Development of photodynamic diagnosis and objective screening methods for oral squamous cell carcinoma using 5-aminolevulinic acid and a lumine plate reader

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Preface

This article is based on a main reference paper, "Development of a Photodynamic Diagnosis Method for Oral Squamous Cell Carcinoma Using 5-Aminolevulinic Acid and a Luminescence Plate Reader" in the Open Journal of Stomatology (vol.11 No.9, 325-340, 2021), reference papers, "Establishment of an objective screening method for oral malignant tumors using a photodynamic technique involving a fluorescence plate reader" in the Journal of Japanese Society for Evidence and the Dental Professional scheduled to be published.

Abstract

Purpose

<u>Study 1)</u> To establish a simple and accurate photodynamic diagnosis (PDD) method for human oral squamous cell carcinoma (OSCC).

<u>Study 2)</u> To accumulation of PpIX by combining 5-ALA with the heme synthesis inhibitor deferoxamine (DFO) and using a fluorescence plate reader to measure fluorescence intensities efficiently in fewer cells.

<u>Methods</u>

In Study 1, human OSCC cell lines HSC-2, HSC-3, HSC-4, and Sa3, and oral keratinocytes (HOK) as a control were used. First, we examined the number of cancer cells needed to divide the different fluorescence intensities between OSCC and HOK for PDD. The fluorescence intensities of OSCC cells and the control were comprised with/without 5-ALA.

In study 2, the experimental groups were then defined as 5-ALA (ALA groups) and 5-ALA with 5 mM DFO (ALA+DFO groups), respectively. Cells were irradiated with a wavelength of 405 nm, which is the excitation wavelength of the PpIX produced from 5-ALA, and the emitted fluorescence at a wavelength of 635 nm was measured as the fluorescence intensity.

<u>Results</u>

In study 1, the fluorescence intensities at 1×10^6 cells/mL in 5-ALA (+) groups increased in a metabolic time dependent manner; the highest increase was shown in HSC-2, followed by HSC-4, HSC-3, Sa3, and HOK. Fluorescence intensities were significantly enhanced after 40 min in HSC-2, HSC-3, and HSC-4, after 60 min in Sa3, and after 100 min in HOK compared to the respective 5-ALA (-) groups. Moreover, all OSCC cell lines increased significantly in their fluorescence intensities after 40min compared to a control.

In study 2, the fluorescence intensities of OSCC cell lines and HOK increased in both ALA and ALA+DFO groups in a metabolic time dependent manner, with a significant increase in the ALA+DFO groups compared to the ALA groups in all cell lines. The fluorescence intensities of the OSCC cell lines at 1×10^6 cells exceeded that of HOK after 40 min of metabolism. Furthermore, HSC-2 demonstrated a significant increase in fluorescence intensities after 40 min of metabolism, HSC-4 after 120 min, and HSC-3 and Sa3 after 200 min. In addition, the fluorescence intensities of the OSCC cell lines at 5×10^5 cells exceeded

that of HOK after 120 min of metabolism and increased significantly in all cell lines. Finally, the fluorescence intensities of 1×10^5 cells of the OSCC cell lines was significantly higher than that of HOK after 280 min in HSC-2 and HSC-4, and exceeded that of HOK in all cell lines in metabolism after 440 min.

Conclusion

The combination of 5-ALA and DFO increases the accumulation of PpIX, and in addition, the fluorescence intensity is measured with high sensitivity using a luminescence plate reader. This application may help early detection of oral cancer on the chair side even if a small number of cells.

Keywords: Luminescence Plate Reader, 5-Aminolevulinic Acid (5-ALA), Photodynamic Diagnosis (PDD), Oral Squamous Cell Carcinoma (OSCC), Deferoxamine (DFO)

Introduction

Deaths from oral cancer have been on the rise for about 20 years in Japan [1]. This trend is expected to continue to increase with Japan's super-aging society. Approximately 90% of malignancies occurring in the oral cavity are squamous cell carcinomas [2]. Generally, surgical resection, chemotherapy, and radiation therapy are the treatments of choice for malignant tumors, and oral malignant tumors are no different. Therefore, advanced oral cancer can cause serious dysfunction and aesthetic problems after treatment. The quality of life of patients may become significantly impaired by these factors. For these reasons, early detection and early treatment of oral malignant tumors are extremely important. From the viewpoint of early detection, there is a significant difference between oral malignant tumors and tumor in other parts of the body: The oral cavity can be viewed and reached directly due to its location, so it is possible to determine the local pathological condition by inspection or palpation; however, this is not possible for tumors arising in other areas. Furthermore, when compared to other areas of the body, it is relatively easy to collect specimens from the oral cavity for testing. Despite these huge advantages when compared to tumors in other parts of the body, the number of deaths from oral cancer is on the rise due to the lack of early detection in Japan. A reason for this is the differences in diagnostic techniques used among medical professionals [3] [4]. The basic methods used for the early detection of oral cancer are inspection, palpation, medical consultation, and imaging diagnosis. However, diagnostic evaluation depends on the experience and subjectivity of medical professionals, which can vary greatly between individuals.

Exfoliative cytology is used as an adjunctive diagnostic method to reduce the differences in evaluations among medical professionals. Exfoliative cytology has been reported to be useful in the dental field due to the simplicity of specimen collection, its low invasiveness, and the speed of obtaining test results [5]. However, due to the unique characteristics of the oral cavity, the evaluation of exfoliates from the oral cavity also varies depending on the experience of the cell screener, and the agreement rate with histology results varies greatly depending on the skill level of the individual performing the test. Therefore, it is extremely important to establish a visual quantitative diagnostic method for the early detection of oral cancer that can reduce the disparity in evaluations among medical professionals.

Photodynamic diagnosis (PDD) has been applied for the diagnosis of malignancy and for the intraoperative diagnosis of cancers, and its ability to provide an objective evaluation via fluorescence intensity measurements for determining malignancy has been in the spotlight

in the medical field. PDD is a method of diagnosing tumors by detecting the fluorescence wavelength generated by a photosensitizer (PS) that has been administered into the body and taken up specifically by the tumor; the PS can be detected by irradiation with excitation light [6]. PDD has been investigated as a new technique in several tumors, and the clinical application of PDD using 5-aminolevulinic acid (5-ALA) as the PS has begun in the fields of gastroenterology, urology, and neurosurgery [7] [8] [9] [10]. 5-ALA is naturally present in small amounts in the human body, but it does not fluoresce by itself. When 5-ALA is administered intravitreally, it is taken up by tumors, and protoporphyrin IX (PpIX), a metabolite of 5-ALA, accumulates in the tumor cells (Figure 1). Under an excitation wavelength of 405 nm, PpIX emits fluorescence at 630 nm. PDD using fluorescence detection is effective in diagnosing the presence of tumors. However, in many malignant tumors, the lesions are located in areas that cannot be directly seen or reached, and the procedure is performed using an endoscope or similar device with limited vision and instrumentation.

Although 5-ALA is considered to be a substance with little phototoxicity or biotoxicity since it is naturally present in small amounts in the human body, and is only administered orally beforehand, there remains a possibility that 5-ALA may show some toxicity when administered into the body. Therefore, it is also important to ensure that its uptake is as efficient and harmless as possible.

This led us to focus on the use of exfoliative techniques as specimens from the oral region. We also thought that it would be useful to quantify the fluorescence intensity using a luminescence plate reader to eliminate subjectivity from individual diagnosticians, and to clarify the diagnostic criteria. PDD using a fluorescent plate reader on an exfoliated sample has not been previously reported in the Japanese or foreign literature. Therefore, we believe that the combined use of oral exfoliative cytology and PDD, and the development of quantitative diagnostic criteria for oral squamous cell carcinoma (OSCC) may enable the establishment of a safe and highly accurate chairside method for the diagnosis of oral cancer.

In study 1, we performed PDD using 5-ALA on cultured human OSCC derived cell lines and normal human oral keratinocytes (HOK) to develop a safe and accurate method for oral cancer diagnosis, and measured the fluorescence intensity of each cell line over time by irradiating them with excitation light and measuring the fluorescence intensity with a luminescence plate reader.

In study 2, we aimed to induce the accumulation of PpIX by combining 5-ALA with the heme synthesis inhibitor deferoxamine (DFO) and using a luminescence plate reader to measure fluorescence intensity efficiently in fewer cells.

<u>**Study 1 : Development of a photodynamic diagnosis method for oral squamous cell carcinoma using 5-aminolevulinic acid and a luminescence plate reader**</u>

<u>**Materials & Methods**</u>

1. Cell culture

In this study, we used the human OSCC cell lines HSC-2, HSC-3, HSC-4, and Sa3 (RIKEN, Tokyo, Japan), and human primary cultures of oral keratinocytes (HOK, ScienCell Research Laboratories, Carlsbad, CA, USA) as a normal cell.

Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum and 100 µg/mL penicillin-streptomycin (Gibco, St. Louis, MO, USA) was used for HSC-2, HSC-3, HSC-4, and Sa3, while oral keratinocyte medium containing 5 mL of HOK growth supplement and 5 mL of penicillin-streptomycin solution (ScienCell Research Laboratories) was used for HOK.

The cultures were maintained in an atmosphere of 5% CO_2, 90% N_2, and 5% O_2 at 37˚C. The culture media were changed every 3 days.

When the cultured cells had reached confluence, they were washed twice with phosphate-buffered saline, detached with 0.05% trypsin-0.53 mmol/L ethylenediaminetetraacetic acid-4 sodium solution (Wako Pure Chemical Industries Ltd., Osaka, Japan) in an incubator, and collected by centrifugation. For experimental use, the cells of the 3rd to 6th passages were counted using a hemocytometer, then adjusted to the concentrations of 1×10^4, 1×10^5, and 1×10^6 cells/mL, and 10 µL trypan blue was added to an equal volume of each cell suspension.

2. PS and fluorescence intensity measurement device

5-ALA (MW: 167.6, NeoPharma Japan, Tokyo, Japan) was used as the PS. The 5-ALA was added to Dulbecco's modified Eagle's medium at the final concentration of 2 mM based on a previous report [11]. The fluorescence intensities were measured using a 1420 Multilable/Luminescence Counter ARVO MX (Perkin Elmer Japan Co., Ltd., Kanagawa, Japan). Cells were irradiated with a wavelength of 405 nm, which is the excitation wavelength of the PpIX produced from 5-ALA, and the emitted fluorescence at a wavelength of 635 nm was measured as the fluorescence intensities.

3. Measurement of fluorescence intensity of each cell line by PDD

Each cell line was divided into two groups: A group treated with 5-ALA (5-ALA (+)) and another group without 5-ALA treatment (5-ALA (−)) as the control group. For each cell line, 100 µL serially diluted cell suspension (1×10^4, 1×10^5, or 1×10^6 cells/mL) was added to a 96-well luminescence plate (ParkinElmer Japan Co., Ltd. Kanagawa, Japan), and the fluorescence intensities were measured at 60 min intervals for 240 min. (Figure 2).

4. Statistical analysis

The fluorescence intensities of the cell lines were shown as the mean ± standard deviation (mean ± SD), and comparisons between the 5-ALA (−) group and the 5-ALA (+) group were performed using the Student's *t*-test. For comparisons among cell lines, Scheffé's multiple comparison test was used after analysis of variance. P values < 0.05 were considered to be statistically significant. Analyses were performed using IBM SPSS Statistics ver. 22 (IBM, Tokyo, Japan).

