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Investigation of the antioxidant effects of Trolox on HT-29 colon cancer cells

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ABSTRACT

Aim: To elucidate the effect of Trolox on HT-29 colon cancer cells and shed light on its therapeutic potential in the treatment of colorectal cancer.

Methods: HT-29 cells were obtained from Atatürk University, Department of Medical Pharmacology (Erzurum, Turkey). Trolox doses (0.1, 1, 10, 100, and 1000 µM) were added to HT-29 cells under cell culture conditions. After 24 hours, cell viability was determined by 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazoliumbromide (MTT). Total antioxidant capacity (TAC), total oxidant status (TOS), superoxide dismutase (SOD), and lactate dehydrogenase (LDH) were analyzed. MTT and ELISA data were evaluated using the One-Way Analysis of Variance (One-Way ANOVA) technique with GraphPad 9.5 software.

Results: While the viability of the control group was 100%, the viability of the other groups was proportional. Viability decreased at different Trolox dosages. Viability rates were lowest at 100 µM and 1000 µM concentrations. The IC50 value of Trolox in HT-29 cells was calculated as 866.26 µM. Statistical analysis showed significant findings. MTT results were consistent with our TAC, TOS, SOD and LDH analyses.

Conclusion: Our study demonstrates that high concentrations of Trolox exert a cytotoxic effect on HT-29 cells by depleting antioxidant defenses and inducing oxidative stress, acting as a pro-oxidant.

Keywords: HT-29, Trolox, antioxidant, oxidant.

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1. Introduction

Colorectal cancer (CRC) is the second most prevalent cause of cancer-related deaths globally and the third most common kind of cancer overall [1,2]. Due to its high morbidity and mortality rates, CRC has a dangerous place among cancer types with its potential to spread to the lungs, liver, ovaries and other organs of the gastrointestinal tract. Most cases of CRC are sporadic and associated with variable lifestyle risk factors [3,4]. These include lack of physical activity, excessive alcohol consumption, high-fat and fiber-free diets, old age and family history. Genetic mutations and epigenetic changes play an important role in cancer

development [5,6]. In addition, inflammatory bowel diseases such as diabetes, ulcerative colitis and Crohn's disease also contribute to CRC formation [5].

Oxidative stress and inflammation are crucial in the pathogenesis of CRC. Overproduction of reactive oxygen species (ROS) in cells causes oxidative damage to cellular components such as DNA, lipids and proteins, which plays an important role in the growth, proliferation and metastasis of tumor cells [7]. Imbalance in ROS production has been highlighted as an important factor, especially in the development of inflammatory bowel diseases [8]. In this context, the therapeutic potential of antioxidants in preventing the damage caused by oxidative stress has been widely investigated [8].

Antioxidants are important defense molecules that protect cells against oxidative damage by neutralizing free radicals [9,10]. Antioxidant enzymes including endogenous superoxide dismutase (SOD), catalase and glutathione peroxidase constitute the first line of defense against ROS [10]. However, since the effectiveness of endogenous antioxidants is limited, exogenous antioxidants from the diet have an important role in increasing the antioxidant capacity of cells [11]. Numerous investigations have been carried out about the possible anti-cancer properties of natural antioxidants such as vitamins, carotenoids, and flavonoids [7].

Trolox, a vitamin E analog (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid), is a widely used compound with its hydrophilic structure and strong antioxidant properties [12]. Trolox, known as a chain-breaking antioxidant, terminates chain reactions by giving hydrogen to peroxyl radicals and prevents oxidative damage caused by free radicals [13,14]. Trolox has been extensively studied in terms of its effects on oxidative stress and inflammation-related diseases and has attracted attention especially with its neuroprotective, antiinflammatory and antioxidative properties [13].

This study aims to examine the antioxidant effects of Trolox using the HT-29 cell line as a human colon cancer model. This study's results may enhance the knowledge of Trolox's potential therapeutic actions against cancer, given the significance of oxidative stress and inflammation in the etiology of colorectal cancer.

