Pharmacology Biochemistry & Behavior, Vol. 30, pp. 5–11. © Pergamon Press plc, 1988. Printed in the U.S.A. 0091-3057/88 $3.00 + .00

Neuropharmacology of Several β-Carboline Derivatives and Their 9-Acetylated Esters. In Vivo Versus In Vitro Studies in the Rabbit

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Received 25 August 1987

MELE, L., M. MASSOTTI AND F. GATTA. *Neuropharmacology of several β-carboline derivatives and their 9-acetylated esters. In vivo versus in vitro studies in the rabbit.* PHARMACOL BIOCHEM BEHAV **30**(1) 5–11, 1988.—The effects of 3-methoxycarbonyl- (β-CCM, I_a), 3-ethoxycarbonyl- (β-CCE, I_c), 3-propoxycarbonyl- (PrCC, I_e), 3-N-methylcarboxamido- (FG-7142, I_g) β-carboline and 2-acetyl-3-methoxycarbonyl-1,2-dihydro-β-carboline (II_a) as well as of their corresponding 9-acetyl derivatives (I_b, I_d, I_f, I_h and II_b) have been studied in rabbits. In addition, the effects of 6,7-dimethoxy-4-ethyl-3-methoxycarbonyl-β-carboline (DMCM) have also been studied. In in vitro studies, these drugs compete with ^{3}H-diazepam to benzodiazepine (BDZ) receptor in membrane preparations from brain cortex. The values of IC_{50} are in the nanomolar range without significant differences between the acetyl derivatives and their congeners only compound I_f shows a 10-fold decrease of the binding capacity in respect to its congener I_e. In the presence of 10^{-5} M GABA, a decrease in the binding capacity for DMCM, I_a, I_c and I_g and an increase for I_f are observed. In vivo studies show that DMCM, I_a, I_b, II_a and II_b elicit three dose-dependent stages of electrocortical changes (trains of slow waves, trains of spike-and-wave complexes and "grand-mal" seizures). Compounds I_c, I_d and I_g elicit only the first two stages. Compound I_h elicits only the first stage. While compound I_e does not affect the EEG pattern, its 9-acetyl derivative I_f induces changes (cortical spindles and disruption of the hippocampal theta waves) characteristic of agonist ligands of BDZ receptor. These findings confirm that the efficacy of compounds DMCM, I_a, I_c, I_d, I_g and I_h as inverse agonists of BDZ receptor in the EEG paradigm parallels the reduction of their apparent binding affinity in the presence of GABA. The 9-acetylated compounds may be more inverse agonist in vivo than predicted from the in vitro findings, due to hydrolysis in the plasma.

Inverse benzodiazepine agonists β-Carboline 9-Acetyl-β-carboline ^{3}H-Diazepam binding EEG Rabbits

A certain number of compounds chemically unrelated to the 1,4-benzodiazepines (BDZ) compete with ^{3}H-BDZ at central BDZ receptors. Among them, a large body of interest has been addressed to the new class of the so-called "inverse agonists" (see [11]). The first representative has been the ethyl ester of the β-carboline-3-carboxylic acid (I_c) [2], endowed with proconvulsant [7, 17, 24] and proconflict effects [8,19]. The N-methylamide derivative of the β-carboline-3-carboxylic acid (I_g) induces similar but longer-lasting effects [15]. The two methyl esters, methyl-β-carboline-3-carboxylate (I_a) and methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM) produce similar effects as well as convulsions [3, 17, 23].

Earlier structure-activity studies pointed out that the presence of a carbonyl group at the 3-position of the β-carboline (βC) molecule is a prerequisite for compounds endowed with binding affinity in low nanomolar range [5]. Additional studies indicate that the introduction of substituents at the 4-position of the βC skeleton is often associated with a considerable increase in receptor binding capacity [16]. On the contrary, either the introduction of a substituent both at the 1- and 7-positions or the methyl substitution at the N-9 position strongly reduces the binding capacity of the βC derivatives [5].

