An Approach for Assaying Benzodiazepine Receptor Binding with Radioiodinated Ligand: ^{125}I -Labeled Diazepam Derivative

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^{125}I 2'-Iododiazepam (IDZ) was prepared and its application in a benzodiazepine receptor binding assay was studied. ^{125}I 2'-IDZ binds to the rat cortical membrane with a high affinity (K_d , 0.66 nM). Various benzodiazepines showed competition with ^{125}I 2'-IDZ for the binding sites in the rat cortical membrane, and the specificity of its binding correlated well with that of ^3H diazepam ($r=0.992$, $p<0.001$). These findings suggested that ^{125}I 2'-IDZ binds to the same sites as ^3H diazepam and indicated that ^{125}I 2'-IDZ can be used in a benzodiazepine receptor assay.

Keywords radioiodinated diazepam; receptor binding assay; benzodiazepine receptor; iodine-125; tritiated diazepam

Introduction

The radioreceptor binding assay has been used widely as an important technique in pharmacological, neurological, and biological studies of the benzodiazepine receptor system.¹⁾ In the benzodiazepine receptor assay, ^3H -labeled 1,4-benzodiazepines such as ^3H diazepam and ^3H flunitrazepam are commonly used as the radioligand.¹⁾ However, the procedures involving such ^3H -labeled ligands are complicated and time consuming, since they require the use of a liquid scintillation counter for the measurement of radioactivity.²⁾ Thus, there is a need for a ^{125}I -labeled ligand because, using a NaI well scintillation counter (gamma counter), ^{125}I can be quantitated more easily than in a liquid scintillation counter.^{1e,2)}

On the basis of the structure-activity relationships between benzodiazepines, we recently designed 2'-iododiazepam (IDZ), a diazepam derivative iodinated at the ortho position of the C-5 phenyl ring, as an *in vivo* imaging agent for nuclear medical studies of brain benzodiazepine receptors.³⁾ We are currently investigating the application of ^{125}I -labeled 2'-IDZ to an *in vitro* benzodiazepine receptor binding assay. In this study, ^{125}I 2'-IDZ was prepared and its displacement from ^{125}I 2'-IDZ binding sites by various drugs was compared with that of ^3H diazepam.

Materials and Methods

^{125}I NaI (specific radioactivity: 81.4 TBq/mmol) was purchased from Amersham International Plc. ^3H diazepam (3.15 TBq/mmol) was purchased from Du Pont New England Research Products, and non-radioactive diazepam was added to it to produce a solution with the same specific activity of 740 GBq/mmol as ^{125}I 2'-IDZ. Flunitrazepam, Ro 15-1788, Ro 11-3128, and Ro 11-3624 were generous gifts from F. Hoffman-La Roche. The other chemicals used were of reagent grade.

Synthesis of 2'-Bromodiazepam and 2'-IDZ We synthesized 2'-bromodiazepam (BDZ) and non-radioactive 2'-IDZ from 2-bromobenzoic acid and 2-iodobenzoic acid, respectively, by addition of *N*-(4-chlorophenyl)-*N*-methylethylenediamine, followed by cyclization and oxidation, according to the method previously reported for the corresponding fluoro analog, fludiazepam.⁴⁾ The details of the synthesis and characterization of these compounds will be reported elsewhere.

Radiosynthesis of ^{125}I 2'-IDZ The radio-iodination of 2'-IDZ was accomplished by a halogen-exchange reaction with ^{125}I NaI. Eighty-five micrograms of 2'-BDZ was dissolved in 107 μl of dimethylformamide (DMF). This solution was added to a mixture of ^{125}I NaI (10 μl , 37 MBq), non-radioactive sodium iodide (0.8 μg /0.25 μl 0.1 N NaOH), 1-naphthalenesulfonic acid dihydrate (0.26 mg/5 μl DMF), and copper sulfate pentahydrate (0.26 mg/5 μl DMF) in a sealed vial. The reaction mixture

was heated for 1.5 h at 100–105 °C. After cooling, 400 μl of 10% Na_2CO_3 solution was added to the reaction mixture, and the product was extracted with chloroform. The combined organic layers were evaporated under a stream of nitrogen gas, and the residue dissolved in methanol. The final product was purified by HPLC (Lichrosorb RP-18, methanol/water/4 mM phosphate buffer (pH 7.4)=11/5/4, flow rate: 1.5 ml/min).

