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Pharmacologic and Behavioral Effects of EMD 28422: A Novel Purine Which Enhances (^{3}H) Diazepam Binding to Brain Benzodiazepine Receptors

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SKOLNICK, P., K.-L LOCK, B. PAUGH, P. MARANGOS, R. WINDSOR AND S. PAUL. *Pharmacologic and behavioral effects of EMD 28422: A novel purine which enhances (^{3}H) diazepam binding to brain benzodiazepine receptors.* PHARMAC. BIOCHEM. BEHAV. 12(5) 685–689, 1980.—A novel purine, (N^6-2-(4-chlorophenyl)-bicyclo 2.2.2-octyl-(3)-adenosine) EMD 28422 increases the binding of (^{3}H) diazepam to benzodiazepine receptors *in vivo* within 10 min after intraperitoneal administration. This increase in (^{3}H) diazepam binding is due to an increase in the number of benzodiazepine receptors (B_{max}) rather than an altered affinity of the radioligand for receptor (K_d). EMD 28422 protects mice against pentylenetetrazole and caffeine-induced seizures and potentiates the anticonvulsant action of subeffective doses of diazepam in a dose-dependent fashion. Furthermore, EMD 28422 also produces a significant increase in punished responding in a conflict situation (rats), and a long-lasting, dose-dependent decrease in spontaneous motor activity (mice). In contrast, neither EMD 39011 nor adenosine (the two component molecules of EMD 28422) possess anticonvulsant properties at doses up to five mole-equivalents of EMD 28422. These data indicate that the purine EMD 28422 produces a spectrum of pharmacologic effects similar to the benzodiazepines, yet in contrast to the benzodiazepines (and other purines), increases benzodiazepine receptor number. Thus, EMD 28422 may represent the prototype of a class of synthetic purines exerting a unique neurochemical effect on benzodiazepine receptors and possessing several therapeutic actions of the benzodiazepines.

Benzodiazepine receptors Purines Diazepam EMD 28422

THE presence of high affinity, saturable, and stereospecific binding sites for benzodiazepines in the mammalian central nervous system (CNS) has been described in both *in vivo* and *in vitro* [3, 11, 20]. The good correlations obtained between the potencies of a series of benzodiazepines in displacing (^{3}H) diazepam from binding sites *in vitro* and the clinical potencies of these compounds as anxiolytics, muscle relaxants, and anticonvulsants [3, 10, 17] strongly suggests these sites may be receptors which mediate the therapeutic actions of the benzodiazepines.

The identification of the purines inosine and hypoxanthine [1, 12, 15] as putative endogenous ligands of the benzodiazepine receptor prompted us to study the minimum structural requirements necessary for a purine to bind to this receptor [8]. During the course of these studies, it was observed that EMD 28422, an N^6-substituted derivative of adenosine (N^6-[2-(4-chlorophenyl)-bi-cyclo[2.2.2.]-octyl-(3)-adenosine) elicited a concentration dependent increase in the apparent number of (^{3}H) diazepam binding sites (B_{max}) *in vitro* with no concomitant increase in the affinity of (^{3}H) diazepam for the receptor [16]. A series of experiments were designed to determine if this effect occurred following *in vivo* administration of EMD 28422 and if these increases in the number of benzodiazepine receptors were associated with pharmacologic or behavioral effects of this compound.

We now report that *in vivo* administration of EMD 28422 to mice elicits a significant increase in (^{3}H) diazepam binding and produces pharmacologic and behavioral effects reminiscent of the benzodiazepines. Parenteral administration of EMD 28422 produces a dose-dependent sedation in mice, a protective effect against both pentylenetetrazole (PTZ) and caffeine-induced seizures, and a significant anti-conflict effect in a standard rat-conflict paradigm. In contrast, administration of up to five-mole equivalents of either the free base (2-(4-chorophenyl)-bicyclo-2.2.2.-octylamine) (EMD 39011) of EMD 28422 or the parent purine (adenosine) did not produce either an anticonvulsant effect or a potentiation of the pharmacologic effects of diazepam.

