JBUON 2014; 19(4): 1055-1061
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
E-mail: editorial_office@jbuon.com

ORIGINAL ARTICLE

Enhanced cytotoxicity and apoptosis by thymoquinone in combination with zoledronic acid in hormone- and drug-resistant prostate cancer cell lines

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Summary

Purpose: *Thymoquinone (TQ), an active ingredient of black seed oil (Nigella Sativa), has been shown to possess cytotoxic activity against a variety of cancer cell lines. Our purpose was to investigate if the cytotoxic and apoptotic effect of zoledronic acid (ZA) can be enhanced by the addition of the TQ in hormone- and drug-refractory prostate cancer cells PC-3 and DU-145.*

Methods: *XTT cell proliferation assay was used to assess cytotoxicity; DNA fragmentation and caspase 3/7 activity were also measured.*

Results: *The combination of TQ and ZA resulted in a significant synergistic cytotoxic activity and DNA fragmentation when compared to any single agent alone, in a dose- and time-dependent manner. In addition, TQ and ZA combination increased the caspase 3/7 activity in PC-3 cell line, while this activity could not be demonstrated in DU-145 cell line.*

Conclusion: *TQ and ZA had minimal hematological and non-hematological toxicity profile compared to cytotoxic agents. So, this combination may be an alternative approach for patients who are unable to be treated by conventional treatments because of poor performance status.*

Key words: *apoptosis, prostate neoplasm, thymoquinone, zoledronic acid*

Introduction

Prostate cancer is the most common cancer in men and the second leading cause of cancer-related deaths in men in the United States [1]. The clinical behavior of prostate cancer ranges from a microscopic, well-differentiated tumor to an aggressive, invasive cancer that finally results in metastases. The most common sites for prostate cancer metastases are local lymph nodes, bones and the lungs [2]. While androgen deprivation therapy (ADT) is the initial systemic therapy of metastatic prostate cancer, for men with castration-resistant prostate cancer (CRPC) potential options include interference with androgenic stimulation of tumor growth (abiraterone), and taxane-based chemotherapy that all have been demonstrated to improve overall survival [3,4]. However, in men with castration-resistant prostate cancer, especially the post-docetaxel regimens cannot delay disease progression for a long period of time. Management of patients with advanced prostate cancer involves the sequential use of agents like cabazitaxel and abiraterone with the goals of prolonging survival and maintaining a good quality of life. The complexity and high costs of these treatment options prompted researchers to seek more efficient and cost-effective therapies [5].

Osteoclast inhibitors are an important adjunct to reduce the risk of skeletal complications

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Received: 09/10/2013; Accepted: 12/06/2014

of bone metastases. ZA, a nitrogen-containing biphosphonate, shows a great effect in slowing down the progression of skeletal-related events in CRPC patients [6]. In addition to having bone resorption inhibiting properties, ZA also exhibits anti-tumor effects [7]. The anti-tumor effects of ZA were interpreted as intervention in the mechanisms of osteoclastogenesis, apoptosis, angiogenesis inhibition and inhibition of tumor cell adhesion [8]. Biphosphonates were usually combined with chemotherapeutics in order to enhance cytotoxicity. Another benefit of combination therapies is that they reduce development of drug resistance since a tumor is less likely to be resistant to multiple drugs simultaneously.

TQ is one of the major bioactive component (30-48%) of *Nigella sativa L*, a plant that is named as black seed or black cumin [9]. The steam-distilled essential oil of it was investigated for its antioxidant and anti-inflammatory properties [10]. TQ has also been shown to have anti-neoplastic activity against a variety of cancer cell lines [11]. Its cytotoxic and pro-apoptotic effects were predominantly demonstrated in prostate cancer cell lines such as PC-3, LNCaP and DU-145 [12,13].

The aim of this study was to evaluate the additive and/or synergistic cytotoxic and apoptotic effects of TQ and ZA combination on PC-3 (hormone resistant and chemotherapy sensitive) and DU-145 (hormone and chemotherapy resistant) prostate cancer cell lines.

