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Central Benzodiazepine Receptors: *In Vitro* Efficacies and Potencies of 3-Substituted 1,4-Benzodiazepine Stereoisomers

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SUMMARY

3-Acyloxy-, 3-methoxy-, and 3-alkyl-substituted derivatives of the benzodiazepine (BZ) agonist desmethyl-diazepam (DMD) were resolved, and the stereochemical properties of binding to central BZ receptors were investigated in synaptosomal membrane preparations of rat brain. Decreasing potency and stereoselectivity of 3-methyl, 3-ethyl, and 3-isopropyl derivatives in displacement of [^{3}H]diazepam binding can be attributed to differential susceptibilities for steric hindrance of 3-axial versus 3-equatorial substituents of the binding conformation M. Chirality in the α-methyl-β-phenyl-propionic acyl moiety of oxazepam, the 3-OH-derivative of DMD, was noncritical in binding, whereas the β-phenyl substituent selectively increased the binding of the 3*S*-stereoisomer. Changing the pH from 7.4 to 5.6 significantly increased the IC_{50} of (3*R*)-oxazepam acetate but not those of (3*R*)-methyl-DMD and diazepam. Binding data led to a steric model of the BZ binding site with the postulation of an additional hydrogen-bond-donating moiety, probably histidine in the "ceiling" of the receptor cavity, that binds the 3-carbonyloxy groups and hinders the 3-alkyl ones. *In vitro* efficacies of 3-substituted BZs were estimated by allosteric binding interactions within the γ-aminobutyric acid$_A$ ($GABA_A$) receptor-ionophore complex. Nonequilibrium enhancement of *t*-butyl-bicyclophosphoro[^{35}S]thionate binding by the BZ agonist oxazepam was stereoselectively antagonized by (3*S*)-oxazepam-(*S*)-α-methyl-β-phenyl-propionate, suggesting a mixed agonist-antagonist character. GABA enhanced the [^{3}H]diazepam-displacing potencies of the 3*S*-enantiomers of the acetate, hemisuccinate, and (*S*)-α-methyl-β-phenyl-propionate esters of oxazepam by a factor of about 1.5–1.6, whereas the GABA shifts for 3*R*-esters were about 1.2. UV affinity labeling with flunitrazepam resulted in a significantly smaller decrease in the displacing potency of (3*R*)-oxazepam acetate than in that of the 3*S*-enantiomer. GABA shifts of successively 3-methylated DMD derivatives were also compared. The GABA shifts of DMD and its (3*S*)-methyl and 3,3-dimethyl derivatives were all characteristic of full agonists (2.4–2.7), whereas that of (3*R*)-methyl-DMD was 1.5. The 3-methoxy enantiomers of DMD displayed stereoselectivity and GABA shift values intermediate between those of 3-methyl and 3-acetoxy derivatives. These allosteric interactions suggest that 3-carbonyloxy derivatives in general, as well as (3*R*)-BZ enantiomers bound with axial 3-alkyl and 3-alkyloxy groups, decrease the agonist efficacies of 1,4-BZs to modulate the $GABA_A$ receptor complex.

The central BZ receptor is one of the most well known subsites of the $GABA_A$ receptor-ionophore complex. The inherent asymmetry of classical 1,4-BZ drugs and the stereoselectivity in receptor binding of 3-substituted 1,4-BZs have not yet been fully exploited to elucidate the steric structure of this binding site.

Fig. 1 shows the structures of 1,4-BZs and their conformations known to exist in aqueous solution (1, 2). 3-Substituents prefer the quasiequatorial orientation and, accordingly, shift the equilibrium of M and P conformations of free molecules. Stereoselectivity for (3*S*)-methyl enantiomers has been attributed to the preferential receptor binding of conformation M via fused rings and conformational restriction (3). We have recently studied enantiomeric 3-alkyl-BZs and proved both conformational recognition and steric hindrance to binding of the axial 3-methyl substituent greater than that of the equatorial one (2). Here we compare 3-alkyl-, 3-alkyloxy-, and 3-acyloxy-BZ enantiomers to find the major factors regulating the interaction of the 3-substituents with the receptor. Different orientations of the 3-substituents of bound enantiomers add a third dimension to previous models of BZ receptors. Due to the relatively planar structures of recent non-BZ ligands, most of the BZ receptor models have been planar, with a few recent exceptions (4).

Another intriguing feature of the BZ site is its bidirectional allosteric modulatory role in the $GABA_A$ receptor complex, e.g., GABA enhances and decreases the receptor binding of GABAergic facilitating BZ agonists and GABAergic inhibitory inverse agonists, respectively (5). Such allosteric *in vitro* binding interactions can be used to characterize the efficacies of BZ receptor ligands to modulate the $GABA_A$ receptor complex.

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ABBREVIATIONS: BZ, benzodiazepine; DMD, 1-desmethyl-diazepam; GABA, γ-aminobutyric acid; TBPS, *t*-butylbicyclophosphorothionate.

Because a structural heterogeneity of the compared ligands may obscure such correlations, chemical modifications of BZs were restricted to position 3. Recent models of BZ receptors have postulated hydrogen bond formation or, more generally, charge transfer interactions and differentiated the binding of BZ agonists and antagonists (6–10). Some esters of oxazepam that had been planned as prodrugs were found later to possess intrinsic antagonist properties (11). The ester enantiomers, with their carbonyloxy groups in different orientations wher bound, prove to be useful as vectorial determinants to test the receptor models.

Experimental Procedures

Materials

1,4-BZ esters were prepared as described (12). Chiral 3-alkyl-1,4-BZs were synthesized from α-amino acids (13). [^{3}H]Ro-15-4513 (20.1 Ci/mmol) and [^{35}S]TBPS (20–50 Ci/mmol) were purchased from Du Pont-NEN (Dreieich, FRG); [^{3}H]diazepam (86 Ci/mmol) was from Amersham (UK).

