"Supporting Information"

A novel selective $GABA_A$ α1 receptor agonist displaying sedative and anxiolytic-like properties in rodents.

Silvia Selleri,[§*] Fabrizio Bruni,[§] Camilla Costagli,[§] Annarella Costanzo,[§] Gabriella Guerrini,[§] Giovanna Ciciani,[§] Paola Gratteri,[§] François Besnard,[‡] Barbara Costa,["] Marina Montali,["] Claudia Martini,["] Jonna Fohlin,[#] Gaetano De Siena, [#] Petra Malmberg Aiello.[#]

Experimental section, including, binding studies, pharmacological methods, elemental analyses.

Experimental Section.

Binding Studies. [^{3}H]Ro 15-1788 (specific activity 70.8 Ci/mmol) was obtained from NEN life Sciences products. All other chemicals were of reagent grade and obtained from commercial suppliers. Bovine cerebral cortex membranes were prepared as previously described.[1,2] The membrane preparations were subjected to 50 mM tris-citrate buffer pH 7.4, and then used in the binding assay. Protein concentration was assayed by the method of Lowry et al.[3] [^{3}H]Ro 15-1788 binding studies were performed as previously reported.[4] Clonal mammalian cell lines, kindly provided by François Besnard (Synthélabo Recherche, France), expressing relatively high levels of $GABA_A$ receptor subtypes ($\alpha_1\beta_2\gamma_2$, $\alpha_2\beta_2\gamma_2$, $\alpha_3\beta_2\gamma_2$, $\alpha_5\beta_3\gamma_2$) were maintained, as previously described,[5] in Minimum Essential Medium Eagle with EBSS, supplemented with 10% fetal calf serum, L-glutamine (2mM), penicillin (100 units/ ml) and streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO_2/95% air at 37 °C. After removal, the cells were harvested by centrifugation at 500xg. The crude membranes were prepared after homogenization in 10 mM potassium phosphate, pH 7.4, and differential centrifugation at 48,000xg for 30 min at 4 °C. The pellets were washed twice in this manner before final resuspension in 10 mM potassium phosphate, pH 7.4, containing 100 mM potassium chloride.[5] [^{3}H]Ro 15-1788 binding assays to transfected cell membranes were carried out as previously described.[5] In brief, the cell line membranes were incubated in a volume of 500 μl which contained [^{3}H]Ro 15-1788 at a concentration of 1-2 nM and test compound in the range 10^-

^{9}-10^{-5} M. Non specific binding was defined by 10^{-5} M diazepam. Assays were incubated to equilibrium for 1 h at 4°C. The compounds were dissolved in DMSO. The level of DMSO did not exceed 1% and was maintained constant in all tubes. At least six different concentrations of each compound were used. The data of n=5 experiments carried out in triplicate were analyzed by an iterative curve-fitting procedure (program Prism, GraphPad, San Diego, CA), which provided IC_{50}, K_i, and SEM values for tested compounds, K_i values being calculated from the Cheng and Prusoff equation.[6]

The potencies of the new synthesized compounds to inhibit [^{3}H]Ro 15-1788 binding in the presence and absence of GABA were compared. Differences obtained were expressed as GABA shifts (namely the ratios of the K_i values obtained in the absence of GABA over the K_i values obtained in the presence of GABA) have given indications about agonist, partial agonist, antagonist, inverse agonist pharmacological activity.

Pharmacological methods. The experiments were carried out in accordance with the Animal Protection Law of the Republic of Italy, DL n. 116/1992, based on the European Communities Council Directive of 24 November 1986 (86/609/EEC). All efforts were made to reduce the number of animals used and to minimize their suffering.

In our experiments we used male CD-1 albino mice (25g) and male Wistar rats (200g). Both the mice and rats were from Harlan Italy. Maximum twelve mice

and three rats were housed per cage and fed a standard laboratory diet, with tap water *ad libitum* for 12 h/12 h light/dark cycles (lights on at 7.00 h). The conditions in the facilities were as follows: the temperature was 22-23 °C, air changed in the room 15 times/hour and the air-humidity was maintained at 50±5%. The cages were brought into the experimental room the day before the experiment for acclimatization purposes. All experiments were performed between 10:00 and 17:00 h.

