Liver enzyme delayed clearance in rat treated by CSF1 receptor specific antagonist Sotuletinib

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A B S T R A C T

Sotuletinib (BLZ945), a CSF1-R specific kinase inhibitor developed for the treatment of Amyotrophic Lateral Sclerosis, induced liver enzyme elevation in absence of hepatocellular lesions in preclinical rat and monkey studies. The monocytic cell family, including Kupffer cells, e.g., the liver-resident macrophages, are dependent upon CSF1 pathway activation for their survival, proliferation, and differentiation. Kupffer cells act as the main body compartment responsible for elimination of some blood-borne proteins, like ALT, AST, and few others. The depletion of Kupffer cells through CSF1 pathway inhibition has already been hypothesized as responsible for apparent liver enzyme elevation without detectable corresponding liver damage. However, a release of these biomarkers from unseen hepatic lesions or from other organs cannot be excluded. In order to eliminate a potential contribution of ALT elevation from an internal organ source, we injected recombinant his-Tagged ALT1 into rats pretreated with Sotuletinib. The elimination rate of the exogenous ALT1 was significantly lower in treated animals, demonstrating a delayed clearance independently of any potential organ lesions.

Introduction

The Colony Stimulating Factor 1 Receptor (CSF1-R) pathway is central to proliferation, differentiation and survival of the monocytic cell family, including tissue resident macrophages, amongst which, Kupffer cells and brain microglia (Jones and Ricardo, 2013; Wynn et al., 2013). As such, CSF1-R is an attractive pharmacological target for a number of diseases where modulation of macrophage biology (Barca et al., 2021; Hume and MacDonald, 2012), and particularly their pro- or anti-inflammatory phenotypes (M1 vs M2), may impact diseases outcome (Liu et al., 2021). Deactivation of microglia has been shown to have positive effects on neurodegenerative diseases (Ilan et al., 2022) and in particular in the case of amyotrophic lateral sclerosis (ALS) by reducing neuroinflammation (Martinez-Muriana et al., 2016). Sotuletinib is a highly specific and potent CSF1-R kinase inhibitor (IC50 1.2 nM) being currently developed for the treatment of ALS by reducing microglia inflammation (ClinicalTrials.gov, identifier: NCT04066244).

The purpose of our study was to evaluate the liver enzyme delayed clearance in the rat treated by Sotuletinib. It is well known that mouse Lactate Dehydrogenase Virus induces higher level of LDH compared to healthy animals, without obvious hepatotoxicity, by depleting the population of Kupffer cells (Coutelier, 2014; Dillberger et al., 1987; Hayashi et al., 1988; Smit et al., 1988).

Abbreviations: CSF1, Colony Stimulating Factor 1; CSF1-R, CSF1 Receptor; ALT1, Cytoplasmic ALT; KC, Kupffer cell.

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were treated with 150 mg/kg/day Sotuletinib, either for 43 days. Application volume was 5 mL/kg. Elevation of liver enzymes were also been proposed as the mechanism underlying elevation of liver enzymes in absence of observable hepatic lesions (Radi et al., 2011). Here we report experiments investigating delayed clearance of ALT independent of any potential organ lesions, after a 8-day Sotuletinib treatment in rat depleting Kupffer cell.

Materials and methods

Test compounds

Sotuletinib was obtained from Novartis. Recombinant His-tagged rat His-tagged alanine aminotransferase type 1 (ALT-1) (Cat. No./Lot No.: Gpt-294R / 709611) was purchased from Creative BioMart, Shirley NY, USA.