Resolution

Aliphatic esters of oxazepam and oxazepam methyl ether were resolved by albumin chromatography (14). Briefly, the racemates were dissolved in ethanol and chromatographed on human serum albumin immobilized on CNBr-activated Sepharose 4B (Pharmacia), using UV detection. The eluted enantiomers were freeze-dried, extracted with chloroform, and dissolved in ethanol. The concentrations were determined by UV spectroscopy. Phenyl-substituted esters of oxazepam could not be resolved this way because of their strong hydrophobic binding to albumin. α-Methyl-β-phenyl-propionic acid was resolved via its quinine salt. The diastereomeric esters of oxazepam (*S*)-α-methyl-β-phenyl-propionate were separated by silica gel chromatography. Oxazepam hemisuccinate was resolved via its (−)-ephedrine salt. The optical purity of stereoisomers was determined by CD spectroscopy, albumin chromatography, and high performance liquid chromatography on a chiral column (Chiralcel OC; Daicel, Düsseldorf, FRG).

Membrane Preparation

Extensively washed synaptosomal membranes were prepared from whole brains of male Wistar rats (15). Brains were homogenized in 0.32 M sucrose and centrifuged at 1,000 × *g* for 10 min. The supernatant was centrifuged at 20,000 × *g* for 20 min. The pellet was dispersed in distilled water with an Ultra-Turrax for 15 sec and centrifuged at 8,000 × *g* for 20 min. Its supernatant and the buffy coat of the pellets were centrifuged at 48,000 × *g* for 20 min. The membrane pellets were washed twice by similar centrifugations in distilled water and frozen.

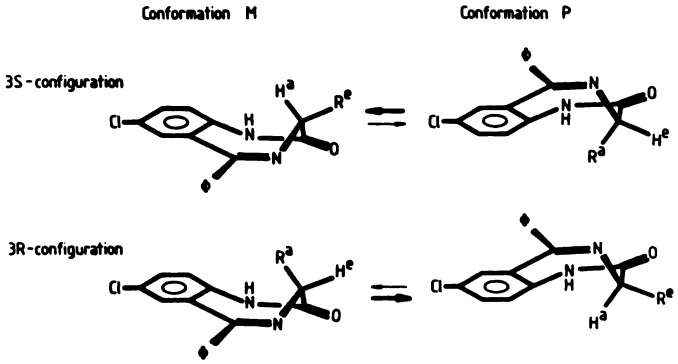

Fig. 1. Structures and M and P conformations of 1,4-BZs. For 3-substituted derivatives, the equilibrium is shifted towards the conformation in which the substituent is oriented quasiequatorially (*R**) (2). R is hydrogen for DMD and methyl, ethyl, or isopropyl for 3-alkyl-BZs. R is OH for oxazepam; the OH group is either methylated or acylated.

On the next day, the membranes were thawed, suspended in 50 mM Tris-citrate (pH 7.1), centrifuged at 48,000 × *g* again, and frozen. On the day of the assay, they were thawed, centrifuged in Tris-citrate buffer, and used for [^{3}H]diazepam binding.

For [^{35}S]TBPS binding, synaptosomal membranes were prepared from rat cerebral cortex (16, 17). Briefly, cortices were homogenized in 50 mM Tris·HCl (pH 7.4) for 20 sec by Ultra-Turrax. The suspension was centrifuged at 48,000 × *g* for 10 min. The pellet was washed with 50 mM Tris·HCl four times, by similar centrifugations, and frozen. Before the binding assay, the thawed suspension was centrifuged similarly and suspended in 50 mM Tris·HCl containing 300 mM NaCl.

UV Affinity Labeling

The synaptosomal membranes of whole brain were incubated in 50 mM Tris·HCl (pH 7.4) with 5 nM flunitrazepam for 40 min on ice, irradiated for 8 min with a UV lamp from a distance of 28 cm, and washed by three centrifugations (20,000 × *g*, 10 min).

Binding Studies

[^{3}H]BZs. Displacing potencies of oxazepam esters were determined with [^{3}H]diazepam or [^{3}H]Ro-15-4513 instead of [^{3}H]Ro-15-1788, the higher affinity of which would have required longer incubations for binding. Membrane suspensions of whole brain in 50 mM Tris-citrate (pH 7.1) were incubated with 1 nM [^{3}H]diazepam and BZs on ice and filtered. Incubation time for oxazepam esters was 25 min, unless stated otherwise, to minimize hydrolysis (quasiequilibrium binding). Nonspecific binding was determined in the presence of 10^{-6} M clonazepam. Because ≥30 μM (3*R*)-oxazepam α-methyl-β-phenyl-propionate (close to its aqueous solubility) enhanced nonspecific [^{3}H]diazepam binding, these ester concentrations were also included for the determination of nonspecific binding. BZs were added in dimethyl sulfoxide or ethanol, the final concentrations of which were 0.1 and 1%, respectively.

Membrane suspensions in 50 mM Tris·HCl were incubated with 1 nM [^{3}H]Ro-15-4513 and BZs for 30 min on ice (quasiequilibrium binding) and filtered. For nonspecific binding, 10^{-6} M β-carboline-3-carboxylic acid methyl ester was applied, the binding of which is insensitive to photoaffinity labeling.

[^{35}S]TBPS. Membrane suspensions of cerebral cortex in 50 mM Tris·HCl containing 0.3 M NaCl were incubated with 2.5 nM [^{35}S]TBPS and BZs for 20 min at 22° (nonequilibrium conditions) and filtered. Nonspecific binding was determined with 40 μM picrotoxin.