Drugs: The following commercial drugs were used: Diazepam (Valium 10-Roche, Italy), Flumazenil (Sigma, Steinheim, Germany), Penthylenetetrazole (PTZ) (Sigma, Steinheim, Germany), Zolpidem (Tocris, Bristol, UK). All the drugs, except PTZ, were suspended in 1% carboxymethylcellulose sodium salt and sonicated immediately before use (20 s at 400 W). All benzodiazepine receptor ligands were administered p.o., except flumazenil that was injected i.p.. PTZ was dissolved in isotonic (NaCl 0.9%) saline solution and injected s.c.

Hole board test

To evaluate the effects of drugs on mice explorative behaviour and curiosity, the hole board was used (Ugo Basile, Italy). Both plane (80x80x72cm) and holes (n=16) were provided with photoelectric cells. When the animal moved on the board and sticked its head into the holes, this caused an interruption of light beams, that were recorded as exploration counts. Mice were placed individually on the board and left there free to explore both panel and holes for 5 minutes, 30 minutes after drug administration.

Rota-Rod test

The integrity of motor coordination was assessed with a rota-rod treadmill for mice (Ugo Basile, Italy). A plastic rod, 3 cm in diameter, and 30 cm long, with non-slippery surface and 15 cm over the base, was used. This rod was divided into 5 equal sections by 6 discs, thus enabling 5 mice to walk on the rod at the same time. The speed of the rotating rod was 24 r.p.m. The number of falls from the rod in 30 s were counted.

Mouse light/dark box test

To evaluate the effects of drugs on mouse anxiety the light/dark box test (Ugo Basile, Italy) was used. The apparatus (length 50 cm, width 20 cm and height 20 cm) consisted of two equal acrylic compartments, one dark and one white. The top-open white compartment was illuminated by a 60 W bulb lamp and separated from the black compartment by a divider with a 10 x 3 cm opening at floor level. Each mouse was tested by placing it in the centre of the white area, facing away from the dark one, and was allowed to explore the novel environment for 5 min. The number of transfers from one compartment to the other and the time spent in the illuminated side were measured. This test exploited the conflict between animals tendency to explore a new environment and its fear of bright light.

Unconditioned conflict procedure in rats (Vogel test)

The test was conducted in a 30 x 30 x 25 cm cage (Coulbourn Instruments) with a stainless steel grid floor. The lateral walls of the cage were of transparent Plexiglas, while the two other walls and the ceiling were of aluminium. The

apparatus contained a drinking tube connected to an external 50 ml drinking bottle filled with tap water. The spout of the drinking bottle protruded into the cage at a height of 3 cm above the grid floor, extending about 2 cm into the box from the wall. The apparatus was equipped with an optical lickometer, which was used to measure licking/drinking. A photocell and light source were mounted on either side of the spout. The animals tongue breaked the light beam on each lick and the response was registered. A drinkometer circuit is connected between the drinking tube and the grid floor of the apparatus, so that the rat completes the circuit whenever it licks the tube. Aversive shock was delivered to the feet of animal from a shocker which applies an unscrambled shock between the drinking tube and the grid floor. Vogel (1971) was the first one to describe this method to test anxiolytics. The rats are deprived of water for 48 hours before the experiment, but they have free access to food all the time. We have noted, that the time for the animals to explore the cage and to find the drinking spout after being placed in the experimental cage is very different, and therefore, to decrease this variability, the procedure we used was a modification of the technique described by Vogel. Water was taken away from the rats (180-200g) 48 hours before the test, but at 24 hours, the rats were placed, one at a time in the apparatus and allowed to drink for one minute without shocks being delivered. With this preliminary training, the rats learned where to find the water, and on the day of the experiment, the variability in the latency to start drinking after being placed in the cage was decreased. The trial was started after the animals

tongue entered in contact with the drinking tube for the first time. An mild electric shock (a single, 1 ms constant current pulse of 0.3 mA intensity) was delivered through the spout to the tongue after every 20 licks. The number of shocks was recorded for 3 minutes after the animal had received its first shock. Experiments were performed 30 min after drug administration. In this experimental procedure the anxiolytic-like effect is measured as an increased rate of responding suppressed by punishment.

Mouse grip strength test

To evaluate the effects of the compounds on mouse muscle strength, the grip strength meter was used. The grip strength meter (Ugo Basile-Italy) apparatus consisted of a base plate of black sand blasted Perspex, a grasping-trapeze (t-shaped), 10 cm long, and 2 mm in diameter fitted to a force transducer of adjustable height (14 cm), provided with connection cable and connector to the peak amplifier. The mouse was grasped by the base of the tail, and was allowed to grasp the bar with its forelimbs. The mouse was gently pulled away from the grasping bar until the pulling force overcame its grip strength and the grip was broken. After the animal had lost its grip, the peak amplifier automatically stored the peak pulling force. The grip force was expressed in grams and shown on the display. 3 consecutive readings were taken for each mouse with an inter trial interval of 4 s.

