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Relationship between elevated levels of the alpha 1 acid glycoprotein in chronic myelogenous leukemia in blast crisis and pharmacological resistance to imatinib (Gleevec®) *in vitro* and *in vivo*

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Abstract

The Abl tyrosine kinase inhibitor imatinb is becoming a standard for the treatment of chronic myelogenous leukemia (CML). However, Bcr-Abl gene mutations have been reported mainly in relapsing or resistant patients. In primary resistant patients, only few mutations have been documented so far, suggesting alternative mechanisms. We aimed to investigate if alpha 1 acid glycoprotein (AGP), an acute phase drug binding protein, could be a biological marker for pharmacological resistance to imatinib in nine patients in acute phase CML. All patients (3/3) with high AGP dosages (2.31 ± 0.17 mg/mL; normal values, 0.5–1.3 mg/mL) were primary resistant to imatinib whereas an early clinical response was observed for the six patients with normal AGP levels (1.13 ± 0.2 mg/mL). No mutation in the adenosine triphosphate domain of Abl were detected before the initiation of imatinib therapy. By using *in vitro* tests combining various imatinib concentrations (1–10 μM) with purified human AGP (1 and 3 mg/mL), we demonstrate that imatinib-induced apoptosis of K562 or fresh leukemic CML cells is abrogated or reduced. The same effect was observed using sera from donors with high AGP levels (1.9–3.28 mg/mL). In patients with CML in blastic phase, AGP levels could reflect pharmacological resistance to imatinib, suggesting that increased dosage of imatinib or the use of a competitor to drug binding should be recommended to optimize the therapeutic effect of the drug.

Keywords: Chronic myelogenous leukemia; Imatinib mesylate; Alpha 1 acid glycoprotein; Drug resistance

1. Introduction

The Abl tyrosine kinase inhibitor imatinib (Gleevec®) is becoming a major agent for the treatment of chronic myelogenous leukemia (CML) [1,2]. Large clinical studies in late chronic phase [3], accelerated phase [4], and blastic phase [5,6] of the disease have recently confirmed the substantial activity of the drug, although responses are not sustained in advanced phase. Primary or acquired mechanisms of resistance have been yet suggested by the observation that patients could be either refractory or in relapse after an initial period of response. First studies have focused on acquired drug resistance mechanisms in cell lines models showing Bcr-Abl overexpression or increased efflux of imatinib by Pgp [7–9]. Other mechanisms of resistance were described in relapsing patients where mutations in the Abl adenosine triphosphate (ATP) binding region of Bcr-Abl or in the activation loop domain was

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Abbreviations: AGP, alpha 1 acid glycoprotein; CML, chronic myelogenous leukemia; CP, chronic phase; BC, blast crisis.

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found to abrogate imatinib efficacy [10–15]. At the same time, a Bcr-Abl independent mechanism of resistance was identified in a preclinical mice model based on imatinib binding to alpha 1 acid glycoprotein (AGP) [16], suggesting that AGP could interfere with imatinib pharmacology in humans.

AGP is a serum acute phase reactant synthesized mainly in liver parenchymal cells which levels has been found to be elevated in advanced phase CML and several other inflammatory conditions [17,18]. Using commercially available purified human protein, Gambacorti-Passerini *et al.* [16] have demonstrated that AGP tightly bind imatinib *in vitro* and mediate drug resistance *in vivo*. In a recent study, Jorgensen *et al.* [17] reported opposite results with an AGP purified from plasma by a low-pressure liquid chromatography method pointing out the differential properties of AGP glycoforms for imatinib binding.

To test the hypothesis that AGP could represent a marker of early clinical resistance, we investigated AGP levels in sera of nine patients with CML in BC. Interestingly, patients with high AGP levels failed to respond to imatinib therapy during the first 3 months. Contrarily, all six patients with normal AGP experienced an early hematological response. We confirmed *in vitro* that AGP impaired imatinib-induced apoptosis in K562 cell line and in fresh leukemic cells from CML acute phase patients. As clinical results in accelerated and blastic phase CML emphasized that better response rates was observed using imatinib 600 mg daily compared to 400 mg daily, our results suggest that an impaired imatinib tissue distribution could in part contribute to the emergence of a resistant clone.

