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Discovery of a synthetic Aminopeptidase N inhibitor LB-4b as a potential anticancer agent

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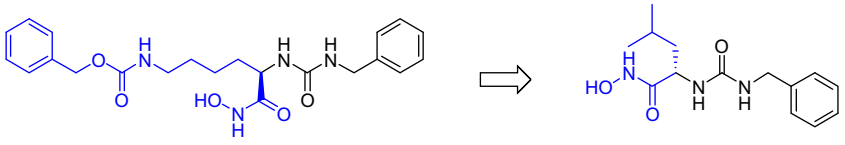
ABSTRACT

APN inhibitors have been considered as potential anticancer agents for years. LB-4b is the first synthetic APN inhibitor to be evaluated for both of its anti-invasion and anti-angiogenesis effects. As a potent synthetic APN inhibitor (IC_{50} = 850 nM, versus bestatin of 8.1 μM), LB-4b was determined to have more significant block effects to cancer cell invasion and angiogenesis than bestatin. Besides, it is able to be easily synthesized with a high total yield, while the reported synthetic methods of bestatin are much more complex.

Aminopeptidase N (APN/CD13, EC 3.4.11.2) is a type II transmembrane zinc-dependent metalloproteinase belonging to M1 family, and it is widely expressed on various cell types and tissues.[1] It is a multiple functional protein involved in cell survival, cell migration, cell invasion, virus invading, pathological regulation and angiogenesis.[2] Dysregulation of APN is found in ovary, prostate, colon, kidney, and lung cancers.[3] Recently, researchers point out that APN plays significant roles in cancer proliferation, metastasis, and cancer induced angiogenesis.[4] As an exopeptidase, APN prefers to cleaving neutral amino acids from the N-terminal of peptides. Extra cellular matrix (ECM) is one of its natural substrates.[5] ECM is the natural barrier to block malignant cell invasion, and ECM degradation is the first step of cancer metatstasis.[6] Besides, various cytokines are stored in ECM, such as VEGF and bFGF, which stimulate angiogenesis.[7] Additionally, in the angiogenesis process, ECM degradation promotes not only cytokines releasing but also endothelial cell invasion. According to those research results, APN could be seen as a therapy target related to both of cancer proliferation and metastasis.[8]

So far, reported APN inhibitors can be divided into natural products and synthetic compounds. Among the natural products, bestatin ((2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl-L-leucine, Ubenimex) as the only one on the market should be the most studied one. It is a dipeptidomimetic initially isolated from a culture filtrate of *Streptomyces olivoreticuli*.[9] As an immunomodulator, bestatin can mediate cancer cell death through T-cell, macrophage and NK cell activiation.[10] Anticancer research also demonstrates that bestatin exhibits significant inhibitory effects



1 (Compound 2a)
IC_{50} = 16.4 μM (Bestatin 5.87 μM)

2 (LB-4b)

Figure 1. Structures of compound **2a** and LB-4b.

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Table 1
IC_{50} and GI_{50} values of LB-4b[a]

Compound	IC_{50} (μM) to towards APN (porcine kidney)	IC_{50} (μM) to towards APN (ES-2 cell surface)	IC_{50} (μM) to towards MMP-2	GI_{50} (μM) towards ES-2
LB-4b	0.85 ± 0.06	3.0 ± 0.37	>500	>1000
Bestatin	8.1 ± 0.60	40 ± 3.8	156 ± 11	350 ± 36

[a] All of the compounds were assayed 3 times, and their inhibition results are expressed as the mean values with standard deviations.

