Novel Acridine-Based N-Acyl-homoserine Lactone Analogs Induce Endoreduplication in the Human Oral Squamous Carcinoma Cell Line SAS

Hongbo Chai, a Masaharu Hazawa, b Yoichiro Hosokawa, a Jun Igarashi, c Hiroaki Suga, c and Ikuo Kashiwakura a,c

a Department of Radiological Life Sciences, Hirosaki University Graduate School of Health Sciences; 66–1 Honcho, Hirosaki, Aomori 036–8564, Japan; b Research Center for Radiation Emergency Medicine, National Institute of Radiological Sciences; 4–9–1 Anagawa, Inage-ku, Chiba 263–8555, Japan; and c Research Center for Advanced Science and Technology, The University of Tokyo; 4–6–1 Komaba, Meguro-ku, Tokyo 153–8904, Japan.

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The cytotoxicity of novel acridine-based N-acyl-homoserine lactone (AHL) analogs was investigated on the human oral squamous carcinoma cell line SAS. One analog induced G2/M phase arrest at 5.3–10.6 µM and induced polyploidy at a higher dose (21.2 µM). Importantly, treatment of SAS cells with a combination of the AHL analog and the Jun N-terminal kinase (JNK) inhibitor, SP600125, prevented mitosis and induced polyploidy. The AHL analog synergized with X-irradiation to inhibit clonogenic survival of SAS cells; however, its radiosensitizing effects were relative to not X-irradiation-induced apoptosis but mitotic failure following enhanced expression of Aurora A and B. These results suggest that the active AHL analog showed growth-suppressive and radiosensitizing effects, which involve polyploidy followed by G2/M accumulation and atypical cell death in the SAS cell line.

Key words Quorum sensing; N-acyl-homoserine lactone; acridine; SP600125; endoreduplication; oral squamous carcinoma cell

Quorum sensing (QS) is a form of cell-to-cell communication mediated by the production, secretion, and response to small, diffusible signaling molecules known as autoinducers. 1,2 In Gram-negative bacteria, members of the N-acyl-homoserine lactone (AHL) family of molecules are used as autoinducers for QS. AHLs generally contain a homoserine lactone moiety and a fatty acyl side chain (Fig. 1A), which may vary in chain length, oxidation state, and degree of carbon saturation. 1,3–5) AHLs and synthetic analogs have been shown to interfere with the bacterial QS system, 6,7) and also have biological effects on human cells. 8–13) It is generally considered that a long acyl chain and oxygen on the third carbon (C3) of the acyl chain are required for biological activity. 12,13)

Acridine, a poly cyclic heteroaromatic compound, and its derivatives are known to exhibit a broad spectrum of biological activities, including antiprotozoal, antibacterial, antiviral, and antitumor activity. 16) We recently reported that acridine-based AHL analogs showed antiproliferative activity against human oral squamous carcinoma cells (OSCC), independently of the structural characteristics described above. 15) However, the underlying mechanisms by which acridine-based AHL analogs exert their cytotoxic effects have not yet been elucidated.

OSCC is a typical squamous cell carcinoma that occurs at various sites in the oral cavity, including the lip, hard palate, gum, and tongue. 16) The ultimate goal of treatment is to eradicate the cancer, preserve or restore form and function at the affected site, minimize the sequelae of treatment, and prevent the development of new primary cancers. Surgery is the standard initial mode of treatment for the majority of oral cancers, but can seriously affect the quality of life of advanced OSCC patients. Radiotherapy is often employed in conjunction with surgery, and is most often offered as post-operative treatment. Conventional chemotherapies such as cisplatin generally are not useful for long-term control of primary OSCCs. 17) Therefore, identification of new druggable targets and effective chemotherapeutic medicines for OSCC is highly desirable.

In the present study, we evaluated several acridine-based AHL analogs for their cytotoxicity towards and potential to radiosensitize OSCC SAS cells.

