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Effect of cell death induced by deferriferrichrysin on oral squamous cell carcinoma cells

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Certain siderophores have recently been reported to inhibit the growth of cancerous cells. Here, we assessed the cytotoxic effects of two siderophores, deferriferrichrysin (Dfcy) and bisucaberin, on the human oral squamous cell carcinoma (OSCC) cell lines HSC-3 and SAS. The iron-chelating ability of Dfcy and bisucaberin was measured using an iron assay kit utilizing the ferrozine chromogenic method. Cell viability was measured using the WST-8 assay, and caspase-3 activity was determined using a caspase-3 assay kit. Apoptotic, anti-apoptotic, and cell cycle markers were assessed by western blot analysis. Dfcy chelated ferric iron in a dose-dependent manner. In addition, 10 μg/mL bisucaberin chelated approximately 75% of the iron. Dfcy and bisucaberin inhibited the proliferation of HSC-3 and SAS cells in a dose-dependent manner. Bisucaberin-treated SAS cells exhibited significantly increased caspase-3 activation. Cleaved poly (ADP-ribose) polymerase (PARP) was also increased in bisucaberin-treated HSC-3 and SAS cells and in Dfcy-treated SAS cells. Bisucaberin treatments alone upregulated cleaved caspase-3 in SAS cells. However, Dfcy had no effect on OSCC cell apoptosis. The results show that bisucaberin exerts potential anticancer effects against SAS cells by inducing apoptosis. (J Osaka Dent Univ 2023; 57: 89-97)

Key words: Deferriferrichrysin; Bisucaberin; Siderophore; Apoptosis; Oral squamous cell carcinoma

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the most common type of head and neck cancer, with an estimated 377,713 new cases and 177,757 deaths worldwide in 2020.[1] OSCC is the most common type of oral cancer, accounting for approximately 90% of oral malignancies; the remaining 10% involve rare histological subtypes (minor salivary gland carcinomas, lymphomas, and melanoma).[2, 3] Standard treatment methods for oral cancer include surgery and radiation therapy. Chemotherapy is another treatment option; however, systemic chemotherapy has off-target effects and harms normal cells in addition to cancer cells. Therefore, new molecular targeted therapies for oral cancer are essential.[4, 5]

Iron is an important trace element that is involved in numerous cellular processes, including energy generation, oxygen transport, and DNA synthesis.[6] Compared to noncancerous cells, cancer cells have a higher demand for iron, which is needed to sustain rapid DNA synthesis and growth. Therefore, iron chelators may be useful as anti-cancer agents.[7] Recently, certain iron chelators such as siderophores have been reported to decrease the growth of cancerous cells.[8]

Deferriferrichrysin (Dfcy) is a cyclic hexapeptide siderophore produced by *Aspergillus oryzae* and other related species. *A. oryzae* is an important filamentous fungus that has traditionally been used by the Japanese fermentation industry for the produc-

tion of sake (rice wine), shochu (distilled spirits), shoyu (fermented soy sauce), and miso (fermented soybean paste). Dfcy binds specifically to ferric ions to become its iron-chelated form, ferrichrysin (Fcy).[9] Dfcy has three consecutive Nd-acetyl-Nd-hydroxy-L-ornithine (Aho) residues; this structure has been shown to have high iron (III) coordination activity among the naturally occurring siderophore peptides.[10]

Bisucaberin is a compound produced by *Aeromonas haloplanktis* strain SB-1123 isolated from deep-sea mud, which enhances tumor killing by tumor-associated macrophages *in vivo*.[11] The macrocyclic bis-hydroxamate structure of bisucaberin is highly amenable to ferric iron binding, and has a much higher affinity for ferric iron than for acyclic bis-hydroxamate ligands.[12] Siderophores are generally classified based on the coordinating groups that chelate Fe (III) ions: catecholates, hydroxamates, and carboxylates.[13] Dfcy and bisucaberin belong to the hydroxamate group, while desferrioxamine (DFO) and ferrichrome also belong to the hydroxamate group. These compounds inhibit tumors by promoting apoptosis *in vitro*.[14, 15] Siderophores are largely unexplored in the field of cancer research. In this study, we investigated the cytotoxic effects of Dfcy and bisucaberin in HSC-3 and SAS human OSCC cell lines.