2. Materials and methods

2.1. Patients

Patients were included in the Novartis CSTI571 0115 (myeloid BC, N = 9) and CSTI571 0113 (CP, N = 19) clinical trials. AGP dosage were performed by immunonephelemetry twice before and three times during the first month of treatment as part of a systematic follow up of acute phase reactants. Clinical response for blast crisis patients was evaluated at the end of the third month of therapy. Patients were classified as early responders if two consecutive blood cell counts showed no circulating peripheral blood blasts in the absence of disease progression during the first 3 months. Blood samples for *in vitro* studies were obtained after informed consent.

2.2. Reagents

Imatinib mesylate (2-phenylaminopyrimidine derivative) was kindly provided by Novartis. Purified human AGP (Sigma Chemical) was dissolved in distilled water (25 mg/mL) and used at a final concentration of 1 or 3 mg/mL within 24 hr. Erythromycin (Sigma Chemical) was used at 50 μM.

2.3. Cell line and fresh leukemic cells culture conditions

The human leukemia Bcr-Abl-positive cell line K562 was maintained in RPMI-1640 medium (BioWhittaker) supplemented with 10% heat-inactivated fetal calf serum, 2% L-glutamine, and 1% penicillin–streptomycin, at 37° with 5% CO_2. K562 cells were exposed to various concentrations of imatinib, AGP and erythromycin for 3 days. Cells were also grown in the presence of sera obtained from nonhematological patients presenting various concentrations of AGP. Mononuclear cells were separated by Ficoll–Hypaque centrifugation from peripheral blood of eight patients with CML in myeloid BC at diagnosis and before initiation of any treatment. Leukemic cells were then cultured in RPMI-1640–10% FCS medium in the presence or absence of imatinib and AGP or in autologous sera. Cell number and viability were determined by trypan blue exclusion test.

2.4. Flow cytometry analysis

Detection of phosphatidylserine on the outer leaflet of apoptotic cells was performed using AnnexinV-Fluos (Roche Diagnostics) and PI according to the manufacturer's recommendations. Briefly, 5×10^5 to 1×10^6 cells were washed in PBS, resuspended in 100 μL staining solution (AnnexinV fluorescein and PI in HEPES buffer), incubated at room temperature for 15 min. Cells were then analyzed by flow cytometry on a FACScan apparatus with the aid of the Cell Quest software (Becton Dickinson).

2.5. Real-time quantitative RT-PCR

MDR-1 and β2 microglobin (β2m) RNA were amplified by RT-PCR and cloned using TOPO II TA cloning Kit (Invitrogen) following the manufacturer's recommendations. Cloned products were digested with *Eco*RI (Invitrogen), extracted from 2% agarose gel and purified with the PCR purification Kit (Qiagen). Standard curves for MDR-1 and β2m were generated using serial dilutions of cloned products ranging from 10^8 to 10^1 molecule/μL. To evaluate the relative expression of MDR-1, real time quantitative PCR was performed using LightCycler (Roche Diagnostics) and expression levels of MDR-1 were normalized by the housekeeping β2m gene. Selection of primers and fluorescent probes for MDR-1 and β2m were performed using Primer Express software (PE Applied Biosystems). Quantitative PCR reaction were carried out with an aliquot of 1/20th of the resulting cDNA in a 20 μL volume using 100 nM of the specific hydrolyse probe, 200 nM of the probe flanking appropriate primer pairs, and 18 μL of LC fast start DNA master mix (Roche Diagnostics). The

expression of MDR-1 was assessed in normal and leukemic cells obtained, respectively, from peripheral blood of healthy donors and patients. All experiments were performed in duplicate.

2.6. Phosphorylation protein pattern

After incubation with different doses of imatinib (0.1, 0.5, 1, 5, 10 μM), tyrosine phosphorylated proteins were detected by cytofluorometry. Cells were fixed in 1% paraformaldehyde/PBS for 10 min at room temperature, permeabilized with 0.3% saponin, and stained with the PY-99 anti-phosphotyrosine antibody followed by a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Becton Dickinson) as the secondary reagent. Appropriate isotypic controls were used in all experiments. Stained cells were analyzed with an XL coulter instrument (Coultronics).

