An essential oil blend significantly modulates immune responses and the cell cycle in human cell cultures

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Abstract: In the current study, we examined the biological activities of an essential oil blend (EOB) in validated human cell cultures, which model the molecular biology of autoimmune diseases and chronic inflammation. EOB is primarily composed of essential oils from wild orange, clove, cinnamon, eucalyptus, and rosemary. These disease models allow the measurement of changes in protein biomarkers induced by EOB treatment. Four T cell autoimmune disease systems and one skin cell system were used for biomarker analysis. Biomarkers levels were measured both before and after EOB treatment for statistic analysis. EOB exhibited significant effects on the levels of protein biomarkers that are critically involved in inflammation, immune modulation, and tissue remodeling processes. The overall inhibitory effect of EOB on these protein biomarkers suggests that it has anti-inflammatory and immune modulating properties. EOB also showed significant anti-proliferative activity against these cells. We next investigated the effect of EOB on genome-wide gene expression in a skin disease model. EOB significantly modulated global gene expression in the skin disease model. Further analysis showed that EOB robustly affected signaling pathways related to inflammation, immune function, and cell cycle control. This study documents the biological activities of EOB in complex human disease models, and indicates that EOB affects various biological and physiological processes in human cells.

Dr Han's group primarily studies the health benefits of essential oils. We are specifically interested in the efficacy and safety of essential oils and their active components. We work closely with research institutes, hospitals, and clinics to develop quality essential oil products with therapeutic benefits. The research work discussed in this paper represents one part of a large research project, which was designed to extensively examine the effect of essential oils on human cells. This study, along with others, will further the understanding of the health benefits of essential oils for a wide research audience. Besides essential oils, we are also interested in studying the health benefits of herbal supplements and skin care products. Dr Han holds a PhD in Biological Sciences and is an elected Fellow of the American College of Nutrition. Parker holds a PhD in Nutritional Sciences. Dorsett holds a MS in Health Sciences.

Essential oils have become popular globally for health reasons. Our study examined the effects of an essential oil blend (EOB) on human cell systems that mimic different diseases. These effects of the EOB were determined by measuring biomarkers that are linked to inflammation, immune function, and wound healing. We found that the EOB had strong anti-proliferative, anti-inflammatory, immune modulatory, and wound healing activities. The effect of the EOB on gene expression in human skin cells was also studied. The EOB robustly affected genes and processes related to inflammation, immune function, and cell cycle control. The study findings suggest that essential oils affect various biological and physiological processes in human cells. Exploration of the health benefits of essential oils may lead to viable options for fighting many diseases. Thus, this study provides an important stepping stone for further research on essential oils and their health benefits for humans.
human cells. This study suggests that EOB possesses significant anti-inflammatory and immune modulating properties.

Subjects: Biochemistry; Pharmaceutical Science; Pharmacology; Immunology

Keywords: anti-proliferation; inflammation; genome-wide gene expression; immune response; cell cycle control; wild orange; clove; cinnamon; eucalyptus; rosemary

1. Introduction

Traditional medicine has long employed the use of botanical preparations to address human ailments. “Aromatherapy,” a term coined by René Maurice Gattefossé in 1920, is one such modality. It involves the therapeutic use of essential oils, complex mixtures of volatile aromatic compounds naturally found in plants, to improve physical, emotional, and spiritual well-being (Vigan, 2010). In addition to being used as natural remedies, essential oils and their respective major constituents are commonly used in cleaners, perfumes, cosmetics, dentistry, agriculture, and food preservatives. Essential oils can be diffused aromatically, consumed internally, or applied topically to the skin to achieve a desired benefit. Emerging scientific evidence supports these traditional uses of essential oils as effective therapeutic tools. A better understanding of the biological activities of essential oils is needed, especially regarding their effects on the human immune system and their potential anti-cancer properties.

