ANTI-ANXIETY- AND ANTIDEPRESSANT-LIKE EFFECTS OF THE COMBINATION OF 6-NITRO-2(1H)-QUINOXALINONE AND 2(1H)-QUINOXALINONE IN WISTAR RATS

Redouan NAKACHE1*, Aboubaker ELHESSNI1, Ali OUICHOU1, Mohamed EL FAYDY2, Inssaf BERKIHS1, Youssef BAHBITI1, Brahim LAKHRISST2, Bouchra BENAZZOUZ3, Abdelhalem MESFIOU1

1Laboratoire de Génétique, Neuroendocrinologie et Biotechnologie, Faculté des Sciences, Kenitra, Maroc.
2Laboratoire de Chimie d’ Agroressources et Génie des Procédées, Faculté des Sciences, Kenitra, Maroc.
3Département de Biologie, Faculté des Sciences, Université Mohamed V- Rabat, Maroc.

ABSTRACT
To augment the effect of classical anti-anxiety and/or antidepressant drugs by combination therapy, which refers to the addition of drugs to enhance their effects, reduce their dose with better efficacy and lower side effects, has been a critical choice and an emerging treatment strategy. In this study, the anti-anxiety and antidepressant potential effect of intraperitoneal co-administration of 6-nitro-2(1H)-quinoxalinone (NQu) and 2(1H)-quinoxalinone (Qu) in the open field test (OFT), elevated plus maze (EPM) and forced swimming test (FST), was evaluated. The combination of NQu (10mg/kg) and Qu (10 mg/kg) exhibited anti-anxiety-like potential synergistic effect in the OFT, whereas, this combination caused no effect in the duration of immobility in the FST. While 20 mg/kg of NQu and Qu separately administered was ineffective in exhibiting the anti-anxiety-like effect in the OFT and EPM. On the contrary, 20 mg/kg of Qu significantly decreased the duration of immobility in the FST. By contrast, Diazepam (Dz) (1 mg/kg) was not influenced by the co-administration of NQu/Qu (10 mg/kg). None of the drugs used in the present study had any effects on the locomotor activity test at the doses applied. The results show that co-administration of NQu and Qu exhibits a synergistic effect and that no potential interaction occurred by association...
of NQu/Qu and Dz. Taken together, these results show that reducing the dose of tested molecules does not remove their anxiolytic effect when combined suggesting the existence of a synergistic interaction between the two molecules.

**KEYWORDS:** anxiety, depression, synergistic effect, diazepam, 6-nitro-2(1H)-quinoxalinone, 2(1H)-quinoxalinone, wistar rat, OFT, EPM, FST.

1. **INTRODUCTION**

Quinoxaline is also called as benzopyrazine, it is heterocyclic compound containing benzene ring and pyrazine ring. The quinoxalinone or quinoxalin-2-one core has received much attention in recent year as active compound. Such compounds are reported to possess diverse biological properties. Moreover, the quinoxalinone skeleton is also used as an intermediate in the designing of novel quinoxalinone derivatives displaying antidepressant\(^1\), \(^2\) antianxiety\(^3\), \(^2\); 5-HT3 receptors antagonists\(^4\); glycine/NMDA receptor antagonists\(^5\); MAO-A inhibitor\(^7\) activities and anticonvulsant activity in animal models of epilepsy\(^8, \)\(^9\). The heterocyclic core of the quinoxalinone pharmacophore and derivatives thereof have received increasing attention owing to their diverse biological properties, which have been extensively studied in recent years. In reviewing their pharmacological activities and structure-activity relationship studies and to seek for new drugs adopting strategy that involves structure modification, we thought it is worthwhile to synthesize some new quinoxalinone derivatives and screen them for their eventual anxiolytic and/or antidepressant activities with lower toxicity and superior efficacy. At an elevated dose (30mg/kg), our molecules showed anxiolytic and antidepressant effects\(^2\). Knowing that a molecule can be anxiolytic at a certain dose and be antidepressant at another one. Starting from our previous study\(^2\) we demonstrated that 6-nitro-2(1H)-quinoxalinone (NQu) compound maintaining an electron withdrawing group (NO2) displayed both anxiolytic and antidepressant effects, however, 2(1H)-quinoxalinone (Qu) compound displayed only a strong antidepressant effect.

