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Auraptene has neuroprotective and memory enhancing effects in a rat model of Alzheimer's disease

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Abstract

Background: Auraptene is a simple coumarin that exhibits multiple protective activities in the brain. Alzheimer's disease is a complex, multifactorial, and progressive neurodegenerative disease. Microinjection of the β-amyloid peptide (Aβ) into the hippocampus of rat has been recognized as a reliable and stable animal model of Alzheimer's disease, which mimics the memory deficits. In the present study, the memory enhancing effects of auraptene were studied in rats that Aβ was injected into their hippocampus to create a model of Alzheimer's disease. *Methods:* Different doses of auraptene (5, 10 and 25 mg/kg) were administered intraperitoneally to male Wistar rats. The spatial memory performance was tested by Morris water maze after Alzheimer`s induction. The hippocampal expression of pro-apoptotic Bax and anti-apoptotic Bcl-2 proteins were calculated for evaluating the neuroprotective and anti-apoptotic effects of Auraptene in the brain tissue. *Results:* In comparison with the control group, auraptene significantly decreased the escape latency time in the treated rats. In addition, auraptene increased the percentage of time spent and traveled pathway in the target quadrant. Molecular data showed that auraptene attenuated the Bax/Bcl-2 ratio in the hippocampus of rats. *Conclusion:* This study demonstrated the memory enhancing effect of Aur after Aβ injection, which could be through inhibiting the apoptotic pathways in the hippocampus of rats.

Keyword: Spatial memory, auraptene, rat/Wistar, bax protein

INTRODUCTION

Dementia is a progressive neurodegenerative disorder that affects about 1 to 2 percent of the population over the age of 65.[1] Different types of dementia include Alzheimer's disease, cerebrovascular dementia, lewy body dementia, frontotemporal dementia and Parkinson`s disease.[2] The most common type is Alzheimer's disease, which usually occurs with gradual progression of learning and memory loss.[1] There are several hypotheses in the pathogenesis of Alzheimer's disease, including beta-amyloid peptide hypothesis, inflammation, Tau protein, cholinergic hypothesis, oxidative stress, hypoxia, calcium imbalance, abnormal accumulation of metals and accumulation of β amyloid in mitochondria and bone marrow.[3] Although the pathological changes commonly found in Alzheimer's disease include amyloid plaques, neurofibrillary tangles, and loss of neurons, the most important accepted mechanism in Alzheimer's pathophysiology is the amyloid cascade hypothesis.[1] In pathologic conditions, the amyloid precursor protein (APP) is not properly broken down by β- and γ-secretase. The incorrect processing of the APP gene results in the production of modified β-amyloid peptides, which results in the formation of extracellular β-amyloid deposits.[4] With regards to the multiple roles of beta amyloid in the neuronal degeneration mechanisms, a compound that would be able to prevent β amyloid toxicity in various ways could have a better effect on controlling the progression of Alzheimer's disease.[5]

Apoptosis has two major pathways; the extrinsic and intrinsic pathways. The Bcl-2 family members have a role in the intrinsic (mitochondrial) pathway. The Bcl-2 family proteins include antiapoptotic (such as Bcl-2)

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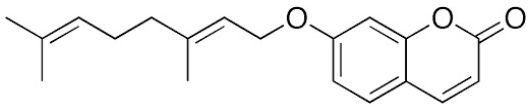

Figure 1. Chemical structure of auraptene

and proapoptotic proteins (such as Bax).[6] It has been shown that the ratio of Bcl-2 to Bax plays a pivotal role in cell survival and death.[7] Neuronal apoptosis is induced beyond the pathological changes in Alzheimer`s disease.[7] This apoptosis can be caused by β-amyloid protein in transgenic mice and neuronal cell culture. β-amyloid could induce the translocation of Bax protein to the mitochondria, which can increase the Bax/Bcl-2 ratio and initiate the activation of the intrinsic apoptotic pathway.[8,9]

Auraptene (Aur, $C_{19}H_{22}O_3$) (Figure 1) is a terpenyloxy coumarin in the citrus family, which is found mainly in their skin.[10] This compound, which forms the bulk of coumarin in citrus,[11] is found in the orange and grapefruit essential oil.[10] Several studies have shown that Aur has valuable effects, including anti-inflammatory[12,13], anti-carcinogenic[14,15], anti-Helicobacteria[16], as well as some regulatory activities in the metabolism of liver fats.[17,18]

Based on previous studies that showed the neuroprotective and memory enhancing effects of Aur[11,19-23], in the present work, we investigated the neuroprotective and memory enhancement effects of Aur in a rat model of Alzheimer's disease induced by β-amyloid injections.