METHOD

Animals

Male C3H/HeN, C57Bl/6N or General Purpose mice (20–25 g) were obtained from the Veterinary Resources Branch, NIH. Rats (male, Wistar) were obtained from Royalhart Farms.

Determination of (^{3}H) Diazepam Binding

General Purpose mice were administered EMD 28422 intraperitoneally in diluted Emulphor. Emulphor was mixed 1:1 with absolute ethanol, diluted 1:10 with phosphate buffered saline and injected in a volume of 0.1 ml. At appropriate intervals, animals were decapitated and the forebrains (tissue rostral to an oblique knife cut from the superior colliculi (dorsal) to the mammilary bodies (ventral) removed and homogenized in 0.32 M sucrose. The binding of (^{3}H) diazepam to hypotonically lysed membranes prepared from crude synaptosomes was performed as previously described [14].

Determination of Spontaneous Motor Activity

Groups of six C57 Bl/6N or NIH General Purpose mice were placed in lucite chambers (42×25×16.5 cm) and allowed to adapt for approximately 20 min to the novel environment. Animals were then injected with 5–30 mg/kg of EMD 28422 (IP) or vehicle and locomotor activity monitored with an Animex Motility Meter Type M (LKB Instruments, Hagersten, Sweden). In some experiments, animals were aroused 45 min after injection by lifting them by the tail and twirling.

Determination of Anticonvulsant Activity

C_3H/HeN mice were injected with 20–40 mg/kg of EMD 28422, IP. Ten minutes later, animals were challenged with 290 mg/kg (IP) of caffeine (administered in a volume of 0.2 ml). The number of animals displaying tonic-clonic seizures was noted. In other experiments, General Purpose mice were injected with EMD 28422 (15–60 mg/kg, IP) or vehicle. Thirty minutes later, the mice were injected with diazepam (500 μg/kg, IP) or vehicle. Mice were challenged 30 min later with pentylenetetrazole (PTZ) (100 mg/kg, IP) and the number of animals seizing was noted. In one series of experiments, mice were injected with 30 mg/kg of EMD 28422 and challenged with PTZ after 10 min.

Conflict Procedure

An unconditioned conflict procedure was used in this study which was identical to a previously reported technique [6] except a 700 μA current rather than 200 μA was applied through the drinking spout.

Materials

Pentylenetetrazole (PTZ) was purchased from K and K Laboratories, Plainview, NY. Caffeine and adenosine were purchased from Sigma, St. Louis, MO. EMD 28422 and EMD 39011 were provided by Drs. K. Irmscher and O. Saiko, E. Merck, Darmstadt, W. Germany. Emulphor EL-620 was donated by GAF Corporation, New York, NY and benzodiazepines by Hoffman-La Roche, Nutley, NJ.

RESULTS

Effects of In Vivo Administration of EMD 2844 on (^{3}H) Diazepam Binding

Intraperitoneal administration of 30 mg/kg EMD 28422 resulted in a rapid increase in (^{3}H) diazepam binding in lysed synaptosomal membranes prepared from mouse forebrain. Specific binding of (^{3}H) diazepam was slightly elevated by 10 min after injection and was significantly elevated between 30 and 240 min post-injection. During this interval, specific binding ranged between 110–125% of control (Fig. 1 and unpublished data). Statistically significant increases in (^{3}H) diazepam binding were observed at doses of between 15 and 60 mg/kg of EMD 28422 when measured at 60 min post-injection. At 120 mg/kg, a slight but statistically significant decrease in (^{3}H) diazepam binding was observed (Fig. 2). Scatchard analyses of a typical experiment (45 mg/kg IP administered 30 min prior to sacrifice) indicated this increase in (^{3}H) diazepam binding resulted from an increased B_{max} rather than an alteration in apparent affinity (K_d) (unpublished observations).