Many studies have examined the biological activities of individual essential oils or their isolated constituents. For example, clove oil has been shown in vitro to increase both primary and secondary humoral responses (Halder, Mehta, Mediratta, & Sharma, 2011) and exhibit antiviral activity against herpes simplex virus (Tragoolpua & Jatisatienr, 2007). Several other essential oils, as well as their major constituents, have been found to possess medicinal properties, including antibacterial, anti-fungal, anti-proliferative, anti-inflammatory, antioxidant, and anesthetic properties. However, many of these studies have only utilized single cell lines or rodent models (Chong, Alegre, Miller, & Fairchild, 2013). Cell lines alone do not model primary disease biology, and rodent models do not accurately reflect regulatory complexities of human disease (Mak, Evaniew, & Ghert, 2014). Human cell coculture systems can help address these limitations by combining healthy host cells, diseased cells (e.g. tumor cells), and disease-relevant stimuli to mimic host-disease micro-environments (Bergamini et al., 2012). In this study, we evaluated the biological activities of a commercially available essential oil blend (EOB), in well-validated human cell coculture systems. EOB is primarily a mixture of essential oils from wild orange (Citrus sinensis), clove (Eugenia caryophyllata), cinnamon (Cinnamomum zeylanicum), eucalyptus (Eucalyptus globulus), and rosemary (Rosmarinus officinalis).

2. Materials and methods

All experiments were conducted in the Biologically Multiplexed Activity Profiling (BioMAP) platform, a set of primary human cell systems designed to model disease biology in a robust and reproducible way. The systems consist of three components, a cell type or cell types (many systems involve cocultures), stimuli to create the disease environment, and then a set of biomarker (protein) readouts to examine how treatments affect that disease environment. See Table S1 in Supplementary Material for a glossary of BioMAP systems used in the study.

2.1. Cell cultures

Primary human (H) cells (i.e. neonatal dermal fibroblasts (HNDFs), umbilical vein endothelial cells (HUVECs), peripheral blood mononuclear cells (PBMCs), and B cells) were obtained as previously described (Bergamini et al., 2012). HNDFs were plated in low serum conditions 24 h before stimulation with cytokines. HUVECs were obtained from Cascade Biologics (Portland, OR, USA) and were cultured in endothelial cell growth medium-2 containing manufacturer-provided supplements and 2% heat-inactivated fetal bovine serum (Hyclone, Logan, UT, USA). HUVECs were subcultured with 0.05% trypsin-0.53 mM ethylenediaminetetraacetic acid (EDTA, Mediatech, Herndon, VA, USA) according to
the manufacturer’s instructions. PBMCs were prepared from buffy coats (Stanford Blood Bank, Stanford, CA, USA) by centrifugation over Histopaque-1077 (Sigma-Aldrich, St. Louis, MO, USA).

Stimulatory molecules for these cell coculture systems were as follows: A mixture of interleukin (IL)-1β, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), and platelet-derived growth factor (PDGF) for the HDF3CGF system (HNDFs); T-cell receptor (TCR) ligands (1×) for the SAg system (PBMCs + HUVECs); immunoglobulin M (IgM) antigens and TCR ligands (0.001×) for the BT system (B cells + PBMCs); TCR ligands (0.001×) for the HDFSAg system (HNDFs + PBMCs); and IL-2 and TCR ligands (0.1×) for the /TH2 system (T helper cell 2 blasts + HUVECs). Cell culture and stimulation conditions for the HDF3CGF, SAg, BT, HDFSAg, and /TH2 coculture assays have been described in detail elsewhere, and were performed in 96-well format (Bergamini et al., 2012).

2.2. Protein-based readouts
An enzyme-linked immunosorbent assay (ELISA) was used to measure the biomarker levels of cell-associated and cell membrane targets. Soluble factors from supernatants were quantified using homogeneous time-resolved fluorescence detection, bead-based multiplex immunoassay, or capture ELISA. Overt adverse effects of test agents on cell proliferation and viability (cytotoxicity) were measured by sulforhodamine B (SRB) for adherent cells, and alamarBlue staining for cells in suspension. For proliferation assays, individual cell types were cultured at subconfluence and measured at time points optimized for each system (48–96 h). Detailed information has been described elsewhere (Bergamini et al., 2012). Measurements were performed in triplicate wells, and a glossary of the biomarkers used in this study is provided in Supplementary Table S2.

Quantitative biomarker data are presented as the mean log10 relative expression level (compared to the respective mean vehicle control value) ± standard deviation of triplicate measurements. Differences in biomarker levels between EOB- and vehicle-treated cultures were tested for significance with the unpaired Student’s t test. A p-value < 0.05, outside of the significance envelope, with an effect size of at least 10% (more than 0.05 log10 ratio units), was considered statistically significant.