2. Materials and methods

2.1. Cell Cultures: HT-29 human colorectal adenocarcinoma cell line (passage no.: 6) was kindly provided by the Department of Medical Pharmacology, Atatürk University (Erzurum, Turkey). Following rapid thawing, the cryopreserved cells were centrifuged at 1200 rpm for 5 minutes and resuspended in fresh culture medium. The cells were maintained in Dulbecco's Modified Eagle Medium-high glucose (DMEM-HG) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) and 1% antibiotics (penicillin, streptomycin, and amphotericin B) (Gibco, USA), and this medium was also used to resuspend the cells [15]. The cells were then put in a 25 cm^2 flask and kept at 37°C with 5% CO_2 in an incubator. When approximately 80% confluency was reached, trypsin-EDTA (0.25% trypsin–0.02% EDTA) (Gibco, USA) was added to the flask. The cell suspension was then centrifuged, and after discarding the supernatant, HT-29 cells were seeded into 96-well tissue culture plates at a density of 10,000 cells per well in 100 µl of medium. All experimental procedures were performed using cells from the same passage to ensure

consistency and minimize variability due to passage-related cellular changes [16-18].

2.2. Drug Administration: When the HT-29 cells seeded in 96-well plates reached approximately 80% confluency, Trolox stock solutions were prepared at final concentrations of 0.1, 1, 10, 100, and 1000 µM. After Trolox was dissolved, it was added to the wells, and the cells were incubated at 37°C with 5% CO_2 for 24 hours. Each concentration was tested in 10 replicates. After the incubation period, the culture media were carefully collected and stored at –20°C for subsequent SOD and LDH analysis. Cell viability was then assessed directly on the adherent cells using the MTT assay.

2.3. MTT Assay: Cell viability was assessed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Sigma, USA), which relies on the conversion of MTT to insoluble purple formazan crystals by metabolically active cells. After 24 hours of treatment, 10 µL of MTT solution (5 mg/mL in PBS) was added to each well and incubated at 37°C for 4 hours. Subsequently, 100 µL of dimethyl sulfoxide (DMSO; Sigma, USA) was added to solubilize the formazan crystals. Absorbance was measured at 570 nm using a microplate spectrophotometer (Quant, Bad Friedrichshall, Biotek, Germany). (19, 20).

2.4. Oxidative Stress Analysis

Total Oxidant Level (TOS) and Total Analysis Antioxidant Capacity (TAC):

TOS and TAC levels were measured using commercial colorimetric assay kits (Rel Assay Diagnostics, Gaziantep, Türkiye) according to the manufacturer's instructions. Culture media collected from treated HT-29 cells were used as samples. Before kit analysis, the cell media were centrifuged (at 1000 x g for 10 minutes), and only the supernatant was collected. TOS results were expressed in µmol H_2O_2 equivalent/L, and TAC values were expressed in mmol Trolox equivalent/L. Absorbance measurements were performed at 520 nm for TOS and 660 nm for TAC, and standard curves were generated using the calibration standards provided with the kits (Quant, Bad Friedrichshall, Biotek, Germany) (19).

2.5. Superoxide Dismutase (SOD) Test: SOD is a key enzymatic component of the cellular antioxidant defense system that catalyzes the dismutation of superoxide radicals into hydrogen peroxide and molecular oxygen. In this study, SOD level was measured using a human-specific commercial ELISA kit (Superoxide Dismutase, BT Lab, Bioassay Technology Laboratory, Zhejiang, China; Cat. No.: E0918Hu), following the manufacturer's instructions. Culture media collected from the experimental groups were used as samples. Before kit analysis, the cell media were centrifuged (at 1000 x g for 10 minutes), and only the supernatant was collected. Results were expressed in ng/mL, based on the standard curve generated with the calibration standards provided in the kit. Absorbance was measured at 450 nm using a microplate reader (Quant, Bad Friedrichshall, Biotek, Germany).

2.6. Lactate Dehydrogenase (LDH) Test: LDH release was evaluated to assess membrane integrity and necrotic cell death. In this study, LDH level was determined in the culture media of the experimental groups using a human-specific commercial ELISA kit (L-lactate dehydrogenase, BT Lab, Bioassay Technology Laboratory, Zhejiang, China; Cat. No.: E5591Hu), following the manufacturer's instructions. Before kit analysis, the cell media were centrifuged (at 1000 x g for 10 minutes), and only the supernatant was collected. Results were expressed in ng/mL based on the standard curve generated using the calibration standards provided in the kit. Absorbance was measured

at 450 nm using a microplate reader (Quant, Bad Friedrichshall, Biotek, Germany).