Results

Diazepam-Displacing Potencies

Displacing potencies of resolved oxazepam esters on [^{3}H]diazepam binding are summarized in Table 1. Binding is stereoselective, preferring the 3*S*-enantiomers. Fig. 2 is a logarithmic plot of the IC_{50} values for the 3*S*-enantiomers of higher affinity (eutomers) and the IC_{50} ratios of the distomers (weaker enantiomers) and eutomers. The eudismic index on the ordinate represents stereoselectivity of binding. This plot, called eudismic-affinity correlation (18), also contains the displacing potencies for 3-alkyl derivatives of diazepam and DMD published recently (2). Whereas the stereoselectivities of 3-alkyl derivatives increase with increasing potencies (positive slope), those for the corresponding 3-acyloxy derivatives tend to decrease.

If we extend the linear correlation of 3-alkyl derivatives for 3-nonsubstituted BZs, we can estimate the binding stereoselectivities of their M and P conformations. The IC_{50} value of 4.6 nM for diazepam (2) corresponds to a concentration of 2.3 nM for its M conformation, resulting in an eudismic index value of 3.57, i.e., a stereoselectivity of 3720. Similarly, the IC_{50} value of 10.0 nM for DMD (2) corresponds to a stereoselectivity of 5750. Fig. 2 clearly shows that increases in size and branching of the

TABLE 1

Displacing potencies of oxazepam ester enantiomers for [^{3}H]diazepam binding and the effect of GABA

Synaptosomal membranes of rat whole brain were incubated in 50 mM Tris-citrate buffer with 1 nM [^{3}H]diazepam and five or six concentrations of oxazepam esters, in the presence or absence of 10^{-6} M GABA. Because the concentration of [^{3}H]diazepam was well below its K_D value, IC_{50} values can be practically considered as K_i values, according to the Cheng-Prusoff equation. Data are mean ± standard deviation of three to eight experiments. The IC_{50} value of oxazepam was 20 ± 2 nM in the presence of GABA.

Configuration of oxazepam	3-Substituent	Ester[a]	IC_{50} −GABA	IC_{50} +GABA	GABA shift
3S	$OCOCH_3$	Ac[b]	45 ± 2 nM	29 ± 2	1.55 ± 0.12
3R			79 ± 7	69 ± 8	1.15 ± 0.09
3S	$OCO(CH_2)_2COOCH_3$	Succ-Me		160 ± 36	
3R				780 ± 176	
3S	$OCOC(CH_3)_3$	Piv		295 ± 75	
3R				1600 ± 300	
3S	$OCO(CH_2)_2COOH$	Succ	1170 ± 450	710 ± 170	1.64 ± 0.37
3R			4500 ± 1100	3600 ± 600	1.25 ± 0.16
3S	$OCOCH(CH_3)CH_2C_6H_5$	αMeβPheProp[c]	80 ± 28	54 ± 20	1.48 ± 0.03
3R			6500 ± 2200[d]	3900 ± 800[d]	1.7

[a] See the legend to Fig. 2 for the names of the esters.
[b] Oxazepam formation during the binding assay was determined as described (21) for 10 μM [2-^{14}C]oxazepam acetate and was 2%.
[c] Esterified with S-acid.
[d] The 3R-stereoisomer contained 3–4% contamination with the 3S-enantiomer; therefore, the values are underestimated.

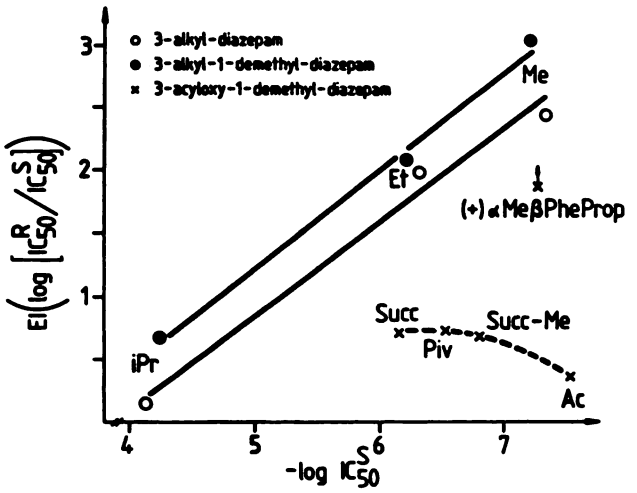

Fig. 2. Eudismic-affinity correlation of 3-substituted 1,4-BZ derivatives. Eudismic index (*Ei*) is the logarithm of the ratio of IC_{50} values for weaker and stronger enantiomers. Oxazepam esters used: succinate (*Succ*), methyl-succinate (*Succ-Me*), pivaloate (*Piv*), acetate (*Ac*), and (*S*)-α-methyl-β-phenyl-propionate (*αMeβPheProp*). IC_{50} values originate from Table 1, in the presence of GABA. IC_{50} values for methyl (*Me*), ethyl (*Et*), and isopropyl (*iPr*) derivatives of diazepam and DMD are taken from Ref. 2.

