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# Molecular Determinants of Benzodiazepine Receptor Affinities and Anticonvulsant Activities

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## SUMMARY

*In vivo* convulsant activities profiles and receptor binding studies together with the techniques of theoretical chemistry were used to characterize 15 compounds, from five different chemical families, known to bind to the BDZ receptor. The experimental goals of this study were to determine the affinity of these analogs for this receptor, the effect of γ-aminobutyric acid on the affinity, and, in a self-consistent manner, the nature of the activity, agonist (anticonvulsant), antagonist, or inverse agonist (proconvulsant, convulsant), elicited by binding to this receptor. To these ends, *in vivo* studies were made to determine the proconvulsant, convulsant, and anticonvulsant activities and antagonism to anticonvulsant activities of the 15 analogs. Their receptor affinities at 25° were also determined by competitive inhibition of [$^3$H] flunitrazepam and [$^3$H]Ro15-1788 in the absence and presence of γ-aminobutyric acid. The goal of the theoretical studies was to identify and calculate molecular properties that modulate these affinities and types of activities and from them to develop a model of receptor recognition and activation that could consistently explain observed behavior and predict new results. Thus, molecular orbital calculations were carried out for all analogs, using semiempirical quantum mechanical methods. In addition to the optimization of structures, a number of electronic properties, such as polarizations, partition coefficients, and proton and electron affinities were computed and examined for their ability to modulate relative affinities and modes of activation of the receptor. From these studies, a model for receptor recognition involving two anchoring hydrogen bond-acceptor sites and for activation involving interaction of the most lipophilic aromatic region of each compound with the receptor was developed, which could systematically account for the three different types of behavior, agonist, antagonist, and inverse agonist, observed for these analogs. Electronic rather than structural properties were found to be the principal modulator of both recognition and activation. A possible mechanism of agonist activation of the receptor involving electron transfer to the agonist, as well as a possible induced conformational change in the receptor, is also suggested by these results. Finally, by complementarity, some steric and electronic characteristics of the receptor binding site could be deduced.

BDZs are widely used therapeutic agents with anxiolytic, anticonvulsant, muscle relaxant, and sedative/hypnotic activity. The benzodiazepine binding site has been identified as part of the GABA receptor/$Cl^-$ ionophore supercomplex (1, 2), although other BDZ effects that are unrelated to GABA have been demonstrated (3–5). Although initial studies indicated that BDZs bind to the α subunit and that GABA binds to the β subunit of the GABA/BDZ receptor complex (6), it was later found that the $\gamma_2$ subunit contributes to the formation of a functional BDZ site (7). Multiple α subunits of this receptor have been found (8) and receptor heterogeneity has been detected in binding studies (9–11). However, the evidence from binding studies is not as robust as in other receptor families and heterogeneity, although observed at low temperatures (9–11), is difficult to detect at a physiological temperature (12–14).

In addition to the classical 1,4-benzodiazepine ligands, diverse chemical families have now been shown to bind with high affinity to the BDZ binding site(s) and also exhibit a similar spectrum of *in vivo* activities. Some members of those families have been shown to be BDZ receptor antagonists. In addition, strikingly, among BDZ ligands are also those compounds that have been reported to elicit an opposite *in vivo* endpoint, such as anxiogenic (15) or convulsant activity (16). Thus, based on

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**ABBREVIATIONS:** BDZ, benzodiazepine; GABA, γ-aminobutyric acid; CL218,872 (3-methyl-6-[3-trifluoromethylphenyl]-1,2,4-triazolo[4,3-b]pyridazine); CGS 9896 (2-(4-chlorophenyl)-2,5-dihydropyrazolo[4,3-*c*]quinolin-3-(3*H*)-one), CGS 9895 (2-(4-methoxy)phenyl-2,5-dihydropyrazolo[4,3-*c*]quinolin-3(3*H*)-one), CGS 8216 (2-phenyl-2,5-dihydro-pyrazolo[4,3-*c*]quinolin-3(3*H*)-one); Ro15-1788 (8-fluoro-3-carboethoxy-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-a]1,4-benzodiazepine); DMCM, (3-carbomethoxy-4-ethyl-6,7-dimethoxy-9*H*-pyrido[4,3-*b*]indole) or (3-carbomethoxy-4-ethyl-6,7-dimethoxy-β-carboline); β-CCN, (3-cyano-β-carboline); β-CCM, (3-carbomethoxy-β-carboline); β-CCE, (3-carboethoxy-β-carboline); FG 7142(3-(N methyl)-carboxamide-β-carboline; ZK 91296, (3-carboethoxy-5-benzyloxy-4-methoxymethyl-β-carboline; ZK 93426, (3-carboethoxy-5-*i*-propyloxy-4-methyl-β-carboline; PTZ, pentylenetetrazol.

different reported studies of *in vivo* activities, BDZ ligands have come to be labeled as agonist, antagonist, or inverse agonist (17).

Despite the growing evidence for these three broad classifications of compounds, most previous structure-activity studies have only focused on identifying molecular determinants of relative affinities (18–20). In past studies, we have also considered the requirements for high affinity at the BDZ receptor sites, for both BDZ agonists (21) and β-carboline antagonists (22), using quantum chemical studies combined with experimental observation. For both families, we proposed a receptor recognition model involving H-bonding receptor interactions. For the BDZ, three sites were postulated, an amine nitrogen and the carbonyl oxygen in the seven-membered ring and the $R_7$-substituents in the condensed ring. For the β-carbolines, two sites were found instead, the β-nitrogen and the proton-accepting residues on C3. No evidence of van der Waals interactions was found.

Since then, a very elaborate binding site model, based on the superposition of BDZ-like compounds and β-carbolines with known crystal structures with seven conformationally mobile binding points, was proposed (20). Finally, Borea and coworkers (23) proposed a very interesting model in which different families of compounds occupied different nonoverlapping regions of a flexible expandable recognition site.

Although various models for receptor recognition have been proposed, there have been very few studies that address the question of molecular determinants of receptor activation. To this end, we report here the use of the techniques of theoretical chemistry together with *in vivo* pharmacological and receptor binding studies to identify and characterize different modes of ligand binding to the BDZ receptor leading to the three qualitatively different effects on receptor activation.

A crucial part of this study is the consistency of the pharmacological data to be used as a basis for categorizing compounds into these three classes.

In the study presented here, we have chosen for our experimental and theoretical investigation 15 compounds from diverse chemical families, shown in Fig. 1, including BDZ, β-carbolines, pyrazoloquinolones (CGS), and triazolopyridazine (CL) compounds. In all but the last family, we have included analogs that have been reported in diverse studies to have a spectrum of pharmacological effects with respect to anticonvulsant activity. To provide a self-consistent base for these compounds, we have used anticonvulsant behavior, a common activity, as a criterion to systematically classify BDZ ligands in a consistent manner as anticonvulsant agonists, antagonists to anticonvulsant activity, or inverse agonists with convulsant or proconvulsant behavior.

We have also verified by receptor binding studies that all analogs studied bind to the BDZ receptor, which presumably initiates their action. This data were then used together with theoretical chemistry methods to identify and characterize these compounds that are reliable determinants of the qualitatively different agonist, inverse agonist, and antagonist activites and to develop a model for BDZ receptor recognition and activation.

## Materials and Methods

[$^3$H]Flunitrazepam and [$^3$H]Ro15-1788 were from Dupont NEN; β-CCM, DMCM, and FG 7142 from Research Biochemicals Inc. Diazepam, flunitrazepam, β-CCE, and Ro15-1788 were the kind gift of Hoffmann-La Roche. β-CCN was synthesized in our laboratory, and a report describing this analog has appeared (24). CGS 9896, CGS 9895, and CGS 8216 were kindly provided by the Research Department of the Pharmaceutical Division of Ciba-Geigy Corp. (Summit, NJ). ZK 91296 and ZK 93426 (Schering, Berlin), prazepam (Sigma Chemical Co.), and CL218,872 (Lederle Laboratories) were also provided *gratis*.

**Receptor binding.** The binding assay method has previously been described by Toll *et al.* (9), except that [$^3$H]Ro15-1788 was used instead of [$^3$H]ethyl-β-carboline-3-carboxylate (β-CCE) and an incubation was performed at a temperature of 25°.

