Low dosage of arsenic trioxide inhibits vasculogenic mimicry in hepatoblastoma without cell apoptosis

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Abstract. Hepatoblastoma (HB) is the most common type of pediatric liver malignancy, which predominantly occurs in young children (aged <5 years), and continues to be a therapeutic challenge in terms of metastasis and drug resistance. As a new pattern of tumor blood supply, vasculogenic mimicry (VM) is a channel structure lined by tumor cells rather than endothelial cells, which contribute to angiogenesis. VM occurs in a variety of solid tumor types, including liver cancer, such as hepatocellular carcinoma. The aim of the present study was to elucidate the effect of arsenic trioxide (As₂O₃) on VM. In vitro experiments identified that HB cell line HepG2 cells form typical VM structures on Matrigel, and the structures were markedly damaged by As₂O₃ at a low concentration before the cell viability significantly decreased. The western blot results indicated that As₂O₃ downregulated the expression level of VM-associated proteins prior to the appearance of apoptotic proteins. In vivo, VM has been observed in xenografts of HB mouse models and identified by periodic acid-Schiff/CD105 channels lined by HepG2 cells without necrotic cells. As₂O₃ (2 mg/kg) markedly depresses tumor growth without causing serious adverse reactions by decreasing the number of VM channels via inhibiting the expression level of VM-associated proteins. Thus, the present data strongly indicate that low dosage As₂O₃ reduces the formation of VM in HB cell line HepG2 cells, independent of cell apoptosis in vivo and in vitro, and may represent as a candidate drug for HB targeting VM.

Introduction

Hepatoblastoma (HB) is a common type of liver malignancy in young children, which is typically diagnosed in those <5 years (1). HB accounts for >90% of malignant liver tumors in childhood (2). The incidence of HB is >1.2 million children every year globally, with high incidences in Japan, China and USA (3). The etiology of HB remains unclear, and low birth weight, preterm birth and maternal gestational age (<20 years and >35 years) may be associated with the morbidity of HB (1,4). HB is hypothesized to originate from primitive hepatic stem cells and it is not associated with hepatic virus infection, cirrhosis or other liver disease, which is different from malignant liver tumors in adults. The primary treatment strategies for HB are surgical resection and chemotherapy. However, the prognosis of HB remains poor, as HB is a tumor of hypervascularity, the majority of patients are asymptomatic and many cases are diagnosed at a late stage. In addition, the treatment of HB continues to be challenging due to drug resistance and the occurrence of metastasis, such as lung, brain and lymph gland metastasis (5,6). Thus, there is an urgent requirement to investigate the pathogenesis of HB and develop novel therapeutic targets for the treatment of patients with HB.

Malignant solid tumors must acquire sufficient blood supply to promote their survival, proliferation and metastasis. There are three dominant tumor microcirculation patterns, including angiogenesis, mosaic vessels and vasculogenic mimicry (VM). VM is first reported in melanoma by Maniotis et al (7) in 1999, which described the specific capacity of tumor cells to form extracellular matrix (ECM)-rich networks. It is distinct from angiogenesis and predominantly occurs in the early stage of tumorigenesis (8). VM is lined by tumor cells, which may provide the tumor with oxygen and nutrients, and has been observed in different types of cancer, and is associated with high tumor grade, invasion, metastasis and short survival (9-18). Li et al (12) reported that patients with VM were at a higher risk for hematogenous metastasis and distant recurrence when compared with patients without VM (12). Antiangiogenic drugs have been demonstrated to be unsatisfactory in the treatment of malignant tumors during clinical trials and animal testing over the past decades (19-21). This may be attributed to the particular structure of the VM, which is lined by tumor cells rather than endothelial cells, meanwhile...
the tumor cells engaged in VM are exposed directly to the blood flow and thus more easily spread throughout the circulation. Therefore, VM may be an important target for novel cancer therapeutic strategies to treat tumors.

