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Antiangiogenic effects of butyric acid involve inhibition of VEGF/KDR gene expression and endothelial cell proliferation

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Abstract

The formation of new blood vessels from pre-existing ones is required for the growth of solid tumors and for metastasis. Interaction of tumor-secreted vascular endothelial growth factor (VEGF) with its receptor(s) on endothelial cells triggers endothelial cell proliferation and migration, which facilitate tumor angiogenesis. Butyric acid (BuA), a fermentation product of dietary fibers in the colon, is shown to alter gene expression and is postulated to be anticarcinogenic. The results presented in this paper indicate that BuA can be antiangiogenic *in vivo* by inhibiting angiogenesis in chorioallantoic membrane assay. BuA was not cytotoxic to endothelial cells but was a potent antiproliferative agent besides being proapoptotic to endothelial cells as verified by FACS analysis. Conditioned media from BuA-treated Ehrlich ascites tumor cells showed a 30% decrease in VEGF concentration when compared with untreated cells. The decrease in VEGF mRNA and its receptor, KDR mRNA levels in EAT and endothelial cells respectively, suggests that the VEGF-KDR system of angiogenesis is the molecular target for the antiangiogenic action of BuA. (Mol Cell Biochem **243**: 107–112, 2003)

Key words: butyrate, endothelial cells, apoptosis, angiogenic ligand-receptor, gene expression, antiangiogenesis

Introduction

Tumor cells require large amounts of oxygen and nutrients and need to recruit new blood vessels by angiogenesis to grow beyond a critical size and for metastasis. The pivotal role of VEGF and its receptors in tumor angiogenesis has been well established. Production of VEGF in abundance by many tumor cell lines and tumors and upregulation of VEGF receptor expression in endothelial cells of tumor blood vessels reflect on the involvement of VEGF in tumor neoangiogenesis [1–3]. Neutralization of VEGF or its receptors either by antibodies or by use of dominant-negative mutants or soluble receptors, profoundly decreased tumor growth in mice [4, 5]. The expression of VEGF is regulated by a plethora of external factors. VEGF production is potentiated by factors like FGF-4, PDGF, TNF-α, TGF-β, KGF, IGF-1, IL-1β and IL-6 [6]. Other cytokines such as IL-10 and IL-13 have been reported to inhibit the release of VEGF [7]. Wild-type tumor suppressor proteins, vHL, p53 and p73 have been shown to inhibit VEGF gene expression [8–11]. Among synthetic, small molecular weight inhibitors, fumagillin was identified as an angiogenesis inhibitor, since it inhibits endothelial cell proliferation *in vitro*, angiogenesis in the chorioallantoic membrane (CAM) assay and tumor growth and angiogenesis in the mouse [12]. Tyrosine kinase inhibitors, SU1498 and PTK787, selective for VEGF receptor-2 (VEGFR-2/Flk-1/KDR) kinase, show antiangiogenic effect in the CAM assay and bring about regression of tumor growth [13]. Thrombospondin 1 (TSP1), angiostatin and transforming growth factor-β (TGF-β) inhibit endothelial cell proliferation, migration and induce endothelial cell apoptosis to bring about their antiangiogenic effect [14–16].

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Dietary fibers (DF) have been shown to have beneficial effects against a variety of diseases, such as colon cancer, diabetes, coronary heart diseases, rheumatoid arthritis etc. [17]. The DF are fermented by the microflora present in the colon to short chain fatty acid (SCFA) such as acetate, propionate and butyrate. The SCFAs enter circulation and modulate activities of many key regulatory enzymes. Butyric acid (BuA) has been implicated in the modulation of gene expression and induction of apoptosis [18, 19]. BuA is known to induce apoptosis in a variety of tissue types including cancer cells [20, 21]. BuA has been postulated to bring about its anticarcinogenic action in different ways like induction of apoptosis, induction of cell differentiation, inhibition of histone deacetylase leading to altered gene expression and repression of specific cell death genes [22].

In this paper, we describe the molecular targets for the antiangiogenic effect of BuA. BuA inhibits the expression of angiogenic ligand and receptor genes and has an antiproliferative and proapoptotic effect on endothelial cells and can thus act as a potent antiangiogenic compound.

