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Original Article

Cytotoxicity and Anti-*Plasmodium berghei* Activity of Emodin Loaded Nanoemulsion

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Abstract

Background: Malaria parasites cause a tremendous burden of disease in both the tropics and subtropics areas. Growing of drugs resistance in parasites is one of the most threats to malaria control. The aim of study was to investigate the anti-malarial activity of nano-emodin isolated from *Rhamnus cathartica* on *Plasmodium berghei* in mice to evaluate parasites inhibition rate using *in-vivo* test.

Methods: The study was conducted in the School of Public Health, Tehran University of Medical Sciences, during 2020. Nano- emodin particles were prepared from *Rhamnus cathartica,* and confirmed by Zeta Potential Analyzer, DLS and electron microscopy techniques. Mice were infected with *P. berghei* and treated by emodin nanoparticles. Parasitemia was evaluated in each group in comparison with control group. Toxicity test was done using twice the highest concentration of emodin extract on a separate group of mice and ED50 was calculated.

Results: Emodin extract was significantly effective in all concentrations on D4 ($P<0.05$). The most effective on parasitemia was observed in 400 mg/kg of Liquid Nano-emodin and solid (non-Nano) emodin. ED50 for emodin extract was determined 220 mg/kg. Toxicity test showed no toxic effect on the subjects.

Conclusion: The emodin extract is safe, lack of side effects. So, it can be used for more and longer period of time and in higher doses. Emodin extract, either in form of liquid and nanoparticle or in a solid form, has the same therapeutic effect on *P. berghei* in infected Balb/c mice.

Introduction

Malaria is one of the most important and widespread protozoan parasitic diseases in the world, especially in tropical and subtropical countries. The causative agent of malaria in humans is the *Plasmodium* spp. parasite, which is often transmitted via the bite of infected blood-feeding female Anopheles mosquitoes. About 228 million people worldwide were infected and the African region has the highest incidence of about 94% (1).

Chloroquine has been an effective drug against malaria parasites. Besides, in cases of drug resistance, fansidar (Pyrimethamine/Sulfadoxine) is also used in combination form with artemisinin derivatives. At present, the emergence of resistance in *P. falciparum* and even in some places in *P. vivax*, against many anti-malarial drugs attention has been drawn to novel and effective replacing antimalarial drugs (2). The development of resistance to antimalarial drugs poses the greatest threat to malaria control and results in increased malaria morbidity and mortality. Since most chemical drugs have many side effects, high drug resistance has been reported in different areas, and some of these drugs are contraindicated in pregnant mothers, therefore, use of drugs with no side effects as well as high antiparasitic effects comes to necessity (3).

In recent years, the use of herbal medicines for the treatment of malaria is increased due to little or no side effects, having antioxidant properties, being cheaper, more accessible, and no cytotoxicity due to high consumption (4, 5). Two species of these herbal medicines derived from plants and cinchona (Quina Quina) and *Artemisia annua* (Sweet wormwood) have been stabilized and used against malaria infection (6). *In-vivo* drug resistance to artemisinin and its derivatives has been reported, so such an idea can guide us to investigate another plant for the treatment of malaria (7, 8). Emodin (6-methyl-1, 3, 8-trihydroxyanthraquinone) is an extract or a chemical compound derived from various plants (9). Emodin as one of the internal compounds of the *Rhamnus cathartica* plant can inhibit the enzymes as well as have antibacterial and anti-cancer activities (10). At present, emodin is used as a reference drug against Giardia protozoa and it has been a very promising drug. This extract has strong anti-inflammatory and antioxidant effects that can be useful in the treatment of osteoporosis, and cardiovascular diseases, CNS, liver, metabolic and respiratory reactions (11, 12).

Nano-particles are a new form of material with outstanding biological properties and low toxicity with a high potential to cross the physiological barrier of the body to reach the target tissue of the body (13). Nano-particles can be derived from many materials with low toxicity than the average toxic doses for the liver and other relevant organs, so nano-particles are very suitable for targeting different cells for drug delivery or genetic factors and diagnostic factors (14).

In this study, for the first time, the antimalarial activity of the Nano-emodin isolated from the *R. cathartica* plant was investigated on the *P. berghei* as a model for studying human malaria in Balb/c mice and the rate of parasitism and inhibition of *in-vivo* parasite growth was assessed (15, 16).

