

## RESEARCH ARTICLE

# Primary investigation on effects of *Pinus massoniana* bark extract inducing senescence of hepatoma HepG2 cells

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**Abstract:** *Pinus massoniana* bark extract (PMBE) is a traditional Chinese medicine used for the treatment of various health disorders. Previous studies have demonstrated that PMBE may induce the apoptosis of hepatoma and colon cancer cells, and one of the potential mechanisms is by activating *p53* to up-regulate the expression of *p21*. The P53/P21 signaling pathway is also one of the main mechanisms to regulate cell senescence. Therefore, we wonder if PMBE is able to induce hepatoma cells into senescence by inhibiting their growth. In the current study, the effects of PMBE on the viability of human hepatoma HepG2 cells were detected using an MTT assay. The phenotypes of HepG2 cells with PMBE treatment were detected with  $\beta$ -Galactosidase staining assay. The results revealed that the growth of HepG2 cells was inhibited by PMBE at dose-dependent manner when PMBE concentration is above 50  $\mu\text{g/ml}$ , furthermore, PMBE could induce HepG2 cells into senescence at concentration of 40  $\mu\text{g/ml}$ . These findings indicated that PMBE significantly inhibited the growth of HepG2 cells and induced them into senescence, while the potential mechanism and its safety in normal cells require further investigation.

**Keywords:** *Pinus massoniana* bark extract, cell senescence, HepG2 cells

## 1 Introduction

*Pinus massoniana* bark extract (PMBE) is a natural product from *pinus massoniana* lamb, an important tree for afforestation in South China<sup>[1]</sup>. Its needles and roots are widely-used traditional Chinese medicinal material, and the trunk are a source of resin and tannin<sup>[1]</sup>.

In recent years, PMBE has attracted more and more attention from domestic and foreign scholars. Standard PMBE contains 26.0-28.3% flavonoids (mainly proanthocyanidins, PAs) as important bioactive substances<sup>[2]</sup>. PAs are primarily known for their powerful anti-oxidative properties<sup>[3]</sup> and also show other biological activities, such as anti-cancer<sup>[4]</sup>, anti-inflammation<sup>[5]</sup>, anti-microbe<sup>[6]</sup>, cardiovascular protection<sup>[7]</sup>, and nerve protection<sup>[8]</sup>. According to their connection modes be-

tween monomers, PAs can be classified as A-type and B-type. A-type PAs are doubly linked by a 4 $\beta$ -8 (B-type) and 2 $\beta$ -O-7 inter-flavanoid bonds, while B-type PAs are only linked by C4-C8 or C4-C6 bonds<sup>[9]</sup>. B-type PAs are the main constituents of PMBE and that may determine the chemical activity of the mixture<sup>[10]</sup>. PMBE has been shown strong effects for antioxidant, anti-cancer, cell cycle arrest, anti-migration, antimicrobial, anti-virus and pro-apoptosis<sup>[1,11]</sup>. PMBE broadly inhibits the growth of cancer cells from eight major systems of human body<sup>[12]</sup>. Some scientists found that PMBE can promote hepatoma cell apoptosis through down-regulating the expression of Bcl-2<sup>[13,14]</sup>.

The *p53/p21* signaling pathway is reported one of the main mechanisms regulating cell senescence mainly by implementing widespread changes in gene expression<sup>[15]</sup>. The *p53* is master transcriptional regulator and *p21* is its downstream effector. The *p53/p21* pathway is cyclin-dependent kinase inhibitor (CDKI) and potent negative regulator of cell cycle progression<sup>[16]</sup>. Chronic activation or over-expression of *p53* or *p21* is generally sufficient to induce a growth arrest of senescence<sup>[16]</sup>. Cellular senescence refers to the essentially irreversible arrest of cell proliferation (growth) that occurs when cells experience potentially oncogenic stress<sup>[17]</sup>. The permanence of the senescent growth arrest enforces the idea that the senescence response evolved at least in part to suppress the development of cancer<sup>[18,19]</sup>.

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There are many researches on PMBE inducing cell cycle arrest and apoptosis. However, we have found few studies on cellular senescence by PMBE. Based on these findings, we hypothesized that PMBE may induce senescent cells to apoptosis by up-regulating the expression of P53 or P21 genes. Therefore, this present study aimed to evaluate the effect of PMBE on cell senescence of hepatoma with HepG2 as cell model.

