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Stereochemical Features Controlling Binding and Intrinsic Activity Properties of Benzodiazepine-Receptor Ligands

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SUMMARY

Benzodiazepine-receptor ligands belong to several different chemical classes. All of them bind to the receptor but display a variety of biological effects ranging from agonist to inverse agonist to antagonist. The properties of the most representative compounds for each class are briefly reviewed as concerns their receptor binding affinities, γ-aminobutyric acid ratios, photoaffinity labeling ratios, and pharmacological properties. Their geometries, as obtained by X-ray crystallography, are discussed and missing crystal and molecular structures of two of them (zopiclone and CL 218-872) are reported. Binding and intrinsic activity properties of series of benzodiazepines and β-carbolines are extensively analyzed and correlated with their molecular structures. A general stereochemical model accounting for both binding abilities and kinds of biochemical and pharmacological activities for all benzodiazepine-receptor ligands is proposed. This is based on the assumption of a rather diffuse and substantially planar recognition site where the main drug-receptor interactions are mediated by the drug carbonylic or iminic groups via hydrogen bonding and the observed differences in pharmacological profiles are accounted for by the different localization of the different ligands inside this unique binding site.

BDZs are among the most widely used drugs as a consequence of their broad spectrum of anxiolytic, sedative-hypnotic, anticonvulsant, and muscle relaxant properties. A few years ago, high affinity, saturable, stereospecific binding sites for ^{3}H-DIA in the central nervous system of mammals were discovered (1, 2) which are supposed to be the substrate by which BDZs exert their pharmacological effects. Arguments have been raised in favor of the idea that BDZ receptors are constituents of a GABA-BDZ-chloride ionophore receptor complex (3).

More recently, several new drugs, chemically unrelated to BDZs, were found which can interact with high affinity with BDZ receptors but which display pharmacological properties often different from those of BDZs (4). They show a spectrum of biological activity ranging continuously from compounds having full BDZ-like properties (AGs) to those having completely opposite actions (the so-called IAGs) to a third class of compounds (ANTs) able to bind to BDZ receptors without producing any definite pharmacological effect.

It has been hypothesized that the BDZ receptor might be, from a functional point of view, similar to an enzymatic allosteric site. The role of the enzyme would be played by the GABA-receptor-chloride channel complex and the BDZs would correspond to positive heterotropic effectors enhancing GABA-induced neurotransmission indirectly (4). In this model the

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ABBREVIATIONS: BDZ, benzodiazepine; GABA, γ-aminobutyric acid; AG, agonist; IAG, inverse agonist; ANT, antagonist; PAL, photoaffinity labeling. *Benzodiazepines:* FLU, flunitrazepam (5-(2-fluorophenyl)-1,3-dihydro-1-methyl-7-nitro-2*H*-1,4-benzodiazepin-2-one); DIA, diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl-2*H*-1,4-benzodiazepin-2-one); OXA, oxazepam (7-chloro-1,3-dihydro-3-hydroxy-5-phenyl-2*H*-1,4-benzodiazepin-2-one); R48, Ro 5-4864 (4′-chlorodiazepam); MED, medazepam (7-chloro-2,3-dihydro-1-methyl-5-phenyl-1*H*-1,4-benzodiazepine); CHL, chlordiazepoxide (7-chloro-2-methylamino-5-methyl-3*H*-1,4-benzodiazepine 4-oxide). *Triazolobenzodiazepines*: TRI, triazolam (6-(2-chlorophenyl)-1-methyl-8-chloro-4*H*-*s*-triazolo[4,3-*a*]1,4-benzodiazepine. *Cyclopirrolones*: ZOP, zopiclone ([6-(5-chloro-2-pyridil)-6,7-dihydro-7-oxo-5*H*-pyrrolo[3,4-b]pyrazin-5-yl]4-methyl-1-piperazine carboxylate); SUR, suriclone ([6-(7-chloro-1,8-naphthyridin-2-yl)-7-oxo-2,3,6,7-tetrahydro-5*H*-1,4-dithiino[2,3-*c*]pyrrol-5-yl] 4-methyl-1-piperazine carboxylate). *Triazolopyridazines*: CL72, CL 218-872 (3-methyl-6-[3-trifluoromethylphenyl]-1,2,4-triazolo[4,3-*b*]pyridazine). *Phenylquinolines*: P84, PK 9084 (phenyl-2[piperidinyl-3)-2-ethyl]-4 quinoline). *Pyrazoloquinolines*: CG96, CGS 9896 (2-(4-chlorophenyl)-2,5-dihydro-pyrazolo[4,3-*c*]quinolin-3(3*H*)-one); CG16, CGS 8216 (2-phenyl-2,5-dihydro-pyrazolo[4,3-*c*]quinolin-3(3*H*)-one). *Imidazobenzodiazepines*: RO88, Ro 15-1788 (8-fluoro-3-carboethoxy-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-*a*]1,4-benzodiazepine). *β-Carbolines*: DMCM (3-carbomethoxy-4-ethyl-6,7-dimethoxy-9*H*-pyrido[3,4-*b*]indole) = (3-carbomethoxy-4-ethyl-6,7-dimethoxy-β-carboline); βCCM (3-carbomethoxy-β-carboline); βCCE (3-carboethoxy-β-carboline); PrCC (3-carbo-*n*-propyloxy-β-carboline); ZK93, ZK 93426 (3-carboethoxy-5-*i*-propyloxy-4-methyl-β-carboline); ZK91, ZK 91296 (3-carboethoxy-5-benzyloxy-4-methoxymethyl-β-carboline); NHA, norharman (β-carboline); MCCE (1-methyl-β-CCE); THE (1,2,3,4-tetrahydro-β-CCE); THN (1,2,3,4-tetrahydro-norharman); THCA (3-carboxylic acid-1,2,3,4-tetrahydroharmane).

BDZ receptor is assumed to occur in two conformations in equilibrium which could be called the active and inactive forms in the sense that the active form increases the probability that the GABA receptor/chloride channel is activated by its neurotransmitter (GABA), whereas the inactive form reduces such a probability. Thus, AGs, acting as positive effectors, would stabilize the active form and IAGs the inactive one, whereas the ANTs would not affect the equilibrium.

From a stereochemical point of view such an allosteric model seems to suggest that AGs, ANTs, and IAGs must have a common chemical moiety responsible for binding at the same receptor site (different binding abilities), whereas AGs and IAGs should possess additional and different stereochemical features able to trigger the opposite biological effects (different efficacies). The aim of this paper is to verify that such a stereochemical model can be fitted by the experimental data on the geometries of the most representative compounds involved. It will be shown that this seems to be the case and that continuously changing geometrical features can account for changes of both binding affinities and efficacies. As the molecular geometries of two important agonists (zopiclone and CL218-872) had never been determined, we report also the X-ray crystallographic determinations of their structures.

Experimental Procedures and Results

Selection and Properties of Compounds

Compounds have been selected according to the following criteria.

1) There is a very large number of BDZs displaying AG properties. The selection includes representative BDZs belonging to four different chemical subclasses: 5-phenyl-1,4-benzodiazepine-2-ones (FLU, DIA, and OXA with high binding affinities and R48 which is a very weak binder), 5-phenyl-1,4-benzodiazepines (MED), 5-phenyl-1,4-benzodiazepine-4-oxide derivatives (CHL), and 6-phenyl-triazolo(4,3-*a*)1,4-benzodiazepines (TRI).