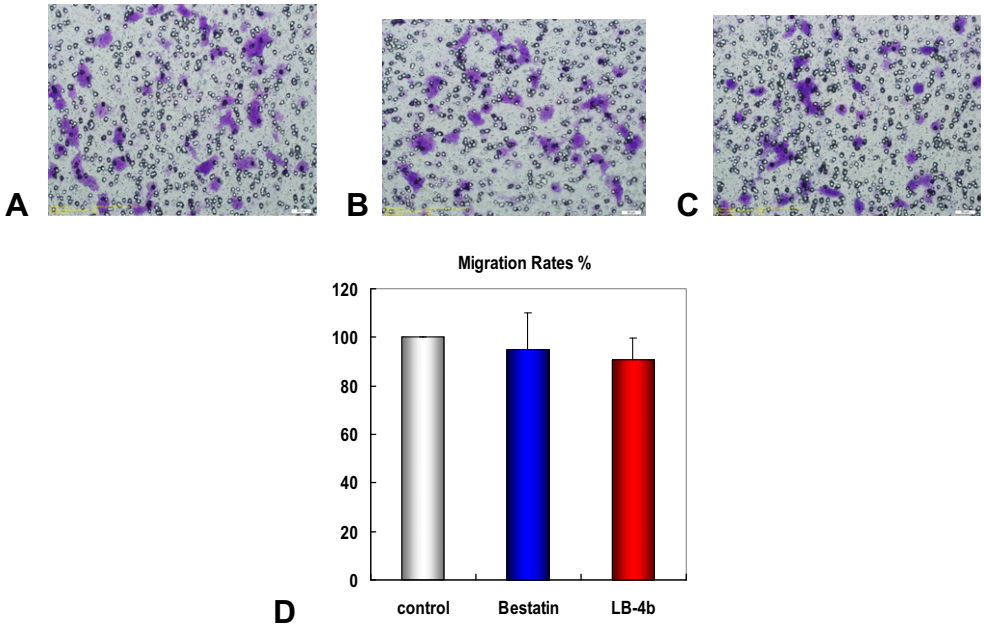

Figure 2. The effects of the compounds on ES-2 cells migration. (A) Control. (B) Bestatin 100 μg/mL. (C) LB-4b 100 μg/mL. (D) The migration rates of the compounds on ES-2. Each column represents the mean values with SD for three independent experiments.

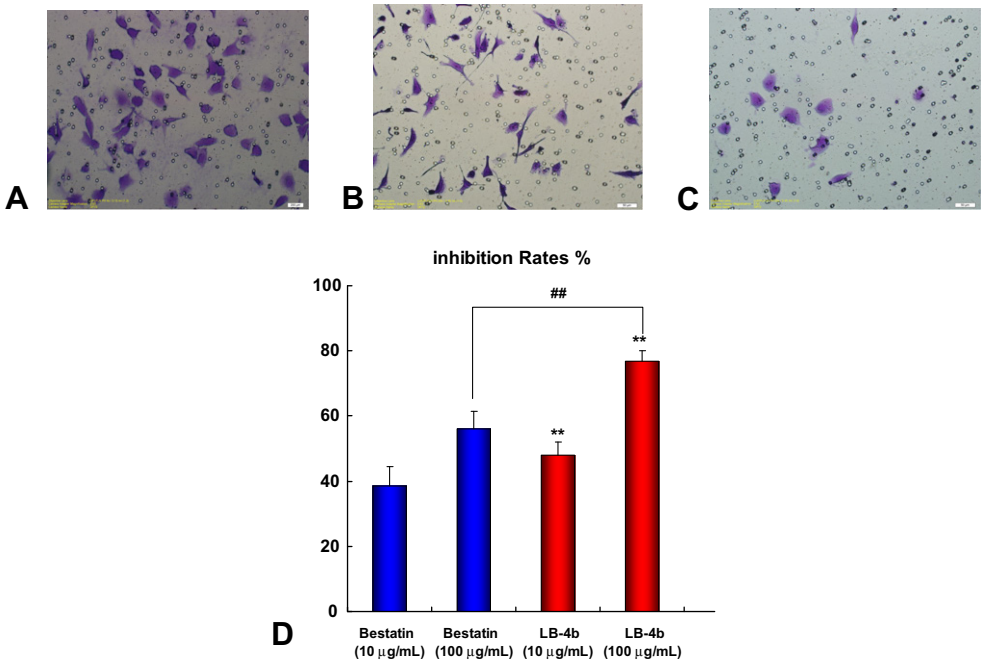

Figure 3. The inhibitory effects of the compounds on ES-2 cell invasion. (A) Control. (B) Bestatin 100 μg/mL. (C) LB-4b 100 μg/mL. (D) The invasion inhibition rates of the compounds on ES-2. Each column represents the mean values with SD values for three independent experiments. $^{**}P$ <0.01, versus the control. $^{\#\#}P$ <0.01, versus Bestatin treated groups.

