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Ligands of the $GABA_A$ Receptor Benzodiazepine Binding Site

Qinmi Wang, Yifan Han, and Hong Xue

Department of Biochemistry, Hong Kong University of Science and Technology, Clear Water Bay, Hong Kong

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INTRODUCTION

Benzodiazepine (BZ) binding site ligands are important central nervous system (CNS) drugs. Their numbers and our knowledge of how they interact with the BZ-binding site of $GABA_A$ receptors are both rapidly expanding.

The $GABA_A$ receptor is a member of the ligand-gated ion channel superfamily. In general, it consists of an assembly of transmembrane pentamers of different subunit compositions (1). To date, at least 15 types of subunits (α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε, and π) have been identified using molecular cloning techniques that combine in various combinations to give rise to the different $GABA_A$-receptor subtypes (1–3). The central BZ-binding site is a part of the $GABA_A$-receptor-associated chloride ion channel. Most functional subtypes of the $GABA_A$ receptor contain α, β, and γ subunits, with the different receptor subtypes displaying marked variations in their binding capacity to different BZ-binding site ligands (4,5). When GABA, the major inhibitory transmitter in the CNS, binds to a $GABA_A$ receptor, the chloride ion flux through the channel is increased. This leads to membrane hyperpolarization that results in a reduction in the excitability potential of the neuron (6). As a consequence, $GABA_A$ receptors are the molecular targets of a variety of pharmacologically and clinically important drugs, such as the anxiolytic, anticonvulsant, sedative-hypnotic benzodiazepines, some anxiogenic, convulsant β-carbolines, and the convulsants bicuculline or picrotoxinin. Furthermore, multiple recognition sites that exist within the three-dimensional structure of the various $GABA_A$-receptor subtypes possess the capacity to interact with a host of different ligands (6). These sites include: the GABA site; the benzodiazepine site; the barbiturate site; the general anesthetic site; the picrotoxinin site; and the storage site for GABA. BZ-binding site ligands act through mechanisms

Address correspondence and reprint requests to Dr. Hong Xue, Department of Biochemistry, Hong Kong University of Science and Technology, Clear Water Bay, Hong Kong. Fax: (852) 2358-1552. E-mail: hxue@ust.hk.

Abbreviations: SAR, structure-activity relationship; QSAR, quantitative structure-activity relationship; BZ, benzodiazepine; CNS, Central nervous system; DI, diazepam insensitive; DS, diazepam sensitive; AChE, acetylcholinesterase.

which modulate the inhibitory effects of GABA. The BZ-binding site of the $GABA_A$ receptor is located at some distance from the GABA binding site. Binding of ligands to the BZ-binding site mediates an allosteric modulation of the structure of the receptor. These conformational changes are transmitted intramolecularly to the GABA binding site, which in turn gives rise to a receptor phenotype that displays an altered affinity for GABA. Electrophysiological experiments in many different neuronal systems have indicated that BZs, such as diazepam or flunitrazepam, enhance the actions of GABA at the $GABA_A$ receptor by increasing the frequency of Cl-channel opening with little effect on either the opening time or its conductance (6).

BZ-binding site ligands are classified according to their spectrum of intrinsic efficacy towards the $GABA_A$ receptor, and thus, act as either an agonist, antagonist or inverse agonist (6–8). The agonists allosterically modulate the binding of GABA with the receptor, exerting a positive cooperative effect that results in increased frequency of Cl-channel opening. Therapeutically, they are used as anxiolytic, anticonvulsant, sedative-hypnotic, and muscle relaxant drugs. In contrast, the inverse agonists exert a negative cooperative effect on GABA binding to its receptor, thereby decreasing GABAergic transmission to produce a proconvulsant, anxiogenic effect. The antagonists have no intrinsic efficacy of their own but can inhibit either the positive effects of agonists or the negative effects of inverse agonists. BZ-binding site antagonists are medically useful compounds that are often employed as an effective treatment for patients that have been diagnosed with complications associated with a BZ overdose. While some compounds are designated agonists, antagonists or inverse agonists, the majority of BZ-binding site ligands are known to possess either positive or negative effects (which are intermediate in nature) on GABA binding and are pharmacologically designated as partial agonists or partial inverse agonists, respectively.

A large number of BZ-binding site ligands, both classical benzodiazepine-type and nonbenzodiazepine-type compounds, have been chemically synthesized. Their structure-affinity (or activity) relationships have been elucidated, and several pharmacophore models have been postulated (9–13). Some of these models can be employed to predict the binding affinities and the intrinsic efficacies of BZ-binding site ligands. However, these pharmacophore models have met only with limited success, and further development with respect to these models is clearly needed. This paper reviews the developments related to the structure-activity and structure-efficacy relationships that surround various BZ-binding site ligands, along with their relevant pharmacophore models. These relationships and models can provide useful signposts for the study of the structural properties of the BZ-binding site and their associated interactions with ligands.

BENZODIAZEPINES

In the basic structure of 1,4-BZ (Fig. 1), early SAR studies indicated that the seven-membered imino ring B was essential for its affinity towards the BZ-binding site (14). Further QSAR and SAR studies (15–19) found that the molecular lipophilicity properties of numerous BZs played a significant role in their corresponding receptor affinity. Additionally, the carbonyl group at position 2, and the 4,5 double bond within the ligand have also been shown to substantially contribute to the binding affinity of the compound. The removal of carbonyl group results in a decrease in the binding affinity of the BZ by two

orders of magnitude, while saturation of the 4,5 double bond results in a complete loss of *in vitro* affinity.

Recently, the QSAR analyses of 57 1,4-BZs with different substituents at positions 1, 3, 7, 8, 2′, and 6′ (Fig. 1) were performed by three research groups (20–22), which reported very similar structure-affinity relationships among the various ligands. The primary chemical moieties of the compounds, which contribute to high receptor binding affinity, are restricted to positions 7, 2′, and 1. Position 7 is the most effective location in these molecules for enhancing the affinity of the compound for the BZ-binding site. Increases in the lipophilicity and electronic charge of substitutions at position 7 are directly related to an increased affinity of the ligand for the binding site, while substitutions at position 2′ represent the second most important functional group location associated with receptor affinity.

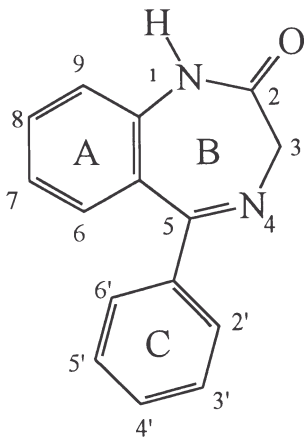


Fig. 1. Classical 1,4-benzodiazepine.

