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ARYL HYDROCARBON RECEPTORS IN INDOLE DERIVATIVE TREATED MICE: NEUROPHARMACOLOGICAL PERSPECTIVES

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Abstract. Aim/objective. *When applied in pharmacological doses, the indole derivative melatonin exhibits neuroactive and neuroprotective effects. Indoles and their metabolites, such as kynurenine, are ligands of aryl hydrocarbon receptors (AhR). This study aimed to evaluate the antiepileptic and analgesic activity of melatonin and two synthetic melatonin derivatives. The possible involvement of AhR and kynurenine in their neuropharmacological effects were also tested.* ***Methods.*** *The tested substances were: melatonin, two melatonin derivatives bearing aryl hydrocarbon moiety with either furyl or thienyl substitute (3e and 3f), and alpha naphthoflavone (ANF), an antagonist of AhR. After intraperitoneal injection of 30, 100, or 300 mg/kg of the tested agents for seven days, male mice ICR (25-30 g) were subjected to a corneal kindling seizure model. Two tests for analgesia, i.e., the hot plate test and the formalin test, were also applied. AhR and kynurenine concentrations were evaluated in brain homogenates.* ***Results.*** *Substances 3e and 3f demonstrated an antiepileptic activity comparable to that of melatonin. Some analgesic activity was also shown, albeit lower than that of melatonin in equivalent doses. For melatonin and 3f treated mice, dose-dependent increases in AhR and kynurenine levels in brain homogenates were recorded. The antagonist ANF neither blocks the antiseizure activity of the tested indoles, nor demonstrated analgesic activity.* ***Conclusion.*** *Melatonin and the two tested melatonin-aroylhydrazone derivatives bearing either furyl or thienyl substitute exhibit antiepileptic and analgesic activity. Our results did not support the involvement of AhR in the demonstrated neurobiological activity. Further studies are needed to elucidate their exact molecular mechanisms.*

Key words: *melatonin, indole derivatives, corneal kindling, analgesia, aryl hydrocarbon receptors, kynurenine*

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INTRODUCTION

The aryl hydrocarbon receptor (AhR) is a highly conserved ligand-activated transcription factor of the basic helix-loop-helix Per-Arnt-Sim (PAS) family among vertebrates. Upon binding to its ligands, cytoplasmic AhR is transferred to the nucleus. After heterodimerization with aryl hydrocarbon receptor nuclear translocator (ARNT), it mediates numerous biological and toxicological effects by transcribing various AhR-responsive genes [1]. Well-known targets for AhR are CYP1A1, CYP1B1, ABCG2, ALDH3A1, NRF2, and UDP-glucuronosyltransferases (UGTAs) genes, and many other genes found to be either up- or down-regulated by AhR activation [2]. Further, AhR regulates a variety of biological processes involved in cell division, differentiation, apoptosis, sex hormone activity, reproduction, and immunity [3]. It was also shown that AhR possesses the intrinsic E3 ubiquitin ligase function that is important for proteasomal degradation [4].

AhR-ligands range from industrial substances, pharmaceuticals, or microbiome products, to natural compounds from plant origin [5]. The majority of high-affinity AhR-ligands are synthetic substances formed as a result of a non-biological activity. They are halogenated aromatic hydrocarbons and polycyclic aromatic hydrocarbons [6]. Natural sources of ligands, such as flavonoids, carotenoids, and glucobrassicin, are derived from fruits and vegetables [7].

Exogenous diet-derived indoles, such as tryptophan, constitute a significant source of AhR ligand precursors, produced via four pathways: i) hydroxylation to serotonin and melatonin; ii) decarboxylation to tryptamine; iii) transamination to indole pyruvic acid; and iv) the kynurenine pathway to kynurenine and its metabolites [8]. Pathways for induction of the tryptophan catabolite (TRYCAT) are commonly associated with the inflammation, and oxidative stress resulting from increased kynurenine levels. Kynurenine is an agonist of AhR; however, its metabolites kynurenic acid and quinolinic acid are AhR antagonists. Melatonin also acts as an antagonist to AhR [9]. Alpha-naphthoflavone (ANF) is a well-known synthetic antagonist of AhR, and it is widely used for the evaluation of toxicological and pharmacological AhR-mediated mechanisms [10]. Besides ligand-modulation, AhR can be activated in a ligand-independent manner by cyclic 3'5' adenosine monophosphate (cAMP) and protein kinase A (PKA) [11].