MATERIALS AND METHODS

Reagents The AHL analogs used in this study were provided by Otsuka Chemical Co., Ltd (Tokushima, Japan), and their structures are shown in Fig. 1B. Each compound was dissolved in dimethyl sulfoxide (Wako Pure Chemical Industries, Ltd., Osaka, Japan) to prepare stock solutions of 10 and 20 mM. The mitogen activated protein kinase (MAPK) 1/2 (MEK1/2) inhibitor (PD98059) and Jun N-terminal kinase (JNK) inhibitor (SP600125) were purchased from Sigma-Aldrich (Tokyo, Japan), and the p38 MAPK inhibitor (SB203580) was purchased from Merck (Darmstadt, Germany). For Western blot analyses, anti-caspase-3 (rabbit, #9662), anti-poly(ADP-ribose) polymerase-1 (PARP) (rabbit, #9542), anti-Aurora A (rabbit, #3079), anti-Aurora B (rabbit, #3094), and anti-phospho-Aurora A/Aurora B/Aurora C (rabbit, #2914) antibodies (Abs) were from Cell Signaling Technology (Tokyo, Japan), and the anti-actin Ab (goat, C-11, sc-1615) was from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.).

Cell Culture and Growth Inhibition Assay The human OSCC SAS cell line was obtained from RIKEN Bio-Resource Center (Tsukuba, Japan). SAS cells were cultured in RPMI-1640 medium (Sigma, St. Louis, MO, U.S.A.) supplemented with penicillin (100 U/mL) streptomycin (100 µg/mL), and 10% heat-inactivated fetal bovine serum (FBS), Bioserum, UBC, Tokyo, Japan, at 37°C in a 5% CO2 humidified atmosphere.

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* To whom correspondence should be addressed. e-mail: ikashi@cc.hirosaki-u.ac.jp

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To determine compound 50% inhibitory concentrations (IC50), SAS cells were cultured for 48 h in 35-mm culture dishes (Iwaki, Chiba, Japan) with 1–20 µM final concentrations of AHL analogs. Cells were harvested and the number of viable cells was enumerated microscopically by the trypan blue exclusion test. The IC50 values were calculated using the Boltzmann function.

In Vitro Irradiation SAS cells were seeded in a 60-mm culture dish (Iwaki) and AHL analogs were added at 50% of the IC50 concentration for 1 h. Cells were then X-irradiated (150 kV, 20 mA) with 0–8 Gy using 0.5-mm aluminum and 0.3-mm copper filters at a distance of 45 cm from the focus, at a dose rate of 1.0–1.05 Gy/min (MBR-1520R; Hitachi Medical Corporation, Tokyo, Japan). During exposure, the dose intensity was evaluated using the ionization chamber probe. After irradiation, cells were placed back in culture at 37°C, and 8 d later cell colonies consisting of at least 50 cells were counted using an inverted light microscope. Survival rate were determined by dividing the colony numbers after incubation with compound plus 4 Gy X-irradiation by the colony numbers after incubation with compounds alone. Values are the mean±S.D. of at least 3 experiments. *p < 0.05, by Mann–Whitney’s U-test. (D) Radiation sensitizing effects are shown as survival curves obtained by X-irradiation alone and a combination of X-irradiation with compound #3 (○, X-irradiation alone; ●, X-irradiation+compound #3). SAS cells were seeded in a 35-mm dish and compound #3 was added at half of the IC50 dose before irradiation within 1 h. Values are the mean±S.D. of 3 experiments. *p < 0.05 by the Student’s t-test.

**Fig. 1. Structure and Activity of AHL Analogs on SAS Cells**

(A) Basic structure of AHLs. (a) Homoserine lactone (HSL), (b) acyl chain. (B) Dose–response of 5 AHL analogs with different acyl chain lengths after culture with SAS cells for 48 h. The 50% inhibition concentration (IC50) values were determined using the Boltzmann function. Values are the mean±S.D. of at least 3 experiments. (C) X-Irradiation experiments were performed using half of the IC50 concentrations of 5 AHL analogs. Colony analysis was performed after 7 d. Values were determined by dividing the colony numbers after incubation with compound plus 4 Gy X-irradiation by the colony numbers after incubation with compounds alone. Values are the mean±S.D. of at least 3 experiments.*p < 0.05, by Mann–Whitney’s U-test. (D) Radiation sensitizing effects are shown as survival curves obtained by X-irradiation alone and a combination of X-irradiation with compound #3 (○, X-irradiation alone; ●, X-irradiation+compound #3). SAS cells were seeded in a 35-mm dish and compound #3 was added at half of the IC50 dose before irradiation within 1 h. Values are the mean±S.D. of 3 experiments. *p < 0.05 by the Student’s t-test.

