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Effect of Combined Treatment Using Wilfortrine and Paclitaxel in Liver Cancer and Related Mechanism

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Background: Liver cancer is a common malignant tumor with high mortality. Currently, effective medicines against liver cancer are still lacking. Paclitaxel is a wide-spectrum anti-tumor agent, while wilfortrine has been shown to have an inhibitory effect on the proliferation of liver cancer cells. This study thus investigated the potential effect of paclitaxel combined with wilfortrine on cultured liver cancer cells and related mechanisms, in order to provide evidence for pathogenesis and treatment of liver cancer.

Material/Methods: Liver cancer cell line HpeG2 was divided into control, paclitaxel, wilfortrine, and combined treatment groups. Cell proliferation was tested by MTT, while invasion was detected in Transwell chamber assay. Apoptotic protein Bcl-2 and Bax expression levels were further quantified using real-time PCR and Western blotting.

Results: Both of those 2 drugs can effectively inhibit cancer cell proliferation, depress invasion ability, increase Bcl-2 expression, and elevate Bax expression levels ($p<0.05$ in all cases). The combined therapy had better treatment efficacy compared to either of those drugs alone ($p<0.05$).

Conclusions: The combined treatment using wilfortrine and paclitaxel can inhibit proliferation and invasion of liver cancer cells via down-regulating Bcl-2 and up-regulating Bax, with better efficacy than single use of either drug.

MeSH Keywords: Carcinoma, Hepatocellular • Cellulose 1,4-beta-Cellobiosidase • Paclitaxel

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Background

Liver cancer is one of the most common solid malignant tumors in the digestive tract. It is now the fifth most common malignant tumor and the second deadliest tumor after pulmonary carcinoma. The pathogenesis of liver cancer is a complicated process involving multiple steps and factors, including environment, genetics, and lifestyles. Various risk factors and toxicants, including aflatoxin, ethanol, sex steroid, hepatitis B virus (HBV), hepatitis C virus (HCV), liver cirrhosis, unclean water, nitrosamine, and trace elements are all related with liver cancer, with a close relationship with HBV infection. Having the worlds largest population of HBV carriers, China has a high incidence of liver cancer, accounting for about 55% of patients worldwide. The study of pathogenesis and progression of liver cancer, and related diagnosis and treatment, are therefore a major issue in biomedical research. Paclitaxel (or Taxol) can work as a first-line or second-line drug for various tumors, including breast cancer, ovary cancer, and non-small cell lung cancer. It has been reported to modulate the occurrence and progression of liver cancer cells. Wilfortrine is a small-molecule compound with bioactivity and has been demonstrated to have an inhibitory effect on proliferation of liver cancer cells. This study thus investigated the effect of paclitaxel-wilfortrine combined therapy on cultured liver cancer HepG2 cells, whose proliferation and invasion were observed, along with related mechanisms.

Material and Methods

Cell culture

Human liver cancer cell line HepG2 (ATCC cell bank, USA) were resuscitated at 37°C in a water bath. Cells were then centrifuged at 1 000 g for 3 min, followed by adding 1 mL DMEM medium (Hyclone, USA) to re-suspend cells, which were removed into a 25-mL culture flask with 4 mL DMEM medium. Cells were kept in a 37°C in a humidified chamber with 5% CO_2. After 48-h incubation, cells were inoculated into 6-well plates at 1×10^5 cells per mL density, using 90% high-glucose DMEM medium (with 100 U/mL penicillin and 100 μg/mL streptomycin, Hyclone, USA) and 10% fetal bovine serum (FBS, Hyclone, USA) for continuous culture. Log-phased cells after 3~8 generations were used and randomly divided into 4 groups: Control; Wilfortrine, with 40 mM wilfortrine for 48 h; Paclitaxel, with 20 mM paclitaxel for 48 h; Combined treatment group, with 40 mM wilfortrine; and 20 mM paclitaxel for 48 h.