In parallel experiments, the same procedure was followed but a solution of 100 μM GABA was included in order to study the effect of GABA in the binding of these compounds. A temperature of 25°, rather than the more common one of 0° that was previously used (9), was selected because the effect of GABA on BDZ ligand binding was reported to be enhanced at higher temperatures and also in order to come closer to physiological conditions (14).

The $K_D$ values were obtained using a nonlinear least squares regression analysis embodied in the program LIGAND (25), in a manner described in detail elsewhere (9). The data used to obtain these results consisted of duplicate determinations of the competitive inhibitions by each nonradioactive drug of [$^3$H]flunitrazepam and [$^3$H]Ro15-1788 without GABA and one determination with GABA.

**Behavioral tests.** Drug-naive male Swiss-Webster mice weighing 27–31 g were used. PTZ (Sigma) was dissolved in 0.9% saline. Microfine ground powders of the test compounds were suspended by sonication in a solution of 0.3% Tween-80 and 0.9% saline and were injected intraperitoneally in a volume of 10 ml/kg of body weight.

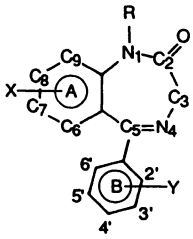
In all tests, after drug injection animals were placed in individual cages and observed for 30 min. For each test, 8 to 14 mice/dose level were used. The median effective dose ($ED_{50}$), the median effective antagonist dose ($AD_{50}$), and 95% confidence limits were computed according to the graphic probit method of Litchfield and Wilcoxon (26). All $ED_{50}$ and $AD_{50}$ values are reported as μmol/kg, to facilitate comparison with binding data.

**Test for convulsant activity.** The test compound was administered intraperitoneally to mice that were then observed for 30 min for occurrence of clonic seizure activity. Most of the chemicals were evaluated at a maximal dose of 100 mg/kg but in the case of FG 7142 and β-CCM lower doses were also tested, because a biphasic dose-response curve for both compounds have been reported (16, 27, 28).

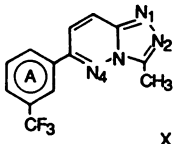
**Test for proconvulsant activity.** Mice were injected with the test compound intraperitoneally, 30 min or 6 sec before administration of a subconvulsant dose of PTZ (40 mg/kg, subcutaneously), and the number of animals per dose level displaying clonic seizures within 30 min of the last injection was recorded.

**Test for anticonvulsant action.** The ability of the test compound to prevent clonic convulsions was tested by intraperitoneal injection of the test compound 30 min or 6 sec before the subcutaneous injection of PTZ (85 mg/kg). Mice were then observed for 30 min and the number of animals not showing clonic seizures was recorded.

**Antagonism of BDZ anticonvulsant activity.** A previously published protocol was used to measure antagonism of anticonvulsant activity of diazepam (29). Mice were given injections of 1 mg/kg diazepam (intraperitoneally), at a standard volume of 10 ml/kg. Thirty minutes later, a test substance was injected (intraperitoneally) and immediately followed (6-sec delay) by 85 mg/kg PTZ (subcutaneously). The total number of animals that developed seizures was tallied at each dose level. The percentage of mice that convulsed at each dose level was plotted against log dose. The $AD_{50}$ was defined as an estimated dose at which 50% of the population were expected to convulse. The time of peak antagonist effect was measured for all pure antagonists, β-CCN, ZK 93426, Ro15-1788, and CGS 9895, by varying the interval between administration of the test substance and PTZ. In each case, simultaneous (i.e., test compound given 6 sec before PTZ) injection led



| | X | $R_1$ | Y |
|---|---|---|---|
| Flunitrazepam | 7-$NO_2$ | $CH_3$ | 2'F |
| Diazepam | 7-Cl | $CH_3$ | H |
| Prazepam | 7-Cl | $CH_2$  | H |



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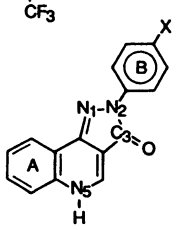
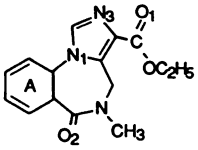
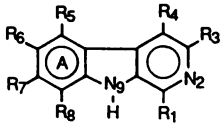


| | X |
|---|---|
| CGS8216 | H |
| CGS9895 | -O-$CH_3$ |
| CGS9896 | -Cl |



RO15-1788



| | $R_3$ | $R_4$ | $R_5$ | $R_6$ | $R_7$ |
|---|---|---|---|---|---|
| β-CCN | CN | H | H | H | H |
| β-CCM | $COOCH_3$ | H | H | H | H |
| β-CCE | $COOC_2H_5$ | H | H | H | H |
| ZK91926 | $COOC_2H_5$ | $CH_2OCH_3$ | $OCH_2$-B | H | H |
| ZK93426 | $COOC_2H_5$ | $CH_3$ | O-CH(-$CH_3$)$_2$ | H | H |
| DMCM | $COOC_2H_5$ | $C_2H_5$ | H | -O-$CH_3$ | -O-$CH_3$ |
| FG7142 | $COONHCH_3$ | H | H | H | H |

**Fig. 1.** Chemical structures and formulas of 15 BDZ receptor ligands included in the present study.

to maximum antagonist activity. For CGS 9895, antagonist activity did not significantly change for 30 min after administration.

**Theoretical studies.** The structures of all compounds studied were fully optimized using the MNDO/H method (30), which provides a reliable description of inter- and intramolecular hydrogen bonds. The planarity of $\pi$-conjugated systems was preserved for the $\beta$-carbolines, thus overcoming a known failure of MNDO. Most of the structures are similar to those previously described (21, 22), and all are available upon request.

Heats of protonation at competing sites of each analog were calculated. These quantities are defined as the difference between the heat of formation of the protonated and unprotonated form of the drug and can be used as a measure of the strength of interaction of different proton-accepting atoms in the drug with proton-donating receptor subsites.

Polarization volumes of the ligands were evaluated using an interpolation procedure described by Fraga (31) and the net atomic charges obtained by MNDO calculations. Polarizabilities were also evaluated in a few cases using an alternative procedure, embodied in the AMPAC program (32, 33). The calculated values of polarization obtained by both procedures were consistent, with a maximum disparity of only 10%. The main advantage of using Fraga's method is that it allows the decomposition of the total polarization into individual atoms and, thus, the polarizability of different regions in the molecule can be calculated. Regional polarization values were used as an indication of the optimum mode of stacking or other dispersion interactions of a fragment of the molecule with aromatic moieties of the receptor. Without this decomposition, because the total polarizability increases with the number of atoms in the molecule, the contributions to polarization from different regions of the system can be masked by side chains or substitutents that would not participate in the interaction.

The extent of hydrophobic interactions was estimated from partition coefficients, which were computed using atomic parameters developed for evaluating this quantity (34). This procedure was selected over others because it yielded values of partition coefficients in closest agreement with those experimentally obtained for diazepam and CGS-related compounds. Differences between experimental and calculated values of ±0.4 units were found, which are similar to the experimental error.

