

# Protein tyrosine phosphatase controls breast cancer invasion through the expression of matrix metalloproteinase-9

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**The expression of matrix metalloproteinases (MMPs) produced by cancer cells has been associated with the high potential of metastasis in several human carcinomas, including breast cancer. Several pieces of evidence demonstrate that protein tyrosine phosphatases (PTP) have functions that promote cell migration and metastasis in breast cancer. We analyzed whether PTP inhibitor might control breast cancer invasion through MMP expression. Herein, we investigate the effect of 4-hydroxy-3,3-dimethyl-2H benzo[g]indole-2,5(3H)-dione (BVT948), a novel PTP inhibitor, on 12-O-tetradecanoyl phorbol-13-acetate (TPA)-induced MMP-9 expression and cell invasion in MCF-7 cells. The expression of MMP-9 and cell invasion increased after TPA treatment, whereas TPA-induced MMP-9 expression and cell invasion were decreased by BVT948 pretreatment. Also, BVT948 suppressed NF- $\kappa$ B activation in TPA-treated MCF-7 cells. However, BVT948 didn't block TPA-induced AP-1 activation in MCF-7 cells. Our results suggest that the PTP inhibitor blocks breast cancer invasion via suppression of the expression of MMP-9. [BMB Reports 2013; 46(11): 533-538]**

## INTRODUCTION

Breast cancer is the most frequently diagnosed cancer, it is also the leading cause of cancer death in females worldwide. Approximately 90% of breast cancer patients die as a result of

the invasive and metastatic growth of cancer (1). An essential process in forming distant metastases is the degradation of the extracellular matrix (ECM), this permits tumor cells to invade local tissue, to intravasate and extravasate blood vessels and allows new metastatic tumor formation. This process is primarily influenced by the activity of proteinases secreted by the tumor and stromal cells (2-4).

Matrix metalloproteinases (MMPs) are capable of degrading ECM components, and have been implicated in several aspects of tumor cell growth and invasion (5). The MMP gene family consists of at least 20 members and is associated with tumor progression and metastasis through its ability to degrade type IV collagen, the main component of basement membranes, as such it is thought to play an important role in breast cancer invasion (6). In particular, MMPs produced by cancer cells are of critical importance in tumor invasion and metastasis (7). MMPs can be stimulated by the inflammatory cytokine tumor necrosis factor (TNF)- $\alpha$ , growth factors, and phorbol esters through activation of intracellular signaling pathways (8).

Protein-tyrosine phosphatases (PTPs) are involved in the regulation of a diverse range of cellular processes, and function as positive or negative regulators of intracellular signaling. Many reports have demonstrated that PTP can promote cell migration in mammalian cells (9). Furthermore, it has recently been shown that PTPs induce MMP-9 expression in MCF-7 breast cancer cells (10), suggesting that PTPs might regulate breast cancer cell invasion through MMP-9 expression.

In this study, we investigated the effect of 4-hydroxy-3,3-dimethyl-2H benzo[g]indole-2,5(3H)-dione (BVT948), a novel PTP inhibitor, on 12-O-tetradecanoyl phorbol-13-acetate (TPA)-induced MMP-9 expression and cell invasion in MCF-7 cells. This study shows the first evidence that PTP inhibitor, BVT948, blocks breast cancer cell invasion via suppression of the expression of MMP-9.

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<http://dx.doi.org/10.5483/BMBRep.2013.46.11.053>

