Introduction

Oral cancer is a major worldwide public health problem that may modify any part of the oral cavity, including the lips, tongue, mouth, and throat (1, 2). Despite the introduction of novel therapeutic modalities to treat oral cancer, improvements in the long-term survival rates have been modest (3). Even with early oral cancer detection, survival rates have not significantly changed over the past two decades (2, 3). Therefore, understanding of the underlying mechanisms of oral cancer is necessary for increasing these survival rates and the development of novel therapeutics agents are warranted.

Amino acids are essential for protein synthesis, which is required for cell growth and proliferation (4, 5). Amino acid transport across the plasma membrane is mediated via amino acid transporters located on the plasma membrane in normal and transformed cells (4, 5). Among the known amino acid transport systems, the system L amino acid transporter, as a Na\(^{+}\)-independent neutral amino acid transport system, is a major route for providing living cells, such as cancer cells, with neutral amino acids.
including several essential amino acids (4, 6).

Kanai et al. identified system L-type amino acid transporters 1 and 2 (LAT1 and LAT2), which are the first and second isoforms of the system L amino acid transport system (7 – 9). LAT1 and LAT2 were predicted to be 12-membrane-spanning proteins that mediate Na\(^+\)-independent amino acid exchange (7 – 9). They require an additional single-membrane-spanning protein, which is a heavy chain of 4F2 antigen (4F2hc), for their functional expression in the plasma membrane (7 – 10). LAT1 is expressed only in restricted organs, such as the brain, spleen, placenta, and testis (7, 8, 11, 12). Interestingly, LAT1 is expressed strongly in a variety of tumors, presumably to support their continuous growth and proliferation (7, 8, 13, 14). LAT2 has very low, if any, expression in tumor cells but is expressed ubiquitously at high frequency in normal tissues (9, 15 – 17).

Based on the characteristics of LAT1 and LAT2, it is proposed that the manipulation of the system L activity, particularly that of LAT1 as a molecular target for cancer therapy, would have therapeutic implications in cancer treatment (18). Suppressing the activity of LAT1, thereby depleting the intracellular neutral amino acids, would be helpful for inducing the inhibition of cancer cell growth (18). When the LAT1-selective activity in tumor cells becomes blocked completely, normal cell growth and proliferation would presumably still be possible due to the presence of LAT2 (18). On the other hand, the mechanisms by which LAT1-selective inhibitors suppress cancer cell growth or trigger cytotoxicity are not entirely clear.

2-Aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH) has been a model compound to study amino acid transporters because it is a broad system L inhibitor and inhibits both LAT1 and LAT2 (7 – 9, 19, 20). Therefore, BCH lacks selectivity toward LAT1. It is well established that system L amino acid transporters transport neutral amino acids, including several essential amino acids (4, 6). In addition, normal cells are damaged when these transporters are blocked in living cells via a broad L-type amino acid transporter inhibitor such as BCH (18, 21). Damage would be caused specifically by the deprivation of amino acids necessary for protein synthesis, cell growth, and proliferation (18, 21). Unfortunately, BCH has low cytotoxicity in several cancer cells (21). Therefore, it remains an excellent compound for the design of more effective analogs with high LAT1 selectivity.

This study examined the effects of JPH203, a novel tyrosine analog with high LAT1 selectivity (22, 23), on cell growth as well as its mechanism for cell growth suppression in YD-38 human oral cancer cells. The results showed that JPH203 can effectively induce the suppression of cell growth and cell apoptosis in YD-38 human oral cancer cells.

Materials and Methods

Materials

\(^{[14]}\text{C}\)-leucine was purchased from PerkinElmer Life Sciences, Inc. (Boston, MA, USA). BCH and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Anti-caspase-3, -7, and -9, poly (ADP-ribose) polymerase (PARP), and β-actin antibodies were supplied by Cell Signaling Technology, Inc. (Danvers, MA, USA). JPH203 HCl salt (Fig. 1, purity > 99%) was a kind gift from J-Pharma, Tokyo. All other reagents were of analytical grade.

