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Induction of Apoptosis by JPH203, an L-type Amino Acid Transporter 1 Selective Compound, in YD-38 Human Oral Cancer Cells

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이 논문을 치의학 박사학위신청 논문으로 제출함.

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윤 대 웅

지도교수: 오지수

조선대학교 대학원 치의학과

아미노산은 단백질 합성의 기질이 되며, 효소, 호르몬 및 신경전달물질들의 합성에 필수적이다. 세포에서 필요한 아미노산의 수송은 세포막에 위치한 아미노산 수송체를 통하여 이루어진다. 아미노산 수송계 L은 여러 필수아미노산을 포함한 중성아미노산을 수송하는 세포막 단백질로서, 암세포를 포함한 대부분의 세포에서 중성아미노산의 주 경로가 되는 아미노산 수송체이다. 아미노산 수송계 L은 L-type amino acid transporter 1(LAT1)과 L-type amino acid transporter 2(LAT2)의 두 아형이 존재한다. LAT1은 암세포와 같이 세포 내 대사가 특이적으로 항진되거나 계속적인 증식과 성장이 필수적인 세포에서 과발현되어 세포의 계속되는 성장에 중요한 역할을 한다고 알려져 있으며, LAT2는 정상조직에서 주로 발현한다. 따라서 본 연구에서는 사람 구강암 세포 YD-38을 이용하여 LAT1의 선택적 화합물인 JPH203의 구강암세포 성장억제에 미치는 효과와 세포성장 억제기전을 밝히고자 하며, 아울러 아미노산 수송체LAT1 억제를 통한 구강암 치료의 효용성을 제시하고자 한다.

본 연구에서 사람 구강암 세포 YD-38을 이용하여 LAT1의 선택적 화합물인 JPH203의 구강암세포 성장억제에 미치는 효과와 세포성장 억제기전을 밝히기 위해, YD-38 세포에서 RT-PCR 분석, 정량 PCR 분석, 아미노산 수송분석, MTT 분석, flow cytometry 분석 및 immunoblotting 등을 시행하여 다음과 같은 결과를 얻었다.

1. YD-38 세포에서 아미노산 수송체 LAT1 및 그 보조인자 4F2hc의 발현은 확인 할 수 있었으나, LAT2의 발현은 관찰할 수 없었다.
2. YD-38 세포에서 L-leucine의 수송은 LAT1 선택적 화합물인 JPH203과 아미노산 수송계 L 억제제인 BCH에 의해 완전히 차단되었으며, 그 효능은 JPH203이 더 뚜렷하였다.
3. JPH203과 BCH는 YD-38 세포의 성장을 억제시켰으며, 그 효능은 JPH203이 더 뚜렷하였다.
4. YD-38 세포에 JPH203을 처리한 실험군에서 활성화된 caspase-3, -7, -9 및 PARP의 증가를 확인할 수 있었다.
5. JPH203은 YD-38 세포의 apoptosis를 유도하였다.

본 연구의 결과로서 사람 구강암 세포 YD-38에서 LAT1 선택적 화합물인 JPH203은 LAT1 활성을 억제하여 세포성장에 필수적인 L-leucine 등 중성아미노산의 세포 내 고갈을 유도함으로서 YD-38 세포의 apoptosis를 유도하는 것으로 사료된다. 또한 본 연구의 결과로 LAT1 선택적 화합물을 이용한 구강암 세포의 성장억제에 관한 하나의 방향을 제시 할 수 있을 것으로 사료된다.

중심어: JPH203, L-type amino acid transporter, Cell death, Apoptosis, Oral cancer cells, Anti-cancer therapy

I. INTRODUCTION

Oral cancer is one of the major worldwide public health problems that can affect any part of the oral cavity, including the lips, tongue, mouth and throat.[1,2] Despite the introduction of novel therapeutic modalities to the treatment of oral cancer, improvements in the long-term survival rates have been modest.[3] Despite the earlier detection of oral cancer, the survival rates have not changed significantly over the past two decades, and this cancer remain among the worst of all cancer sites.[2,3] Therefore, improved understanding of the underlying mechanisms of oral cancer is necessary for increasing the survival rates.

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 for 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 activity in tumor cells is blocked completely, the growth and proliferation of normal cells would still possible due to the presence of LAT2.[18] On the other hand, the mechanism by which the inhibition of LAT1 can cause cancer cell growth suppression or the cytotoxicity of cancer cells is not entirely clear.

