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A N I N T E R N A T I O N A L J O U R N A L

Developmental Toxicity of (4S)-2- (4-hydroxy-3-methoxyphenyl) thiazolidine-4-carboxylic acid in Zebrafish (*Danio rerio*)

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ABSTRACT

(4S)-2-(4-hydroxy-3-methoxyphenyl)thiazolidine-4-carboxylic acid is new synthesized substance obtained from cysteine and valine. Thiazolidine derivates have important biological responses so scientists work intensively on these compounds in recent years. It is obvious that thiazolidine contained compounds will be used in future in the pharmaceutical industry to treat important diseases. Median lethal concentrations (LC50) for 48 h and 96 h were found as 1.106±0.052 mM and 0.804mM ± 0.102 respectively. According to LC50, exposure doses were determined as control, 0.4 mM, 0.2 mM and 0.1 mM (4S)-2-(4-hydroxy-3-methoxyphenyl)thiazolidine-4-carboxylic acid. Developmental toxicity and apoptotic features on zebrafish development were evaluated in this study. The results of this study indicate that (4S)-2-(4-hydroxy-3-methoxyphenyl)thiazolidine-4-carboxylic acid exposure cause developmental defects like pericardial edema, bent spine, tail malformation, blood accumulation, yolk sac edema but on the other hand concentration-dependent decrease in apoptotic rate. Likewise, concentration-dependent decrease in hatching and increase in mortality of embryos were also detected.

Key words: thiazolidine, toxicity, development, zebrafish, apoptosis

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INTRODUCTION

Five membered nitrogen-containing heterocyclics are found in many natural products and pharmaceutical structure. Additionally, many of them have been used as synthetic intermediates, auxiliary reagents, ligands or asymmetric synthesis catalyst [1]. Thiazolidines are the heterocyclic organic compounds which are generally used in biology and medicine. Thiazolidines can be synthesized by condensation of thiol with aldehide or ketone. This process is reversible. Thiazolidines decompose to aldehide and thiol in water and they are sulfur analogs of oxazolidines [2].

The significance of thiazolidine ring systems has increased in recent years because of possessing broad spectrum of bioactivities [3]. Thiazolidine derivates have several important biological and pharmacological features. In many research, antimicrobial activity of thiazolidine derivates have been reported. Bhoot et al. [4] found antimicrobial activity of 2-(ptolylimino)-3-(4-tolyl)-5-[5-(3,4- dichlorophenyl)-2-furylidene]-4- thiazolidinone and derivates. The antimicrobial activity were observed by using several bacterial strains such as *Bacillus megaterium*, *Staphylococcus aureus*, *Escherichia coli*, *Proteus vulgaris* and fungi such as *Aspergillus niger*. Similarly Shanmugapandiyan et al. [5] observed antibacterial and antifungal activity of 2-[(Thiazolidin-4- one)phenyl]-1H-phenylbenzimidazole towards gram positive and gram negative bacterial as well as fungal species. As is known, peptidoglycan is an important component at gram-positive and gram-negative bacterial cell wall. Andres et al. [6] have found that 4-Thiazolidinones act as inhibitors of the bacterial enzyme Mur B which is essential for peptidoglycan biosynthesis.

The biological effects of thiazolidine derivates are not limited to microbial activity. Thiazolidin-4-ones with 2,3 substitution are reported to possess significant hypoglycaemic [7], anti-inflammatory [8], choleretic [9], anti-HIV [10], and anticonvulsant activities [11]. Gududuru et al. [12] showed the antiproliferative activity of 2-aryl-4-oxothiazoilidin-3-yl amides against prostate cancer lines (DU-145, PC-3, LNCaP, PPC-1 and TSU).

Thiazolidines have minimal side effects and important biological features so they have been usually used in drug discovery for years. Biological effects of thiazolidine derivates are more efficient than currently used drugs. So, it is possible that thiazolidine derivates will be take part in plenty of medication. In this study evaluation of the toxicological effects of (4S)-2-(4-hydroxy-3-methoxyphenyl)thiazolidine-4-carboxylic acid on zebrafish embryos was done.

