RESEARCH ARTICLE

Dabrafenib, an inhibitor of RIP3 kinase-dependent necroptosis, reduces ischemic brain injury

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Abstract

Ischemic brain injury triggers neuronal cell death by apoptosis *via* caspase activation and by necroptosis through activation of the receptor-interacting protein kinases (RIPK) associated with the tumor necrosis factor-alpha (TNF-α)/death receptor. Recent evidence shows RIPK inhibitors are neuroprotective and alleviate ischemic brain injury in a number of animal models, however, most have not yet undergone clinical trials and safety in humans remains in question. Dabrafenib, originally identified as a B-raf inhibitor that is currently used to treat melanoma, was later revealed to be a potent RIPK3 inhibitor at micromolar concentrations. Here, we investigated whether Dabrafenib would show a similar neuroprotective effect in mice subjected to ischemic brain injury by photothrombosis. Dabrafenib administered intraperitoneally at 10 mg/kg one hour after photothrombosis-induced focal ischemic injury significantly reduced infarct lesion size in C57BL6 mice the following day, accompanied by a markedly attenuated upregulation of TNF-α. However, subsequent lower doses (5 mg/kg/day) failed to sustain this neuroprotective effect after 4 days. Dabrafenib bl ocked lipopolysaccharides-induced activation of TNF-α in bone marrow-derived macrophages, suggesting that Dabrafenib may attenuate TNF-α-induced necroptotic pathway after ischemic brain injury. Since Dabrafenib is already in clinical use for the treatment of melanoma, it might be repurposed for stroke therapy.

Key Words: *ischemic brain injury; inflammation; macrophage; Dabrafenib; tumor necrosis factor-alpha; photothrombosis; receptor-interacting protein kinases; necroptosis; microglia; stroke; neural regeneration*

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Introduction

Stroke following ischemic brain injury is a leading cause of death worldwide. Ischemic brain injury resulting from disruption of blood supply to the brain triggers immediate cell death by necrosis, but also activates additional cellular mechanisms, including caspase-dependent apoptosis and caspase-independent necroptosis that propagate a wave of delayed cell death (Tovar-y-Romo et al., 2016).

Release of glutamate from dying neurons and astrocytes triggers excitotoxicity (Schock et al., 2008), a hyper-excitation of glutamatergic receptors that disrupts intracellular Ca^{2+} homeostasis, increases production of reactive oxygen species and release of cytochromce c from mitochondria that activates caspases (Degterev et al., 2003). Injured or dead neurons also activate local brain-resident macrophages (microglia) that secrete chemokines and inflammatory cytokines, including tumor necrosis factor-alpha (TNF-α), to recruit more immune cells in attempt for tissue repairs. However, inflammatory cytokines secreted from immune cells also induce cell death through the activation of the Fas/TNFR family of death-domain receptors (DRs).

Although activation of the caspase family of cysteine proteases is essential to mediate DR signal transduction and execution of apoptosis (Degterev et al., 2003), many studies have shown that caspase inhibitors, including the pan-caspase inhibitor zVAD.fmk, fail to block DR-induced cell death (Schulze-Osthoff et al., 1994; Kawahara et al., 1998; Vercammen et al., 1998; Khwaja and Tatton, 1999; Holler et al., 2000; Matsumura et al., 2000; Chan et al., 2003). Emerging evidence points to non-apoptotic caspase-independent cell death pathway called necroptosis, in which DRs activate receptor-interacting protein kinase 1 (RIPK1) and a signaling cascade involving the phosphorylation of RIPK3 by RIPK1 (Weinlich et al., 2017). Ischemic brain injury activates necroptotic cell death by activating RIPK3 (Vieira et al., 2014). The RIPK1 inhibitor necrostatin-1 limits experimentally induced ischemic brain injury in mice (Degterev et al., 2005; Degterev et al., 2008), but necrostatin-1 has not yet undergone phase 1 clinical trials for safety and tolerance in humans.

It has emerged that Dabrafenib, an inhibitor of B-raf currently approved for the treatment of melanoma (Hauschild et al., 2012), is also an effective high affinity inhibitor of RIPK3 that blocks TNF-α-induced necroptosis (Li et al., 2014b). In human vascular endothelial cells, Dabrafenib markedly attenuates the activation of TNF-α (Jung et al., 2016). Here, we tested whether Dabrafenib has a neuroprotective effect in an experimentally induced stroke model.

