The two components were presented in an alternating fashion. The two side keys in the chamber remained dark during the entire session. Responses on the side keys were not counted and had no consequence. The session terminated after three presentations of the VI90 component and three presentations of the VI90 (pun) component, approximately 30 min.

The effects of **27** on the matching-to-sample performance in pigeons are shown in Figure 18. This ligand **27** had no significant effect on the mean or maximum delay achieved during the session or on percent accuracy. In contrast, rate of responding was decreased at doses of 0.1 mg/kg and higher. Thus, **27** failed to decrease matching accuracy at doses which had marked effects on rate of responding. These effects are similar to those produced by Ro 15-4513 (**9**, Figure 19); Ro 15-4513 (**9**) produced decreases in response rates at all doses tested (0.03−3 mg/kg). However, no significant effects were observed on mean or maximum delay or on percent accuracy.

RY 24 (**27**) had no significant effects on mean delay or maximum delay values in squirrel monkeys responding under the titrating matching-to-sample schedule (Figure 20). Percent accuracy was decreased at 0.3 mg/kg but not at 1 mg/kg. However, because of marked decreases in response rates at 1 mg/kg, the data from only one of the three monkeys are included in the data points shown for this dose. The mean rate of responding was decreased at 0.3 and 1 mg/kg. These effects are slightly different from those observed following Ro 15-4513 (**9**) (Figure 21) which produced no significant effects on any parameter of performance over the dose range tested (0.1−3 mg/kg). A trend toward a decrease in response rate was observed at 1 and 3 mg/kg, but these decreases failed to achieve statistical significance. However, the failure to see significant decreases may be due to the small n employed in these studies.

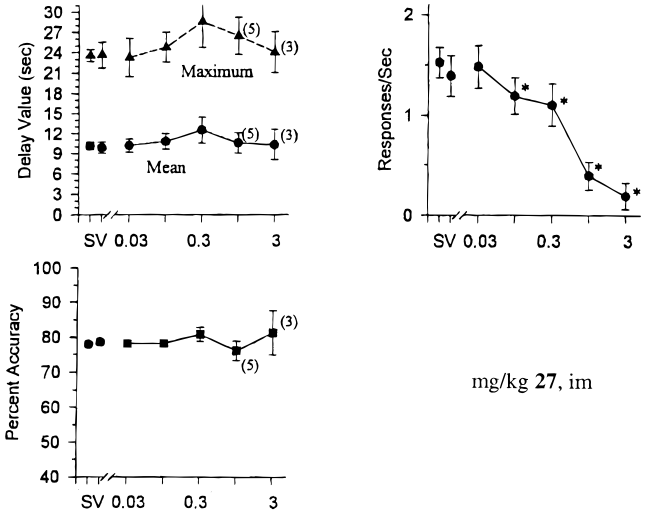


Figure 18. Effect of **27** in pigeons responding under the titrating matching-to-sample schedule of reinforcement. Abscissa: dose in mg/kg on a log scale. Ordinate: upper left, delay value achieved during the session in seconds; upper right, rate of responding upon the sample stimulus in responses per second; lower left, matching accuracy as a percentage of total trials. Points and brackets above S and V represent the saline and vehicle injection control mean ± SE, respectively. Data points and parentheses for the effects of **27** represent the mean of individual determinations in each of 8 pigeons. Where subjects have not been included in the mean because of marked response rate decreases (see Methods in the Experimental Section), the number contributing to the mean is indicated by (*n*). Statistical significance from saline control is indicated by *.

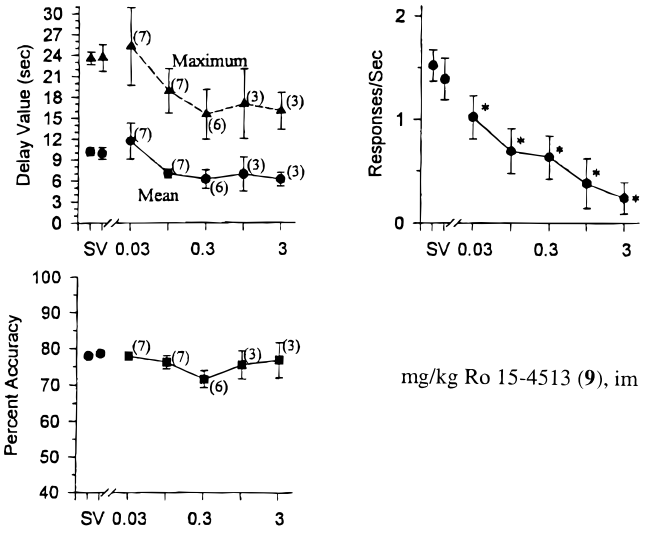


Figure 19. Effect of Ro 15-4513 (**9**) in pigeons responding under the titrating matching-to-sample schedule of reinforcement. Data are presented as in Figure 18. Data points and parentheses for the effects of **9** represent the mean of individual determinations in each of 8 pigeons. Where subjects have not been included in the mean because of marked response rate decreases (see Methods in the Experimental Section), the number contributing to the mean is indicated by (*n*). Statistical significance from saline control is indicated by *.

The effects of **27** and Ro 15-4513 (**9**) on punished and unpunished responding in pigeons are very similar (Figure 22). Both compounds produced decreases in rates of punished and unpunished responding at the same doses. It could be argued that punished responding

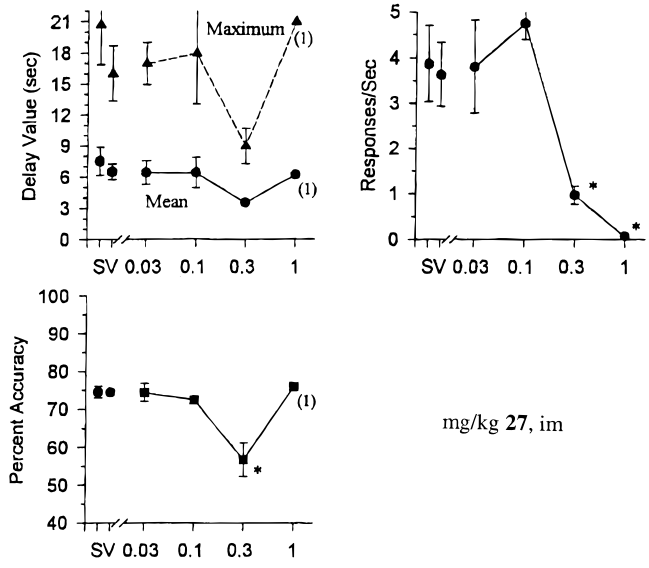


Figure 20. Effect of **27** in squirrel monkeys responding under the titrating matching-to-sample schedule of reinforcement. Data are presented as in Figure 18. Data points and parentheses for the effects of **27** represent the mean of individual determinations in each of 3 squirrel monkeys. Where subjects have not been included in the mean because of marked response rate decreases (see Methods in the Experimental Section), the number contributing to the mean is indicated by (*n*). Statistical significance from saline control is indicated by *.

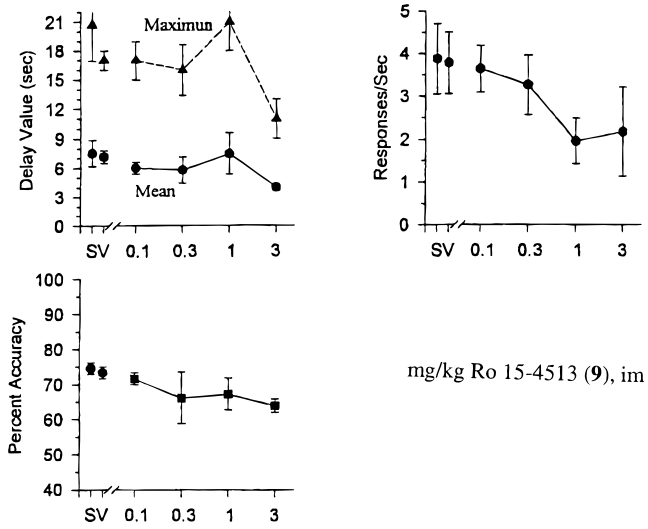


Figure 21. Effect of Ro 15-4513 (**9**) in squirrel monkeys responding under the titrating matching-to-sample schedule of reinforcement. Data are presented as in Figure 18. Data points and parentheses for the effects of **9** represent the mean of individual determinations in each of 3 squirrel monkeys. Where subjects have not been included in the mean because of marked response rate decreases (see Methods in the Experimental Section), the number contributing to the mean is indicated by (*n*). Statistical significance from saline control is indicated by *.

was decreased more than unpunished responding as a percentage of the control values. However, it is difficult to make a case for selectivity since the same doses decreased both behaviors.

