EFFECT OF FLAVONE AND ITS MONOHYDROXY DERIVATIVES ON ANIMAL MODELS OF DEPRESSION IN SWISS ALBINO MICE

SHANMUGAPRIYAN S*, JAIKUMAR S, VISWANATHAN S, PARIMALA K, RAJESH M

Department of Pharmacology, Meenakshi Medical College and Research Institute, Meenakshi Academy of Higher Education and Research,Kanchipuram, Tamil Nadu, India. Email: priyan.vellore. 87@gmail.com

ABSTRACT

Objectives: This research was designed to investigate the antidepressant activity of a few structurally related flavones (flavone, 3-hydroxyflavone, and 7-hydroxyflavone) and the possible mechanisms involved.

Methods: Antidepressant activity was evaluated in mice by subjecting them to forced swim test and tail suspension test. The involvement of adrenergic, serotonergic, nitric oxide (NO), and opioid mechanisms was investigated using suitable interacting chemicals.

Results: Flavone, 3-hydroxyflavone, and 7-hydroxyflavone exhibited a significant and dose-dependent reduction in total time of immobility in the forced swim test and tail suspension test. Pre-treatment with alpha-methyl-para-tyrosine and parachlorophenyl alanine attenuated the reduction in immobility period produced by flavone and its derivatives in forced swim test. Naloxone pre-treatment partially reversed the effect of flavone while L-arginine pre-treatment did not alter their effect.

Conclusion: The investigated flavones exhibited promising antidepressant activity in both the animal models of depression. However, the flavone compounds did not alter the motor coordination and ambulatory behavior in the Rotarod and locomotor activity test. The participation of serotonergic, adrenergic, and opioid mechanism in the antidepressant activity of these compounds was elucidated from the results, and the role of NO pathway was excluded.

Keywords: Antidepressant activity, Flavone, Forced swim test, Tail suspension test.

INTRODUCTION

Depression is often under diagnosed and undertreated. The current antidepressant drugs such as monoamine oxidase inhibitors, selective serotonin reuptake inhibitors (SSRIs), tricyclic antidepressants, and inhibitors of norepinephrine and serotonin uptake delay the onset of resolution of depressive symptoms and cause serious adverse effects. An estimated 29–46% of patients treated for major depressive disorder have either a partial response or no response at all to antidepressive treatment. Hence, there is a continued need to search for an antidepressant drug which is more effective and safe [1]. *Hypericum Perforatum,* a European traditional medicine, has been extensively investigated and clinically evaluated in comparison with standard antidepressant drugs [2]. The medicinal plants are not only always free of side effects, for example, *H. perforatum* though exhibiting antidepressant-like actions, but also has some of its serious side effects [3]. Many plants used in the Indian traditional system of medicine have also been scientifically investigated for their effect in animal models of depression, and the mechanisms of action have also been explored [4]. This observation kindled an interest whether flavonoids could be a potential source for antidepressant drugs [5]. Flavonoids such as rutin [6], quercetin [7-9], and naringenin[10] that are widely distributed in many plants have been investigated for potential antidepressant effects. Most of the above compounds are polysubstituted flavones and it was considered interesting to explore the antidepressant activity of the basic flavone nucleus and its simpler derivatives. Hence, in this research, flavone and two of its mono-hydroxy derivatives have been investigated for considerable antidepressant effect in mice by subjecting them to forced swim test and tail suspension test. In addition, the possible mechanisms involved in the antidepressant effect of these compounds were also considered for investigation.

METHODS

Animals

For the current study, male adult Swiss albino mice weighing 25–30 g were selected and were sourced from the institutional animal house. On a natural day and night cycle, the animals were housed in a controlled environment with food and water available *ad libitum*. In all the experiments, each group consisted of six animals. Each animal was used only once. All the experiments were carried out between 9:00 and 13:00 h to avoid circadian variation and to maintain uniformity. The experimental protocol was approved by the Institutional Animal Ethics Committee (BR/HOS/85/2013/F-378 and KN/COL/3401/2014).

