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Determination of biological activity of adinazolam and its metabolites

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Adinazolam and its metabolites inhibit [^{3}H]flunitrazepam binding in-vitro. The binding affinities of these compounds is significantly enhanced in the presence of 10–5 M muscimol, indicating that both adinazolam and its metabolites are benzodiazepine agonists. In-vivo metabolism of adinazolam results in the formation of active metabolites.

Adinazolam (8-chloro-1-((dimethylamino)methyl)-6-phenyl-4H-s-triazolo(4,3-a)-(1,4)benzodiazepine methanesulfonate) has been reported in preclinical studies to exhibit both antidepressent and anxiolytic properties (Hester et al 1980: Lahti et al 1982). Antidepressant activity with low sedative potential has been confirmed in clinical studies (Pyke et al 1983). In rodents, the parent compound is extensively metabolized with multiple *N*-dealkylation steps leading to the removal of the dimethyl-aminomethyl side chain. The benzodiazepine ring and the attached 6-phenyl group may also be hydroxylated in this species. However, in man the major metabolite found in the urine is the monomethylaminomethyl analogue of adinazolam. In the in-vitro benzodiazepine receptor binding assay, adinazolam is significantly less potent that diazepam, but in in-vivo tests like protection against pentetrazol (leptazol, metrazol)- and nicotine-induced seizures and against hypoxic stress in mice, adinazolam is nearly as potent as diazepam (Sethy et al 1983). These results are consistent with the observation that adinazolam is extensively metabolized in rodents into a compound which may have greater anxiolytic activity than the parent compound. The activity of adinazolam metabolites in both in-vitro and in-vivo tests is not completely investigated. We have undertaken a study to determine the biological activity of adinazolam and its metabolites on benzodiazepine receptors using [^{3}H]flunitrazepam ([^{3}H]FNZ) binding to a crude rat brain membrane preparation. The binding studies were done in the presence and absence of muscimol to determine if any metabolite has benzodiazepine antagonist properties (Braestrup & Nielson 1981; Skolnick et al 1982). The results of in-vitro [^{3}H]FNZ binding assays have been compared with the in-vivo ED50's of these compounds required to protect the mice against pentetrazol- and bicuculline-induced seizures and against hypoxic stress.

Methods

In-vitro [^{3}H]FNZ binding to crude rat brain membrane preparations for the determination of inhibition constants (K_i) of adinazolam and its metabolites was by the method of Sethy & Harris (1982) with slight modifications (Braestrup et al 1979). Male Sprague-Dawley rats were decapitated, the brain quickly removed and the bilateral cerebral cortex dissected out, weighed, and homogenized in 25·0 ml of iced cold (4 °C) 50 mM Tris-citrate buffer, pH 7·1, using a Brinkman Polytron PCU-2-110 homogenizer for 25 s at setting No 6. The homogenate was centrifuged at 48 000*g* for 10 min and the pellet was washed six times by resuspension and recentrifugation as described above. The final pellet was frozen at −70 °C for a minimum of 18 h. On the day of the assay, the pellet was thawed in 25 ml of Tris-citrate buffer, pH 7·1, and was washed once again as described above. The final pellet was suspended in 50 volumes of the same buffer.

[^{3}H]FNZ binding was carried out by incubating 1·0 ml aliquots of membrane suspension in triplicate with 0·1 ml of [^{3}H]FNZ (spec. act. 76·9 Ci mmol^{-1}) to give a final concentration of 0·98 nM, 0·1 ml of distilled water or drug as indicated, and 0·8 ml of Tris-citrate buffer, pH 7·1, to give a final volume of 2 ml. Each drug was investigated at five concentrations. The final concentrations of adinazolam ranged between 10^{-5}–10^{-9} M. Diazepam, U-compounds, RO 15-1788, β-CCE (3-carboethoxy-β-carboline), and β-CCM (3-carbomethoxy-β-carboline) were studied at 10^{-7}–10^{-11} M. Muscimol (0·1 ml) at a concentration of 10^{-5} M was used for the studies involving the presence of a GABA agonist. The volume of buffer was adjusted accordingly. The mixture was incubated for 100 min at 4 °C and then filtered under vacuum through a Whatman GF/B filter. The incubation tube was rinsed with 5 ml of ice cold buffer and this rinse was applied to the filter. The filter was washed three times with 5 ml aliquots of buffer. Finally, the filter paper was placed in a scintillation vial to which 15 ml of Amersham Searle ACS Cocktail was added. The radioactivity was counted by liquid scintillation spectrometry. Each drug was studied in two independent experiments.

