CAY10591, a SIRT1 Activator, Suppresses Malignancy of Gingival Epithelial Carcinoma Cells

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This thesis is composed by an article ahead of print and two additional data listed below.

1. Takahisa Murofushi, Hiromasa Tsuda, Yoshikazu Mikami, Yoko Yamaguchi, and Naoto Suzuki. CAY10591, a SIRT1 Activator, Suppresses Cell Growth, Invasion, and Migration of Gingival Epithelial Carcinoma Cells. ***J Oral Sci***, ***in press.***

2. miRNA-expression data suggesting that CAY10591 induces expression of microRNA which reportedly inhibits cell invasion/migration.

3. Released matrix metalloproteinases data suggesting that CAY10591 inhibits release of MMP-3 and MMP-9 to the extracellular space.

ABSTRACT

SIRT1 is a NAD-dependent histone deacetylase that plays an important role in a wide diversity of physiological/pathophysiological process. Although many studies regarding the relationship between SIRT1 and cancer have been reported, the role of SIRT1 in tumor malignancy remains disputed. In the present study, the effects of CAY10591, a SIRT1 activator, in gingival epithelial carcinoma Ca9-22 cells were examined. CAY10591 treatment caused an increase in SIRT1 and activity in dose and time-dependent manners. The treatment decreased cell growth and induced cell-cycle repressor p21. In addition, dimethyl sulfoxide significantly decreased cellular invasion and migration and CAY10591 enhanced this decrease. qPCR analysis showed decreased expression of several invasion/migration promoter genes and induction of repressor genes and microRNAs upon CAY10591 treatment. Moreover, CAY10591 treatment suppressed release of active MMP-3 and MMP-9, which play a role in cancer cell invasion into connective tissues. These data suggest that CAY10591 suppresses malignancy of gingival squamous cell carcinoma Ca9-22 cells.

Introduction

In the oral cavity, squamous cell carcinoma (SCC) represents >90% of all malignant neoplasms (1,2). These types of cancer cells are characterized by rapid cell growth and progressive invasion/migration. SCCs are most commonly located in the lower lip, tongue, and the floor of mouth, whereas gingival SCC accounts for <10% intraoral carcinoma (3,4). Considering that the clinical findings of gingival SCC are diverse and similar to benign conditions such as periodontal disease, it is thus very difficult to diagnose; resulting in delay of SCC diagnosis and subsequent poor prognosis (4-7). Therefore, useful methods for preventing gingival SCC are needed.

Sirtuins have gained attention because of their diverse physiological and pathological roles, such as life-span extension, heart disease, neurodegenerative disease, obesity, diabetes, inflammation, and cancer (8-15). Sirtuin family members are well conserved from yeast to mammals, and they belong to class-III histone deacetylases (HDACs) (16). The Sirtuin family of proteins is composed of seven members (SIRT1–7) in mammals with SIRT1 being the most extensively studied family member (17-19). SIRT1 is activated in a NAD^+-dependent manner, and the enzyme deacetylates not only acetyl-histones but also other non-histone proteins, including acetyl-p53. Thus, SIRT1 exhibits diverse functions by changing multiple signals (20-23).

Although extensive studies have reported a role of SIRT1 in cancer, there is still controversy regarding the mechanism of action of SIRT1 in cancer. It is under debate as to whether SIRT1 plays a role as a tumor promoter or suppressor. For example, SIRT1 is significantly elevated in human prostate cancer, primary colon cancer, acute myeloid leukemia, and SCC (24-27). In addition, overexpression of SIRT1 decreases expression and/or activity of tumor suppressors and DNA-damage repair pathways, suggesting that SIRT1 promotes cancer growth and progression (21,23,28-32). By contrast, there is much evidence that suggests a tumor-suppressive role of SIRT1. For example, SIRT1 gene expression was attenuated in prostate carcinoma, glioblastoma, and bladder carcinoma (13). In addition, transgene-induced overexpression of SIRT1 in APC-heterozygous knockout mice, a mouse model of colon cancer, decreases cancer growth (33). As described above, the roles of SIRT1 on cancer are dependent on the cancer cell types and the context of experiments. Therefore, it is important to examine the role of SIRT1 in gingival SCC. In the present study, the effect of CAY10591 was examined (Fig. 1) (34), an activator of SIRT1, on the malignant potential of Ca9-22 gingival SCC cells.

Materials and Methods

Antibodies and Reagents

Rabbit anti-p21 and SIRT1, goat anti-matrix metalloproteinase (MMP)-3 and MMP-9, and mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) immunoglobulin (Ig) G were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-p53, acetyl-p53 (Lys377/382), and biotin-conjugated anti-mouse and rabbit IgGs were obtained from ABNOVA (Taipei, Republic of China), Millipore (Billerica, MA), and Zymed Laboratories (San Francisco, CA), respectively. CAY10591 and dimethyl sulfoxide (DMSO) were purchased from Cayman Chemical (Ann Arbor, MI) and WAKO (Osaka, Japan), respectively.

Cell culture

Human gingival SCC Ca9-22 cells purchased from RIKEN Bio Resource Center (Tsukuba, Japan) were maintained in αMinimum Essential Media (αMEM, WAKO) containing 10% fetal bovine serum (FBS, Biofill, Victoria, Australia) and 1% penicillin/streptomycin (WAKO) at 37°C in a 5% CO_2 atmosphere.

Western blot

Ca9-22 cells (1×10^6 cells/dish) in 6-cm dishes were cultured overnight in 10% FBS αMEM. Cells were treated with increasing concentrations of CAY10591 or DMSO in 10% FBS αMEM for increasing periods of time. After washing with cold PBS, the cells were scraped in 200 μL of ice-cold lysis buffer, sonicated, and centrifuged. The supernatants were used as cell lysates; 10−20 micrograms of total protein in the lysates were subjected to SDS-PAGE (5−20% gradient gel) and transferred to PVDF membranes using Trans-Blot Turbo blotting system (Bio-Rad, Hercules, CA). The membranes were blocked by EzBlock blocking reagent (ATTO, Tokyo, Japan) and incubated for 1 h to overnight with desired primary antibodies. After three times washing in tris-buffered saline containing 0.1% Tween20 (TBS-T, pH7.5), they were treated with corresponding biotin-conjugated secondary antibodies. Following streptavidin-peroxidase (KPL, Gaithersburg, MD) treatment for 30 min, the membranes were washed three times with TBS-T. The membranes were further washed three times with TBS-T and protein bands were visualized by Clarity Western ECL Substrate (Bio-Rad) and photographed using ChemiDoc XRS Plus (Bio-Rad).

