Supporting Information

Suppression of hepatocellular carcinoma by inhibition of overexpressed ornithine aminotransferase

Ehud Zigmond,[1] Ami Ben Ya'acov,[1] Hyunbeom Lee,[2] Yoav Lichtenstein,[1] Zvi Shalev,[1] Yoav Smith,[3] Lidya Zolotarov,[1] Ehud Ziv,[4] Rony Kalman,[4] Hoang V. Le,[2] Hejun Lu,[2] Richard B. Silverman,*[2] and Yaron Ilan*[1]

[1]Liver, [3]Genomic Data Analysis, and [4]Diabetes Units, Faculty of Medicine, Hebrew University-Hadassah Medical Center, Jerusalem, Israel; [2]Department of Chemistry, Department of Molecular Biosciences, Chemistry of Life Processes Institute, and Center for Molecular Innovation and Drug Discovery, Northwestern University, Evanston, IL 60208, United States

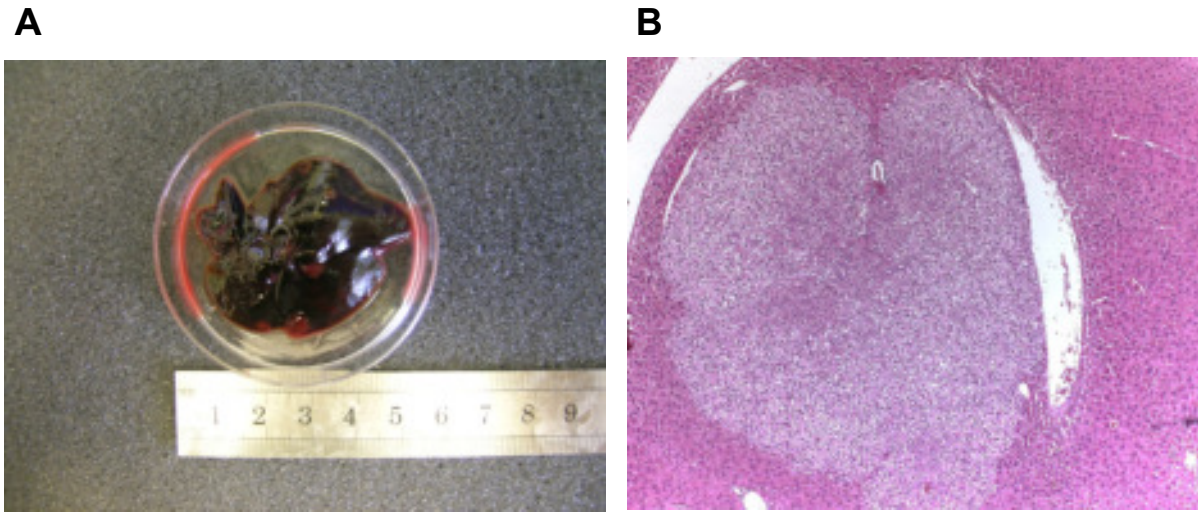
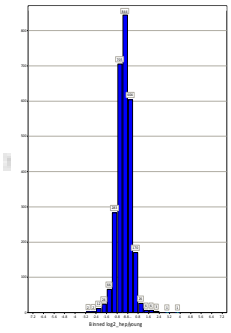
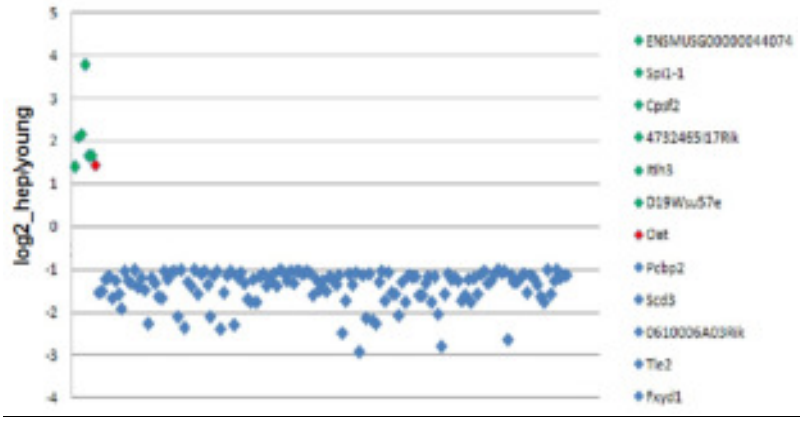


