Full Paper

Anti-tumor Effect of Polysaccharides From Scutellaria Barbata D. Don on the 95-D Xenograft Model via Inhibition of the C-Met Pathway

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Abstract. Polysaccharides isolated from Scutellaria barbata (PSB) have been reported to have anti-tumor effects. To investigate the underlying mechanism, a highly invasive, metastatic and phospho-c-Met overexpression lung carcinoma cell, 95-D cell line was used. The results showed that in vitro, PSB not only could inhibit the proliferation of 95-D cell line (IC₅₀ = 35.2 μg/mL), but also down-regulated the expression of phospho-c-Met and its downstream signaling molecules including phospho-Erk and phospho-Akt. In vivo, PSB inhibited tumor growth in the 95-D subcutaneous xenograft model in a dose-dependent manner; after once-daily intraperitoneal injection for 3 weeks, tumor growth inhibition T/C ratio for 100 and 200 mg/kg treatments was 42.72% and 13.6%, respectively. In the end of the in vivo study, tumor tissues were harvested for further evaluation of the phosphorylation level of c-Met, AKT, and ERK. Ex vivo results demonstrated that the phosphorylation of c-Met and its downstream signaling molecules were also significantly inhibited by PSB. Immunohistochemistry analysis showed dose-dependent inhibition of tumor cell proliferation (Ki67) and reduction of microvessel density (CD31). In summary, the results indicated that PSB exerted anti-tumor growth activity on human lung cancer 95-D in vitro and in vivo by directly regulating the c-Met signaling pathway and the anti-tumor effects were mainly based on its anti-proliferation and anti-angiogenesis action.

Keywords: polysaccharides Scutellaria barbata (PSB), c-Met, 95-D cell line, lung cancer, receptor tyrosine kinase

Introduction

C-Met is a transmembrane tyrosine kinase receptor expressed in epithelia, mesenchymal, hematopoietic cells, myoblast, and spinal motor neurons. Its main function is mediating morphogenetic embryonic development and tissue repair in vertebrates. The natural ligand for this receptor is the hepatocyte growth factor (HGF), produced by stromal and mesenchymal cells, that acts primarily on c-Met-expressing epithelial cells in an endocrine and/or paracrine fashion (1, 2). Going more into detail, when HGF binds to the c-Met receptor, c-Met major autophosphorylation sites (located within the tyrosine kinase domain) are phosphorylated, with subsequent intrinsic catalytic activation of multiple signaling cascades involved in cell proliferation, survival, angiogenesis, morphogenesis, cell scattering, motility, migration, and invasion (3).

C-Met gene amplification and gene product overexpression have been identified in different types of human tumors, including lung, breast, colorectal, prostate, pancreatic, head and neck, gastric, liver, ovarian, renal, glioma, melanoma, and a number of sarcomas (4, 5). C-Met receptor is overexpressed in both Small Cell Lung Cancer (SCLC) and NSCLC, mainly in the non-squamous histotype (6, 7). The tumor microarray expression analysis demonstrated a 72% c-Met expression in human lung cancer tissues and 40% c-Met
Materials and Methods

Reagents

Scutellaria barbata was purchased from the Hehuachi Market of Traditional Chinese Herbs and identified by Professor Yong Chen of Chengdu Academy of the Chinese Materia Medica (Chengdu, China). A voucher specimen of this herb (S-2011-0901) was kept in the institutional herbarium. PSB was prepared by the method described previously by Song et al. (15) with minor modification. In brief, the crude polysaccharides were extracted by the traditional technique of water extraction and alcohol precipitation. The dried and crushed S. barbata was extracted 4 times (each extraction period lasting 2 h) with deionized water by decoction at 90°C. After filtration, the resulting extract was mixed with 4 volumes of dehydrated ethanol (ethanol final concentration, 80%) and kept overnight at 4°C in a refrigerator. Thereafter, the mixture was centrifuged at 4000 rpm/min for 10 min, washed 4 times with dehydrated ethanol, and the precipitate was collected as crude PSB. Subsequently, DEAE-52 column chromatography was used to purify the crude PSB with NaCl solution (0 – 2 mol/L). PSB was identified as a homogeneous polysaccharide component by SePhadexG-200 gel chromatograph, which was mainly composed of rhamnose, arabinose, xylose, mannose, glucose, and galactose (14).