In order to check if our molecules can change state with dose modification, and in order to minimize the adverse effects of high doses, this study aims to combine these two molecules at lower doses: 20 mg/kg or 10 mg/kg (combination).
2. Experimental Procedures

2.1 Animals
All experimental procedures were performed according to the NIH Guide for the Care and Use of Laboratory Animals. The animals used in this study, rats from the wistar strain weighing 225 ± 23 g at the beginning of the experiments, were born, bred and housed in groups of rats in acrylic cages (35 × 56 × 19 cm) in aerated room, according to standard animal care protocols. All rats were maintained on a 12 h light/dark cycle (lights on at 07:00) under constant temperature (≈22°C), with food and water ad libitum. The rats were randomly shared out into eight experimental groups (n = 5). Rats assigned to control group were administered by the vehicle solution, whereas the experimental groups were administered by different drugs for the remainder of the study. Animals were acclimatized to laboratory conditions before the experiment. Each animal was used only once. All the experiments were carried out between 10:00 and 16:00. The cages were cleaned regularly every 24 h.

2.2 Experimental Design
The rats were daily injected intraperitoneally; the anxiolytic and antidepressant standard group rats were administered with dizepam (Dz) (1 mg/kg), (Laboratoires PHARMA5) or clomipramine (Clmp) (2 mg/kg), (Manufactured by NYCOMED Austria GmbH, Linz, Austria for Novartis Pharma AG, Basle, Switzerland) respectively. In the experiment using quinoxaline derivatives, rats were administered (dose: 20 mg/kg) with compound Qu or compound NQu and 10 mg/kg when associated (NQu: 10mg/kg + Qu: 10mg/kg) or combined with Dz (NQu/Qu: 10mg/kg + Dz: 1mg/kg). Appropriate control (T) studies were performed administering vehicle (NaCl 0.9% + 10% DMSO) in place of drug. All drugs were administered dissolved in normal saline and dimethyl sulphoxide (DMSO) on each day of the experiment. After two weeks in treatment conditions, the rats underwent a battery of behavioral tests to measure anxiety- and depressive-like responses. All testing occurred during the light phase between 10:00 and 16:00. Testing occurred in the following order to minimize stress effects in the most sensitive tests: open field, elevated-plus maze, Porsolt forced swim test. Rats naive to the apparatus and treatments were used for all the experiments and each rat was used only once. Rats were allowed to acclimate to the testing room 1 h before testing began. To eliminate any lingering olfactory cues, the pieces of test equipment were thoroughly cleaned by using water and 20% alcohol followed by thorough drying before each rat was tested.
2.3 Behavioral Testing

2.3.1. Open Field Test (OFT)

The investigatory behavior was tested in a wooden made apparatus (100 cm × 100 cm). As previously reported,[11] it was enclosed with 40 cm high walls and placed under strong illumination (100 watt, 2 m above the apparatus). The area was divided into 25 squares (20 cm × 20 cm), defined as nine central and sixteen peripheral squares. At the beginning of the 10-min test, the animal was placed in the center of the apparatus and its behavior was videotaped for subsequent analysis. The device was cleaned after each individual rat session. The quantified parameters were the time spent in the center of the area (TCA), the number of returns to the nine square central sections (NRC) and number of total squares crossed (NTS). Central perimeter residence time is used as a measure of anxiety.[12] The number of returns to the central area is also an indicator of the emotional reactivity.[11] The central area of a novel environment is anxiogenic and aversive and the behavioral inhibition appears therefore as an avoidance behavior towards the central zone of the open field.[13]

2.3.2 Elevated Plus-Maze Test (EPM)

The EPM is an ethological model of anxiety in rodents provoked by the novelty and repulsion as a result of elevation and illumination of the maze.[14] This test is based on the creation of a conflict between the exploratory drive of the rat and its innate fear of open and exposed areas; it has been validated for the detection of emotional responses to anxiogenic and anxiolytic substances.[15] Thus, increased open-arms exploration indicates reduced anxiety-related behavior. The EPM consists of a wooden plus-shaped platform elevated 70 cm above the floor. Two of the opposing arms (50 cm × 10 cm) are closed by 40 cm high side and end walls, having an open roof. In order to avoid fall, the other two arms (open arms) were surrounded by 0.5 cm high edge, the four arms had at their intersection a central platform (10 cm × 10 cm).[12] A 100-W lamp was placed exactly over the central platform. At the beginning of the test, the rats were placed on the central area of the maze facing an open arm. The following parameters of anxiety-related behavior were measured during the 5 min testing period: 1) entries into open arms (EOA), 2) time spent on the open arms (TOA), and 3) number of total entries into the arms (TEA).[16] Decreased anxiety-like behavior is illustrated by a significant statistical increase of parameters in open arms (time and/or entries). Although, entries in closed arms and total entries reflect the motor component of the exploratory activity.[12, 17] To eliminate any lingering olfactory cues, the apparatus was cleaned between each examination using 70% ethyl alcohol.
2.3.3 Forced Swimming Test (FST)
To assess depressive-like responses, rats were individually placed in a cylinder (height = 50 cm; diameter = 30 cm) containing 27 cm water (22°C) from which they could not escape. The rats were placed in the water for 6 min and the total duration of immobility (T-Im) was recorded during the 6-min period. A decrease in the duration of immobility is indicative of an antidepressant-like effect. The latency to the first bout of immobility was also recorded starting immediately after placing the rats in the cylinders. A rat was judged immobile when it ceased all active behaviors (i.e. struggling, swimming and jumping) and remained passively floating or making minimal movements necessary to maintain the nostrils above water. High percent time floating is interpreted as an increased depressive-like response.\(^{[18]}\)