We recently reported a series of newly synthesized molecular hybrids bearing two highly promising pharmacophores, i.e., indole and aroylhydrazone moieties. For two of the substances, i.e., furan or thiophene substituted (corresponding respectively to the 3e- and 3f-substance) – Fig. 1, we have already discovered an acute anticonvulsant activity potentially mediated by the melatonin M1 receptors [12].

With the present study, we wanted to test 1) the potential chronic antiseizure activity; 2) the possible analgesic activity, and 3) the potential involvement of AhR. Substances with anticonvulsant activity are often tested for potential analgesic effects, thus trying to satisfy the patient's need for pain relief with better safety profile [13]. Electrical kindling is a model using subthreshold electrical stimulation of rodents, making them prone to develop spontaneous seizures. The pharmacological profile of the corneal kindling test in mice corresponds to partial epilepsy in humans and effectively identifies the anticonvulsant potential of active drugs under similar conditions, e.g., levetiracetam [14]. Additionally, we performed analgesic tests. We characterized the effect of indole-containing substances melatonin, 3e and 3f, and alpha-naphthoflavone, a known AhR antagonist. The level of AhR and kynurenine in total brain homogenates were also evaluated.

MATERIALS AND METHODS

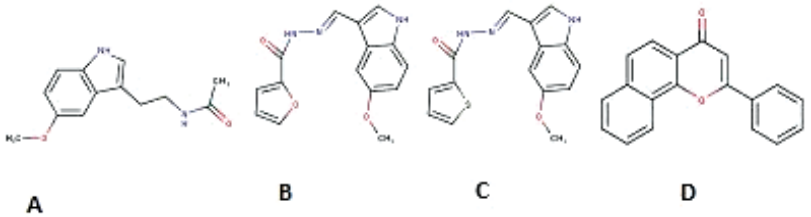

Fig. 1. Chemical structure of tested substances: A. Melatonin; B 3e; C 3f; D Alpha naphthoflavone

Drugs and Chemicals

ANF and melatonin were purchased from Merck KGaA, Darmstadt, Germany. The two tested substances 3e and 3f were synthesized in the Chemistry Department of the Faculty of Pharmacy of the Medical University of Sofia. Their synthesis and spectral verifications were already reported elsewhere [12]. Three of these substances, i.e., melatonin, 3e, and 3f, have an indole structure. ANF was selected for this project as an AhR antagonist. All tested compounds were dissolved in saline and injected at various concentrations of 30, 100 and 300 mg/kg. Each animal participated only once in an experiment.

Animals

Adult male ICR mice (25-35 g) were obtained from the Vivarium of the Institute of Neurobiology of Bulgarian Academy of Sciences in Sofia. They were kept at a controlled temperature (23 ± 2ºC) and relative humidity (50-70%), at 12-12 hr light-dark cycle and free access to rodent chow and water. The animal models were tested under "the Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals', Washington DC, National Academy Press. Approval N 246/08.10.2019 was received by the Bulgarian Food Safety Agency under the National Regulations.

Induction of kindling by corneal electrical stimulation

Electrical kindling was performed with ECT Unit 57800 (Ugo Basile, Gemonio, Italy). Mice were electrically stimulated twice daily with 3-second corneal stimulation with a current of 3 mA, 60 Hz for 14 days. After the 12 days of the 2-time kindling procedure, their seizure-prone activity reaches a plateau, and no further increases of severity can be expected. Seizure severity was ranked as follows: 1. Mild facial clonus and eye blinking. 2. Sever facial clonus, head nodding, chewing. 3. Unilateral or alternating forelimb clonus. 4. Bilateral forelimb clonus and rearing. 5. Bilateral forelimb clonus with rearing and falling. 6. Tonic hind-limb extension. Reaching stage 3 and more severity score was accepted as sufficient kindle.

After twice-daily corneal stimulation, mice typically reach stage 5 in 10-14 days. Twice daily stimulation was continued for each mouse until stage-5 seizures were reached when it was assumed that the mouse had achieved complete kindling. Successfully kindled mice kept kindling throughout the day until all mice in the group have reached the state of “complete kindling.” The substances were not tested in mice, who did not achieve complete kindling.

Drug experiment

The drug testing was performed in two sets of experiments. In the first set, drugs were applied to successfully kindled mice for testing their anticonvulsant activity.