Materials and Methods

Ischemic brain injury by photothrombosis in mice

C57BL6 adult male mice aged 2–3 months, weighing approximately 33 g, were fed with regular chow, and randomly assigned to experimental groups. All procedures were carried out with the approval of the animal care and use committee of the University of Ottawa (OGHRI-49), according to animal use guidelines of the Canadian Council on Animal Care (www.

ccac.ca/en/standards/guidelines/). Photothrombosis-induced focal ischemic brain injury was carried out in two-month-old mice, using the protocol of Watson et al. (1985) with the modifications we previously described (Cruz et al., 2017). Mice were anesthesized in an induction chamber ventilated with 5% isoflurane gas (AbbVie Corporation, St-Laurent, Quebec, Canada), transferred to a stereotaxic frame and anesthesia was maintained with 1.5% isoflurane with a nose mask. A midline incision of the scalp exposed a 1 × 1 mm^2 region of the skull. Rose Bengal (Sigma-Aldrich, Oakville, Ontario, Canada, 10 mg/mL in PBS, made fresh by vortexing and filtered) was administered by i.p. injection (100 mg/kg) and five minutes later, a laser was used to induce thrombosis. A collimated green laser (532 nm wavelength at 20 mW, Beta Instruments, Zaventem, Belgium) was placed 2 cm above the skull, positioned 0.7 mm anteroposterior and 2 mm mediolateral relative to the Bregma to target the sensorimotor cortex and used to illuminate the skull for 10 minutes. The skin wound was closed using cyanoacrylate glue, and mice were monitored until they regained consciousness. Sham operated mice received Rose Bengal and underwent surgery without laser illumination.

One hour after photothrombosis, Dabrafenib (10 mg/kg, Selleck, Houston, TX, USA) or vehicle (dimethyl sulfoxide, DMSO) was delivered by i.p. injection in 150 μL 80% DMSO in saline. Daily maintenance doses of Dabrafenib (5 mg/kg) were administered, prior to sacrifice on day 4.

Quantification of infarction

Mice were sacrificed and perfused with saline followed by 4% paraformaldehyde and brains were isolated and sectioned on a cryostatmicrotome for histology. Infarct volumes were determined by cresyl violet staining to reveal Nissl bodies of living neurons in 10 coronal sections (20 μm thick) sampled every 10 sections over a 2 mm distance overlapping the area of infarction, as previously described (Schock et al., 2008). Infarct volume was calculated by stacking infarct areas in serial sections using the ImageJ software (NIH, Bethesda, MD, USA). Lesion volumes were normalized to brain volumes of the corresponding sections to control for edema. Investigators were blinded to genotype.

Immunofluorescence

Areas surrounding the infarction were visualized with primary antibodies incubated at 4°C overnight to the macrophage marker Iba1 (WAKO Chemicals, Richmond, VA, USA, #019-19741, rabbit anti-rat,1:500 dilution) and neuronal marker NeuN (Millipore Canada Ltd., Etobicoke, Ontario, Canada, MAB377, mouse anti-mouse, 1:500 dilution) by immunofluorescence as previously described (Chen et al., 2007). Cy2 and Cy3 conjugated secondary antibodies were obtained from Jackson Laboratories (Bar Harbour, ME, USA) and used at 1:1,000 dilution. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Iba1-positive cells were imaged on a Zeiss (Oberkoken, Germany) AxioImager Z1 fluorescence microscope and counted in three independent fields at 20× magnification from six sections per mouse.