Comparative in vivo and in vitro studies in the rat have shown that while the 9-methyl derivative of the βC exhibits a

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100-fold decrease in the binding affinity, it is only three-fold less potent than the paired compound in antagonizing the anticonvulsant effect of diazepam [12]. It was therefore interesting to explore the effects of βC derivatives having substituents at the 9-position. To our knowledge no studies have been performed with compounds having substituents at this position other than the methyl group. Since the introduction of an acetyl radical into the βC skeleton is rather easy to perform and this possibility can occur at the 9-position only, we concentrated our attention upon the effects of several 9-acetyl derivatives of the βC.

In vitro competition studies have been carried out in order to explore the ability of several βC and their 9-acetyl derivatives to bind at the central BDZ receptor. Since in the past we have described that on the basis of the electrocortical changes in the rabbit it is possible to classify ligands of the central BDZ receptor [14], electroencephalographic (EEG) studies have also been performed.

METHOD

Male mongrel rabbits, weighing 2.0–2.4 kg, were used in both in vivo and in vitro studies.

Binding Studies In Vitro

The animals were sacrificed by cervical translocation. The brain was immediately removed and the cortex was rapidly dissected out on ice. Then the tissue was homogenized in 20 volumes of 0.32 M sucrose with a polytron for 30 sec. The suspension was centrifuged at 3,500 rpm for 10 min, then the supernatant was collected and centrifuged at 12,500 rpm for 20 min. The supernatant was then discarded and the residual pellet was resuspended in the same volume of H_2O, then centrifuged at 15,000 rpm for 20 min. The pellet was resuspended with the same volume of 50 mM Tris-HCl buffer, pH 7.4, and centrifuged. The last step was repeated twice, and the final suspension was frozen at −20°C for at least 12 hours. Then, the frozen suspension was thawed, centrifuged and the supernatant was discarded. The pellet was resuspended in the same volume of 50 mM Tris-HCl buffer, incubated at 37°C for 30 min, then washed 5 times by centrifugation with Tris buffer and used for binding assay.

Binding assays were performed at +4°C in an incubation mixture of 50 mM Tris-HCl buffer (0.5 ml, final volume) containing the membrane suspension (0.2–0.3 mg of membrane proteins), ^{3}H-diazepam (76.7 Ci/mmole), the competing drugs and, in a series of experiments, gamma-aminobutyric acid (GABA). Following one hour of incubation, the reaction was terminated by rapid filtration under vacuum on GF/B glassware filters (Wathman). The filters were washed with 14 ml of cold Tris-HCl buffer. The radioactivity was dissolved in Aquasol and measured by a liquid scintillation counter. Specific binding was obtained by subtracting from the total binding that binding which occurred in the presence of cold diazepam (10^{-6} M, final).

In the competition experiments, the ratio between the values of IC_{50} found in the absence over the ones found in the presence of 10^{-5} M GABA (thereafter defined GABA shift) was calculated for each competing compound.

EEG Studies

Acute preparations were used. Rabbits were prepared under local anesthesia (2% xylocaine). Six screw electrodes were implanted in the skull at the level of the frontal, parietal and occipital cortices of both hemispheres. In a limited number of experiments, hippocampal electrical activity was recorded by means of a concentric deep electrode, according to the method described by Longo [13].

The EEG was continuously recorded starting at 30 min before the injection of the testing drug, until it returned to the pre-drug pattern. During the recording session, the animals were partially restrained (for details see [13]).

Drugs, Solutions and Injection Route

The 9-acetyl derivatives of I_a, I_c, I_e and I_g were prepared by acetylation of the corresponding 9H-βC in refluxing acetic anhydride. The products, crystallized from n-propanol, showed the following melting points: I_b, 200–203°C; I_d, 162–164°C; I_f, 111–113°C; I_h, 271–273°C.

The 2,9-diacetyl-3(methoxycarbonyl)1,2-dihydro-β-carboline (II_b) was obtained by oxidation with SeO_2 of the 2-acetyl-3(methoxycarbonyl)-1,2,3,4-tetrahydro-β-carboline, followed by cyclization in boiling acetic anhydride. This compound heated under reflux in 30% acetic acid solution was 9-deacetylated to give 2-acetyl-3(methoxycarbonyl)-1,2-dihydro-β-carboline (II_a) [10].