Eight animals by the group were evaluated. All drug experiments were performed between 8:00 and 11:00 a.m. to minimize the circadian variance of the seizure susceptibility. Drugs were dissolved in saline and injected i.p. 30 min before electrostimulation. Several doses of the substances were evaluated.

To test the hypothesis about the involvement of AhR in the anticonvulsant effect of the studied substances, in some of the experiments, we injected the 100 mg/kg, i.p. ANF 30 minutes before the application of melatonin, 3e or 3f. The dose of ANF was based on the pharmacokinetic study performed by other authors [15]. Pre- and post-drug vehicle control experiments were done one day before and one day after the test. Each animal was used in the trial only once. Animal behavior was observed and assessed by two independent observers, unaware of the applied treatment.

For evaluation of AhR and kynurenine in brain homogenates, we tested kindling-naïve mice. Data from other authors demonstrate that the corneal kindling test is related to an oxidative stress [16], and this factor is known to increase the kynurenine level. This part of our study aimed to evaluate AhR and kynurenine levels without the kindling procedure’s interference. Mice were injected consecutively with the corresponding drug/dose group (n = 6) for 7 days. AhR and kynurenine were measured in brain homogenates.

Hot plate test

All experiments were performed after one week of adaptation, between 9.00 and 12.00 a.m. The mice were deprived from food the night before the experiment but had free access to water. Each animal was tested only once, 30 minutes after intraperitoneal injection of saline dissolved test compounds, at concentrations of 30, 60, or 100 mg/kg. The control group was injected with the vehicle only. Acute thermal pain was elicited using a temperature of 47° C. The test mice were placed in an open cylinder above the hotplate equipped with a thermostat and observed for two types of reactions – paw licking and jumping. The latency period for antinociceptive behavior was measured. This test was performed 30 minutes after the injection of the test substance.

Formalin test

Subdermal injection of 0.5% formalin was performed in the plantar region of the right mouse hind paw, thus triggering a respective 2 phase behavioral response. Immediately after injection, the mouse starts licking the paw for 5-10 minutes, corresponding to the acute phase of the test that is mediated by the activation of local C-fibers. A short latency period follows (usually less than 5 minutes) with no or minimal activity and a more prolonged inflammatory second phase (about 20-30 min) of licking, mediated mainly by inflammatory mechanisms and by the development of sensitization in the dorsal spine nerves.

Animals were monitored to observe the effects of the test substances in both acute and inflammatory stages of persistent pain. Before administering the test substance, each mouse underwent a 15-minute conditioning period in one of several standard Plexiglass cages. The mice (n = 8 in the treatment group) were then monitored throughout the experiment. After the conditioning period, the test substance was administered intraperitoneally at different doses, and the mouse returned to the cage. Formalin was injected subcutaneously into the plantar part of the hind paw. After that, each animal was monitored by two independent investigators, and time in the first and second phases was counted in seconds.

Brain homogenates

The whole brains were harvested, weighed, and homogenized in ice with phosphate buffer saline (1:12 g: ml) in an ultrasound homogenizer GX-10 (Bueno Biotech Ltd, Nanjing, China) with 1 second per impulse, 44 W, time-off interval 1 sec, 120 cycles for 240 seconds. The homogenate was after that centrifuged at 0°C for 10 min in 15000 rpm. The supernatant was stored at -80°C for further evaluation of AhR and kynurenine.

AhR

Mouse AhR ELISA kit was purchased from Wuhan Fine Biotech Co. Ltd, Wuhan, China. The principle of this kit is based on sandwich enzyme-linked immune-sorbent essay technology. The biotin-conjugated anti-AhR antibody was used as a detection antibody. Horseradish peroxidase (HRP)-streptavidin conjugate was added, and unbound conjugates were washed away with wash buffer. 3,3',5,5'-Tetramethylbenzidine (TMB) substrates were used to visualize HRP enzymatic reaction, read at 450 nm with ELx800 Biotech, Bad Friedrichshall, Germany.

Kynurenine

Mouse Kynurenine ELISA kit was purchased from ImmuSmal, Talence, France. The test is based on the competitive enzyme immunoassay method for the quantitative determination of L-kynurenine. In preparation for the examination, the samples were mixed with a derivatization reagent for the containing L-kynurenine. Subsequently, the derivatized sample and a polyclonal kynurenine antiserum in an ELISA plate coated with L-kynurenine derivative (tracer) were incubated. During the incubation, the target antigen competes with the tracer bound for binding the polyclonal antibody. In the second incubation step, a peroxidase-labeled secondary antibody was added, which binds to the L-kynurenine antibody. After a washing step to remove unbound components, the peroxidase substrate TMB was added. The enzyme reaction was visualized by HRP enzymatic reaction, changing the color from blue to yellow, which was read at 450 nm with ELx800 Biotech, Bad Friedrichshall, Germany.