2.7. Statistical Analysis: Statistical analyses were performed using GraphPad Prism version 9.5 (GraphPad Software, San Diego, CA, USA). Data are presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to assess differences between groups, followed by Tukey's post-hoc test for multiple comparisons. A *p*-value of less than 0.05 was considered statistically significant. The IC50 value was calculated using a non-linear regression (log(inhibitor) vs. normalized response) mathematical regression model with GraphPad Prism 9.5 software.

3. Results

3.1. MTT Assay: The MTT method was used to determine the cell viability of Trolox on the HT-29 cell line (Figure 1). Accordingly, after cells were exposed to Trolox for 24 hours, the negative control group and the 0.1-1-10-100 and 1000 µM concentration groups were compared with each other. The lowest dose (0.1 µM) showed a mild but statistically significant decrease (*p < 0.05), while all higher doses (1–1000 µM) resulted in highly significant reductions (**p < 0.001). The half-maximal inhibitory concentration (IC_{50}) of Trolox on HT-29 cells was calculated as 866.26 µM.

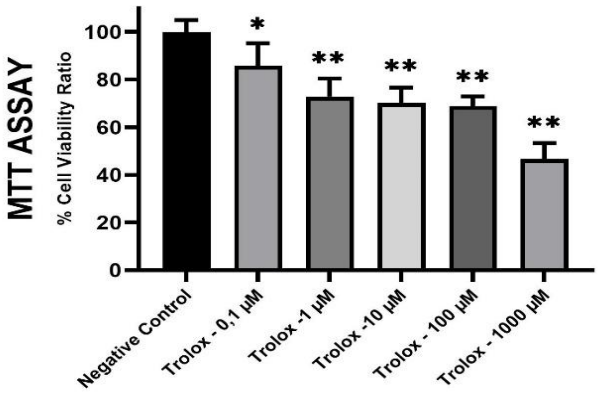

Figure 1. MTT Test Results. Results are means ± SD. (*p <0.05, **p <0.001, vs control, one-way ANOVA with Tukey's post-hoc test).

3.2. TOS and TAC: The effects of Trolox on oxidative balance in HT-29 cells were evaluated by measuring total oxidant status (TOS) and total antioxidant capacity (TAC) (Figure 2A and 2B, respectively and Table 1). In the TOS assay, only the highest concentration of Trolox (1000 µM) resulted in a statistically significant increase in oxidant levels compared to the negative control group (*p <0.05), while no significant changes were observed at lower concentrations (0.1–100 µM). In contrast, TAC analysis revealed a significant decrease in antioxidant capacity at 1000 µM (**p <0.001), with no significant differences observed at the lower doses. The lower doses did not result in any significant change when compared to the control.

