Entinostat reverses P-glycoprotein activation in snail-overexpressing adenocarcinoma HCC827 cells

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Abstract

Epithelial-to-mesenchymal transition (EMT) in cancer cells facilitates tumor progression by promoting invasion and metastasis. Snail is a transcriptional factor that induces EMT, while P-glycoprotein (P-gp) is an efflux transporter involved in anticancer drug resistance, and P-gp efflux activity is stimulated in Snail-overexpressing lung cancer cells with EMT characteristics. Since the histone deacetylase (HDAC) inhibitor entinostat (Ent) reverses EMT features, our aim in this study was to determine whether Ent also suppresses P-gp activation in Snail-induced cells. First, we confirmed that Ent treatment reduced migration activity, downregulated E-cadherin and upregulated vimentin at the mRNA level in Snail-overexpressing cells, thus inhibiting EMT. Efflux and uptake assays using rhodamine123 (Rho123), a fluorescent P-gp substrate, showed that Ent also inhibited Snail-induced activation of P-gp. Moreover, P-gp activity was more strongly inhibited by Ent in Snail-overexpressing cells than in Mock cells. When we evaluated the uptakes of Rho123 by LLC-PK1 cells and P-gp-overexpressing LLC-GA5COL150 cells, Rho123 accumulation in LLC-GA5COL150 cells was significantly decreased compared with that in LLC-PK1 cells. Coincubation with Ent had no effect on Rho123 accumulation in either of the cell lines. Thus, Ent appears to be an inhibitor, but not a substrate, of P-gp at low concentration. Our results suggest that Ent treatment might suppress not only Snail-induced cancer malignant alteration, but also P-gp-mediated multidrug resistance.

Introduction

Lung cancer is a leading cause of cancer death worldwide [1], in part because of its high metastatic potential [2], which is related to the occurrence of epithelial-to-mesenchymal transition (EMT) of cancer cells [3] [4]. Conversion of epithelial cancer cells to mesenchymal cancer cells (i.e., EMT) involves down-regulation of epithelial markers such as E-cadherin [5], occludin [6]
and claudin [7] and up-regulation of mesenchymal markers such as vimentin [8] and ZEB1 [9]. Snail is a transcriptional factor that regulates cancer EMT through the inhibition of E-cadherin expression [10]. It is also reported that EMT triggers cancer multidrug resistance (MDR) by causing changes in the activities of drug-metabolizing enzymes [11] and drug transporters [12]. Among these transporters, P-glycoprotein (P-gp) mediates efflux of drugs, toxic compounds, xenobiotics and metabolites, and plays a key role in cancer MDR [13]. We have shown that the efflux activity, though not the expression level, of P-gp is enhanced in Snail-overexpressing lung adenocarcinoma HCC827 cells [12]. Therefore, we considered that inhibitors of cancer EMT might also be useful for overcoming cancer MDR mediated by P-gp. Entinostat (Ent) is a histone deacetylase (HDAC) inhibitor that is under clinical trial for the treatment of various cancers, such as breast cancer [14], non-small cell lung cancer [15], melanoma [16] and various solid tumors [17]. Shah et al. have revealed that Ent reverses cancer EMT characteristics in vitro. Moreover, they also indicated that the mechanism of EMT inhibition by Ent partially depended on Snail down-regulation [18]. Therefore, we considered that Ent might be a useful drug not only for inhibition of EMT, but also for inhibition of P-gp activation in Snail-overexpressing cells. In this context, the aim of the present study was to establish whether or not Ent inhibits P-gp activation, as well as EMT, in Snail-overexpressing cells.

Materials and methods

Materials and cells

Ent was purchased from MedChem Express (QLD, Australia), Rhodamine123 (Rho123) from Sigma-Aldrich (St. Louis, MO), and elacridar (Elc) from Santa Cruz Biotechnology (Santa Cruz, CA). All other reagents were commercial products of reagent grade.