Pentylenetetrazole-induced seizures

Anticonvulsant activity was studied using PTZ for chemically-induced seizures. 5 min after the rota-rod test, each mouse was injected with PTZ (90 mg/kg s.c.). The occurence of clonic generalized convulsions was noted over a 30 minute period.

Step through passive avoidance tests

The apparatus (50 cm long, 20 cm wide, 20 cm high) consisted of two equal compartments, one dark and one white, lighted with a 60W light bulb, and separated by a divider with a 10x3-cm opening at floor level. The dark compartment had a pitfall floor. Punishment consisted of a fall (40 cm) into cold water (10 °C), instead of a painful electric foot shock. Each mouse was placed in turn in the lighted compartment, facing away from the dark one. When the mouse entered the dark compartment, it fell into the water. Treatment was soon after the training session. Twenty-four hours later, a retention trial was performed. The step-through latency for entering the dark compartment was again recorded. In the retention trials, if the mouse did not enter the dark compartment within 120 s, the test was interrupted and the step-through latency was recorded as 120 s.

Effect on ethanol-induced sleeping time

Ethanol (4 g/kg i.p) was injected 30 min after administration of compound under study. The duration of a loss of the righting reflex was measured as the sleep time. If the mice slept more than 2.5 h, the test was stopped and the sleep time was recorded as 150 min.

Statistical analysis.

Results are given as the mean ± SEM. The results were analysed by using the one-way analysis of variance (ANOVA) followed by Fishers LSD post hoc test for multiple comparison. The data were analysed using a computer program (Number Cruncher Statistical System, Version 6.0/2001). For percentage values in the PTZ test, χ_2 (Chi-square) analysis was used in accordance with Tallarida & Murray (1984). *P* values of less than 0.05 were considered significant.

References

1. Martini, C.; Lucacchini, A.; Ronca, G.; Hrelia, S.; Rossi, C. A. Isolation of Putative Benzodiazepine Receptors From Rat Brain Membranes by Affinity Chromatography. *J. Neurochem.* **1982**, *38*, 15-19.
2. Primofiore. G.; Da Settimo F.; Taliani, S.; Marini, A. M.; Novellino, E.; Greco, G.; Lavecchia, A.; Besnard, F.; Trincavelli, L.; Costa, B.; Martini C. Novel N-(arylalkyl)indol-3-ylglyoxylylamides targeted as ligands of the benzodiazepine receptor: synthesis, biological evaluation, and molecular modeling analysis of the structure activity relationships. *J Med. Chem.* **2001**, *44*, 2286-2297.
3. Lowry, O. H.; Rosenbrough, N. J.; Farr, A. L.; Randali, R. J. Protein Measurement with the folin reagent. *J. Biol. Chem.* **1951**, *193*, 265-275.
4. Costanzo A., Guerrini G., Ciciani G., Bruni F., Selleri S., Costa B., Martini C., Lucacchini A., Malmberg Aiello P., Ipponi A., Benzodiazepine Receptor Ligands. 4. Synthesis and Pharmacological Evaluation of 3-Heteroaryl-8-

chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-Oxides. *J. Med. Chem.* **1999**, *42*, 2218-2226.

5. Besnard, F.; Even, Y.; Itier, V.; Granger, P.; Partiseti, M.; Avenet, P.; Depoortere, H.; Graham, D. Development of Stable Cell Lines Expressing Different Subtypes of $GABA_A$ Receptors. *J. Recept. Signal Transduct. Res.* **1997**, *17*, 99-113.
6. Cheng, Y.; Prusoff, W. H. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (IC_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **1973**, *22*, 3099-3108.

Elemental analyses

Compd	MW (MF)	Anal. Calcd.			Anal. Found.		
		C	H	N	C	H	N
1	(217.22) $C_{12}H_{11}NO_3$	66.35	5.10	6.45	66.02	5.20	6.25
2	(231.25) $C_{12}H_{13}N_3O_2$	62.33	5.67	18.17	62.16	5.48	18.30
3	(281.31) $C_{16}H_{15}N_3O_2$	68.31	5.37	14.94	68.52	5.29	14.76
4	(308.42) $C_{18}H_{20}N_4O$	70.11	6.54	18.17	69.93	6.78	18.35