Zebrafish is a well known vertebrate model for reproduction and development studies. There are some advantages for studying with zebrafish. Their genome shares significant homology with the human genome and they have small size and high productivity rate. Zebrafish can survive fairly severe environmental changes without succumbing, surviving long enough to show developmental defects. Transparent embryos, low maintance cost, high fecundity and short generation time making them an ideal animal model for research laboratories with limited funding. Consequently, zebrafish is a model organism that has been widely used in toxicological studies [13] and all of these characteristics have contributed to making zebrafish model of choice in this study.

MATERIAL AND METHODS

Synthesis of Chemical

4-hydroxy-3-methoxybenzaldehyde (2.55 g, 10 mmol) was dissolved in EtOH. L-Cysteine hydrochloride (1.57 g, 10 mmol) and NaOAc (0.98 g, 12 mmol) dissolved

in water (5ml) were added to the solution. Mixture was stirred for 24 hours at room temperature. The precipitate was then separated by filtration and washed several times with EtOH to give the product in 72% yield (Fig. 1).

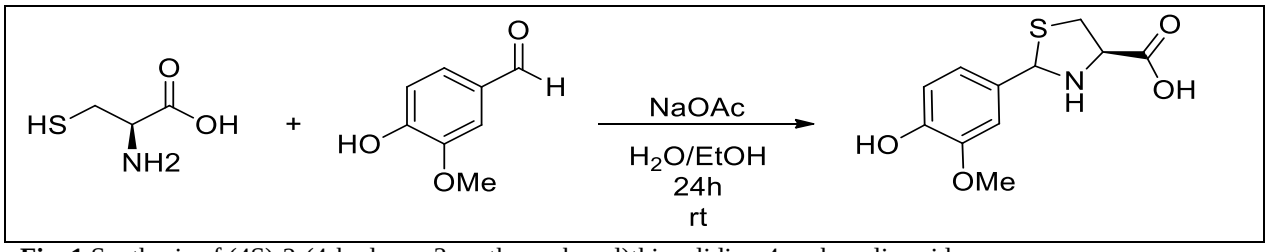

Fig. 1. Synthesis of (4S)-2-(4-hydroxy-3-methoxyphenyl)thiazolidine-4-carboxylic acid

Zebrafish Maintance and Embryo Collection

Adult zebrafish individuals with 1:2 for female to male ratio were maintained in 20 L capacity spawning aquarium. Dechlorinated tap water was used in recirculating chamber. Culture conditions were as following: 14 h light/10 h dark photoperiod, 28.5 ± 1°C temperature, 7.0 ± 0.5 pH and 6.0 mg/L dissolved oxygen. They were fed with *Artemia sp.* twice a day. When the light was turn on, spawning was induced and embryos were collected immediately. Under a stereo microscope, fertilized and unfertilized eggs were separated. Embryos were washed with distilled water (dH_2O) three times and fertilized embryos were transferred into petri dishes.

Experimental Exposure

EPA toxicity secreening method was performed for zebrafish embryos [14] After 4 hour post fertilization (hpf) embryos which had developed normally and reached blastula stage, were seperated under stereo microscope. The embryos were exposed to different concentrations (2.5 mM, 1.5 mM, 1mM, 0.85 mM, 0.5 mM, 0.35 mM, 0.1 mM) of (4S)-2-(4-hydroxy-3-methoxyphenyl)thiazolidine-4-carboxylic acid to calculate median lethal concentration (LC50). The embryos were incubated at 28 ± 0.5 °C in 24 well plates with 3 ml solution in each plate for 5 days. Control group only received embryo medium. Each well contained 1 embryo. The solutions were changed in each well in every 24 hours and dead embryos removed immediately. Same conditions were performed for each exposure groups and three replicate were used for each concentration. According to LC50 value, one control and 3 sub-lethal exposure groups (0.1 mM, 0.2 mM and 0.4 mM) were formed. Developmental abnormalities, hatching, mortality, apoptotic rate and morphology of embryos were observed under stereo microscope. Conditions were same as above and three replicate were performed.