Cell line and cell culture

Normal human oral keratinocytes (NHOKs) were purchased from the ScienCell Research Laboratories (Carlsbad, CA, USA). The NHOKs were maintained in KGM and a supplementary growth factor bullet kit (Clonetics Corp., San Diego, CA, USA). The YD-38 human oral cancer cells were provided by the Korean Cell Line Bank (Seoul, Korea). The YD-38 human oral cancer cells were grown in RPMI 1640 containing 10% fetal bovine serum (FBS). The HT-29 human colorectal adenocarcinoma cells were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). The HT-29 cells were cultured in McCoy’s 5A modified medium in the presence of 10% FBS and penicillin/streptomycin (Invitrogen Life Technologies, Carlsbad, CA, USA). The cells were maintained as monolayers in plastic culture plates at 37°C in a humidified atmosphere containing 5% CO\(_2\).

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

RT-PCR analysis was performed using the previously described method (18, 20). A pair of primers, 5′-CAAGGACATCTTTCTCCGTAC-3′ (1242-1263 bp)
and 5′-AGCCACTTGCTTTGTTT-3′ (1526-1509 bp), were used for the PCR amplification of human LAT1. A pair of primers, 5′-AAAGGGAGTGCTGGAGAATG-3′ (932-951 bp) and 5′-GACCCATGTGAGGAGCAATAA-3′ (1271-1251 bp), were used for the PCR amplification of human LAT2. A pair of primers, 5′-GGTGGAGCTGAATGAGTTAGAG-3′ (594-1054 bp) and 5′-CGACATCATCTCTGGTTTT-3′ (1075-1054 bp), were used for the PCR amplification of human 4F2hc.

Real-time quantitative RT-PCR (qRT-PCR)

Real-time qRT-PCR analysis was performed using the previously described method (24). For LAT1, the forward and reverse primers were 5′-CAAGGACATCTTCTCCGTCATC-3′ and 5′-AGCCACTTGCTTTGTTT-3′, respectively. For LAT2, the forward and reverse primers were 5′-AAAGGGAGTGCTGGAGAATG-3′ and 5′-GACCCATGTGAGGAGCAATAA-3′, respectively. For 4F2hc, the forward and reverse primers were 5′-GGTGGAGCTGAATGAGTTAGAG-3′ and 5′-CGACATCATCTCTGGTTTT-3′, respectively.

Immunofluorescence in YD-38 cells and KHOKs

YD-38 cells and NHOKs were incubated and then washed with phosphate-buffered saline (PBS). The cells were fixed and permeabilized in ice-cold methanol/acetone (1:1) for 10 min at −20°C and then washed twice with PBS (25). After blocking with normal goat serum, the cells were incubated with affinity-purified anti-LAT1 polyclonal antibody (1:100) (Kumamoto Immunochemical Laboratory, Transgenic Inc., Kumamoto) or affinity-purified anti-LAT2 polyclonal antibody (1:100) (Kumamoto Immunochemical Laboratory, Transgenic Inc.) overnight at 4°C and then with mouse anti-human 4F2hc monoclonal antibody (1:100) (Ancell, Bayport, MN, USA) for 2 h at room temperature. Thereafter, they were treated with TRITC-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h and then, FITC conjugated rabbit anti-mouse IgG (Santa Cruz Biotechnology) for 1 h at room temperature. The cells were examined under the Nikon Eclipse E2000 fluorescence microscope (Nikon Instruments, Melville, NY, USA).

Transport measurements in YD-38 cells

To examine the inhibition of amino acid transport by JPH203 and BCH in YD-38 cells and NHOKs, uptake experiments were performed using [14C]-l-leucine, which is a prototypical system L substrate and a model neutral amino acid, as described previously (21). To determine the IC50 values of JPH203 and BCH, the uptake of 1.0 μM [14C]-l-leucine was measured in the presence of JPH203 (0 – 100 μM) and BCH (0 – 3000 μM), respectively.