In in vivo studies, the drugs were dissolved with a solution of 20% polyethylenglycol 300 in acidified (0.1 N HCl) saline, then injected by slow (1 ml/min) intravenous route (IV) through the ear vein, in a volume of 1 ml/min. In in vitro studies, a 10^{-2} M solution was obtained by dissolving the various drugs with two drops of ethanol, then the volume was adjusted with 50 mM Tris-HCl buffer. The lower concentrations were made by diluting the 10^{-2} M solution with the Tris-HCl buffer.

RESULTS

Biochemical Studies

In membrane preparations from rabbit brain cortex subjected to freezing-thawing and incubation at 37°C, ^{3}H-diazepam binding (7 concentrations: 1.5–48 nM, final) shows the following binding parameters: K_d, 21.2±3.2 nM and B_{max}, 1.59±0.22 pmol/mg prot. The binding capacity increases in the presence of GABA starting from the concentration of 10^{-7} M. In the presence of a maximally stimulating concentration of GABA (10^{-5} M), the following binding parameters are noticed: K_d, 9.7±0.9 nM ($p<0.001$, in respect to those found in the absence of GABA, paired t-test) and B_{max}, 1.49±0.3 pmol/mg prot (not significant). The resulting value of the GABA shift is 2.10±0.71. Comparative studies have shown that these values are similar to those observed using membrane suspensions from rat brain subjected to the same purification procedure (data not shown).

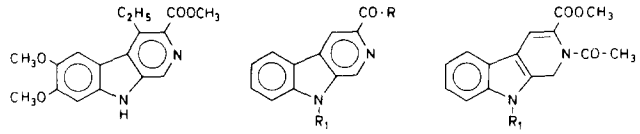
Table 1 lists the values of the IC_{50} found for the various βC derivatives in competition studies using 1.5 nM ^{3}H-diazepam as ligand, and 12 concentrations (10^{-11}–10^{-4} M, final) of the competing drugs. The values of the GABA shift found for each compound are reported in Table 2.

DMCM, a known convulsant βC, competes at BDZ receptor with an IC_{50} of 18.1±2.4 nM. The value of GABA shift is 0.21±0.07

No statistically significant differences of the IC_{50} values are observed between compound I_b (9.0±0.3 nM) and the corresponding 9H-βC I_a (6.4±1.2 nM). On the other hand, in the presence of 10^{-5} M GABA, the IC_{50} of I_b is almost unchanged (8.4±0.4 nM), whereas the one of I_a shifts to 15.8±2.3 nM. The resulting values of the GABA shift for I_a and I_b are 0.34±0.08 and 1.07±0.06, respectively.

TABLE 1

RECEPTOR-BINDING AFFINITIES IN THE ABSENCE AND IN THE PRESENCE OF 10^{-5} GABA OF SEVERAL β-CARBOLINE DERIVATIVES



DMCM I II

Compounds	R	R_1	N	IC_{50} (nM) −GABA	IC_{50} (nM) +GABA
DMCM	—	—	6	18.1 ± 2.4	86.2 ± 12.4‡
I_a	-O-CH_3	H	3	6.4 ± 1.2	15.8 ± 2.3†
I_b	-O-CH_3	-CO-CH_3	4	9.0 ± 0.3	8.4 ± 0.4*
I_c	-O-C_2H_5	H	3	10.4 ± 2.4	19.3 ± 3.4†
I_d	-O-C_2H_5	-CO-CH_3	4	9.1 ± 0.7	9.1 ± 0.9*
I_e	-O-C_3H_7	H	3	6.4 ± 0.8	5.4 ± 0.1
I_f	-O-C_3H_7	-CO-CH_3	5	66.1 ± 8.1*	29.9 ± 1.9*‡
I_g	-NH-CH_3	H	5	961 ± 140	1,438 ± 240†
I_h	-NH-CH_3	-CO-CH_3	5	902 ± 194	910 ± 133*
II_a	—	H	4	9.1 ± 0.5	12.9 ± 1.7
II_b	—	-CO-CH_3	4	13.3 ± 1.7	13.5 ± 0.9

I_a=β-CCM; I_c=β-CCE; I_e=PrCC; I_g=FG 7142.

