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Study on the apoptosis of Raji cell line induced by arsenic trioxide and its correlation with Survivin gene*

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Abstract ***Objective:*** To investigate the apoptosis induction by arsenic trioxide (As_2O_3) in Raji cells and its correlation with cell cycle arrest and expression of the Survivin gene. ***Methods:*** After Raji cells were treated with As_2O_3 in different concentrations (1, 2, 4 and 8 μM), for 24, 48 and 72 h, respectively, and cell proliferation was tested by MTT assay. Apoptosis was observed with electron microscope and DNA electrophoresis. The distribution of cell cycles and cell apoptosis were detected by flow cytometry. Expression of the Survivin gene was determined by real-time quantitative RT-PCR. ***Results:*** As_2O_3 (1–8 μM) inhibited Raji cells growth effectively in a dose- and time-dependent manner. As_2O_3 at 2–8 μM could induce cell apoptosis and cell cycle arrest. However, As_2O_3 (1 μM) inhibited Raji proliferation only by cell cycle arrest, without any symptoms of cell apoptosis. At the same time, Survivin gene expression was down-regulated after the treatment. ***Conclusion:*** As_2O_3 could induce substantial proliferation inhibition, cell cycle arrest and apoptosis in Raji cell. Cell cycle arrest might be a reason why apoptosis occurs. As_2O_3 can markedly down-regulate expression of the Survivin gene in a dose- and timedependent manner. The down-regulated Survivin gene might be leading to cell apoptosis by As_2O_3.

Key words arsenic trioxide (As_2O_3); lymphoma; apoptosis; Survivin gene

It is shown [1] that the incidence of lymphoma in the world has increased by years. Chemotherapy-resistance and recrudescence are two main barriers, despite the development of therapeutic methods and improvement of curative effects. Searching for a safe and effective therapy approach is the current research focus [2]. Molecular targeting therapy that can induce cell apoptosis has become the noticeable therapeutic method and been applied to cure lymphoma gradually [3].

Recent studies have shown that Survivin, a newly discovered member of the inhibitor of apoptosis protein (IAP) family, plays a dual role in suppressing apoptosis and regulating cell division [4]. Survivin is regulated in a highly cell cycle-dependent manner, with a remarkable increase in the G2/M phase. Also Survivin gene overexpressed in most common human cancers but is generally undetectable in normal tissues [5]. The selective overexpression of Survivin has also been associated with cancer occurrence and development, resistance to therapy and poor clinical outcome in cancer patients [6]. However, it is not clear whether the Survivin gene has been involved in apoptosis induced by As_2O_3 in lymphoma cells [7]. In the present study, we used human Burkitt's lymphoma cell line as a model, *in vitro*, and evaluated the effects of As_2O_3 on Raji cells and their relation with cell cycle arrest and expression of the Survivin gene.

Materials and methods

Reagents and drugs

As_2O_3 injection (1.0 g/L) was provided by Harbin Yida Co. Ltd (China). It was solubilized with distilled water to 1, 2, 4 and 8 μM before use. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, RPMI1640 and fetal bovine serum were purchased from Hangzhou Sjiqing Biological Engineering Materials Co. Ltd (China). PI (Propidium iodide) was obtained from Sigma Chemical Co. (USA). RNA extracted and reverse transcribe reagents were provided by Fermentas Co. SYBR-Green Ⅰ dye was

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Cell culture

Raji cell (human Burkitt's lymphoma cell line) was purchased from the Cell Bank of Shanghai Institutes of Life Sciences under the Chinese Academy of Science. Cells were cultured in RPMI-1640 medium and supplemented with 15% fetal bovine serum and 5% CO_2 at 37 ℃. When cells were in the log growth period that could be used for studies, we adjusted the cell concentration and experimented.

MTT assay

Cells (2×10^5 cells/mL in 96-wells microplate) were incubated without or with different concentrations (1, 2, 4 and 8 μM) of As_2O_3 for different times (24, 48 or 72 h). At the end of drug exposure, 10 μL of MTT was directly added to microplate and kept at 37 ℃ for 4 h. The formazan converted from tetrazolium salt by viable cell was solubilized by 100 μL of lysis solution containing 10% SDS, 5% isobutanol and 0.012 M HCl. Cell proliferation was measured in terms of optical absorbance (OD) per well by a micro plate reader at a wave length of 570 nm. Both the growth inhibition rate and the 50% growth-inhibitory concentration (IC_{50}) value for As_2O_3 were calculated.