Materials and Methods

Ethics Approval

This experimental-interventional study performed in the National Malaria Laboratory of School of Public Health, Tehran University of Medical Sciences with research code of IR.TUMS.SPH.REC.1398.295, approved by Research Ethical Committee and supported

financially of Tehran University of Medical Sciences No. 99-1-99-46111.

Chemical supply

Standard solution of emodin with a concentration of 1 Milli-molar (Sigma Aldrich, USA, CAS Number 518-82-1) was used for quantitative evaluation of emodin.

Plant collection and extraction

Preparation and purification of emodin from *R. cathartica* plant using protocols performed by previous studies (10,17).

Preparation and characterization of Nano-particles

Nano-particles and their surface charge were measured by dynamic light scattering (DLS), Zeta Potential Analyzer and electron microscopy techniques, for which samples were sent to Parto Rayan Rastak Company, Tehran, Iran (Figs.1,2) (11).

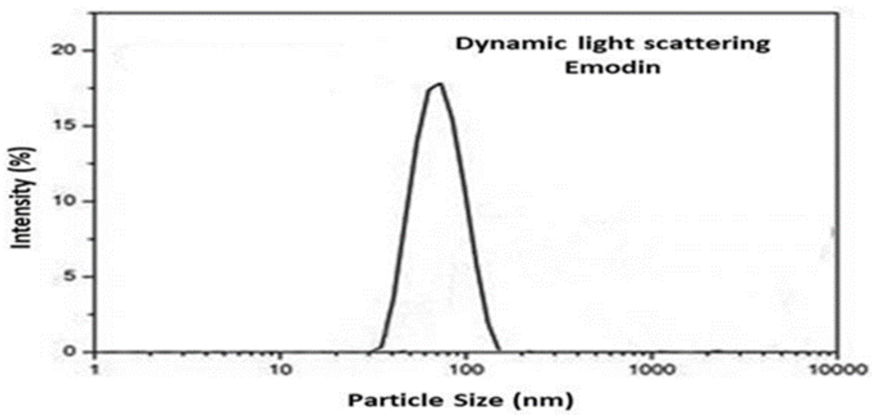

Fig. 1: DLS showing the size distribution of emodin nanoparticles

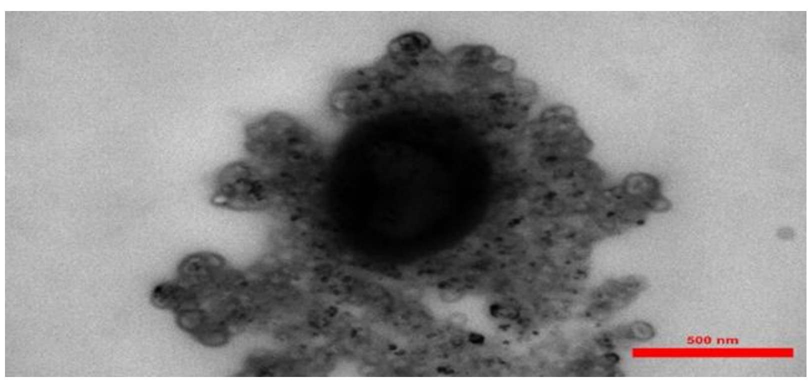

Fig. 2: SEM image of emodin nanoparticles showed proper morphology

Laboratory Animals

Forty-five male Balb/c mice with age of 8-10 weeks and weight 20 ±2 grams were divided in nine groups including five mice in each group for *In-vivo* evaluation. The mice were obtained from the Faculty of Pharmacology and were kept in special cages under the normal daylight and fed with mouse meal and tap water (18).

Parasites

Rodent malaria parasite of *P. berghei* NICD strain was used for antimalarial screening. The parasite was derived out from Liquid nitrogen tank with safe cautions, and placed at room temperature to be steady, and then the sample was injected intraperitoneally to a number of mice. Three to five days after injection, when clinical symptoms in mice became appearance, blood samples were obtained from the mice via cardiac puncture to determine the rate of parasitemia and prepare the right percentage to inject to the study groups.

