Bioorganic & Medicinal Chemistry Letters 21 (2011) 824–828

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Biological study of a somatostatin mimetic based on the 1-deoxynojrimycin scaffold

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ARTICLE INFO

Article history:
Received 11 October 2010
Revised 17 November 2010
Accepted 18 November 2010
Available online 3 December 2010

Keywords:
Peptidomimetic
Somatostatin
Angiogenesis
Cell cycle
Apoptosis
Migration
Stress fiber

ABSTRACT

Previously the synthesis of novel somatostatin mimetic from 1-deoxynojirimycin (DNJ) led to identification of a compound with affinity for human somatostatin receptor subtypes 4 and 5 (hSSTR4 and hSSTR5). Here we examined the properties of this peptidomimetic in a human umbilical vein endothelial cell (HUVEC) based assays. The peptidomimetic prevented capillary tube formation based on HUVECs. It also inhibited HUVEC proliferation by inducing G1 phase cell cycle arrest and apoptosis. Stress fiber assembly and cell migration in HUVECs was markedly suppressed by the somatostatin receptor ligand.

Angiogenesis provides new blood vessels to growing and developing tissue.[1] Pathological angiogenesis occurs in tumor formation, and also in a range of diseases that include rheumatoid arthritis, diabetic retinopathy, atherosclerosis, Crohn's disease, and diseases of the nervous system. Angiogenesis is believed to be a factor which facilitates the metastasis of tumor cells and so the development of angiogenesis inhibitors is of interest in cancer therapy[2] and a number of inhibitors have been approved for clinical use.[3]

Somatostatin (Fig. 1) is a peptide that is widely distributed throughout the central nervous system and peripheral tissues. There are two naturally occurring, bioactive forms of somatostatin, the 14-amino-acid-containing somatostatin (somatostatin 14) and its N-terminal extended precursor somatostatin 28. Both of these forms bind to somatostatin receptors that are coupled to heterotrimeric guanine nucleotide-binding proteins. There are five distinct somatostatin receptor subtypes (SSTR1 through SSTR5) which are present in human tissues. Somatostatin 14 was used in early clinical trials to control peptide hypersecretion, gut motility, and a variety of other physiologic processes. Somatostatin has poor bioavailability and pharmacokinetics and this led to research into the development of peptidal and non-peptidal mimetics of this hormone. The presence of somatostatin receptors on tumor cells and on the proliferating vascular endothelium has led to several

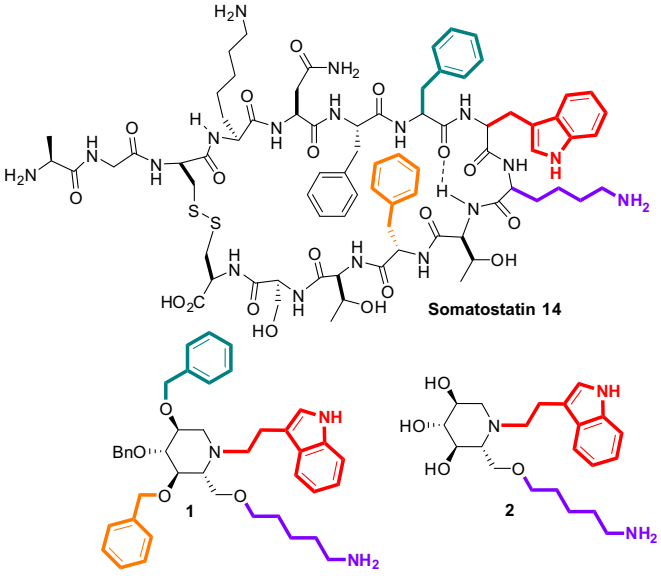

Figure 1. Structure of somatostatin 14 and peptidomimetics **1** and **2**.