Arsenic trioxide (As$_2$O$_3$) has been used to successfully treat acute promyelocytic leukemia (22,23) and other types of cancer cell lines (24-28). As$_2$O$_3$ induces cell apoptosis, and reduces the invasive and metastatic activities in cancer cells (24,25,29), which involve the caspase-3 signaling pathway, reactive oxygen species, DNA damage, oxidative stress and cell cycle arrest. However, As$_2$O$_3$, as a vascular disrupting agent, delayed tumor growth in colon cancer and gastric cancer cells at a dose of >2.5 mg/kg in a mouse model (30,31). However, the effectiveness of As$_2$O$_3$ is based upon a high concentration or long exposure and cell toxicity, which leads to undesirable side effects and limits the application of this drug. A previous study reported that As$_2$O$_3$ exerted its antitumor activity via depletion of regulatory T cells in a murine model of colon cancer at a low concentration without side effects (32). To the best of our knowledge, whether the rapid progression of HB is associated with VM formation and whether a low dosage of As$_2$O$_3$ exerts inhibitory effects on VM formation in HB have not been reported. The aim of the present study was to investigate the impact of a low dosage of As$_2$O$_3$ on VM formation in HB and the relative mechanisms in order to develop novel therapeutic strategies for HB.

Materials and methods

Chemicals. The As$_2$O$_3$ solution was purchased from Heilongjiang Harbin Medical Pharmaceutical Co., Ltd. (Heilongjiang, China). Matrigel was purchased from BD Biosciences (Franklin Lakes, NJ, USA), the CDDO-antibody was purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA), and anti-vascular endothelial (VE)-cadherin, anti-Bcl-2-associated X protein (Bax) and anti-B-cell lymphoma 2 (Bcl-2) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Matrix metalloproteinase (MMP)-2 and MMP-9 antibodies were purchased from Nanjing KeyGen Biotech Co., Ltd. (Nanjing, China), and the animals were obtained from Hunan Slack Jingda Experimental Animals Co., Ltd. (Hunan, China).

Cell culture and animal treatment. The HB-derived cell line HepG2 cells were donated by the Department of General Surgery of the First Affiliated Hospital of Harbin Medical University (Harbin, China). Cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (both from Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin. All cells were maintained at 37°C in a humidified incubator with 5.0% carbon dioxide.

The present study was approved by the Ethics Committee of The First Affiliated Hospital of Harbin Medical University (Heilongjiang, China). A total of 10 healthy male nude mice (age, 4-6 weeks; weight, 18-22 g) were used in the present study. The animals were maintained under specific pathogen-free conditions using a laminar airflow rack. The animals had access to sterilized food and autoclaved water ad libitum, and experiments commenced after 1 week of acclimatization. Mice were injected subcutaneously in the right flank with 1x10$^6$/ml HepG2 cells suspended in 200 µl serum-free DMEM, and were randomly allocated into two groups following the injection of tumor cells. Then, 2 ml As$_2$O$_3$ (2 mg/kg) or an equivalent volume of phosphate-buffered saline (PBS) was administered intraperitoneally every 2 days for a total of 20 days. Tumor size was measured using Vernier calipers every 2 days, and tumor volume (TV) was calculated using the formula, TV (mm$^3$) = (L x W$^2$)/2, where L and W were the longest and shortest tumor dimensions, respectively. Growth curves of TV were drawn for the two groups. The mice were carefully monitored for symptoms of toxicity and were weighed twice a week. The mean mouse body weight of each group was calculated and used as a parameter of toxicity, as previously described (33). The mice were anesthetized before the tumors were removed, and the tumors were weighed and fixed with 10% formaldehyde for the pathological tissue sections.

Methylthiazol tetrazolium (MTT) cell viability assay. MTT assay was conducted to assess the effect of As$_2$O$_3$ on the viability of HepG2 cells. Cells were seeded in 96-well plates at a density of 5,000 cells/well, experiments were performed as follows: Varying concentrations of As$_2$O$_3$ for 24 h and 2 µM As$_2$O$_3$ for varying durations. A culture medium without As$_2$O$_3$ served as the control. Following treatment, the medium was aspirated and the cells were rinsed with PBS. 15 ml MTT solution (5 mg/ml in PBS) was added to each well. After a 4-h incubation, 100 µl dimethyl sulfoxide was added to dissolve the formazan crystal and incubated for another 10 min at room temperature. The absorbance value was measured at 490 nm using an Epoch microplate spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA).

