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POLYCLONAL ANTIBODIES TO AGONIST BENZODIAZEPINES

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Abstract—Benzodiazepine-binding, immunoglobulin G class antibodies have been raised in three rabbits immunised with a conjugate of kenazepine coupled to keyhole limpet haemocyanin. The antibodies were assayed by [^{3}H]flunitrazepam binding, followed by adsorption onto *Staphylococcus aureus* cells. Measurement of the rates of association and dissociation of [^{3}H]flunitrazepam binding, together with saturation analysis of equilibrium binding, revealed varying degrees of heterogeneity in the affinity constants of the three rabbit antisera (equilibrium K_D values 0.18 to 4.13 nM at 20–22°). Specificity of the antibodies was investigated by testing a wide variety of compounds (at concentrations of up to 10–100 μM) for their ability to inhibit [^{3}H]flunitrazepam binding. Only benzodiazepines known to act as agonists at their receptor sites in the central nervous system (CNS) caused an inhibition of binding. The rank orders of the IC_{50} values of these drugs for inhibition of [^{3}H]flunitrazepam binding to IgG from two out of the three rabbits correlated significantly with that previously published for displacement of CNS receptor binding. The agonist β-carboline derivative ZK 93423, the anxiolytic cyclopyrrolones suriclone and zopiclone and the purines inosine and hypoxanthine all failed to inhibit antibody binding, supporting previous suggestions that these drugs may bind at non-benzodiazepine recognition sites on the CNS receptor.

The antibodies described are expected to provide useful reagents for raising anti-idiotypic antibodies directed against the CNS receptor and for the identification and purification of possible endogenous benzodiazepine receptor agonists in the CNS.

Anxiolytic, anticonvulsant, sedative and muscle relaxant actions of the benzodiazepines are thought to be mediated by specific receptor binding sites in the central nervous system (CNS; [1, 2]). Demonstration of these binding sites has prompted the discovery of a variety of benzodiazepine receptor ligands, some of which share the above pharmacological actions of the conventional benzodiazepine tranquillisers and act as agonists at the receptor, whereas others have proconvulsant and anxiogenic actions and have become known as inverse agonists [3]. Ligands have also been found which appear to bind as pure antagonists at the receptor (see Discussion).

In the present paper, we report the development of polyclonal rabbit antibodies specific for those benzodiazepines which act as agonists at the CNS receptor. These antibodies have been raised for two reasons; (1) To aid in the detection and affinity purification of possible endogenous benzodiazepine receptor ligands in CNS tissue. (2) To act, in turn, as immunogens for the development of anti-idiotypic antibodies [4] directed against the recognition site of the CNS benzodiazepine receptor. In order for the above approaches to succeed, the idiotypic, benzodiazepine-binding antibodies should bind these drugs in much the same way as the receptor itself. We have tried to ensure such an interaction by using as our hapten the alkylating benzodiazepine kenazepine [5] coupled to the antigenic protein keyhole limpet haemocyanin through position 1 of the benzodiazepine nucleus, the only position on which substituents have little effect on receptor binding affinity [6–8].

Rabbit antisera to benzodiazepines have been raised before [9, 10] and have recently been supplemented by the availability of mouse monoclonal antibodies [11]. However, all these antibodies have been raised against haptens coupled to an immunogenic protein through positions 3, 7, 3′ or 4′ of the benzodiazepine nucleus (Fig. 1), substitution on any of which is known to influence agonist potency (see Discussion). In comparison to the antibodies described in the present paper, such antibodies display fewer similarities to the recognition site of the CNS benzodiazepine receptor.

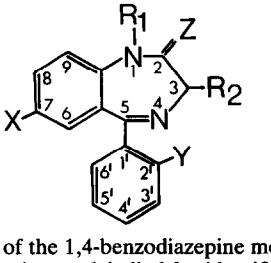

Fig. 1. Structure of the 1,4-benzodiazepine molecule, with positions and substituents labelled for identification of the compounds listed in Table 1.