## Results and Discussion

**Binding studies.** $K_D$ values, with and without GABA, at 25° for each of the 15 compounds are given in Table 1. This 2 × 15 matrix of data was analyzed simultaneously with the assumption of one receptor site, yielding the set of dissociation constants given in Table 1. Attempts to probe receptor heterogeneity at this temperature with these labeled ligands, although somewhat decreasing the root mean square values indicating a better fit to the data, did not yield a unique or reproducible set of dissociation constants for each ligand at each receptor or of maximum receptor capacities for each. Thus, we report only the $K_D$ values of a one-site model. The $K_D$ values

TABLE 1

**BDZ receptor affinities at 25° and effect of GABA on families of compounds with diverse convulsant end points**

| | $K_D$[a] | | Ratio, (−)/(+) |
|---|---|---|---|
| | −GABA[b] | +GABA[c] | |
| | nM | | |
| Agonists | | | |
| CGS 9896 | 0.62 ± 0.3 | 0.47 ± 0.04 | 1.32 |
| ZK 91296 | 1.7 ± 0.12 | 1.40 ± 0.13 | 1.21 |
| Flunitrazepam | 4.3 ± 0.7 | 3.0 ± 0.2 | 1.43 |
| Diazepam | 15.1 ± 0.7 | 8.3 ± 0.8 | 1.82 |
| CL218,872 | 277.0 ± 16 | 145.0 ± 13 | 1.91 |
| Prazepam | 344.0 ± 15 | 238.0 ± 16 | 1.44 |
| Antagonists | | | |
| CGS 9895 | 0.06 ± 0.003 | 0.07 ± 0.007 | 0.86 |
| ZK 93426 | 1.80 ± 0.08 | 3.0 ± 0.3 | 0.60 |
| Ro15-1788 | 2.3 ± 0.1 | 2.3 ± 0.2 | 1.00 |
| β-CCN | 12.7 ± 0.5 | 14.5 ± 0.4 | 0.88 |
| Inverse Agonists | | | |
| CGS 8216 | 0.15 ± 0.008 | 0.16 ± 0.02 | 0.94 |
| β-CCE | 4.8 ± 0.2 | 5.6 ± 0.4 | 0.86 |
| DMCM | 4.8 ± 0.2 | 3.6 ± 0.3 | 1.33 |
| β-CCM | 5.9 ± 0.3 | 7.1 ± 0.7 | 0.83 |
| FG 7142 | 303 ± 15.4 | 256.0 ± 23 | 1.18 |
| $B_{max}$ (pmol/g) | 66.7 ± 2 | 70.0 ± 4 | |

[a] All results using [$^3$H]flunitrazepam and [$^3$H]Ro15-1788.
[b] Without GABA.
[c] Adding 100 μM GABA.

(25°) obtained for the compounds are given in order of decreasing affinities for compounds grouped according to the results of our *in vivo* studies of their convulsant/anticonvulsant behavior.

In contrast to the present results, previous studies in our laboratory, at 0°, using [$^3$H]flunitrazepam and [$^3$H]βCCE, did allow a definitive identification of two receptor sites. Differences between the previous results and present ones could be due to the increased temperature used here or use of [$^3$H]Ro15-1788 instead of [$^3$H]β-CCE. Other investigators have reported a similar apparent decrease in receptor heterogeneity from 0° to 37° (12, 13). Because the receptor normally functions at physiological temperature, our results would indicate that these compounds bind to a single receptor or nonselectively to multiple receptors.

In addition to having more physiological relevance, one of the reasons we chose to do the binding studies at 25° rather than 0° was because of literature reports (14) that the effect of GABA on BDZ ligand binding is enhanced at the higher temperature. Early studies appeared to show that GABA modulated BDZ ligand binding, enhancing agonist, not affecting antagonist, and diminishing inverse agonist affinity (35). The reported effects were not very large, most often within 20–50% and never exceeding a factor of 2. As shown in Table 1, given the uncertainty in results of receptor binding studies in general, the observed effects of GABA in modulating BDZ ligand binding, even at 25°, are not very robust. Nevertheless, we did find a systematic increase of the affinity for agonists, in agreement with previous studies. However, contrary to the report of no effect (35), GABA seems to somewhat reduce the affinity of antagonists, whereas we found no common reproducible trend for inverse agonists, i.e., compounds with either proconvulsant or convulsant activity. Thus, the binding of GABA to its binding site does not appear to produce an obvious systematic differential effect on the affinities of the three different types of BDZ ligands.

**Behavioral studies.** Results of the evaluations of the 15 compounds in the convulsant, proconvulsant, and anticonvulsant agonism and antagonism tests are summarized in Table 2. Six compounds, from four different chemical classes, were found to be agonists. These compounds are listed in order of decreasing anticonvulsant agonist activity. The pure anticonvulsant agonist activity of ZK 91296 is unusual, because most β-carbolines have been reported to have antagonist or inverse agonist activity, as is the case for the remaining β-carbolines studied here.

Our results also showed four analogs that are pure antagonists of diazepam anticonvulsant activity with no significant anticonvulsant, convulsant, or proconvulsant activity when administered intraperitoneally. The time of peak antagonist activity of all of these occurred with simultaneous administration of test drug and PTZ, but for CGS 9895 there was no significant decrease if it was administered 10, 15, or 30 min before PTZ. In general, antagonist activity of the β-carbolines and Ro15-1788 is short-lived, with only the CGS 9895 effect lasting more than 30 min.

Whereas two β-carbolines and CGS 9895 were among the pure antagonists, in both series related analogs with small chemical modifications were not only potent antagonists but also had measurable proconvulsant activity. Specifically, the CGS analog 8216, with only one substituent change from CGS 9895, and two β-carbolines, β-CCE and β-CCM, were all found to be potent antagonists but with some proconvulsant activity at much higher concentrations. Whereas the β-carbolines are quick acting and of short duration, the CGS compounds have a longer duration of action and a longer onset time. For example, the potency of CGS 8216 as a proconvulsant is 100-fold weaker ($ED_{50}$ = 107 μmol/kg) when it is administered simultaneously rather than 30 min before PTZ. In addition to the time interval between administration of the test drug and PTZ, the route of administration also affects apparent proconvulsant activity. Interestingly, when it was administered intravenously, the proconvulsant activity of β-CCM increased 100-fold ($ED_{50}$ = 2.7 μmol/kg). The most obvious explanation is more efficient delivery of intact compound to the BDZ receptors and one would expect proportional increases in antagonist activity. All results reported in Table 2 are for intraperitoneal administration of the drug.

Unlike β-CCE, CGS 8216, and β-CCM, the proconvulsant activity of FG 7142 far exceeds its antagonist activity and it would be difficult to measure at a concentration with no proconvulsant activity. It is surprising, however, that this potent proconvulsant does not manifest any significant convulsant activity even at the very high doses administered. It, thus, seems to have only marginal ability to activate BDZ receptors in a mode leading to convulsive action and, hence, is a partial inverse agonist. Of all the analogs tested, DMCM is the only pure and complete inverse agonist with only convulsant activity.

The $ED_{50}$ values obtained in all four behavioral tests for all compounds agree with the published data, when available, with a few exceptions. Yokoyama *et al.* (36) failed to find proconvulsant action of CGS 8216 and also found that CGS 9895 had anticonvulsant properties. However, our results for CGS 8216 are in agreement with those of File (37) and for CGS 9895 with those of Brown *et al.* (38). In our tests of FG 7142, the $ED_{50}$ for proconvulsant action was 0.14 μmol/kg, which is much less

TABLE 2

**Convulsant, proconvulsant, and anticonvulsant agonist, and anticonvulsant antagonist potencies of diverse BDZ ligands**

| | Agonist[a], $ED_{50}$ | Antagonist,[b] $AD_{50}$ | Convulsant,[c] $ED_{50}$ | Proconvulsant[d] $ED_{50}$ |
|---|---|---|---|---|
| | | μmol/kg | | |
| Agonist | | | | |
| Flunitrazepam | 0.07 (0.05–0.10) | | | |
| Diazepam | 1.5 (1.3–1.9) | | | |
| CGS 9896 | 3.0 (2.0–5.0) | | No | No |
| Prazepam | 7.0 (4.0–11.0) | | | |
| CL218,872 | 12.0 (11.0–15.0) | | No | No |
| ZK 91296 | 22.0 (14.0–34.0) | No | | No |
| Antagonist | | | | |
| ZK 93426 | No[e] | 0.9 (0.5–1.6)[f] | No | No |
| Ro15-1788 | No[e] | 1.0 (0.6–1.6)[f] | No | No |
| CGS 9895 | No,[e,g] | 7.0 (4.0–11.0)[f] | No | No[h] |
| β-CCN | No[e] | 18.0 (9.0–35.0)[f] | No[i] | No[h,j] |
| Inverse agonist, antagonist | | | | |
| β-CCE | | 0.03 (0.01–0.06)[k] | No | 145.0[h] (103.0–205.0) |
| CGS 8216 | No[e] | 1.1 (0.8–1.6)[f] | No | 12.0 (40.0–35.0) |
| β-CCM | No[e] | 0.09 (0.06–0.14)[k] | 330[m] | 86.0[h] (46.0–163.0) |
| FG 7142 | No[e] | | No[n] | 0.14[h] (0.08–0.24) |
| DMCM | | | 11.0 (10.0–12.0) | |