2.7. Mutations detection

Total RNA was extracted from peripheral blood and bone marrow specimens collected and reverse-transcribed according to the manufacturer's instructions (First Strand cDNA Synthesis Kit; Roche Diagnostics). The cDNA was PCR-amplified to isolate a 1298-bp fragment including a part of Bcr and the entire tyrosine kinase region of Abl including the ATP-binding site of the Bcr-Abl allele. Primers for the former amplification were 5′-TGTGAAACTCCAGACTGTCCAC-3′ (sense) and 5′-GATGGAGAACTTGTTGTAGGCC-3′ (antisense). A second stage of hemi-nested PCR used forward primer Abl kinase 5′-CTGTCTATGGTGTGTCCCCC-3′ and the same antisense primer as described above. A fragment of 569 bp was sequenced in the forward and reverse directions; (GeneBank accession number M14752) using the ABI Prism Rhodamine Terminator Ready Reaction Kit on the ABI Prism 377 (PE Applied Biosystem).

3. Results

3.1. Early clinical response to imatinib is associated with normal AGP plasmatic level

AGP dosages were performed in 9 BC and 19 CP patients at day 0 and day 30 of therapy (Fig. 1). Mean AGP values of patients in BC (1.48 ± 0.6 mg/mL) differed from that in the CP group (0.94 ± 0.2 mg/mL, $P < 0.01$) as previously reported [17]. Among the BC patients, AGP levels were elevated in three (mean 2.31 ± 0.17 mg/mL) and in the normal range in six (1.13 ± 0.2 mg/mL).

Baseline characteristics were similar in the two groups for age (61 vs. 51), peripheral blast count (21 G/L vs. 49 G/L, $P > 0.05$), disease and performance status at the time of study (ECOG performance status 0–1 for all patients except one in the low AGP group). All patients were free of documented bacteriological, fungal, or virological infection. Four patients in the normal AGP group and two in the high AGP group displayed clonal evolutions at inclusion. The three patients with high AGP levels failed to achieve an early clinical response or progressed ($P < 0.01$; Table 1). Among the six responders, two were in persistent complete hematological response beyond the sixth month and four subsequently relapsed.

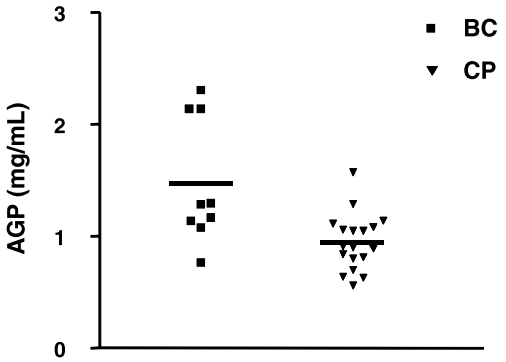

Fig. 1. AGP levels in chronic phase or blastic phase CML patients. Results are given as mean values of AGP dosage at day 0 and during the first 3 months. Mean AGP level was 0.94 ± 0.2 mg/mL (N = 19) in patients in CP and 1.48 ± 0.6 mg/mL in patients with BC (N = 9).

Mutations in the ATP-binding region of Bcr-Abl and MDR gene expression profile were also studied as they represent intrinsic mechanisms of resistance. No mutation could be evidenced for all eight patients in myeloid BC at diagnosis. In the only patient with lymphoid BC, a T315I mutation was detected at the time of relapse. These results are consistent with the phosphorylation patterns showing a decrease of tyrosine phosphorylation after imatinib incubation. All patients but one had a higher MDR-1 relative expression (range 9.4–1561) compared to control mean value (17; N = 25). Thus, in our experiment, MDR gene quantification does not appear as a predictive marker for response to imatinib. These data suggest that high AGP levels during the first month of imatinib therapy in acute phase CML patients could represent a pharmacological mechanism of resistance.

