Pharmacology Biochemistry & Behavior, Vol 14, pp 487–491, 1981 Printed in the U S A

In Vivo Effects of Two Novel Alkylating Benzodiazepines, Irazepine and Kenazepine

EVAN F. WILLIAMS,* KENNER C. RICE,† MARIENA MATTSON,*
STEVEN M. PAUL‡ AND PHIL SKOLNICK*

**Laboratory of Bioorganic Chemistry and †Laboratory of Chemistry, NIAMDD,*
‡Clinical Psychobiology Branch, NIMH, National Institutes of Health, Bethesda, MD 20205

Received 20 October 1980

WILLIAMS, E F, K. C. RICE, M. MATTSON, S. M. PAUL AND P. SKOLNICK. In vivo *effects of two novel alkylating benzodiazepines, irazepine and kenazepine* PHARMAC. BIOCHEM. BEHAV. **14**(4) 487–491, 1981 — Intracerebroventricular administration of the alkylating benzodiazepines irazepine or kenazepine (20 nmol) resulted in a complete protection against convulsant doses of pentylenetetrazole (PTZ) for at least one hour, and a statistically significant protection for at least two and four hours, respectively. In contrast, administration of the non-alkylating parent benzodiazepine Ro-7/1986 or diazepam (20–60 nmol) resulted in no detectable anticonvulsant effects at fifteen minutes post-injection, the earliest interval examined. These results suggest that alkylating benzodiazepines which bind to brain benzodiazepine receptors in a non-competitive (covalent) fashion *in vitro* may exert a long lasting anticonvulsant effect by a similar mechanism.

Irazepine Kenazepine Benzodiazepine receptors Pentylenetetrazole

IN VITRO studies of two alkylating benzodiazepines, irazepine [1-(2-isothio-cyanatoethyl)-7-chloro-1,3-dihydro-5-(2-fluorophenyl)-2H-1,4-benzodiazepine-2-one] [4] and kenazepine [1-(2-bromoacetamidoethyl)-7-chloro-1,3-dihydro-5-(2-fluorophenyl)-2H-1,4-benzodiazepine-2-one] [8], demonstrated a non-competitive and mixed-type (competitive and non-competitive components) inhibition of [^{3}H] diazepam binding to benzodiazepine receptors, respectively. In contrast, non-alkylating benzodiazepines (e.g. diazepam, clonazepam) are competitive (reversible) inhibitors of the binding of radiolabelled benzodiazepines to their receptor sites in the CNS [4,7]. The present studies were undertaken to determine the effects of covalent binding of benzodiazepines to receptors *in vivo*.

We now report that both alkylating benzodiazepines afford a long-lasting protection against pentylenetetrazole (PTZ)-induced seizures when administered intracerebroventricularly (ICV). In contrast, benzodiazepines which are competitive inhibitors of benzodiazepine binding *in vivo* afford no protection against seizures at comparable doses, despite the high affinity of these compounds for the benzodiazepine receptor *in vitro*. This long lasting protection observed after intracerebroventricular administration of the alkylating benzodiazepines is accompanied by a significant reduction in [^{3}H] diazepam binding in the forebrain of treated mice. No comparable inhibition of [^{3}H] diazepam binding was observed following administration of either diazepam or Ro-7/1986.

METHOD

Intracerebroventicular Injection Technique

Drugs were injected into the right lateral ventricle of NIH General Purpose mice (23–25 g) using a modification of the method of Noble, *et al.* [2]. Animals were lightly anesthetized with ether and an incision made in the scalp to expose the bregma. A burr hole for insertion of a 27-ga needle was made through the skull with a dissecting needle approximately 1 mm caudal and lateral to the bregma. The animals were allowed to recover for at least 20 min prior to ICV injection of compounds. Compounds were injected with a 27-ga needle fitted with a stainless steel jacket recessed to expose 3.25 mm of needle. The needle was inserted perpendicularly to the skull and a volume of 10 μl was injected. The needle was held in place for 15 sec after injection to minimize leakage. After administration of the alkylating benzodiazepines, both sedation and muscle relaxation were observed in all animals.

Antagonism of PTZ-Induced Seizures

At varying intervals following ICV administration of benzodiazepines or an equal volume of vehicle, animals were injected with 100 mg/kg PTZ (IP) and observed for the appearance of tonic-clonic convulsions. Mice were counted as being protected against seizures if no convulsive episodes were observed for 15 min after injection of PTZ.