[a] Test drug administered intraperitoneally 6 sec before 85 mg/kg PTZ (subcutaneously).
[b] Antagonism to 1 mg/kg diazepam anticonvulsant activity. Test compound administered intraperitoneally 30 min after diazepam and 6 sec before 85 mg/kg PTZ (subcutaneously).
[c] No, no convulsant activity at maximum injectable dose of 100 mg/kg.
[d] Proconvulsant activity. Test compound administered intraperitoneally 30 min before 40 mg/kg PTZ (subcutaneously).
[e] Test drug administered intraperitoneally 30 min before 85 mg/kg PTZ (subcutaneously). No anticonvulsant effect at highest dose administered (100 mg/kg except for β-CCN at 30 mg/kg, because of insolubility).
[f] Time course performed. Antagonist potency found to be maximum if the test substance was administered 6 sec before injection of PTZ.
[g] Maximum effective agonism occurred at 137 μmol/kg, where 37% of the animals were protected. With lower and higher doses, decreases in agonist activity were observed.
[h] Proconvulsant activity. Test compound administered intraperitoneally 6 sec before 40 mg/kg PTZ (subcutaneously).
[i] Maximum injectable dose at 155 μmol/kg (solubility).
[j] At the maximally injectable dose of 155.4 μmol/kg (saturated concentration), 30% of the animals showed clonic convulsions.
[k] No time course measured. Assumed to be same as for other β-carboline antagonists, 6 sec before injection of PTZ.
[l] Time course performed. Peak time of antagonist activity 30 min before PTZ administration.
[m] Biphasic. Approximate $ED_{50}$ = 330. Higher and lower doses decreased activity.
[n] At maximum injectable dose, 100 mg/kg, 10% convulsion.

than that reported by Little *et al.* (27) to achieve maximal proconvulsant action in combination with a higher subconvulsant dose of PTZ. The differences can be attributed to the difference in protocol, bearing in mind the fact that the peak effect of the β-carbolines seems to occur almost immediately following administration. A marked strain difference in sensitivity to the convulsant actions of β-CCM has been noted (25). This sensitivity, in addition to large differences in $ED_{50}$ values obtained by different routes of administration, may account for the wide variation in the published effective doses (16, 28). There is also some disagreement whether Ro15-1788 is a pure antagonist or has some partial agonist properties, especially as pertains to anticonvulsant action. In our hands, Ro15-1788 appeared to be a pure antagonist. However, anticonvulsant activity when administered against a lower dose of PTZ (55 mg/kg subcutaneously) has been demonstrated by other investigators (39).

In addition to classification of the compounds, in Table 3 we have ordered the agonist according to their decreasing affinity and decreasing activity. As seen in this table, this rank order is different, implying different ability to activate the $GABA_A$ receptor. We have expressed this difference in effectiveness as the ratio of $K_D/ED_{50}$. This ratio is not, however, a true measure of effectiveness, because binding activity was not measured in the same system and apparent *in vivo* activity is influenced by many factors, such as pharmacokinetic and pharmacodynamic factors or the ability to cross the blood-brain barrier (40, 41).

TABLE 3

**Rank order of agonist by affinity, activity and effectiveness**

| Receptor affinity | | Agonist activity | | Efficacy | |
|---|---|---|---|---|---|
| Agonist | $K_D$ | Agonist | $ED_{50}$ | Agonist | $K_D/ED_{50}$ |
| | nM | | μmol/kg | | |
| CGS 9896 | 0.62 | Flunitrazepam | 0.07 | Flunitrazepam | 60 |
| ZK 91296 | 1.7 | Diazepam | 1.53 | Prazepam | 50 |
| Flunitrazepam | 4.3 | CGS 9896 | 2.77 | CL218,872 | 22 |
| Diazepam | 15.1 | Prazepam | 6.90 | Diazepam | 10 |
| CL218,872 | 277 | CL218,872 | 12.5 | CGS 9896 | 0.2 |
| Prazepam | 344 | ZK 91296 | 21.8 | ZK 91296 | 0.08 |

With these reservations in mind, the results obtained (Table 3) show that, because affinities and activities do not vary together, the six pure agonists have very disparate "apparent abilities" to activate the BDZ receptor. This rank order was used as a guide in determining possible activation mechanisms to be tested by further studies.

**Molecular modulators and model of ligand-receptor recognition and activation.** In these studies, our goal was to identify and calculate molecular properties of the compounds studied that would be reliable indicators of both receptor recognition and activation leading to the three qualitatively different types of activity initiated by binding to the receptor. The calculated molecular properties examined as candidate indicators were 1) a single distance parameter previously proposed (42) to distinguish agonism, antagonism, and inverse agonism (Table 4); 2) polarization volumes (Table 5); 3) partition coef-

TABLE 4

**Fryer distance index for BDZ receptor ligands grouped on the basis of *in vivo* studies and ranked according to their median effects**

| Drug | Fryer index *r* |
|---|---|
| | Å |
| Agonists | |
| Flunitrazepam | 4.873 |
| Diazepam | 4.286 |
| CGS 9896 | 6.255 |
| Prazepam | 6.807 |
| CL218,872 | 5.546 |
| ZK 91296 | 7.235 |
| Antagonists | |
| ZK 93426 | 7.080 |
| Ro15-1788 | 7.269 |
| CGS 9895 | 6.254 |
| β-CCN | 6.878 |
| Inverse agonists | |
| β-CCE | 6.052 |
| CGS 8216 | 6.255 |
| β-CCM | 5.972 |
| FG 7142 | 6.797 |
| DMCM | 6.881 |

TABLE 5

**Polarization volumes, total value (α) and continuation of different rings**

Rings A and B as shown in Fig. 1.

| Drug | αTotal | α(A) | α(B) |
|---|---|---|---|
| Agonists | | | |
| Flunitrazepam | 42.8 | 13.9 | 13.8 |
| Diazepam | 38.6 | 13.2 | 13.1 |
| CGS 9896 | 40.0 | 12.6 | 13.7 |
| Prazepam | 47.2 | 13.1 | 13.4 |
| CL218,872 | 34.7 | 16.4 | |
| ZK 91296 | 56.4 | 14.1 | 13.4 |
| Antagonists | | | |
| ZK 93426 | 47.2 | 13.2 | |
| Ro15-1788 | 39.9 | 12.8 | |
| CGS 9895 | 41.7 | 12.5 | |
| β-CCN | 28.4 | 10.8 | |
| Inverse agonists | | | |
| β-CCE | 34.2 | 13.2 | |
| CGS 8216 | 39.0 | 12.6 | |
| β-CCM | 31.5 | 13.2 | |
| FG 7142 | 33.0 | 13.2 | |
| DMCM | 43.5 | 13.5 | |

ficients (Table 6); 4) heats of protonation at different proton-accepting sites in each analog (Table 7); 5) calculated dipole moments of CGS analogs in these tautomeric forms (Table 8); 6) the relative orientation of three proposed key sites of receptor interaction expressed as an angle defined by the three points (Table 9); and 7) the electron-accepting ability of the agonist, measured by the energy of the lowest unoccupied molecules (Table 10).

