Anti-HIV activity of olive leaf extract (OLE) and modulation of host cell gene expression by HIV-1 infection and OLE treatment

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Abstract

We investigated the antiviral activity of olive leaf extract (OLE) preparations standardized by liquid chromatography-coupled mass spectrometry (LC-MS) against HIV-1 infection and replication. We find that OLE inhibits acute infection and cell-to-cell transmission of HIV-1 as assayed by syncytia formation using uninfected MT2 cells co-cultured with HIV-1-infected H9 T lymphocytes. OLE also inhibits HIV-1 replication as assayed by p24 expression in infected H9 cells. These anti-HIV effects of OLE are dose dependent, with EC50s of around 0.2 μg/ml. In the effective dose range, no cytotoxicity on uninfected target cells was detected. The therapeutic index of OLE is above 5000. To identify viral and host targets for OLE, we characterized gene expression profiles associated with HIV-1 infection and OLE treatment using cDNA microarrays. HIV-1 infection modulates the expression patterns of cellular genes involved in apoptosis, stress, cytokine, protein kinase C, and hedgehog signaling. HIV-1 infection up-regulates the expression of the heat-shock proteins hsp27 and hsp90, the DNA damage inducible transcript 1 gadd45, the p53-binding protein mdm2, and the hedgehog signal protein patched 1, while it down-regulates the expression of the anti-apoptotic BCL2-associated X protein Bax. Treatment with OLE reverses many of these HIV-1 infection-associated changes. Treatment of HIV-1-infected cells with OLE also up-regulates the expression of the apoptosis inhibitor proteins IAP1 and 2, as well as the calcium and protein kinase C pathway signaling molecules IL-2, IL-2Rα, and ornithine decarboxylase ODC1.

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Keywords: Anti-HIV agent; Olive leaf extract; Antiviral; cDNA microarray; Gene expression profile; LC-MS
We prepared and standardized OLE using LC-MS and tested these OLE preparations for activity against HIV-1 infection and replication. To define molecular events in viral pathogenesis and to identify cellular targets for OLE, we conducted cDNA microarray studies to profile gene expression patterns associated with HIV-1 infection and OLE treatment. Our results demonstrate that HIV infection modulates the expression of host genes and that treatment with OLE can reverse these changes. These results define the anti-HIV activity of OLE and provide insight into its molecular mechanisms of action. They also shed light on the cellular and viral events involved in HIV-1 pathogenesis and potential new targets for the treatment of HIV-1 infection.

Materials and methods

Preparation of olive leaf extract. Dried olive leaves (Olea europea) were rinsed thoroughly with sterile distilled water to remove dust, insecticides, and contaminating material. The leaves were ground into small pieces and extracted twice with sterile distilled water for 12 h at 80 °C, at a ratio of 40 ml water to 1 g leaf. Insoluble material was removed by centrifugation at 20,000g for 30 min. The clear supernatant was concentrated by lyophilization, reconstituted with water to 0.1 g starting material per ml, sterilized by Millipore filtration with a 0.45 micron filter, and stored at −20 °C until use.

Identification of major anti-HIV components in OLE. OLE prepared as above was subjected to HPLC using a Waters two solvent delivery system with photodiode array detector. A Symmetry C18 column (5 μm, 3.9 × 250 mm) with a Sentry Guard 3.9 μm (20 mm) insert was used. The mobile phase was 79% distilled water and 21% acetonitrile (HPLC grade), both acidified to pH 3 with 0.1 M orthophosphoric acid. This solvent system is designed for resolution and quantitation of polyphenolic compounds. The flow rate was 1 ml/min and the injection volume was 20 μl. Polyphenolic compounds were monitored by measuring the absorbance at 280 nm.

Identification of compounds using thin-layer chromatography. Thin-layer chromatography (TLC) was carried out on silica gel 60 F254 with chloroform/methanol/acetic acid (70/30/10). Scoriocidins and flavonoids were detected by visualization under UV light at 254 nm with 10% ferric chloride and 10% aminooxydine diphenylborate spray, using known standards. HPLC with known compounds was also performed to confirm chemical identity.