MATERIALS AND METHODS

The kenazepine was prepared by bromacetylation of Ro7-1986 essentially according to Williams *et al.* [5]. Briefly, the reaction was carried out as follows. One hundred milligrams of Ro7-1986 together with a 6 molar excess of sodium bicarbonate was partitioned between 10 ml water and 25 ml chloroform by vigorous stirring for 15 min at room temperature. The mixture was then cooled in an ice bath. Bromoacetic anhydride (1.4 molar excess to Ro7-1986) was then added in 2 ml chloroform and the reaction was allowed to proceed for 30 min on ice. The organic phase of the reaction mixture was separated, dried over anhydrous sodium sulphate, and evaporated to dryness to afford essentially pure kenazepine. Kenazepine was dissolved in phosphate buffered saline (PBS) and 120 μl was added to 750 μl of keyhole limpet haemocyanin in the same buffer. The final concentration of kenazepine in the reaction mixture was 5 mM and the concentration of keyhole limpet haemocyanin was 14.4 μM. The reaction was allowed to proceed overnight at 30°. Progress of the reaction was monitored by the assay of free kenazepine remaining in the reaction mixture by extraction of 10 μl aliquots into 200 μl ethyl acetate and subsequent quantitation by HPLC, after dilution of the organic phase with an equal volume of methanol. The separation was carried out on a 250 mm × 3 mm i.d. RP-8 column with an elution buffer consisting of 60% 70 mM sodium phosphate buffer, pH 7.0, containing 25 mM B8 ion-pairing reagent and 0.1 mM EDTA together with 40% methyl cyanide. The column was run at room temperature at a flow rate of 2 ml min^{-1}; under these conditions the kenazepine had a retention time of 6.0 min. The detection was by u.v. at 250 nm. We estimated, using an appropriate control reaction mixture lacking only the keyhole limpet haemocyanin, that the final conjugate contained approximately 90 molecules of kenazepine to 1 molecule of keyhole limpet haemocyanin.

Immunisation. Serum samples were obtained from three New Zealand Red rabbits, bred in the animal house at University College London and aged 6 months at the time of first immunisation. All three animals received initial intradermal injections of the kenazepine-keyhole limpet haemocyanin (KNZ-KLH) conjugate (total dose 500 μg) dissolved in 1 ml of PBS and emulsified with 1 ml of Freund's complete adjuvant. Seven weeks later 250 μg of the conjugate was given in the same volume of incomplete adjuvant. A first bleed was taken 11 days after the latter injection. The animals were left for six weeks and then given an intravenous injection of 25 μg KNZ-KLH conjugate dissolved in 0.2 ml PBS, preceeded by 0.1 ml of a 10 mg ml^{-1} solution of chlorphèniramine B.P. A second bleed was taken 13 days later.

Immunoassays. Serum dilutions were incubated at room temperature (20–22°) in PBS containing bovine serum albumin (0.1%, w/v), to reduce non-specific adsorption and thimerosal (0.01%, w/v), to prevent microbial growth (PAM buffer). Benzodiazepine binding was detected by addition of the ligand [^{3}H]flunitrazepam. Incubations containing no serum dilution served as assay blanks, which always amounted to less than 2% of the total ligand bound.

The [^{3}H]flunitrazepam bound to antibody was separated from free ligand in two ways: (1) by the addition of *Staphylococcus aureus* cells to adsorb immunoglobulin G (IgG) class antibodies [12], as described below; (2) by the addition of $(NH_4)_2SO_4$ to 50% of saturation to precipitate all immunoglobulins [13]. In the latter case, bovine γ-globulin (0.1 mg ml^{-1}) was included in the incubations as a carrier protein. The incubation was mixed thoroughly with an equal volume of saturated $(NH_4)_2SO_4$ solution and left for at least 30 min to ensure complete precipitation of immunoglobulins. The precipitate was then pelleted by centrifugation (12,000 *g*; 4 min), washed by resuspension in 1 vol. of 50% saturated $(NH_4)_2SO_4$ solution, pelleted again and then redissolved in 1 vol. of PBS, 50 μl of which was taken for measurement of bound radioactivity. In the majority of experiments, however, bound ligand was separated by adsorption of the IgG class antibodies onto formalin-fixed, heat-killed *S. aureus* cells, prepared as a 10% (v/v) suspension according to Kessler [12] and stored at −20°. Before use the cells were thawed and washed by centrifugation (12,000 *g*; 1 min) and resuspension in PBS containing 0.5% (v/v) Nonidet P-40. The cells were left in this detergent solution for 15 min at room temperature, then washed three times in PBS and finally resuspended in PAM buffer, in which they could be stored at 4° for several weeks. On the day of each assay, the cells were washed again three times in PBS before use.

To estimate the titre of benzodiazepine binding IgG, serum dilutions (1:100 to 1:1,000,000) were incubated for 3 hr with [^{3}H]flunitrazepam (0.5 nM) in a total volume of 0.5 ml. To each tube was then added 50 μl of a 10% (v/v) suspension of *S. aureus* cells. Contents of the tubes were mixed well and left for at least 10 min. The IgG adsorbed onto the *S. aureus* could then be isolated by addition of 3 ml ice-cold PBS to each tube followed by immediate filtration through glass-fibre filters under vacuum. Tubes were rinsed with a further 3 ml of PBS and each filter washed with an additional 5 ml of this ice-cold buffer. Filters were then placed in plastic insert vials and 3 ml of detergent/scintillant mixture added for measurement of radioactivity by scintillation counting. These experiments served to estimate the appropriate dilution of serum for 50% binding of added [^{3}H]flunitrazepam (at 0.5 nM) to the anti KNZ-KLH IgG (see Results), serum dilutions which were employed in subsequent assays to investigate the kinetics and specificity of benzodiazepine binding. For reasons of economy, when assaying these more dilute serum samples the *S. aureus* suspension was added to a final concentration of only 0.01% (v/v), pilot experiments having shown that this concentration of cells was sufficient to adsorb all of the [^{3}H]flunitrazepam binding IgG.