Competition binding studies are performed using 1.5 nM ^{3}H-diazepam (final) and 12 concentrations (10^{-11}–10^{-4} M, final) of each competing compound (for details, see text).

*$p \leq 0.05$ in respect to the non-acetylated congener, and †$p \leq 0.05$ or ‡$p \leq 0.01$ in respect to the −GABA values, according to the paired t-test.

Compound I_d shows identical IC_{50} values (9.1 nM) both in the absence and in the presence of 10^{-5} M GABA. On the contrary, its 9H-congener I_c elicits IC_{50} values of 10.4±2.4 nM and 19.6±3.4 nM, respectively. Statistically different values of the GABA shift are noticed, namely, 1.01±0.12 for I_d and 0.51±0.09 for I_c.

The IC_{50} values for the propyl-β-carboline- 3-carboxylate (I_e) are 6.4±0.8 nM in the absence and 5.4±0.1 nM in the presence of 10^{-5} M GABA. The 9-acetyl derivative I_f shows a ten-fold decrease of the binding capacity (IC_{50}, 66.1±8.1 nM) in respect to the congener. The IC_{50} value decreases to 29.9±1.9 nM in the presence of 10^{-5}M GABA (Table 1). The resulting value of the GABA shift for compound I_f (2.23±0.05) is significantly higher than the one of compound I_e (1.18±0.09) (data not shown).

The IC_{50} values for compound I_g and its acetyl derivative (I_h) are 961±140 nM and 902±194 nM, respectively. In the presence of 10^{-5} M GABA, a slight but significant change of IC_{50} value (to 1,438±240 nM) occurs for the former, but not for I_h. The resulting value of the GABA shift for compound I_h (0.98±0.2) is slightly but significantly higher than the one of I_g (0.67±0.07).

The 2-acetyl-3(methoxycarbonyl)-1,2-dihydro-β-carboline (II_a) has an IC_{50} value of 9.1±0.5 nM. This value shows a slight but not significant increase (to 12.9±1.7 nM) in the presence of 10^{-5} M GABA. The 2,9-diacetyl derivative (II_b) shows a similar binding capacity (IC_{50}, 13.3±0.9 nM), which does not change in the presence of 10^{-5} M GABA. No statistically significant difference in the resulting values of the GABA shift are noticed between the two congeners (0.71±0.08 for compound II_a and 0.98±0.14 for compound II_b).

EEG Studies

In preliminary studies (2 animals for each drug), a 0.1% solution of the compounds has been infused IV at the speed rate of 1 ml/min with 0.1% solution in order to determine the EEG changes and the range of the effective doses (data not shown). The experiments have been then carried out using single doses, calculated on the basis of the previous results. Table 2 lists the EEG changes and the range of the active doses for each compound, as well as the number of animals used.

As previously reported in the rabbit [14,15], within a few sec after injection, both DMCM and compound I_a elicit EEG changes characteristic enough to be classified into three dose-dependent stages: STAGE 1, trains of slow waves in the occipital cortex; STAGE 2, trains of spike-and-wave complexes in the frontal and parietal cortices; STAGE 3, "grand-mal" seizures. Behaviourally, the latter is associated with tonico-clonic convulsions, whereas STAGE 1 and STAGE 2 are associated with a state of alert. The compounds I_c and I_g only elicit, within a few sec and 5–10 min after injection respectively, STAGE 1 and STAGE 2. These drugs, when injected at very large doses induce a slowing of the record made up of large amplitude 1–4 Hz waves with occasional spikes. The finding that spike-and-wave com-

TABLE 2

RELATIONSHIP BETWEEN THE EEG MODIFICATIONS IN VIVO AND GABA SHIFT IN VITRO INDUCED BY SEVERAL β-CARBOLINE DERIVATIVES IN THE RABBIT