OLE was standardized by oleuropein content because it is the major known bioactive component of OLE. Both TLC and HPLC with known standards were used to measure oleuropein content. The oleuropein content of our OLE preparation was standardized to 12% by weight. Standardized OLE was used in all of our experiments.

Analysis of OLE by LC-MS. HPLC purified OLE was further subjected to LC-MS analysis. Pure oleuropein from Extrasynthese (Genay, France) was used as the standard. The standard and purified oleuropein were prepared in sterile water to a final concentration of 1 mg/ml. For comparison, crude OLE prepared as above at 100 mg/ml was used. All samples were Millipore filtered and stored at −20 °C until use.

LC-MS was performed using an HP1100 equipped with diodearray detector and ESI-mass spectrometer. LC was done using a C18 column (4 × 20 mm), with 4-min elution using a gradient of 5–95% CH3CN (containing 1% acetic acid)-H2O (containing 1% acetic acid). The diodearray recordings were made at 280 and 230 nm, and the ESI mass spectrum was made in negative detection mode. The standard and purified oleuropein were diluted to a final concentration of 0.5 mg/ml and OLE was diluted to 5 mg/ml. The injection volumes were all 5 μl.

Experiments were optimized by infusion of the standards in negative scan mode to investigate the [M-H] ion of oleuropein (m/z 539).

Cell lines and viruses. MT2 cell line was used as indicator cell for the microtiter syncytia formation assay. The H9 cell line was used for p24 expression assay. The cell lines were cultured in RPMI medium 1640 containing 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine, and 10% heat-inactivated fetal calf serum. HIV-1IIIB virus was prepared and stocked as described previously [20–22]. The cell lines and the virus stock were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. MT2 from D. Richman [23, 24], H9 and HIV-1IIIB from R. Gallo [25, 26].

Syncytium formation assay of acute HIV infectivity. The effect of OLE on acute HIV infectivity was measured by the syncytia formation assay [20, 21]. H9 cells infected with HIV-1IIIB were plated into microtiter wells at 50,000 cells in 50 μl/well. Cells were treated with 50 μl OLE at various concentrations for 2 h. One hundred microliters of MT2 target cells at 10° cells/ml was then added to each well. After co-culture for 24 h, focal syncytium formation was scored under an inverted microscope.

p24 assay of HIV replication. The effect of OLE on HIV-1 replication in vitro was tested by viral core protein p24 expression using commercial ELISA kit (Coulter, Hialeah, FL) as described previously [21, 22]. Briefly, H9 cells were inoculated with a titered stock of HIV-1IIIB virus at a multiplicity of infection of 0.005. Cells were incubated at 5 × 10^5/ml at 37 °C for 60 min to allow viral absorption. The cells were then washed to remove unbound virus and plated at 1 × 10^5/ml with or without the addition of OLE for the duration of the experiment. In this assay, at the multiplicity of infection used, viral production peaks at day 4. Thus, p24 expression was assayed in cell-free supernatants harvested at day 4.

Determination of OLE cytotoxicity. Cytotoxicity of OLE was evaluated by the MTT assay. Uninfected H9 and MT2 cells were grown in the absence or presence of various concentrations of OLE for 1, 2, and 4 days. The cells were then exposed for 3 h to MTT tetrazolium salt (CellTiter®, Promega, Madison, WI) with phenazine methosulfate as described by the manufacturer. The viability of drug-exposed cells is reflected in the activity of mitochondrial hydrogenases of the cells converting MTT into color-dense formazan. Optical density was determined in a plate reader set to record the absorbance at 490 nm and compared to absorbance values of the control cells cultured without drugs.

cDNA microarray analysis of HIV-1 infection and OLE treatment. Profiling the effects of HIV-1 infection and OLE treatment on target cell gene expression was carried out by cDNA microarray analysis. Cellular total RNAs were prepared and characterized as previously described [27]. Pathway specific arrays were obtained from Super Array (Bethesda, MD). Five microarrays of total RNA was used as template for 32P cDNA probe synthesis using a mixture of pathway specific primers. The cDNA probe was hybridized to the cDNA array, washed at a stringency of 0.1× SSC, 0.5% SDS at 60 °C, and exposed to film. Each array is composed of 96 marker genes in tetra-spot format. Four housekeeping genes are included as positive controls: GAPDH (glyceraldehyde-3-phosphate dehydrogenase), PPIA (peptidylprolyl isomerase A), RPL13A (ribosomal protein L13a), and ACTB (β-actin). The pUC 18 plasmid is included as a negative control.

