Biochemical Pharmacology, Vol. 32, No. 4, pp. 603–607, 1983.
Printed in Great Britain.

0006-2952/83/040603-05 $03.00/0

BRAIN RECEPTOR BINDING AND LIPOPHILIC CHARACTER OF BENZODIAZEPINES

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(*Received* 3 *May* 1982; *accepted* 9 *August* 1982)

Abstract—A series of measurements of the potency of 26 benzodiazepines (BDZs) to inhibit the binding of [^{3}H]diazepam to rat brain synaptosomal membranes has been undertaken. All compounds studied are NO_2 or Cl 7-substituted, a class of BDZs known to have the highest biological activities and binding affinities (expressed as K_i). The results show that: (a) 2′-unsubstituted BDZs display a parabolic dependence of $-\log K_i$ values on lipophilic character of the molecule (expressed by means of the chromatographic R_m value); (b) 2′-halogen substituted BDZs show quite high binding affinities (k_i values in the range 2–5 nM) giving rise to another class of BDZs whose dependence on the lipophilic character remains to be studied; and (c) BDZs lacking the carbonyl oxygen at position 2 (see Table 1), and having an oxygen at position 4, show low or very low binding affinities (K_i values in the range 600–7000 nM). Moreover for the compounds under examination, statistically significant correlations have been obtained between $-\log K_i$ values and psychopharmacological activity data.

Benzodiazepines (BDZs) are a class of compounds widely employed in therapy due to their anxiolitic, sedative–hypnotic, anticonvulsant and muscle relaxant properties.

Recently, high-affinity, saturable, stereospecific binding sites for [^{3}H]diazepam displacement have been reported [1, 2] and it has been suggested that they represent a substrate by which BDZs produce their pharmacological actions. Several experimental findings have been reported in support of the hypothesis that these binding sites represent the receptors which *in vivo* mediate various physiological effects of BDZs: (a) the displacement of [^{3}H]diazepam is obtained only by pharmacologically active BDZs and the order of affinity for several of them to the specific recognition sites has been correlated in a statistically significant way [1, 3–6] to their potency *in vivo* in animal tests, many of the exceptions being connected with BDZs having metabolization problems; (b) the concentration of BDZs necessary for half saturation of the binding site membranes corresponds to the human plasma concentration during their clinical use [7]; and (c) no other neurotransmitter or psychotropic drug similar in action to BDZs interacts in a significant way with their recognition sites [8].

In recent years a great deal of work has been carried out on BDZs' structure–activity relationships. The results are not quite satisfactory and the main reasons are to be found in the fast metabolization of BDZs *in vivo*, with formation of derivatives which often display, in their turn, the same kind of activity as the parent drugs. To avoid this drawback, we measured the potency of several BDZs to inhibit the binding of [^{3}H]diazepam to synaptosomal rat brain membranes, a kind of experiment in which metabolization has been proven not to occur [5].

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This was done in order to correlate the binding affinities to the lipophilic character (expressed by means of the chromatographic R_m values [9, 10]) and to the structural features of the BDZs under examination.

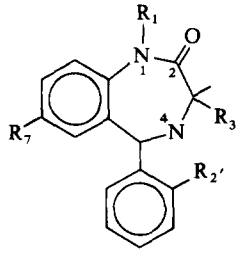
MATERIALS AND METHODS

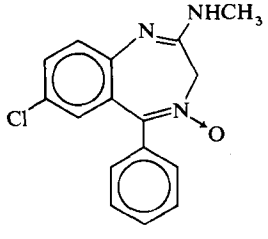
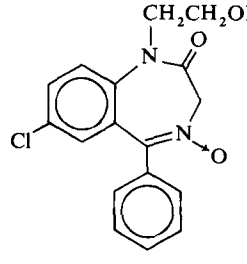
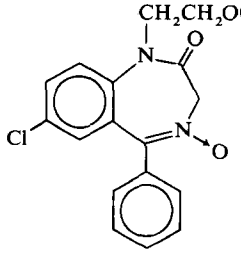
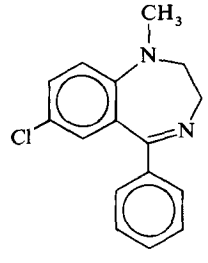
Assay for specific [^{3}H]*diazepam binding inhibition.* The tissue preparation and the binding assay have been carried out essentially according to Squires and Braestrup [1]. Crude synaptosomal membranes were prepared from whole forebrains, excluding cerebellum and pons-medulla, of 100–120 g Wistar male rats by homogenization in 15 vols of 0.32 M sucrose using a Teflon–glass apparatus. The crude P_2 synaptosomal fraction was obtained by centrifuging the homogenate at 1000 *g* for 10 min and by recentrifuging for 20 min at 20,000 *g*; the P_2 fraction was carefully suspended in 25 vols of 50 mM Tris–HCl buffer (pH 7.4). The binding assay consisted of 0.7 ml of P_2 suspension, 2.84 nM [^{3}H]diazepam [New England Nuclear (80 Ci/mmole)] and the compound to be tested. The total assay volume was 1 ml and the final Tris–HCl (pH 7.4) buffer concentration was 50 mM.