Human non-small cell lung cancer cell line HCC827 was purchased from American Type Culture Collection (Manassas, VA). Porcine kidney epithelial cell line LLC-PK1 and its P-gp-overexpressing variant LLC-GA5COL150 (MDR1 gene-transfected cells) were purchased from RIKEN Cell Bank (Ibaraki, Japan). The cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (FBS), 100 units/mL penicillin, 0.1 mg/mL streptomycin. LLC-GA5COL150 cells were cultured in the same medium supplemented with 500 μg/mL geneticin and 150 ng/mL colchicine. All cells were cultured in a humidified atmosphere of 5% CO₂ at 37°C.

Adenoviral vector infection and Ent treatment of HCC827 cells

Mock or Snail human adenovirus serotype 5 vectors (Ad) were gifts from Masahiro Kajita [19]. Purification and particle counting of Ad were carried out as described [12].

HCC827 cells were seeded at 0.5 × 10⁵ on 24-well culture plates. One day after seeding, cells were infected with Ad at 1000 VPs/cell. After 3 days, the medium was changed to fresh medium with or without 10 nM Ent. After 4 days, cells were used for RNA extraction, Rho123 efflux assay and uptake assay.

Quantitative real-time polymerase chain reaction

Total RNA was isolated from HCC827 cells using TRIzol™ reagent (Invitrogen, Carlsbad, CA) and 1 μg of extracted total RNA was used for cDNA synthesis with ReverTra Ace® (Toyobo, Osaka, Japan).

Quantitative real-time polymerase chain reaction (qRT-PCR) was carried out using Power SYBR™ Green PCR Master Mix (Applied Biosystems, Carlsbad, CA) with an Mx3000P qPCR system (Agilent Technologies, Santa Clara, CA). The values of fold change in mRNA
expression were calculated by the 2-ΔΔ threshold cycle (Ct) method, normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Oligonucleotide primers for E-cadherin, vimentin, Snail, P-gp and GAPDH were as follows: E-cadherin (159 bp), F: 5’-CAGCACGTACACAGCCCTA A-3’, R: 5’-ACCTGAGGCTTTGGAT TCCT-3’, vimentin (105 bp), F: 5’-CGGGAGAAATTGCAGAGGA-3’, R: 5’-AAGGTCAA- GACGTGCCAGAG-3’, Snail (249 bp), F: 5’-GAAGGCTTCCACGTCAA-3’, R: 5’-TGACATCTGAGTGGGTCG-3’, P-gp (157 bp), F: 5’-CCCATCATGGAATCCCAGGA-3’, R: 5’-GTGTCGACGTCCCAGA-3’, GAPDH (87 bp), F: 5’-GGTCAAACTTCTTGCT-3’, R: 5’-GCCATGGACTGTGTCATGAG-3’.

**Cell viability assay**

One day after HCC827 cell seeding at 1 × 10^4 cells/well on a 96-well culture plate, the medium was replaced with medium containing 0–100 μM Ent. After 4 days, alamarBlue® reagent (AbD Serotec, Dusseldorf, Germany) was added and the absorbance was measured at 570 and 600 nm. The viability was calculated from these values in accordance with the manufacturer’s instructions.

**Western blot analysis**

One day after HCC827 cell seeding at 2.5 × 10^5 cells/well on a 6-well culture plate, the medium was replaced with medium containing 0–1000 nM Ent. After 4 days, the cells were washed twice with ice-cold phosphate-buffered saline (PBS), collected by trypsinization, washed twice with ice-cold PBS, and lysed with RIPA Buffer (Wako, Osaka, Japan) containing Complete Mini, EDTA-free (Roche, Basel, Switzerland) for 30 min on ice. The lysate was centrifuged at 15,000 g for 30 min at 4˚C and the supernatant was collected. Subsequent processing was carried out as described elsewhere [12]. Briefly, a 30 μg aliquot of protein was separated on 4–20% polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane. The membrane was blocked with 5% skim milk and blotted with primary antibodies [anti-histone H3 (BioLegend, San Diego, CA) or anti-acetyl-histone H3 (Lys9) (Cell Signaling Technology, Danvers, MA)] overnight at 4˚C. The next day, the membrane was washed and reacted with goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hr at room temperature. The band intensity was measured using ECL substrate (GE Healthcare, Little Chalfont, Buckinghamshire).

