Immunomodulatory Effect and an Intervention of TNF Signalling Leading to Apoptotic and Cell Cycle Arrest on ORL-204 Oral Cancer Cells by Tiger Milk Mushroom, *Lignosus rhinocerus*

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**SUMMARY**

*Research background.* Tiger milk mushroom (*Lignosus rhinocerus*) is a medicinal mushroom that is geographically distributed in the region of South China, Thailand, Malaysia, Indonesia, Philippines and Papua New Guinea. Consumption of its sclerotium has been reported to treat various ailments. However, its anticancer potential towards oral cancer cell lines is yet to be determined considering the traditional method of its consumption by biting/chewing of the sclerotium.

*Experimental approach.* Mushroom sclerotial powder of cultivar TM02* was extracted and fractionated in a chromatographic column prior to cytotoxicity testing against a panel of human oral cancer cell lines. The capability of the identified bioactive fraction in regulating several molecules associated with its tumour necrosis factor (TNF) pathway was investigated.

*Results and conclusions.* 2,5-Diphenyl-2H-tetrazolium bromide (MTT) proliferation assay indicated that cell lines ORL-48 (derived from gingiva), ORL-188 (derived from the tongue) and ORL-204 (derived from buccal mucosa) were inhibited by cold water extract of *L. rhinocerus* sclerotia and its high-molecular-mass fraction (HMM) in varying degrees with ORL-204 being most affected. Hence, the treatment of ORL-204 with HMM mushroom extract was further investigated. HMM mushroom extract induced apoptosis and G0/G1 phase cell cycle arrest through caspase-3/7 cleavage. Activities of MIP2 and COX-2 were downregulated by 0.2- and 4.6-fold respectively in the HMM mushroom extract-treated ORL-204 cells.

*Novelty and scientific contribution.* Using ORL-204, we showed that HMM mushroom extract may act via the TNF pathway at various network sites as a potential dietary compound for cancer prevention and natural adjunct therapeutic to conventional cancer treatment.

*Keywords:* *Lignosus rhinocerus*; oral cancer; apoptosis; cell cycle; COX-2; MIP2

**INTRODUCTION**

*Lignosus rhinocerus* or tiger milk mushroom (also known locally as ‘cendawan susu harimau’) has well-recorded medicinal values (1). Its sclerotium is traditionally used as a health tonic or treatment regime for asthma, bronchitis, various cancer ailments as well as discomforts caused by fright, fever, cough, vomiting or injury (2,3). It is consumed in the form of decoction, in a betel quid, and other preparations where the sclerotium is pounded with raw rice, infused and then taken as a drink (4,5). A technique mimicking cold water extraction has been described by Chan (6) where the sclerotium is grated on a hard surface such as granite plate with some water and the resulting mixture is further diluted with water before consumption. There is also a practice of biting/chewing of the sclerotium by local indigenous communities during their journeys in the wild (7).

Previous omics studies reported the presence of lectins, fungal immunomodulatory proteins, superoxide dismutase, aegerolysin and laccases in *L. rhinocerus* that could be involved in various bioactivities, including immunomodulatory properties...
(8–10), which play a pivotal role in diseases such as cancer. In fact, the anticancer properties of *L. rhinoceros* in numerous cell lines have previously been reported. A polysaccharide-protein complex from *L. rhinoceros* sclerotium has been shown to inhibit the growth of several leukaemic cell lines induced by a G2 phase cell cycle arrest (11), while a cold aqueous extraction preparation derived from cultivar KUM61075 exhibited cytotoxicity against a panel of human cancer cell lines. The cytotoxic component(s) was/were speculated to be thermostable, water-soluble protein/peptide(s) (12). On the other hand, Lee et al. (13) demonstrated the anticancer properties of a cold water extract of TM02*®* sclerotia, a protein- and carbohydrate-rich extract, against breast cancer MCF7 and lung cancer A549 cell lines. The efficacy of its molecular mass fractions and a partially purified cytotoxic serine protease-like protein against MCF-7 cells via a cross-talk in between intrinsic and extrinsic apoptotic routes has also been reported (14). However, despite the growing scientific data of beneficial therapeutic effects of *L. rhinoceros* against various cancer cell lines, its anticancer potential towards oral cancer cell lines remains unknown. In this study, we investigate the anticancer activity of TM02*®* sclerotial extracts on a panel of human oral cancer cell lines and their possible mode of action.