The radiochemical purity of the product was more than 99% as determined by TLC (chloroform/acetone=4/1, R_f =0.62–0.68) and analytical HPLC (Lichrosorb RP-18, methanol/water/4 mM phosphate buffer (pH 7.4)=11/5/4, flow rate: 1.5 ml/min, t_R =62 min). The specific radioactivity was 6.66 TBq/mmol, as estimated by the ultraviolet absorbance at 284 nm. For the receptor assay described below, non-radioactive 2'-IDZ was added to the ^{125}I 2'-IDZ solution to produce a solution with a specific radioactivity of 740 GBq/mmol.

Preparation of Rat Cortical Membranes Preparation of synaptosomal membranes from the rat cortex was carried out according to the method of Möhler and Okada.^{1a)} The cerebral cortex of a male Wistar rat (150–200 g) was homogenized in 20 volumes of ice-cold 0.32 M sucrose using a Teflon-glass homogenizer. The homogenate was centrifuged at 1000 $\times g$ for 10 min at 4 °C, and the supernatant was recentrifuged at 20000 $\times g$ for 20 min at 4 °C. The pellet was resuspended in ice-cold 50 mM Krebs-Tris buffer (50 mM Tris-HCl buffer, pH 7.4, 118 mM NaCl, 4.8 mM KCl, 1.2 mM CaCl_2 , 1.2 mM MgCl_2) to yield a synaptosomal membrane suspension with a protein concentration of 1.0 mg protein/ml. The protein concentration was measured by the method of Lowry *et al.*⁵⁾

Saturation Study of ^{125}I 2'-IDZ Binding to Rat Cortical Membranes One milliliter of the cortical membrane preparation (1 mg protein) was incubated at 4 °C with various concentrations of ^{125}I 2'-IDZ in 1.0 ml of 50 mM Krebs-Tris buffer (pH 7.4). After a 15-min incubation, the samples were rapidly filtered through a Whatman GF/B filter, and the filter was washed twice with 5 ml of ice-cold assay buffer. Then the radioactivity bound to the filter was measured in a NaI well scintillation counter. Nonspecific binding was determined in the presence of 1 μM diazepam. All incubations were done in duplicate, and the results are presented as the average of three separate experiments.

The effects of incubation time, temperature and pH on ^{125}I 2'-IDZ binding were also studied.

Inhibition of ^{125}I 2'-IDZ and ^3H diazepam Binding to Rat Cortical Membranes by Various Drugs One milliliter of the cortical membrane preparation (1 mg protein) was incubated at 4 °C with different concentrations of competing drugs and the radioligand (0.6 nM ^{125}I 2'-IDZ or ^3H diazepam) in 1 ml of 50 mM Krebs-Tris buffer (pH 7.4). After a 15-min incubation, the samples were rapidly filtered through a Whatman GF/B filter, and the filter was washed twice with 5 ml of ice-cold assay buffer. For ^{125}I 2'-IDZ, the radioactivity bound to the filter was measured in a NaI well scintillation counter. For ^3H diazepam, the filter was placed into a 20 ml scintillation vial containing 10 ml of ACS II (Amersham), and the radioactivity bound to the filter was measured in a liquid scintillation counter. Nonspecific binding was determined in the presence of 1 μM diazepam. IC_{50} values were determined from the displacement curves for the percentage inhibition of radioligand binding versus inhibitor concentration using the curve-fitting computer program

LIGAND (Elsevier-Biosoft, Cambridge, UK). K_i values were calculated by the method of Cheng and Prusoff.⁶⁾ To calculate K_i , the value of 3.6 nM obtained by Möhler and Okada was used as the K_d for diazepam,^{1a)} and the value obtained in this study (0.66 nM) was used as the K_d for 2'-IDZ. Also pseudo-Hill coefficients were calculated from drug displacement data.⁷⁾ The results are presented as the average of 3–5 separate experiments.