2.3. RNA isolation
Total RNA was isolated from cell lysates using the Zymo Quick-RNA MiniPrep kit (Zymo Research Corporation, Irvine, CA, USA) according to the manufacturer’s instructions. RNA concentration was determined using a NanoDrop ND-2000 (Thermo Fisher Scientific, Waltham, MA, USA). RNA quality was assessed with a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) and an Agilent RNA 6000 Nano Kit. All samples had an A260/A280 ratio between 1.9 and 2.1 and an RNA integrity number score greater than 8.0.

2.4. Microarray analysis for genome-wide gene expression
A 0.01% (v/v) concentration of EOB was tested for its effect on expression of 21,224 genes in the HDF3CGF system after 24 h treatment. Samples for microarray analysis were processed by Asuragen, Inc. (Austin, TX, USA) according to the company’s standard operating procedures. Biotin-labeled cRNA was prepared from 200 ng of total RNA with an Illumina TotalPrep RNA Amplification kit (Thermo Fisher Scientific) and one round of amplification. The cRNA yields were quantified using ultraviolet spectrophotometry, and the distribution of transcript sizes was assessed using the Agilent Bioanalyzer 2100. Labeled cRNA (750 ng) was used to probe Illumina Human HT-12 v4 Expression BeadChips (Illumina, Inc., San Diego, CA). Hybridization, washing, staining with streptavidin-conjugated Cyanine-3, and scanning of the Illumina arrays were carried out according to the manufacturer’s instructions. Illumina BeadScan software was used to produce the data files for each array; raw data were extracted using Illumina BeadStudio software.

Raw data were uploaded into R and analyzed for quality-control metrics using the beadarray package. Data were normalized using quantile normalization, then were re-annotated and filtered.
to remove probes that were non-specific or that mapped to intronic or intragenic regions (Barbosa-Morais et al., 2010). The remaining probe sets comprised the data-set for the remainder of the analysis. Fold-change expression for each was calculated as the log₂ ratio of EOB to vehicle control. These fold-change values were uploaded to Ingenuity Pathway Analysis (IPA, Qiagen, Redwood City, CA, www.qiagen.com/ingenuity) to generate the networks and pathway analyses.

2.5. Reagents
The EOB (commercial name On Guard, dōTERRA International LLC, Pleasant Grove, UT, USA) was diluted in dimethyl sulfoxide (DMSO) to 8X the specified concentrations (final DMSO concentration was no more than 0.1% [v/v]); 25 μL of each 8 × solution was added to the cell culture to obtain a final volume of 200 μL. DMSO (0.1%) served as the vehicle control. Gas chromatography–mass spectrometry (GC-MS) analysis of EOB showed that its main (≥2%) chemical constituents were d-limonene, eugenol, 1,8-cineole, and cinnamaldehyde. GC-MS results of EOB will be made public via the supplier’s website www.sourcetoyou.com, as part of its transparency program.

3. Results
We studied EOB’s activities in disease-mimicking cell culture systems. Key activities were designated if biomarker values were significantly different (p < 0.05) from vehicle controls at studied concentrations, outside of the significance envelope, with an effect size of at least 10% (0.05 log units). Only biomarkers designated as key activities are discussed below.

3.1. Bioactivity profile of EOB in the autoimmune systems, SAg, BT, HDFSAg, and /TH2
To explore the biological activities of EOB in immune systems, we analyzed four different concentrations (0.01, 0.004, 0.0014, and 0.0004% [v/v]) of EOB in four different T cell autoimmune systems (i.e. SAg, BT, HDFSAg, and /TH2). EOB at 0.01% was overtly cytotoxic and was excluded from further analyses. The lower two concentrations of EOB did not have a significant effect on the biomarkers, so the 0.004% EOB was used for further analysis and is discussed below. For subsequent cell systems, the highest EOB concentration that did not demonstrate cytotoxicity was utilized.