The assay was initiated by the addition of [^{3}H]diazepam followed by a 20-min incubation at 4° and was terminated by filtration through Whatman GF/B glass filters under suction. The filters were washed 3 times with 5 ml of ice-cold buffer, dried and counted in 10 ml of acidified Instagel (Packard) by liquid scintillation counting. Non-specific binding was determined by incorporation of 3 μM unlabeled diazepam in the assay and represents 7–9% of the total binding. To determinate IC_{50} values (i.e. values for 50% inhibition of specific [^{3}H]diazepam binding) of the BDZs under examination the compounds were added, in triplicate, to the binding assay at at least six different concentrations.

The IC_{50} values were calculated by probit analysis.

Table 1. Structure, R_m* and K_i values of benzodiazepines



Compound No.	R_7	R_1	R_3	$R_{2'}$	R_m	K_i (nM)
1 Nitrazepam	NO_2	H	H	H	1.470	13.6
2	NO_2	$CONHCH_3$	H	H	1.457	13.6
3 Diazepam	Cl	CH_3	H	H	1.952	14.0
4	Cl	H	H	H	1.772	15.0
5 Temazepam	Cl	CH_3	OH	H	1.580	38.0
6	Cl	H	$OCOCH_2CH_2COOH$	H	1.332	38.0
7 Oxazepam	Cl	H	OH	H	1.510	38.0
8	Cl	CH_2CH_2OH	OH	H	1.345	67.9
9	Cl	CH_2CH_2Cl	OC_2H_5	H	2.322	95.1
10	Cl	H	$OCOC_6H_5(OCH_3)_3$	H	2.430	119.6
11	Cl	CH_2CF_3	H	H	2.540	135.8
12 Prazepam	Cl	CH_2—cC_3H_5	H	H	2.410	137.8
13	NO_2	CH_2CH_2OH	OH	H	0.868	353.3
14	Cl	H	$OCOCH(CH_2CH_2CH_3)_2$	H	3.012	1712
15	Cl	H	H	Cl	1.939	2.40
16	Cl	CH_3	H	F	1.905	2.45
17	Cl	H	OH	F	1.481	2.45
18 Lorazepam	Cl	H	OH	Cl	1.645	2.72
19	Cl	CH_3	OH	Cl	1.818	2.72
20	Cl	CH_3	OH	F	1.621	3.53
21	Cl	CH_3	H	Cl	2.150	4.35
22	Cl	H	H	F	1.732	5.10
23 Chlordiazepoxide					1.813	610.0
24					1.000	2000
25					0.797	7000
26 Medazepam					2.648	3710

* The R_m values are taken from Ref. 9.