Received 8 March 2013, Revised 19 March 2013,  
Accepted 26 March 2013

**Keywords:** MCF-7, Metastasis, MMP, NF- $\kappa$ B, PTP

## RESULTS

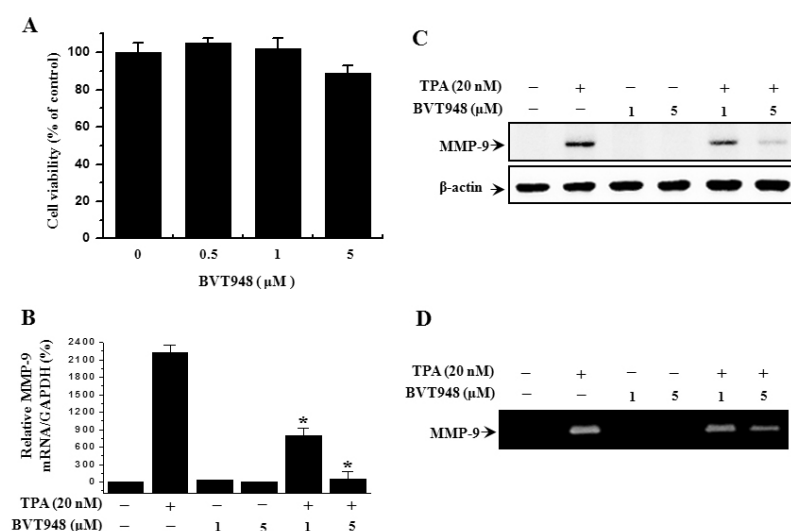
### Effect of BVT948 on MCF-7 cell viability

In order to investigate the cytotoxicity of BVT948 on MCF-7 cells, the cells were seeded into 96-well culture plates at a density of  $1 \times 10^5$  cells/plate. The influence of BVT948 on MCF-7 cellular toxicity was then analyzed using the MTT assay. Treatment of MCF-7 cells with 0.5, 1 or 5  $\mu\text{M}$  of BVT948 for 24 h did not cause any significant changes in cell viability (Fig. 1A). Therefore, upon subsequent experimentation, nontoxic concentrations (1 and 5

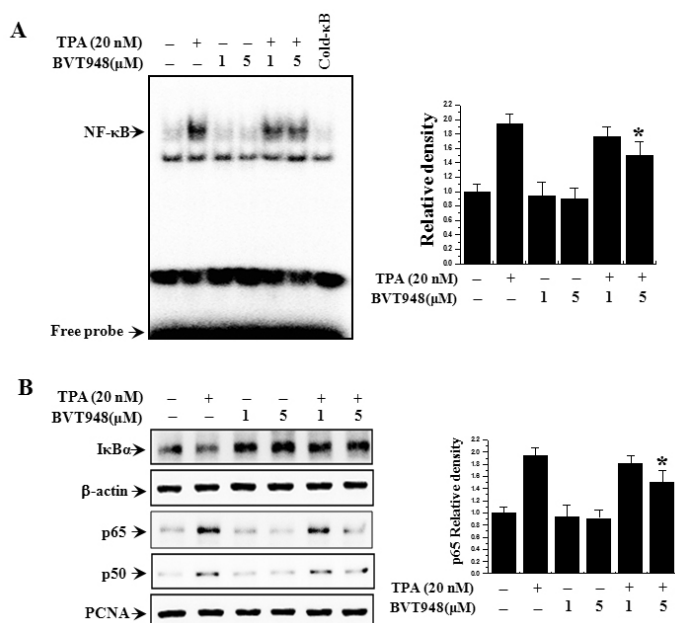
$\mu\text{M}$ ) of BVT948 were used.

### Effect of BVT948 on TPA-induced MMP-9 expression in MCF-7 cells

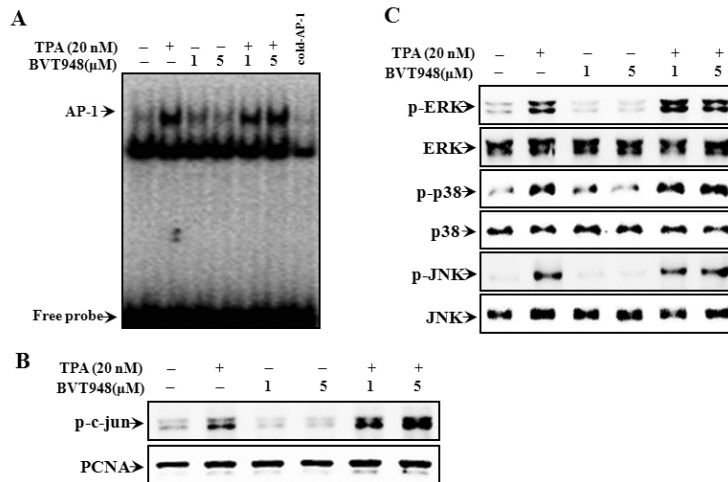
To investigate the effect of BVT948 on TPA-induced MMP-9 expression, western blot, real-time PCR and zymography were performed in MCF-7 cells. Real-time PCR revealed an increase in the MMP-9 level by TPA, and also revealed that BVT948 inhibited TPA-induced MMP-9 up-regulation in a dose-dependent manner (Fig. 1B). Western blot analysis revealed that BVT948