Scutellaria barbata D. Don, a perennial herb belonging to the family Lamiaceae, is widely distributed throughout China and Korea, which has been traditionally used in folk medicine as anti-tumor and anti-inflammatory agents (8, 9). Scutellaria barbata D. Don herb is known in traditional Chinese medicine as Ban-Zhi-Lian, which is known to contain a large number of polysaccharides, flavones, alkaloids, and neo-clerodane diterpenoids (10, 11). It is reported that Polysaccharides isolated from S. barbata could significantly inhibit the growth of S180 tumor model (12, 13). In our previous study, we also found that PSB could inhibit invasion, metastasis, and proliferation in 95-D cells (14). To further elucidate the mechanism of the anti-tumor activity of PSB, here we investigated its anti-tumor activity in vivo as well as the inhibitory effect on the c-Met pathway.

Cell culture

95-D cells were incubated in 96-cell plates (NUNC, Roskilde, Denmark) at a density of 1 × 10^4 cells/mL. The cells were cultured in RPMI 1640 medium supplemented with 15% FBS, 100 mg/L streptomycin, 100 IU/mL penicillin, and 0.03% L-glutamine (Hyclone, Logan, UT, USA) and maintained at 37°C with 5% CO₂ in a humidified atmosphere.

Cell growth inhibition assay

95-D cells were treated with different concentrations of PSB for 48 h. Cell growth was measured with a plate reader (TECAN SPECTRA, Wetzlar, Germany) by MTT. The percentage of cell growth inhibition was calculated as follows:

\[
\text{Cell growth inhibition} (%) = \frac{A_{570} \text{(Vehicle)} - A_{570} \text{(PSB)}}{A_{570} \text{(Vehicle)}} \times 100.
\]

C-Met kinase activity assay

The kinase reaction buffer was composed of 67 mM of HEPES (pH 7.4), 0.013% Triton X-100, 27 mM MgCl₂, 0.67 mM MnCl₂, and 1.25 mM DTT. The enzymatic reaction contains 0.2 μg/mL of recombinant c-Met catalytic domain, 25 μg/mL of Poly E4Y substrate, 5 μL of tested compound diluted in 5% DMSO, and 10 μM of ATP. All components were diluted in assay buffer. The final concentration of DMSO in the reaction was 4%. ATP was the last component to be added, for initiating the reaction. The reaction mixture was incubated at 25°C for 45 min. Then the ADP detection mixture was added.

570
and incubated at 25°C for an additional 1 h. Fluorescence polarization is measured with the TECAN SPECTRA at excitation of 610 nm and emission of 670 nm. Produced ADP concentration was calculated using a standard curve obtained in the same study based on the recommendation of the manufacturer. Inhibition rate of PSB at each point was calculated according to following equation and IC_{50} is calculated using XL-Fit 2.0 software: Inhibition (%) = (100 − [ADP]_{PSB} / [ADP]_{Vehicle}) × 100, where, [ADP]_{PSB} represents the ADP concentration in the wells treated with PSB and [ADP]_{Vehicle} represents the ADP concentration in the wells treated with 5% of DMSO instead of PSB.

Western blotting analyses

The expressions of p-c-Met, p-AKT, p-ERK, c-Met, AKT, and ERK were analyzed by western blotting in vitro. The 95-D cells were treated with different concentrations of PSB for 24 h. Then, cells were harvested in lysis buffer and homogenized by sonification. Then equal amounts of protein (40 μg) were separated by sodium dodecyl sulfate / polyacrylamidegel electrophoresis (SDS/PAGE) on 8% gels; blotted on polyvinylidene difluoride (PVDF); and probed with p-c-Met, p-AKT, p-ERK, c-Met, AKT, and ERK rabbit monoclonal antibody and subsequently with goat anti-rabbit (HRP), and detected by chemiluminescence. To measure protein loading, antibodies directed against β-actin were used. The results were analyzed by Quantity One software (Version 4.4.0.36; Bio-Rad, Hercules, CA, USA).