In order to study the rate of onset of [^{3}H]flunitrazepam binding to anti KNZ-KLH IgG, serum was first diluted in PAM buffer containing *S. aureus* (0.01%, v/v). Association with [^{3}H]flunitrazepam could then be initiated by the addition of this ligand at a final concentration of 0.5 nM and 500 μl samples

of the continuously mixed incubation taken at various time intervals for vacuum filtration through prewashed glass-fibre filters. Dissociation of bound [^{3}H]flunitrazepam was studied by pre-incubating serum dilutions in PAM buffer containing *S. aureus* (0.01%, v/v) and [^{3}H]flunitrazepam (0.5 nM) for 2 hr. At time zero, an excess of unlabelled flunitrazepam (1 μM) was added and 500 μl samples taken for vacuum filtration at various time intervals thereafter. For both the onset and offset experiments, filtration of incubation mixture was followed by a wash with 2 × 0.5 ml ice-cold PBS and filters prepared for scintillation counting as described above. Blanks containing the above reagents but no serum were included at all time points.

Saturation of equilibrium binding was investigated by incubating serum dilutions for 2 hr in 0.5 ml PAM buffer containing a range (0.04–45.22 nM) of [^{3}H]flunitrazepam concentrations. Binding was determined by the addition of 50 μl *S. aureus* suspension at 0.1% (v/v) followed by vacuum filtration, as described above for the measurements of IgG titre. Concentrations of free, unbound [^{3}H]flunitrazepam were measured in a parallel series of incubations, which were centrifuged (12,000 *g*, 4 min), rather than filtered, after the addition of the *S. aureus* cell suspension. Samples (50 μl) of supernatant fluid could then be taken for measurement of unbound radioactivity. These estimates agreed well with those obtained by subtracting bound [^{3}H]flunitrazepam from the total amount added.

The ability of a variety of drugs and putative transmitters to inhibit [^{3}H]flunitrazepam binding to the anti KNZ-KLH IgG was estimated by pre-incubating serum dilutions for at least 2 hr, in 0.4 ml PAM buffer, containing the unlabelled compound under investigation. [^{3}H]Flunitrazepam was then added to a final concentration of 0.5 nM in 0.5 ml and the incubation continued for a further 2 hr. The IgG was isolated by the addition of 50 μl *S. aureus* suspension at 0.1% (v/v) followed by vacuum filtration, as described above.

Analysis of data. Results from saturation binding assays were fitted by an iterative non-linear least squares procedure [14] to give estimates of the predominant equilibrium dissociation constant(s) or K_D and maximal number of [^{3}H]flunitrazepam binding sites (B_{max}) in each serum sample. The rate of association of [^{3}H]flunitrazepam with antibody could then be plotted as $\log_{10}L_0/Ab_0$ $[(Ab_0\text{-}AbL)/(L_0\text{-}(AbL + BlL)]$ against time, a second order kinetic [15], where Ab_0 is the initial concentration of antibody binding sites given by the above B_{max} value, L_0 is the initial concentration of free ligand, AbL the concentration of antibody–ligand complex and BlL the concentration of blank ligand binding. These plots were only attempted for the initial 2 min of the reaction, when dissociation of bound ligand was known to be minimal. Kinetics of dissociation were plotted as $\log_{10}$ % initial AbL against time. Another non-linear least squares fitting programme (Simplex algorithm) was used to resolve these association and dissociation plots into the optimum number of components and provide estimates of association (k_f) and dissociation (k_r) rate constants.

Data from experiments in which [^{3}H]flunitrazepam binding was displaced by unlabelled compounds over a range of concentrations could be transformed by the Hill equation [16] into linear plots. A linear least-squares fit of such plots gave estimates of the concentrations for 50% displacement (IC_{50}), with 95% confidence limits.

Significance of the correlations between potencies for displacement of [^{3}H]flunitrazepam binding to antibody and to CNS receptor was evaluated by calculating the Spearman rank correlation coefficient [17].

Materials. [^{3}H]Flunitrazepam (77.4–87.3 Ci/mmol; 2.86–3.23 TBq/mmol) was purchased from Amersham or New England Nuclear. The scintillation fluid employed for measurement of radioactivity was Optiphase X (L.K.B.) and the glass microfibre paper used for filtration assays was Whatman GF/B. Heat-killed, formalin-fixed suspensions of *Staphylococcus aureus*, prepared according to Kessler [12], were a gift from M. Ginsburg (I.C.R.F. Laboratories, Lincoln's Inn Fields, London) and Freund's complete and incomplete adjuvant were obtained from Miles Laboratories. All other reagents were A.R. grade except for bovine serum albumin (Fraction V, B.D.H.), bovine γ-globulin (Fraction II, labile enzyme free, Miles Laboratories), Nonidet P-40 (octylphenol ethylene oxide condensate, B.D.H.), thimerosal (sodium ethylmercurithiosalicylate, Sigma) and the drugs listed in the following section.