Results

The fluorescence intensities of cells at 1×10^6 cells/mL, which was the concentration that showed the strongest fluorescence intensities, were measured at 20 min intervals for 700 min under the same conditions (n = 7)

The fluorescence intensities obtained from 1×10^4 to 1×10^6 cells/mL for each cell line were shown as the ratio between the 5-ALA (+) groups and the 5-ALA (−) groups (Figures 3A-D).

In all cell lines, the 5-ALA (+)/5-ALA (−) ratios increased in a cell concentration-dependent manner: It was the highest at the concentration of 1×10^6 cells/mL, and the lowest at the concentration of 1×10^4 cells/mL at the metabolic time of 240 min.

At the concentration of 1×10^6 cells/mL, HOK showed a metabolic time dependent increase in the fluorescence intensities in the 5-ALA (+) groups, but not in the 5-ALA (−) groups (Figure 4A). Furthermore, there was a significant difference in the intensity after 100 min. The intensities in the 5-ALA (+) group was increased by about 2.3-fold at 700 min when compared to that immediately after 5-ALA administration. The intensities of HSC-2, HSC-3, and HSC-4 cells showed metabolic time dependent increase in the 5-ALA (+) groups, but not in the 5-ALA (−) groups (data not shown). There were significant differences in the intensity between both after 40 min. Furthermore, the intensities of HSC-2, HSC-3, or HSC-4 cells in the 5-ALA (+) group was increased 20.4-, 9.8-, and 24.4-fold, respectively, at 700 min in

comparison to that immediately after 5-ALA administration (Figures 4B-D). Similarly, in Sa3, the intensities changed in a metabolic time dependent manner in the 5-ALA (+) group, but not in the 5-ALA (−) group (Figure 4E). There was a significant difference in the intensities between both after 60 min. In addition, the intensity in the 5-ALA (+) group was increased by 10.2-fold at 700 min when compared to that immediately after 5-ALA administration.

Among 5-ALA (+) groups, the increase in the fluorescence intensity was the highest in HSC-2, followed by HSC-4, HSC-3, Sa3, and HOK (Figure 5). A significant difference was shown between the HOK and the OSCC cells after 40 min.

<u>Study 2 : Establishment of an objective screening method for oral malignant tumors using a photodynamic technique involving a fluorescence plate reader</u>

<u>Materials & Methods</u>

1. Cell culture

 The human oral squamous cell carcinoma (OSCC) derived cell lines, HSC-2, HSC-3, HSC-4, and Sa3, were obtained from RIKEN, and human primary cultured cells, human oral keratinocytes (HOK), were obtained from ScienCell Research Laboratories.

 The OSCC cell lines were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 10% fetal bovine serum and 100 µg/mL penicillin streptomycin (Gibco) in 10 cm diameter dishes. HOK were cultured in containing 5 mL HOK growth supplement and 5 mL penicillin/streptomycin solution HOK medium (ScienCell Research Laboratories). Then, the cultures were maintained in an atmosphere of 5% CO_2, 90% N_2, and 5% O_2 at 37 °C. The culture media were changed every 3 days.

2. Reagents and fluorescence intensity measuring device

 Next, 5-ALA (Neopharma Japan) as a porphyrin derivative was dissolved in DMEM at a final concentration of 2 mM. The iron-chelating agent DFO (MW: 656.8) was obtained from Cayman Chemical Company (Ann Arbor, MI, USA). A multilabel/luminescence microplate reader (Perkin Elmer Japan Co.) was used to measure fluorescence intensity. The excitation light was irradiated at 405 nm, and a 632 nm filter was set for the fluorescence measurement.

3. Cell viability assay

 Cell viability was determined using a Cell Counting Kit-8 ® (Dojindo Laboratories, Kumamoto, Japan) following the manufacturer's instructions. In brief, the OSCC cell lines (HSC-2, HSC-3, HSC-4, and Sa3) and HOK were seeded into 96-well plates (1×10^4 cells/200 µL medium per well) and maintained for 24 h in an incubator to allow attachment of the cells before adding DFO at final concentrations of 1, 10, 100, 500 µM, and 5 mM, respectively. The control received no DFO. Then, optimal density was measured at 450 nm wavelengths at 6 and 12 h after the addition of DFO and compared with the control.

4. Fluorescence intensity measurement of 5-ALA or 5-ALA with DFO on each cell line

When the OSCC cell lines and HOK reached confluence, the cells were detached with 0.05% trypsin-0.53 mmol/L ethylenediaminetetraacetic acid-4 sodium (Wako) solution and subcultured in culture dishes.

For the experiments, the cells were adjusted to 1×10^5, 5×10^5, and 1×10^6 on a hemocytometer using trypan blue. The experimental groups were then defined as 5-ALA (ALA group) and 5-ALA with 5mM DFO (ALA+DFO group), respectively. Each OSCC cell line and HOK (1×10^5, 5×10^5, and 1×10^6 cells/well) were seeded into 96-well luminescence plates (ParkinElmer Japan Co., Ltd.), and the fluorescence intensities were measured in the above three groups at 20 min intervals for 600 min. The results were shown as a ratios of the increase in fluorescence intensities to the time of metabolic initiation and compared between the ALA and ALA+DFO groups. Next, we compared the fluorescence intensities of HOK and the OSCC cell lines obtained from their respective cell numbers.

5. Statistical analysis

All statistical analyses were performed using SPSS Statistics ver. 22 (IBM, Tokyo, Japan). Dunnett's test was used to compare the results of cell viability on DFO, the fluorescence intensities of HOK and the OSCC cell lines obtained from their respective cell numbers. The difference in fluorescence intensity in each cell between the ALA and ALA+DFO groups was analyzed using Student's *t* test. The level of significance was set at 5% for all analyses.

Results

1. Cell viability assay

Figure 6 showed the cell viability of different concentrations of DFO applied to HOK and the OSCC cell lines for 6 or 12 h. No significant reduction in cell viability was seen in any cell line at 6 h. At 6 h, cell viability was significantly increased by 100, 500 µM and 5mM DFO in Sa3. At 12 h, cell viability was significantly increased by 5 mM DFO in HOK. In addition, no significant difference in cells viability were observed in HSC-2 and HSC-3. However, a significant decrease in cell viability was observed in HSC-4 by addition of DFO.

2. Measurement of fluorescence intensity of ALA only or ALA+DFO in each cell line

The fluorescence intensities of HOK and the OSCC cell lines (1×10^5, 5×10^5, and 1×10^6 cells) increased in a metabolic time dependent manner in the ALA and ALA+DFO groups.

In HOK, the fluorescence intensities ratios at 600 min after the start of metabolism differed the ALA (1.03-, 1.44- and 2.03-fold) and ALA+DFO groups (1.24-, 2.57- and 4.83-fold) at 1×10^5, 5×10^5, and 1×10^6 cells, respectively (Figure 7). Also, a significant difference was observed in the fluorescence intensity ratio between ALA and ALA+DFO after 200, 60, 120 min at 1×10^5, 5×10^5, and 1×10^6 cells, respectively.

In HSC-2, the fluorescence intensities ratios at 600 min after the start of metabolism differed the ALA (1.2-, 3.7-, and 17.1-fold) and ALA+DFO groups (1.8-, 6.4-, and 22.7-fold) at 1×10^5, 5×10^5, and 1×10^6 cells, respectively. At 1×10^5 cells, the fluorescence intensity ratios of the ALA+DFO groups were significantly higher than that of the ALA groups after100 min. Similarly, the fluorescence intensities ratios between both were significantly increased after 40 min for 5×10^5 and 1×10^6 cells (Figure 7B).

In HSC-3, the fluorescence intensities ratios at 600 min after the start of metabolism differed the ALA (1.5-, 3.5-, and 8.6-fold) and ALA+DFO groups (1.8-, 6.7-, and 9.1-fold) at 1×10^5, 5×10^5, and 1×10^6 cells, respectively. At 1×10^5 cells, the fluorescence intensity ratios of the ALA+DFO groups were significantly higher than that of the ALA groups after 260 min. Similarly, the fluorescence intensities ratios between both were significantly increased after 60 min for 5×10^5 cells. However, no significant difference in the fluorescence intensity ratio at 1×10^6 cells were observed between the ALA and ALA+DFO groups (Figure 7C).

In HSC-4, the fluorescence intensities ratios at 600 min after the start of metabolism differed the ALA (2.5-, 10.6-, and 15.5-fold) and ALA+DFO groups (4.2-, 16.5-, and 17.3-fold) at 1×10^5, 5×10^5, and 1×10^6 cells, respectively. At 1×10^5 cells, the fluorescence intensities ratios of the ALA+DFO groups were significantly higher than that of the ALA groups after 120 min. Similarly, the fluorescence intensities ratios between both were significantly increased after 60 min for 5×10^5 cells and after 260, 280, or 420 min for 1×10^6 cells (Figure 7D).

In Sa3, the fluorescence intensities ratios at 600 min after the start of metabolism differed the ALA (1.4-, 3.5-, and 6.7-fold) and ALA+DFO groups (1.8-, 6.7-, and 17.9-fold) at 1×10^5, 5×10^5, and 1×10^6 cells, respectively. At 1×10^5 cells, the fluorescence intensities ratios of the ALA+DFO groups were significantly higher than that of the ALA groups after 260 min. Similarly, the fluorescence intensity ratios between both were significantly increased after 80 min for 5×10^5 cells and after 440 min for 1×10^6 cells (Figure 7E).

The fluorescence intensities of the different cell numbers of the OSCC cell lines and HOK in the ALA+DFO group are shown in Figure 8. First, the fluorescence intensity of the OSCC cell lines at 1×10^6 cells exceeded that of HOK after 40 min of metabolism. In addition, significant increases in the intensity were shown after 40, 120 and 200 min in HSC-2, HSC-4, HSC-3 and respectively (Figure 8A).

Similarly, the fluorescence intensities of the OSCC cell lines at 5×10^5 cells exceeded that of HOK after 120 min of metabolism in all cell lines (Figure 8B). Finally, the fluorescence intensities of 1×10^5 cells of the OSCC cell lines were significantly higher than that of HOK after 280 min in HSC-2 and HSC-4, and exceeded that of HOK in all cell lines in metabolism after 440 min (Figure 8C).

Discussion

Recently, the use of 5-ALA to fluorescently label tumors has attracted much attention [12]. PpIX, a metabolite of 5-ALA, is thought to play a major role in enhancing the fluorescence intensity of tumor cells. The maximum excitation wavelength of PpIX is around 405 nm (blue light), and it fluoresces red light at around 630 nm when excited by excitation light [7] [8] [9] [10]. 5-ALA is a type of amino acid that is found in living organisms. It is a raw material for porphyrins, such as heme, vitamin B12, and chlorophyll, but it is not light-sensitive by itself. After passing through various metabolic pathways in the cytoplasm, 5-ALA becomes PpIX in the mitochondria, and divalent iron ions chelate with PpIX to form heme by the action of ferrochelatase [13]. The expression of ferrochelatase is regulated by the amount of iron in the cytoplasm, and the heme keeps the heme concentration constant via negative feedback [14]. However, in tumor cells, the synthesis of heme from PpIX is insufficient due to decreased activity of ferrochelatase [7]. Therefore, PpIX accumulates in tumor cells, and is observed as strong fluorescence under excitation wavelengths. In addition, the enzymatic activity required for the biosynthesis of PpIX is higher in malignant tumor cells than in normal cells, which may also contribute to the enhanced fluorescence intensity of tumor cells.