Statistics

In cases where the data obtained showed normality of distribution and uniformity of variations, ANOVA was applied. In cases where these two conditions were not met, a non-parametric one-factor Kruskal-Wallis analysis was used, followed by a Tukey test for group comparisons to identify statistically significant differences. In the analgesia test, a Dunnett test was applied to compare with the Wilcoxon control group. A repeated comparison test with the control was used to evaluate the severity of seizures (SS) of the exposed mice. A significance level of $p < 0.05$ was accepted. Analyzes were performed with Sigma Plot 11.0.

RESULTS

The anticonvulsant activity of the indole derivatives in corneal kindling seizure model and lack of antagonistic effect by alpha naphthoflavone.

Mice were subjected to 3-second electrical stimulation at 3 mA 60 Hz and corneal electrodes to achieve five consecutive stage-5 seizures (facial cloning and shaking of the head, progressing to a clone of the forelimbs, and finally rising and falling, accompanied by generalized clonic seizure). After twice-daily corneal stimulation, mice typically reach stage 5 in 10-14 days. Twice daily stimulation was continued for each mouse until the criterion of 5 consecutive stage-5 seizures was reached when it was considered that complete kindling was achieved. The electrical treatment was continued until all mice in the group have reached the state of "complete kindling." The substances were not tested in mice, where full kindling was not achieved. The increase of the seizure severity during the kindling procedure is shown in Fig. 2.

The measurement of severity scores for the spontaneous seizures of kindled mice demonstrated effective suppression by melatonin (30, 100 and 300 mg/kg), 3e (30 and 100 mg/kg), and 3f (100 and 300 mg/kg) – Figure 3. The antagonist ANF in a dose of 100 mg/kg did not abolish the seizure activity, indicating that AhR is not involved in the mechanism of action of the studied substances.

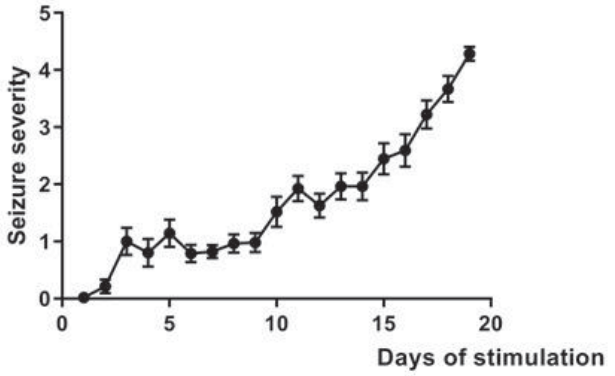

Fig. 2. The severity of epileptic seizures in the course of kindling. Data are presented through mean and error