Animal experimentation

All animals received humane care according to the criteria outlined in the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86–23 revised 1985). Male Wistar Hannover Rats; Crl: WI(Han) were obtained from Charles River Laboratories, Raleigh, NC which were at the initiation of dosing approximately 15 weeks old. Sotuletinib was administered at a dose of 150 mg/kg/day orally per gavage, dissolved in vehicle [50:50 (v:v) 200 mM Sodium Chloride, Inj. USP to 0.06 mg/mL and was administered to Sprague Dawley rats depleting Kupffer cells in liver using silica (Hayashi, et al., 1988) or gadolinium chloride in vivo and in vitro (Hardonk et al., 1992; Lee et al., 2004). Furthermore, it has been demonstrated that Kupffer cells were the main macrophages involved in removal of ALT, AST, CK, LDH, adenylate kinase and malate dehydrogenase from blood circulation (Bijsterbosch et al., 1983; Horiiuchi et al., 1985; Kamimoto et al., 1985; Smit et al., 1988; Smit et al., 1987; Smit et al., 1986). Therefore, depletion of Kupffer cells could lead to a delayed clearance of liver and other organ released protein biomarkers like muscle CK (Pognan et al., 2019). The co-occurrence of Kupffer cells inhibition through the CSF1 pathway modulation and elevation of liver functional tests (LFTs) endpoints has also been proposed as the mechanism underlying elevation of liver enzymes.

For endogenous ALT elevation experiments, 2 groups of 12 animals were treated with 150 mg/kg/day Sotuletinib, either for 43 days (continuous treatment group) or for 16 days and further followed after compound withdrawal, during another 8-day duration (‘withdrawal’ group) for a total of 24 days of ALT monitoring. The control group was composed of 8 animals that received the vehicle till day 43. The dose of 150 mg/kg/day was chosen because it was clinically well tolerated in other studies and already observed to induce significant ALT elevation. The duration was chosen in order to follow the raise of ALT and determine if a plateau of ALT level could be reached. For the withdrawal group, 16 days of treatment were deemed to be enough for a high enough elevation to monitor the time of return to baseline if at all. From control and Sotuletinib treated animal’s blood was collected on day 1 (pre-dose), 2, 3, 4, 5, 7, 9, 11, 12, 13, 15, 16, 17, 18, 19, 20, 22, 24, 25, 29, 32, 36, 39 and 43. Plasma was frozen at −70 °C until used for biochemical investigations.

Automated methodologies for clinical chemistry

Small RNA was extracted from 200 μL of serum by using the miRNAesy/MinElute kit and following the instructions of the manufacturer (Qiagen). One hundred ng of serum small RNA were reverse transcribed using the Megaplex RT primer rodent pool A and B (Applied Biosystems / Life Technologies, reference 4,399,970 and 4399972) and Taqman MicroRNA Reverse Transcription kit according to manufacturer instructions (Applied Biosystems / Life Technologies, reference 4366596). cDNA was preamplified using the Megaplex PreAmp primer rodent pool A and B (Applied Biosystems / Life Technologies, reference 4,399,203 and 4401011) and the Taqman PreAmp Master mix (Applied Biosystems / Life Technologies, reference 4391128). Single microRNA were amplified from preamplified cDNA by using commercially available validated Taqman assays dilute in the Taqman MasterMixII (Applied Biosystems / Life Technologies, assay #002245) and following the instructions of the manufacturer. The housekeeping gene used for comparison and normalization of gene expression data in tissues and serum was the small nuclear RNA U6 (assay #001973). The relative quantification of gene expression changes was performed using the standard curve method to generate expression values as number of molecules. Statistical significance was assessed using the two-sample unequal variance, two-tailed distribution Student’s t-test (homoscedastic Student’s t-test). Coefficients of variation with a p-value < 0.05 were considered to be statistically significant.

Brightfield microscopy and quantification of Kupffer cells

Animals from the ALT clearance study (Sotuletinib administered to 10 male rats at a dose of 150 mg/kg/day orally for 10 days along with 10 vehicle control rats) were used for pathology evaluation and Kupffer cells quantitation. For each animal on study, the liver was sampled, fixed in 10 % neutral buffered formalin, the left lateral, right medial and right lateral liver lobes were trimmed and processed to paraffin embedded histology blocks and 5 μm sections were generated. For each animals a section was stained with hematoxylin and eosin (H&E) for histopathology evaluation and another section was submitted to anti-CD68

1990). These effects have been reproduced by experimentally depleting Kupffer cells in liver using silica (Hayashi, et al., 1988) or gadolinium chloride in vivo and in vitro (Hardonk et al., 1992; Lee et al., 2004).

Clinical observations, body weight and food consumption determinations were performed for all animals. Clinical laboratory (hematology and clinical chemistry) evaluations were performed on pretest day 1, treatment day 7 and 9. Microscopic examinations were performed from liver sections after H&E staining.