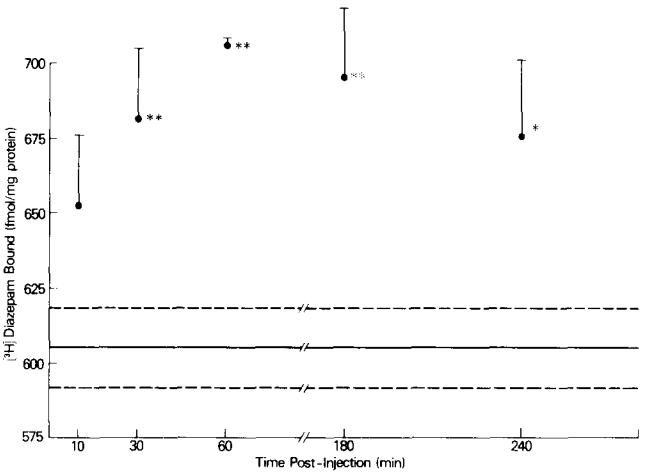

FIG. 1. Time Course of (^{3}H) Diazepam Binding Following 30 mg/kg of EMD 28422: Following intraperitoneal administration of EMD 28422, NIH General Purpose mice were sacrificed at the times indicated and the forebrains removed. Values represent mean ± SEM of 4 mice per time point. Mean ± SEM of (^{3}H) diazepam binding in vehicle injected animals (n=12) is indicated by solid line and two dashed lines. The concentration of (^{3}H) diazepam used in this experiment was 4.2 nM. Symbols: $^{**}p<0.01$; $^{*}p<0.05$ compared with vehicle injected mice.

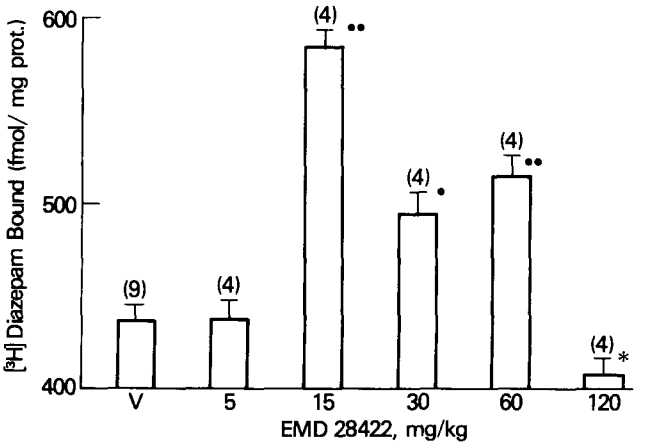

FIG. 2. Effects of Parenterally Administered EMD 28422 on (^{3}H) Diazepam Binding in Mouse Forebrain: NIH General Purpose mice were administered vehicle or 5–120 mg/kg of EMD 28422. Mice were sacrificed 60 min later and the forebrains removed and examined for (^{3}H) diazepam binding as described in *Method*. Values are the mean ± SEM with the number of mice used in parentheses. The concentration of (^{3}H) diazepam in this experiment was 3.9 nM. Symbols: ●●$p<0.001$; ●$p<0.01$; $^{*}p=0.05$.

Effects of EMD 28422 on Spontaneous Motor Activity

Intraperitoneal injection of EMD 28422 elicited a dose-dependent decrease in spontaneous motor activity which was fully manifest by 10 min after injection (Fig. 3a, 4). Mice appeared sedated, yet rapidly responded to tactile stimulation. Following stimulation, the animals appeared to rapidly return to a sedated state, while uninjected animals had a sustained increase in locomotor activity following identical stimulus (unpublished observations). The effects of a single dose of EMD 28422 (30 mg/kg) on spontaneous behavioral activity were long lived. Cumulative activity recorded during a 6-hr observation period were significantly depressed compared with vehicle injected controls (Fig. 3b) despite the adaptation of both groups of animals to the novel environment resulting in an almost complete cessation of activity.