From the results of study1, we estimated that a concentration of at least 1×10^6 cells/mL is necessary for evaluation using PDD with a luminescence plate reader, because a remarkable fluorescence intensity is needed for improved diagnostic accuracy. We measured the changes in the fluorescence intensity obtained by incorporating 5-ALA into various cultured OSCC cell lines and HOK at 1×10^6 cells/mL and irradiating them with excitation light. Although there were some differences among the OSCC cell lines, a significant increase in fluorescence intensity was observed from 40 to 60 min after the administration of 5-ALA. Although an increase in fluorescence intensity was also observed in HOK, it took longer to detect a significant difference, and the difference was smaller than those of the various cultured OSCC cell lines. These differences may be attributable to the fact that there was no decrease in ferrochelatase activity in the HOK, meaning that metabolism was normal and small amount of PpIX was remain. This would explain why the fluorescence intensity increased with the passage of time, albeit only weakly.

The differences in the increase in fluorescence intensity among the cultured OSCC cell lines were considered to be due to the differences in the primary sites, the sites of collection, and the degree of tumor differentiation. Regarding the differentiation levels of the tumors in this study, HSC-2, HSC-4, and Sa3 cells were highly differentiated cell lines [15] [16] [17] [18], while HSC-3 was a poorly differentiated cell line, suggesting that the fluorescence

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intensity is higher in highly differentiated cells (Table 1). However, Uekusa et al. [19] reported that the fluorescence intensity was enhanced in poorly differentiated cells, which is in contrast with the results of this study. To clarify this point, we believe that further investigations using many kinds of cell lines are necessary.

Furthermore, in study 1, the concentration of cultured cells required to observe a significant difference in fluorescence intensity was 1×10^6 cells/mL. However, we estimate that the number of cells that can be clinically harvested from a lesion with a cytobrush is less than 1×10^6 cells/mL. Related to this, some studies have reported that the combination of 5-ALA with DFO increased the accumulation of PpIX. DFO, which is an iron-chelating agent, inhibits the synthesis of PpIX into heme, promotes intracellular PpIX accumulation, and enhances the fluorescence intensity [19] [20] [21] [22]. Therefore, in study 2, we aimed to induce the accumulation of PpIX by combining 5-ALA with the heme synthesis inhibitor DFO and using a fluorescence plate reader to measure fluorescence intensity efficiently in fewer cells. DFO is not only the most effective agent for the removal of excess ferric ions (Fe^{3+}) as an antidote for iron poisoning [23][24], but also is known to be an inhibitor compound of heme synthesis in mitochondria. DFO promotes PpIX accumulation in mitochondria, which has led to the widespread use of PDD and photodynamic therapy for a variety of malignancies [25]. On the other hand, iron metabolism plays an important role in cell maintenance/homeostasis. This means that either a deficiency or an excess of iron can trigger cell damage and cause cell death. Therefore, since our ultimate goal was to apply DFO against exfoliative cytology, we examined the cytotoxicity of DFO against HOK.

The results showed that even at the maximum concentration of 5 mM, the results differed among cell lines; only HSC-4 showed a significant decrease in cell viability at the 12 h value. Since this experiment measured fluorescence intensity for up to 10 h and none of the cell lines showed a significant decrease in the value after 6 h, 5 mM DFO was used in this study.

Next, we examined the behavioral changes in the fluorescence intensity ratio with and without DFO against each cell line and different cell numbers. The difference in the fluorescence intensity ratios was greater in the ALA+DFO than in the ALA alone group for all cell lines. This result is consistent with previous reports [21][22], suggesting that DFO inhibits both ferrochelatase and the conversion of PpIX to heme, resulting in an increase in the intracellular PpIX concentration and thereby an increase in fluorescence intensity by excitation. However, it is interesting that the addition of DFO caused differences in the fluorescence intensity ratio depending on the cell line and number. In brief, at 1×10^6 cells, no significant difference in the fluorescence intensity ratio was found between the ALA and

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ALA+DFO groups for HSC-3 only; however, at lower cell numbers, a significant increase in the fluorescence intensity ratio was observed in the ALA+DFO group. We hypothesize that these phenomena may be due to differences in the optimal concentration of DFO for each cell line or to differences in the primary lesion, considering the degree of differentiation of the OSCC cell lines.

Study 1 showed that when 5-ALA was added to the OSCC cell lines and HOK, and the fluorescence intensity was compared with a group without 5-ALA, a significant increase was found in the group with 5-ALA, because it is not necessary to add DFO at 1×10^6 cells.

The aim of this study was to measure stable fluorescence intensity using a luminescence plate reader with a smaller number of cells because it is difficult to collect 1×10^6 cells from an oral lesion. In this regard, we compared the fluorescence intensity of HOK and each OSCC cell line at 1×10^5 or 5×10^5 cell numbers. The fluorescence intensity obtained from 1×10^5 cells showed a significant increase in HSC-2 and HSC-4 after 20 min and 280 min of metabolism, respectively, compared to that of HOK. Also, the fluorescence intensity obtained from 5×10^5 cells showed a significant increase only at 20 min after the start of metabolism for HSC-2, and at 120 min for the other OSCC cell lines. These results suggest that the combination of 5-ALA and DFO can be used for screening using a luminescence plate reader with a smaller number of cells than 1×10^6 cell numbers.

As the reason for the significant increase in fluorescence intensity of HSC-2 and HSC-4 in a small number cells, it is considered to be largely due to the differences in the degree of tumor differentiation. HSC-2, HSC-4 and Sa3 cells were highly differentiated cell lines, while HSC-3 was a poorly differentiated cell line [15][16], suggesting that the fluorescence intensity is higher in highly differentiated cells. Sa3 is also a highly differentiated cell type, but no significant difference in early metabolic time was observed. However, the number of cells at 5×10^5 and 1×10^6 significantly increased the late metabolic time, considering that the appropriate concentration of DFO may differ among cell lines. On the other hand, several other studies have reported enhanced levels of fluorescence intensity in poorly differentiated cells [19], which is in contrast to the results of the present study. Thus, it is necessary to investigate more cell lines.

Igawa et al. [26] examined the number of cells that could be collected using a brush for each area of the oral mucosa, and reported that the brushes able to collect the most cells were the hard palate ($< 1 \times 10^5$ cells), lower gingiva ($< 2 \times 10^5$ cells), upper gingiva ($< 1 \times 10^5$ cells), buccal mucosa ($< 2 \times 10^5$ cells), tongue ($< 2 \times 10^5$ cells), and floor of the mouth ($< 5 \times 10^5$ cells).

In study 1, the 5-ALA-PDD method using a fluorescent plate reader required at least 1 $\times$ 10^6 cells, but with the addition of DFO, stable and significant fluorescence intensity was obtained with half that number. However, it is still necessary to collect cells safely from lesions suspected of being oral cancer. We believe that more stable fluorescence must be encouraged at a cell number level of 1×10^5, referring to the report by Igawa et al. [26] as one guide.

As a strategy to increase the fluorescence intensity with a smaller number of cells, the accumulation of PpIX in cancer cells is related to the balance between the intracellular generation of PpIX from 5-ALA and the extracellular excretion of PpIX. Kitajima et al.[27] reported that the accumulation of PpIX was enhanced in culturing cells by fumitremorgin C (FTC) and the dynamin inhibitor myristyl trimethyl ammonium bromide (MTAB) in combination with 5-ALA. FTC is a known inhibitor of the drug resistance transporter ATP binding cassette subfamily G member 2, which is involved in the excretion of PpIX, and MTAB is a known inhibitor of dynamin, which functions to separate vesicles, also involving PpIX, from the cell membrane in clathrin mediated endocytosis [27][28] (Figure 9).

In the future, we expect that the combination of FTC, MTAB, and DFO with ALA will further promote the accumulation of PpIX, and the luminescence plate reader will be able to obtain fluorescence intensity using fewer cells.

Conclusion

The combination of 5-ALA and DFO increases the accumulation of PpIX, and in addition, the fluorescence intensity is measured with high sensitivity using a luminescence plate reader. This application may help early detection of oral cancer on the chair side even if a small number of cells.