Drugs. Drugs were dissolved at 1 or 10 mM in water, PBS, methanol or dimethylformamide and then diluted in PBS to the concentrations given in the text. Sources of drugs are listed below and grouped according to the initial solvent used. Structures of benzodiazepines are given in Table 1.

Dissolved in water: γ-aminobutyric acid (Sigma), bicuculline methobromide (Cambridge Research Biochemicals), chlordiazepoxide HCl (Roche; gift), clorazepate diK (Boehringer Ingelheim; gift), harmaline HCl and harmane HCl (Sigma), harmine (Koch-Light), hypoxanthine and inosine (Sigma), midazolam maleate (Roche; gift), pentobarbital Na (Sigma), picrotoxin (Koch-Light), Ro14-7437 (Roche; gift), strychnine SO_4 (Sigma), valproate Na (Reckitt & Colman; gift).

Dissolved in PBS: flurazepam HCl (Roche; gift).

Dissolved in methanol: 2-amino-5-nitrobenzophenone (Aldrich), bromazepam (Roche; gift), β-carboline-3-carboxylate ethyl ester (βCCE; synthesised by I. L. Martin), cartazolate (Abbot Laboratories; gift), CGS 8216 (2-phenylpyrazolo [4.3-c] quinolin-3[5H]-one and CL 218,872 (3-methyl-6-[3-(trifluoromethyl)phenyl]-1,2,4-triazolo [4,3-b] pyridazine) (Cyanamid; gift), clobazam (Hoechst; gift), clonazepam, diazepam and flunitrazepam (Roche; gift), glycyl-glycyl-*N*-methylamino-5-nitrobenzophenone (Shionogi Research Laboratories; gift), lorazepam and lormetazepam (Wyeth Research; gift), medazepam and nitrazepam (Roche; gift), metaclazepam HCl (Kali Chemie; gift), 5-nitroanthranilinonitrile (Aldrich), prazepam (Warner Lambert; gift), Ro5-4864, Ro5-2181, Ro5-3448, Ro7-1986, Roll-6893/6896, Roll-7800, Ro15-1788 (Roche; gift), R5135 (3-hydroxy-16-imino-5-17-aza-androston-11-one) (Roussel; gift), tifluadom HCl

(Sandoz; gift), ZK 93423 (6-benzyloxy-4-methoxymethyl-β-carboline-3-carboxylate ethyl ester) (Schering; gift).

Dissolved in dimethylformamide: suriclone (6-(2-chloro-7-naphtyridine[1, 8]yl)-5-[(4-methyl-1-piperazinyl)carbonyloxy]-7-oxo 2,3,6,7-tetrahydro dithiinno [1,4] [2,3-c] pyrrole) and zopiclone (6-(5-chloro-2-pyridyl)-5-(4-methyl-1-piperazinyl)-carbonyl-oxy-7-oxo-6,7-dihydro-5*H*-pyrrolo [3,4-b] pyrazine) (Rhône-Poulenc; gift).

RESULTS

Production of benzodiazepine binding antibodies

All three rabbits immunised with the kenazepine–keyhole limpet haemocyanin (KNZ–KLH) conjugate yielded [^{3}H]flunitrazepam binding antibodies at a high titre. This binding was detected by adsorption of immunoglobulins onto *S. aureus* cells and so reflected the content of immunoglobulin G (IgG) class antibodies. Only a slight increase in titre was seen between the first and second bleeds and all subsequent experiments employed serum from the later bleed. Using this bleed, serum dilutions for 50% binding of [^{3}H]flunitrazepam, added at 0.5 nM, were approximately 1:5000, 1:2000 and 1:4500 for rabbits 1, 2 and 3 respectively. These serum dilutions were used in subsequent experiments to determine the kinetics and specificity of benzodiazepine binding. At these dilutions, pre-immune serum from all three rabbits bound less than 0.06% of the ligand. Comparison of [^{3}H]flunitrazepam binding activity measured in immune serum by precipitation of all immunoglobulins with 50% $(NH_4)_2SO_4$ with that detected by adsorption of IgG onto *S. aureus* cells, indicated that IgG class antibodies accounted for all of the high affinity binding in the antiserum of rabbit 1, 90% in rabbit 2 and 88% in rabbit 3. For convenience, the latter adsorption method has been employed in further assays.

Kinetics of [^{3}H]*flunitrazepam binding*

Figure 2a shows the onset of [^{3}H]flunitrazepam binding to the anti KNZ–KLH IgG of rabbit 2 to be rapid, reaching at least 50% of maximum within 3 min of addition of the ligand (at 0.5 nM; 20–22°), and equilibrating within 2 hr. Also shown in this figure is a second order kinetic plot of the initial rates of binding, suggesting a multiplicity of association rate constants. Dissociation of binding was slower, 30 min being required for 50% displacement of [^{3}H]flunitrazepam with an excess (1 μM) of unlabelled ligand (Fig. 2b). As found for the onset of binding, analysis of the offset data suggested a range of dissociation rate constants. Comparable results (not shown) were obtained with the anti-KNZ–KLH IgG from rabbits 1 and 3.