Cell growth assay

Cell growth of Ca9-22 wild-type cells and its mutant cells were measured using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). Cells were seeded in a 96-well plate at a density of 1,000 cells/well. After overnight incubation at 37°C, media was changed to 100 μL of fresh 10% FBS αMEM and cells were further incubated for 0, 48, and 72 h. At the each time point, supernatants were replaced with 100 μL of prewarmed 10% FBS αMEM containing 1/20 volume of CCK-8 reagent. After 1 h incubation at 37°C, optical density (450 nm) of each well was measured using the Benchmark plus microplate spectrophotometer (Bio-Rad).

Cell invasion assay

Ca9-22 cells were seeded at a density of 2×10^5 cells /well in a 24-well plate. After overnight incubation at 37°C, linear scratch wounds were created in cell-monolayers using a Cell Scratcher scratch stick (AGC TECHNO GLASS, Tokyo, Japan). After washing twice with PBS to remove cell debris, cell layers were treated for 24 h with 0, 2.5, 5, 10, and 20 μM of CAY10591 and then stained with crystal violet. DMSO was used (0.25, 0.5, 1, and 2 μg/mL) as a vehicle control for each concentration of CAY10591. Time zero exhibited freshly scratched wounds. Widths of the remaining

uncovered scratched areas were measured using a measuring grid accompanied by a Cell Scratcher scratch stick. Measurements were performed three times, and the data were displayed as the average ± standard deviation.

Cell-migration assay

Cell-migration assays were performed using transwell cell-culture inserts with small pores (Fig. 2). A conditioned culture medium of human fibroblast was used as chemoattractant (35). One milliliter of condition medium was placed into each well of a 24-well plate, and an 8-μm-pore-size CHEMOTAXICELL cell-culture chamber (KURABO, Osaka, Japan) was placed over each well. Then, 500 μL of cell suspension (4×10^4 cells/mL) was added to upper-side of the chambers. After 24 h incubation at 37°C, chambers were removed and supernatants in the lower wells were aspirated. Cells attached to the bottom of the lower wells were stained with crystal violet (WAKO), and the cell numbers in each well were counted under microscopy.

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) assay

Ca9-22 cells (4.3×10^5 cells/dish) in 3.5-cm dishes were incubated overnight at 37°C and then treated for 12 h with 20 μM CAY10591 or 2 μL/mL DMSO. Total RNA was extracted using the RNAiso plus (TAKARA, Otsu, Japan) according to the manufacturer's instruction. Total RNA (1 μg) was reverse-transcribed using the

ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan). qPCR was performed using the THUNDERBIRD SYBR qPCR Mix (TOYOBO) and the TP900 Thermal Cycler Dice (TAKARA). This involved an initial incubation at 95°C for 1 min, followed by 40 cycles at 95°C for 10 s and 60°C for 30 s. Relative expression of target mRNA was determined using the ΔΔCT method. The β-2-microglobulin gene (*B2M*) was used as a reference gene. The primer sequences used for quantification of invasion/migration related genes are listed in Table 1. Experiments were performed twice and similar results were obtained from each independent experiment. For microRNA (miRNA) quantification, cDNA was created using microScript microRNA cDNA Synthesis Kit (NORGEN biotek, Thorold, Canada), according to the manufacturer's instruction (Fig. 3). Briefly, miRNAs in total RNA (1 μg) were polyadenylated by poly(A) polymerase at their 3′-end. cDNA was then reversely transcribed using oligo(dT) primer conjugating adapter sequence at the 5′-end [oligo(dT)-adapter] to obtain cDNA. PCR was performed using miRNA-specific forward and universal PCR reverse primers. miRNA-specific forward primers used in this study are listed on Table 2. qPCR was performed using the THUNDERBIRD SYBR qPCR Mix and the TP900 Thermal Cycler Dice. This involved an initial incubation at 95°C for 1 min, followed by 40 cycles at 95°C for 5 s and 60°C

for 30 s. Relative expression of target mRNA was determined using the ΔΔCT method. The *RNU48*, a non-coding RNA gene was used as a reference.

Analysis of released MMP-3 and MMP-9

Ca9-22 cells (1×10^6 cells/dish) were seeded in 6 cm-dishes and incubated overnight at 37℃. Cells were treated for 0, 12, 24, 36, and 48 h with 20 μM or 2 μL/mL of CAY10591 or DMSO, respectively, in serum free αMEM. Culture supernatants were collected and subsequently concentrated 10 times using centricon 10 centrifugal filter units (Amicon, Danvers, MA). Twenty microliters of the concentrated culture supernatants were applied to gelatin zymography and western blot for MMP-3 and MMP-9 detection. In the gelatin zymography, 2 native-PAGE gels containing gelatin were prepared and the gels were soaked in 2.5% Triton X-100 for 15 min to substitute SDS to a weaker detergent. One gel was transferred to calcium assay buffer [CAB; 50 mM Tris-HCl (pH7.4) containing 0.2 M NaCl, 5 mM $CaCl_2$, 0.05% Briji35, and 0.02% NaN_3] and the other was to CAB containing 20 mM EDTA. Gels were allowed to incubate for 4 h at 37℃ as enzymatic reactions and stained with CBB G-250. White spots which exhibit gelatin digestion were observed on an X-ray film illuminator and photographed using IXY DIGITAL 920IS (Canon, Tokyo, Japan).

Statistical analysis

The data obtained are presented as means ± standard deviation and analyzed by an unpaired Student's *t*-test to determine statistical significance.