Matrigel tube formation assay. Matrigel tube formation assay was performed to determine the VM capacity of HepG2 cells and the effect of As$_2$O$_3$ on VM channels. Each well of a 96-well plate was coated with 80 µl Matrigel and allowed to solidify at 37°C for 30 min. Cells were seeded into the coated wells and incubated in a humidified incubator at 37°C with 5% CO$_2$ for 24 h. The effect of As$_2$O$_3$ on tube formation was evaluated using varying concentrations of As$_2$O$_3$ for 24 h and 2 µM As$_2$O$_3$ for varying durations. Cells in the control group were cultured in complete medium without As$_2$O$_3$ for 24 h. Finally, cells were imaged using an inverted microscope (Olympus, Tokyo, Japan; magnification, x200) and tube numbers were counted.

Western blot analysis. Following treatment with As$_2$O$_3$, cells were harvested and lysed using RIPA buffer (Beijing Solarbio Science and Technology Co., Ltd., Beijing, China) containing 1% phenylmethylene sulfonyl fluoride protease inhibitors. Equivalent quantities of proteins were separated by SDS-PAGE using a 4.5% stacking gel and an 8% running gel, and the resolved proteins were transferred to polyvinylidene fluoride membranes using the semi-dry transfer system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were blocked with 5% skimmed milk for 2 h and incubated with primary antibodies overnight at 4°C. The primary antibodies included: MMP-2 (cat. no. KG22546, 1:500) and MMP-9 (cat. no. KG22551, 1:500) (both from Nanjing KeyGen Biotech Co., Ltd., China). The membranes were incubated with HRP-conjugated secondary antibodies (1:1,000) for 1 h at room temperature. The bands were visualized by chemiluminescence.
HepG2 cells form typical tubular structures in vitro. HepG2 cells were plated on Matrigel in 96-well plates and 1 h later changes in cell morphology were observed, including shape changes, elongation and the formation of needle-like structures (Fig. 1A). After 12 h, typical tubular networks emerged that represented VM (Fig. 1B).

Immunohistochemical (IHC) and histochemical double-staining. Tissue sections (4 µm) were sliced from the paraffin-embedded tumor samples, and were stained with hematoxylin and eosin according to routine protocols. All slides were dewaxed in xylenes, rinsed in graded ethanol solutions and finally rehydrated in double-distilled water. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol at an ambient temperature for 20 min. Antigen retrieval was performed by heating the slides in 0.01 M sodium citrate buffer for 3 min. After blocking with 3% normal goat serum for 30 min to reduce non-specific binding, the slides were incubated in a humidified chamber overnight at 4˚C with the following primary antibodies: CD105 (cat. no. MS-1290, 1:150; Thermo Fisher Scientific, Inc.), MMP-2 (cat. no. KG22546, 1:150), MMP-9 (cat. no. KG22551, 1:150) (both from Nanjing KeyGen Biotech Co., Ltd.), VE-cadherin (cat. no. sc-9989, 1:50; Santa Cruz Biotechnology, Inc.), and GAPDH (cat. no. KC-5G4, 1:1,000; Shanghai Kangcheng Bioengineering Co., Ltd., Shanghai, China). The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (cat. no. ZB-2301, 1:3,000; OriGene Technologies, Inc., Beijing, China) for 1 h at room temperature and the protein bands on the membranes visualized using an ECL detection system (GE Healthcare Life Sciences, Little Chalfont, UK).

Statistical analysis. Statistical analysis was performed using Graph Pad Prism 5 (GraphPad Inc., San Diego, CA, USA). The differences were analyzed using a non-parametric Mann-Whitney t-test. All experiments were performed in triplicate, data were expressed as means ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