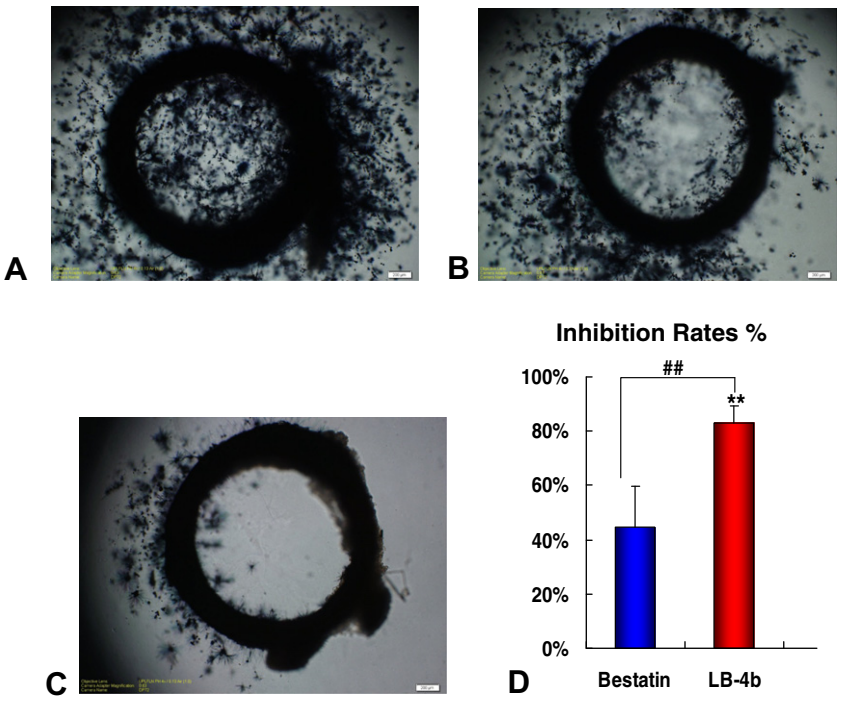

Figure 4. The inhibitory effects of the compounds on microvessel outgrowth arising from rat aortic ring. (A) Control. (B) Bestatin 100 μg/mL. (C) Lb-4b 100 μg/mL. (D) The inhibition rates on microvessel outgrowth arising from the rat aortic ring. Each column represents the mean values with SD for three independent experiments. $^{**}P$ <0.01, versus the control. $^{\#\#}P$ <0.01, versus Bestatin treated groups.