MTT assay

Log-phased HepG2 cells were counted and seeded into 96-well plates (3 000 cells per well). After 48 h of drug incubation, 5 g/L MTT solution (Gibco, USA) was added into each well. After 4-h incubation, supernatants were removed with addition of 150 μL DMSO. The plate was vibrated for 10 min until complete dissolving of violet crystals. A microplate reader quantified absorbance value (A value) at 570 nm in each well. Each experiment was performed in triplicate for calculating cell proliferation rate.

Transwell assay

All cells were cultured in serum-free medium for 24 h before the experiment. Transwell chambers were pre-coated with 50 mg/L Matrigel solution on both bottom and upper membranes, and dried at 4°C. In each well, serum-free medium containing 10 g/L bovine serum albumin (BSA) was added. Transwell chambers containing 0.1 mL HepG2 cell suspensions were placed into 24-well plates with 0.5 mL DMEM medium (with 10% FBS) in the well. A parallel control group was performed using a Transwell chamber without Matrigel pre-coating. After 48-h incubation in triplicate, chambers were removed, rinsed in PBS, and cleaned for cells on the upper surface. After fixation in cold ethanol, the chamber was stained by crystal violet for 30 min. The number of cells that migrated to the bottom layer was counted under an inverted microscope from 10 randomly selected fields. All experiments were performed in triplicate.

Real-time PCR

Total RNA were firstly extracted from HepG2 cells using Trizol reagent (Invitrogen, USA) and were then used to synthesize cDNA by reverse transcription kit (Axygen, USA). Real-time PCR was then performed to detect the expression of target genes using specific primers (Table 1) under the following conditions: 90°C denature for 30 s, 58°C annealing for 50 s, and 72°C elongation for 35 s, repeated for 35 cycles. On a

Table 1. Primer sequence.

Target gene	Forward primer (5'-3')	Reverse primer (5'-3')
GADPH	AGTGCCGTCTCCTCAGCATAG	CGACTTGCTTGACGTGGGTAG
Bcl-2	CCTATGGAAATGACTAAGCCG	ATTCGCTAGCGACTCTCCG
Bax	GTGCTAAGCCCCTAAAATGAG	GCTATAGCGACTCTCCGGTA

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fluorescent quantitative PCR platform, CT values of both target genes and GAPDH were collected for quantitative analysis according to $2^{-\Delta Ct}$ method.

Western blotting

Total proteins were extracted from HepG2 cells using lysis buffer. In brief, cells were mixed with lysis buffer on ice for 30-min incubation. Ultrasonic rupture was then performed briefly, followed by centrifugation at 10 000 g for 15 min. The supernatant was transferred to a new tube and kept at –20°C for further use. In Western blotting, proteins were separated by 10% SDS-PAGE and were transferred to PVDF membrane (Pall Life, USA). Non-specific binding sites were blocked by 5% defatted milk powder for 2 h. Primary antibody against Bcl-2 (1:1 000, Cell Signaling, USA) or Bax (1:2 000, Cell Signaling, USA) was then applied for 4°C overnight incubation. On the next day, the membrane was rinsed in PBST, and was incubated with goat anti-rabbit secondary antibody (1:2 000, Cell Signaling, USA) for 30-min incubation. ECL reagent (Amersham Bioscience, USA) was used to develop the membrane, which was then exposed to X-rays. The optical density of each protein band was processed by Quantity One software. All experiments were performed in replicates (N=4).

Statistical analysis

SPSS 16.0 software was used to process all collected data, which are presented as mean ± standard deviation (SD). Analysis of variance (ANOVA) was performed to compare means across multiple groups. A statistical significance was defined when $p<0.05$.

Results

Cancer cell proliferation

After 48-h incubation of wilfortrine or paclitaxel alone, the proliferation of HepG2 cells was significantly inhibited compared to the control group ($p<0.05$, Figure 1). No difference was observed between wilfortrine and paclitaxel groups. The combined treatment using both drugs can further potentiate these inhibitory effects ($p<0.05$ compared to either of wilfortrine or paclitaxel groups, Figure 1).

