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The Novel Anxiolytic U-101017: In Vitro and Ex Vivo Binding Profile and Effect on Cerebellar cGMP

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SETHY, V. H. AND H. WU. *The novel anxiolytic U-101017: In vitro and ex vivo binding profile and effect on cerebellar cGMP.* PHARMACOL BIOCHEM BEHAV **58**(2) 609–613, 1997.—The binding affinities (K_i) of U-101017 and diazepam for the $GABA_A$ receptor in rat cortical membranes were determined using [^{3}H]flunitrazepam ([^{3}H]FNZ) as the ligand. The inhibition constants of U-101017 and diazepam were 3.78 nM and 6.36 nM, respectively. Brain uptake of U-101017 was studied by the ex vivo [^{3}H]FNZ binding assay. A significant ex vivo inhibition of [^{3}H]FNZ binding was observed 10 min after oral administration of U-101017, and the effect lasted for at least 240 min (the last time point of investigation). The potential anxiolytic activity of U-101017 and diazepam was investigated in nonstressed and stressed (electric foot shock) mice by quantitative estimation of cerebellar cyclic 3′,5′-guanosine monophosphate (cGMP). Both U-101017 and diazepam dose-dependently decreased cGMP and attenuated stress-induced elevations in cGMP. These effects were antagonized by the $GABA_A$ receptor antagonist flumazenil. U-101017 was about two orders of magnitude more potent in stressed animals than in controls. The results of our investigation indicate that the anxiolytic-like activity of U-101017 is mediated via $GABA_A$ receptors. © 1997 Elsevier Science Inc.

U-101017 Stress cGMP Anxiolytic $GABA_A$ receptor

U-101017 (7-chloro-5-[*cis*-3,5-dimethylpiperazine)carbonyl]-imidazole [1,5*a*] quinoline-3-carboxylate (Fig. 1) was synthesized in an attempt to discover a novel anxiolytic that is devoid of the clinical liabilities of benzodiazepines, including sedation, muscle relaxation, potentiation of alcohol effects, and physical dependence. Benzodiazepines bind to $GABA_A$ receptors with affinities that correlate with their anxiolytic and muscle relaxant properties (16,23). Recently, molecular biological investigations have led to the identification and cloning of genes for the different subunits of $GABA_A$ receptors and the recognition that the receptors may vary in different brain regions in terms of their subunit composition and affinities for various compounds (18,19). Anxiolytics selective for particular subtypes of $GABA_A$ receptors may lack some of the side effects of benzodiazepines.

U-101017 has been previously reported to produce a biphasic effect on GABA-induced increases in Cl^- flux using either $\alpha_1\beta_2\gamma_2$ or $\alpha_3\beta_2\gamma_2$ $GABA_A$ receptor complexes. At low concentrations, U-101017 potentiated, and at high concentrations inhibited, GABA-induced Cl^- current in these subtypes of $GABA_A$ receptors. Flumazenil, an antagonist of the $GABA_A$ receptor, blocked only the potentiating effect of U-101017 on GABA-induced Cl^- current (11). Consistent with this unique neurophysiological effect, U-101017 was reported to have a limited disinhibitory effect in the punished behavioral paradigm of Vogel and in Geller's conflict test, and this compound blocked diazepam-induced muscle relaxation. However, U-101017 was found to be very effective in attenuating the stress-induced elevation in plasma corticosterone in rats (25,27). Thus, U-101017 appears to be a partial agonist of $GABA_A$ receptors.

Anxiolytics decrease cerebellar cGMP (1,4,14) and attenuate stress-induced increases in this nucleotide (7). U-78875, a mixed agonist/antagonist of $GABA_A$ receptors (24), was found to have high binding affinity for benzodiazepine receptors (22). This compound was also as efficacious as diazepam in

