Icariin Enhances Cytotoxicity of Doxorubicin in Human Multidrug-Resistant Osteosarcoma Cells by Inhibition of ABCB1 and Down-Regulation of the PI3K/Akt Pathway

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Multidrug resistance is one of the major causes limiting the efficacy of chemotherapeutic agents used to control osteosarcoma. Multidrug resistance protein 1 (MDR1 or ABCB1) was considered to play a critical role in multidrug resistance. Agents from traditional Chinese medicines (TCMs) have great potential to prevent the onset or delay the progression of the carcinogenic process, and also to enhance the efficacy of mainstream antitumor agents. Herein, we investigated the effect and mechanism of icariin in the human osteosarcoma doxorubicin (DOX)-resistant cell line MG-63/DOX. In this study, icariin exhibited significant effects in sensitization of the resistant cancer cells at a concentration non-toxic to doxorubicin. It also increased the intracellular doxorubicin accumulation and retention in MG-63/DOX cells. In addition, an increase in Rh123 accumulation and a decrease in Rh123 efflux were observed in MG-63/DOX cells treated with icariin, indicating a blockage of the activity of MDR1. Furthermore, icariin enhanced the apoptosis induced by doxorubicin and down-regulated the expression of MDR1. The mechanism involves the inhibition of phosphatidylinositol 3-kinase (PI3K)/Akt signaling. In conclusion, icariin possesses a reversal effect on multidrug resistance in MG-63/DOX cells through down-regulation of the MDR1 and the PI3K/Akt pathway, and has the potential to be an adjunct to chemotherapy for osteosarcoma.

Key words osteosarcoma; icariin; multidrug resistance (MDR); phosphatidylinositol 3-kinase (PI3K)/Akt pathway

Chemotherapy is regarded as an important line of defense against osteosarcoma which is the most common primary mesenchymal malignant tumor of bone tissue in human, especially in children and adolescents. However, on account of drug resistance especially multidrug resistance (MDR), only a limited proportion of cancer patients respond favorably to commonly used chemotherapeutic drugs. With respect to the mechanisms of multidrug resistance, ATP-binding cassette transporters, such as ABCB1 (MDR1), P-glycoprotein (P-gp), ABCC1/multidrug resistance-associated protein 1 (MRP1) and ABCG2/breast cancer resistance protein (BCRP), mediate energy-dependent drug efflux and play a main role in chemoresistance. And central to the mechanism is the overexpression of MDR1, which extrudes (certainly but not only) Vinca alkaloids, anthracyclines, epipodophyllotoxins, and taxanes out of cancer cells.

Inhibiting drug transporter and modulating MDR are one of the most important strategies in the field of cancer chemotherapy. First-generation inhibitors of P-gp such as verapamil (VPL) and cyclosporine were combined with a range of chemotherapy regimens for many cancers, but the results were not convincing. The second-generation inhibitors of P-gp such as PSC 833 and VX-710 were attempted in subsequent clinical trials, but the results of these trials were largely negative, failing in some cases because of pharmacokinetic interaction between the chemotherapeutic agents and the P-gp inhibitors. The third-generation inhibitors of P-gp and MRPI such as tariquidar (XR9576), Zosuquiar (LY335979), Laniquidar (R102933), and ONT-093 (OC144-193) may be more effective in certain patients with MDR. However, none of them was approved for clinical use due to their unwanted side effects.

Therefore, the screening of more potent chemosensitizers with desirable pharmacology is of paramount clinical importance. Agents derived from plant origin are being increasingly utilized in drug discovery and drug development programs. Identification of natural compounds capable of circumventing MDR with minimal adverse side effects is an attractive goal. For example, flavonoids are a group of compounds which have been extensively studied as chemosensitizers in a number of cancer cell lines. Icariin (Fig. 1), one of the most abundant flavonoids in Herba Epimedii, has lots of pharmacological and biological activities, including preventing osteoporosis, pencil erection, anti-cancer and anti-depression. But its effect on anti-MDR and the reversal mechanism has not been reported. In this study, we found that icariin showed the potent MDR

Fig. 1. The Structure of Icariin

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MATERIALS AND METHODS

**Chemicals** Doxorubicin (DOX) was from Santa Cruz Biotechnology (TX, U.S.A.). Icarin (C_{37}H_{50}O_{15}, molecular weight (MW): 676.66, purity ≥98%) was from J&K Scientific (Beijing, China), Verapamil (Vera), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), rhodamine 123 (Rh123), RNase A, sodium pyruvate and paraformaldehyde were from Sigma-Aldrich (MO, U.S.A.). LY294002 was from KeyGEN (Jiangsu, China).