## 2 Materials and methods

### 2.1 Chemicals and cell line

PMBE powder was purchased from N.B.C. Biological Material Co. Ltd. (Huzhou, Zhejiang, China) and contained more than 95% PAs, which consist of 24.18% monomers, 50.11% dimers, 20.32% trimers, and 5.39% polymers as determined by HPLC<sup>[20]</sup>.

Sorafenib-naive human hepatocellular carcinoma cell line (HepG2) and normal hepatic cell line (L02) was purchased from the cell banks of Chinese Academy of Sciences Committee Type Culture Collection. They were cultured in Dulbecco's modified Eagle's medium (DMEM; Solarbio, Beijing, China) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco), 100 units/ml penicillin, and 100  $\mu\text{g/ml}$  streptomycin. The penicillin/streptomycin mixture was purchased from Beyotime (Shanghai, China). Cells were maintained at 37°C in a humidified incubator containing 5% CO<sub>2</sub>. All other chemicals were of analytical grade. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium (MTT), dimethylsulfoxide (DMSO), trypsin, Senescence  $\beta$ -Galactosidase Staining Kit were purchased from Beyotime, Shanghai, China.

### 2.2 Preparation of PMBE stock and working solutions

PMBE powder was dissolved in dimethyl sulphoxide (DMSO; Beyotime, Shanghai, China) to make a 10 mg/ml stock solution, which was then sterilized by being passed through a 0.22  $\mu\text{m}$  micro-filter (Beyotime, Shanghai, China). Finally stored at -20°C until required. DMSO was not expected to exhibit any effect on cells under its final concentration in the working solution lower than 0.25%<sup>[20]</sup>.

### 2.3 The cell growth curve

HepG2 and L02 cells cultured in 10% FBS-DMEM media were digested with 0.25% trypsin (Gibico) and counted, then seeded into 24-well plates at a density of  $1 \times 10^4$  cells per well, respectively. The average cell density of the next three wells was calculated on each subsequent day. The data were collected to plot the cell growth

curve, and the logarithmic growth period of HepG2 and L02 cells was found for the following experiments.

### 2.4 Determination of suitable concentration of PMBE by MTT assay

HepG2 and L02 cells were cultured in 10% FBS-DMEM media till to logarithmic growth further above 90% confluency, then digested with 0.25% trypsin (Gibico) in sterile 1 $\times$ phosphate buffered saline (PBS; HyClone), and finally suspended in complete culture media. Then, cells were seeded into a 96-well plate at a density of  $2 \times 10^3$  cells per well. When the cells had grown into the logarithmic growth phase, they were divided into several groups. Different concentrations of PMBE (0-130  $\mu\text{g/ml}$ ) were added into each well. After 48h, the growth inhibition rate of each concentration was obtained by 2-(3,5-diphenyltetrazol-2-ium-2-yl)-4,5-dimethyl-1,3-thiazole bromide (MTT) assay (Beyotime, Shanghai, China).

### 2.5 Senescence $\beta$ -Galactosidase Staining

Two suitable concentrations of PMBE (70  $\mu\text{g/ml}$  and 40  $\mu\text{g/ml}$ ) were obtained through the above experiments. HepG2 and L02 cells were seeded in 6-well plates at a density of  $1 \times 10^4$  cells per well and cultured for 3 days and entered the logarithmic growth phase. Then cells were treated with 70  $\mu\text{g/ml}$  and 40  $\mu\text{g/ml}$  PMBE for 48 h, respectively. Then, senescence assay was performed according to the protocol of senescence  $\beta$ -galactosidase staining kit from Beyotime, Shanghai, China<sup>[21]</sup>. Observed under the ordinary optical microscope, the percentage of blue-stained cells was calculated from ten randomly selected viewing fields according to the following equation: senescence rate (%) =  $N_{\text{senescent cells}}/N_{\text{all cells}} \times 100$ , where N stood for the total cell number in all viewed fields.