MATERIALS AND METHODS

Reagents

Dfcy was provided by the Research Institute of Gekkeikan Sake Co., Ltd, Kyoto, Japan. A 200 mg/mL stock solution of Dfcy was prepared by dissolving the Dfcy in phosphate-buffered saline (PBS). The solution was stored at −20°C. Bisucaberin was purchased from the Institute of Microbial Chemistry, Tokyo, Japan. A 10 mg/mL stock solution of bisucaberin was prepared by dissolving it in dimethyl sulfoxide. It was stored at −20°C.

Chelation ability test

Fe^{3+} iron standard stock solution (Wako Pure Chemical Industries, Osaka, Japan) was used to examine the binding capacity of various iron chelators. An iron diluent (200 μg/dL) was prepared from the Fe^{3+} standard stock solution, and the iron chelators Dfcy, Fcy, and bisucaberin were added. An iron assay kit (Metallogenics, Chiba, Japan) utilizing the ferrozine chromogenic method was used to measure iron content after iron chelation according to the manufacturer's protocol.

Cell culture

Cancer cells of the human oral cancer cell lines HSC-3 and SAS were maintained in MEM or RPMI (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (Biosera, Tokyo, Japan) and a 1% penicillin-streptomycin solution (FUJIFILM Wako Chemicals, Osaka, Japan) at 37°C under 5% CO_2.

Cell viability

HSC-3 and SAS cells were seeded in 96-well plates at a density of 10,000 cells/well. The following day, cells were treated with Dfcy, Fcy, or bisucaberin for 48 h. Cell viability was monitored using WST-8 reagent (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. The absorbance was measured at 450 nm.

Caspase-3 activity assay

A total of 3×10^6 cells were plated in a 6-well plate for a 24 h growth period. After 24 h of Dfcy or bisucaberin treatment, the medium was replaced with RPMI or MEM. Cells were harvested after 48 h. Caspase-3 activity was determined using a caspase-3 assay kit (ab 39401; Abcam, Cambridge, UK).

Western blotting analysis

After treatment with Dfcy or bisucaberin, whole cell lysate was obtained using RIPA buffer (Nacalai Tesque) and total protein concentrations were determined using a BCA protein assay kit (Takara, Shiga, Japan). Twenty micrograms of total protein from each sample were separated using 10-15% SDS-PAGE and transferred onto nitrocellulose membranes (Merck Millipore, Burlington, MA, USA). The membranes were blocked in 5% BSA in Tris-buffered saline with 0.1% Tween-20 (TBST) for 1 h

and subsequently incubated with primary antibodies (Cell Signaling Technology, Danvers, MA, USA), including rabbit anti-cleaved caspase-3, rabbit anti-caspase-3, rabbit anti-cleaved poly(ADP-ribose) polymerase (PARP), rabbit anti-PARP, anti-cyclin D1, and rabbit anti-β-actin (Genetex, Irvine, CA, USA), diluted in 5% BSA in 0.01 M TBST at 4°C overnight. The membranes were washed in TBST and further incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Dallas, TX, USA) diluted in 0.01 M TBST at 1:5000 for 1 h at room temperature. Images were viewed using a ChemiDoc™ MP Imaging System (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

Data are presented as the mean and standard deviation (SD) from three experiments (n＝3). Statistical analyses were performed using the Student's t-test with the significance level set at 1%.

RESULTS

Iron chelating abilities of Dfcy, Fcy, and bisucaberin

Dfcy chelated Fe^{3+} in a dose-dependent manner (Fig. 1). Fcy did not show significant iron chelation compared to the control. A dose of 10 μg/mL bisucaberin chelated approximately 75% of the iron, while 10 μg/mL Dfcy chelated approximately 50%.

Cell viability in HSC-3 and SAS cells treated with Dfcy or bisucaberin

We examined the effects of Dfcy and bisucaberin treatment on the viability of HSC-3 and SAS cells using the WST-8 reagent. WST-8 viability assays revealed that Dfcy and bisucaberin significantly inhibited the growth of HSC-3 and SAS cells in a dose-dependent manner (Fig. 2). Fcy had no effect on the growth of HSC-3 and SAS cells.