**Migration assay**

Migration assay was carried out according to the Boyden chamber method in Transwells with polycarbonate membranes having 8-μm pore size (Corning, MA, USA). Briefly, cells were seeded at 1 × 10^6 cells/dish on 10 cm cell culture dishes. The next day, cells were infected with Ad at 1000 VPs/cell. After 3 days, cells were suspended in FBS-free medium and seeded at 5 × 10^4 cells/chamber on the Transwell in the presence or absence of 10 nM Ent. Medium including 10% FBS with or without 10 nM Ent was added under the chamber. After 4 days, cells were washed twice with ice-cold phosphate-buffered saline (PBS) and fixed with 3.7% formaldehyde for 2 min at room temperature. Cells were washed twice with ice-cold PBS and permeabilized with methanol for 20 min at room temperature. Cells were stained with crystal violet solution for 15 min at room temperature. Cells on the chamber were removed with a cotton swab to identify migrated cells, which were observed by microscopy.
Rho123 efflux assay and uptake assay

Rho123 efflux assay was carried out as described [12], with some modifications. After Ad infection and Ent treatment, HCC827 cells were washed twice with ice-cold PBS. Ice-cold 10 μM Rho123 in Opti-MEM® was then added. The cells were incubated for 15 min at 4˚C, then washed twice with ice-cold PBS, and further incubated in Opti-MEM® for 30 min at 37˚C. The cells were washed three times with ice-cold PBS and dried. Initial uptake of Rho123 in each group was evaluated using cells incubated with Rho123 for 15 min at 4˚C. The efflux rate was calculated according to the following formula.

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\text{Efflux rate (nmol/min/g protein)} = \frac{\text{Initial uptake of Rho123 (nmol/g protein)} - \text{Residual amount of Rho123 after 30 min incubation (nmol/g protein)}}{30 \text{ (min)}}
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Rho123 uptake assay was carried out as described [12]. After Ad infection and Ent treatment, HCC827 cells were washed twice with Hank’s balanced salt solution (HBSS) buffered with 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) (pH 7.4) (HBSS-HEPES) at 37˚C. Then, HBSS-HEPES including 10 μM Rho123 was added. The cells were incubated for 1 hr at 37˚C, then washed three times with ice-cold PBS, and dried.

Rho123 uptake assay with LLC-PK1 and LLC-PK1COL150 cells

LLC-PK1 or LLC-PK1COL150 cells were seeded (5 × 10^4 cells/well) on 24-well culture plates. After 4 days, the medium was changed to fresh medium without genetin and colchicine. The next day, uptake experiments were carried out using 10 μM Rho123 with or without 10 nM Ent or 10 μM Elc as a P-gp inhibitor. The uptake experiments were performed as described for Rho123 uptake assay. Cells were incubated for 30 min at 37˚C.

Determination of intracellular concentrations of Rho123 and Ent

Intracellular Rho123 concentration in efflux or uptake assays was determined according to the following protocol. After experiments, cells were lysed with 0.1 N NaOH and the lysate was transferred to a 96-well black microplate. Rho123 fluorescence was measured with an ARVO™MX (PerkinElmer, Waltham, MA) at wavelengths of 485 nm (excitation) and 538 nm (emission). Protein concentration in each lysate was evaluated with the DC™ Protein Assay (BIO-RAD, Hercules, CA).

Evaluation of ATPase activity in P-gp-expressing vesicles

The ATPase activity of P-gp was determined using a PREDEASY™ ATPase assay kit in accordance with manufacturer’s protocol and a previous report [20]. Briefly, solutions of various concentrations of Ent (0.001–100 μM) in 2% DMSO were used for activation and inhibition assays. A suspension of P-gp-expressing membrane vesicles derived from Spodoptera frugiperda ovarian cells (Sf9) was incubated with the Ent solution in the presence of 2 mM MgATP for 10 min at 37˚C. Vanadate was used in the activation study to evaluate background activity and verapamil was used in the inhibition study as an ATPase activator for P-gp-mediated transport. The assays were stopped by addition of the developer solution. Blocker solution was then added, and the samples were incubated for 30 min. The optical density (OD) was measured at 590 nm with a microplate reader Sunrise™ rainbow RC-R (TECAN, Kanagawa, Japan). Percentage ATPase activity was calculated from the OD values.
Statistical analysis

All data are presented as mean ± standard deviation (S.D.). Statistical analysis was undertaken using the Williams test or Holm test.