Drugs and chemicals

The compounds used in the study are flavone, 3-hydroxyflavone, and 7-hydroxyflavone, and its hydroxy derivatives were prepared as a fine suspension in 2% carboxymethyl cellulose and injected i.p. in doses of 5, 10, and 20 mg/kg, 30 min before the test procedures. The doses were selected based on the pilot study. Imipramine (Torrent Pharmaceuticals, India), para-chlorophenylalanine (PCPA) (Sigma-Aldrich, St. Louis, MO, USA), alpha-methyl-para-tyrosine (AMPT) (Sigma-Aldrich, St. Louis, MO, USA), naloxone hydrochloride (Endo Labs USA), L-arginine (Tokyo Chemical Industry, Japan), and diazepam (Neon Laboratories Ltd., Mumbai) were used in the present study.

Ambulatory behavior by actophotometer test

Mice were observed for ambulatory activity using an actophotometer (INCO, India). The activity of the animals was recorded by interruption of light beams passing through the square chambered apparatus. Mice were treated with flavone, 3-hydroxyflavone, or 7-hydroxyflavone in a dose of 20 mg/kg i.p. or vehicle and placed in the actophotometer after 30 min. The number of beam breaks was

recorded for 5 min [11]. The instrument was cleaned with cotton swab dipped in spirit in between the observations. Diazepam (2 mg/kg i.p) was used for comparison in a separate group of mice.

Motor coordination by Rotarod test

The effect on motor coordination was evaluated by treating the mice with flavone, 3-hydroxyflavone, or 7-hydroxyflavone in a dose of 20 mg/kg i.p. or vehicle, 30 min before being tested on a Rotarod [12]. The apparatus (INCO, India) consists of a bar with a diameter of 2.5 cm subdivided into two compartments. The bar rotates at a constant speed of 15 revolutions/min and the ability of the animal to remain on the rotating rod (balancing time) was measured and compared with vehicle-treated animals. The cut-off time employed was 120 s. A group of mice was treated with diazepam 2 mg/kg i.p. for comparison.

Antidepressant activity by forced swim test[13]

In this test, the mouse is forced to swim inside a vertical open cylindrical container (height 25 cm and diameter 10 cm) with 19 cm of water (depth) and the temperature was maintained at 25 ± 1°C. Initially, the mouse swims vigorously in circles and tries to climb the wall or dive to the bottom. After a minute, the activity begins to subside and replaced with phases of immobility or floating of increasing length, with only minimal movements to keep its head above the water level. The total duration of immobility in seconds was recorded during a 6-min period in which the 1st min was given as the time for the animal to get adapted to the new environment. Hence, no readings were taken during the 1st min. A decline in the duration of immobility is indicative of an antidepressant effect. Flavone, 3-hydroxyflavone, or 7-hydroxyflavone was administered i.p in doses of 5, 10, or 20 mg/kg to different groups of mice, 30 min before the test. Imipramine 20 mg/kg i.p was used for comparison in a separate group of mice. The doses of flavone compounds selected for the study were based on the pilot study conducted.

Antidepressant activity by tail suspension test[14]

In this method, a mouse is suspended in a rod of 10 cm length attached perpendicularly to the vertical iron stand, 50 cm above the floor by adhesive tape placed approximately 1 cm from the tip of the tail in a dim light and silent environment. The animal remains immobile after active and unsuccessful attempts to escape when suspended by tail. The animal was observed for 6 min and the immobility time was recorded. Effective antidepressants reduce the immobility time of the animals. Flavone, 3-hydroxyflavone, or 7-hydroxyflavone was injected i.p to different groups of mice in doses of 5, 10, or 20 mg/kg. Animals were subjected to the test 30 min after administration of the test compounds. Imipramine 20 mg/kg i.p was used as a standard drug for comparison.

Investigations on the mechanism of action

Further, experiments were tried to elucidate the mechanisms by which flavone and its derivatives exerted their antidepressant activity. A single dose of flavone (20 mg/kg), 3-hydroxyflavone (20 mg/kg), or 7-hydroxyflavone (20 mg/kg) was used for this purpose in the forced swim test in mice.

Serotonergic system

To assess the possible role of serotonergic system in the antidepressant effect of flavone and its derivatives, animals were pretreated with a 5-HT synthesis inhibitor, PCPA, 100 mg/kg i.p, for 4 consecutive days [6]. On the 5th day, that is, 24 h after the last PCPA injection, the animals were

given an injection of flavone or its derivatives in a dose of 20 mg/kg i.p and were employed to forced swim test after 30 min.