Specific binding was defined as the total binding minus binding in the presence of 10 μM flurazepam. Specific binding represented over 95% of total binding. The IC50 was obtained by a logit-log plot of the data. The inhibition constant (K_i) was calculated by the following equation: $K_i = IC50/(1 + c/K_d)$, where c = concentration of ligand (0·98 nM) and K_d = dissociation constant (1·09 nM). The ED50 for protection of mice against pentetrazol and bicuculline-induced seizures and against hypoxic stress was determined by the

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method described by Gall et al (1978) and Moffett et al (1978).

Results and discussion

In the rat and mouse, adinazolam is metabolized first by *N*-dealkylation to form the mono-*N*-demethyl derivative, U-42,352, which is subsequently dealkylated to di-n-demethyl analogue, U-41,128. Deamination of U-41,128 results in the formation of a postulated intermediate which undergoes either α-hydroxylation (U-40,125) or cleavage of the side chain (U-33,737, estazolam) (Fig. 1). U-40,125 and U-33,737 constitute the major metabolites in mice. In man, U-42,352, the mono-*N*-demethyl compound, is the major metabolite.

The inhibition constant of adinazolam in [^{3}H]FNZ binding assay is 208 nM. In comparison to the parent compound, the metabolites of adinazolam are 20–40 times more potent in inhibiting the binding of [^{3}H]FNZ. However, in the in-vivo tests, adinazolam and its metabolites are found to be equipotent in protecting mice against pentetrazol- and bicuculline-induced seizures and against hypoxic stress (Table 1). These results indicate that adinazolam may undergo metabolism, leading to the formation of more active compounds in-vivo.

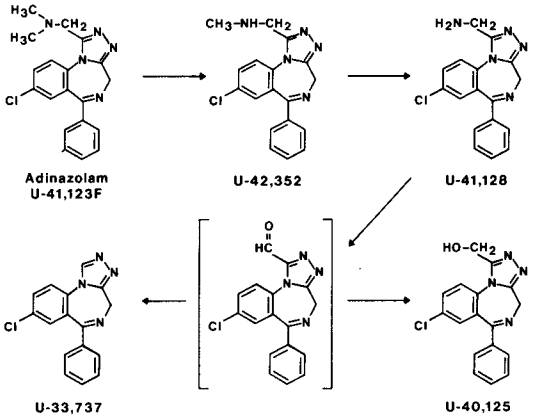


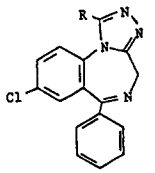
FIG. 1

Table 2. Effect of muscimol (10^{-5} M) on K_i of benzodiazepin antagonists in [^{3}H]flunitrazepam binding assays.

Compound	K_i (nM) Without muscimol	With muscimol
RO 15-1788	0·91	1·0
β-CCE	1·4	1·7
β-CCM	2·0	3·1

Like diazepam, the binding affinities of adinazolam and its metabolites are significantly enhanced in the presence of 10^{-5} M muscimol as demonstrated by the decrease in K_i value (Table 1). The binding affinity of RO 15-1788 was not altered by the presence of muscimol. However, the presence of muscimol decreased the binding of β-CCE and β-CCM and increased K_i values by 21·4 and 55%, respectively (Table 2). The presence of muscimol increases the binding affinities of benzodiazepine agonists like alprazolam, diazepam and CL 218872. In contrast to the agonists, the binding affinities of benzodiazepine antagonists like β-CCE and β-CCM are significantly reduced by muscimol (Braestrup & Nielsen 1981; Braestrup et al 1982; Skolnick et al 1982). The binding affinities of adinazolam and its

Table 1. Effect of adinazolam and its metabolites on [^{3}H]flunitrazepam binding to a crude rat brain membrane preparation in-vitro and its correlation with in-vivo pharmacological tests.



Compound	R	K_i (nM) Without muscimol	With 10^{-5} M muscimol	ED50: mg kg^{-1} i.p. Pentetrazol	Bicuculline	Hypoxic stress
Adinazolam	CH_2-N(CH$_3$)(CH$_3$)	208	138	0·9	0·7	1·0
U-42,352	CH_2-N(CH$_3$)(H)	6·96	4·92	0·4	0·5	1·0
U-41,128	CH_2-N(H)(H)	4·17	3·07	0·35	0·7	0·8
U-40,125	CH_2-OH	10·4	7·34	0·36	0·2	0·2
U-33 737	H	6·6	4·29	0·9	0·2	0·8
Diazepam	—	7·9	6·1	0·7	1·9	0·2

metabolites are significantly enhanced in the presence of muscimol, suggesting that these compounds may not be benzodiazepine antagonists.