Discussion

Analysis of these data failed to show that RY 24 (**27**, given im) induced increases in short-term memory

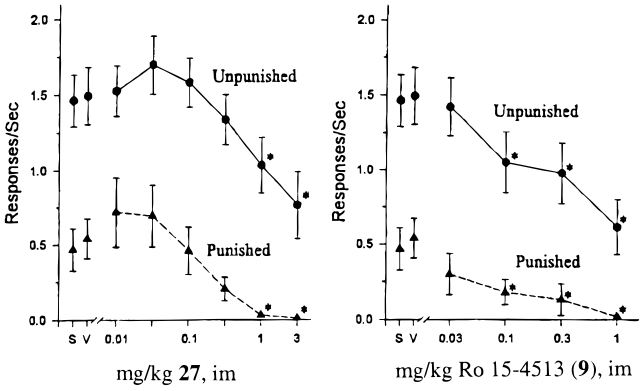


Figure 22. Effect of **27** (left panel) and Ro 15-4513 (**9**) (right panel) on the rate of responding of pigeons responding under a mult VI90 (pun) schedule. Abscissa: dose in mg/kg on a log scale. Ordinate: rate of responding in responses per second. Points and parentheses above S and V represent the saline and vehicle injection control mean ± SE, respectively. Data points and parentheses for the effects of **27** and **9** represent the mean of individual determinations in each of 7 pigeons. Statistical significance from saline control is indicated by *.

function in either pigeons or squirrel monkeys in contrast to that observed by Helmstetter[141] on intrahippocampal injection of **27**. There were no increases in the mean delay or maximum delay values achieved during the session or percent accuracy in either species over the range of doses studied. While it could be argued that the dose range selected for the study did not include active doses, doses were included that decreased the rate of responding to the sample stimulus in a dose-related fashion in both species. Alternatively, it could be argued that the doses were too high and that lower doses should have been studied. While this possibility cannot be totally ruled out, doses were included that did not affect the rate of responding in either species. Interestingly, it should be noted that Ro 15-4513 (**9**) also failed to improve short-term memory function in these studies. Ro 15-4513 (**9**) failed to statistically decrease response rates in the monkeys, but the failure to see significant decreases over the dose range studied may be due to the small *n* employed in these studies.

The effects of RY 24 (**27**) and Ro 15-4513 (**9**) on punished and unpunished responding in pigeons were remarkably similar. The rates of responding were decreased in both schedule components at the same doses in both species. The rate decreases, as a percentage of the control performance, were greater in the punished component, but no dose decreased punished responding without also significantly decreasing unpunished responding. Thus, there is little evidence of selectivity in the effects observed. It should be noted that Britton et al.[152] saw decreases in unpunished responding of rats at lower doses than those required to decrease punished responding. This would also argue against a selective effect on punished responding.

In conclusion, although no improvements in short-term memory performance were observed (im), the effects of RY 24 (**27**) in pigeons and squirrel monkeys responding under a matching-to-sample baseline were very similar to those of Ro 15-4513 (**9**). In addition, the effects of RY 24 (**27**) in both pigeons and monkeys responding under a multiple schedule having both punished and unpunished components were nearly identical to those observed for Ro 15-4513 (**9**). Both compounds produced effects which are drastically different from those of diazepam and other GABA agonists under matching-to-sample procedures[166−168] and under punished responding schedules.[169−172] Thus, the results certainly suggest that RY 24 (**27**) does not exhibit potent agonist properties when given intramuscularly, and its similarity to Ro 15-4513 (**9**) further supports the previous reports[32,77,104] that it is an inverse agonist at the GABA channel complex.

α5-Selective ligand **27** when injected directly into the hippocampus did enhance memory in one paradigm;[141] however, systemic administration of **27** into animals did not provide an observable enhancement. This result is in complete agreement with the observation of Liu.[147] It has been shown[147,149] that in the CNS of the rat (as well as monkeys and pigeons) there are several native subtypes of the $GABA_A$ receptor which exhibit different functions, regional distributions, and neuronal locations. These receptors require different membrane potentials to be activated and display different sensitivity to BzR agonists and inverse agonists.[173] Although **27** binds more potently at $\alpha5\beta2\gamma2$ receptor subtypes and is clearly an inverse agonist,[32,147] it is possible that this ligand acts as an agonist at one or more subtypes much the same as the previously studied and structurally similar inverse agonist RY 80 (**11**).[147] Liu clearly pointed out that a number of imidazobenzodiazepines were negative modulators at one subunit and agonists at another.[147] Therefore, selectivity for a particular subtype at this point is not sufficient to rule out some physiological effect at other $GABA_A$/BzR subtypes. The inability of **27** to potentiate memory when given systemically is again in support of this hypothesis, especially since $\alpha1\beta2\gamma2$ subtypes are distributed throughout the brain.[149] In addition, relative efficacy at a specific receptor subtype also complicates BzR pharmacology. The activity of **27** is a perfect example wherein low doses given intrahippocampally provide one effect (inverse agonist) and high doses appear to produce agonist-like effects when administered into the hippocampus. This is in agreement with previous reports on Ro 15-1788 (**2**) by File.[174] Although **2** functions primarily as an antagonist, in some paradigms it exhibits weak inverse agonist actions and in others appears to be a weak agonist. Recent results by June et al.[142] on allene **10** and by Liu[147] on **2** (efficacy in oocytes) as well as with **11** are in agreement with this possibility. Certainly the possibility exists that the efficacy of a ligand at different receptor subtypes may not be the same and one outweighs the other at higher concentrations.[147] A drug delivered systemically is far more likely to interact with all subtypes than one delivered to a specific brain region. The observation that **27** potentiates memory when delivered into the hippocampus versus the lack of effect when given systemically supports the previous work of Liu with imidazobenzodiazepines.[32,147] It is clear that BzR ligands can potentiate the effects of GABA at one subtype and negatively modulate it at another.[147,175] This conundrum provides further support for the design of more subtype-selective ligands for BzR in order to accurately define their pharmacology, one key to the design of new drugs with fewer side effects.

Experimental Section

Chemistry. The syntheses of the ligands employed in this study have been reported elsewhere.[20,21,30,32,34−36,44,51,66,70,71,77,105,109,112,114,127,128,135,147,176]

In Vitro Binding. In brief, the affinity of compounds at GABA$_A$/BzR subtypes was measured by competition for [^{3}H]-Ro 15-1788 (83 Ci/mmol; NEN) binding to Ltk$^-$ cells expressing human receptors of composition $\alpha1\beta3\gamma2$, $\alpha2\beta3\gamma2$, $\alpha3\beta3\gamma2$, $\alpha5\beta3\gamma2$, and $\alpha6\beta3\gamma2$. Cells were removed from culture by scraping into phosphate-buffered saline, centrifuged at 3000*g*, and resuspended in 10 mL of phosphate buffer (10 mM KH_2PO_4, 100 mM KCl, pH 7.4 at 4 °C) for each tray (25 cm^2) of cells. Radioligand binding assays were carried out in a volume of 500 μL which contained 100 μL of cells, [^{3}H]Ro 15-1788 at a concentration of 1−2 nM, and the test compound in the range 10^{-9}−10^{-5} M. Nonspecific binding was defined by 10^{-5} M diazepam and typically represented less than 5% of the total binding. For cells expressing $\alpha6\beta3\gamma2$, [^{3}H]Ro 15-4513 was employed as the radioligand. Assays were incubated to equilibrium for 1 h at 4 °C and harvested onto GF/B filters (Brandel) by filtration using a Tomtec cell harvester and washing with ice-cold assay buffer. After drying, filter-retained radioactivity was detected by liquid scintillation counting. K_i values were calculated using the least-squares iterative fitting routine of RS/l analysis software (BBN Research System, Cambridge, MA) and are the means of two determinations which differed by less than 10%. Recombinant receptors expressed with either a $\beta3$ or $\beta2$ subunit have been shown to exhibit the same BzR ligand affinities.[32,177]

Methods. Subjects: Three groups of animals were used for this study. One group of animals consisted of three adult male squirrel monkeys (*S. sciureus*). The second and third groups consisted of a total of 15 White Carneau pigeons (8 in group 2 and 7 in group 3). The squirrel monkeys were maintained at 80% of their free feeding weight by postsession feeding of Purina monkey chow biscuits (Purina Mills, St. Louis, MO), supplemented with access to fresh fruit or vitamin supplements 4 days/week. The pigeons were also maintained at 80% of their free feeding weight by postsession feeding with pigeon checkers (Purina Mills, St. Louis, MO). All animals were maintained on ad lib water in their home cages. A 12-h light: dark cycle (lights were on from 7:00 a.m. − 7:00 p.m.) was utilized for both species, and all experimental sessions occurred during the light cycle. The pigeons had received acute administration of a variety of drugs prior to the start of this experiment, but the monkeys were drug-naive prior to these studies.