[1]Send reprint requests to Dr Phil Skolnick, Laboratory of Bioorganic Chemistry, NIAMDD, NIH, Building 4, Room 212, Bethesda, MD 20205

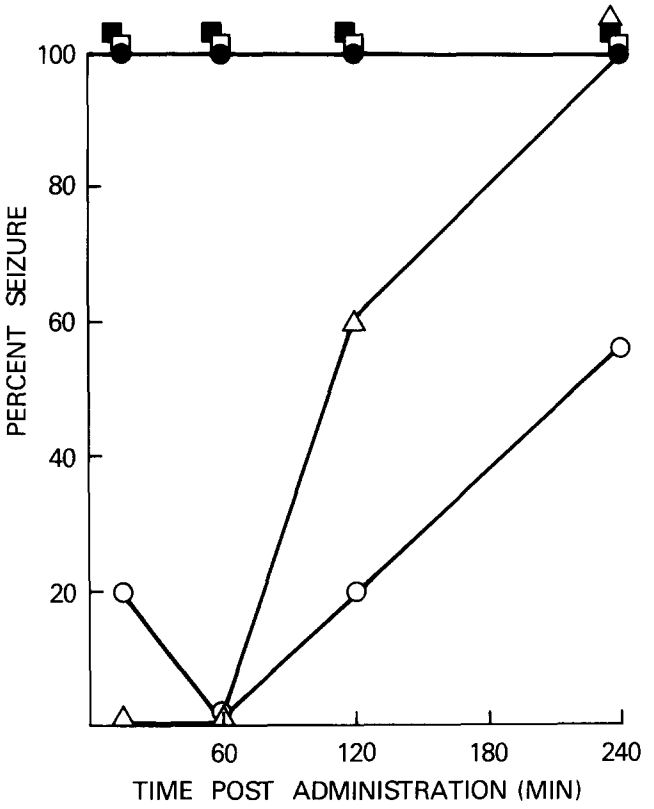

FIG. 1 Antagonism of pentylenetetrazole-induced seizures by benzodiazepines. Mice were administered benzodiazepine (20 nmols) ICV followed by pentylenetetrazole (100 mg/kg IP) at the indicated time intervals Mice were not protected against pentylenetetrazole-induced tonic-clonic seizures by Ro-7/1986/1 (■) or diazepam (□) Full or partial protection was observed with kenazepine (○) and irazepine (△) as described in Results Vehicle-injected mice (●)

Measurement of [^3H] *diazepam Binding* in vitro

Mice were decapitated at 5, 15, 30, 60, 120 and 240 min following the ICV administration of drug; mice injected with vehicle were used to determine basal values of [^{3}H] diazepam binding. The forebrains (tissue rostral to an oblique cut from the superior colliculus to the mammillary bodies and without the olfactory bulbs) were removed and crude synaptosomal fractions (P_2) were prepared as previously described [3]. This fraction was hypotonically lysed in 50 mM Tris-HCl buffer, pH 7.4, with gentle homogenization, and the binding of [^{3}H] diazepam to these synaptosomal membrane fragments was determined as previously described [3] using a total incubation volume of 1.5 ml.

Materials

Drugs for intracerebroventricular (ICV) administration were dissolved in diluted Emulphor vehicle. Emulphor-EL 620, a polyoxyethylated vegetable oil (GAF Corp., New York, N.Y.), was diluted 1·1 with 95% ethanol and one part of this mixture diluted with nine parts of phosphate buffered saline (0.85% NaCl, pH 7.4). Kenazepine and irazepine were prepared as previously described [4,8]. Diazepam and Ro-7/1986 were the generous gifts of Dr. W. Scott, Hoffman-LaRoche, Nutley, N.J.

RESULTS

Antagonism of PTZ-Induced Seizures by Benzodiazepines

Tonic-clonic convulsions were induced by PTZ in all vehicle-treated mice. Administration of kenazepine and irazepine (20 nmol, the maximum amount of these drugs soluble in 10 μl of vehicle) protected 100% of the mice challenged with PTZ at 60 min after ICV injection. At 120 min post-injection, kenazepine protected 80% and irazepine protected 40% of the mice challenged with PTZ. At 240 min post-injection, kenazepine protected 45% and irazepine afforded no protection against PTZ-induced seizures. Administration of 20–60 nmol/mouse of either the parent benzodiazepine Ro-7/1986 or diazepam afforded no protection against PTZ-induced seizures at 15 min after ICV injection, the earliest interval examined (Fig. 1 and unpublished observations).