2) The β-carbolines chosen belong to two different groups. Those belonging to the first (DMCM, βCCM, βCCE, PrCC, ZK93, and ZK91) are all high affinity ligands which display a continuous spectrum of biological activity ranging from full IAGs to partial AGs to ANTs; the second group includes β-carbolines which, for different reasons to be discussed later, are weak or very weak binders to BDZ receptors (NHA, THN, THE, MCCE and THCA).

3) The remaining compounds are representative of other known classes of BDZ-receptor ligands chemically unrelated to BDZs: ZOP and SUR (cyclopyrrolones), CL72 (triazolopyridazine), P84 (phenylquinoline), CG96 and CG16 (pyrazoloquinolines), and RO88 [6-oxo-imidazo(1,5-*a*)1,4-benzodiazepine]. All of these compounds display the full spectrum of pharmacological properties.

Table 1 is a synopsis of pharmacological and biochemical data for the compounds relevant to the following discussion. IC_{50} values are not homogeneous as they are derived from different sources (5–8) and refer either to inhibition of ^{3}H-DIA or ^{3}H-FLU binding from rat whole brain or cortex membranes; however, they can be considered quite suitable for qualitative comparison purposes. GABA ratio and PAL ratio values (7–10) have been chosen as "*in vitro*" indicators of the IAG-ANT-AG properties according to the suggestions of different authors (9, 10). As for the pharmacological profile, the classification is taken from Refs. 4, 7, 8, and 11 and references therein and is the result of the following tests: pentylenetetrazole-induced convulsions, audiogenic seizures in DBA/2 mice, increase of conflict behavior, and suppression of conflict behavior. For the sake of simplicity, very weak AGs and IAGs are classified as ANTs.

Fig. 1 reports chemical formulas of compounds listed in Table 1. Their molecular geometries can be derived from X-ray crystallographic structure determinations. As regards BDZs, structures for FLU (12), DIA (13), OXA (14), MED (15), CHL (16), and the ANT RO88 (17) have been reported; structures of other BDZs can be easily extrapolated from those.

The skeleton of β-carbolines can be derived from the structures (18, 19) of βCCM and *N*-ethyl-3-carbamoyl-β-carboline, and that of 1,2,3,4-tetrahydro-β-carbolines can be derived from the crystal structure of THCA. Structures of CG96 and CG16 (pyrazoloquinolines) have been recently determined (20). No structures have been reported for the cyclopyrrolones, triazolopyridazines, and phenylquinolines until now, and in the present paper we present the crystal and molecular structures of ZOP and CL72 as compounds representative of the first two classes. As for phenylquinolines, their geometries must be derived from tabulated data as any crystallization attempt has been unsuccessful.

Crystal Structure Determinations

CL72. Crystals were kindly provided by Dr. J. W. Epstein, American Cyanamid, Lederle Laboratories (Pearl River, NY) and recrystallized from ethanol. A crystal of dimensions 0.20 × 0.20 × 0.40 mm was submitted to analysis by means of an Enraf-Nonius CAD4 diffractometer with monocromated MoKα radiation.

Crystal data. $C_{13}H_9N_4F_3$, formula weight = 278.24; orthorombic, space group *Pccn*; a = 22.624(2), b = 13.073(2), c = 8.385(1) Å; U = 2440.6(6) Å^3, D_c = 1.51 g/cm^3 for Z = 8; μ(MoKα) = 0.91 cm^{-1}. Of 2661 reflections collected ($2° < \theta < 27°$), 1160 having $I > 3\sigma(I)$ were used in the refinement. The crystal was stable during data collection. The structure was solved by direct methods (21) and refined by weighted full matrix least squares with anisotropic non-H and isotropic H atoms to R = 0.038, Rw = 0.045, maximum shift/error = 0.06 and S = 1.6.[1] A sketch of the molecule is reported in Fig. 2.

ZOP. Crystals, provided by Drs. J. Mollé and J. C. Blanchard, Rhone-Poulenc Recherches (Vitry-sur-Seine, France), were recrystallized from various solvent systems. Only a very small crystal of dimensions 0.15 × 0.15 × 0.20 mm was obtained from ethanol and submitted to X-ray analysis under the same conditions as for CL72.

Crystal data. $C_{17}H_{17}N_6O_3Cl$, formula weight = 388.82; orthorombic, space group $P2_12_12_1$, a = 5.567(3), b = 8.852(2), c = 35.677(17) Å, U = 1758(1) Å^3, D_c = 1.47 g/cm^3 for Z = 4; μ(MoKα) = 2.5 cm^{-1}. Of 1846 reflections collected ($2° < \theta < 25°$), 915 with $I > 1.5\sigma(I)$ were used in the refinement. The crystal was stable during data collection. The structure was solved by direct methods (21) and refined by weighted full matrix least squares with anisotropic non-H and calculated H atoms. Final parameters: R = 0.060, Rw = 0.068, maximum

[1] Final coordinates and bond distances and angles are available on request to the authors (G. Gilli).

TABLE 1

Synopsis of pharmacological and biochemical data for a selection of BDZ-receptor ligands

	Chemical class[a]	Code	IC_{50} of ^{3}H-BDZ binding inhibition[b]	GABA ratio[c]	PAL ratio[c]	Pharmacological profile	Crystal structure (reference)
			nM				
Higher affinity ligands							
Flunitrazepam	BDZ	FLU	5	2.45	104	Agonist	12
Diazepam	BDZ	DIA	16	2.30	166	Agonist	13
Oxazepam	BDZ	OXA	38	2.35	97	Agonist	14
Triazolam	tBDZ	TRI	1.9			Agonist	
Zopiclone	CYP	ZOP	36	1.53	5.5	Agonist	present work
Suriclone	CYP	SUR	2.2			Agonist	
CL 218-872	TPA	CL72	140	1.98	6.1	Agonist	present work
PK 9084	PHQ	P84	459	1.7	4.2	Partial agonist	
CGS 9896	PQ	CG96	0.6	1.20	1.2	Partial agonist	20
ZK 91926	βC	ZK91	1.1	1.23	1.7	Partial agonist	
RO 15-1788	iBDZ	RO88	3.3	1.22	1.1	Antagonist	17
ZK 93426	βC	ZK93	0.4	1.39	1.5	Antagonist	
PrCC	βC	PrCC	12	1.11	1.4	Antagonist	
CGS 8216	PQ	CG16	0.3	0.90	1.2	Antagonist	20
βCCE	βC	βCCE	7	0.86	0.9	Partial inverse agonist	
βCCM	βC	βCCM	8	0.61	1.1	Inverse agonist	18
DMCM	βC	DMCM	10.9	0.46		Inverse agonist	
Lower affinity ligands							
Chlordiazepoxide	BDZ	CHL	1,120	2.23		Agonist	16
Medazepam	BDZ	MED	6,800			Agonist	15
RO 5-4864	BDZ	R48	160,000				
Norharmane	βC	NHA	12,000				
Tetrahydronorharmane	βC	THN	920,000				
THE	βC	THE	4,900				
MCCE	βC	MCCE	4,900				
Tetrahydronorharmane-3-carboxilic acid	βC	THCA	400,000				19

[a] tBDZ, iBDZ, and BDZ, triazolo-, imidazo-, and benzodiazepines; CYP, cyclopyrrolones; TPA, triazolopyridazines; PHQ, phenylquinolines; PQ, pyrazoloquinolines; βC = β-carbolines.