2-Aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH) is a model compound for a study of amino acid transporters because it is a system L selective inhibitor, and inhibits both LAT1 and LAT2.[7-9,19,20] Therefore, it lacks selectivity toward LAT1. The system L amino acid transporters transport neutral amino acids, including several essential amino acids.[4,6] The cells would be damaged if these transporters are blocked in living cells by a specific 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 to 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 induce the suppression of cell growth and cell apoptosis in YD-38 human oral cancer cells.

Ⅱ. MATERIALS AND METHODS

1. Materials

[^{14}C]L-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 (St Louis, MO, USA). Anti-caspase-3, anti-caspase-7, anti-caspase-9, poly (ADP-ribose) polymerase (PARP) and β-actin antibodies were supplied by Cell Signaling Technology, Inc. (Danvers, MA, USA). JPH203 HCl salt (purity > 99%) was obtained from J-Pharma (Fig. 1, Tokyo, Japan). All other reagents were of analytical grade.

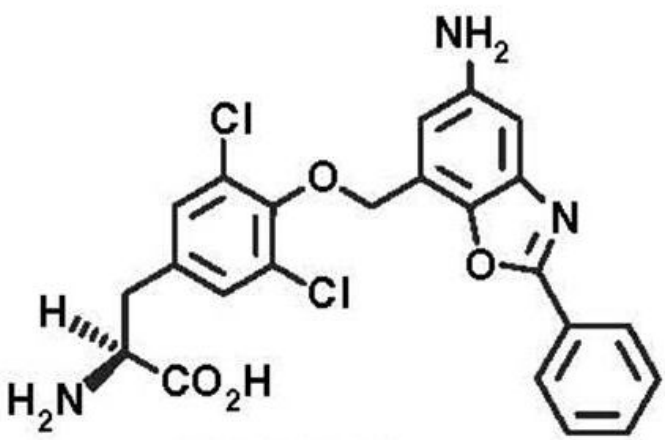

Fig. 1. Chemical structure of JPH203.

2. 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 (CloneticsCorp., 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, CA, USA). The NHOKs, YD-38 cells and HT-29 cells were maintained as monolayers in plastic culture plates at 37°C in a humidified atmosphere containing 5% CO_2.

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

The total RNA was prepared from YD-38 cells and HT-29 cells maintained in a growth medium at 37°C using a RNA preparation kit (Isogen, Nippon-Gene, Japan) according to the manufacturer's instruction. For RT-PCR analysis, the first-strand cDNA was prepared from the cell total RNAs using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies Inc., CA, USA) with an oligo dT primer, and used as a template for PCR amplification. PCR amplification was performed using Taq polymerase Amplitaq Gold (Roche Molecular

Systems, Inc., Germany) according to the following protocol: 94°C for 12 min, followed by 25 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 45 sec and a final extension step of 72°C for 10 min. A pair of primers, 5′-CAAGGACATCTTCTCCGTCATC-3′ (1242-1263 bp) and 5′-AGCCACTTGGGC TTGTTT-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′-GGTGGAGCTGAATGAGTT AGAG-3′ (594-1054 bp) and 5′-CGACATCATCCTTCTGGTTCTT-3′ (1075-1054 bp), were used for the PCR amplification of human 4F2hc.

4. 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′-AGCCACTTGGGCTTGTTT-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′-CGACATCATCCTTCTGGTT CTT-3′, respectively. The qRT-PCR results, which were recorded as the threshold cycle numbers (Ct), were normalized against the internal control (GAPDH). The deviations in the samples were obtained from the results of three separate experiments.

5. 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 [^{14}C]L-leucine, one of the substrates of system L amino acid transporters and a model neutral amino acid, as described previously.[21] Because the uptake of amino acids induced by system L amino acid transporters is not dependent on Na^+,[21] the uptake experiments were performed in Na^+-free uptake solution (125 mM choline-Cl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM HEPES, 1.2 mM KH_2PO_4, 5.6 mM glucose, pH 7.4). The cells were maintained in growth medium at 37°C in 5% CO_2. The cells were collected and seeded on 24-well plates (1 X 10^5 cells/well) in fresh growth medium. The uptake measurements were performed when the cells reached approximately 85~95% confluence on 24-well plates. After the removal of growth medium, the cells were washed three times with Na^+-free uptake solution and pre-incubated for 10 min at 37°C. Then, the medium was replaced by the uptake solution containing [^{14}C]L-leucine. Because the uptake of [^{14}C]L-leucine induced by system L amino acid transporters was time-dependent and exhibited a linear dependence on the incubation time up to 1 min,[21] the uptake measurements were performed for 1 min. Uptake was terminated by removing the uptake solution followed by washing three times with ice-cold uptake solution. Then, cells were solubilized with 0.1 N NaOH, and radioactivity was counted by liquid scintillation spectrometry. The values are expressed as percentage of activity. For the measurement of the uptake of [^{14}C]L-leucine, four to six wells of cells were used for each data point. To confirm the reproducibility of the results, four separate

experiments were performed for each measurement. Results from the representative experiments are shown. To determine the IC_{50} values for JPH203 and BCH, the uptake of 1 μM [^{14}C]L-leucine was measured in the presence of JPH203 (0, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30 and 100 μM) and BCH (0, 1, 3, 10, 30, 100, 300, 1000 and 3000 μM), respectively.