Results and Discussion

Figure 1 shows the effects of incubation time, temperature, and pH on specific [125 I]2'-IDZ binding. As the incubation time increased, the amount of specifically-bound [125 I]2'-IDZ also increased, and equilibrium was reached after about 15 min incubation (Fig. 1A). Since [3 H]diazepam binding has been reported to be temperature-dependent,^{1a)} the effect of incubation temperature was also studied. Similarly to the findings reported for [3 H]diazepam,^{1a)} the specific binding of [125 I]2'-IDZ increased as the temperature fell, reaching a maximum at 4–15 °C (Fig. 1B). In contrast, specific [125 I]2'-IDZ binding was almost constant over the pH range of 6 to 9 (Fig. 1C), unlike that of [3 H]diazepam, for which there is a pH optimum between pH 7.0–7.4.^{1a)} The cause of the difference in pH-dependency between the two compounds is not clear at

present. On the basis of these results, saturation and displacement studies were performed as described in Methods. When analyzed by HPLC, the [125 I]2'-IDZ was stable under these experimental condition.

The specific binding of [125 I]2'-IDZ to rat cortical membranes was saturable, and nonspecific binding amounted to only 2–7% of the total binding (Fig. 2). The data for specific [125 I]2'-IDZ binding gave a linear Scatchard plot, which indicated the involvement of a single population of binding sites. The apparent equilibrium dissociation constant (K_d) for [125 I]2'-IDZ was 0.66 nM and the maximum number of binding sites (B_{max}) was

Fig. 2. Binding of [125 I]2'-IDZ to Rat Cortical Membranes

Fig. 1. Effect of Incubation Time (A), Temperature (B), and pH (C) on the Specific Binding of [125 I]2'-IDZ

Fig. 3. Inhibition of [125 I]2'-IDZ Binding to Rat Cortical Membranes by Various Benzodiazepines

A: $\blacktriangle$, 2'-IDZ; $\bullet$, diazepam; $\circ$, fludiazepam; $\blacksquare$, medazepam; $\square$, Ro 15-1788. B: $\triangle$, clonazepam; $\blacklozenge$, nitrazepam; $\diamond$, flunitrazepam; $\blacksquare$, Ro 11-3128; $\square$, Ro 11-3624.

TABLE I. Inhibition of [³H]Diazepam or [¹²⁵I]2'-IDZ Binding to Rat Cortical Membranes by Various Drugs

Drug	Competing for [³ H]diazepam		Competing for [¹²⁵ I]2'-IDZ	
	<i>K_i</i> (nM) ^{a)}	<i>n_H</i> ^{b)}	<i>K_i</i> (nM) ^{a)}	<i>n_H</i> ^{b)}
2'-IDZ	0.64 ± 0.04	1.0 ± 0.02	0.72 ± 0.04	1.0 ± 0.01
Ro 15-1788	0.90 ± 0.3	1.0 ± 0.1	0.93 ± 0.01	0.9 ± 0.05
Clonazepam	1.9 ± 0.6	0.9 ± 0.01	1.7 ± 0.1	0.9 ± 0.1
Flunitrazepam	2.3 ± 1.1	1.0 ± 0.03	3.5 ± 0.2	1.0 ± 0.1
Ro 11-3128	2.9 ± 0.7	0.9 ± 0.1	4.1 ± 2.0	0.9 ± 0.1
Fludiazepam	3.3 ± 0.4	1.0 ± 0.03	1.9 ± 0.5	1.0 ± 0.02
Diazepam	5.7 ± 1.5	0.9 ± 0.1	5.4 ± 0.1	0.9 ± 0.1
Nitrazepam	7.4 ± 0.3	1.0 ± 0.2	5.5 ± 0.2	1.0 ± 0.2
Ro 11-3624	360 ± 60	1.0 ± 0.05	290 ± 30	1.0 ± 0.05
Medazepam	1300 ± 800	1.0 ± 0.01	590 ± 40	1.0 ± 0.1