Electronic microscope

After treatment without and with 1–8 μM As_2O_3 for different times (24, 48 or 72 h), cells were washed, fixed, dehydrated and embedded in paraffin, sliced into sections, and dyed with uranium acetic acid and lead nitrate according to routine procedure. Raji cells were then examined under an electronic microscope (Philips, Tecnai 10).

DNA electrophoresis

After 72 h, DNA of all treated and untreated cells (5×10^6 cells/group) were extracted with phenol-chloroform. An identical amount (20 μL) of DNA samples were run on one percent agarose gel at 100 V for 1 h. After electrophoresis, DNA electrophoresis was visualized by UV light illumination.

Flow cytometry

A flow cytometric evaluation of apoptosis and the cell cycle status of the treated and untreated cells were performed. Briefly, the cells (10^6 cells/group) were centrifuged, washed twice with PBS and fixed with a cold 70% ethanol liquid for at least 24 h, at which point they were stained with a PI solution for 30 min. The sample was read on a Coulter Epics XL flow cytometer (Beckman-Coulter, Inc., USA) and the percentage of cells in the apoptotic sub-G1 G1 phase, S phase and G2/M phases were calculated using CELL Quest software. At least 10 000 cells were counted every time.

Real-time PCR

Total RNA were isolated from Raji cell line that were treated by different As_2O_3 concentrations and negative control group by using TRIZOL reagent. Quantification of RNA was determined by UV-Spectrometer, and cDNA were synthesized with 2 μg total RNA by Fermentas corporation reagent. The primer of Survivin gene and internal reference gene (β-actin) were synthesized by Shanghai Sango Co. (China). The Survivin primers were 5'-TTCTTGGAGGGCTGCGCCT-3' (forward) and 5'-CCTGGTAGTGGTGCAGCCA-3' (reverse), the products were 401 bp. The β-actin primers were 5'-AACGGCTCCGGCATGTGCAA-3' (forward) and 5'-CTTCTGACCCATGCCCACCA-3' (reverse), the products were 117 bp. Amplication condition were 94 ℃ for 5 min, 94 ℃ for 30 s, 55 ℃ for 30 s, 72 ℃ for 30 s, and 90 ℃ for 5 s for 35 cycles, followed by incubation at 72 ℃ for 5 min (ABI 9600 PCR appearance). The amplified Survivin cDNA of 401 bp and β-actin of 117 bp were separated on 1% agarose gel and shown by UV light illumination. Both of the PCR products were sequenced by Beckman Co. (USA). The negative control group cDNA, extracted from untreated Raji cells, was also amplified in the above mentioned methods. Quantification of PCR production was determined by the UV-spectrometer. According to quantification, we calculated the Survivin gene copies in per microlitre PCR products. Then PCR products were diluted from 10^9 to 10^3 copies/μL as quantitative template to generate a standard curve.

Amplication systems as follows: SYBR-Green I dye (including TaqE) 25 μL; P1 1 μL; P2 1 μL; template 2 μL; dH_2O 21 μL.

Amplication condition were 94 ℃ for 5 min, 94 ℃ for 30 s, 55 ℃ for 30 s, 72 ℃ for 30 s for 35 cycles, followed by incubation at 89℃ for 5 s, 72℃ for 5 min (ABI 5700 PCR appearance).

After real time FQ-PCR amplification, the standard curve established by 10^8, 10^7, 10^6, 10^5, and 10^4 copies/μL templates showed a fine linear relationship between threshold cycle and template concentration. Then both of the treated Raji cell's cDNA (1, 2, 4 and 8 μM As_2O_3) and the positive template (10^8, 10^7, 10^6, 10^5, and 10^4 copies/μL) that were extracted from different periods (24, 48, or 72 h) were amplified by the real time FQ-PCR to calculate the Survivin gene quantity.

Statistical analysis

The software package SPSS 10.0 was used for statistical analysis. The data was presented as mean ± standard deviation (SD). The F test was used to compare the means of two groups. P value less than 0.05 was considered statistically significant.