Bone marrow-derived macrophage cultures

Bone marrow-derived macrophages (BMDM) were cultured for 6 days in HyClone™ Dulbecco's Modified Eagles Medium (GE Healthcare Life Sciences, Missassauga, Ontario, Canada) conditioned with medium from L926 cells, as we described (Chen et al., 2015) and treated with lipopolysaccharides (LPS; Sigma-Aldrich, Oakville, Ontario, Canada, 100 ng/mL) with or without Dabrafenib (10 μM) for 4 hours. RNA was isolated for quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis.

qRT-PCR

Total RNA from BMDM or frozen brain tissue was extracted using the Qiagen (Germantown, MD, USA) RNeasy Mini Kit (Hari et al., 2017). qRT-PCR was conducted as described previously (Pandey et al., 2013; Qin et al., 2015), and the results were normalized to beta-actin. In brief, 1 μg of RNA was used for cDNA synthesis (ABM 5x All-in-one RT MasterMix, #AG490), and 4 μL of 1:4 diluted cDNA was used for qPCR with the following primers: TNF-α: (F) 5′-CCA CCA CGC TCT TCT GTC TAC-3′, (R) 5′-AGG GTC TGG GCC ATA GAA CT-3′ and normalized to beta-actin: (F) 5′-CCT TCT GAC CCA TTC CCA CC, (R) 5′-GCT TCT TTG CAG CTC CTT CG-3′ for cultured BMDM ornormalized to GAPDH: (F) 5′-TGT TCC TAC CCC CAA TGT GT-3′, (R) 5′-TGT GAG GGA GAT GCT CAG TG-3′ for brain tissue.

Statistical analysis

All results are presented as the mean ± SEM. For infarct volumes, percentages were normalized by arcsine transformation. For between-group comparisons of fold changes in TNF-α expression, values were normalized by log transformation. Two-way analysis of variance (ANOVA) was used to compare the main effects of treatment (vehicle *versus* Dabrafenib) and time (1 day *versus* 4 days) and the interaction (treatment *versus* time) following photothrombosis on infarct volumes. For experiments in BMDM, two-way ANOVA was used to compare the effects of treatment (vehicle *versus* Dabrafenib) and immune stimulation (with or without LPS challenge) as well as the interaction on TNF-α mRNA levels. For *post-hoc* analysis, the Bonferroni correction was applied for multiple pairwise testing using two-tailed Student's *t*-test. Differences in means were considered significant at $P < 0.05$.

Results and Discussion

Dabrafenib (10 mg/kg) administered one hour after photothrombosis-induced focal ischemic injury significantly reduced infarct lesion size in C57Bl6 mice one day after infarction (**Figure 1**). Two-factor ANOVA revealed a main effect of Dabrafenib ($F = 16.458$, $P = 0.00036$) and time ($F = 10.131$, $P = 0.0035$) to reduce infarction volume. Administration of a daily maintenance dose of Dabrafenib (5 mg/kg) for 3 days did not further reduce the infarction on day 4. Ischemic injury induced infiltration of Iba1-positive cells (microglia/macrophages), as revealed by immunofluorescence (**Figure 2A**). Although Dabrafenib treatment did not result in a significant reduction in $Iba1^+$ microglia recruitment to the site of injury (**Figure 2B**), Dabrafenib treatment attenuated up-regulation of TNF-α mRNA levels one day after photothrombosis (**Figure 3A**, Dabrafenib effect: $F = 5.479$, $P = 0.037$; time effect: $F =$