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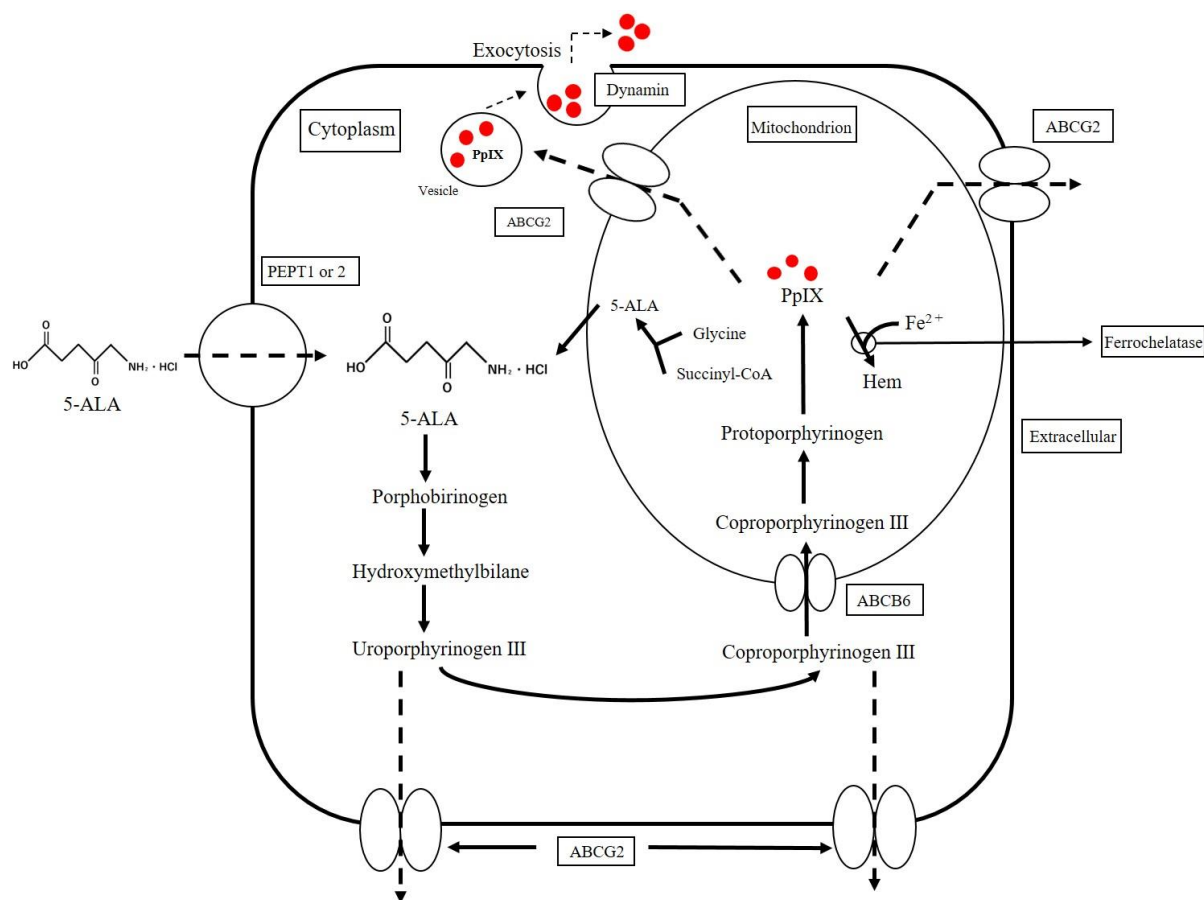
Figure 1. Metabolic pathway of exogenous 5-ALA administration. (Taken from the literature with some modifications [7] [9]). When 5-ALA is administered exogenously, it goes through various metabolic pathways in the cytoplasm, then becomes PpIX in the   mitochondria. After that, Fe^{2+} forms a chelate complex with PpIX by the action of ferrochelatase to form heme.
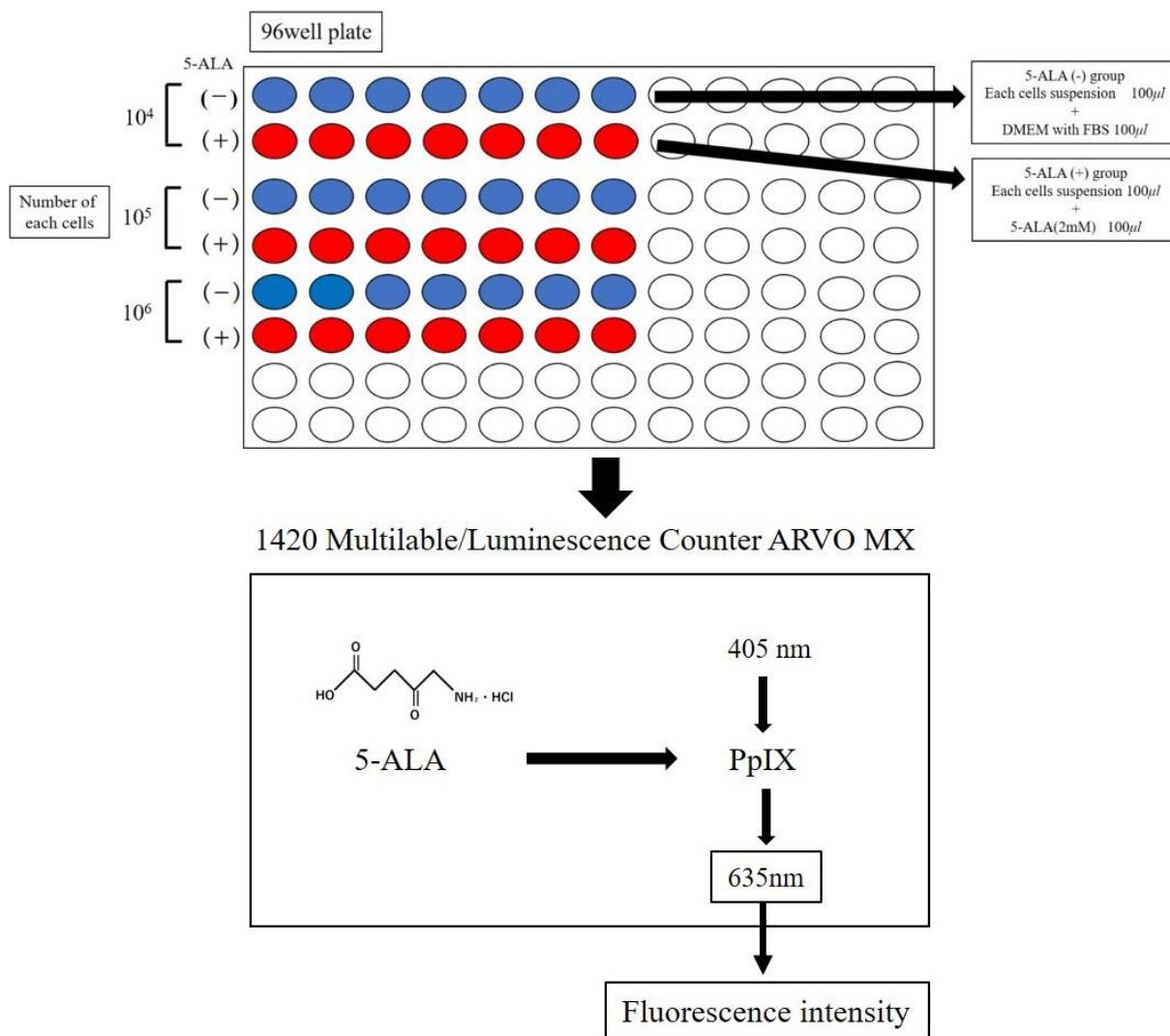
Figure 2. Measurement of the fluorescence intensity of each cell line using PDD. For each cell line, 100 μL of each of the tested concentrations of cells (1×10^4, 1×10^5, and 1×10^6 cells/mL) was added to 96-well luminescence plate, and the fluorescence intensities were measured at 60-min intervals for 240 min. Cells were irradiated with a wavelength of 405 nm, which is the excitation wavelength of the PpIX produced from 5-ALA, and the emitted fluorescence at a wavelength of 635 nm was measured as the fluorescence intensities.

A
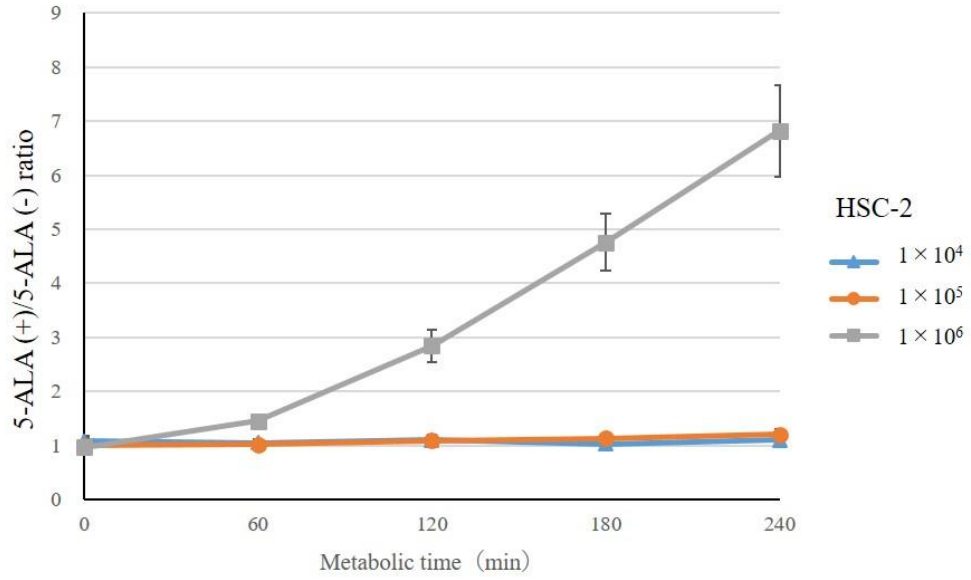
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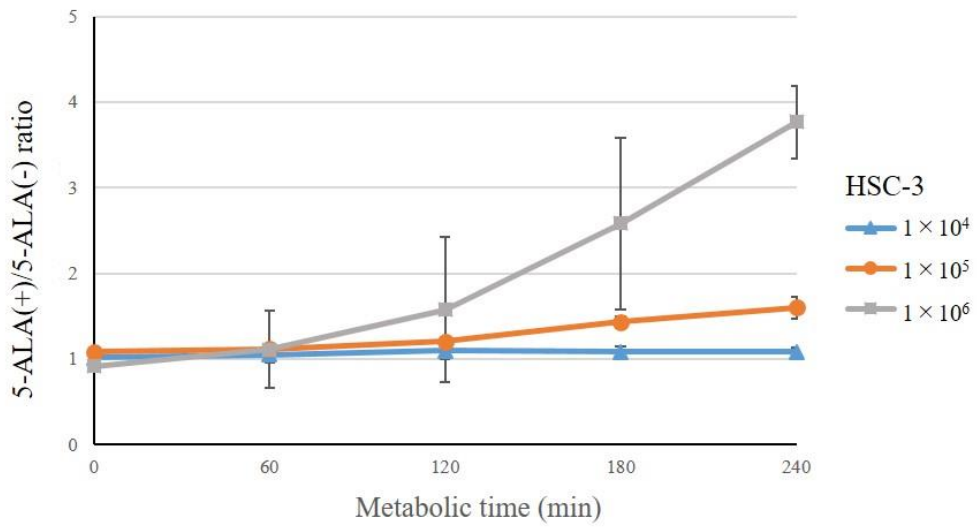
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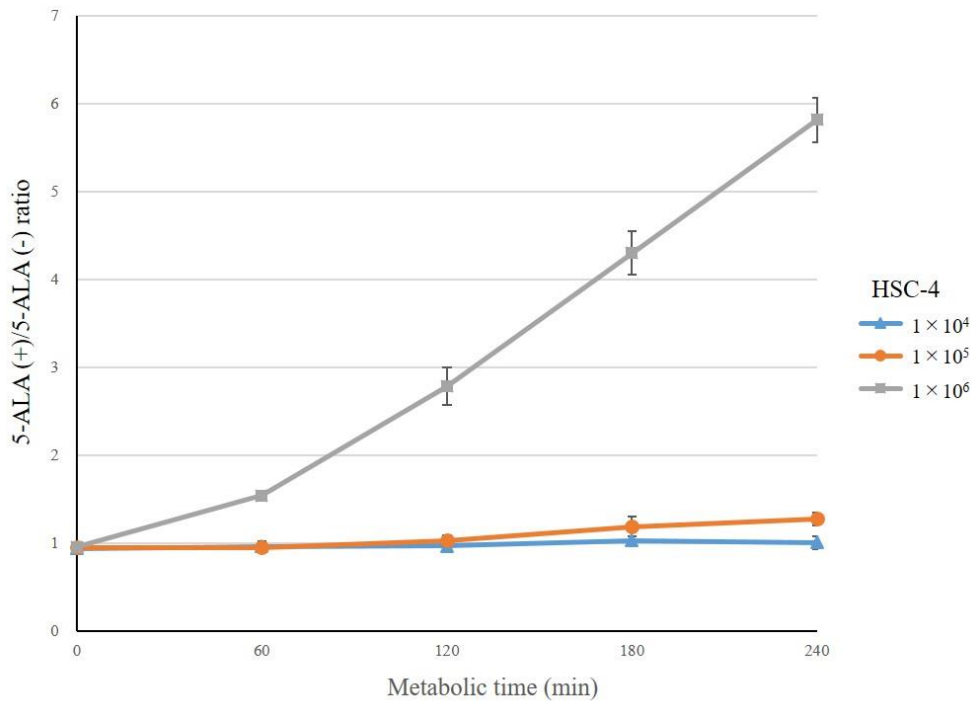
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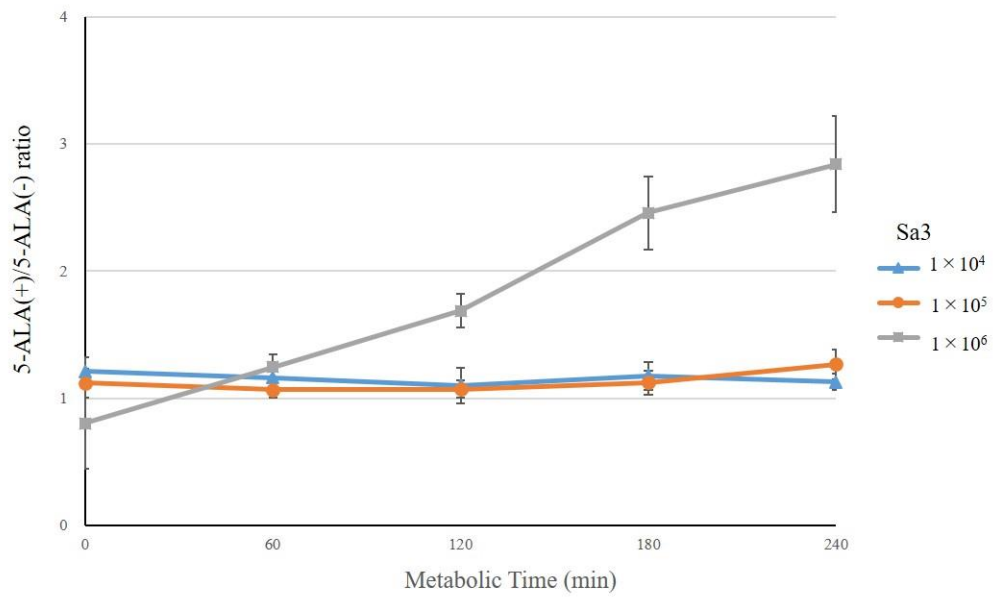
Figure 3. The 5-ALA (+)/5-ALA (−) ratio of each cell line at different cell concentrations (1 × 10^4, 1 × 10^5, and 1 × 10^6 cells/mL). The 5-ALA (+)/5-ALA (−) ratios of HSC-2 (A), HSC-3 (B), HSC-4 (C), and Sa3 (D) cells are shown. The y-axis shows the ratio of the fluorescence intensity of the 5-ALA (−) group to that of the 5-ALA (+) group. The x-axis indicates the metabolic time. The blue, orange, and gray lines indicate 1 × 10^4, 1 × 10^5, and 1 × 10^6 cells/mL, respectively.