**Cell Lines and Cell Culture** The human osteosarcoma cell line MG-63 (purchased from Typical culture preservation commission cell bank, Shanghai, China) and its doxorubicin resistant subline MG-63/DOX (established and maintained in our laboratory), were cultured in flasks with MEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2.2 g/L NaHCO₃ and 0.11 g/L sodium pyruvate at 37°C in a humidified atmosphere containing 5% CO₂.

**Establishment of DOX-Resistant MG-63 Subline** A DOX-resistant subline, MG-63/DOX, was established by increasing the concentration gradient of DOX in a stepwise manner.16 In brief, the initial concentration (2 μM) was increased gradually increasing concentrations of DOX. The passaging was repeated for 6 months, and displayed 29.37-fold resistance compared with their corresponding parental sensitive cell lines.17 All experiments were performed with cells in the logarithmic growth phase.

**Cytotoxicity Assay** MG-63/DOX or MG-63 cells were seeded into 96-well culture plates at 5×10³ cells/well. For resistance index assay, serial dilutions of conventional chemotherapeutic drugs were added to the wells. For MDR reversal activities assay, a full range of concentrations of conventional chemotherapeutic drugs with or without icariin (verapamil as a positive control) were added to the cells. After 48 h, absorbance in each well was read by a microplate reader. IC₅₀ values were calculated from survival curves by using the Bliss method.18 The resistance index (RI) was represented as the following formula: RI=IC₅₀ (MG-63/DOX)/IC₅₀ (MG-63).19 It exhibited high resistance when RI is greater than 15. IC₅₀ values of doxorubicin were calculated from plotted results using untreated cells as 100%.

**Cell Viability Assay** Cells were seeded into 96-well plates at a density of 5×10³/well. For the cell viability assay, cells were incubated with different concentration of icariin for 48 h, followed by MTT assay as described.18 Data were collected by reading at 570 nm and 630 nm with a model Spectra max PLUS 384 (Molecular Devices Corp., U.S.A.). The percentage of cell survival was calculated by the following formula: percentage of cell survival=(mean absorbance in test wells)/mean absorbance in control wells)x100%.20

**Intracellular Doxorubicin Fluorescence Assay by Flow Cytometry** Intracellular doxorubicin accumulation was measured by FACS analysis as described previously.21 In brief, both MG-63/DOX and MG-63 cells were exposed to 10 μM doxorubicin for 3 h in the absence or presence of 10 μM icariin. Cells were suspended in PBS followed by FACS analysis with BD FACSScan System (Becton Dickinson, NJ, U.S.A.).

**Intracellular Accumulation and Retention of Doxorubicin** The doxorubicin accumulation and retention analyses were evaluated by laser scanning confocal microscopy (LSCM), as previously described.22 Briefly, in accumulation assay, cells were incubated for 24 h in a medium containing doxorubicin (5 μM) with or without icariin (10 μM). Verapamil (10 μM) was used as a positive control. In retention experiment, the MG-63/DOX cells were incubated for 24 h in a medium containing doxorubicin (5 μM), and then washed with warm culture medium three times. The cells were incubated for another 24 h in the present or absent of 10 μM icariin (10 μM verapamil as a positive control). Images were analyzed with Image Pro Plus 6.0. Cells were measured and values were expressed as “mean light intensity per cell ± S.D.”