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(RO 15-1788 is: ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a][1,4]-benzodrazepine-3-carboxylate.)

REFERENCES

Braestrup, C., Nielsen, M. (1981) Nature (London) 294: 472–474

Braestrup, C., Nielsen, M., Krogsgaard-Larsen, P., Falch, E. (1979) Nature (London) 280: 331–333

Braestrup, C., Schmiechen, R., Nielsen, M., Petersen, E. N. (1982) in: Usdin, E. et al (eds), Pharmacology of Benzodiazepines, MacMillan Press, London, 71–85

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

Hester, J. B., Rudzik, A. D., Von Voigtlander, P. F. (1980) J. Med. Chem. 23: 392–402

Lahti, R. A., Cohn, J. B., Feighner, J. P., Smith, W. T., Pyke, R. E. (1982) Neuro. Sci. 8, Abstract No. 128.3, p. 463

Moffett, R. B., Kamdar, B. V., Von Voigtlander, P. F. (1978) J. Med. Chem. 21: 1290–1294

Pyke, R. E., Cohn, J. B., Feighner, J. P., Smith, W. T. (1983) Psychopharmacol. Bull. 19: 96–98

Sethy, V. H., Harris, D. W. (1982) J. Pharm. Pharmacol. 34: 115–116

Sethy, V. H., Russell, R. R., Daenzer, C. L. (1983) Ibid. 35: 524–526

Skolnick, P., Schweri, M. M., Williams, E. F., Moncada, V. Y., Paul, S. M. (1982) Eur. J. Pharmacol. 78: 133–136

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Mianserin: is the intraperitoneal route of administration the best?

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A 1 h pretreatment with s.c. mianserin (0·5 mg kg^{-1}) markedly blunted the oedema of the rat paw induced by the subplantar injection of 5-HT (5 μg). In contrast, i.p. mianserin (same dose) failed to modify 5-HT-induced oedema. However, pretreatment with SKF-525-A (40 mg kg^{-1}) resulted in a profound potentiation of the action of i.p. mianserin, the effect of the combination of SKF-525-A and i.p. mianserin rivalling that of s.c. mianserin. It is concluded that i.p. mianserin is metabolized by the rat liver to compounds which possess a reduced propensity to block peripheral 5-HT receptors and that the i.p. route of administration is not to be recommended when mianserin is being studied as a 5-HT receptor antagonist in the rat.

The tetracyclic antidepressant mianserin is a potent 5-hydroxytryptamine (5-HT) receptor antagonist (Vargaftig et al 1971). In the course of studying this property of the drug it was observed that the ability of mianserin to block the resultant oedema of the rat paw following the subplantar injection of 5-HT was markedly dependent on its route of administration and it was decided to pursue this observation.

Method

Male Sprague-Dawley rats, 200–250 g, were used. The rear left paw received a subplantar injection (0·1 ml) of 5 μg of 5-HT creatinine sulphate. The contralateral paw received vehicle (0·9% NaCl). Paw thickness was measured 15 min after 5-HT injection using the apparatus described by Bonta & de Vos (1965). The recorded reading represented an approximate sevenfold amplification in the actual paw thickness. The dose of 5-HT used approximately doubled paw thickness. Mianserin hydrochloride was dissolved in distilled water and was injected either i.p. or s.c. 1 h before 5-HT. Doses of both mianserin and 5-HT refer to the free base. For each rat the thickness of both rear paws was measured and the difference in recorded values was expressed as percentage increase in thickness. Each result is the mean ± s.e.m. of at least six observations and statistical significance was determined using Student's *t*-test (two-tailed).

Results

The i.p. injection of mianserin, 0·5 mg kg^{-1}, was devoid of effect on the 5-HT-induced swelling of the rat paw (Table 1). Increasing the dose to 1 mg kg^{-1} resulted in a modest, statistically significant inhibition. In contrast, the s.c. injection of mianserin, 0·5 mg min^{-1}, markedly blunted the response to 5-HT. Hence, the response to mianserin, 0·5 mg kg^{-1}, is markedly dependent on the route of administration. The s.c. injection of 0·05 and 0·1 mg kg^{-1} of mianserin also significantly antagonized the 5-HT-induced oedema. The inability of mianserin, 0·5 mg kg^{-1} i.p., to attenuate the paw swelling evoked by 5-HT was reversed by a 1 h pretreatment with SKF-525-A, 40 mg kg^{-1} i.p., the inhibitory action of the combination rivalling that of mianserin, 0·5 mg kg^{-1} s.c.

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