Results

CAY10591 induces SIRT1 protein levels and activity in Ca9-22 gingival SCC

CAY10591 is often used as a SIRT1 activator (34,36). To examine whether CAY10591 treatment affect to SIRT1 production of Ca9-22 gingival epithelial SCC, western blot analysis was performed. Although Ca9-22 did not produce SIRT1 protein in the absence of CAY10591, the compound caused an induction of SIRT1 protein >4 h after stimulation (Fig. 4A). The maximum induction was observed at 12 h after CAY10591 stimulation (Fig. 4A). The inducible effect was observed in a dose-dependent manner (Fig. 4B). In contrast to CAY10591, DMSO, which is used as a vehicle control, did not induce SIRT1 protein levels (Figs. 4A and 4B). To measure the effect of CAY10591 on SIRT1 activity, acetylation of p53 protein at Lys377 and Lys383 was further examined. SIRT1 not only deacetylates histone proteins but also deacetylates p53 (23). Although DMSO decreased p53 acetylation 4 h after treatment, acetylation of p53 was recovered after >8 h (Fig. 4C). By contrast, CAY10591 decreased acetylated p53 in a time-dependent manner (Fig. 4C).

CAY10591 treatment inhibits cell growth and increases level of p21

Aggressive cell growth is one of the most important characteristics of cancer. Thus, the effects of CAY10591 on the rate of cell growth were examined. DMSO treatment did

not affect the growth rate of Ca9-22 cells (left panel of Fig. 5A). By contrast, CAY10591 significantly and dose-dependently inhibited growth rate of Ca9-22 cells (right panel of Fig. 5A). It is well documented that p21 protein suppressively regulates the cell cycle (37-41). Therefore, the effect of CAY10591 on p21 production by Ca9-22 cells was examined. Although both CAY10591 and DMSO induced p21 protein at 24 h after treatment, the induction of p21 protein levels was much higher with CAY10591 than with DMSO (Fig. 5B). In addition, CAY10591 increased p21 levels in a dose-dependent manner 16 h after treatment (Fig. 5C).

CAY10591 represses invasion and migration abilities of Ca9-22 cells

Other characteristics of epithelial SCC cells include aggressive invasion of cells into the connective tissue. Therefore, cell-scratch assays were used to determine cell invasiveness of Ca9-22 cells. DMSO (0.5, 1, and 2 μL/mL) treatments caused a small but significant reduction in cell invasiveness of Ca9-22 cells into scratched area (Figs. 6A and 6B). By contrast, treatment with CAY10591 exhibited a significant reduction in cell invasion into the area scratched in a dose-dependent manner (Figs. 6A and 6B). The results of the cell-scratch assay demonstrate cellular abilities of growth and movement. However, it does not include an ability of migration toward chemoattractants contained in connective tissue, such as laminins (35). To examine the effects of CAY10591 on the

migratory ability of Ca9-22 cells, a cell-migration assay was further utilized. DMSO treatment dose-dependently inhibited cellular migration to the conditioned medium of human periodontal fibroblasts, which was used as a chemoattractant (Fig. 6C). The number of migrating cells treated with CAY10591 decreased in a dose-dependent manner, and the reduction rates were significantly higher than those treated with DMSO (Fig. 6C).

CAY10591 suppresses expression of cell invasion/migration genes and epithelial-mesenchymal marker genes

To explore the mechanisms related to CAY10591-induced reduction of invasion/migration-abilities, gene expressions related to invasion and migration were examined. The expression of *CSNK2A2*, *FRA1*, *ACTB*, and *SLUG*, which are reportedly promote in invasion/migration (42,43), in CAY10591 treated cells were significantly lower than those in vehicle-treated cells (Fig. 7A). In addition, expressions of genes related to suppression of invasion/migration, such as *NEDD9* and *FMN1* genes (42), were induced by CAY10591 treatment (Fig. 7B). However, expression of *VEGFB* and *RADIXIN* (invasion/migration promoter genes) (42,44) and *CSNK1G1* and *ADCK1* (invasion/migration suppressor genes) (42) were unchanged and *ENPP5* (an invasion/migration promoter gene) (42) significantly increased upon CAY10591

treatment (Figs. 7A and 7B). It is reported that carcinoma cells often change their epithelial characteristics to mesenchymal features, and the phenomenon has been documented as the epithelial–mesenchymal transition (EMT) (45). Mesenchymal cells have more migratory characteristics than epithelial cells. Therefore, it was examined the expression levels of *E-cadherin* and *N-cadherin* as a marker of epithelial cells and mesenchymal cells, respectively. Although expression of *E-cadherin* gene was not affected by CAY10591 treatment, *N-cadherin* expression was significantly decreased by CAY10591 treatment compared to DMSO treatment ($P > 0.05$, Fig. 7C).

CAY10591 induces expression of cell invasion/migration-suppressing miRNA

It has been reported that several miRNA control cell invasion/migration (46-48). Thus, the effect of CAY10591 on cell invasion/migration-inhibiting miRNA expressions was examined using qRT-PCR. Compared to DMSO-treated cells, CAY10591-treated cells exhibited 1.72, 1.33, and 1.17 times higher expression level of hsa-miR-194-5p, hsa-miR-200c-3p, and hsa-miR-214-3p, respectively (Fig. 8). hsa-miR-194-5p expression of CAY10591-treated cells showed a significant induction ($P > 0.01$), although the others did not (Fig. 8).

CAY10591 treatment inhibits release of active MMP-3 and MMP-9 from Ca9-22 cells

During penetration of SCC cells into adjacent connective tissues, SCC cells secrete enzymes which digest extracellular matrix proteins, such as matrix metalloproteinases (MMPs) (49,50). Thus, the effects of CAY10591 on release of MMP-3 and MMP-9 from Ca9-22 cells were examined. Western blot analysis exhibited that MMP-3 and MMP-9 were released after 12 h in the supernatant of Ca9-22 cell culture treated with DMSO (Fig. 9A). CAY10591 treatment strongly suppressed release of the both proteinases (Fig. 9A). To further examine the activities of released MMPs, the gelatin zymography was performed. Two white bands which exhibit gelatin digestion were observed at the same molecular weights which were observed in western blot (Fig. 9B) in the gel incubated in an EDTA-free condition [panel (a) of Fig. 9B]. By contrast, no white band was observed in the gel incubated in an EDTA-containing condition [panel (b) of Fig. 9B].

