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Pyrazolo[1,5-a]pyrimidines: Receptor Binding and Anxiolytic Behavioral Studies

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LOEW, G., L. TOLL, J. LAWSON, E. UYENO AND H. KAEGI. *Pyrazolo[1,5-a]pyrimidines: Receptor binding and anxiolytic behavior studies.* PHARMACOL BIOCHEM BEHAV **20**(3)343–348, 1984.—Pyrazolo[1,5-a]pyrimidines (PZP) have been reported to be specific anxiolytic agents which do not potentiate ethanol or barbiturates. To further investigate these compounds, three of the most promising analogs were synthesized and a tritium-labeled analog of one of them prepared by a new synthetic procedure. These analogs did not compete with [^{3}H]flunitrazepam or [^{3}H]β-carboline ethyl ester binding nor did they potentiate the [^{3}H]flunitrazepam binding. Receptor binding studies with the [^{3}H]PZP revealed a low affinity receptor site, distinct from that of the benzodiazepines, but with only a small fraction (20%) of specific binding. Behavioral tests using three different animal models for anxiety: muricide, approach/avoidance conflict and two-chamber exploration tests gave conflicting results, positive in the first and negative in the latter two. Furthermore, these compounds were found not to be antagonists of diazepam's anticonvulsant activity. Taken together, these results, while provocative, do not support evidence that these analogs are promising specific anxiolytic agents.

Pyrazolo[1,5-a]-pyrimidines Approach-avoidance conflict Two-chambered exploration
Benzodiazepine Receptors

IT is now generally accepted that the benzodiazepines (BZ) mediate their pharmacological actions by interacting with specific receptor sites in the brain [16,22]. Although the major therapeutic uses of benzodiazepines are for their anxiolytic and mild sedative properties, they are also potent anticonvulsants and muscle relaxants. Initial BZ receptor studies indicated a single benzodiazepine binding site [16, 20, 22]. However, the binding of some non-benzodiazepine compounds has subsequently demonstrated the possibility of BZ receptor heterogeneity [3, 13, 17, 21, 29]. The methyl, ethyl and propyl esters of β-carboline-3-carboxylate which antagonize many BZ actions, and the anxiolytic 1,2,4-triazolo[4,3-b]pyridazine, (Cl 218,872), all inhibit [^{3}H]flunitrazepam and [^{3}H]diazepam binding. However, the competition curves are shallower than a mass-action curve with Hill coefficients of 0.5–0.7 [3,29]. Furthermore, a marked regional variation in the slope of the inhibition curves has led to the hypothesis of two BZ subtypes that may mediate different pharmacological effects. Accordingly, it has been suggested that the β-carbolines and Cl 218,872 have high affinity for type I receptors, while the benzodiazepines have high affinity for type I and II receptors [13,15]. This could explain the observation that Cl 218,872 has potent anticonflict and anticonvulsant activities, but is not a sedative or a muscle relaxant. Recently, however, binding studies done at physiological temperatures have shown no evidence of multiple binding sites, but possibly different states of a single receptor [7,8]. Furthermore, behavioral studies have demonstrated an antagonism by Cl 218,872 of some but not all of diazepam's actions [6]. Clearly the nature and number of BZ binding sites and the relationship to their multiple activities is not well understood.

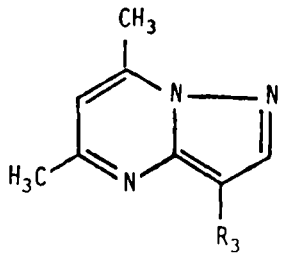
Currently, there is great interest in so-called "anxioselective" anxiolytics, both as clinically relevant drugs and as selective ligands to study BZ receptor heterogeneity. Recently a class of compounds, pyrazolo[1,5-a]pyrimidine (PZP) (Fig. 1), which were originally synthesized as phosphodiesterase inhibitors, were reported to possess anxiolytic activity with similar efficacy to diazepam [12]. These compounds differed from the benzodiazepines in that they lacked sedative-hypnotic, anticonvulsant and muscle relaxant activity. Furthermore, they were reported not to potentiate the depressant effects of alcohol and barbiturates. No receptor binding studies were described. In the work reported here we have continued to investigate the most active analogs of this long-ignored but potentially interesting class of compounds. Specifically, the three compounds shown in Fig. 1 were synthetized and a tritiated analog of *1b* prepared. The behavioral effects of these compounds were re-evaluated and receptor binding studies conducted to determine whether these seemingly specific and potent anxiolytics bind to a subset of benzodiazepine receptors or to a separate anxiolytic site altogether.