The distribution of [^{3}H]flunitrazepam binding affinities in the three rabbit antisera was also evaluated by performing saturation assays, in which the serum was incubated to equilibrium with increasing concentrations of [^{3}H]flunitrazepam (from 0.04 to 45.22 nM). Scatchard plots revealed a greater heterogeneity in rabbit 2 than in rabbits 1 and 3. Indeed, estimates of binding parameters for these Scatchard plots gave a best fit for a single site model for rabbit

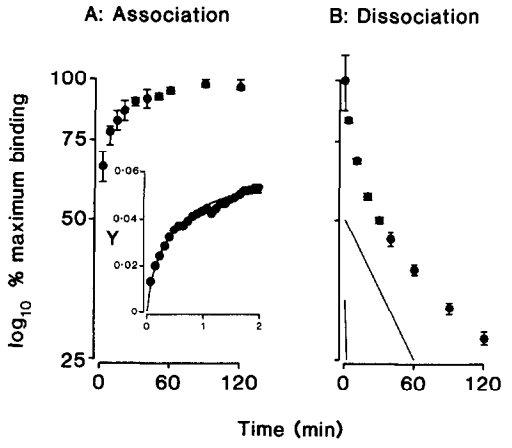

Fig. 2. Association and dissociation of [^{3}H]flunitrazepam binding to anti KNZ–KLH IgG from rabbit 2. The percentage of maximal binding at equilibrium is plotted on a logarithmic scale against time (A) after addition of the ligand (0.5 nM) or (B) after addition of excess unlabelled flunitrazepam (1 μM) to a previously equilibrated mixture of the radioactive ligand and antiserum. Each point is the mean of four determinations performed in triplicate and vertical bars indicate standard error (SE) of the mean. A second order association kinetic plot of the initial rates of binding is inset, with each point determined in triplicate and with $Y = \log_{10}L_0/Ab_0[(Ab_0 - AbL)/L_0 - (AbL + BlL)]$ (see Methods). Curves have been fitted to a two-site model which gave the following rate constants. Association: K_{f1} $8.55 \times 10^7\ M^{-1}\ sec^{-1}$ (23%), K_{f2} $1.00 \times 10^4\ M^{-1}\ sec^{-1}$ (77%). Dissociation: K_{r1} $0.012\ min^{-1}$ (50%), K_{r2} $0.172\ min^{-1}$ (34%).

1 (K_D 0.67 nM) while rabbits 2 and 3 yielded a best fit for two classes of binding sites (see Fig. 3).

Specificity of benzodiazepine antisera

A variety of drugs and putative neurotransmitters were tested for their ability to inhibit [^{3}H]flunitrazepam binding to the anti KNZ–KLH IgG. In

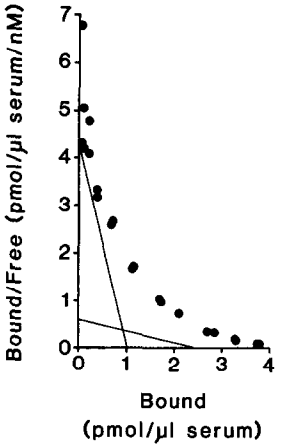

Fig. 3. Scatchard plot of [^{3}H]flunitrazepam binding to anti KNZ-KLH IgG from rabbit 2. The results were obtained from two experiments and each point was determined in triplicate. Lines were fitted by a non-linear least squares analysis (see Methods), which gave the following parameters: K_{D1} 4.13 nM, K_{D2} 0.24 nM, B_{max1} 2.48 pmol μl^{-1} serum, B_{max2} 1.01 pmol μl^{-1} serum.

the initial screening experiments, 78 substances known to act at receptors for acetylcholine, excitatory and inhibitory amino acids, catecholamines, histamine, 5-hydroxytryptamine, opiates, purines and steroids were tested at 10 or 100 μM (results not shown). Only the benzodiazepine receptor ligand diazepam caused a clear displacement of binding, less than 0.01% of ligand being bound in the presence of 100 μM diazepam. Compounds thought to act at sites other than the benzodiazepine recognition site on the GABA/benzodiazepine receptor complex in the CNS (see Discussion), namely GABA itself, bicuculline methobromide, picrotoxin, pentobarbitone, valproate, R5135 and strychnine all failed significantly to displace [^{3}H]flunitrazepam binding to the anti KNZ–KLH IgG. Further experiments were therefore performed in which the different benzodiazepine derivatives listed in Table 1, benzodiazepine fragments and non-benzodiazepine ligands at the CNS benzodiazepine receptor were tested over a range of concentrations, in order to estimate the concentrations for 50% inhibition (IC_{50}) of anti KNZ–KLH IgG [^{3}H]flunitrazepam binding. These IC_{50} values revealed a significant correlation ($P < 0.01$) between the ranked abilities of benzodiazepines to inhibit [^{3}H]flunitrazepam binding to the anti KNZ–KLH IgG from rabbits 1 and 2 and their published IC_{50} values for inhibition of [^{3}H]diazepam binding to the benzodiazepine receptor in rat brain membranes. Figure 4 displays this correlation for rabbit 2. Compounds which do not appear on Fig. 4 (with IC_{50} values in brackets) include metaclazepam (0.4 μM), Ro5-2181 (0.2 μM), tifluadom (8.3 μM), glycyl-glycyl-*N*-methylamino-5-nitrobenzophenone (47.1 nM), 2-amino-5-nitrobenzophenone (17.8 μM), and 5-nitroanthranilinonitrile (>1 mM).