Results

Selection of a suitable concentration of Ent for studies of its effect on P-gp activation in HCC827 cells

We assessed the maximum non-cytotoxic concentration and the maximum non-effective concentration of Ent using alamarBlue® assays and western blotting of acetylated histone H3 (AcH3), respectively, in order to find a suitable concentration for reversing Snail-induced EMT in HCC827 cells without the need to consider either P-gp activity changes owing to Ent-induced cytotoxicity or altered AcH3 status. AlamarBlue® assays revealed that 10 and 100 μM Ent exposure significantly reduced HCC827 viability (89.9 ± 3.6% in 10 μM and 42.7 ± 0.9% in 100 μM respectively). At concentrations under 1 μM, Ent did not show cytotoxicity (Fig 1A). Moreover, whereas 1000 nM Ent treatment increased AcH3 expression, 100 nM Ent had no

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**Fig 1.** Effects of Ent on cell viability (A) and AcH3 expression (B) in HCC827 cells. (A) HCC827 cells were treated with Ent for 4 days in vitro. Cell viability was determined using alamarBlue® reagent. Each value is the mean ± S.D. (n = 5). Statistical significance was evaluated with the Williams test: **p<0.01. (B) Western blot analysis of AcH3 and H3 in HCC827 whole cell lysate. Cells were treated with Ent for 4 days in vitro. Band densities were determined with a Luminescent Image Analyzer LAS-3000, and AcH3 densities were normalized by H3.

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effect on AcH3 compared with the control (Fig 1B). Therefore in subsequent studies, we used 10 nM Ent to reverse Snail-induced EMT in HCC827 cells.

**Effect of Ent on migration activity of HCC827 cells**

Firstly, we evaluated whether Ent treatment inhibited migration activity of HCC827 cells by means of migration assay. Snail-overexpressing cells showed high migration activity. The Ent-treated Snail-overexpressing cells tended to show lower migration activity than Snail-overexpressing cells without Ent treatment, whereas the migration activity of Mock cells was not affected by Ent (Fig 2).

**Reversing effect of Ent on Snail-induced EMT in HCC827 cells**

Next, we confirmed that Ent reverses Snail-induced EMT in HCC827 cells. In Snail-overexpressing cells, E-cadherin expression was reduced and vimentin expression was increased compared with Mock cells (0.63 ± 0.09 and 5.54 ± 1.34 relative to Mock cells, respectively), in agreement with Mock cells (0.63 ± 0.09 and 5.54 ± 1.34 relative to Mock cells, respectively), in agreement

![Fig 2. Effects of Ent on migration activity of HCC827 cells.](image)

Migration activity of Mock and Snail-overexpressing cells was assessed at day 4 with or without Ent treatment by means of Transwell® assay. Cells were stained with crystal violet. Scale bars indicate 200 μm.

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with our previous findings [12]. Ent treatment restored E-cadherin expression and reduced vimentin expression in Snail-overexpressing cells (0.92 ± 0.11 and 4.13 ± 0.98 relative to Mock cells, respectively) (Fig 3A and 3B). On the other hand, Snail mRNA expression was unaffected by Ent (1.03 ± 0.27 in Mock cells, 406.57 ± 225.05 in Snail-overexpressing cells, 0.76 ± 0.15 in Mock cells with Ent, and 322.06 ± 47.64 in Snail-overexpressing cells with Ent) (Fig 3C). Moreover, Snail-overexpressing cells showed lower P-gp mRNA expression than Mock cells. However, Ent treatment did not affect P-gp expression in either Mock or Snail-overexpressing cells (1.01 ± 0.18 in Mock cells, 0.65 ± 0.24 in Snail-overexpressing cells, 1.17 ± 0.39 in Mock cells with Ent, and 0.46 ± 0.18 in Snail-overexpressing cells with Ent) (Fig 3D).

