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Verapamil enhances the sensitivity of oxaliplatin to tumor cells by influencing the PARP pathway

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PARP is a DNA damage-modifying enzyme present in most eukaryotic cells. In this study, reverse docking showed that verapamil (Vera), which can effectively bind PARP1/2, could significantly inhibit PARP1/2 activity inside and outside the system. Moreover, it could enhance the sensitivity of oxaliplatin to low-expression P-glycoprotein (P-gP) tumor cells and strengthen its apoptosis-inducing effect on tumor cells under the reverse drug resistance concentration of tumor cells. Vera, which can reverse chemotherapy resistance of tumor cells, showed no simple correlations with oxaliplatin drug resistance or P-gP expression and could enhance the anti-tumor effect of platinum chemotherapeutic agents by influencing the PARP pathway.

1. Introduction

Chemotherapy resistance is the main factor causing comprehensive tumor treatment failure and high death rate. Therefore, developing a reversion method of chemotherapy resistance on the basis of the chemotherapy resistance mechanism of tumors is important. As clinically used chemotherapeutic agents, platinum-based drugs are generally applied to tumor treatment, but their use can be limited by toxicities and resistance (Lai et al. 2018; Oun et al. 2018; DiSogra et al. 2019). The action mechanism of platinum drugs is that they cause DNA damage and cell death through alkylation after being embedded into the DNA double strands (Dilruba et al. 2016). However, some DNA repair enzymes are activated in tumor cells to protect DNA from damage induced by platinum drugs, weakening their chemotherapeutic efficacy (Galluzzi et al. 2012). In these DNA repair enzymes, polymerase (ADP-ribose) 1/2 is highly expressed in multiple types of tumor cells and can significantly degrade the clinical therapeutic effect of platinum drugs on hepatic carcinoma (McQuade et al. 2018). Therefore, inhibiting PARP1/2 expression and activity can be used to solve or improve the resistance of tumors against platinum chemotherapeutics.

Verapamil (Vera) is a calcium channel inhibitor. Recent studies found that Vera can improve the sensitivity of multiple tumor cells to chemical drugs (Guo et al. 2017; Zhang et al. 2017). The P-glycoprotein (P-gP) multidrug resistance (MDR) protein 1 is known as the main target of Vera for reversion of tumor drug resistance (Jin et al. 2012). Vera allows the energy released by ATP hydrolysis to couple with the transport function of P-gP, which causes the inefficient hydrolysis of ATP and the loss of P-gP's discharge function; alternatively, Vera competes with drugs for P-gP so that drugs excreted out of cells are reduced (Wang et al. 2011; Tsubaki et al. 2014). However, some studies did not detect high MDR1 expression in taxol-resistant cell strains of ovarian carcinoma and adriamycin-resistant cell strains of gastric carcinoma (Kang et al. 2004). Chiu et al. (2010) also verified that the Vera reversion of lung carcinoma cell MDR was uncorrelated with P-gP expression. In an early-stage study, we found that P-gP expression levels in two groups of lesion tissues were not different in the treatment combining Vera and transhepatic arterial chemotherapy and embolization; furthermore, Vera had a reversal effect on platinum chemotherapeutic agents in hepatic carcinoma cells (HCCs) with low P-gP expression, indicating that other pathways may exist for the VER reversion of platinum chemotherapeutic resistance, which may be related to its repair effect on DNA damage (Zhang et al. 2017). In the docking of related targets of tumor drug resistance in this study, Vera could bind with PARP1/2 very well. Therefore, HCCs with low P-gP expression were considered as study objects. The ability of Vera to promote the anti-tumor effect of the sensitization chemotherapeutic drug oxaliplatin by influencing the PARP pathway was evaluated in this study.

2. Investigations and results

2.1. Influence of Vera combined with oxaliplatin on HCC proliferation

The toxicity of Vera combined with oxaliplatin to multiple HCC lines was detected through the CCK8 test. As shown in Fig. 1, Vera could significantly reverse the drug resistance of three HCC lines, namely, BEL7402, HeG2, and QGY-7703, against oxaliplatin concentration of 4.91 mg/mL. The expression levels of P-gP-coded gene MDR1 in three HCC lines were detected through qRT-PCR. The results showed that P-gP expression levels in the three HCC lines were different, and the expression of the MDR1 gene in QGY-7703 cell was low. This result indicated that MDR1/P-gP expression level had no simple correlation with Vera's ability in antagonizing drug resistance.