Results

As$_2$O$_3$ significantly inhibits the tube formation of HepG2 cells prior to cell viability decrease in vitro. In Matrigel tube formation assay, the mean value of tube-like structure numbers in 10 fields under an inverted microscope was obtained, and divided by the optical density (OD) value. The results indicated that As$_2$O$_3$ induced depolymerization of tube-like structures in a concentration and time-dependent manner. Significant decreases were observed at 2, 3, 4 and 5 µM, respectively (P<0.05; Fig. 2A and C); however, the number of tubes was only moderately reduced at 1 µM for 24 h (P>0.05; Fig. 2A and C). When the tubes were exposed to 2 µM As$_2$O$_3$ for varying durations, the number of tubes was decreased notably in all groups except the 6 h group when compared with the control (P<0.05; Fig. 2B and D). Furthermore, in the As$_2$O$_3$ groups, the walls of the tubes became thinner, more fragile and irregular, particularly at concentrations of 4 and 5 µM for 24 h. Furthermore, the tubular structures were severely damaged and cells could not form typical structures correctly compared with the remaining groups, whereas in the control group the wall of the tubes became thicker (Fig. 2A). Additionally, the structures diminished gradually with the extension of time at 2 µM As$_2$O$_3$ (Fig. 2B).

Although the viability of cells was reduced by As$_2$O$_3$, the number of channels was divided by the OD value, hence the
inhibition of tube structures was not affected by the reduction of total cell numbers. In addition, only when the cells were exposed to 4 and 5 µM \( \text{As}_2\text{O}_3 \) for 24 h and 2 µM for >36 h, the cell viability was reduced significantly (\( P<0.05 \); Fig. 3), but did not decrease notably with 3 µM for 24 h and 2 µM within 24 h. No significant differences were identified when compared with the control group (\( P>0.05 \); Fig. 3). Therefore, the inhibition of tube channels was earlier and more obvious than the reduction in cell numbers.

\( \text{As}_2\text{O}_3 \) inhibits the VM-associated molecules before apoptosis proteins emerge in vitro. MMP-2 and MMP-9 are two important molecules in ECM remodeling, which are vital in the formation of VM. VE-cadherin is the key molecule in the malignant tumors capable of VM formation and associated with tumor invasion (35,36). The western blot results indicate that the expression levels of MMP-2 and MMP-9 (Fig. 4A), and VE-cadherin (Fig. 4C) decreased significantly and in a concentration-dependent manner when cells were treated with 2, 3, 4 and 5 µM \( \text{As}_2\text{O}_3 \) for 24 h (\( P<0.05 \)). The ratio of Bax/Bcl-2 was upregulated significantly at 4 and 5 µM for 24 h (\( P<0.05 \); Fig. 4C). When cells were exposed to 2 and 3 µM \( \text{As}_2\text{O}_3 \) for 24 h, the Bax/Bcl-2 ratio was increased slightly, but no statistical difference was identified when compared with the control group (\( P>0.05 \)). The MMP-9 (Fig. 4B) and VE-cadherin (Fig. 4D) expression levels decreased significantly and in a time-dependent manner when the cells were exposed to 2 µM \( \text{As}_2\text{O}_3 \) for varying durations compared with the control (\( P<0.05 \)), and MMP-2 expression levels decreased significantly at 24 and 36 h (\( P<0.05 \); Fig. 4B). However, the ratio of Bax/Bcl-2 was not significantly increased within 24 h (\( P>0.05 \)), although a significant difference was identified when the duration was prolonged to 36 h (\( P<0.05 \); Fig. 4D), which indicated that
the inhibition of VM was associated with the decrease of VM-associated proteins rather than cell apoptosis.

Figure 3. Effect of As$_2$O$_3$ on cell viability of HepG2 cells was evaluated by methylthiazol tetrazolium assay. (A) HepG2 cells were treated with the indicated concentration of As$_2$O$_3$ for 24 h. (B) HepG2 cells were incubated with 2 µM As$_2$O$_3$ for varying durations. *P<0.05, **P<0.01 and ***P<0.001 vs. control (cells cultured on Matrigel without As$_2$O$_3$ for 24 h). As$_2$O$_3$, arsenic trioxide; NS, not statistically significant (P>0.05).