Table 1 Effects of As_2O_3 on the growth of Raji cells ($\overline{\chi} \pm SD$, $n = 3$)

As_2O_3 concentration (μM)	Inhibitory rate (%)		
	24 h	48 h	72 h
8	54.46 ± 4.29	73.41 ± 5.08	92.85 ± 2.81
4	34.18 ± 4.51	49.46 ± 5.20	82.49 ± 4.97
2	22.65 ± 4.02	31.90 ± 1.87	53.68 ± 9.79
1	10.28 ± 1.31	22.04 ± 2.93	38.74 ± 6.54
IC_{50}	6.84 ± 0.38	3.60 ± 0.44	1.51 ± 0.21

Raji cells were treated with 1–8 μM As_2O_3 for 24, 48 and 72 h, the inhibitory rates of different groups were analyzed by F test. $F = 58.161$, $P < 0.05$. The differences were significant

Results

The inhibitory effects of As_2O_3 on the growth of Raji cells were measured by MTT assay

To evaluate the inhibitory effects of As_2O_3 on the growth of Raji cells, the cells were treated with 1–8 μM of As_2O_3 for 24, 48 and 72 h, and the cell viability was determined by MTT assay. As_2O_3 inhibited the growth of Raji cells in a time- and dose-dependent manner. IC_{50} of 48 h time course were 3.60 ± 0.44 μM (Table 1 and Fig. 1).

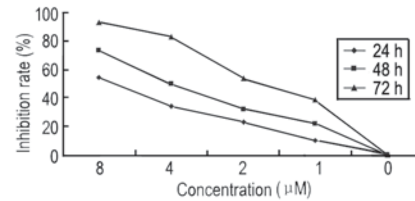

Fig. 1 Effects of different concentrations As_2O_3 on Raji cells

Apoptosis was observed with an electronic microscope

Being treated with different concentrations (1–8 μM)of As_2O_3, many Raji cells visualized remarkably altered shape and most exhibited apoptotic bodies, a few of them even appeared necrotic changes. After 72 h, however, under 1 μM Raji cells presented no obvious change in morphology. Chromatins of untreated cells (control group) showed asymmetry (Fig. 2a). Chromatins of treated cells twisted and some vacuole structures could be seen. Then the nucleolus agglomerated and behaved a crescent shape (Fig. 2b). The whole nucleolus exhibited condensation. There was a clear border between the cytoplasm and the nucleolus membrane. At last, the nucleolus was divided into two or more fragments (Fig. 2c). Apoptosis bodies came into being (Fig. 2d).

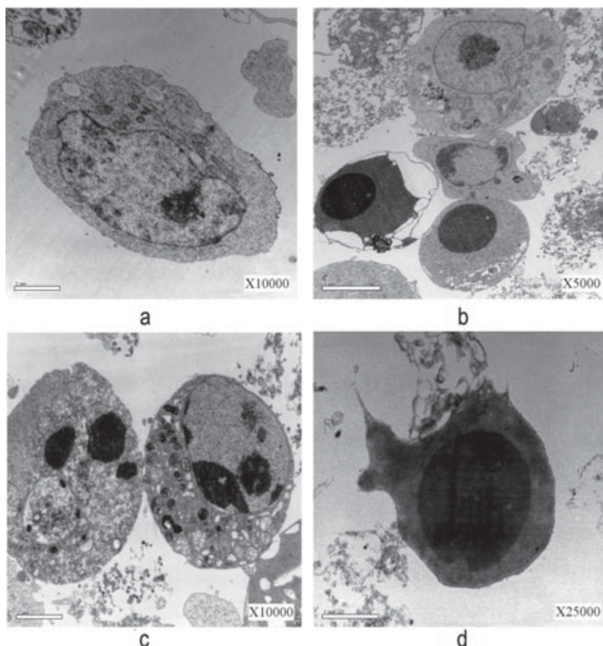

Fig. 2 (a) Nomal Raji cell; (b) Apoptosis Raji cell – chromatin agglomerated; (c) Apoptosis Raji cell – nucleolus disintegrated; (d) Apoptosis Raji cell – apoptosis body

Agarose gel electrophoresis DNA of the apoptosis Raji cells

Apoptosis is characterized by a fragment of the genomic DNA. These DNA fragments have a length of about 180 base pairs or multiples (360, 540, 720, ...). In agarose gel electrophoresis these DNA fragments form a distinctive ladder pattern. In 72 h, the ladder was visible only after treatment with 8μM As_2O_3 group, other groups could not be seen (Fig. 3).