6. 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 X 10^3 cells/well in 24-well plates. After 24 hours 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.

7. Annexin V-fluorescein isothiocyanate (V-FITC), propodium iodie (PI) and flow cytometry analysis

Apoptosis was determined using an Annexin V-FITC 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-FITC and 7-amino-actinomycin D (BD Biosciences, San Diego, CA, USA) were added to the cells, which were then incubated in the dark for 15 minutes. Subsequently, the cells were resuspended in 400 $\mu\ell$ 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, San Jose, CA, USA).

8. Immunoblotting

To determine the level of activated caspase-3, -7, -9 and PARP in YD-38 cells treated with JPH203, the proteins were extracted, as described previously.[18] Briefly, YD-38 cells were cultured in 6-cm dishes. After the cell reached confluence, they were treated with 3 mM of JPH203 for 24 hours. After incubation, cells were washed twice with ice-cold PBS and lyzed using a RIPA buffer for protein extraction following the instructions provided by the manufacturer. Total protein concentrations were determined by a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA). An equal amount of protein was resolved by 10% SDS-PAGE and transferred to nitrocellulose membrane for immunoblotting as described previously.[18] The anti-caspase-3, -7, -9 (1:1000 dilution) and PARP (1:2000 dilution) antibodies were used as the primary antibody. Immunoreactivity was visualized using the ECL system (Amersham Biosciences, Piscataway, NJ, USA) and the Signal Visual Enhancer System (Pierce, Rockford, IL, USA) to magnify the signal.

9. Data Analysis

All experiments were performed at least in triplicate. The results were

presented as the mean ± SEM. Statistical significance was analyzed using a Student's t-test for two groups and one way analysis of variance for multi-group comparisons using StatView version 5.0 for Windows. A P value <0.05 was considered statistically significant.

Ⅲ. RESULTS

1. 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).

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. 2C). The mRNA levels of LAT1 and 4F2hc were significantly higher in the HT-29 cells than in the YD-38 cells (Fig. 2D).

2. Inhibition of L-leucine uptake by JPH203 in YD-38 cells

To examine which JPH203 and BCH concentration interacts with the L-leucine uptake mechanism in YD-38 cells and NHOKs, the [^{14}C]L-leucine (1 μM) uptake was measured in the presence of various JPH203 concentrations (0～100 μM) and BCH concentrations (0～3000 μM). As shown in Fig. 3A, JPH203 (0.001～