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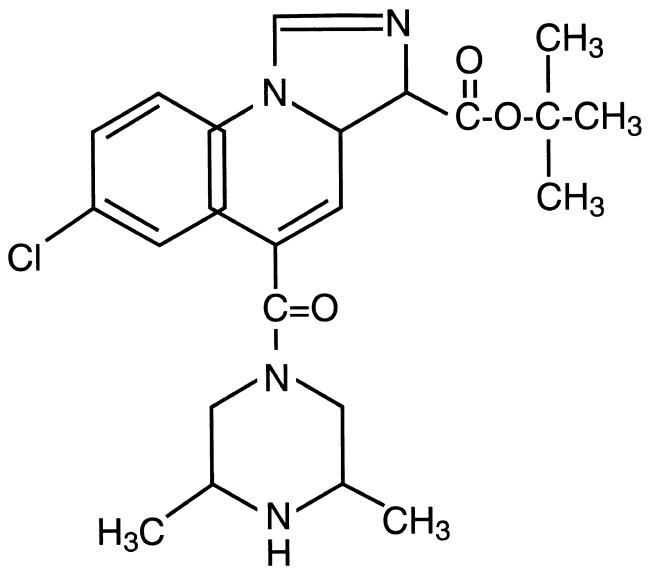

FIG. 1. Structure of U-101017.

decreasing baseline cerebellar cGMP and in attenuating the stress-induced response (22). Because U-101017 is a biolog of U-78875, we decided to further investigate the neurochemical profile of this new partial $GABA_A$ agonist. Some of the results with U-101017 have been compared with those obtained with diazepam under similar conditions.

METHODS

Animals

Male Sprague–Dawley rats (125–180 g) and male CF-1 mice (18–20 g), bred at Pharmacia & Upjohn, Inc., were used in this study. Animals were kept under constant diurnal (12 L: 12 D cycle) lighting and temperature conditions for 3–5 days prior to their use. They were sacrificed between 0800 and 1100 h for the neurochemical investigations.

In Vitro [^{3}H]Flunitrazepam ([^{3}H]FNZ) Binding

In vitro [^{3}H]FNZ binding to crude rat cerebral cortical membrane preparation for determination of inhibition constants (K_i) of U-101017 and diazepam was carried out with a slight modification of the method previously described (21). Rats were decapitated, the brain was quickly removed from the skull, and the cerebral cortex was dissected out bilaterally. The tissue was weighed and then homogenized in 25 ml of ice cold (0°C) 50 mM Tris-HCl buffer, pH 7.4, using a Brinkman Polytron homogenizer for 30 s at setting 6. The homogenate was centrifuged at 48,000 × *g* for 10 min, and the pellet was washed once, followed by resuspension and recentrifugation. The final pellet was then resuspended in 100 volumes (w/v) of the same buffer.

Inhibition of binding of [^{3}H]FNZ was determined by incubating 0.4 ml of membrane suspension, 0.05 ml of [^{3}H]FNZ (final concentration of ligand 0.98–1.0 nM, specific activity 87 Ci/mmol), and 0.05 ml of drug or distilled water to give a final volume of 0.5 ml. Each drug was investigated at five to seven concentrations in triplicate. The mixture was incubated at 0°C for 60 min. The binding reaction was terminated by vacuum filtration of the mixture through a Whatman GF/B filter using the Tom Tech™ system. The ligand–receptor complex was trapped in the filter paper, and the unbound ligand was removed by washing the filter paper twice with buffer. The filter was air dried and the radioactivity was counted with a liquid scintillation counter.

Specific binding was defined as total binding minus binding in the presence of 10 μM flurazepam. Specific binding represented more than 95% of total binding. The IC_50 was obtained by logit–log plot of the data. K_is were calculated by the following equation: $K_i = IC_50/(1 + c/K_D)$, where c is the concentration of ligand (0.98–1 nM) and K_D is the dissociation constant of the ligand (0.94 nM).

Ex Vivo [^{3}H]Flunitrazepam Binding

U-101017 was dissolved in 0.25% carboxymethylcellulose and then administered orally to mice. Control mice received an equal volume of the vehicle (1 ml/100 g). Animals were sacrificed at 1, 3, 10, 30, 60, 120, and 240 min after treatment. Whole brain minus cerebellum was quickly removed from the skull, immediately frozen on dry ice, and then stored at −80°C until used for the binding assay. Determination of ex vivo inhibition of [^{3}H]FNZ binding by previously administered U-101017 was carried out by the procedure described in the past (20). The results are expressed as percent inhibition of binding, which is directly related to the concentration of the parent drug plus its active metabolites in the brain.