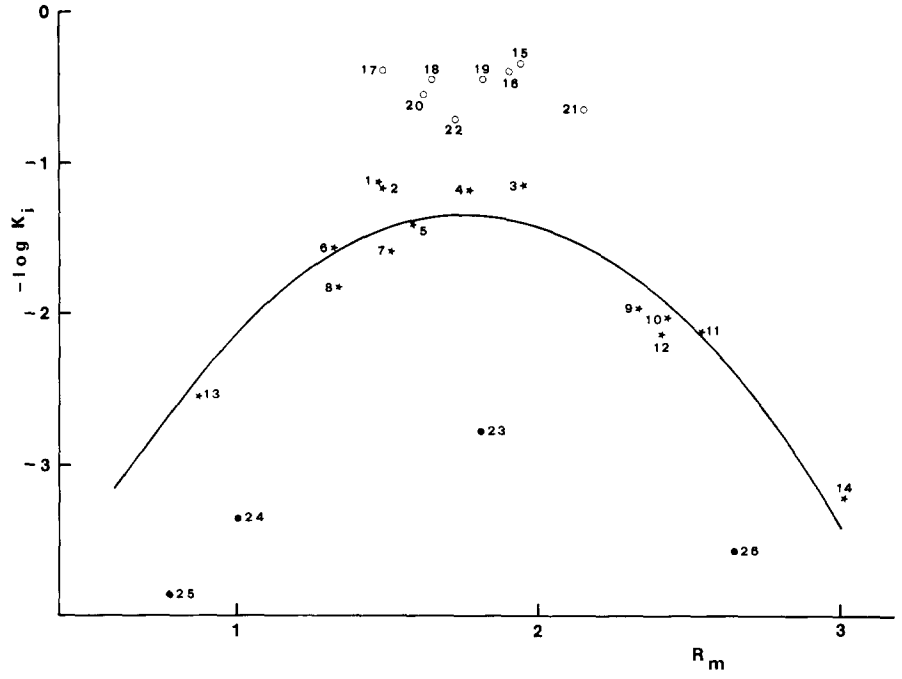


Fig. 1. Correlation between R_m and K_i values (ordinate $-\log K_i$) for [^{3}H]diazepam displacement. ★ = 2′-unsubstituted benzodiazepin-2-one derivatives; ○ = 2′-halogen (Cl or F) substituted benzodiazepin-2-one derivatives; ● = benzodiazepines lacking the C_2=O group and/or N_4-substituted. The numbering scheme corresponds to that of Table 1. The curve shown in the figure belongs to compounds 1–14 only.

The competitive nature of ligand interaction was tested, for compounds under examination, according to Mohler and Okada [11]. So, instead of IC_{50} values, we could use K_i, inhibitor constant, values. K_i values were calculated using the equation $K_i = IC_{50}/(1 + C/K_d)$, where C = concentration of the radioligand (2.84 nM) and K_d = affinity constant (3.4 nM). They present, with respect to the IC_{50} values, the advantage of being independent of the radioligand concentration used; low K_i values mean high receptor affinities. The BDZs used in the experiments were a kind of gift of Drs M. Gaiardi and M. Bartoletti, Istituto di Farmacologia, University of Bologna, Italy.

R_m *values.* The R_m values for BDZs under examination, obtained by Biagi, coworkers and one of the present authors, were taken from Ref. 9. They were measured by means of a reversed-phase TLC which allowed the partitioning of BDZs between a polar mobile phase and a non-polar stationary phase. The mobile phase consisted of H_2O in various mixtures (v/v) with acetone. The stationary phase was obtained by impregnating with 5% (v/v) silicon oil solution in ether a layer of silica gel GF_{254} (Merck Co., Darmstadt, F.R.G.). R_m values, together with the K_i values and the chemical structure of BDZs are reported in Table 1.

RESULTS AND DISCUSSION

The 26 BDZs we have taken into account can be, from a chemical point of view, divided into three sets: set A (compounds 1–14 of Table 1) (consisting of benzodiazepin-2-one derivatives unsubstituted at position 2′); set B (compounds 15–22 of Table 1) [consisting of 2′-halogen (Cl or F) substituted benzodiazepin-2-one derivatives]; and set C [consisting of BDZs lacking the carbonyl group at position 2 of the diazepine ring (medazepam and chlordiazepoxide) as well as N_4-substituted BDZs (compounds 24 and 25 of Table 1)].

All BDZs studied were NO_2 or Cl 7-substituted, a class of compounds known to have the highest biological activities and binding affinities [1, 3, 12] and were variously substituted in positions 1 and 3 of the diazepine ring.

In Fig. 1 the $-\log K_i$ values vs the R_m values for the 26 compounds under examination are plotted.

Fig. 1 shows that for BDZs belonging to set A the receptor affinities seem to be related in a parabolic way to their lipophilic character; for the other compounds no clear relationship appears to exist between $-\log K_i$ and R_m values; all BDZs belonging to set B show very high receptor affinities and lie above the parabola traced for compounds 1–14 while all BDZs belonging to set C, on the contrary, display lower receptor affinities and lie below the same parabola.

If we carry out a regression analysis to correlate in a quantitative way the $-\log K_i$ values for compounds 1–14 with their R_m values, we obtain the following equation:

$$-\log K_i = -5.542 + 4.751(\pm 0.720)R_m - 1.344(\pm 1.842)R_m^2; \quad (1)$$

$n = 14$; $s = 0.236$; $r = 0.934$; $F = 37.48$; $P < 0.001$.