Methods

Cell lines and reagents

Human prostate cancer cell lines (PC-3 and DU-145) were obtained from ICLC (Genova, Italy). The cells were grown as monolayer adherent cell lines and were routinely cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin-streptomycin in 75 cm^2 polystyrene flasks (Corning Life Sciences, UK) and maintained at 37 °C in a humidified atmosphere with 5% CO_2. Growth and morphology were monitored and cells were passaged when they had reached 90% confluence. Cell culture supplies were obtained from Biological Industries (Israel). TQ was purchased from Sigma-Aldrich St. Louis, MO and a stock solution of TQ (10 μM) was prepared in dimethyl sulphoxide (DMSO). ZA was a generous gift from Novartis Pharmaceuticals Inc (Basel, Switzerland) and prepared in distilled water (10 μM). Final dilutions were made immediately before use, and new stock solutions were prepared for each experiment. DMSO concentration in the assay was <0.1%, which had no cytotoxic effect on the tumor cells. All other chemicals, unless mentioned, were purchased from Sigma Chemical Co.

XTT viability assay

For the viability assay, after verifying cell viability using the trypan blue dye-exclusion test in a Cellometer automatic cell counter (Nexcelom Inc., Lawrence, MA, USA), cells were seeded at 10^4/well in 200 μl into 96-well flat-bottom microtitre plates with or without different concentrations of the drugs. Plates were incubated at 37 °C in a 5% CO_2 incubator for the indicated periods. At the end of incubation, 100 μl of XTT (2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide) (Roche Applied Science, Mannheim, Germany) were added to each well, and plates were incubated at 37 °C for a further 4 hrs. Absorbance was measured at 450 μM against a reference wavelength at 650 μM using a microplate reader (DTX 880 Multimode Reader, Beckman Coulter, Fullerton, CA, USA). The mean of triplicate experiments for each dose was used to calculate the 50% inhibitory concentration (IC_{50}) and the combination index (CI) values.

Evaluation of apoptosis by DNA fragmentation analysis

Apoptosis was measured with a Cell Death Detection ELISA Plus Kit (Roche Applied Science, Germany) according to the manufacturer's instructions. The relative amounts of mono- and oligo-nucleosomes generated from the apoptotic cells were quantified using monoclonal antibodies directed against DNA and histones by ELISA. Briefly, the cytoplasmic fraction of the untreated control, TQ-, ZA- and combination treated cells were transferred onto a streptavidin-coated plate and incubated for 2 hrs at room temperature with a mixture of peroxidase conjugated anti-DNA and biotin labeled anti-histone. The plate was washed thoroughly, incubated with 2,29-azino-di-[3-ethylbenzthiainesulphonate] diammonium salt, and the absorbance was measured at 405 μM with a reference wavelength at 490 μM (DTX 880 Multimode Reader, Beckman Coulter, Fullerton, CA, USA).

Caspase 3/7 activity assay

Caspase 3/7 activity was evaluated by using the Caspase-Glo 3/7 Assay (Promega, Madison, WI) according to the manufacturer's instructions. Briefly, 10^4 prostate cancer cells/well were plated in a 96-well plate in 100 μl culture medium with increasing concentrations of trabectedin for 72 hrs. Then 100 μl of Caspase-Glo 3/7 reagent were added to each well and the plates were incubated at room temperature for 1 h. Finally, the luminescence of each sample was measured by DTX 880 Multimode Reader (Beckman Coulter, Miami, FL).

Statistics

All experiments were conducted in triplicate and the results expressed as the mean ± SD, with differenc-

Table 1. The IC_{50} value of TQ and ZA on human prostate cancer cells

Cell line	*TQ (μM)*	*ZA (μM)*
PC-3	55.3	95.0
DU-145	51.0	72.9

es assessed statistically by Student's t-test. Statistical significance was set at $p<0.05$. The median dose-effect analysis was used to assess the interaction between agents. Determination of the synergistic vs additive vs antagonistic cytotoxic effects of the combined treatment of cells with TQ and ZA were assessed by Biosoft CalcuSyn program (Ferguson, MO, USA). CI was used to express synergism (CI <1), additive effect (CI=1), or antagonism (CI >1) [14].