Figure S1. A. Spontaneous hepatic pre-neoplastic tissue and hepatomas from a 12-month old *Psammomys obesus*. **B.** Histologic examination of nodules containing hepatocytes characterized by malignant changes including excessive pleomorphism, loss of trabecular pattern, and tumor penetration across hepatic vein walls

A



B



C

ENSMUST	Spi1-1	NM_00924	M12586
ENSMUST00000060584			
ENSMUST	Cpsf2	NM_01685	BC007163
ENSMUST	4732465I17	NM_17257	BC031398
ENSMUST	Itih3	NM_00840	X70393
ENSMUST	Gldc	NM_13859	BC017135
ENSMUST	Oat	NM_01697	X64837

Figure S2. A. DNA microarray-based gene expression analysis performed on spontaneous HCC and compared with normal livers for RNA expression levels. **B.** Analysis of microarray data that identified seven genes whose expression levels were increased and 143 genes whose expression levels were decreased in tumor tissues compared with normal livers. The distribution along the diagonal line demonstrates identical behavior in the results of both the original and die swap set of arrays. **C.** Listed are genes with overexpressed mRNA in tumors, one of which is OAT.

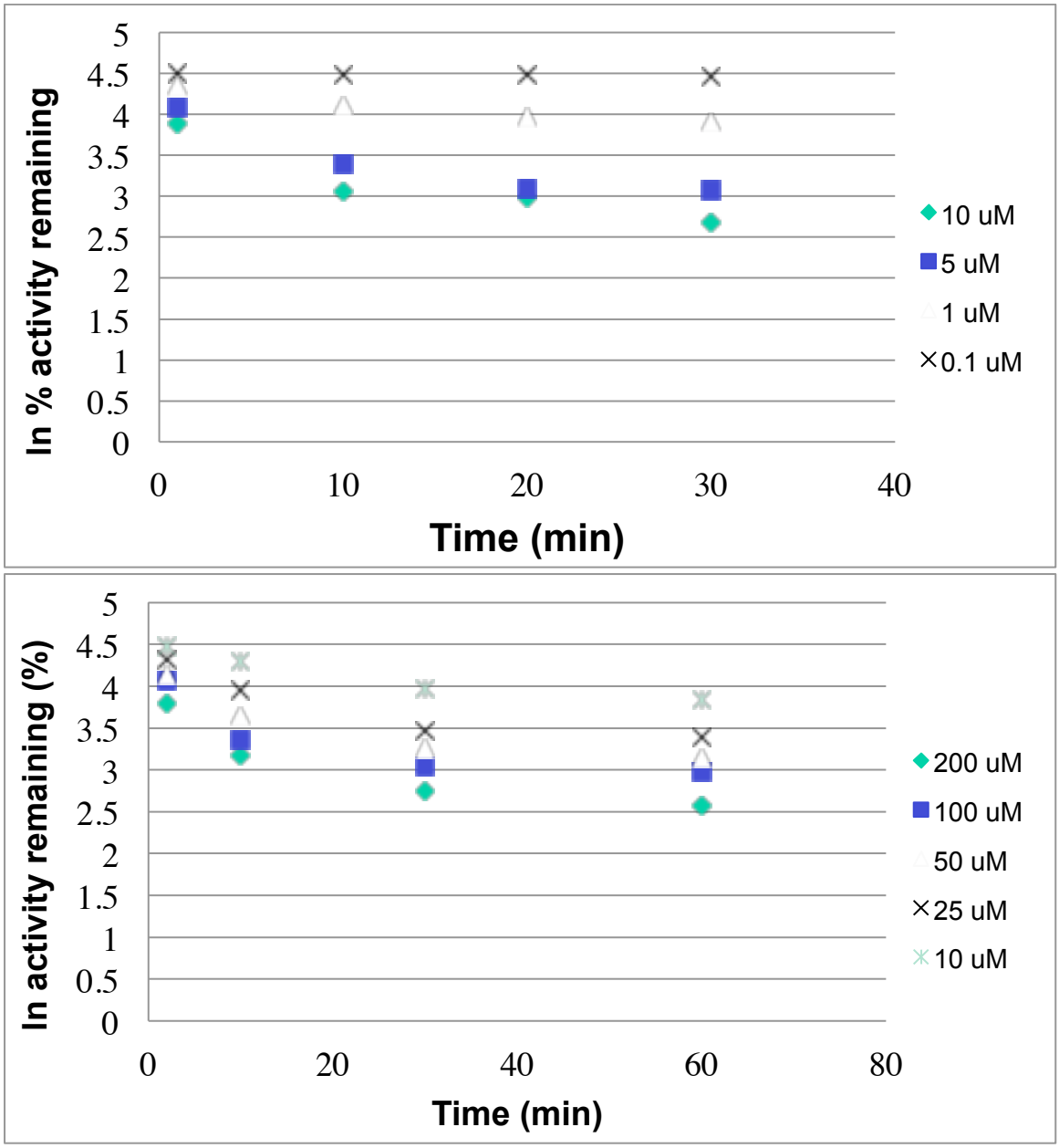
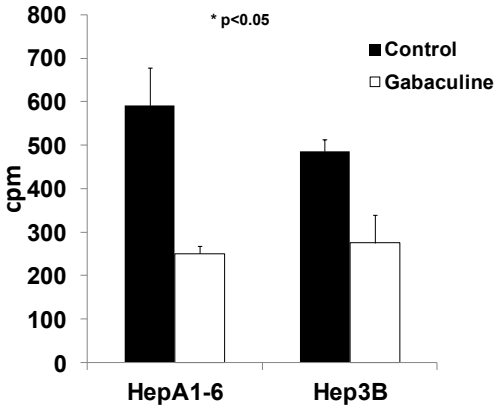