MTT assay

The cell viability test was performed using the method described previously (21). The YD-38 cells and NHOKs were seeded at a concentration of 5 × 10^4 cells/well in 24-well plates. After 24 h of growth, the cells were treated with JPH203 or BCH at a range of concentrations and incubation times. The cell viability was assessed using a MTT assay. Three or four separate experiments were performed for each concentration/exposure time combination.

Annexin V–FITC, propodium iodide (PI), and flow cytometry analysis

Apoptosis was determined using an Annexin V–fluorescein isothiocyanate assay. The YD-38 cells were washed in phosphate-buffered saline twice and resuspended in a binding buffer (BD Biosciences, San Diego, CA, USA). Annexin V–fluorescein isothiocyanate and 7-amino-actinomycin D (BD Biosciences) were added to the cells, which were then incubated in the dark for 15 min. Subsequently, the cells were resuspended in 400 μl of binding buffer. The cells were analyzed by fluorescence activated cell sorting Calibur flow cytometry (Becton Dickinson, San Jose, CA, USA). Data analysis was performed using standard Cell Quest software (Becton Dickinson).

Immunoblotting

To determine the level of activated caspase-3, -7, and -9 and PARP in the YD-38 cells treated with JPH203, the proteins were extracted, as described previously (18). The anti-caspase-3, -7, and -9 (1:1000 dilution) and PARP (1:2000 dilution) antibodies were used as the primary antibody.

Data analyses

All experiments were performed at least in triplicate. The results were presented as the mean ± S.E.M. Statistical significance was analyzed using a Student’s t-test for two groups and one way analysis of variance for multi-group comparisons. A P-value < 0.05 was considered significant.

Results

Detection of the system L amino acid transporters in YD-38 cells and HT-29 cells

In RT-PCR analysis, the PCR products for LAT1 and its associating protein 4F2hc were detected in YD-38 cells, whereas LAT2 was not detected in the YD-38 cells.
(Fig. 2A). RT-PCR suggested that LAT1 but not LAT2 is present together with 4F2hc in YD-38 cells. Real-time qRT-PCR detected LAT1 and 4F2hc in YD-38 cells (Fig. 2B). Consistent with the result from RT-PCR analysis (Fig. 2A), LAT2 was not detected in the YD-38 cells by real-time qRT-PCR (Fig. 2B). In the immuno-
fluorescence microscopic analyses, LAT1 or LAT2 protein with 4F2hc was detected in YD-38 cells or NHOKs, respectively (Fig. 2C). They were found to be mainly co-localized in the plasma membrane.

To compare the expression of LAT1, LAT2, and 4F2hc in YD-38 cells and HT-29 cells, RT-PCR and real-time qRT-PCR analyses were performed in HT-29 cells. LAT1 was present with 4F2hc in HT-29 cells but LAT2 was not detected (Fig. 2D). The mRNA levels of LAT1 and 4F2hc were significantly higher in the HT-29 cells than in the YD-38 cells (Fig. 2E).

Inhibition of l-leucine uptake by JPH203 in YD-38 cells

As shown in Fig. 3A, JPH203 (0.001 – 100 μM) inhibited the [14C]-l-leucine (1.0 μM) uptake slightly in the NHOKs. In contrast, JPH203 (0.001 – 100 μM) inhibited the [14C]-l-leucine (1.0 μM) uptake in a concentration-dependent manner in the YD-38 cells with an IC50 value of 0.79 ± 0.06 μM (mean ± S.E.M., n = 4 separate experiments). As shown in Fig. 3B, the BCH (1 – 3000 μM) inhibited the [14C]-l-leucine (1.0 μM) uptake in the YD-38 cells and NHOKs in a concentration-dependent manner with IC50 values of 92.6 ± 8.9 μM (mean ± S.E.M., n = 4 separate experiments) and 206.7 ± 29.7 μM (mean ± S.E.M., n = 4 separate experiments), respectively.