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Cell Cycle Analysis SAS cells were treated with compound alone, 4 Gy X-irradiation alone, or a combination of both. The compound was added 1 h before X-irradiation, as described above. After each treatment, the cells were incubated for 12, 24, 48, or 72 h at 37°C. Cells were harvested, washed, and resuspended in phosphate-buffered saline (PBS) containing 0.1% Triton X-100 (Wako) and propidium iodide (PI) 50 µg/mL (Sigma) to stain DNA. The cell cycle distribution analysis was performed with a Cell Lab Quanta™ SC MPL flow cytometer (Beckman Coulter, Fullerton, CA, U.S.A.).
Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting

SDS-PAGE and Western blotting were performed as described previously. In brief, treated SAS cells were harvested and lysed on ice for 30 min in 50 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (Hepes)–HCl (pH 7.4), 100 mM NaCl, 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride (PMSF) (Wako), then sonicated twice for 30 s at 4°C. After a 20-min centrifugation (12,000 rpm at 4°C), the supernatant was removed and the protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad Lab, Hercules, CA, U.S.A.) and a SmartSpecTM Plus spectrophotometer (Bio-Rad). An equal volume of sample buffer (625 mM Tris–HCl, pH 6.8, 20% SDS, 2% 2-mercaptoethanol, and 2% glycerol) was added to the supernatant, which was then boiled for 5 min. Proteins (40–80 µg/lane) were separated by SDS-PAGE and transferred to nitrocellulose membranes (Advantec Toyo, Tokyo, Japan). The membranes were blocked with Tris-buffered saline (10 mM Tris–HCl, pH 7.4, 100 mM NaCl, 0.1% Tween-20) supplemented with 5% non-fat milk or bovine serum albumin, and then incubated with primary Abs in blocking buffer for 1 h at room temperature. Membranes were incubated with horseradish peroxidase-conjugated secondary Abs (donkey anti-rabbit immunoglobulin G-horseradish peroxidase (IgG-HRP), anti-goat IgG-HRP Ab; Santa Cruz Biotechnology) and the blots were developed with Pierce ECL Western blotting substrate (Pierce, Rockford, IL, U.S.A.).

Combined Effects of AHL Analogs and MAPK Inhibitors

Cells were treated with 20 µM MEK 1/2 inhibitor PD98059, 10 µM JNK inhibitor SP600125, or 15 µM p38 inhibitor SB203580 for 2 h before addition of the AHL analogs. After 48 h, cells were harvested, viability was determined by the trypan blue exclusion test, and cell cycle distribution was analyzed by flow cytometry following PI staining. The optimal inhibitor concentrations were determined according to previous studies.

Statistical Analysis

Each experiment was performed at least 3 times. Differences between the control and experimental groups were evaluated by the Mann–Whitney’s U-test, the Student’s t-test, or the Scheffé’s F-test. Statistical significance was defined as a p-value of less than 0.05.

RESULTS

The Antiproliferative Activity and Radiosensitizing Effects of AHL Analogs

The antiproliferative activity of compounds #1–5 was evaluated by measuring the IC50 for inhibition of SAS cell viability (Fig. 1B), which ranged from 1.5 to 18 µM. The radiosensitizing effect was determined by treating cells with the compounds followed by 4 Gy X-irradiation. AHL analogs #2–5 were found to radiosensitize SAS cells, as measured by the synergistic inhibition of clonogenic survival (Fig. 1C). To clarify the radiosensitizing effect in more detail, the most effective compound (compound #3) was applied to the culture together with 0–8 Gy X-irradiation. A significant radiosensitizing effect was observed on combination of both treatments (Fig. 1D). The following experiments were performed with this compound.

Effects on Cell Cycle Distribution

The distribution of SAS cells within the cell cycle was quantified by PI staining.
and flow cytometry at 12, 24, 48, and 72h of culture. Treatment of cells with analog #3 at 10.6 $\mu$M induced a G2/M phase block, and at 21.2 $\mu$M induced G2/M accumulation and polyploidy resulting from the failure to undergo mitosis (Fig. 2).

**Combined Effects of Active Compounds and MAPK Inhibitors**

To determine if MAPK signaling is involved in the cytotoxic mechanism of compound #3, we analyzed the effects of treatment with PD98059, SP600125, and SB203580, inhibitors of MEK1/2, JNK, and p38, respectively. Cells treated with compound #3 at twice the IC$_{50}$ showed obvious G2/M accumulation and polyploidy resulting from the failure to undergo mitosis (Fig. 2).