**Ent suppresses P-gp activation in Snail-overexpressing HCC827 cells**

We previously showed that P-gp is activated in EMT-induced HCC827 cells [12]. Therefore, we evaluated the ability of Ent to block P-gp activation. Firstly, we carried out Rho123 efflux

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**Fig 3.** Effects of Ent on E-cadherin (A), vimentin (B), Snail (C) and P-gp (D) mRNA expression levels in HCC827 cells. Cells were infected with Ad vector for 3 days and then treated with 10 nM Ent-containing medium for 4 days in vitro. Each value is the mean ± S.D. (n = 4 for E-cadherin, n = 5 or 6 for vimentin, Snail and P-gp). Statistical significance was evaluated with the Holm test: * \( p<0.05 \), ** \( p<0.01 \). N.S. indicates no significant difference.

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experiments. The Rho123 efflux rate was increased in Snail-overexpressing cells. On the other hand, P-gp activity was decreased by Ent treatment in both Mock and Snail-overexpressing cells (2.61 ± 0.48 nmol/min/g protein in Mock cells, 6.54 ± 0.08 nmol/min/g protein in Snail-overexpressing cells, 1.66 ± 0.48 nmol/min/g protein in Mock cells with Ent, and 3.13 ± 0.08 nmol/min/g protein in Snail-overexpressing cells with Ent) (Fig 4A). Rho123 uptake experiments revealed that the accumulation of Rho123 in Snail-overexpressing cells was decreased compared with that in Mock cells (0.103 ± 0.002 mL/mg protein in Mock cells and 0.077 ± 0.006 mL/mg protein in Snail-overexpressing cells). The decrease of Rho123 accumulation in Snail-overexpressing cells was reversed by Ent treatment (0.095 ± 0.014 mL/mg protein). However, Ent treatment had no effect on Rho123 accumulation in Mock cells (0.088 ± 0.001 mL/mg protein) (Fig 4B).

**Evaluation of the influence of Ent on P-gp efflux activity**

Finally, we examined whether 10 nM Ent inhibits Rho123 transport by P-gp. The accumulation of Rho123 in LLC-GA5COL150 cells was significantly decreased compared with that in LLC-PK1 cells. Coincubation with Ent had no effect on Rho123 accumulation in either of the cell lines. However, the P-gp inhibitor Elc significantly increased Rho123 uptake in LLC-GA5COL150 cells (Fig 5).

Furthermore, we evaluated whether or not Ent is a substrate of P-gp using P-gp-expressing Sf9 vesicles. Activation assay showed that 10 μM or more Ent increased ATPase activity, while in inhibition assay, 10 μM or more Ent inhibited ATPase activity (Fig 6).
Discussion

In this study, we examined whether or not the EMT inhibitor Ent suppresses P-gp hyper-activation in Snail-overexpressing cells. Various reports have indicated that exposure to high concentrations of cytotoxic agents or HDAC inhibitors induces P-gp mRNA expression [21] [22] [23]. Based on these reports, we explored the concentration range of Ent within which it does not show cytotoxicity or induce AcH3 accumulation, and we found that less than 100 nM Ent had no effect on cytotoxicity or AcH3 accumulation (Fig 1A and 1B). Lillico et al. reported that the IC$_{50}$ values of Ent in K562 (chronic myelogenous leukemia) and HEK293 (human embryonic kidney) cells were 0.97 ± 0.04 μM and 5.4 ± 0.2 μM respectively [24]. Therefore, it is unlikely that Ent would have affected normal cell survival in this study.
Migration assay and mRNA expression analysis both indicated that treatment with 10 nM Ent partially suppressed Snail-induced EMT, as reflected by the reversal of E-cadherin down-regulation and vimentin up-regulation (Fig 2, Fig 3A and 3B), without changing Snail mRNA expression (Fig 3C). Therefore, our data suggest that the reversing effect of Ent on Snail-induced EMT does not depend on suppression of Snail expression.