[^3H] *diazepam Binding* in vitro *after* in vivo *Administration of Benzodiazepines*

Both kenazepine and irazepine elicited statistically significant inhibitons of [^{3}H] diazepam binding between 5–240 min post-injection ($p<0.05$ to $p<0.001$, see Fig. 2). In contrast, administration of diazepam (20 nmol) resulted in a statistically significant inhibition of [^{3}H] diazepam binding only at 5

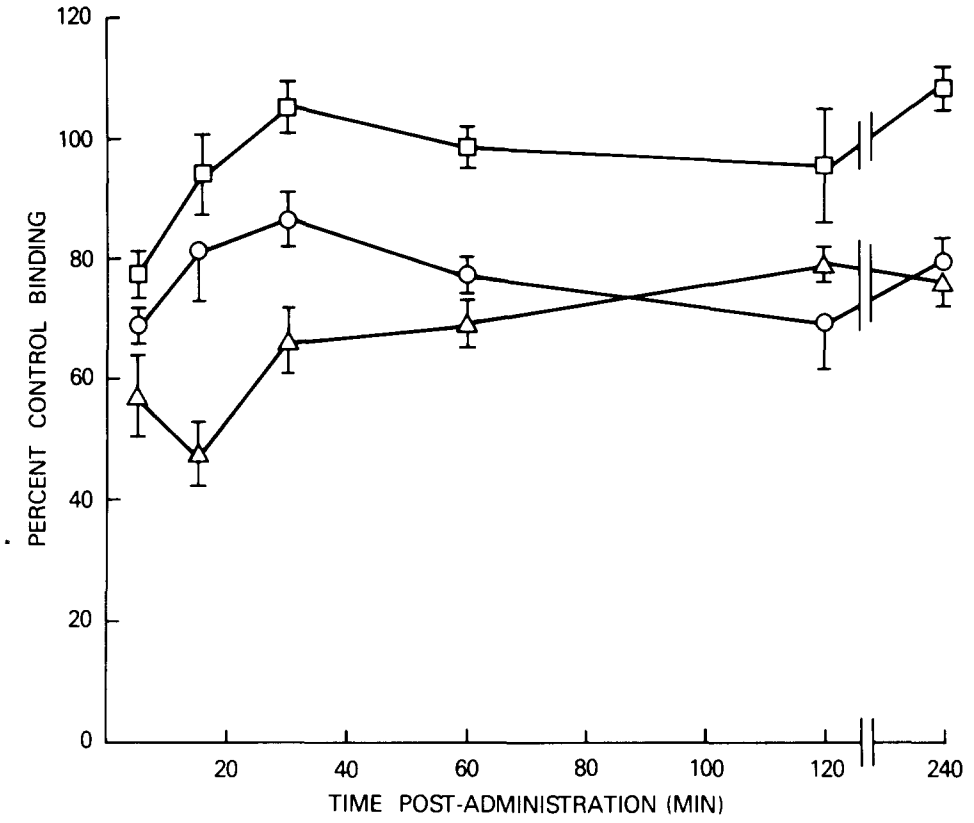

FIG. 2. Effects of *in vivo* administration of irazepine and kenazepine on [^{3}H] diazepam binding Mice were administered benzodiazepines (20 nmols) ICV and decapitated at the indicated time intervals. Mouse forebrain synaptosomal membranes were prepared and assayed for [^{3}H] diazepam binding as described in Method Symbols. diazepam (□), kenazepine (○), and irazepine (△). Values represent mean±SEM of between 3 and 16 animals per group at each time point. In a representative experiment the binding of [^{3}H] diazepam in vehicle injected groups was 280 ± 13 5 fmols/mg protein, (n=12) at 1 7 nM. Mice injected with diazepam had significantly lower ($p<0.01$) levels of [^{3}H] diazepam bound only at 5 min post-injection. Mice injected with kenazepine had significantly lower levels of [^{3}H] diazepam bound ($p<0$ 05–$p<0.01$) at all time points between 5–240 min Irazepine treated mice had lower levels of [^{3}H] diazepam binding ($p<0.01$–$p<0.001$) from 5–120 min Significance was determined using a statistical analysis for groups of unequal sample sizes (Snedecor and Cochran, 1974).

min post-injection, the earliest interval examined (Fig. 2). The temporal pattern of inhibition elicited by kenazepine differed from irazepine during the first 40 min post-injection. The inhibition produced by kenazepine was greatest (30%) at the earliest interval examined, declined to less than a 20% inhibition at 30 min, and then stabilized at 25–35% inhibition between 60 and 240 min. In contrast, irazepine produced a profound inhibition at 15 min post-injection (55%) which was reduced to approximately 25% by 240 min (Fig. 2). Scatchard analysis of the effects of irazepine and kenazepine on [^{3}H] diazepam in forebrain at 240 min post-injection revealed a non-competitive inhibition of [^{3}H] diazepam binding (Fig. 3).

