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Anxiolytic Natural and Synthetic Flavonoid Ligands of the Central Benzodiazepine Receptor Have No Effect on Memory Tasks in Rats

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SALGUEIRO, J. B., P. ARDENGHI, M. DIAS, M. B. C. FERREIRA, I. IZQUIERDO AND J. H. MEDINA. *Anxiolytic natural and synthetic flavonoid ligands of the central benzodiazepine receptor have no effect on memory task in rats.* PHARMACOL BIOCHEM BEHAV **58**(4) 887–891, 1997.—The naturally occurring flavonoids, chrysin (5,7-dihydroxyflavone) and apigenin (5,7,4′-trihydroxyflavone), and the synthetic compound, 6,3′-dinitroflavone have been recently reported to selectively bind with high affinity to the central benzodiazepine receptor, and to exert powerful anxiolytic and other benzodiazepine-like effects in rats. Their chemical analog, quercetin, shares none of these effects. In the present article we find that, in contrast to diazepam, chrysin, apigenin, and 6,3′-dinitroflavone have no amnestic effect on acquisition or retention of three different learning tasks (inhibitory avoidance, shuttle avoidance, and habituation to an open field), even when given at doses higher than those previously reported to be anxiolytic. Apigenin had a slight enhancing effect on training session performance and, when given posttraining, on test session retention, of crossing responses in the open field and hindered retention of inhibitory avoidance, and showed no anxiolytic action in an elevated plus maze. Unlike diazepam, none of these drugs had any analgesic effect in the tail-flick test. The data suggest that chrysin, apigenin, and 6,3′-dinitroflavoine, three flavonoids derivatives possessing anxioselective effects acting on central benzodiazepine receptors, may deserve clinical trials as anxiolytic agents. © 1997 Elsevier Science Inc.

Apigenin Chrysin Dinitroflavone Quercetin Diazepam Anxiolytic action Effects on memory Effects on analgesia

CENTRAL benzodiazepine receptor (BDZ-R) agonists have been known for years to induce anxiolytic, myorelaxant, anticovulsant, hypnotic, and amnestic effects (1,6). The amnesia is viewed as an unwanted side effect for most of the therapeutic applications of these substances and is seen both in anxiogenic and in nonanxiogenic tasks (6). Recently, some natural and synthetic flavonoids have been found to bind specifically and competitively to BDZ-Rs and to possess anxiolytic effects (4,5,7,12–15). Among these substances, the two naturally occurring flavonoids, chrysin [5,7-dihdroxyflavone] (7,12,14) and apigenen [5,7,4′-trihydroxyflavone] (12,13) and the synthetic compound, 6,3′-dinitroflavone (4,15) (Fig. 1) are perhaps the most interesting, considering their pharmacological profiles.

All the three flavonoids exhibit clear-cut anxiolytic effects in the elevated plus-maze test in rodents without evidencing anticonvulsant, myorelaxant, or sedative actions. Moreover, the specific antagonist of the BDZ-R, RO 15-1788, blocks the anxiolytic effects of these flavonoids (13–15). Based on their biochemical and pharmacological profiles, it has been hypothesized that they behave as partial agonists of the BDZ-R. In

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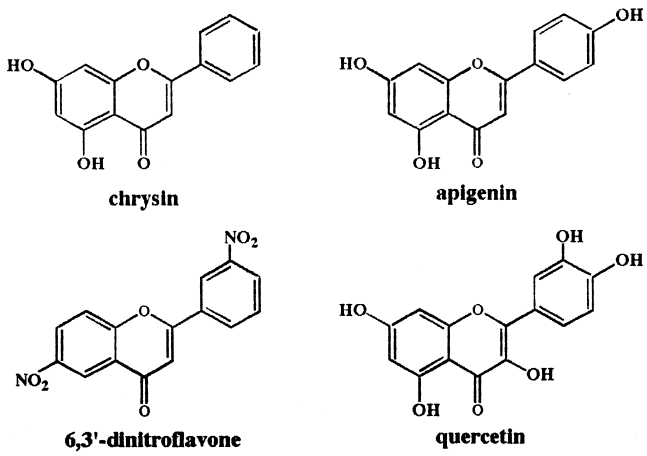

Fig. 1. Chemical structures of chrysin, apigenin, 6,3′-dinitroflavone, and quercetin.

contrast, quercetin, a derivative of the flavone nucleus with strong chemical resemblance to these compounds that is widely distributed in nature, has no affinity for BDZ-Rs (5).