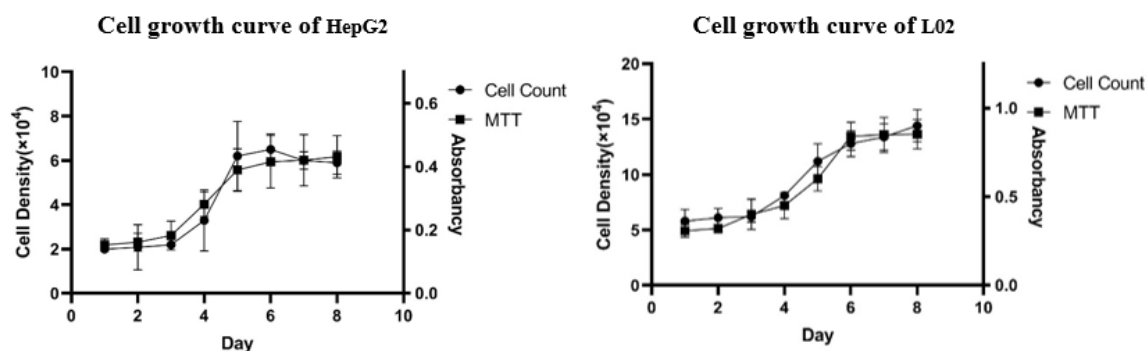
### 2.6 Statistical analysis

Statistical analyses were performed with Microsoft Excel 2010 and SPSS 20.0 using the *t* test for linear regression analysis. Results were expressed as the mean  $\pm$  standard deviation (SD) of *n* independent experiments. “\*” indicates that the calculated *p* value was less than 0.05 (*p* < 0.05) versus the corresponding control, which was regarded as significant.

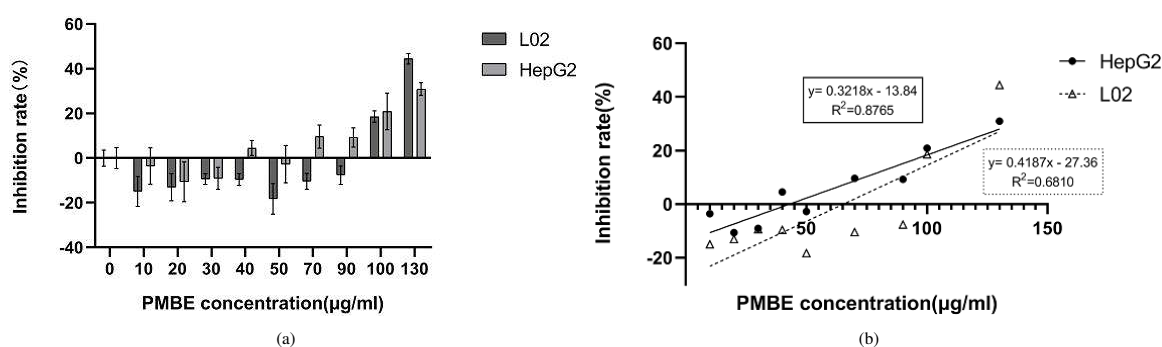
## 3 Results

### 3.1 The cell growth curve

HepG2 and L02 cells were seeded into 24-well plates at a density of  $1 \times 10^4$  cells per well and counted using blood



**Figure 1.** Growth curve of HepG2 and L02 cells. Both HepG2 and L02 cells entered the logarithmic growth phase on the fourth day. In terms of mechanism, cell counting method is more reliable than MTT method



**Figure 2.** Effect of PMBE on the growth of L02 and HepG2 cells. (a) Inhibition rate and standard deviation of L02 and HepG2 cells at different concentrations. Mean  $\pm$  standard deviation ( $n = 6$ ); (b) Statistical analysis of inhibition rate. Results showed obvious concentration-dependent effect, however, no obvious regularity was found between adjacent concentration gradients. Under concentrations of 40  $\mu\text{g/ml}$  and 70  $\mu\text{g/ml}$ , PMBE inhibited the growth of HepG2 cells rather than L02 cells

counting chamber and MTT method, respectively. The results showed that both HepG2 and L02 cells entered the logarithmic growth phase on the fourth day (Figure 1).