Terminal deoxynucleotide transferase-mediated deoxy-UTP nick end labelling (TUNEL)

For apoptotic cell visualization Milipore ApopTag® Peroxidase In Situ Apoptosis Detection Kit was used. 10 embryos were selected from each group at the stage of 24 hpf. Embryos were fixed 4% paraformaldehyde at 4°C overnight. After dechorionating embryos they rinsed three times in phosphate buffered saline (PBS). After incubation 3% hydrogen peroxide in methanol for 15 minutes in room temperature embryos were incubated in TUNEL mixture [terminal deoxynucleotidyl transferase (TdT) enzyme+reaction buffer] at 37°C for 1 hour. Then embryos rinsed with PBS and embryos incubated in peroxidase for 1 hour and washed in PBS. Eventually embryos were stained with diaminobenzidine for 30 minutes and then

TUNEL positive (apoptotic) cells were counted and photographed under stereo microscope.

Statistical analysis

All statistical analysis were done using SPSS 15.0. Probit analysis were used to determine median lethal concentrations (LC50) of (4S)-2-(4-hydroxy-3-methoxyphenyl) thiazolidine-4-carboxylic acid on zebrafish embryos for 48 h and 96 h. For determining the effects of (4S)-2-(4-hydroxy-3-methoxyphenyl)thiazolidine-4-carboxylic acid on developmental defects, the presence of significant differences between dose groups from control were determined with one-way analysis of variance (ANOVA). For further analysis TUKEY HSD post-hoc test were performed. In addition, apoptotic effects, after counting TUNEL positive cells the presence of significant differences between mean values from control were determined with ANOVA.

RESULTS AND DISCUSSIONS

Development

Median lethal concentrations (LC50) for 48 h and 96 h were found as 1.106±0.052 mM and 0.804mM ± 0.102 respectively with probit analysis. So, exposure doses were determined as control, 0.4 mM, 0.2 mM and 0.1 mM. Developmental stages of zebrafish embryos at control and exposure groups (0.1 mM, 0.2 mM and 0.4 mM) were observed during 5 days. In control group, zebrafish individuals were developed normally (Figure 2A, 2B, 2C). Any developmental abnormalies couldn't seen. In 0.1 mM exposure group, few developmental abnormalities were detected. Pericardial edema (Figure 2D) and tail malformation (Figure 2E) were monitored. Bent spine (Figure 2F), blood accumulation (Figure 2F, Figure 2G), pericardial edema and yolk sac accumulation (Figure 2G) were observed at 0.2 mM exposure group. In 0.4 mM exposure group, developmental abnormalities were severe compared with other groups. Blood accumulation, yolk sac edema (Figure 2H), pericardial edema (Figure 2I, 2J) and tail malformation (Figure 2I) were monitored. According to TUKEY HSD test, we proved (4S)-2-(4-hydroxy-3-methoxyphenyl)thiazolidine-4-carboxylic acid cause developmental abnormalities ($P<0.05$) (Table 1), concentration-dependent decrease in hatching (Figure 3) and increase in mortality (Figure 4) compared with control group.