A

B

C

D
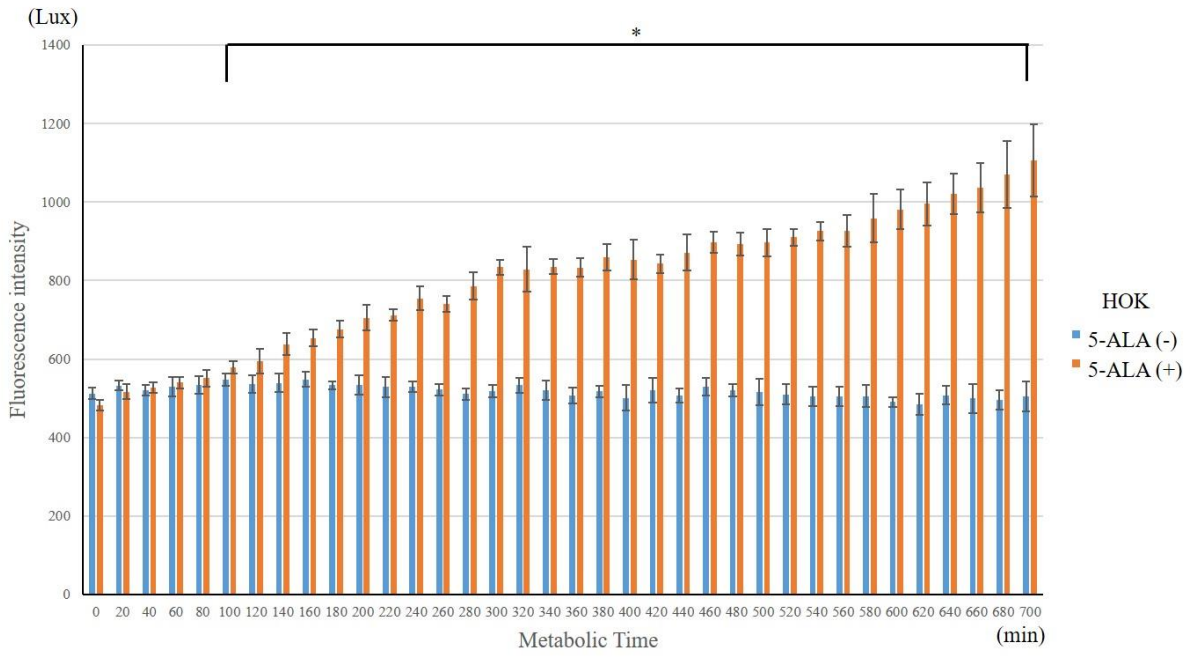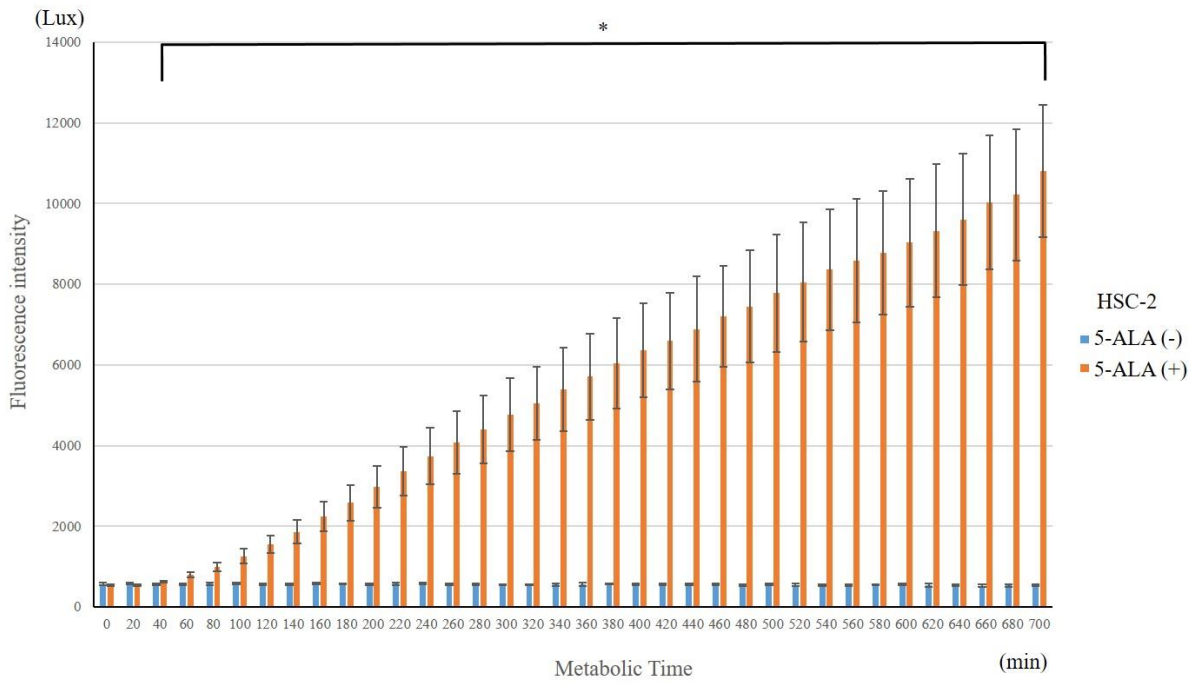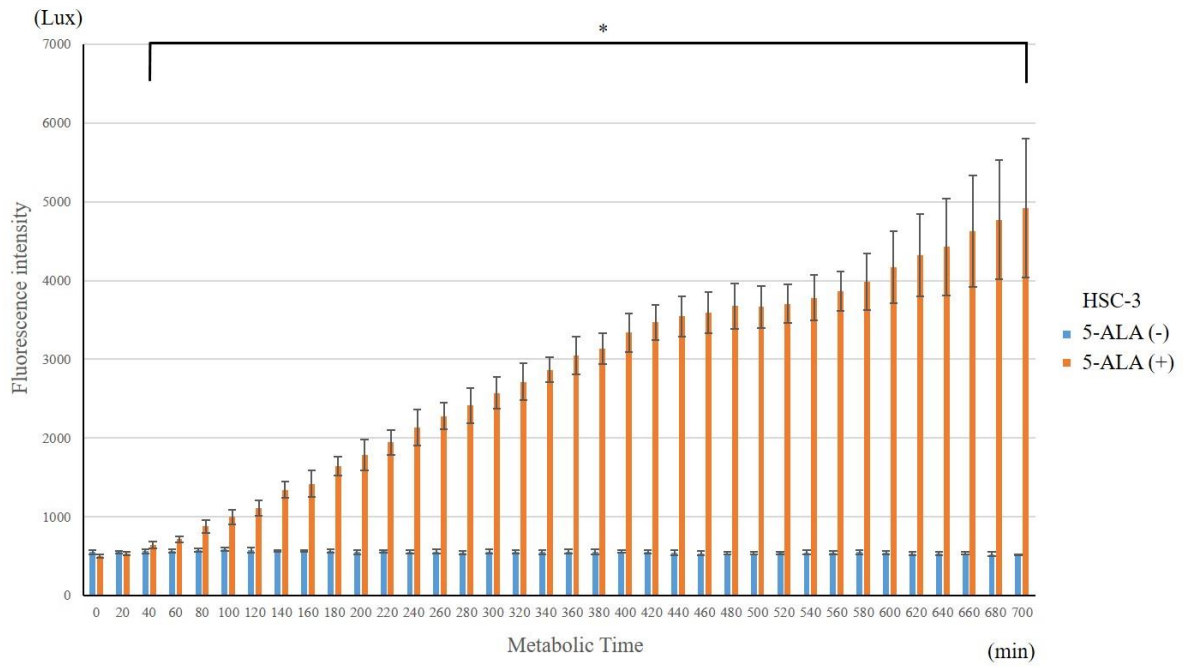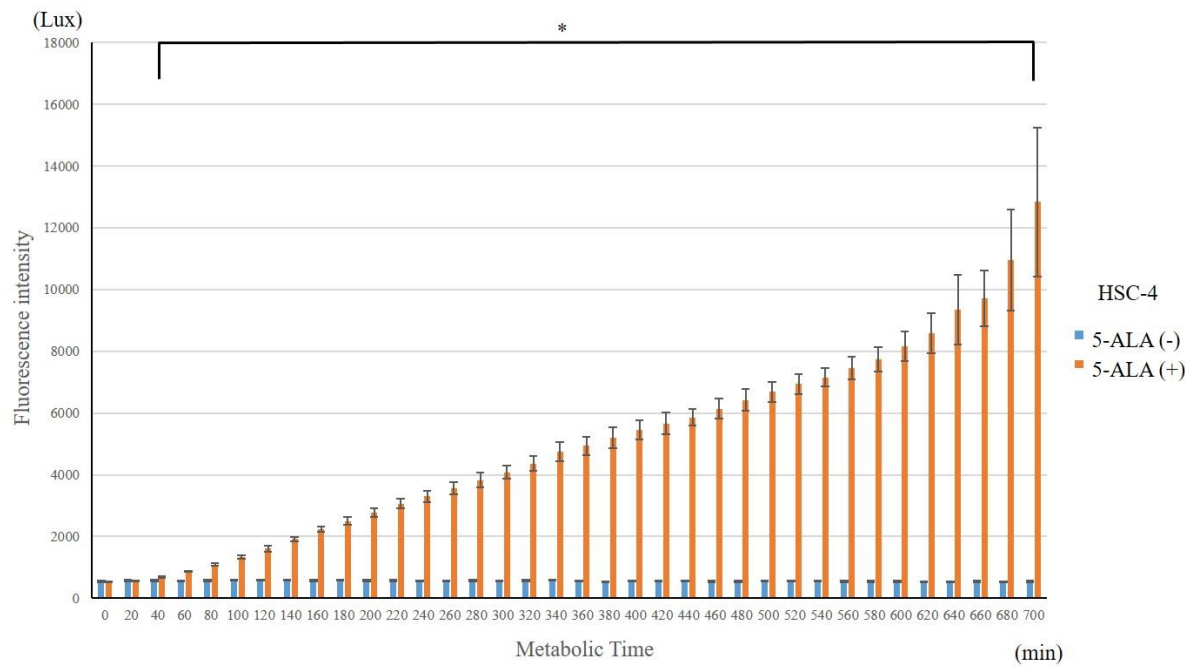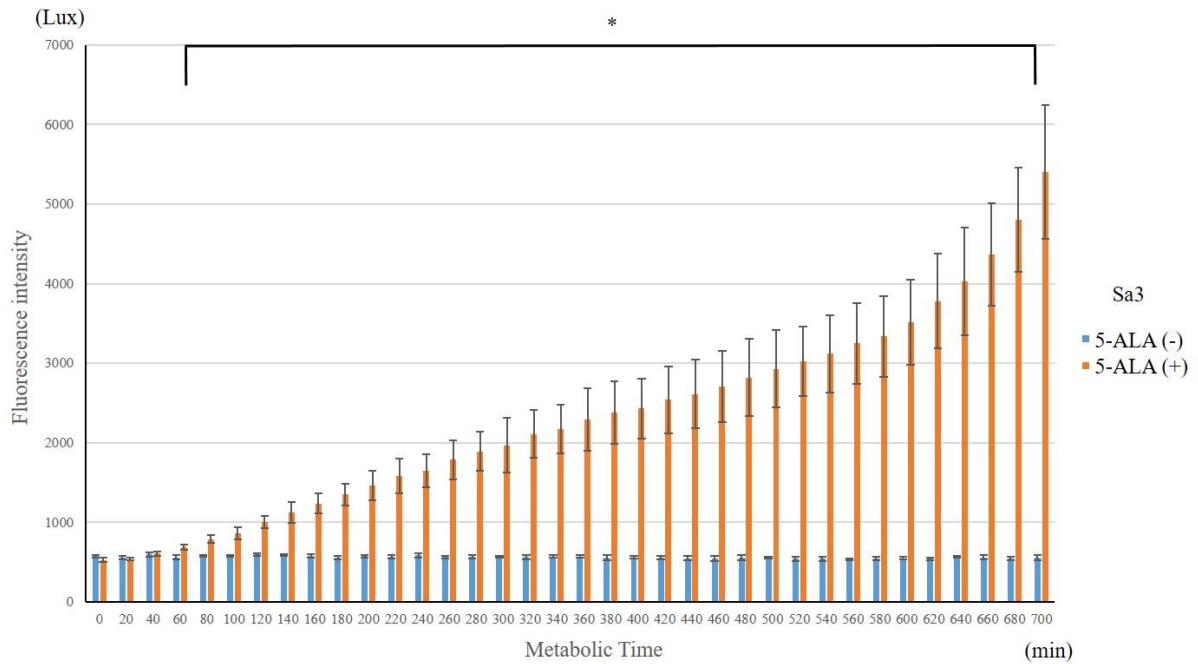
Figure 4. The fluorescence intensities of various types of cultured cells at 1×10^6 cells/mL. The fluorescence intensities in HOK (A), HSC-2 (B), HSC-3 (C), HSC-4 (D), and Sa3 (E) cells are shown. The y-axis shows the fluorescence intensity of each cell line that was irradiated with 405-nm light and emitted fluorescence at a wavelength of 635 nm. The x-axis shows the metabolic time of 5-ALA. The blue and orange bars indicate the fluorescence intensity of the 5-ALA (−) and 5-ALA (+) groups, respectively. Data are expressed as mean±SD in multi-replicated (n=7) experiments (*vs. 5-ALA (−), *P* < 0.05).

