Structure-Affinity Relationships Between Several New Benzodiazepine Derivatives and $^3$H-Diazepam Receptor Sites

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Abstract—Several new benzodiazepines were studied with respect to their ability to bind specifically to benzodiazepine receptor sites in rat cerebral cortex membrane fraction. The IC50 values of new benzodiazepines were compared to that of diazepam. A group of triazolo-[1,4] benzodiazepines displaced $^3$H-diazepam very effectively. The most potent of this group was brotizolam. Its potency was about ten times higher than that of diazepam. In this study, camazepam, which differs from diazepam in its C-3 substitution, had the lowest affinity to the benzodiazepine receptor site. This potency was about 0.006 that of diazepam. CM 7116 bound with the highest affinity to the benzodiazepine receptor site among the metabolites of CM 6912. The length of the side chain at the C-3 position of this compound is shorter than that of the other metabolites of CM 6912. These results indicated that the long side chain at the C-3 position might inhibit a close interaction between the receptor site and the substrate molecule, thereby leading to low-affinity binding.

Materials and Methods

Membrane preparation: Male Sprague-Dawley rats weighing 200–250 g were decapitated, and the brain removed rapidly, dissected into several regions on ice, and frozen at $-80^\circ$C. For this assay, frozen rat brain cortex tissue was homogenized in 10 volumes of ice-cold 0.32 M sucrose with a Polytron homogenizer (Brinkman Ins.: setting 6, 5 sec, 3X; 300 mg = 3.00 ml). The homogenate was centrifuged at $1,000 \times g$ (3,500 PRM, JA-20 rotor, Beckman Model J-21 C) for 10 min at a constant temperature of 4°C. After centrifugation, the resulting supernatant was recentrifuged at 30,000 $\times g$ (19,650 RPM, JA-20 rotor) for 30 min at 4°C. The resultant membrane pellet was resuspended in ice-cold 50 mM Tris-HCl
buffer (pH=7.4 at 4°C) and centrifuged (3.0 ml buffer, 30,000×g). After centrifugation, the pellet was frozen at −80°C, for 1–30 days before use.

**Binding assay:** The frozen P2 pellet prepared previously was resuspended in 3.0 ml of Tris-HCl buffer (pH=7.4 at 4°C). Various concentrations of drugs (50 µl) were placed into labelled tubes, followed by addition of 50 µl of protein (−250 µg) and enough Tris-HCl buffer so that the final incubation volume of each tube, after addition of the radioisotope, would be 0.500 ml. After preparing the tubes, 50 µl of 16 nM 3H-diazepam (specific activity =86.6 GB/mmol, New England Nuclear) was added to each tube at 30 sec intervals. After incubation for 30 min in an ice-water bath (−4°C), 2×5 ml of ice-cold Tris-HCl buffer (50 mM, pH=7.4) was added to the incubation mixture immediately, and the entire contents were vacuum filtered using a Milipore filter apparatus on Whatman GF/B glass fiber filters, which had been prewetted with 3.0 ml of buffer. The filters were then immediately washed with an additional 2×5 ml of ice-cold buffer. After filtration and drying for 10 min, the filters were placed into polyethylene scintillation vials (Kimble). To each vial was added 10.0 ml of Aquasol II (New England Nuclear), followed by moderate shaking for 15 min. The radioactivity bound to each filter was counted by liquid scintillation spectrometry (Searle, Delta 300) for 10 min per sample, at a counting efficiency of 40–45%.

Specific binding of 3H-diazepam was obtained by subtracting the degree of non-specific binding (3H-diazepam binding which occurred in the presence of 3 mM diazepam) from the total binding of 3H-diazepam (3H-diazepam binding in the absence of unlabelled diazepam).

The 5 percent inhibitory concentration (IC50) was calculated by log probit analysis. An aliquot of the resuspended P2 pellet was used to measure protein concentration according to the method of Lowry et al. (11).

