Title

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A NOVEL PYRAZOLOQUINOLINE THAT INTERACTS WITH BRAIN BENZODIAZEPINE RECEPTORS: CHARACTERIZATION OF SOME IN VITRO AND IN VIVO PROPERTIES OF CGS 9896.

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Summary

The novel pyrazoloquinoline, CGS, 9896, was a potent inhibitor of specific [^{3}H]-flunitrazepam binding in several brain regions with subnanomolar K_I values. The inhibition of [^{3}H] propyl beta-carboline-3-carboxylate ([^{3}H]-PCC) binding by CGS 9896 was enhanced by gamma-aminobutyric acid (GABA) but not by chloride ion. GABA enhancement of CGS 9896 inhibition of [^{3}H]-PCC binding predicts this compound has benzodiazepine (BZD) agonist-type activity. Behavioral studies support this prediction. CGS 9896 was found to protect mice against bicuculline and metrazol induced seizures at doses that did not induce ataxia or sedation. CGS 9896 may represent a class of compounds with potential therapeutic value. The high affinity of this non-BZD compound suggests that CGS 9896 may also be of value as a high affinity ligand for the continued study of BZD receptors.

A substantial body of evidence suggests that the pharmacological actions of the benzodiazepines (BZD) are mediated through specific receptors located on brain neurones (1,2). The relative in vitro affinity of certain pharmacologically active BZDs for these receptors is significantly correlated with their behavioral and clinical potency (3,4). Certain non-BZD compounds produce pharmacological effects similar those elicited by BZDs and have been shown to interact specifically with brain BZD receptors (5,6). This observation provides further support for the hypothesis that the BZD receptor is pharmacologically relevant. Among the non-BZD compounds, the triazolopyridazine, CL 218872, produced BZD-like effects in conflict tests and showed anti-metrazol activity (7). CL 218872 was also an effective inhibitor of brain BZD receptor binding. However, inhibition curves obtained from CL 218872 inhibition of specific [^{3}H]-flunitrazepam ([^{3}H]-FLU) binding were inconsistent with classic mass action behavior (5). Direct radioligand binding studies using [^{3}H]-CL 218872 yielded curvilinear Scatchard plots (8). The deviation from the law of mass action and the regional specificity of these observations provided part of the initial evidence for BZD receptor heterogeneity. Subsequently, several beta-carboline carboxylates have been shown to inhibit specific [^{3}H]-BZD binding in the brain (9,10). These compounds pharmacologically antagonize the effects of the BZDs. Radioligand binding assays of rat cerebral cortex using [^{3}H]-propyl beta-carboline-3-carboxylate ([^{3}H]-PCC) yielded curvilinear Scatchard plots, which suggests that this compound can also discriminate between BZD receptor subtypes (10). Thus, the findings derived from the use of non-BZD compounds have provided a clear demonstration of BZD receptor heterogeneity.

Recently, [^{3}H]-CGS 8216 (2-phenylpyrazolo[4,3-c]quinolin-3(5)-one), a

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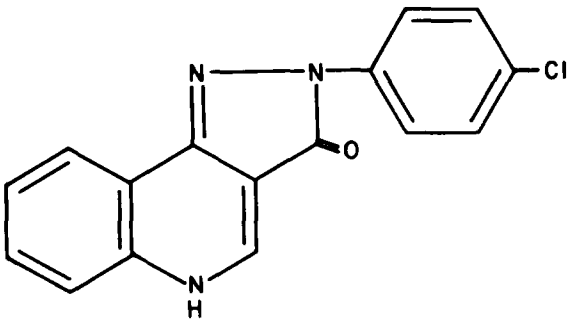
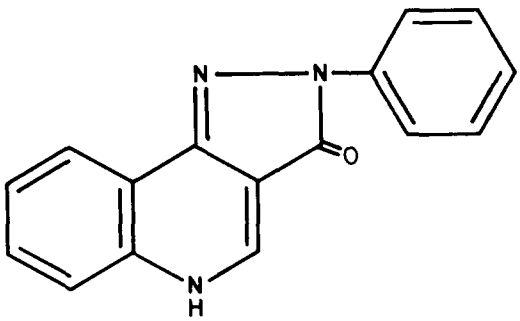
compound belonging to the pyrazoloquinoline class, was reported to interact specifically with BZD receptors in the rat brain (11). [^{3}H]-CGS 8216 was bound specifically and with high affinity to BZD receptors in rat cerebral cortical membranes. Scatchard analysis of equilibrium binding of [^{3}H]-CGS 8216 indicated that this compound did not discriminate between receptor subtypes in cortical membranes. *In vivo*, CGS 8216 was inactive in pharmacological screens for BZD-like activity (12). However, this pyrazoloquinoline was a potent antagonist of diazepam in the roto-rod test, antimetrazol screen and the test for prolongation of hexobarbital sleep time.