METHODS

Chemicals

Aur was obtained from Golexir Company (495-02-3, Mashhad, Iran). To prepare the drug, Aur was dissolved in dimethyl sulfoxide (DMSO) and polyethylene glycol (PEG) 300 at a ratio of 1: 1. Based on previous studies, this concentration of DMSO is not toxic for animal.[20] β amyloid 1-42 (Aβ1-42) (Sigma-Aldrich, SCP0038, Germany, 1 mg vial) was incubated at 37 ° C for a week after adding 200 ml distilled water (0.005mg/ml) to it.

Animals

Male Wistar rats (200-250 g) aging 60-65 days were taken from the animal house of the Sabzevar University of Medical Sciences and maintained at 22-25 ° C under light conditions (12 hours of light and 12 hours of darkness). Rats had free access to food and water. All animal experiments were performed according to the rules of the Ethics Committee of Sabzevar University of Medical Sciences (IR.MEDSAB.REC.1396.87).

Surgery

For the stereotaxic surgery, the rats were anesthetized with ketamine and xzylazine (80 and 20 mg/kg, i.p., respectively). Then, the animals were placed in a stereotaxis device. A longitudinal split was created in the posterior part of the skull and the canulla was injected into the hippocampus of the brain in accordance with the Paxinos Atlas (nose bar, –3.3, AP, –4.0; ML, ±2.9; DV, –1.8 mm from dura).[24] The incisor bar was set 3.3 mm below interaural line. The cannula was fixed on the skull with a dental acrylic.

Groups and study design

Based on a statistical analysis, 48 rats were selected. Animals were randomly divided into 6 groups: (1) Sham group: In this group only cannulation was performed by a stereotaxic surgery. Animals in this group did not receive a drug or a drug solvent (n = 8); (2) Alzheimer`s disease (Alz) group: In this group, beta amyloid (Aβ) was injected (by the stereotaxic method and intra-hippocampal injection). This group was designated to confirm the progression of Alzheimer's disease after the administration of beta amyloid injection (n = 8); (3) Aβ+Vehicle group: In this group beta amyloid (Aβ) (using the stereotaxic method and intra-hippocampal injection) with DMSO and PEG300 (Aur solvent) (1: 1 by intraperitoneal injection) were injected as planned (n = 8); (4) Alz + Aur (4,5,6) groups: In this group Aβ with Aur (5, 10 and 25 mg/kg by intraperitoneal injecton) were injected (n = 8 for each group).

In order to induce Alzheimer's disease, 30 ng of Aβ1-42 in 3 µl distilled water was injected into the hippocampus.[25] To do so, after a stereotaxic surgery and creating a hole on the skull with a special drill, beta amyloid was injected with a Hamilton syringe. Twelve days after the injections the animals were evaluated to confirm the creation of Alzheimer's disease by the Morris water maze (MWM). Aur solvent and Aur (5, 10 and 25 mg/kg/day) were injected intraperitoneally for 12 days after the surgery. This schedule is based on previous studies.[26,27] After the last dose of the drug administration, animals were evaluated for learning and spatial memory using the MWM method (Figure 2).

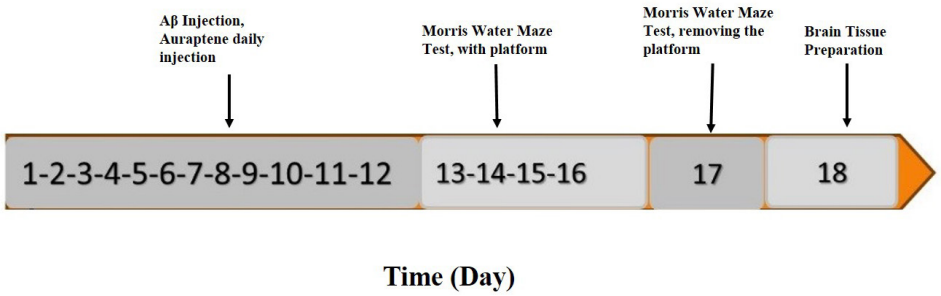

Figure 2. Timeline diagram showing the experimental protocol used in groups.