Chloroquine

Chloroquine in form of diphosphate salt with the formula (C18 H26 C1N3.2H3PO4) (MW: 515.87 mg) was used as a gold standard to treat–control group with concentration of 20 mg/kg.

Infecting the Studied Mice with P. berghei Parasite

First, 10^6 parasitized erythrocytes were injected intraperitoneally into several mice prepared as a ready suspension in physiological saline with a final volume of 0.2 ml. Afterward, the blood was diluted to the required amount with sterile physiological saline, to reach 10^6 parasitic erythrocytes per 0.2 ml of the suspension. The suspension containing parasitic erythrocytes was inoculated intraperitoneally to different groups of mice to start the next step (21, 22).

Treatment of infected mice

Treatment was performed subcutaneously and continued for 4 days, with concentrations of 100, 200, 400, and 800 mg/kg of liquid Nano-emodin emulsion respectively, to groups 1 to 4, moreover, ordinary solid (non-Nano) emodin emulsion in the concentration of 400 mg/kg was injected into group 5 and 20 mg/kg chloroquine was injected into group 6 as a positive control (gold standard), respectively. Group7 was received physiological serum as a placebo and remained negative group (untreated group). Group 8 in order to evaluate the cytotoxic effects of the studied drugs, a concentration of 800 mg/kg of Nano-liquid emodin was injected for two weeks. Group 9 was left in the animal house without infection and treatment (Null) (Table 1).

Table 1: Groups of mice based on the different types of treatment provided for each group

Groups	*Infected with P. berghei*	*Extract Concentrations*
1	*	100 mg/kg of LNE [1]
2	*	200 mg/kg of LNE
3	*	400 mg/kg of LNE
4	*	800 mg/kg of LNE
5	*	400 mg/kg of SE [2]
6	*	20 mg/kg Chloroquine (PC) [3]
7	*	Placebo (NC) [4]
8	Non –Infected	800 mg/kg of LNE (Cytotoxicity of Drug)
9	Non – Injected	NULL (Animal House Study)

* Infected with *P. berghei*
1. LNE: Liquid Nano-emodin
2. SE: Solid emodin (Non-Nano)
3. PC: Positive Control
4. NC: Negative Control

Preparing blood slides

Characteristics of each mouse were recorded on the slide with a pencil, all slides were stained with Giemsa stain, and eventually they were examined with a light microscope with total magnification of 1000 x (23).

Parasite Counting

The parasite-infected erythrocytes were counted against 10,000 red blood cells and converted to a percentage; as the following equation;

$$\text{Parasite } \% = \frac{\text{number of infected erythrocytes}}{10000 \text{ RBCs}} \times 100$$

Evaluating the effectiveness of emodin extract on P.berghei

Evaluation of effectiveness of the extract was conducted based on peters' method (24).

Cytotoxicity assay

The cytotoxicity group which was concluded five healthy mice, treated with twice the highest dose (An amount of 800 mg/kg of Liquid Nano-emodin was considered as the highest dose) of drug for two weeks.

Possible hemolysis of Human Blood by the different concentration of Nano-emodin

Evaluation of the blood hemolysis was performed based on existing protocols (25, 26). Different concentration of Liquid Nano-emodin including were implemented in evaluation of hemolysis effect.

ED50 calculation

ED50 (50% Effective dose) means the concentration of the agent (emodin extract) that can kill 50% of the parasites or the concentration that inhibit 50% growth of parasites compared to the control group; It was calculated as follows (27):

$$\textit{Inhibition of Parasite Growth } [\%] = \frac{\text{Parasitemia rate in control group} - \text{Parasitemia rate in test group}}{\text{Parasitemia rate in control group}} \times 100$$

To identify ED50, different concentration of Liquid Nano-emodin including 100, 200, 400 and 800 mg/kg were tested on *P. berghei* infected mice. Finally, LD50 was calculated based on the percentage of parasitemia in different group.

Data analysis

The obtained data in this study were statistically analyzed using SPSS software version 16 (Chicago, IL, USA) including Kolmogorov-Smirnov Z, one-way analysis of variance (abbreviated one-way ANOVA), Post Hoc analysis and Kruskal–Wallis test.