[b] IC_{50} for inhibition of specific BDZ binding from rat synaptosomal membranes; taken from Refs. 1–7.

[c] GABA ratio = IC_{50} without GABA/IC_{50} with GABA; PAL ratio = IC_{50} in photoaffinity-labeled membranes/IC_{50} in non-photoaffinity-labeled membranes. Both GABA and PAL ratio values are from Refs. 4, 5, and 7–10.

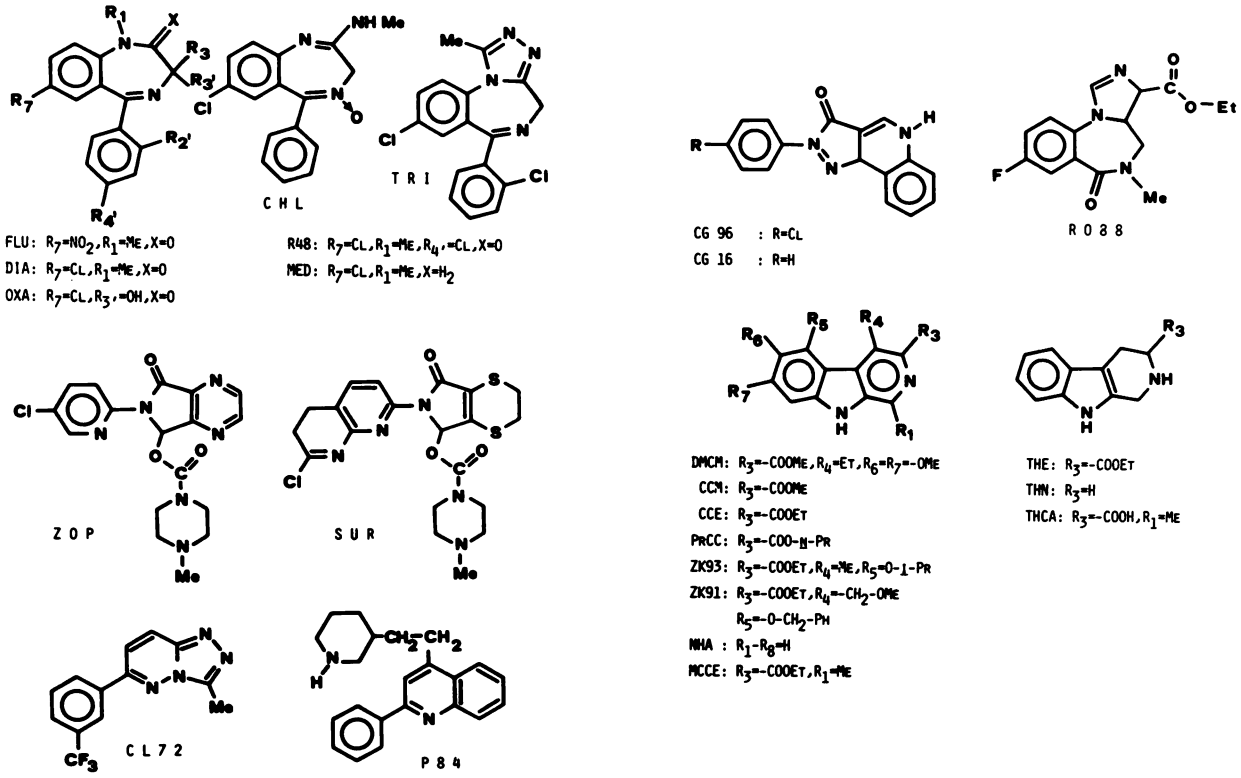

Fig. 1. Chemical formulas of all BDZ-receptor ligands taken into account here. *CCM* and *CCE* represent β-CCM and β-CCE, respectively.

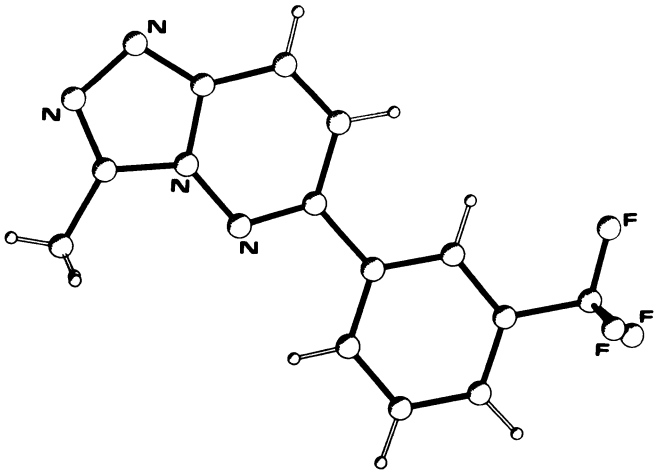

Fig. 2. A view of the molecule of CL72 as determined by X-ray crystallography.

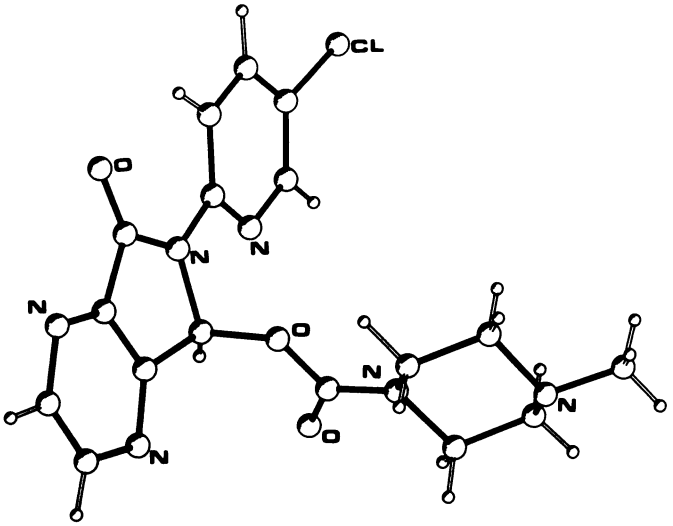

Fig. 3. A view of the molecule of ZOP as determined by X-ray crystallography.

shift/error = 0.06, S = 1.6.[2] A sketch of the molecule is reported in Fig. 3.

Criteria for Development of a Model of BDZ-Receptor Interaction

The development of a general model accounting for both binding abilities (affinities) and type of biological or pharmacological activity (efficacy), taking into account all BDZ-receptor ligands, encounters two main objective difficulties. 1) Common structure-activity studies deal with receptorial drug sets having the same effect. In such a case it has to be assumed that efficacy is a necessary consequence and then functionally related to the binding ability. This is well represented by classical BDZs where structure-activity relationships are, in general, strictly paralleled (1, 2) by the structure-affinity ones. There are, however, many other ligands binding with high affinity to the BDZ receptor for which the situation is much more complicated, as is very well exemplified by β-carbolines, which are good binders displaying a wide spectrum of efficacies, ranging continuously from pure IAGs (DMCM) to partial AGs (ZK91) to ANTs (ZK93). Thus, an effective stereochemical model encompassing all the ligands must deal with affinities and efficacies as not necessarily related variables. 2) Another difficulty arises from the possible heterogeneity of BDZ-receptors. Different authors (e.g., Ref. 22) have raised evidence in favor of the existence of two receptor subclasses, called BZ_1 and BZ_2, having slightly different recognition properties and differently distributed in different brain regions [cerebellum almost exclusively BZ_1, cerebral cortex about 75% BZ_1 and 25% BZ_2, hippocampus 50–60% BZ_1 and 50–40% BZ_2 (5)]. It has been reported (5) that, whereas BDZs are unable to distinguish between the two subclasses, other compounds such as triazolopyridazines (e.g., CL72) and βCCE express a 5- to 10-fold higher affinity for BZ_1 receptors. Other authors (23) argue, on the grounds of kinetic arguments, that the apparent receptor multiplicity may be due to the presence of two different conformations on only one class of receptors. However, even assuming the existence of two BZ_1 and BZ_2 subpopulations (or of two differently behaving conformations), the differences between them cannot be dramatic as both recognize, even if to a different extent, all BDZ-receptor ligands. A similar position has been assumed, for example, by Skolnick and Paul (24) on the grounds that equilibrium binding studies with ^{3}H-β-carbolines do not result in Scatchard plots clearly resolvable into multiple components. It seems, therefore, reasonable to hypothesize that these small differences do not prevent, *a priori*, the search of a unique stereochemical model able to account, at least as a first approximation, for both recognition and intrinsic activity properties of the different BDZ-receptor ligands.