Materials and methods

Materials

Fertilized eggs were purchased from the Government poultry farm, Mysore, India. EAT cells were obtained from American type cell culture, Rockville, USA. VEGF-ELISA kit was purchased from R & D Systems, Germany. Endothelial growth medium (EGM), endothelial basal medium (EBM) and fungizone were obtained from Clonetech, NCTC-135, FCS, Penicillin and streptomycin were purchased from Gibco, Germany. RNeasy kit & QIAshredder were procured from Qiagen, Hilden, Germany. Quickhyb, RNase and BuA were procured from Sigma-Aldrich, St. Louis, USA. All other reagents were of analytical grade. EZ4U kit for cytotoxicity assay was obtained from Biomedica, Austria. T7-Quickprime kit, Kodak X-Omat X-ray film, Nick-column and α-^{32}P labeled CTP were procured from Amersham Pharmacia Biotech, Germany. The cDNA was retrieved from the backbone vector using EcoR1/BamH1 for human $VEGF_{165}$ and Bgl II for human KDR.

Methods

Chorioallantoic membrane (CAM) assay

The fertilized eggs were incubated for 5 days at 37°C in a humidified atmosphere. A window was made under aseptic conditions on the eggshell to check for proper development of the embryo. The window was resealed and the embryo was allowed to develop further. On the 11th day, either saline or BuA (1 mM) was air dried on a glass cover slip. The window was reopened and the cover slip was inverted over the CAM. The window was closed again and the eggs were incubated for another 2 days. The window was opened on the 13th day and inspected for changes in the microvessel density in the area under the cover slip and photographed.

In vitro *butyric acid treatment*

EAT cells were grown in NCTC-135 medium with 10% FCS and penicillin/streptomycin (1 mg/ml). HUVECs were grown in gelatin coated flasks using EGM with 5% FCS and penicillin/streptomycin (1 mg/ml). After the cells were 80% confluent, they were treated with various concentrations of BuA (0–20 mM) for various time periods (0–24 h). Then, the cells were trypsinized and collected before being used for further experiments.

Cytotoxicity assay

Cytotoxicity assay for BuA was done using the EZ4U kit as per the manufacturer's instructions. In brief, 50,000 HUVECs were plated in 96-well plates and grown overnight. After addition of varying concentrations of BuA (0–20 mM), 20 µl of substrate (Tetrazolium compound) was added to each well and incubated for 4 h at 37°C. Absorption at 450 nm was measured.

Assay for proliferation of HUVECs

Cells (HUVECs) were plated at a concentration of 100,000 cells/well in 6-well cluster plates. Twenty-four h after plating, cells were treated with varying concentrations of BuA (0–10 mM) and further cultured for 2 days at 37°C and 5% CO_2. Thereafter, cells were trypsinized and counted using a Coulter Counter.

Fluorescence activated cell sorter (FACS) analysis

HUVECs were grown to 80% confluence and treated with 10 mM BuA for various time intervals (0–24 h). The cells were harvested by trypsinization and fixed in ice-cold methanol overnight at 4°C. The cells were centrifuged, rehydrated using PBS, washed once and transferred to FACS tubes. The cells were then treated with 5 µl of RNase (10 mg/ml) and 20 µl of propidium iodide (1 mg/ml) and incubated in the dark at room temperature for at least 30 min, before processing for FACS analysis using a FACSCalibur machine. 20,000 events were counted. Data were analyzed using Win MDI 2.8 software.

Vascular endothelial growth factor-enzyme linked immunosorbent assay

VEGF-ELISA was performed using a kit method as per the manufacturer's instructions. In brief, 50 µl of each of assay

diluent and samples were added to a 96-well microplate precoated with mouse VEGF polyclonal antibody. Recombinant mouse VEGF was used to set up the standard curve. After 2 h of incubation, the wells were washed before adding 100 µl of polycolonal antimouse VEGF conjugated to horseradish peroxidase. After incubation for 2 h at room temperature and washing, 100 µl of substrate solution was added and incubated for 30 min at room temperature. The reaction was stopped and the O.D. at 450 nm (correction wavelength set at 510 nm) was measured in a Multiskan Ascent v22. A standard graph was generated using the Multiskan Ascent software with four-parameter logistic fit.