2.2. Interaction between Vera and PARP

Reverse docking has been certified as an effective tool used to directly seek for the target (Huang et al. 2018). As shown in Fig. 2A, some targets correlated with tumor drug resistance, such as P-gP, PARP1, PARP2, PLK1, and sorcin, were selected to establish a database for the reverse docking of Vera. On the basis of the analysis of binding free energy, Vera had good binding effect with both DNA repair enzyme PARP1/2 and protein crystal (Table). Docking results showed that Vera could promote binding action between Vera and PARP1/2 active pocket region through VDW force, hydrophobic effect, p–p stacking, and hydrogen-bond interaction (Figs. 2B and 2C). Two

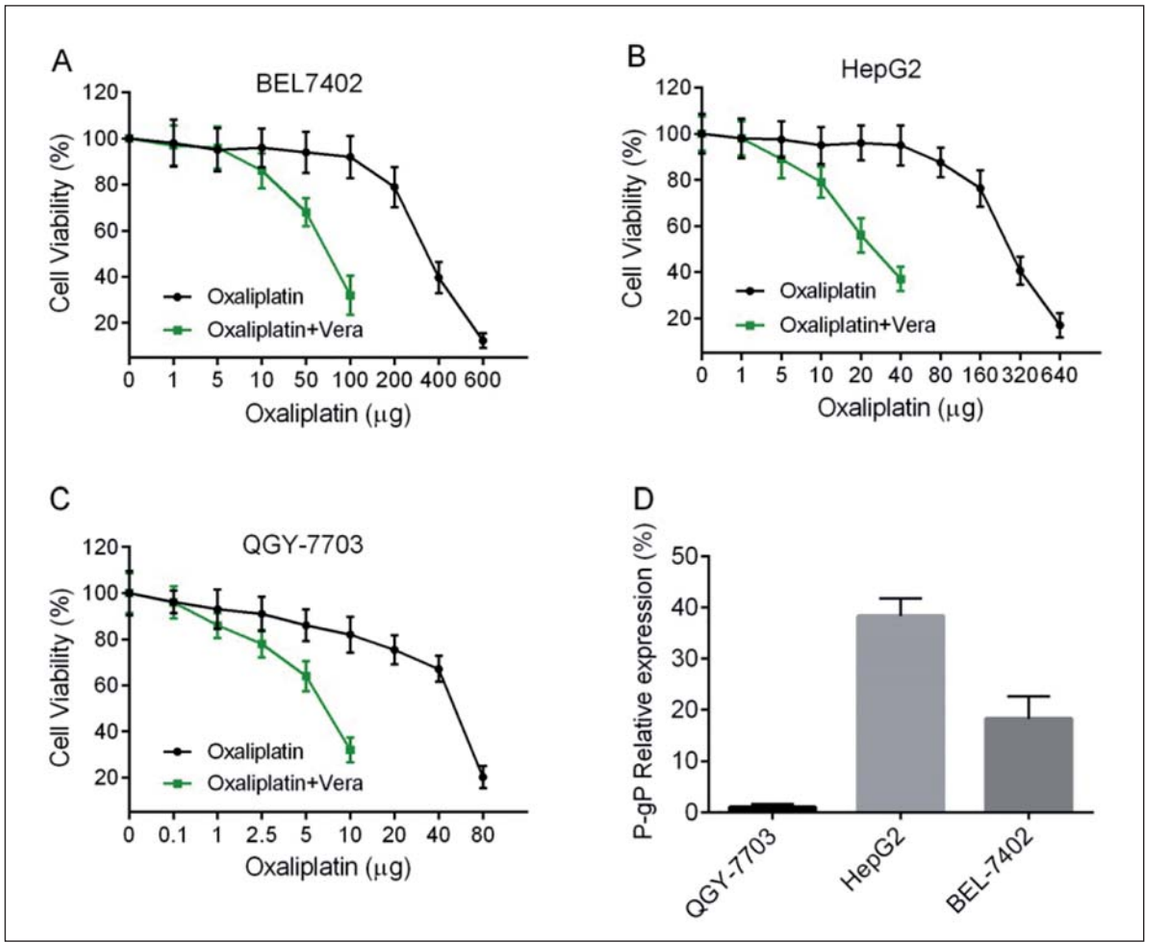

Fig. 1: Sensitivity test of Vera combined with oxaliplatin to the HCC lines, namely, BEL7402 (A), HeG2, (B) and QGY-7703 (C), and the detection of expression levels of MDR1/P-gP in the three cell lines (D).