2.4 Statistics
Data are expressed as the means ± standard error of the means (S.E.M.). To determine the differences between experimental groups statistical analysis was performed by analysis of variance (ANOVA) 1st order followed by a post-hoc tests (Fisher LSD) or Student test “t”. Inter-group differences were considered significant when p < 0.05, very significant when p < 0.01 and highly significant at p < 0.001.

3. RESULTS
3.1. Anxiolytic-Like Effect
3.1.1. Open Field Test
3.1.1.1. Number of Returns to the Center (NRC): Figure 1a
Analysis by ANOVA test showed no significant difference between the treatment groups, p = 0.29. So the ECC parameter was not influenced by the treatment of co-administration of the two tested drugs on the one hand and by the association with diazepam on the other (Fig.). However, there is a tendency to increase this parameter by the treatment groups Qu20 (44%) and NQu (46%) compared to the control group. We also observed that the administration of both derivatives associated with a reduction in their dose of 50% kept the same effect as these one separately administered at the dose of 20mg/kg, taken together, these results suggest a possible synergistic effect of the two quinoxaliniques derivatives.

3.1.1.2. Time Spent in the Central Area (TCA): Figure 1b
Chronic treatment with the combination of the two quinoxalinic derivatives NQu (10mg/kg) and Qu (10mg/kg) significantly increased the time spent in the central area TCC of the OF test in both groups NQu and Qu compared to control T (p = 0.003), and to the same
derivatives administered separately NQu (20mg/kg) (p = 0.000) Qu (20mg/kg) (p = 0.013), confirming the synergistic effect shown by the CC parameter. Co-administration with diazepam has no significant effect. The ANOVA showed no significant difference between the Dz (1mg/kg) and Dz associated with NQu (10mg/kg), p = 0.087 or Qu (10mg/kg), p = 0.891.

3.1.1.3. Number of total squares crossed (Locomotor Activity: NTS): Figure 1c One way ANOVA showed no significant effect between the different groups of chronic treatment with NQu and Qu derivatives (10mg/kg), p = 0.478.

We also note that the combination of the two derivatives showed no significant effect on locomotor activity in rats.
Figure 1. Mean ± (S.E.M.) (a) the number of return into center area of the arena in the open-field behavior apparatus (NRC); (b) the total amount time spent in the center of the open field (TCA) and (c) the number of total squares (NTS); by rats treated by (NaCl 0.9%); Dz (1mg/kg); NQu (20mg/kg); Qu (20mg/kg); (NQu:10mg/kg + Qu:10mg/kg); (NQu:10mg/kg + Dz:1mg/kg) or (Qu:10mg/kg + Dz:10mg/kg). *p < 0.05, **p < 0.01, ***p < 0.001.

3.1.2. Elevated Plus Maze

3.1.2.1. Entry to Open Arms (EOA): Figure 2a

Analysis of the EOA parameter results by ANOVA showed no significant difference of NQu (p = 0.689) and Qu (p = 0.403) derivatives compared to the control in the EPM test. However, the interaction between the two molecules NQu and Qu co-administered at dose of 10mg/kg each, has significantly reduced the number of visits to open arms compared to the same compounds administered separately NQu: 20mg/kg (p = 0.03) Qu: 20mg/kg (p = 0.408). No significant difference was observed by the combination of Dz with NQu (10mg/kg, p = 1) on the one hand and Qu (10mg/kg, p = 0.64) on the other, compared to the Dz administered alone, so this result eliminates the hypothesis of a possible interaction of inhibition between Dz and quinoxalinic derivatives in EPM test.