The specificity and high affinity of CGS 8216 for brain BZD receptors suggested that other structural analogues of this compound may also possess pharmacologically interesting properties. CGS 9896 (2-(4-chlorophenyl)-2,5-dihydropyrazolo[4,3-c]quinoline-3(3H)-one) is an analogue different from CGS 8216 only by the presence of a chloro- group in the para- position (Figure 1). This report describes some of the *in vitro* interactions of CGS 9896 with brain BZD receptors and some *in vivo* properties of this novel pyrazoloquinoline.



CGS 9896



CGS 8216

FIG. 1

Chemical structures of CGS 9896 and CGS 8216

Methods

Tissue preparation: Brains from male Sprague-Dawley rats (200-300g Hilltop Labs) were rapidly removed following sacrifice. Various brain regions were dissected over ice and immediately homogenized in 100 vol. of 50mM Na/K phosphate buffer, pH 7.4. Tissue homogenates were washed 3X in the same buffer by centrifugation at 48,000xg for 10 min. Brain homogenates were washed 5X in the experiments assessing the effect of gamma-aminobutyric acid (GABA) and chloride ion modulation of BZD receptor binding.

Preparation of drug solutions: Dilutions of [^{3}H]-FLU(83.6Ci/mmole, New England Nuclear Inc.) and [^{3}H]-PCC(48.3Ci/mmole) were made in H_2O. CGS 9896, CL 218872, lorazepam, clonazepam and flunitrazepam were initially solubilized in absolute ethanol, followed by serial dilution with H_2O. Dilutions were made using a "Pipetman" (Gilson Co.) automatic pipetor. Pipet tips were changed after each serial dilution. Fresh dilutions of radiolabeled ligand and unlabeled drugs were prepared immediately prior to each assay and all solutions were kept in glass vials.

[^{3}H]-FLU and [^{3}H]-PCC binding studies: A rapid filtration assay similar to the method described by Speth et.al. (13) and Ehlert et.al. (10) was used to measure [^{3}H]-FLU and [^{3}H]-PCC binding, respectively. Briefly, 100 µl aliquots of brain homogenate were incubated with [^{3}H]-FLU for 90 min at 0°C in 50mM Na/K phosphate buffer, pH 7.4, with the total incubation volume being 2 ml. [^{3}H]-FLU or [^{3}H]-PCC was incubated with tissue homogenate for 30 min at 37°C in studies measuring GABA (100µM) or chloride ion (100mM NaCl) modulation of BZD receptor binding. Binding in the presence of 1 uM clonazepam was defined as non-specific. All binding asays were performed in triplicate. Proteins were assayed by the method of Lowry et.al. (14).

Anticonvulsant activity: Male Swiss-Webster mice (25-30g) were used in studies assessing the effect of CGS 9896 and diazepam on (+) bicuculline (Sigma Chem. Co.) and pentylenetetrazol (Sigma Chem. Co.) induced seizures. Both (+)bicuculline and pentylenetetrazol were dissolved in normal saline immediately prior to use. CGS 9896 and diazepam were dissolved in propylene glycol:normal saline (1:10) solutions. Mice were injected with various doses of CGS 9896 or diazepam i.p., 10 min prior to the injection of (+)bicuculline (6mg/kg) or pentylenetetrazol (120mg/kg). The ED_{50} doses of CGS 9896 and diazepam that prevented seizures 15 min after the injection of (+)bicuculline or pentylenetetrazol were calculated by the method of Litchfield and Wilcoxon (15).