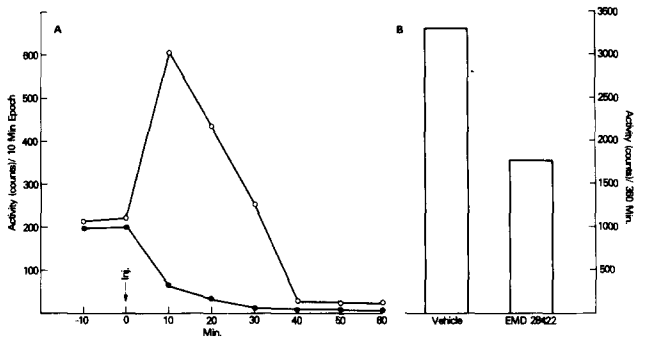

FIG. 3. Effects of EMD 28422 on Spontaneous Motor Activity: Groups of six NIH General Purpose mice were placed in lucite boxes (42×16.5×25 cm), and allowed to adapt to this environment until the locomotor activity in both groups were nearly equal. The mice were then injected with EMD 28422 (30 mg/kg, IP) or vehicle and returned to the lucite chambers. A) Activity (counts) per 10 min epoch in (○) vehicle and (●) EMD 28422 treated mice; B) Cumulative activity recorded over a 360 min interval. Experiments were replicated 2 or 3 times.

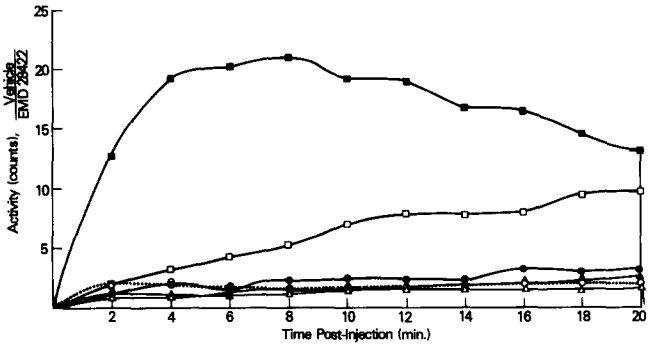

FIG. 4. Effects of EMD 28422 on Spontaneous Motor Activity: Groups of six NIH General Purpose mice were injected with 30 (■), 15 (□), 7.5 (●), 5 (▲), 2.5 (○), and 0.75 (△) mg/kg IP, and spontaneous locomotor activity was recorded during a 20 min period. Vehicle injected animals received an equal volume of diluted Emulphor and spontaneous motor activity was recorded simultaneously with the EMD 28422 injected groups. The ordinate is the ratio of activity recorded in vehicle compared with EMD 28422 injected mice. For example, a ratio of 10 would represent a 90% reduction in spontaneous motor activity.

Effects of EMD 28422 on PTZ and Caffeine-Induced Seizures

Sixty minutes after injection of EMD 28422 (15–60 mg/kg IP; 0.031–0.124 mmol/kg) a marginal protection against PTZ-induced seizures (100 mg/kg) was observed in General Purpose mice (Fig. 5). In one series of experiments, EMD 28422 (30 mg/kg) did not protect animals against 100 mg/kg PTZ when administered 10 min prior to administration. However, administration of a subeffective anticonvulsant dose (0.5 mg/kg) of diazepam 30 min after EMD 28422 resulted in a marked potentiation of the anticonvulsant activity of EMD 28422. The increase in the percent of animals protected was directly related to the dose of EMD 28422 between 15–60 mg/kg (Fig. 5). In contrast, administration of up to 0.197 mmol/kg of EMD 39011 or 0.51 mmol/kg of adenosine resulted in no protection of mice against PTZ-induced seizures alone or in combination with 500 μg/kg of diazepam (unpublished data). In one series of experiments using the CFW-1 strain of mice, 30 mg/kg of EMD 28422 protected all animals from PTZ-induced seizures (50 mg/kg, SC) at both 30 and 240 min after injection (G. Gladding, ADD Program, NINCDS, personal communication).

Ten minutes after administration of EMD 28422 (20 or 40 mg/kg IP), a statistically significant increase in the number of C_3H/HeN mice protected against caffeine-induced seizures was observed (Table 1). Furthermore, a highly significant decrease in post-ictal mortality was also observed in those animals undergoing seizures (Table 1).