Table 2. Combination index (CI) values of TQ or ZA alone and their combination on growth inhibition of PC-3 and DU-145 cells. Combination index (CI) values were calculated from the XTT cell proliferation assays, according to CalcuSyn software. The CI was used to express synergism (CI<1), additive effect (CI=1), or antagonism (CI>1), where CI < 0.5 represents strong synergism

	CI value - Interpretation	
Combination	*PC-3*	*DU-145*
40 TQ + 60 ZA	0.429-strong synergism	0.467- strong synergism
60 TQ + 40 ZA	0.421- strong synergism	0.535- synergism
60 TQ + 60 ZA	0.404- strong synergism	0.429- strong synergism

Results

Effects of TQ and ZA on the viability of PC-3 and DU-145 prostate cancer cells

To evaluate the cytotoxic effects of ZA and TQ alone on the viability of human prostate cancer cells, PC-3 and DU-145 were exposed to increasing concentrations of ZA (20 to 120 μM) and TQ (1 to 120 μM) for 24, 48 and 72 hrs, and XTT cell proliferation assay was performed. ZA and TQ alone decreased the cell viability in a time- and dose-dependent manner both in PC-3 and DU-145 cells. There was 18.5, 36.5, and 85 % decrease in cell viability of PC-3 cells exposed to 40, 100, and 120 μM of ZA, respectively, when compared to untreated controls at 72 hrs ($p<0.05$). In addition, there was 35, 58.5, and 59 % decrease in cell viability of DU-145 cells exposed to 40, 100, and 120 μM of ZA, respectively, when compared to untreated controls at 72 hrs ($p< 0.05$). Highest cytotoxicity was observed at 72 hrs and IC_{50} values of ZA were found to be 95.0 and 72.4 μM in PC-3 and DU-145 cells, respectively (Table 1).

We also examined the effect of TQ on PC-3 and DU-145 cells. There was 10, 13, and 54 % decrease in cell viability of PC-3 cells exposed to 10, 20, and 40 μM of TQ, respectively, when compared to untreated controls at 72 hrs ($p<0.05$). In DU-145 cells, there was 6, 12, and 36 % decrease in cell viability of DU-145 cells exposed to 10, 20, and 40 μM of TQ, respectively ($p<0.05$). Highest cytotoxicity was observed at 72 hrs and IC_{50} values of TQ in PC-3 and DU-145 cells were found to be 55.3 and 51 μM in PC-3 and DU-145 cells, respectively.

Synergistic effects of TQ and ZA in PC-3 and DU-145 prostate cancer cells

To examine the possible synergistic/additive effects of ZA and TQ combination, PC-3 and DU-145 cells were exposed to different concentrations of each agent alone, and in combination of both for 24, 48 and 72 hrs. Figures 1A and 1B clearly demonstrate the synergism between TQ and ZA on prostate cancer cells. We also calculated the CI values for these agents from the XTT cell proliferation assay by using the CalcuSyn software. Our results indicated that 60 μM ZA and 40 μM TQ achieved 22 and 60% decrease, respectively, in cell viability of PC-3 cells but the combination of both resulted in 80% decrease in cell viability ($p<0.05$) (Figure 1A). In DU-145 cells, 40 μM ZA and 60 μM TQ showed 18.5% and 59% decrease, respectively, in cell viability, but the combination of both resulted in 79% decrease in cell viability ($p<0.05$) (Figure 1B). The concentrations for each agent, found to be strongly synergistic in PC-3 and DU-145 cells, are presented in Table 2.

Effects of the sequential treatment

Combination of ZA and TQ resulted in significant synergism at 72 hrs. Sequential administration of the agents were carried out to see if either of these agents enhances the other one's effect and to understand whether the synergism depended on which agent applied first. We examined the effect of sequential treatment of PC-3 and DU-145 cells with either ZA or TQ and subsequent treatment with the second agent. Pretreatment of tumor cells with ZA for 36 hrs, washing and then treating them for an additional 36 hrs with TQ resulted in synergistic cytotoxicity in PC-3 and DU-145 cells. Also, pretreatment of tumor cells with TQ for 36 hrs, washing and then treating them for an additional 36 hrs with ZA resulted in synergistic cytotoxicity in PC-3 and DU-145 cells (data not shown). We concluded that synergistic cyto-