The present discussion is articulated in three main topics. The first two deal with structure-activity relationships in the only two classes of BDZ-receptor ligands (BDZs and β-carbolines) for which extensive series of derivatives have been synthesized and biologically tested. The third is devoted to developing a common model of drug-receptor interaction accounting for the properties of the largest number of BDZ-receptor ligands.

Criteria for Molecular Comparison

The criteria used for superimposing the different molecules (Figs. 4–6) were as follows. All drawings were done on the same scale by the use of graphic programs PLUTO (25) or ORTEP (26) on the plane of maximum atomic resolution; both spokes and ball and stick representations were used and sometimes superimposed in the same figure. Some graphical signs, such as double bonds or aromaticity circles were subsequently added by hand to increase the intelligibility of the figures. For the same reason Van der Waals envelopes were retraced in the final figures using a different sign. All molecules were drawn from crystallographic coordinates. The βCCM molecule in Fig. 4 is almost perfectly planar (18), the methoxycarbonyl group included, and this has been interpreted on the grounds of chemical bonding considerations (18). Both N2 and O3-*trans* and -*cis* conformations have been experimentally observed (18, 19, 27) and can be supposed to exist in equilibrium in solution; the *cis* was chosen as the only one capable of giving a reasonable model. Dashed groups in zones A and B of Fig. 4 were added on the grounds of simple atom-atom potential energy calculations by the use of the program EENY (28) and potentials were taken from Giglio (29). 6,7-Dimethoxy groups were placed

[2] Final coordinates and bond distances and angles are available on request to the authors (G. Gilli).

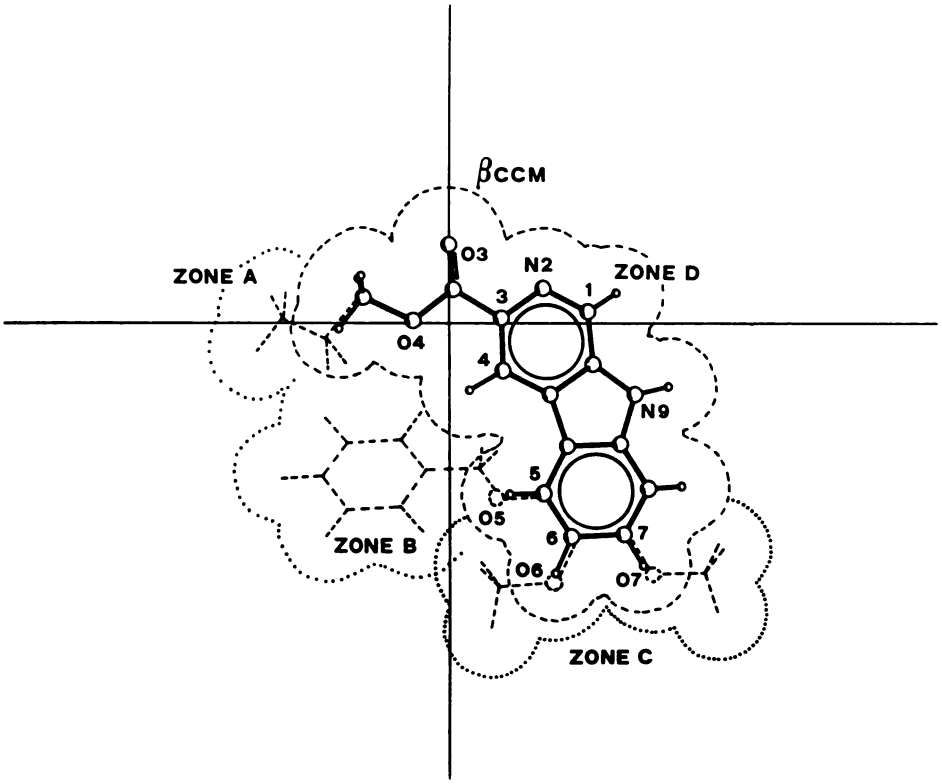

Fig. 4. A sketch of the molecular structure of βCCM and its van der Waals envelope (– – –). Substitution of different groups modifies the molecular volume in the way shown (· · ·).

based on the crystal structure of DMCM (27). All molecules of Fig. 5 were experimentally found to be essentially planar with the exception of the *N*-carboxypiperazine group in ZOP and the C5 phenyl group and bow C3 atom in OXA. Crystallographic data (Ref. 20 and present structures) show that the pyrazoloquinoline (in CG96), pyrrolopyrazine (in ZOP), and triazolopyridazine (in CL72) fragments are planar and that the dihedral angles between this group and the phenyl ring are 10.2, 16.6, and 7.4° in CG96, ZOP, and CL72, respectively. Only in CG16 was this angle found to be higher (41.2°), but results for CG96 clearly indicate that the completely planar form is allowed. All known BDZs (30) are found to adopt similar conformations as far as the seven-membered ring is concerned. Atoms N1 and C5 (see numbering scheme of Fig. 5c) are coplanar with the phenylene ring; this plane makes an angle of nearly 30° with the plane containing N1, C3, N4, and C5 atoms, causing a displacement of the O2 atom from the first plane of about 0.5 Å. Conversely the C2, C3, N4 plane is nearly perpendicular to the first plane. In conclusion, all atoms of the benzodiazepinone fragment lie on the same plane with maximum displacements of 0.2–0.3 Å, with the exception of C3 pointing up and the C5-phenyl group pointing down or vice versa. Both up and down conformations are isoenergetic, being conformational enantiomers. Although crystallographically observed conformations do not necessarily correspond to those of absolute energy minimum, they can be considered to be good indicators of possible relatively low energy conformations. Accordingly, all molecules of Fig. 5 were considered to be superimposable in their completely planar conformations with the exception of the moieties filling the AG2 zone which are certainly rotated. Here the superimposition of the 5-phenyl ring in BDZs and of the *N*-carboxypiperazine group of ZOP is not good (see transverse view in Fig. 8); however, conformational energy calculations by EENY on this ZOP fragment by rotation around the >CH—O— and —O—CO— bonds [the torsion angle around the —CO—N (piperazine) bond was taken as constant in view of the amide resonance] show a rather flat energy map with several interconnected minima (including the crystallographically observed one) which practically allow any positioning of the group.