Cancer cell invasion

The 48-h incubation using wilfortrine or paclitaxel alone significantly inhibited the invasion ability of HepG2 cells as compared to the control group ($p<0.05$, Figures 2, 3). Paclitaxel had a more potent inhibitory effect on cell invasion when compared to wilfortrine ($p<0.05$, Figures 2, 3). The combined application of both drugs further enhanced this inhibitory effect ($p<0.05$ compared to either wilfortrine or paclitaxel group, Figures 2, 3).

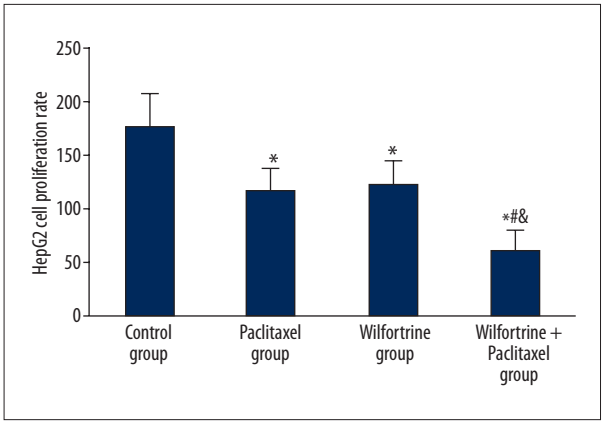

Figure 1. Proliferation of liver cancer cells. * $p<0.05$ compared to control group; # $p<0.05$ compared to paclitaxel group; & $p<0.05$ compared to wilfortrine group.

mRNA levels of Bcl-2 and Bax

We further tested the gene expression level of Bcl-2 and Bax in HepG2 cells. RT-PCR was found to significantly depress anti-apoptotic gene Bcl-2 expression but enhanced apoptotic gene Bax mRNA levels in both the wilfortrine and paclitaxel groups as compared to the control group ($p<0.05$, Figures 4, 5). No difference was observed between wilfortrine and paclitaxel groups. The combined application further enhanced Bcl-2 inhibition and Bax potentiation ($p<0.05$ compared to either of wilfortrine or paclitaxel group, Figures 4, 5).

Bcl-2 and Bax protein expressions

Western blotting revealed similar patterns of Bcl-2 and Bax protein expressions as those in RT-PCR: The single use of wilfortrine or paclitaxel effectively decreased Bcl-2 and increase Bax protein levels ($p<0.05$, Figures 6, 7). No difference was observed between wilfortrine and paclitaxel groups. The combined use of wilfortrine and paclitaxel further potentiated Bcl-2 inhibition and Bax potentiation ($p<0.05$ compared to either of wilfortrine or paclitaxel groups, Figures 6, 7). In a further analysis of Bcl-2/Bax ratio, it was shown that either of those 2 drugs lowered this ratio, which was further decreased in the combined treatment group (Figure 8).

Discussion

Although various approaches, including surgery, radiotherapy, chemotherapy, immune therapy, and intervention therapy, have been developed for use against liver cancer, its recurrence rate and metastatic incidence are still high, causing short life-spans and lower quality of life. The search for effective anti-tumor drug is thus of critical importance [8]. Paclitaxel is a terpenoid compound extracted from *Taxus chinensis*. It is now accepted