Our studies have failed to demonstrate consistent anxiolytic activity, or benzodiazepine antagonism. Furthermore, these compounds neither have an effect on [^{3}H]flunitrazepam receptor binding nor demonstrated appreciable specific binding to rat brain membranes.

METHOD

Materials

[^{3}H]Flunitrazepam and [^{3}H]ethyl-β-carboline-3-carboxyl-



1a R_3 = H
1b R_3 = Cl
1c R_3 = Br
1d R_3 = Cl ^{3}H-LABELED

FIG. 1. Pyrazolo[1,5-a]pyrimidine analogs synthesized and studied.

ate ([^{3}H]β-CCE) were purchased from New England Nuclear. Gifts of diazepam and chlordiazepoxide from Hoffmann-LaRoche are gratefully acknowledged.

Animal Behavior Tests

PZP compounds *1a*, *1b*, and *1c* were tested for anxiolytic activity in three animal paradigms: the muricide, approach/avoidance conflict, and two-chamber exploration tests. In order to reproduce or further corroborate published results, the procedure and rationale for the muricide and approach/avoidance conflict tests were the same as that in the previous work on this series of compounds [12].

Muricide test [12]. In a preliminary test male Long-Evans hooded rats weighing between 300 g and 500 g, were housed individually in isolation cages. One mouse was introduced into each rat cage to determine which rats would kill the mice. Twenty-four animals that killed the mice were selected for the main test and were assigned to five groups (five or four animals/group). Groups I, II, III, IV, and V were orally injected with 80 mg/kg of *1a*, *1b*, *1c* and diazepam, and with the vehicle, respectively. The compounds were partially dissolved and suspended in a solution of 8% alcohol and sesame oil. A standard volume of 2 ml/kg was administered. One mouse was placed in each rat cage 1, 2, 4, and 6 hours after drug administration. A two-minute period was allowed for the kill and at the end of the session the mouse was removed. The rats served as their own controls.

Approach/avoidance conflict test [26]. Male Sprague-Dawley rats were deprived of water for 48 hours prior to the test session. They were injected intraperitoneally with a test compound or vehicle, thirty minutes before the test. Each animal was placed individually in a test chamber (38×38×32 cm), provided with a drinking spout. An animal was allowed to find the drinking tube and complete 20 licks. Immediately after the 20th lick, it was given a mouth shock for two sec. The shock was administered by a shock generator reading 1.8 mA. In accordance with the suggestion of Dr. A. S. Lippa of American Cyanamid Company [14], an analysis of the actual intensity of the current accepted by a rat was conducted by inserting a resistance of 220 kohms (an average resistance of 140 to 150 g rats) between the drinking tube and the grid floor. At the same level of current which measured 1.8 mA on the external meter, the presence of the resistance reduced the ammeter reading to 0.3 mA. This current was selected so that the number of shocks taken by control animals would be significantly less than expected ceiling effects of ~35–40 shocks per session. At the termination of the shock a three-minute timer was automatically activated. During the three-minute period, a shock was delivered following each twentieth lick. The total number of shocks delivered during the three-minute session was recorded for each subject.

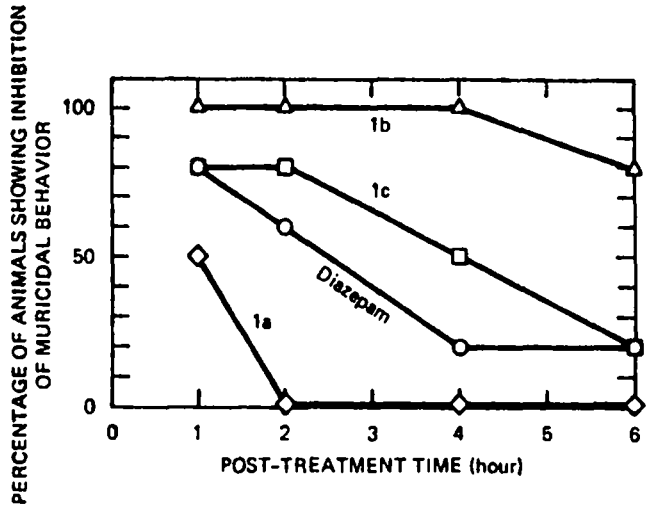


FIG. 2. Results of muricide test for 80 mg/kg oral dose of analogs *1a*, *1b*, and *1c*, and diazepam.