The presence of an electrophilic and bulky substituent at position 2′ results in a strong increase in receptor binding affinity of the corresponding compounds. On the other hand, molar refractivity is the most important parameter at position 1, suggesting that the molecular size of the substituent needs to be restricted at position 1 for effective ligand binding. The effects of substitutions at positions 6′, 3, and 8 on the affinity of the ligand for the BZ-binding site are less pronounced than those at positions 7, 2′, and 1. However, these studies documented that the molecular size of the substituent has to be restricted at position 6′, whereas electrostatic influences are important at positions 3 and 8 in order to maintain ligand binding.

According to the above QSAR analysis (20) the optimal functional groups at positions 7, 2′, and 1 are as follows: position 7: C > H_2CF_3 > I > Br > CF_3 > Cl > $C(CH_3)_3$ > NO_2 > F > N_3 > CH = CH_2; position 2′: NO_2 > F CN > Cl > CF_3; position 1: OH > F > NH_2 > H > NHOH > Me > Cl > CF_3 > Br > Et.

Substitutions at positions 1 and 2 of 1,4-BZ with either an imidazo- or triazolo-ring lead to a significant increase in receptor affinity for only the classic 1,4-BZ which initially possessed a relatively low affinity. This was not true for the high affinity class of 1,4-BZ compounds (23). Figure 2 presents the core structure of the various imidazobenzodiazepines.

Imidazobenzodiazepines were identified as one of several chemical families, including imidazobenzodiazepines, pyrazoloquinolinones, and β-carbolines, that exhibit high-to-moderate potency for diazepam-insensitive (DI) $GABA_A$ subtypes (24,25). The DI receptor subtype is characterized by a low affinity for the prototypical 1,4-BZs, such as diazepam or flunitrazepam, which exhibit a high affinity for the diazepam-sensitive (DS) subtype (24,25).

The importance of the ester group substituent at position 3 (Fig. 2) and the high affinity of the ligand for the DI $GABA_A$ subtypes were indicated by SAR and QSAR analyses of imidazobenzodiazepines (26–28). A DI $GABA_A$ receptor pharmacophore (Fig. 3A) was proposed (28) and compared to the DS $GABA_A$ receptor pharmacophore (Fig. 3B) as previously described (27). An alkyl ester group possessing an optimal size at position 3 (from ethyl to *tert*-butyl ester) is required for high-affinity binding to DI $GABA_A$ receptors

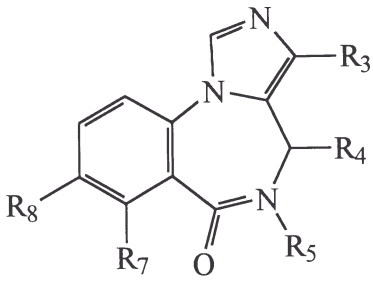


Fig. 2. Imidazobenzodiazepines.

(26,27). This observation suggests that a lipophilic pocket within the DI $GABA_A$ receptors is juxtaposed to, and interacts with the functional moiety at position 3 of the compound as the ligand leads to the receptor. The oxygen atoms on the 3-alkyl ester group are important because one of these oxygen atoms is thought to be involved in the formation of a hydrogen bond with the H_2 site (Fig. 3B) on the receptor. Furthermore, changing the substituent at position 8 from an electron-withdrawing to an electron-donating group did not substantially alter either the affinity of the ligand or its selectivity for DI $GABA_A$ receptors.

The DI $GABA_A$ receptor pharmacophore (Fig. 3B) shares common features with those of the DS pharmacophore (Fig. 3A). In particular, while the L_1, H_1, and H_2 structural elements, were similar between these compounds, the size and location of the lipophilic pockets within the corresponding receptor subtype represented the major difference between the DS and DI ligand binding sites (29).

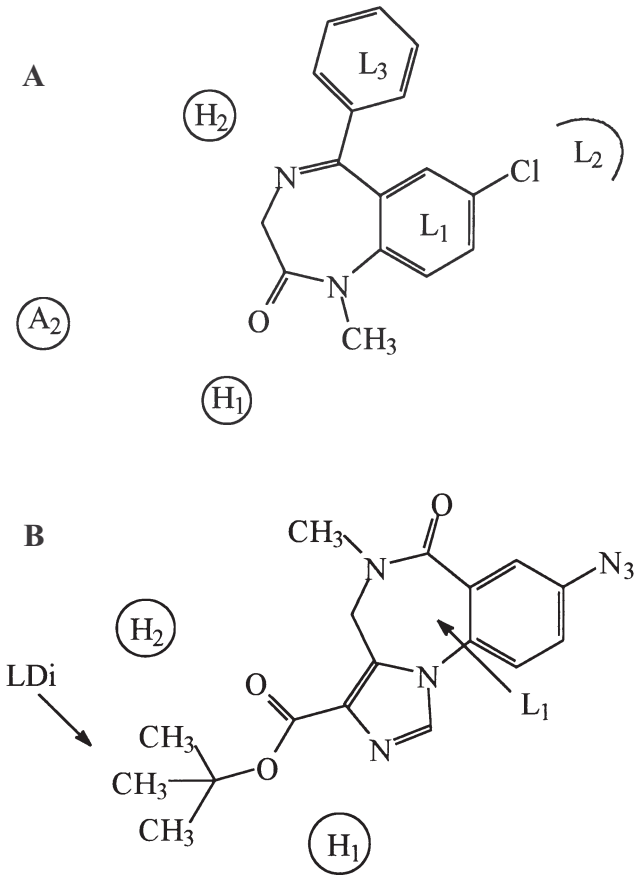


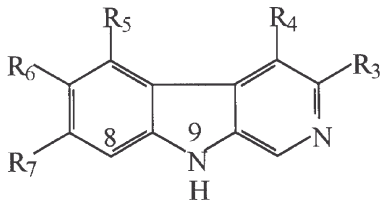
Fig. 3. Pharmacophore models for diazepam-sensitive (DS) and -insensitive (DI) $GABA_A$ receptor subtypes. A. The DS subtype pharmacophore, in which the template is diazepam. H_1 and H_2 are two hydrogen bond donating sites on the receptor. A_2 is a hydrogen bond accepting site. L_1, L_2, and L_3 are three lipophilic pockets on the receptor. The agonist pharmacophore is characterized by H_1, H_2, L_1, L_2, and L_3. The inverse agonist pharmacophore requires A_2, H_1, and L_1. B. The DI subtype pharmacophore, in which the template is imidazobenzodiazepine Ro15-4513. It consists of two hydrogen donating sites (H_1 and H_2) and two lipophilic pockets (L_1 and LD_i) on the receptor.