Equation (1) shows that the receptor affinities for BDZs under examination depend parabolically in a statistically significant way on the R_m values ($t_{R_m} = 6.69$, $P < 0.001$: $t_{R_m^2} = 7.37$, $P < 0.001$), an expression of the lipophilic character of the compounds [10].

Owing to the fact that the parabolic nature of the curve depends strongly on BDZs 13 and 14 (see Fig. 1), to check further the goodness of the hypothesis of a parabolic relationship between R_m and $-\log K_i$ values, we carried out the same regression analysis for BDZs 1–12 only. The following equation was obtained:

$$-\log K_i = -7.117 + 6.619(\pm 2.214)R_m - 1.866(\pm 0.572)R_m^2; \quad (2)$$

$n = 12$; $s = 0.227$; $r = 0.863$; $F = 13.08$.

The statistical parameters are in this case obviously worse (due to the narrower range of R_m and $-\log K_i$ values); however, the regression is significant at the $P < 0.01$ level.

The positive coefficient associated with R_m can be explained by the following considerations: when an organic drug molecule is placed in aqueous solution a loose envelope of water molecules surrounds it and when the drug molecule is removed from the aqueous phase into a lipidic phase, this orderly shell of water molecules is disrupted with a consequent entropy increase which is well known as one of the prominent factors of hydrophobic binding [13].

The negative R_m^2 term, which takes into account the descending branch of the parabola, is to be explained by considering the non-specific adsorption on the lipidic membranes which increases with the increasing of the drug's lipophilic character.

Partial differentiation of equation (1) with respect to R_m leads to a value of $R_{m\max}$ of 1.767 where $R_{m\max}$ is the value of R_m corresponding to the maximum of binding, a value very close to that suggested by Hansch as the optimum of lipophilicity for CNS active drugs [13].

All BDZs of set B (compounds 15–22) show affinities for the recognition sites much higher than those displayed by compounds belonging to sets A and C (K_i values in the range 2–5 nM) and this is in agreement with the fact that BDZs substituted at position 2′ by electron-withdrawing atoms or groups of small steric hindrance behave, from a quantitative point of view, in a different way from the 2′-unsubstituted ones. It is well known that they display, under the same conditions, greater receptor affinities and greater biological activities [3, 12, 14]. Compounds 15–22 can be included into the regression analysis carried out for BDZs belonging to set A by the use of an indicator variable ($I_{2'}$) taking into account the effect of halogen substitution at position 2′. Such a variable assumes the values of 1 when a chlorine or fluorine atom is present at position 2′ and of 0 otherwise. The indicator variables are usually introduced into multiple regression equations when the number of substituents in a given position of the drug molecule is too small to allow the use of a continuous variable.

By taking into account BDZs 15–22 we obtain the following regression equation:

$$-\log K_i = 5.520 + 4.725(\pm 0.610)R_m - 1.337(\pm 0.155)R_m^2 + 0.914(\pm 0.100)I_{2'}; \quad (3)$$

$n = 22$; $s = 0.204$; $r = 0.973$; $F = 105.42$; $P < 0.001$.

Equation (3) shows a highly significant dependence of $-\log K_i$ values on R_m ($t_{R_m} = 7.75$, $P < 0.001$),