Adrenergic system

To witness the contribution of adrenergic system, mice were pretreated with AMPT, an inhibitor of the enzyme tyrosine hydroxylase, 100 mg/kg i.p. [6]. After 4 h, they received flavone or its hydroxy derivatives in a dose of 20 mg/kg i.p and were subjected to forced swim test, 30 min later.

Opioid mechanism

The participation of opioid system in the antidepressant action of flavone and its derivatives was investigated by pre-treating the mice with naloxone 5 mg/kg i.p [15], and after 15 min, the animals received an injection of flavone or its derivatives in a dose of 20 mg/kg i.p. The antidepressant effect was recorded 30 min later using forced swim test.

Nitric oxide (NO) mechanism

Pre-treatment with L-arginine (750 mg/kg i.p), a NO precursor, 15 min before flavone or its derivatives was attempted to investigate the role of NO pathway in the antidepressant effect of these compounds [16]. Flavone and its derivatives were used in a dose of 20 mg/kg i.p.

Statistical analysis

One-way ANOVA followed by Dunnett's t-test was used using SPSS 16 software to analyze the data obtained from various experiments. A probability value <5% was considered to be statistically significant. Results are as mean ± standard error mean.

RESULTS

Ambulatory behavior and motor coordination

The balancing time of mice on a Rotarod and the activity score in the actophotometer were significantly reduced by diazepam (2 mg/kg i.p) treatment. Flavone and its hydroxy derivatives in a dose of 20 mg/kg i.p did not alter the activity score of mice in the actophotometer or the balancing time on the Rotarod apparatus when compared to vehicle-treated animals. Hence, the ambulatory behavior and motor coordination of mice were not significantly influenced by flavone and its hydroxy derivatives (Table 1).

Forced swim test

The immobility period recorded in vehicle-treated control animals was 195.07 ± 5.8 s. A significant reduction in immobility period was noted in imipramine (20 mg/kg)-treated mice and the mean value was 3.47 ± 1.49 s. A dose proportionate reduction in immobility period compared to vehicle treatment was observed after treatment with different doses (5, 10, and 20 mg/kg i.p) of flavone, 3-hydroxyflavone, and 7-hydroxyflavone. The percentage reduction in immobility period after treatment with different doses of the above test compounds was calculated in comparison with the vehicle treatment. The maximum reduction in immobility period in a dose of 20 mg/kg of flavone, 3-hydroxyflavone, and 7-hydroxyflavone was 88.49%, 45.48%, and 55.88%, respectively, while imipramine treatment produced 98% inhibition (Table 2).

Tail suspension test

The mean period of immobility in control group of animals was 206.85 ± 5.76 s which was decreased to 14.84 ± 1.41 s in imipramine (20 mg/kg)-treated animals and the reduction was 92%

Table 1: Effect of flavone and its monohydroxy derivatives on ambulatory behavior and motor performance of mice

Treatment groups (mg/kg i.p)	Activity score in the actophotometer (5 min)	Balancing time on the Rotarod (120 s)
Vehicle	464.33±9.34	120.00±0.00
Diazepam (2)	163.83±2.73[a]	3.66±0.66[a]
Flavone (20)	446.00±9.23	120.00±0.00
3-hydroxyflavone (20)	452.00±7.42	120.00±0.00
7-hydroxyflavone (20)	467.50±8.12	120.00±0.00

[a]p<0.05 in comparison to vehicle (one-way ANOVA followed by Dunnett's t-test)

when compared to vehicle-treated animals. A dose-dependent reduction in immobility period was observed in animals treated with increasing doses of flavone, 3-hydroxyflavone, and 7-hydroxyflavone (5, 10, and 20 mg/kg i.p). The percentage reduction in immobility period was calculated in comparison with the vehicle treatment. The maximum reduction in immobility period in a dose of 20 mg/kg of flavone, 3-hydroxyflavone, and 7-hydroxyflavone was 73.97%, 51.77% and 59.27%, respectively (Table 3).

Investigations on the mechanism of action

Involvement of serotonergic system

Flavone, 3-hydroxyflavone, and 7-hydroxyflavones (20 mg/kg) *per se* significantly decreased the immobility time of mice in forced swim test compared with vehicle treatment. However, PCPA pre-treatment completely reversed the reduction in immobility period produced by the above compounds in forced swim test (Table 4).