Figure S3. A. Time- and concentration dependent inhibition of OAT by gabaculine (**1**). **B.** Time- and concentration dependent inhibition of OAT by **2**. The natural logarithm of the percentage of remaining OAT activity is plotted against the preincubation time at each inhibitor concentration to obtain the k_{obs} (slope) value for each concentration. k_{obs} is the rate constant describing the inactivation at each inhibitor concentration.

A



B

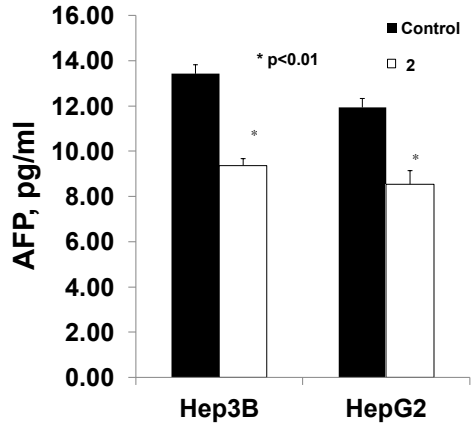


Figure S4. A. Gabaculine (50 μg/mL, white bars) was tested in vitro on HCC cell lines HepA1-6 and Hep3B. Forty-eight hours following exposure to 20000 umol/L of gabaculine, cell proliferation was assessed with a ^{3}H-thymidine assay and compared with untreated controls (black bars). Gabaculine significantly suppressed proliferation ($p<0.05$). **B.** In vitro effect of **2** (50 μg/mL) on HCC cell lines was determined by measuring AFP secretion in vitro from two hepatoma cell lines, Hep3B and HepG2. White bars represent treated cells and black bars the control.

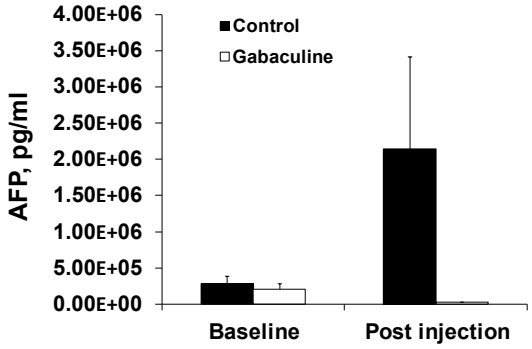


Figure S5. Administration of a single dose of **1** (0.1 mg/kg, white bars) suppressed AFP levels *in vivo* as compared with untreated controls (black bars). Within seven days of a single dose administration to HCC harboring mice, AFP serum levels were tested using an ELISA.