Behavioral study

Thirteen days post-surgery and maintenance of animals under appropriate conditions, the memory impairment was investigated using MWM. The MWM used in this study was consisted of a black metallic pond with a diameter of 210 cm and a height of 60 cm, water filled up to 32 cm with a temperature of 21 ± 2 ° C. The pond situated in a dark room with visual cues on the room walls. The pool was divided into four geographical quadrants: Southwest (SW), southeast (SE), northwest (NE) and northeast (NE). Four points [east (E), west (w), north (N) and south (S)] were designed as a swimming starting position. A hidden platform with a diameter of 10 and a length of 30 cm, one cm below the water level, was located at the center of one of the quadrants. A camera was mounted on top of the maze that recorded the animal behavior using a tracker software. The test lasted for as long as 60 seconds, during which the animals accidentally found and placed on a hidden platform that was located underwater. The animals were allowed to stay on the platform for 20 seconds and identify its position by looking at the signs around it. If a rat could not find the platform within 60 seconds, the animal was slowly directed to the platform and permitted to rest on it for 20 seconds. The start positions and platform locations were balanced within and between subjects. During the training sessions, each rat was placed in the maze. Each session lasted 5 consecutive days during which 4 training sessions took place each day with 1-minute intervals. At this stage of testing, some parameters were detected by the software. For example, 1) the swimming speed 2) distance traveled to find the hidden platform and 3) time elapsed until finding the hidden platform. The final trial test was performed on day 17 at which the platform was removed from the pool and then the testing was performed. This step took 60 seconds, and after the end of the period the rats were removed from the pool. This stage of the experiment was performed for each rat once, and the total distance traveled, the swimming rate, the percentage of entering the target quadrant, the percentage of distance traveled in the target quadrant, and the percentage of time elapsed in the target quarter were considered as the scales for learning and remembrance.[28]

Molecular studies

After the final behavioral study test on day 18th, the rats of each group were anesthetized with inhaled CO_2 and decapitated by a specialized guillotine. Their hippocampus was separated and placed in a microtube. Then, it was snap-frozen in liquid nitrogen and stored in –80° C until the biochemical experiments were performed on them. The tissues were homogenized and centrifuged 1000 rate/minute for 10 minutes. The supernatants were collected and total the protein concentration was determined using the Bradford method.[24] Twenty micrograms of the total protein was used to determine the Bax and Bcl-2 concentrations. The Bcl-2 and Bax protein levels were determined using the Rat ELISA Kits (ZB-10826 and 1825-H9648, ZellBio, Germany, respectively).

Statistical analysis

The Prism 5 software was used to calculate the statistical data. The results were reported as Mean ± SEM. Analyzing the data obtained from the time to reach the platform was performed using the Mixed Model ANOVA and Bonferroni's follow-up test. The results of swimming speed, percentage of time and distance traveled in the target quadrant and the results of molecular studies were evaluated by one-way ANOVA with post-hoc Turkey HSD Test ($p < 0.05$ was considered statistically significant).