Results

Findings related to the rate of parasitemia and inhibition of parasite growth

There was a significant difference in the amount of parasitemia between tests groups and control group on day four (D4) (P< 0.001); however, the difference in the rate of parasitemia was regarded as no significant between the results of the drug-receiving groups with the drug-free control groups, on day seven (D7), (P= 0.638) (Table 2).

Table 2: Evaluation parasitemia rate between tests and control groups using One- way ANOVA test on D4 and D7

Variable	*Mean% ± SD**							
Row and Name of Groups**	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	*P. value*
Parasitemia on D4	6.6 ±2.5	6.8 ±3.8	6.2 ±4.6	8.4 ±1.8	4.2 ±1.7	0	13 ±2.4	<0.001
Parasitemia on D7	29 ±0.84	26.5 ±5.06	25.6 ±4.1	26.4 ±7.8	27.2 ±8.9	0	34 ±7.5	0.638

*Mean ± Standard deviation (SD)

** **Group 1**: 100 mg/kg of L.NE, **Group 2**: 200 mg/kg of L.NE, **Group 3**: 400 mg/kg of L.NE, **Group 4**: 800 mg/kg of L.NE, **Group 5**:400 mg/kg of S.E, **Group 6**: P.C, **Group 7**: N.C

Emodin extract was significantly effective in all concentrations studied among which concentrations of 400 mg/kg of Liquid Nano-emodin with 52/30% growth inhibition and 400 mg/kg of Solid (Non-Nano) emodin with 67/69% growth inhibition were more effective. Besides, no significant difference in reduction of parasites was observed between these two extracts (Fig.3. A-B).

ED50 determination of emodin extract on fourth day

ED50 for emodin extract was determined 220 mg/kg and this amount of emodin extract can reduce 50% of parasites in the host, (Fig.3. C).