Table S1. Kinetic Constants for GABA analogs against OAT

Compound	k_{inact} (min^{-1})	K_I (mM)	k_{inact}/K_I (min^{-1} mM^{-1})	K_i (mM)
Gabaculine (1)	0.05	0.0021	23.8	-
2	0.018	0.0064	2.8	-
3	0.097	0.116	0.83	-
4	0.023	0.69	0.03	-
5	0.027	0.74	0.04	-
6				46
7	0.15	0.86	0.17	-
8	0.20	0.95	0.21	-
9	-	-	-	>10
10	-	-	-	0.15
11	-	-	-	>10
12	0.11	3.5	0.03	-
13	0.02	7.8	0.003	-
14	0.03	6.2	0.005	-
15	0.04	13	0.003	-
16	-	-	-	3.5
17	-	-	-	>5
18	-	-	-	>5
19	-	-	-	>5
20	-	-	-	2.2
21	-	-	-	>5
22	0.17	0.2275	0.75	-
23	-	-	-	>10
24	-	-	-	>10
25	-	-	-	>10

Table S2. Kinetic constants for the analogs against GABA-AT

Compound	k_{inact} (min^{-1})	K_I (mM)	k_{inact}/K_I (min^{-1} mM^{-1})	K_i (mM)
Gabaculine (1)[i]		2.86×10^{-3}		-
2[a]	-	-	-	4.2
3[b]	0.18	0.031	5.7	-
4[c]	0.25	0.25	1	-
5[c]	0.74	0.53	1.4	-
6[a]	0.24	0.85	0.28	-
7[a]	6.96	39.23	0.18	-
8[a]	2.89	7.74	0.37	-
9[a]	-	-	-	2.8
10[a]	0.68	4.11	0.17	-
11[e]	-	-	-	$IC_{50} > 10$ mM
12[d]	0.18	49	0.0013	4.2 ± 0.3
13[d]	-	-	-	0.19 ± 0.03
14[d]	0.02	16	0.0037	3.6 ± 0.4
15[d]	-	-	-	12 ± 2
16[f]	-	-	-	1.64 ± 0.44
17[f]	-	-	-	1.4 ± 0.14
18[f]	-	-	-	2.27 ± 0.51
19[f]	-	-	-	1.35 ± 0.08
20[f]	-	-	-	2.49 ± 0.28
21[f]	-	-	-	1.59 ± 0.14
22[g]	0.5	0.395	1.27	-
23[h]	-	-	-	$IC_{50} > 23$ mM
24[h]	-	-	-	$IC_{50} > 23$ mM
25[h]	-	-	-	$IC_{50} > 23$ mM

[a]Lu and Silverman[1]
[b]Pan et al[2]
[c]Pan et al[3]
[d]Qiu and Silverman[4]
[e]Yuan and Silverman[5]
[f]Hawker and Silverman[6]
[g]Silverman and Levy[7]
[h]Juncosa et al.[8]
[i]Rando[9]

EXPERIMENTAL SECTION

Experimental compounds. Gabaculine (**1**) was purchased from Sigma-Aldrich. All other compounds were prepared previously in the Silverman group and their preparation methods have been published as follows: compounds **2** and **6-10** were prepared by Lu and Silverman,[1] compound **3** by Pan et al.,[2] **4** and **5** were prepared by Pan *et al*.,[3] compound **11** was prepared by Yuan and Silverman,[5] compounds **12-15** were prepared by Qiu *et al*.,[4] compounds **16-21** were prepared by Hawker and Silverman,[6] compound **22** was prepared by Silverman and Levy,[7] and compounds **23-25** were prepared by Juncosa *et al*.[8]

Sand rats. Twenty-four month old male *Psammomys obesus* from the Jerusalem colony were purchased from Harlan laboratories (Jerusalem, Israel). Animals were maintained in a closed barrier and housed in solid bottom polypropylene cages equipped with water bottles and white pine woodchip bedding and were fed a standard artificial diet. Animal experiments were carried out in accordance with guidelines of the Hebrew University-Hadassah Institutional Committee for Care and Use of Laboratory Animals and with the committee's approval.