DISCUSSION

The observation that alkylating benzodiazepines elicit both a long-lasting protection of mice against PTZ-induced seizures and inhibition of [^{3}H] diazepam binding is consistent with previous studies [4,8] on the effects of these compounds on benzodiazepine receptors *in vitro*. Preliminary *in vivo* experiments demonstrated that parenteral administration of either irazepine (20–50 mg/kg, IP) or the parent benzodiazepine Ro-7/1986 (5 mg/kg, IP) failed to protect mice against PTZ-induced seizures despite the high affinity of these compounds for the benzodiazepine receptor *in vitro* (K_i values of 66 and 5.4 nM, respectively) [4]. The lack of effect of these drugs after parenteral administration was thought to be due to insignificant penetration of these compounds into the CNS, since Ro-7/1986 is a primary amine, and irazepine contains a highly reactive isothiocyanate moiety, which reacts with bionucleophiles (such as -SH and -NH_2 groups) resulting in a sequestration of the compound in peripheral tissues. Therefore, in the present study both drugs were administered directly into the CNS.

Intracerebroventricular administration of irazepine and kenazepine afforded mice significant protection against PTZ-induced seizures for at least 120 and 240 min, respectively. In contrast, neither diazepam nor Ro-7/1986 afforded any significant protection against PTZ-induced seizures even at 15 min post-injection. These observations are consonant

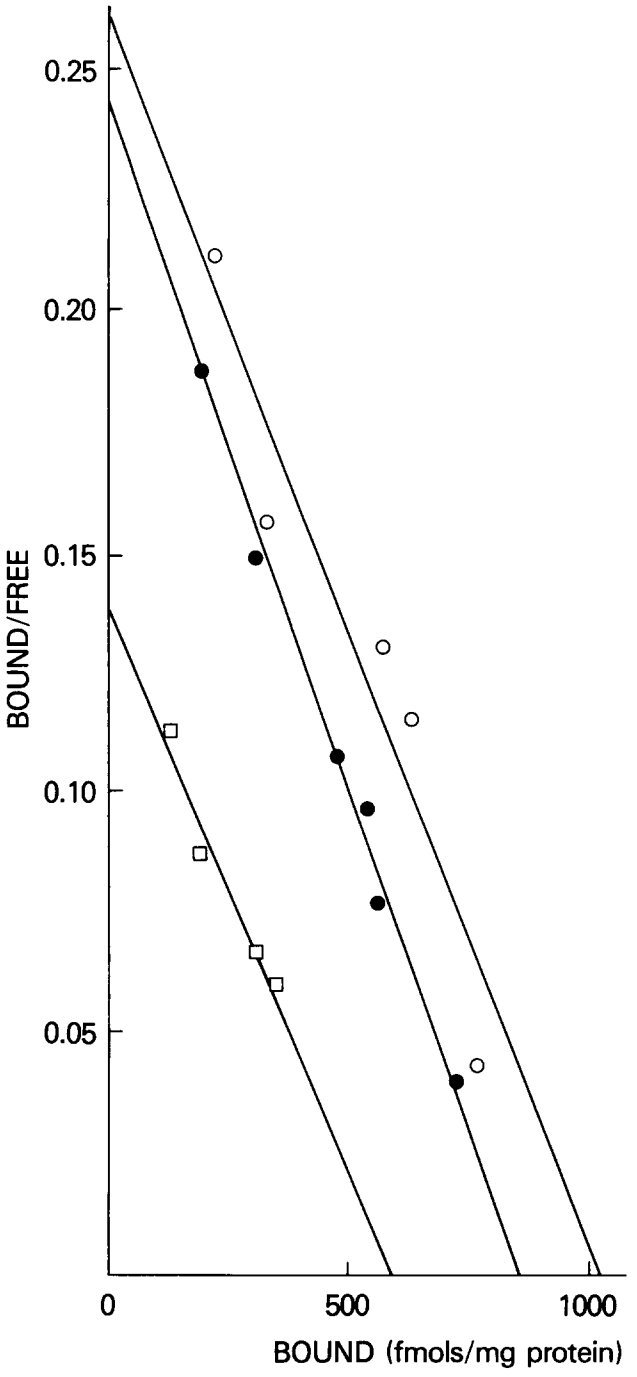

FIG. 3. Scatchard analysis of the effects of irazepine and kenazepine (20 nmol) on [^{3}H] diazepam binding at 240 min post-injection. Mice were decapitated at 240 min following intracerebroventricular administration of the compounds Crude synaptosomal forebrain membranes were pooled (3 mice per group) and assayed for [^{3}H] diazepam binding as described in Method. Symbols (○) control, (●) irazepine, (□) kenazepine This is a representative experiment

with the findings of Cook (L. Cook, personal communication) that at least 75 μg (260 nmol) of diazepam is needed to protect rats against PTZ-induced seizures following central administration.