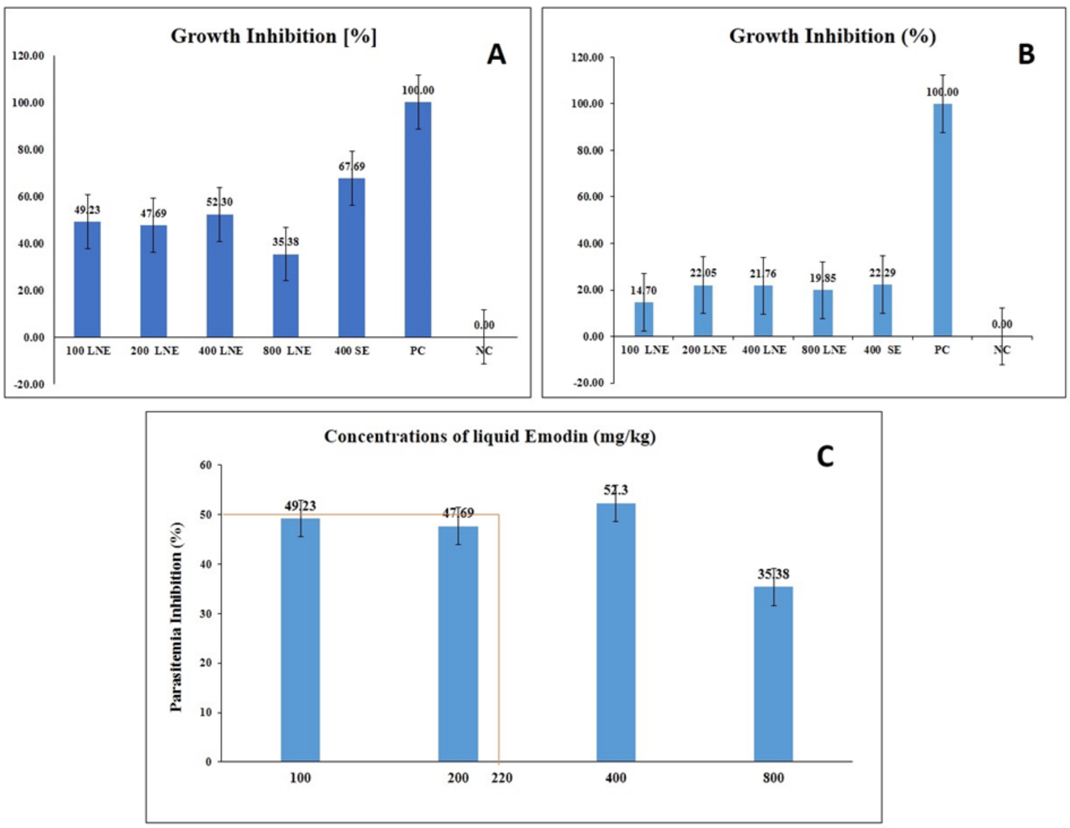

Fig. 3: A: Growth inhibition percentage in mice treated with different concentrations of emodin extract on **D4. B**: Growth inhibition percentage in mice treated with different concentrations of emodin extract on **D7. C**: Determination diagram of ED50 for Parasitaemia Inhibition Percentage of *P. berghei* – Concentration (mg/kg) on **D4**

Hepatomegaly, liver enzymes and Cytotoxicity assay

Post-test of one-way ANOVA demonstrated that emodin extract in different concentrations has no effect on the liver of mice in terms of cytotoxicity. Besides, by comparing the changes in liver length, especially in cytotoxicity group, negative control, the positive group and the null group, showed that the extract has no toxic effect on mice's liver (Table 3). Meanwhile, results of measuring liver enzymes (ALT, AST, ALP) showed that there was not any significant difference among the cytotoxicity group, negative control, positive control and null group in amount of liver enzymes (Table 4).

Table 3: Comparison of mice liver length using One – way Anova test

Comparison of the length of mice's liver	***Mean ± Standard Deviation (SD) [%]***	***P. value***
Group Names	Liver Length [mm]	
Cytotoxicity	24.6±3.2	
N.C	24.2±3.8	
P.C	22.2±1.9	
NULL	21.8±1.3	0.236

Table 4: Assessment of liver enzymes between tests and control groups using One –Way Anova test

Comparison of the amount of Enzymes	***Mean ± Standard Deviation (SD) [%]***				***P.value***
	N.C	P.C	Cytotoxicity	NULL	
AST	3.46±1.39	32.20±1.48	8.54±1.33	7.83±1.41	0.248
ALT	68.14±5.66	73.80±16.14	60.16±13.55	71.6±6.95	0.288
ALP	8.15±5.92	39.54±8.46	8.24±3.48	18.57±8.24	0.192

AST: Aspartate transaminase; ALT: Alanine transaminase; ALP: Alkaline Phosphatase

Splenomegaly in tested mice

Results of Kruskal-Wallis test showed that any significant difference in the length of spleen among those mice that were treated with emodin extract in comparison with control group (*P*= 0.514). Moreover, comparing the mean of cytotoxicity group, negative group, positive group and null group asserted that emodin extract has no cytotoxicity effect on the spleen of mice since the mean size of the spleens are close to each other and have not much differences. (In this test, a significance level greater than 0.05 is considered meaningless) (Table 5).

Table 5: Kruskal - Walli's test results for comparing spleen length between tests and control groups

Group Names	***Mean Rank Value [%]***	***Kruskal-Wallis Test Results***		
		Chi-Square Test [χ^2 test]	Degree of Freedom	*P.value*
Cytotoxicity	13.2			
N.C	10.5			
P.C	10.5			
NULL	9	2.293	3	0.514

Discussion

Drug resistance has created many problems in the treatment of malaria in recent years and has been the biggest challenge in the pathway of malaria control. Spreading drug-resistant strains of *Plasmodium* in malarious areas has led to an increased rate of malaria mortality, especially in children (5-8). Moreover, drug resistance may induce some malaria outbreaks, especially because of falciparum malaria.

Since many chemical drugs have shown many side effects and are contraindicated in malaria-infected pregnant mothers, replacing safe, novel and more effective antimalarial drugs are anticipated. In recent years, the use of herbal medicines for the treatment of some infections has been considered due to few or no side effects, having antioxidant properties, low cytotoxicity, being cheaper, and more accessible (4, 5). The use of herbal medicine against malaria fevers has a long history and that is widely believed that nano-particles can facilitate reasonable effectiveness of medicines, so in this study, we decided to use nanotechnology to investigate about a medicinal plant, emodin, on *P. berghei*.