Most of the benzodiazepine derivatives were dissolved in methanol. CM6913 was freshly dissolved in 50 mM phosphate buffer (pH 7.4). This drug, which is a metabolite of CM6912, is unstable in acidic or alkaline solutions and the use of phosphate buffer circumvents possible decarboxylation to CM7116, which could occur in acidic or alkaline media.

**Results**

Structures of the benzodiazepines investigated are presented in Fig. 1.

Figure 2 demonstrates the inhibitory effects of several benzodiazepine derivatives on the 3H-diazepam binding sites in rat cortex.

IC50 values were calculated for each benzodiazepine and they are presented in Table 1. Linear regression coefficients of all test drugs were approximately equal, indicating that the same receptor was involved.

Three triazola-[1, 4] benzodiazepines were studied. All three displaced 3H-diazepam very effectively; the most potent in the group of benzodiazepines tested was brotizolam, which bound with the highest affinity to the 3H-diazepam binding site. The potency of brotizolam was about ten times higher than that of diazepam (Fig. 1, Table 1).

Of the typical benzodiazepine derivatives, lorazepam had the highest affinity to the 3H-diazepam binding site, having a potency that was three times higher than that of diazepam. In contrast, the potency of camazepam was about 0.006 that of diazepam, and this value was the lowest in the group of benzodiazepines tested. On the other hand, the potency of temazepam which is one of metabolites of camazepam was about 74 times higher than that of camazepam. Both clobazam, which is a [1, 5] benzodiazepine, and CM6913, which is a metabolite of CM6912, were weakly bound to the 3H-diazepam binding site. The potency of each was about 0.025 and 0.017 that of diazepam, respectively.

**Discussion**

Several benzodiazepines were studied with respect to their ability to bind specifically to benzodiazepine receptor sites in rat cerebral cortex tissue. In this report, we studied 3 major different types of benzodiazepine
derivatives: triazolo [1, 4] benzodiazepines, [1, 5] benzodiazepines, and typical [1, 4] benzodiazepines (Fig. 1).

Brotizolam, which is one of the triazolo [1, 4] benzodiazepines, was the strongest displacer at the 3H-diazepam binding site (Table 1). This drug is a new thienodiazepine and has been reported to be a potent hypnotic-sedative in man (12–14). Another derivatives of this type, alprazolam (TUS-1), also effectively displaced the 3H-diazepam binding site. This potency was about 3 times higher than that of diazepam. Interesting anxiolytic activity for alprazolam has already been demonstrated (15).

Among the compounds tested, differences in binding affinity can be related to structural differences. CM-7116 and flurazepam are very similar in structure, differing only in their position 1 substitution (Fig. 1). CM-7116 binds nearly five times more strongly than does flurazepam (Table 1); the amine side chain from position 1 in flurazepam most likely is protonated at physiological pH and thus would affect the lipophylicity of the molecule as compared to the more neutral molecule CM-7116.

The high binding affinity of lorazepam, as
Fig. 2. Inhibition of $^3$H-diazepam binding in rat cortex by a series of benzodiazepine derivatives. Assays contained approximately 250 μg of synaptosomal membrane protein and 1–6 nM $^3$H-diazepam in 50 mM Tris-buffer (pH 7.4 at 0°C). Each point represents a mean of between 3–4 experiments.

Table 1. Inhibition of specific $^3$H-diazepam binding (1.6 nM) to rat brain cortex membranes by benzodiazepines