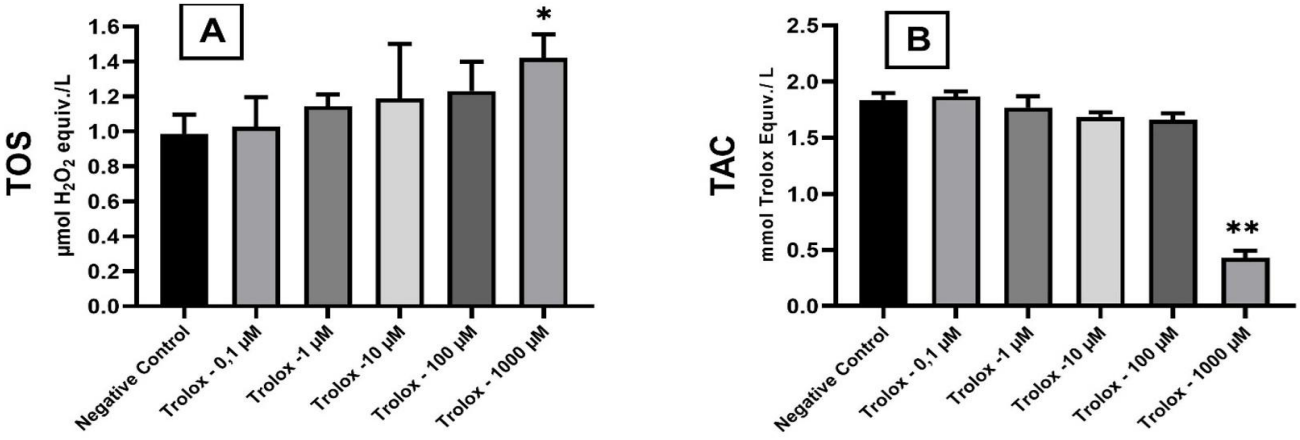

Figure 2. A) TOS Results **B)** TAC Results. Results are means ± SD. (*p <0.05 vs control, **p <0.001, vs control and others, one-way ANOVA with Tukey's post-hoc test).

3.3. SOD Test and LDH Test: The effects of Trolox on oxidative stress markers were evaluated by measuring superoxide dismutase activity and lactate dehydrogenase release in HT-29 cells (Fig. 3A and 3B, respectively and Table 1). According to the SOD assay results, Trolox treatment at concentrations of 10, 100, and 1000 µM significantly decreased SOD levels compared to the control group (*p <0.05). However LDH levels were significantly elevated at the same concentrations (*p <0.05) compared to control group.

and fueling tumor progression [21,22]. The persistent imbalance between ROS production and antioxidant defenses damages DNA, lipids, and proteins, thereby accelerating carcinogenesis via mutagenesis, lipid peroxidation, and disruption of key cellular functions [23]. Additionally, ROS modulate redox-sensitive signaling pathways that regulate transcription factors and apoptotic resistance, supporting tumor growth and metastasis [24]. Given this dual role of ROS as both damaging agents and signaling molecules,

Table 1. The values of TOS, TAC, SOD, and LDH levels in HT-29 cells following 24-hour Trolox treatment.

Group	TOS (µmol/L)	TAC (mmol/L)	SOD (ng/mL)	LDH (ng/mL)
Control	0.022 ± 0.002	0.359 ± 0.01	3.399 ± 0.18	1.443 ± 0.15
Trolox 0.1 µM	0.023 ± 0.003	0.364 ± 0.01	2.763 ± 0.49	2.101 ± 0.44
Trolox 1 µM	0.026 ± 0.001	0.346 ± 0.03	2.452 ± 0.16	2.607 ± 0.06
Trolox 10 µM	0.027 ± 0.007	0.333 ± 0.01	2.259 ± 0.11	3.093 ± 0.31
Trolox 100 µM	0.028 ± 0.003	0.328 ± 0.01	2.027 ± 0.21	3.160 ± 0.65
Trolox 1000 µM	0.032 ± 0.003	0.114 ± 0.01	2.105 ± 0.54	3.847 ± 0.50