**Effects of Icariin on the Function and Expression of MDR1, MRP1 and BCRP1** The function of MDR1 was assessed by measuring intracellular accumulation and efflux of Rh123 as previously described.10 Briefly, cells were pretreated with or without 10 μM icariin for 1 h and then incubated with 5 μM Rh123 in the dark for another 1 h. Then cells were subjected to FACS to record the fluorescence produced by Rh123 for accumulation analysis. In the efflux study, MG-63/DOX and MG-63 cells were first cultured with medium containing 5 μM Rh123 at 37°C for 1.5 h, washed three times, then incubated in the absence or presence of 10 μM icariin at 37°C for another 1.5 h. The fluorescence was recorded. The cell-surface MDR1, MRP1 and BCRP1 levels were analyzed by FACS using FITC conjugated mouse anti-human monoclonal antibody against MDR1, MRP1 and BCRP1 respectively. MG-63/DOX cells were pretreated with or without 10 μM icariin (10 μM Vera as a positive control) for 24 h, and then harvested, washed twice with PBS, labeled with MDR1 monoclonal antibody according to manufacturer’s instruction.

**Apoptosis Assay** Annexin V-propidium iodide (PI) double staining apoptosis detection kit (KeyGEN, China) was used to detect cell apoptosis and its protocol was followed as the reference and manufacturer’s instructions described.23 Briefly, the MG-63/DOX cells were maintained in medium containing doxorubicin (1 μM) with or without icariin (10 μM) for 48 h. Vera (10 μM) was used as a positive control. Cells were harvested by the method described above, washed twice with PBS and resuspended in a volume of 500 μL binding buffer. The cells were stained with Annexin V-FITC (5 μL) and PI (5 μL) for 15–30 min. Analysis was performed on the flow cytometer (BD Biosciences). The percentage of the early apoptosis plus necrosis was calculated by the dimension of Annexin V positive and PI positive.
Quantitative Real-Time Polymerase Chain Reaction (PCR) Quantitative real-time PCR was carried out using the LightCycler 480 (Roche Molecular Biochemicals, Mannheim, Germany), as previously described. The primer pairs for PCR are listed in Table 1. The relative expression level of each gene was normalized to that of respective glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Western Blot Analysis Cells were incubated in the presence or absence of drugs for an appropriate time interval and then lysed by radio immunoprecipitation assay (RIPA). Protein levels were determined by standard Western blotting. Antibodies against p-Akt-Ser473, p-Akt-Thr308, AKT, PI3K-p85α, MDR1, MRP1, BCRP1, caspase-9, cleaved-caspase-9, cleaved-poly(ADP-ribose)polymerase (PARP), cleaved-caspase-3 and cleaved-caspase-3 (Cell Signaling Technology, MA, U.S.A.); p-PI3K-p85α (Santa Cruz, CA, U.S.A.); GAPDH (Sigma-Aldrich, MO, U.S.A.) were used as primary antibodies. Rabbit anti-goat, goat anti-rabbit and anti-mouse immunoglobulin G-horseradish peroxidase (IgG-HRP)-conjugated antibody (Jackson Laboratories, PA, U.S.A.) were used as secondary antibodies.

Statistical Analysis All experiments were performed at least three times. Statistical analysis was performed with the Prism software package (GraphPad v5). One-way ANOVA was used to compare each parameter. Student’s unpaired t-test was performed to identify which group differences accounted for significant overall ANOVA results. All quantitative data were reported as “mean ± S.D.” and statistical significance was expressed as ** or *** p<0.01 or 0.01< p<0.05.

RESULTS The Effect of Icariin on Cell Viability of MG-63 and MG-63/DOX Cells We first examined the cell viability and cytotoxicity of icariin itself on the doxorubicin-resistant cell line MG-63/DOX and its parental cell line MG-63. Both cell lines were pretreated with increasing concentration of icariin from 2.5 to 80 µM for 48 h, respectively. There was no obvious cytotoxicity on MG-63/DOX and MG-63 treated by varying dose of icariin (Fig. 2). Cell survival was more than 90% in both MG-63 and MG-63/DOX cells when exposed to 10 µM or lower concentrations of icariin. Therefore, icariin concentrations of 1, 5 and 10 µM were used in following experiment.