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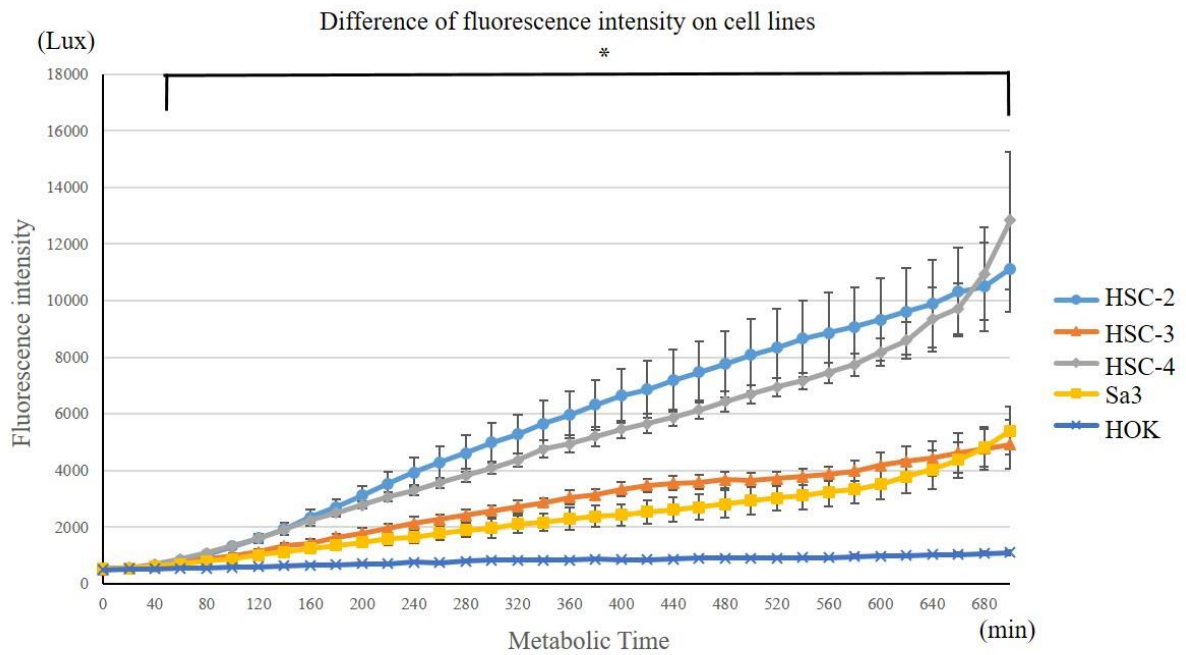
Figure 5. Differences in the fluorescence intensities of cell lines.

The y-axis shows the fluorescence intensity of each cell line that was irradiated with 405-nm light and emitted fluorescence at a wavelength of 635 nm. The x-axis shows the metabolic time of 5-ALA. The light blue, orange, gray, yellow, and blue lines indicate the fluorescence intensities of HSC-2, HSC-3, HSC-4, Sa3, and HOK, respectively. Data are expressed as mean±SD in multi-replicated (n=7) experiments (*vs. HOK, $P < 0.05$).

A

B

C

D

E
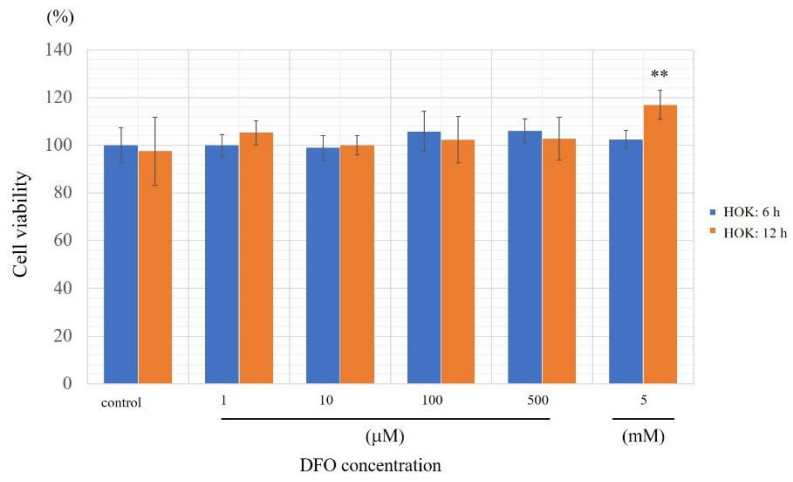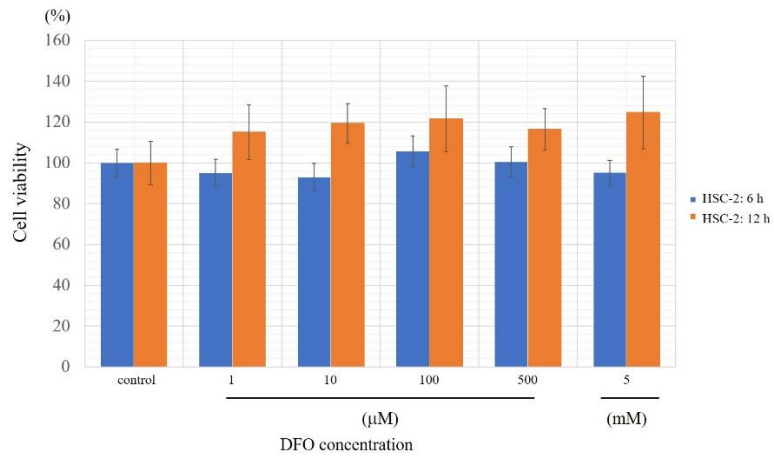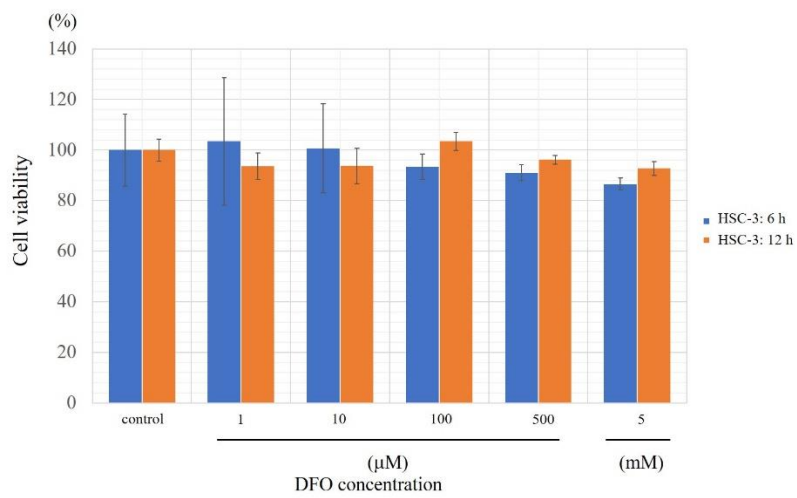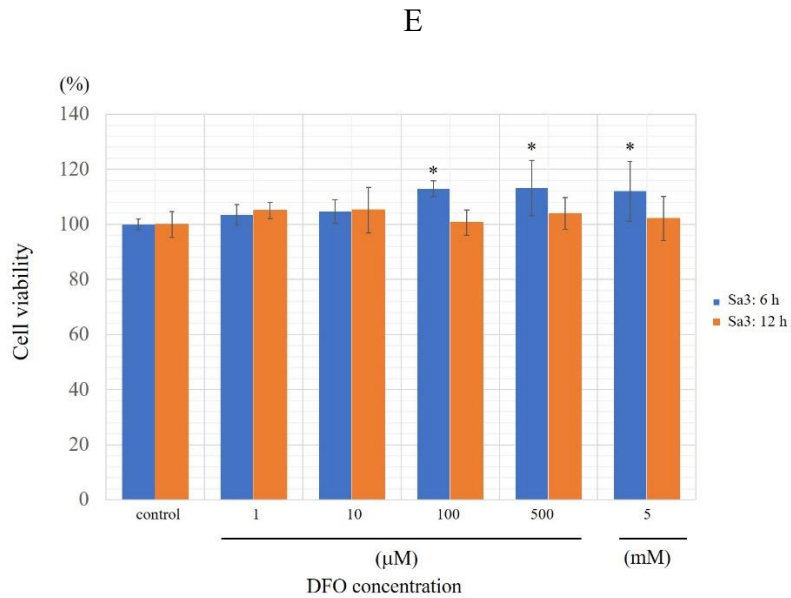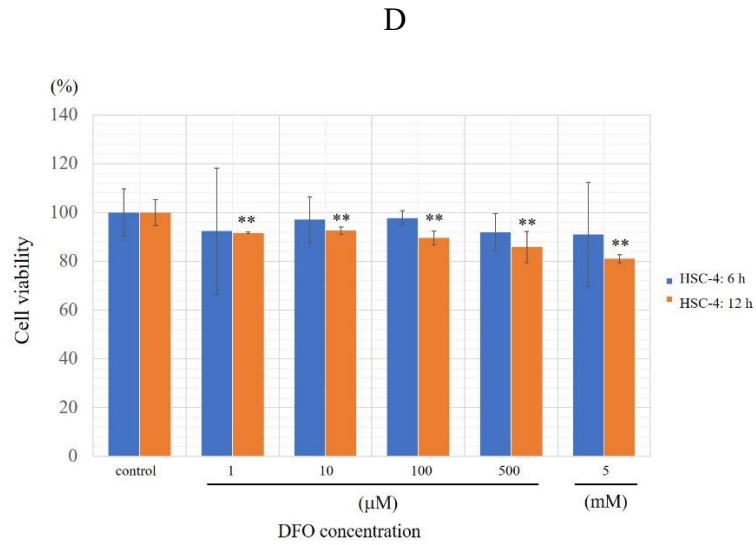
Figure 6. The cell viability of different concentrations of deferoxamine (DFO) applied to HOK and the OSCC cell lines for 6 or 12 h. The cell viabilities of HOK (A), HSC-2 (B), HSC-3 (C), HSC-4 (D), and Sa3 (E) cells are shown. The x- and y-axes indicate the DFO concentration and cell viability, respectively. Data are expressed as mean±SD in multi-replicated (n=5) experiments (*$P < 0.05$ vs. 6h control, **$P < 0.05$ vs 12h control).