A QSAR analysis (30) designed to assess the effect of substitutions at position 3 on the oxadiazole ring of imidazobenzodiazepines (Fig. 2) showed that an increase in the size of the substituent at this position decreased the receptor-binding affinity of these molecules. Meanwhile, bulky substituents at position 7, which were offset by smaller substituents at position 8, led to enhanced binding affinities for the resultant ligands.

In addition to the structure-affinity relationship of BZs, a structure-efficacy relationship analysis of imidazobenzodiazepines was also performed. In general, ester groups at position 3 reduced the intrinsic efficacy of the ligands to levels that are associated with antagonists and inverse agonists. In contrast, the oxadiazolyl ring at position 3 increased the intrinsic efficacy of the ligand to levels that are associated with those of an agonist (31,32).

β-CARBOLINES

β-Carbolines (Fig. 4) possessing a carbonyl group substitution at position 3 show a higher *in vitro* activity towards GABA receptors than unsubstituted compounds (33). Some β-carboline-3-carboxylates exhibit an affinity approximately three orders of magnitude higher than their parent compounds (β-carbolines-3-carboxylic acid) (34) One study suggested that the maximum binding affinity of β-carboline (containing a 3-substituent with a carbonyl group) was achieved when the carbonyl group at position 3 was attached directly to the aromatic pyridine ring (35). However, insertion of two atoms between the carbonyl group and the pyridine ring resulted in a marked decrease in the affinity of the compound for the BZ-binding site. Furthermore, this site preferentially recognized the s-cis conformation of the 3-carbonyl group (36). Moreover, in the 3-substituted alkoxy-β-carboline series, the affinity for the BZ-binding site increased as the chain length was increased from methoxy to n-propoxy (37).



	R_3	R_4	R_5	R_6	R_7	Efficacy
β-CCM	COOMe	H	H	H	H	PIA
β-CCE	COOEt	H	H	H	H	PIA
β-CCPr	COO^nPr	H	H	H	H	ANT
β-CCBu	COO^tBu	H	H	H	H	ANT
ZK93423	COOEt	CH_2OMe	H	OCH_2Ph	H	A
ZK91296	COOEt	CH_2OMe	OCH_2Ph	H	H	PA
ZK95962	COOEt	CH_2OMe	O–⟨	H	H	PA
ZK93426	COOEt	Me	O–⟨	H	H	ANT

Fig. 4. β-carbolines.

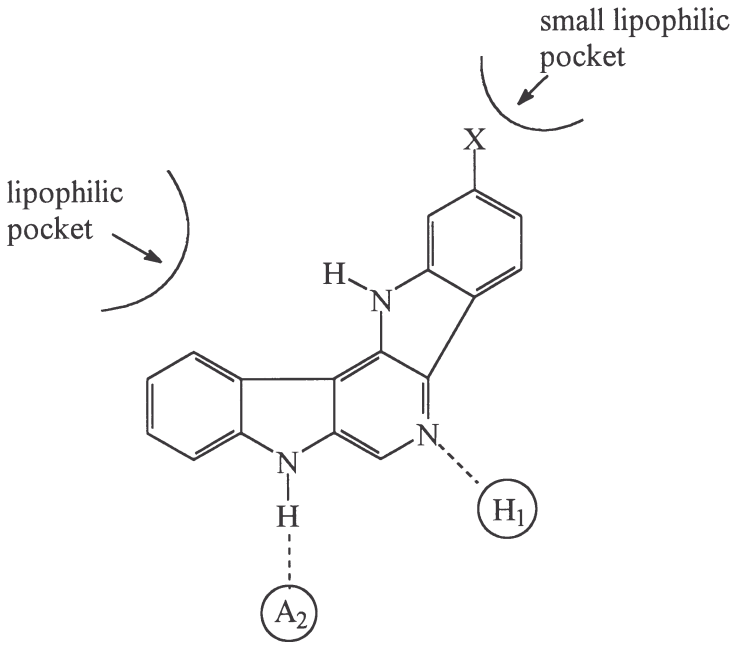


Fig. 5. An inverse agonist/antagonist pharmacophore model based on β-carbolines at the benzodiazepine binding site. A rigid and planar inverse agonist pyridodiindole is used as a template. A_2 is a hydrogen bond accepting site at benzodiazepine binding site; H_1 is a hydrogen bond donating site at the benzodiazepine binding site.

With respect to substitutions at position 6 of β-carboline (38,39) the benzyl oxygen and benzylamino nitrogen have been closely correlated to the potency of the compound via a recent study which employed a series of 6-benzyloxy and 6-benzylamino analogs to study such effects. Additionally, the phenyl ring of the β-carboline has been shown to play an important role in its hydrophobicity. In these compounds, substitution of a methyl group at the N9-position disrupted ligand-receptor binding because the replacement of the hydrogen atom with a methyl group may have prevented the formation of an essential hydrogen bond. When the pyridine ring was saturated to form tetrahydro-β-carbolines, the affinity of the ligand for the $GABA_A$ receptor was reduced compared to that of the corresponding fully aromatic β-carbolines (33).

A pharmacophore model (Fig. 5) for ligand binding to an inverse agonist/antagonist domain within the BZ-binding site was postulated by Allen et al. (34). Key features of the model include two hydrogen bonding sites (A_2 and H_1) and a hydrophobic pocket. A_2 is a hydrogen bond accepting site in the BZ-binding site, and H_1 is a hydrogen donating site. This model is based on a template of the rigid and planar inverse agonist pyridodiindole as shown in Fig. 5.