| Benzodiazepine      | IC50 (nM) | $r^*$ | Relative potency** |
|---------------------|-----------|-------|--------------------|
| Brotizolam (WE-941) | 0.6±0.1   | 0.993 | 9.50               |
| Lorazepam           | 1.8±0.4   | 0.989 | 3.17               |
| CM-7116             | 2.1±0.6   | 0.990 | 2.71               |
| Alprazolam (TUS-1)  | 2.9±0.4   | 0.994 | 1.97               |
| Diazepam            | 5.7±0.3   | 0.996 | 1.00               |
| Nitrazepam          | 6.2±0.5   | 0.990 | 0.92               |
| Estazolam           | 6.3±0.7   | 0.996 | 0.90               |
| Flurazepam          | 9.9±1.3   | 0.993 | 0.58               |
| Temazepam           | 12.2±0.8  | 0.994 | 0.47               |
| CM-6912             | 28.9±0.4  | 0.995 | 0.20               |
| Clobazam            | 225±4     | 0.995 | 0.025              |
| CM-6913             | 331±52    | 0.978 | 0.017              |
| Camazepam           | 901±62    | 0.991 | 0.006              |

Specific $^3$H-diazepam binding to the crude synaptosomal preparation from rat cerebral cortex was assayed as described in the text. Inhibition of specific binding by benzodiazepines was determined at four separate concentrations (in triplicate). The 50 percent inhibitory concentration (IC50) was calculated by log probit analysis. The IC50 values represented are the means of three experiments ± S.E.M. $^*$Linear regression coefficient from log probit analysis. $^{**}$Compared to unlabelled diazepam.
compared to that of temazepam, can be explained by the added significance of a halo group in the 2' position and by the fact that the N-1 position is unsubstituted in lorazepam (Table 1). Camazepam, which is a precursor of temazepam, possesses binding with the lowest affinity to the benzodiazepine receptor site. Camazepam differs from diazepam in its C-3 substitution; camazepam and its long carbamate side chain at the C-3 position might inhibit close interaction of the receptor site and the substrate molecule, thereby leading to low-affinity binding. It has been documented that the benzodiazepine receptor site is stereoselective for chiral C-3 position enantiomers (3); it can be postulated, then, that the molecular positioning of the substrate in the receptor has very specific geometric requirements and that for overall effective binding, a small substituent (if any) must be at the C-3 position to maximize the binding interaction.

Of the two metabolites of CM-6912 tested, CM-6913, and CM-7116, both have an F-substitution at the C-2 position. Steric effects at position 3 might explain why CM-6912 and CM-6913 bind with lower affinity than CM-7116 (Table 1); however, CM-6913 binds most weakly of the three possibly due to its free carboxyl group, which would be in the ionized form at physiological pH. Following ester hydrolysis and decarboxylation, CM-6912 is converted to its metabolite CM-7116, a drug which binds with very high affinity to the benzodiazepine receptor site.

One atypical benzodiazepine derivative was studied, clobazam, a [1, 5] benzodiazepine. This drug exhibited very weak binding affinity, 1/40th that of diazepam (Table 1). Clobazam differs from the others most notably in the geometric conformation of its seven-membered ring and the presence of two carbonyl groups at positions 2 and 4.

Although it is not possible to correlate directly binding affinities of the new benzodiazepines investigated with their associated pharmacological activity, structure-activity relationships document that of those benzodiazepines studied (3, 4, 9, 10) (e.g., diazepam, nitrazepam, flurazepam), there is a relationship between a drug's binding affinity and its potency in vivo, suggesting there would be a similar correlation in the series of new benzodiazepines under investigation. These findings are supported from data in a separate study where a number of drugs used in the present investigation were examined with respect to preventing pentylentetrazol induced convulsions. A good positive correlation between these data and the IC50 values on 3H-diazepam binding sites in rat cortex was noted.

Using the technique of receptor binding, one can potentially find new and better drugs based upon their ability to bind with the appropriate affinity to a specific receptor. Additionally, by investigating binding differences among drugs and how the structure of each drug might affect the affinity of the receptor site for the substrate molecule, the receptor protein may be more fully characterized.

Finally, using the technique of receptor binding, coupled with animal testing, it may be possible to distinguish in a given drug which moiety(ies) of the molecule are responsible for specific pharmacologic effects. Potentially, one could then find a new benzodiazepine with any degree of binding affinity which demonstrates the desired pharmacological profile.

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