Drugs were prepared as suspensions in agar so that each ml contained 1 kg of body weight dosage.

Two-chamber exploration test [4,5]. Male NIH albino general purpose mice were individually put into the lighted section of a two-chambered cage (44×21×21 cm), placed on an Automex locomotor activity monitor (Columbus Instruments). The total locomotor activity scores and total number of transitions across the partition were recorded for each animal during the ten-minute session. *1b* (10 and 20 mg/kg) and chlordiazepoxide (5 and 10 mg/kg) were dissolved in a vehicle consisting of 2% ethyl alcohol and 4% propylene glycol in 0.9% saline solution. The chemicals were injected intraperitoneally in volumes of 5 ml/kg, 30 minutes before testing.

Benzodiazepine antagonism test [10, 19, 25]. The effects of chloro-PZP on the anticonvulsant activities of diazepam were studied by injecting Swiss-Webster mice with 5 mg/kg, IP of diazepam and forty-five minutes later with chloro-PZP or the vehicle (5% gum acacia). Fifteen minutes after PZP administration the animals were dosed with 120 mg/kg (IP) of pentylenetetrazole. β-CCE and RO 15-1788 were evaluated as reference compounds.

Receptor Binding Studies

For [^{3}H]flunitrazepam binding, whole rat brain was homogenized in a Polytron Homogenizer (Brinkman Instruments Inc.) in 40 volume 50 mm Tris-HCl pH 7.4. The homogenate was centrifuged at 48,000 × g for 10 min, rehomogenized in Tris buffer and centrifuged again. This washing procedure was carried on six times to remove any endogenous GABA. After six washes, the tissue was resuspended in Tris buffer, frozen and then used within one month.

To measure specific binding [^{3}H]flunitrazepam or [^{3}H]β-CCE (usually 0.2 nM) and unlabeled substances were added to the brain homogenate in a final incubation volume of 2 ml containing approximately 9.0 mg wet weight of tissue. Incubation was carried out on ice for 90 min and terminated by rapid filtration over Whatman GF/B filters and two additional 5 ml washes. Radioactivity trapped on the filters was counted in 8 ml Scintisol (Isolabs Inc.) at 50% efficiency after 12 hr at room temperature. Unlabeled β-CCE (10^{-6} M) was

TABLE 1

EFFECTS OF 3-CHLORO- AND 3-BROMO-5,7-DIMETHYLPYRAZOLO [1,5-a] PYRIMIDINES AND DIAZEPAM ON SUPPRESSED DRINKING RESPONSE OF SPRAGUE-DAWLEY RATS

	Dose mg/kg IP	Shocks Taken During the 3-Minute Session Mean ± S.E.M.
1b	40	8 ± 1.8
	20	10 ± 1.6
	10	11 ± 1.9
1C	40	8 ± 1.9
	20	10 ± 1.9
	10	10 ± 1.9
Diazepam	20	19 ± 2.4†
	10	20 ± 2.4†
	5	18 ± 2.3†
	2.5	16 ± 2.0*
	1.25	13 ± 2.4
AGAR (Vehicle)	—	10 ± 1.3

At each dose level 10 animals were tested.
*$p<0.02$, †$p<0.005$, two-tailed *t*-test comparison with the control group.

used to define nonspecific binding and represented less than 5% of total binding.

For [^{3}H]PZP, *1d*, saturation binding studies were attempted by a filtration assay similar to that just described, and by a centrifugation assay as described by Yamamura *et al.* [29]. No differences in binding were apparent at 0° or 25°C for times ranging from 30–90 min or with Tris or Krebs/Hepes buffer.

Chemistry

Melting points were determined in open capillary tubes on a Mel-Temp apparatus and are uncorrected. NMR spectra were determined on a Varian XL-100 and T-60 spectrometer in $CDCl_3$ with Me_4Si internal standard. All compounds synthesized were single spots by TLC and known compounds showed the expected melting point and NMR described in the literature [18]. Elemental analyses were correct to 0.4% CHN.