Growth inhibition of YD-38 cells and NHOKs by JPH203

To examine the effects of JPH203 and BCH on the viability of YD-38 cells and NHOKs, the cells were treated with JPH203 or BCH at various concentrations and incubation times and then analyzed using an MTT assay. As shown in Fig. 4A, when the cells were treated with 0.01 – 30 mM JPH203 for 1 – 4 days, JPH203 completely inhibited the proliferation of YD-38 cells in a dose- and time-dependent manner. In contrast, JPH203 only slightly inhibited the proliferation of NHOKs, which are the primary normal oral keratinocytes in the human oral cavity (Fig. 4B). From 0.1 to 30 mM BCH, the inhibition of YD-38 cell growth was dependent on the BCH treatment time and concentration (Fig. 4C). When the NHOKs were treated with BCH at 0.1, 0.3, 1.0, 3.0, 10, and 30 mM, BCH inhibited the proliferation of NHOKs in a dose- and time-dependent manner (Fig. 4D). Table 1 lists the IC50 values of the JPH203 and BCH in YD-38 cells and NHOKs at set time points.

Apoptosis induction of YD-38 cells by JPH203

To determine if JPH203-induced cell death is associated with the induction of apoptosis, the YD-38 cells were stimulated with 3.0 mM JPH203 for 24 h and then co-stained with Annexin V–FITC, an apoptotic marker, and PI, a necrotic marker (26). As shown in Fig. 5, the percentage of Annexin V–FITC-positive cells at both the early and late stages of apoptosis was increased up to 16.5% by JPH203 compared to the control.

Activation of PARP and caspases by JPH203 in YD-38 cells

The immunoblotting assays for the expression of the apoptotic factors, such as PARP and cleaved caspase-3,
Fig. 4. Effects of JPH203 and BCH on the cell viability in YD-38 cells and NHOKs. The YD-38 cells and NHOKs were treated with various concentrations of JPH203 (A: YD-38 cells, B: NHOKs) or BCH (C: YD-38 cells, D: NHOKs) for 1 – 4 days. The cell viabilities were determined by MTT assays. The percentage cell viability was calculated as a ratio of the A570 nm of JPH203-treated cells and untreated control cells. Each data point represents the mean ± S.E.M. of 4 experiments. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. control (the control cells were measured in the absence of JPH203 or BCH treatment).

Table 1. Anti-proliferative effect of JPH203 and BCH in YD-38 cells and NHOKs

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<tr>
<th>days</th>
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<td>YD-38 cells</td>
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<td>1</td>
<td>3.50 ± 0.42</td>
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<td>2</td>
<td>0.69 ± 0.08</td>
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<td>3</td>
<td>0.19 ± 0.02</td>
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<td>4</td>
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The IC50 values represent the mean ± S.E.M. for 4 experiments.
were performed to confirm that JPH203-induced apoptosis in YD-38 oral cancer cells. As shown in Fig. 6, the level of cleaved PARP (85 kDa) was increased by JPH203 compared to the control. In addition, cleaved caspase-3, -7, and -9 were expressed strongly by JPH203 compared to the control (Fig. 6). These results suggest that JPH203-induced apoptosis was regulated by the activation of the caspases cascade in the YD-38 oral cancer cells.

**Discussion**

JPH203 is a novel tyrosine analog with established LAT1 selectivity (22, 23). This study examined the effects of JPH203 on cell growth as well as its growth suppression mechanism in human oral cancer cells. RT-PCR and real-time qRT-PCR analyses (Fig. 2: A and B) showed that the YD-38 cells express LAT1, an isoform of system L amino acid transporter, together with its associating protein 4F2hc, but do not express the other system L isoform LAT2. Also, immunofluorescence microscopic analysis showed that LAT1 protein or LAT2 protein is present in the plasma membrane of YD-38 cells or NHOKs with 4F2hc protein, respectively (Fig. 2C). In previous studies, the KB human oral cancer cells and C6 rat glioma cells expressed only LAT1 with 4F2hc in the plasma membrane of YD-38 cells or NHOKs with 4F2hc protein, respectively (21, 24, 29, 30). In another study, in contrast to the YD-38 and KB cells, NHOKs expressed both LAT1 and LAT2 together with 4F2hc, and the expression of LAT1 was particularly weak in the NHOKs (31). In another study, the expression of the LAT1 protein increased as the tissues pro-
progressed, from normal to hyperplasia and from dysplasia to squamous cell carcinomas (32). Moreover, the LAT1 protein was observed in most of the tumor cells in squamous cell carcinomas (32). Besides, Sakata et al. proposed that elevated LAT1 expression in biopsy samples of prostate cancers is a novel biomarker for high-grade malignancy, and LAT1 immunostaining of prostate cancer may be a useful approach for predicting clinical outcome before surgery (33). These results correspond to those of previous studies (21, 24, 29 – 32). This shows that the YD-38 human oral cancer cells, as cancer cells, express only LAT1, but not LAT2, along with 4F2hc in the plasma membrane, and NHOKs, as normal cells, express both LAT1 and LAT2 with 4F2hc in the plasma membrane but the expression of LAT1 is quite weak.