Reliable IC_{50} values (>1 mM) for displacement of antibody binding could not be estimated for a number of other compounds, such as the β-carboline derivatives, harmaline, harmane, harmine, β-carboline-3-carboxylate ethyl ester (βCCE) and ZK 93423, the pyrazoloquinoline CGS 8216, the purines inosine and hypoxanthine, and the cyclopyrrolones suriclone and zopiclone, all of which are known to inhibit CNS benzodiazepine receptor binding. The CNS benzodiazepine receptor antagonists Ro15-1788 and Ro14-7437 were also inactive. For

Table 1. Benzodiazepines used in the present study, with substituents labelled according to Fig. 1. The drugs are numbered for identification in Fig. 4

No.	Ligand	R1	R2	X	Y	Z	Other
1	Ro5-3448	CH_3	H	Cl	Cl	O	
2	Ro15-1788	4-carboethoxy imidazole	H	F	C = O at position 5	—	N-CH_3 at position 4
3	Ro14-7437	$R_1 \leftrightarrow Z$	H	H		—	
4	Roll-7800	3-aminomethyl-1,2,4-triazole $R_1 \leftrightarrow Z$	H	—	Cl	—	Thienodiazepine
5	Lormetazepam	CH_3	OH	Cl	CH_3	O	
6	Clonazepam	H	H	NO_2	Cl	O	
7	Midazolam	2-methyl-imidazole $R_1 \leftrightarrow Z$	H	Cl	F	—	
8	Flunitrazepam	CH_3	H	NO_2	F	O	
9	Lorazepam	H	OH	Cl	Cl	O	
10	Roll-6893(−)	CH_3	CH_3	NO_2	F	O	
11	Roll-6896(+)	CH_3	CH_3	NO_2	F	O	
12	Ro7-1986	$C_2H_4NH_2$	H	Cl	F	O	
13	Diazepam	CH_3	H	Cl	H	O	
14	Nitrazepam	H	H	NO_2	H	O	
15	Flurazepam	$C_2H_4N(C_2H_5)_2$	H	Cl	F	O	
16	Bromazepam	H	H	Br	—	O	2′-Pyridino at position 5
17	Oxazepam	H	OH	Cl	H	O	
18	Clorazepate	H	$CO_2^-K^+$	Cl	H	O	
19	Ro5-3464	CH_3	H	H	H	O	
20	Prazepam	CH_2-(c-C_3H_5)	H	Cl	H	O	
21	Clobazam	CH_3	H	Cl	H	O	N at position 5 C = O at position 4
22	Chlordiazepoxide	H	H	Cl	H	$NHCH_3$	N → O at position 4
23	Medazepam	CH_3	H	Cl	H	H_2	
24	Ro5-4864	CH_3	H	Cl	4′ − Cl	O	
25	Metaclazepam	CH_3	H	Br	F	CH_2OCH_3	
26	Tifluadom	CH_3	H	H	F	Thiophenoamin	
27	Ro5-2181	H	H	Cl	H	O	N–H at position 4

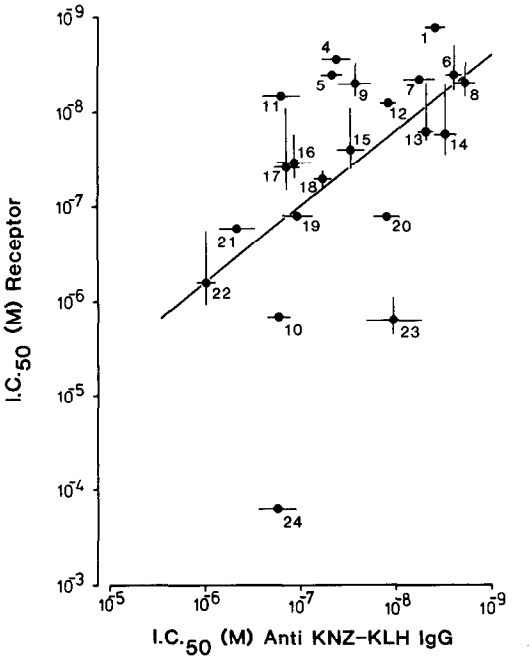

Fig. 4. Correlation between IC_{50} values for benzodiazepine inhibition of [^{3}H]diazepam binding to rat brain membranes ([8, 47] and I. L. Martin, unpublished; vertical bars indicate SE of the mean where available) and of [^{3}H]flunitrazepam binding to the anti KNZ–KLH IgG from rabbit 2 (horizontal bars indicate 95% confidence limits). Benzodiazepines are numbered according to Table 1.

cartazolate, displacement of antibody binding was detectable only in rabbit 1 (IC_{50} 32 μM) and for CL 218,872, only in rabbits 1 and 3 (IC_{50} 157 and 125 μM, respectively).