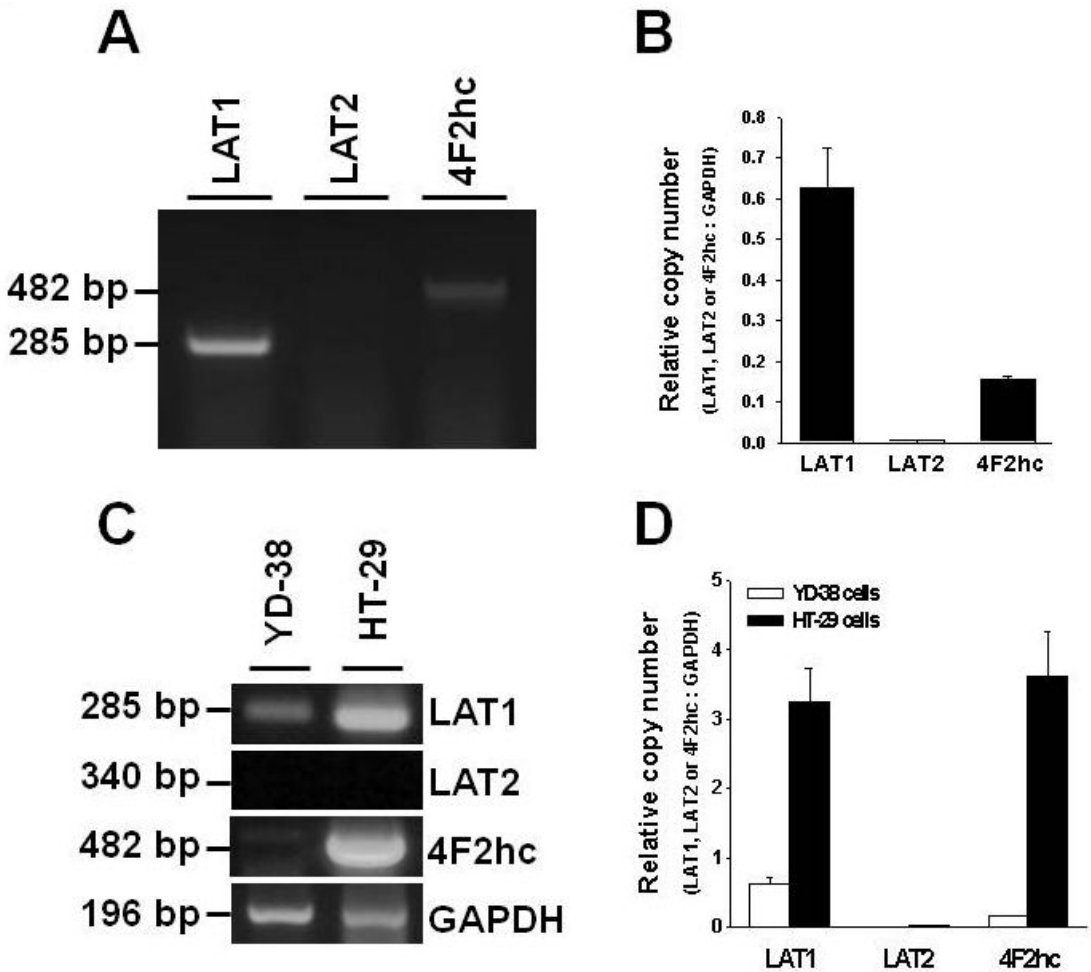

Fig. 2. Detection of LAT1, LAT2 and 4F2hc by RT-PCR and real-time qRT-PCR in YD-38 cells and HT-29 cells. (A) Detection of LAT1, LAT2 and 4F2hc by RT-PCR in YD-38 cells. The first strand cDNA prepared from the YD-38 cell total RNA was used as a template for PCR amplification. The PCR products were electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide. The LAT1-specific PCR product

(285 bp) and 4F2hc-specific PCR product (482 bp) were obtained from YD-38 cells. (B) Detection of LAT1, LAT2 and 4F2hc by real-time qRT-PCR in YD-38 cells. The qRT-PCR was performed as described in "MATERIALS AND METHODS". The qRT-PCR results, which were recorded as threshold cycle numbers (Ct), were normalized against an internal control (GAPDH). The deviations in the samples represent three separate experiments. (C) Comparison of the expression of LAT1, LAT2 and 4F2hc by RT-PCR in YD-38 cells and HT-29 cells. (D) Comparison of the expression of LAT1, LAT2 and 4F2hc by real-time qRT-PCR in YD-38 cells and HT-29 cells.

100 μM) inhibited the [^{14}C]L-leucine (1 μM) uptake slightly in the NHOKs. In contrast, JPH203 (0.001～100 μM) inhibited the [^{14}C]L-leucine (1 μM) uptake in a concentration-dependent manner in the YD-38 cells with an IC_{50} value of 0.79 ± 0.06 μM (mean ± SEM of four separate experiments). As shown in Fig. 3B, the BCH (1～3000 μM) inhibited the [^{14}C]L-leucine (1 μM) uptake in the YD-38 cells and NHOKs in a concentration-dependent manner with IC_{50} values of 92.6 ± 8.9 μM (mean ± SEM of four separate experiments) and 206.7 ± 29.7 μM (mean ± SEM of four separate experiments), respectively.

3. 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 analyzed using a MTT assay. As shown in Fig. 4A, when the cells were treated with 0.01 to 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, 3, 10 and 30 mM, BCH inhibited the proliferation of NHOKs in a dose- and time-dependent manner (Fig. 4D). Table 1 lists the IC_{50} values of the JPH203 and BCH in YD-38 cells and NHOKs at set time points.