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doi:10.1016/j.bmcl.2010.11.088

in vitro and in vivo studies to investigate the antiproliferative and antiangiogenic effects of somatostatin analogues. Currently available data suggest that somatostatin analogues might inhibit angiogenesis directly through their interaction with somatostatin receptors present on endothelial cells and also indirectly through the inhibition of growth factor secretion such as IGF-I and vascular endothelial growth factor (VEGF) and reducing monocyte chemotaxis.[4]

The synthesis of the somatostatin mimetic **1** from 1-deoxynojirimycin (DNJ) has been described and **1** has been shown to have affinity for the human somatostatin receptor subtype 4 (hSSTR4; K_i = 4.4 μM) and hSSTR5 (K_i = 5 μM).[5] Compound **2** showed affinity for hSSTR4 but not hSSTR5. The aim of this study was to investigate the effects of **1** in a human umbilical vein endothelial cell (HUVEC) culture system, in an attempt to define the multifaceted role of **1** in HUVEC proliferation, apoptosis and migration and the results are described herein.

It has been previously reported that HUVECs, express endogenous somatostatin hSSTR2 and hSSTR5 receptor and HUVEC cell growth can be inhibited with the peptidal somatostatin mimetics octreotide and SOM230, both of which have hSSTR5 activity.[6] The study herein focused on **1** as it displayed affinity for hSSTR5 in the previous study.[5] The inhibition of HUVEC growth by **1** was therefore first determined using the MTT assay.[7] Based on the average of three independently performed experiments, **1** was shown to inhibit growth of HUVECs with an IC_{50} of 12.6 μM.

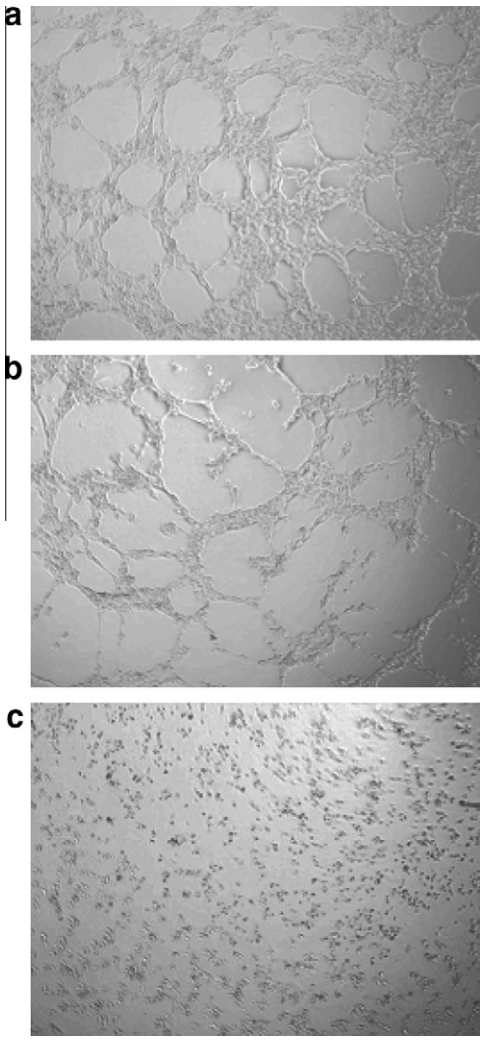

Figure 2. Tube formation. (a) HUVECs cultured on polymerized matrigel organize into tube-like structures. (b) Compound **1** inhibits tube formation partially (20 μM). (c) Compound **1** inhibits tube formation completely (40 μM).