Results

CGS 9896 and two other compounds that are known to specifically inhibit BZD receptor binding were screened for their potency in inhibiting [^{3}H]-FLU binding (Table I). Competition curves indicate CGS 9896 is a very potent inhibitor of [^{3}H]-FLU binding in all brain regions examined with K_I values in the subnanomolar range. In the cerebral cortex and dorsal hippocampus, CGS 9896 was approximately 4-6 thousand times more potent than CL 218872, another non-BZD compound. CGS 9896 was also significantly more potent than flunitrazepam in all brain areas studied. Inhibition of specific radiolabeled BZD binding by non-labeled BZDs are typically characterized by competition curves with Hill slopes of one suggesting adherence to classic mass action behavior. Both flunitrazepam and CGS 9896 showed this type of behavior in the cerebral cortex and cerebellum (Table I). However, the Hill slope for CGS 9896 in the dorsal hippocampus was significantly less than that observed with flunitrazepam ($P<0.025$, Student's t-test). The Hill slopes for CL 218872 also

showed a regional dependence consistent with previous reports (5). Similar to CGS 9896, the most shallow Hill slope was observed in the dorsal hippocampus while the slope for the cerebellum was approximately one.

Scatchard analysis of specific [^{3}H]-FLU binding in cerebral cortex in the absence or presence of various concentrations of CGS 9896 was performed so that the type of inhibition produced by CGS 9896 could be determined. CGS 9896 produced typical competitive-type inhibition of [^{3}H]-FLU binding with the apparent K_D being increased and no change in the B_{max} when compared to control (Fig. 2A). In comparison, lorazepam also competitively inhibited [^{3}H]-FLU binding in cerebral cortex (Fig. 2B).

TABLE I

Inhibition of [^{3}H]-flunitrazepam binding in various regions of the rat brain by CGS 9896, CL 218872 and flunitrazepam.

Brain region	Hill slope	K_I(nM)
Cerebral cortex		
CGS 9896 (4)	0.85±0.06	0.035±0.006*
CL 218872 (5)	0.67±0.01	136.8±22.8*
Flunitrazepam (5)	0.92±0.03	0.80±0.11
Dorsal hippocampus		
CGS 9896 (4)	0.73±0.05	0.040±0.001*
CL 218872 (5)	0.62±0.04	243.0±26.0*
Flunitrazepam (3)	0.91±0.03	1.0±0.1
Cerebellum		
CGS 9896 (4)	0.92±0.03	0.026±0.002*
CL 218872 (5)	0.85±0.03	41.0±7.3*
Flunitrazepam (5)	0.86±0.02	0.90±0.20

[^{3}H]-FLU was used in a final concentration of 0.5nM and tissue concentrations were 20mg original wet weight/ml. When CGS 9896 was used as the inhibitor, tissue and [^{3}H]-FLU concentrations were 5mg original wet weight/ml and 2nM, respectively. K_I values were determined from the equation $K_I = IC_{50}/(1+[L]/K_D)$ where L is the free tritiated ligand concentration and K_D is the independently determined apparent dissociation constant. The K_D used in these experiments was 1.5nM. The IC_{50} and Hill slope values were derived from the inhibition data by least squares linear regression of log $(B_{max}/B-1)$ vs log [I] where I is the concentration of inhibitor in moles/liter, B_{max} is the amount of specific [^{3}H]-FLU bound at equilibrium and B is the amount of specific [^{3}H]-FLU bound at a given concentration of inhibitor. All values represent the mean±SEM. Numbers in ( ) equal the number of determinations. Significantly different from flunitrazepam at *$P<0.005$ by Student's t-test.

Both GABA and chloride ion have been shown to modulate the binding of BZDs to their specific receptors (16,17). Consequently, the effects of these modulators on CGS 9896 inhibition of BZD receptor binding were examined (Table II).