The known PZP's *1a*, *1b*, *1c* were resynthesized as described by Novison, *et al.* [18].

3-Chloro-5,7-bis-(dibromomethyl)pyrazolo[1,5-a]pyrimidine

A mixture of 3-chloro-5,7-dimethylpyrazolo[1,5-a] pyrimidine (*1b*) [18] (1.35 g, 7.4 mmole) and N-bromosuccinimide (NBS) (8.0 g, 44.9 mmole) in 350 ml tetrachloromethane (CCL_4) was stirred under N_2 in a 500 ml pyrex flask and irradiated externally with a Hanovia lamp in a pyrex filter. This procedure initiated the bromination without causing the reaction mixture to warm above 50°C. The reaction was complete in 4 hours. The crude product was purified on a Waters LC-500 on silica gel, eluting with 10% ethyl acetate in hexane. The isolated product *2* (1.23 g) was a yellow gum which was crystallized from dichloromethane/hexane 1:1, then recrystallized from cyclohexane to afford 0.93 g (25%) of analytically pure product mp 104–105°C; nmr ($CDCL_3$) four singlets in a ratio of 1:1:1:1 at δ 6.73 (H at 2 position), 7.37 (H on 5-$CHBr_2$), 8.00 (H on 7-$CHBr_2$), and 8.27 (H at 6 position), mass spectrum, m/e (relative intensity) 497 (M^+, 20), 418, 416 (M-Br, 100, 90), 337 (M^-Br_2, 44). *Anal.* ($C_8H_4N_3Br_2Cl$) C,H,N. Analysis of the by-products from this reaction indicates major components are tribromo-derivatives.

[^{3}H]-labeled-3-Cl,-5,7-Dimethylpyrazolo [1,5-a]pyrimidine (1d)

To a solution of 49 mg (0.1 mmole) of the tetrabromide *2* and 0.05 ml (36 mg, 0.36 mmole) of triethylamine in 5 ml of ethyl acetate was added 25 mg of palladium (black) and 10 Ci of carrier-free tritium gas. After stirring at room temperature for 4 hours, the hydrogenolysis was completed with hydrogen gas. The reaction mixture was filtered through a cellite pad, evaporated to dryness, and the residue (39 mg) was taken up in dichloromethane, washed with an aqueous solution of sodium bicarbonate, and dried over magnesium sulfate. Evaporation of the solvent gave crude product *1d* which was purified on preparative TLC plates (silica gel, hexane-ethyl acetate 3:2) to give 8.7 mg (48%) of pure produce *1d* (98% radiochemical purity) with a specific activity of 77.15 m Ci/mg or 14 Ci/mmole.

RESULTS AND DISCUSSION

Behavioral Tests

As shown in Fig. 2, results for the muricide test on three analogs (*1a*, *1b*, *1c*) were positive and reproduced the reported results. All compounds suppressed muricidal responses. At one-hour post treatment, 80 mg/kg of *1a*, *1b*, *1c*, and diazepam inhibited the muricidal behavior of 50%, 100%, 80%, and 80% of the rats tested, respectively. Two hours after injection all the chemicals except *1a* continued to inhibit the killing. By six hours post administration the repressive effects of all the substances, except *1b* were substantially decreased.

The results of the approach/avoidance conflict are summarized in Table 1. In each case the mean number of shocks taken by the group treated with 2.5, 5, 10, or 20 mg/kg of diazepam were significantly greater than that of the control group, indicating the antianxiety effects of diazepam. However, the mean number of shocks accepted by each of the three groups injected with *1b* was not significantly different from the control mean. Similarly, the average shock score of each of the three groups administered *1c* were not significantly different from the control mean, implying that the results of Kirkpatrick *et al.* [12] were not reproducible. In our attempt to determine whether or not *1b* and *1c* had any antianxiety activity, we tested hundreds of other rats (i.e., Long-Evans and Holtzman rats) injected intraperitoneally or orally with 5 to 80 mg/kg of *1b* or *1c*. In all the experiments the test compounds failed to give positive results; whereas 5 to 20 mg/kg of diazepam produced positive results repeatedly.