### 3.2 Determination of suitable selective concentration of PMBE by MTT assay

HepG2 and L02 cells with logarithmic growth were treated with different concentrations of PMBE for 48 h. The inhibition rate for cell growth was detected by MTT assay and calculated by the following equation: inhibition rate (%) =  $(1 - A_{\text{Treatment}}/A_{\text{Control}}) \times 100$ , where A was the absorbance at 570 nm. By calculating the inhibition rate, two concentrations (40  $\mu\text{g/ml}$  and 70  $\mu\text{g/ml}$ ) only inhibiting HepG2 cells were found (Figure 2).

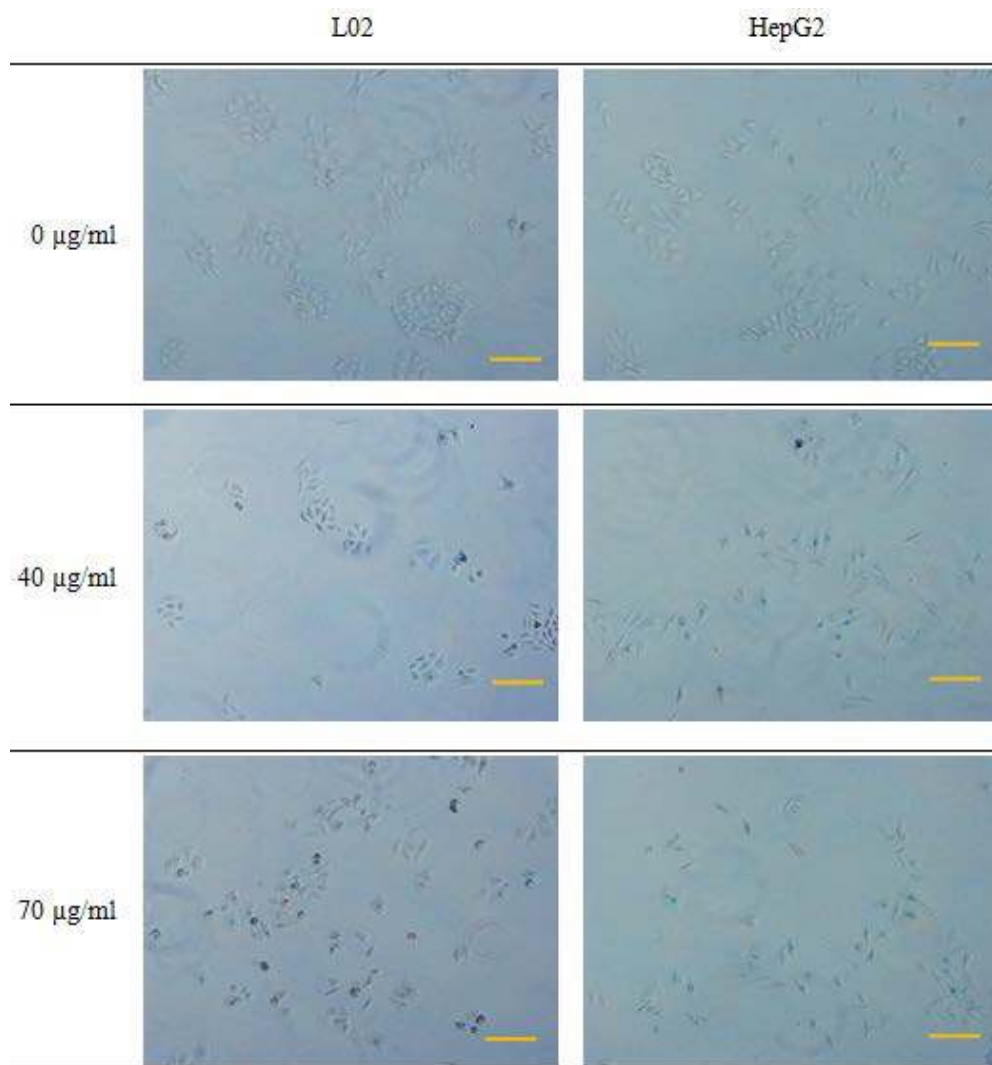
### 3.3 Senescence $\beta$ -Galactosidase Staining assay