Compounds	GABA Shift	N	EEG Changes: Slow Waves (Occ.Ctx)	EEG Changes: Spike-and-Waves (Sens-Mot.Ctx)	EEG Changes: Grand Mal
DMCM	0.21 ± 0.07	16	0.4–1.0	(0.8–1.0)	1.0–2.0
I_a	0.34 ± 0.08	12	0.2–1.2	1.2–2.0	2.0–4.0
I_b	1.07 ± 0.06*	13	0.2–0.7	0.8–1.2	1.2–2.0
I_c	0.51 ± 0.09	14	0.2–0.5	0.5–2.0	—
I_d	1.01 ± 0.12*	16	0.5–2.0	[2.0–6.0]	—
I_g	0.67 ± 0.07	11	2.0–10	[10–20]	—
I_h	0.98 ± 0.20*	11	1.0–4.0	—	—
II_a	0.71 ± 0.08	12	0.7–1.2	1.2–2.0	2.0–4.0
II_b	0.98 ± 0.14	14	0.5–2.0	2.0–6.0	6.0–10

For details on GABA shift and EEG Stages, see text. N=number of animals.

Trains of spike-and-wave complexes occur either in 40–50% of the animals injected (brackets) or occasionally (parentheses) with the above reported doses of the compounds. See text for details.

Occ.Ctx=associative cortex, Sens-Mot.Ctx=sensorimotor cortex. *$p<0.05$ from its non-acetylated congener, according to the paired t-test.

plexes occur occasionally after DMCM and in about 50% of the animals after I_g, has also been confirmed.

The acetyl derivative I_h elicits EEG changes similar to those of the congener I_a. STAGES 1, 2 and 3 are observed in the range of doses of 0.2–0.7 mg/kg IV (4 out of 4 animals), 0.8–1.2 mg/kg IV (5 out of 5 animals) and 1.2–1.8 mg/kg IV (4 out of 4 animals), respectively. STAGE 1 occurs within 1 min after the injection, STAGE 2 within 2 min and STAGE 3 within 3–5 min (Fig. 1). The effects last about 30 min. When STAGE 3 occurs, during the period of the onset EEG abnormalities characteristic of STAGE 1 and 2 are noticed. Seizures occur as a single fit, lasting 20–80 sec. The fit is followed by a short period of quiescence (10–15 sec) and long-lasting large amplitude low frequency waves, with occasional presence of manifestations characteristic of STAGE 1 and 2. It was surprising that the compound I_h, in three additional experiments carried out with the doses of 2.2, 4.0 and 6.5 mg/kg IV, induces EEG changes referred to as STAGE 2. On the contrary, compound I_a fails to induce this paradoxical effect up to the dose of 15 mg/kg IV.

Compound I_d elicits, within 3 min after the injection, a desynchronized pattern with manifestations referred to as STAGE 1 in 5 out of 5 animals injected with 0.5–2.0 mg/kg IV. Doses of 2.0–6.0 mg/kg IV induce STAGE 1 in 4 out of 7 animals and STAGE 2 in the other 3. The doses of 8 mg/kg IV (2 animals) and 10 mg/kg IV (2 animals) give rise to EEG patterns similar to those recorded with large doses of its non-acetylated congener. These effects last 20–30 min.

The acetyl derivative I_h elicits, within 1 min after the injection, STAGE 1 in 6 out of 6 animals challenged with the doses of 1–4 mg/kg IV. The doses of 6 mg/kg (3 animals) and 10 mg/kg (2 animals) induce EEG changes similar to those reported with very large doses of compounds I_c, I_d and I_g. These effects last 20–30 min.

The injection of propyl-β-carboline-3-carboxylate (I_e) at the doses of 1–10 mg/kg IV does not induce major EEG and/or behavioural changes, but beginning from the dose of 0.2 mg/kg IV, it antagonizes the effect of a dose of 10 mg/kg IV of diazepam. In contrast, the acetylated compound I_f (0.6–5 mg/kg IV) elicits, within 1–2 min after injection, a lengthening of the spindle bursts more sustained at the frontal cortex, associated to disruption of the hippocampal theta waves. These effects last 30–80 min, depending upon the doses. Signs of either slight sedation or weak muscle relaxation are observed.