**Combined Effects of Active Compounds and X-Irradiation**

We next evaluated the effects on SAS growth of compound #3 combined with 4 Gy X-irradiation. X-Irradiation alone blocked the cells at the G2/M phase, and this was enhanced by the addition of compound #3 (Fig. 3B). Moreover, the combination of compound #3 at its IC$_{50}$ and SP600125 at 10 $\mu$M showed significant antiproliferative activity, compared to either #3 alone or SP600125 alone (Fig. 3C).
of the specific executor proteins of apoptosis, caspase-3 and PARP. As expected, the expression of cleaved caspase-3, the activated form of caspase-3, and cleaved PARP were observed after treatment with X-irradiation alone. However, there was no further induction of cleaved caspase-3 or PARP when X-irradiation was combined with AHL analog treatment (Fig. 4B).

Although the toxicity of compound #3 did not require JNK activation, it significantly induced synergistic polyploidy in the presence of SP600125 (Fig. 3). To elucidate the reason for this discrepancy, we investigated Aurora kinases, which are potent targets of SP600125. Expression of Aurora A and B was increased in all treatments (Fig. 4C). Activation of Aurora A and B was inhibited by X-irradiation or combined treatments.

**DISCUSSION**

In the present study, we analyzed the mechanisms by which acridine-based AHL analogs of different acyl chain lengths exert their cytotoxic effects on OSCC SAS cells. In general, long acyl chains (C12) are considered important to the stability of AHL liquids, and are also important for AHL activity. However, we found that the cytotoxic activity of AHL analogs was independent of acyl chain length (Fig. 1B). Therefore, acridine is likely to play a role not only in the cytotoxicity of these compounds, but also in their stability. In addition, the acyl chain length of acridine analogs might be important for effects on drug targets.

The cell cycle is strictly regulated by phase-specific factors that coordinate the division of intact DNA, and defects in these regulatory molecules or damage to DNA can prevent cell cycle progression. In this study, compound #3 led not
only to G2/M accumulation but also to polyploid induction (Fig. 2), which is considered to be result from endoreplication, in which cells exit S phase, bypass mitosis, and double their DNA content.23 However, compound #3 was not involved in induction of apoptosis (Figs. 2, 4B). Importantly, endoreplication and growth inhibition were increased synergistically only by the combination of compound #3 and SP600125 (Fig. 3); however, we could detect no activation of JNK by compound #3 over 12 and 48 h of treatment (data not shown). This discrepancy could be explained by the additional pharmacological effects of SP600125, as demonstrated in a previous report, which showed that SP600125 induced endoreplication independent of JNK activity through inhibition of Cdk1 activity, and subsequent suppression of Aurora A kinase and PLK1 kinase.24 To the best of our knowledge, there are no reports of the relationship between endoreplication and MAPK. Taken together, our data suggest that compound #3 does not affect any of the MAPK tested, and rather it regulates another suggested target of SP600125, Aurora kinases.

Overexpression of the epidermal growth factor receptor (EGFR) in OSCC is associated with poor disease control.25 Hence, EGFR inhibition using cetuximab (anti-EGFR monoclonal antibody) is considered a potential therapeutic strategy.26 Although it is known that EGFR signaling stimulates 3 of the major signaling pathways for cell survival (phosphatidylinositol 3-kinase (PI3K)-Akt, nuclear factor-kB (NF-kB), and Raf-MEK-Erk), Raf-MEK-Erk signaling is not involved in radiation sensitivity of epithelial cells.27 This observation is consistent with our results that compound #3 had no effect on Erk (Figs. 3A, 3B), but did have a radiosensitizing effect on the SAS cells (Fig. 1C). A recent report suggests the potential importance of Aurora B expression as a diagnostic indicator and therapeutic target in OSCC.28 Aurora kinases are key regulators of mitosis and multiple signaling pathways;29 hence, their overexpression leads to polyploidy and subsequent cell death followed by mitotic failure.30 Moreover, a correlation between mitotic catastrophe and radiation sensitization has been suggested.31 In accordance with previous reports, compound #3 showed cytotoxicity and radiosensitizing effects which could be related to the enhanced expression of Aurora A and B (Fig. 4C).

In conclusion, we showed that compound #3, an active AHL analog, induced atypical mitotic cell death followed by mitotic failure and polyploidy in OSCC SAS cells. This may be also related to compound #3 sensitized SAS cells to irradiation. Additional investigation will be required to determine the potential therapeutic value of compound #3 for treatment of OSCC.

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