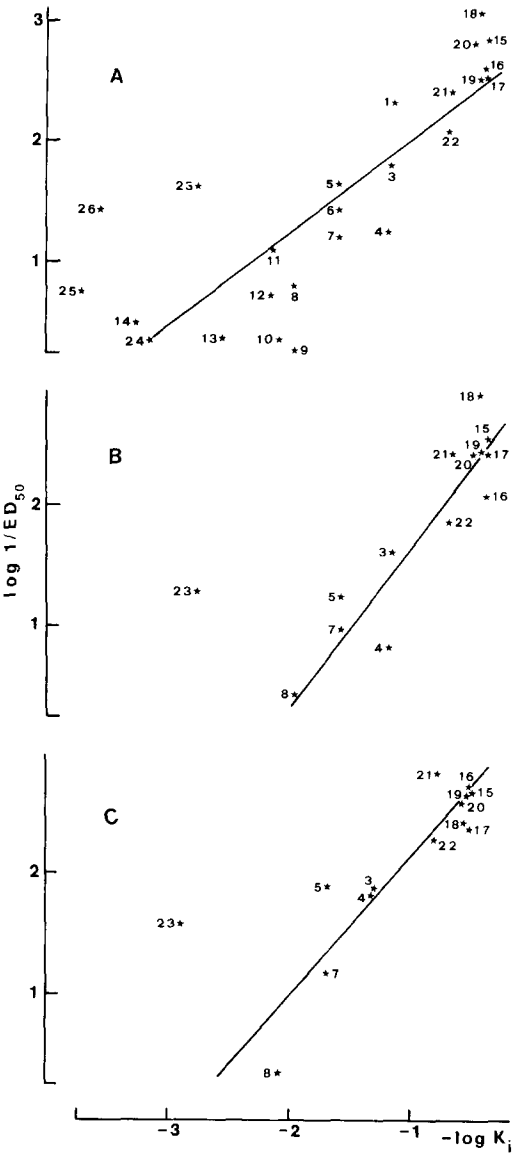


Fig. 2. Correlation of K_i for [^{3}H]diazepam displacement on the one hand (abscissa $-\log K_i$) and (A) exploratory behaviour test [ED_{50} (mmoles/kg i.p.)]; including all compounds (N = 25), $r = 0.750$; excluding medazepam and chlordiazepoxide, $r = 0.870$. (B) Conflict behaviour test (unpunished schedule) [ED_{50} (mmoles/kg i.p.)]; including all compounds (N = 14), $r = 0.778$; excluding chlordiazepoxide, $r = 0.919$. (C) Conflict behaviour test (punished schedule) [ED_{50} (mmoles/kg i.p.)]; including all compounds, $r = 0.773$; excluding chlordiazepoxide, $r = 0.902$. The numbering scheme corresponds to that of Table 1. Pharmacological activity data were taken from Ref. 9.

Table 2. Correlation between '*in vitro*' inhibition of [^{3}H]diazepam binding to rat brain synaptosomal membranes (K_i) and '*in vivo*' tests of pharmacological potency of several benzodiazepines

Test for benzodiazepine potency*	Spearman rank order correlation coefficient	Probability (one-tailed test)
Exploratory behaviour (N = 25)	0.830	<0.01
Conflict behaviour (unpunished schedule) (N = 14)	0.846	<0.01
Conflict behaviour (punished schedule) (N = 14)	0.792	<0.01

* Pharmacological activity data were taken from Ref. 9.

R_m^2 ($t_{R_m^2} = 8.61$, $P < 0.001$) and $I_{2'}$ ($t_{I_{2'}} = 9.13$, $P < 0.001$) terms and the positive sign associated with the $I_{2'}$ term indicates, as already mentioned, the positive influence on binding of halogen substitution at position 2'.

Partial differentiation of this equation with respect to R_m leads to the same $R_{m\max}$ of 1.767 as obtained by differentiation of equation (1). It may be of some interest to remark that a very similar regression equation has been obtained by Biagi *et al.* [9] by correlating the physicochemical properties of a slightly different series of BDZs with psychopharmacological activity data. All compounds belonging to set C display low or very low receptor affinities (K_i values in the range 600–7000 nM). This fact is consistent with the hypothesis [15, 16, *] according to which the primary BDZ–receptor interaction would be determined by electrostatic interaction acting via a hydrogen bonding mechanism implying that the $C_2{=}O$ group and N_4 atom of the diazepine ring are hydrogen bond acceptors.

The fact that compounds, like medazepam and chlordiazepoxide, belonging to set C are biologically active *in vivo* can be explained by pharmacokinetic data: it has been proved that these compounds are metabolized to the corresponding benzodiazepin-2-one derivatives and that the oxygen of the N→ O group is lost [17].

As a last step in this study, we have correlated the K_i values with psychopharmacological activity data as obtained in the rat. Activity data have been taken from Ref. 9. Fig. 2 reports the correlations obtained for [^{3}H]diazepam displacement (abscissa $-\log K_i$) and: (A) exploratory behaviour test, (B) conflict behaviour test (unpunished schedule), and (C) conflict behaviour test (punished schedule). The rank order correlation between the *in vivo* tests and the *in vitro* potency of BDZs is reported in Table 2. The positive statistically significant correlations obtained are strong further evidence that the brain BDZ receptors mediate the psychopharmacological effects of these compounds [6, 18, 19].

The largest discrepancies between *in vivo* and *in vitro* data (see, for example, medazepam and chlordiazepoxide) are, also in this case, most properly explained by considering the pharmacokinetics of these compounds.

Acknowledgements—This work was financially supported by C.N.R. (Rome); Progetto Finalizzato Chimica Fine e Secondaria. The authors thank Mrs G. Bertocchi and R. Rescazzi for technical assistance.

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