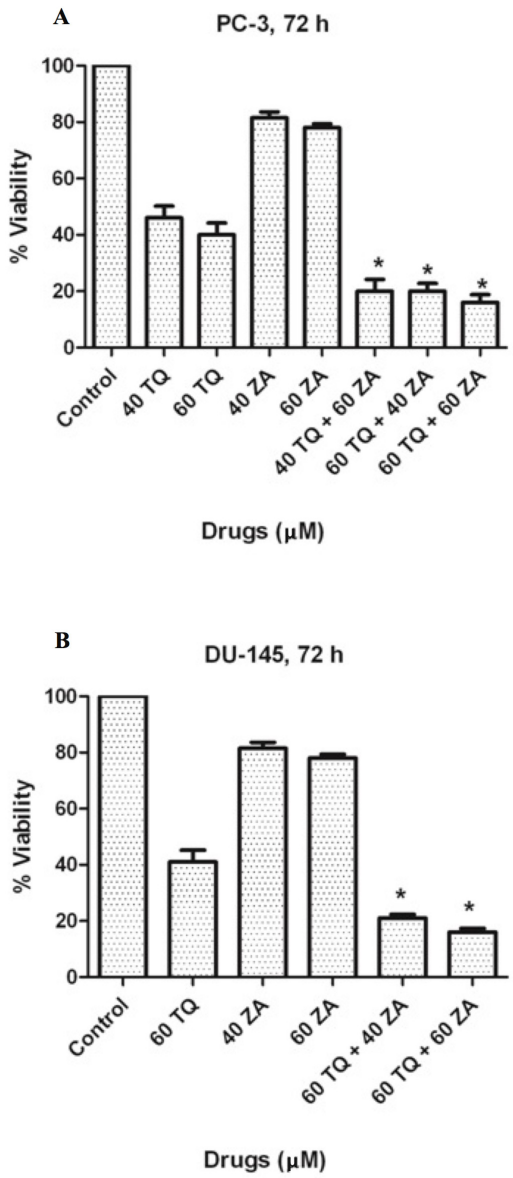

Figure 1. Effects of combinatorial treatment of ZA with TQ on the viability of PC-3 (**A**) and DU-145 (**B**). Cytotoxicity was determined by the XTT cell viability test at 72 hrs. *p<0.05 compared to the control group.

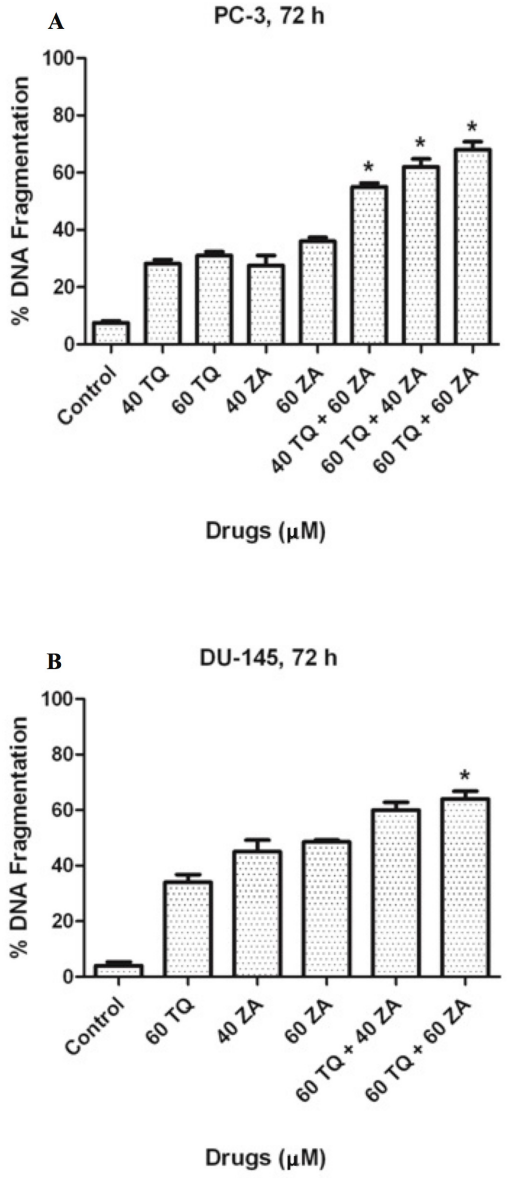

Figure 2. Effects of combinatorial treatment of ZA with TQ on the percent changes in DNA fragmentation of PC-3 (**A**) and DU-145 (**B**) cells were determined to show apoptosis. The bar graphs values represent the means ±SD of three experiments. *p<0.05 compared to each agent alone.

toxicity was observed no matter which agent was applied first in both cell lines.