Involvement of adrenergic system

Pre-treatment with AMPT did not significantly alter the period of immobility in vehicle-treated control animals. However, in AMPT-pretreated animals, the magnitude of reduction in immobility time due to flavone and its derivatives was significantly attenuated (Table 5).

Involvement of opioid system

Naloxone (5 mg/kg i.p) treatment *per se* did not significantly alter the immobility period of mice in forced swim test. However, a partial but significant reversal of the reduction of immobility period of mice was evident in animals treated with flavone and its derivatives (Table 6).

Involvement of L-arginine-NO system

L-arginine (750 mg/kg i.p) pre-treatment did not significantly modify the immobility period of vehicle-treated mice in forced swim test. The reduction in immobility period showed by flavone and its hydroxy derivatives was also not significantly modified by treatment with L-arginine (Table 7).

DISCUSSION

Mood and anxiety disorders are the most common psychiatric disorder widely presented to clinicians. Evidence are accruing that some natural products such as S-adenosine methionine, 1-methylfolate, and omega-3 fatty acids may be useful in depression [17]. Thus, there appear to be multiple novel targets for the new drug development to

the treatment of depressive illness. It is pertinent to mention here that flavonoids are ubiquitous natural compounds endowed with many potential therapeutic benefits. Their opioid-like analgesic effect has been identified earlier [18]. A few recent studies have identified some flavone compounds such as rutin [6-9] and naringenin[10] to be effective in animal models of depression. This research was undertaken to investigate the antidepressant activity of basic flavone nucleus and two of its monohydroxy derivatives.

The investigated compounds, such as flavone, 3-hydroxyflavone, and 7-hydroxyflavone, did not alter either the ambulatory activity or the balancing time of mice on a Rotarod. Assessment of motor coordination and ambulatory activity is essential because the important parameter monitored for antidepressant activity in both forced swim test and tail suspension test is the period of immobility. If the test compounds are going to affect the motor coordination and ambulatory activity, it may produce false positive/negative results [19]. The present results on flavone derivatives exclude such a possibility.

The results of the present study clearly depict that the time of immobility in the forced swim test as well as in the tail suspension test is decreased in a dose-dependent fashion in mice treated with flavone, 3-hydroxyflavone, or 7-hydroxyflavone (Tables 2 and 3). The reduction in immobility time was evidently significant even in a dose of 5 mg/kg of all the tested compounds in forced swim test. In the tail suspension test also, a similar trend has been observed except for 7-hydroxyflavone. The above findings clearly indicate an antidepressant-like effect of flavone, 3-hydroxyflavone, and 7-hydroxyflavone. It is important to point out that the antidepressant effect for these compounds was clearly evident even in a dose as low as 5 mg/kg. Further, increase in doses augmented the antidepressant effect of these flavones as observed from the results of forced swim test and tail suspension test (Tables 2 and 3).

Mechanisms involved in the antidepressant effect of investigated flavones

It was considered interesting to investigate the possible mechanisms through which the antidepressant effects of flavones are mediated. The probable roles played by serotonergic, adrenergic, opioid, and NO pathways in the antidepressant effect of flavones have been investigated in the present study. PCPA, a tryptophan hydroxylase enzyme inhibitor when administered for 4 days in mice, is known to deplete the endogenous stores of serotonin by about 60% without altering the levels of noradrenaline and dopamine [20,21]. Pre-treatment with PCPA in

Table 2: Effect of flavone and its monohydroxy derivatives on mice in forced swim test

Dose (mg/kg i.p)	Period of immobility (s)		
	Flavone	3-hydroxyflavone	7-hydroxyflavone
Vehicle	195.07±5.8	195.07±5.8	195.07±5.8
Imipramine (%)	3.47±1.49 (98)	3.47±1.49 (98)	3.47±1.49 (98)
5 mg/kg	138.86±2.68[a] (40.42)	157.69±1.78[a] (19.16)	171.80±4.23[a] (11.92)
10 mg/kg	119.40±1.77[a] (38.79)	137.48±8.7[a] (29.52)	111.16±4.69[a] (43.01)
20 mg/kg	22.45±3.85[a] (88.49)	106.34±6.03[a] (45.48)	86.05±2.14[a] (55.88)