In vivo studies

Animals: BALB/c female nude mice, obtained from Experimental Animal Center of the Third Military Medical University (Chongqing, China), were used when they were 7 – 9 weeks old. The health of all animals was monitored daily by gross observation, and the experimental animals were housed in the laminar frame. All animals were allowed to acclimate and recover from any shipping related stress for at least three days prior to experimental manipulation. Autoclaved water and irradiated food (also obtained from Experimental Animal Center of the Third Military Medical University) were provided ad libitum, and the animals were maintained in a 12-h light and dark cycle. Cages, bedding, and water bottles were autoclaved before use and were changed twice weekly. All animal experiments were performed in accordance with protocols approved by the Experimental Animal Center of the Third Military Medical University Animal Care and Use Committee.

95-D subcutaneous xenograft model establishment: 95-D cells were harvested, pelleted by centrifugation at 450 × g for 5 – 10 min, and resuspended in sterile serum-free medium supplemented with 30% – 50% Matrigel. Cells (5 × 10^6 in 100 μL) were subcutaneously implanted into the hind-flank region of each mouse and allowed to grow to the designated size before the administration of PSB.

C-Met signaling transduction studies: Nude mice bearing 95-D tumors (150 – 250 mm³) were given PSB in normal saline by i.p. injection at designated dose levels. At designated times following PSB administration, mice were humanely euthanized, and tumors were resected. Tumors were snap frozen and pulverized using a liquid nitrogen cooled cryomortar and pestle, protein lysates were generated, and protein concentrations were determined using a BSA assay (Pierce, Madison, MI, USA). The level of total and phosphorylated protein was determined using the western blot method.

Efficacy studies: Daily treatment with PSB given in normal saline by i.p. injection was initiated when tumors were 150 to 250 mm³ in volume. Tumor volume was determined by measurement with electronic vernier calipers, and tumor volume was calculated by the formula: TV = Length × Width² / 2. Tumor volume was expressed on indicated days as the median tumor volume ± S.D. indicated for groups of mice. Tumor growth inhibition T/C ratio is calculated by the following equation:

\[
\text{T/C ratio} = \frac{(V_t - V_0)_{drug\ treated}}{(V_t - V_0)_{vehicle\ control}} \times 100\%
\]

Immunohistochemistry: Tumor specimens were fixed in 10% buffered formalin for 24 h before being transferred to 70% ethanol. Tumor samples were subsequently paraffin-embedded, and 4 mm sections were cut and baked onto microscope slides. Slides were incubated with the primary antibodies, followed by secondary antibodies, and then visualized using a colorimetric method (DAB kit; DAO Envision-HARP, Carpentaria, CA, USA). All of the immunostained sections were counterstained using hematoxylin. An automated Ventana Discovery XT Staining Module (Ventana Medical Systems, Inc., Tucson, AZ, USA) was used to conduct histologic staining. Stained sections were analyzed using an Olympus microscope, and quantitative analysis of section staining was done using the ACIS system (Automated Cellular Imaging; Clarient, Irvine, CA, USA). The number of Ki-67–positive nuclei was counted regardless of the immunointensity in 4 random fields at × 100 magnification (60% center field). For quantification of vascular area in lung tumors, up to 4 random fields for each tumor section at × 100 magnification (60% center field) were captured after staining with anti-CD31 antibody. Vascular area was calculated using Image Pro software (Media Cybernetics, Inc., Rockville, MD, USA).

Statistical analyses of the data

All data was expressed as the mean ± S.D. and statistical
analysis of all results was performed by Student’s t-test for a mean comparison. The test material can be demonstrated as an effective compound if T/C ratio ≤ 42% and a value of $P < 0.05$ was taken as being statistically significance in the tumor volume calculation.