Discussion

In the present study, the effects of CAY10591 on cell proliferation and invasion/migration in gingival SCC Ca9-22 cells were demonstrated. CAY10591 has been used as a specific activator of SIRT1 (34,36,51) and treatment of Ca9-22 cells with the compound decreased p53 acetylation in a time-dependent manner (Fig. 4C). In addition, CAY10591 induced SIRT1 protein levels in a dose and time-dependent manner (Figs. 4A and 4B). These data suggest that CAY10591 not only activates SIRT1 activity but also induces SIRT1 protein levels. Taking this point into account, it was subsequently considered whether SIRT1 acts as a cancer promoter or repressor. CAY10591 treatment resulted in decreased cell proliferation rate (Fig. 5A) and inhibited invasion/migration activities in Ca9-22 cells (Fig. 6). These data suggest that SIRT1 acts as a tumor suppressor in Ca9-22 gingival SCC cells.

Cell proliferation rates of CAY10591-treated Ca9-22 cells were lower than those of vehicle-treated cells (Fig. 5A), and p21 protein levels were induced by CAY10591 treatment (Figs. 5B and 5C). p21 protein is a well characterized cyclin-dependent kinase inhibitor (41), and it inhibits entry into S-phase from G_1-phase and S-phase progression of the cell cycle (40,41,52,53). Therefore, it is hypothesized that CAY10591-treatment may inhibit cell growth rate by the induction of p21 and

subsequent G_1-phase and S-phase arrest. Similar to the hypothesis, Yang *et al.* (54) demonstrated that resveratrol, a famous SIRT1 activator, decreased cell growth rate of gastric carcinoma cell lines through G_1-phase and S-phase arrest. In addition, resveratrol dose-dependently induced p21, and the decreased cell growth and p21 induction were inhibited by SIRT1 silencing (54). Thus, it is concluded that CAY10591 may suppress cell growth through p21 induction. CAY10591 decreased gene expression that is involved in promotion of cell invasion/migration (*CSNK2A2*, *FRA1*, *ACTB*, and *SLUG*, Fig. 7A) and increased expression of cell invasion/migration inhibitory genes and miRNA (*NEDD9*, *FMN1*, and hsa-miR-194-5p, Figs. 7B and 8). These data are consistent with studies that show CAY10591 treatment decreased cell invasion into scratched areas and migration toward chemoattractants released from fibroblasts compared to vehicle treatment (Fig. 6). However, expression of *VEGFB*, *RADIXIN*, *ENPP5*, *CSNK1G2*, *ADCK1*, hsa-miR-200c-3p, and hsa-miR-214-3p did not result in the expected outcome (Figs. 7A, 7B, and 8). Numerous SIRT1 substrates have been found. SIRT1 removes acetyl group from not only acetylated histone but also acetylated-p53, p300, Ku70, FoxO3, and PPARγ (23,55-57). In addition, Kim *et al.* demonstrated that SIRT1 regulates the deacetylation of 70 proteins in mouse liver using acetylome analysis (58). Moreover, >6,800 acetylation sites that can be modified by

deacetylases were found by peptide microarray analysis (59). The complexities of SIRT1 substrates may result in complicated expression patterns of invasion/migration-related genes. Therefore, expression patterns of invasion/migration promoter and repressor genes and miRNAs may determine invasion/migration activities of the cells. In the present model I demonstrated, CAY10591 induces SIRT1 expression and activity, and may subsequently regulate expression of genes and miRNAs involved in cell invasion/migration toward an invasion/migration-suppressive state.

EMT often occurs in carcinoma cells and results in a loss of intercellular adhesions and increased cell mobility; this phenomenon contributes to the malignancy of carcinoma cells (60). qPCR analysis in the present study showed that CAY10591 treatment repressed expression of *N-cadherin* (a mesenchymal marker) but did not alter expression of *E-cadherin* (an epithelial marker, Fig. 7C). These results suggest that CAY10591-treated cells may attenuate mesenchymal characteristics and subsequent invasion/migration abilities. Chen *et al.* overexpressed SIRT1 in oral SCC cells and demonstrated that upregulation of SIRT1 inhibited the invasion/migration ability of carcinoma cells (61). This phenotype change was accompanied by an increase in expression of *E-cadherin* and a decrease in expression of *N-cadherin* (61). Thus, the results of this study may be consistent with the report by Chen *et al*.

As shown in Figure 9A, MMP-3 and MMP-9 were released by Ca9-22 cells in the control condition, and CAY10591 inhibited release of them. In gelatin-zymography, white bands that indicate digested gelatin were observed at the same molecular weight of MMP-3 and MMP-9 (upper panel of Fig. 9B). These bands were disappeared in the gel incubated in the EDTA-containing condition (lower panel of Fig. 9B). Therefore, these gelatin digesting bands were confirmed as active MMP-3 and MMP-9. MMPs, which digest extracellular matrix, also play a role in the invasion of carcinoma cells into adjacent connective tissues (49,50). Thus, the suppression of the release of MMPs by CAY10591 may suggest that CAY10591 negatively controls the progression of carcinoma-cell infiltration into environmental tissues in addition to the suppressive effects of the compound onto cellular motility.

As demonstrated in this study, DMSO was used as a solvent for CAY10591 stock solutions. Treatment with DMSO significantly decreased cell invasion and migration (Fig. 6). During the treatment, SIRT1 production was not affected (Figs. 4A and 4B), suggesting the existence of independent mechanisms other than SIRT1 signaling. Thus, combined use of CAY10591 and DMSO acted in an additive manner and might be a strong tool for inhibition of malignancy of gingival SCC.