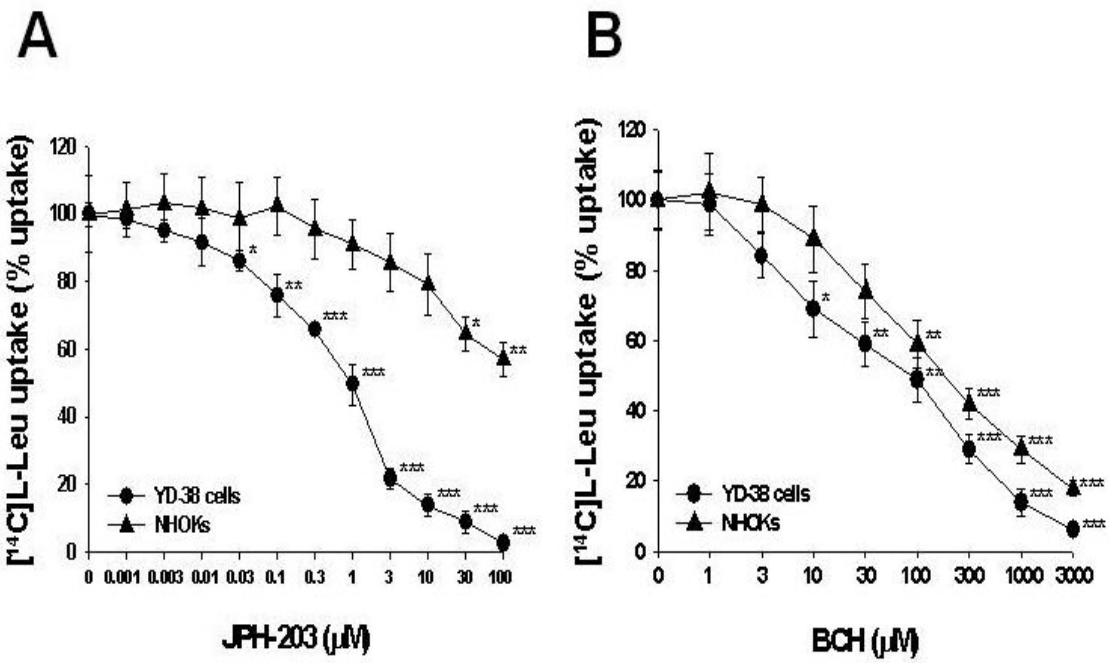

Fig. 3. **Concentration-dependent inhibition of [^{14}C]L-leucine uptake by JPH203 and BCH in YD-38 cells and NHOKs.** [^{14}C]L-leucine uptake (1 μM) was measured for 1 min in the presence of various JPH203 (A) and BCH (B) concentrations in YD-38 cells and NHOKs, and was expressed as a percentage of the control L-leucine uptake in the absence of JPH203 or BCH. Each data point represents the mean ± SEM for four experiments. $^{*}P<0.05$ vs. control, $^{**}P<0.01$ vs. control and $^{***}P<0.001$ vs. control (the control cells were measured in the absence of JPH203 or BCH treatment).