Drug administration: Both RY 24 (**27**) and Ro 15-4513 (**9**) (graciously supplied by Hoffman LaRoche, Nutley, NJ) were dissolved in a vehicle consisting of 40% propylene glycol, 10% ethanol, and 50% saline. For both pigeons and monkeys, drug or saline or vehicle was injected intramuscularly immediately prior to being placed into the experimental chamber (15 min prior to the start of the test session) in a volume equivalent to 1 mL/kg of body weight. Drugs and vehicle were typically administered on Tuesdays and Fridays with saline given on Thursdays. All doses were calculated and are expressed as the free base. Doses of drug were administered to both pigeons and monkeys in a mixed sequence such that each pigeon or each monkey received a different dose on a given day.

Data analysis: In both species responding under the titrating matching-to-sample schedule, the following data were collected for each session: the mean and maximum delay values that were achieved during the session, the percentage of trials in which a correct matching response was made (accuracy), the number of completed trials, and the overall response rate on the center key during the observation phase. The SE of the saline control mean was derived by dividing the total standard deviation ($n - 1$) by the square root of the number of animals participating in the studies (monkeys = 3, pigeons = 8). If an animal failed to complete at least 10 trials during the session, the data for the session were not included in the calculation of the group mean for the mean and maximum delay values nor the percent accuracy.

For the experiments with pigeons responding under the mult VI90 VI90 (pun) schedule of reinforcement, the following data were collected: the total number of responses in each component of the schedule and the total amount of time that the center key stimulus lights were illuminated during both components of the schedule.

For all experiments, statistical significance was determined by utilizing an analysis of variance (ANOVA) with a post-hoc Dunnett's test ($p < 0.05$).

Acknowledgment. This work was supported by a grant from the NIMH (MH-46851). The authors thank Mr. Dean Wright and Mr. William Hardwick for their excellent technical assistance.

Supporting Information Available: Details of apparatus and procedures of the matching-to-sample experiments for RY 24 (**27**) and Ro 15-4513 (**9**) not included in the Experimental Section. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Möhler, H.; Okada, T. Benzodiazepine receptors: demonstration in the central nervous system. *Science* **1977**, *198*, 849−851.
(2) Squires, R. F.; Braestrup, C. Benzodiazepine receptors in rat brain. *Nature* **1977**, *266*, 732−734.
(3) Gavish, M.; Snyder, S. H. Soluble benzodiazepine receptors: GABAergic regulation. *Life Sci.* **1980**, *26*, 579−582.
(4) Gavish, M.; Snyder, S. H. Gamma-aminobutyric acid and benzodiazepine receptors: copurification and characterization. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 1939−1942.
(5) Ninan, P. T.; Insel, T. M.; Cohen, R. M.; Cook, J. M.; Skolnick, P.; Paul, S. M. Benzodiazepine receptor-mediated experimental "anxiety" in primates. *Science* **1982**, *218*, 1332−1334.
(6) Wong, G.; Skolnick, P. High affinity ligands for 'diazepam-insensitive' benzodiazepine receptors. *Eur. J. Pharmacol. Mol. Pharmacol. Sect.* **1992**, *225*, 63−68.
(7) Mendelson, W. B.; Cain, M.; Cook, J. M.; Paul, S. M.; Skolnick, P. A benzodiazepine receptor antagonist decreases sleep and reverses the hypnotic actions of flurazepam. *Science* **1983**, *219*, 414−416.
(8) Venault, P.; Chapouthier, G.; de-Carvalho, L. P.; Simiand, J.; Morre, M.; Dodd, R. H.; Rossier, J. Benzodiazepine impairs and β-carboline enhances performance in learning and memory tasks. *Nature* **1986**, *321*, 864−866.
(9) Sieghart, W. Structure and pharmacology of γ-aminobutyric acid$_A$ receptor subtypes. *Pharm. Rev.* **1995**, *47*, 181−234.
(10) Davies, P. A.; Hanna, M. C.; Hales, T. G.; Kirkness, E. F. Insensitivity to anaesthetic agents conferred by a class of GABA$_A$ receptor subunit. *Nature* **1997**, *385*, 820−823.
(11) Verdoorn, T. A.; Draguhn, A.; Ymer, S.; Seeburg, P. H.; Sakmann, B. Functional properties of recombinant rat GABA$_A$ receptors depend on subunit composition. *Neuron* **1990**, *4*, 919−928.
(12) Sigel, E.; Baur, R.; Trube, G.; Möhler, H.; Malherbe, P. The effect of subunit composition of rat brain GABA$_A$ receptors on channel function. *Neuron* **1990**, *5*, 703−711.
(13) Mertens, S.; Benke, D.; Möhler, H. GABA$_A$ receptor populations with novel subunit combinations and drug binding profiles identified in brain by $\alpha5$- and δ-subunit-specific immunopurification. *J. Biol. Chem.* **1993**, *268*, 5965−5973.
(14) Benavides, J.; Peny, B.; Ruano, D.; Vitorica, J.; Scatton, B. Comparative autoradiographic distribution on central ω (benzodiazepine) modulatory site subtypes with high, intermediate, and low affinity for zolpidem and alpidem. *Brain Res.* **1993**, *604*, 240−250.
(15) Chang, Y.; Wang, R.; Barot, S.; Weiss, D. S. Stoichiometry of a recombinant GABA$_A$ receptor. *J. Neurosci.* **1996**, *16*, 5415−5424.
(16) Sieghart, W.; Eichinger, A.; Richards, J. G.; Möhler, H. Photoaffinity labeling of benzodiazepine receptor proteins with the partial inverse agonist [^{3}H]Ro 15-4513: A biochemical and autoradiographic study. *J. Neurochem.* **1987**, *48*, 46−52.
(17) Wisden, W.; Herb, A.; Wieland, H.; Keinanen, K.; Luddens, H.; Seeburg, P. H. Cloning pharmacological characteristics and expression patterns of the GABA$_A$ receptor $\alpha4$-subunit. *FEBS Lett.* **1991**, *289*, 227−230.
(18) Gu, Z. Q.; Wong, G.; Dominguez, C.; de-Costa, B. R.; Rice, K. C.; Skolnick, P. Synthesis and evaluation of imidazo[1,5-*a*][1,4]-benzodiazepine esters with high affinities and selectivities at diazepam insensitive (DI) benzodiazepine receptors. *J. Med. Chem.* **1993**, *36*, 1001−1006.
(19) Fryer, R. I.; Zhang, P.; Lin, K.-Y.; Upasani, R. B.; Wong, G.; Skolnick, P. Conformational similarity of diazepam-sensitive and -insensitive benzodiazepine receptors determined by chiral pyrroloimidazobenzodiazepines. *Med. Chem. Res.* **1993**, *3*, 183−191.

(20) Wong, G.; Koehler, K. F.; Skolnick, P.; Gu, Z. Q.; Ananthan, S.; Schönholzer, P.; Hunkeler, W.; Zhang, W.; Cook, J. M. Synthetic and computer-assisted analysis of the structural requirements for selective, high affinity ligand binding to 'diazepam-insensitive' benzodiazepine receptors. *J. Med. Chem.* **1993**, *36*, 1820−1830.

(21) Zhang, P.; Zhang, W.; Liu, R.; Harris, B.; Skolnick, P.; Cook, J. M. Synthesis of novel imidazobenzodiazepines as probes of the pharmacophore for "diazepam-insensitive" $GABA_A$ receptors. *J. Med. Chem.* **1995**, *38*, 1679−1688.

(22) Pritchett, D. B.; Luddens, H.; Seeburg, P. H. Type I and type II $GABA_A$-benzodiazepine receptors produced in transfected cells. *Science* **1989**, *245*, 1389−1392.

(23) Wong, G.; Sei, Y.; Skolnick, P. Stable expression of type I γ-aminobutyric $acid_A$/benzodiazepine receptors in a transfected cell line. *Mol. Pharmacol.* **1992**, *42*, 996−1003.

(24) Skolnick, P.; Wong, G. Drug-receptor interactions in anxiety in imidazopyridines. In *Anxiety disorders: a novel experimental and therapeutic approach*; Bartholini, G., Garreau, M., Morselli, P., Zivkovic, B., Eds.; Raven Press: New York, 1993; pp 23−32.