[a]$p<0.05$ in comparison to vehicle (one-way ANOVA followed by Dunnett's t-test). The values in brackets indicate the percentage reduction of immobility duration compared to vehicle

Table 3: Effect of flavone and its monohydroxy derivatives on mice in tail suspension test

Dose mg/kg i.p	Period of immobility (s)		
	Flavone	3-hydroxyflavone	7-hydroxyflavone
Vehicle	206.85±5.76	206.85±5.76	206.85±5.76
Imipramine (%)	14.84±1.41 (92)	14.84±1.41 (92)	14.84±1.41 (92)
5 mg/kg	171.52±3.83[a] (17.08)	197.15±1.56 (4.68)	174.18±2.66[a] (15.79)
10 mg/kg	154.75±5.81[a] (25.18)	184.57±7.67[a] (10.77)	140.13±2.97[a] (32.25)
20 mg/kg	53.84±4.85[a] (73.97)	99.76±7.68[a] (51.77)	84.23±4.14[a] (59.27)

[a]$p<0.05$ in comparison to vehicle (one-way ANOVA followed by Dunnett's t-test). The values in brackets indicate the percentage reduction of immobility duration compared to vehicle

Table 4: Effect of PCPA pre-treatment on flavone and its monohydroxy derivatives on mice in forced swim test

Treatment (mg/kg i.p)	Immobility period (s) without PCPA	Immobility period (s) with PCPA
Vehicle	195.07±5.8	205.92±5.35
Flavone (20)	22.45±3.85[a]	156.05±1.64[b]
3-hydroxyflavone (20)	106.34±6.03[a]	192.43±1.80[b]
7-hydroxyflavone (20)	86.05±2.14[a]	201.48±0.43[b]

[a]$p < 0.05$ in comparison to vehicle (one-way ANOVA followed by Dunnett's t-test), [b]$p < 0.05$ compared to PCPA 100 mg/kg i.p pre-treated animals (one-way ANOVA followed by Dunnett's t-test). PCPA: Parachlorophenylalanine

Table 5: Effect of AMPT pre-treatment on flavone and its monohydroxy derivatives on mice in forced swim test

Treatment (mg/kg i.p)	Immobility time (s) without AMPT	Immobility time (s) with AMPT
Vehicle	195.07±5.8	205.92±5.35
Flavone (20)	22.45±3.85[a]	156.05±1.64[b]
3-hydroxyflavone (20)	106.34±6.03[a]	192.43±1.80[b]
7-hydroxyflavone (20)	86.05±2.14[a]	201.48±0.43[b]

[a]$p < 0.05$ in comparison to vehicle (one-way ANOVA followed by Dunnett's t-test). [b]$p < 0.05$ compared to AMPT-pretreated animals 100 mg/kg i.p (one-way ANOVA followed by Dunnett's t-test). AMPT: Alpha-methyl-para-tyrosine

Table 6: Effect of naloxone pre-treatment on flavone and its monohydroxy derivatives on mice in forced swim test

Treatment (mg/kg i.p)	Immobility time (s) without naloxone	Immobility time (s) with naloxone
Vehicle	195.07±5.8	196.95±0.97
Flavone (20)	22.45±3.85[a]	35.10±2.33[b]
3-hydroxyflavone (20)	106.34±6.03[a]	136.73±3.99[b]
7-hydroxyflavone (20)	86.05±2.14[a]	117.11±2.43[b]

[a]$p < 0.05$ in comparison to vehicle (one-way ANOVA followed by Dunnett's t-test). [b]$p < 0.05$ compared to naloxone pretreated animals 5 mg/kg i.p (one-way ANOVA followed by Dunnett's t-test)

Table 7: Effect of L-arginine pre-treatment on flavone and its monohydroxy derivatives on mice in forced swim test

Treatment (mg/kg i.p)	Immobility time (s) without L-arginine	Immobility time (s) with L-arginine
Vehicle	195.07±5.8	200.57±2.93
Flavone (20)	22.45±3.85[a]	25.67±0.68
3-hydroxyflavone (20)	106.34±6.03[a]	110.29±2.05
7-hydroxyflavone (20)	86.05±2.14[a]	79.32±3.18