**Fig. 1.** Effects of BVT948 on the viability of MCF-7 cells and TPA-induced MMP-9 expression. Cells were cultured in 96-well plates until 90% confluence, and various concentrations of BVT948 were then added to cells for 24 h. An established MTT assay was used to detect the viability of the cells (A). MCF-7 cells were treated with the indicated BVT948 concentrations in the presence of TPA for 24 h. MMP-9 mRNA levels were analyzed by real-time PCR, and GAPDH was used as an internal control (B). Cell lysates were analyzed by Western blot with an anti-MMP-9 antibody. The blot was retaken with anti  $\beta$ -actin to confirm equal loading (C). Conditioned medium was prepared and used for gelatin zymography (D). Each value represents the mean  $\pm$  SEM of three independent experiments. \* $P < 0.01$  vs. TPA.



**Fig. 2.** BVT948 blocks TPA-induced NF- $\kappa$ B activation in MCF-7 cells. Cells were treated with BVT948 in the presence of TPA. Following 3 h incubation, nuclear extracts were prepared. NF- $\kappa$ B DNA binding was analyzed by EMSA (A). The translocation of p65 and p50 to the nucleus and I $\kappa$ B $\alpha$  degradation in the cytoplasm were determined by Western blotting.  $\beta$ -actin and PCNA were used as loading controls for cytoplasmic and nuclear proteins, respectively (B). Each value represents the mean  $\pm$  SEM of three independent experiments. \* $P < 0.01$  vs. TPA.



**Fig. 3.** BVT948 doesn't block TPA-induced AP-1 and MAPK signaling activation in MCF-7 cells. Cells were treated with BVT948 in the presence or absence of TPA. Following 3 h incubation, nuclear extracts were prepared. AP-1 DNA binding was analyzed by EMSA (A). The phosphorylation of c-Jun, a major subunit of AP-1 was determined by Western blotting and PCNA was used as loading control for nuclear protein (B). Cells were pre-treated with BVT948 for 15 min in the presence or absence of TPA. Cell lysates were prepared for Western blotting with specific p-ERK, ERK, p-p38, p38, p-JNK, and JNK antibodies (C).

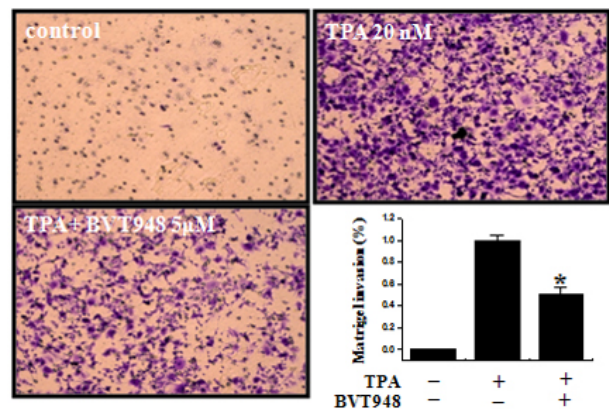
treatment of MCF-7 cells blocked the up-regulation of TPA-induced MMP-9 protein expression (Fig. 1C). To determine the effect of BVT948 on TPA-induced MMP-9 secretion a zymography analysis was carried out, this demonstrated TPA increased MMP-9 secretion from MCF-7 cells. However, BVT948 significantly diminished TPA-induced MMP-9 secretion (Fig. 1D). These results indicate that BVT948 is a potent inhibitor of TPA-induced MMP-9 expression in MCF-7 cells.