The cell apoptosis was detected by flow cytometry

Flow cytometric analysis showed a hypodiploid apoptotic peak before the G1 phase of cell cycle in Raji cells after 2, 4, 8 μM treatments from 24 to 72 h. The peak

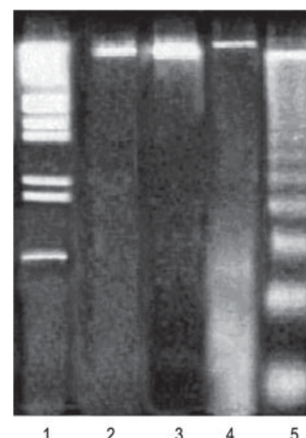

Fig. 3 DNA fragments of apoptosis Raji cell treated with As_2O_3 in various concentrations for 72 h were electrophoresed in agarose gell. Lane 1: 100 bp DNA marker; Lane 2: 1 μM; Lane 3: 2 μM; Lane 4: 4 μM; Lane 5: 8 μM

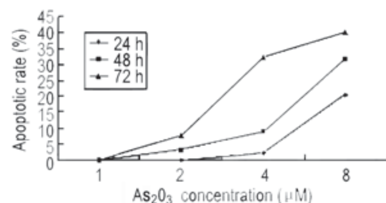

Fig. 4 Effects of different concentrations As_2O_3 on the apoptotic rate of Raji cells

value increased with the elevation of As_2O_3 concentration. The apoptosis cells increased too. But the Raji cells treated with 1 μM As_2O_3 had not shown hypodiploid apoptotic peak within 24 to 72 h (Fig. 4 and 5).

The distribution of cell cycles was detected by flow cytometry

In 1, 2 μM As_2O_3, we found that Raji cells were arrested at the G2/M phase of treatment compared to the control group. But the percentage of the cells at G2/M and S phase significantly decreased when treated with 4–8 μM of As_2O_3 in a dose dependent manner for hours. And the percentage of the cells at the G0/G1 phase increased gradually. Apoptotic rates increased too (Table 2).

The result of Survivin mRNA expression determined by FQ-PCR

The amplified Survivin cDNA of 401 bp and β-actin cDNA of 117 bp were separated on a agarose gel and visualized by UV light illumination (Fig. 6). Different expression levels of Survivin mRNA could be observed between different As_2O_3 treated concentrations and times by FQ-PCR. Expression of the Survivin gene could be down-regulated by 1–8 μM As_2O_3. Also, along with the time elapsed, Survivin expression decreased more than the control group. The above data indicated that As_2O_3 (1–8 μM) could inhibit Raji cells growth effectively in a dose- and time-dependent manner. The down-regulated Survivin gene might be lead to the cell apoptosis by As_2O_3 (Fig. 7).

Discussion

Survivin is a recently characterized IAP protein [8], which is found abundantly expressed in solid and hematology malignancies, but which is undetectable in most normal adult differentiated tissues. There is accumulating evidence that Survivin is associated with both cancer progression and drug resistance [9]. However, the mechanism by which Survivin blocks apoptosis has remained controversial. Some researchers demonstrated that Survivin could bind to the effector caspase-3/7 and caspase-9 *in vitro* and proposed that Survivin may block apoptosis through inhibiting caspase activity *in vivo* [10]. And it has