DISCUSSION

Immunisation with the KNZ-KLH conjugate stimulated the production of a high titre of IgG class, benzodiazepine binding antibodies in each of the three rabbits used for the present investigation. As found with other hapten antibodies [18], binding of the ligand [^{3}H]flunitrazepam was rapid in onset, giving a range of association rate constants from 10^4 to $10^8\,M^{-1}\,sec^{-1}$, the upper values probably approaching those determined by the diffusion-limited encounter of antibody and ligand [19]. The range of both association and dissociation rate constants estimated for each serum sample suggested, as might be expected for polyclonal antibodies, a heterogeneity of binding affinities. Independent estimates of equilibrium dissociation constants by saturation analysis concurred in giving the most curvilinear Scatchard plot and therefore the greatest heterogeneity, for rabbit 2.

The aim of the present investigation was to produce antibodies with a binding specificity comparable to the recognition site of the CNS benzodiazepine receptor. We therefore used kenazepine as a hapten which would couple to the immunogenic protein through position 1 of the benzodiazepine nucleus. Substituents on this position have been known for some time to have little effect on pharmacological potency [20] or affinity for the CNS receptor site [6–8]. Indeed, benzodiazepines coupled through position 1 to inert supports have successfully been used for the affinity purification of solubilised benzodiazepine receptors [21, 22]. Thus, our KNZ–KLH conjugate should present a benzodiazepine to the immune system in a similar orientation to that recognised by the receptor. A comparison of the structure–activity relations illustrated in Fig. 4 for inhibition of anti KNZ–KLH IgG and CNS receptor binding by various benzodiazepines suggests this to be the case.

The contribution of various substituents on the benzodiazepine nucleus to the pharmacological potency and receptor binding affinity of benzodiazepine agonists has been reviewed elsewhere [6–8, 20, 23, 24]. Only at two positions on this nucleus did we find substituents to introduce changes in potency for inhibition of anti KNZ–KLH IgG binding that were not paralleled by comparable changes in potency for inhibition of CNS receptor binding. Firstly, unsymmetric introduction of a methyl group at position 3, which gives the compounds of Roll-6896 and Roll-6893, (+)- and (−)-enantiomers, respectively. As can be seen from Fig. 4, these enantiomers differ by over two orders of magnitude in their potencies for inhibition of CNS receptor binding, but show no significant differences in potency for inhibition of [^{3}H]flunitrazepam binding to the anti KNZ–KLH IgG from rabbit 2. However, the kenazepine hapten employed for immunisation had no stereospecificity at position 3 and so we did not expect to raise stereospecific antibodies. The second position at which substituents were found to bring about differences in binding specificity between the antibodies and the CNS receptor was at C4′ of the 5-phenyl ring. Previous studies have shown the introduction of this 4′-chloro substituent to cause a greater than 1000-fold increase in the IC_{50} for displacement of neuronal benzodiazepine receptor binding in rat brain homogenates [2]. However, the IC_{50} values for inhibition of [^{3}H]flunitrazepam binding to the anti KNZ–KLH IgG increased only 6-fold in rabbit 1, 37-fold in rabbit 2 and 22-fold in rabbit 3. Models of the neuronal benzodiazepine receptor based on the analysis of distance geometry [24] or the binding energies contributed by different groups [6] generally assume that the 4′-chloro substituent causes steric hindrance of entry of the 5-phenyl ring into its hydrophobic slot. Presumably the fit at this point in the majority of benzodiazepine antibodies is not so precise and can accommodate the relatively small addition of a chlorine atom at C4′.

As fragments of the benzodiazepine nucleus we tried 2-aminobenzophenones, which are key intermediates in the preparation of the 5-phenyl-1,4-benzodiazepines [20]. Although these compounds have a low affinity at the CNS receptor, they appear to act as pro-drugs *in vivo* [25]. The compound 2-amino-5-nitrobenzophenone, which can be considered as a ring-opened derivative of nitrazepam, was considerably less potent than this benzodiazepine as an inhibitor of antibody binding, although the addition of a dipeptide at N1 to give peptidoamino-benzophenone [26] increased potency. Another fragment tested was 5-nitroanthranilinonitrile, which showed no detectable dis-

placement of antibody binding. Apart from the open ring, this fragment also lacks the equivalent of the 5-phenyl group on the intact benzodiazepine nucleus that appears to be required for antibody recognition, since Ro15-1788 and Ro14-7437 were also inactive.