Figure 4. Effect of As$_2$O$_3$ on VM-associated proteins by western blot analysis. (A) Different concentrations of As$_2$O$_3$ for 24-h treatment downregulated the expression levels of MMP-2 and MMP-9 in HepG2 cells. (B) As$_2$O$_3$ (2 µM) inhibits the expression level of MMP-2 and MMP-9 in a time-dependent manner. (C and D) As$_2$O$_3$ inhibits the expression level of VE-cadherin in a concentration- and time-dependent manner. The ratio of Bax/Bcl-2 was upregulated by As$_2$O$_3$ at high concentrations and prolonged exposure. GAPDH served as the loading control. *P<0.05, **P<0.01, ***P<0.001 vs. control. As$_2$O$_3$, arsenic trioxide; VM, vasculogenic mimicry; MMP, matrix metalloproteinase; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2; VE, vascular endothelial; NS, not statistically significant (P>0.05).

VM structures exist in HB xenografts from mice models, and low-dose As$_2$O$_3$ inhibits VM channels and VM-associated
molecules in vivo. In vivo experiments demonstrated that VM structures existed in the HepG2 cell tumor xenografts, which were lined by tumor cells without participation of endothelial cells and surrounded by a PAS\(^+\) material (stained pink; Fig. 5A; magnification, x200), which was secreted by tumor cells. PAS\(^+\)/CD105\(^-\) channels were validated by red blood cells and tumor cells in the channel. This was predominantly observed in the marginal zone of the tumor tissues.

In previous studies, high-dose As\(_2\)O\(_3\) significantly inhibited tumor growth. In the present study, low concentration of As\(_2\)O\(_3\) (2 mg/kg) was administrated intraperitoneally to the mice to observe the effect on VM; this dosage is markedly lower than that used in previous studies, which may lead to cell toxicity. An equal quantity of PBS served as the control. The results indicate that the VM structure was observed in all the control specimens (5/5); however, in the As\(_2\)O\(_3\) group, the
cells were depressed and could not form channels correctly, and VM structures were only observed in two tissue sections. The number of VM channels in the As$_2$O$_3$ group (1.0±0.95) was decreased by 72% compared with the control group (3.56±1.81), and a statistically significant difference was observed (P<0.01; Fig. 5B). No obvious necrotic areas were observed in the visual fields under the microscope. Additionally, IHC analysis demonstrated that the staining intensity of VM-associated markers in the control group was brown, which was stronger than that of the As$_2$O$_3$ group, and the percentage of positive cells in the control group was markedly more than that of the As$_2$O$_3$ group (Fig. 5C). The staining index in the As$_2$O$_3$ group was significantly lower than that of the control (P<0.05; Fig. 5D). These results indicate that As$_2$O$_3$ inhibits the VM structures by decreasing VM-associated proteins in vivo at a low concentration.

**Discussion**

HB is the most common type of pediatric liver malignancy, typically affecting children aged <5 years. HB are divided into two histological subtypes based on the level of cell differentiation (37), including epithelial and mixed epithelial mesenchymal. The majority of HB is epithelial, consisting of embryonal and fetal cells, such as HepG2 cells. HB is a tumor characterized by hypervascularity (38) and the incidence of HB has increased in the past 30 years (39). The overall survival of this disease remains poor and, due to its rarity, there is little experience of treating HB. Therefore, there is an urgent requirement to establish an effective treatment strategy for HB.

VM is associated with high tumor grade, invasion, metastasis and short survival, and is a marker of poor clinical prognosis in adult liver cancer, such as hepatocellular carcinoma (34,40). In the present study, the epithelial HB cell line HepG2 cells (41,42) were found to form the typical structure of VM in vivo and in vitro, which may indicate why HB tends to metastasize and relapse.