The 3D-QSAR (9) and SAR (40) analyses were performed on several compounds to further test the model and define the inverse agonist domain within the BZ-binding site. Substituents at position 3 of the β-carbolines (Fig. 4) were observed to have a strong influence on both the affinity and type of activity of the ligand. The binding of β-carbolines to the BZ-binding site are stabilized by favorable interactions between hydrophobic sub-

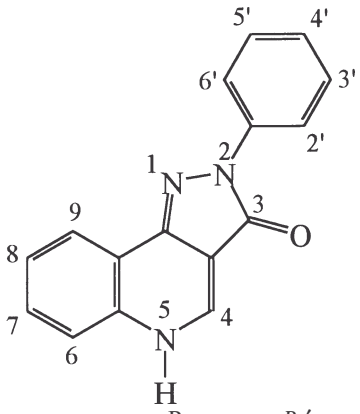
stituents at position 3 and a hydrophobic pocket associated with the BZ-binding site of the receptor (Fig. 5). It has been proposed that the hydrogen bond accepting site A_2 interacts with the N9 hydrogen atom of the β-carbolines or the N7 hydrogen nuclei of the diindoles when the ligand binds to the receptor. Likewise, the hydrogen bond donating site H_1 is thought to interact with the N2 nitrogen atom of the β-carbolines or the N5 nitrogen atom of the diindoles. Moreover, six new 3-substituted β-carbolines for the BZ-binding site were reported by Allen et al. (40). Of these compounds, analogs possessing γ-branched alkyl substituents at position 3 displayed a significantly higher affinity for the BZ-binding site than either the β- or δ-branched derivatives. Furthermore, all β-carbolines with substituents at position 3 with chain lengths exceeding five atoms were shown to have relatively poor receptor binding affinities. Thus, the hydrophobic pocket associated with the BZ binding domain of the receptor appears to place rather restrictive dimensional parameters on the nature of the substituents at position 3 of the ligand in order to maintain the binding potential of the ligand (Fig. 5).

A QSAR analysis (41,42) designed to study the correlation between the potency of the ligand and its physicochemical properties as well as the effects of substitutions at positions 3 and 1 (Fig. 4) showed that both the hydrophobic and electronic characteristics of the 3-ester group are important for receptor affinity. The hydrophobic effect of the alkyl (or phenyl) group as well as the steric effect of substituents at position 1 contribute significantly to the binding affinity of the ligand. Appropriate substitutions at positions 1 or 3 (such as an amide at position 3 and a methyl moiety at position 1) are tolerated with respect to the agonist activity of β-carboline but, in sharp contrast, are not well tolerated for retention of inverse agonist activity of the β-carboline. Moreover, the 4-methoxymethyl group has been shown to be essential for the agonist activity of the β-carboline (43).

Following the discovery of β-carboline-based antagonists and agonists, a number of research groups began to study the structure-efficacy relationships of these compounds (44). The ligand β-CCM (3-methyl ester β-carboline) has been identified as an inverse agonist which possess an efficacy greater than that of β-CCE (3-ethyl ester β-carboline) (Fig. 4) (7). Increasing the bulk of the alkyl ester group at position 3 further reduced the inverse agonist properties of the ligand. Interestingly, β-CCPr (the propyl ester) and β-CCBu (the *tert*-butyl ester) are in fact antagonists (44,45). β-carbolines containing bulky substituents at position 3, such as pentyl, hexyl, heptyl, and octyl esters, are all effective anticonvulsants possessing efficacies against leptazol-induced seizures. This observation suggests that these compounds function, to some degree, as agonists (46). Therefore, as the bulk of the alkyl ester moiety of the β-carboline increases, the intrinsic efficacies of the corresponding ligands transverse a spectrum of activities moving in a direction from inverse agonist to antagonist to agonist.

Replacement of the ester group at position 3 of β-carboline with an oxadiazolyl ring results in analogs which retain their affinity for the BZ-binding site on $GABA_A$ receptors. All ethyl-1,2,4-oxadiazole-5-yl derivatives of the ligand consistently showed an elevated agonist efficacy in comparison to their methyl or ethyl ester counterparts. Likewise, substitution of the ester group at position 3 with an oxadiazolyl ring increased the agonist efficacy of the ligand.

A number of analogs of the partial inverse agonist β-CCE, which possess substitutions at position 4, 5, or 6, resulted in ligands with an enhanced intrinsic efficacy compared to



	R_8	R_4'
CGS8216	H	H
CGS9896	H	Cl
CGS9895	H	OCH_3

Fig. 6. Pyrazolo[4,3-c]quinolin-3-ones.

the parent compound. For example, ZK93423 (Fig. 4) is a full agonist associated at the BZ-binding site (7); the activities of ZK9129 and ZK95962 (Fig. 4) are suggestive of partial agonist activity (47,48), while ZK93426 (Fig. 4) is an extensively studied BZ antagonist (7,47). Therefore, the corresponding intrinsic efficacies of the β-carboline series, including β-CCM, β-CCE, β-CCPr, and β-CCtBu, can be increased by appropriate substitutions at position 4, 5, or 6.

The interaction of a ligand with both A_2 and H_1 sites of the BZ-binding domain of the receptor (Fig. 5) appears obligatory for inverse agonist activity (39,40). It has also been reported that smaller lipophilic substituents at position 3, which lie close to the plane of the β-carboline ring system, tend to yield inverse agonists, while analogs carrying longer or bulkier substituents at the same position possess antagonist activity (38).

PYRAZOLOQUINOLINONES AND DERIVATIVES

Comparison of a series of pyrazolo[4,3-c]quinolin-3-ones (Fig. 6) gave rise to the hypothesis that the pharmacological profiles of these compounds may be correlated to the nature of the substituent in the *para* position of the 2-phenyl ring (49). The steric effects of substituents on the 2-phenyl ring as well as the lipophilicity of the 2-phenyl ring have been shown to influence the receptor affinity of the pyrazoloquinolinone ligands (50). Their pharmacological profiles appear to be modulated by the electronic effects of substituents on the 2-phenyl ring (50).

CGS8216, CGS9895, and CGS9896 (Fig. 6) are closely related 2-phenyl analogs of pyrazoloquinolines. CGS8216 was originally considered to be an antagonist for the BZ-binding site (49,51), but recent studies suggest that it possesses a weak inverse agonist efficacy (7,52). CGS9895 and CGS9896 are partial agonists (51,53); however, CGS9895 displays a lower agonist efficacy than CGS9896 (53). The structure-efficacy relationship of these three analogs further suggests that unsubstituted 2-phenyl pyrazoloquinolines (such as CGS8216) are characterized by a lower intrinsic efficacy than substituted 2-phenyl pyrazoloquinolines (such as CGS9895 and CGS9896).