Values: mean ± SD

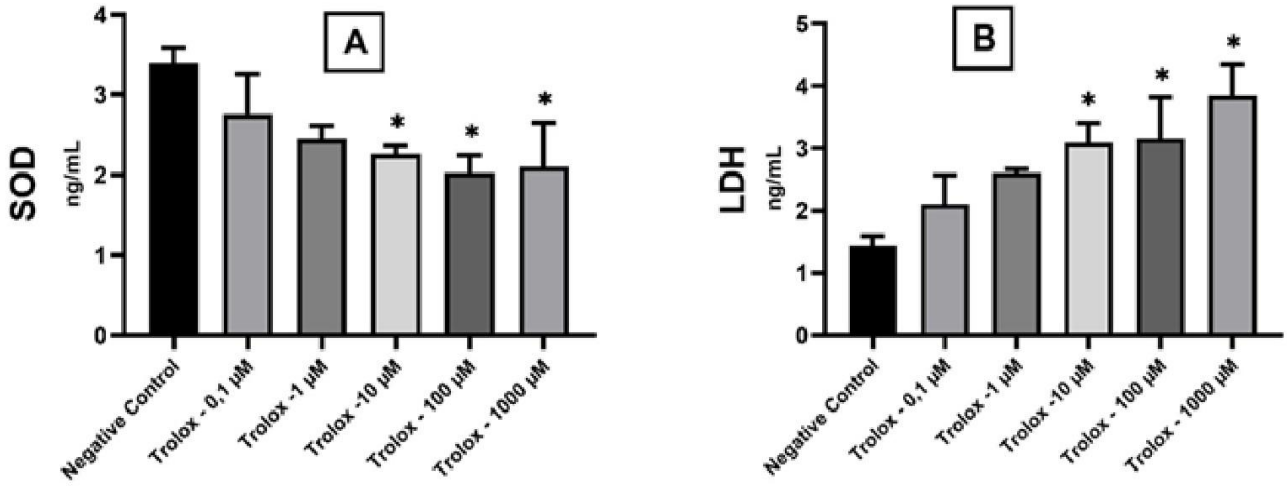

Figure 3. A) SOD test results **B)** LDH Test results. Results are means ± SD. (*p <0.05, **p <0.001, vs control, one-way ANOVA with Tukey's post-hoc test).

4. Discussion

Oxidative stress has emerged as a central driver in colorectal cancer pathogenesis, fundamentally altering cellular homeostasis

investigating redox-modulating compounds in CRC models has become increasingly relevant. In this context, our study aimed to evaluate the oxidative stress-related effects of Trolox, a known antioxidant, on HT-29 colorectal cancer

cells by assessing key oxidative parameters such as TOS, TAC, SOD, and LDH levels.

Due to their elevated metabolic activity, CRC cells (derived from the rapidly renewing intestinal epithelium) exhibit increased sensitivity to oxidative stress [25]. CRC cells, which originate from the rapidly renewing intestinal epithelium, exhibit increased vulnerability to oxidative stress due to their high metabolic activity [25]. Given this shared origin, CRC cells may retain certain vulnerabilities of their epithelial precursors, including sensitivity to redox imbalance. Previous studies have demonstrated that poorly differentiated colonocytes are particularly sensitive to ROS, indicating that oxidative stress not only contributes to tumor initiation but also shapes intratumoral heterogeneity and progression [26,27]. In our study, the dose-dependent changes in oxidative parameters (TOS, TAC, and SOD) observed in HT-29 cells support the relevance of redox imbalance in CRC. These findings highlight the potential of redox-modulating agents like Trolox as therapeutic candidates to mitigate oxidative stress–induced cellular damage.

The interaction between ROS and antioxidants is critical in cancer development [28,29]. Given this importance, regulating ROS levels is thought to be important for anticancer strategies. These techniques may inhibit ROS-induced carcinogenesis and cancer development by promoting oxidative damage and ROS-mediated cell death. Partial inhibition of apoptosis is a defining characteristic of colorectal cancer (CRC) and confers a survival advantage to tumors, rendering existing treatments ineffective [30]. The primary therapeutic modalities used for the treatment of colorectal cancer (CRC) include surgical intervention, chemotherapy, and radiation [31]. Nevertheless, owing to the adverse side effects of chemotherapeutic drugs and unfavorable prognoses after such treatments, there exists a need to innovate novel anticancer methodologies and substances characterized by minimal side effects and enhanced effectiveness.

Vitamin E serves as a crucial chain-breaking antioxidant in tissues and provides protective effects on membranes against lipid peroxidation. It is lipid-soluble and mostly situated in the plasma membrane, where it works as a scavenger of ROS and engages with free radicals, thus offering protection against oxidative stress [32].

Improved antioxidant activity as a result of greater cell permeability is the hallmark of trolox, a hydrophilic analogue of vitamin E. As a result, it prevents oxidative processes in water [33]. Rozanowska et al. showed that Trolox can slow down lipid peroxidation much better than its analogs even at low doses (34). Many in vivo and in vitro studies have shown that trolox reduces oxidative stress and inhibits cancer metastasis [11,35]. Moreover, Trolox specifically amplifies arsenic-induced apoptosis in many cancer cell types, including myeloma and breast cancer cells [36,37].