Results

Proliferation inhibitory effect of PSB on 95-D cells

PSB induced 95-D cell death was shown in a concentration-dependent manner. PSB from 10 to 80 $\mu$g/mL exerted a potent inhibitory effect on 95-D cell growth. By 48 h after treatment with 40 $\mu$g/mL PSB, cell death rate reached to almost 70%. The IC$_{50}$ for 48-h PSB treatment was 35.2 $\mu$g/mL. Therefore, 24-h incubation with PSB seemed to be sufficient for half induction of cell death (Table 1).

Inhibitory effect of PSB on c-Met kinase activity

Inhibition of PSB on c-Met kinase activity was measured by the Transcreener$^{\text{TM}}$ KINASE Assay that is developed to detect ADP generated in the enzyme reaction. PSB were tested in a 10-point series dilution. The result demonstrated that PSB is a potent inhibitor of human c-Met, which inhibited c-Met kinase activity in a dose-dependent manner, with an IC$_{50}$ was 13.261 $\mu$g/mL (Table 2).

Effect of PSB on expressions of p-c-Met, p-AKT, and p-ERK in 95-D cells

To ascertain whether the expression of p-c-Met in 95-D cells would be affected by PSB, the cells were treated with 20, 40, and 60 $\mu$g/mL PSB for 24 h. The results indicated that the expressions of p-c-Met of the 95-D cells treated with PSB for 24 h were all down-regulated in a concentration-dependent manner compared with the vehicle group. Middle-dose of PSB (40 $\mu$g/mL) could completely inhibited p-c-Met expressions. Meanwhile, the expressions of downstream molecules p-AKT

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration ($\mu$g/mL)</th>
<th>OD$_{570}$</th>
<th>Inhibition (%)</th>
<th>SD (%)</th>
<th>IC$_{50}$ ($\mu$g/mL)</th>
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<tr>
<td>PSB</td>
<td>80</td>
<td>0.303</td>
<td>93.8**</td>
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<tr>
<td></td>
<td>60</td>
<td>0.305</td>
<td>93.5**</td>
<td>0.5</td>
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<tr>
<td></td>
<td>40</td>
<td>0.489</td>
<td>67.9**</td>
<td>0.8</td>
<td>35.2</td>
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<td></td>
<td>20</td>
<td>0.891</td>
<td>12.2</td>
<td>0.5</td>
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<td></td>
<td>10</td>
<td>0.901</td>
<td>10.1</td>
<td>0.3</td>
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<tr>
<td>Vehicle (0.5% DMSO solution)</td>
<td></td>
<td>0.979</td>
<td>**$P &lt; 0.01$</td>
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<td></td>
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<tr>
<td>Background</td>
<td></td>
<td>0.258</td>
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Data are reported as the mean ± S.D. (n = 5), **$P < 0.01$.

<table>
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<tr>
<th>PSB ($\mu$g/mL)</th>
<th>Mean of [ADP] ($\mu$M)</th>
<th>Inhibition of [ADP] (%)</th>
<th>SD (%)</th>
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<td>0.015</td>
<td>1.44</td>
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Fig. 1. PSB inhibited the expressions of p-c-Met, p-AKT, and p-ERK in 95-D cells. PBS at 20, 40, and 60 $\mu$g/mL could significantly inhibit the expression of p-c-Met, p-AKT, and p-ERK in 95-D cells.
and p-ERK in the 95-D cells were also significantly down-regulated in a concentration-dependent manner compared to the vehicle group (Fig. 1).

**PSB inhibited tumor growth in 95-D subcutaneous xenograft model**

In order to examine the anti-tumor efficacy of PSB in an in vivo model, we used a p-c-Met overexpression 95-D s.c. xenograft model. Daily i.p. injection of PSB could inhibit 95-D s.c. xenograft tumor growth in a dose-dependent manner up to 21 days. T/C ratio for 100 and 200 mg/kg PSB treatment was 41.2% ($P < 0.05$) and 13.6% ($P < 0.01$), respectively (Fig. 2: A and B). During the duration of the efficacy study, there was no body weight loss in any group (Fig. 2C).