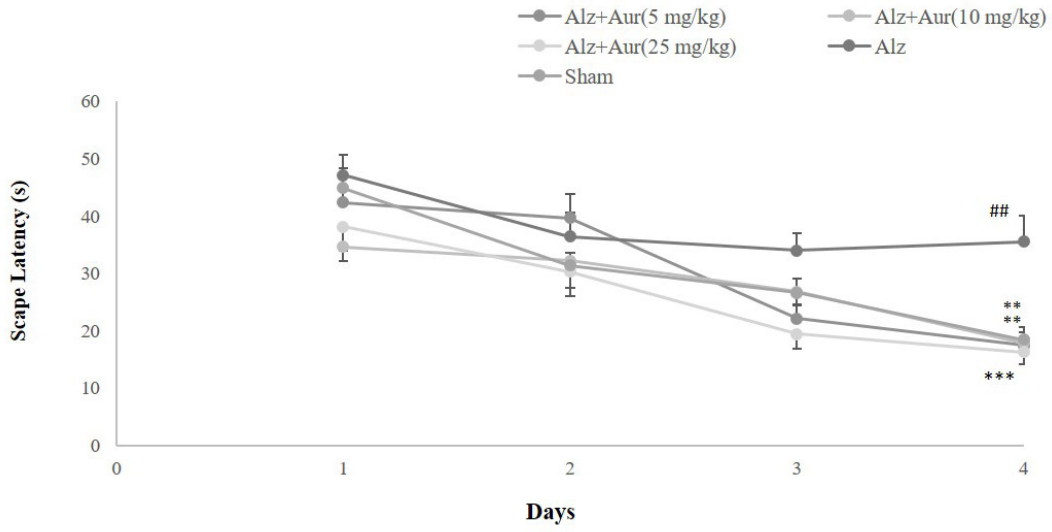

Figure 3. Neuroprotective effect of Aur administration on the Aβ-induced spatial memory impairment in the MWM task. Escape latency time to access platform during the final trials test. The values are shown as mean ± S.E.M. Significant differences between the Aβ group versus the sham group (## p <0.01) and the therapeutic groups versus the Aβ group (** p <0.01, ***p <0.001) were detected.

RESULTS

Auraptene improves the spatial memory of rats and their function in Morris-water-maze

The spatial learning based performance of rats was measured by MWM. All groups were trained to find the platform for 4 days, which was accompanied by a shortening of the time needed to find the platform during the study. Time spent looking at the platform in the Alz group was significantly more than the sham group (p <0.01). Different doses of Aur (5, 10 and 25 mg/kg) in the Alz+ Aur groups significantly reduced the time of finding the platform compared to the Alz group (p <0.05). There was no significant difference between the sham and Alz+ Aur groups (p> 0.05) (Figure 3). In the last test of the final day, the platform was removed from the quadrant and the percentage of the time and distance traveled in the target quadrant was used to assess the spatial memory. Compared to the Alz group, sham and Alz+ Aur groups swam longer times in the target quarter significantly (p> 0.01). There was no significant difference between the sham group and the Alz+ Aur groups (p>0.01) (Figure 4A). The results of the statistical analysis showed a significant increase in the distance traveled in the target quadrant for the Alz+ Aur (p<0.01) and sham (p<0.05) groups compared to the Alz group. There was no significant difference between the sham and Alz+ Aur groups (Figure 4B). There was no

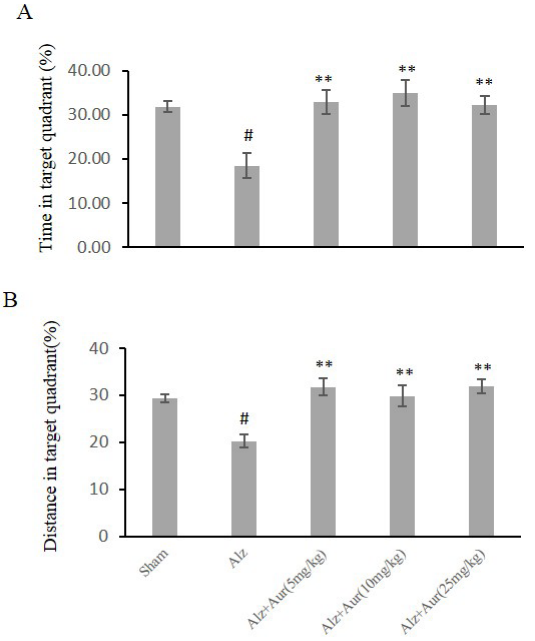

Figure 4. Neuroprotective effect of auraptene administration on the Aβ-induced spatial memory impairment in the Morris water maze (MWM) task. Percentage of time spent in target quadrant (A) and percentage of distance traveled in target quadrant (B) during the final trials test. The values are shown as mean ± S.E.M. Significant differences between the Aβ group versus the sham group (#p <0.05) and the therapeutic groups versus the Aβ group (** p <0.01) were detected.

significant difference between Aβ+vehicle group and Alz group in all the behavioral functions. Our interventions did not affect the locomotor activity of rats. As it is shown in Figure 5, there is no significant differences in the swimming rate between the groups on day 17th (p> 0.05).