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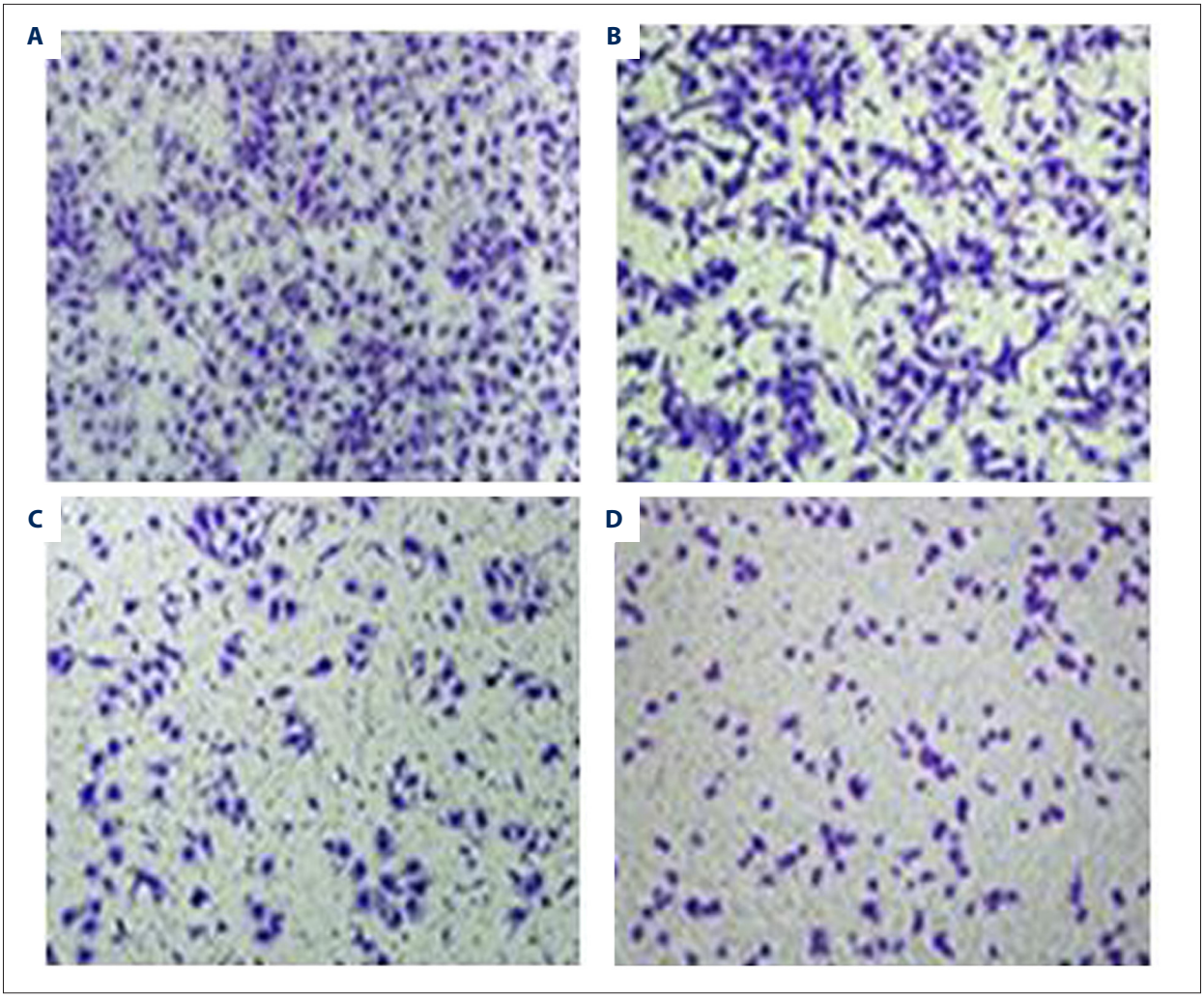

Figure 2. HepG2 cell invasions. (**A**) Control group; (**B**) Wilfortrine group; (**C**) Paclitaxel group; (**D**) Wilfortrine + Paclitaxel group.

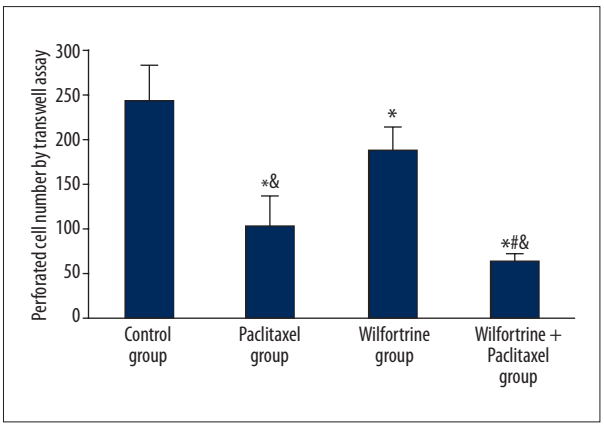

Figure 3. Number of invasion cells. * p<0.05 compared to control group; # p<0.05 compared to paclitaxel group; & p<0.05 compared to wilfortrine group.

as one of the most potent natural compound for tumor treatment [10]. Although paclitaxel can exert certain roles against liver cancer cells, the clinical trial is still at an initial stage, with some evidence questioning its treatment efficacy when used alone [16]. *Tripterygium Wilfordii* Hook, also named *Gelsemium elegans* Benth, contains multiple alkaloids, among which wilfortrine is extracted from Celastraceae family woody climber plants [14]. Previous studies have illustrated the inhibition of liver cancer cell proliferation by wilfortrine [17,18]. The combined effect of wilfortrine and paclitaxel on liver cancer cells and related mechanism, however, remains unknown.