Results

OLE inhibits HIV-1 acute infection

Acute infection and cell-to-cell transmission of HIV-1 were assayed by the syncytia formation assay. This assay is based on the interaction between fusigenic virus-infected cells expressing the HIV envelope gene products gp120 and gp41, and uninfected nearby target cells
OLE is not toxic to uninfected cells

Cytotoxicity of OLE was evaluated using the MTT assay. This assay is based on the activity of mitochondrial hydrogenases of viable cells converting MTT into color-dense formazan that can be measured using a microtiter plate reader. Target cells were grown in 96-well plates in the absence or presence of various concentrations of OLE for 1, 2, and 4 days. The cells were then exposed for 3 h to MTT tetrazolium salt with phenazine methosulfate. Cell viability was determined by measuring the absorbance at 490 nm. As shown in Fig. 2B, OLE exhibits no detectable reduction in the MTT activity in uninfected H9 and MT2 target cells over a 10,000-fold concentration from 0.1 to 1000 μg/ml. The therapeutic index of OLE is above 5000.

HIV-1 infection and OLE treatment modulate host cell gene expression profile

To probe cellular targets associated with HIV-1 infection and OLE treatment, we carried out pathway-specific signal transduction cDNA microarray analysis. The Pathway Finder gene expression arrays are designed to monitor the modulation of 18 signal transduction pathways with 96 marker genes. RNA samples were prepared from uninfected MT2 cells, HIV-1-infected MT2 cells, and OLE-treated HIV-1-infected MT2 cells.

Our results indicate that cellular genes involved in survival, stress, TGFβ, p53, apoptosis, protein kinase C, and hedgehog signaling pathways are modulated in HIV-infection and OLE treatment. These include the inhibitor of apoptosis proteins BIRC2 (BIRC3) in the survival pathway; heat shock proteins hsp27 (HSPB1) and hsp90 (HSPCA) in the stress pathway; cyclin-dependent protein kinase inhibitor 1A, p21/waf1/Cip1 (CDKNIA) in the TGFβ pathway; the BCL2-associated X protein Bax, DNA-damage-inducible transcript gadd45, and p53-binding protein mdm2 in the p53, and apoptosis pathways; IL-2, IL2RA, and ornithine decarboxylase (ODC1) in the calcium and protein kinase C pathways, and the patched protein (PTCH1) in the hedgehog pathway. HIV-1 infection up-regulates the expression of hsp27, hsp90, gadd45, mdm2, and patched 1 genes while it down-regulates Bax.

Treatment with OLE reverses these HIV-1 infection-associated changes. Treatment of HIV-1-infected cells with OLE also up-regulates the expression of BIRC2, BIRC3, CDKNIA, IL-2, IL-2Rα, and ODC1 genes. HIV infection and OLE treatment cause no significant changes in the expression of the housekeeping genes GAPDH, PPIA, RPL13A, and β-actin. The bacterial plasmid pUC18 was not expressed in either control, HIV-infected or OLE-treated target cells. These results are summarized in Table 2 and Fig. 3.
To identify OLE components responsible for anti-HIV activity, we analyzed OLE by HPLC. Fig. 4 shows the elution profile, which contains seven major peaks. The solvent front and excluded material appear before peak 1, with a retention time less than 6 min. The major components were identified by TLC and HPLC with the elution profile.