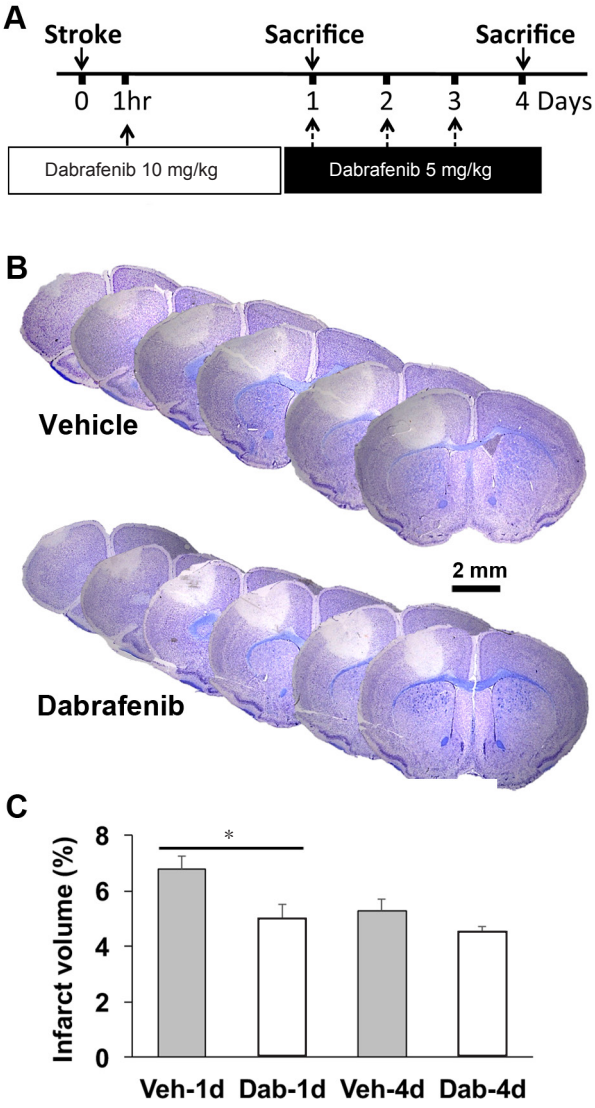

Figure 1 Dabrafenib attenuated ischemic brain injury in mice.
(A) Dabrafenib (Dab) was intraperitoneally injected after photothrombosis-induced ischemic brain injury as shown in the diagram. (B) Infarction volumes (white areas) were measured by Cresyl violet staining at one day and four days after infarction and expressed as a percentage of total brain volume (C). $*P < 0.05$. *t*-test was used with Bonferroni correction ($n = 8$ mice per group). Veh: Vehicle.

15.412, $P = 0.002$). This result suggests that the effect of Dabrafenib on reduction of infarct volume is related to attenuated local inflammation after ischemic injury.

To test whether Dabrafenib affects TNF-α activation in macrophages, bone marrow-derived macrophages were stimulated with LPS in the absence or presence of Dabrafenib. Dabrafenib blocked LPS-induced activation of TNF-α expression in macrophages (**Figure 3B**; Dabrafenib effect: $F = 642.54$, $P = 8.632E-12$; LPS effect: 465.39, $P = 5.735E-11$; interaction: $F = 220.49$, $P = 4.36E-9$). This *in vitro* data may explain why markedly lower TNF-α mRNA level was detected one day after Dabrafenib treatment (10 mg/kg, 1 hour after photothrombosis) despite similar numbers of Iba1-positive microglia/macrophages recruited to the ischemic site. However, we are puzzled by the lack of a sustained effect of Dabrafenib 4 days after stroke. This suggests that the maintenance dose (5 mg/kg/day) we used was not sufficient. It is noteworthy that in humans, Dabrafenib is administered in two 150 mg doses daily (equivalent to 4 mg/kg body weight per day, given a 75 kg average weight) for the treatment of melanoma (Hauschild et al., 2012). On the other hand, in mice, daily oral administration of 10 mg/kg was effective at suppressing tumor growth (King et al., 2013), a dose we also found to be effective when administered intraperitoneally. Future studies are needed to test whether higher maintenance doses of Dabrafenib can further reduce infarct volumes and favor recovery.

Of note, vehicle treated mice showed regression of the infarction volume (expressed as a percentage of brain volume to correct for edema) at 4 days compared to day 1. Regression of infarction volume after photothrombosis has been reported by us (Cruz et al., 2017) and others (Li et al., 2014a). Local inflammation likely contributes to the size of infarction, since we previously showed that mice with sustained inflammation have delayed regression of infarction (Cruz et al., 2017).This would be consistent with the acute anti-inflammatory effect of the higher dose of Dabrafenib we observed 1 day after stroke.

Since the tumour necrosis factor receptor (TNFR) is a major death receptor involved in the activation of necroptosis, we propose a model whereby inhibition of TNF-α production from macrophages/microglia likely accounts for part of the protective effect by Dabrafenib to limit stroke injury (see diagram; **Figure 4**). Ischemic stroke results in an abrupt deprivation of nutrient supplies that quickly leads to irreversible damage in the core of the affected area. Damage-associated molecular patterns (DAMPS) released from dying cells are sensed by Toll-like receptors (TLRs). TLRs are a receptor family broadly expressed in many cell types including neurons and glia cells. Activation of TLRs triggers gene expression through several transcription factors. Interferon regulatory factors (IRFs) are the main targets of TLRs that initiate the innate immune response. Activation of TLRs/IRFs increases inflammatory cytokine expression, including TNF-α, leading to the recruitment of immune cells, like macrophages, to the site of injury for tissue repair (Kariko et al., 2004). On the other hand, TNF-α activates the TNF receptor on neurons and causes programmed cell death via caspase-dependent and independent pathways (Degterev et al., 2003).