(25) Duncalfe, L. L.; Carpenter, M. R.; Smillie, L. B.; Martin, I. L.; Dunn, S. M. The major site of photoaffinity labeling of the gamma-aminobutyric acid type A receptor by [^{3}H]flunitrazepam is histidine 102 of the alpha subunit. *J. Biol. Chem.* **1996**, *271*, 9209−9214.

(26) Davies, M.; Martin, I. L.; Bateson, A. N.; Hadingham, K. L.; Whiting, P. J.; Dunn, S. M. Identification of domains in human recombinant $GABA_A$ receptors that are photoaffinity labeled by [^{3}H]flunitrazepam and [^{3}H]Ro 15-4513. *Neuropharmacology* **1996**, *35*, 1199−1208.

(27) Doble, A.; Martin, I. L. Multiple benzodiazepine receptors: no reason for anxiety. *Trends Pharmacol. Sci.* **1992**, *13*, 76−81.

(28) Lippke, K. P.; Schunack, W. G.; Wenning, W.; Müller, W. E. β-Carbolines as benzodiazepine receptor ligands. 1. Synthesis and benzodiazepine receptor interaction of esters of β-carboline-3-carboxylic acid. *J. Med. Chem.* **1983**, *26*, 499−503.

(29) Haefely, W.; Martin, J. R.; Schoch, P. Novel anxiolytics that act as partial agonists at benzodiazepine receptors. *Trends Pharmacol. Sci.* **1990**, *11*, 452−456.

(30) Hagen, T. J.; Guzman, F.; Schultz, C.; Cook, J. M. Synthesis of 3,6-disubstituted β-carbolines which possess either benzodiazepine antagonist or agonist activity. *Heterocycles* **1986**, *24*, 2845−2855.

(31) Allen, M. S.; Tan, Y. C.; Trudell, M. L.; Narayanan, K.; Schindler, L. R.; Martin, M. J.; Schultz, C.; Hagen, T. J.; Koehler, K. F.; Codding, P. W.; Skolnick, P.; Cook, J. M. Synthetic and computer-assisted analyses of the pharmacophore for the benzodiazepine receptor inverse agonist site. *J. Med. Chem.* **1990**, *33*, 2343−2357.

(32) Liu, R.; Hu, R. J.; Zhang, P.; Skolnick, P.; Cook, J. M. Synthesis and pharmacological properties of novel 8-substituted imidazobenzodiazepines: High-affinity, selective probes for α5-containing $GABA_A$ receptors. *J. Med. Chem.* **1996**, *39*, 1928−1934.

(33) Gee, K. W.; Brinton, R. E.; Yamamura, H. I. CL 218872 antagonism of diazepam induced loss of righting reflex: Evidence for partial agonistic activity at the benzodiazepine receptor. *Life Sci.* **1983**, *32*, 1037−1040.

(34) Allen, M. S.; Hagen, T. J.; Trudell, M. L.; Codding, P. W.; Skolnick, P.; Cook, J. M. Synthesis of novel 3-substituted β-carbolines as benzodiazepine receptor ligands: probing the benzodiazepine receptor pharmacophore. *J. Med. Chem.* **1988**, *31*, 1854−1861.

(35) Martin, M. J.; Trudell, M. L.; Diaz-Arauzo, H.; Allen, M. S.; Deng, L.; Schultz, C. A.; Tan, Y.-C.; Bi, Y.; Narayanan, K.; Dorn, L. J.; Koehler, K. F.; Skolnick, P.; Cook, J. M. Molecular yardsticks: rigid probes to define the spatial dimensions of the benzodiazepine receptor binding site. *J. Med. Chem.* **1992**, *35*, 4105−4117.

(36) Trudell, M. L.; Lifer, S. L.; Tan, Y. C.; Martin, M. J.; Deng, L.; Skolnick, P.; Cook, J. M. Synthesis of substituted 7,12-dihydropyrido[3,2-*b*:5,4-*b*′]diindoles: rigid planar benzodiazepine receptor ligands with inverse agonist/antagonist properties. *J. Med. Chem.* **1990**, *33*, 2412−2420.

(37) Trudell, M. L.; Basile, A. S.; Shannon, H. E.; Skolnick, P.; Cook, J. M. Synthesis of 7,12-dihydropyrido[3,4-*b*:5,4-*b*′]diindoles. A novel class of rigid, planar benzodiazepine receptor ligands. *J. Med. Chem.* **1987**, *30*, 456−458.

(38) Arbilla, S.; Depoortere, H.; George, P.; Langer, S. Z. Pharmacological profile of the imidazopyridine zolpidem at benzodiazepine receptors and electrocorticogram in rats. *Naunyn-Schmiedebergs Arch. Pharmacol.* **1985**, *330*, 248−251.

(39) Yokoyama, N.; Ritter, B.; Neubert, A. D. 2-Arylpyrazolo[4,3-*c*]-quinolin-3-ones: novel agonist, partial agonist, and antagonist of benzodiazepines. *J. Med. Chem.* **1982**, *25*, 337−339.

(40) Clements-Jewery, S.; Danswan, G.; Gardner, C. R.; Matharu, S. S.; Murdoch, R.; Tully, W. R.; Westwood, R. (Imidazo[1,2-a]-pyrimidin-2-yl)phenylmethanones and related compounds as potential nonsedative anxiolytics. *J. Med. Chem.* **1988**, *31*, 1220−1226.

(41) Haefely, W.; Kyburz, E.; Gerecke, M.; Möhler, H. Recent advances in the molecular pharmacology of benzodiazepine receptors and in the structure−activity relationships of their agonists and antagonists. In *Advances in Drug Research*; Testa, B., Ed.; Academic Press: New York, 1985; pp 165−322.

(42) Tarzia, G.; Occelli, E.; Toja, E.; Barone, D.; Corsico, N.; Gallico, L.; Luzzani, F. 6-(Alkylamino)-3-aryl-1,2,4-triazolo[3,4-*a*]phthalazines. A new class of benzodiazepine receptor ligands. *J. Med. Chem.* **1988**, *31*, 1115−1123.

(43) Petke, J. D.; Im, H. K.; Im, W. B.; Blakeman, D. P.; Pregenzer, J. F.; Jacobsen, E. J.; Hamilton, B. J.; Carter, D. B. Characterization of functional interactions of imidazoquinoxaline derivatives with benzodiazepine-γ-aminobutyric $acid_A$ receptors. *Mol. Pharmacol.* **1992**, *42*, 294−301.

(44) Allen, M. S.; LaLoggia, A. J.; Dorn, L. J.; Martin, M. J.; Costantino, G.; Hagen, T. J.; Koehler, K. F.; Skolnick, P.; Cook, J. M. Predictive binding of β-carboline inverse agonists and antagonists via the CoMFA/GOLPE approach. *J. Med. Chem.* **1992**, *35*, 4001−4010.

(45) Braestrup, C.; Schmiechen, R.; Neff, G.; Nielsen, M. C.; Peterson, E. N. Interaction of convulsive ligands with benzodiazepine receptors. *Science* **1982**, *216*, 1241−1243.

(46) Braestrup, C.; Honore, T.; Nielsen, M. C.; Peterson, E. N.; Jensen, L. H. Ligands for benzodiazepine receptors with positive and negative efficacy. *Biochem. Pharmacol.* **1984**, *33*, 859−862.

(47) MacDonald, R. L.; Twyman, R. E. Kinetic properties and regulation of $GABA_A$ receptor channels. In *Ion Channels*; Narahashi, T., Ed.; Plenum Press: New York, 1992; pp 315−343.

(48) Barrett, J. E.; Brady, L. S.; Witkin, J. M.; Cook, J. M.; Larscheid, P. Interactions between the benzodiazepine receptor antagonist Ro 15-1788 (flumazenil) and the inverse agonist β-CCE: behavioral studies with squirrel monkeys. *Life Sci.* **1985**, *36*, 1407−1414.

(49) Takada, K.; Winger, G.; Cook, J.; Larscheid, P.; Woods, J. H. Discriminative and aversive properties of β-carboline-3-carboxylic acid ethyl ester, a benzodiazepine receptor inverse agonist, in rhesus monkeys. *Life Sci.* **1986**, *38*, 1049−1056.

(50) Shannon, H. E.; Hagen, T. J.; Guzman, F.; Cook, J. M. β-Carbolines as antagonists of the discriminative stimulus effects of diazepam in rats. *J. Pharmacol. Exp. Ther.* **1988**, *246*, 275−281.

(51) Cox, E. D.; Diaz-Arauzo, H.; Huang, Q.; Reddy, M. S.; Ma, C.; Harris, B.; McKernan, R.; Skolnick, P.; Cook, J. M. Synthesis and evaluation of analogues of the partial agonist 6-(propyloxy)-4-(methoxymethyl)-β-carboline-3-carboxylic acid ethyl ester (6-PBC) and the full agonist 6-(benzyloxy)-4-(methoxymethyl)-β-carboline-3-carboxylic acid ethyl ester (ZK 93423) at wild type and recombinant $GABA_A$ receptors. *J. Med. Chem.* **1998**, *41*, 2537−2552.