In the SAg system, EOB reduced the level of cell differentiation 40 (CD40), which is a cell surface adhesion receptor related to immunomodulatory activity (Figure 1(A)). In the BT system, EOB significantly decreased the level of an inflammation biomarker, soluble tumor necrosis factor alpha (sTNFα). In the same system, it also significantly decreased the levels of three immunomodulatory biomarkers, namely secreted immunoglobulin G (slgG), soluble interleukin 17A (sIL-17A), and soluble interleukin 17F (sIL-17F) (Figure 1(B)). In both systems, EOB showed significant anti-proliferation activity and cytotoxic activity against PBMCs (Figure 1(A) and (B)).

In the HDFSAg system (Figure 1(C)), EOB reduced the levels of three inflammation-related biomarkers, vascular cell adhesion molecule 1 (VCAM-1), interferon gamma-induced protein 10 (IP-10), and sTNF-α, as well as the levels of four immunomodulatory biomarkers, sIL-17A, sIL-17F, sIL-2, and sIL-6 (Figure 1(C)). EOB also slightly increased the level of vascular endothelial growth factor (sVEGF). In the /TH2 system, EOB significantly decreased the levels of Eotaxin-3 and P-Selectin, which are both important inflammation-related biomarkers (Figure 1(D)). The immunomodulatory biomarker CD40 was also significantly decreased by EOB in the /TH2 system (Figure 1(D)).

3.2. Bioactivity profile of EOB in the dermal fibroblast system, HDF3CGF
The study results from autoimmune systems showed promising anti-inflammatory and immune function-modulating effects of EOB. We then analyzed its activity in a dermal fibroblast cell system, HDF3CGF, which features the microenvironment of inflamed human skin cells. Four different concentrations (0.01, 0.0033, 0.0011, and 0.00037% [v/v]) of EOB were initially analyzed for viability assays. None of them was overtly cytotoxic to these cells, and thus, the 0.01% EOB was included for further analysis and is discussed below.
EOB showed significant anti-proliferative activity in dermal fibroblast cells. The levels of several inflammation-related biomarkers, including VCAM-1, IP-10, interferon-inducible T-cell alpha (I-TAC), and monokine induced by gamma interferon (MIG), decreased in response to EOB (Figure 2). Macrophage colony-stimulating factor (M-CSF) and plasminogen activator inhibitor 1 (PAI-1), also decreased in response to EOB.

3.3. Effects of EOB on genome-wide gene expression in the HDF3CGF system

To further explore the effect of EOB in human cells, we analyzed the effect of 0.01% (v/v) (i.e. the highest tested concentration that was non-cytotoxic to these cells) on the RNA expression of 21,224 genes in the HDF3CGF system. The results showed a robust and diverse effect of EOB on regulating human genes, with many genes being upregulated and many others being downregulated (Table S3). Among the 200 most-regulated genes by EOB (log2 the fold change ratio of expression over vehicle control ≥1.5), the vast majority (172 out of 200 genes) were significantly downregulated.

IPA showed that the bioactivity of EOB significantly overlapped with many canonical pathways from the literature-validated database (Figure 3). Many of these pathways are critically involved in the processes of inflammation, immune response, cell cycle control, DNA damage response, and cancer biology. The significant matches of EOB bioactivity suggest that it affects various biological and physiological functions in human cells. For example, all the top four matched signaling pathways play important roles in the process of inflammation and cell cycle control (Tables S4–S7).

4. Discussion

Although essential oils have been used to prevent and treat diseases for many centuries, the scientific understanding of their biological action remains elusive. Therefore, we chose to study an EOB in the BioMAP platform to explore the biological activities of EOB in human cells. The BioMAP platform is a set of primary human cell systems designed to robustly model disease biology and ascertain the effect of therapeutic interventions on that biology.

4.1. EOB’s effects on inflammation, immune function, and tissue remodeling

Inflammation is a protective response that involves immune cells, blood vessels, and molecular mediators. The purpose of inflammation is to eliminate the initial cause of cell injury, remove...
necrotic cells and tissues damaged by the injury, and initiate tissue repair. Chronic inflammation may lead to various diseases, ranging from hay fever to periodontitis, atherosclerosis, rheumatoid arthritis, and cancer. Wound healing is a complex process composed of several phases: blood clotting (hemostasis), inflammation, growth of new tissue (proliferation), and remodeling of tissue (maturation). The wound healing process is fragile, and is susceptible to disruption, leading to the formation of non-healing chronic wounds or scar tissue. Both inflammation and wound healing processes are closely related to the immune function of the host.