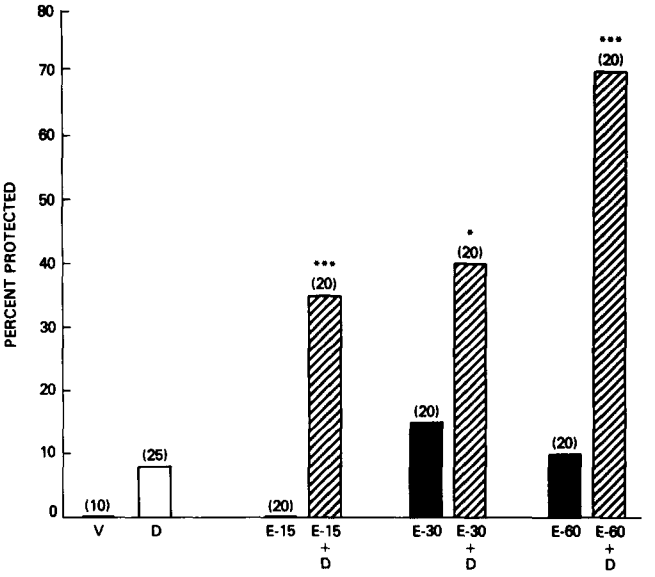

FIG. 5. Effects of EMD 28422 and Diazepam on PTZ-induced Convulsions: NIH General Purpose mice were injected with EMD 28422 (15–60 mg/kg, IP) or vehicle. Thirty minutes later, mice were injected with vehicle or diazepam (500 μg/kg, IP). Animals were challenged with PTZ (100 mg/kg, IP) 30 min after the diazepam injection. Ordinate (percent protected): the percent of animals not displaying tonic-clonic seizure activity after this dose of PTZ. Symbols: V—vehicle treatment; D—diazepam; E—15, 30, or 60, EMD 28422, 15, 30, or 60 mg/kg, respectively. ***$p<0.001$; *$p<0.05$, using a Chi-Square analysis comparing the *sum* of the protective effects of diazepam and each dose of EMD 28422 with the protective effects of a combination of EMD 28422 and diazepam. The number of mice used per point is in parentheses.

TABLE 1

EFFECTS OF EMD 28422 ON CAFFEINE-INDUCED SEIZURES

Dose EMD 28422 (mg/kg)	No. mice tested	No. mice seizing	Deaths
0	12	9	6
20	12	5*	0*
40	11	4*	1*

Male C_3H/HeN mice were injected with vehicle or EMD 28422 10 min prior to administration on caffeine. The number of animals displaying generalized tonic-clonic seizures and the number of deaths was noted. *$p<0.01$ compared with vehicle injected animals, Chi-Square Analysis.

Anti-Conflict Properties of EMD 28422

Thirty minutes after IP administration of 20 mg/kg of EMD 28422 a significant increase in the number of shocks taken by food and water deprived rats was observed compared with vehicle-injected animals (Table 2). A standard anxiolytic dose (12.5 mg/kg) of the benzodiazepine, chlordiazepoxide, was included in this study and produced a comparable increase in the number of shocks taken compared with control.

DISCUSSION

The recent discovery of high affinity, saturable and stereospecific binding sites for benzodiazepines, coupled with the excellent correlations obtained between the *in vitro* binding of benzodiazepines to these sites and the pharmacologic actions of these compounds [13,18] strongly supports the hypothesis that these sites are directly involved in the pharmacologic actions of the benzodiazepines. Pharmacologically active benzodiazepines have been demonstrated to inhibit the binding of radiolabelled benzodiazepines to these sites in a competitive fashion, suggesting occupation of the receptor may be the initial step in a sequence of events resulting in a therapeutic effect.

The isolation and identification of the purines inosine and hypoxanthine as endogenous inhibitors of (^{3}H) benzodiazepine binding [1, 12, 15], and the benzodiazepine-like electrophysiologic [7] and pharmacologic [9,18] effects of inosine led us to examine the minimum structural requirements necessary for a purine to interact with the benzodiazepine receptor. During the course of these studies we observed that the adenosine derivative EMD 28422 increased the number of benzodiazepine receptors *in vitro without* increasing the affinity of (^{3}H) benzodiazepine for the receptor ([16] also cf. [2,19]).