**PSB could significantly inhibit the expressions of Ki-67 and CD31 in 95-D xenograft model**

PSB was also evaluated for its effect on tumor mitotic index (Ki67) using immunohistochemical methods. A significant 2 – 3 fold decrease in Ki67 levels was observed at 2 h after administration of 100 and 200 mg/kg PSB in the 95-D tumor tissues (Fig. 3: B, C), which is correlated with the maximum anti-tumor efficacy.

In order to investigate the anti-angiogenic activity of PSB, we performed IHC studies using the endothelial cell marker CD31. The results demonstrated that a significant dose-dependent reduction of CD31–positive endothelial cells was observed at 2 h after administration of 100 and 200 mg/kg PSB in 95-D tumor tissues (Fig. 4: B, C). These data indicate that PSB is able to inhibit tumor growth by inhibiting c-Met pathway–mediated tumor growth and angiogenesis.

**Effect of PSB on signal transduction pathways in 95-D xenograft model**

To investigate the effect of PSB on critical c-Met–dependent signaling transduction events, Western blot analysis of phosphorylation of molecules that regulate c-Met signaling transduction was done using tumor tissues. In these studies, marked inhibition of phosphorylated c-Met, AKT, and ERK levels was observed in 95-D...
tumors following i.p. injection of PSB (Fig. 5: A, B, and C). Inhibition of these signaling events was dose-dependent and was observed at pharmacologically relevant PSB dose levels, which correlated with the inhibition of c-Met phosphorylation, anti-tumor efficacy, and other mechanistic end points in vivo.

Discussion

According to the report, lung carcinoma is one of the leading causes of cancer deaths in both men and women worldwide (16). In the present study, PSB could significantly inhibit the growth of 95-D, a highly invasive, metastatic, and p-c-Met-overexpressing non-small cell lung carcinoma cell line, in vitro and in vivo. Lung cancers are classified as small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC constitutes approximately 75% of lung cancer cases. NSCLC is further classified into adenocarcinoma (40%), including bronchoalveolar carcinoma, squamous cell carcinoma (30%–35%), and large cell carcinoma (5%–15%). Current therapy for NSCLC is surgery and adjuvant chemotherapy for early-stage disease and palliative chemotherapy (and/or radiation therapy) for

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**Fig. 3.** PSB could significantly inhibit the expressions of Ki-67 in the 95-D xenograft model. Fresh tumors in each group were collected after treatment with PSB for 2 h. A: vehicle control. B: mice were dosed with 100 mg/kg PSB for 2 h. C: mice were dosed with 200 mg/kg PSB for 2 h. Representative Ki-67 staining of lung tumors (brown) viewed at ×100 magnification, 60% center field. Tumor proliferation (% of Ki-67-positive cells per total tumor cells) is shown. **P < 0.01 vs. vehicle group.

**Fig. 4.** PSB could significantly inhibit the expressions of CD31 in the 95-D xenograft model. Fresh tumors in each group were collected after treatment with PSB for 2 h. A: vehicle control, B: mice were dosed with 100 mg/kg PSB for 2 h, C: mice were dosed with 200 mg/kg PSB for 2 h. Representative CD31 staining of lung tumors (brown) viewed at ×100 magnification, 60% center field, and vascular area (area of CD31-positive objects per field area × 100%) are shown. **P < 0.01 vs. vehicle group.
late-stage disease (17).