Structure-Activity Relationships in BDZs

The first crystal structure of a BDZ was that of DIA published by Camerman and Camerman (13) in 1972. They hypothesized that the pharmacologically active part of the molecule, at least with regard to anticonvulsant properties, was related to the presence of two electron donating groups, O_2 and N_4, and two hydrophobic moieties, that is, the two phenyl rings, in a fixed reciprocal orientation. Qualitative and quantitative (Ref. 30 and references therein) structure-activity studies using the results of a variety of neurological and behavioral tests have shown that activities are increased by electrophilic 7-substituents, by the overall lipophilicity of the molecule, and by the presence of electrophilic substituents of small steric hindrance at position 2′, whereas it collapses with any substituent in position 4′. After the discovery (1, 2) of BDZ receptors, binding affinity data for a large number of BDZs became quickly available and many researchers switched to structure-affinity studies (6, 31–33). These have the advantages that metabolization does not occur in *in vitro* binding experiments and pharmacological activities are, as a rule, strongly correlated with receptor binding parameters, the few exceptions being connected with compounds having metabolization problems. The results can be summarized as follows (33).

1) Affinities of 1-alkylated or dealkylated BDZs are very

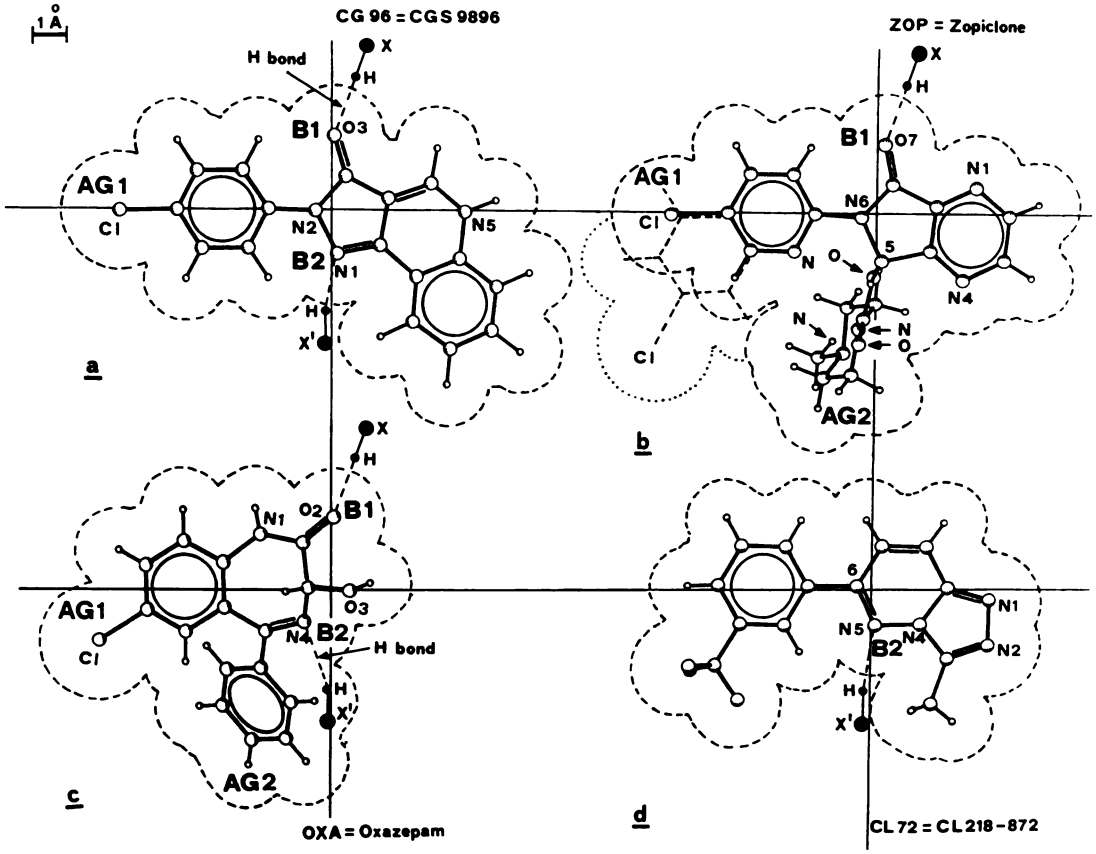

Fig. 5. Molecular structures and van der Waals envelopes for CG96 (a), ZOP (b), OXA (c), and CL72 (d). The *dotted* envelope in b shows the main molecular volume modifications in SUR. The *horizontal* and *vertical coordinate axes* are the same in the four figures and are intended to allow the superposition of all molecules. *B1* and *B2* are the main binding points and *AG1* and *AG2* are the regions responsible for agonistic properties. *X—H* and *X′—H* groups are hypothesized hydrogen-bonding donors.

similar [e.g., K_i[3] (DIA) = 8.9 nM, K_i(demethyl-DIA) = 8.9 nM (1)], showing that the main drug receptor interaction is not mediated by the N_1-H group.

2) N_4-substituted BDZs or BDZs lacking the $C_2{=}O_2$ group show low or very low receptor binding affinities [e.g., K_i(MED) = 3850 nM, K_i(CHL) = 574 nM (1)] suggesting that these two points can be considered the basic requirements for *optimal* BDZ receptor binding.

3) Previous structure-activity results are confirmed as regards the positive effects of overall lipophilicity, of small electrophils in 2′-, and the strong negative effect of 4′-substituents.

4) *R* and *S* stereoisomeric 3-substituted BDZs display a dramatic affinity difference [e.g., K_i(RO11-6896,3*S*) = 4.8 nM, K_i(RO11-6893,3*R*) = 1040 nM (34)], and this has been interpreted as an indication of the presence of at least a third noncoplanar point of drug-receptor interaction, which is to be identified in the phenyl ring in position 5. The effect of 7-substitution has not been investigated as far as binding affinities are concerned (binding assays have been carried out almost exclusively on 7-Cl/NO_2/CF_3 "active" BDZs), so that it is presently unknown whether the lack of electrowithdrawing substituents in such a position keeps the drug from binding or prevents the effect.

[3] All K_i values refer to the inhibition of ^{3}H-DIA binding from brain synaptosomal membranes.

Structure-Activity Relationships in β-Carbolines

Up to now no exhaustive attempt has been carried out in the field of structure-activity relationships of β-carbolines, with the exception of an elegant theoretical study carried out by Loew *et al.* (35). This is not surprising because, although an increasing number of receptor binding affinities (5, 35) is presently available to them, a quantitative scale of intrinsic activities is quite difficult to be defined. This situation is well represented by data of Table 1, where actual figures are reported for affinity data, but only a loose classification of efficacy, derived from a variety of biological tests, is shown.

A few simple remarks on binding can be summarized by saying that β-carbolines display high binding affinities for the BDZ receptor only if all of the following conditions are fullfilled *at the same time*: the presence of an esteric (sometimes amidic) function in position 3 [e.g., IC_{50}(NHA)=12,500 nM, IC_{50} (βCCM)=8 nM]; full aromaticity of both six-membered rings [e.g., IC_{50} (THE)=4900 nM, IC_{50} (βCCE)=7 nM), that is planarity of the three-ring system; and no substituents in position 1 [e.g., IC_{50} (MCCE)=4900 nM, IC_{50} (βCCE)=7 nM].