#### Effect of BVT948 on TPA-induced NF-κB and AP-1 activation

To clarify the mechanism by which BVT948 inhibits MMP-9 expression, the effect of BVT948 on TPA-induced activation in NF-κB and AP-1 was evaluated using EMSA. As shown in Fig. 2A and 3A, TPA substantially increased NF-κB and AP-1 binding activities. Treatment with BVT948 inhibited TPA-stimulated NF-κB binding activity, but not AP-1 binding activity. We examined whether BVT948 affects the degradation of IκBα and the nuclear translocation of the NF-κB p65 and p50 subunits. The increased level of IκBα degradation and translocation of p65 and p50 as a result of TPA stimulation were significantly suppressed by treatment with BVT948 (Fig. 2B). As shown in Fig. 3B, we also determined whether BVT948 affects the TPA-induced phosphorylation of c-Jun, which indicates the activation of AP-1(11). The phosphorylation of c-Jun was not affected by BVT948. These results indicate that BVT948 inhibits NF-κB activation by suppressing IκBα degradation and the nuclear translocation of NF-κB in TPA-treated MCF-7 cells.

#### Effects of BVT948 on TPA- induced MAPK activation

To investigate which inhibitory activities of BVT948 are mediated by MAPK (ERK, p38 and JNK), TPA-induced MAPK activation was determined using western blot analysis. BVT948 did not affect the MAPK phosphorylation by TPA. These results suggest that the MAPK pathways are not involved in the inhibition of TPA-induced MMP-9 expression by BVT948 (Fig. 3C).



**Fig. 4.** BVT948 inhibits TPA-induced Matrigel invasion. A Matrigel migration assay was carried out with BVT948 in the presence of TPA (20 nM). Cells were seeded onto the upper chamber and TPA and BVT948 were placed in the well. After 24 h incubation, cells on the bottom of the filter were fixed, stained and counted. Each value represents the mean  $\pm$  SEM of three independent experiments. \*P < 0.01 vs. TPA.

#### Effect of BVT948 on TPA-induced MCF-7 cell invasion *in vitro*

It has been reported that the up-regulation of MMP-9 expression contributes to the invasion of cancer cells (12-14). An *in vitro* invasion assay was used to investigate the inhibitory effects of BVT948 on the invasive potency of MCF-7 breast cancer cells. Treatment with TPA-induced a 10-fold increase in MCF-7 cell invasion when compared with untreated control cells, as determined by a Matrigel invasion assay. However, treatment with BVT948 diminished the TPA-induced cell invasion by 50% (Fig. 4).

## DISCUSSION

This paper is the first evidence that PTPs might control breast

cancer invasion via suppression of the expression of MMP-9. In this study, we demonstrated that BVT948, a novel PTP inhibitor blocks TPA-induced MMP-9 expression and cell invasion in MCF-7 cells. BVT948 blocked the TPA-mediated activation of NF- $\kappa$ B, but not that of AP-1 in MCF-7 cells. These findings suggest that the PTP inhibitor blocks cancer cell invasion through the suppression of NF- $\kappa$ B-mediated MMP-9 expression. Thus, the PTP inhibitor may be a potential candidate in the development of novel therapeutics to prevent breast tumor invasion and metastasis.

It has been well known that a number of important signaling pathways are modulated by reversible tyrosine phosphorylation, which is regulated by the opposing actions of protein-tyrosine kinases (PTKs) and PTPs (15). Thus, PTPs are important signaling enzymes that serve as key regulatory components in signal transduction pathways. Defective or inappropriate regulation of PTP activity leads to aberrant tyrosine phosphorylation, which contributes to the development of many human diseases, including cancers (16). Recently, the involvement of certain PTPs in cancer metastasis has been extensively studied (17). PTP1B overexpression is a common phenotypic manifestation in human breast cancers (18). SHP2 knockdown in established breast tumors blocked their growth and reduced metastasis. The SHP2 that is simultaneously activated in a large subset of human primary breast tumors is associated with invasive behavior and poor prognosis (19). Together, these reports indicate that PTPs are important in metastasis, and so, affect the prognosis of breast cancer patients.