Mice. Eight-week old athymic male Balb/c mice were purchased from Harlan Laboratories (Jerusalem, Israel). Animals were kept in laminar flow hoods in sterilized cages in 12 h light/dark cycles.

Cell cultures. The HBsAg-expressing human hepatoma cell line, Hep-3B, was grown in culture as monolayers in a medium supplemented with non-essential amino acids and 10% heat inactivated fetal bovine serum.

DNA microarray-based gene expression. A DNA microarray-based gene expression profiling study was performed to compare HCC in a spontaneous HCC-developing sand rat model and normal liver tissue. A mouse 70-mer oligonucleotide microarray was used (Operon); sand rats are rare, and there are no microarrays available, but the mouse microarray is similar. The set includes 31,769 probes representing 24,878 genes. RNAs were purified from three tumoral and normal livers and were used to prepare fluorescent Cy-3 or Cy-5 labeled cDNA probes. Each Cy-5-labeled cDNA probe was combined with a Cy-3-labeled reference probe, and the mixture was hybridized to the microarray. The fluorescence ratio of each gene was quantified and reflected the relative abundance of RNA in the tumors compared with normal liver tissue. To exclude labeling biases, total RNAs from each pool of tumors were labeled with the reciprocal fluorochrome in a duplicate experiment (dye swap). Experiments were conducted with two pools of RNA, each from three different tumor sources.

Enzyme assays. All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Human recombinant ornithine-δ-aminotransferase (OAT) and human recombinant pyrroline 5-carboxylate reductase (PYCR1) were purchased from US Biologicals (Salem, MA, USA). OAT assays were recorded on a Synergy H1 hybrid multi-mode microplate reader (Biotek, USA) with transparent 96-well plates.

Time-dependent inhibition of OAT by gabaculine and GABA analogs. OAT activity assays were carried out using a modification of the coupled assay reported.[10] OAT (0.25 μg) was incubated with various concentrations of gabaculine (0.1 μM, 1 μM, 5 μM, 10 μM) or GABA analogs (10 μM, 25 μM, 50 μM, 100 μM, 200 μM) in 100 mM potassium pyrophosphate buffer, pH 8.0, containing 1 mM α-ketoglutarate in a total volume of 20 μL at room temperature. At time intervals, 80 μL of assay solution containing PYCR1 (0.5 μg), 12.5 mM α-ketoglutarate, 1 mM NADH, 0.03 mM PLP, and 25 mM L-ornithine in 100 mM potassium pyrophosphate buffer, pH 8.0, was added to the incubation mixture and assayed for OAT activity.

For the determination of K_I and k_{inact} values, the natural logarithm of the percentage of remaining OAT activity was plotted against the preincubation time at each inhibitor concentration to obtain the k_{obs} (slope) value for each concentration. The k_{obs} is the rate constant describing the inactivation at each inhibitor concentration. k_{obs} is replotted against the inhibitor concentration using nonlinear regression analysis (Graph-Pad Prism 6; GraphPad Software Inc.). k_{inact} and the K_I were estimated from the equation below:

$$k_{obs} = \frac{k_{inact} \times [I]}{K_I + [I]}$$

where k_{inact} is the maximal rate of inactivation, K_I is the inhibitor concentration required for half-maximal inactivation, and [I] is the preincubation concentration of inhibitor. The initial rate constants for the inactivation at various concentrations of the two compounds were determined using nonlinear regression analysis.