Moreover, substitution of the $COOCH_3$ group in position 3 by bulkier groups such as $COOC_2H_5$, $COOC_3H_7$ does not significantly affect affinities [e.g., IC_{50} (βCCM)=8 nM, IC_{50} (βCCE)=7 nM, IC_{50} (PrCC)=12 nM], and introduction of various substituents in positions 4, 5, 6, 7 likewise has little effect on this property (5).

Regarding the intrinsic activities of β-carbolines, we believe

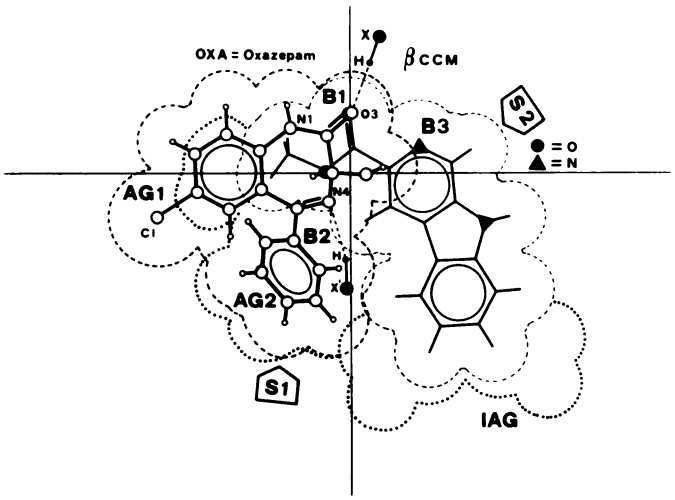

Fig. 6. Superposition of the structures of OXA and βCCM binding in different positions at the same receptor site. *B1*, *B2*, *AG1*, *AG2*, *X*—H, and *X′—H* have the same meaning as defined in the legend to Fig. 5. *B3* is an additional binding point for β-carbolines and *S1* and *S2* are regions of steric hindrance for the binding of BDZs and β-carbolines, respectively. *IAG* could be a region whose occupation increases IAG properties (see DMCM).

that they can be rationalized according to a scheme which is depicted in Fig. 4. The molecule which is sketched by heavy lines is that of βCCM as determined by X-ray diffraction (18). The surrounding space has been divided in four A–D subdomains corresponding to the different biological properties the molecule is granted by substitution in the four zones themselves.

Zone A. Substitution by groups of increasing steric hindrance shifts the pharmacological profile from full IAGs (βCCM) to partial IAGs (βCCE) to ANTs (PrCC), in agreement with the parallel change in GABA ratio (0.61, 0.86, and 1.11, respectively).

Zone B. Substitution in this zone causes the most remarkable effect on intrinsic activity. We can compare βCCE, a well known partial inverse agonist, with its 4,5-substituted derivatives. ZK93, which is the 4-methyl,5-O-*i*-propyl derivative, is an antagonist, and ZK91, which is substituted by bulkier substituents (4-CH_2-O-CH_3,5-O-CH_2-phenyl), displays partial agonist properties. It should be remarked that 4,6 substitution also shifts properties in the same direction as that represented by ZK93423 (not reported in Table 1; 4-CH_2-O-CH_3, 6-O-CH_2-phenyl-βCCE) which likewise displays agonist properties.

Zone C. Substitution in 7 seems to increase the IAG properties of β-carbolines. The only example is given by DMCM (4-ethyl-6,7-dimethoxy-βCCM) which is considered a more powerful inverse agonist than βCCM.

Zone D. Substitution here practically hinders the binding. In conclusion, experimental findings are not in disagreement with the following general statements: The recognition site is most probably a planar cleft (see effect of rings hydrogenation) where the main drug-receptor interaction is mediated by the carbonyl group of the esteric or amidic function, which is a typical hydrogen-bonding acceptor. The role played by the two ring nitrogens should be accessory as shown by the very low binding

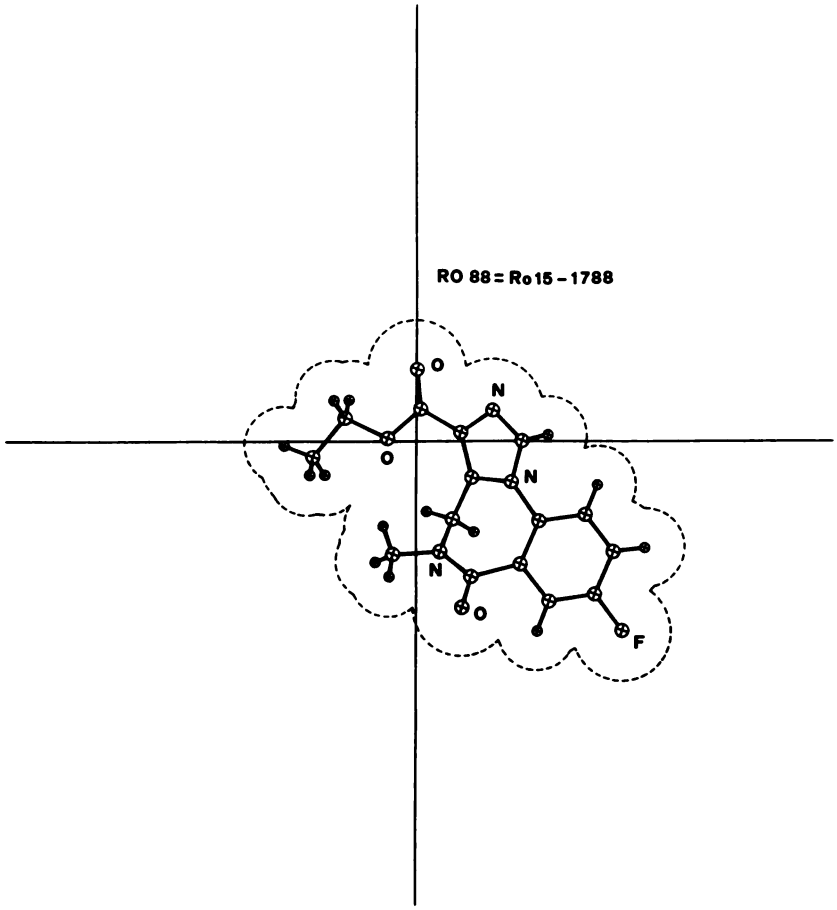

Fig. 7. The hypothesized superposition of the antagonist RO88 in the usual reference frame. The figure refers to the N(imidazole),O(carbonyl)-*cis*-conformation of the molecule. Another quite reasonable superposition can be obtained for the N,O-*trans*-conformer and, in this case, the seven-membered ring points upward.