**HPLC analysis and identification of OLE components**

To identify OLE components responsible for anti-HIV activity, we analyzed OLE by HPLC. Fig. 4 shows the elution profile, which contains seven major peaks. The solvent front and excluded material appear before peak 1, with a retention time less than 6 min. The major components were identified by TLC and HPLC with...
Table 2
Expression profile of signal transduction-related genes in MT2 cells and its modulation by HIV-1 infection and OLE treatment

<table>
<thead>
<tr>
<th>UniGene</th>
<th>GenBank</th>
<th>Gene symbol/name</th>
<th>Array location</th>
<th>MT2</th>
<th>MT2 + H9-HIV-1</th>
<th>MT2 + H9-HIV-1 + OLE</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS.159428</td>
<td>L22474</td>
<td>BAX/Bax</td>
<td>1-C</td>
<td>M</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HS.289107</td>
<td>U45879</td>
<td>BIRC2/IAP2</td>
<td>1-H</td>
<td>VL</td>
<td>VL</td>
<td></td>
</tr>
<tr>
<td>HS.127799</td>
<td>U37546</td>
<td>BIRC3/IAP1</td>
<td>2-A</td>
<td>M</td>
<td>M</td>
<td></td>
</tr>
<tr>
<td>HS.179665</td>
<td>L47233</td>
<td>CDKN1A/P21, Cip1</td>
<td>2-H</td>
<td>VL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HS.80409</td>
<td>M60974</td>
<td>GADD45/gadd45</td>
<td>5-D</td>
<td>VL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HS.76067</td>
<td>Z23090</td>
<td>HSPB1/hsp27</td>
<td>6-D</td>
<td>L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HS.289088</td>
<td>X15183</td>
<td>HSPCA/hsp90</td>
<td>6-E</td>
<td>L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HS.89679</td>
<td>U25676</td>
<td>IL-2</td>
<td>6-H</td>
<td>L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HS.1742</td>
<td>X01057</td>
<td>IL2RA/IL-2 Ra</td>
<td>7-A</td>
<td>H</td>
<td>H</td>
<td></td>
</tr>
<tr>
<td>HS.170027</td>
<td>Z12020</td>
<td>MDM2/mdm2</td>
<td>8-C</td>
<td>VL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HS.75212</td>
<td>M16650</td>
<td>ODC1/Odc</td>
<td>9-C</td>
<td>VL</td>
<td>VL</td>
<td></td>
</tr>
<tr>
<td>HS.159526</td>
<td>U43148</td>
<td>PTCH1/Ptc</td>
<td>10-B</td>
<td>VL</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

VL, very low expression; L, low expression, M, moderate expression, H, high expression, VH, very high expression, ↑, up-regulation, and ↓, down-regulation.

Fig. 2. Effects of OLE on p24 expression and cytotoxicity. (A) Effects of OLE on HIV core protein p24 expression in HIV-infected H9 cells. The production of p24 was assayed by ELISA and expressed in nanograms per milliliters. The value in control culture without the addition of OLE was 2186 ng/ml. The EC50 (concentration at 50% inhibition) of OLE in this assay is 0.23 μg/ml. (B) Cytotoxicity assayed by the MTT assay. Over a 10,000-fold concentration from 0.1 to 1000 μg/ml, OLE shows no inhibition on the proliferation of uninfected H9 and MT2 cells. Comparable results were obtained for both cell lines.

Fig. 4. HPLC chromatogram of OLE. The elution profile of OLE was recorded at 280 nm. Seven peaks are identified under the HPLC conditions used. The identity and the anti-HIV activity of the peaks are summarized in Table 3. Peak 6 corresponds to oleuropein and contains the bulk of the anti-HIV activity.
known standards. The identities of the polyphenolic compounds in each peak are shown in Table 3. Fractions from each peak were pooled, concentrated, and quantitated.

We assayed the anti-HIV activity of the material in these fractions by the p24 anti-HIV and MTT cytotoxicity assays. As summarized in Table 3, the bulk of the anti-HIV activity was found in peak 6, corresponding to the polyphenolic glucoside oleuropein, with EC_{50} in the range of 40ng/ml. Considerable activity with similar EC_{50} was also found in peak 7, corresponding to oleuroside. No significant anti-HIV activity was detected in peaks 1–5.