Inactivation of OAT by 2 and dialysis of the inactivated enzyme. OAT (30 μg) was preincubated for 24 h with 2 mM **2** in 100 mM pyrophosphate buffer (pH 8.0) containing 5 mM α-ketoglutarate in a total volume of 60 μL at room temperature. OAT incubated without the inactivator served as a control. After 24 h, the enzyme solutions were transferred to a D-Tube™ Mini dialyzer and exhaustively dialyzed against the buffer (100 mM pyrophosphate buffer containing 0.1 mM α-ketoglutarate and 0.1 mM PLP, pH 8.0) at 4 °C protected from light. The dialysis buffer was changed three times every 4 h and left overnight. After 48 h of dialysis, the remaining OAT activity in each of the solutions was assayed.

Inhibition of L-aspartate aminotransferase by 2. Microtiter plate wells were loaded with 90 μL of an assay mixture containing 100 mM potassium phosphate at pH 7.4, 5.55 mM α-ketoglutarate, 1.11 mM NADH, 5.55 mM *L*-aspartate, 5.55 units of malic dehydrogenase, and various concentrations of **2**. After incubation of the mixture at room temperature for a few minutes, 10 μL of Asp-AT (2.0 units/mL in 100 mM potassium phosphate at pH 7.4) was added. The plate was shaken at room temperature for 1 min, and the absorbance was measured at 340 nm every 10 s for 90 min. All assays were performed in duplicate.

Inhibition of L-alanine aminotransferase by 2. The assay was identical to that with L-aspartate aminotransferase except *L*-alanine was used as the substrate and lactate dehydrogenase was the enzyme.

Statistical analysis. All analyses were performed using Excel 2007 (Microsoft, Redmond, WA, USA). The variables are expressed as mean ± standard deviation (SD). The comparison of two independent groups was performed using the Student's t-test. All tests applied were two-tailed. P value of 0.05 or less was considered to be statistically significant.

In vitro effect of 1 on HCC growth. Gabaculine (**1**) was tested *in vitro* on Hep3b, HepA1-6, and HepG2 HCC cell lines as described.[11,12] Cells were plated in triplicates 1 x 10^5 cells/well, and incubated at 37 °C for 48 h with **1** (0.1, 1.0, 10, and 50 μg/mL). Cell proliferation and alpha fetoprotein (AFP) secretion were evaluated by a standard ELISA as described.[11]

Assessment of the effect of 2 on HCC growth *in vitro*. Compound **2** was tested for its effect on HCC growth *in vitro* in two hepatoma cell lines: Hep3B and HepG2 by measuring AFP secretion. Cells were plated in triplicates 1 x 10^5 cells/well, and incubated at 37 °C for 48 h with **1** (0.1, 1.0, 10, and 50 μg/mL). Cell proliferation and alpha fetoprotein (AFP) secretion were evaluated by a standard ELISA as described.[11]

In vivo effect of 1 on AFP levels. Athymic Balb/C mice were conditioned with sub-lethal radiation (400 cGy). At 24 h after irradiation, animals were injected subcutaneously in the right shoulder with 5 x 10^6 human hepatoma Hep3B cells as described.[11] Blood samples were obtained weekly by retrobulbar puncture, and serum was separated and frozen at -20 °C until assayed. On day 45 the mice were divided into two groups (n = 8 per treated and controls) and baseline serum AFP was measured. The experimental group was injected intra-peritoneally once with 500 microgram/kg of **1**. Mice in the control group were injected with saline. AFP serum levels, which correlate with tumor growth, were measured on day 52 using a standard ELISA.[11]

In vivo effect of 2 on AFP levels and HCC growth. Athymic Balb/C mice (n = 10) underwent total body sub-lethal irradiation (400 rad), followed three days later by subcutaneous transplantation of 5 x 10^6 Hep3b cells. Oral treatment was started on day 25, and all mice were sacrificed on day 53. Control mice were fed orally three times a week with vehicle (PBS). Two groups of treated mice received 20 μg (1 mg/kg) or 2 μg (0.1 mg/kg) of **2** three times a week, orally, per mouse, for five weeks. Mice in all groups were followed for serum AFP levels and tumor volume. At the end of the experiment the tumors were biopsied for determination of the degree of tumor apoptosis and necrosis using a phosphatidylserine detection kit (RocheDiagnostics GmbH, Penzberg, Germany).[13] The exposure of phosphatidylserine on the outside of the cell was monitored in cell suspensions using fluorochrome labeled-Annexin V by flow cytometry.

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