hydrogen-bond interactions were produced between the O atoms of the methoxy group with the SER904, and cyano group with the HIS862 in PARP1-ligand complexes. One π–π stacked interaction generated in benzene ring with TYR907. Two hydrogen-bond interactions were also generated by connecting the O atoms of the methoxy group with SER430 and ASN434 in PARP2-ligand complexes. One π–π stacked interaction was generated in benzene ring with TYR473.

2.3. Effect of Vera on PARP1/2 activity

The inhibitory activity of Vera for PARP1/2 was detected through an *in vitro* enzyme test. The results indicated that at 4.91 mg/mL, the inhibitory effect of Vera on PARP1/2 reached 76.3±3.6% and 81.6±4.2%, respectively. Olaparib was used as positive control (Fig. 3A).

To further evaluate the inhibitory effect of Vera on PARP1/2, we performed Western blot analysis to detect the expression level of gH2A inside cells, which reflects PARP enzymatic activity, PAR expression level, and cellular DNA damage (Hu et al. 2018). The results show that after QGY-7703 cells were processed using Vera for 24 h, the PAR expression levels in cells markedly decreased, whereas the gH2AX expression levels increased. This finding indicates that Vera could inhibit PARP activity in tumor cells to obstruct intracellular DNA damage repair and enhance the sensitivity of chemotherapeutic agents to tumor cells, as shown in Figs. 3B and C.

2.4. Effect of Vera combined with oxaliplatin on the apoptosis of QGY-7703 cells

Cell apoptosis, a biochemical regulation pathway of cell death, is also called "programmed cell death" or "cell suicide." The Bcl2 protein family (Bax and Bcl2) is an important substrate for cell apoptosis (Aird et al. 2019). Caspases are cysteine proteases that digest cell proteins in the final cell apoptosis phase (Orrenius et al. 2003). Cells are decomposed through apoptosome formation, and nuclear DNA is cut by endonuclease into oligonucleosome fragments. Obstructing PARP activation is greatly important for normal cell apoptosis (Kyle et al. 2008). Therefore, detecting downstream apoptosis-related proteins of PARP is the main task of Vera when exerting the inhibitory effect on PARP. The influences of annexin V-PI double staining and oxaliplatin of Western blot analysis or Vera and oxaliplatin co-treatment on cell apoptosis and apoptosis-related proteins (Bax, Bcl2 and Caspase 3) were analyzed. The results are shown in Fig. 4A. Annexin V-PI double staining showed that Vera could add to anaphase cell apoptosis and death relative to oxaliplatin. Figs. 4B and 4C show that oxaliplatin decreased Bcl2 expression while increasing the expression levels of bax and cleaved caspase-3, but Vera significantly enhanced oxaliplatin's effect on apoptosis-related proteins. These findings further indicated that Vera could enhance the sensitivity of oxaliplatin to QGY-7703 cells by inhibiting PARP activity to activate the mitochondrial apoptosis pathway.

2.5. Conclusion

Verapamil (Vera) is the first clinically used reversal agent for tumor cell MDR, and its mechanism is especially complicated. In this study, CCK8 test and qRT-PCR showed that at 4.91 mg/mL, Vera could reverse the resistance of multiple HCCs against oxaliplatin and this was not correlated with P-gP expression level in cells. The reverse docking of some targets of Vera and drug resistance of tumor cells showed that Vera could effectively bind the DNA repair enzyme PARP1/2. Under the concentration for the reversion