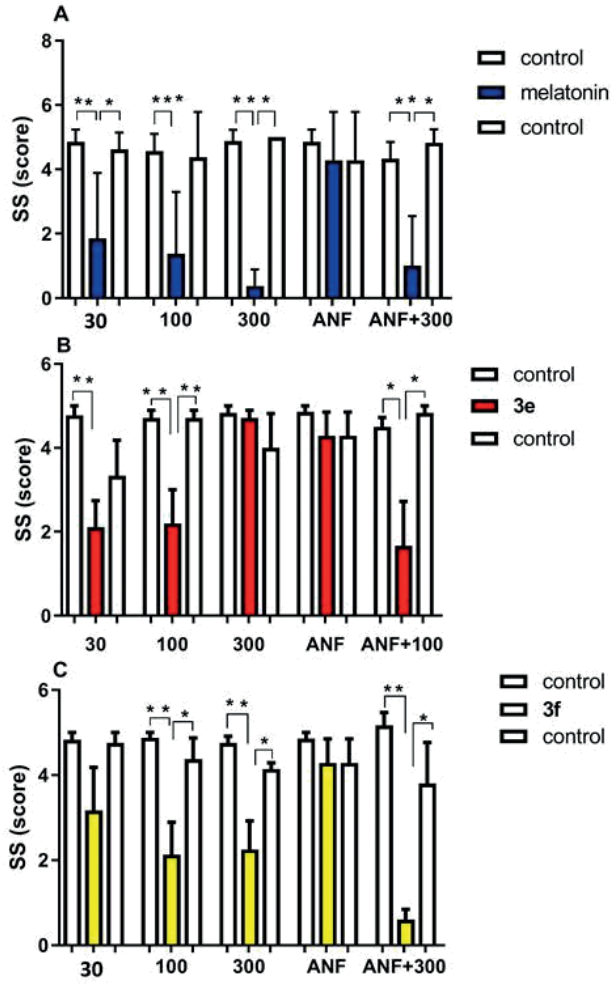

Fig. 3. Measurement of seizure severity with or without administration of 100 mg/kg ANF. The explanations are given in the text

Hot plate test

After assessing normality of distribution and equality of variance, a one-way ANOVA was applied, followed by a Dunnett test for comparison with the control group with $p < 0.05$ acceptable level of significance. None of the treatment groups differed significantly from the control group – Figure 4.

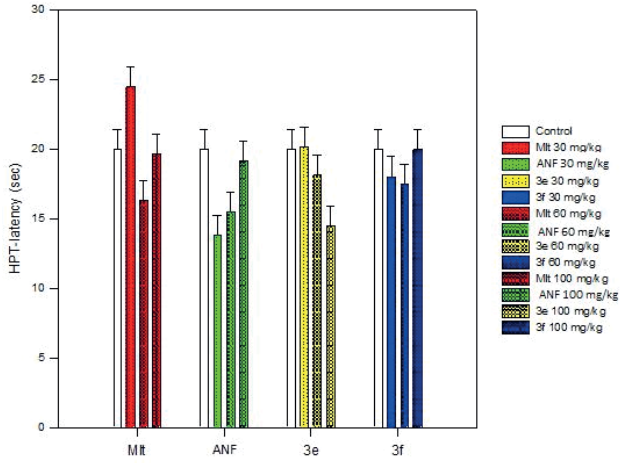

Fig. 4. Hot plate test – antinociceptive behavior latency (sec). n = 6 in each group; $p > 0.05$

Formalin test – phase I (0-5 min)

A two-factor ANOVA of the two independent variables (test substance, dose) of the latency period before antinociceptive behavior was applied – Figure 5. All effects were statistically significant at significance levels of 0.05. The main effect of the test substance gave an F ratio of $F(3, 80) = 13.772$, $p < 0.001$, indicating a significant difference between the groups. The dose's main effect gave an F ratio of $F(3, 80) = 7.769$, $p < 0.001$, indicating that the dose-effect was significant. The interaction effect was significant; $F(9, 80) = 4.866$, $p < 0.001$.

In the first phase of the formalin test, melatonin demonstrated the most potent analgetic effect in a dose-dependent manner. 3e at the lowest tested dose of 30 mg/kg, even increased the antinociceptive behavior compared to melatonin.

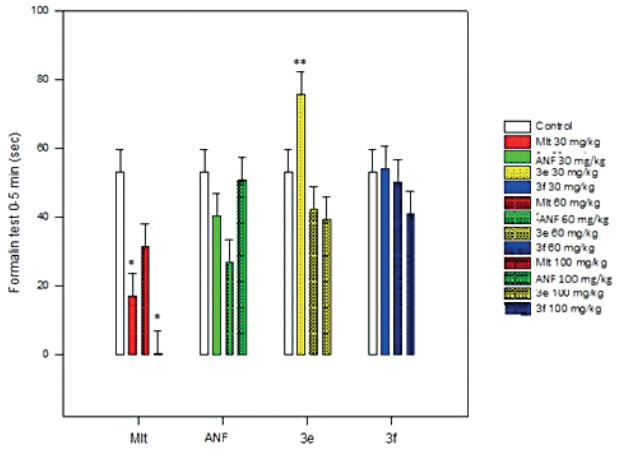

Fig. 5. Results of the first phase of the formalin test. The explanations are given in the text

Formalin test phase II

A two-factor ANOVA was used for both factors – administered substances and doses. Statistically,

a significant interaction was identified between two factors, F (9, 80) = 5,505. P < 0.001 Melatonin, both substances 3e and 3f showed statistically significant lower values (analgesic effect) than controls (p < 0.05). ANF did not show analgesic effect – Figure 6.