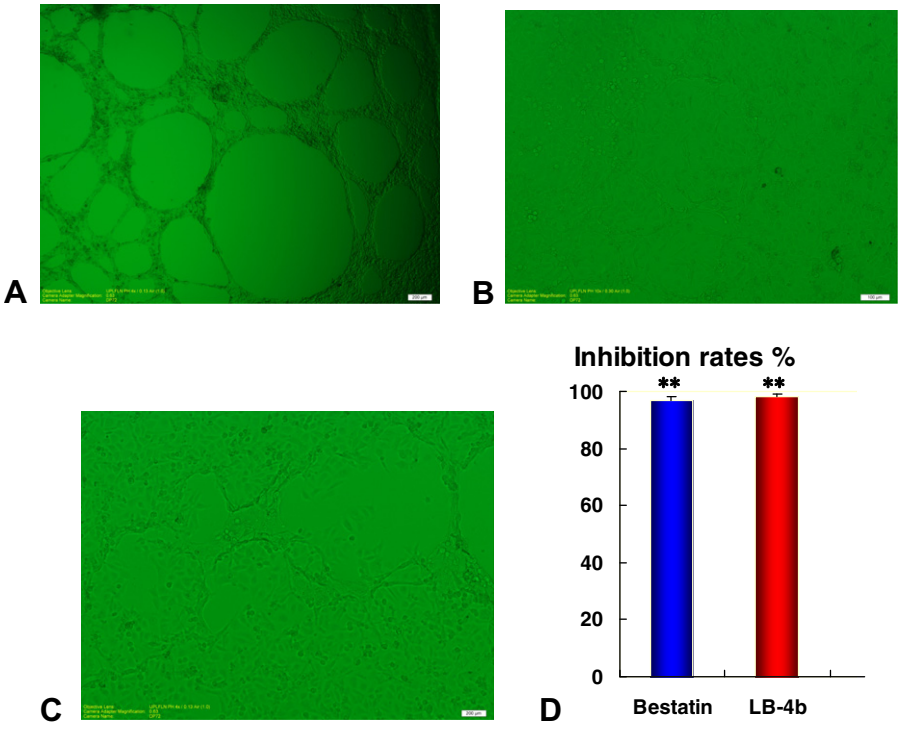

Figure 5. The inhibitory effects of the compounds on HUVEC capillary tube like structure formation. (A) Control. (B) Bestatin 100 μg/mL. (C) LB-4b 100 μg/mL. (D) The inhibition rates of the compounds on the tubular structures formation of HUVECs, column represents the mean values with SD for three independent experiments. $^{**}P$ <0.01, versus the control.

on APN activity, B16BL6 murine melanoma cell,[11] Lewis lung carcinoma cell and SN12P human renal cancer cell invasion.[12] Besides, bestatin can block human umbilical vascular endothelial cell (HUVEC) tubular structure formation and B16BL6 induced angiogenesis in C57BL/6 mice.[13] Furthermore, there are also similar reports on other natural occurring APN inhibitors such as AHPA-Val, amastatin, phebestin and lapstatin, etc.[14] But those natural products are difficult to be synthesized, because most of them have more than one chiral centers, such as (2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanic acid (AHPA) subunit in bestatin, probestin, and AHPA-Val, etc. Several total synthesis methods of bestatin have been reported, but the yields are far from satisfaction.[15] There are also various synthetic inhibitors reported, such as AHPA derivatives or analogs, organophosphonates, flavones, and hydroxamic acids, but only their APN inhibitory activity were evaluated.[16] In addition, bestatin is moderately potent. Therefore, more potent and easy-obtained synthetic APN inhibitors should be developed, and more profound studies on their anti-invasion and anti-angiogenesis effects are needed.

In our previous work, a series of L-lysine ureido derivatives were designed, synthesized and preliminarily evaluated for their APN inhibitory activity and anticancer potency.[17] Among them, several compounds, such as **1**, showed moderate potency toward APN (Fig. 1). In order to improve the potency, we made some modification, and discovered **2** (LB-4b) with approximately ten-fold improvement compared to **1** (Table 1). And **2** displayed quite high selectivity to APN over MMP-2 at the assay concentration. In our efforts to investigate the anti-invasion effect of this synthetic APN inhibitor, we selected ES-2 human ovarian clear cell carcinoma cell line, an advanced matastatic cancer, with high APN expression. Then the inhibitory activity of LB-4b towards APN on ES-2 cell surface was tested, and found it 10-fold more potent than bestatin as the positive control. Besides, it showed very weak influence on ES-2 cell survival (Table 1).

Cancer cell invasion includes ECM degradation and cell migration, etc. So both of ES-2 cell migration and invasion were assayed. Transwell chambers with 8 μm micropores were used in cell migration assay, on which cancer cells cross through the membrane with amoeboid movement. From Figure 2 we can see that both of bestatin and LB-4b did not exhibit obvious inhibitory effects on ES-2 cell migration. Transwell chambers coating with Matrigel were used in cell invasion assay. As an advanced metastatic cancer cell line, ES-2 could freely invade Matrigel and migrate to the other side of the chamber within 8 h, but it can be significantly inhibited by both of bestatin and LB-4b with a dose-dependent tendency, and LB-4b was more potent than bestatin (P <0.01) (Fig. 3). As the results shown above, LB-4b could inhibit APN activity on ES-2 cells and block ES-2 cell invasion without obvious influence on MMP-2 inhibition, ES-2 cell survival and migration.