TABLE 2

EFFECTS OF EMD 28422 AND CHLORDIAZEPOXIDE ON CONFLICT RESPONDING

Drug/dose	Shocks received (X ± SEM)	*p*
Vehicle	9.9 ± 4.2	
Chlordiazepoxide, 8 mg/kg	25.2 ± 7.2	<0.01*
EMD 28422, 20 mg/kg	19.3 ± 7.5	<0.05*

Animals were placed in the conflict situation 30 min after drug administration. Values represent X ± SEM using 8 animals (adult, male Wistar rats) per regimen.

*Evaluated using a Mann-Whitney U test.

Administration of EMD 28422 resulted in a dose-dependent sedation in mice, manifested in the present study as a decrease in spontaneous motor activity (Fig. 3). Nonetheless, animals were easily aroused following mild stimulation, a property also observed following low doses of benzodiazepines.

The anticonvulsant effects of this compound were examined using several different convulsant paradigms. A highly significant protective effect of EMD 28422 was observed against caffeine-induced seizures at doses as low as 20 mg/kg. EMD 28422 was only marginally effective as an anticonvulsant with General Purpose mice receiving a large (100 mg/kg) intraperitoneal dose of PTZ. However, EMD 28422 exerts a long lasting anticonvulsant effect when examined in a PTZ-sensitive strain (CFW-1) challenged with 50 mg/kg pentylenetetrazole, SC. Under these conditions, mice were fully protected against seizures at both 30 min and 4 hr following drug-administration. A dramatic potentiation of the anticonvulsant effects of subeffective doses of diazepam was also observed between 15–60 mg/kg of EMD 28422. Although a potentiation of the anticonvulsant effects of diazepam is observed with many compounds (e.g., ethanol), neither EMD 39011 nor adenosine (the two constituent molecules of EMD 28422) exerted an anticonvulsant effect alone or in concert with diazepam at doses of up to 5 mole equivalents of EMD 28422 (unpublished observations), suggesting the potentiation observed with EMD 28422 was specific, and emphasizing the necessity of the intact molecule for pharmacologic activity.

The mechanism by which EMD 28422 exerts its pharmacologic and behavioral effects are not known. However, the increases in (^{3}H) diazepam binding observed *in vitro* [16] and *in vivo* (Fig. 1, 2) at doses where anticonvulsant anxiolytic, and sedative effects were manifest suggests these increases may be responsible for the pharmacologic effects observed. The neuropharmacologic character of the increased number of benzodiazepine receptors elicited by EMD 28422 is currently under investigation (cf. ref. [16]).

The sedative action of EMD 28422 was apparent at doses lower than those necessary for increasing the apparent number of receptors, suggesting these two phenomena may be unrelated. Alternatively, increases in receptor number below the sensitivity of the experimental methods may have occurred. However, in contrast to the anxiolytic, anticonvulsant, and muscle relaxant properties of the benzodiazepines, the sedative effects of benzodiazepines do not appear to correlate well with their Ki values for the receptor *in vitro*, suggesting that the sedation produced by benzodiazepines may not be *directly* mediated by interaction with this receptor.

Both the doses of EMD 28422 necessary to manifest anxiolytic and anticonvulsant effects and the duration of action appear to be related to the increases in receptor number observed following *in vivo* administration of this compound. Although the increases in apparent number of (^{3}H) diazepam binding sites were modest (10–25%), they were comparable to the increases observed following either pharmacologic or behavioral manipulation of rats [4, 5, 14]. The recent demonstration that occupation of only a small fraction (less than 20%) of benzodiazepine receptors by diazepam results

in both a significant anticonvulsant [13] and anxiolytic [6] action suggests that small changes in benzodiazepine receptor number or their occupation could be important for the observed pharmacologic effects of EMD 28422.

The observation that the adenosine derivative EMD 28422 possesses a pharmacologic spectrum reminiscent of the benzodiazepines suggests it may be possible to treat conditions presently requiring benzodiazepines by increasing receptor number rather than competitive occupation of receptors. This novel hypothesis merits further investigation.