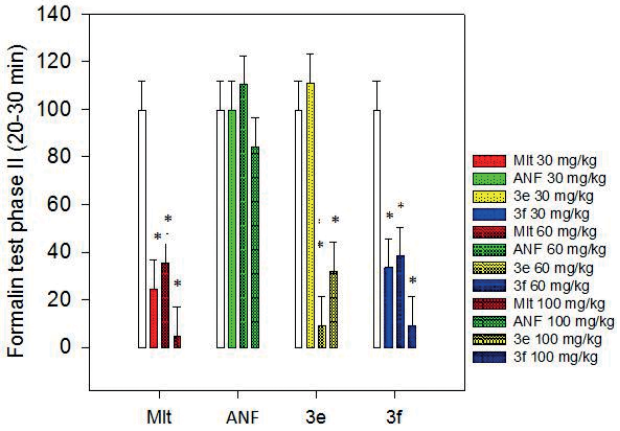

Fig. 6. Results of the second phase of the formalin test. The explanations are given in the text

AhR levels in brain homogenates

After 7-day treatment with melatonin or 3f, a dose-dependent increase in the level of AhR in the brain homogenates was detected. After applying the Kruskal-Wallis test for melatonin, H = 9.266 with 3 degrees of freedom, p = 0.026, and for substance 3f and H = 10.325 with 3 degrees of freedom, p = 0.016, Figure 7.

Higher AhR levels were recorded in the 300 mg/kg dose group in melatonin treated mice, and this was statistically significant (higher than the 30 mg/kg treatment group (p < 0.05)). For substance 3f, AhR levels in the 300 mg/kg treated group also showed statistically significant higher AhR levels than the control and 30 mg/kg treated groups (p < 0.05).

Kynurenine level in brain homogenates

In determining kynurenine levels, significant changes were observed after administering different doses of melatonin and substance 3f. In contrast, no significant differences were observed after administering different doses of ANF and substance 3e. After one way administration of different doses melatonin ANOVA, F (3.20) = 7.171, p = 0.002 was found. Significantly higher levels were found at doses of 100 mg/kg p < 0.05. Regarding substance 3f, the administration of ANOVA also showed that different treatment doses had a significant effect differences – F (3,20) = 3,643, p = 0.030. Significantly higher levels were found at doses of 100 mg/kg p < 0.05 – Figure 8.

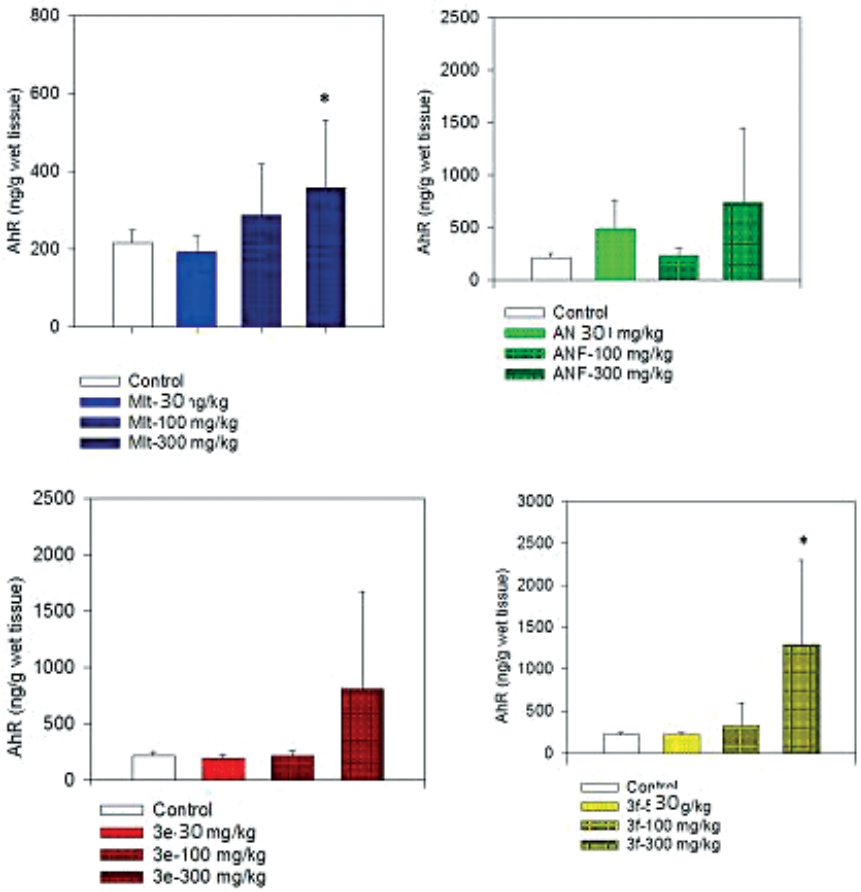

Fig. 7. AhR levels in brain homogenates after seven days of treatment with melatonin, ANF, 3e or 3f. * P < 0.05