Cerebellar cGMP

U-101017 and diazepam were administered orally (PO), whereas flumazenil was injected intraperitoneally (IP). Thirty minutes after treatment with U-101017, diazepam, flumazenil, or U-101017 plus flumazenil, mice were sacrificed by a beam of microwave radiation focused on the skull for 0.6 s (Metabostat™, model 4094, developed by Gerling-Moore). The procedure for estimation of cGMP was the same as that described by Burkard et al. (4). The cerebellum was quickly removed from the skull, weighed, and then homogenized in 10 volumes of 1% perchloric acid, using a Brinkman Polytron PCU-110 homogenizer for 15 s at setting 6. The samples were kept on ice for 30 min, boiled for 5 min, and then centrifuged at 17,000 × *g* for 20 min. The content of cGMP in the supernatant was measured by radioimmunoassay. Statistical analysis of data was done by one-way analysis of variance and subsequently by Student's *t*-test. The results are expressed as percent of control.

Electric Foot Shock Stress

U-101017, either alone or in combination with flumazenil, was administered to mice 30 min before stress induction. The animals were subjected to inescapable electric foot shock (0.5

TABLE 1

INHIBITION OF [^{3}H]FNZ BINDING IN RAT CEREBRAL CORTICAL MEMBRANES BY U-101017 AND DIAZEPAM

Drug	K_i (nM)
U-101017	3.37 ± 0.22
Diazepam	6.36 ± 0.87

K_i values are mean ± SE of three observations, each in triplicate.

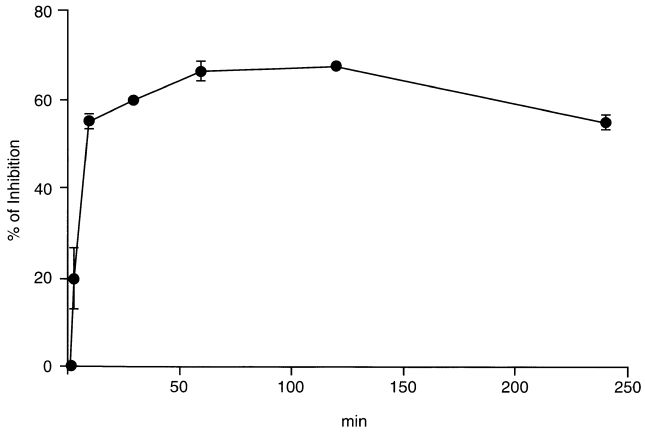

FIG. 2. Inhibition of ex vivo [^{3}H]FNZ binding to the mouse cortical membrane after oral administration of U-101017 (30 μmol/kg).

mA for 10 s) stress in a behavioral box with a stainless steel grid floor and then were immediately sacrificed for estimation of cerebellar cGMP.

RESULTS

U-101017 and diazepam concentration-dependently inhibited the binding of [^{3}H]FNZ to the membrane preparation of rat cerebral cortex in vitro. U-101017 was significantly ($p < 0.05$) more potent in inhibiting [^{3}H]FNZ binding than was diazepam; K_i values are shown in Table 1.

Oral administration of U-101017 (30 μmol/kg) time-dependently blocked [^{3}H]FNZ binding to the mouse cerebral cortex. At 10 min, a significant ($p < 0.01$) inhibition of ex vivo binding was observed, and a significant blockade of [^{3}H]FNZ binding persisted at 240 min, the last time point of investigation. The peak inhibition with U-101017 was observed at 60 min (Fig. 2).

TABLE 2

ED_{50}s FOR U-101017 AND DIAZEPAM IN DECREASING CEREBELLAR cGMP IN NONSTRESSED AND FOOT SHOCK-STRESSED MICE

Drug	ED_{50}, μmol/kg PO (95% CL)		Nonstressed/ Stressed
	Nonstressed	Stressed	
U-101017	260.0 (163–425)	0.37 (0.12–1.04)	703
Diazepam	2.08 (0.92–4.48)	0.08 (0.03–0.21)	26

In control mice, cerebellar cGMP ranged between 412 and 523 pmol/g of tissue ($n = 6$). These cerebellar cGMP levels are consistent with those reported by Burkard et al. (4). Both U-101017 and diazepam dose-dependently decreased the levels of this nucleotide (Fig. 3). U-101017 was significantly ($p < 0.05$) less potent than diazepam in decreasing cGMP; ED_{50}s with 95% confidence limits are shown in Table 2.

In stressed mice, cerebellar cGMP ranged between 976 and 1,042 pmol/g of tissue ($n = 5$); this is about a twofold increase in cGMP level compared with nonstressed animals. U-101017 and diazepam dose-dependently attenuated the stress-induced increases in cGMP. Although U-101017 was significantly ($p < 0.05$) less potent than diazepam, it appeared to be more efficacious in reducing stress-induced elevations in cGMP (Fig. 4); ED_{50}s with 95% confidence limits are shown in Table 2.