TNF-α further activates IRF1 (Jaruga et al., 2004) that increases expression of RIPK proteins and amplifies TNF-α-induced RIPK3 activation and necroptosis (McComb et al., 2014). Consistent with the activation of TNF-α in ischemic brain injury, IRF1 expression is also elevated in ischemic neurons in mice after experimentally-induced stroke and in post-mortem neurons of stroke patients (Alexander et al., 2003). In line with these observations, mice that lack IRF1 have a smaller infarction after experimental focal brain ischemia (Iadecola et al., 1999).

Blocking necroptosis with the RIPK1 inhibitor necrostatin-1was found to be neuroprotective in ischemic brain injury (Degterev et al., 2005). However, necrostatin-1 and related RIPK1 inhibitors also exhibit some toxicity (Takahashi et al., 2012) and most have not undergone clinical trials for safety in humans. Recently, Dabrafenib was identified as a RIPK3 in-

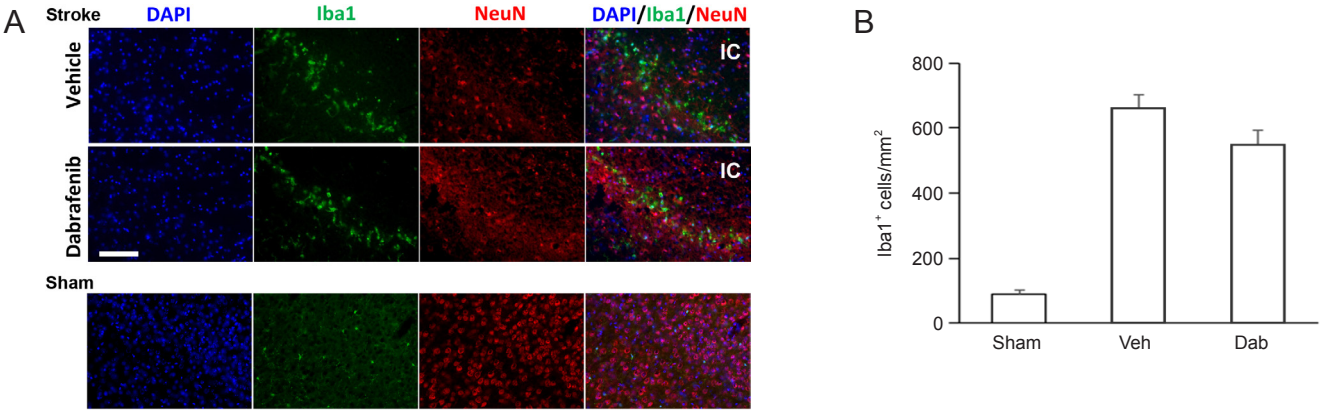

Figure 2 Immunofluorescence did not reveal an obvious difference in microglial recruitment between vehicle (Veh) and Dabrafenib (Dab)-treated mice one day after ischemic injury.
(A) The peri-infarct area shows Iba1-immunopositive (Iba1$^+$) macrophages/microglia recruited to the area surrounding the ischemic core (IC). Scale bar: 100 μm. Sham-operated control shows few Iba$^+$ cells in the same cortical region. (B) Iba1$^+$ cell counts. n = 4 mice per group. NeuN: Neuronal marker. DAPI: 4′,6-diamidino-2-phenylindole, nuclei marker; Iba1: ionized calcium binding adaptor molecule 1.