Oral cancer is selected as it is one of the more common cancers in the world with estimated 354 864 new cases diagnosed, and 177 384 deaths reported in the year 2018 (15). Furthermore, tiger milk mushroom has been consumed by chewing and kept in mouth for a considerable amount of time, prior to swallowing. Hence, it is intriguing to find out its cytotoxicity in the oral cavity. More than 90 % of oral cancer that occur on the lips and in the oral cavity are squamous cell carcinoma (16). It is believed that oral squamous cell carcinoma (OSCC) develops through stages, from increasing severity of histological changes of premalignant lesions to malignancy. OSCC is life-threatening and with a mere five-year survival rate for stages 3 and 4. Early signs of oral cancer often go unnoticed and have been frequently discovered during routine dental examinations. Many cases of oral cancer may have advanced to an untreatable stage where the cancer cells have become aggressive and unresponsive to therapeutic drugs (17,18). In general practice, oral cancer is treated with either surgery, radiotherapy and/or chemotherapy. The treatment outcomes may include disease recurrence and post-treatment morbidity owing to the non-specific damages of these treatments to the function of healthy cells. Several determining factors that may increase the risk of cancer have been identified and these include massive exposure to chemical carcinogens such as tobacco and alcohol, solar ultraviolet radiation by excessive exposure of lips to the sun, human papillomavirus infection and a weakened immune system (19). Therefore, research that focused on natural immunomodulators to impede side effects of cytotoxic drugs has been gaining limelight in recent years. Natural compounds with simultaneous targeting of cancer pathways may often result in efficient and selective killing of cancer cells, which could be an added advantage for treatment of the disease (20,21). Thus, it is of tremendous interest if the anticancer properties reported for this medicinal mushroom are also effective for oral cancer.

**MATERIALS AND METHODS**

**Extraction and fractionation of TM02*®* sclerotial powder**

The freeze-dried sclerotial powder TM02*®* (reg no. MAL 11035004TC) was provided by LiGNO® Biotech Sdn. Bhd. (Balakong Jaya, Selangor). Preclinical toxicological study determined that the product was not associated with any toxicity concerns. No-observed-adverse-effect level dose was more than 1000 mg/kg. The powder also did not cause detectable adverse effect on rats’ fertility, teratogenic and genotoxicity effects (22). Hot water, cold water and methanol extractions were carried out in a mass to volume ratio 1:20 (g/mL) as described earlier (23). Cold water extract of TM02*®* sclerotia was further fractionated by Sephadex® G-50 (fine) (Sigma-Aldrich, Merck, St. Louis, MO, USA) gel filtration chromatography column equilibrated with 0.05 M ammonium acetate (Sigma-Aldrich, Merck) buffer. Eluted fractions were subsequently grouped based on their molecular masses.

**Cell culture and maintenance**

ORL-48, ORL-204 and ORL-188 oral cancer cell lines isolated respectively from gingiva (gum), buccal mucosa (lining of the cheeks and back of the lips) and tongue were obtained from untreated primary human oral squamous cell carcinomata of the oral cavity as in vitro models to study a disease prevalent in Asia. Their growth characteristics, epithelial origin and molecular alterations were previously characterized (24,25). Genetic information and clinical data associated with these ORL cell lines are available at https://genipac.cancerresearch.my/ (26). ORL-48, ORL-204 and ORL-188 cells were cultured and maintained in DMEM/F-12 medium (Nacalai Tesque Inc., Kyoto, Japan) supplemented with 10 % foetal bovine serum (Nacalai Tesque Inc.) and 0.1 % penicillin-streptomycin (Sigma-Aldrich, Merck) at 37 °C and 5 % CO2.