The present article examines the effect of these four compounds on several learning paradigms in rats in which diazepam and other benzodiazepine receptor ligands have long been known to induce amnesia (1,6).

METHODS

A total of 484 male Wistar rats (age, 70–90 days; weight, 240–280 g) was used. They were submitted to the following behavioral tasks: step-down inhibitory avoidance (2), habituation to an open field (3), shuttle avoidance (9), elevated plus maze (8), and the tail-flick test (11). The first three tasks were used for the study of learning and memory. In these, the animals received the vehicle or drug treatments either 1 h prior to training, or immediately posttraining, and a retention test of the same task was carried out 24 h later. The tail-flick procedure was used for the study of potential analgesic effects. The doses were 10 mg/kg for the natural compounds, chrysin, apigenin, and quercetin, 0.1 mg/kg for 6,3′-dinitroflavone, and 2 mg/kg of diazepam. Except for quercetin, these doses were above those found to exhibit anxiolytic properties in the elevated plus maze (4,13–15), quercetin has no effect on this test (10). Pretraining treatments were given 1 h prior to the behavioral procedures, and posttraining treatments were given immediately after completion of the training task. All injections were IP at a volume of 0.1 ml/100 g body weight. The vehicle was DMSO 40% in NaOH 0.1 N (7:3, v/v) (12,14).

Briefly, the experimental procedures were as follows:

Inhibitory Avoidance

A 50 × 25 × 25 cm plastic box with a frontal glass wall and whose floor was made of parallel 10-mm caliber bronze bars was used. The left end of the grid was occupied by a 7-cm wide, 2.5-cm high formica platform. The animals were gently placed on the platform facing the rear wall and their latency to step down placing their four paws on the grid was measured with an automatic device. In the training session, after stepping-down the animals received a 0.4 mA, 2-s scrambled footshock and were immediately withdrawn from the cage. In the test session, 24 h later, the procedure was repeated but the footshock was not given. Test session step-down latency was taken as a measure of retention.

Habituation to the Open Field

The animals were exposed twice, with a 24-h interval, to a 40 × 50 × 60 cm open field whose brown linoleum floor was divided into 12 equal quadrangles by white lines. In both sessions, the animals were placed in the rear left quadrangle and left to explore freely for 5 min, during which the number of line crossings and rearings were counted.

Shuttle Avoidance

Animals were placed in one corner of a 50 × 25 × 25 cm automated shuttle avoidance box, whose floor was a series of