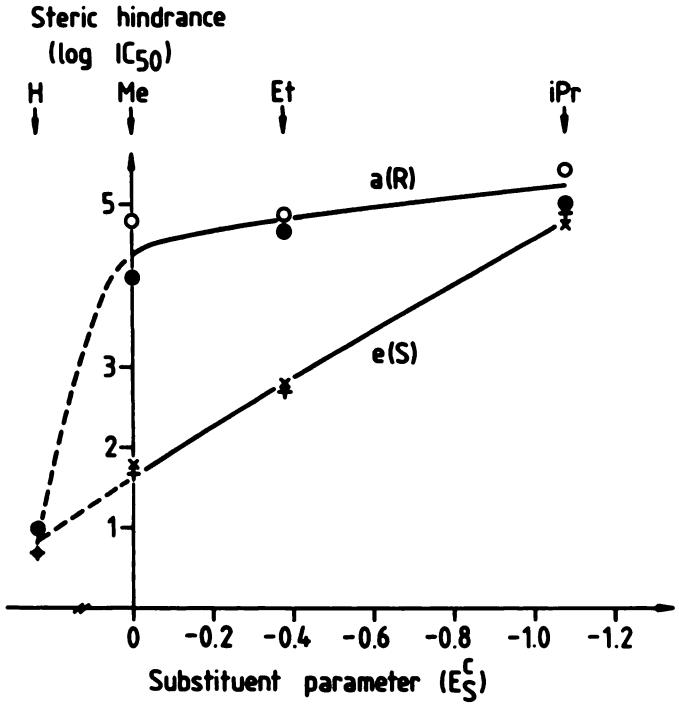

Fig. 3. Correlation between steric hindrance to binding to BZ receptors and a steric parameter of the 3-alkyl substituents. Axial (*a*) and equatorial (*e*) orientations correspond to bound 3*R*- and 3*S*-enantiomers, respectively. Enantiomers of 3-alkyl-diazepam (○, R; +, *S*) and 3-alkyl-DMD (●, R; ×, *S*) are shown. IC_{50} values, expressed in nM, are taken from Ref. 2. E_S^c values are taken from Ref. 19. Because the origin of the steric parameter E_S^c is set to the methyl groups, we cannot attribute a numerical abscissa value to the nonsubstituted compounds (for H). Therefore, the starting point of the curves may move horizontally and the curves are tentative (*dashed*) up to the value of methyl (*Me*). *Et*, ethyl; *iPr*, isopropyl.

3-alkyl substituents decrease the affinity of BZs for their binding sites. This can be attributed to steric hindrance to binding, as shown in Fig. 3, which depicts the displacing potencies as a function of the steric parameter E_S^c of the 3-alkyl groups. E_S^c is Taft's steric parameter modified by Hancock to characterize the size and branching of a substituent (19). Steric hindrance to binding, characterized by log IC_{50} values, increases gradually from hydrogen to isopropyl for the 3*S*-enantiomers (Fig. 3), whereas steric susceptibility is greater, i.e., the dependence from hydrogen to methyl is steeper, for the 3*R* enantiomers.

Although the predominant role of 3-alkyl substituents in BZ receptor affinity seems to be steric hindrance, the 3-acyloxy groups also have a substantial binding contribution. This is especially true for oxazepam α-methyl-β-phenyl-propionate. Table 1 shows that the β-phenyl group increases the displacing potency and stereoselectivity of this ester. In fact, 3–4% contamination of the 3*R*-ester by the more potent 3*S*-stereoisomer must have contributed to an underestimation of its IC_{50} value and stereoselectivity (Table 1).

Racemic oxazepam was also esterified with resolved *S*-(+)- and *R* (−)-enantiomers of α-methyl-β-phenyl-propionic acid,

and the IC_{50} values for [^{3}H]diazepam displacement were 257 ± 48 nM for the *S* (+)- and 419 ± 120 nM for the *R* (−)-diastereomer (mean ± standard deviation, three experiments, in the presence of 10^{-5} M GABA). The small stereoselectivity of 1.61 ± 0.18 indicates that orientation of the α-methyl group is not critical in the binding of the acyl moiety.

Accessory binding of the 3-acyl group of oxazepam might have resulted in differential displacement for BZ receptor subtypes, which cannot be observed for the parent compound. Therefore, the displacing potencies of (3*S*)-oxazepam-(*S*)-α-methyl-β-phenyl-propionate were compared in hippocampal and cerebellar membrane preparations. However, the IC_{50} values were not different (40 ± 7 nM in hippocampus and 41 ± 6 nM in cerebellum, three experiments, in the presence of 10^{-5} M GABA).

Displacing potencies of oxazepam methyl ether enantiomers were investigated to differentiate the contribution of 3-oxy and 3-carbonyloxy groups (Table 2). The potency of the (3*S*)-methoxy derivative was similar to that of the isosteric (3*S*)-ethyl derivative (0.61 μM) (2), but the 3*R*-enantiomer was not as weak as the (3*R*)-ethyl derivative (73 μM) (2).

The effect of pH on the displacing potencies of three representative BZs was examined. Table 3 shows that acidification to pH 5.6 doubled the IC_{50} of (3*R*)-oxazepam acetate but did not change significantly those of diazepam and (3*R*)-methyl-DMD.

Allosteric Interactions Within the $GABA_A$ Receptor Complex

GABA shift. Displacing potencies of three esters, oxazepam acetate, hemisuccinate, and (*S*)-α-methyl-β-phenyl-propionate, were determined for [^{3}H]diazepam binding in the presence and absence of GABA. Table 1 shows that the ratios of the IC_{50} values (GABA shift) were intermediate (1.5–1.6) for the 3*S*-enantiomers. In contrast, those for the 3*R*-enantiomers were closer to unity. The GABA shift value for (3*R*)-oxazepam-(*S*)-α-methyl-β-phenyl-propionate suggested the dominant contribution of its contamination by the 3*S*-isomer. GABA shift values for all other esters were similar within each enantiomeric group (Table 1). This suggests that substituents beyond the 3-carbonyloxy moiety (the carboxylate and phenyl groups in the ω-position) do not significantly modulate the efficacies of BZs.

3-Methylation of DMD resulted in the highest stereoselectivity reported thus far for BZ receptor binding (2). Table 4 contains the displacing potencies and GABA shifts of successively 3-methylated DMD derivatives. The high GABA shift value of DMD is in agreement with its full agonist efficacy. The GABA shift of its (3*S*)-methyl derivative was as high as that of DMD. However, the (3*R*)-methyl enantiomer displayed not only very low displacing potency but also an intermediate value of GABA shift (Table 4). The displacing potency of the 3,3-dimethyl derivative was not as weak. Its GABA shift was not significantly different from that of DMD (Table 4), as has also been reported for [^{3}H]flumazenil (2).

GABA shift values for oxazepam methyl ether are given in Table 2. The 3*S*-enantiomer resulted in a value intermediate between those of the corresponding enantiomers of 3-methyl and 3-acetoxy derivatives, whereas (3*R*)-methoxy-DMD displayed a lower GABA shift value, equal to that of the (3*R*)-methyl derivative (Table 4).