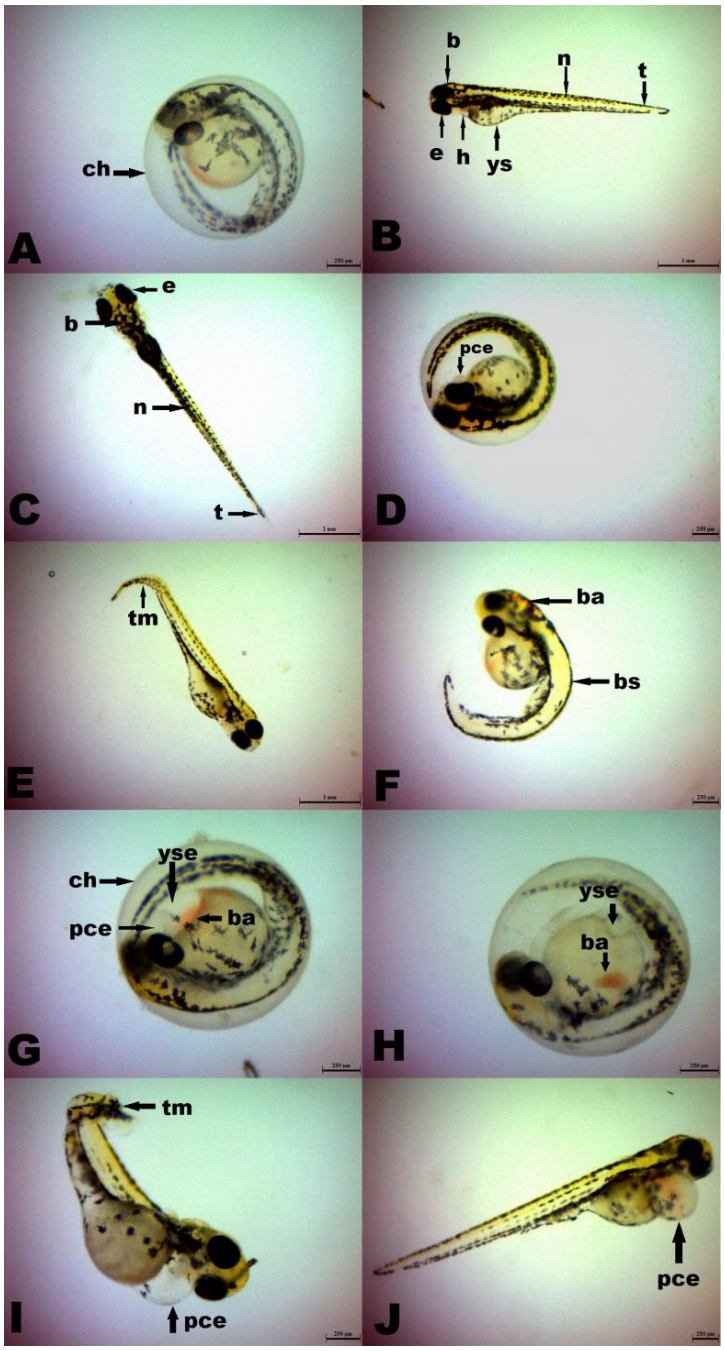

Figure 2: A- control group 2 days post fertilization (dpf), B- control group 4 dpf, C- control group, 5 dpf. D- 0.1mM 4dpf, E- 0.1 mM 5dpf, F, G- 0.2mM 3dpf, H- 0.4 mM 2dpf, I-0.4 mM 3 dpf, J- 0.4 mM 4dpf, ch: chorion, e: eye, b:brain, n:notocord, h: heart, ys: yolk sac, t: tail, pce: pericardial edema, tm: tail malformatiom, ba: blood accumulation, bs: bent spine, yse: yolk sac edema.

Table1: Dose-abnormality mean values obtained from zebrafish embryo which exposed to different concentrations of (4S)-2-(4-hydroxy-3-methoxyphenyl)thiazolidine-4-carboxylic acid for 5 days.

	24 h	**48 h**	**72 h**	**96 h**	**120 h**
Control	3.6±0.8^{*a}	0.2±0.4^{*a}	0±0^{*a}	0±0^{*a}	0±0^{*a}

0,1 mM	4±3.1^{*ab}	0.2±0.4^{*b}	0±0^{*b}	0.8±1.6^{*b}	2.4±4.8^{*b}
0,2 mM	2±0.89^{*bc}	0±0^{*bc}	0.4±0.4^{*b}	0.4±0.4^{*c}	2.8±4.6^{*b}
0,4 mM	4±2.52^{*c}	0±0^{*c}	0.2±0.4^{*c}	0.2±0.4^{*d}	6.2±8.6^{*c}