a) *K_i* value were derived from IC₅₀ values according to the equation, $K_i = IC_{50} / (1 + C/K_d)$, where *C* is the radioligand concentration and *K_d* is its dissociation constant (*K_d* for diazepam = 3.6 nM,^{1a)} *K_d* for 2'-IDZ = 0.66 nM (measured value)). b) *n_H*: pseudo-Hill coefficient. Each value is the mean ± S.E.M. of 3–5 independent experiments.

Fig. 4. Correlation between the *K_i* Values for the Inhibition of Specific [³H]Diazepam and [¹²⁵I]2'-IDZ Binding by Various Benzodiazepines

1, 2'-IDZ; 2, Ro 15-1788; 3, clonazepam; 4, flunitrazepam; 5, Ro 11-3128; 6, fludiazepam; 7, diazepam; 8, nitrazepam; 9, Ro 11-3624; 10, medazepam.

1.07 pmol/mg protein. This low *K_d* value indicated a high affinity of 2'-IDZ for the cortical membrane. The *B_{max}* value of [¹²⁵I]2'-IDZ was similar to that obtained with [³H]diazepam (0.88 pmol/mg protein).^{1a)}

Figure 3 shows the displacement of bound [¹²⁵I]2'-IDZ from the rat cortical membrane by various benzodiazepines, and Table I shows the *K_i* values calculated from the IC₅₀ values together with those for [³H]diazepam. Among the tested compounds in this study, 2'-IDZ showed the lowest *K_i* value, i.e., the highest affinity, and its affinity was approximately 8 times higher than that for diazepam, the parent compound. This finding is in agreement with a re-

port that substitution with electron-withdrawing groups such as halogens at position 2' increases receptor affinity in the 1,4-benzodiazepine molecule.⁸⁾ In addition, the pharmacologically potent (*S*)-enantiomer (Ro 11-3128) had a more than 70-fold higher displacement potency than its pharmacologically weak (*R*)-enantiomer (Ro 11-3624), demonstrating that the binding sites for [¹²⁵I]2'-IDZ were stereospecific. Competition curves for these compounds tested had pseudo-Hill coefficients close to 1, which indicated the absence of negative or positive co-operativity.⁷⁾ Figure 4 shows the correlation between *K_i* values for [¹²⁵I]2'-IDZ binding and [³H]diazepam binding. The correlation was high with a correlation coefficient of *r* = 0.992 (*p* < 0.001), indicating that [¹²⁵I]2'-IDZ binds to the same receptor as [³H]diazepam.

The present study indicates that [¹²⁵I]2'-IDZ can be used in a benzodiazepine receptor assay.

We used [¹²⁵I]2'-IDZ with the same specific activity (740 GBq/mmol) as that of [³H]diazepam to compare the two ligands in the binding assay. The theoretical maximum specific radioactivity of a ¹²⁵I-labeled ligand is 81.4 TBq/mmol under carrier-free conditions. The acquisition of such a high specific radioactivity would permit the use of small tissue samples and the detection of very low levels of binding, i.e., the development of a highly sensitive radioreceptor assay.^{1e,2)} Accordingly, the synthesis of [¹²⁵I]2'-IDZ under carrier-free conditions and its application to a highly sensitive radioreceptor assay are currently in progress.

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