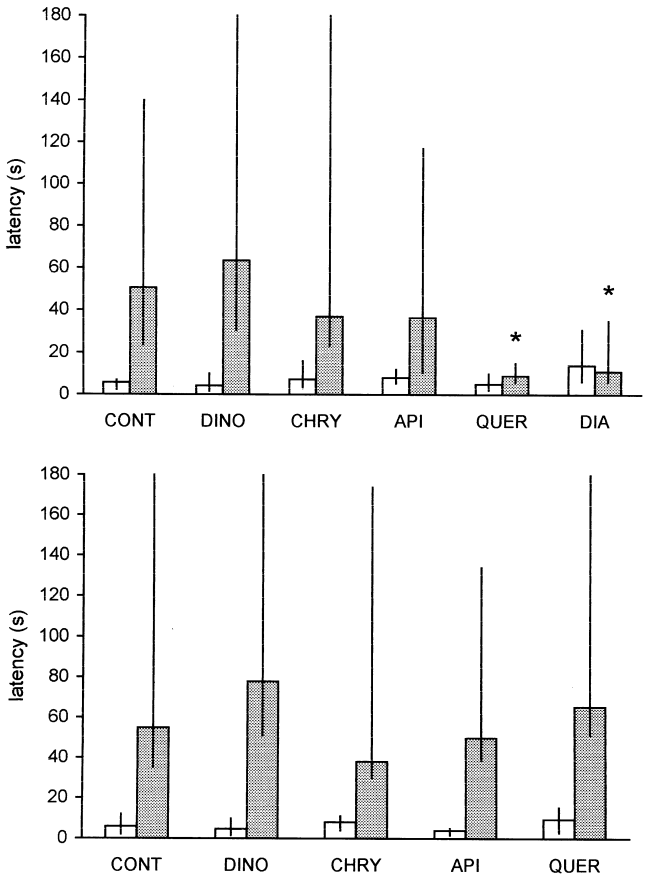

Fig. 2. Effect of the IP administration of vehicle (CONT), 6,3′-dinitroflavone, 0.1 mg/kg (DINO), chrysin, 10 mg/kg (CHRY), apigenin, 10 mg/kg (API), quercetin, 10 mg/kg (QUER), and diazepam, 2 mg/kg (DIA), given 1 h before training (upper graph) or immediately after training (lower graph), on training (white columns) and test session performance (gray columns) in a step-down inhibitory avoidance task. Diazepam was not studied by posttraining injection. Data expressed as median (interequartile range) step-down latencies. Asterisks indicate a significant difference of test session values as compared with the CONT group at $p < 0.02$ level in a two-tailed Mann–Whitney *U*-test. Quercetin and diazepam caused anterograde amnesia for this task without significantly altering training session performance.

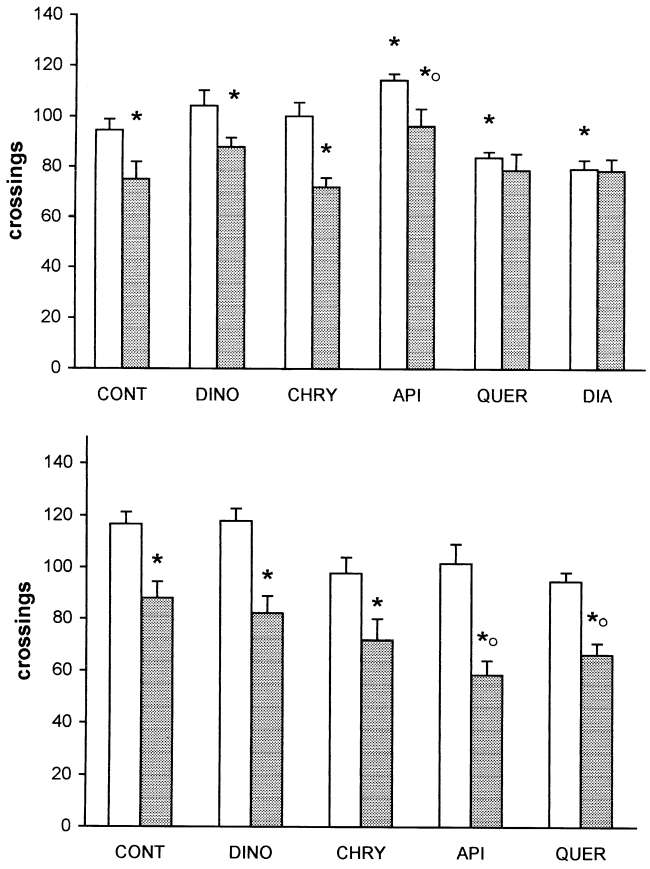

FIG. 3. Same as in Fig. 2, but for the number of crossing responses in a 10-min exposure to an open field. Data expressed as means ± SEM. Asterisks placed on the test session columns indicate significant differences between training and test session performance at $p < 0.02$ level in Duncan multiple range tests. In the pretraining-treated groups (upper graph), these differences were significant in all groups except those treated with quercetin and diazepam, which were, therefore, amnestic. In the groups receiving posttraining treatments, all training–test differences were significant. Asterisks placed on the training session columns indicate significant differences relative to the control group. Pretraining apigenin increased the number of crossings in the training session, and quercetin and diazepam reduced it ($p < 0.02$ in Duncan test). Open circles on the test session columns indicate significant differences in performance in this session relative to the control group ($p < 0.02$ in a Duncan test). Test session performance was higher than that of controls in the animals pretreated with apigenin, and lower than that of controls in those treated with posttraining apigenin and quercetin.