Automated methodologies for hematology was investigated by Advia 2120 Automated Hematology Analyzer (flow cytometry methodology), with the software version: Version 5.5.1 – MS. As technical reference served the Advia Online Operator’s Manual. Instrument manufacturer was Siemens Medical Solutions Diagnostics.
immunohistochemistry (IHC), anti-C668 IHC labelling was performed on the Ventana XT Discovery Platform. Slides were baked at 60 °C for 1 hr prior to loading on the Ventana XT. Slides were de-paraffinized using EZ Prep (cat#950-100) followed by heat-induced epitope retrieval for 20 min with CC1 (pH 8 at 95–100 °C, cat#950-124). Tissues were blocked prior to primary antibody incubation for 12 min (S Block, cat#760-4212). Primary antibody (CD68 abcam cat# ab31630) was incubated for 32 min, and detected using Omnimap Multimer (Omnimap anti-MS HRP cat#760–4310) with DAB Chromomap kit (cat#760-159). Hematoxylin and bluing (cat#760-2021, cat#760-2037) were used as counterstains.

Quantiﬁcation of Kupffer cells via automated image analysis was conducted on the liver sections labelled with anti-C668 antibody. IHC-stained slides were digitalized using the Scanscope XT console from Aperio at 20X magnification. The three standardized cross-sections per liver per animal were analyzed, using the Definiens Developer image analysis software. Briefly, the liver sections were identiﬁed as the regions of interest (ROIs), excluding vessel lumen areas, and total liver area was measured (mm²). Subsequently, Kupffer cells were identiﬁed and quantitated. Data were expressed as number of Kupffer cells per unit area of total liver surface analyzed. Statistical data analysis was performed using the GraphPad Prism software. Unpaired t-test was used to determine statistical signiﬁcant differences between Sotuletinib-treated and control-vehicle groups.

Capture and recovery of histidine labeled proteins

The HIS-Select® nickel affinity gel (Sigma, P6611-5ML) was used to capture the His-tagged ALT1 or His-tagged CKM from lithium heparin rat or monkey plasma. The protocol from the HIS-Select nickel affinity gel was followed exactly, with the exception by using Pierce Spin Columns (Thermo Scientiﬁc, 69705). The plasma samples were incubated by the 10-fold of 1500 mM sucrose, 5 mM TCEP (Thermo Scientiﬁc, 77720) and a 1/10th concentration of HALT solution (Thermo Scientiﬁc, 78443). 200 µL of the plasma was added to the nickel column and shaken in a MixMate shaker at 1200 rpm for 15 min. The columns were spun at 1500 × g for 1 min and then washed four times with PBS (pH 8) containing 0.3 M NaCl, with shaking for 3 min before each wash spin. 70 µL of the HIS-Select Elution buffer containing 250 mM imidazole was added. After shaking in the MixMate at 1000 rpm for 12 min, the columns were spun using fresh 1.5 mL Eppendorf tubes to collect the eluate, to which the HALT preservative solution was added at 1:10 vol to maintain His-ALT1 enzymatic activity. Samples were frozen and stored at –80 °C until used for the enzyme assay activity. A standard protocol was used to measure His-tagged ALT1 or His-tagged CKM enzymatic activity (Thermo Protocol TR18515). The enzymatic activity of His-captured ALT1 or CKM was determined based on standard curves to obtain ALT or CK enzymatic activity expressed as units per liter (U/L), and reported values are means of triplicate determinations (average % CV = 1.90). The His-tagged enzymatic activities of both enzymes were stable during 72 h in plasma.

PDPK modelling

The non-compartmental PK analysis (NCA) and population analysis were performed using Phoenix WinNonlin Version 6.3 (Certara, St. Louis, MO). Pharmacokinetic parameters were derived using non-compartmental analysis, and parameter of mean plasma Cmax, area under the curve (AUClast and AUCinf), volume of distribution at steady-state (Vss), half-life (t1/2), and clearance (CL) were calculated for His-ALT1 based on an elimination proﬁle obtained after single intravenous administration of His-ALT1. The pharmacokinetics of injected His-ALT1 were further analyzed using a population model, which was built upon a two-compartment PK model.