Photo shift. UV photoaffinity labeling with flunitrazepam decreased the affinity of BZs, and this shift correlated with their agonist efficacies (5, 20). Table 5 shows the displacing potencies of oxazepam acetate enantiomers for [^{3}H]Ro-15-4513 binding after UV irradiation of the synaptosomal membranes in the presence and absence of flunitrazepam. Photo shifts were

TABLE 2

Displacing potencies of oxazepam methyl ether enantiomers for [^{3}H]diazepam binding and the effect of GABA

Synaptosomal membranes of rat whole brain were incubated with 1 nM [^{3}H]diazepam and five concentrations of oxazepam derivatives, in the presence or absence of 10^{-5} M GABA, for 40 min on ice and filtered. Data are mean ± standard deviation of *n* experiments.

Enantiomer	IC_{50}		GABA shift
	−GABA	+GABA	
	μM		
3*S* (*n* = 6)	1.02 ± 0.27	0.47 ± 0.16	2.2 ± 0.4
3*R* (*n* = 5)	10.0 ± 2.8	6.5 ± 1.8	1.5 ± 0.2

TABLE 3

Effect of pH on the displacing potencies of diazepam and (3*R*)-substituted BZs

Synaptosomal membranes of rat whole brain were incubated, in 50 mM Tris-phosphate buffer containing 200 mM KCl, with 0.7 nM [^{3}H]diazepam and five or six concentrations of its displacers, in the presence of 10^{-5} M GABA, at 0–4° for 50 min. Specific binding of [^{3}H]diazepam at pH 5.6 was 82 ± 9% of that at pH 7.4. Data are mean ± standard deviation of five or six experiments.

BZ	IC_{50}	
	pH 7.4	pH 5.6
	nM	
Diazepam	6.9 ± 1.7 nM	9.5 ± 1.3
(3*R*)-Methyl-DMD	57,000 ± 15,000	48,000 ± 10,000
(3*R*)-Oxazepam acetate	42 ± 13	83 ± 9[a]

[a] Significantly ($p < 0.001$) higher than for pH 7.4.

TABLE 4

Displacing potencies and GABA shifts of successively 3-methylated DMD derivatives

Freeze-thawed membranes of rat whole brain were incubated, in 50 mM Tris-citrate, with 1 nM [^{3}H]diazepam and five concentrations of displacers on ice for 40 min, in the presence or absence of 10^{-5} M GABA, and filtered. Data are mean ± standard deviation of *n* experiments.

BZ	IC_{50}		GABA shift
	−GABA	+GABA	
	nM		
DMD (*n* = 7)	16 ± 5	6.5 ± 1.1	2.5 ± 0.9
(3*S*)-Methyl-DMD (*n* = 6)	51 ± 14	19 ± 7	2.7 ± 0.5[a]
	μM		
(3*R*)-Methyl-DMD (*n* = 5)	96 ± 20	66 ± 13	1.5 ± 0.2[a]
3,3-Dimethyl-DMD (*n* = 7)	39 ± 11	16 ± 4	2.4 ± 0.6

[a] Significantly different from that of the other enantiomer by *t* test ($p < 0.001$).

TABLE 5

UV affinity labeling with flunitrazepam and the displacing potencies of oxazepam acetate enantiomers on [^{3}H]Ro-15-4513 binding

Freeze-thawed synaptosomal membranes of rat whole brain were UV-irradiated in the absence (control) or presence of 5 nM flunitrazepam, washed, and incubated for [^{3}H]Ro-15-4513 binding for 30 min on ice. Specific [^{3}H]Ro-15-4513 binding after flunitrazepam labeling was 119 ± 23% of the UV control. Photo shift is the ratio of IC_{50} values for flunitrazepam-labeled versus control membranes. Data are mean ± standard deviation of four experiments.

Enantiomer	IC_{50}		Photo shift
	UV control	UV + flunitrazepam	
	nM		
3*S*	61 ± 4	1043 ± 332	17.1 ± 4.9[a]
3*R*	104 ± 3	1405 ± 409	13.4 ± 3.5[a]

[a] Significantly different ($p < 0.05$) in a paired Student's *t* test.

high and variable, but the 3*R*-enantiomer displayed a significantly lower value in a paired *t* test (Table 5).

Nonequilibrium modulation of [^{35}S]TBPS binding. BZ receptor ligands allosterically modulate [^{35}S]TBPS binding to the convulsant site of the $GABA_A$ receptor complex (5). Under nonequilibrium conditions of binding, BZ agonists enhance and inverse agonists decrease TBPS binding (5). The effect of BZ antagonists can be studied in combination with an agonist (17). Oxazepam was applied as an agonist at 0.3 μM, equivalent to its brain concentration required to cause 50% protection from metrazol convulsions (11). This agonist concentration resulted in near maximal enhancement of nonequilibrium TBPS binding *in vitro* (17), which was completely antagonized by Ro-15-1788, a specific antagonist of central BZ receptors (17). Fig. 4 shows the allosteric effect of oxazepam (*S*)-α-methyl-β-phenyl-propionate stereoisomers. The enhancing effect of oxazepam was stereoselectively antagonized by its ester. The more potent 3*S*-stereoisomer eliminated the enhancement of TBPS binding, and concentrations above 10^{-6} M decreased binding below control. Antagonism by higher concentrations of the 3*R*-isomer can be quantitatively accounted for its contamination by the more potent 3*S*-enantiomer. The ester stereoisomers displayed a biphasic effect on TBPS binding in the absence of oxazepam (Fig. 4).

Discussion

Methodological limitations in binding studies with oxazepam esters should be kept in mind. To minimize hydrolysis of the esters by membrane-bound esterases, freeze-thawed membranes, radioligands with rapid binding kinetics, and shorter quasiequilibrium incubations at low temperature were used (21). Consequently, oxazepam formation was minimal, even for oxazepam acetate (Table 1), which is most sensitive to brain esterases (21). In contrast, in previous studies oxazepam formation *in vitro* could have decreased the IC_{50} for oxazepam hemisuccinate (reviewed in Ref. 21) to values lower than those in Table 1, and stereoselective esterases could have resulted in different apparent binding stereoselectivities (21).