Apoptotic caspase-3 activity assay

Bisucaberin-treated SAS cells demonstrated a significant 2.5-fold increase in caspase-3 activation compared with the controls (Fig. 3), whereas the bisucaberin-treated HSC-3 cells showed no significant increase in caspase-3. No significant increase in caspase-3 was observed in the Dfcy-treated HSC-3 and SAS cells, compared with the vehicle-treated controls.

Evaluation of the effects of Dfcy and bisucaberin on PARP, cleaved PARP, caspase-3, cleaved caspase-3, and cyclin D1 expressions in HSC-3 and SAS cells

We performed western blot analysis to compare levels of apoptotic markers between treatment groups (Fig. 4). The expression of cleaved PARP, a DNA repair enzyme, increased after treatment with bisucaberin in both HSC-3 and SAS cells. However, it increased after treatment with Dfcy in only the SAS cells. Only bisucaberin treatment increased cleaved caspase-3 levels in SAS cells,

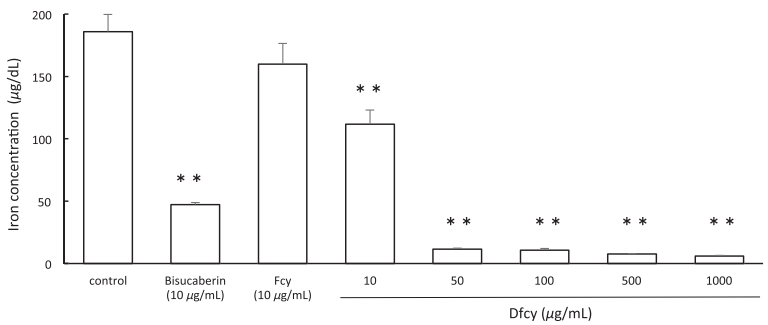


Fig 1 Chelating abilities of deferriferrichrysin (Dfcy), ferrichrysin (Fcy), and bisucaberin were determined using a standard Fe^{3+} water solution and the ferrozine chromogenic method. Dfcy, Fcy, and bisucaberin were added to the 200 μg/dL standard Fe^{3+} solution (**$p < 0.01$ for the difference between bisucaberin and Dfcy, and the control by Student's t-test, Mean±SD).