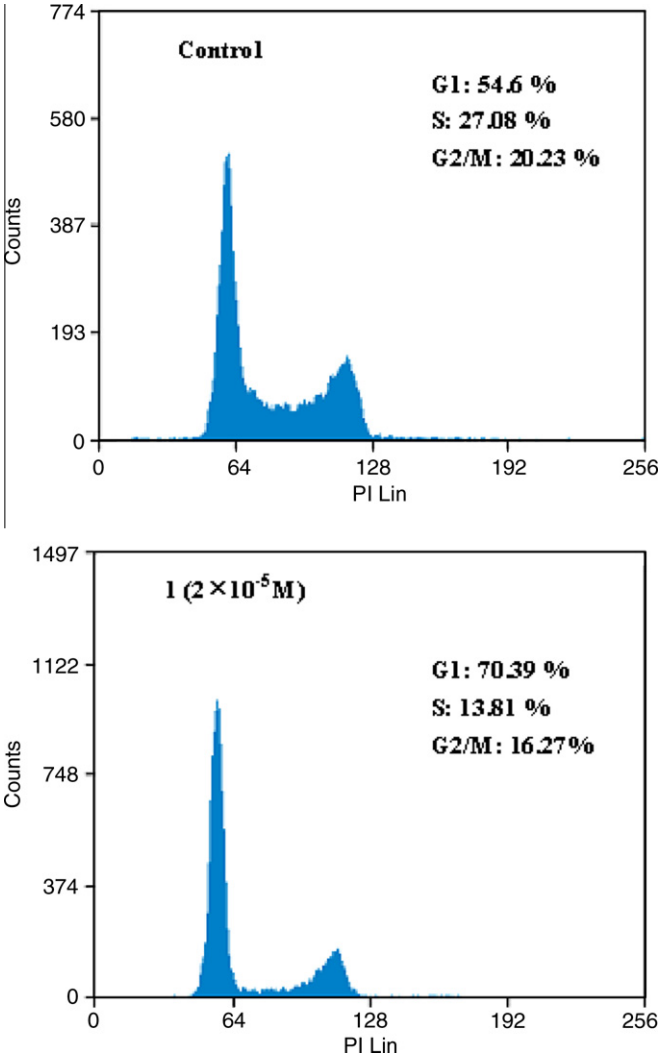

Figure 3. Effect of **1** on HUVEC cell cycle progression. HUVECs were treated with or without **1** for 24 h, respectively. Cells were then stained with propidium iodide, and the nuclei were analyzed for DNA content by flow cytometry. A total of 10,000 nuclei were analyzed from each sample, and the percentages of cells within G1, S, and G2/M were determined. Representative profiles are shown, and the % of cells in G1, S, and G2/M are shown in the upper right corner of the profiles.

The iminosugar derivative **1** was next evaluated for its ability to inhibit angiogenesis in vitro using the endothelial cell tube formation assay.[8] Endothelial cells which are induced to undergo capillary tube formation change their architecture and form cell-cell contacts that lead to branched networks that are similar to capillary-like blood vessels. When HUVECs are cultured on polymerized matrigel, they organize into such tube-like structures (Fig. 2a). However, when HUVECs were cultured on polymerized matrigel in the presence of **1** (40 μM, Fig. 2c) the cells failed to organize into these capillary-like structures. When the cells were treated with **1** at a concentration of 20 μM the tube formation was partially inhibited (Fig. 2b).

Next the mechanism by which the growth of HUVEC endothelial cells was altered by **1** was studied.[9] The effect of **1** on cell cycle distribution was analyzed and the result of a typical experiment is shown in Figure 3. As determined by flow cytometry, the exposure of HUVEC cells to **1** (20 μM doses) after 24 h, resulted in a clear