In this study, cultured liver cancer HepG2 cells were tested for proliferation and invasion using wilfortrine, paclitaxel, or both drugs. Our results showed either of those two agents can effectively inhibit the proliferation of liver cancer cells. The combined use further enhances such inhibitory effects. In a cell invasion analysis, we found more potent effect from paclitaxel compared to wilfortrine alone. The combined effect further enhanced the inhibition on cell invasion. Our results suggested the blocking of liver cancer cell proliferation and invasion

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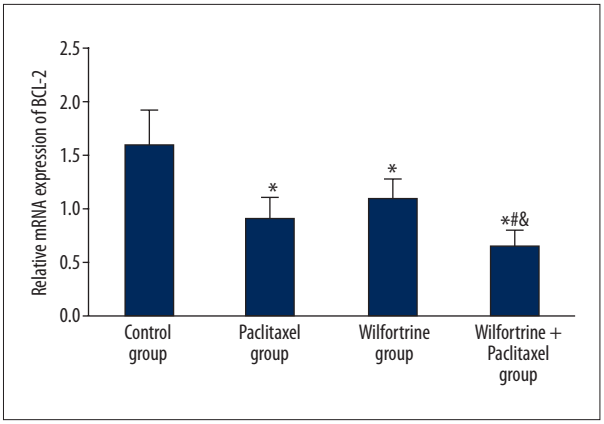

Figure 4. Bcl-2 mRNA level. * p<0.05 compared to control group; # p<0.05 compared to paclitaxel group; & p<0.05 compared to wilfortrine group.

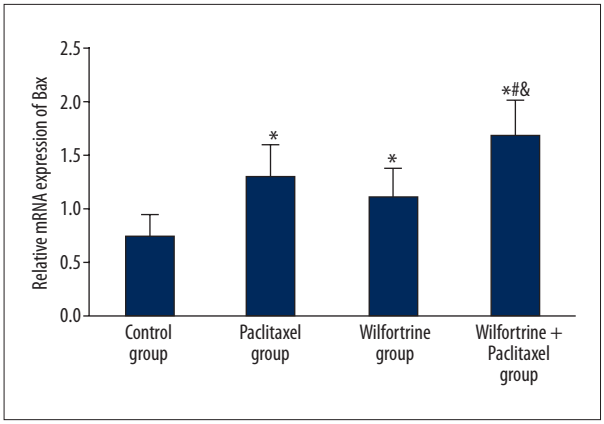

Figure 5. Bax mRNA level. * p<0.05 compared to control group; # p<0.05 compared to paclitaxel group; & p<0.05 compared to wilfortrine group.

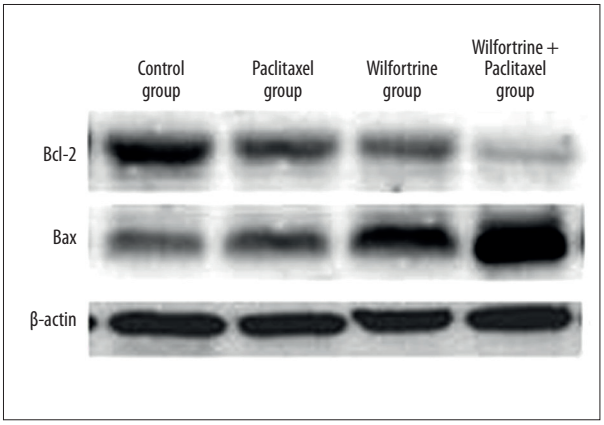

Figure 6. Western blotting of Bcl-2 and Bax proteins.