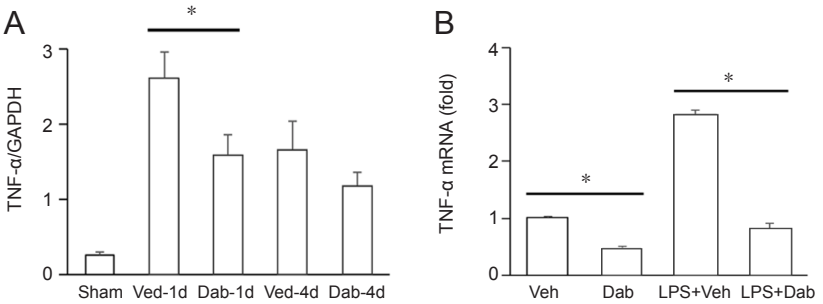

Figure 3 Dabrafenib (Dab) attenuated tumor necrosis factor-alpha (TNF-α) activation in the brain.
(A)TNF-α mRNA levels in the brain measured by quantitative reverse transcription-polymerase chain reaction are elevated after photothrombosis. Dab treatment attenuated this effect 1 day after initial dose (10 mg/kg). n = 4 mice per group. (B) Dab (10 μM) blocked TNF-α mRNA expression in lipopolysaccharides (LPS)-stimulated bone marrow-derived macrophages. n = 4 independent cultures per group. $*P$ < 0.05. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; Veh: Vehicle.

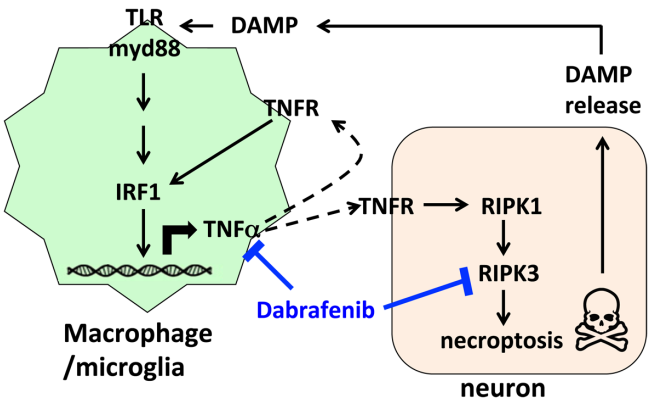

Figure 4 Diagram of Dabrafenib action to block TNF-α-mediated activation of necroptosis induced after ischemic injury.
Dabrafenib is proposed to inhibit tumor necrosis factor-alpha (TNF-α) production from macrophages and microglia. At the same time, Dabrafenib can also inhibit RIPK3 in neurons and prevent necroptosis. TNFR: TNF-α-receptor; RIPK: receptor-interacting protein kinase; DAMP: damage-associated molecular patterns; TLR: toll-like receptors; IRF1: interferon regulatory factor-1; myd88: myeloid differentiation primary response gene 88.

hibitor (Li et al., 2014b). Importantly, Dabrafenib has already undergone clinical trials and is an FDA approved drug for the treatment of melanoma in humans (Hauschild et al., 2012). Thus, to the best of our knowledge, our study is the first to demonstrate that Dabrafenib attenuates stroke injury in a preclinical mouse model. Our finding is exciting because it suggests that Dabrafenib could readily be repurposed for stroke therapy.

Whether the effect of Dabrafenib is mediated by its inhibition of RIPK3 remains to be demonstrated. Another RIPK3 inhibitor was recently shown to protect cultured neurons against injury caused by oxygen and glucose deprivation (Fayaz et al., 2016). However, it should be pointed out that ablation of RIPK3 had no effect on brain injury caused by middle cerebral artery occlusion followed by reperfusion (Newton et al., 2016). The discrepancy between the RIPK3 knockout study and our findings could arise from compensatory effects of genetic ablation of RIPK3, or from a difference in stroke models, since photothrombosis does not involve reperfusion. We also cannot exclude that B-raf inhibition by Dabrafenib may itself be beneficial to stroke injury, as has been shown for other B-raf inhibitors (Ahnstedt et al., 2011). Lastly, additional behaviour studies will be required to test whether higher or more frequent doses of Dabrafenib can improve sensorimotor functional recovery after ischemic brain injury.

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Research ethics: *All procedures were carried out with the approval of the animal care and use committee of the University of Ottawa (OGHRI-49), accord-*

ing to animal use guidelines of the Canadian Council on Animal Care (www.ccac.ca/en/standards/guidelines/).

Data sharing statement: *Datasets analyzed during the current study are available from the corresponding author on reasonable request.*
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