In the present study, JPH203 completely and slightly inhibited the l-leucine uptake in YD-38 cells (IC$_{50}$ value: 0.79 μM) and NHOKs (IC$_{50}$ value: > 100 μM), respectively (Fig. 3A). BCH inhibited the l-leucine uptake in YD-38 cells (IC$_{50}$ value: 92.6 μM) and NHOKs (IC$_{50}$ value: 206.7 μM) (Fig. 3B). The affinity for JPH203 and BCH against l-leucine transport was higher in the YD-38 cells than in the NHOKs. Interestingly, the affinity for JPH203 against l-leucine transport was much higher than that for BCH in YD-38 cells. The apparent potency of JPH203 was more than 117 times higher than that of BCH. YD-38 cells expressed only LAT1 as the system L amino acid transporter, whereas, the NHOKs expressed both LAT1 and LAT2 (31). In NHOKs, however, the level of LAT1 expression was quite low (31). These results suggest that LAT1 plays an important role in neutral amino acid transport, including l-leucine transport, in YD-38 cells as cancer cells that strongly express LAT1 only. On the other hand, LAT2 plays an important role in neutral amino acid transport in NHOKs, which are normal cells that strongly express LAT2 (31). Moreover, these results confirm that JPH203 has high selectivity to LAT1.

In the MTT assay, JPH203 completely and slightly inhibited cell growth in YD-38 cells and NHOKs, respectively (Fig. 4: A and B, Table 1), and each affinity of JPH203 against cell growth inhibition was higher in the YD-38 cells than in the NHOKs. BCH also inhibited cell growth in YD-38 cells and NHOKs (Fig. 4: C and D, Table 1). In particular, the affinity for JPH203 against cell growth inhibition was higher than that for BCH in YD-38 cells, which corresponded to the results of the l-leucine uptake experiments in this study. The apparent potencies of JPH203 were > 19-fold (2 days after compound treatment), > 30-fold (3 days after compound treatment), and > 26-fold (4 days after compound treatment) higher than that of BCH. LAT1 is up-regulated in cancer cells to support their continuous growth and proliferation (7, 8, 13, 14). LAT1 was also expressed strongly in YD-38 cells, and neutral amino acids were transported for cell growth and proliferation. If the activity of LAT1 is inhibited by JPH203, the intracellular depletion of neutral amino acids, including the essential amino acids necessary for cell growth, may be induced (34). The results suggest that cancer cell growth was inhibited.

Although 100 μM JPH203 inhibited the l-leucine uptake completely (IC$_{50}$ value: 0.79 μM) in YD-38 cells, it was not enough to suppress cell growth (IC$_{50}$ value: 69 μM) (Figs. 3A and 4A); this represents an 87-fold difference in susceptibility. Oda et al. reported similar results in HT-29 human colorectal adenocarcinoma cells and is likely a result of highly protein-bound drug resulting in much lower free fraction concentrations (22). JPH203 inhibited HT-29 cell growth, generating an apparent IC$_{50}$ of 4.1 μM, but the JPH203 IC$_{50}$ concentration (0.06 μM) needed to inhibit the l-leucine uptake did not inhibit HT-29 cell growth, which represents a 68-fold difference in susceptibility (22). From this result, Oda et al. suggested that the differences in the IC$_{50}$ value are due to the amino acid concentrations (the substrates of LAT1) used in the incubation medium (22). In addition, they also suggested that JPH203 inhibits LAT1 by competing with its substrates (22). Therefore, the degree of inhibition was greatly influenced by the concentration of the substrates (22). As the results in this study correspond well to those in the study of Oda et al. (22), it is believed that the uptake experiment is a short term view, whereas cell growth inhibition and apoptosis induction is a long term view because JPH203 competes with extracellular amino acids and slowly induces cell growth inhibition and apoptosis via caspase activation. The blockade of LAT1 by JPH203 as a selective compound to LAT1 in the YD-38 cells might induce the intracellular depletion of neutral amino acids containing the essential amino acids necessary for cell growth. As the results, it was thought that the inhibition of YD-38 cell growth was induced.