Auraptene decreases the Bax/Bcl-2 ratio in the brain's hippocampus

Compared to the sham group, there was a significant increase in the expression of Bax protein in the Alz group (p <0.05). The Aur injections led to a significant decrease in the expression of Bax protein in the hippocampus tissue compared to the Alz group (p <0.01 for 5mg/kg of Aur and p <0.001 for 10 and 25mg/kg of Aur). Furthermore, there was no significant difference in the hippocampal Bax content between the sham and Alz+ Aur groups (p> 0.05) (Figure 6A).

Compared to the sham group, the Bcl-2 content of the hippocampus was significantly decreased in the Alz group (p <0.01). The Aur administration at doses of 5, 10 and 25 mg/kg significantly increased the Bcl-2 content in the hippocampus tissue compared to the Alz group (p <0.01 for 5mg/kg of Aur and p <0.001 for 10 and 25mg/kg of Aur). There was no significant difference in the hippocampal Bcl-2 content between the sham and Alz+ Aur groups (p> 0.05) (Figure 6B).

Our study showed that compared to the sham group, the Bax/Bcl-2 ratio increased significantly in the hippocampal tissue of Alz group (p <0.001). The intraperitoneal injection of Aur showed a significant decrease in the Bax/Bcl-2 ratio (p <0.01 for 5mg/kg of Aur and p <0.001 for 10 and 25mg/kg of Aur). (Figure 6C).

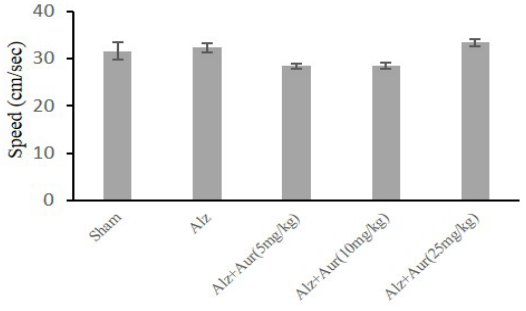

Figure 5. Swim speed in the Morris water maze (MWM) test in Aβ induced memory impairment and therapeutic groups. The values are shown as mean ± S.E.M. There was no significant difference between groups.

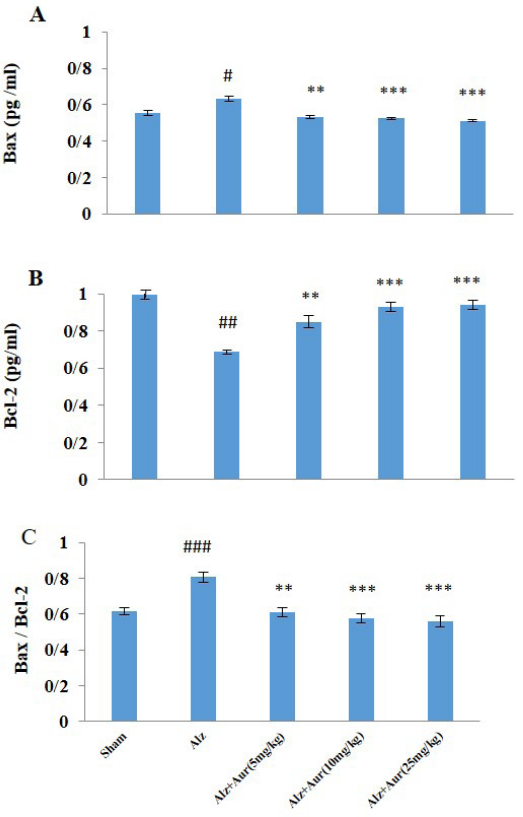

Figure 6. Effect of auraptene on expression of Bax (A), Bcl-2 (B) protein and Bax/Bcl-2 ratio (C) in hippocampal tissue using ELISA method. The values are shown as mean ± S.E.M. Significant differences between the Aβ group versus the sham group (#p <0.05, ## p <0.01 and ### p <0.001) and the therapeutic groups versus the Aβ group (** p <0.01, ***p <0.001) were detected.