Flumazenil (6 μmol/kg IP), an antagonist of $GABA_A$ receptors, had no significant effect on cGMP in nonstressed mice, but pretreatment with flumazenil significantly blocked U-101017 (10 μmol/kg PO)-induced reductions in cGMP (Table 3). In stressed mice, flumazenil (3 and 6 μmol/kg IP) was found to be ineffective in altering cerebellar cGMP, but pretreatment with these doses of flumazenil significantly ($p < 0.01$) blocked U-101017-induced attenuation of stress-induced elevations in cGMP (Table 4).

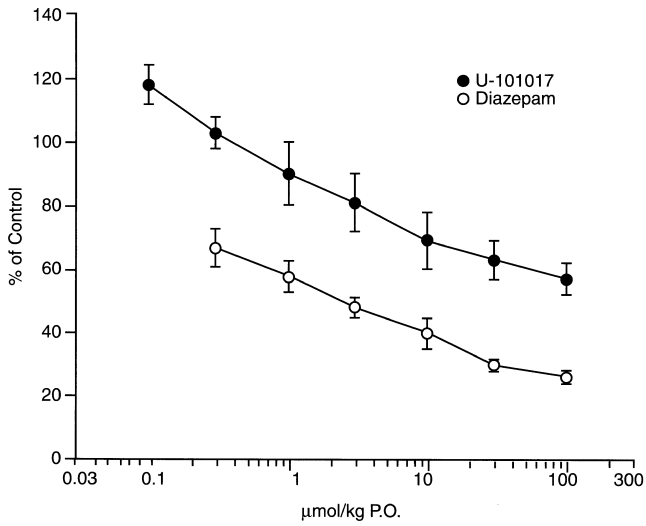

FIG. 3. Effect of U-101017 and diazepam on cerebellar cGMP in mice. Each point is the mean ± SE of four to six observations.

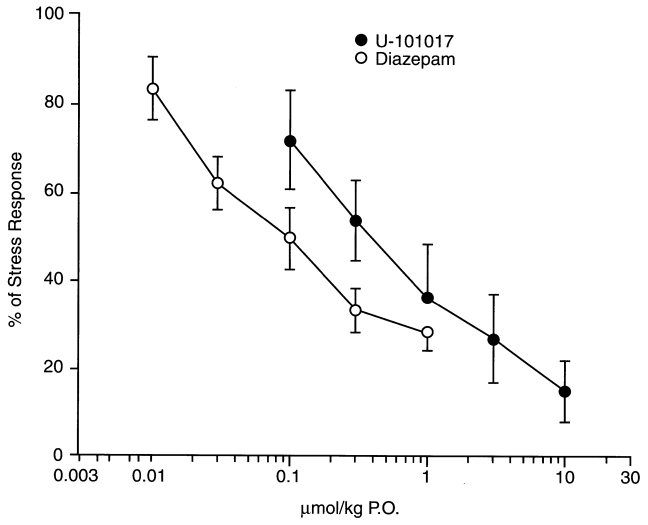

FIG. 4. Effect of U-101017 and diazepam on stress-induced increases in cerebellar cGMP in mice. Stress-induced increases in cGMP (about twofold) were considered 100%. Each point is the mean ± SE of four to six observations.

TABLE 3

EFFECT OF FLUMAZENIL ON U-101017-INDUCED REDUCTIONS IN CEREBELLAR cGMP IN MICE

Treatment	Dose (μmol/kg)	cGMP:% of Control
Control	—	100 ± 9
Flumazenil	6	99 ± 4
U-101017	10	44 ± 3**
Flumazenil + U-101017	6, 10	73 ± 3***

Cyclic GMP levels are expressed as percentage of control level, mean ± SE of four to six observations. $^{**}p < 0.01$ compared with control; ***p < 0.01 compared with U-101017.