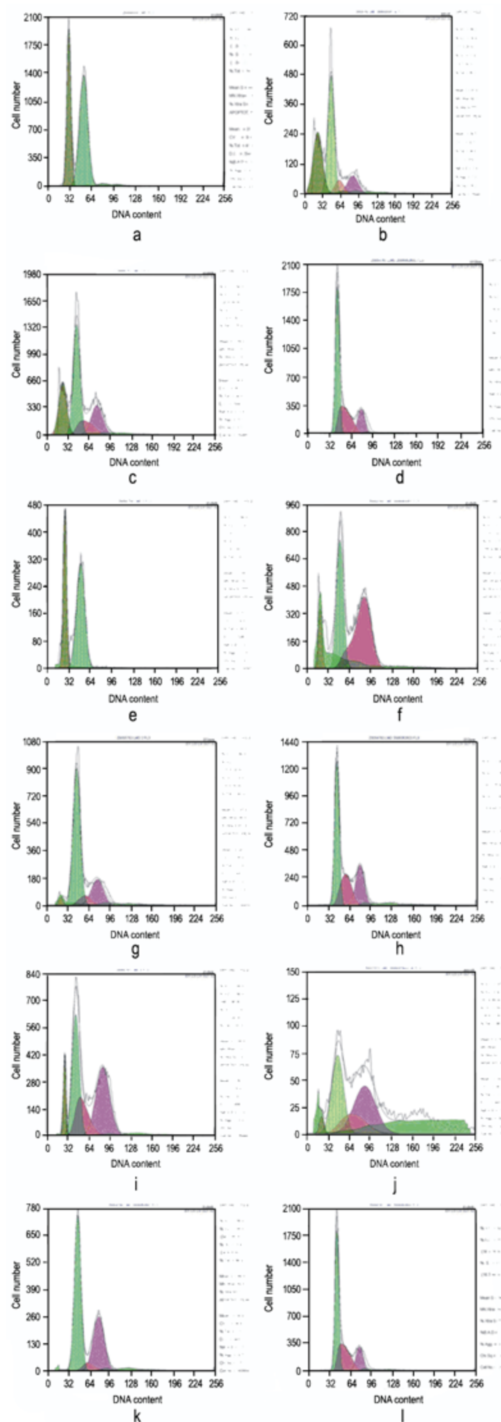

Fig. 5 Determination of cell apoptotic rate with PI by FCM. (a) Raji cell treated with 8 μM As_2O_3 for 72 h; (b) Raji cell treated with 8 μM As_2O_3 for 48 h; (c) Raji cell treated with 8 μM As_2O_3 for 24 h; (d) Raji cell treated with 8 μM As_2O_3 for 0 h; (e) Raji cell treated with 4 μM As_2O_3 for 72 h; (f) Raji cell treated with 4 μM As_2O_3 for 48 h; (g) Raji cell treated with 4 μM As_2O_3 for 24 h; (h) Raji cell treated with 4 μM As_2O_3 for 0 h; (i) Raji cell treated with 2 μM As_2O_3 for 72 h; (j) Raji cell treated with 2 μM As_2O_3 for 48 h; (k) Raji cell treated with 2 μM As_2O_3 for 24 h; (l) Raji cell treated with 2 μM As_2O_3 for 0 h

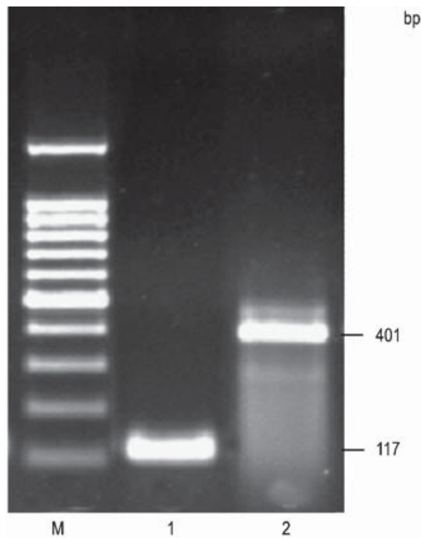

Fig. 6 PCR products of Survivin gene and β-actin were electrophoresised. M: 100 bp ladder DNA marker; Lane 1: Survivin gene; Lane 2: β-actin

a close relationship with some factors such as IL-3, Fas (CD95), Bax, VEGF, TNF-α, Bcl-2, P53, and so on [11–13]. Consequently, Survivin has emerged as an ideal diagnostic and therapeutic target for cancer [14]. On the one hand, it can strengthen the chemotherapy medicine's effects, on the other hand it can supplement other cure methods' defect [15].