The present concept of cellular senescence stems from the fact that it is an arrest of cellular cycle resulting in a permanent loss of proliferative potential in spite of viable signals (which fails to reinitiate cell cycling). Using the classical senescence-associated- $\beta$ -galactosidase (SA- $\beta$ -gal) staining in the HepG2 and L02 cells, we observed that PMBE induced senescence up to  $3.5 \pm 4\%$ ,  $31.3 \pm 8\%$  for

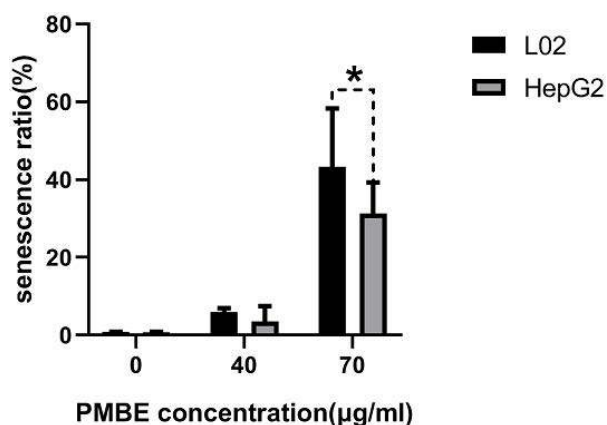
HepG2 at dose of 40  $\mu\text{g/ml}$  and 70  $\mu\text{g/ml}$ , respectively, in contrast to  $6.0 \pm 1\%$ ,  $43.3 \pm 15\%$  for the control group (L02). Besides SA- $\beta$ -gal staining, the small and wizened morphology which is marked as an important hallmark of apoptotic cell was also prominently observed in the treated cells with 70  $\mu\text{g/ml}$  PMBE. We inferred that with the increase of the concentration of PMBE, the percentage of stained cells gradually increased, showing an obvious concentration-dependent effect. PMBE was a highly potent therapeutic agent which diminishes the cancer cells' proliferative potential. However, under 40  $\mu\text{g/ml}$  there was no difference in the senescence-inducing effect of PMBE on HepG2 and L02 cells ( $p = 0.56$ ,  $>0.05$ ), and under 70  $\mu\text{g/ml}$  the percentage of stained cells of L02 was significantly greater than that of HepG2 ( $p = 0.043$ ,  $<0.05$ , Figure 3).

## 4 Discussion

*Pinus massoniana* is a tree species native to Southern China<sup>[14]</sup> and *P. massoniana* bark extract (PMBE) is an established traditional Chinese medicine used for the treatment of rheumatism, arthralgia, inflammation and cancer<sup>[22,23]</sup>. HPLC analysis indicated that PMBE contains several polyphenolic compounds, such as taxifolin



(a)



(b)

**Figure 3.** PMBE's effect on cell senescence of HepG2 cells. (a) The senescence induced by PMBE was quantified by counting SA- $\beta$ -Gal positive cells (scale bar 10  $\mu$ m); (b) Both L02 and HepG2 cells showed different degrees of senescence at 40  $\mu$ g/ml concentration, while apoptotic cells were even observed at 70  $\mu$ g/ml concentration. PMBE induced senescence up to 3.5  $\pm$  4%, 31.3  $\pm$  8% for HepG2 at dose of 40  $\mu$ g/ml and 70  $\mu$ g/ml respectively in contrast to 6.0  $\pm$  1%, 43.3  $\pm$  15% for the control group (L02). Mean  $\pm$  standard deviation (n = 3); \*p < 0.05



epicatechin and epigallocatechin gallate<sup>[14]</sup>. PMBE is reported to have many biomedical properties, such as antioxidant, anticarcinogenic, antimutagenic, antimicrobial, anti-inflammatory, and cardio-cerebrovascular protective, etc.<sup>[11]</sup>.

Even though, the anticancer effect has always been a hot topic in PMBE research. PMBE can inhibit the growth of eight different tumor cells *in vitro*<sup>[12]</sup> and selectively inhibit the proliferation of several cancer cells, such as human hepatoma BEL-7402 cells and HepG2 cells. But PMBE seldom influences normal cells<sup>[2,14]</sup>. Previous studies have demonstrated that PMBE selectively induce the apoptosis of hepatoma cells without impacting the growth of normal liver cells<sup>[14]</sup>, the investigation of the mechanism indicated that activation of extrinsic and intrinsic Caspases, inhibition of NF- $\kappa$ B activation and decrease of the antiapoptotic protein Bcl-2 and the intact Bid protein were involved<sup>[14]</sup>. In addition, PMBE was found to have obvious antiproliferation and apoptosis-inducing effects on colon cancer LoVo cells, mainly by inhibiting the growth of cancer cells by activating *p53* to up-regulate the expression of *p21*, and/or promoting the apoptosis of cancer cells by down-regulating the expression of *bcl-2* gene<sup>[24]</sup>.