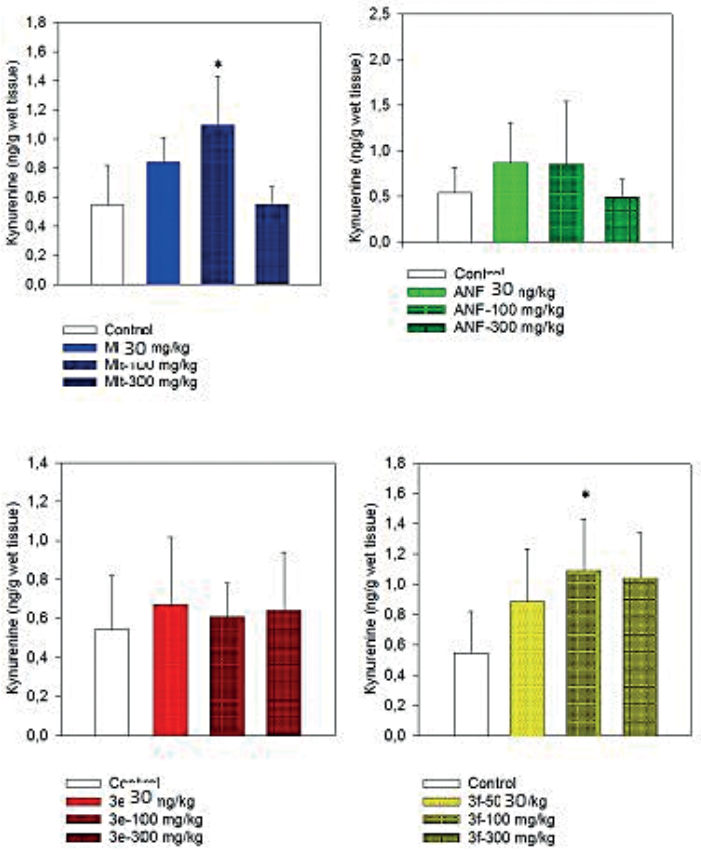

Fig. 8. Kynurenine levels in brain homogenates after a 7-day course of treatment with melatonin, ANF, 3e or 3f. *P < 0.05

DISCUSSION

We conducted a neuropharmacological screening of two indole derivatives with an aroylhydrazone moiety, comparing them with melatonin (indole derivative) and alfa-naphthoflavone (AhR). The tested substances (melatonin, ANF, 3e, and 3f) showed a lack of neurological and muscular toxicity with the rotarod test. For the compounds 3e and 3f, we found antiepileptic activity comparable to one of melatonin after kindling test administration and some analgesic activity, albeit weaker, than that of melatonin in equivalent doses. Although melatonin and substance 3f showed dose-dependent increases in AhR and kynurenine levels in brain homogenates, our results do not support our initial hypothesis for AhR involvement in the analgesic or antiepileptic mechanisms of test substances. Further studies are needed to evaluate the effects of AhR and kynurenine in the neuropharmacological activity of the indole derivatives, with or without the aroylhydrazone moiety.

In recent years, many research teams have implemented targeted syntheses and development of melatonin analogs with potential pharmacological activity similar to endogenous melatonin and with advantages associated with prolonged action. Scientific studies focus on the development of melatonin analogs for various diseases, such as depression, Alzheimer's disease, anxiety, circadian rhythm disorders, and insomnia [17].