Among MMPs, it well known that MMP-9 plays a critical role in the breakdown of ECM in normal physiological processes, such as embryonic development, reproduction and tissue remodeling, as well as in disease processes such as tumor metastasis (3, 20). MMP-9 activation has been shown to be associated with tumor progression and invasion, including that of mammary tumors (21). In previous reports, inflammatory cytokines, growth factors, and phorbol esters have been shown to stimulate MMP-9 by activating different intracellular-signaling pathways in breast cancer cells (22-24). The PKCs can be activated by phorbol esters *in vitro* and TPA acts as a potential inducer of tumor invasion and migration in various tumor cells. Upregulation and activation of PKCs are highly correlated with increased invasiveness in breast carcinomas (25-27). The inhibitory effects on MMP-9 expression are important for the development of a therapeutic experimental model of tumor metastasis.

The three major MAPKs families: JNK, ERK and p38 kinase are expressed in the MCF-7 cell and active phosphorylated forms of these proteins have also been detected in these cells (28). The role of MAPKs as upstream modulators of NF- $\kappa$ B in the activation of MMP-9 expression is well known (29, 30). However, this study has shown that BVT948 did not inhibit the phosphorylation of MAPKs in TPA-mediated signaling pathways, indicating that BVT948 is not involved in the TPA-stimulated MAPK/NF- $\kappa$ B pathway. Thus, it suggests that other pathways may be associated with the upstream modulators of NF- $\kappa$ B in the inhibitory activities of BVT948.

The activating NF- $\kappa$ B transcription factor is reported to occur in the regulation of MMP-9 gene expression (29-31). NF- $\kappa$ B comprises of a family of inducible transcription factors that regulate host inflammatory and immune responses. Diverse signal transduction cascades mediate NF- $\kappa$ B pathway stimulation (32). NF- $\kappa$ B is an inducible dimeric transcription factor that belongs to the Rel/NF- $\kappa$ B family and consists of two major polypeptides, p65 and p50 (33). NF- $\kappa$ B is initially located in the cytoplasm, in an inactive form, complexed with I $\kappa$ B - an inhibitory factor of NF- $\kappa$ B. Consequently, we identified the molecular mechanisms of NF- $\kappa$ B and AP-1 signals and the inhibitory effects of BVT948 pathways in breast cancer cells. The results show that BVT948 is a potent inhibitor of TPA-induced MMP-9 expression. However, BVT948 blocks only the NF- $\kappa$ B activation in MCF-7 cells, but not AP-1. Our results show that BVT948 blocks MMP-9 expression of breast cancer cells by inhibiting the TPA-stimulated NF- $\kappa$ B pathway.

## MATERIALS AND METHODS

### Cells and materials

MCF-7 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in high glucose containing Dulbecco's modified Eagle's medium (DMEM), this was supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics at 37°C in a 5% CO<sub>2</sub> incubator. BVT948 was purchased from Tocris Bioscience (Ellisville, Missouri 63021, USA) and was dissolved in dimethyl sulfoxide (DMSO). 12-O-tetradecanoylphorbol-13-acetate (TPA), 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and anti- $\beta$ -actin antibody were obtained from Sigma-Aldrich (St. Louis, MO, USA). The antibody related to p38, phosphorylated p38 (p-p38), c-Jun N-terminal kinase (JNK), p-JNK, extracellular signal-regulated kinase (ERK) and p-ERK were purchased from Cell Signaling Technology (Beverly, MA, USA). The antibody related to MMP-9, p50, p65, proliferating cell nuclear antigen (PCNA), I $\kappa$ B $\alpha$ , and horseradish peroxidase (HRP)-conjugated IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). [ $\alpha$ -<sup>32</sup>P]dCTP was obtained from Amersham (Buckinghamshire, UK). High glucose-containing DMEM, FBS and phosphate-buffered saline (PBS) were obtained from Gibco-BRL (Gaithersburg, ME, USA).