**MTT cytotoxicity assay**

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was used to determine the anti-proliferative activity of the extracts, where the yellow tetrazolium dye was reduced to purple crystalline formazan in metabolically active viable cells by NAD(P)H-dependent cellular oxidoreductases. ORL cell lines were seeded in monolayer and were allowed to adhere overnight prior to treatment with TM02*®* samples at different concentrations from 31.25 to 1000 μg/mL. Following 72 h of incubation, 20 μL of
5 mg/mL MTT solution (Calbiochem®, Sigma-Aldrich, Merck, San Diego, CA, USA) in phosphate buffered saline (Oxoid, Bas- ingstoke, UK) were added to each well. The plate was then incubated for 4 h at 37 °C to promote the formation of purple formazan crystals. All the solutions were then aspirated and 200 µL of dimethyl sulfoxide (Sigma-Aldrich, Merck, St. Louis) were added to dissolve the attached formazan crystals. Absorbance was read at 570 nm after incubation for 10 to 30 min in the dark. Cell viability (%) was calculated and plotted against the extract concentration curve.

Caspase activity measurement

Caspase-3/-7, -8 and -9 activities were measured using re- spective Caspase-Glo® 3/7, Caspase-Glo® 8 and Caspase-Glo® 9 assay systems (Promega, Fitchburg, WI, USA) according to the manufacturer’s protocol. Cells were seeded in monolayer into a 96-well plate and treated with high-molecular- mass (HMM) fractions of TM02® cold water extract at 75 µg/mL for 24, 48 and 72 h. After treatment, luminescent signal proportional to caspase activity was measured an hour after the addition of Caspase-Glo® reagent, which relies on the properties of a proprietary thermostable luciferase (Ultra-Glo™ Recombinant Luciferase, Promega), to the post-treat- ed cells in 1:1 ratio.

Flow cytometry analysis of cell cycle

Cell cycle distribution of treated cells was quantified us- ing Muse™ cell cycle kit (Millipore, Burlington, MA, USA) ac- cording to the manufacturer’s protocol. In brief, cells were treated with HMM extracts at 40 and 250 µg/mL (IC50) for 72 h prior to 70 % ethanol fixation and staining with Muse™ cell cycle reagent, a nuclear DNA stain containing propidium io- dice, prior to the analysis on Muse™ cell analyzer (Millipore). The number of cells (in %) in the G0/G1, S and G2/M cell cycle phases, which differ in DNA content, were quantified by fluo- rescence-activated cell sorting (FACS) analysis with the con- figuration of 532 nm green laser line and three detection channels.

ELISA assay

Selected modulators (TIMP1 and MIP2) were quantified using ELISA kit (Elabscience, Wuhan, PR China) according to the manufacturer’s protocol. In brief, cells were treated for 72 h with 10 (IC50) and 40 µg/mL of HMM extract. A total of 100 µL of standard or sample (the collected spent media from treated cells) was then added into each pre-coated well and incubated for 2.5 h at 37 °C to combine with the specific ant- body. Specific biotinylated detection antibody was then added into each well and incubated for 1 h at 37 °C. This was followed by washing three times and then the addition of avidin-horseradish peroxidase conjugate for an hour incuba- tion. Unbound components were washed off and substrate solution was added to each well. The enzyme-substrate reaction was terminated by the addition of stop solution (Elabscience). Absorbance was measured at 450 nm using Ep- och microplate spectrophotometer (BioTek Instruments Inc., Winooski, VT, USA).

Cyclooxygenase assay

Cyclooxygenase (COX) activity assay kit (fluorometric), purchased from Abcam, Cambridge, UK, served to detect the peroxidase activity of COX. A fresh set of standards was pre- pared and the supernatant of cells treated with 40 µg/mL of HMM extract for 72 h was collected according to the manu- facturer’s protocol. Samples were kept on ice for downstream processing. Standard and reaction wells of samples and positive control were prepared and 10 µL of diluted arachidonic acid/NaOH solution were added into each reaction well. Fluoro- scence was measured (Ex/Em=535/587 nm) in a kinetic mode once every 15 s for 30 min.

Statistical analysis

SPSS Statistics v. 21.0 (27) with one-way ANOVA followed by LSD’s post hoc test for multiple comparisons was used to compare the mean values. A p-value of less than 0.05 was considered as statistically significant.