In a recent attempt to identify a molecular property that modulates activity, Fryer *et al.* (42) suggested that the distance between a proton-acceptor group and a $\pi$ aromatic ring, two common features of all BDZ ligands, could serve as an indication of the type of activity elicited by binding to the BDZ receptor site. Specifically, they proposed that, if the distance between the center of the aromatic ring and the proton-accepting group was shorter than 6 Å ($r < 6$ Å), the compound would be an agonist. Increasingly larger distances were proposed to lead to antagonists ($7.5 < r < 6$ Å) and, at the largest separations, to inverse agonists ($r > 7.5$ Å). When two or more $\pi$ rings

TABLE 6

**Logarithm of the calculated partition coefficient (*P*) between octanol and water**

| Drug | Log *P* | Most lipophilic ring |
|---|---|---|
| Agonists | | |
| Flunitrazepam | 2.95 | B |
| Diazepam | 3.08 | B |
| CGS 9896 | 3.04 | B |
| Prazepam | 3.47 | B |
| CL218,872 | 4.25 | A |
| ZK 91296 | 3.82 | A |
| Antagonists | | |
| ZK 93426 | 2.75 | A |
| Ro15-1788 | 1.86 | A |
| CGS 9895 | 2.35 | A |
| β-CCN | 2.60 | A |
| Inverse agonists | | |
| β-CCE | 2.43 | A |
| CGS 8216 | 2.49 | B |
| β-CCM | 2.09 | A |
| FG 7142 | 1.93 | A |
| DMCM | 2.41 | A |

TABLE 7

**Heats of protonation at different nucleophilic sites in BDZ ligands**

Numbering of atoms is as shown in Fig. 1.

| Drug | Heat of protonation | | | $H_1^+,H_2^{+a}$ |
|---|---|---|---|---|
| | Site I | Site II | Site III | |
| | kcal/mol | | | Å |
| Agonists | | | | |
| Flunitrazepam | 167 (N4) | 176 (O) | 183 (N1) | 3.488 |
| Diazepam | 154 (N4) | 161 (O) | 184 (N1) | 3.513 |
| CGS 9896[b] | 162 (N5) | 168 (N1) | | 4.070 |
| Prazepam | 154 (N4) | 160 (O) | | 3.564 |
| CL218,872 | 167 (N2) | 190 (N4) | | 3.515 |
| ZK 91296 | 135 (=O;$R_3$) | 150 (O;$R_4$) | 152 (N2) | 3.550 |
| Antagonists | | | | |
| ZK 93426 | 145 (N2) | 155 (=O;$R_3$) | | 3.550 |
| Ro15-1788 | 160 (N3) | 163 (=O1) | 167 (O2) | 3.400 |
| CGS 9895 | 141 (=O) | 154 (N1) | | 3.480 |
| β-CCN | 158 (N2) | 172 (≅N;$R_3$) | | 3.670 |
| Inverse agonists | | | | |
| β-CCE | 154 (N2) | 160 (=O;$R_3$) | | 3.591 |
| CGS 8216 | 142 (=O) | 157 (N1) | | 3.489 |
| β-CCM | 154 (N2) | 161 (=O;$R_3$) | | 3.589 |
| FG 7142 | 146 (=O;$R_3$) | 157 (N2) | | 3.532 |
| DMCM | 146 (N2) | 156 (=O;$R_3$) | | 3.580 |

[a] Distance between two most favorable proton accepting sites.
[b] Value given for enol form.

TABLE 8

**Calculated dipole moments of CGS analogs in three tautomeric forms**

See Fig. 3 for structures.

| | Dipole moment | | |
|---|---|---|---|
| | I | II | III |
| | debye | | |
| CGS 9896 | 6.5 | 2.7 | 4.4 |
| CGS 9895 | 4.5 | 3.7 | 8.9 |
| CGS 8216 | 4.8 | 3.8 | 3.9 |

are present, the choice of which one to use for this distance measurement was made on the basis of maximum overlap of the structures. Table 4 shows the values for Fryer's index for the compounds studied here, using optimized geometries. Clearly, this hypothesis cannot explain the qualitative differences in behavior elicited. This is particularly evident when

TABLE 9

**Distances between the two proton-accepting atoms ($H_1^+$,$H_2^+$) and the center of the lipophilic ring (Ir)**

$\theta$ is the angle between the lipophilic ring, $H_1^+$, and $H_2^+$.

| Drug | $D_{r,H}$ | $D_{r,H}$ | $\theta$ |
|---|---|---|---|
| | Å | | |
| Agonists | | | |
| Flunitrazepam | 6.776 | 3.665 | 142° |
| Diazepam | 6.800 | 3.669 | 143° |
| CGS 9896 | 7.418 | 3.685 | 141° |
| Prazepam | 7.004 | 3.735 | 147° |
| CL218,872 | 7.185 | 3.947 | 148° |
| ZK 91296 | 7.273 | 3.864 | 150° |
| Antagonists | | | |
| ZK 93426 | 7.289 | 5.597 | 103° |
| Ro15-1788 | 7.289 | 5.022 | 115° |
| CGS 9895 | 6.254 | 3.906 | 115° |
| β-CCN | 7.615 | 5.570 | 114° |
| Inverse agonists | | | |
| β-CCE | 6.910 | 5.570 | 95° |
| CGS 8216 | 6.255 | 4.559 | 97° |
| β-CCM | 6.902 | 5.570 | 95° |
| FG 7142 | 6.990 | 5.581 | 99° |
| DMCM | 6.881 | 5.560 | 95° |

TABLE 10

**Comparison of calculated energy of the lowest unoccupied molecular orbital for agonists (ELUMO) and their apparent effectiveness**

| Drug | $K_D/ED_{50}$ | ELUMO |
|---|---|---|
| | | *atomic units* |
| Flunitrazepam | 61 | −1.608 |
| Prazepam | 50 | −0.970 |
| CL218,872 | 22 | −1.108 |
| Diazepam | 10 | −0.846 |
| CGS 9896 | 0.2 | −0.766 |
| ZK 91296 | 0.08 | −0.668 |

compounds of the same family with different activities are compared, such as the β-carbolines or CGS compounds. The single distance parameter used for such series does not significantly change. In general, no single distance, which gives the relative position of only two points in a ligand, can be a useful property in describing the receptor interaction. In addition, the use of maximum overlap of structures to determine this distance implies a rigid receptor site with little flexibility to accommodate disparate molecular shapes. Although steric factors are important in determining ligand-receptor interactions, these interactions are also determined by electrostatic interactions, hydrophobic interactions, and dispersion forces, which could also make significant relative contributions.

The diversity of the structures that bind significantly to the BDZ receptor site suggests a rather flexible binding region, so that a rigid approach that emphasizes maximum spatial overlap is not necessarily the most appropriate. Binding kinetic experiments on rat cerebral cortical membranes that indicate a conformationally flexible receptor site have also been put forward (43). Thus, for the families of compounds considered, a systematic examination of electronic properties that could determine modes of interaction and activation of the receptor appeared to be more relevant.

One such property that was calculated was the polarizability of these compounds, given in Table 5. This quantity can be taken as a measure of the contribution of the dispersion energy when the molecule binds to its receptor. As seen in this table, there is no significant evidence of correlation of polarizabilities with the observed binding or activities. As discussed in Materials and Methods, this quantity increases with the number of atoms in the molecule. However, even the variations in the partial polarization volumes attributed to the different rings do not vary with either the affinities or the type of activity. Hence, it appears that the contribution of dispersion interactions is not a determining factor in modulating either recognition or activation of the receptor. This deduction is in agreement with our previous results for the specific case of β-carboline interactions with model receptors, using an empirical energy method (22).

A common measure of hydrophobic interactions is the partition coefficient between octanol and water. The calculated values of this quantity in Table 6 show that the agonists tend to be more lipophilic than the other compounds that bind to the BDZ receptor. This difference could affect the optimum position of binding in a membrane-bound receptor and suggests that agonists may bind to a more buried site within the membrane, compared with the inverse agonists or antagonists. The fraction of administered drug that reaches the receptor site could also be greater. However, calculated values of partition coefficients indicate that all the drugs under consideration should cross the blood-brain barrier easily, because they are much more soluble in a hydrophobic phase.[2]

In spite of their general hydrophobic nature, all ligands that bind significantly to the receptor also have at least two strong proton-accepting groups in a hydrophilic region, which could participate in specific hydrogen-bonding interactions with proton-donating receptor subsites. In order to determine which of the several different competing proton-accepting groups in each ligand would interact most favorably with receptor sites, we have calculated their proton affinities. Table 7 shows the resulting heats of protonation of each ligand at different competing proton-accepting sites. These quantities are calculated as the difference in heats of formation between the cation resulting from the protonation and the neutral ligand. In this table, the smaller the energy, the better the proton acceptor at that position.

There are several interesting aspects of these results. For example, among the β-carbolines, the only agonist, ZK 91296, has a unique proton-accepting site, somewhat better than the aromatic nitrogen. Thus, we found three, rather than two, prospective sites of protonation, the carbonyl oxygen, the aromatic nitrogen ($N_2$), and either of the two ether oxygens (Table 7; Fig. 2). The third protonation site is shown as the $R_4$ ether oxygen substituent in Fig. 2, because it is more exposed on the molecular surface. As can also be seen, the $R_5$ ether oxygen participates in stabilizing this protonation.