Furthermore, we also investegated the anti-angiogenesis effects of LB-4b with bestatin as the positive control. Rat aortic ring microvessel growth and HUVEC tubular structure formation were the most commonly used angiogenesis models in vitro. Both of the two assays were performed on Matrigel, because the cytokines released from Matrigel could promote endothelial cell proliferation, and the vascular tubule formation process involved the invasion of endothelial cells into Matrigel. Therefore, by inhibiting APN activity, LB-4b should exhibit anti-angiogenesis effects. As shown in Figure 4, we can see notable new microvessel growth in 6 days, and it could be significantly suppressed by LB-4b, much more obviously than bestatin (P <0.01). As shown in Figure 5, HUVECs formed tubular structure within 8 h, and both of bestatin and LB-4b could block this phenomenon obviously. During the assay time, no acute cytotoxicity of the samples treated with bestatin or LB-4b was observed under microscope.

LB-4b as an APN inhibitor with significantly more potent anti-invasion and anti-angiogenesis effects than bestatin, can be easily obtained by the synthetic method shown in Figure 6. With benzylamine as the start material, it was transformed into benzyl isocyanate with triphosgene, and then coupled with L-leucine methyl ester to form the ureido group. Finally, the methyl ester was transformed into hydroxamic acid as the zinc binding group (ZBG). Compared with bestatin, the synthetic method of LB-4b is much easier, and the intermediates could be used in next steps without further purification, so the total yield is high (78%).

In order to compare the binding mode of LB-4b with bestatin to *Escherichia coli* APN (PDB ID: 2DQM), molecular docking was adopted. As shown in Figure 7, LB-4b chelated the zinc ion with its hydroxamate and ureido carbonyl groups. Similar to bestatin, LB-4b formed hydrogen bonds with Glu 264 and His 301 with the distance of 2.66 and 2.98 Å (for bestatin 2.63 and 2.73 Å), and formed hydrophobic interaction with Met 260, Gly 261, Met 263 Tyr 376 and Tyr 381. Figure 7B and C also showed that LB-4b could also form hydrogen bonds between its hydroxyl group of and His 297 of APN, as well as its phenolic hydroxyl hydrogen of Tyr 381, with the distance of 3.27 and 3.03 Å, respectively. We can also see LB-4b had hydrophobic interaction with Met 263, Tyr 376, Met 260 and Glu 261 residues of the target.

APN inhibitors have been considered as potential anticancer agents for decades. Natural occurring APN inhibitors are limited in development and application because of their rare sources and synthetic difficulty. Hydroxamic acids as important synthetic zinc-dependent metalloproteinase inhibitors have been developed as anticancer candidates for years, some of which have been on the market, such as Zolinza. LB-4b as a potent APN inhibitor, could be easily synthesized, and showed more significant anti-invasion and anti-angiogenesis effects than bestatin. Such outcomes prompt our continued efforts toward the studies of developing synthetic APN inhibitors as anticancer agents and revealing their potential mechanism of action.

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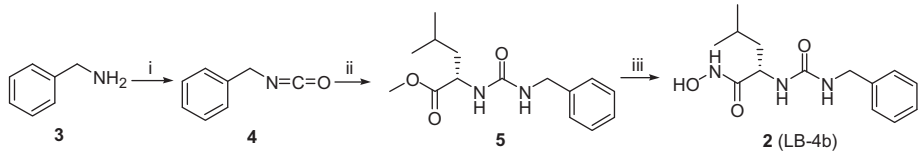

i) triphosgene, toluene, reflux; ii) L-leucine methyl ester, TEA, DCM, r.t.; iii) NH_2OK, MeOH, r.t.

Figure 6. Synthetic method of **2** (LB-4b).