RESULTS AND DISCUSSION

In this study, pulverized Lignosus rhinoceros TM02® was extracted with hot water, cold water and methanol to coerce its carbohydrates, proteins and secondary metabolites, re- spectively. The yield and composition of these extracts were previously reported by our lab (23). Hot water extract con- sisted almost entirely of carbohydrates with 56 % α-glucans, while cold water extract retained most of the extractable pro- teins as the extraction at low temperature of 4 °C had pre- vented the excessive degradation of thermolabile constitu- ents including proteins and peptides. The major constituent of carbohydrates in the cold water extract was mainly glu- cose, which makes up the glucans in linear polysaccharides with 1,4-linkage. On the other hand, methanol extract con- sisted mainly of secondary metabolites (terpenoids) with no detectable level of proteins (23,28).

The ORL-48, ORL-204 and ORL-188 oral squamous cell car- cinoma (OSCC) cell lines were selected as they represent some of the most common areas for oral cancer with 100 % orthotopic take rate and are highly hyperplastic (24). These cells were treated with 31.25−1000 µg/mL hot water, cold wa- ter and ethanol extracts for 72 h. Cytotoxic effect of the crude extracts was then measured with MTT assay. The reduction in the number of cells indicates inhibition of cell growth, and their sensitivity to drugs is further specified as the drug concentration needed to reach 50 % inhibition of cell growth (IC50). ORL-48 and ORL-204 were more responsive towards cold water extract (Fig. 1) and the observed differences could be attributed to genetic predisposition resulting from site
variation as ORL-48 and ORL-204 were isolated from gingiva and buccal mucosa while ORL-188 was extracted from tongue (24).

Owing to its antiproliferative potential, cold-water extract was further fractionated via Sephadex® G-50 column to high, medium- and low-molecular mass (termed HMM (>30 kDa), MMM (7–30 kDa) and LMM (<7 kDa), respectively) extracts. In our previous study, we reported that HMM extract contained the highest amount of carbohydrates and proteins, without detectable level of β-glucans, suggesting the existence of polysaccharide-protein complexes in the fraction. Our group reported that HMM extract contained the glycosidic linkage (1,3,6-Glcp) in the prospective polysaccharide-protein complexes (28). On the other hand, LMM extract had a lower amount of carbohydrates with very few proteins, but higher number of secondary metabolites such as phenolics and terpenoids. MMM extract had a moderate amount of the macromolecules and secondary metabolites. It contained highly branched glucans with mixed linkages including 1,4,6-, 1,3,6- and 1,2,4,6-Glcp (28,29).

HMM, MMM and LMM extracts were tested on the OSCC cell lines with concentrations ranging from 16 to 500 µg/mL for 72 h (Table 1). A lower concentration range of crude extracts was selected since their stronger toxicity was expected. The range of IC50 values was determined to be 40–115 µg/mL for HMM extract, 125–175 µg/mL for MMM extract and above 400 µg/mL for LMM extract. HMM extract was found to be more cytotoxic to OSCC cell lines, specifically ORL-204, which originated from buccal mucosa cancer patient. Further testing of these extracts and fractions on an in-house isolated primary human fibroblast culture indicated that they are not toxic to normal tissues (Table 1). As HMM extract consists mainly of carbohydrates and proteins, the present result suggests that the bioactive component(s) responsible for its cytotoxic activity could be of proteoglycan nature and/or carbohydrate-protein complex derivatives.

Table 1. Cytotoxicity of Lignosus rhinoceros TM02* against various human cell lines

| Mushroom extract | IC50 (µg/mL) |
|------------------|--------------|
| CWE              | 230          |
| HMM              | 115          |
| MMM              | 125          |
| LMM              | >400         |

IC50=half maximal inhibitory concentration value. It was determined from a mean plot of cell viability (in %) against concentration curve (N=2). CWE=cold water extract; HMM=high molecular mass, MMM=medium molecular mass, LMM=low molecular mass, NP=not performed.

We further determined if HMM extract induced apoptosis in ORL-204 by investigating the regulation of the key effectors in cell death signalling pathway. ORL-204 cell line was treated with HMM extract at 75 µg/mL (35 µg/L above IC50) for 24, 48 and 72 h. This concentration was selected for better capture of caspase activity that spans from 24 h onwards. At 24 h, HMM extract increased caspase-8 and -9 activities significantly in the treated cells (Fig. 2a). It is predicted that the active caspase-8- and -9 had subsequently activated the downstream executioner caspase as demonstrated in Fig. 2b, where caspase-3/7 activity increased up to 2-fold over a period of 72 h in HMM extract-treated cells as compared to the untreated control. This suggests that HMM extract induced apoptosis in a caspase-dependent manner via both the extrinsic and intrinsic signalling pathways. The cleavages of caspase-3/7 will presumably lead to the activation of endonuclease and protease as well as a series of cyt morphological changes.