In this study, we took the human Burkitt lymphoma cell line (Raji) as the treated target. As expected, we found that the Survivin gene overexpressed in Raji cell, about 10^6–10^7 copies/μL through FQ-PCR. It is suggested that high expression of survivin often exists in haematopoietic tumor, which was coincided with other reported

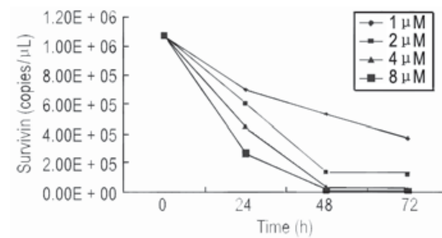

Fig. 7 Effects of different concentrations of As_2O_3 on copies of the Survivin gene of Raji cells

[16]. After treatment with different concentrations (1, 2, 4 and 8 μM) of As_2O_3, expression of Survivin gene was down-regulated. Additionally, the number of Raji cells in G2/M phase decreased, but in G1 stage increased. This gave us a hint to the Survivin gene expression in a cell cycle-dependent manner: high expression in G2/M stage and low expression in G1 stage, respectively. Down-regulation of Suvvivin gene has, to a certain extent, correlated with the aforementioned observation of FCM and the electron microscope. We conclude that the down-regulation of Survivin gene expression is one of the mechanisms of As_2O_3-induced apoptosis of Raji cells.

Moreover, we noticed the treated Raji cell with 1 μM As_2O_3 did not appear apoptosis apparently but block cells cycles. This was consistent with Zhu's reports [17]. In our present study, the cells cycle's retardarce have occurred ahead of apoptosis. Along with the cells cycles retardarce progression, the cells proliferation time prolonged, and the apoptotic ratio increased gradually leading to our speculation that cells cycle's hindrance is restrained by cells proliferation and induced apoptosis.

The Survivin gene has exerted an anti-apoptosis quality in multiple courses such as cell cycles, cell apoptosis and

Table 2 Effects of As_2O_3 on the cell cycle distribution of Raji cells ($\overline{\chi}$ ± SD, *n* = 3)

Concentration (μM)	Time (h)	Apoptotic rate (%)	Cell cycle (%)		
			G0/G1	G2/M	S
8	0	0	50.3 ± 1.1	26.2 ± 3.4	23.5 ± 2.2
	24	20.4 ± 1.1	56.9 ± 2.3	25.7 ± 2.6	17.4 ± 1.8
	48	31.8 ± 1.4	69.2 ± 2.2	18.9 ± 2.2	11.9 ± 1.4
	72	39.9 ± 1.6	72.9 ± 4.3	16.1 ± 0.5	11.0 ± 0.6
4	0	0	49.6 ± 2.4	24.3 ± 1.1	26.1 ± 3.1
	24	2.2 ± 1.6	50.7 ± 4.3	22.1 ± 1.1	27.2 ± 2.1
	48	8.8 ± 1.2	63.5 ± 2.1	16.0 ± 2.5	20.5 ± 2.3
	72	31.8 ± 1.4	68.9 ± 0.1	14.0 ± 2.1	17.1 ± 1.1
2	0	0	52.7 ± 0.9	25.8 ± 0.3	21.5 ± 1.6
	24	0.1 ± 2.2	55.7 ± 0.6	29.0 ± 2.3	15.3 ± 2.1
	48	3.1 ± 2.1	49.4 ± 3.6	31.6 ± 0.5	19.0 ± 2.2
	72	7.9 ± 3.8	48.9 ± 1.8	33.3 ± 2.3	17.8 ± 3.4
1	0	0	51.2 ± 0.3	20.9 ± 2.2	27.9 ± 4.1
	24	0	52.1 ± 4.3	23.6 ± 3.4	24.3 ± 4.1
	48	0	49.7 ± 2.1	29.1 ± 2.3	21.2 ± 3.2
	72	0	53.7 ± 2.3	31.7 ± 2.1	14.6 ± 3.2

cancer development. This is surprisingly familiar with As_2O_3 anti-cancer therapeutic mechanisms. We think application of medicine that is aimed at the Survivin gene may increase lymphoma cells apoptosis. So Survivin appears to be a targeted gene and the methods of As_2O_3 inducing cells apoptosis will become a new mean of treatment in the lymphoma therapy field. Of course, there should be more *in vivo* and *vitro* experiments done to support and improve this before it are used in any clinic.

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