Apoptotic effects of TQ and ZA alone or in combination in PC-3 and DU-145 prostate cancer cells

To examine the induction of apoptosis in response to ZA and TQ alone or in combination, human prostate cancer cells were exposed to increasing concentrations of drugs for 72 hrs and the levels of mono- and oligo-nucleosome fragments were quantified using Cell Death Detection Kit. Our results clearly showed that ZA and TQ alone induced apoptosis in a dose-dependent manner. In 60 μM ZA or 40 μM TQ alone exposed PC-3 cells, 4.8- or 3.76-fold increase respectively was observed, but in the combined treatment 7.3-fold increase was noticed in DNA fragmentation as compared to untreated controls (p<0.05); when PC-3 cells was exposed to 40 μM ZA or 60 μM TQ alone, 3.6- or 4.13-fold increase in DNA fragmentation was observed respectively, but their combined treatment resulted in 8.26-fold increase

in DNA fragmentation as compared to untreated controls ($p<0.05$). In addition to 60 μM ZA or 60 μM TQ alone exposed PC-3 cells, 4.8- or 4.13-fold increase in DNA fragmentation was observed respectively, but their combined treatment resulted in 9.06-fold increase in DNA fragmentation as compared to untreated controls ($p<0.05$) (Figure 2A). There were 6.4- or 8.3-fold increase in DNA fragmentation in 60 μM ZA or 60 μM TQ exposed in DU-145 cells respectively, while the combination treatment resulted in 8.53-fold increase in DNA fragmentation as compared to untreated controls ($p<0.05$). However, there was 6- or 8.3-fold increase in DNA fragmentation in 40 μM ZA or 60 μM TQ alone exposed DU-145 cells respectively, but 8.0-fold increase was observed in the combined treatment as compared to untreated controls ($p>0.05$) (Figure 2B).

Caspase 3/7 enzyme activity in PC-3 and DU-145 cells exposed to combination treatment

Next, we investigated the molecular mechanisms underlying apoptosis induced by combination treatment in prostate cancer cells. For this aim, we evaluated the possible role of the executioner DEVD caspases 3/7. The combination of ZA with TQ induced caspase 3/7 activity in a synergistic manner as compared to any agent alone in PC-3 cells at 72 hrs ($p<0.05$). However, the combination of ZA with TQ did not affect the activation of caspase 3/7 activity in DU-145 cells ($p>0.05$).

Discussion

This study provided evidence that treatment of PC-3 and DU-145 prostate cancer cells with a combination of TQ and ZA resulted in a significant synergistic cytotoxic activity and apoptosis. This effect was observed in a dose- and time-dependent manner. Besides, this is the first study in the literature evaluating the efficiency of TQ and ZA combination in prostate cancer cell lines.

The anticancer effects of TQ depend on the inhibition of proliferation, angiogenesis, invasion and metastasis [15]. TQ reduces DNA synthesis in PC-3, DU-145 and LNCaP cell lines in a dose- and time-dependent manner while it has no effect in benign prostatic hyperplasia (BPH-1) epithelial cell line. In this study, TQ ceased the LNCaP cells between G1 and S phase and this inhibition was found to be related with the cell cycle regulatory protein E2F-1 and androgen receptor suppression [16]. Another study investigated the mechanism of action of TQ in androgen receptor (AR)-independent (C4-2B) and AR naive (PC-3) prostate cancer cells. Exposure to TQ inhibited the growth of both C4-2B and PC-3 cells, with IC_{50} values of approximately 50 and 80 μmol/L, respectively. Within one hour, TQ increased reactive oxygen species (ROS) levels (3-fold) and decreased glutathione (GSH) levels (60%) in both cell types. TQ did not increase the activity of caspases and the caspase inhibitor z-VAD-FMK did not decrease TQ-induced apoptosis. Furthermore, Koka et al. concluded that TQ-induced cell death was primarily due to increased ROS generation and decreased GSH levels, and was independent of AR activity [12]. Zubair et al. observed that TQ even at lower concentrations (5-10 μM) showed cytotoxicity in PC-3 and LNCaP cell lines and its cytotoxic effect was mediated through apoptotic pathways [17]. They also proved the dependency of TQ mediated apoptosis on ROS as several antioxidants like catalase, superoxide dismutase and thiourea were used to inhibit the apoptotic effect.