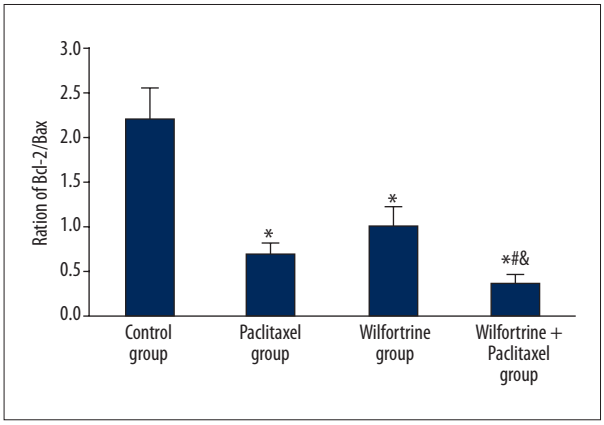

Figure 8. Bcl-2/Bax ratio in HepG2 cells. * p<0.05 compared to control group; # p<0.05 compared to paclitaxel group; & p<0.05 compared to wilfortrine group.

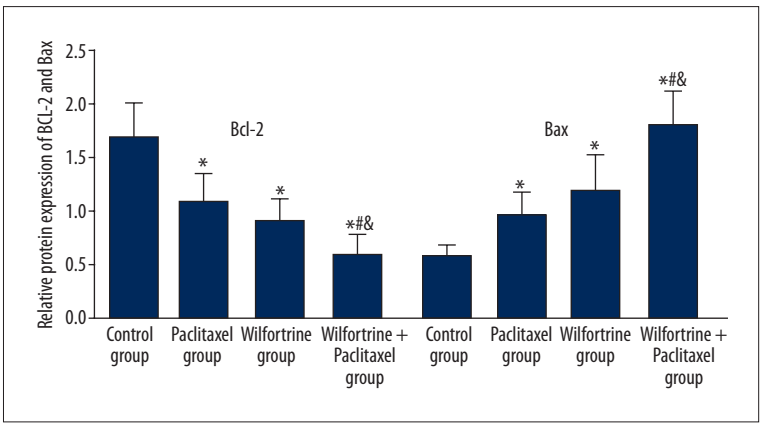

Figure 7. Relative expression levels of Bcl-2 and Bax proteins in HepG2 cells. * p<0.05 compared to control group; # p<0.05 compared to paclitaxel group; & p<0.05 compared to wilfortrine group.

by paclitaxel and wilfortrine, for further modulating on tumor occurrence and progression.

Cell apoptosis is known to play a crucial role in regulating the occurrence and progression of liver cancer [19]. The over-expression of Bcl-2 and inhibition on apoptotic protein Bax are closely correlated with the imbalance of anti-apoptosis/apoptosis axis in liver cancer cells [20]. As a regulatory mechanism for body homeostasis, cell apoptosis can inhibit tumor growth and retard its occurrence. In over-expression

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of Bcl-2, those injured cells can resist apoptosis, thus initiating downstream proliferation/growth signals and genes, all of which may promote tumor transformation [21]. The over-expression of apoptotic protein Bax, however, can activate apoptotic signals and antagonize Bcl-2 protein expression. Therefore, the dynamic balance and relative ratio of Bax and Bcl02 determines the cell fate, as predominant Bcl-2 proteins or their dimers expression inhibit cell apoptosis, while Bax protein/dimers induce programmed cell death. The imbalance of those 2 proteins thus causes dysregulation of apoptosis [22]. This study demonstrated the inhibited Bcl-2 and facilitated Bax expressions by wilfortrine/paclitaxel or both drugs, for further potentiating cell apoptosis.

Conclusions

The combined use of wilfortrine and paclitaxel can inhibit proliferation and invasion of liver cancer HepG2 cells via inhibiting Bcl-2 and enhancing Bax expressions, with better efficacy than single use. This study provided further information of the pathogenesis mechanism of liver cancer, in addition to providing a novel treatment approach for clinicians.

Disclosure of conflict of interest

None.

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