[a]$p < 0.05$ compared to vehicle (one-way ANOVA followed by Dunnett's t-test)

mice has been shown to alter the response to SSRIs such as fluoxetine and citalopram [22]. The results of the present research indicate that PCPA pre-treatment effectively reversed the reduction in immobility period produced by flavone, 3-hydroxyflavone, and 7-hydroxyflavone in the forced swim test (Table 4). This observation clearly indicates the role of serotonergic mechanisms in the antidepressant effect of all the three investigated flavones. Flavonoids such as rutin [6], vitexin [23], liquiritin, and isoliquiritin[24] and flavonoids from *H. perforatum*[2] have been earlier shown to involve serotonergic pathways in mediating their antidepressant effect. The present result is in agreement with these findings, and serotonergic mechanism may be considered as an important mode of action in the antidepressant effect of flavonoids in general.

The role of adrenergic system in the effect of antidepressant drugs is generally studied by employing tyrosine hydroxylase inhibitor AMPT, which has been shown to reduce the levels of dopamine and nonadrenaline to an extent of 57 and 53%, respectively, in mice without altering the levels of serotonin [25]. The results of the present study indicate that prior administration of AMPT significantly reversed the decrease in immobility period of mice produced by flavone, 3-hydroxyflavone, and 7-hydroxyflavone treatment in forced swim test (Table 5). This observation shows the valid role for adrenergic system in the antidepressant effect of the above flavones. Previous studies on flavonoids such as rutin 4, liquiritin and isoliquiritiin [24], and vitexin[23] and flavonoids from *H. perforatum*[2] indicated an involvement of adrenergic mechanism in their antidepressant effect. The present results on flavone and its monohydroxy derivatives are in agreement with the earlier reports.

The μ-opioid receptors are implicated in depression and stress states due to their dense distribution in brain areas associated with stress and emotion. Serum β-endorphin levels have been shown to be decreased in patients with severe depression [26,27]. Amentoflavone and hyperoside present in *H. perforatum* have κ antagonist activity *in vitro*, and Katavic also suggested flavonoid core as a future new scaffold for the development of opioid receptor ligands [28]. Moreover, central administration of endogenous peptides such as endomorphin-1 and endomorphin-2 has been shown to possess antidepressant activity in mice [15]. All these evidence indicate a prominent role for opioid system in the development of depression. Flavone and many of its hydroxy derivatives were found to possess opioid-mediated analgesic action in mice. Hence, it was considered interesting to investigate the possible participation of opioid mechanism in the antidepressant effect of the investigated flavones. Naloxone, a non-specific opioid antagonist, was used for this purpose. It is clear from the results (Table 6) that naloxone administration partially reversed the reduction in immobility period produced by flavone, 3-hydroxyflavone, and 7-hydroxyflavone. There is no complete suppression of the effect of flavones with naloxone pre-treatment. The results, thus, depict that the antidepressant effect of the investigated flavones at least in part could be due to the involvement of opioid mechanism.

The participation of L-arginine-NO pathway in the modulation of depression has been reported earlier by Da Silva *et al.* [29]. Melatonin has been shown to exert an antidepressant effect through an interaction with NMDA receptors and L-arginine-NO pathway [15]. A flavanone glycoside hesperidin exhibited antidepressant activity involving NO -GMP pathway [30]. These authors provided evidence that NO donor L-arginine could attenuate the antidepressant effect of hesperidin. A recent report identified the role of NO modulatory mechanism in the neuroprotective effect of rutin, and L-arginine pre-treatment has been shown to significantly reverse the protective effect of rutin against immobilization stress-induced anxiety-like behavior [31]. In light of aforementioned information, it was considered interesting to investigate the role of NO in the antidepressant effect of flavones. A NO donor L-arginine was employed for this purpose [32]. However, pre-treatment with L-arginine did not significantly modify the reduction in immobility period affected by flavone, 3-hydroxyflavone, or 7-hydroxyflavone. The unaltered response of the above flavones in the presence of L-arginine indicates that NO mechanism may not play a role in the antidepressant effect of these compounds.

CONCLUSION

The present study has identified the novel antidepressant activity of the basic flavone scaffold and its monohydroxy derivatives. The major participation of serotonergic and adrenergic mechanisms and partial role of opioid mechanism in the antidepressant effect of flavone compounds were also revealed. Since multiple neurotransmitter systems are involved in the antidepressant effect of the flavone compounds, the serious side effects are inevitable. Hence, the safety profile of the compound needs to be evaluated.

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