*****Significantly different from control group P<0.05

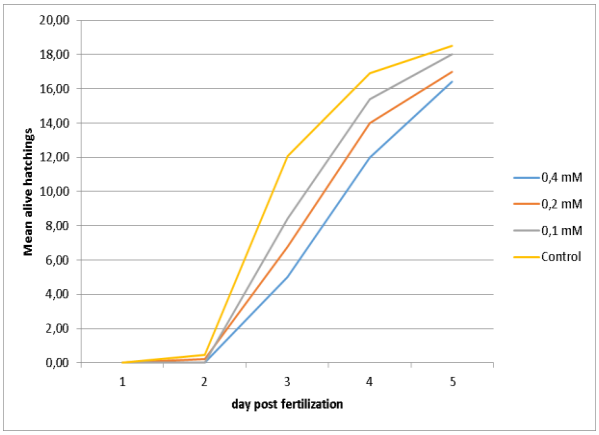

Figure 3: Hatching mean values of zebrafish embryos

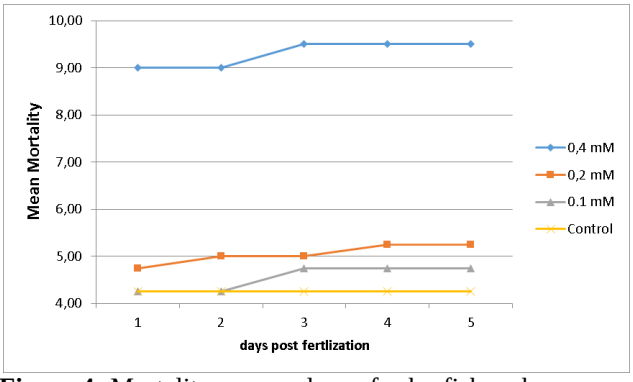

Figure 4: Mortality mean values of zebrafish embryos

Apoptosis

Apoptotic effects of (4R)-thiazolidinecarboxylic acid were evaluated with TUNEL assay (Figure 5). As a result of TUNEL analysis, decrease at apoptotic rate at exposure groups were detected. Apoptotic cells were counted under stereo microscope. In exposure groups, decrease in the number of apoptotic cells when compared with control group. Values given with Mean± SE, results were considered significantly different P<0.05 (Table 2)

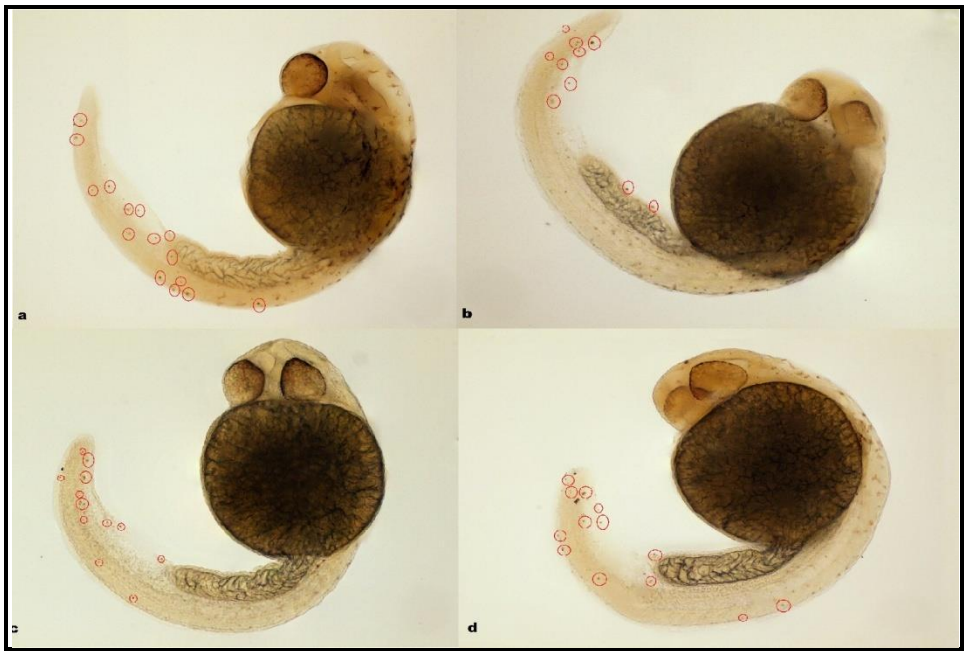

Figure 5: TUNEL analysis of 24 hpf zebrafish embryos, apoptotic cells showed with red circle, a) control group, b) 0.1 mM (4R)-thiazolidinecarboxylic, c) 0.2 mM (4R)-thiazolidinecarboxylic, d) 0.4 mM (4R)-thiazolidinecarboxylic.

Table2: Apoptotic cell numbers at 24 hpf zebrafish embryo

Control	0.1 mM	0.2 mM	0.4 mM
14.50±0.69	11.00±1.12^{*a}	11.60±1.49^{*b}	3.60±0.50^{**c}

*Significantly different from control group P<0.05.
**Significantly different from control group P<0.01.

