REFERENCES

Björkman, S., Lewander, T., Zetterström, T. (1980) J. Pharm. Pharmacol. 32: 296–297

Bligh, J. (1979) Neuroscience 4: 1213–1236

Chang, K., Cuatrecasas, P. (1979) J. Biol. Chem. 254: 2610–2618

Chiu, S., Mishra, R. K. (1979) Eur. J. Pharmacol. 53: 119–125

Dickinson, S. L., Slater, P. (1980) Peptides 1: 293–299

Ireson, J. D. (1970) Br. J. Pharmacol. 40: 92–101

Kastin, A. J., Olson, R. D., Ehrensing, R. H., Berzas, M. C., Schally, A. V. (1979) Pharmacol. Biochem. Behav. 11: 721–723.

Lotti, V. J. (1973) The Pharmacology of Thermoregulation. Karger, Basel, pp 382–394

Pleuvry, B. J., Tobias, M. A. (1971) Br. J. Pharmacol. 43: 706–714

Plotnikoff, N. P., Kastin, A. J. (1975) Pharmacol. Biochem. Behav. 9: 759–762

Slater, P., Blundell, C. (1980) Naunyn-Schmiedeberg's Arch. Pharmacol. 313: 125–130

Turnbull, M. J., Wheeler, H. (1980) J. Pharm. Pharmacol. 32: 803–804

Van Ree, J. M., De Wied, D. (1976) Life Sci. 19: 1331–1340

Witter, A., Schottens, H., Verhoef, J. (1980) Neuroendocrinology 30: 377–381

J. Pharm. Pharmacol. 1982, 34: 115–116
Communicated July 21, 1981

0022–3573/82/020115–02 $02.50/0

Determination of biological activity of alprazolam, triazolam and their metabolites

VIMALA H. SETHY*, DOUGLAS W. HARRIS, *The Upjohn Company, CNS Research, Kalamazoo, Michigan 49001, U.S.A.*

The benzodiazepines are psychoactive drugs with wide therapeutic applications as anxiolytics, anticonvulsants, and muscle relaxants (Zbinden & Randall 1967). Their site and mechanism of action have recently become the subject of extensive investigations because of the availability of tritium-labelled diazepam and flunitrazepam with high specific activity. Using these ligands, in vitro and in vivo binding studies have indicated that a specific high affinity binding site, the 'benzodiazepine receptor', in the brain may be relevant to the pharmacological action of the drugs (Mohler & Okada 1977; Squires & Braestrup 1977; Tallman et al 1979).

Triazolobenzodiazepines, like alprazolam and triazolam, have been found to be both anxiolytic and hypnotic in man (Fabre & McLendon 1979; Chatwin & John 1976) in whom these drugs are metabolized to hydroxylated compounds and benzophenones, which are excreted in urine (Eberts et al 1980, 1981). The activity of alprazolam, triazolam and their metabolites on the benzodiazepine receptor is not known. We have undertaken to determine the activity of these compounds on benzodiazepine receptors by using [^{3}H]flunitrazepam ([^{3}H]FNZ) binding to crude rat brain membrane preparations. The results of in vitro [^{3}H]FNZ binding assays were compared with the in vivo ED50s of these compounds required to protect the mice against leptazol(pentetrazol)- and nicotine-induced seizures and against hypoxic stress.

Benzodiazepine receptor binding assays were carried out according to Speth et al (1979). Male Sprague-Dawley rats (180–200 g) were decapitated, their brains quickly removed and, minus cerebellum, homogenized in 10 volumes of cold (4 °C) distilled water, using a Brinkman Polytron PCU-110 homogenizer for 30 s at setting No 6, and centrifuged at 48 000 g at 4 °C for 10 min. The pellet was washed three times by resuspension and recentrifugation, as described above. The final pellet was then suspended in 50 volumes of distilled water.

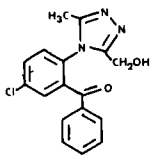
[^{3}H]FNZ binding was measured by incubating 1·0 ml aliquots of membrane suspension with 0·1 ml of [^{3}H]FNZ (specific activity 87·9 Ci mmol^{-1}) to give a final concentration of 0·7 nM for IC50 determinations (or in the range of 0·1–3·2 nM for Scatchard analysis), 0·1 ml of water or drug, as indicated, and 0·8 ml of phosphate-buffered saline (81 mM, Na_2HPO_4, 9·5 mM KH_2PO_4, and 100 mM sodium chloride), pH 7·4, to give a final volume of 2 ml. The mixture was incubated for 100 min at 0–4 °C and then filtered under vacuum through a Whatman GF/B filter. The incubation tube was rinsed with 5 ml of ice cold buffered saline and this rinse was applied to the filter. The filter was finally washed three times with 5 ml amounts of buffered saline. The filter paper was placed in a scintillation vial containing 15 ml of Amersham Searle ACS^R cocktail and the radioactivity was counted by a liquid scintillation spectrometer.

Specific binding was defined as the total binding minus binding in the presence of 100 μM flurazepam. Specific binding represented over 90% of the total binding. Data were subjected to Scatchard and Hill analyses to determine the dissociation constant (K_d), maximum number of binding sites (B_{max}), and Hill coefficient. The IC50 was obtained by logit-log plot of the data. Inhibition constant (K_i) was calculated by the following equation: IC501 + (c/K_d), where c = concentration of ligand (0·7 nM) and K_d = dissociation constant (1·5 nM). The ED50 dose for protection of mice against leptazol- and nicotine-induced seizures and against hypoxic stress was determined by the method of Gall et al (1978) and Moffett et al (1976).

The high affinity specific binding of [^{3}H]FNZ was

* Correspondence.