Recently, a new series of CGS8216 analogs bearing different substituents at positions 6, 7, 8, 9, 2′, 3′, and 4′ have been subjected to structure-activity and structure-efficacy relationship analyses (54). The results of the SAR and QSAR analyses for these ligands are consistent with a previously postulated pharmacophore model (Fig. 7) (13). The key features of this model include: hydrogen bond donor sites (H_1 and H_2); a hydrogen bond acceptor site (A_2); hydrophobic sites (L_1 and L_2); a lipophilic region reached by the 5-phenyl ring of classical BZs (L_3); and a sterically inaccessible region (S_1).

The aryl or heteroaryl substituents at the N2 position (Fig. 6) (occupying the L_1 and L_2 regions associated with the receptor) (Fig. 7) are crucial for the high affinity of the ligand towards the BZ-binding site. Substitution of the ligand with a methyl group at its N5 position strongly diminishes its receptor affinity by preventing the formation of a hydrogen bond between the compound and the A_2 site of the receptor. An *ortho* substitution (partial occupation of the "sterically inaccessible" S_1 region) to the 2-phenyl ring of the ligand reduces its activity. In comparison, small electron donor substituents with the phenyl ring at both the *meta* and *para* positions favor the activity of the compound. Furthermore, *para* substitutions produce compounds which are more active than their corresponding *meta* analogs. Substitutions at position 6 were observed to be less tolerated in terms of the functional activity of the ligand compared to substitutions at positions 7, 8, and 9. This finding may result from the inability of these analogs to form a hydrogen bond between the N5–H group of the ligand and the A_2 site of the receptor due to the presence of bulky groups at position 6. The activity of ligands with a monosubstitution of the quinoline ring is higher than for ligands produced from multiple substitutions. In particular, substitutions at position 9 are most advantageous for the biological activity of the ligand.

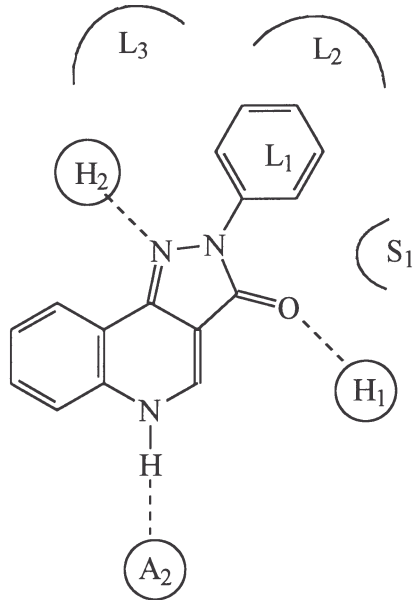
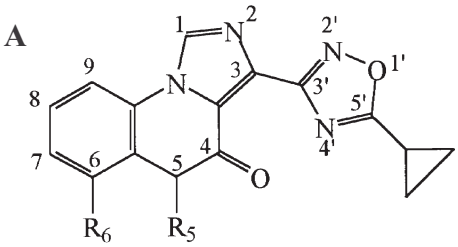


Fig. 7. The pharmacophore model based on pyrazoloquinolinones at benzodiazepine binding site. CGS8216 is used as a template. The interaction sites between receptor and ligand are H_1, H_2 (hydrogen bond donating sites), A_2 (hydrogen bond accepting site), L_1, and L_2 (hydrophobic site). L_3 (another hydrophobic site) does not interact with pyrazoloquinolinones but can be reached by the 5-phenyl ring of classical BZs. S_1 is a sterically inaccessible region. A_2 and H_2 are not necessary for inverse agonist binding.

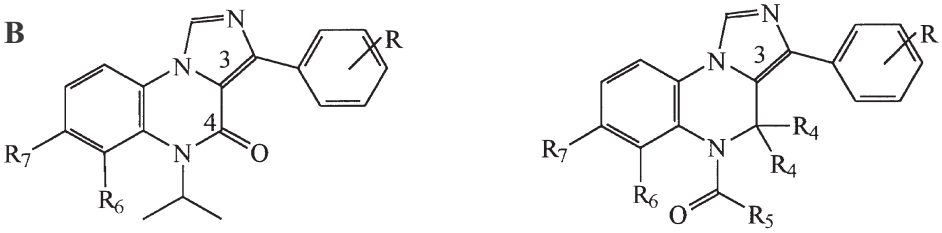
IMIDAZOQUINOXALINES

U78875 and its derivatives (Fig. 8A) were characterized by *in vitro* binding assays and electrophysiological measurements as well as computational three-dimensional structural modeling (55). The *in vitro* assays showed that U78875 was an antagonist with high affinity for the BZ-binding site. Substitutions at the R5 and R6 positions of the ligand produced only marginal changes in the affinity of the ligand for the BZ-binding domain. However, the efficacy of these compounds was markedly dependent on the nature of the various substituents. For example, changing the chemical moiety of the R5 position of the ligand from isopropyl to *tert*-butyl and that of the R6 position from a H to a Cl or an OCH_3 moved the intrinsic efficacy of the corresponding compounds to a type characteristic of an agonist.

On the basis of the structure-activity relationship of U78875, a new class of imidazo[1,5-a]quinoxalines with an open-chain structure (Fig. 9A) and a close-chain



	R_5	R_6
U78263	Methyl	H
U78280	Ethyl	H
U78875	Isopropyl	H
U82249	t-Butyl	H
U79098	Isopropyl	Cl
U81139	Methyl	CF_3
U89320	Isopropyl	OCH_3



3-phenylimidazo[1,5-a]quinoxaline-4-ones 3-phenylimidazo[1,5-a]quinoxaline ureas

Fig. 8. A. U78875 and its derivatives. B. 3-phenylimidazo[1,5-a]quinoxaline-4-ones and 3-phenylimidazo[1,5-a]quinoxaline ureas.

structure (Fig. 9B) were discovered (56–58). Additionally, both structures were capable of binding to the BZ-binding site of the receptor with high affinity although their intrinsic efficacy encompassed a wide spectrum of activities.

The SAR studies involving the open-chain imidazoquinoxalines (Fig. 9A) showed that changes in the position 5 substituent had minimal effects on the binding affinity of the compound for the BZ-binding domain of the receptor; however, dramatic differences were reported for their intrinsic efficacy. The 5-amide-, carbamate-, and thiocarbamate-substituted imidazoquinoxalines tend to be full agonists while the urea-substituted compounds function as partial agonists. Carbamate substitutions at the R5 position of the ligand were shown to elevate the intrinsic efficacy of the compounds as the size of the appended group increased from methyl to isopropyl and then to the "super agonist" *tert*-butyl moiety. With regard to amide derivatives, addition of benzoamide to the R5 position yielded compounds with lower efficacy than those produced by acetamide addition.