Potency

Since the pioneering conclusion that 3-substituents decrease the pharmacological activities of 1,4-BZs (22), 3-substituted 1,4-BZs have been primarily used as prodrugs. For oxazepam esters, too, displacing potencies decrease with increasing size of the 3-acyloxy groups (21). The unprecedented recovery of affinity for (3*S*)-oxazepam-(*S*)-α-methyl-β-phenyl-propionate (Table 1) proved that the recognition site of BZs is not hindered in the equatorial direction of the 3-substituent. However, there does seem to exist a "ceiling" of the receptor cavity, which hinders axial 3-alkyl substituents more than equatorial ones (Fig. 3).

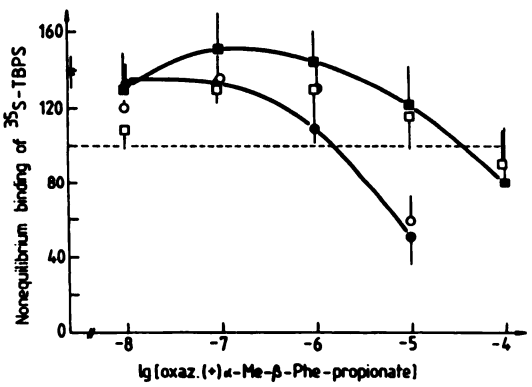

Fig. 4. Effect of (3*S*)- and (3*R*)-oxazepam-(*S*)-α-methyl-β-phenyl-propionate stereoisomers on the nonequilibrium binding of [^{35}S]TBPS. Effect of 3*R* (□) and 3*S* (○) stereoisomers alone and in the presence of 0.3 μM oxazepam (■, 3*R*;; ●, 3*S*). ×, Effect of 0.3 μM oxazepam alone. Data are mean ± standard deviation of three to eight experiments.

This study confirmed the conclusion of previous reports that the BZ receptor preferentially binds conformation M of 1,4-BZs. It is supported by the following major observations. 1) Stereoselective binding prefers conformation M constrained by ring annellations (4). 2) The quantitative treatment of successively 3-methylated BZs also showed the highly preferred recognition of conformation M (2). 3) Stereoselectivity for (3*S*)-alkyl BZs (Fig. 2) may be extrapolated for 3-nonsubstituted BZ conformations.

Assuming that BZs are bound to their central receptors predominantly in conformation M, the curves in Fig. 3 represent differential steric susceptibilities of axial versus equatorial 3-substituents in binding. With the appearance of the 3-methyl group, the axial substituent is presumed to be less well tolerated by the ceiling of the BZ receptor cavity. However, the same high levels of steric hindrance for 3-isopropyl-BZ enantiomers suggest that the isopropyl group may reach this ceiling also from the quasi-equatorial position.

Different eudismic-affinity dependences in Fig. 2 suggest an interaction of the 3-carbonyloxy groups that is different from that of 3-alkyl groups. The higher displacing potencies of 3-acyloxy, compared with 3-alkyl and 3-alkyloxy, derivatives may result from accessory binding of the 3-carbonyloxy group; e.g., the insertion of a 3-carbonyl group into oxazepam methyl ether, in spite of the increased size, decreased the IC_{50} values from 0.5–10 μM (Table 2) to 29–79 nM for oxazepam acetate (Table 1). Although steric hindrance is stronger for axial 3-alkyl groups (Fig. 3), binding is stronger for 3*R*-enantiomers bound with axial 3-acyloxy groups. This is especially true for (3*R*)-oxazepam acetate. The relative concentration of its M conformation is very small, due to the unfavorable axial 3-acetoxy group, yet it was only slightly less potent than (3*S*)-oxazepam acetate (Table 1, in the absence of GABA), which exists predominantly in the binding conformation M. Accessory binding of the esters can be attributed to their 3-carbonyloxy moieties, because the 3-alkyl moieties of the acyl groups of esters decrease affinity (21) (Table 1). Steric hindrance from the acid chains is stronger in the axial direction (in 3*R*-enantiomers). Further addition of a phenyl substituent to the acyl group results in enhanced affinity of (3*S*)-oxazepam-(*S*)-α-methyl-β-phenyl-propionate, probably because the equatorial phenyl group can produce accessory binding in the plane of the BZ molecule (Fig. 5), thereby preventing its spacer alkyl group from hindrance by the ceiling of the receptor cavity.

This evaluation of the contributions of 3-substituents to the potencies of BZs for displacement at their binding sites is summarized in Table 6. It differentiates positive (binding) and negative (steric hindrance) contributions of dissected moieties of the 3-substituents. The increments are distinguished vectorially, along axial and equatorial directions.

Several recent models for BZ receptors have postulated that carbonyl groups of BZs contribute to binding via hydrogen

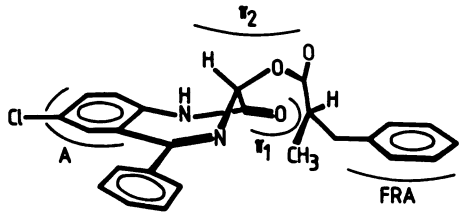

Fig. 5. Steric model of the BZ binding site, containing the hypothetical binding conformation of (3*S*)-oxazepam-(*S*)-α-methyl-β-phenyl-propionate. π_1 and π_2 refer to hydrogen-bond donor groups (6, 7); locus A binds the aromatic ring A of BZs, and FRA binds the freely rotating aromatic ring (6, 23). A histidine moiety is assumed for π_2. Based on the saturation of steric hindrance for 3-isopropyl-BZs, Dreiding stereochemical models suggest the height of the receptor cavity to be about 4–5 Å.