The compound II_a induces EEG manifestations characteristic of STAGE 1, STAGE 2 and STAGE 3 in the ranges of doses of 0.7–1.2 mg/kg IV (4 out of 4 aminals), 1.2–2.0 mg/kg IV (4 out of 4 animals) and 2.0–4.0 mg/kg IV (4 out of 4 animals). Its acetylated derivative (II_b) also induces EEG changes referred to as STAGE 1 (3 out of 5 animals), STAGE 2 (3 out of 4 animals) and STAGE 3 (4 out of 5 animals) at the doses of 0.5–2.0, 2.0–6.0 and 6.0–10 mg/kg, respectively. These effects initiate within 30 sec after the injection of the drugs and last 15–30 min, depending upon the doses. In this case, also, seizures consist of a single fit, lasting 30–90 sec. Recovery occurs within 10–20 min. Three animals tested at doses of 0.5, 1.2 and 3.0 mg/kg exhibit within a few sec a slowing of the EEG pattern, and in the following 10 min a flattened record occurs followed by death.

DISCUSSION

Competition binding studies in vitro, using ^{3}H-diazepam as ligand and membrane suspension from rabbit brain cortex, show that βC derivatives presently investigated bind at the BDZ receptor in the nanomolar range. This suggests that these compounds initiate their pharmacological activity at these receptors. When binding studies are performed in the presence of GABA, a decrease (DMCM, I_a, I_c and I_g) or no change (I_b, I_d, I_h, II_a and II_b) of the binding capacity are noticed. Moreover, the resulting values of the GABA shift for these compounds are lower than those found for pure antagonists of central BDZ receptors, such as Ro 15-1788 and Ro 15-3505 (1.20±0.25 and 1.15±0.32, respectively; unpublished data), and I_e (1.18±0.09). On the contrary, GABA