(52) Zhang, W.; Koehler, K. F.; Zhang, P.; Cook, J. M. Development of a comprehensive pharmacophore model for the benzodiazepine receptor. *Drug Des. Discov.* **1995**, *12*, 193−248.

(53) Zhang, W.; Diaz-Arauzo, 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*; Choudhary, M. I., Ed.; Harwood Academic Publishers: New York, 1996; pp 303−373.

(54) Villar, H. O.; Davies, M. F.; Loew, G. H.; Maguire, P. A. Molecular models for recognition and activation at the benzodiazepine receptor: a review. *Life Sci.* **1991**, *48*, 593−602.

(55) Villar, H. O.; Uyeno, E. T.; Toll, L.; Polgar, W.; Davies, M. F.; Loew, G. H. Molecular determinants of benzodiazepine receptor affinities and anticonvulsant activities. *Mol. Pharmacol.* **1989**, *36*, 589−600.

(56) Crippen, G. M. Distance geometry analysis of the benzodiazepine binding site. *Mol. Pharmacol.* **1981**, *22*, 11−19.

(57) Ghose, A. K.; Crippen, G. M. Modeling the benzodiazepine receptor binding site by the general three-dimensional structure-directed quantitative structure−activity relationship method REMOTEDISC. *Mol. Pharmacol.* **1990**, *37*, 725−734.

(58) Codding, P. W.; Muir, A. K. S. Molecular structure of Ro 15−1788 and a model for the binding of benzodiazepine receptor ligands. *Mol. Pharmacol.* **1985**, *28*, 178−184.

(59) Muir, A. K. S.; Codding, P. W. Structure−activity studies of β-carbolines. 3. Crystal and molecular structures of methyl β-carboline-3-carboxylate. *Can. J. Chem.* **1985**, *63*, 2752−2756.

(60) Fryer, R. I. Ligand interactions at the benzodiazepine receptor. In *Comprehensive Medicinal Chemistry*; Emmett, J., Ed.; Pergamon Press: Oxford, U.K., 1989; pp 539−566.

(61) Borea, P. A.; Gilli, G.; Bertolasi, V.; Ferretti, V. Stereochemical features controlling binding and intrinsic activity properties of benzodiazepine-receptor ligands. *Mol. Pharmacol.* **1987**, *31*, 334−344.