TABLE 4

EFFECT OF FLUMAZENIL ON U-101017-INDUCED REDUCTIONS IN CEREBELLAR cGMP IN FOOT SHOCK-STRESSED MICE

Treatment	Dose (μmol/kg)	cGMP:% of Control
Control	—	100 ± 4
Stress	—	173 ± 8
Flumazenil + stress	3	177 ± 4
Flumazenil + stress	6	171 ± 4
U-101017 + stress	1	122 ± 6**
Flumazenil + U-101017 + stress	3, 1	154 ± 3***
Flumazenil + U-101017 + stress	6, 1	177 ± 7***

Cyclic GMP levels are expressed as percentage of control level, mean ± SE of four to six observations. $^{**}p < 0.01$ compared with stressed control; $^{***}p < 0.01$ compared with U-101017.

DISCUSSION

The anxiolytic benzodiazepines bind to $GABA_A$ receptors in vitro (15,23), potentiate GABA-induced chloride flux (12), and are brain penetrable (10), resulting in both in vivo and ex vivo inhibition of [^{3}H]FNZ binding (3,20). They decrease cerebellar cGMP (1) and block stress-induced elevations in this nucleotide (7). In the present investigations, the $GABA_A$ partial agonist U-101017 was found to have a neurochemical profile similar to the benzodiazepine anxiolytics. The compound was found to enter the brain rapidly and to produce a long duration of inhibition of ex vivo [^{3}H]FNZ binding. This profile of ex vivo $GABA_A$ receptor occupancy by U-101017 is similar to that described for diazepam (10,20). The prolonged duration of receptor occupancy may be due to the presence of U-101017 or the combination of parent compound and the active metabolites in the brain.

U-101017 decreases cerebellar cGMP in animals miantained within normal physiological conditions, an effect that is blocked by the $GABA_A$ receptor antagonist flumazenil. Likewise, flumazenil has been reported to antagnonize diazepam-induced reduction in cGMP (4,22). The results of the present investigations indicate that U-101017 has an agonist effect on $GABA_A$ receptors, which may be responsible for the observed decrease in cerebellar cGMP.

Flumazenil (6 μmol/kg IP) by itself had no significant effect on cerebellar cGMP in the present investigations. Likewise, even at high doses (32 and 100 μmol/kg), flumazenil was reported to have no effect on cerebellar cGMP (13). Although flumazenil has been described as a partial agonist of $GABA_A$ receptors in some behavioral tests (5,26), we and others have failed to demonstrate any neurochemical agonist activity. Thus, the neurochemical profiles of U-101017 and flumazenil appear to be dissimilar.

Acute stress induced by electroconvulsive shock, forced swimming in ice-cold water, or immobilization has previously been found to increase cerebellar cGMP levels (6,7,9) Similarly, brief electric foot shock was found to elevate cerebellar cGMP in the present study. Pretreatment with U-101017 or diazepam blocked electric foot shock-induced increases in cGMP. The ED_{50} doses required to block stress-induced elevations for U-101017 and diazepam were 703- and 26-fold respectively, lower than those needed to lower cGMP in nonstressed animals. Doses of U-101017 that were ineffective in modulating cGMP in nonstressed mice completely prevented the perturbation induced in these animals by the stress of electric foot shock. Thus, the blockade of stress-induced elevations in cerebellar cGMP seems to be a sensitive neurochemical correlate of anxiolytic-like activity.

Flumazenil, a weak anxiogenic (8), had no significant effect on stress-induced elevations in cerebellar cGMP. Consistent with this observation, flumazenil has been shown to be ineffective in altering stress-induced elevations in plasma corticosterones (2,17). In addition, flumazenil was found to be inactive in modulating the stress-induced increase in striatal and limbic forebrain homovanillic acid levels. Thus, flumazenil has no antistress activity as determined by neurochemical assessments. However, flumazenil blocked the attenuating effect of diazepam on stress-induced rises in homovanillic acid (14). In agreement with this observation, flumazenil was found to antagonize the attenuating effect of U-101017 on stress-induced elevations in cerebellar cGMP. These data further indicate that U-101017 acts as an agonist of $GABA_A$ receptors, which regulate cerebellar cGMP levels.

In summary, U-101017 binds to $GABA_A$ receptors in vitro and ex vivo. In nonstressed mice, U-101017 is less efficacious than diazepam in decreasing cerebellar cGMP, suggesting that it may be a partial agonist under normal physiological conditions. However, U-101017 appears to be as efficacious as diazepam in attenuating stress-induced increases in cGMP, indicating that it is a full agonist of the $GABA_A$ receptor under a perturbed physiological state. The pharmacological profile of U-101017 is consistent with that observed for clinically used anxiolytics.

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