Since the important details of the evaluation methods were not described in the report of Kirkpatrick *et al.* [12], it is difficult to determine whether some possible subtle differences between their and our test procedures produced the discrepancy. Moreover, it is difficult to fully evaluate the reported results, as only the minimal effective doses are given for their four most potent compounds, and no data are

presented to evaluate the statistical significance of their results. One difference to be noted is that their sample of rats was considerably irresponsive to diazepam, since a dose of 20 mg/kg or higher was required to produce "a statistically significant difference in the number of shocks accepted by treated and control animals" [12]. However, in the experiment of Vogel *et al.* [26] and that of ours, only 2 mg/kg and 2.5 mg/kg of diazepam, respectively, were required to increase the suppressed drinking response significantly. Since the animals observed by Kirkpatrick *et al.* [12] reacted to diazepam in such an unusual manner, it seemed that their sample of subjects were considerably biased and manifested an uncommon response to the PZP administration.

The disparate results obtained from these two different animal models of anxiety illustrate one of the major difficulties in the search for a specific anxiolytic agent without the multiple activities of the BZs: the lack of an animal test that will definitively correlate with human antianxiety for such behavior. The BZs themselves were not screened in animals for their anxiolytic activity, but for their anticonvulsant, muscle-relaxant, and sedative-hypnotic effects. These activities, particularly the anticonvulsant activity against pentylenetetrazole (PTZ), were then found to correlate with human antianxiety activity when these drugs were clinically evaluated [23].

Since contradictory results were obtained from the muricide and approach/avoidance conflict tests, a third model of animal anxiety was investigated, the so-called two-chamber exploration test, very recently described by Crawley and Goodwin, [5] and by Crawley [4]. Some difficulty was encountered, in that the level of activity and response of control mice varied considerably with strain. However, when using the same strain of mice reported (NIH general-purpose), we obtained significantly positive results for 5 and 10 mg/kg chlordiazepoxide HCl, but negative results for PZP analog *1b* (Fig. 3).

The two most reliable screens for the evaluation of anxiolytics (the approach/avoidance conflict and two-chamber exploration tests) revealed that *1b* and *1c* were not anxiolytics. Although the muricide test indicated that 80 mg/kg of the test substances and 80 mg/kg of diazepam apparently inhibited the muricidal activity of the isolated rats, the effect may simply be due to sedation produced by the administration of the very high dose. Horovitz *et al.* [9] also noted that the nonspecific inhibition of muricidal effects of chlordiazepoxide and diazepam paralleled the depressant effects on locomotor coordination. Since some tranquilizers, sedatives, antidepressants, antihistamines, and amphetamine-like compounds have blocking action on muricide, [9] it seemed that the nonspecific muricide test was not an effective assay to determine anxiolytic activity.

Recently Gee *et al.* have suggested that the selective anxiolytic activity of the 1,2,4-triazolo[4,3-b]pyridazine, (CI 218,872), may be due to antagonism of some of diazepam's actions [6]. If, as reported, compounds *1a*, *1b*, and *1c* are anxiolytic but not anticonvulsant, [12] it is possible they would antagonize the anticonvulsant activity of diazepam. However, when injected intravenously, the known antagonists RO 15-1788 and β-CCE had antagonist ED_{50}s of 0.13 and 3.40 mg/kg respectively, while compound *1b* was inactive up to 40 mg/kg.

Binding Studies

Analogs *1a*, *1b*, and *1c* were tested to determine whether they competed for [^{3}H]flunitrazepam-binding sites in rat brain homogenates. No inhibition of [^{3}H]flunitrazepam or [^{3}H]β-CCE binding was observed up to concentrations of 10^{-5} M. These compounds were also tested to determine whether they increased [^{3}H]flunitrazepam binding since such potentiation has been demonstrated by a number of classes of compounds including GABA, [11, 24, 27] and pyrazolopyridines [1, 2, 28]. In particular, the pyrazolopyridines are anxiolytic compounds whose activity is thought to be related to their ability to increase benzodiazepine binding [28]. However, as shown in Table 2, at temperatures ranging from 0–25°C analogs *1b* or *1c* did not increase [^{3}H]flunitrazepam binding, while GABA did.