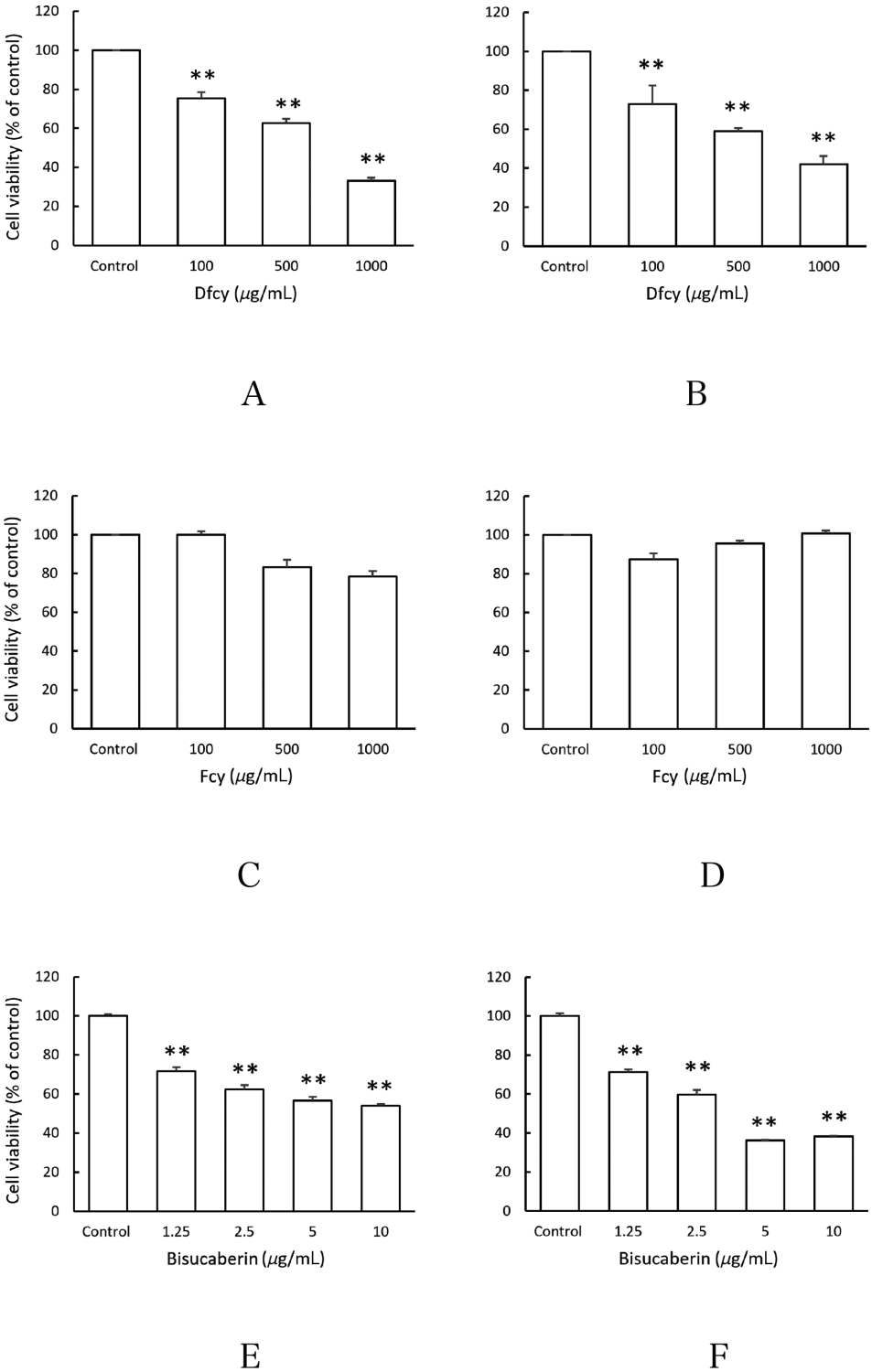


Fig. 2 Inhibitory effects of deferriferrichrysin (Dfcy), ferrichrysin (Fcy), and bisucaberin on OSCC cells *in vitro.* Cultured (A, C and E) HSC-3 and (B, D and F) SAS cells were treated with Dfcy, Fcy and bisucaberin for 48 h, and cell viability was evaluated using the WST-8 assay. The cell viability in the absence of treatment was set as 100%. Results represent the mean of three independent experiments (**$p < 0.01$ between the (A and B) Dfcy-, (C and D) Fcy-, or (E and F) bisucaberin-treated groups, and the control by Student's t-test).