The Reversal Effect of Icariin on Resistance to Doxorubicin in MG-63/DOX Cells In order to investigate whether icariin modulated the sensitivity of cells to doxorubicin, MG-63 or MG-63/DOX cells were incubated with 1, 5 and 10 µM icariin (10 µM verapamil as a positive control) and a full range of concentrations of doxorubicin for 48 h. The IC50 and RI of doxorubicin, docetaxel and vincristine in the different treated cells were evaluated by MTT assay. As demonstrated in Table 2, the resistant index of DOX, DOC and VCR in MG-63/DOX cells were 29.37, 15.17 and 16.95, respectively. It was clear that the sensitivity to doxorubicin in MG-63 cells was significantly more than that in MG-63/DOX cells, and icariin could effectively reverse the drug resistance.

Table 1. Primers Used for Real-Time PCR Assays Performed on the LC480 System

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward primer (5’–3’)</th>
<th>Reverse primer (5’–3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>GAAAGCCTGCCGTGTACTAA</td>
<td>AAGAAAAAGCATACCCCGGAG</td>
</tr>
<tr>
<td>MDR1</td>
<td>AGAGTCAGAGCATGTCCTC</td>
<td>AACGTCAGAGTTCACCTGCGG</td>
</tr>
<tr>
<td>MRP1</td>
<td>TATCCTCGCCATGACTCCA</td>
<td>ACTTGTTCCGGACCTGTCTTC</td>
</tr>
<tr>
<td>BCRP</td>
<td>AGGATGAGACGGCGGGAAG</td>
<td>CACCCGGACCTTCAACAA</td>
</tr>
</tbody>
</table>

Table 2. The IC50 and RI for MG-63 and MG-63/DOX Cell Lines

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Drugs</th>
<th>IC50 (nM)</th>
<th>RI</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG-63</td>
<td>DOC</td>
<td>3131.84</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>VCR</td>
<td>730.28</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>DOX</td>
<td>1760.38</td>
<td>1</td>
</tr>
<tr>
<td>MG-63/DOX</td>
<td>DOC</td>
<td>47520.37</td>
<td>15.17a</td>
</tr>
<tr>
<td></td>
<td>VCR</td>
<td>12384.47</td>
<td>16.95b</td>
</tr>
<tr>
<td></td>
<td>DOX</td>
<td>51699.34</td>
<td>29.37</td>
</tr>
<tr>
<td></td>
<td>DOX +1 µM icariin</td>
<td>43793.37</td>
<td>24.88</td>
</tr>
<tr>
<td></td>
<td>DOX +5 µM icariin</td>
<td>22171.43</td>
<td>12.59</td>
</tr>
<tr>
<td></td>
<td>DOX +10 µM icariin</td>
<td>3507.24</td>
<td>1.99</td>
</tr>
<tr>
<td></td>
<td>DOX +10 µM Vera</td>
<td>4574.00</td>
<td>2.60</td>
</tr>
</tbody>
</table>

Note: All values are mean of three independent experiments. a) The resistance index of docetaxel in MG-63/DOX cells. b) The resistance index of vincristine in MG-63/DOX cells.
dose-dependently. Thus, the doxorubicin resistant subline was successfully established from MG-63 cells and icariin displayed potential anti-MDR activities in MG-63/DOX cell line.

**The Effect of Icariin on the Expression of MDR1, MRP1 and BCRP1** Since the overexpression of ABC transporters in cancer is considered to be a primary determinant of the MDR phenotype, we detected the expression of three main ABC transporters by FACS (Fig. 3A), qRT-PCR (Fig. 3C) and Western blotting (Fig. 3A) to find whether icariin reversed the drug resistance to DOX through mediating these ABC transporters. It was indicated that MDR1, MRP1 and BCRP1 expression were significantly up-regulated on the surface of MG-63/DOX cells. But the expressions of MRP1 and BCRP1 have not obvious changes in different treated MG-63/DOX cells. In contrast, the expression of MDR1 was dose-dependently decreased by icariin in MG-63/DOX cells. Therefore, unless otherwise stated, icariin at 10µM was used for the subsequent studies.