Formation of VM is a complex process that involves various mechanisms, including EPH receptor A2 (EphA2), cancer stem cells, epithelial-mesenchymal transition, the phosphoinositide 3-kinase signaling pathway, tumor cell plasticity, remodeling of the ECM and microenvironments (such...
as hypoxia). ECM degradation and remodeling are important in the formation of VM channels, a variety of molecular and signaling pathways, including MMPs and VE-cadherin, participate in remodeling of the ECM (35,36,43,44). Among MMPs, MMP-2 and MMP-9 are two important enzymes, which are involved in the remodeling of ECM and are key mediators of invasion, metastasis, tumor angiogenesis and facilitate VM formation (45,46). MMP-2 overexpression may be associated with lymph node metastasis from gastric carcinoma and has been reported to be linked to the recurrence of breast cancer (47,48). VE-cadherin is associated with tumor invasion and is considered to be essential for VM network formation. Plastic tumor cells lacking VE-cadherin are incapable of VM tube formation (36). By contrast, upregulation of VE-cadherin, EphA2, MMP-2 and MMP-9 promotes the formation of VM (49). Therefore, VM-targeting strategies should include these markers to combat the recurrence and metastasis of malignant tumors. Certain experimental evidence indicates that curcumin, thalidomide and gene deletion techniques may successfully inhibit VM and tumor growth. However, the 5-year survival rate of patients with VM remains poor, and there are few effective and feasible methods to treat tumors exhibiting VM.

To the best of our knowledge, this is the first study discussing the effect of As$_2$O$_3$ on VM formation. In the present study, a low concentration of As$_2$O$_3$ was found to inhibit the formation of VM structures in vivo and in vitro, and As$_2$O$_3$-treated HepG2 cells exhibited a significantly lower VM capacity than those of the control group. As$_2$O$_3$ destroys the formation of the tubular structures in vitro at a concentration of <3 µM within 24 h before cell toxicity appeared, in a time- and concentration-dependent manner. During the in vivo experiments, the VM channels were decreased markedly following treatment with 2 mg/kg As$_2$O$_3$ twice a day for a total of 20 days, which was markedly lower than that of clinically tolerable concentrations (50). Additionally, tumor growth was suppressed significantly without obvious adverse reactions.

Furthermore, the expression levels of VE-cadherin, MMP-2 and MMP-9 were decreased significantly with the treatment of low dosage As$_2$O$_3$ in vivo and in vitro. Previous studies indicated that As$_2$O$_3$ upregulates the ratio of Bax/Bcl-2 and induces cell apoptosis (29,51,52); the present in vitro results had the same conclusion, although the ratio of Bax/Bcl-2 increased significantly only when exposed to higher concentrations and longer exposure of As$_2$O$_3$ (in cells exposed to 2 and 3 µM As$_2$O$_3$ for 24 h, the ratio of Bax/Bcl-2 increased only slightly). However, the expression levels of VE-cadherin, MMP-2 and MMP-9 were decreased notably in cells exposed to 2 and 3 µM As$_2$O$_3$ for 24 h, which indicated that As$_2$O$_3$ inhibits tube structures in HepG2 cells via inhibition of VM-associated proteins rather than by inducing cell apoptosis <3 µM within 24 h. IHC results from the in vivo study indicated that the staining intensity of VE-cadherin, MMP-2 and MMP-9 were obviously weaker in the 2 mg/kg As$_2$O$_3$ group when compared with that of control group, and no necrotic areas were observed in the tissues. These results indicate that As$_2$O$_3$ inhibits VE-cadherin, MMP-2 and MMP-9, and thus the formation of VM and tumor growth at low concentrations without cell toxicity.

Complete surgical resection of the tumor is essential for survival of patients with HB (53,54). HB is a chemotherapy-sensitive tumor and preoperative chemotherapy effectively improves the surgical resectability of the tumor and prolongs the survival time of patients, even in cases of metastasis (55). In previous decades, As$_2$O$_3$ treatment has exerted therapeutic effects on adult liver malignant tumors and induced attenuation of invasion potential; however, the application of this drug has been limited due to the dose-dependent heart, liver and kidney toxicity (56), therefore the therapeutic window of As$_2$O$_3$ is particularly narrow. Establishing the appropriate dosage of As$_2$O$_3$ without significant adverse reactions has become a breakthrough in the treatment of malignant tumors. Furthermore, the drug resistance of HB often occurs after four cycles of chemotherapy (57), therefore avoiding this issue presents a therapeutic challenge.

In conclusion, the present results demonstrate that low-dose As$_2$O$_3$ is effective in reducing the formation of VM channels and tumor growth in HB mouse models by inhibiting VM-associated proteins without causing cytotoxicity. Therefore, As$_2$O$_3$ may present as a promising candidate drug to treat HB by targeting VM.

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