Some studies have been conducted to find antimalarial activity in plants (4). For instance, in a study (28), the effect of alcoholic extract of *A. annua* was evaluated on *P. berghei* and the best therapeutic concentration was reported as 1100 mg/kg. In addition, *A. annua* showed toxic effects in mice in higher concentrations, while in the present study; toxic effects were not observed in any of the concentrations of emodin. Furthermore, all concentrations of emodin were effective to reduce the parasitemia, without any adverse effects on the subject mice, especially the concentration of 400 mg/kg of liquid Nano-emodin and 400 mg/kg of solid (Non-Nano) emodin (28).

Chabra et al (11) considered the effect of emodin, chitosan and nano-chitosan *in-vitro* on *G. lamblia* parasite. The studied drugs had acceptable effects on cysts and trophozoite of *G. lamblia* parasite compared to the control group. The emodin with a concentration of 200 micrograms per milliliter was able to remove 100% of *G. lamblia* parasites after 180 minutes. The lethal effect of emodin on *Giardia* reveals its antiparasitic properties, at the same time.

Emodin may have a specific mechanism of effect depending on the type of parasite, which requires further research in this area. As it mentioned all concentrations in this study, especially the concentration of 400 mg/kg of liquid Nano-emodin and 400 mg / kg of solid (Non-Nano) emodin, had the best effect on *P. berghei* parasite on fourth day. The lethal effect of emodin on *Giardia* reveals its antiparasitic properties, at the same time; the emodin may have a specific mechanism of effect depending on the type of parasite, which requires further research in this area (11). Batista et al (29) used emodin extract on Zika virus to evaluate antiviral effects of the extract against the virus for 24, 48 and 72 hours. The infectious effect of Zika virus was reduced about 83.3% with concentration of 40 microliters per millimeter of the emodin. Dynamic light scattering data showed that the emodin significantly reduced the hydrodynamic radius of virus particles in solution, so that the natural compound (emodin) had strong power against Zika virus. In the present study, concentrations of 400 mg / kg of emodin extract in both forms of liquid Nano-emodin and solid (Non-Nano) emodin were more effective on the *P. berghei*. Therefore, it seems that emodin has therapeutic effects on both viruses and parasites with different mode (29).

Nateghpour et al. investigated the effect of alcoholic extract of *Otostegia persica* on *P. berghei*. A concentration of 450 milligrams per mouse body weight of the plant extract could significantly reduce parasitemia in infected mice. While Chloroquine showed more effective than the plant extract, no significant difference was determined between the results statistically (30). Heydarian et al. investigated the effectiveness of the alcoholic extract of *Curcuma longa* plant, sporadically in combination with the chloroquine against sensitive strains of *P. berghei*. The ED50 results for Chloroquine and *C. longa* was 1.4 mg/kg and 1250 mg/kg respectively. The combination of alcoholic extract of the *C. longa* with chloroquine in the ratio of 80:20 had the highest effect with 71.75% inhibition of the growth of *P. berghei* and demonstrated that it had a synergetic reaction in a combination treatment method. Although ED50 of *C. longa* plant was reported as 1250 mg/kg, in our study the ED50 results for emodin was obtained as 220 mg/kg. This matter can imply that emodin with lower concentration has more effect on parasites in comparison with *C. longa* (31).

Combination between emodin and chloroquine was not investigated in the present study; more investigation needs to identify this effect on the parasite.

Conclusion

The effect of emodin against *P. berghei* was significant but disappeared quickly, probably due to its short half-life. Moreover, emodin extract was completely safe, due to its non-toxicity and lack of side effects, so it can be used for more and longer period of time and in higher doses. Emodin extract, either in form of liquid and nanoparticle or in a solid form, has a therapeutic effect on *P. berghei in* infected BALB/c mice and it seems that due to its short half-life, this agent shall be used for more than four days and in higher doses or with more repetitions to be able to stabilize its effect. This extract in comparing with chloroquine did eliminate the parasite merely, but it did not bear a toxic effect like chloroquine on the internal organs of the host and did not affect the liver enzyme also. Finally, it is recommended to evaluate the therapeutic effect of emodin extract by increasing its concentration or in combination with chloroquine or separate the different fractions of the extract and examine them separately in order to eliminate the disturbing factors (such as hemolytic effect) and purify the effective factors.

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Conflicts of interest

The authors declare that they have no conflict of interest that affects this study.

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