TABLE 6

Positive (binding) and negative (steric hindrance) contributions of axial and equatorial 3-substituents in binding to BZ receptors

+, Enhanced binding; −, steric hindrance. Double signs, stronger effects. =, Equipotent with methylene. The increments are attributed to corresponding moieties along axial (a) and equatorial (e) directions of the 3-substituents. Ph, phenyl; Q, alkylene; Y, alkyl groups.

	3-Alkyl-BZs[a]	3-Acyloxy-BZs[b]
BZ − Y		BZ - O - CO - Q - Ph
3*S* (e)	−	= + − +
3*R* (a)	−−	+ ++ −−

[a] Symbols relate to a comparison of Y and H.

[b] Symbols for O-CO are defined with respect to alkyl groups of similar size, whereas those for Q and Ph are compared with potencies without these substituents.

bonds or charge transfer interactions (6–10, 23). Fig. 5 shows attachment point π_1 for the 2-oxo group described before (6–10). Another accessory binding interaction needs to be invoked for the 3-carbonyloxy group, which might be identical to π_2 postulated recently (8). The ceiling of the cavity might be involved in axial 3-acyl groups being bound more tightly than equatorial ones, as deduced above. This polar hydrogen donor residue of the receptor for π_2 might be the same as that which strongly hinders the receptor fit of axial 3-alkyl BZ groups. Other attachment points indicated in Fig. 5 have been introduced in previous models, locus A for binding of the aromatic ring A of BZs (6–8) and FRA for attachment of freely rotating aromatic rings (23) such as the β-phenyl group of the oxazepam ester in Fig. 5 and those of non-BZ ligands, e.g., CGS 8216 (6).

The question of which part of the 3-carbonyloxy groups can possibly participate in accessory binding was approached via the 3-methoxy-BZ enantiomers (Table 2). The equal displacing potencies of (3*S*)-methoxy- and (3*S*)-ethyl-BZ enantiomers suggest that the equatorial ether bond has a steric hindrance similar to that of the methylene bridge. However, because (3*R*)-ethyl-DMD showed about one order of magnitude weaker potency than the corresponding (3*R*)-methoxy derivative, the axial ether bond either resulted in less steric hindrance or contributed to accessory binding. Consequently, the 3-oxy unit may contribute to stronger binding of the axial 3-carbonyloxy groups, with respect to the axial 3-alkyl moieties (Table 6).

The carbonyloxy groups appear to form hydrogen bonds with π_2 located in the ceiling of the receptor cavity (Fig. 5). Modification of BZ receptors by diethyl pyrocarbonate, a reagent that is rather selective for histidine at pH 6, was selectively prevented by BZ ligands (24, 25). The rate of this modification was also changed around pH 6, as was the affinity of oxazepam acetate (Table 3), suggesting the involvement at π_2 of residues protonated around pH 6, probably histidine (24, 25). The BZs in Table 3 do not contain groups that are protonated significantly between pH 7.4 and 5.6 (26). The pH-related differences in the IC_{50} values for diazepam and (3*R*)-methyl-DMD were not significant (Table 3). This suggests that the basic recognition properties of the BZ receptors and steric hindrance from the 3-axial direction were not significantly altered by this change in pH. On the other hand, the IC_{50} value was doubled for (3*R*)-oxazepam acetate (Table 3), the axial 3-carbonyloxy group of which is presumed to form the strongest accessory binding to the histidine (π_2) moiety.

Accessory binding of the β-phenyl-substituted 3-acyloxy group did not create heterogeneity in binding to cerebellar and hippocampal membranes, representing type I and II BZ receptors, respectively (27). However, the recently proposed heterogeneity of BZ receptors emerging from the cloning of different subunits of the $GABA_A$ receptor complex (e.g., Ref. 28) may restrict the validity of the model in Fig. 5.

Efficacy *in vitro*

After the discovery of the BZ ester-type antagonist Ro-15-1788 (29), a slowly hydrolyzing ester prodrug, oxazepam α,α-dimethyl-β-phenyl-propionate, was reported to require an elevated brain level of oxazepam in order to elicit a certain level of anticonvulsant effect against metrazol convulsions in mice (11). A Schild plot of this antagonism resulted in a pA_2 value of about 7 (11). Oxazepam α-methyl-β-phenyl-propionate also showed this type of *in vivo* antagonism (30). However, evaluation of the intrinsic effect of these esters as BZ antagonists has been hampered by the agonist effects of oxazepam formed *in vivo*.

The bidirectional allosteric modulatory effect of BZ receptor ligands on the binding of the cage convulsant TBPS to the $GABA_A$ receptor-ionophore complex correlated with the efficacies of BZs (5). Under nonequilibrium conditions of TBPS binding, the enhancing effect of the agonist oxazepam on TBPS binding has been used to characterize *in vitro* the antagonist potency of racemic oxazepam α,α-dimethyl-β-phenyl-propionate (17). Fig. 4 shows that the antagonism by oxazepam α-methyl-β-phenyl-propionate resides predominantly in its 3*S*-stereoisomer, because in the concentration range of 10^{-7} to 10^{-6} M it prevented the enhancing effect of oxazepam. This *in vitro* potency corresponds to the brain concentrations that antagonize the antimetrazol effect of oxazepam in mice (30). All displacing potencies and *in vitro* efficacies of the 3*R*-stereoisomer (Table 1 and Fig. 4) can be accounted for by its contamination by the 3*S*-stereoisomer.

It is difficult to interpret the dual (enhancing and displacing) effects of oxazepam α-methyl-β-phenyl-propionate alone on TBPS binding (Fig. 4). Similar concentration-dependent dual effects were found for the α,α-dimethyl-β-phenyl-propionic ester of oxazepam (17) and for the quinolines PK 8165 and PK 9084 (31) and were explained by dual agonist and inverse agonist efficacies (31). A mixed agonist-antagonist character recently reported for some BZ esters (6) is compatible with the enhancement and antagonism seen in Fig. 4. The small GABA shift values of oxazepam esters (Table 1) also support a partial agonist or mixed agonist-antagonist character of the esters. A recent interesting model of the BZ receptors (10) might also account for the dual character of BZ esters by dual modes of fitting to the binding sites.