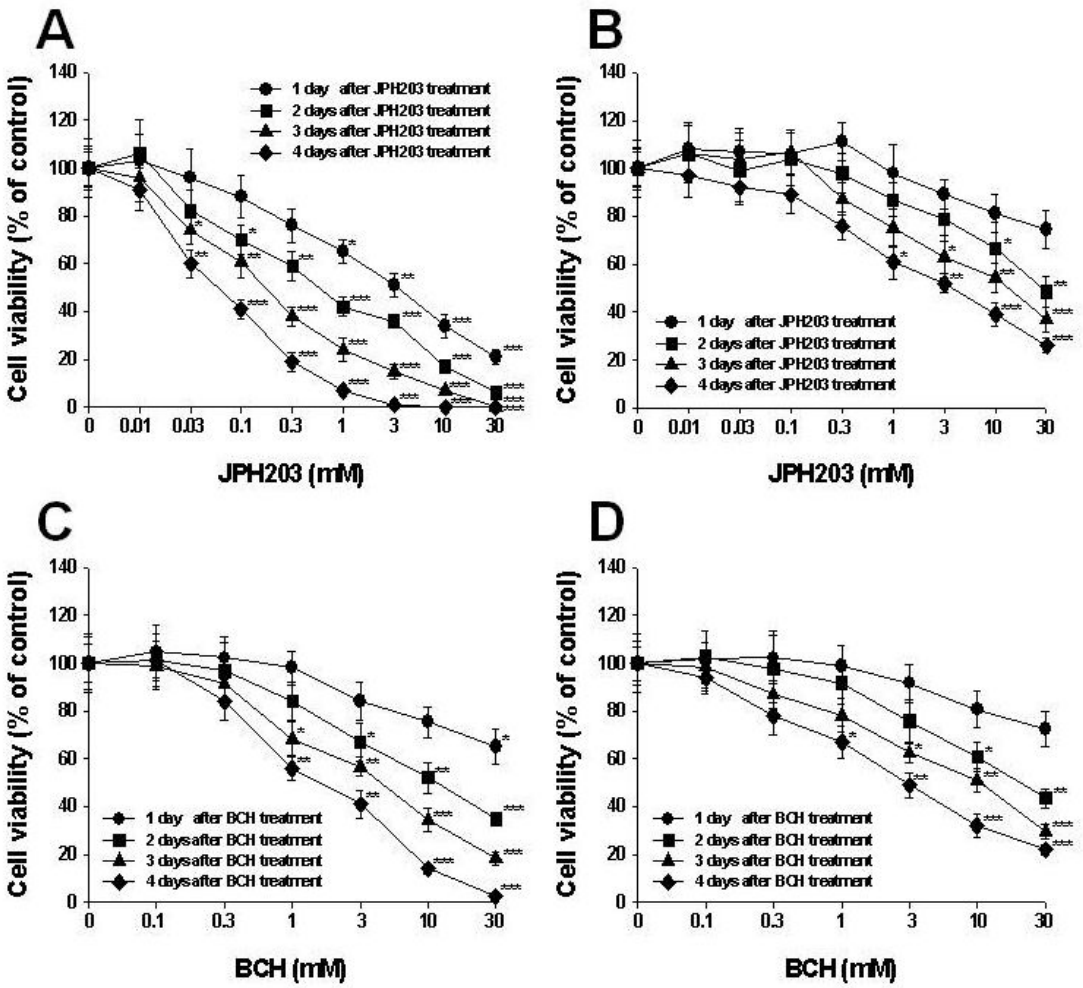

Fig. 4. Effects of JPH203 and BCH the cell viability in YD-38 cells and NHOKs. The YD-38 cells and NHOKs were treated with various concentrations of JPH203 (A; YD-38 cells, B; NHOKs) or BCH (C; YD-38 cells, D; NHOKs) for 1~4 days. The cell viabilities were determined by MTT assays. The percentage cell viability was calculated as a ratio of the A570 nms of JPH203 treated cells and untreated control cells. Each data point represents the mean ± SEM of four experiments. $^{*}P<0.05$ vs. control, $^{**}P<0.01$ vs. control 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

days	IC_{50} (mM)			
	JPH203		BCH	
	YD-38 cells	NHOKs	YD-38 cells	NHOKs
1	3.50 ± 0.42	> 30	> 30	> 30
2	0.69 ± 0.08	28.3 ± 5.9	13.1 ± 3.1	22.4 ± 5.4
3	0.19 ± 0.02	15.8 ± 2.9	5.7 ± 0.9	11.9 ± 2.2
4	0.069 ± 0.007	4.1 ± 0.7	1.8 ± 0.3	3.3 ± 0.5

The IC_{50} values represent the mean ± SEM for four experiments.

4. 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 mM JPH203 for 24 hours and then co-stained with Annexin V-FITC, an apoptotic marker, and PI, a necrotic marker.[25] 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 16.53% by JPH203 compared to the control.

5. 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/-7/-9,[26,27] were performed to confirm the 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.