(62) Tebib, S.; Bourguignon, J. J.; Wermuth, C. G. The active analogue approach applied to the pharmacophore identification of benzodiazepine receptor ligands. *J. Comput.-Aided Mol. Des.* **1987**, *1*, 153−170.
(63) Gardner, C. R. A review of recently developed ligands for neuronal benzodiazepine receptors and their pharmacological activities. *Prog. Neuro-Psychopharmacol. Biol. Psychiat.* **1992**, *16*, 755−781.
(64) Codding, M. G.; Roszak, A. W.; Szkaradzinska, M. B.; Cook, J. M.; Aha, L. J. Modeling the benzodiazepine receptor using structural and theoretical characterization of novel β-carbolines. In *Trends in Medicinal Chemistry'88*; van der Goot, H., Domány, G., Pallos, L., Timmerman, H., Eds.; Elsevier Science Publishers: Amsterdam, 1989; pp 109−120.
(65) Naryanan, K.; Cook, J. M. Molecular yardsticks: synthesis of higher homologues of 7,12-dihyropyrido[3,4-*b*:5,4-*b*′]diindole. Probing the dimensions of the benzodiazepine receptor inverse agonist site. *Heterocycles* **1990**, *31*, 203−208.
(66) Hollinshead, S. P.; Trudell, M. L.; Skolnick, P.; Cook, J. M. Structural requirements for agonist actions at the benzodiazepine receptor: studies with analogues of 6-(benzyloxy)-4-(methoxymethyl)-β-carboline-3-carboxylic acid ethyl ester. *J. Med. Chem.* **1990**, *33*, 1062−1069.
(67) Diaz-Arauzo, H.; Evoniuk, G. E.; Skolnick, P.; Cook, J. M. The agonist pharmacophore of the benzodiazepine receptor. Synthesis of a selective anticonvulsant/anxiolytic. *J. Med. Chem.* **1991**, *34*, 1754−1756.
(68) Cook, J. M.; Diaz-Arauzo, H.; Allen, M. S. Inverse agonists: probes to study the structure, topology and function of the benzodiazepine receptor. *Natl. Inst. Drug Abuse Res. Monogr.* **1991**, 133−139.
(69) Diaz-Arauzo, H.; Koehler, K. F.; Hagen, T. J.; Cook, J. M. Synthetic and computer assisted analysis of the pharmacophore for agonists at benzodiazepine receptors. *Life Sci.* **1991**, *49*, 207−216.
(70) Zhang, W.; Koehler, K. F.; Harris, B.; Skolnick, P.; Cook, J. M. The synthesis of benzofused benzodiazepines employed as probes of the agonist pharmacophore of benzodiazepine receptors. *J. Med. Chem.* **1994**, *37*, 745−757.
(71) Liu, R.; Zhang, P.; Gan, T.; McKernan, R. M.; Cook, J. M. Evidence for the conservation of conformational topography at five major GABA$_A$/benzodiazepine receptor subsites. Potent affinities of the (S)-enantiomers of framework-constrained 4,5-substituted pyrroloimidazobenzodiazepines. *Med. Chem. Res.* **1997**, *7*, 25−35.
(72) Huang, Q.; Zhang, W.; Liu, R.; McKernan, R. M.; Cook, J. M. Benzo-fused benzodiazepines employed as topological probes for the study of benzodiazepine receptor subtypes. *Med. Chem. Res.* **1996**, *6*, 384−391.
(73) Huang, Q.; Liu, R.; Zhang, P.; He, X.; McKernan, R. M.; Gan, T.; Bennett, D. W.; Cook, J. M. Predictive models for GABA$_A$/benzodiazepine receptor subtypes: Studies of quantitative structure−activity relationships (QSAR) for imidazobenzodiazepines at five recombinant GABA$_A$/BzR subtypes [$\alpha x\beta 3\gamma 2$ (x = 1−3, 5 and 6)] via CoMFA. *J. Med. Chem.* **1998**, *41*, 4230−4142.
(74) Huang, Q.; Cox, E. D.; Gan, T.; Ma, C.; Bennett, D. W.; McKernan, R. M.; Cook, J. M. Studies of molecular pharmacophore/receptor models for GABA$_A$/benzodiazepine receptor subtypes: Binding affinities of substituted β-carboline ligands for $\alpha x\beta 3\gamma 2$ (x = 1−3, 5, 6) subtypes and quantitative structure−activity relationship studies via comparative molecular field analysis. *Drug Des. Discov.* **1999**, *16*, 55−76.
(75) Yu, S.; He, X.; Ma, C.; McKernan, R.; Cook, J. M. Studies in search of α2 selective ligands for GABA$_A$/BzR receptor subtypes. Part I. Evidence for the conservation of pharmacophoric descriptors for DS subtypes. *Med. Chem. Res.* **1999**, *9*, 186−202.
(76) Ligands related to loprazolam are not included because subtype selectivity data was not available.
(77) Liu, R.; Zhang, P.; McKernan, R. M.; Wafford, K.; Cook, J. M. Synthesis of novel imidazobenzodiazepines selective for the $\alpha 5\beta 2\gamma 2$ (Bz5) GABA$_A$/benzodiazepine receptor subtype. *Med. Chem. Res.* **1995**, *5*, 700−709.
(78) Bertolasi, V.; Feretti, V.; Gilli, G.; Borea, P. A. Structure of methyl-β-carboline-3-carboxylate (βCCM), $C_{13}H_{10}N_2O_2$. *Acta Crystallogr., Sect. C: Cryst. Struct. Commun.* **1984**, *40*, 1981−1983.
(79) Camerman, A.; Camerman, N. Stereochemical basis of anticonvulsant drug action. II. Molecular structure of diazepam. *J. Am. Chem. Soc.* **1972**, *94*, 268−272.
(80) Ferretti, V.; Bertolasi, V.; Gilli, G.; Borea, P. A. Stereochemistry of benzodiazepine-receptor ligands. II. Structures of two 2-arylpyrazolo[4,3-c]quinolin-3-ones: CGS-8216, $C_{16}H_{11}N_3O$, and CGS-9896, $C_{16}H_{10}ClN_3O$. *Acta Crystallogr., Sect. C: Cryst. Struct. Commun.* **1985**, *C41*, 107−110.
(81) Hempel, A.; Camerman, N.; Camerman, A. Benzodiazepine stereochemistry: Crystal structures of the diazepam antagonist Ro 15-1788 and the anomalous benzodiazepine Ro 5-4864. *Can. J. Chem.* **1987**, *65*, 1608−1612.
(82) Neidle, S.; Webster, G. D.; Jones, G. B.; Thurston, D. E. Structures of two DNA minor-groove binders based on pyrrolo-[2,1-*c*][1,4]-benzodiazepines. *Acta Crystallogr.* **1991**, *C47*, 2678−2680.
(83) Halgren, T. A. Merck molecular force field. I. Basis, form, scope, parametrization, and performance of MMFF94. *J. Comput. Chem.* **1996**, *17*, 490−519.
(84) Halgren, T. A. Merck molecular force field. II. MMFF94 van der Waals and electrostatic parameters for intermolecular interactions. *J. Comput. Chem.* **1996**, *17*, 520−552.
(85) Halgren, T. A. Merck molecular force field. III. Molecular geometrics and vibrational frequencies for MMFF94. *J. Comput. Chem.* **1996**, *17*, 553−586.
(86) Halgren, T. A.; Nachbar, R. B. Merck molecular force field. IV. Conformational energies and geometries for MMFF94. *J. Comput. Chem.* **1996**, *17*, 587−615.
(87) Halgren, T. A. Merck molecular force field. V. Extension of MMFF94 using experimental data, additional computational data, and empirical rules. *J. Comput. Chem.* **1996**, *17*, 616−641.
(88) Mohamadi, F.; Richards, N. G. J.; Guida, W. C.; Liskamp, R.; Caufield, C.; Chang, G.; Hendrickson, T.; Still, W. C. Macro-Model-An integrated software system for modeling organic and bioorganic molecules using molecular mechanics. *J. Comput. Chem.* **1990**, *11*, 440−467.
(89) Gaussian92, Gaussian, Inc., Carnegie Office Park, Bldg. 6, Pittsburgh, PA 15106.
(90) Gaussian94, Gaussian, Inc., Carnegie Office Park, Bldg. 6, Pittsburgh, PA 15106.
(91) Andzelm, J.; Klobukowski, M.; Radzio-Andzelm, E. Compact contracted Gaussian-type basis sets for halogen atoms. Basis-set superposition effects on molecular properties. *J. Comput. Chem.* **1984**, *5*, 146−161.
(92) The M4 basis set for Br and I, which is then split to obtain the 6-31G quality basis set (SV4) and d functions are added to obtain the 6-31G* basis set, also referred to as the SV4P basis sets. Personal communication with Dr. Misra Ashutosh.
(93) McGrath, M. P.; Radom, L. Extension of Gaussian-1 (G1) theory to bromine-containing molecules. *J. Chem. Phys.* **1991**, *94*, 511−516.
(94) Glukhovtsev, M. N.; Pross, A.; McGrath, M. P.; Radom, L. Extension of Gaussian-2 (G2) theory to bromine- and iodine-containing molecules: Use of effective core potentials. *J. Chem. Phys.* **1995**, *103*, 1878−1885.
(95) Glukhovtsev, M. N.; Pross, A.; McGrath, M. P.; Radom, L. Erratum: Extension of Gaussian-2 (G2) theory to bromine- and iodine-containing molecules: Use of effective core potentials. *J. Chem. Phys.* **1996**, *104*, 3407.
(96) Benavides, J.; Peny, B.; Dubois, A.; Perrault, G.; Morel, E.; Zivkovic, B. In vivo interaction of zolpidem with central benzodiazepine binding sites (as labeled by ^{3}H-Ro 15-1788) in the mouse brain. Preferential affinity of zolpidem for the ω_1 (BZD$_1$) subtype. *J. Pharmacol. Exp. Ther.* **1988**, *245*, 1033−1041.
(97) Wafford, K. A.; Whiting, P. J.; Kemp, J. A. Differences in affinity and efficacy of benzodiazepine receptor ligands at recombinant γ-aminobutyric acid$_A$ receptor subtypes. *Mol. Pharmacol.* **1993**, *43*, 240−244.
(98) SYBYL6.4, Tripos, Inc., 1699 S. Hanley Rd., St. Louis, MO 63144-2913.
(99) Boobbyer, D. N.; Goodford, P. J.; McWhinnie, P. M.; Wade, R. C. New hydrogen-bond potentials for use in determining energetically favorable binding sites on molecules of known structure. *J. Med. Chem.* **1989**, *32*, 1083−1094.
(100) Ippolito, J. A.; Alexander, R. S.; Christianson, D. W. Hydrogen bond stereochemistry in protein structure and function. *J. Mol. Biol.* **1990**, *215*, 457−471.
(101) Murray-Rust, P.; Glusker, J. P. Directional hydrogen bonding to sp2 and sp3-hybridized oxygen atoms and its relevance to ligand-macromolecular interactions. *J. Am. Chem. Soc.* **1984**, *106*, 1018−1025.
(102) Tintelnot, M.; Andrews, P. Geometries of functional group interactions in enzyme-ligand complexes: guides for receptor modelling. *J. Comput.-Aided Mol. Des.* **1989**, *3*, 67−84.
(103) Vedani, A.; Huhta, D. W. A new force field for modeling metalloproteins. *J. Am. Chem. Soc.* **1991**, *113*, 5860−5862.
(104) Skolnick, P.; Hu, R. J.; Cook, C. M.; Hurt, S. D.; Trometer, J. D.; Liu, R.; Huang, Q.; Cook, J. M. [^{3}H]RY-80: A high affinity, selective ligand for GABA$_A$ receptors containing α5 subunits. *J. Pharmacol. Exp. Ther.* **1997**, *283*, 488−495.
(105) Yu, S.; Ma, C.; He, X.; McKernan, R.; Cook, J. M. Study in the search for α5 subtype selective agonists for GABA$_A$/BzR sites. *Med. Chem. Res.* **1999**, *9*, 71−88.
(106) Skolnick, P.; Schweri, M.; Kutter, E.; Williams, E.; Paul, S. Inhibition of [^{3}H]diazepam and [^{3}H]3-carboethoxy-β-carboline binding by irazepine: evidence for multiple "domains" of the benzodiazepine receptor. *J. Neurochem.* **1982**, *39*, 1142−1146.