3.2. AGP inhibits imatinib-induced apoptosis in K562 cells

In order to test whether AGP could reduce the bioavailability of imatinib, we investigated the *in vitro* effect of AGP from different origins in Bcr-Abl-positive cells. Imatinib at 1 and 10 μM induce apoptosis in 25 and 56% of the K562 cells, respectively (Fig. 2). When purified human AGP 1 mg/mL was added in the culture, imatinib-induced apoptosis was abrogated at 1 μM, and partially conserved at 10 μM (29.7% of apoptotic cells). This residual activity was not observed with AGP concentrations similar to that in resistant patients *in vivo* (3 mg/mL). We also tested the naturally glycosylated protein rather than human purified

Table 1
Clinical characteristics under imatinib therapy according to AGP levels and analysis of intrinsic mechanisms of resistance

Patients	Diagnosis	Dose of imatinib (mg/day)[a]	AGP levels (normal values 0.5–1.3 mg/mL)[a]	Response at third month[b]	Response at sixth month	Tyrosine phosphorylation expression (%)[c]		MDR gene amplification[d]	Abl tyrosine kinase mutation
						Imatinib (1 μM)	Imatinib (10 μM)		
1	MBC	600	1.11	ER	CHR	54	34	32.5	NO
2	MBC	600	1.15	ER	Progression	73	63	9.4	T315I[e]
3	LBC	450	1.29	ER	CHR	81	49	31	NO
4	MBC	600	1.17	ER	Progression	NA	NA	274	NO
5	MBC	600	0.77	ER	Progression	77	48	751	NO
6	MBC	600	1.3	ER	Progression	NA	NA	1561	NO
7	MBC	600	2.48	F	Progression	68	46	791	NO
8	MBC	600	2.14	F	Progression	NA	NA	172	NO
9	MBC	600	2.31	F	Progression	NA	NA	20.3	NO

Abbreviations: MBC, myeloid blast crisis; LBC, lymphoid blast crisis; CHR, complete hematological response; ER, early response; F, failure.
[a] Mean during the first month.
[b] Defined as complete disappearance of blastic cells in blood smears.
[c] Compared to untreated cells.
[d] Expressed as number of MDR copies/number of β2m 10^6 copies.
[e] Mutation detected 3 month after initiation of imatinib therapy.

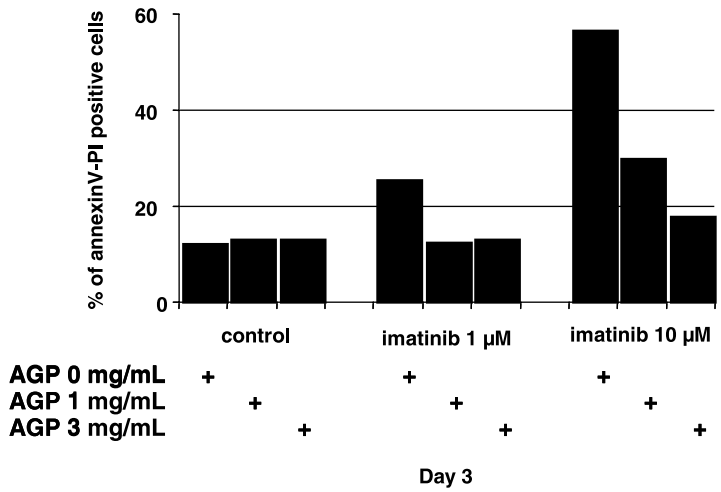

Fig. 2. Inhibition of imatinib-induced apoptosis by purified human AGP. Percentage of K562 apoptotic cells is assessed by flow cytometry after staining with fluorescein-labeled AnnexinV and propidium iodide. K562 Bcr-Abl-positive cells were cultured for 3 days in medium alone or in the presence of various concentration of imatinib (1 and 10 μM) and/or purified human AGP (1 and 3 mg/mL). These results were reproduced in two identical experiments.

AGP. A similar experience was performed with K562 cells incubated either in sera from donors with normal (range 0.64–1.23 mg/mL; normal values 0.5–1.3 mg/mL) and high (range 1.9–3.28 mg/mL) AGP levels. Sera with high AGP levels also inhibited sensitivity to STI571, in concordance with the results previously obtained with purified AGP (Fig. 3).

The inhibitory effect of AGP 1 mg/mL was overcome by adding erythromycin at 50 μM in the culture (data not shown). Contrarily, at the same concentration, this drug that is known to bind AGP was unable to restore imatinib sensitivity when AGP was used at 3 mg/mL.