Statistics

Differences in His-tagged protein kinetics between treatment groups were assessed using repeat-measured two-way ANOVA with post-hoc Sidak multiple comparison test (α = 0.05), and p-values<0.05 were considered to be statistically signiﬁcant. Kupffer cell counts from Sotuletinib treated animals compared to controls were evaluated for statistically signiﬁcant differences by unpaired t-test.

Results

Han-Wistar rats were treated for either 16 or 43 continuous days with 150 mg/kg/day Sotuletinib. Animals treated for 16 days (withdrawal group – Fig. 1A) were from day 17 onward, dosed with vehicle only.

Some haematological effects were noted in treated animals compared to controls at day 9 of treatments as shown in Table 1. Moderate increases in serum AST and ALT activities were noted in rats dosed with Sotuletinib at 150 mg/kg/day for 1 week when results were compared to the concurrent controls.

Drain, the whole GI, heart, liver, skeletal muscle, and the injection site of his-tag ALT1 were sampled and processed for histopathology. Treatment-related microscopic changes were noted in the stomach and liver. In the stomach, there was a combination of erosion (glandular), ulceration (glandular and nonglandular), parakeratosis (non-glandular) and inflammation in 4/10 rats administered BLZ945 at 150 mg/kg/day. The stomach observations were attributed to the acidic formulation used in the present studies, and not to the compound directly. This was later conﬁrmed when using a neutral pH formulation in further studies not reported here. The inﬂammation might correlate with the increased platelet count noted in clinical pathology. In the liver, there was an increased incidence of glycogen depletion in treated rats (1/10 in control, 10/10 in treated rats). There were no hepatic microscopic ﬁndings reﬂective of clinical pathology alterations in ALT or AST. All other microscopic ﬁndings were considered incidental and unrelated to treatment.

In the withdrawal group, ALT levels started to revert on day 18 and were back to control levels by day 24 (Fig. 1A). In the continuous group, ALT elevation reached a plateau by day 25, at an averaged value of about 520 % above controls. This elevation was not accompanied by liver lesions at observation by histopathology or by elevation of liver-specific micro-RNA miR-122 (Fig. 1B), which were in the range of published normal variations for both controls and treated animals (Starkey Lewis et al., 2012).

Treatment of male Han-Wistar rats with 150 mg/kg/day Sotuletinib for 9 days induced elevation of endogenous serum ALT by 247 % and 337 %, and of AST by 268 % and 233 % at day 7 and 9 respectively, compared to time-matched controls. At day 9, histopathology examination of liver H&E stained sections, revealed only an increased incidence of glycogen depletion, in absence of any clear observations that could relate to the above enzyme elevations. In order to better characterize Kupffer cells, histological sections were submitted to anti-C668 immunohistochemistry (IHC), C668 being a specific marker of liver macrophages/Kupffer cells (KC) (Tomita et al., 1994). Sotuletinib-related decrease Kupffer cells was evident by semi-quantitative pathology evaluation (Fig. 2A) and was substantiated via automated image analysis demonstrating a statistically significant 4.17-fold reduction of KC number (Fig. 2B). On day 8 of the study, all animals, controls and Sotuletinib-treated, were injected with a single dose of recombinant rat his-tag ALT1, while oral dosing with Sotuletinib was maintained until necropsy at day 9 in the treated group.

From day 7 to day 9, periodic blood samplings were performed to establish kinetics of his-tag ALT1 serum values. To calculate the total activity in U/L corresponding to the amount of ALT1 injected into each animal, a standard curve was derived from a plot of amount of ALT1 versus activity following His-capture recovery (activity in U/L) (see
Materials and Methods). From these elimination profiles, mean plasma $C_{\text{max}}$ areas under the curve ($AUC_{\text{last}}$ and $AUC_{\text{agg}}$), volume of distribution at steady state ($V_{\text{ss}}$), half-life ($t_{1/2}$), and clearance ($Cl$) were calculated for each treatment group and summarized in Table 2.