FG 7142, similar to ZK 91296, is also more likely to be protonated at the carbonyl oxygen than at the aromatic ($N_2$) nitrogen. Protonation of $N_2$ is less favored in this β-carboline because $N_2$ forms a competing internal hydrogen bond with the amide hydrogen, which must be broken to allow external $N_2$ protonation. This competition could account for the lower binding affinity of FG 7142 compared with other similar β-

[2] This property per se is not an indicator of the type of activity. However, partition coefficients were calculated adding contributions per atom, which allowed the identification of the most lipophilic regions for every compound. Local interactions of such sites with the receptor could be important discriminants.

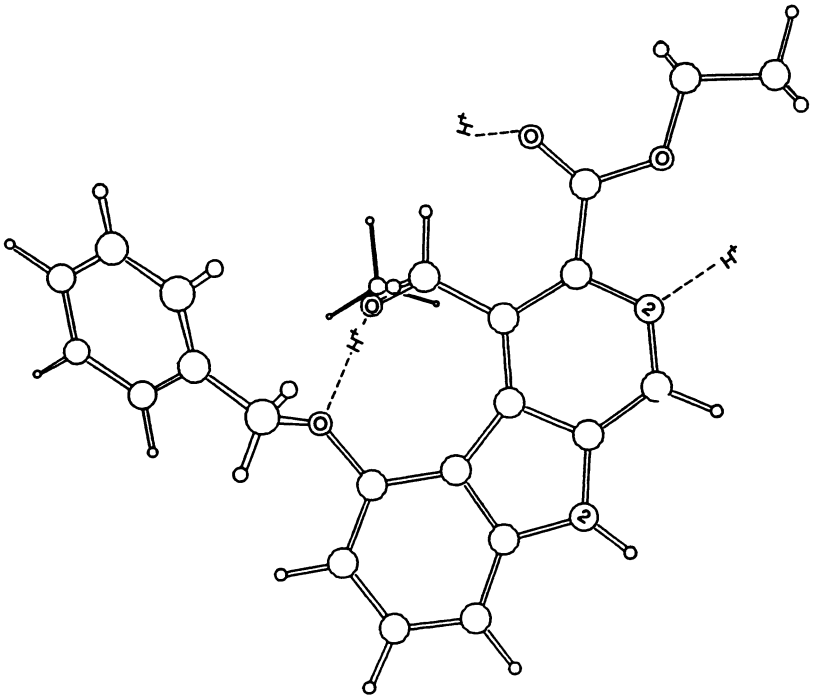


**Fig. 2.** Three favorable protonation sites in ZK 91296. Note the unique ether oxygen site, in addition to the ester oxygen and aromatic nitrogen common to all β-carboline esters.

carboline structures such as β-CCM, where such an internal hydrogen bond is not possible.

In addition to β-carbolines, another interesting family in which small chemical modifications lead to different types of activity is the series of pyrazoloquinolones CGS 9896, CGS 9895, and CGS 8216. These three analogs differ only in the substitutent in the *para*-position of the phenyl ring, being H, $OCH_3$, and Cl, respectively (Fig. 1). It is very challenging to understand why such apparently minor changes lead to qualitatively different activities, i.e., agonist, antagonist, and inverse agonist, respectively. It has previously been suggested (20, 23) that the size of the varying substituent could explain the difference in efficacies, with the larger the substitutent the stronger the agonist character displayed. However, the Van der Waals radii of the substitutents increase from CGS 8216 to CGS 9896 to CGS 9895, which is not the qualitative order of their pharmacological actions. If it were, CGS 9895 would be the most potent full agonist, contrary to our findings. Neither is this biological profile modulated by relative hydrophobicity (CGS 9895 < CGS 8216 < CGS 9896). The studies made here reveal that the origin of the qualitatively different ability of these three compounds to activate the BDZ receptor is much more complex and lies in inherent differences in calculated electronic properties.

One important property of these compounds that was explored was the possibility of tautomeric isomerism (see Fig. 3). X-ray studies (44) show Tautomer I to be more stable, as do NMR results in dimethyl sulfoxide, a polar nonprotic solvent. Our calculated results show that Tautomer III has the highest energy and would not be a candidate for the bioactive form ($\Delta E$ ~8 kcal/mol compared with Tautomer I). This destabilization is caused by the nonplanarity of the fused tricyclic ring resulting from lone pair-lone pair repulsion of the contiguous N atoms that break the extension of the $\pi$ conjugation. However, the keto (I) and enol (II) forms are closer in energy and both are candidates for binding to the receptor. Because the relative energies were calculated *in vacuo*, the dipole moment of each

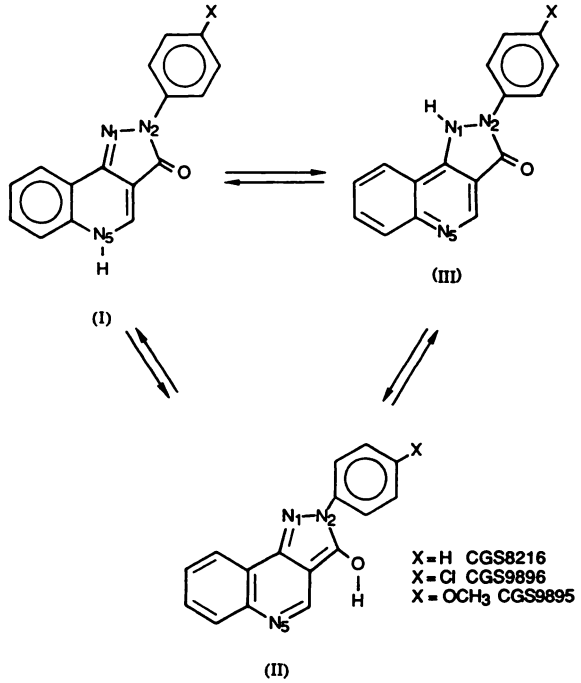



**Fig. 3.** Three possible tautomeric forms of pyrazoloquinoline (CGS) compounds, two keto forms, one protonated at $N_5$ (I) and the other at $N_1$ (III), and an enol form (II).

form provides a measure of the differential stabilization of each form in polar and nonpolar environments. These dipole moments for the keto and enol forms of the three analogs are given in Table 8. As seen in this table, the enol and keto forms of two compounds, CGS 8216 and CGS 9895, have similar dipole moments. Thus, the keto form, which is more stable *in vacuo*, should still be more stable in both polar and nonpolar environments and is the candidate bioactive form.

The striking result obtained is that for CGS 9896 there is a large difference in dipole moment values between the keto form (I) and enol form (II) (6.5 versus 2.7 debye). Thus, for this

analog only, the enol form would be preferentially stabilized in a nonpolar environment. For this analog only then, we proposed that it is the enol form that preferentially binds to the receptor, while the keto form remains in the aqueous phase, stabilized by its much larger dipole moment.

The possibility that in a nonpolar environment the enol form of only CGS 9896 is the bioactive species is further enhanced by the deduction of a possible reaction mechanism for its formation. We have observed that the compound is largely dissociated in polar aprotic environments, according to $^1H$ NMR relaxation studies. This is not the case for CGS 8216 or CGS 9895. Moreover, calculations of the molecular electrostatic potentials for the anion of CGS 9896 show that the carbonyl oxygen is the strongest proton acceptor. These results taken together provide a feasible mechanism for tautomerization, rapid transfer of the amine hydrogen to the solvent, leaving an anionic CGS 9896, then from the solvent to the carbonyl oxygen. The implication is that, whereas the keto form is thermodynamically preferred (has a lower heat of formation), the formation of the enol is kinetically favored due to a stronger coulombic attraction.

Paradoxically then, a combination of our calculated and spectroscopic results indicate surprisingly large qualitative differences in the electronic structure of these seemingly very similar compounds. The bioactive form of CGS 9896 is predicted to be an enol form (Tautomer II), whereas that of both CGS 8216 and CGS 9895, a keto form (Tautomer I). These latter two analogs are further distinguished by a change caused by the substitutent in their most lipophilic regions, the condensed ring of CGS 8216 and the phenyl ring of CGS 9895 (Fig. 1). As described below, these large differences have been incorporated in a consistent model for recognition and activation that explains the qualitatively different actions that these compounds elicit at the BDZ receptor. Histamine offers a similar case, where tautomerism in the imidozole portion of the molecule can be directly involved in a receptor activation mechanism (45).