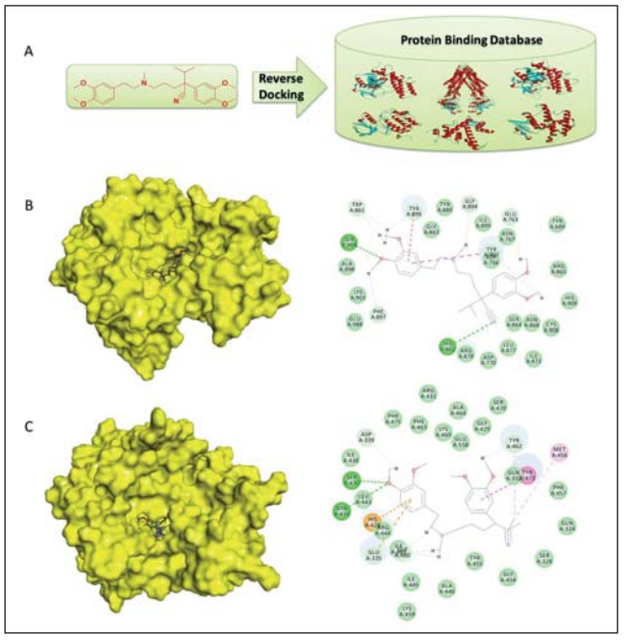

Fig. 2: (A) Reverse docking route. (B) 3D and 2D docking models of Vera and PARP1. (C) 3D and 2D docking models of Vera and PARP2. The green color represents intermolecular hydrogen bonds, the light green color represents VDW force, the light blue color represents hydrophobic effect, and the purple color represents p–p stacking effect.

Table: Docking score of Vera and target combination

Name	PDB ID	LibDock Score
P-gP	4q9h	94.5818
PARP1	4und	133.298
PARP2	4zzx	144.279
PLK1	4j52	126.233
Sorcin	4upg	82.8832

3. Experimental

3.1. Materials

Vera and oxaliplatin were purchased from Jingchun Reagents Co., Ltd., Shanghai. CCK8 kit was obtained from DOJINDO, Japan. RNA extraction and reverse transcription kits and 2× SYBR Green universal-type qPCR Master Mix were acquired from TIANGEN. Anti-PAR antibody, anti-gH2AX antibody, anti-PARP1/2 antibody, anti-b-actin antibody, anti-BAX antibody, and anti-Bcl2 antibody were obtained from Cell Signaling Technology, USA. DNA, PARP1/2, and LipofectamineTM 2000 were purchased from Sigma-Aldrich, USA. Annexin V-PI double staining kit was acquired from Double Care, Beijing, China. Primer design and synthesis were performed by Shine Gene, Shanghai.

3.2. CCK8 Test

Cells in logarithmic phase were obtained, and a single-cell suspension was prepared using high-glucose DMEM after 0.25% trypsinization. The cells were inoculated into a 96-pore culture plate (100 µL/pore) at a cell density of 2×10^5 cells/mL. The mixture was cultured in a 5% CO_2 incubator under 37 °C for 12 h. Different concentrations of chemotherapeutic agents, which were diluted with high-glucose DMEM, were added. Three multiple pores were set for each dosage (control group was divided into PBS treatment group and blank control group). After 48 h of culture, CCK8 reagent was added. After 1 h, the PBS treatment group served as the control to determine the OD_{450} value of each group. A concentration–effect curve was drawn by taking drug concentration as x-coordinate and OD_{450} as y-coordinate.

3.3. P-gP gene expression in cells through real-time quantitative PCR

RNA extraction and quantification were carried out in accordance with the manufacturer's instructions. A reaction mixture was prepared according to the following reaction system: 12.5 mL 2× SYBR Green universal-type qPCR Master Mix, 1.5 mL of upstream primer and 1.5 mL of downstream primer, and 3 mL of cDNA. Double-distilled water was added until the final volume of 25 mL was reached. The corresponding volume was prepared according to the quantity of detection specimens and added into PCR plate (25 mL in each pore). The reaction mixture was tossed to the tube bottom through inching centrifugation. PCR was implemented under the following reaction conditions: 15 min pre-modification at 95 °C, PCR reaction (10 s modification at 95 °C, 32 s annealing/extension at 60 °C for 40 cycles). A solubility curve was then established. Finally, the data were directly read from the real-time quantitative PCR instrument. The primer sequence was as follows: (5′-3′), ABCB1: F: 5′-TTTTCATGCTATAATGCGAC-3′, R: 5′-TCCAAGAACAGGACTGATGG-3′.