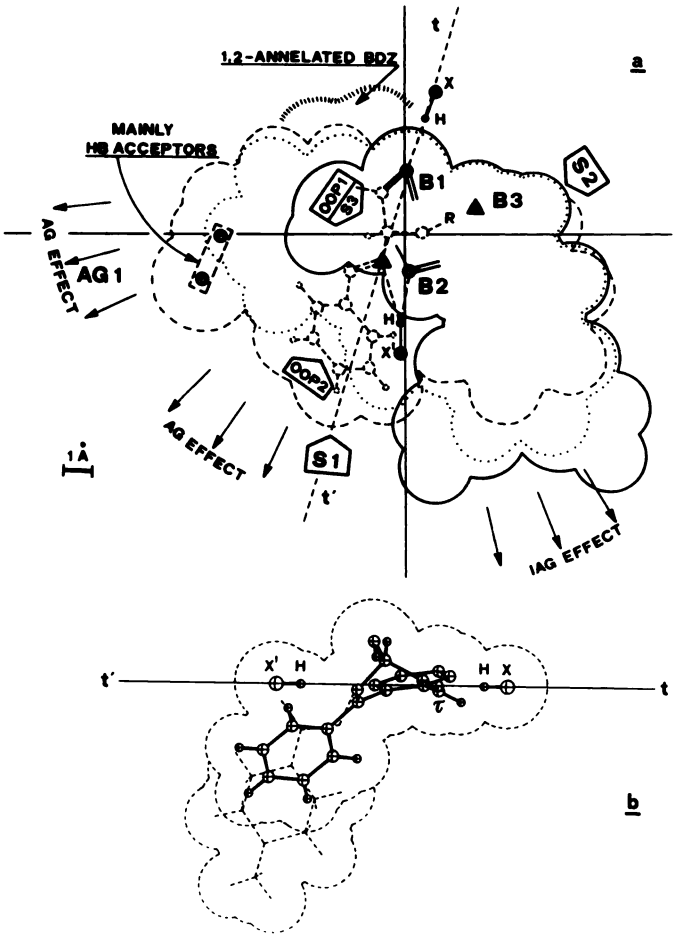

Fig. 8. A final comprehensive sketch of the model proposed. —, DMCM; – – –, van der Waals combined envelope of the two AGs OXA and CG96; . . ., van der Waals combined envelope of the two ANTs PrCC and RO88. The binding site is essentially planar except for the zones of out-of-plane displacement marked as *OOP1* and *OOP2*. *B1*, *B2*, *B3*, *S1*, *S2*, *S3*, *X—H*, and *X′—H* have the usual meaning. The rectangular area indicated as *Mainly HB Acceptors* marks the positions of strong electrophilic groups (Cl, F, NO_2. . .) in AG molecules. The *arrows* show that the bound molecule causes AG or IAG effects by pushing the receptor macromolecule in the directions marked *AG EFFECT* or *IAG EFFECT*. Perfect balancing of these opposite forces produces ANT behavior. b is a cross section of a along the dashed diagonal *t–t′* line; the molecule shown in b is OXA and the dashed fragment added refers to ZOP.

affinities displayed by non-3-carboxylated β-carbolines. In contrast, the fact that 1-substitution (zone D) prevents binding can be taken as indirect evidence that the main interaction region is located on the C═O, N_2, N_9 line. The effect of other physicochemical parameters is not well known; it may be remarked that the overall lipophilicity contribution to binding seems to be rather small in this class of compounds, as shown by the fact that compounds of very different molecular volume can display similar binding ability [e.g., IC_{50}(ZK91) = 1.1 nM, IC_{50}(βCCE) = 7 nM). As far as intrinsic activities are concerned, the van der Waals envelope of βCCM (see Fig. 4) can be considered to represent the spatial arrangement of a typical inverse agonist, properties being shifted by increasing steric hindrance in zones A and B toward those of antagonists and partial agonists, whereas the inverse agonist effect is possibly potentiated by substitution in zone C.

Towards a General Model of BDZ-Receptor Ligand Interaction

We may start from the comparison of CG16 and CG96. The structure of the latter (20) is shown in Fig. 5a and that of CG16 differs only in the presence of a phenyl instead of a *p*-Cl-phenyl group. As CG96 is a partial agonist whereas CG16 can be roughly classified as an antagonist, it could be concluded that increasing substitution in the Cl region shifts the properties in the agonist direction. Let us call this pro-agonist region AG1 (Fig. 5a).

ZOP (Fig. 5b) is perfectly superimposable to CG96 for a large part of its molecular structure and differs mainly in the addition of the 4-methyl-1-piperazine carboxylate fragment. As ZOP is a more potent agonist than CG96 (and, in fact, it displays AG properties very similar to those of BDZs), it may be concluded that the region indicated as AG2 confers further agonistic properties.[4]

A plausible superposition of a generic 1,4-benzodiazepin-2-one to the previous compounds is shown in Fig. 5c. It *makes correspond* the AG1 and AG2 zones with the two BDZ aromatic rings (in agreement with the high agonistic properties of BDZs) and superimposes the carbonyl groups as a common moiety, B1, responsible for the drug-receptor binding. The B1 interaction point had already been hypothesized for BDZs (33) and the data of Table 1 definitely indicate that compounds lacking the B1 point display lower binding affinities (e.g., IC_{50} values for CG16, CG96, ZOP, OXA, and FLU are 0.3, 0.6, 36, 38, and 5 nM, respectively, whereas IC_{50} values for CL72, CHL, and MED are 140, 1120, and 6800 nM, respectively). In the figures the B1 interaction point is shown to interact via hydrogen bonding with a generic X—H donor group. The different orientation in space of the C═O bond in OXA when compared with that in CG96 and ZOP is justified by the following considerations. In an extensive review (37) on the geometrical features of the >C═O— — —H—N hydrogen bond in crystals it was found that the hydrogen tends to be situated on the carbonyl plane, most likely forming a C═O— — —H—N angle of 20–50°. Seen the other way round, this means that a N—H group aligned as in Fig. 5 and pointing downwards has exactly the same probability of hydrogen bonding formation with the two differently oriented >C═O groups in OXA or CG96 and ZOP. In the figures another possible interaction point (B2) is marked, which is supposed to be a hydrogen bond donated by a generic X′—H group and accepted by the iminic atoms N1 for CG16 and CG96, N4 for BDZs, and N5 for CL72 (see below). This zone also has been shown (33) to be of importance in BDZs.

CL72 is classified as a full AG. It does not seem to be of great use in determining the receptor geometry. A reasonable superimposition is shown in Fig. 5d. The iminic N5 nitrogen could be localized in the B2 site and the CF_3 group situated in a region intermediate between the two agonistic zones AG1 and AG2. Its relatively low binding ability can be accounted for by the lack of proper substituents in the B1 region.

Such a provisional model of agonist binding and efficacy (i.e., AG1, AG2, B1, and B2 sites) has to be connected with that

[4] For the sake of completeness it must be said that Trifiletti and Synder (36) have recently suggested, based on binding kinetic evidence, that ZOP (and SUR) may also bind to a further allosteric site of the GABAergic macromolecular complex. The present stereochemical evidence shows, however, that binding at the BDZ receptor is quite credible; further biochemical experimentation will definitively clarify the problem.

already given in Fig. 4 for β-carbolines. A hint for doing that comes from PAL experiments. The labeling is obtained by irreversibly binding FLU molecules by means of near ultraviolet light irradiation. The PAL ratio is given by the quotient IC_{50} in PAL-labeled membranes/IC_{50} in nonlabeled membranes. This PAL ratio is very high for BDZs (of the order of 100), indicating that the BDZ site is definitely hindered by such treatment. It tends to be higher for typical AGs (e.g., 5.5 and 6.1 for ZOP and CL72, respectively). PAL ratios for β-carbolines are always very small (0.9–1.7), smaller values being tendentially associated with IAGs and geater ones with ANTs. From a stereochemical point of view they increase with the increasing bulkiness of the esteric part of R_3 (see Table 1) and with increasing substitution in positions 4 and 5. All of this seems to indicate that β-carbolines occupy in the receptor site a zone not overlapping that of BDZs and that the overlapping increases by increasing substitution in the carboxyalkyl region.