Northern blot analysis

Total RNA was extracted from confluent HUVECs and EAT cells using an RNeasy kit according to the instructions from the manufacturer. Total RNA (15 µg) was separated by 0.8% agarose-formaldehyde gel electrophoresis and blotted onto nylon membrane which was baked and hybridized with $\alpha(^{32}P)$-dCTP-labelled $VEGF_{165}$ or KDR cDNA. The hybridized blot was processed and subjected to autoradiography at –70°C. All blots after autoradiography were stripped and reprobed for expression of GAPDH as an internal control using labeled GAPDH cDNA.

Results

Angioinhibitory effect of BuA

In vivo angioinhibitory effect of BuA was clearly evident from results obtained in chorioallantoic membrane (CAM) assay. Results shown in Fig. 1 indicate that when compared to the extensive angiogenesis seen in normal CAM, angiogenesis in the CAM at the site of application of BuA was reduced significantly.

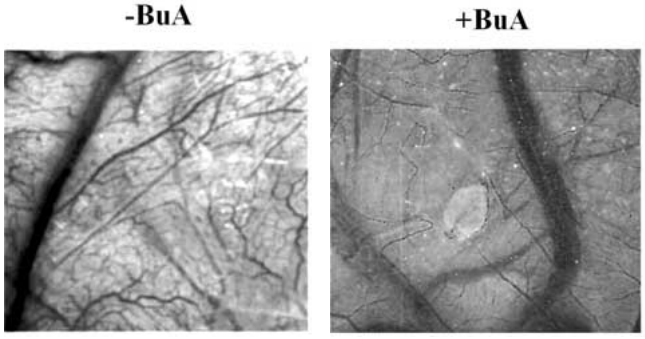

Fig. 1. Angioinhibitory effect of BuA. Either saline or BuA (1 mM) was dried on cover slips and applied onto the CAM of the developing chick embryo through a cut window. The CAM was observed for inhibition of neovascularization. The data shown represents the result of an experiment which was done using a minimum of six eggs in each group.

Kinetics of gene expression of VEGF and KDR in response to BuA treatment

The angioinhibitory effect of BuA could be attributed to its effect on either angiogenic ligand/receptor gene expression and/or to its effect on endothelial cell proliferation and apoptosis. The results in Fig. 2 show that over a period of 24 h in BuA-treated EAT cells, the VEGF mRNA levels decrease considerably. KDR mRNA levels in BuA-treated HUVECs were also significantly reduced in a time dependent manner. The decrease in VEGF gene expression was corroborated by the reduction in the amount of VEGF protein as estimated by VEGF-ELISA in the conditioned media of cells treated with BuA.

BuA inhibits VEGF production in EAT cells

Figure 3 compares the amount of VEGF in conditioned media at different time intervals over a period of 24 h for untreated and BuA (10 mM)-treated EAT cells. The results indicate that BuA has a time-dependent inhibitory effect on the release of VEGF in EAT cells under *in vitro* conditions. When compared to control, approximately 30% inhibition is seen in the amount of protein expressed (at 8 h and above) in BuA-treated cells.

Antiproliferative effect of BuA

The proliferation of endothelial cells induced by an angiogenic ligand determines the extent of angiogenesis. The possibility of BuA influencing the growth of endothelial cells therefore reflects on its antiangiogenic effect. The results in Fig. 4B indicate that BuA inhibited the proliferation of HUVECs *in vitro* in a dose-dependent manner. To rule out the possibility that the antiproliferative effect of BuA on HUVECs was not due to cytotoxicity of BuA, a cytotoxicity test was performed. BuA did not show any cytotoxicity towards HUVECs at the concentration of 0–10 mM *in vitro* (Fig. 4A).

BuA induces apoptosis of HUVECs

The results on inhibition of growth of HUVECs *in vitro* clearly indicate the involvement of the process of apoptosis. To verify this, HUVECs were subjected to FACS analysis, where significant increase of events in the sub-G1 phase in BuA-treated cells confirmed induction of apoptosis in HUVECs by BuA (Fig. 5).