(A) HOK
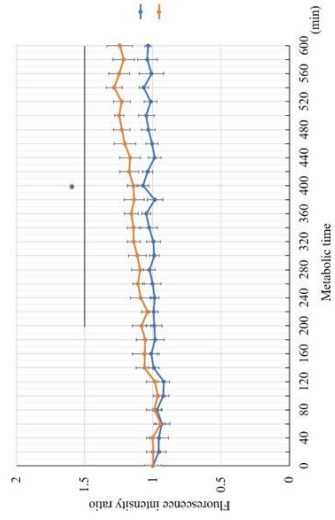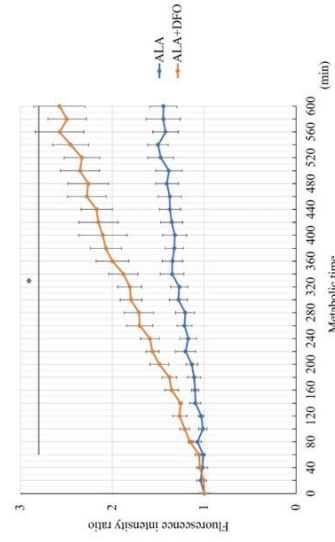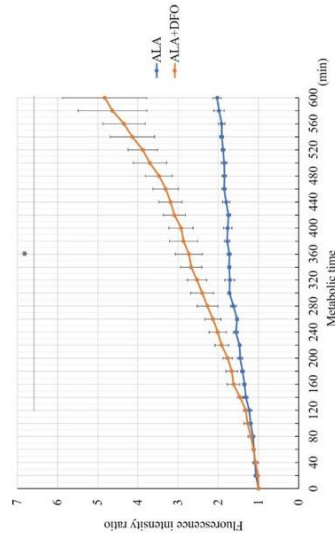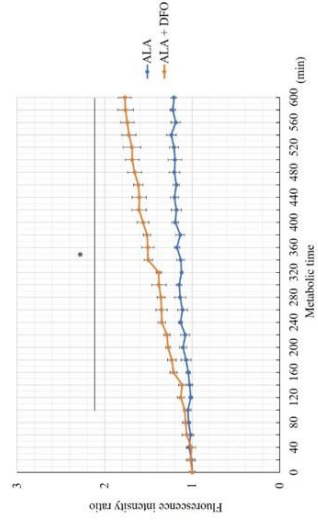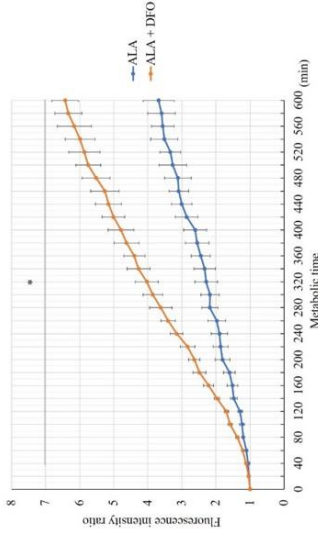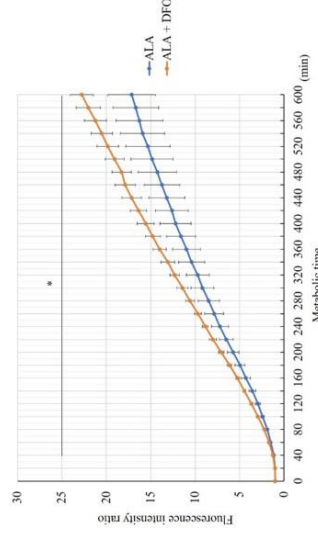
(B) HSC-2
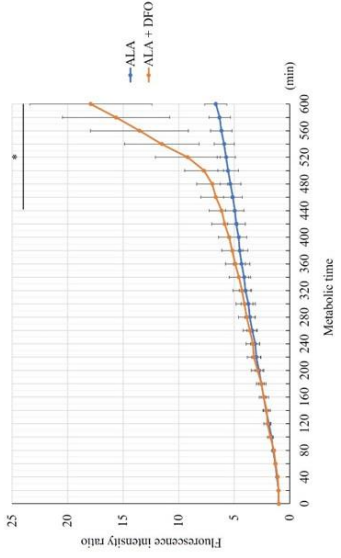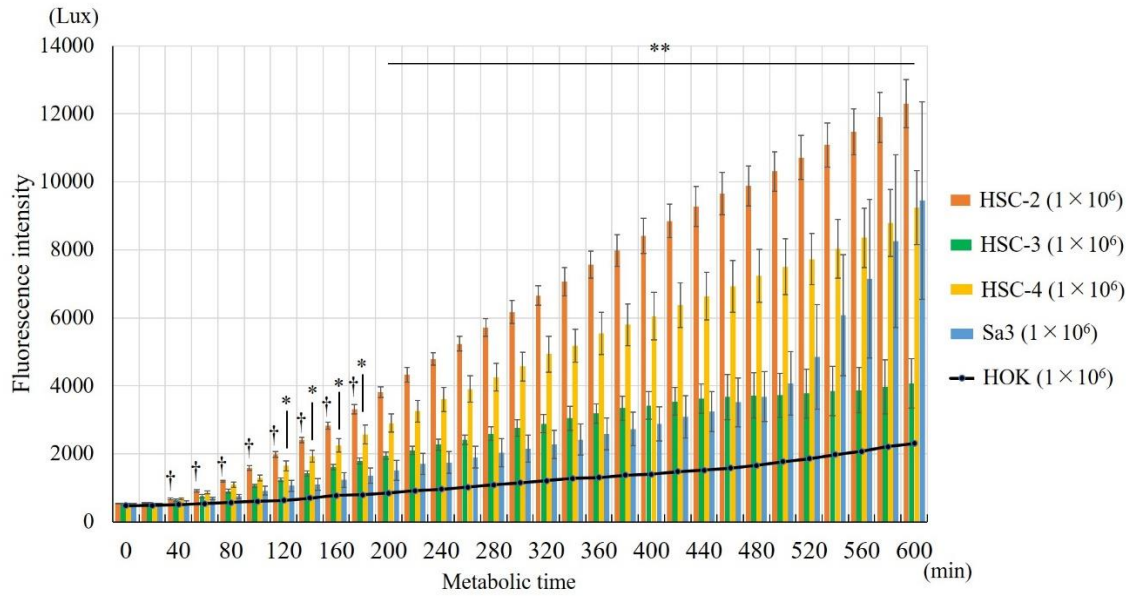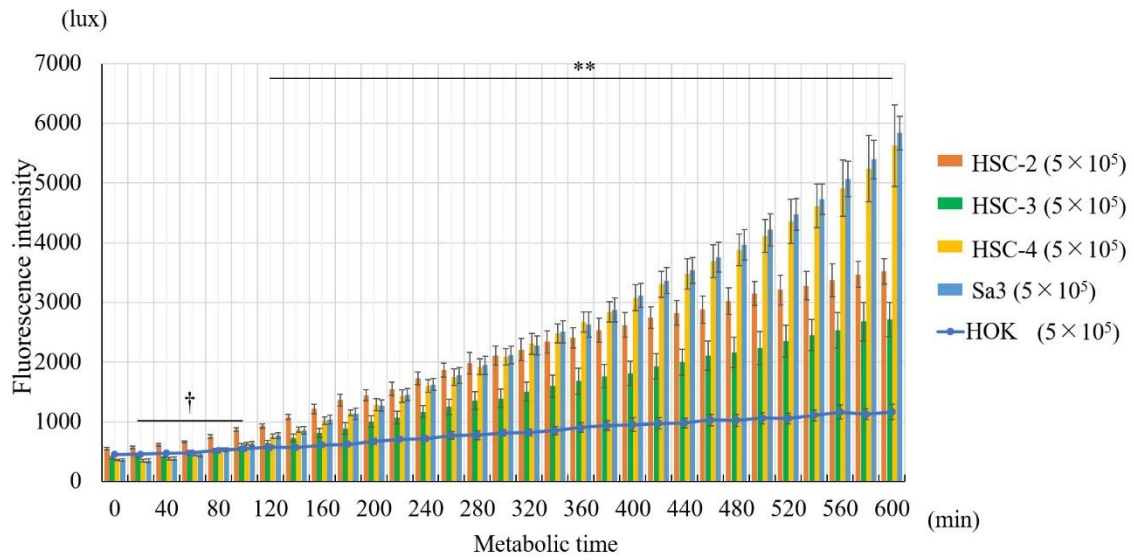
(C) HSC-3
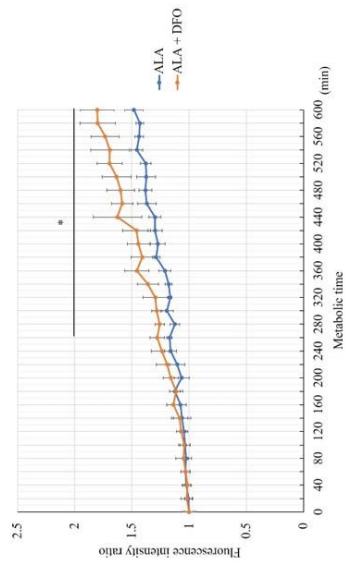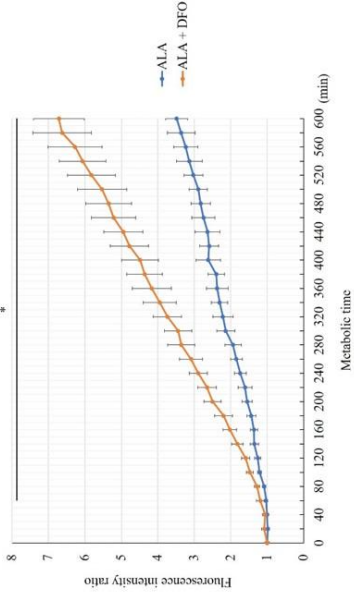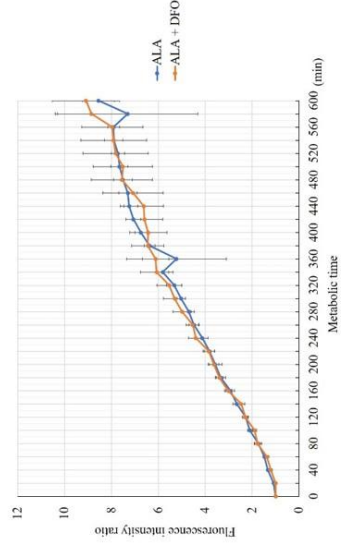
(D) HSC-4
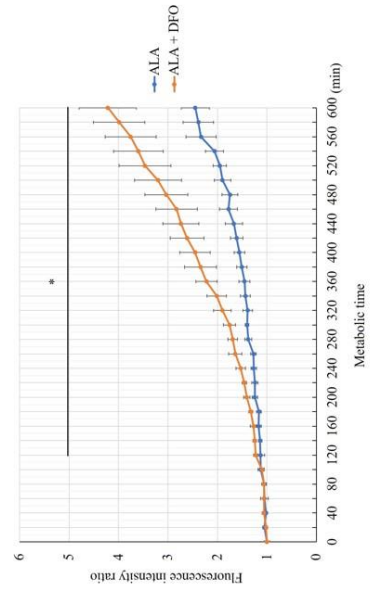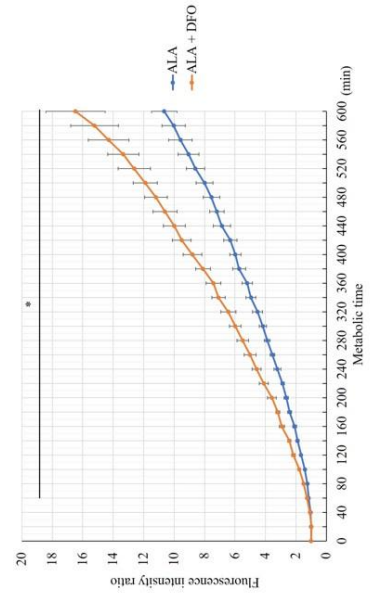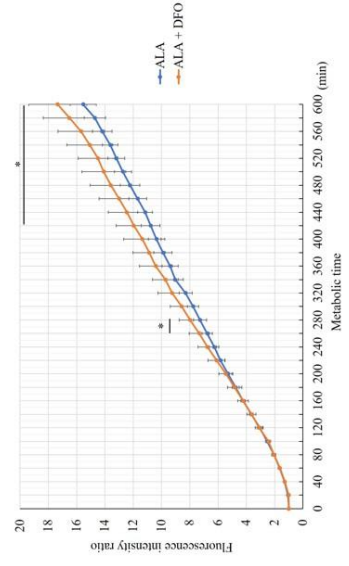
(E) Sa3
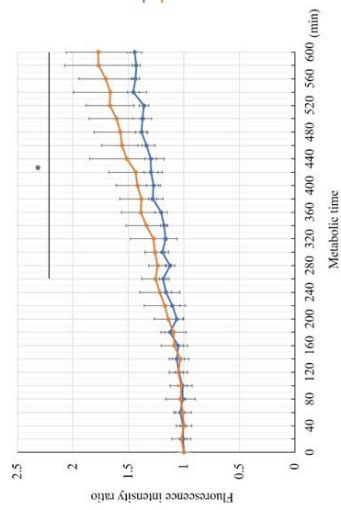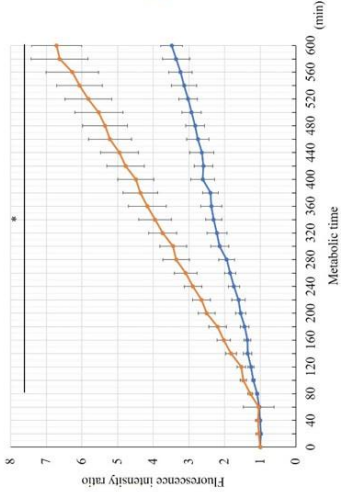
Figure 7. Comparison of fluorescence intensities between the ALA and ALA+DFO groups in each cell line and number. The x- and y-axes indicate the metabolic time and fluorescence intensities ratios calculated as follows the fluorescence intensity at experimental time that at 0 min, respectively. Data are expressed as mean±SD in multi-replicated (n=7) experiments (* $P < 0.05$).
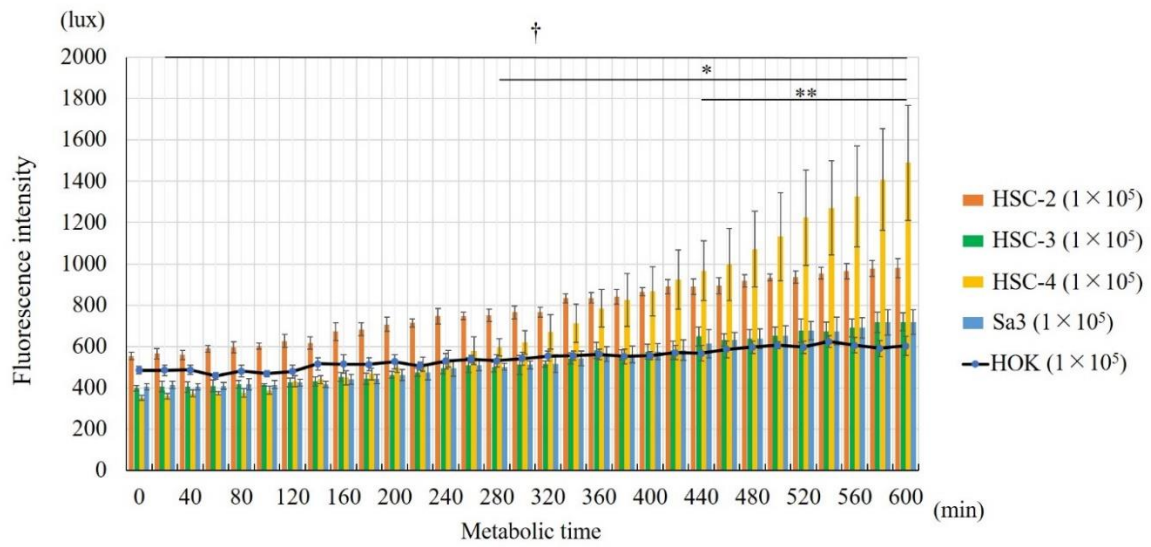
A