(107) Malminiemi, O.; Korpi, E. R. Diazepam-insensitive [^{3}H]Ro 15-4513 binding in intact cultured cerebellar granule cells. *Eur. J. Pharmacol.* **1989**, *169*, 53−60.
(108) Wong, C.-G.; Langer, T.; Kamath, P. G.; Gu, Z.-Q.; Skonick, P.; Fryer, R. I. Computer-aided molecular modeling, synthesis, and biological evaluation of 8-(benzyloxy)-2-phenylpyrazolo[4,3-*c*]-quinoline as a novel benzodiazepine receptor agonist ligand. *J. Med. Chem.* **1995**, *38*, 950−957.
(109) Zhang, W. Ph.D. Thesis. Part I: Chemical and Computer Assisted Development of an Inclusive Pharmacophore for the Benzodiazepine Receptor. Studies Directed Toward the Synthesis of Anxioselective Anxiolytics. Part II: Molecular Yardsticks: Probes to study the Actual Dimensions of Benzodiazepine Receptors. University of Wisconsin−Milwaukee, 1994.
(110) Nielsen, M.; Gredal, O.; Braestrup, C. Some properties of ^{3}H-diazepam displacing activity from human urine. *Life Sci.* **1979**, *25*, 679−686.
(111) Schweri, M.; Cain, M.; Cook, J.; Paul, S.; Skolnick, P. Blockade of 3-carbomethoxy-β-carboline induced seizures by diazepam and the benzodiazepine antagonists, Ro 15-1788 and CGS 8216. *Pharmacol. Biochem. Behav.* **1982**, *17*, 457−460.
(112) Cain, M.; Weber, R. W.; Guzman, F.; Cook, J. M.; Barker, S. A.; Rice, K. C.; Crawley, J. N.; Paul, S. M.; Skolnick, P. β-Carbolines: synthesis and neurochemical and pharmacological actions on brain benzodiazepine receptors. *J. Med. Chem.* **1982**, *25*, 1081−1091.
(113) Fryer, R. I.; Cook, C.; Gilman, N. W.; Walser, A. Conformational shifts at the benzodiazepine receptor related to the binding of agonists, antagonists, and inverse agonists. *Life Sci.* **1986**, *39*, 1947−1957.
(114) Hagen, T. J.; Skolnick, P.; Cook, J. M. Synthesis of 6-substituted β-carbolines that behave as benzodiazepine receptor antagonists or inverse agonists. *J. Med. Chem.* **1987**, *30*, 750−753.
(115) Trullas, R.; Ginter, H.; Jackson, B.; Skolnick, P.; Allen, M. S.; Hagen, T. J.; Cook, J. M. 3-Ethoxy-β-carboline: a high affinity benzodiazepine receptor ligand with partial inverse agonist properties. *Life Sci.* **1988**, *43*, 1193−1197.
(116) Petersen, E. N.; Jensen, L. H.; Honore, T.; Braestrup, C. Differential pharmacological effects of benzodiazepine receptor inverse agonists. *Adv. Biochem. Psychopharmacol.* **1983**, *38*, 57−64.
(117) Jensen, L. H.; Petersen, E. N. Bidirectional effects of benzodiazepine receptor ligands against picrotoxin- and pentylenetetrazol-induced seizures. *J. Neural Transm.* **1983**, *58*, 183−191.
(118) Nielsen, M. C.; Braestrup, C. Ethyl β-carboline-3-carboxylate shows differential benzodiazepine receptor interaction. *Nature* **1980**, *286*, 606−607.
(119) Braestrup, C.; Nielsen, M. C. GABA reduces binding of [^{3}H]-methyl β-carboline-3-carboxylate to brain benzodiazepine receptors. *Nature* **1981**, *294*, 472−474.
(120) Haefely, W. E. Pharmacology of the allosteric modulation of $GABA_A$ receptors by benzodiazepine receptor ligands. In *Allosteric Modulation of Amino Acid Receptors: Therapeutic Implications.*; Barnard, E. A., Costa, E., Eds.; Raven Press: New York, 1989; pp 47−70.
(121) Stephens, D. N.; Schneider, H. H.; Kehr, W.; Andrews, J. S.; Rettig, K. J.; Turski, L.; Schmiechen, R.; Turner, J., D.; Jensen, L. H.; Petersen, E. N.; Honore, T.; Bondo-Hansen, J. Abecarnil, a metabolically stable, anxioselective β-carboline acting at benzodiazeoine receptors. *J. Pharmacol. Exp. Ther.* **1990**, 334−343.
(122) Cox, E. D.; Hagen, T. J.; McKernan, R. M.; Cook, J. M. Bz_1 receptor subtype specific ligands. synthesis and biological properties of BCCt, a Bz_1 receptor subtype specific antagonist. *Med. Chem. Res.* **1995**, *5*, 710−718.
(123) Personal communication with Dr. Harry June.
(124) Wong, G.; Gu, Z. Q.; Fryer, R. I.; Skolnick, P. Pyrazolo[4,3-c]-quinolines with high affinities for ′diazepam-insensitive′ (DI) $GABA_A$/benzodiazepine receptors. *Med. Chem. Res.* **1992**, *2*, 217−224.
(125) Fryer, R. I.; Zhang, P.; Rios, R.; Gu, Z. Q.; Basile, A. S.; Skolnick, P. Structure−activity relationship studies at benzodiazepine receptors (BzR): a comparison of the substituent effects of pyrazoloquinolinone analogues. *J. Med. Chem.* **1993**, *36*, 1669−1673.
(126) Takada, S.; Shindo, H.; Sasatani, T.; Chomei, N.; Matsushita, A.; Eigyo, M.; Kawasaki, K.; Murata, S.; Takahara, Y.; Shintaku, H. Thienylpyrazoloquinolines: potent agonists and inverse agonists to benzodiazepine receptors. *J. Med. Chem.* **1988**, *31*, 1738−1745.
(127) Allen, M. S.; Skolnick, P.; Cook, J. M. Synthesis of novel phenyl-2*H*-pyrazolo[4,3-*c*]isoquinolin-3-ols: topological comparisons with analogues of 2-phenyl-2,5-dihydropyrazolo[4,3-*c*]quinolin-3(3*H*)-ones at benzodiazepine receptors. *J. Med. Chem.* **1992**, *35*, 368−374.
(128) Huang, Q. Ph.D. Thesis, University of Wisconsin−Milwaukee, 1998.
(129) He, X.; Huang, Q.; Yu, S.; Ma, C.; McKernan, R.; Cook, J. M. Studies of molecular pharmacophore/receptor models for $GABA_A$/BzR subtypes: binding affinities of symmetrically substituted pyrazolo[4,3-*c*]quinolin-3-ones at recombinant $\alpha x\beta 3\gamma 2$ subtypes and quantitative structure−activity relationship studies via a comparative molecular field analysis. *Drug Des. Discov.* **1999**, *16*, 77−91.
(130) Braestrup, C.; Nielsen, M. C.; Olsen, C. E. Urinary and brain β-carboline-3-carboxylates as potent inhibitors of brain benzodiazepine receptors. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 2288−2292.
(131) Haefely, W. E.; Kulcsar, A.; Möhler, H.; Pieri, L.; Polc, P.; Schaffner, R. Possible involvement of GABA in the central actions of benzodiazepines. In *Advances in Biochemical Psychopharmacology*; Costa, E., Greengard, P., Eds.; Raven Press: New York, 1975; pp 131−151.
(132) Squires, R. *GABA and Benzodiazepine Receptors;* CRC Press: Boca Raton, FL, 1988.
(133) Atack, J. R.; Smith, A. J.; Emms, F.; McKernan, R. M. Regional Differences in the Inhibition of Mouse in vivo [^{3}H]Ro15-1788 Binding Reflect Selectivity for α_1 verses α_2 and α_3 Subunit-containing $GABA_A$ Receptors. *Neuropsychopharmacology* **1999**, *20*, 255−262.
(134) Sanger, D. J.; Benavides, J.; Perrault, G.; Morel, E.; Cohen, C.; Joly, D.; Zivkovic, B. Recent developments in the behavioral pharmacology of benzodiazepine (ω) receptors: Evidence for the functional significance of receptor subtypes. *Neurosci. Biobehav. Rev.* **1994**, *18*, 1.
(135) Zhang, W.; Liu, R.; Huang, Q.; Zhang, P.; Koehler, K.; Harris, B.; Skolnick, P.; Cook, J. M. Synthesis of 5-thienyl and 5-furyl substituted benzodiazepines: Probes of the pharmacophore for benzodiazepine receptor agonists. *Eur. J. Med. Chem.* **1995**, *30*, 483−496.
(136) Lippa, A. S.; Coupet, J.; Greenblatt, E. N.; Klepner, C. A.; Beer, B. A synthetic nonbenzodiazepine ligand for benzodiazepine receptors: A probe for investigating neuronal substrates of anxiety. *Pharmacol. Biochem. Behav.* **1979**, *11*, 99−106.
(137) McKernan, R. M.; Wafford, K.; Quirk, K.; Hadingham, K. L.; Harley, E. A.; Ragan, C. I.; Whiting, P. J. The pharmacology of the benzodiazepine site of the $GABA_A$ receptor is dependent on the type of gamma-subunit present. *J. Recept. Signal Transduct. Res.* **1995**, *15*, 173−183.
(138) Villiger, J. W.; Taylor, K. M.; Gluckman, P. D. Characteristics of type 1 and type 2 benzodiazepine receptors in the bovine brain. *Pharmacol. Biochem. Behav.* **1982**, *16*, 373−375.
(139) Langer, S. Z.; Arbilla, S. Imidazopyridines as a tool for the characterization of benzodiazepine receptors: A proposal for a pharmacological classification as omega receptor subtypes. *Pharmacol. Biochem. Behav.* **1988**, *29*, 763−766.
(140) Private communication with Dr. Mireille Sevrin of Synthélabo, National ACS Meeting, Dallas, TX, spring of 1998.
(141) Bailey, D. J.; Tetzlaff, J. E.; Cook, J. M.; He, X.; Helmstetter, F. J. Effects of hippocampal injections of a novel ligand selective for the $\alpha 5\beta 2\gamma 2$ subtype of the $GABA_A$/benzodiazepine receptor on Pavlovian conditioning. Submitted.
(142) June, H. L.; Cason, C. R.; Cheatham, G.; Liu, R.; Gan, T.; Cook, J. M. $GABA_A$-benzodiazepine receptors in the striatum are involved in the sedation produced by a moderate, but not an intoxicating ethanol dose in outbred Wistar rats. *Brain Res.* **1998**, *794*, 103−118.
(143) Suzdak, P. D.; Glowa, J. R.; Crawley, J. N.; Schwartz, R. D.; Skolnick, P.; Paul, S. M. A selective imidazobenzodiazepine antagonist of ethanol in the rat. *Science* **1986**, *234*, 1243−1247.
(144) Draski, L. J.; Deitrich, R. A. Initial effects of ethanol on the central nervous system. In *Pharmacological Effects of Ethanol on the Nervous System*; Deitrich, R. A., Erwin, V. G., Eds.; CRC Press: Boca Raton, FL, 1996; pp 277−294.
(145) Jackson, H. C.; Nutt, D. J. Inverse agonists and alcohol. In *Benzodiazepine Receptor Inverse Agonists*; Sarter, M., Nutt, D. J., Lister, R. G., Eds.; Wiley-Liss: New York, 1995; pp 113−138.
(146) June, H. L.; Duemler, S. E.; Greene, T. L.; Williams, J. A.; Lin, M.; Devaraju, S. H. A.; Lewis, M. J.; Murphy, J. M. Effects of benzodiazepine inverse agonist Ro19-4603 on the maintenance of tolerance to a single dose of ethanol. *J. Pharmacol. Exp. Ther.* **1995**, *274*, 1105−1112.
(147) Liu, R. Ph.D. Thesis, University of Wisconsin−Milwaukee, 1996.
(148) Koob, G. F. Drug abuse: anatomy, pharmacology and function of reward pathways. *Trends Pharmacol. Sci.* **1992**, *13*, 177−184.
(149) Wisden, W.; Laurie, D. J.; Monyer, H.; Seeburg, P. H. The distribution of 13 $GABA_A$ receptor subunit mRNAs in the rat brain. I. telencephalon, diencephalon, mesencephalon. *J. Neurosci.* **1992**, *12*, 1040−1062.
(150) Overton, D. A. Major theories of state dependent learning. In *Drug Discrimination and State Dependent Learning*; Ho, B. T., Richards III, D. W., Chute, D. L., Eds.; Academic Press: New York, 1978; pp 283−318.