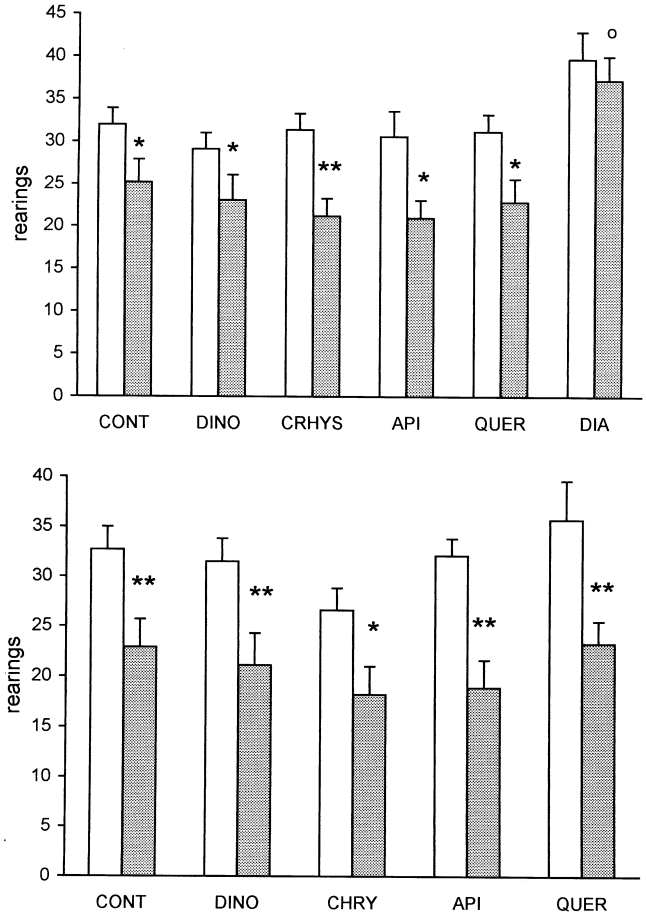

FIG. 4. Same as Fig. 3, for rearing responses in the open field. Double asterisks indicate significant training–test differences at $p < 0.002$ level in Duncan test; single asterisks and open circles have the same meaning as in Fig. 3. None of the drugs had nay effect on this measure, when given either prior to training (upper graph) or posttraining (lower graph), except for diazepam, which exerted its usual anterograde amnestic effect (upper graph).

1-mm caliber bronze bars. Unlike the apparatus used for inhibitor avoidance (see above), this box had no platform and the floor grid was divided at the middle by a 1-cm high acrylic hurdle. The conditioned stimulus was a 5-s, 70 dB, 1 kHz tone delivered by a loudspeaker attached to the rear wall of the box at the midline. Each tone was immediately followed by a 2-s, 0.5 mA footshock (unconditioned stimulus). Training and test sessions were procedurally identical: animals were left to explore the box freely for 3 min, after which they received 20 tone-footshock trials; the intertrial interval was varied at random between 10 and 50 s. Animals avoided shocks by crossing the hurdle during the tone (conditioned responses).

Tail-Flick Test

Rats were gently wrapped in a towel and placed on the apparatus (11). The light source position below the tail was focused on a point 2.3 cm rostral to the tip of the tail. Deflection of the tail activated a photocell and automatically terminated the trial. Light intensity was adjusted so as to obtain a baseline tail-flick latency of 3 to 6 s. A cutoff time of 10 s was used so as to prevent eventual tissue damage. Briefly, the general procedure was as follows: a baseline tail-flick latency value was obtained for each animal. Following this, the rats were placed alone in a waiting cage. Tail-flick latency value was measured 1 h after an IP injection of vehicle or drug treatments.

Statistics

The data from the inhibitory avoidance task and the tail-flick test were nonparametric, because both procedures involved a cutoff time. They are expressed as medians (interquartile ranges) and we analyzed by individual two-tailed Mann–Whitney *U*-tests. The data from the habituation and the shuttle avoidance tasks and the elevated plus maze test are

expressed as means ± standard error and were examined by one-way ANOVAs followed by a Duncan multiple range test.