**Icariin Increases Intracellular Doxorubicin Accumulation and Decreases Doxorubicin Efflux** By pumping drug substrates out of cells, MDR tumor cells retain less anticancer drug than their drug-sensitive cells. The ability of icariin to

![Fig. 3. Effect of Icariin on ABC Transporters Expression of MG-63/DOX Cells](image)

(A) The membrane protein MDR1, MRP1 and BCRP1 expressions of MG-63/DOX detected by FACS, cells were treated icariin(10 µM) or Vera, respectively, for 24h; **p<0.01 compared with MG-63 group; ***p<0.01 compared with MG-63/DOX group. (B) The membrane protein MDR1, MRP1 and BCRP1 expressions of MG-63/DOX detected by Western blotting, cells were treated with PBS or different dose of icariin (1, 5, 10 µM, respectively) for 24h. The densities of the bands were quantified by a Bio-Rad Laboratories, Inc. imaging Lab, and calibrated by the internal standard GAPDH. Data are mean±S.D. of three independent experiments; *p<0.05, **p<0.01. (C) Cells were pretreated with different dose of icariin (1, 5, 10 µM) or Vera for 24h, respectively. The expressions of MDR1, MRP1 and BCRP1 were determined by quantitative real-time PCR (40 cycles). GAPDH was used as internal reference in each treatment group; (D) The effusion and accumulation of Rh123 in MG-63 and MG-63/DOX cells. Data are mean±S.D. of three independent experiments; *p<0.05, **p<0.01.
inhibit P-gp-mediated transport of anticancer drug was first evaluated by examining their effects on the accumulation of doxorubicin in MG-63/DOX and MG-63 cells using FACS. As shown in Fig. 4B, doxorubicin accumulation was higher (36.25-fold) in sensitive MG-63 cells than in MG-63/DOX cells. Icariin could significantly increase intracellular DOX concentration in MG-63/DOX cells (6.25-fold). Doxorubicin is an auto-fluorescent compound, which enables the direct visualization of its intracellular presence by LSCM. Figure 4A showed that compared with doxorubicin or verapamil, icariin could significantly increase intracellular doxorubicin accumulation and decrease doxorubicin effusion. The cellular accumulation of doxorubicin in icariin and verapamil group was 2.8 and 1.4-fold as compared with DOX group.

**Icariin Inhibits the Function and Down-Regulates the Expression of MDR1 in MG-63/DOX Cells**

We then investigated the effects of icariin on the MDR1 function by detecting accumulation and the efflux of Rh123 (a MDR1 special substrate) in MG-63 and MG-63/DOX cells. FACS analyses indicated that MG-63/DOX cells maintained much less Rh123 than MG-63 cells. And the accumulation of Rh123 in MG-63/DOX cells treated with icariin was higher (8.49-fold) than MG-63/DOX cells treated with Rh123 alone. In Rh123 efflux experiment, a rapid decrease of intracellular Rh123 level was observed in MG-63/DOX cells after incubation in Rh123-free medium for 1.5h. Treatment with icariin in MG-63/DOX cells suppressed Rh123 efflux to an extent larger (3.92-fold) than cells incubated with Rh123 only, suggesting its inhibitory effect on MDR1-mediated active transport process (Fig. 3D).

**Icariin Enhances DOX-Induced Apoptosis in MG-63/DOX Cells**

To test if icariin was able to enhance DOX-induced apoptosis, we used the Annexin-V/PI analyses by FACS. Furthermore, we measured the caspase-9, cleaved caspase-9, cleaved caspase-3, caspase-3 and cleaved PARP as indicators for activation and onset of apoptosis. We observed a notable increase in the early and later apoptotic phase when the cells were treated with DOX and icariin combined (Fig. 5), and the levels of cleaved caspase-9, cleaved caspase-3 and cleaved PARP were also higher than other groups (Fig. 6A). It is obvious that icariin is able to augment the potency of DOX, and its effect is stronger than verapamil.