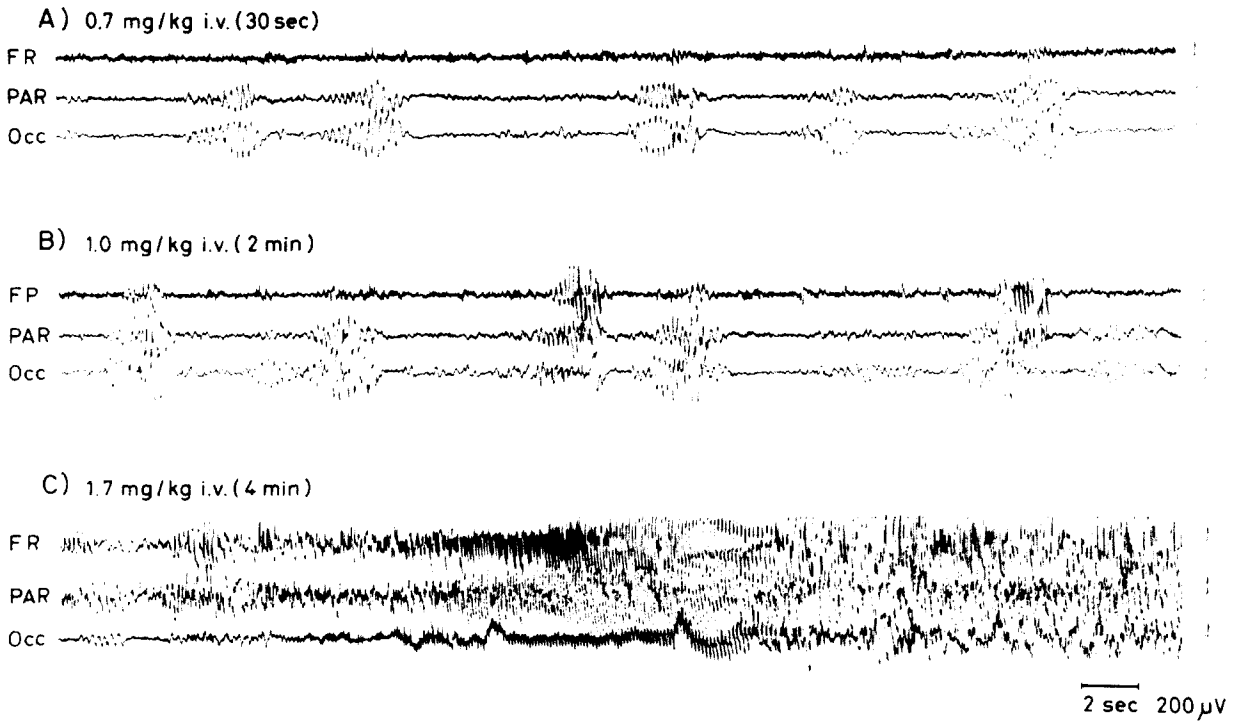


FIG. 1. EEG abnormalities induced by 9-acetyl-3(methoxycarbonyl)-β-carboline (I_h) in the rabbit. (A) The dose of 0.7 mg/kg IV induces 30 sec after injection the appearance of trains of 2–4 Hz slow waves (STAGE 1) mainly at the level of the optic cortex. (B) The dose of 1.0 mg/kg IV elicits, 2 min after injection, the appearance of trains of 4–6 Hz spike-and-wave complexes (STAGE 2) mainly at the level of the sensorimotor cortex, accompanied but not always synchronous with the trains of slow waves. (C) The dose of 1.7 mg/kg IV induces, 4 min after injection, EEG "grand-mal" seizures (STAGE 3), associated with behavioural tonico-clonic convulsions. Leads: FR, left-right anterior sensorimotor cortex; PAR, left-right posterior sensorimotor cortex; OCC, left-right optic cortex.

shift value for compound I_f is higher than those of pure antagonists and close to that found with diazepam.

GABA shift in vitro has been considered as an index of the ability of a given compound to modify GABA receptor activity. Drugs that either potentiate or antagonize GABA-mediated transmission, acting at postsynaptic junction, enhance or decrease GABA shift value, respectively [4,11]. Therefore, according to the classification of Polc *et al.* [20], compounds I_e and I_f can be included into the groups of the antagonists and of the agonists of BDZ receptor, respectively, and the other drugs into the group of the inverse agonists.

Present in vivo findings confirm that the inverse agonists elicit in the rabbit electrocortical abnormalities which can be divided into three dose-dependent stages (Table 2) similar to those found after the so-called "supraspinal convulsants" (see also [14,15]), according to the classification of Bovet and Longo [1]. As mentioned in previous papers using compounds DMCM, I_a, I_c and I_g [14,15], these EEG abnormalities are reminiscent of those induced by the GABA antagonist bicuculline [9] as well as by the inhibitors of the opening of GABA-associated chloride channel picrotoxin, pentamethylenetetrazol [13] and the convulsant BDZ Ro 5-3663 [21]. This confirms that compounds that stabilize the GABA-BDZ-chloride channel complex in a conformation unfavourable for GABA binding share common EEG patterns. It is interesting to mention that behavioral convulsions in squirrel monkeys [22] and EEG spike-and-wave complexes in rats [23] have been described after β-CCE (I_c).

Comparison of the in vivo and in vitro findings after several inverse agonists indicate a positive correlation between the efficacy to progress through the three stages (DMCM and I_a induce all three stages; I_c and I_g the first two; I_h only the first) and the extent of reduction of their binding affinity in the presence of GABA (DMCM > I_a >> I_c > I_g >> I_h). In contrast, no correlation seems to exist between the in vivo efficacy and the binding capacity for these drugs (see Table 1). This hypothesis is corroborated by the findings that, in the rabbit, the pyrazoloquinoline derivative CGS 8216, which possesses antagonist-inverse agonist property at BDZ receptor, induces EEG manifestations characteristic of STAGE 1 only [14,15] and exhibits IC_{50} value of 3.32±0.7 (unpublished data). This value, although it exceeds by about one order of magnitude the one reported in the literature [7], is the lower found among the various antagonists and inverse agonist. The GABA shift value (0.98±0.10), instead, is intermediate between those of the I_c and I_g, and those of the BDZ antagonists Ro 15-1788, Ro 15-3505 (see above) and I_e (Table 1). All these data suggest that GABA shift value might not predict the pharmacological activity of a BDZ ligand.

The introduction of an acetyl substituent at the 9-position of the βC skeleton does not induce significant changes in the binding capacity for compound I_a, I_c, I_g and II_a. A ten-fold decrease of the binding affinity is instead noticed for the compound I_f only, the acetyl derivative of pure antagonist I_e.