3.3. Sensitivity to imatinib of fresh leukemic cells from patients in blast crisis is modulated by plasmatic AGP levels in vitro

In order to correlate the results obtained with the K562 cell line, primary fresh leukemic cells obtained from peripheral blood of the eight patients in myeloid BC were incubated with 1 and 10 μM imatinib. In all eight cases, cells were exquisitely sensitive to imatinib with $61.5 \pm 6.9\%$ and $91.8 \pm 3\%$ of AnnexinV-PI-positive cells, respectively, compared to $26.6 \pm 8.9\%$ in the control culture at day 3 ($P < 0.01$; Fig. 4) confirming data previously described [19]. As observed with K562 cells, purified AGP at 1 and 3 mg/mL inhibited cell death induced by 10 μM imatinib ($65.6 \pm 15\%$ and $84.2 \pm 8.7\%$ of inhibition of apoptosis, respectively, data not shown). When fresh leukemic cells were incubated with 10 μM imatinib in autologous sera, a significant difference in apoptosis was noted between the normal AGP level group ($83 \pm 11\%$) and the high AGP level group ($35 \pm 2.6\%$, $P < 0.05$; Fig. 5). Thus, AGP at concentrations observed in imatinib-resistant CML patients inhibits *in vitro* imatinib-induced apoptosis of CML cells.

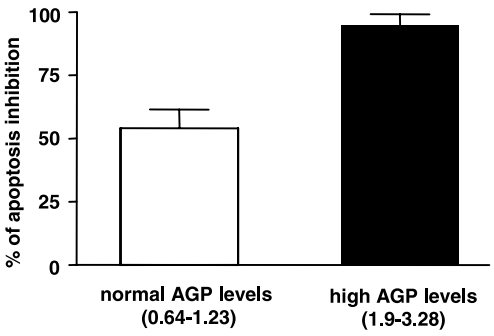

Fig. 3. Sera from donors with high AGP levels inhibit K562 apoptosis induced by imatinib. K562 cells were incubated during 3 days in the presence of imatinib 10 μM and sera from donors (N = 5) with either normal (0.64–1.23 mg/mL, white bars) or high (1.9–3.28 mg/mL, black bars) AGP levels. Results are expressed as the percentage of apoptosis inhibition compared to the value obtained in control culture (imatinib 10 μM/RPMI-1640–10% FCS).

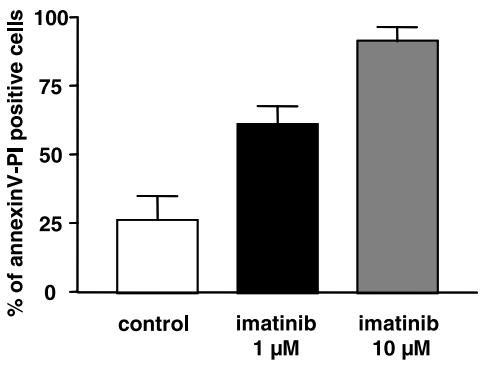

Fig. 4. Fresh leukemic cells from myeloid blast crisis patients at diagnosis are sensitive to imatinib *in vitro*. Mononuclear cells were separated by Ficoll–Hypaque centrifugation from peripheral blood of eight patients with CML in myeloid blast crisis at diagnosis and before initiation of any treatment. Cells were cultured in the presence or absence of imatinib (1 and 10 μM). Histograms plot the percentage of AnnexinV-PI-positive cells at day 3.

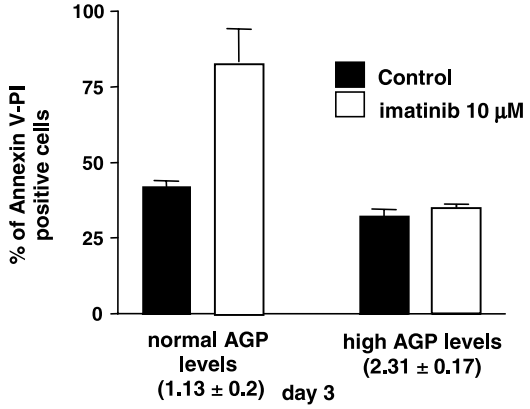

Fig. 5. Decreased imatinib sensitivity of fresh leukemic cells cultured in autologous sera depends on AGP levels. Histograms represent the percentage of apoptotic cells for two groups of patients; one with normal AGP levels (N = 3, 1.13 ± 0.2 mg/mL), one with high AGP levels (N = 3, 2.31 ± 0.17 mg/mL) after 3 days of culture in the presence or absence of STI 10 μM.