DISCUSSION

Alzheimer's disease is the most common neurodegenerative disease and the leading cause of dementia in elderly. There are several animal models for studying the memory impairment and dementia. Some of them are bilateral permanent common carotid ligation or two vessel occlusion (2VO), disruption of memory by scopolamine injection and microinjection of Aβ into the hippocampus. Microinjection of Aβ into the hippocampus of animals has been recognized as a reliable and stable method to create animal models of Alzheimer`s disease, which mimics the alterations known for AD patients including memory deficits.[30-32]

The neuroprotective effect of Aur was shown in different studies. A number of studies showed that Aur has its neuroprotective effect by AChE

inhibition, Anti-inflammatory and Anti-apoptotic activity.[11,20,22] In an in vitro study, Furukawa *et al*. showed that Aur could induces the activation of extracellular signal-regulated kinases (ERK1/2) in PC12 cells, which is a model system for studies on neuronal proliferation and differentiation. Based on other studies, ERK1/2 are involved in synaptic plasticity and in the development of long-term memory in the central nervous system (CNS).[11] Okuyama et al. showed that Aur has anti-inflammatory effects in the mouse brain by suppression of microglial activation and cyclooxygenase (COX)-2 expression in astrocytes and neuronal cell death.[20,33,34] In addition, Aur could reverse the scopolamine- induced passive avoidance memory retention impairments in a mouse model of Alzheimer`s disease.[22]

In our study, a rat model of Alzheimer's disease was created by the injection of Aβ in the hippocampus to investigate the neuroprotective effects of Aur. Although we did not study the pathological changes in the hippocampus, we observed significant differences between the Alz and sham groups in the spatial memory performance. Our statistical data showed that Aur, in comparison with the Alz group, could significantly reduce the escape latency time during the training days. Also, Aur increased the percentage of time spend and traveled pathway in the target quadrant. According to the swimming speed data, Aβ microinjection into the hippocampus did not cause any motor dysfunction in operated rats and there were no differences between groups in motor activity. Our behavioral data are supporting the findings of another previous study.[23] Ghanbarabadi *et al*. showed that Aur could significantly decrease the scape latency time in the treated rats. They showed that Aur increased the percentage of time spent and traveled pathway in target quadrant on the final trial test day.[23]

Several studies showed that Aβ induces the translocation of Bax to the mitochondria and activation of intrinsic apoptotic pathway.[9,35] The activation of Aβ inhibited the anti-apoptotic pathway by a significant enhancement of Bax expression and reduction of Bcl-2 expression.[36] Wang *et al*. showed that Aβ could increase the Bax/Bcl-2 ratio in hippocampus.[8] In agreement with those, our molecular results showed that the Aβ injection could induce the apoptosis by increasing the Bax and decreasing the Bcl-2 protein levels. As a result, the Bax to Bcl-2 ratio significantly increased in the hippocampal neurons. These data are in line with other previous studies.[8,9,37] Moreover, we showed that Aur could reverse these changes and decrease the Bax protein expression, increase the Bcl-2 protein expression and decrease the Bax/Bcl-2 ratio in the hippocampus of a rat model of Alzheimer`s disease.

In contrast to our results, the previous studies showed that Aur is an apoptotic-inducing agent in vitro and in vivo.[38,42] Kohno *et al*. showed that dietary feeding of Aur (0.01 and 0.05%) for 17 weeks to mice bearing carcinogenesis in their colon significantly inhibited the occurrence of colonic adenocarcinoma and increased the apoptotic index in the colonic malignancies.[38] In another study feeding transgenic rats by a diet that had Aur led to developing adenocarcinoma of the prostate (500 p.p.m.) and effectively reduced the size of lesions in the prostate.[39] These findings in comparison with our findings interestingly showed that Aur could induce/inhibit the apoptotic dose-dependency. More detailed studies about this concept should be done in the future.

In the present study, the results showed that Aur could protect neuronal cells from some aspects of apoptosis after the injection of Aβ into the hippocampus. Molecular data indicated that the Bax/Bcl-2 ratio decreased in the hippocampus subfields in the Aur treated rats. The molecular results are in accordance with the behavioral data in the Morris water maze. These results demonstrated the potential of Aur in preventing or minimizing neuronal damage and enhancing the spatial memory performance in a rat model of Alzheimer`s disease.

DISCLOSURE

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Conflict of interest: None

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