There was a significant difference in ALT1 elimination time in Sotuletinib treatment (Fig. 3). The mean ALT1 $C_{\text{max}}$ values were similar between the vehicle and Sotuletinib treatment groups. The mean predicted AUC of the Sotuletinib treatment group was about 40 % higher than the value of the vehicle group, and the predicted clearance (CI) with Sotuletinib treatment was about 20 % lower than the value of the vehicle group. Volume of distribution at steady state ($V_{\text{ss}}$) in the Sotuletinib treatment group was determined to be about 30 % larger than that of the vehicle group, and calculated $t_{1/2}$ with Sotuletinib treatment was approximately 60 % higher compared to vehicle treatment. The increased exposure ($AUC$) was likely directly associated with reduced clearance (CI) and increased distribution volume ($V_{\text{ss}}$) in the Sotuletinib treatment group.

Discussion and conclusion

ALT and AST are highly expressed in the liver and their releases into blood stream are considered as specific biomarkers of hepatotoxicity, although increased serum values could also derive from skeletal muscle, heart, fat tissues, brain or GI damage after a toxic insult (Yang et al., 2009). Rats treated with the specific CSF-1 receptor inhibitor Sotuletinib, displayed elevation of liver enzymes in absence of observable tissue damage, including hepatotoxicity, and absence of liver-specific toxicity marker miR-122 elevation (Fig. 1). miR-122 levels were in the range of expected background variations and well below those observed in case of hepatic lesions (Starkey Lewis et al., 2012). Upon Sotuletinib withdrawal, ALT values rapidly returned to those of control animals. Sotuletinib treated animals exhibited a significantly reduced number of Kupffer cells (KCs) after only 9 days of treatment. KCs have long been shown to largely participate to elimination of such circulating enzymes, such as ALT, AST, LDH and CK amongst few others (Bijsterbosch et al., 1989; Horiuchi et al., 1985; Kamimoto et al., 1985; Smit et al., 1988). However, a direct and unique causal relationship between these two events, depletion of KCs and increased serum biomarkers as previously reported (Radi et al., 2011), could not be ascertain. In order to alleviate the issue of a possible unseen hepatic or non-hepatic source responsible for the observed plasmatic liver enzymes elevation, we took an approach to exogenously inject rat recombinant his-tag ALT1 (cytoplasmic isoform of ALT) into Sotuletinib-treated rats. These animals displayed a significant reduction of Kupffer cell count as expected from the inhibition of the CSF1-R after 9 days at 150 mg/kg/day Sotuletinib treatment (Fig. 2). The half-life of exogenous ALT1 of control male Wistar rats was approximately 12 h, compared to 18.6 h in the treated group, or a 1.55-fold lower clearance rate (Fig. 3). This reduced rate resulted in an increased ALT1 AUC by 1.37-fold over 48 h. If this rate were cumulative independently of the clearance rate, this would lead to an approximative 11-fold elevation after 16 days as opposed to roughly a 5-fold real increase (Fig. 1A). However, an equilibrium is reached after about day 25, showing that the clearance rate will not be constant throughout the course of a long kinetics. The observed delayed clearance of single intravenous injected ALT1 is consistent with the Sotuletinib-induced depletion of Kupffer cells and with increased level of endogenous ALT accumulating every day of treatment. Since the apparent increase of exogenous his-tag ALT1 in Sotuletinib-treated animals compared to time-matched controls cannot arise from a hidden internal source, it is reasonable to conclude of a delayed clearance rather than of an unseen organ damage. This delay could allegedly be hypothesized to result from the increase of endogenous ALT saturating the elimination rate and therefore hindering the his-tag enzyme removal. However, the endogenous ALT clearance is plateauing around day 25 of treatment, where the maximum elimination rate is reached. Oppositely, at day 9 when the his-tag ALT1 kinetic was run, the system is still in the linear increase phase and therefore should not skew the outcome (Fig. 1A). Considering in addition that KCs have been shown to largely participate to elimination of such circulating proteins, the relationship between increased circulating liver enzyme or such other blood-born proteins, and depletion of KCs in presence of CSF1-R inhibitors, is not the result of organ insults, but from reduced
clearance. Sotuletinib affects KCs as well as circulating and other tissue macrophages, which all could play a role in the overall protein clearance process. Bone marrow and spleen macrophages have also been shown to participate to circulating enzyme clearance, however to a much lower extent (Bijsterbosch, et al., 1983; Smit, et al., 1988). Overall, the reduction of monocytic lineages, either circulating or tissue resident, is inducing elevation of liver enzyme by reduced clearance and not through hepatotoxicity. This corroborates a similar finding of CK delayed clearance in cynomolgus monkey treated with an antibody against CSF1 ligand (Pognan, et al., 2019) as opposed to a CSF1 receptor antagonist in the present study. In this case, exogenous his-tag cynomolgus CKM enzyme was injected in monkey treated with an antibody depleting the CSF1 ligand. Kupffer cell depletion was also effective and led to ALT and AST enzymes rise in addition to CK elevation, also in absence of relevant organ lesions. A similar delayed clearance of his-tag CKM was observed and corroborated with asymptomatic CK elevation in Phase I clinical trial (Pognan, et al., 2019). Therefore, it is likely that elevation of blood enzyme toxicity biomarkers in absence of evident organ toxicity, is bound to CSF1 pathway inhibition, independently of the drug mode of action. This is corroborated in further clinical trials where CSF1 pathway-targeting drugs, small chemicals as well as antibodies, are reported to induce ALT, AST and CK increases as being the most frequent adverse events, however without associated symptoms or evidence of organ damage (Lin, 2021). There is a reasonable likelihood that this phenomenon may apply to other blood-borne enzymes for which the mechanism of clearance is unknown but may be processed at least in part by Kupffer cells. It would be therefore advisable to explore such a possibility with CSF1-pathway inhibitors in case of asymptomatic protein markers elevation, as well as with any drug candidates depleting Kupffer cells by other mechanisms.