Clinical and experimental data in several animal models, including the temporal lobe epilepsy model, confirm the anticonvulsant role of the pineal hormone melatonin [18]. Its advantages are good safety profile (being an endogenous compound), potent antioxidant activity, contribution to degeneration protection of neurons against the mutagenic and carcinogenic effect of oxidants, and above all, its essential biological function to synchronize natural rhythms. Melatonin alleviates sleep disorders of various origins in both children [19] and adults with epilepsy [20]. In most cases, it has been found that improving sleep quality is associated with a decrease in seizure activity in young patients with resistant epilepsy and patients receiving antiepileptic treatment [21]. While some of the routinely used anticonvulsants such as lamotrigine, levetiracetam, valproate, gabapentin do not show improvements in the architectonics of sleep, other drugs such as benzodiazepines, barbiturates and phenytoin impair the quality of sleep in epilepsy patients [22].

Substances with anticonvulsant activity are often tested for analgesic effects, trying to find alternatives to satisfy patients' needs for pain relief with favorable safety profile [23]. Melatonin is a hormone with several neurophysiological and antioxidant properties that have been increasingly studied recently. In supra-physiological doses, it is authorized for use as a medicine in cases of insomnia associated with disturbed circadian rhythms. Due to its favorable safety profile, it is widely used as a nutritional supplement.

Numerous preclinical studies indicate the analgesic potential of melatonin, administered alone or in addition to well-established analgesics, where it shows synergism with analgesia [24]. There are promising, but still limited data on the administration of melatonin in humans to respond to pain [25].

The AhR is most widely expressed in the human placenta, followed by the lungs, heart, pancreas, and liver. It was also detected in the kidney, the CNS, and the skeletal muscles [26]. In the brain, AhR is thought to mediate the host-microbiome interaction with a particular implication in the neurodevelopment [27]. The brain expression of AhR shows low region specificity [28]; therefore, we evaluated the concentration of the AhR in whole brain homogenates.

Other authors investigated the dysregulation of the AhR as a cause for tumorigenesis, autism spectrum disorders, multiple sclerosis, depression, leaky gut and blood-brain barrier, stroke, epilepsy, and the universal mechanism seems to be suboptimal mitochondrial functioning. One plausible cause of the mitochondrial dysfunction could be an increasing N-acethylserotonin/melatonin ratio due to AhR activation [29]. As it is already known, all body cells can produce melatonin, a circadian regulator, and powerful antioxidant, which synthesis occurs in mitochondria [30]. Its synthetic pathway can be diverted to the kynurenine pathway with the production of kynurenic acid and quinolinic acid, substances with the opposite to melatonin effects in terms of neuroprotection and neuronal overexcitation. One of the significant consequences of the neurovascular activation by AhR is an increasing N-acethylserotonin/melatonin ratio and modulating mitochondrial function [31].

Besides ligand-modulation, AhR can be activated in a ligand-independent manner by cyclic 3'5' adenosine monophosphate (cAMP) and protein kinase A (PKA) [32]. PKA is a ubiquitously expressed member of the serine-threonine kinases implicated in multiple cellular processes, including neuronal excitability [33]. As reported by other authors, repeated injection of an initially sub convulsive dose of cAMP into the rat amygdala produced progressive seizure development, like that of electrical kindling [34]. We hypothesized that the application of an inhibitor of AhR could, after that, suppress such activity. However, our results did not support this hypothesis.

The effect of melatonin on circadian rhythms is very well described. However, we do not know if our two tested substances, 3e and 3f, being M1 agonists, according to our previous work, also interfere with the circadian rhythms. We do know, however, from other authors that both AhR and ARNT proteins display diurnal changes in several evaluated tissues [35]. Although the AhR has no effect on the circadian rhythms in the absence of exogenous agonists, upon activation, AhR may affect the ability of the endogen timekeeping system to adjust the canonical clock genes to alterations [36]. AhR activation in mice and hamsters alters rhythms of feeding and activity [37], shifts the surge of corticosterone, and reduces the level of prolactin [38] and melatonin [39].

CONCLUSION

In this study we have confirmed the analgesic and anticonvulsant potential of melatonin. In addition, we performed neuropharmacological studies of two substances, melatonin analogs with the aroylhydrazone moiety, and reported objectifying results with respect to the activities tested. The theoretical contribution is with regard to the reporting of data on AhR and kynurenine levels in brain homogenates after seven days of treatment with the test substances. These data could be used in future studies regarding AhR and its relationship to the kynurenine metabolic pathway. Indoles derived from an exogenous diet, such as tryptophan, are a major source of endogenous AhR ligand precursors. It is known from studies by other authors that kynurenine itself is an AhR agonist; while its metabolites kynurenic acid and quinoline acid and also melatonin are AhR antagonists.

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