Suzuki *et al.* (62) demonstrated the effects of SRT1720, a SIRT1 activator, using an *in vivo* mouse model. In this report, tumor weight and size were slightly decreased by SIT1720 administration. However, the agent increased metastatic score and recovered from cisplatin-decreased expression of *ANGPTL4*, a gene that encodes a protein involved in angiogenesis. The ambivalent effects of SRT1720 in malignancy of breast cancer may also be derived from the complexity of SIRT1-induced signal transduction. Therefore, the *in vivo* effects of CAY10591 are unpredictable, suggesting further research and considerate discussions are needed for use of this potential agent to prevent cancer or as cancer therapy.

Conclusion

All data in this manuscript suggest that CAY10591-treatment of gingival epithelial carcinoma cells inhibits their malignancy, such as cell growth, invasion/migration.

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Table 1. Primer sequences used for quantitation of gene expressions

Genes	Forward primers	Reverse primers
Invasion/migration promoter genes		
CSNK2A2	5′-AAACTTCTGCGATACGACCATCAAC-3′	5′-TCTGCACAAGGCTGGGACTG-3′
FRA1	5′-CAGCTCATCGCAAGAGTAGCA-3′	5′-CAAAGCGAGGAGGGTTGGA-3′
ACTB	Primers provided in Human Housekeeping Gene Primer Set (TAKARA, Japan)	
SURUG	5′-ATCTGCGGCAAGGCGTTTTCCA-3′	5′-GAGCCCTCAGATTTGACCTGTC-3′
VEGFB	5′-AAGGACAGTGCTGTGAAGCCAG-3′	5′-TGGAGTGGGATGGGTGATGTCA-3′
RADIXIN	5′-TATGCTGTCCAAGCCAAGTATG-3′	5′-CGCTGGGGTAGGAGTCTATCA-3′
ENPP5	5′-TCTCCAGTAGCAGCCATCTTGC-3′	5′-GCCACCTTTCTGGAACGTCTTC-3′
Invasion/migration suppressor genes		
NEDD9	5′-CCCATCCAGATACCAAAAGGACG-3′	5′-CACTGGAACTGAAAACACAGGGC-3′
FMN1	5′-CATGAGAAGGAGTCGCTAAGAGC-3′	5′-TGAAGTCTGCCAGGAGTCCTGT-3′
CSNK1G2	5′-AAGGAGCGGTACCAGAAGATCG-3′	5′-GAAGAGCTTCCGCAGGTAGTCA-3′
ADCK1	5′-GGGAAAGGCGGAGATTGTC-3′	5′-CAGAGGTGGCAGTAATTCAGG-3′
Epithelial/mesenchymal marker genes		
E-cadherin	5′-TGCCCAGAAAATGAAAAAGG-3′	5′-GTGTATGTGGCAATGCGTTC-3
N-cadherin	5′-ACAGTGGCCACCTACAAAGG-3′	5′-CCGAGATGGGGTTGATAATG-3′
Reference gene		
B2M	Primers provided in Human Housekeeping Gene Primer Set (TAKARA, Japan)	

Table 2. Primer sequences used for quantitation of miRNA expressions

miRNAs and a reference gene	miRNA-specific forward primers
miRNAs related to cell migration/invasion	
hsa-miR-194-5p	5′-TGTAACAGCAACTCCATGTGGA-3′
hsa-miR-200c-3p	5′-TAATACTGCCGGGTAATGATGGA-3′
hsa-miR-214-3p	5′-ACAGCAGGCACAGACAGGCAGT-3′
Reference gene	
RNU48	5′-ACTCTGAGTGTGTCGCTGATG-3′

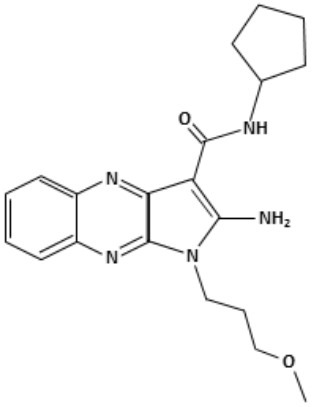

Fig. 1. The structural formula of CAY10591

CAS registry No.: 839699-72-8
Formal name: 2-amino-N-cyclopentyl-1-(3-methoxypropyl)-1H-pyrrolo[2,3-b] quinoxaline-3-carboxamide
Molecular formula: $C_{20}H_{25}N_5O_2$
Molecular weight: 367.5

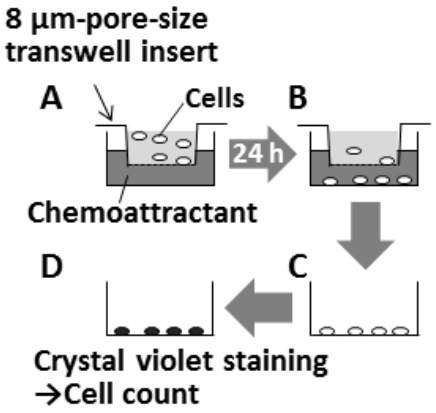

Fig. 2. A schematic of the cell-migration assay

Ca9-22 cells were treated for 24 h with CAY10591 or DMSO. (A) Medium containing chemoattractants was placed into the lower chamber. After an 8-µm-pore-size transwell insert was placed, a suspension of cells treated with CAY10591 for 24 h was placed into the upper chamber. (B) During incubation for 24 h at 37°C, cells migrated through transwell membrane and adhered onto the bottom of the well. (C) After aspiration of medium in the lower chamber following removal of the insert, (D) adherent cells were gently washed with PBS and stained with crystal violet. Stained cells were counted under microscopy.