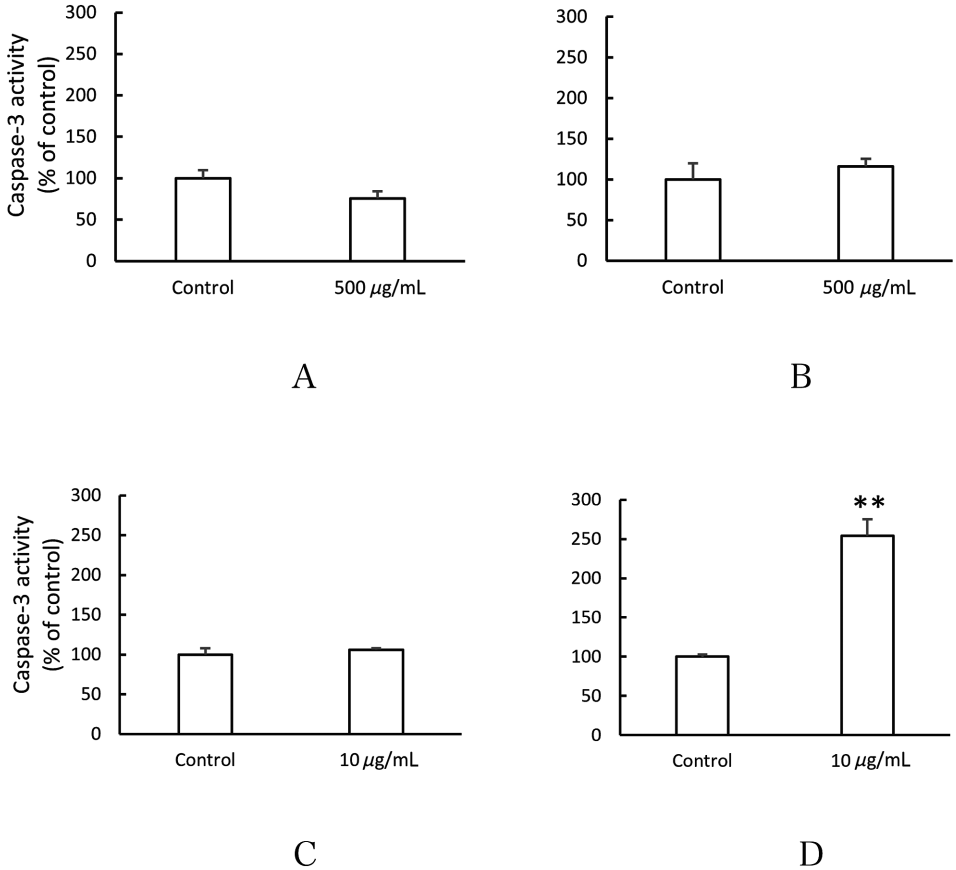
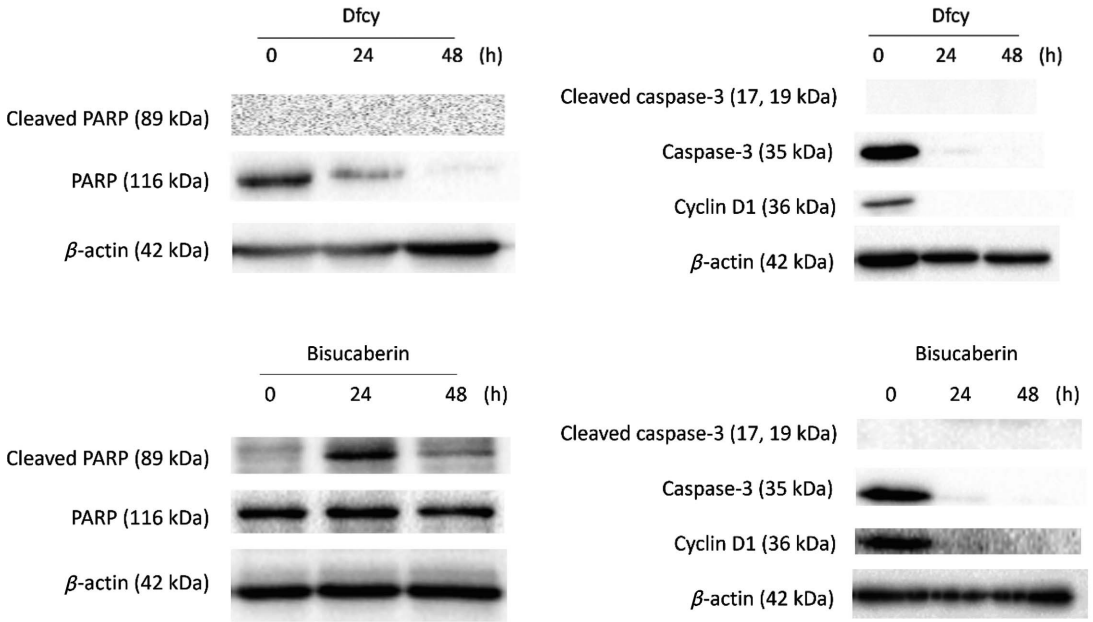


Fig. 3 Apoptosis levels of (A and C) HSC-3 and (B and D) SAS cells treated with (A and B) deferriferrichrysin (Dfcy) or (C and D) bisucaberin for 48 h, as assessed using the caspase-3 activity kit. Results represent the mean of three independent experiments (**$p < 0.01$, between the (D) bisucaberin-treated groups and the control group, as analyzed by Student's t-test).