**Discussion**

OLE has been used for medicinal purposes for centuries [12,28]. Compounds found in OLE have direct microbicidal activity against bacteria, mycobacteria, and fungi [3–7,29]. OLE also affects macrophage function and modulates the inflammatory response, two effects that may also contribute to activity against infectious agents [14,15]. OLE has also been noted to lower blood pressure and inhibit lipid oxidation [8,10,30–33]. OLE is known to contain a mixture of polyphenolic compounds, among them oleuropein and hydroxytyrosol, both of which are readily absorbed and bioavailable. The biological activities of OLE are mainly derived from these compounds [12,17].

It has been estimated that up to half of patients with HIV currently use some form of complementary or alternative medicine, in addition to conventional medicine [34]. HIV patients use OLE for a variety of reasons, including strengthening the immune system, relieving chronic fatigue, boosting the effects of anti-HIV medications, and treating HIV-associated Kaposi’s sarcoma and HSV infections. However, a search of MEDLINE reveals no publications in the medical literature about the anti-HIV effects of OLE or of the effects of OLE on anti-HIV medications currently in use. There has been one anecdotal report that OLE augments the activity of the HIV-RT inhibitor 3TC [19]. However, the anti-HIV effects of OLE have not been systematically studied or defined, nor have its viral and cellular targets been identified at the molecular level.

<table>
<thead>
<tr>
<th>Peak</th>
<th>Rt (min)</th>
<th>Compound</th>
<th>% (w/w)</th>
<th>Anti-HIV</th>
<th>Cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Excl.</td>
<td>&lt;6</td>
<td>Solvent front</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>1</td>
<td>6.6</td>
<td>Rutin</td>
<td>0.34</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>7.8</td>
<td>Verbascoside</td>
<td>0.38</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>3</td>
<td>8.9</td>
<td>Luteolin7-glucoside</td>
<td>0.68</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>4</td>
<td>16.0</td>
<td>Apigenin7-glucoside</td>
<td>0.18</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>5</td>
<td>17.0</td>
<td>Flavonoid x</td>
<td>0.56</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>6</td>
<td>22.8</td>
<td>Oleuropein</td>
<td>12.8</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>7</td>
<td>34.0</td>
<td>Oleuroside</td>
<td>0.51</td>
<td>+</td>
<td>—</td>
</tr>
</tbody>
</table>

Compounds were identified by TLC and HPLC with known standards. Peak number corresponds to HPLC peak position in Fig. 4. Rt (min) represents HPLC retention time. Anti-HIV activity and cytotoxicity were measured by HIV-1 p24 expression and the MTT assays.
The major objective of our study was to define and characterize the anti-HIV activity of olive leaf extract (OLE) using standardized OLE formulations with well-established bioassays. Our results demonstrate that OLE inhibits acute HIV-1 infection and cell-to-cell transmission, as assayed by syncytia formation. OLE
also inhibits HIV-1 replication, as assayed by p24 expression in HIV-1-infected cells. These effects of OLE are dose dependent and occur at an EC$_{50}$ of 0.2 µg/ml. No cytotoxicity on uninfected target cells was detected over a 10,000-fold concentration from 0.1 to 1000 µg/ml. Thus, OLE by itself has direct anti-HIV activity in these assays. The therapeutic index of our OLE preparations is well above 5000.

In addition, our microarray results indicate that OLE may modulate the host response to infection. Our results show that HIV-1 infection modulates the expression profile of cellular genes involved in survival, stress, TGFβ, p53, apoptosis, calcium/protein kinase C, and hedgehog signaling pathways. HIV-1 infection up-regulates the expression of the heat-shock proteins, hsp27 and hsp90, the DNA damage inducible transcript 1 gadd45, the p53-binding protein mdm2, and the hedgehog signal protein patched 1 (Ptc1), while it down-regulates the expression of the anti-apoptotic BCL2-associated X protein, Bax. Treatment with OLE reverses many of these HIV-1 infection-associated changes. Treatment of HIV-1-infected cells with OLE also up-regulates the expression of the apoptosis inhibitor proteins IAP1 and 2, IL-2, IL-2Rα, and ODC1 genes. These results provide insight into the molecular mechanisms of HIV-1 pathogenesis as well as the anti-HIV action of OLE. They further indicate that anti-HIV activity of OLE is mediated in part by effects on the host, rather than solely by effects on viral pathogenesis in infected cells.