**Fig. 1.** Cytotoxicity of Lignosus rhinoceros TM02* sclerotial cold water extracts after 72 h of treatment of ORL-48, -188 and -204 cells. Viability of the cells was determined by calculating the relative cell viability in MTT assay. Values are mean±S.D. CWE=cold water extract of TM02* sclerotia

**Fig. 2.** Upregulation of a) caspase-8, -9 and b) -3/7 activities in ORL-204 cells after treatment with high molecular mass (HMM) fraction at 75 µg/mL over a period of 72 h. Data were expressed as fold change compared to the untreated control which was set as 1 (mean values±S.D., N=2; *p<0.05)
Table 2. Effect of high molecular mass extract on ORL-204 cell cycle distribution

| Cell treatment | G₀/G₁ phase (%) | S phase (%) | G₂/M phase (%) |
|----------------|-----------------|------------|----------------|
| Untreated      | 43.8±1.2        | 12.4±2.9   | 37.8±1.3       |
| IC₅₀           | (47.5±2.3)*     | 12.4±3.0   | (23.1±3.2)*    |
| IC₇₀           | (49.1±1.7)*     | 13.6±2.9   | (17.9±1.9)*    |

Cells were treated with 40 (IC₅₀) and 250 µg/mL (IC₇₀) of HMM extract for 72 h. Cell distribution (%) in G₀/G₁, S and G₂/M phases is expressed as mean value±S.D. (N=3; *p<0.05)

Apoptosis is often linked to proliferation as they share the same set of regulators such as c-Myc, p53, pRb, Ras, PKA, PKC, Bcl-2, NF-κB, CDK, cyclins and CKI (31). This knowledge prompted us to look into the cell cycle profile of ORL-204. Cells were treated with HMM extract at IC₅₀ and IC₇₀ (250 µg/mL) for 72 h prior to staining and data acquisition via FACS analysis. Cell death is prominent in the group of cells treated with HMM extract at IC₅₀, with the accumulation of cells in the sub-G₀/G₁ peak, which may be indicative of DNA fragmentation due to apoptosis (Table 2). Furthermore, our earlier demonstration of upregulated caspase-3 activities in HMM extract-treated cells is indicative that the extract can mediate the cleavage and inactivation of p21 that converts cancer cells from growth arrest to apoptosis (32). HMM extract arrested ORL-204 at G₀/G₁ phase in the cell cycle where there was a minor but statistically significant increment of the cell population and subsequently a decreasing trend in the G₂/M phase (Table 2). OSCC cell lines have been shown to overexpress cdk4 and cdk6, the key players in G₁ phase (33), thus suggesting that these cell lines are more sensitive to G₁ inhibitor. HMM extract may act as a cdk inhibitor that impedes downstream functions.

A previous work done using wild type L. rhinoceros revealed a novel water-soluble polysaccharide-protein complex that could potentially be immunomodulatory agent for cancer immunotherapy (34). Sum et al. (28) have reported that L. rhinoceros TM02® regulated the release of several cytokines/chemokines which are associated with tumourigenesis by RAW 264.7 murine macrophages, in particular the MIP2 and TIMP1. We proceeded to question if TM02® also demonstrated comparable immunomodulating properties in oral cancer in addition to its selective antiproliferative property. We investigated the regulation of the release of these cytokines in ORL-204 over a period of 72 h after treatment with HMM extract at 10 (IC₅₀) and 40 µg/mL (IC₇₀). HMM extract significantly inhibited the release of MIP2 from ORL-204 by 30 to 80 % in a dose-dependent manner, while no effect was observed for TIMP1 (Fig. 4a). In most cancers, MIP2 expression is upregulated for cell proliferation promotion and metastasis (35). Its secretion inhibition by HMM extract therefore suggests a repressive effect of the extract towards ORL-204 growth, while its associative role in the alteration of osteoclastic activity remains unknown. It has been reported that TNF-α mediated the increase of MIP2 mRNA via NF-κB/MAPK, a caspase-3 signalling pathway in macrophages (36), but the question whether similar mechanisms are applicable to our current study remains to be answered. However, in view of its anticancer effect by promoting caspase-3/7 activities and suppressing MIP2 secretion, it is suggestive that HMM extract has antagonizing and/or multiple involvements in the TNF signalling pathway.