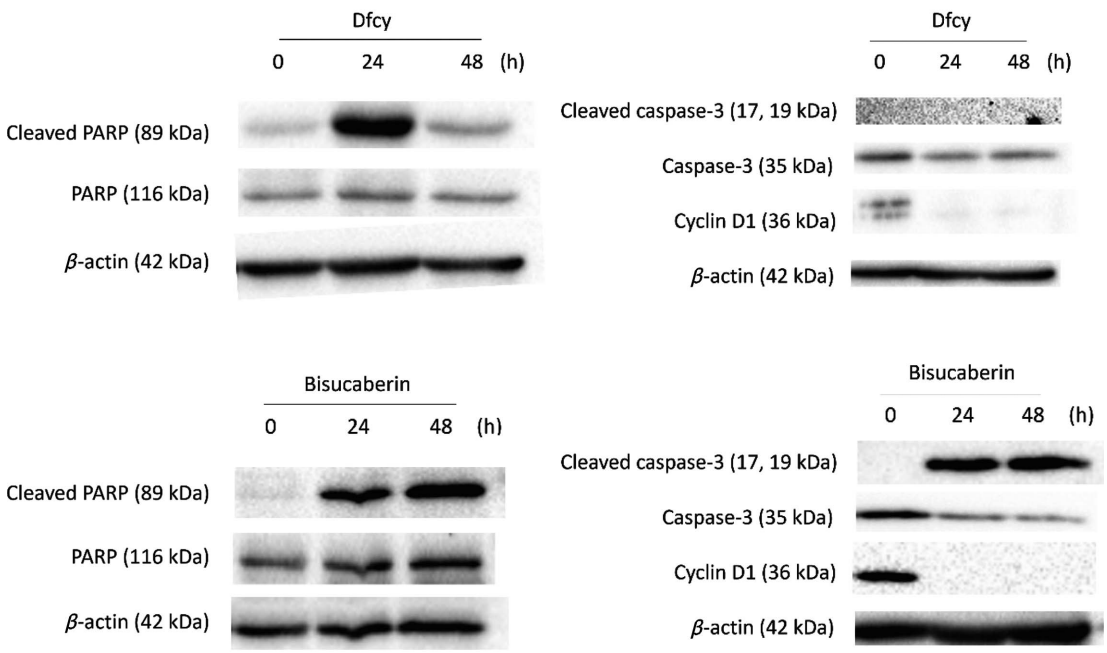
whereas Dfcy treatment did not alter cleaved caspase-3 levels in either HSC-3 or SAS cells. Cyclin D1 protein content decreased in a dose-dependent manner after treatment with Dfcy and bisucaberin in both HSC-3 and SAS cells.

DISCUSSION

Siderophores are high-affinity ferric ion-specific chelators with a molecular weight of less than 10 kDa. Siderophores are excreted under iron starvation by various microorganisms such as bacteria, fungi, and some plants. Iron is an essential micronutrient for biological and metabolic cellular functions, but has limited availability in the environment,[16] as it is oxidized to insoluble oxyhydroxide polymers under biological pH and aerobic conditions.[17] Siderophores form complexes with free iron and transport it into the cell via membrane receptor molecules, which are encoded by five genes in the operon and are turned off by sufficient intracellular iron levels.[18] Disruption of iron homeostasis has been associated with various diseases, including cancer.[19, 20] Therefore, augmentation of iron metabolism likely affects cancer growth, proliferation, and metastasis. Natural and synthetic siderophores have been extensively studied as potential therapeutic agents for different cancers because of their iron-chelating capabilities.[21] One of the most popular bacterial siderophores in cancer therapy research is deferoxamine (desferal, desferrioxamine, desferrioxamine-B, or DFO), a water-soluble trihydroxamate hexadentate siderophore secreted by many *Streptomyces* species.[22] DFO is reported to promote apoptosis *in vitro* by upregulating the tu-



A



B

Fig. 4 Effects of deferriferrichrysin (Dfcy) and bisucaberin on protein expression of poly(ADP-ribose) polymerase (PARP), cleaved PARP, caspase-3, cleaved caspase-3, and cyclin D1 in (A) HSC-3 and (B) SAS cells. Both cell lines were treated with Dfcy (500 μg/mL) and bisucaberin (10 μg/mL), as indicated for 0, 24 and 48 h. The expression of PARP, cleaved PARP, caspase-3, cleaved caspase-3, cyclin D1, and β-actin was determined by western blot assays.

mor suppressor gene *p53,* increasing the expression of the pro-apoptotic genes *Bax* and *Fas,* and reducing the expression of the anti-apoptotic gene *Bcl-2* in leukemia.[14] However, DFO is a hydrophilic molecule with many therapeutic limitations, including poor membrane permeability, poor oral viability, and a short plasma half-life of approximately 12 min.[23] Mixed results of DFO as an antitumor agent have encouraged the use of other effective iron chelators, with a particular focus on chelator lipophilicity, membrane permeability, and selective antitumor activity. Therefore, the present study aimed to determine whether Dfcy and bisucaberin could be used as alternatives to DFO as anticancer agents.