Oda et al. reported that JPH203 inhibited the l-leucine uptake (IC$_{50}$: 0.06 μM) and cell growth (IC$_{50}$: 4.1 μM) in HT-29 human colorectal adenocarcinoma cells (22). In the present study, JPH203 also inhibited the l-leucine uptake (IC$_{50}$: 0.79 μM) and cell growth (IC$_{50}$: 69 μM). On the other hand, the affinity for JPH203 against cell growth inhibition in YD-38 cells was lower than in HT-29 cells, which represents a 17-fold difference in susceptibility (22). As shown in Fig. 2, the mRNA levels of LAT1 and 4F2hc in HT-29 cells were higher than those of LAT1 and 4F2hc in YD-38 cells. These results suggest the affinity difference for JPH203 against cell
growth inhibition between YD-38 cells and HT-29 cells came from the difference in mRNA levels between LAT1 and 4F2hc in two cell types and the distinction of the cell type. More research will be needed to determine the affinity difference for JPH203 against cell growth inhibition between YD-38 cells and HT-29 cells.

In this study, the percentage of Annexin V–FITC-positive cells at both the early and late stages of apoptosis were increased significantly compared to the control in the YD-38 cells stimulated with JPH203, as shown in Fig. 5. These results suggest that JPH203 significantly induced YD-38 cell apoptosis.

The activation of a family of intracellular cysteine proteases, called caspases, plays an important role in the initiation and execution of apoptosis induced by a range of stimuli (35, 36). Among the caspases identified in mammalian cells, caspase-3, -7, and -9 may serve as effector caspases of apoptotic cell death (26, 35, 36). Caspase-3, -7, and -9 were synthesized as inactive proenzymes (sizes 32, 35, and 47 kDa, respectively), which require proteolytic activation to cleaved enzymes (sizes 17, 20, and 37 kDa, respectively) (26, 35, 36). In this study, the immunoblotting assays for the expression of the apoptotic factors (PARP and cleaved caspase-3, -7, and -9) were performed to confirm the JPH203-induced apoptosis in YD-38 cells. As shown in Fig. 6, JPH203 upregulated the population of apoptotic YD-38 cells through the activation of apoptotic factors associated with the apoptosis signaling pathway, such as caspase-3, -7, and -9, and PARP. These results suggest that JPH203 induces the apoptotic cell death of YD-38 cells through caspase-3, -7, and -9-dependent processing. On the other hand, the mechanisms of apoptosis induced by JPH203 in YD-38 cells are not completely understood. Further studies will be needed to reveal the precise cellular and molecular mechanisms of apoptosis induced by JPH203.

In conclusion, inhibition of the amino acid transporter LAT1 by JPH203 led to apoptotic cell death in YD-38 human oral cancer cells by inducing the intracellular depletion of essential neutral amino acids, such as L-leucine. To our knowledge, this study using human oral cancer cells is the first report to demonstrate the in vitro anti oral cancer effects of JPH203, which is a selective LAT1 inhibitor. Future work will describe the additional JPH203 in vitro and in vivo studies as well as its potential development as a chemotherapeutic agent for the management of oral cancer.

Acknowledgements

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science, and Technology (No. 2012R1A1A2001175).

Conflicts of Interest

The authors have no conflict of interest to disclose.

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