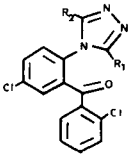
Table 1. Effect of alprazolam and its metabolites on [^{3}H]flunitrazepam binding to crude rat brain membrane preparations.



1

Compound	K_i (nM)	ED50 mg kg^{-1} i.p. Leptazol	Nicotine	Hypoxic stress
Diazepam	5·3	0·7	0·11	0·2
Alprazolam	3·4	0·13	0·056	0·07
α-Hydroxyalprazolam	4·4	0·22	0·08	0·1
4-Hydroxyalprazolam	24	0·7	0·2	0·6
α, 4-Di-hydroxyalprazolam	34	28	3·7	6·2
Comp. 1	510	12·6	40	>50

Table 2. Effect of triazolam and its metabolites on [^{3}H]flunitrazepam binding to crude rat brain membrane preparations.



2–4

Compound	K_i (nM)	ED50 mg kg^{-1} i.p. Leptazol	Nicotine	Hypoxic stress
Diazepam	5·3	0·7	0·11	0·2
Triazolam	0·76	0·009	0·004	0·035
α-Hydroxytriazolam	0·92	0·018	0·01	0·05
α, 4-Di-hydroxytriazolam	1·9	4	0·7	30
4-Hydroxytriazolam	3·2	10	2·5	>50
Comp. 2 R_1 = CH_2OH R_2 = Me	1500	>50	>50	>50
Comp. 3 R_1 = H R_2 = Me	1450	45	>50	>50
Comp. 4 R_1 = CH_2OH R_2 = CH_2OH	3600	N.T.	N.T.	N.T.

saturable with respect to ligand concentration. The Scatchard analysis of whole brain minus cerebellum gave a straight line with a regression coefficient of 0·99, a dissociation constant of 1·50 ± 0·10 nM, and a B_{max} of 445 ± 30 fmols mg^{-1} protein (mean ± s.e. n = 12). A Hill plot gave a coefficient of 1·01, indicating that there was no positive or negative cooperativity in ligand binding. These observations agree with those reported by Speth et al (1979).

The relative potency of diazepam, alprazolam and its metabolites is shown in Table 1. The K_i of diazepam was 5·3 nM. This value is identical to that reported by Chang & Snyder (1978). Alprazolam (K_i = 3·7 nM) and α-hydroxyalprazolam (K_i = 4·2 nM) had high affinity for binding to benzodiazepine receptors. Both of these compounds were also found to be very potent in protecting mice against leptazol- and nicotine-induced seizures and against hypoxic stress. 4-Hydroxyalprazolam and α-4-dihydroxyalprazolam had relatively low affinity for binding to benzodiazepine receptors with K_i's of 24 and 34 nM respectively. Both of these hydroxylated metabolites of alprazolam also had low protective activity against in vivo tests. The benzophenone metabolite, compound 1, was practically inactive in the receptor binding assay and was very weak in in vivo tests.

Triazolam and α-hydroxytriazolam were potent inhibitors of [^{3}H]FNZ binding, with K_i's of 0·54 and 0·73 nM, respectively. In agreement with this, both triazolam and α-hydroxytriazolam were potent in protecting mice against leptazol- and nicotine-induced seizures and against hypoxic stress. α-4-Dihydroxytriazolam and 4-hydroxytriazolam had 37 and 18% receptor binding activity, respectively, compared with the parent compound. In the in vivo test also, these compounds had low protective activity compared with triazolam. The benzophenone metabolites, compounds 2, 3 and 4, were inactive in [^{3}H]FNZ binding assays. Correspondingly, compounds 2 and 3 were also inactive in leptazol- and nicotine-induced seizures and in hypoxic stress tests in mice (Table 2).

The high binding affinities of alprazolam and triazolam, as determined by [^{3}H]FNZ binding assays, indicate that both of these compounds may mediate their biological actions by binding to benzodiazepine receptors. The decreased binding affinities of the metabolites of alprazolam and triazolam correlates well with their decreased protective activity against leptazol- and nicotine-induced seizures and against hypoxic stress. The lowered biological activity of alprazolam and triazolam metabolites as determined by both the in vitro and in vivo tests indicates that these triazolobenzodiazepines undergo a biological deactivation. The in vitro [^{3}H]FNZ binding assay serves as a quick and simple method for determining the biological activity of triazolobenzodiazepines and any change that may occur in their activity as a result of metabolism.

REFERENCES

Chang, R. S., Snyder, S. H. (1978) Eur. J. Pharmacol. 48: 213–218

Chatwin, J. C., John, W. L. (1976) Curr. Ther. Res. 21: 207–214

Eberts, F. S., Jr., Philopoulos, Y., Reineke, L. M., Vliek, R. W. (1980) Pharmacologist 22: 279

Eberts, F. S., Jr., Philopoulos, Y., Reineke, L. M., Vliek, R. W. (1981) Clin. Pharm. Ther. 29: 81–93

Fabre, L. F., McLendon, D. M. (1979) Curr. Ther. Res. 25: 419–426

Gall, M., Kamdar, B. V., Collins, R. J. (1978) J. Med. Chem. 21: 1290–1294

Moffett, R. B., Kamdar, B. V., VonVoigtlander, P. F. (1976) J. Med. Chem. 19: 192–193

Mohler, H., Okada, T. (1977) Sci. 198: 849–851

Speth, R. C., Bresolin, N., Yamamura, H. I. (1979) Eur. J. Pharmacol. 59: 156–160

Squires, R., Braestrup, C. (1977) Nature (London) 266: 732–734

Tallman, J. R., Thomas, J., Gallager, D. (1979) Life Sci. 24: 873–880

Zbinden, G., Randall, L. O. (1967) Adv. Pharmacol. 5: 213–291