4. Discussion

In this report, we have focused our experiments on the role of AGP levels in patients in blastic phase treated with imatinib. We have confirmed that fresh leukemic cells obtained from patients prior imatinib therapy did not exhibit any intrinsic mechanism of resistance. 8/8 myeloid blast crisis samples at diagnosis were *in vitro* sensitive to 1 μM imatinib and no point mutations could be evidenced after sequencing the ATP-binding region of Bcr-Abl. The pattern of tyrosine phosphorylation was studied in four of these patients. In all cases, a decrease of phosphorylation was observed after incubation with 1–10 μM imatinib demonstrating that leukemic cells were not intrinsically resistant. The only patient for whom a point mutation (T315I) was detected was resistant to imatinib (defined as a loss of response), which is consistent with previous report [14]. Chemoresistance is one of the characteristic of CML blast crisis cells [20]. In the LAMA84-r cell line, imatinib resistance is mediated by Pgp overexpression [7]. However, in our *in vivo* study, quantification of MDR-1 RNA levels was not associated with clinical resistance.

To investigate whether AGP could interfere with imatinib pharmacology, we have compared AGP from three different origins: AGP commercially purified AGP in sera from non hematologic patients and AGP in sera from subjects in BC-CML. K562 Bcr-Abl-positive cells sensitivity to imatinib 10 μM was abrogated in the presence of AGP from the first two mentioned origins. Moreover, sera from patients with high AGP levels lead to the same pattern of imatinib-induced apoptosis inhibition in fresh leukemic cells from BC-CML patients. The ability of AGP to bind and to mediate *in vitro* resistance to imatinib is still controversial [17,21,22]. Previously published data on binding studies were obtained by using the commercially purified AGP [16]. These results have not been reproduced with AGP purified from CML patients by low-pressure liquid chromatography [17]. As a consequence, no significant inhibition was observed *in vitro* when culturing Bcr-Abl-positive cells in the presence of imatinib. The discordance observed between these results and the one we obtained using the whole patient's sera could be explained by the different origins of AGP or by the possible binding of imatinib with other acid plasma proteins. In our conditions, we can assume that AGP conformation and glycosylation is not modified by any procedure. Our *in vitro* results indicate that AGP could be a suitable biological marker of primary imatinib resistance.

To test the prognostic significance of AGP levels, we have analyzed both primary and secondary hematological response and survival in blastic phase patients included at our institution in the Novartis clinical trial STI0115. AGP levels were significantly higher in BC-CML compared with chronic and accelerated phase ($P < 0.01$). We found that the mean value of AGP levels during the first month of treatment is associated with early clinical response. Of note, high AGP levels do not correlate with the tumor mass. Recent clinical trials clearly demonstrate that a daily dose of 600 mg imatinib/day improve the outcome in AP patient [4]. We correlated the *in vitro* inhibitory effect of AGP with *in vivo* observations of primary resistance. *In vitro*, AGP 3 mg/mL inhibits imatinib-induced apoptosis at 1 and 10 μM. Pharmacological data showed that a mean maximal concentration of 4 μg/mL (6.8 μM) was reached at steady state by once-daily administration of 600 mg of imatinib. This *in vivo* concentration is thus insufficient to reverse AGP mediated resistance in patients with high AGP levels.

These results suggest that achieving a maximal cellular concentration of the drug led to a marked increase of clinical efficacy. Most of the published reports focused on resistance mediated by point mutations. However, the circumstances favoring the occurrence of mutations are largely unknown. A recent report indicates that mutations in the tyrosine kinase domain of Bcr-Abl can pre-exist to the onset of imanitib treatment [23]. We suggest that high AGP levels could facilitate the selection of a resistant clone by impairing intracellular concentrations of imatinib. The predictive value of AGP at diagnosis has to be tested in a larger cohort of patients in advanced phase CML. This potential deleterious effect of AGP could however be overcome either by using a competing ligand or high dose imatinib.

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