(151) Cole, B. J.; Hillmann, M. Effects of benzodiazepine receptor ligands on the performance of an operant delayed matching to position task in rats: Opposite effects of FG 7142 and lorazepam. *Psychopharmacology (Berlin)* **1994**, *115*, 350−357.
(152) Britton, K. T.; Ehlers, C. L.; Koob, G. F. Is ethanol antagonist Ro 15-4513 selective for ethanol? *Science* **1988**, *239*, 648−649.
(153) Forster, M. J.; Prather, P. L.; S. R., P.; Lal, H. The benzodiazepine receptor inverse agonist Ro 15-3505 reverses recent memory deficits in aged mice. *Pharmacol. Biochem. Behav.* **1995**, *51*, 557−560.
(154) Anglade, F.; Bizot, J. C.; Dodd, R. H.; Baudoin, C.; Chapouthier, G. Opposite effects of cholinergic agents and benzodiazepine receptor ligands in a passive avoidance task in rats. *Neurosci. Lett.* **1994**, *182*, 247−250.
(155) Yasui, M.; Kawasaki, K.; Matsushita, A.; Satoh, M. Benzodiazepine inverse agonists augment long-term potentiation in CA1 and CA3 of guinea pig hippocampal slices. *Neuropharmacology* **1993**, *32*, 127−131.
(156) Mello, N. K. The effect of alcohol on short-term memory in rhesus monkeys. *Psychopharm. Bull.* **1971**, *8*, 20−22.
(157) Roberts, W. A.; Grant, D. S. Studies of short-term memory in the pigeon using the delayed matching to sample procedure. In *Processes of Animal Memory*; Medlin, D. L., Roberts, W. A., Davis, R. T., Eds.; Lawrence Earlbaum Associates: New York, 1976; pp 79−112.
(158) Pontecorvo, M. J.; Clissold, D. B.; Conti, L. H. Aged-related cognitive impairments as assessed with an automated repeated measures memory task: Implication for the possible role of acetylcholine and norepinephrine in memory dysfunction. *Neurobiol. Aging* **1988**, *9*, 617−625.
(159) Glick, S. D.; Jarvik, M. E. Impairment by d-amphetamine of delayed matching performance in monkeys. *J. Pharmacol. Exp. Ther.* **1969**, *169*, 1−6.
(160) Schulze, G. E.; Slikker, W. J.; Paule, M. G. Multiple behavioral effects of diazepam in rhesus monkeys. *Pharmacol. Biochem. Behav.* **1989**, *34*, 29−35.
(161) Wenger, G. R.; Wright, D. W. Disruption of performance under a titrating matching-to-sample schedule of reinforcement by drugs of abuse. *J. Pharmacol. Exp. Ther.* **1990**, *254*, 258−269.
(162) Roche, J. D.; Cherek, D. R.; Spiga, R.; Bennett, R. H.; Cowan, K. A.; Yingling, J. Benzodiazepine-induced impairment of matching-to-sample performance in humans. *Pharmacol. Biochem. Behav.* **1990**, *36*, 945−952.
(163) Hudzik, T. J.; Wenger, G. R. Effects of drugs of abuse and cholinergic agents on delayed matching-to-sample responding in the squirrel monkey. *J. Pharmacol. Exp. Ther.* **1994**, *265*, 120−127.
(164) Hake, D. F.; Azrin, N. H. An apparatus for delivering pain shock to monkeys. *J. Exp. Anal. Behav.* **1963**, *6*, 297−298.
(165) Azrin, N. H. A technique for delivering shock to pigeons. *J. Exp. Anal. Behav.* **1959**, *2*, 161−163.
(166) McMillan, D. E. Effects of chemicals on delayed matching behavior in pigeons. II Tolerance to the effects of diazepam and cross tolerance to phencyclidine. *Neurotoxicology* **1982**, *3*, 138−141.
(167) Pontecorvo, M. J.; Clissold, D. B.; White, M. F.; Ferkany, J. W. *N*-Methyl-D-aspartate antagonists and working memory performance: Comparison with the effects of scopolamine, propranolol, diazepam and phenylisopropyladenosine. *Behav. Neurosci.* **1991**, *105*, 521−535.
(168) Wenger, G. R.; Hudzik, T. J.; Wright, D. W. Titrating matching-to-sample performance in pigeons: Effects of diazepam, morphine, and cholinergic agents. *Pharmacol. Biochem. Behav.* **1993**, *46*, 435−443.
(169) Stein, L.; Wise, C. D.; Berger, B. D. Antianxiety action of benzodiazepines: Decrease in activity of serotonin neurons in the punishment system. In *The Benzodiazepines*; Garattini, S., Mussini, E., Randall, L. O., Eds.; Raven Press: New York, 1973; pp 299−326.
(170) McMillan, D. E. Effects of drug on behavior before and during chronic diazepam administration. *Eur. J. Pharmacol.* **1992**, *215*, 145−152.
(171) McMillan, D. E.; Li, M.; Hardwick, W. C. Discriminative stimulus effects and antipunishment effects of drugs measured during the same session. *Pharmacol. Biochem. Behav.* **1997**, *56*, 161−166.
(172) Shumsky, J. S.; Lucki, I. Differential tolerance to the effects of chlordiazepoxide on unpunished and punished operant responding following chronic treatment. *Pharmacol. Biochem. Behav.* **1996**, *53*, 593−601.
(173) Schmid, G.; Bonanno, G.; Raiteri, M. Functional evidence for two native $GABA_A$ receptor subtypes in adult rat hippocampus and cerebellum. *Neuroscience* **1996**, *73*, 697−704.
(174) File, S. E.; Dingemanse, J.; Friedman, H. I.; Greenblatt, D. J. Chronic treatment with Ro 15-1788 distinguishes between its benzodiazepine antagonist, agonist and inverse agonist properties. *Psychopharmacology (Berlin)* **1986**, *89*, 113−117.
(175) Another possible reason is that there are several obvious differences between Helmstetter's studies and our studies including: species (pigeon and squirrel monkey vs rat), differences in the light:dark cycle (12:12 vs 14:10), route of injection (im vs hipppocampal), Pavlovian vs operant conditioning, and food-maintained responding vs aversive stimulus-produced freeing. There also is the issue of acquisition/learning and 24-h recall vs our matching-to-sample procedure which is usually considered a measure of working or short-term memory. In addition, local differences in concentration of drug in the hippocampus (systemic vs intrahippocampal) may be drastically different depending on peripheral metabolism. Active metabolites might also be generated and further complicated the issue. More work in this area will be required to exclude these possibilities.
(176) Cox, E. Ph.D. Thesis, University of Wisconsin−Milwaukee, 1997.
(177) 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. Role of the β subunit in determining the pharmacology of human γ-aminobutyric acid type A receptors. *Mol. Pharmacol.* **1993**, *44*, 1211−1218.

JM990341R