CRediT authorship contribution statement

François Pognan: Conceptualization, Validation, Formal analysis, Writing – original draft, Visualization, Supervision, Project administration. Chiara Buono: Methodology, Formal analysis. Philippe Couttet: Methodology, Formal analysis. Jean-René Galarneau: Methodology,

Table 2
Mean pharmacokinetic parameters of injected ALT1.

| Treatment Group | Cmax (U/L) | AUC0-24h (U/L*h) | AUC0-Inf (U/L*h) | CL (L/h) | Vss (L/kg) | T1/2 (h) |
|-----------------|-----------|-----------------|------------------|--------|----------|---------|
| Vehicle Mean    | 365       | 4648            | 4834             | 0.31   | 3.99     | 12.0    |
| SEM             | 24.98     | 215.35          | 219.78           | 0.013  | 0.22     | 0.70    |
| Sotuletinib Mean| 367       | 5797            | 6622             | 0.24   | 5.2      | 18.6    |
| SEM             | 23.08     | 338.36          | 391.49           | 0.013  | 0.57     | 1.33    |

Note: Pharmacokinetic parameters were derived using non-compartmental analysis. SEM: standard error of the mean.

Fig. 2. A. Immunohistochemical labeling of liver macrophages (Kupffer cells) with anti-CD68. In control liver numerous Kupffer cells are lining the parenchymal sinusoidal space (A). Marked reduction in the number of Kupffer cells was observed in Sotuletinib-treated animals (B). Portal space identify by asterisks. B. Number of Kupffer cells (KC) CD68 + per square mm of liver observed. ****p < 0.0001: significant difference compared to respective vehicle control using unpaired t tests.
Fig. 3. A. Plasma His-ALT1 activities of vehicle control and Sotuletinib-treated rats (day 7 to 9). Rats were treated once daily at a dose level of 150 mg/kg for 9 days (mean ± SEM, n = 5 per timepoint up to 42 h in order to respect blood volume withdrawal in live animals; n = 10 for 50 h after His-ALT1 injection). His-tagged ALT1 i.v. injection (0.2 mg/kg) was on day 7 of Sotuletinib treatment. B. Endogenous ALT values at predose, day 7 and 9 of Sotuletinib treatment, before injection of His-tag ALT1. **p < 0.01, and ***p < 0.0001: significant difference compared to respective vehicle control using the Sidak-Bonferroni multiple t tests (α = 0.05).

Formal analysis, Investigation. Yoav Timsit: Methodology, Validation, Formal analysis, Investigation, Project administration. Armin Wolf: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Project administration.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: [Financial interests to report are limited to the fact that all authors are or have been employees of Novartis and may possess company shares and/or options.]

Data availability

Data will be made available on request.

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