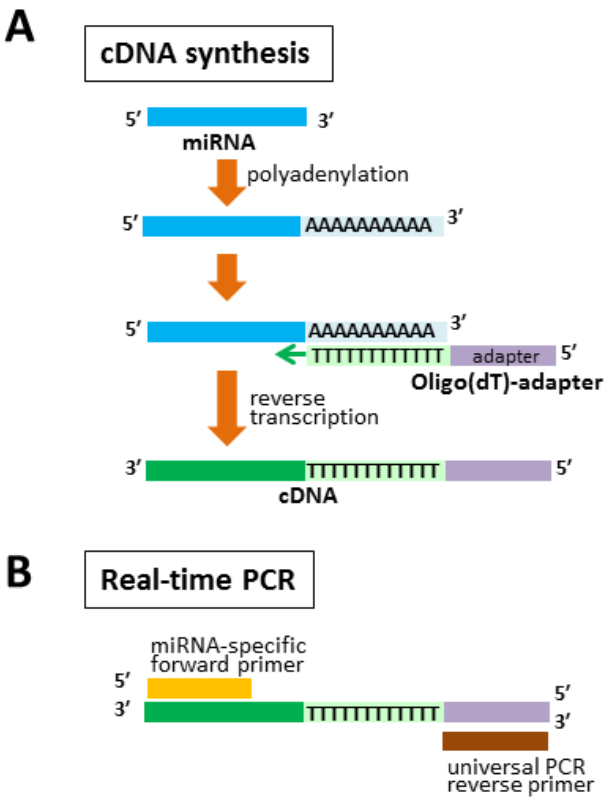

Fig. 3. A schematic of the RT-PCR for miRNA quantification

(A) cDNA synthesis. miRNAs in total RNA were first polyadenylated by poly(A) polymerase at their 3′-end. cDNA was then reversely transcribed using oligo(dT) primer conjugating adapter sequence at the 5′-end [oligo(dT)-adapter] and cDNAs were reversely transcribed. (B) PCR was performed using miRNA-specific forward and universal PCR reverse primers.

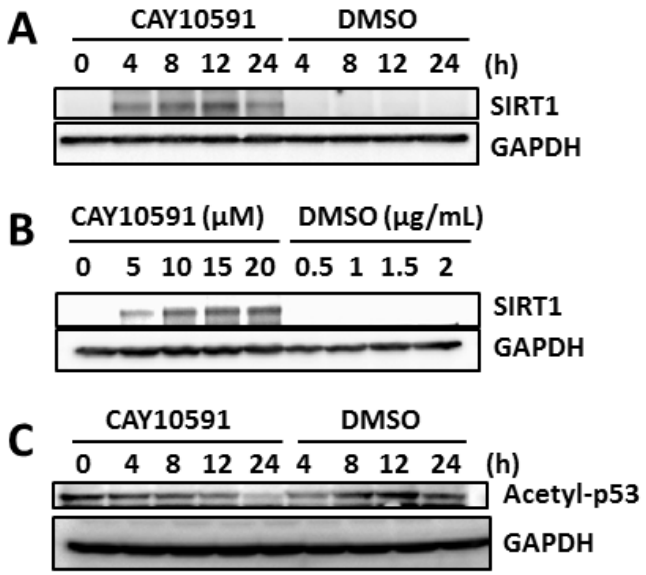

Fig. 4. Induction of SIRT1 protein and activity by CAY10591

(A) CAY10591 treatment induces SIRT1 protein in a time-dependent manner. Ca9-22 cells were treated for the indicated time points with 20 μM CAY10591 or 2 μL/mL DMSO. Cells were lysed and subjected to western blot analysis for SIRT1detection. GAPDH was detected as an internal control. (B) SIRT1 induction by CAY10591 is dose-dependent. Ca9-22 cells were treated for 24 h with CAY10591 or DMSO. Lysed cells were subjected to western blot analysis to detect SIRT1 proteins. GAPDH was detected as a reference control. (C) CAY10591 induces deacetylation activity of SIRT1. Ca9-22 cells were treated for the indicated time points with 20 μM CAY10591 or 2 μL/mL DMSO. Cells were lysed with buffer containing HDAC inhibitors and lysates were subjected to western blot analysis to detect acetylated p53 (Lys377/383). GAPDH was detected as an internal control.