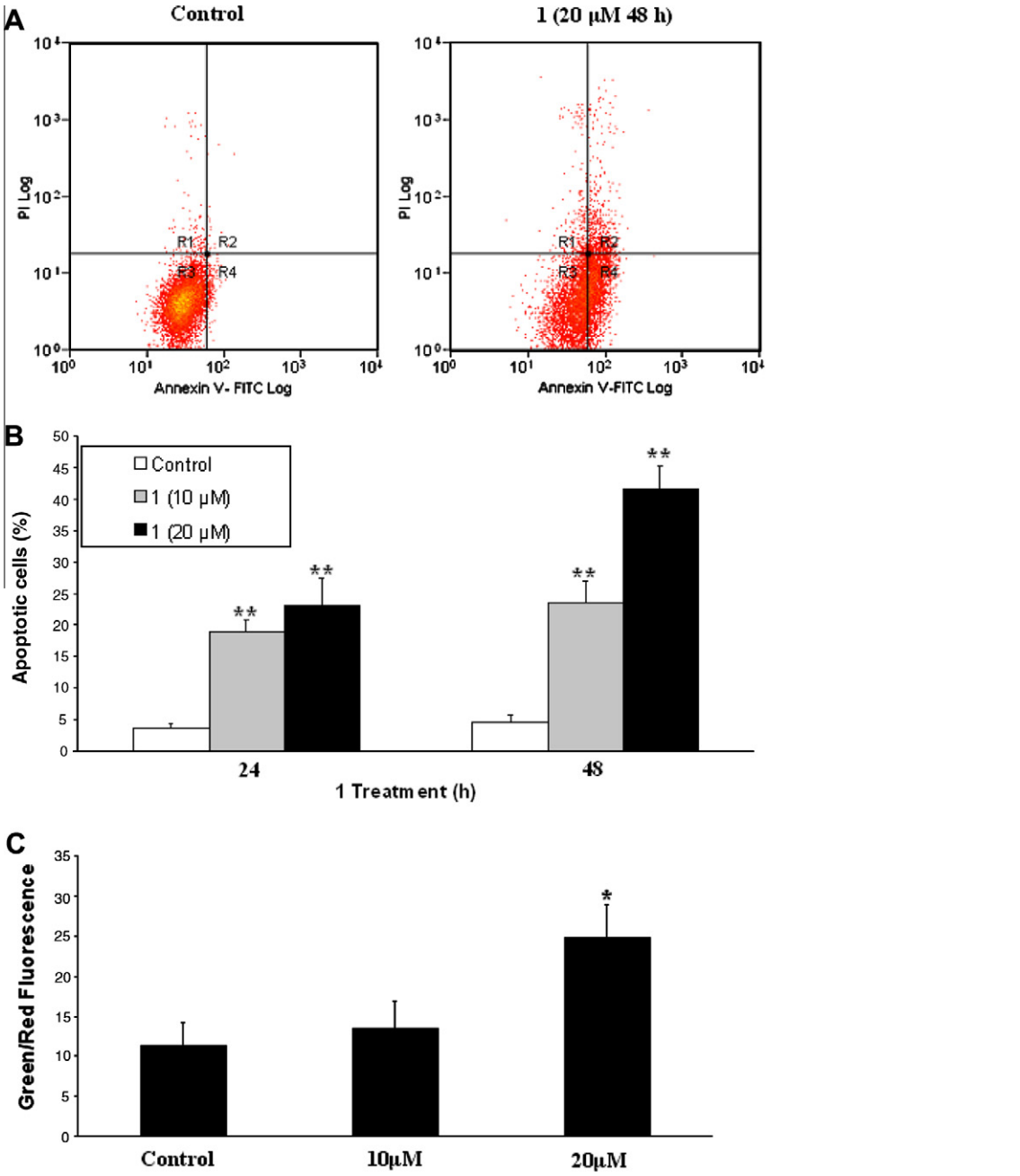

Figure 4. Apoptotic effect of **1** on HUVEC cells. (A) The fluorescence pattern of annexin V-FITC and propidium iodide-stained HUVEC cells after 48 h treatment with **1** (20 μM). (B) HUVEC cells were seeded and next day treated with different doses of **1** in complete medium as indicated. After 24 and 48 h of treatments, cells were harvested and analyzed for annexin V-positive apoptotic cell population as described in References and notes. (C) Effect of **1** on mitochondrial membrane potential in HUVEC. Cells were treated with different doses of **1** for 24 h and then harvested and processed by JC-1 staining followed by flow cytometry analysis. Each bar represents the mean (±SD $n = 3$). $^{*}P$ <0.01, $^{**}P$ <0.001 comparing with control.

increase of the percentage of cells in the G1 phase with a concomitant decrease in the percentage of cells in the S and G2/M phase when compared with the control.