EOB demonstrated its anti-inflammatory potential by reducing many inflammatory biomarkers in already highly inflamed cell systems (Figures 1 and 2). It reduced sTNFα in the BT system; VCAM-1, IP-10, and sTNF-α in the HDFSAg system; and Eotaxin-3 and P-Selectin in the TH2 system. EOB also significantly reduced VCAM-1, IP-10, I-TAC, and MIG in the HDF3CGF system. The anti-inflammatory property of EOB is consistent with previous studies of single oils and their active constituents. Cinnamaldehyde, the major bioactive chemical constituent of cinnamon bark essential oil, has been shown to possess anti-inflammatory properties (Chen et al., 2016; Han & Parker, 2017a; Koh et al., 1998; Mendes et al., 2016; Reddy et al., 2004). Clove oil and its major constituent, eugenol, have been shown to have anti-inflammatory activities, even more effective than NSAIDs (Han & Parker, 2017b; Nogueira de Melo et al., 2011). Rosemary oil has been shown to reduce inflammation by modulating leukocyte migration (Nogueira de Melo et al., 2011).

In vitro and animal studies suggest that d-limonene, the major constituent of orange oil, also possesses anti-inflammatory properties (Nogueira de Melo et al., 2011).

EOB demonstrated the potential to modulate immune responses in the highly inflamed systems (Figures 1 and 2). EOB reduced slgG, sIL-17A, and sIL-17F in the BT system; CD40 in both 5Ag and TH2 systems; sIL-17A, sIL-17F, sIL-2, sIL-10, and sIL-6 in the HDFSAg system; and M-CSF in the HDF3CGF system. EOB’s inhibitory effects on these important immunomodulatory biomarkers suggest its immune-enhancing potential in these pre-inflamed systems. Many of these activities are also likely attributed to the anti-inflammatory properties of these essential oils and their active constituents, as stated above. In addition, it had been previously shown that limonene, a major constituent of EOB, may enhance immune responses and act as an immune modulator (Raphael & Kuttan, 2003).

EOB also demonstrated beneficial effects on tissue-remodeling activities, which are important for proper wound healing (Figures 1 and 2). EOB increased the level of sVEGF in the HDFSAg system. VEGF is a widely expressed growth factor that induces vascular permeability, angiogenesis,
vasculogenesis, and inhibits apoptosis. Increasing VEGF may, therefore, promote better wound healing. Interestingly, EOB decreased PAI-1 in the HDF3CGF system. PAI-1 is the serine proteinase inhibitor and inhibitor of tissue plasminogen activator that is involved in tissue remodeling and fibrinolysis. Decreasing PAI-1 is also potentially beneficial to promote wound healing. A previous study showed rosemary essential oil to be effective at healing diabetic wounds by reducing inflammation and enhancing wound contraction, re-epithelialization, regeneration of granulation tissue, angiogenesis, and collagen deposition in BALB/c diabetic mice (Abu-Al-Basal, 2010).

Microarray analyses also showed evidence supporting the anti-inflammatory, wound healing, and immune modulatory effects of EOB. EOB seemed to regulate many important pathways relevant to anti-inflammation, wound healing, and immune response. For example, EOB's biological activities matched significantly with both the hepatic fibrosis/hepatic stellate cell activation canonical pathway and the antigen presentation canonical pathway. The vast majority of players (including many genes for components of the major histocompatibility complex, cytokines, and collagens) in these pathways were significantly inhibited by EOB, consistent with the potential role of EOB in reducing inflammation, modulating immune responses, and promoting wound healing.
Collectively, EOB shows various potential properties, including anti-inflammatory, immune enhancing, and wound healing promoting, in complex human cell cultures. These effects are putatively attributed to these single oils in EOB and their active constituents. Our results warrant further studies to explore the mechanisms of action in more detail.