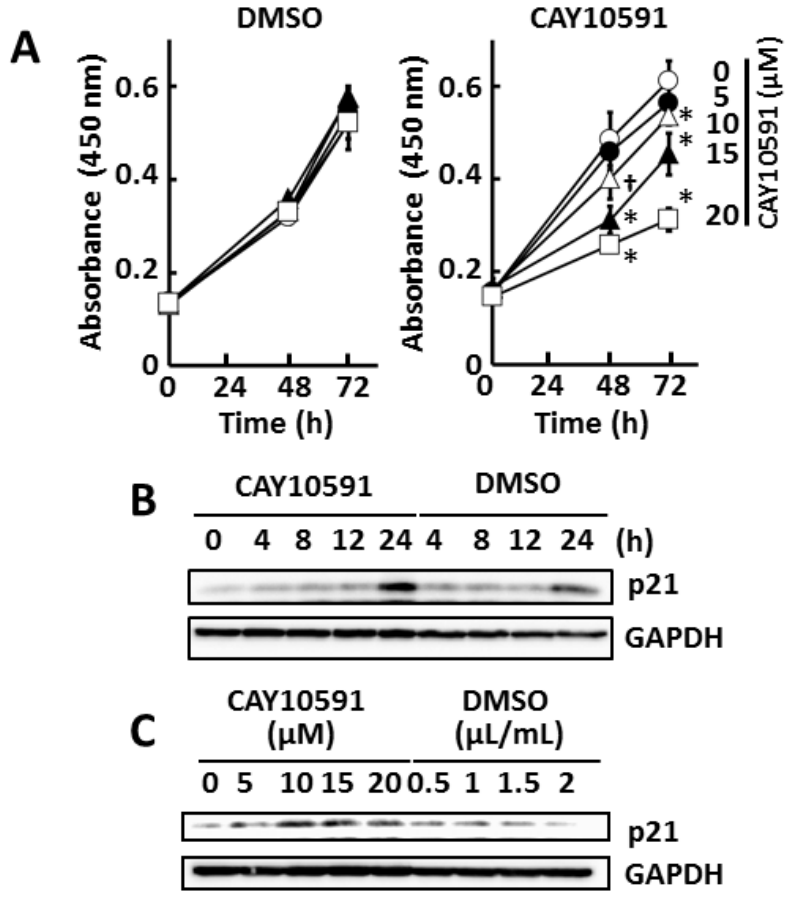

Fig. 5. Suppressing effect of CAY10591 on cell growth

(A) CAY10591 decreased cell growth rate of Ca9-22 cells. Ca9-22 cells were seeded in 96-well plates, incubated overnight, and treated with the indicated concentrations of CAY10591 (○, 0; ●, 5; △, 10; ▲, 15; □, 20 μM, respectively) or DMSO (○, 0; ●, 0.5; △, 1; ▲, 1.5; □, 2 μL/mL, respectively). After incubation for the indicated time points, cell growth was measured using Cell Counting Kit-8. *, $P < 0.01$ (Student's *t*-test); †, $P < 0.05$ (Student's *t*-test) when compared with untreated controls. (B) Time course for the effect of CAY10591 treatment on p21 production. Ca9-22 cells were treated for the indicated time points with 20 μM CAY10591 or 2 μL/mL DMSO. Cells were lysed and subjected to western blot analysis to detect p21 protein. GAPDH was detected as an internal reference. (C) Dose-dependent effects of CAY10591 treatment on p21 protein level. Ca9-22 cells were treated for 16 h with the indicated concentration of CAY10591 or DMSO. Cells were lysed and subjected to western blot analysis to detect p21 protein. GAPDH was detected as an internal control.

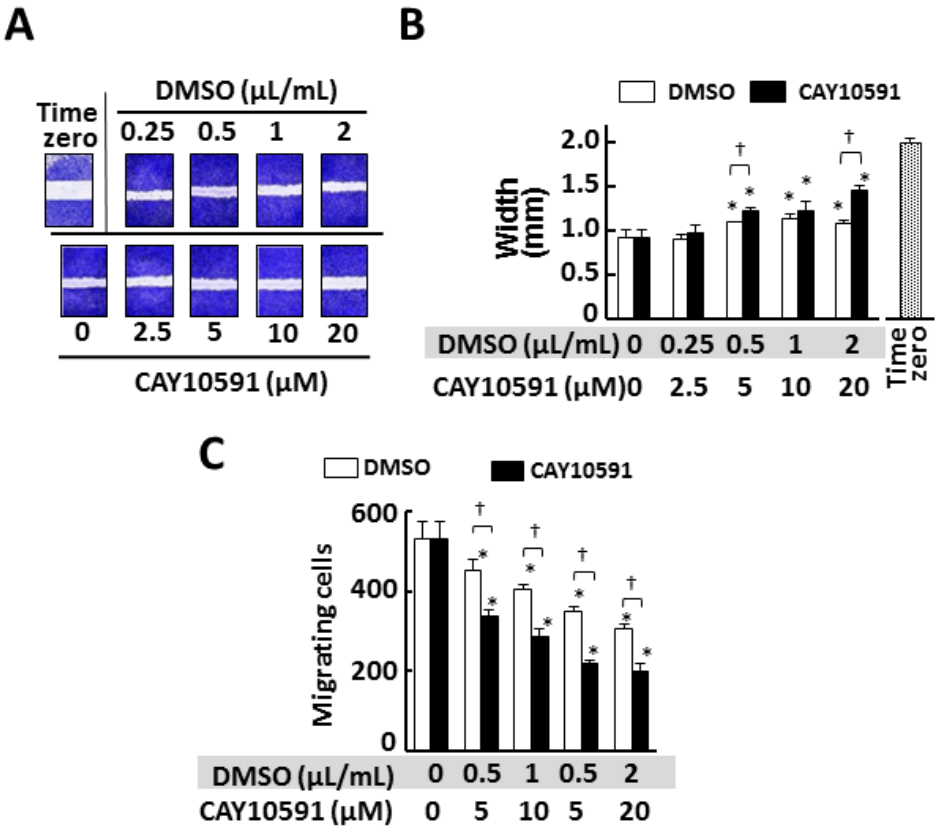

Fig. 6. CAY10591 suppressed invasion/migration of Ca9-22 cells

(A) Ca9-22 cells were seeded in a 24 well plate. After overnight incubation at 37°C, a linear wound was created on the cell monolayer using a Cell Scratcher scratch stick. Cell layers were treated for 24 h with indicated concentrations of CAY10591 or DMSO. Cells were then stained with crystal violet. DMSO was used as a vehicle (the volumes indicated are equivalent to those of CAY10591). At time zero, freshly scratched wounds are seen. (B) Widths of the remaining uncovered wound area were measured three times and the data are displayed as the average ± standard deviation. *, $P < 0.01$ (Student's *t*-test) when compared with time zero; †, $P < 0.01$ (Student's *t*-test). (C) Migrating cells were counted under microscopy. Open bars and closed bars indicate the number of migrating cells treated with DMSO and CAY10591, respectively. *, $P < 0.01$ (Student's *t*-test) when compared with time zero; †, $P < 0.01$ (Student's *t*-test).