We next tested whether apoptosis induction could contribute to the growth inhibitory function of **1** in HUVECs. The ability of **1** to induce apoptosis in HUVECs was thus evaluated by treatment of the cells with different doses of **1** for 24 and 48 h and subsequently subjecting the cells to staining with annexin V and propidium iodide and flow cytometry analysis.[10] The treatment with **1** (10 and 20 μM) caused a considerable apoptotic effect in HUVEC cells in comparison with controls (Fig. 4A and B). To further determine the molecular events leading to apoptosis induced by **1**, we analyzed the mitochondrial membrane potential in HUVEC cells which had been treated with **1**. The damage of mitochondrial integrity and the consequent loss of mitochondrial membrane potential ($\Delta\Psi$m) is an event in the initiation and activation of apoptotic cascades.[11] To determine whether **1** induced mitochondrial disruption in HUVEC cells, we examined the depolarization of the mitochondrial membrane by measuring the fluorescence remission shift (red to green) of the $\Delta\Psi$m sensitive cationic JC-1 (5,50,6,60-tetrachloro-1,10,3,30-tetraethylbenzamidazolocarbocyanin iodide) dye in HUVEC cells. Briefly these cells were treated with **1** for 24 h and subsequently processed and stained with the JC-1 dye and then analyzed by flow cytometry. Cells which had been treated

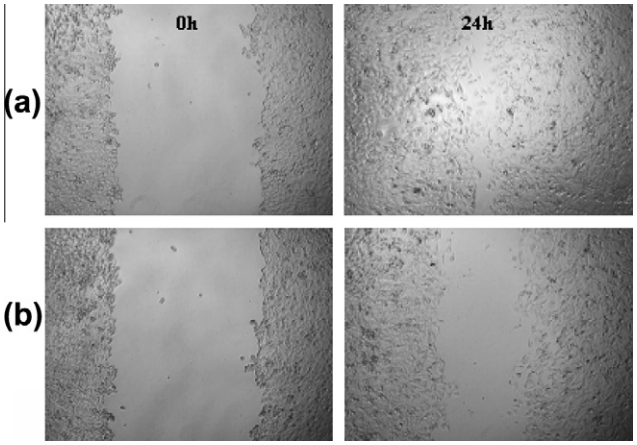

Figure 5. Effect of **1** on HUVEC cell migration. Migration of HUVEC cells was evaluated using the in vitro scratch–wound healing assay in the absence (a) and presence (b) of **1** (2×10^{-5} M).

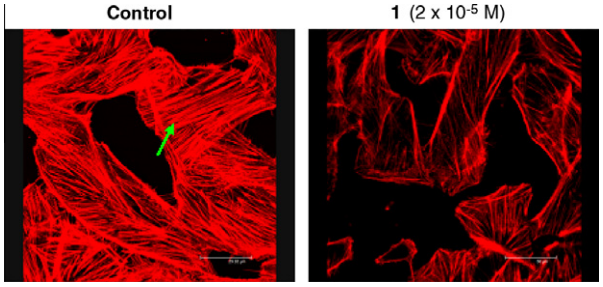

Figure 6. The effect of **1** on actin stress fiber polymerization in HUVEC cells was assessed by fluorescence staining with TRITC-phalloidin, a substance which specifically detects F-actin. In the control, stress fibers are present (green arrow). Incubation of HUVEC cells with **1** (20 μM) led to marked disruption of stress fiber assembly.

with **1** (20 μM) showed an increase in green/red fluorescence intensity indicating increased mitochondrial membrane depolarization (Fig. 4C). Therefore, it was concluded that the induction of apoptosis by **1** in HUVEC cells is closely associated with mitochondrial membrane disruption.

Growth and migration of endothelial cells are critical in the organization of new blood capillaries in tumors.[12] The effect of **1** on HUVEC cell migration was evaluated with the wound healing assay in vitro.[13] As shown in Figure 5, there is a significant reduction in the ability of **1** (20 μM) treated cells to migrate into the empty space. The results indicated that **1** can inhibit HUVEC cell migration (20 μM). This could be at least partially due to the inhibition of proliferation induced by **1**.