GABA shift values for oxazepam esters were similar within

each enantiomeric group, with those for 3*R*-esters being smaller (Table 1). This suggests that the orientation of the 3-substituent in binding rather than the chemical structure of the substituent beyond the 3-carbonyloxy moiety influences the *in vitro* efficacies of BZs. The (3*R*)-methoxy and (3*R*)-methyl derivatives also resulted in smaller GABA shift values than the corresponding 3*S*-enantiomers (Tables 2 and 4). This suggests that an axial orientation of the 3-substituent in binding reduces the agonist efficacies of BZs *in vitro*.

Photoaffinity labeling with flunitrazepam has been shown to reduce the displacing potencies of BZ agonists. The extent of this photo shift correlated with efficacies (5). However, some non-BZ ligands did not obey this correlation, suggesting that the photo shift might be characteristic of the chemical structures of the ligands rather than of the efficacies (32). It is interesting to test this issue with BZ enantiomers of identical chemical composition, where BZ receptor heterogeneity is least likely to obscure the results. The significantly lower photo shift value of the 3*R*-enantiomer, in agreement with its lower GABA shift value, supports lower agonist efficacies for the axial orientation of the 3-substituent in binding *in vitro*.

The distance between the center of the annellated benzo ring A and a proton-accepting moiety of the BZs (mid A-π) has been reported to correlate with the efficacies at BZ receptors; a larger distance appeared to be accompanied by reduced agonist efficacies (6, 7). The mixed agonist-antagonist character of some BZ esters has been attributed to changes in the mid A-π distance, due to the conformational flexibility of the ester group (7, 8). Oxazepam esters also share this flexibility; Dreiding stereochemical models showed that the distance of the ester carbonyl group (π_2) from ring A of oxazepam may vary between 5.2 and 7.2 Å for equatorial 3-substitution, but for the axial ester group it cannot surpass 4.9 Å. Further, the enantiomeric pairs do not have the disadvantage of conformational constraints via ring annellation (3) when the contributions of structural modifications themselves cannot be excluded. For (3*S*)-ester enantiomers, the mid A-π_2 distance is longer in receptor binding, yet the *in vitro* measures suggested greater agonist efficacies. All (3*S*)-oxazepam esters displayed greater GABA shift (Table 1) and photo shift (Table 5) values than the 3*R*-enantiomers. These data contradict the correlation referred to above (6, 7) between efficacies and mid A-π distance. Instead, our data support a very recent model of the BZ receptor, which has suggested the existence of an additional hydrogen bond (π_2) for BZ esters (8). This type of interaction tends to increase the antagonist character of BZs *in vitro*, as supported by the decreasing order of GABA shift values for the corresponding (3*S*)-methyl (2.7) (Table 4), (3*S*)-methoxy (2.2) (Table 2), and (3*S*)-acetoxy (1.5) (Table 1) derivatives. In other words, the binding contribution of the 3-alkoxy and especially the 3-carbonyloxy moieties can be augmented by GABA to a decreasing extent. The GABA shift values of oxazepam methyl ether being smaller than those for full agonists are in accordance with the recent finding that the δ-phenyl-butyl ether of oxazepam acted as a partial agonist *in vitro* on the nonequilibrium binding of TBPS (17).

This study illustrates the importance of ring conformations and group orientations in binding. For 3*R*-enantiomers bound with axial 3-substituents, GABA shift values smaller than those for 3*S*-enantiomers suggest lower agonist efficacies *in vitro* for all three types of 3-substituted BZs. The axial orientation of a 3-substituent in binding does not necessarily decrease potencies and efficacies of BZs simultaneously. This is supported by the GABA shift value for 3,3-dimethyl-DMD given in Table 4. Its low potency was associated with a high GABA shift value, equal to those of full agonists. Therefore, low affinities of BZs bound with axial 3-substituents can be mainly attributed to direct steric hindrance of binding, which exists for both (3*R*)-methyl- and 3,3-dimethyl-DMD. Moreover, the low GABA shift for (3*R*)-methyl-DMD indicates that GABA can alleviate steric hindrance less well in the axial direction. Lower *in vitro* agonist efficacies of (3*R*)-BZs may be related, for example, to the reduced stability of conformation M bound with axial 3-substituents, so that the compounds do not have a chance to elicit the proper conformational change in the receptor produced by agonists. When another 3-methyl substituent is added to the (3*R*)-methyl derivative, it restores the stability of the binding conformation M, and mutual facilitation with the GABA recognition site is also regained (Table 4).

CONCLUSIONS

Potency

Chirality in position 3 regulates the availability and stability of the binding conformation M.

Regarding the structure of the 3-substituent, alkyl groups exert steric hindrance whereas carbonyloxy and β-phenyl groups result in accessory binding.

Regarding the orientation of the 3-substituent, 3*R*-enantiomers bound with axial 3-substituents have greater susceptibilities both in steric hindrance and in accessory binding. The β-phenyl group binds equatorially. The 3-carbonyloxy moieties form additional hydrogen bonds, supposedly with a histidine moiety embedded into the ceiling of the BZ receptor cavity.

Efficacy

In vitro agonist efficacies of BZs are decreased by 3-oxy and 3-carbonyloxy groups, as well as by axial orientation of the 3-substituent upon binding to the receptor. Because the allosteric interactions of the $GABA_A$ receptor complex do not strictly correlate with efficacies of BZs *in vivo*, the conclusions should be restricted to efficacies for modulating the $GABA_A$ receptor complex *in vitro*. However, our data are consistent with the BZ antagonistic properties of phenyl-substituted esters of oxazepam in the antimetrazol test (11, 30).

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