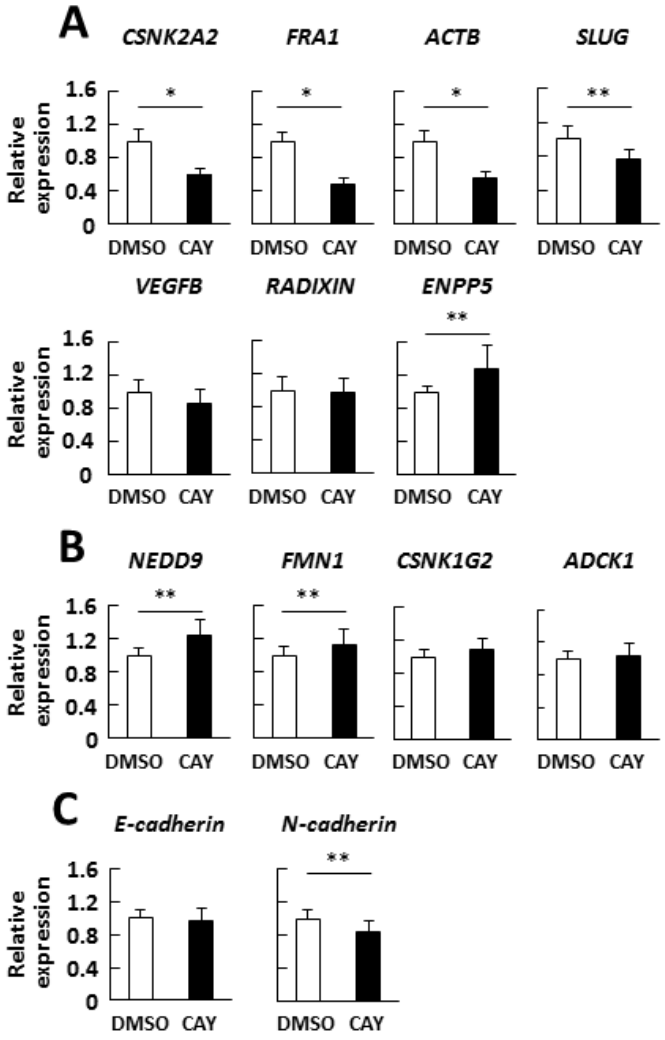

Fig. 7. CAY10591 treatment regulates expression of invasion/migration-related genes

Ca9-22 cells were treated for 12 h with CAY10591 (20 μM) or DMSO (2 μL/mL). Total RNA was extracted and used in qRT-PCR analysis. β-2 microglobulin gene was used as a reference gene. (A) Relative expression of invasion/migration promoter genes. (B) Relative expression of invasion/migration suppressor genes. (C) Relative expression of epithelial/mesenchymal marker genes. DMSO, dimethyl sulfoxide-treated. CAY, CAY10591-treated. *, $P < 0.01$ (Student's *t*-test); **, $P < 0.05$ (Student's *t*-test).

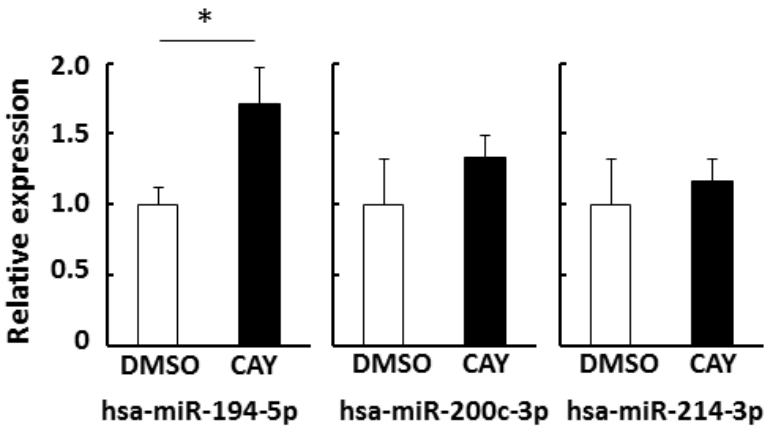

Fig. 8. CAY10591 treatment induces expression of invasion/migration-suppressing miRNAs

Ca9-22 cells were treated for 12 h with CAY10591 (20 μM) or DMSO (2 μL/mL). Total RNA was extracted and used in qRT-PCR analysis. RNU48 gene was used as a reference gene. Relative expression of miRNAs suppressing invasion/migration. DMSO, dimethyl sulfoxide-treated. CAY, CAY10591-treated. *, $P < 0.01$ (Student's *t*-test).

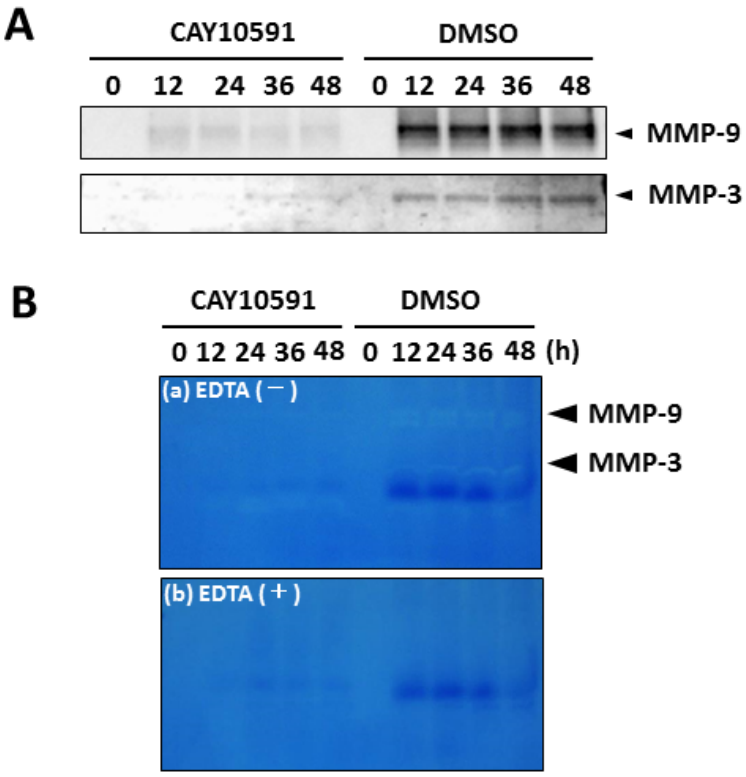

Fig. 9. CAY10591 treatment inhibits release of MMP-3 and MMP-9 from Ca9-22 cells

(A) Ca9-22 cells release MMP-9 and MMP-3 to extracellular milieu and CAY10591 represses them. Ca9-22 cells were incubated with DMSO or CAY10591 in the serum free media and the supernatants were collected at 0, 12, 24, 36, and 48 h. Ten-times concentrated supernatants (20 μL) were applied to Western blot analysis.(B) Confirmation of MMP-activities of MMP-3 and MMP-9 in culture supernatants. Concentrated cell-culture supernatants were also applied to gelatin-zymography. Two native PAGEs gels were prepared. The gels were soaked in 2.5% Triton X-100 for 15 min and one gel was transferred to CAB (a) and the other was to CAB containing 20 mM EDTA (b). Gels were allowed to incubation for 4 h at 37°C as enzymatic reaction, stained in CBB G-250, and destained.