3.1.2.2. Time Spent in Open Arms (TOA): Figure 2b

The TOA parameter was not significantly affected by chronic treatment with Qu (p = 0.887). However, the Fisher’s LSD test showed a significant reduction in the time spent into the open arms between rats treated with NQu (p = 0.032) and controls.
The association of the two molecules showed no significant difference, but there is a tendency to increase the TBO parameter by 5% compared to the control, 3% compared to Qu and 62% compared to NQu.

No significant difference was observed between the group treated with Dz in association with Qu and control group, \( p = 0.676 \). However, NQu showed a significant effect compared to the control when administered in combination with Dz, \( p = 0.006 \). In addition, we note that the rats receiving NQu associated with Dz increased TBO by 10% compared to those administered with Dz.

3.1.2.3. Total Entries in Arms (TEA): Figure 2c

The TEA parameter was not significantly affected by chronic treatment with the two molecules. Application of Fisher LSD test showed no significant difference between control and treated rats with NQu \( (p = 0.151) \) and Qu \( (p = 0.952) \).

Co-administration of the two molecules at dose of 10mg/kg showed no significant difference compared to the same compounds administered separately at a dose of 20mg/kg, \( (p = 0.051) \) NQu \( (p = 0.054) \) and Qu \( (p = 0.585) \) compared to control. However it is noted that these molecules co-administered (10mg/kg), increased the EBT 10% compared to Qu and 320% relative to NQu.

Chronic treatment with NQu associated with Dz has very significantly increased locomotor activity in rats \( (p = 0.005) \), while no significant effect was marked by Qu in comparison to the control, but there is a trend of increasing this activity by 16% (NQu + Dz) and 2.4% (Qu + Dz) compared to Dz (1mg/kg).
Figure 2. Mean ± (S.E.M.) (a) Number of entries in the two exposed arms of elevated plus maze (EOA); (b) Total amount of time spent exploring these arms (TOA) and (c) Total number of arms entries (TEA) by rats treated by (NaCl 0.9%); Dz (1mg/kg); NQu (20mg/kg); Qu (20mg/kg); (NQu: 10mg/kg + Qu: 10mg/kg); (NQu: 10mg/kg + Dz: 1mg/kg) or (Qu:10mg/kg + Dz:10mg/kg). *p < 0.05, **p < 0.01, ***p < 0.001.

3.2 Anti-depressant-like effect

3.2.1. Forced Swimming Test

Immortality Time (TIM): Figure 3
Chronic treatment with Qu significantly affects the parameter T-imm p = 0.001. Application of the Fisher LSD test showed a highly significant difference between the control rats and those treated with Qu (p = 0.001). We then noted a significant drop in the immobility time in this group of rats compared to controls. However, no significant effect was observed by the combination of two molecules compared to control (p = 0.728).
Figure 3. Mean ± (S.E.M.) Immobility time in Forced swim-ming test by rats treated by (NaCl 0.9%); Clmp (2mg/kg); NQu (20mg/kg); Qu (20mg/kg) or (NQu: 10mg/kg + Qu: 10mg/kg). *p < 0.05, **p < 0.01, ***p < 0.001.

DISCUSSION

Depression is a mood disorder that is pervasive and affects almost every part of the world. Globally, it ranked fourth among the leading causes of disability\[19\] and by 2030; it is expected to be the largest contributor to disease burden.\[20\]

Figure 4: common structural requirements for glycine/NMDA and AMPA receptor antagonists. (a) : test compounds, Qu(R=H), NQu(R=NO2)\[21\]; (b): quinoxaline-2,3-diones structure.\[32\]
Depression is often associated with anxiety that share many overlapping symptoms including fatigue, impaired concentration, irritability, sleep disturbance, experiences of nervousness, worry and restlessness.[21]

Depression ranks among the top most co-existing disorders with anxiety and approximately 39% of the patients with mood disorder meet criteria for both generalized anxiety disorder and major depressive disorder.[22, 23, 24] The co-existence of these mood disorders suggests that they may also share a common pathophysiology. According to the classic monoamine hypothesis, an imbalance in the levels of monoamines in certain areas of brain results in depression and anxiety.[25, 26, 27, 22] In this respect, augmentation therapy is a critical choice to discard drug resistance and refers to the addition of drugs so as to enhance the effect of standard antidepressants and/or anti-anxiety, to discover novel drugs with better efficacy and lower side effects. The fact that glutamatergic may be involved in the antidepressant and or anti-anxiety-like effects of quinoxalinone derivatives directed us to perform the present experiments.