The only non-benzodiazepine derivatives for which reliable, although high ($>10^{-5}$ M) IC_{50} estimates could be obtained for inhibition of [^{3}H]flunitrazepam binding to the anti KNZ-KLH IgG were the pyrazolopyridine, cartazolate (rabbit 1) and the triazolopyridazine, CL 218, 872 (rabbits 1 and 3). Both of these agents exhibit anxiolytic and anti-convulsant actions *in vivo* and will inhibit CNS benzodiazepine receptor binding *in vitro* [27–29]. The potency of CL 218, 872 to inhibit CNS receptor binding is reduced after photoaffinity labelling of the receptor with flunitrazepam [30], a procedure which blocks agonist benzodiazepine binding. This observation is consistent with the present results in suggesting that the triazolopyridazines may share some aspects of a 5-phenyl-1,4-benzodiazepine recognition site. The pyrazolopyridines may also interact with this benzodiazepine recognition site, since etazolate is able to protect against irreversible alkylation of the CNS receptor [31]. However, both etazolate and cartazolate appear to bind at additional sites on the receptor, since they are more potent at enhancing GABA-facilitated binding than at displacing the benzodiazepine ligand itself [27, 32].

Other non-benzodiazepine derivatives thought to act at the GABA/benzodiazepine receptor complex in the CNS [33] failed to displace antibody binding. Such compounds included: GABA and the GABA receptor antagonist bicuculline methobromide [34]; pentobarbitone and valproate, which enhance benzodiazepine binding [35]; picrotoxin, which attenuates the pentobarbitone enhancement of benzodiazepine binding [36]; the steroid R5135 and strychnine, both of which antagonise GABA-induced enhancement of benzodiazepine binding [37, 38]; the cyclopyrrolones suriclone and zopiclone, which exhibit a similar pharmacological profile *in vivo* and *in vitro* to the benzodiazepine tranquillisers [39, 40]; the purines inosine and hypoxanthine, which displace benzodiazepine receptor binding at micromolar concentrations and which have benzodiazepine-like electrophysiological and anti-convulsant actions [41–44]; the pyrazoloquinoline benzodiazepine receptor antagonist CGS 8216 [45]; the β-carboline derivatives harmaline, harmane, harmine, βCCE and ZK 93423.

The β-carboline derivatives displace benzodiazepine receptor binding at nanomolar concentrations [46] and can produce inverse agonist (βCCE), and agonist (ZK 93423) effects at this receptor [47, 48]. The fact that neither inverse agonist nor agonist β-carboline derivatives inhibited [^{3}H]flunitrazepam binding to the antibodies suggests that these β-carbolines may bind at different recognition sites on the receptor to the agonist 5-phenyl-1,4-benzodiazepines. A similar argument could be made for the benzodiazepine receptor antagonists Ro15-1788, Ro14-7437 and CGS 8216 [45, 49] which also failed to inhibit antibody binding. Again, our results are consistent with previous observations on CNS benzodiazepine receptors photoaffinity labelled with flunitrazepam; the procedure modifies recognition site characteristics for the agonist benzodiazepines, but not for β-carbolines, Ro15-1788 or CGS 8216 [30, 50, 51]. The cyclopyrrolone tranquillisers suriclone and zopiclone and the purines, inosine and hypoxanthine, which did not inhibit benzodiazepine antibody binding, might also be predicted to act on the receptor at sites other than those specific for agonist benzodiazepines. Further testing of these predictions is likely to emerge from computer-assisted modelling of three-dimensional structures which, as far as the benzodiazepine antagonists are concerned, has already revealed greater similarities to antagonist β-carboline derivatives than to agonist benzodiazepines (P. A. Borea, P. M. Dean, D. J. Danziger and I. L. Martin, unpublished).

In conclusion, the anti KNZ-KLH IgG from rabbits 1 and 2 in the present investigation display a specificity for 5-phenyl-1,4-benzodiazepines which act as agonists at the neuronal benzodiazepine receptor in the CNS. Despite the polyclonal nature of these antibodies, a significant rank correlation was obtained between the potencies of benzodiazepine agonists to inhibit CNS receptor and benzodiazepine antibody binding. This correlation presumably arose not only because the KNZ–KLH conjugate presented a benzodiazepine to the immune system in a similar orientation to that bound by the CNS receptor, but also because the hapten was sufficiently small to preclude the generation of antibodies directed only against fragments of the benzodiazepine nucleus. The variable region of the anti KNZ–KLH IgG might, therefore, be expected to exist in a conformation similar to that adopted by the recognition site of the receptor when binding an agonist benzodiazepine. We anticipate the anti KNZ–KLH IgG to provide a useful reagent for the identification and purification of putative endogenous benzodiazepine receptor agonists in the CNS [52] and for the raising of receptor specific anti-idiotypic antibodies.

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