RESULTS

The three learning tasks studied were sensitive to the amnestic effect of an anxiolytic dose of diazepam (2 mg/kg) given 1 h before. At this dose, diazepam increased training session step-down latency and decreased the retention of inhibitory avoidance (Fig. 2), decreased locomotor activity and reduced the retention of habituation to the open field (Figs. 3 and 4), and caused anterograde amnesia for the shuttle avoidance task (Fig. 5). In addition, diazepam had an analgesic effect revealed in the tail-flick (Fig. 6).

In contrast, pretraining administration of the BDZ-R ligands, chrysin, apigenin and 6,3′-dinitroflavone, at doses higher than those reported to be anxiolytic (4,5,7,12–15), had no deleterious effect on memory; in fact, apigenin induced a slight increase in the number of crossings in the open field, both in the training and in the test session (Figure 3), but did not affect retention of this measure. Posttraining administration of apigenin slightly reduced crossings in the test session of the open field (Fig. 3) and could therefore be considered to have a slight facilitating effect on memory if measured by this parameter; however, it did not affect the performance of test session rearing responses in the same task (Fig. 4).

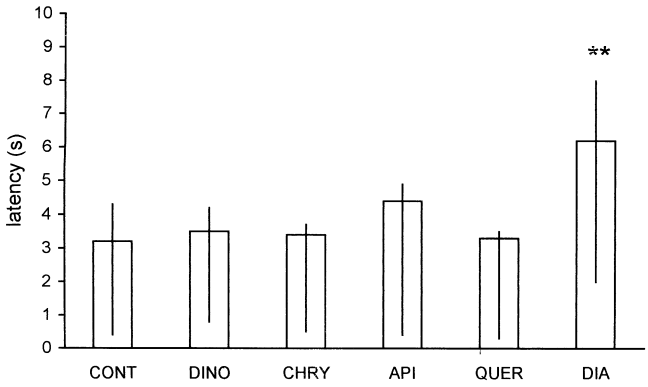

FIG. 6. Effect of the drugs on the tail-flick test. Ordinates: latency, in seconds. Data expressed as medians (interquartile ranges). Only diazepam had an analgesic effect, significant at $p < 0.002$ level in Mann–Whitney *U*-test.