3.4. Molecular docking

Molecular docking was conducted using the Discovery studio 2017 software. Vera structure was drawn with Chemdraw 2010, and optimal hydrogenation was carried out using Discovery studio 2017. Protein docking with the designed protein database was performed through the LibDock program, and the results were analyzed. 2D and 3D binding patterns were obtained, and the bonding effect between small molecules and ligand was analyzed. Protein crystal structure was derived from Protein Data Bank.

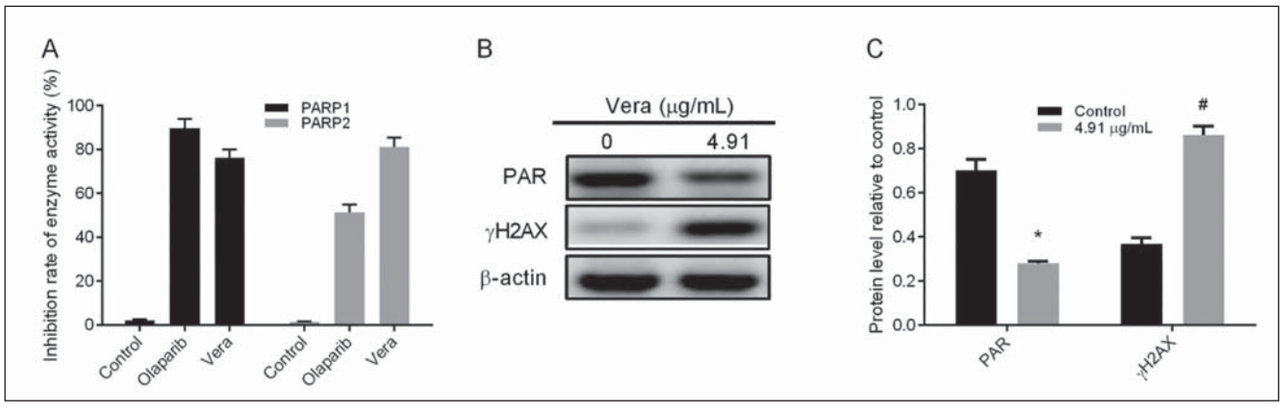

Fig. 3: (A) Inhibitory effect of Vera (4.91 mg/mL) on PARP1/2 activity, with olaparib (5 nM) as positive control. (B) Expression levels of the proteins QGY-7703, PAR, and gH2AX 48 h after Vera administration. (C) Histogram of relative protein expressions. $^*P<0.05$ when the relative PAR expression is compared with the control. $^\#P<0.05$ when the relative-gH2AX protein expression is compared with the control.

of tumor drug resistance, Vera could significantly inhibit PARP1/2 activity; inhibit PAP levels, which reflects PARP1 activity in cells; and elevate γH2A expression levels, which could reflect DNA damage repair in cells. Cell apoptosis detection further verified that Vera could enhance the sensitivity of oxaliplatin to QGY-7703 cells by inhibiting PARP activity to activate the mitochondrial apoptosis pathway. These results provide further theoretical bases for the application of Vera combined with chemotherapeutic agents.

3.5. Western blot analysis

Cells were collected after Vera or Vera + oxaliplatin was incubated for 48 h together with QGY-7703 cells, or cells were independently cultured for 48 h. Transmembrane protein was separated through SDS-PAGE following protein quantification. A buffer solution was prepared using TBS-T, and 5% skimmed milk was used to seal transmembrane protein. After 1 h, the membrane was washed using TBS-T buffer solution