When the oxadiazole ring of the open-chain imidazoquinoxalines (Fig. 9A) was replaced by *tert*-butyl ester at position 3, the intrinsic efficacy of the compounds was reduced. When the 5′ substituent on the oxadiazole ring was derived from bulkier chemical groups, the resultant analogs usually possessed an enhanced efficacy. For example, the 5′-ethyl analog was nearly an antagonist while the *tert*-butyl derivative was a full agonist.

The tetracyclic imidazo[1,5-a]quinoxalines (Fig. 9B) displayed high affinity for the BZ-binding site as determined through binding affinity experiments using [^{3}H]flunitrazepam. The constraint of the bottom ring forces the carbonyl group of these ligands into a relatively planar ring system, which in turn is responsible for imparting to these compounds' intrinsic efficacies, that range from those that are associated with antagonists to those that are associated with agonists. Furthermore, the binding affinity for compounds

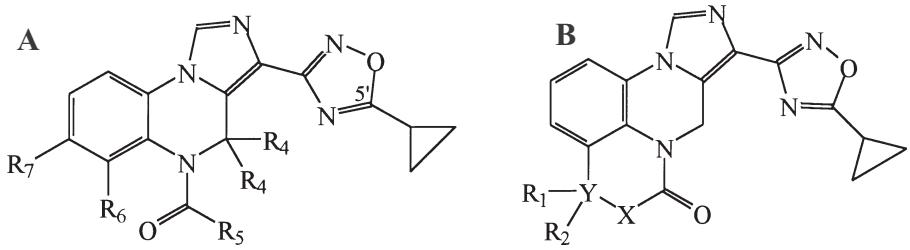


Fig. 9. A. Imidazo[1,5-a]quinoxalines with open-chains. B. Imidazo[1,5-a]quinoxalines with close-chains.

whose bottom ring contains heteroatoms is significantly reduced. Incorporation of a bulkier substituent (such as a phenyl group) into the bottom ring also reduces the binding affinity of the ligand for the BZ binding site of the receptor. The efficacy of the compound is enhanced with an increase in the number of substitutions present in the bottom ring. All the relatively planar analogs are antagonists or partial agonists, whereas those compounds containing out-of-plane groups are all nearly full agonists.

The related 3-phenylimidazo[1,5-a]quinoxaline-4-ones and 3-phenylimidazo[1,5-a]quinoxaline ureas (Fig. 8B) were studied to test the effects of a 3-phenyl substitution on the affinity and *in vitro* efficacy properties of the ligand (54). Substitution of the oxadiazole group with a phenyl ring at position 3 in the imidazoquinoxaline-4-one series results in a notable decrease in the binding affinity of the resultant ligands, as well as a moderate increase in their *in vitro* efficacy. For the imidazoquinoxaline ureas, replacement of the oxadiazole group with a phenyl ring produced analogs that maintained their high affinity in addition to retaining their partial agonist *in vitro* efficacy. These observations were constant, regardless of the nature of the 3-phenyl substituent.

Recently, Gupta and Paleti (60) have performed a QSAR analysis in an attempt to correlate the potency of an imidazoquinoxaline amide and a carbamate series with some other indicator variables (56). The results of the study suggest that the 5-cyclopropyl-1,2,4-oxadiazolyl-3-yl group is preferred over other groups at position 3 for retention of the potency of the ligand. A methyl group at position 4 is detrimental to the activity of the compound, while a fluorine substitution at position 6 or 7 is advantageous for its binding properties.

FLAVONOIDS

Flavonoids (Fig. 10) are a class of natural products that are isolated from a variety of plants. They exhibit a wide range of biological activities, including antiviral, antiinflamatory, vasculoprotector, antithrombotic, spasmolytic, estrogenic, antioxidant, and liver-protecting effects (61). More recently, flavonoids were also found to exhibit binding affinity for the BZ-binding domains of GABA$_A$ receptors (62–66). It is now known that most of the flavonoids with specific affinity for the BZ-binding site are characterized by low to medium receptor affinity *in vitro* and anxiolytic activity *in vivo*, along with possessing minor sedative or myorelaxant effects (66,67).

Häberlein et al. (68) have extracted several flavonoids from Leptospermum scopanum that contain an affinity to the BZ-binding site. Following the identification of these com-

pounds, the research group conducted structure-activity analysis, as well as semiempirical AM1 calculations on the ligands. The results of these studies show that the sterical orientation of the substituents lying coplanar to the aromatic ring is of crucial relevance for the binding affinity of the ligand, especially for substituents at the C5 and C6 positions (Fig. 10). The phenyl ring, the methoxy group at C5, and the carbonyl group at position 4 of the flavonoids were shown to be important for their binding affinity through analysis and comparison of the chemical structures of diazepam, the classical BZ, and the flavonoids [67]. The BZ-binding site contains a pocket that interacts with the aforementioned structural elements of the flavonoids. Hence, the phenyl ring, the π electron system of the carbonyl group, and the substituent on C5 are considered the key structural features that collectively contribute to binding affinity of the flavonoids. Moreover, the double bond between C2 and C3 of the flavone has also been identified to be important in this regard.

Ai et al. (62) have screened 17 flavonoids for the ability of the compounds to inhibit (with IC_{50} of less than 8 μM) [^{3}H]diazepam binding to the rat brain BZ-binding site *in vitro*. Among these compounds, 6-methylflavone was found *in vitro* to be the most potent competitive inhibitor possessing an antagonist activity on both the human and rat BZ-binding sites. The structure-activity relationship of these flavonoids and data available in the literature shows that the basic flavone structure is required for their binding affinity toward the BZ binding domain on $GABA_A$ receptors, since isoflavones and flavanones are inactive. Furthermore, the carbonyl group at position 4 is known to be crucial for the binding affinity of the compound because 4-hydroxyflavone is inactive. Likewise, substitutions at position 6 are of key importance if the resultant analogs are meant to retain their binding affinity. Flavone analogs with methyl, hydroxy, or methoxy substituents at this position have a higher affinity than the parent compound. Substitutions at position 5 or 7 generally diminish the inhibitory activity of the flavonoids, which is mediated through their binding to the BZ binding site of the receptor. Substitutions at position 3 give rise to inactive flavonoids.