Previous studies have shown that ferrichrome, a hydroxamate-containing cyclic hexapeptide siderophore with the same three Aho residues as Dfcy produced by *Lactobacillus casei,* exerts antitumor effects by activating c-Jun N-terminal-DNA damage -inducible transcript 3 signaling in gastric and colon cancer cell lines.[15, 24] As Dfcy and bisucaberin are also cyclic hydroxamate structures, we investigated whether they have the same antitumor effect as ferrichrome. WST-8 assays showed that Dfcy and bisucaberin exerted dose-dependent tumor-suppressive effects on OSCC cells (Fig. 2). Previous reports have shown that iron chelators exert anticancer effects by depleting intracellular iron.[25] The chelation ability test and WST-8 assays did not reveal any increase in the tumor-suppressive effects of Fcy, an iron-chelated form of Dfcy (Figs. 1 and 2). Ijiri *et al.*[24] confirmed that the tumor-suppressive effects of ferrichrome are negated by iron binding. Furthermore, it has been reported that iron-chelated ferrichrome is unable to further bind to additional cellular molecules. Thus, we speculated that the iron-free structure of siderophores may capture unknown molecules in cancer cells through the iron-binding site.

Cyclin D1, a key regulator of cell proliferation, is commonly amplified at the genetic level and has increased expression in oral cancer.[26] Moreover, studies have indicated that cyclin D1 overexpression occurs early in oral tumorigenesis and is strongly associated with advanced tumor stages.[27] Western blotting showed that Dfcy and bisucaberin decreased cyclin D1 expression in OSCC cells (Fig. 4). Previous studies have demonstrated that the iron chelators Dp 44 mT, DFO, and deferasirox significantly inhibit OSCC cell growth and cause cell cycle G1 phase arrest.[28] Based on these observations, further studies using flow cytometry are needed to confirm that Dfcy and bisucaberin can induce G0/G1 phase arrest in OSCC cells.

Apoptosis is programmed cell death induced by signaling cascades in the intrinsic and extrinsic pathways that involve activation of the same target caspases and apoptotic molecules.[29, 30] Our data revealed that only bisucaberin upregulated the expression of cleaved caspase-3 in SAS cells (Figs. 3 and 4). These results suggest that bisucaberin suppresses the growth and proliferation of human cancer cells by inducing cancer cell apoptosis. PARP is referred to as an anti-apoptotic protein owing to its DNA repair ability; however, activated caspase-3 cleaves proteins, including PARP, during apoptosis.[31, 32] Western blotting analysis revealed that the expression of cleaved PARP in Dfcy-treated SAS cells and bisucaberin-treated HSC-3 and SAS cells was significantly increased (Fig. 4). Our results did not provide sufficient evidence that Dfcy inhibits the progression of OSCC cells via apoptosis. Interestingly, a recent study reported that some cancer cells undergo caspase-independent cell death through apoptosis-inducing factor, a mitochondrial oxidoreductase.[33, 34] Whether Dfcy induces cell death in OSCC cells in a caspase-independent manner should be investigated in the future. Regarding the difference in sensitivity to anticancer drugs between HSC-3 and SAS cells, previous reports have shown that cetuximab, a specific anti-epidermal growth factor receptor (EGFR) monoclonal antibody, inhibited proliferation of HSC 3 and HSC 4 cells, but not the proliferation of SAS cells.[35] However, we were unable to find reports on iron chelators. It is likely that different cancer cell lines harboring different oncogenic mutations respond differently to iron chelation.

In conclusion, our study revealed that bisucaberin

could suppress the growth and proliferation of OSCC by inducing apoptosis. Although Dfcy inhibited cell proliferation in OSCC, it did not significantly increase cell death or caspase-3 activity. The antitumor mechanisms of these siderophores, such as cell cycle arrest, should be investigated further.

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