Under the same conditions, we evaluated P-gp activity using Rho123 efflux and uptake assays. Efflux assays revealed that P-gp efflux activity was significantly increased in Snail-overexpressing cells. Moreover, Ent reduced the Rho123 efflux rate by 36% in Mock cells and by 52% in Snail-overexpressing cells (Fig 4A). In accordance with this, Ent increased Rho123 accumulation in Snail-overexpressing cells to the same level as in Mock cells (Fig 4B). However, P-gp mRNA expression was not changed by Ent in either Mock or Snail-overexpressing cells (Fig 3D). Therefore, the results of Rho123 assays were not due to altered P-gp mRNA expression. These results indicate that Ent preferentially suppresses P-gp activity in Snail-overexpressing cells.

Fig 6. Effects of Ent on ATPase activation and inhibition in P-gp-expressing S9 vesicles. P-gp-expressing membrane vesicles were incubated with 1 nM–100 μM Ent for 10 min at 37˚C. Closed circles indicate the results in the activation study and open circles indicate those in the inhibition study. Each point is the mean ± S.D. (n = 2).
In the present study, we also found that Ent at the concentration of 10 nM is not a substrate of P-gp (Figs 5 and 6). This is important because, although various P-gp inhibitors have been shown to overcome cancer MDR, most of them are substrates of P-gp, and therefore they might cause adverse drug-drug interactions at P-gp in normal organs such as the kidney [25]. Thus, Ent may be a candidate for a non-substrate P-gp inhibitor acting preferentially on cancer cells exhibiting Snail-induced EMT.

In this study, we examined the inhibition of Snail-induced EMT and P-gp activation using 10 nM Ent, which was considered not to have any effect on P-gp mRNA expression. Although Ent sufficiently inhibited P-gp activation, it only partially inhibited EMT in Snail-overexpressing cells. Shah et al have reported that 1 μM Ent reversed EMT features in triple-negative breast cancer. Therefore, the use of 10 nM or more Ent should be effective for inhibition of both P-gp activation and EMT. On the other hand, our results also indicated that Ent is a substrate and/or inhibitor of P-gp at concentrations greater than or equal to 10 μM (Fig 6). Further study will be needed to explore the optimal concentration of Ent for inhibition of P-gp activation and EMT only in Snail-overexpressing cells, without inhibiting P-gp function in normal tissues.

Ent treatment inhibited P-gp activation in Snail-overexpressing cells in this study. However, the mechanism involved remains unclear. A previous study revealed that Ent suppresses Snail expression and the binding of Snail to E-cadherin promoter [18]. The binding of Snail to its target gene promoter is required for complex formation with various factors such as HDAC I [26]. In the present study, we used AcH3 accumulation as an indicator of the HDAC inhibition potential of Ent. However, this might not fully reflect the Ent-mediated HDAC inhibition state. Thus, we considered that Ent weakly inhibited HDAC activity, leading to reduced complex formation with Snail and HDAC I. We previously showed that P-gp protein expression of Snail-overexpressing HCC827 is unchanged, even though P-gp activity is up-regulated. These results might indicate that P-gp activation is induced through indirect mechanisms, such as P-gp posttranslational modification. Therefore, Ent might suppress P-gp activation through the modulation of these mechanisms in Snail-overexpressing cells.

It should also be noted that many epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs; e.g. gefitinib, erlotinib), which are key molecules in non-small cell lung cancer treatment [27] [28] [29] [30], are substrates of P-gp. This may be the reason why treatment of P-gp-mediated MDR cancer with these molecules is often unsuccessful. On the other hand, it was reported that Ent overcomes gefitinib resistance in vitro [31]. Moreover, a clinical trial indicated that combined treatment with erlotinib and Ent improved overall survival in non-small cell lung cancer patients who showed high E-cadherin expression levels [15]. Our findings suggest that P-gp inactivation by Ent may underlie this phenomenon, at least in part. Further studies are needed to explore optimal Ent treatment for controlling both EMT and P-gp activity, and to clarify the mechanism of P-gp regulation by Ent.

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