The protection of mice against PTZ-induced seizures by the alkylating benzodiazepines, irazepine and kenazepine may be accomplished by a non-competitive occupation of benzodiazepine receptors since Scatchard analysis of forebrain homogenates at 240 min post-injection demonstrated a non-competitive inhibition of [^{3}H] diazepam binding by these compounds (Fig. 3). The failure of irazepine to protect animals against seizures at 240 min post-injection suggests that irazepine binds a subset of sites not primarily involved in seizures [8]. The temporal differences between irazepine and kenazepine in inhibiting [^{3}H] diazepam binding (Fig. 2) may be explained by recent *in vitro* studies demonstrating a mixed type inhibition elicited by kenazepine and a relatively pure non-competitive inhibition by irazepine. The mixed type inhibition by kenazepine was proposed to be due to a differential reactivity of this compound with a heterogeneous population of benzodiazepine receptors [8], a hypothesis supported by several recent reports [1,6] If kenazepine were to react competitively with one subpopulation of receptors and non-competitively with another, the finding of an initial reduction (i.e. within the first 30 min post-injection) of inhibitory activity could be explained by a reequilibration of the compound in the brain followed by reaction with another population of sites resulting in a uniform non-competitive (covalent) binding of kenazepine receptors at the later time points. The inhibitory pattern produced by diazepam (Fig 2) would appear to support this hypothesis, since an initial inhibition of [^{3}H] diazepam binding was observed which parallels the profile produced by kenazepine for the first 30 min The observation of a difference in the efficacy of these compounds as anticonvulsants during a period (120–240 min) when it is presumed equal numbers of benzodiazepine receptors are occupied by these compounds is currently under investigation Nevertheless, modification of benzodiazepine by addition of alkylating moieties markedly prolongs their pharmacologic activity despite an affinity for benzodiazepine receptors which is comparable to diazepam [4,8]. Alkylating benzodiazepines such as kenazepine and irazepine which have now been demonstrated to have long-lived pharmacologic effects *in vivo*, may prove useful in studying the regulation of the benzodiazepine receptor.

ACKNOWLEDGEMENTS

The authors thank Ms K -L Lock and Ms. V Moncada for technical assistance and Dr W. E Scott, Hoffman-LaRoche, for providing Ro-7/1986 and diazepam.

REFERENCES

1 Klepner, C A., A. Lippa, D. Benson, M Sano and B Beer Resolution of two biochemically and pharmacologically distinct benzodiazepine receptors. *Pharmacol Biochem Behav* **11**: 457–462, 1979.

2 Noble, E , R Wurtman and J Axelrod A simple and rapid method for injecting ^{3}H-norepinephrine into the lateral ventricle of rat brain. *Life Sci* **6**: 281–287, 1967

3. Paul, S. M. and P Skolnick. Acute changes in brain benzodiazepine receptors following experimental seizures. *Science* **202:** 1978, 1978.
4. Rice, K. C , A Brossi, S. M. Paul, J. Tallman and P. Skolnick. Synthesis of irazepine, a noncompetitive, irreversible inhibitor of [^{3}H] diazepam binding *in vitro* *Nature* **278:** 854–855, 1979.
5 Snedecor, G. W. and W. G Cochran. *Statistical Methods* Ames· Iowa State University Press, 1974.
6. Squires, R. F , D. Benson, C. Braestrup, J. Coupet, C. Klepner, V. Myers and B Beer Some properties of brain specific benzodiazepine receptors. New evidence for multiple receptors *Pharmac Biochem Behav.* **10:** 825–830, 1979
7. Tallman, J., S. Paul, P Skolnick and D Gallager. Receptors for the age of anxiety: Pharmacology of the benzodiazepines. *Science* **207:** 274–281, 1980.
8. Williams, E. F., K. C. Rice, S. M Paul and P. Skolnick. Heterogeneity of benzodiazepine receptors in the CNS demonstrated with kenazepine, an alkylating benzodiazepine. *J. Neurochem.* **35:** 591–597, 1980.