TABLE II

The effect of GABA and chloride ion on the inhibition of benzodiazepine receptor binding by CGS 9896 in rat cerebral cortex and cerebellum.[a]

Ligand	Brain region	$\frac{IC_{50}(control)}{IC_{50}(+GABA)}$	$\frac{IC_{50}(control)}{IC_{50}(+NaCl)}$
[^{3}H]-PCC	Cerebral cortex	2.3±0.4*	-
	Cerebellum	2.8±0.2**	-
[^{3}H]-FLU	Cerebral cortex	-	1.3±0.2

All incubations were carried out at 37° for 30 min in the presence of 0.5 nM [^{3}H]-PCC or [^{3}H]-FLU and 50 mM Na/K phosphate buffer, pH 7.4. GABA modulation experiments were performed in the presence of 100mM NaCl. Significantly different from one at *P<0.025 & **P<0.005.

[^{3}H]-PCC was used to label BZD binding sites in the GABA modulation experiments because GABA will not enhance the binding of [^{3}H]-PCC at 37°C so that any effect of GABA will be related to modulation of the affinity for CGS 9896 (10). The potency of CGS 9896 inhibition of [^{3}H]-PCC binding in both cerebral cortex and cerebellum was enhanced in the presence of 100 μM GABA. In contrast, chloride ion did not alter the potency of CGS 9896 inhibition of [^{3}H]-FLU binding in the cerebral cortex at 37°C. However, ^{3}H-FLU binding was decreased approximately 10% in the presence of chloride ion at 37°C. The potency of CGS 9896 inhibition of [^{3}H]-FLU binding was also influenced by temperature. In the cerebral cortex, the K_I for CGS 9896 at 37°C was significantly greater (10 fold) than that observed at 0°C with no change in the Hill slopes (data not shown).

Anticonvulsant activity is a prominent action among the diverse clinical effects of the BZDs (18). Therefore, CGS 9896 was screened for potential anticonvulsant activity by determining the ED_{50} for protection against (+)bicuculline or pentylenetetrazol induced seizures. CGS 9896 was found to be anticonvulsant and significantly (P<0.05) more potent than diazepam in protecting mice against seizures (Table III). No differential potency of CGS 9896 against (+)bicuculline-versus pentylenetetrazol-induced seizures was observed at the doses of convulsant used. Protection against seizures was afforded at doses of CGS 9896 that did not produce ataxia or sedation.

TABLE III

Comparison of the anticonvulsant activities of CGS 9896 and diazepam in mice.

Convulsant agent	CGS 9896 ED_{50}(ug/kg)	Diazepam ED_{50}(ug/kg)
(+)bicuculline	263 (192-362)	705 (420-1184)
pentylenetetrazol	340 (249-466)	1162 (942-1434)

ED_{50} values were determined by the method of Litchfield & Wilcoxon (15). Numbers in ( ) represent the 95% confidence limits.