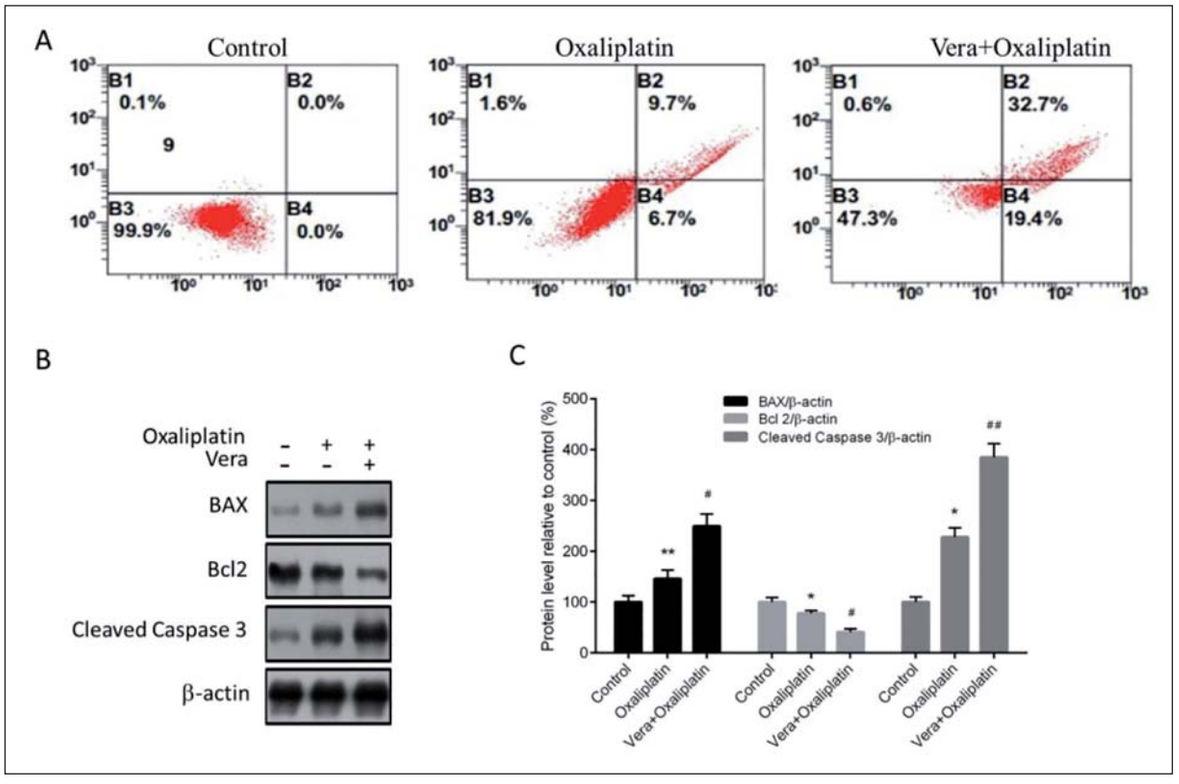

Fig. 4: Effect of Vera (4.91 mg/mL) combined with oxaliplatin (10 mg/mL) on QGY-7703 cell apoptosis. (A) Annexin V-PI double staining; (B) and (C) detection of expression levels of apoptosis-related proteins compared with the control (**P*<0.05, ***P*<0.01) and compared with the L-OHP group ($^{\#}P$<0.05, $^{\#\#}P$<0.01).

for three times. The primary antibody diluted at 1:1000 was added, and the mixture was incubated overnight at 4 °C. Afterwards, the membrane was recovered to room temperature and washed using TBS-T buffer solution for three times. The corresponding secondary antibody diluted at 1:3000 was added and incubated for 1 h. Subsequently, the membrane was taken out, washed with TBS-T buffer solution three 3 times, and then vacuum dried. Chemical luminescence reagent was added for gel-imaging detection, optical density was calculated, and results were analyzed. Bank.

3.6. Annexin V-PI double staining method

Cells were cultured in a 6-pore plate for 24 h by using 10% FBS RMPI-1640 until they were 70% confluent. Pancreatic enzyme effect was terminated using 10% FBS culture medium after trypsinization. The cells were centrifuged at 1000 rpm and 4 °C for 5 min and rinsed twice with precooled PBS. The cells were resuspended in 100 μL of binding buffer and blended with 2 μL of Annexin V-FITC. The mixture was placed on ice for 15 min away from light and then added with 400 μL of PBS. Each specimen was added with 1 μL of PI solution before being placed on the flow cytometry instrument, and HCC apoptosis rate was rapidly detected after the specimen was blown evenly.

3.7. Statistical analysis

Experiments were repeated at least three times. The experimental data were analyzed using SPSS version 12.0 software. One-way analysis of variance was performed followed by a post hoc Tukey's test for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.

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Conflicts of interest: The authors declare no conflict of interest.

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