The roles of some of these genes in HIV pathogenesis are obvious, while those of others are less clear. For example, the expression of ODC1 is barely detectable in MT2 and HIV-infected MT2 cells yet it is significantly up-regulated in OLE treated cells. ODC is the rate-limiting enzyme in the biosynthesis of polyamines, the levels of which must be precisely regulated for cell growth and differentiation. Regulation of ODC is known to be critical to the control of cellular growth, differentiation, and carcinogenesis [35]. Thus, effects of OLE on ODC expression in HIV-infected cells may be important to the anti-HIV activity of OLE.

Another interesting example is the drastic up-regulation of the hedgehog receptor Ptc1 expression in HIV-1 infected MT2 cells and its down-regulation upon OLE treatment. The hedgehog (Hh) signaling pathway is involved in the development of many tissues, including thymocytes [36,37]. Sonic hedgehog (Shh) is critical for the regulation of the development of thymocytes from the earliest CD4$^+$CD8$^-$ double-negative through the CD4$^+$ or CD8$^+$ single-positive to CD4$^+$CD8$^+$ double-positive stages. Hedgehog proteins signal nearby cells through two transmembrane proteins, Patched (Ptc) and Smoothened (Smo). Ptc1 is the primary receptor for Hh, but expression of Smo is required for cells to transduce a hedgehog signal. In the absence of a Hh ligand, Ptc1 inhibits the activity of Smo, which otherwise is constitutively active [38]. The binding of the ligand relieves the inhibition [39], so that Smo can relay the signal into the target cell. Termination of Hh negative signaling is necessary for differentiation from CD4$^+$CD8$^-$ double negative to CD4$^+$CD8$^+$ double-positive thymocytes. Shh is produced by the thymic stroma, while Ptc1 and Smo, the transmembrane receptors for Shh, are expressed in double-negative thymocytes. The upregulation of the Hh receptor Ptc1 by HIV-1 infection and its downregulation upon OLE treatment may be important to immunomodulatory effects on the number of CD4+ and/or CD8+ cells during HIV infection.

Our LC-MS analysis of OLE shows the presence of three major species. These have been identified by mass spectrometry to be oleuropein ([M–H]$^-$ = 539), olenolic acid ([M–H]$^-$ = 377), and hydroxytyrosol ([M–H]$^-$ = 153). Furthermore, peak 6 oleuropein is as pure as the oleuropein standard and accounts for 85–90% of the anti-HIV activity in syncytiata and p24 assays. Evaluations of the biological effects of the other components in OLE are currently ongoing.

These results represent the first characterization of the anti-HIV activity of OLE using well-established bioassays. The microarray results are the first systematic identification of cellular targets associated with HIV-1 infection and OLE treatment. There are several important directions for future study. First, the pharmacokinetics of these compounds remain to be determined. Recent studies suggest that many polyphenolic compounds found in OLE are absorbed through the gastrointestinal tract, resulting in significant levels in the circulation [28,40]. Second, several of these compounds are further metabolized: oleuropein to d-elenolate and hydroxytyrosol to tyrosol. The biological activities of these derivatives need to be characterized. Third, the pharmacodynamic effect of OLE on other medications currently used by HIV-1 patients is unknown. The effects of these and other interactions need to be defined. Finally, given the current limitations of current treatment options for HIV, it will be important to define whether OLE acts in a synergistic or additive manner with currently used anti-retroviral medications. This will allow the rational and evidence-based inclusion of OLE into HIV treatment regimens.

References

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