Antibiotics are the drugs that have been widely used for treatment or preventing disease in human and various animals. Antibiotic residues get through aquatic environment via water. The toxicity of various antibiotics on fish is still unknown. Oliveira et al. [15] investigated the effects of oxytetracycline and amoxicillin on development of zebrafish. They detected edema, spine deformity and early hatching at 48 hpf embryo at 1125 mg/L amoxicillin exposed group. In 150 mg/L and 300 mg/L oxytetracycline exposed group non-hatching embryos were photographed. The results of our study show similarity with amoxicillin group. We also detected edema and spine deformity. Besides, dose-dependent hatching delay and increase in mortality at zebrafish embryos were found.

Zhao et al. [16] investigated toxicological effects of three members of the aminoglycoside antibiotics family, gentamycin, neomycin and streptomycin, on zebrafish embryonic development. The results showed that the lethal effect of all three drugs demonstrated a significant dependence on concentration, and the severity order of the lethal effect was streptomycin > neomycin > gentamycin. In addition, all the three drugs caused the larva trunk bending in resting state at 5 dpf, probably due to their ototoxicity in the physical imbalance and postural abnormalities. Wang et al. [17] performed a study about the toxicity β-diketone antibiotics on the development of embryo-larval zebrafish. They observed developmental malformations such as

hatching delay, pericardial and yolk sac edema, bent spine and uninflated swim bladder. The results are consistent with our study.

Toxicological effects of different concentrations of tetracycline on development of zebrafish were evaluated by Zhang et al. [18]. The larvae display developmental delay phenotypes, including hatching delay, shorter body length, increased yolk sac area and uninflated swim bladder upon exposure to tetracycline. Delayed yolk sac absorption and swim bladder deficiency at 96 hpf were observed in the zebrafish larvae upon exposure to 20 μg/L of tetracycline. No obvious apoptotic cells were observed in control group. In the groups treated with 5, 10 and 20 μg/L of tetracycline, apoptotic cells appeared, mainly around the tail area, but in the groups treated with 20 μg/L of tetracycline, some of the apoptotic cells appeared around the heart area in a few of the larvae. Quite the contrary we detected decrease at the apoptotic cell rate at exposure groups. Except increased yolk sac area findings are not consistent with our study.

In a study, toxicity of functional group of cephalosporins on zebrafish embryo were investigated by Zhang et al. [19]. During the experiment, 2-mercapto-5-methyl-1,3,4-thiadiazole (MMTD), cefazolin sodium (CFZL) and cefazedone (CFZD) were exposed. Problems at pigmentation, opaque yolk sac, heart defects, pericardial edema, poor swimming activity, hemorrhages at brain, and bent spine were monitored at CFZD and CFZL exposed groups. Similarly in MMTD exposed groups, transparent and yellow surface, nomelanin spots, colorless eye, opaque embryonic yolk sac and extension structure, swollen pericardial sac, no redness of the heart (wan heart), slow heart rate, short body length, bending anteroposterior axle, no stress response and low swimming activity were detected at zebrafish embryos. Similarly with this study, pericardial edema, hemorrhages and bent spine were also observed at our study.

Considering overall related studies, antibiotics caused abnormalities such as pericardial and yolk sac edema, hatching delay, hemorrhages, spine deformity has been found. It is clear from the results presented that (4S)-2-(4-hydroxy-3-methoxyphenyl)thiazolidine-4-carboxylic acid Show similar toxicological effects like other antibiotics. Dose-dependent increasing in mortality, hatching time and developmental defect. On the other hand suprisingly we detected dose-dependent decrease in apoptotic rate. Under these conditions, we can specified that after investigating the toxicological and teratological effects on higher animals, it has the potential to be used as a drug in the future.

CONCLUSIONS

Toxicological studies about thiazolidine ring are limited. (4S)-2-(4-hydroxy-3-methoxyphenyl)thiazolidine-4-carboxylic acid is an important thiazolidine derivate chemical which has potential for use as antibiotics in future. Consequently in our study, toxicological aspects of (4S)-2-(4-hydroxy-3-methoxyphenyl)thiazolidine-4-carboxylic acid on zebrafish development were proved. Although (4S)-2-(4-hydroxy-3-methoxyphenyl)thiazolidine-4-carboxylic acid exposure cause some developmental defects more studies are needed to understand toxicological effects of this substances on animal health.

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Erratum

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