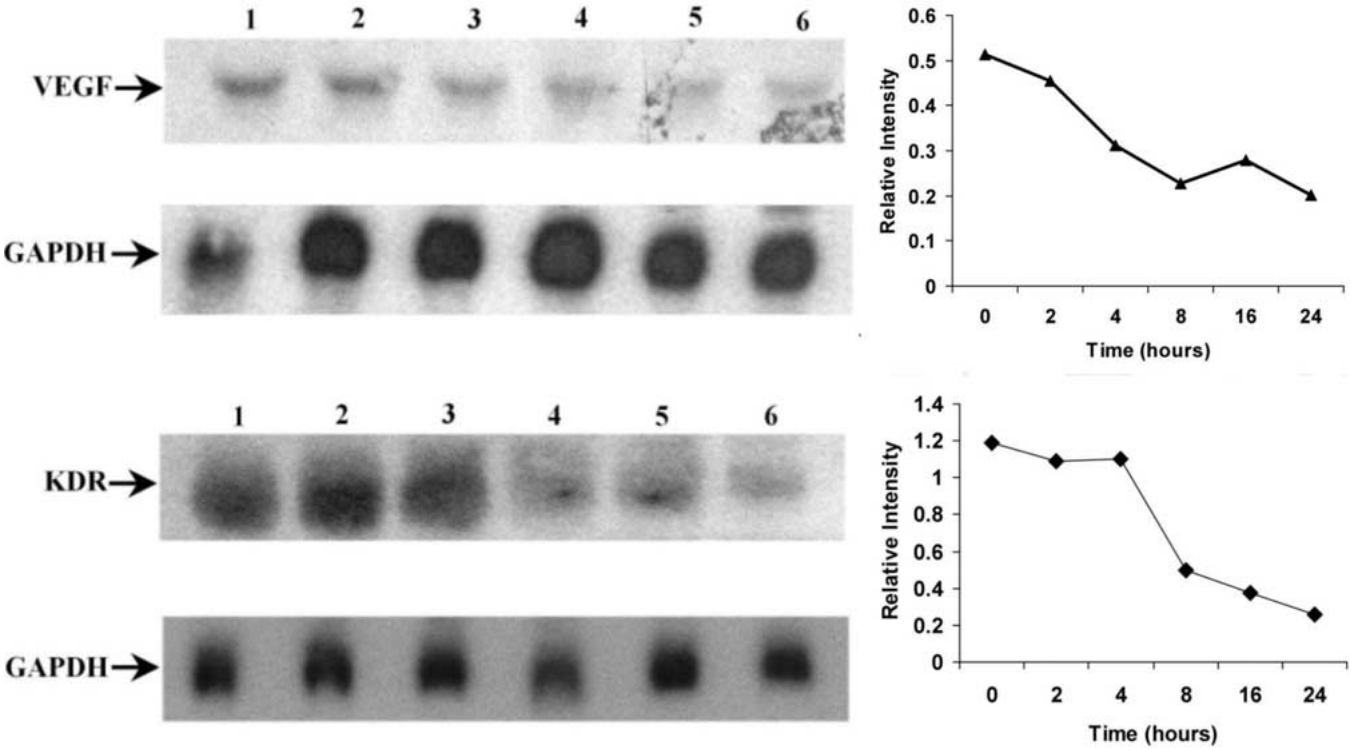

Fig. 2. Effect of BuA on expression of VEGF mRNA in EAT cells and KDR mRNA in HUVECs. Total RNA from EAT cells or HUVECs was isolated after treatment with BuA for varying time periods (Lane 1: Control, Lanes 2–6: 2–24 h) and Northern blot analysis was performed using $VEGF_{165}$ and KDR cDNA labeled probes. GAPDH was used as an internal control. The representative Northern blots and the graph depicting quantitation of VEGF and KDR mRNA is shown. The experiment was performed 3 times.

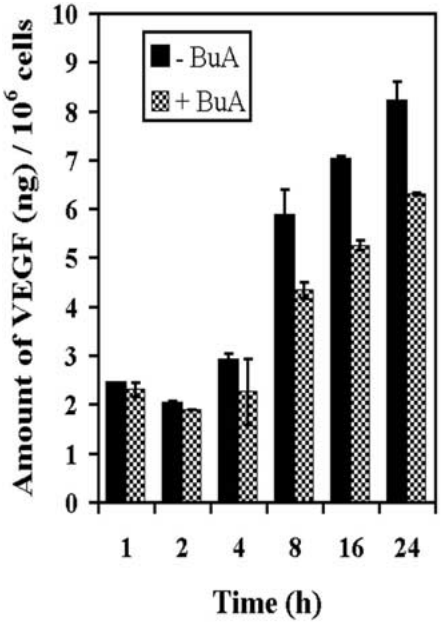

Fig. 3. Expression of VEGF protein by EAT cells in response to BuA. Conditioned media from EAT cells treated with and without BuA for various time periods (0–24 h) was subjected to VEGF-ELISA. Values are normalized to cell number and are expressed as mean ± S.E.M. of experiment done in triplicate.