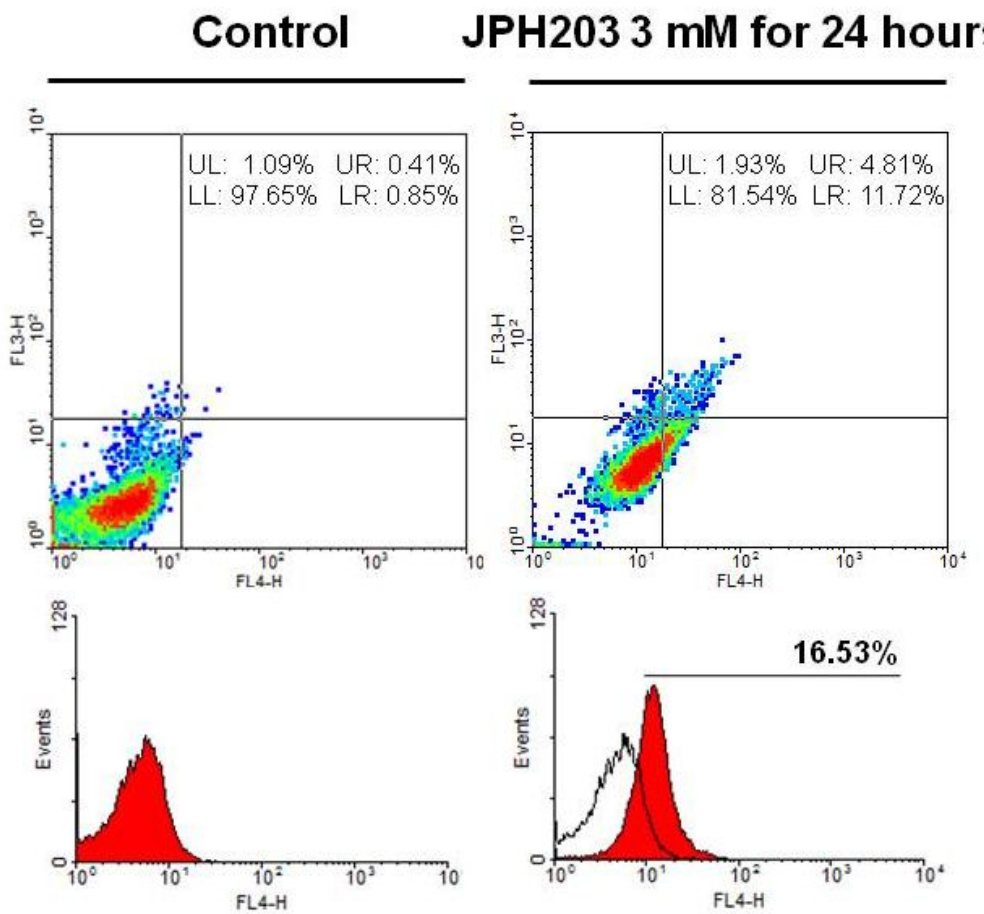

Fig. 5. Apoptotic population induction of YD-38 cells by JPH203. To identify the JPH203-induced YD-38 cell apoptosis, FACS analysis were performed by Annexin-V and PI staining. The YD-38 cells were cultured in complete medium, and stimulated with 3 mM JPH203 for 24 hours. After stimulation, the cells were analyzed by flow cytometry. LL; lower left (normal), UL; upper left (necrosis), LR; lower right (early phase of apoptosis), UR; upper right (late phase of apoptosis).

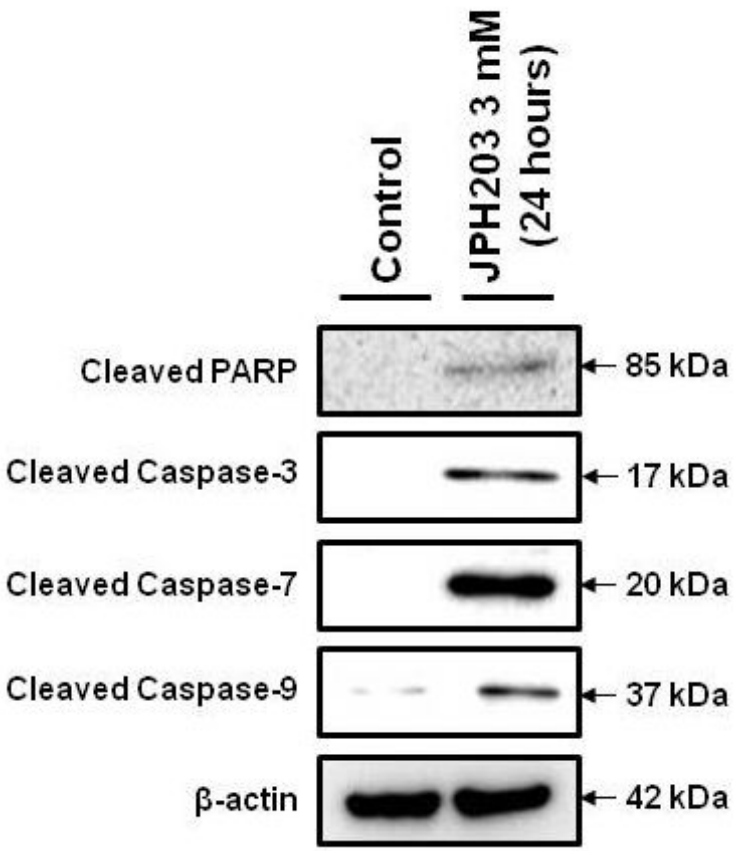

Fig. 6. Expression and activation of the apoptotic proteins by JPH203 in YD-38 cells. The YD-38 cells were stimulated with 3 mM JPH203 for 24 hours. The cell lysate was prepared and analyzed by immunoblotting as described in "MATERIALS AND METHODS".