4.2. EOB’s effect on cell cycle control

In both the BT and SAg systems, 0.01% (v/v) of EOB demonstrated anti-proliferative and cytotoxic activity against PBMCs (Figure 1). This suggests a possible role of EOB in regulating the cell cycle. However, the same concentration of EOB showed little anti-proliferative or cytotoxic activity in other studied systems. Further investigation is needed to elucidate the reason for difference and the mechanisms responsible.

Microarray analyses showed significant matches between EOB’s biological activities and several important cell cycle control pathways. The top two pathways were the mitotic roles of the polo-like kinase canonical pathway and the role of CHK proteins in the cell cycle checkpoint control pathway. Overall, the two pathways and the vast majority of players in these pathways were significantly inhibited by EOB. BRCA1 was one of many important genes shown to be significantly downregulated by EOB. This is consistent with the potential role of EOB to inhibit cell cycle progression in human cells. Generally, cell cycle control plays a critical role in cancer development and progression. Therefore, these results also suggest that EOB might be active in modulating cancer progression, by interacting with cell cycle control events. This property is putatively attributed to the anticancer potential of some single oils of EOB and their active constituents.

Previous studies of the individual essential oils and their major constituents also demonstrated promising anticancer properties. Cinnamon essential oil suppressed tumor growth in a Hep-2 cell xenograft model (Yang, Zheng, Ye, Li, & Chen, 2015). Cinnamaldehyde, a major constituent of cinnamon oil, has been shown to possess anti-mutagenic and anti-tumorigenic properties (de Silva & Shankel, 1987; Imai et al., 2002). In vitro studies in various cancer cell lines have also revealed cinnamaldehyde to be both anti-proliferative (Lin et al., 2013) and pro-apoptotic (Lin et al., 2013; Wu & Ng, 2007). Clove oil has been shown to possess anticancer properties against breast, colorectal, lung, and leukemia cancer cells (Kouidhi, Zmantar, & Bakhrouf, 2010; Kumar, Febriyanti, Sofyan, Luftimas, & Abdulah, 2014; Yoo et al., 2005). Rosemary oil exhibited strong cytotoxicity towards three human cancer cell lines (Wang, Li, Luo, Zu, & Efferteh, 2012), prevented the spread of breast and androgen-sensitive prostate cancer cells (Hussain et al., 2010), and promoted apoptosis of liver cancer cells (Melusova, Slamenova, Kozics, Jantova, & Horvathova, 2014), presumably via cell-membrane disruption (Wei, Liu, Wang, Li, & Luo, 2008). Orange oil exhibited dose-dependent inhibition of cell proliferation and induced apoptosis in colon cancer cells (Chidambara Murthy, Joyaprakash, Patil, & Chidambaram Murthy, 2012). Limonene, a major component of wild orange oil, has also been reported to affect a number of cancer hallmarks (i.e. proliferation, apoptosis, and inflammation) (Miller, Thompson, Hakim, Chow, & Thomson, 2011). More recently, studies (Han & Parker, 2017a, 2017b, 2017c) of clove oil, cinnamon oil, wild orange oil, and rosemary oil in the HDF3CGF system showed that they can potentially impact the process of cancer signaling and cancer biology, suggesting promising anticancer properties.

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Human cell culture systems are in vitro environments and do not reflect all of the complexities of the in vivo situation (e.g. pharmacokinetics). However, the model systems used in this study have been validated by both industrial use and regulatory acceptance. Gene expression levels were measured only after short-term exposure to EOB; how gene expression levels respond to longer-term exposure is still unknown. Finally, additional work (e.g. study in other coculture systems) is needed to better understand the complex mechanisms of action of EOB.

5. Conclusion

EOB significantly affected the levels of biomarkers related to inflammation, immune function, and tissue remodeling in various primary human cell models of diseases. EOB modulated mRNA levels of various signaling pathways (Figure 3), including inflammation, immune modulation, cell cycle...
regulation, and other cellular functions. To the best of our knowledge, this is the first study to explore the biological activities of this EOB in complex human cell cultures. This study provides novel and important findings of how EOB affects inflammation and immune-related biomarkers, and how it modulates genome-wide gene expression in validated human cell cultures.

Supplementary material

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Competing Interest

Xuesheng Han, Tory L. Parker and Jeff Dorsett are employees of doTERRA (Pleasant Grove, UT, USA), where the studied agent, EOB, was manufactured.

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