Our findings show that Trolox has a significant cytotoxic effect on HT-29 colon cancer cells, especially at concentrations of 100 µM and 1000 µM. According to our MTT test results, cell viability decreased to 68.87% at a 100 µM concentration and to 46.70% at a 1000 µM concentration. This dose-dependent cytotoxic effect is in line with similar studies in the literature. It was shown that Trolox showed significant anticancer effects, especially at high doses, and inhibited metastatic spread [35]. Similarly, it was reported that Trolox showed anticancer effects by inducing apoptosis [36].

The cytotoxic effect observed in our study can also be associated with the antioxidant

properties of Trolox. Indeed, the peroxyl radical scavenging property of Trolox and its regulatory effect on oxidative stress can trigger cell death in cancer cells [9]. In addition, the capacity of Trolox to prevent lipid peroxidation may explain its effect on cell membrane integrity [34].

Our MTT results show that Trolox has a limited effect on HT-29 cells at low doses (0.1-10 μM), while it has a significant cytotoxic effect, especially at concentrations of 100 μM and above. This dose-response relationship provides important information for the evaluation of the therapeutic potential of Trolox. The findings we acquired indicate that Trolox may be assessed as a prospective therapeutic agent for colorectal cancer therapy.

TAC and TOS analyses performed in our study confirm the antioxidant capacity of Trolox and clearly reveal its effect on oxidative stress parameters. Particularly, the significant decrease in TAC (**p < 0.001) and the concurrent increase in TOS ($p < 0.05$) observed at the 1000 μM concentration clearly indicate that Trolox loses its antioxidant capacity at high doses. Instead, it triggers a severe pro-oxidant response by depleting the cellular antioxidant reserves and exacerbating oxidative stress.

Changes in SOD activity showed statistically significant decreases, especially at 10, 100, and 1000 μM doses ($p < 0.05$). Superoxide dismutase is a crucial enzyme in cells that neutralizes free radicals by transforming them into hydrogen peroxide. The marked reduction in SOD activity observed in our study indicates that high concentrations of Trolox deplete the endogenous antioxidant defense mechanisms, ultimately inducing a severe pro-oxidant state. This depletion is likely due to the excessive consumption of SOD in an attempt to counteract the overwhelming oxidative stress generated by high-dose Trolox treatment. While previous studies, such as Bai et al. (2014), reported the capacity of Trolox to protect and regulate antioxidant enzyme activities under specific stress conditions, our results clearly demonstrate that in HT-29 colon cancer cells, high doses of Trolox overwhelm these defenses and significantly decrease SOD activity, which contributes to its cytotoxic effect.

The dose-dependent increase in LDH levels (*P<0.05 at 10, 100 and 1000 μM doses) is a significant indicator of changes in cell membrane integrity. This increase in LDH release indicates the effect of Trolox on the cell membrane at high doses and potential necrotic cell death. These membrane-level effects are associated with the capacity of Trolox to prevent lipid peroxidation and modulate membrane integrity [37].

When the changes in these biochemical parameters were evaluated together, it was concluded that Trolox modulated the oxidative stress response in HT-29 cells in a dose-dependent manner and showed a significant antioxidant effect, especially at high doses (100 and 1000 μM). These findings suggest that Trolox may induce oxidative stress-mediated cell death in colorectal cancer cells. The antioxidant and anti-inflammatory properties of Trolox were demonstrated and our study confirms these findings in a colorectal cancer cell line and provides new evidence for its therapeutic potential [9]. In addition, the antitumor activity of Trolox is in line with our findings [10].

4.1. Conclusion

The findings of our study demonstrated the anticancer effect of Trolox at concentrations of 100 and 1000 μM in HT-29 cells. In this way, it may be possible to use it in colon cancer diseases after preclinical and clinical research is carried out in the next stage.

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Ethical Statement: *Since this study was conducted on the HT-29 cell line, an ethics committee decision is not required.*

Language and AI assistance: *During the preparation of this work, the author(s) used Gemini and QuillBot strictly in order to improve English language readability, fix grammatical errors, and enhance the overall flow of the text. After using these tools, the author(s) thoroughly reviewed and edited the content as needed. The AI tools were not used for data generation, analysis, or drawing scientific conclusions. The author(s) take full responsibility for the final content of the publication.*

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