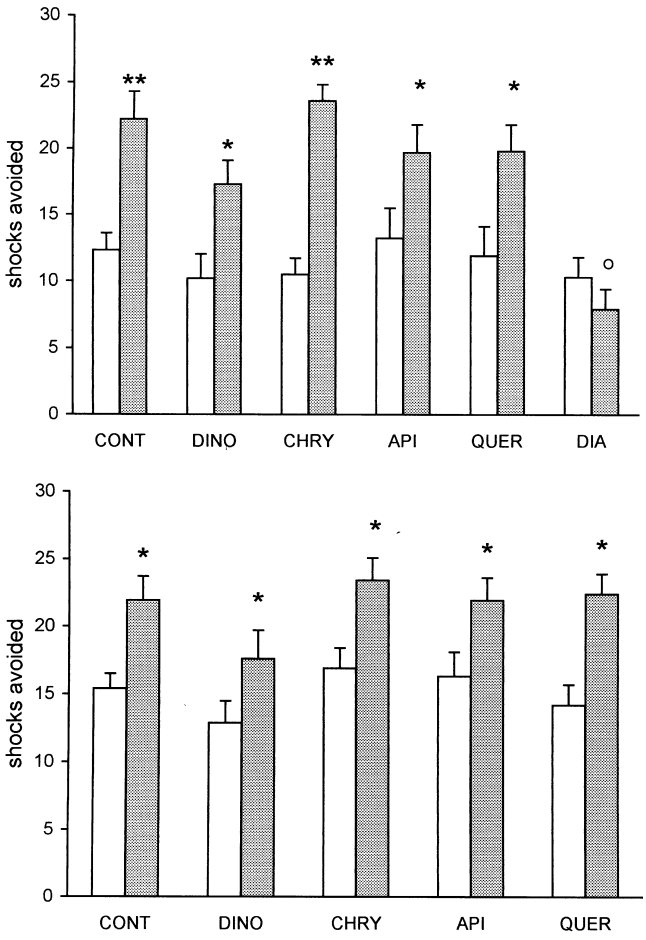

FIG. 5. Same as previous figures, but for shuttle avoidance responses. Data are expressed as means ± SEM. Symbols have the same meanings as in Fig. 4. None of the drugs had any effect on this measure, when given either prior to training (upper graph) or posttraining (lower graph), except for diazepam, which exerted its usual anterograde amnestic effect (upper graph) ($p < 0.02$ in Duncan multiple range test).

In contrast, quercetin, which does not bind to BDZRs (5), was amnestic for the inhibitory avoidance task by not for the others, and had no influence on tail-flick latency (Figs. 2–6). None of the substances tested had any effect on retention of the learning tasks when given posttraining (Figs. 2–5).

DISCUSSION

The main finding of the present study in that chrysin, apigenin, or 6,3′-dinitroflavone, three flavnoids representative of a new family of BDZ-R ligands, had no effect on training or test session performance of inhibitory avoidance, habituation to the open field, or active avoidance. This was in spite of the anxiolytic effect of these substances at doses equal to or lower than those that were used here (4,5,7,12–15), and therefore stands in clear contrast to the well-known amnestic effect of classical BDZ-R ligands in these and many other tasks (3,6, 8,9). The findings with pretraining injections of chrysin, apigenin, or 6,3′-dinitroflavone, particularly in the open field, endorse previous suggestions that they are devoid of other than anxiolytic effects at doses such as were used here (4,5,7,12–15). Quercetin had a depressant effect on locomotion in the open field, and an anterograde amnestic effect restricted to the inhibitory avoidance paradigm.

The lack of effect of chrysin, apigenin, and 6,3′-dinitroflavone was in contrast to diazepam, which was amnestic when given prior to training in all the learning tasks studied and, in addition, decreased locomotion both in the open field and in the training session of inhibitory avoidance. The results with diazepam confirm many previous findings demonstrating that BDZ possess amnestic effects in a variety of learning paradigms [e.g., (8,9)]. This drug was not studied by posttraining injection because it is well known to have no retrograde amnestic effects (3,9).

Further, also in contrast to diazepam, the flavonoids had no effect in the tail-flick test; which suggests that eventual analgesic effects had no influence on the performance of the avoidance tasks.

Chrysin, apigenin, and 6,3′-dinitroflavone were reported previously (4,13,14) to exhibit anxiolytic action in the elevated plus maze at doses several times lower than the ones used in the learning experiments. This effect was blocked by the prior administration of RO 15-1788, a specific antagonist of BDZ-R (13–15). Quercetin has no effect on this test (10).

Therefore, albeit largely negative, the present results contribute importantly to the knowledge of the pharmacological profile of this new class of highly specific BDZ-R ligands. Chrysin, apigenin, and 6,3′-dinitroflavone, whose affinity for the BDZ-R is, respectively 3 μm (7), 4 μM (13), and 12.7 nM (4), are potent anxiolytics, with no sedative, myorelaxant, anticonvulsant (13–15), or, as shown above, amnestic effects. In contrast, quercetin, which has a very low affinity for the BDZ-R [$K_i > 100$ μM] (5), and has no anxiolytic effect (10), shared the amnestic action of an anxiolytic dose of diazepam in the habituation and the inhibitory avoidance tasks, and, also like diazepam, reduced locomotion in these two paradigms.

In conclusion, the present results demonstrate that some natural and synthetic derivatives of the flavone nucleus that possess anxioselective properties acting on BDZ-R, have no effects on the acquisition and consolidation of several learning paradigms. In addition, our findings further endorse the hypothesis that these flavnoids behave as partial agonists of the BDZ-R. Because 6,3′-dinitroflavone is 30–100 times more potent anxiolytic than diazepam (4), and due to its selective pharmacological profile and low intrinsic efficacy (15), this flavonoid may represent an improved therapeutic tool for the treatment of anxiety disorders. Clearly, toxicological and clinical trials are now desirable (4,12,15).

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