Marder et al. (65) synthesized 15 halogenated/nitrated flavone derivatives and measured their affinity for the central BZ-binding site. The SAR analysis of these and other compounds indicates that substitutions at positions 6 and 3′ in the flavone nucleus (Fig. 10) make important contributions to ligands known to have a high affinity for the BZ-binding site. The results of their studies are as follows:

a. Br, Cl, or NO_2 at position 6 plus halogen or NO_2 at position 3′ can greatly increase the affinity of flavonoids. The effectiveness of the different substituents at position 6 is as follows: Br > Cl > NO_2 > F.

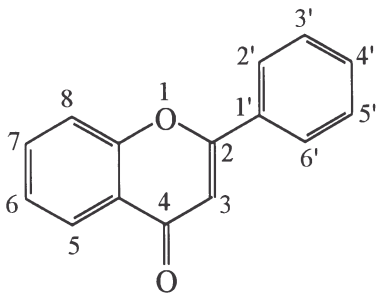


Fig. 10. Flavone.

b. The following substitutions can moderately increase the binding affinity of the resultant analogs: Br, Cl, or NO_2 at position 6; Br at position 6 plus NO_2 at position 2′ or 4; NO_2, Br, or Cl at position 3′; F at position 6 plus NO_2 at position 3′.

c. The following substitutions can greatly decrease the binding affinity of flavonoids: F at position 6; NO_2 at position 2′ or 4′, Br at position 4′; NO_2 at positions 3′ and 5′; at positions 6 and 2′; or

at positions 6 and 4′; Br at position 3 or at positions 6 and 3, or at position 3 plus NO_2 at position 3′.

IMIDAZOPYRIMIDINES AND IMIDAZOQUINOLINES

To date, a large number of imidazopyrimidines and imidazoquinolines (Fig. 11) have been synthesized and studied using behavioral test models. This has made possible investigations focused on the relationships between the structure and efficacy of the ligand (69–70).

Imidazoquinolines show higher agonist efficacy than their dialkyl imidazopyrimidine equivalents (64). When the 2-benzoyl group is replaced by an oxadiazole ring, the affinity of this series of compounds for the BZ-binding site is reduced (70), but their intrinsic efficacy acquires enhanced agonist characteristics. 2-Cyclopropyl imidazoquinolines and imidazopyrimidines have a lower intrinsic efficacy than their 2-oxadiazole or 2-phenyl equivalents. When the 2-cyclopropyl group substitution is combined with a dialkyl substitution of reduced size at positions 5 and 6 of imidazopyrimidines (Fig. 11), both the affinity and intrinsic efficacy of the compounds are diminished (71). Table 1 gives the details of the structure-efficacy relationships of imidazoquinolines and imidazopyrimidines (8,71).

IMIDAZOBENZOTHIAZOLES

By changing the indole NH and the pyridine ring of β-carbolines (Fig. 4) to sulfur and to an imidazo ring, respectively, a series of imidazo[2,1b]benzothiazoles were synthesized which possessed affinity for the BZ-binding site. Of these compounds, the cyclopropylcarbonyl imidazobenzothiazoles (Fig. 12) were found to have good oral bioavailability.

RU33782 (Fig. 12) is a partial inverse agonist at the BZ-binding site of $GABA_A$ receptors (72). RU33862, with a methoxy group at R3, likewise behaves as a partial inverse agonist. However, changing the R3 substitution to benzyloxy group results in RU33894 which displays agonist properties (72). These results suggest that changing R3 from hydrogen to methoxy to benzyloxy increases the agonism of the molecules. The R3 position

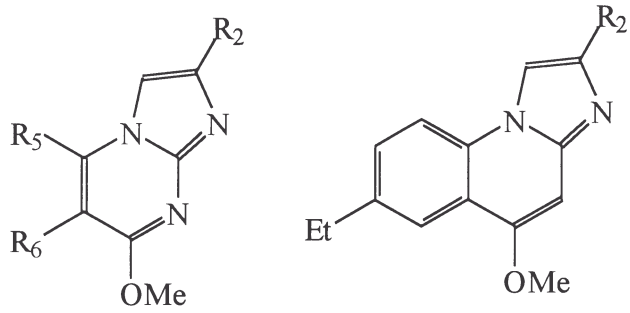


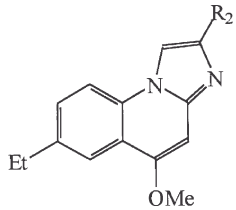
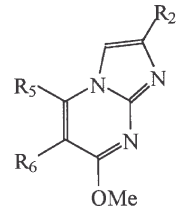
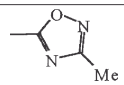
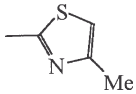
Fig. 11. Imidazo[1,2-a]pyrimidines and imidazo[1,2-a]quinolines.

of the imidazobenzothiazoles is sterically equivalent to position 6 in the β-carboline structure (Fig. 4). Furthermore, these observed efficacy changes of the imidazobenzothiazoles are entirely comparable to the efficacy changes observed with β-carbolines bearing the same or similar substitutions at position 6. Moreover, position R2 of the imidazobenzothiazoles is equivalent to position 4 of β-carbolines. Substitution of R2 with $–CH_2OMe$ and $–CH_2CONH_2$ produces RU34166 and RU34283, respectively (Fig. 12). These compounds have a lower affinity for the BZ-binding site than the unsubstituted analog RU33782 and display shifts in their efficacy for leptazol seizures toward those of an antagonist (RU34166) and agonist (RU34283). These effects on efficacy also parallel those observed for similar substitutions at position 4 of β-carbolines.

In investigating the effects of bulky R1 substitutions on the efficacy of the ligand, several compounds provide useful clues. Increasing the size of the cyclopropyl ring of the partial inverse agonist RU33782 to cyclobutyl produces a compound (RU34225) which reportedly possesses antagonist activity for the BZ-binding site. RU34267, in which its cyclopropyl ring contains a cyano group substituent, is another possible antagonist of $GABA_A$ receptors. RU34256, with a larger substituent on its cyclopropyl ring than that of RU34267, possesses the pharmacological profile of an agonist (73). Therefore, adding bulk to the cyclopropyl ring can result in reduced inverse agonist efficacy and reduced affinity for the BZ binding site.