Discussion

In the present study, we have focused our attention on delineating the pleiotropic effects of BuA, the fermentation product of dietary fiber in the colon, as a potent antiangiogenic compound.

Our results show that there is inhibition of neovascularization by BuA in CAM. CAM assay is an assay commonly used to identify antiangiogenic compounds. Since there is inhibition of neovascularization by BuA, it supports our view that BuA may repress the expression of VEGF-like factors or inhibit the secretion of such factors, thereby inhibiting the formation of new blood vessels. This hypothesis is further confirmed by our results on Northern blot analysis and VEGF-ELISA where BuA was able to inhibit VEGF gene expression effectively in a time dependent manner, at a concentration that it is present in the body. Recent studies suggest that endothelial cell apoptosis is necessary for the repair of damaged blood vessels and for sprouting and branching of capillaries during angiogenesis [23, 24]. Traditionally, regression of neovascular responses has been associated with inhibition of endothelial cell proliferation, migration and adhesion. Recently, the role of endothelial cell survival and death signals

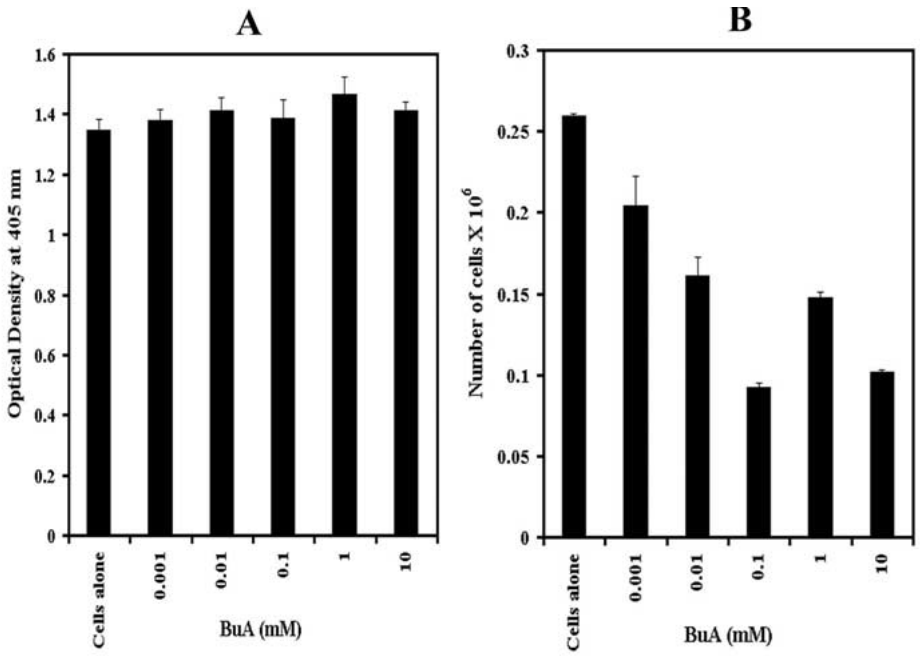

Fig. 4. Antiproliferative effect of BuA. (A) 50,000 cells were plated in 96-well plates and incubated overnight. Various concentrations of BuA were added and incubated for 5 h before the cytotoxicity was assayed. Results are expressed as mean O.D. ± S.E.M. (B) 100,000 cells were plated in 6-well plates and incubated overnight. Cells were treated with varying concentrations of BuA and after 2 days were counted in a Coulter counter. Values are mean of triplicates ± S.E.M.