The presence of the acetyl substituent at this position greatly (2–3-fold) reduces the extent of the decrease for the GABA shift values in in vitro studies. This seems to reflect

changes in the efficacy for compounds I_d and I_h in respect to the congeners I_c and I_g, respectively. A 50% reduction of the incidence of manifestations referred to as STAGE 2 indeed occurs after compound I_d. Compound I_h only elicits EEG abnormalities characteristic of STAGE 1.

The introduction of such a radical seems to influence only in part the potency of compounds I_h and II_h in inducing manifestations referred to as STAGES 2 and 3 when compared to the congeners I_a and II_a, respectively. In addition, the delay of the effects would suggest that an enzymatic removal of the radical at 9-position occurs in vivo shortly after injection of compound I_h. Therefore, effects similar to those observed with its non-acetylated congener appear in EEG studies. This possibility instead seems to be excluded for compound II_h since no difference in the onset is observed in respect to its congener II_a. Paradoxically, both compounds, unlike the others here investigated, exhibit GABA shift values in vitro similar to those found with partial inverse agonists. In contrast, they induce EEG changes in vivo characteristic of the full inverse agonists. Further studies are required to clarify this discrepancy.

Experiments aimed to explore possible enzymatic removal of 9-acetyl radical were carried out by incubating at 37°C, for 1, 2 and 5 min, compounds I_h, I_h and II_h (100 nM) in rabbit's plasma. Compounds were extracted from plasma (alcalinized with ammonium hydroxyde) with ethyl acetate. Thin-layer chromatography (elution with ethyl acetate) revealed the presence of deacetylated paired compounds at the 2nd (I_a and I_g) and 5th (II_a), but never at the 1st min. A spot is observed at the R_f of the deacetylated derivatives, which is about 20–30% in respect to that of the 9-acetyl paired compounds. At the 5th min, the spots for compound I_a and II_a are identical to those of I_h and II_h (unpublished observations). Clearly this method reflects only in part the pharmacokinetic of these compounds in vivo. However, the results can explain at least in part the similar potency and efficacy of these compounds in affecting the EEG activity. In addition, the absence of deacetylated compounds at the first min clearly indicates that degradation does not occur during the preparation of the solution in acidified vehicle.

The introduction of the acetyl at the 9-position of the antagonist I_c modifies the pharmacodynamic profile of the compound toward an agonist. Compound I_f shows indeed a value of the GABA shift [2,23] and an EEG profile (cortical spindles and hippocampal disruption) similar to those of diazepam [14]. Interestingly, compound I_f fails to induce cortical 25–30 Hz waves as the anxiolytic-sedative 1,4-BDZ does. Such an EEG pattern occurs after injection of drugs which bind to the subpopulation of BDZ receptors defined as BDZ_1, such as CL 218,872 and zolpidem (manuscript in preparation).

In conclusion, present data indicate that: (1) the study of the changes of the electrocorticogram in the rabbit in vivo, as well as the GABA shift in vitro, are reliable methods of screening drugs acting at BDZ receptors (see also [14]); (2) the efficacy of DMCM, I_a, I_c, I_d, I_g, I_h and CGS 8216 in the EEG paradigm parallels the extent of the reduction of their affinity in the presence of GABA. This confirms previous data in rats, using behavioral endpoints, such as proconflict effect [19], social interaction [8] as well as convulsant and proconvulsant effects [3, 6, 17, 18, 22, 24]; (3) in general, acetylation at the 9-position of the βC skeleton of the inverse agonists does not seem to affect the binding capacity of these drugs, but seems to lessen the ability of these drugs to antagonize GABA receptor activity in in vitro studies. In contrast, minor changes occur between the acetylated and non-acetylated compounds in in vivo studies; (4) acetylation of the antagonist I_c reduces the binding capacity in vitro and yield a compound endowed with agonist properties both in vitro and in vivo.

ACKNOWLEDGEMENTS

The authors are indebted to Prof. V. G. Longo for his criticism and advice in the course of this investigation. This paper has been supported in part by CNR contract No. 86.02039.56.

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