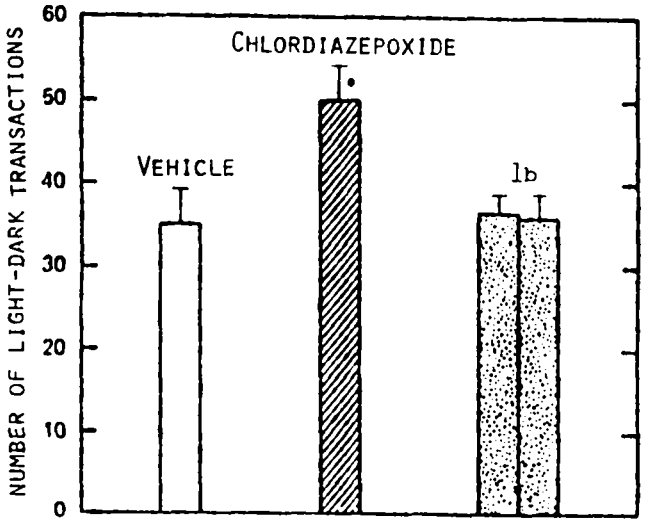


FIG. 3. Results of two-chamber exploration test using NIH general purpose mice. The results show the total number of transitions recorded for each animal for a ten-minute period following intraperitoneal injection of 10 and 20 mg/kg of analog *1b* and 5 mg/kg of chlordiazepoxide compared to vehicle.

Because of the reported specific anxiolytic activity of the PZP compounds, a tritiated [^{3}H]PZP analog, *1d*, was prepared to explore the possibility that this class of compounds binds to its own specific receptor site, distinct from that of the BZs.

As discussed in the Method section, the original protocol attempted was a filtration assay, similar to that used for [^{3}H]flunitrazepam binding. This procedure yielded no specific binding. We then adopted the centrifugation assay as described by Yamamura *et al.* for the binding of a relatively low affinity anxiolytic [^{3}H]CI-218,872 [29]. Under these conditions, we repeatedly observed a very small amount of specific binding (20% of total binding). Binding appeared to be linear with tissue concentration but did not begin to saturate by 150 nM [^{3}H]PZP. Because of the small percentage of specific binding and the marginal results obtained in animal models for anxiolytic activity, the nature and significance of this binding site is uncertain.

CONCLUSIONS

The pyrazolo[1,5-a]pyrimidines were examined as a class of possible specific anxiolytic compounds without the anticonvulsant, depressant or muscle relaxant properties inherent in the benzodiazepines. The compounds were of further

TABLE 2

ATTEMPTED STIMULATION OF [^{3}H]FLUNITRAZEPAM BINDING TO RAT BRAIN MEMBRANES

Incubation Temperature	Drug	(fmol) Specific Binding	% of Control
0°/0°	Control	65.7 ± 1.7	100
	GABA	83.0 ± 5.1	126
	1b	65.5 ± 0.3	100
	1c	64.3 ± 2.1	98
25°/0°	Control	44.1 ± 2.0	100
	GABA	64.9 ± 1.1	147
	1b	41.0 ± 0.6	98
	1c	42.9 ± 0.3	97
25°/25°	Control	27.5 ± 1.1	100
	GABA	57.5 ± 1.7	209
	1b	30.0 ± 1.8	109
	1c	30.6 ± 1.1	111

The incubation was carried out as described in the Method section for 90 min at 0°, then filtered at 0°C, incubated at 25°C for 60 min, then placed on ice for 5 min prior to filtration, or incubated at 25°C for 60 min prior to filtration. The concentration of all drugs was 10^{-5} M.

interest because they were reported not to potentiate the depressant effects of alcohol and barbiturates [26]. In our hands, however, compounds *1a–1c* were not found to be consistently effective anxiolytics, nor did they antagonize the anticonvulsant activity of diazepam. As reported, they were found to inhibit the muricide activity of isolated rats. However, this effect may simply be due to sedation, which we observed, particularly at high concentration.

Binding studies of these compounds failed to demonstrate inhibition of [^{3}H]flunitrazepam or [^{3}H]β-carboline binding up to 10 μm. Nor did these compounds stimulate [^{3}H]flunitrazepam binding or demonstrate a high affinity saturable receptor site distinct from that of the benzodiazepines. The test compounds: *1a*, *1b*, and *1c*, were the most promising anxiolytics reported [26]. However, the three derivatives were not effective in our evaluation. Therefore, we feel that 3-halo-5,7-dimethylpyrazolo[1,5-a] pyrimidines, in general, are not promising anxiolytics.

ACKNOWLEDGEMENTS

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