In the present study, a lower dose (20mg/kg) of NQu and Qu was ineffective but slightly increased the number of returns to the center by rats in the Open field test relatively to control. We also observed that the administration of both derivatives combined with a reduction in their dose of 50% kept the same effect as these one separately administered at the dose of 20mg/kg. In addition, the combination of either lower doses of NQu and Qu significantly increased the time spent in the central area of OFT. These results indicate that there is a synergism between NQu and Qu and that the mechanisms of anti-anxiety like effect of these drugs may interfere at some point. The co-administration of quinoxalinone derivatives 10mg/kg and diazepam, 1mg/kg, a GABA-A receptor agonist, showed no significant effect in the OF and EPM test relatively to the effect of diazepam administered solely 1mg/kg such as the anxiolytic effect of diazepam was not influenced by combination with quinoxalinone compounds NQu and Qu. These results suggest that there is no interaction between Dz and test compounds NQu and Qu, thus, no direct implication of GABAergic system in the mediation of anxiolytic like effect of ours compounds. In fact, the structure–activity relationship studies on quinoxaline-2,3-diones, which have been reported to have glycine/NMDA and AMPA receptor antagonist activities,[28, 29] have revealed that there are remarkable structural similarities in the binding sites of both receptor types.[30, 31] As shown in figure 4, both glycine/NMDA and AMPA antagonists possess a NH proton donor
which binds to a proton acceptor of the receptors, as well as negatively-charged heteroatoms able to form a Coulombic interaction with a positive site of the receptors. Both glycine/NMDA and AMPA receptors can tolerate a polar side-chain called $X$ (figure 4B), and both prefer to accommodate electron-withdrawing group(s) at R1 and/or R2. However, glycine/NMDA or AMPA selectivity can be modulated by varying the nature of R1, R2 and the spatial orientation of the X polar side-chain. Generally, bulky electron-withdrawing NO2, CF3 or Br substituents and a nitrogen-containing heterocycle are preferred by the AMPA receptor in R1 and R2, respectively, while the glycine/NMDA site preferably accommodates chlorine atoms in R1 and R2. Although, the structural similarities existing between test compounds containing hydrogen bond donor NH, negative-charged heteroatom and electron-withdrawing group NO2 in position 6, and glycine/NMDA and AMPA receptors, taken together, these data are in agreement with our results which support the anxiolytic like effect of the test compounds may be mediated by the glutamatergic AMPA and/or glycine/NMDA receptors (figure 4A).

The analysis of data obtained from OF and EPM tests revealed no alteration of motor activity of the rats after administration of test compounds solely or combined at reduced dose (10 mg/kg) and associated with diazepam. These results confirm our previous study that have showed no inhibition of motor activity by test compounds Qu and NQu at the dose used (30 mg/kg). Although, in other literature work they have showed that from total locomotor activity test, all quinoxalinone compounds tested have been found to have strong sedative effect in mice, the most sedative effect was exhibited at 40mg/kg by a compound, which carries an electron-withdrawing group dibenzylsulfonamido at position 6. However, the compound, which carries an azidosulfonyl group (N3SO2-) at position 6, showed only a slight reduction in the locomotor activity at maximum dose of 100mg/kg.[3] According to these data, we concluded that the electron-withdrawing group dibenzylsulfonamido at position 6 is beneficial in exhibiting sedative effect. Moreover, and in spite of the test compound NQu carries an electron-withdrawing group NO2 at position 6, the absence of sedative effect could be due to the lower dose used (10 and 20 mg/kg) which seems to be ineffective in exhibiting sedative property.

The Antidepressant effect was observed in compound Qu at the lower dose selected 20mg/kg, whereas, the same dose was ineffective in decreasing of the duration of immobility in the forced swimming test by the test compound NQu which carries an electron-withdrawal group...
NO₂ at position 6. In addition, the combination of either lower dose of Qu (10 mg/kg) and NQu (10 mg/kg) did not improve the result. These results indicate that there is no synergistic effect between the two test compounds and that the disappearance of the antidepressant-like effect of Qu when combined with NQu is likely due to the lower dose used (10 mg/kg) which seems to be ineffective in the exhibiting of the antidepressant-like effect or to an inhibitory effect of the second compound NQu.

REFERENCES
6. Phillip J. Birch, Carol J. Grossman and Ann G. Hayes. 6,7-Dinitro-quinoxaline-2,3-dion and 6-nitro,7-cyano-quinoxaline-2,3-dion antagonise responses to NMDA in the rat spinal cord via an action at the strychnine-insensitive glycine receptor. European Journal of Pharmacology, 1988; 156: 177-180.