Fig. 6 shows a reasonable overlapping hypothesis in agreement with the above data. Its main interest lies in the good fitting of the carbonylic oxygens in the supposed binding region B1. Conversely, the binding zone B3 is supposed, in this model, to be a peculiarity of β-carbolines and independent of the binding sites of the other drug classes. Additional evidence in support of the model comes from the already mentioned fact that methylation in 1 deletes the binding—most probably by hindering the binding controlled by B1 and B3—and from the overlapping of the substituents in positions 4 and 5 with the proagonist AG2 region, in agreement with the observed shift of properties from pure IAG to AG to ANT caused by substitution here.

The "pure" ANT RO88 obviously can be well superimposed to BDZs; however, it can as well be superimposed on the side of β-carbolines as shown in Fig. 7, and its PAL ratio value suggests that the second hypothesis is to be preferred.

As far as phenylquinolines are concerned (e.g., P84), their precise location in this model is problematic. They could be loosely superimposed in different manners maintaining the fitting of the piperidine ring with the AG2 zone. These drugs bind with relatively low affinity [e.g., IC_{50}(P84) = 459 nM) in consequence of the lack of carbonyl groups binding in B1.

All superpositions of Figs. 4–7 were obtained by best qualitative fitting of both hydrogen bonding interacting sites and van der Waals envelopes. However, more quantitative methods of searching for functional correspondences in molecular structures according to the method of Danziger and Dean (38) have been applied to the problem.[5] Preliminary data show that quantitative superposition among ANTs of all different chemical classes gives results in strict accord with our hypotheses, whereas no fitting is possible between β-carbolines and agonist BDZs. This last failure is, however, an essential feature of the present model which essentially hypothesizes only the B1 binding site in common between these two classes of drugs. In contrast, a new class of β-carbolines-benzodiazepine hybrid molecules recently has been synthetized and it has been shown (39) that one of these, 8,14-dioxo-13,14-dihydro-8*H*-indolo[3′,2′:4,5]pyrido[2,1-*c*][1,4]benzodiazepine, displays high affinity for the BDZ receptor, displacing both ^{3}H-BDZs (IC_{50} = 23 nM) and ^{3}H-β-carbolines (IC_{50} = 47 nM). Although the pharmacological profile of this compound cannot be assessed owing to its rapid metabolization *in vivo*, its GABA ratio of 1 strongly suggests an antagonist behavior. It seems clear that this single experiment adds strong evidence to the above hypothesis that β-carbolines occupy in the receptor site a zone not overlapping but adjacent to that held by BDZs.

[5] I. L. Martin, P. M. Dean, and P. A. Borea, manuscript in preparation.

Conclusions

The following points, in connection with the sketches of Fig. 8, can be considered to summarize the general features of the proposed model.

1) A unique recognition site for all ligands is assumed.

2) The overall envelope of all compounds studied consists of an essentially planar object, but with a definite bump (marked in Fig. 8 as out-of-plane displacement zones OOP1 and OOP2) in the middle. The thickness of the planar part is the usual value for aromatic rings (≃3.54 Å). The bump is asymmetrical, being much more extended in the lower than in the upper part, where the maximum height is nearly 3.68 A from the mean ligand plane at the extreme limit of the van der Waals radius of the hydrogen attached to the bow C3 atom of BDZs. This point can be considered a zone of steric confinement of the ligand (marked S3 in Fig. 8) able to discriminate *R*- and *S*-enantiomers of 3-disubstituted BDZs in the sense that only compounds having the bulkiest substituent in the axial position are allowed to bind with high efficiency. The lower part of the bump is much larger and is associated, typically, with the 5-phenyl group of BDZs or *N*-carboxypiperazine moiety in ZOP and whose actual encumbrance is difficult to define precisely, owing to the great conformational freedom associated with the *N*-carboxypiperazine group in ZOP (see above). In its horizontal plane the overall molecular envelope is an ovoid of about 12 × 18 Å. As Fig. 8 has been drawn on the contour of the largest ligands, the figure can be considered to map the internal borders of the overall binding site.

3) Specific binding determining drug-receptor interaction points would be B1 and B2 for BDZs and pyrazoloquinolines (CGS), B1 and B3 for β-carbolines, RO88 and ZOP and only B2 for CL72. The forces responsible for the binding are most probably originated by hydrogen bonds accepted by the ligands. Drugs not able to bind at B1 display IC_{50} values higher by 1 or 2 orders of magnitude. Assuming the hydrogen bonding donor groups of sites B1 and B2 are X—H and X′—H, as shown in Figs. 5, 6, and 8, the distance between the two donating hydrogen atoms is nearly 7.2 Å.

4) The different ligands bind to the same site, but in different positions, still having a superimposition zone responsible for their ability to displace one another. The different location in the site gives origin to the different kinds of intrinsic activity. With reference to Figs. 6–8, there is a continuous property shift in the order IAG, ANT, AG for a site location going from right to left.

In Fig. 8 the borderlines of pure IAGs, ANTs, and AGs, marked as continuous, dotted, and dashed lines, respectively, are a clear graphical illustration of the previous statements. *In a more functional sense, a generic ligand*, whose binding is rather strictly delimited in the right upper part (both by the binding in B1 and the steric hindrance in S2), *can be considered as an effector able to modify the conformation of the receptor by pushing along the arrows shown in Fig. 8. Pushing leftwards and downwards on the left causes AG behavior, downwards on the right IAG behavior; different balancing of the two forces*

produces any intermediate behavior, pure ANTs representing only the point of perfect equilibrium of the two driving forces.

5) GABA ratio values (Table 1) are consistent with the idea that GABA binding (and/or the consequent opening of the Cl^- channel) causes conformational changes in the BDZ receptor site so as to favor the binding of compounds naturally interacting with the left recognition site with respect to those interacting with the right one. This would be in agreement with the allosteric model already mentioned (4).

The discussion can be extended and detailed by considering a few compounds not reported in Table 1. SUR, a ZOP analogue behaving as a high affinity agonist, indicates that AG1 and AG2 zones do not need to be separated (Fig. 5b). Its structure is mostly superimposable to that of ZOP (and then it should bind much in the same manner), but the presence of the naphthyridine moiety expands the AG1 zone toward the AG2 zone, a situation strictly similar to that already suggested for CL72 (see Fig. 5d). Moreover, the high affinity of 1,2-annelated BDZs, such as triazolo and imidazolo derivatives, indicates that a further zone of possible molecular expansion is located above the amidic group of the diazepine ring (Fig. 8), in agreement also with the observation that several pharmacologically active BDZs carry bulky chains at the amidic nitrogen. Likewise, single 3-substitution in active BDZs does not delete both binding and efficacy as such substituted groups can dispose themselves in the empty region of β-carboline binding.

7) The proposed model is based on the indirect evidence coming from a large variety of biochemical, pharmacological, and stereochemical data, and we do not know of any compound which does not fit in it. We have been able to suggest rather definite dimensions and geometrical specific features for the binding site, and this has been obtained much more by trying to combine all the observed properties of the different ligands within the limits of a definite stereochemical pattern than by looking for purely geometrical superpositions of molecular structures. In this sense the model can be classified as analogical as well as stereochemical and seems to be able, at least in our opinion, to overcome some fundamental objections put down on the pure superposition method by Haefely *et al.* (40), who state that "efforts directed at mapping the receptor site by using X-ray data from ligands implicate that a single receptor . . . interacts with all ligands in the same way, accepting them in the conformation which they adopt in the crystalline state. It seems very unlikely that such static conditions are fulfilled in nature."

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