Ⅳ. DISCUSSION

JPH203 is a novel tyrosine analog with high 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. 2A and 2B) 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. In previous studies, the KB human oral cancer cells and C6 rat glioma cells expressed only LAT1 with 4F2hc in the plasma membrane but LAT2 was not detected, and the Saos2 human osteogenic sarcoma cells expressed mainly LAT1 with 4F2hc as well as weak LAT2 expression.[21,24,28,29] 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.[30] In another study, the expression of the LAT1 protein increased as the tissues progressed, from normal to hyperplasia, and from dysplasia to squamous cell carcinomas.[31] Moreover, the LAT1 protein was observed in most of the tumor cells in squamous cell carcinomas.[31] These results correspond to those of previous studies.[21,24,28-31] This shows that the YD-38 human oral cancer cells, as cancer cells, express only LAT1, but not LAT2, along with 4F2hc, and NHOKs, as normal cells, express both LAT1 and LAT2 with 4F2hc 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 that of BCH. YD-38 cells expressed only LAT1 as the system L amino acid transporter, whereas, the NHOKs expressed both LAT1 and LAT2.[30] In NHOKs, however, the level of LAT1 expression was quite low.[30] 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.[30] Moreover, these results suggest 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. 4A and 4B, Table 1), and the each affinity for 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. 4C and 4D, 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.[32] 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.[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 being greatly influenced by the concentration of the substrates.[22] As the results in this study correspond to the Oda *et al.*'s study,[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 compete with extracellular amino acids and slowly induced 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 ($IC50$ 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.[33,34] Among the caspases identified in mammalian cells, caspase-3, -7 and -9 may serve as effector caspases of apoptotic cell death.[26,33,34] Caspase-3, -7 and -9 were synthesized as inactive proenzymes (sizes 32 kDa, 35 kDa and 47

kDa, respectively), which require proteolytic activation to cleaved enzymes (sizes 17 kDa, 20 kDa and 37 kDa, respectively).[26,33,34] In the present study, the immunoblotting assays for the expression of the apoptotic factors (PARP and cleaved caspase-3/-7/-9) were performed to confirm the JPH203-induced apoptosis in YD-38 cells. As shown in Fig. 6, JPH203 up-regulated the population of apoptotic YD-38 cells through the activation of apoptotic factors associated with the apoptosis signaling pathway, such as caspase-3, -7, -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. 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.

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- ABSTRACT -

Induction of Apoptosis by JPH203, an L-type Amino Acid Transporter 1 Selective Compound, in YD-38 Human Oral Cancer Cells

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Amino acids are required for protein synthesis and energy sources in all living cells. The amino acid transport system L is a major nutrient transport system that is responsible for transport of neutral amino acids including several essential amino acids. The system L is divided into 2 major subgroups, the L-type amino acid transporter 1 (LAT1) and the L-type amino acid transporter 2 (LAT2). In cancer cells, the LAT1 is expressed strongly to support their continuous growth and proliferation. This study examined the effects of JPH203, an LAT1 selective compound, and the mechanism through which JPH203 suppresses cell growth in YD-38 human oral cancer cells.

The effect of JPH203 and the mechanism of JPH203 on cell growth suppression in YD-38 cells were examined by RT-PCR and qRT-PCR analyses,

amino acid transport measurements, MTT assay, flow cytometry analysis and immunoblotting. The results are as follows.

1. The YD-38 cells express LAT1 with its associating protein 4F2 heavy chain (4F2hc), but not LAT2.
2. JPH203 and BCH completely inhibited L-leucine uptake in YD-38 cells, and the affinity for JPH203 against L-leucine transport was much higher than that for BCH.
3. JPH203 and BCH inhibited cell growth in YD-38 cells, and the affinity for JPH203 against cell growth inhibition was higher than that for BCH.
4. The percentage of Annexin V-FITC-positive cells at both early and late stage of apoptosis were significantly increased by JPH203 compared with control in YD-38 cells.
5. JPH203 up-regulated the population of apoptotic YD-38 cells through activation of apoptotic factors such as caspase-3, caspase-7, caspase-9 and PARP.

These results suggest that the inhibition of LAT1 activity by JPH203 leads to apoptotic cancer cell death by inducing intracellular depletion of the neutral amino acids essential for cancer cell growth in YD-38 human oral cancer cells. Overall, JPH203 appears to have potential as a novel anti-oral cancer agent, presumably via selective LAT1 inhibition.

KEY WORDS: JPH203, L-type amino acid transporter, Cell death, Apoptosis, Oral cancer cells, Anti-cancer therapy