The compound **1** inhibited the growth and migration of HUVEC cells and it was thus of interest to explore the effect of **1** on actin polymerization. It is well established that actin polymerization, which leads to stress fiber assembly and depolymerization, plays a crucial role in cell motility.[14,15] In an attempt to explore the molecular events in response to treatment with **1**, the formation of the actin cytoskeleton in HUVEC cells was assessed by staining with TRITC-phalloidin, which specifically binds F-actin.[16] The results showed that stress fiber assembly in HUVEC was abolished by **1** (20 μM) (Fig. 6). Therefore, **1** suppressed the migration of HUVEC at least in part through disrupting actin assembly and stress fiber formation.

In summary, we have demonstrated that the non peptide somatostatin mimetic **1**, prevented capillary tube formation from human umbilical vein endothelial cells, through multiple pathways, including cell cycle arrest, induction of apoptosis and disruption of cell migration. In addition, we found that **1** disrupted cell migration at least in part through preventing stress fiber formation. The effects of **1** on HUVEC cell signal transduction and in an animal model for angiogenesis and cancer are currently being evaluated. Efforts to develop more potent somatostatin mimetics are also underway and will be reported in due course.[17]

Acknowledgments

The research was supported by Shandong Province Science and Technology Program (No. 2009GG10002084) and Science Foundation Ireland (03/IBN/B352) and the HEA Programme for Research in Third Level Institutions Cycle 3.

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7. HUVEC endothelial cells were purchased from American Type Culture Collection and the cells were cultured in F12K medium supplemented with 10% heat inactivated FBS, 0.1 mg/ml heparin and 0.03 mg/ml endothelial cell growth supplement (ECGS), 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, and maintained at 37 °C in a humidified atmosphere of 5% CO_2. For the MTT assay the HUVECs (3×10^3 cells) were seeded on 96-well microtitre plates in F12K medium with 10% FBS and incubated overnight. The cell culture medium was replaced by the different dose of a solution of **1** and the other compounds, and then the cells were cultured for another 72 h. The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reagent was added to the cell supernatant for a final concentration of 0.5 mg/ml of MTT. After 3 h the cell culture medium was removed. Formazan crystals in adherent cells were dissolved in 200 μl DMSO and the absorbance of the formazan solution was measured. The compound was tested in triplicate at each concentration and the experiments were repeated three times.
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9. See Ref. 8. Cells were seeded on six well plates in F12K medium with 10% FBS overnight. Then **1** (20 μM) was added to the cells, which were then cultured for another 24 h. Cells were collected with trypsin–EDTA and washed three times with PBS. The cells were re-suspended and fixed for at least 2 h at −20 °C with 70% ethanol. After washing twice with PBS, cells were incubated at 25 °C with 200 μg/ml RNase A for 30 min. The resulting cells were incubated with 50 μg/ml propidium iodide for 30 min at 4 °C. The treated cells were subjected to flow cytometry and the percentage of cells at each phase of the cell cycle was analyzed. Compound **1** was tested in triplicate and the experiment was repeated three times.
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13. See Ref. 10. Briefly HUVEC cells (3×10^4 cells) were seeded on 96-well microtitre plates in F12K with 10% FBS overnight. The confluent monolayers were wounded with a 200 μl plastic pipette tip. After washing, the chamber slide was incubated at 37 °C in 10% FBS F12K media in the presence or absence of compound. Migration of cells into wounded areas were photographed at 0 and 24 h.
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16. See Ref. [10]. Cells were seeded to the glass coverslips in cell culture dishes (3.5 cm) overnight. Compound **1** was then added and cells were cultured for 24 h. After fixing with 4% paraformaldehyde, cells were treated with 0.1% Triton X-100 and blocked with 1% BSA. Cells were incubated with TRITC-conjugated phalloidin for 60 min and examined under a confocal laser scanning microscope (100×, oil). The experiments were repeated at three times in each case.
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