B

C

Figure 8. Comparison of fluorescence intensities between HOK and the OSCC cell lines at 1 × 10⁶ cells (A), 5 × 10⁵ cells (B) and 1 × 10⁵ cells (C). The x- and y-axes indicate the metabolic time and fluorescence intensity value (lux), respectively. Data are expressed as mean±SD in multi-replicated (n=7) experiments († $P < 0.05$ HOK vs HSC-2, *$P < 0.05$ HOK vs HSC-4, **$P < 0.05$ HOK vs HSC-2, HSC-3, HSC-4, Sa3).
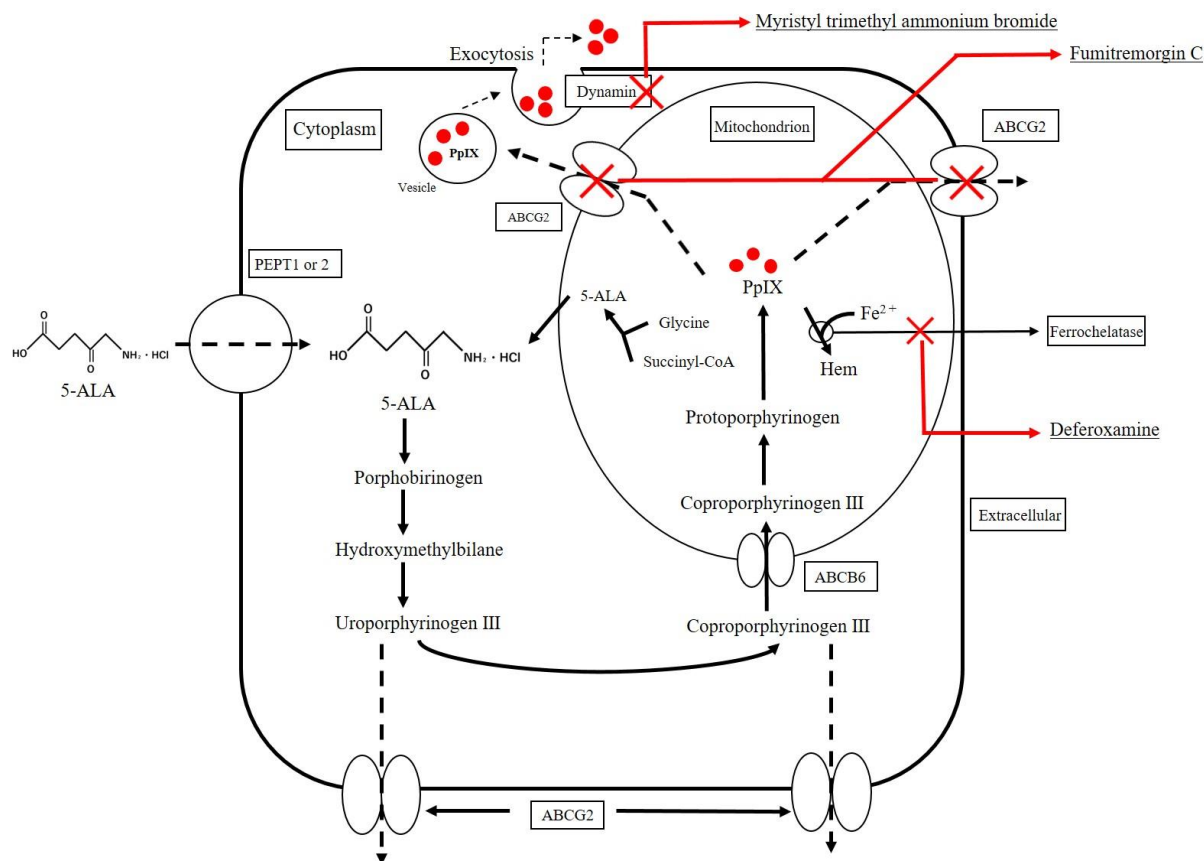
Figure 9. Action points of PpIX-excretion inhibitors. (Taken from the literature with some modifications [9] [19] [20] [21] [22] [28]). Schematic representation of the mechanism by which PpIX accumulation in cells is promoted by PpIX-excretion inhibitors. Deferoxamine, which is an iron-chelating agent, inhibits the synthesis of PpIX into heme, and promotes intracellular PpIX accumulation. Furthermore, the accumulation of PpIX is enhanced when cells are cultured with the dynamin inhibitors myristyl trimethyl ammonium bromide and fumitremorgin C in combination with 5-ALA.

Name	Sa3	HSC-2	HSC-3	HSC-4
Primary lesion	maxillary gingiva	mouth, bottom	tongue	tongue
Site of collection		submandibular lymph node metastases	metastasis in the middle and deep cervical lymph nodes	metastasis in the middle and deep cervical lymph nodes
Degree of differentiation	high	high	low	high

Table 1. Primary site, site of collection, and differentiation level of various human oral squamous cell carcinoma-derived cell lines. (Taken from the literature with some modifications [15])

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