Calculated proton affinities at competing sites for each analog are shown in Table 7, together with the distance between the two most favorable sites. The use of gas proton affinities as indicators of the hydrogen bond-accepting ability by the molecule have been used by us (17) and others (46). Neither the strength nor the nature of the proton-accepting groups appear to be direct modulators of the type of activity elicited by BDZ ligand binding to receptors. Strikingly, however, the distance between the two most favorable proton-accepting sites is approximately constant for all analogs, regardless of the type of compound or the nature of the site. This result strongly suggests that these sites are common anchoring sites involved in receptor recognition of diverse BDZ ligands. This conclusion is consistent with our previous studies, which implicated two such sites in modulating affinities in both BDZ and $\beta$-carbolines (21, 22).

Because hydrophobic aromatic rings are a third common feature of all the families studied, it seemed possible that they could play a role in receptor activation. To test the validity of this model, we have examined the relative spatial arrangement of these three postulated key ligand interaction sites for differences in them among agonists, antagonists, and inverse agonists. If the two candidate recognition sites are superimposed in all compounds, the value of the angle $\theta$ determines the relative position of the most lipophilic aromatic ring. The angle $\theta$ is made by the line between the center of the most lipophilic ring and the closer proton acceptor ($H^+_2$) as the apex, and from it to the second proton acceptor ($H^+_1$). As seen in Table 9, this angle is an excellent indicator of the three qualitatively different responses evoked by these compounds. The different values of $\theta$ obtained for agonists, antagonists, and inverse agonists indicate that, when anchored by common recognition sites, their most lipophilic aromatic rings would occupy different regions in the receptor. Consistently, the similar values of $\theta$ obtained within each group for diverse chemical families indicate that this moiety would occupy similar positions for drugs with the same action. These results strongly support the proposed role of the most lipophilic aromatic ring in receptor activation and provide the basis for a coherent and self-consistent model for the different modes of binding of agonists, antagonists, and inverse agonists, which can account for both similarities in activation among diverse BDZ ligands and differences in activation among closely related analogs. Thus, our proposed model of ligand-receptor interaction involves receptor recognition of the two most favorable proton-accepting centers of BDZ ligands by complementary proton-donating amino acid residues in the BDZ receptor binding site and receptor activation modulated by the interaction of the most lipophilic aromatic ring with key receptor sites.

Fig. 4 illustrates the proposed relative mode of binding of the agonist diazepam, the antagonist Ro15-1788, and the inverse agonist DMCM to the BDZ binding site at the $GABA_A$ receptor. In this pharmacophore, the two postulated recognition sites are maximally superimposed for all analogs, keeping in mind that it is the lone pair of electrons and not the atoms themselves that participates in proton-accepting interaction with the receptor. All molecules considered have an aromatic nitrogen with a common direction for these electrons, and this site could be overlapped exactly. The other recognition site has more flexibility in the different analogs, as indicated by the oblong box in Fig. 4. For example, this site is frequently a carbonyl oxygen with two possible interaction directions, corresponding to its two pairs of nonbonding electrons. In addition, there is also some conformation flexibility at this site with respect to the direction of intermolecular H-bonding.

Fig. 4 shows the consequence of different values of the angle $\theta$ and its significance as a reliable indicator of activation. Different values of $\theta$ mean that the lipophilic rings, postulated to be involved in activation of the receptor, bind to different receptor regions for agonists, antagonists, and inverse agonists. Values of $\theta$ are similar for antagonists and inverse agonists, leading to overlapping regions of their most lipophilic rings, consistent with the finding that small steric differences lead to one or the other type of behavior in many compounds.

In addition to identifying important ligand regions, Fig. 4 also indicates, by complementarity, important receptor sites for interaction with them as well as some steric features of the receptor binding site. For example, as indicated by shaded regions, there is room for proton-donating amino acid side chains of the receptor to interact with the two proton-accepting recognition sites. As also indicated by a shaded region, there appears to be a receptor region that cannot accommodate any portion of the ligands. This region, clearly seen in Fig. 4, corresponds to values of $\theta$ $145° > \theta > 115°$ and is reflected in the fact that values of the angle $\theta$ are not continuous from

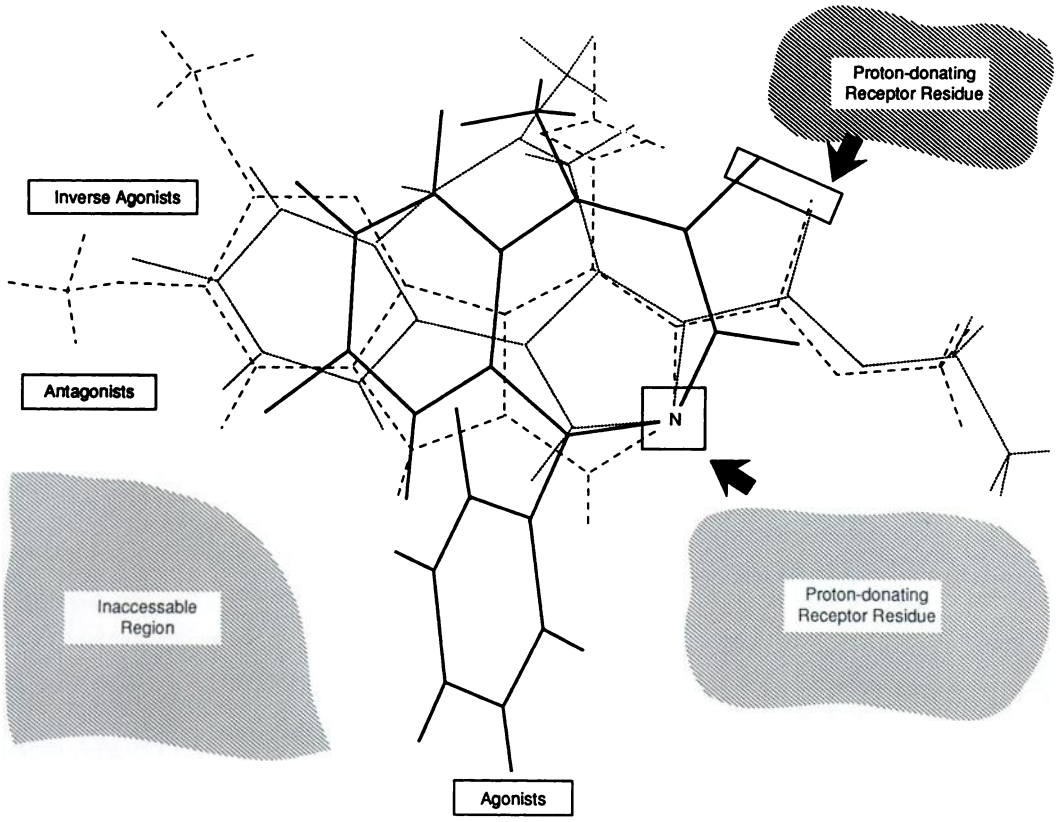



**Fig. 4.** Proposed mode of binding of an agonist (diazepam), antagonist (Ro15-1788), and inverse agonist (DMCM) to the BDZ receptor binding site. The *arrows* indicate the two proposed ligand recognition sites. Also indicated is the lipophilic region of each type of ligand proposed to be involved in receptor activation. The *shaded areas* represent, by complementarity, proton-donating amino acid residues that would be involved in recognition, as well as a sterically inaccessible region of the receptor. Not indicated is the receptor region involved in activation, which would be stacked over the corresponding aromatic regions of the ligands. Most of the figure is planar. Ring A of diazepam as well as the seven-membered ring of Ro15-1788 is out of the plane toward the viewer.

agonist to inverse agonist. One allowed region of binding, $\theta \sim 145°$, leads to agonist activity, whereas the other allowed region, values of $\theta \sim 95–115°$, leads to antagonism or inverse agonism.