In our study, IC_{50} values for TQ were found to be similar with previous published studies (PC-3 IC_{50}:55.3; DU-145 IC_{50}:55) [12,18]. TQ in combination with ZA had a strongly synergistic cytotoxic effect, which was detected with the CalcuSyn software. TQ and ZA combination inhibited the cell viability of hormone- and drug-refractory prostate cancer cells at *in vivo* achievable therapeutic concentrations. Additionally, we demonstrated that there were drug concentration dependent increases in DNA fragmentation and caspase 3/7 enzyme activity in PC-3 cells exposed to TQ / ZA combination but did not observe caspase activation in DU-145 cells. The reason of apoptosis in our DU-145 cell lines may be due to the ROS-dependent apoptotic mechanisms of TQ as there was no role of caspase activation like in the studies of Koka et al. and Zubair et al. [12,17].

For frail or elderly prostate cancer patients, the tolerance of cytotoxic chemotherapeutics may be lower. TQ and ZA combination seems to be less toxic than docetaxel or cabazitaxel-based treatments. So, it may be a more appropriate treatment option for patients with low performance status. Anticancer drugs are generally used in combination in order to prevent drug resistance and increase their efficiency. With respect to this, TQ was combined with various chemotherapeutic drugs in different cancer cell lines. Effenberger-Neidnicht et al. found a significant growth inhibition by doxorubicin in HL-60 leucemia cells and multidrug-resistant MCF-7/TOPO cells when TQ had been added [19]. Another study demon-

strated that combination of TQ and cisplatin was well tolerated and significantly reduced the tumor volume without additional toxicity in non-small and small cell cancer cell lines [20].

Zoledronic acid, either alone or in combination with other drugs, showed cytotoxicity on prostate cancer cells in several studies [21,22]. It also reduced the incidence of skeletal-related events better than placebo in men with castration-resistant metastatic prostate cancer patients [23]. Therefore, ZA is commonly used in all treatment lines of prostate cancer patients with bone metastasis. Despite these benefits, ZA has some serious adverse effects like renal toxicity and jaw osteonecrosis which limits its usage. Renal toxicity of ZA is thought to be due to acute tubular necrosis [24]. On the other hand, TQ has some protective effects on cisplatin-induced nephrotoxicity, significantly reducing serum urea and creatinine, and significantly improving polyuria and creatinine clearance [25]. From this aspect, it is rational to think that TQ in combination with ZA may ameliorate or even prevent ZA-induced renal toxicity. The pathophysiology of jaw osteonecrosis in patients taking bisphosphonates depends on the impairment of bone and vascular repair mechanisms, and impaired angiogenesis [26,27]. Kara et al. evaluated the effect of systemic TQ in a rat rapid maxillary expansion model and demonstrated that systemic use of TQ may be effective in accelerating new bone formation, number of capillaries and the amount of inflammatory cells in maxillary sutures [28]. As a result of these data, in addition to its various chemopreventive effects, TQ may also have a role in preventing the osteonecrosis of jaw which is a complication of long-term, high-dose bisphosphonate therapy [29-31].

Conclusion

This is the first report of a synergistic combination of ZA and TQ, which inhibits cell viability and induces apoptosis in PC-3 and DU-145 prostate cancer cells. The results of this study present the translational potential of our *in vitro* findings and offer a basic rationale for the design of new combinatorial strategies with ZA and phosphatase inhibitors for the treatment of prostate cancer, which may develop resistance to conventional therapy. As prostate cancer is mainly a disease of elderly men, new therapeutic strategies with fewer side effects than cytotoxic treatment are very important. So, this new combination strategy may be an alternative approach for poor performance status patients who are unable to be treated by conventional treatments.

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