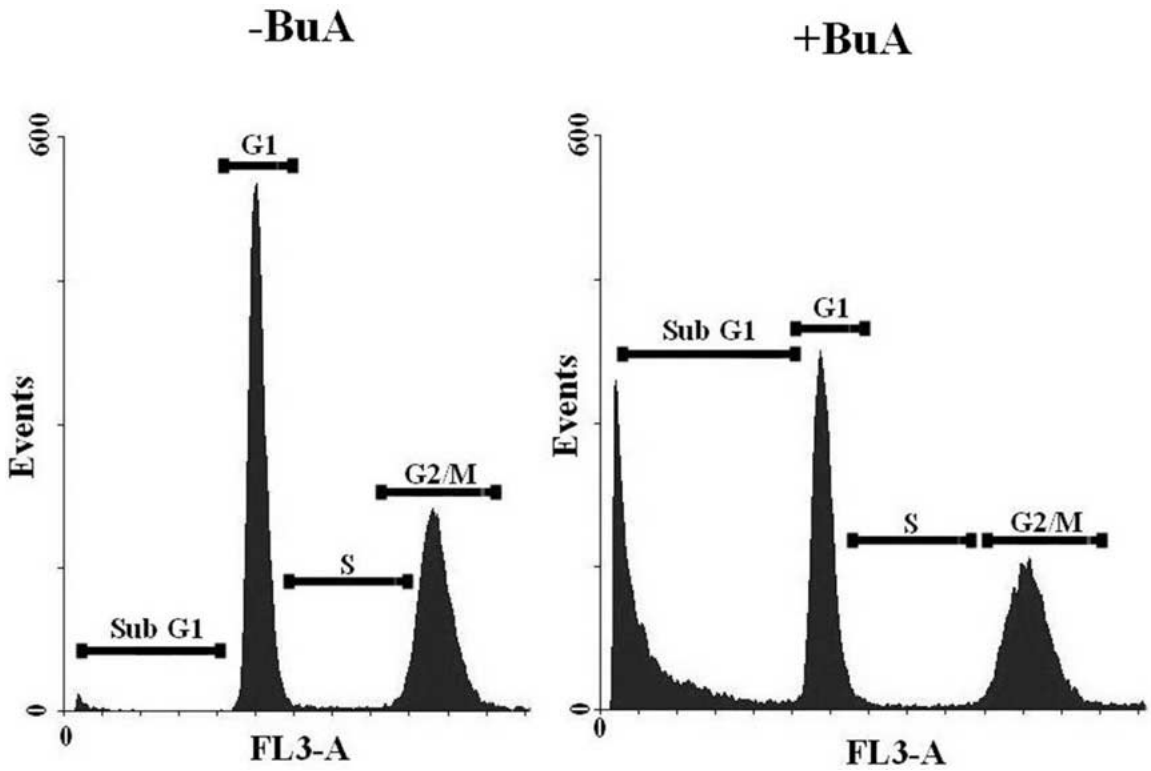

Fig. 5. Analysis of induction of apoptosis in HUVECs by BuA. HUVECs were grown to approximately 80% confluence and treated with or without BuA (10 mM) for 24 h. Cells were trypsinized and processed for FACS analysis.

in sustaining and disrupting neovascularization have been recognized. It is now well established that key regulators of angiogenesis function, at least in part, by modulating the survival of endothelial cells during the process of vessel repair and angiogenesis. Many proangiogenic factors act as endothelial cell survival factors while antiangiogenic factors induce endothelial cell apoptosis [25]. BuA was found to be an antiangiogenic compound [19]. The efficacy of BuA as an inducer of endothelial cell apoptosis was therefore verified. The results of FACS analysis show that the number of events in the sub-G1 phase, which are indicative of apoptotic bodies and condensed nuclei, was increased in BuA treated cells. Apart from being a direct inducer of apoptosis of endothelial cells, BuA may act as a apoptotic agent *in vivo* for endothelial cells by inhibiting the expression of VEGF, which is a survival factor for these cells and this could be one of the mechanisms by which it induces its *in vivo* antiangiogenic effect. Inhibition of angiogenic receptor (KDR) gene expression by BuA indicates that BuA acts at multiple sites to effectively inhibit angiogenesis. Since BuA has a high metabolic turnover rate *in vivo,* structural analogues of BuA with increased stability would be useful as *in vivo* antiangiogenic compounds.

Acknowledgements

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