The P53/P21 signaling pathway is one of the main mechanisms to regulate cell senescence, various stimulators of cell senescence function mainly by activating this pathway<sup>[15]</sup>. Cell senescence refers to the irreversible cell cycle stagnation caused by the activation of a series of intracellular signaling pathways under the stimulation of various internal and external factors<sup>[25]</sup>. Its characteristics mainly include chromatin changes, cell metabolism and morphological changes, and  $\beta$ -galactosidase activity enhancement<sup>[16]</sup>. Inducing cancer cell senescence can inhibit the proliferation of cancer cells, activate the body's immune response, and promote the clearance of cancer cells<sup>[26]</sup>. The present study investigated the senescence-inducing effect of PMBE on hepatoma HepG2 cells and the potential underlying mechanisms.

Firstly, the results of the current study indicated that PMBE was able to significantly affect the growth of hepatoma HepG2 cells with dose-dependent manner. The growth of HepG2 was promoted when PMBE concentration is below 50  $\mu\text{g/ml}$ , while when PMBE concentration is above 50  $\mu\text{g/ml}$ , the growth of HepG2 cells was inhibited by PMBE at dose-dependent manner. As for normal liver L02 cell, PMBE exhibit no inhibitory effect until the concentration was  $> 90 \mu\text{g/ml}$ , which is discrepant with previous studies, Ma *et al.* reported that PMBE exhibited little growth inhibition of L02 cells when its concentration is within 200  $\mu\text{g/ml}$ <sup>[14]</sup>. In addition, another study revealed that PMBE have hepatoprotective activity

against hydrogen peroxide ( $\text{H}_2\text{O}_2$ )-induced damage in normal human liver L02 cells<sup>[27]</sup>. These discrepancies may result from different sources of PMBE and different status of cell lines.

Secondly, the present study demonstrated that PMBE could induce HepG2 cells into senescence at concentration of 40  $\mu\text{g/ml}$ .

To the best of our knowledge, the current study is the first report to indicate that PMBE can induce cell senescence of hepatoma cells and further inhibit its growth, and the latter is similar to previous studies' report. However, the controversial result is that PMBE also exhibit cell senescence-inducing and cell growth-inhibiting effect on normal liver cells. Cui *et al.* ever reported that PMBE could inhibit the growth of human liver cancer cells BEL-7402 while slightly promote that of normal liver cells L02 under 200  $\mu\text{g/ml}$ <sup>[2]</sup>. Here may be some possible reasons. Firstly, the biological characteristics of L02 cell may change during cell passage, which resulted in increased sensitivity to PMBE; secondly, different sources of PMBE and storage conditions of PMBE stock solution may cause changes in its biological activity<sup>[2]</sup>; furthermore, DMSO in PMBE working solutions may exhibit cytotoxic effect, but previous studies demonstrated that DMSO was not expected to exhibit any effect on cultured cells when its concentration in the working solution was lower than 0.25%<sup>[20]</sup>. Therefore, in order to solve this dispute, we need to optimize the experimental scheme and repeat the experiments to obtain more accurate results. In addition, there are some limitations in this study. First, we used HepG2 cells as our target cell line, meaning our conclusions are based on *in vitro* data and the effects have not been examined *in vivo*; second, the conclusions of this study are just at the cellular level, to further understand the comprehensive effect of PMBE on cancer cells, the corresponding specific molecular mechanisms should be explored.

## 5 Conclusion

Summarily, the current study indicated PMBE could induce hepatoma cells into senescence and then inhibit their growth, but more studies should be performed to identify the potential mechanism and its safety in normal cells before its application in treatment of cancer in clinic.

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## Conflict of Interest

The authors declare no conflict of interest.

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