**Effects of Icariin on PI3K/Akt Signaling in MG-63/DOX**

As we all know that LY294002 is the efficient inhibitors of PI3K, we hope to elucidate whether icariin increases DOX-induced apoptosis in MG-63/DOX cells via inhibition of PI3K/Akt pathway. Western blots indicated differences in levels of p-PI3K-p85α, phospho-Akt and Akt in MG-63 and MG-63/DOX cells treated with LY294002, and the reduction is more significant when co-treated with icariin and
LY294002 (Fig. 6B). Thus, icariin could inhibit PI3K activity and then led to a decrease phosphorylation of the downstream target such as Akt.

**DISCUSSION**

Failure of chemotherapy in osteosarcoma has been observed due to the presence and/or development of drug resistance. Drug resistance is a significant factor that limits the effectiveness of current chemotherapeutic drugs. Although resistance can develop through a variety of mechanisms, multidrug resistance due to the overexpression of drug transporters such as ABCB1 (P-gp) is an established cause of drug resistance.\(^{25}\) ABCB1 is a transmembrane protein encoded by the MDR1 gene in human cells. As a member of the ATP-binding cassette family of drug transporters, ABCB1 effluxes a variety of hydrophobic, neutral, and positively charged drugs from the cell. P-gp expression is a component of the normal cellular defense system against xenobiotics.\(^{25}\) However, in some human cancers, the overexpression of P-gp is correlated with decreased survival and poor prognosis.\(^{27,28}\)

Icariin is well known in China as one of the most abundant active flavonoid glycosides isolated from Herba Epimedii.\(^{13}\) However, to our knowledge, no evidence has been reported for the anti-MDR activity of icariin in osteosarcoma. Our data indicated that icariin significantly increased the DOX concentration in MG-63/DOX cells. DOX was mainly concentrated in the nuclei when in combination use with icariin or verapamil, while DOX dispersed in the cytoplasm when only administrated with doxorubicin. Icariin could also enhance the drug-transport function of MDR1 assessed by Rh123. Besides, icariin had the ability to enhance DOX-mediated apoptosis.

Extensive literature indicated that increased PI3K activity was strongly implicated in cells cycle, proliferation, and resistance to chemotherapy-induced neoplastic apoptosis.\(^{29}\) Given these previous findings regarding to PI3K, we aimed to characterize the role of PI3K/Akt signal pathway in the above-mentioned biological behavior of MG-63/DOX cells, and evaluated the therapeutic effects of icariin in vitro. In order to evaluate the effect of icariin on PI3K/Akt pathway, we use

![Graph showing effects of Icariin on DOX-Induced Apoptosis in MG-63/DOX Cells](image)
Western blot to explore the relationship between icariin and Akt phosphorylation firstly. Our Western blot results revealed the decrease of phosphorylated Akt levels. Therefore, our results supported previous studies and suggested that icariin inhibits PI3K activity and decreases Akt phosphorylation in MG-63/DOX cells.

Additionally, we have recently shown that icariin significantly increase DOX-induced apoptosis in MG-63/DOX cells. The cells treated with icariin showed a significant increase in apoptosis versus the DOX group. In our study, we also observed that PARP and caspase-3 were significantly active in treated groups, whereas the apoptotic rate was higher when cells were treated with icariin than that treated with DOX group. But PARP and caspase-3 are activated in the apoptotic cells by mitochondrial and non-mitochondrial death pathway; we continued to investigate the changes of caspase-9 in order to reveal which pathway is involved in this apoptotic process. Caspase-9 is regarded as an important activator related to apoptosis induction via mitochondrion-mediated pathway, wherein it subsequently activates caspases-3/6/7.29) The PI3K/Akt pathway conferred resistance by suppressing caspase-9 cascade.23) Earlier study suggested that the amount of caspase-9 (47 kDa) was significantly reduced in apoptosis.31) Our results also indicated that procaspase-9 (47 kDa) expression became less with icariin treatment, and caspase-9 was the target of PI3K/Akt pathway.

In conclusion, our findings demonstrate for the first time that icariin is able to reverse DOX-induced MDR and enhance DOX-induced apoptosis on MG-63/DOX by directly blocking PI3K/Akt pathway. In addition, icariin could dose-dependently reduce MDR1 in MG-63/DOX cells at non-toxic concentration. Bearing the function and high safety, icariin is one of potentials as a candidate adjuvant for cancer therapy.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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