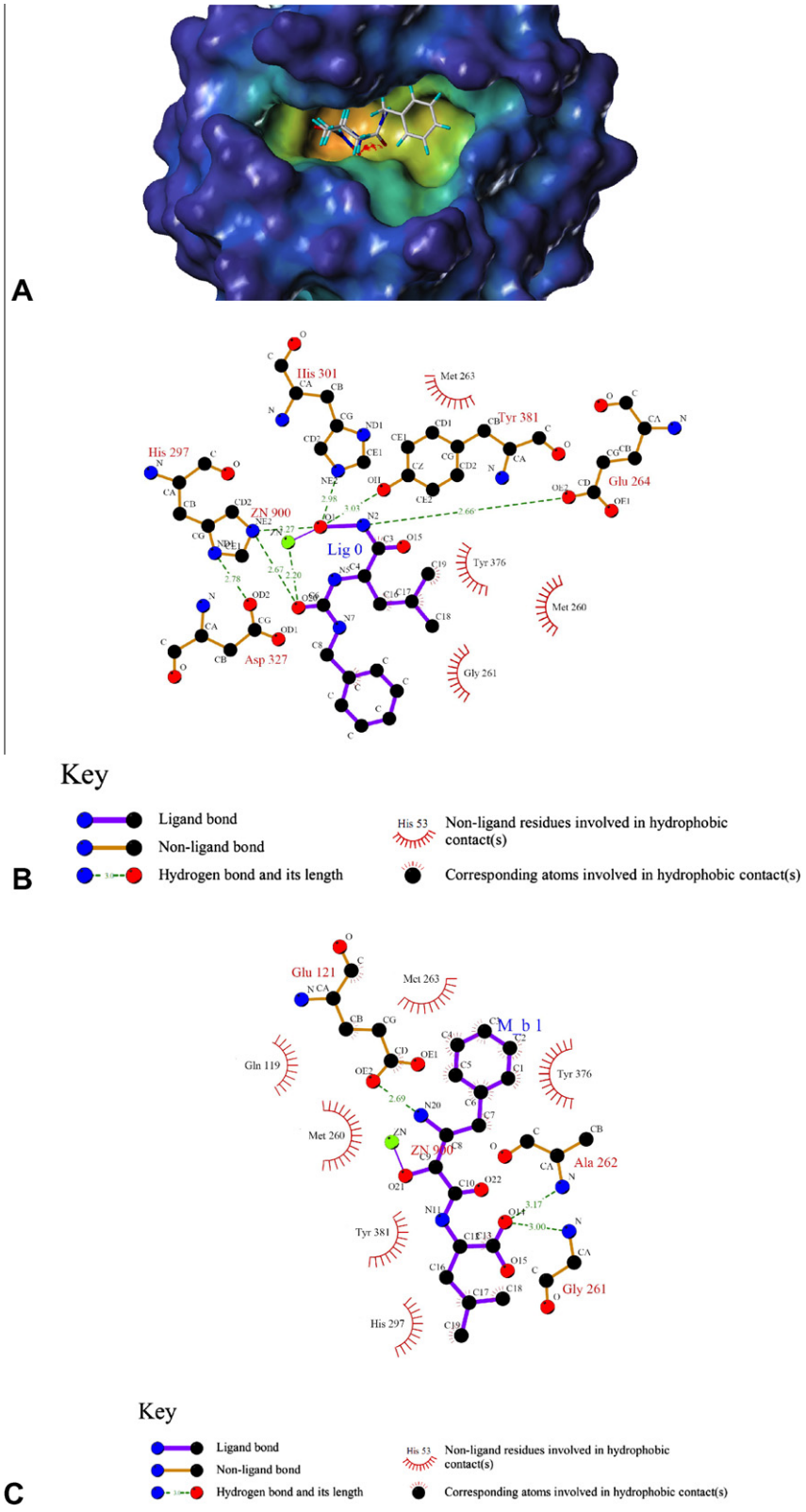

Figure 7. (A) Docking comformation of LB-4b in the APN (*E. coli*) active site. (B) Binding profile of LB-4b with APN (*E. coli*) obtained from Ligplot analysis. (C) Binding profile of Bestatin with APN (*E. coli*) obtained from Ligplot analysis.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.03.021.

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