ACKNOWLEDGEMENTS

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REFERENCES

1. Asano, T. and S. Spector. Identification of inosine and hypoxanthine as endogenous ligands for the brain benzodiazepine receptor binding sites. *Proc. natn. Acad. Sci. U.S.A.* **76**: 977–981, 1979.
2. Beer, B., C. Klepner, A. Lippa and R. Squires. Enhancement of (^{3}H) diazepam binding by SQ65,396: A novel anti-anxiety agent. *Pharmac. Biochem. Behav.* **9**: 849–851, 1978.
3. Braestrup, C., R. Albrechtsen and R. F. Squires. High densities of benzodiazepine receptors in human cortical areas. *Nature* **269**: 702–704, 1977.
4. Gallager, D. W., P. Mallorga and J. F. Tallman. Interaction of diphenylhydantoin and benzodiazepines in the central nervous system. *Brain Res.*, in press.
5. Lippa, A. S., D. Crichett, M. C. Sano, C. A. Klepner, E. M. Greenblatt, J. Coupet and B. Beer. Benzodiazepine receptors: Cellular and behavioral characteristics. *Pharmac. Biochem. Behav.* **10**: 831–843, 1979.
6. Lippa, A. S., C. A. Klepner, L. Yunger, M. C. Sano, W. V. Smith and J. B. Beer. Relationship between benzodiazepine receptors and experimental anxiety in rats. *Pharmac. Biochem. Behav.* **9**: 853–856, 1978.
7. MacDonald, J. F., J. L. Barker, S. M. Paul, P. J. Marangos and P. Skolnick. Inosine may be an endogenous ligand for benzodiazepine receptors on cultured spinal neurons. *Science* **205**: 715–717, 1979.
8. Marangos, P. J., S. M. Paul, F. K. Goodwin, P. Syapin and P. Skolnick. Purinergic inhibition of diazepam binding to rat brain *(in vitro)*. *Life Sci.* **24**: 851–858, 1979.
9. Marangos, P., A. Martino, S. Paul and P. Skolnick. Benzodiazepines and inosine antagonize caffeine induced seizures. *Psychopharm.*, submitted.
10. Mohler, H. and T. Okada. Benzodiazepine receptor: Demonstration in central nervous system. *Science* **198**: 849–851, 1977.
11. Mohler, H. and T. Okada. Properties of (^{3}H)-diazepam binding to benzodiazepine receptors in rat cerebral cortex. *Life Sci.* **20**: 2101–2110, 1977.
12. Mohler, H., P. Polc, R. Cumin, L. Pieri and R. Kettler. Nicotinamide is a brain constituent with benzodiazepine-like actions. *Nature* **278**: 563–565, 1979.
13. Paul, S. M., P. J. Syapin, B. A. Paugh, V. Moncada and P. Skolnick. Correlation between benzodiazepine receptor occupation and anticonvulsant effects of diazepam. *Nature* **281**: 688–689, 1979.
14. Paul, S. M. and P. Skolnick. Rapid changes in brain benzodiazepine receptors after experimental seizures. *Science* **202**: 892–894, 1978.
15. Skolnick, P., P. J. Marangos, F. K. Goodwin, M. Edwards and S. M. Paul. Identification of inosine and hypoxanthine as endogenous inhibitors of (^{3}H) diazepam binding in the central nervous system. *Life Sci.* **23**: 1473–1480, 1978.
16. Skolnick, P., K.-L. Lock, S. M. Paul, P. J. Marangos, R. Jonas and K. Irmscher. Increased benzodiazepine receptor number elicited *in vitro* by a novel purine, EMD 28422. *Eur. J. Pharmac.*, submitted.
17. Squires, R. and C. Braestrup. Benzodiazepine receptors in rat brain. *Nature* **266**: 732–734, 1977.
18. Tallman, J., S. Paul, P. Skolnick and D. Gallager. Receptors for the age of anxiety: Molecular pharmacology of the benzodiazepines. *Science*, in press.
19. Williams, M. and E. A. Risley. Enhancement of the binding of (^{3}H) diazepam to rat brain membranes *in vitro* by Sa 20009, a novel anxiolytic, GABA and muscimol. *Life Sci.* **24**: 833–842, 1979.
20. Williamson, M., S. Paul and P. Skolnick. Labeling of benzodiazepine receptors *in vivo*. *Nature* **275**: 551–553, 1978.