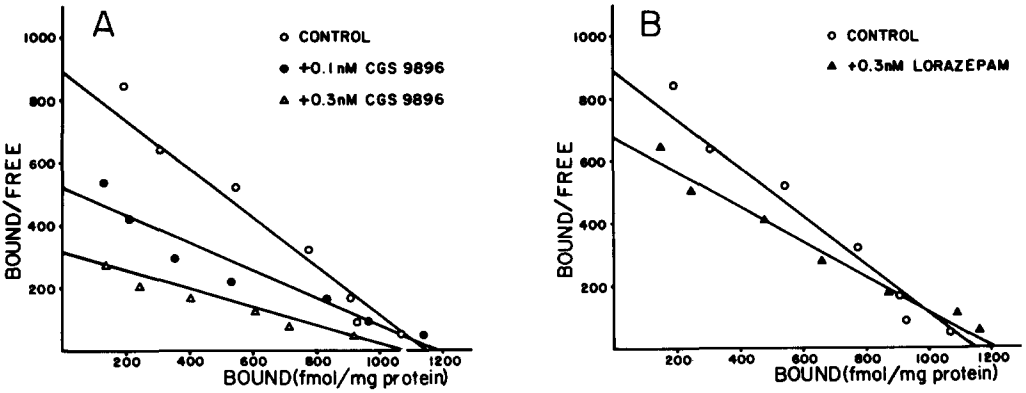


FIG. 2

Scatchard plots of CGS 9896 and lorazepam inhibition of [^{3}H]-flunitrazepam binding in rat cerebral cortex. Triplicate tubes containing 100 ul of 2% cortical homogenate were incubated with increasing concentrations of [^{3}H]-FLU (0.25-20.0nM) and a fixed amount of CGS 9896 or lorazepam at 0°C for 90 min in 50mM Na/K phosphate buffer + 100 mM NaCl, pH 7.4. Non-specific binding was determined in tubes containing 1uM clonazepam. The results of a single experiment are depicted.

Discussion

CGS 9896 is a potent inhibitor of brain BZD receptors with K_I values in the subnanomolar range. The high affinity of CGS 9896 is retained even at physiological temperatures. The potency of CGS 9896 is similar to that reported for its close structural analogue, CGS 8216 (11). Hill slope values derived from inhibition curves suggest tht CGS 9896 may interact with [^{3}H]-FLU binding sites with equal affinity in cerebral cortex and cerebellum. Consistent with this observation, Scatchard analysis in the cerebral cortex indicates typical competitive inhibition of [^{3}H]-FLU binding by CGS 9896. In contrast, a small but significant deviation from a Hill slope of one was observed in the dorsal hippocampus. This may be interpreted as evidence for negative cooperativity or the existence of multiple binding sites for [^{3}H]-FLU in the dorsal hippocampus with different affinities for CGS 9896.

A recent proposal by Ehlert et.al. (10) suggests that GABA will enhance the potency of compounds that interact with BZD receptors and possess BZD agonist-type activity (10). When interpreted within the context of this proposal, GABA enhancement of CGS 9896 inhibition of BZD receptor binding predicts this pyrazoloquinoline will have BZD agonist-type activity. Consistent with this prediction, the affinity of the BZD antagonist, CGS 8216, for BZD receptors was not enhanced by GABA (11). GABA enhancement of CGS 9896 inhibition of [^{3}H]-PCC binding also suggests that this pyrazoloquinoline may have even greater potency in unwashed brain homogenates since endogenous GABA would be present. Further evidence in support of BZD agonist-type activity was provided by the observation that CGS 9896 protects mice against both pentylenetetrazol and (+)bicuculline induced seizures at doses that did not produce ataxia or sedation. Indeed, the most remarkable difference between CGS 9896 and CGS 8216 is their opposite pharmacological effects. The addition of a chloro- group to CGS 8216 results in CGS 9896, a compound that produces BZD agonist instead of antagonist-type actions.

Chloride ion has been shown to enhance the binding of BZDs to their receptors by increasing receptor affinity (17). Under our conditions, chloride ion did not enhance the affinity CGS 9896. This observation suggests that CGS 9896 does not have preferential affinity for the conformational state that may be induced by chloride. A similar phenomenon was observed with the non-BZD, CL 218872 (19). Chloride has also been observed to enhance GABA regulation of BZD receptor binding (10). Alternatively, an effect of chloride on CGS 9896 binding may only be demonstrable in the presence of GABA. Since the tissue used in the chloride modulation experiments were extensively washed, GABA levels may have been too low for a chloride effect to be observed.

The results of the behavioral studies suggest that CGS 9896 is a potent anticonvulsant. The difference in the anticonvulsant potencies of CGS 9896 and diazepam is consistent with their relative in vitro potencies. The presence of antipentylenetetrazol activity is believed to be an accurate predictor of anxiolytic activity (20). Consequently, CGS 9896 may also possess anxiolytic qualities.

In conclusion, CGS 9896 is a very potent inhibitor of BZD receptor binding and has BZD agonist-type activity. This pyrazoloquinoline may be of potential value as a high affinity ligand for the further study of BZD receptors. CGS 9896 may also be a prototype for a class of non-BZD compounds with some very interesting pharmacological effects that may prove to be of therapeutic importance.

Acknowledgements

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