### Determination of cell viability

The effect of BVT948 on cell viability in MCF-7 was determined using an MTT assay. Briefly, cells of  $3 \times 10^4$  cells/well were inoculated in a 96-well plate and were incubated at 37°C for 24 h to allow for attachment. The attached cells were either untreated or treated with 0.5, 1, or 5  $\mu$ M BVT948 for 24 h at 37°C. The cells were then washed with PBS prior to the addition of MTT (0.5 mg/ml PBS), and were incubated at 37°C for 30 min. Formazan crystals were then dissolved with DMSO (100  $\mu$ l/well) and were detected at 570 nm using a model 3550 microplate reader (Bio-Rad, Richmond, CA, USA).

### Western blot analysis

MCF-7 cells ( $7 \times 10^5$ ) were pretreated with 1  $\mu$ M or 5  $\mu$ M BVT948 for 1 h, and were then incubated with 20 nM of TPA for 24 h at 37°C. Cells were lysed with ice-cold M-PER Mammalian Protein Extraction Reagent (Pierce Biotechnology, Rockford, IL, USA). Samples (10  $\mu$ g) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to Hybond™-polyvinylidene fluoride membranes (GE Healthcare Life Sciences, Buckinghamshire, UK). Each membrane was blocked for 2 h with 2% bovine serum albumin or 5% skim milk, and was then incubated overnight at 4°C with 1  $\mu$ g/ml of a 1 : 2,000 dilution of primary antibody. HRP-conjugated IgG (1 : 2,000 dilutions) was used as the secondary antibody. Protein levels were determined using an image analyzer (Fuji-Film, Tokyo, Japan).

### Gelatin zymography assay

Gelatin zymography assay was performed as described previously (34).

### Quantitative real-time polymerase chain reaction

The total RNA was isolated from cells using TRIzol reagent, following the manufacturer's instructions. Total RNA of 1  $\mu$ g was transcribed into cDNA at a final volume of 20  $\mu$ l for the reaction buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 1 mM each dNTP) and 2.4 M oligo-d(T)16-primer, 1 U RNase inhibitor, and 2.5 U M-MLV RNase H-reverse transcriptase by incubation for 15 min at 70°C, 50 min at 42°C and 95°C for 10 min. MMP-9 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression were determined by real-time PCR using the ABI PRISM 7900 sequence detection system and SYBR® Green (Applied Biosystems, Foster City, CA, USA). The primers were: MMP-9 (NM 004994) sense, CCTGGAGACCTGAGAACCAA TCT; antisense, CCACCCGAGTGTAACCATAGC and GAPDH (NM002046) sense, ATGGAAATCCCATCACCATCTT; anti-sense, CGCCCCACTTGATTTTGG. To control for variation in mRNA concentration, all results were normalized to the GAPDH housekeeping gene. Relative quantitation was performed using the comparative C<sub>t</sub> method according to the manufacturer's instructions.

### Electrophoretic mobility shift assay (EMSA)

Nuclear extract of cells was prepared as described previously (34). An oligonucleotide containing the  $\kappa$ -chain ( $\kappa$ B, 5'CCGG TTAACAGAGGGGGCTTCCGAG-3') or AP-1 (5'CGCTTGAT GAGTCAGCCGGAA-3') binding sites were synthesized and used as a probe for the gel retardation assay. The two complementary strands were annealed and labeled with [ $\alpha$ -<sup>32</sup>P] dCTP. Labeled oligonucleotides (10,000 cpm), 10  $\mu$ g of nuclear extracts and binding buffer [10 mM Tris-HCl, pH 7.6, 500 mM KCl, 10 mM EDTA, 50% glycerol, 100 ng poly (dl · dC), 1 mM DTT] were then incubated for 30 min at room temperature in a final volume of 20  $\mu$ l. The reaction mixtures were analyzed by electrophoresis on 4% polyacrylamide gels in

0.5X Tris-borate buffer. The gels were dried and examined by autoradiography. Specific binding was controlled by competition with a 50-fold excess of cold  $\kappa$ B or AP-1 oligonucleotide.

### Invasion assay

Matrigel invasion assay was performed as described previously (34).

### Statistical analysis

Statistical data analysis was performed using ANOVA. Differences with a P < 0.05 were considered statistically significant.

### Acknowledgements

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea Government (MEST) (No. 2012-0006172), and the Korea Research Foundation Grant (KRF-2012040388), Republic of Korea, and Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2012R1A6A3A01040388).

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