Many of the cytokines and mediators of inflammatory pathways are involved in the different steps of tumourigenesis (37). Non-steroidal anti-inflammatory drugs (NSAIDs) have been reported to prevent cancer and stop tumour growth by inhibiting prostaglandin synthesis through COX-2 hindrance (38). We further investigated the potential role of COX in regulating the inhibitory effect of HMM extracts on ORL-204. Cells were treated with HMM extract at IC₅₀ and the COX levels were determined (Fig. 4b). The selective inhibition of HMM extracts towards COX-2 might indicate its potential role against the development of oral lesions by inhibiting...
and/or interrupting the oral carcinogenesis pathway, further strengthening the therapeutic potential of COX-2 inhibitors in oral cancer treatment (39). There has also been increasing evidence that COX-2 produced prostaglandins that intervene in tumour cell proliferation while some selective COX-2 inhibitors such as nabumetone inhibit proliferation of various COX-2-expressing cancer cells by a G_{0}/G_{1} phase cell cycle arrest (40). Our current findings point to a possible linkage between COX-2 inhibitory effect and G_{0}/G_{1} phase arrest in ORL-204. However, due to the limitations of an in vitro study, the association remains to be elucidated.

From this study, we showed that HMM extract of L. rhinocerus induced apoptosis in ORL-204 cells via the activation of caspase-3/7 through the extrinsic and intrinsic signalling pathways. HMM extract further manipulated cell cycle by arresting the cells at G_{0}/G_{1} phase. Several molecules such as MIP2 and COX-2 related to TNF signalling contributed to the anticancer effect of HMM extract towards ORL-204, where both immunomodulators have been implicated in cell proliferation and inflammation (Fig. 5). A halted COX-2 expression has been shown to decrease MIP2 (41). Lee et al. (42) also reported that HMM extract attenuated TNF-α activity in lipopolysaccharide-induced RAW 264.7 cells, signifying an anti-inflammatory/immunosuppressive effect. HMM extract contains abundant amounts on dry mass basis of carbohydrates and proteins (4%) (42), suggesting the existence of polysaccharide-protein complexes in the fraction. The conglomeration of molecules in HMM extract may have contributed to its diverse roles in targeting various signalling pathways such as apoptosis, inflammation and immunomodulation in order to exert its anticancer effect.

**CONCLUSIONS**

This is the first study to reveal the capability of *Lignosus rhinocerus* TM02\(^*\) in aiding oral cancer treatment and/or as a form of preventive measure against tumourigenesis by the intervention of the tumour necrosis factor (TNF) signalling. The action of biting/chewing the sclerotium, which has been practiced traditionally, may now have an insightful implication. Cold water extract of the tiger milk mushroom demonstrated selective cytotoxic effect to ORL-48 and ORL-204 cell lines, with little cytotoxicity towards primary human fibroblast, while high-molecular mass polysaccharide-protein complexes from the cold water extract induced apoptosis and exhibited antiproliferative activity against ORL-204 by G_{0}/G_{1} phase cell cycle arrest and inhibition of several immunomodulators affiliated with the TNF signalling pathway, such as MIP2 and COX-2. Further investigations including in vivo and downstream molecular works are warranted to strengthen the current findings. As a limitation of this study, future work will also incorporate anticancer drug(s) as positive control to test along with purified fractions.

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CONFLICT OF INTEREST
S.T. Ng and C.S. Tan are affiliated with LiGNO Biotech Sdn. Bhd., an industry that commercialises tiger milk mushroom. The authors declare they have no financial interests.

AUTHORS’ CONTRIBUTION
All authors contributed to the study conception and design. H.Y.Y. Yap, B.H. Kong, C.S.A. Yap, S.T. Ng and C.S. Tan prepared the material, collected data and performed analyses. H.Y.Y. Yap wrote the first draft of the manuscript and all authors commented on the previous versions. All authors read and approved the final manuscript.

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