There are no guidelines as yet, however, for extending the proposed model for ligand binding to the BDZ receptor to include other amino acids in the vicinity of the postulated key anchoring sites, because the position of hydrophobic groups near the proton-donating amino acid residues cannot be inferred. Such residues appear to play a subtle role in modulating activity of high affinity analogs, as manifest in the family of $\beta$-carboline esters, $\beta$-CCM, $\beta$-CCE, and the propyl ester $\beta$-CCP. They are all potent antagonists of BDZ anticonvulsant action but with varying amounts of inverse agonism. In all of these compounds, the methyl, ethyl, and propyl groups are in close proximity to the carbonyl oxygen, one of the postulated receptor anchoring points. The different steric requirements needed to accommodate these groups could then slightly alter the relative orientation of this recognition site with respect to the lipophilic region, resulting in shifts of values of $\theta$ found for antagonists to very similar ones for inverse agonists. Thus, it is not surprising that this small modulation of relative orientation could introduce some weak proconvulsant activity in these antagonists. By contrast, much greater differences in orientation are postulated to exist between agonists and antagonists or inverse agonists.

The proposed mode of binding of these 15 ligands explains, for the first time, in a consistent way the particularly puzzling behavior of closely related analogs. For example, we can now understand the origin of the enigmatic agonist behavior of ZK 91296, different from all other $\beta$-carbolines studied, which are either antagonists or inverse agonists. As shown in Table 9, the two most favorable proton-accepting anchoring positions for ZK 91296 are the carbonyl oxygen and the unique ether oxygens, making the relative orientation of the most lipophilic aromatic ring identical to that of other agonists. This result is in contrast to all the other $\beta$-carboline analogs, where an imine N is the second center, making their value of $\theta$ similar to those of antagonists and inverse agonists.

The results also provide a striking explanation for the differences in behavior of the three closely related CGS compounds. Assuming that the active tautomer of CGS 9896 is the least polar enol form, the relative orientation of its most lipophilic aromatic region is similar to that of agonists of other chemical classes. Because the substituent on the rings changes the location of the most lipophilic rings, CGS 9895 is further distinguished from CGS 8216, the former with a value of $\theta$ similar to that of other pure antagonists and the latter with a smaller value common to compounds with inverse agonist activity. Thus, all three CGS analogs have unique features that allow them to bind to the receptor in a different way, accounting for their different activities.

Fig. 4 also illustrates why photoaffinity labeling by BDZs with $NO_2$ at the 7 position of the condensed aromatic ring (47) significantly interferes only with the binding of other BDZs and by a much smaller amount with all other classes of ligands (48, 49), including other agonists such as CL218,872, CGS 9896, or ZK 91296. The position occupied by the BDZ condensed ring, where the attachment occurs, is unique and does not overlap significantly with the binding of any other chemical family considered in this study, because it is out of the plane shown in the figure. Overlap with other compounds occurs largely in the plane associated with the reversible binding. The model would account for the experimental observation if the 1,4-benzodiazepine covalently bound at the $R_7$ position could

be accommodated by the receptor without interfering with the binding region, which depends on the geometry of the receptor outside the recognition area and the conformational flexibility of the residue bound to the BDZ.

If, as we have postulated, the relative position of the lipophilic aromatic group is important in determining receptor activation, the question remains as to what type of activation process could be involved. Two complementary effects of the lipophilic zone, one conformational and the other electronic, could contribute to the mechanism of activation. Specifically, different receptor conformations could be required to accommodate the lipophilic portion in different regions of the receptor, while still allowing hydrogen bond recognition at similar sites. Thus, binding of the lipophilic ring in the region $\theta \sim 145°$ could represent one accessible receptor conformation and binding in the region $\theta = 95 \simeq 115°$, another accessible receptor conformation, with a high energy barrier between them. The different induced receptor conformations could lead to different types of activities. Consistent with this hypothesis is a previous suggestion that conformational changes induced by antagonists in the receptor are different from those induced by agonist (43). Further evidence that a different receptor conformation may be required for agonist and/or antagonist binding is provided by preliminary studies made in our laboratory, which indicate that the receptor cannot accommodate analogs that have lipophilic groups in both agonist and antagonist/inverse agonist regions. An example of this effect is that β-carbolines with aromatic substitutions in the 1-position (Fig. 1) do not bind to the receptor.

In addition to causing a unique conformational change, a second possible role of the lipophilic group in receptor activation by agonists is that it could alter the electronic structure of the binding site by formation of a $\pi-\pi$ charge transfer complex with an aromatic amino acid in the receptor. The absence of an appropriate partner in different regions of the receptor, as indicated by different values of $\theta$, could prevent this charge transfer in the case of antagonists and inverse agonists.

To test this activation mechanism hypothesis, we have calculated the electron-accepting and -donating capabilities of the six agonists and compared them with their rank order of apparent effectiveness ($K_D/ED_{50}$). As shown in Table 10, relative electron-accepting ability is an extremely promising indicator of efficacy when the relative energy of the lowest unoccupied molecular orbital of the ligand is used as a measure of electron affinity. It should be kept in mind that differences in pharmacokinetic profiles of these compounds may obscure true efficacy differences and weaken this relationship. Nevertheless, a plausible initial activation step could involve charge transfer from the receptor to the ligand via a stacked $\pi$ complex. This interaction could also be accompanied by a change in conformation of the receptor that propagated to the GABA binding site, affecting its activity. Further evidence for this proposed mechanism is provided by the recent finding that a histidine residue, a good candidate for charge transfer, is near the BDZ binding site in the receptor. Selective modification of histidine residues was found to block both BDZ and β-carboline binding (50).

Although our model provides a self-consistent explanation of observed behavior, there are other possible sites of ligand-receptor interactions that we have not yet considered. Among these are proton-donating interactions with the receptor. Such interactions are implied by the fact that most BDZ ligands have an acidic hydrogen. The hydrogen is either bound to a heteroatom (as in the CGS compounds and β-carbolines) or an electron-deficient carbon (as in the imidazole ring of Ro15-1788). It is not clear what role these potential H-donor sites play in either receptor recognition or activation. The heats of deprotonation of the compounds follow no particular pattern, compared with their affinities or type of activities. In the particular case of the β-carbolines, reports about its importance in binding are contradictory. On the one hand, the replacement of an acidic hydrogen by a methyl group decreases the binding (22), whereas acetylation seems to induce no significant change (51). Thus, this is a potentially important site of interaction that remains to be explored. In addition, all the compounds that bind to the BDZ receptor site can be in ionic equilibria and, therefore, are likely to be dissociated in the vehicle when injected. The influence of this factor will be further considered in future studies.

The model for recognition that is proposed has some resemblance to the one proposed by Borea *et al.* (23), in the sense that both arrive at the conclusion of common recognition sites for various classes of ligands and of nonoverlapping regions for the lipophilic moieties of agonists and antagonists. However, we have proposed two, rather than one, important recognition sites and have identified them by systematic calculations of electronic properties, particularly the relative abilities of different atoms in the molecule to be involved in direct electrostatic interaction with the receptor. The postulated existence of two common identified anchoring points for all systems greatly reduces the degrees of freedom of receptor complexes with diverse chemical families, leading to more overlap than previously postulated (23). In addition, our results lead for the first time to a model for activation, with an important modulating role for the lipophilic site.

Our results also illustrate two important points, 1) maximum structural overlap does not always provide the best criterion for deducing a model of drug-receptor interaction for diverse classes of ligands; and 2) the ligand may interact with the receptor in an entirely different conformation or form from the one identified in the solid state. For these reasons, models based entirely on the X-ray structures of drugs and maximum structural overlap may be misleading. They omit conformational and isomeric changes, such as the one found to tentatively rationalize the behavior of the CGS compounds, as well as the possible importance of electronic properties in directing and orienting ligands in their binding sites. Structural information, including conformational anlaysis, is best used together with other relevant physical, chemical, and electronic properties that are not deducible from the crystal structure to determine important modulations of both affinity and activation.

If the proposed mechanism of recognition and activation is correct, it should be possible to design analogs with varying efficacy in two ways. The first is electronic modulation by alteration of the electron-accepting ability of the most lipophilic ring. The second is steric control by alteration of the relative position of the recognition sites and the most lipophilic ring. This reorientation can be achieved by changing the position of the recognition sites or by altering competing aromatic rings to change the position of the most lipophilic one. Design of such analogs, together with further studies of a larger set of known analogs and the development of *in vitro* measures of efficacy,

are planned in continuing efforts to test the hypotheses developed here.

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