The similarity between efficacy changes produced at positions R_2 and R_3 of imidazobenzothiazoles and those produced in β-carbolines at position 4 and 6 indicates that these two kinds of compounds share common steric features in binding to the BZ site. This phe-

TABLE 1. Summary of the structure-efficacy relationships of imidazopyrimidines and imidazoquinolines

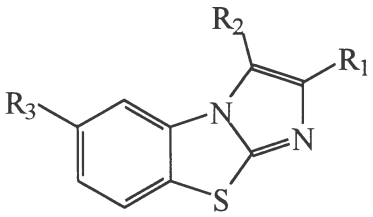
R_2	 imidazoquinolines	 imidazopyrimidines				
	5,6-position benzo-ring	R_5 = Me R_6 = Allyl	R_5 = Me R_6 =	$–(CH_2)4–$	R_5 = Et R_6 = H	R_5 = Me R_6 = H
	33642 SA	33531 SA	33203 SA	33543 MA	34299 MA	
COPh	31719 SA	32734 MA	32698 MA	32514 WA	32696 WA	32635 WA
CO– 	34019 MA	33941 WA	33094 ANT	33697 WIA	34000 SIA	34113 SIA
	33645 MA	33627 WA	33356 WIA	33624 WIA	34317 SIA	

Note: SA, strong agonist; MA, moderate agonist; WA, weak agonist; ANT, antagonist; WIA, weak inverse agonist; SIA, strong inverse agonist.

nomenon may very well shed light on the nature of the BZ-binding site surface and the mode of its interaction with these ligands.

ACETYLCHOLINESTERASE INHIBITORS

Recently, work in our own laboratory (74) provided an indication that bis(7)-THA and THA (Fig. 13), which are selective and potent inhibitors of acetylcholinesterase (AChE), may also exhibit affinity for the BZ-binding site. In both electrophysiological experiments



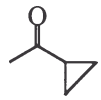
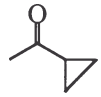
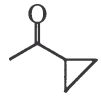
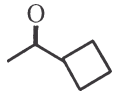
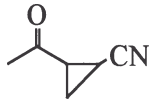
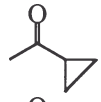
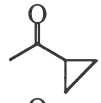
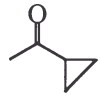
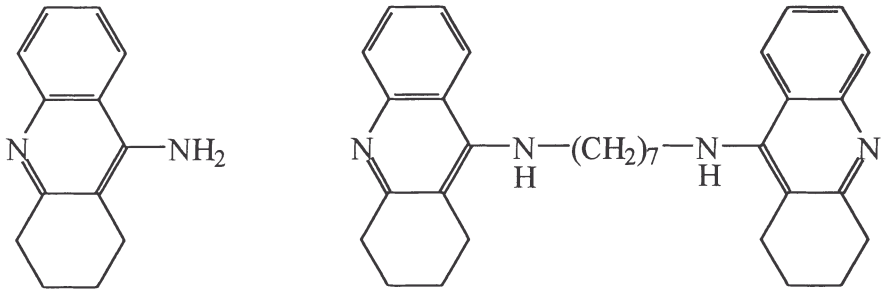
	R_1	R_2	R_3
RU33782		H	H
RU33862		H	OMe
RU33894		H	OCH_2Ph
RU34225		H	H
RU34267		H	H
RU34256		H	H
RU34136		CH_2NHMe	H
RU34166		CH_2OMe	H
RU34283		CH_2CONH_2	H

Fig. 12. Imidazobenzothiazoles.



THA bis(7)-THA

Fig. 13. THA and bis(7)-THA.

and receptor binding assays, bis (7)-THA and THA behave as competitive antagonists for the BZ-binding site. In these experiment, bis (7)-THA was shown to be more potent than THA in this regard. Since the inhibitory effects of bis (7)-THA and THA on AChE appear not to be related to their antagonistic activities toward the BZ-binding site of the $GABA_A$ receptor, the pronounced memory-enhancing effects of bis (7)-THA and THA could be the beneficial result of the combinatorial effects of the compounds on both AChE and the BZ-binding site.

PHARMACOPHORE MODELS

The structure-activity and structure-efficacy relationships embody important advances achieved with respect to the chemical specificities of the BZ-binding sites toward ligand molecules and their response to these molecules. Such specificities are prerequisite to an eventual understanding of the interactions between the BZ-binding site of the $GABA_A$ receptors and the highly diverse, chemical classes of ligands, many of which possess high affinity and specific intrinsic activity toward the site.

A number of BZ-binding site pharmacophore models have been proposed (9–13). Some of these models treat agonist and inverse agonist/antagonist as distinct entities. Given the heterogeneity of the $GABA_A$ receptors in terms of subunit composition, there exists a lack of certainty to whether different subtypes of the receptor indeed may possess distinctive binding affinity spectra toward the different classes of ligands. However, there is growing evidence that the agonist, antagonist, and inverse agonist ligands bind to the same location on the receptor (13). On this basis, Cook et al. have developed an unified pharmacophore model of the BZ-binding site using 136 different ligands spanning ten structural classes of compounds. This model defines the structural requirements needed for the high binding affinity of the ligand to the DS and DI receptor subtypes, the structural requirements needed for DS selectivity, as well as the structural requirements needed for agonist, antagonist, and inverse agonist action.

However, the accuracy of this unified pharmacophore model for predicting ligand affinity and efficacy currently remains open to speculation due in large part to the complexity and heterogeneity of $GABA_A$ receptors. Some of the data from which the structure-activity relationships were derived have also been supported by *in vitro* measurements. Quantitative projections associated with the *in vivo* performance of the ligand could be impacted by factors such as metabolism and pharmacokinetics. Of course, the conformational freedom of the various ligands is also a significant factor that requires consideration in applying any pharmacophore model for predictive purposes.

Pharmacophore models of the BZ-binding site are important tools for rational drug design focused on the theoretical design of novel and potent drugs that are computationally predicted to possess greater selectivity for different $GABA_A$ subtypes. These models are most powerful for predicting the affinities and efficacies of new BZ-binding-site ligands before sufficient structural insight into various receptor subtypes becomes available.

At present, the lack of a high resolution three-dimensional structure for the $GABA_A$ receptor represents a fundamental bottleneck to the rapid identification of protein elements that constitute the different pharmacophore sites. As a result, the usefulness of rational drug design for this receptor system remains limited. Recently, our laboratory has successfully expressed a 131-residue fragment of the human $GABA_A$ receptor α_1 subunit in *Escherichia coli* (74). The fragment forms an integral structural domain of the receptor polypeptide and contains residues previously suggested to be involved in the BZ-binding site. Its well-formed secondary structures are evident from the circular dichroism spectrum. Since the fragment is capable of binding such BZ binding site ligands as Ro1986 and [^{3}H]flunitrazepam (unpublished data), its structure-function characterization may open the way to the eventual identification of relevant pharmacophoric sites in the receptor.

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