The epidermal growth factor–receptor pathway has been shown to play a vital role in the pathogenesis of NSCLC, leading to the development of targeted therapeutic agents using small-molecule inhibitors such as gefitinib and erlotinib (18, 19). However, the response rate of the population responding to epidermal growth factor–receptor inhibition carrying a tyrosine kinase mutation is only 15% (20). At present, microarray expression demonstrated that there is about 72% c-Met expression in human lung cancer tissue and 40% c-Met receptor overexpression; such values are higher than in breast (16%) and ovarian cancer (31%), but lower than in renal (70%) and colorectal cancers (78%) (21). However, p-c-Met expression is found to be at the highest levels in lung cancer (73%), followed by ovarian (33%), breast (23%), and renal (18%) cancer (22). So, c-Met would be the other promising target for treatment of lung cancer. In this study, the expression of p-c-Met in 95-D cells is high, which could be down-regulated by 60 μg/mL PSB completely. In addition, c-Met kinase activity assay showed that PSB could bind to the c-Met kinase catalytic domain, which leads to a direct inhibition of the phosphorylation of c-Met. Ex vivo study demonstrated that PSB also could inhibit the expression of p-c-Met in 95-D tumor tissue isolated from nude mice, which was consistent with the in vitro study.

We have recently shown that phosphatidylinositol 3-kinase (PI3K) is an important signaling pathway downstream of c-Met (23). PI3K is responsible for diverse cellular regulation, including cell adhesion, motility and migration, proliferation, reduced apoptosis, anchorage independence, and intracellular vesicle trafficking/secertion (24, 25). AKT is the main downstream target of PI3K (26), and phosphorylation of AKT leads to enhancement of cell survival. Constitutive activation of PI3K signaling pathway has been reported in SCLC, mediating anchorage-independent proliferation via the PI3K/AKT-dependent pathway (27). Upon activation, c-Met can recruit and associate with PI3K, which eventually leads to the downstream pathway. Activation of AKT in turn regulates cell survival by inhibiting apoptosis (28). Here, we show that the PSB treatment of the 95-D xenograft model led to decreased phosphorylation of AKT at Ser473, thereby leading to tumor growth inhibition. It has been shown that c-Met/hepatocyte growth factor activation protects against cell death via the PI3K- and AKT-dependent pathway in human

Fig. 5. Inhibitory effect of PSB on the phosphorylated c-Met, AKT, and ERK levels in the 95-D s.c. tumor model. The 95-D s.c. xenograft model mice were treated with PBS at the indicated concentration for 2 h, and the tumor tissues in each group were resected. The expression of p-c-Met (A), p-AKT (B), and p-ERK (C) were all down-regulated by PSB, and the inhibitory effects were dose-independent. Quantitation of PSB-induced changes in levels of p-c-Met, p-AKT, and p-ERK in the 95-D xenograft model. *P < 0.05, **P < 0.01 vs. vehicle group, n = 3.
glioblastoma cells treated with cytotoxic agents (29). It would therefore be useful to determine whether there is any cooperation or synergism between PSB and PI3K inhibition. The other downstream signal phospho-ERK was also down-regulated by PSB. Therefore, the mechanism of PSB inhibited 95-D tumor growth may occur via regulation of the PI3K/AKT and ERK pathway.

Traditional Chinese medicines (TCM) have been used for more than a millennium in China to prevent and alleviate a wide variety of diseases. Some agents for treatment of cancer have been derived from TCM (30, 31). A large number of studies showed that the proper use of TCM-based therapies can enhance immune function, speed up recovery, alleviate radiochemotherapy-related toxicities, improve quality of life, and extend survival (32 – 34). However, most of TCM-based treatment only would be categorized as palliative therapy in the clinic. The exact anti-tumor growth mechanism of TCM is unclear, which hinders the usage of TCM in clinical cancer treatment. In our study, we tried to explain the mechanism of anti-tumor growth of PSB in lung cancer, which may help TCM to made a breakthrough in the radical treatment of malignancies. Nevertheless, could PSB exhibit anti-tumor effects by other pathways? It is possible that the combination of PSB with an EGFR inhibitor for the treatment of lung cancer are still unclear.

In conclusion, we demonstrated that PSB inhibited 95-D tumor growth in vitro and in vivo. PSB not only down-regulated the phosphorylation of c-Met level in 95-D but also blocked the downstream pathway, including p-AKT and p-ERK. Ki-67 and CD31 analysis in tumor sections showed that the anti-tumor efficacy of PBS was mainly related to anti-proliferation and anti-angiogenesis.

Acknowledgments

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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