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Anti-proliferative effect of (19Z)-halichondramide, a novel marine macrolide isolated from the sponge *Chondrosia corticata*, is associated with G2/M cell cycle arrest and suppression of mTOR signaling in human lung cancer cells

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ABSTRACT

Five oxazole-containing macrolides isolated from the marine sponge *Chondrosia corticata* were evaluated for their anti-proliferative activity in a panel of human solid cancer cell lines. (19Z)-Halichondramide ((19Z)-HCA), a novel trisoxazole-containing macrolide, exhibited the highest potency among the macrolides, with IC_{50} values in the submicro-molar ranges. Prompted by the high potency of growth inhibition of cancer cells, we investigated the mechanism of action of the anti-proliferative activity of (19Z)-HCA in human A549 lung cancer cells. (19Z)-HCA induced cell cycle arrest in the G2/M phase, and this event was highly correlated with the expression of checkpoint proteins, including the up-regulation of p53 and GADD45α and the down-regulation of cyclin B1, cyclin A, CDC2, and CDC25C. In addition, the growth inhibition by (19Z)-HCA was associated with the suppression of mTOR and its downstream effector molecules 4EBP1 and p70S6K. The modulation of mTOR signaling by (19Z)-HCA was found to be mediated by the regulation of upstream proteins, including the down-regulation of Akt and p38 MAPK and the up-regulation of AMPK. These data suggest the potential of (19Z)-HCA to serve as a candidate for cancer chemotherapeutic agents derived from marine organisms by virtue of arresting the cell cycle in the G2/M phase and the modulation of mTOR/AMPK signaling pathways.

1. Introduction

Natural products have played a crucial role in drug discovery and development (Harvey, 2008). Indeed, plant-based terrestrial natural products have provided valuable therapeutic agents. However, marine natural sources with global biodiversity have recently been explored for the identification of new chemical entities. In our continuous efforts to identify novel small molecules for cancer chemotherapeutic agents from natural products, we recently identified numerous bioactive natural compounds from marine sponges, including *Chondrosia corticata*. In our previous study, oxazole-containing macrolides along with new metabolites were isolated from the marine sponge *C. corticata* (Chondrillidae) (Shin et al., 2004). The oxazole-containing macrolides exhibited potent cytotoxicity and antifungal activity. Although the macrolides showed significant cytotoxicity against human leukemia cells, their anti-proliferative activity against human solid cancer cells and the precise mechanism of action remain to be elucidated. Therefore, in the present study, the anti-proliferative activity of oxazole-containing macrolides from *C. corticata* was evaluated in a panel of human cancer cells. In addition, the plausible mechanism of action of anti-cancer activity by (19Z)-halichondramide ((19Z)-HCA), the most active metabolite of *C. corticata*, was investigated in human non-small cell lung cancer A549 cells.

Lung cancer is the most common cancer and the leading cause of cancer-associated death worldwide (Jemal et al., 2011; Parkin, 2001). As reported by the American Cancer Society, lung cancer is expected to account for 26% of all female cancer deaths and 29% of all male cancer deaths in 2012 (Siegel et al., 2012). Among lung cancers, non-small cell lung cancer (NSCLC) accounts for approximately 80–85% of all lung cancers (Breathnach et al., 2001). Although novel chemotherapeutic agents and regimens have been introduced for NSCLC therapy during the past decade, the two-year survival rate remains lower than 20% in patients with advanced NSCLC (Raez and Lilenbaum, 2006). Therefore, the search for novel chemotherapeutic agents might be important for the treatment of lung cancer.

Herein, we report for the first time that (19Z)-HCA, a trisoxazole-containing macrolide derived from *C. corticata*, exhibits potent anti-proliferative activity in human non-small cell lung cancer cells, which was associated with G2/M cell cycle arrest and the suppression of Akt/mTOR signaling pathways.

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2. Materials and methods

2.1. Materials

RPMI 1640 medium, fetal bovine serum (FBS), antibiotic–antimycotic solution, and trypsin–EDTA were purchased from Invitrogen (Grand Island, NY, USA). Bovine serum albumin (BSA), sulforhodamine B (SRB), trichloroacetic acid, propidium iodide (PI), ribonuclease A (RNase A), HEPES, mouse monoclonal anti-β-actin antibody, ellipticine, nocodazole, paclitaxel and other agents unless otherwise indicated were purchased from Sigma–Aldrich (St. Louis, MO, USA). Mouse monoclonal anti-phospho-ERK (Tyr 204) and anti-p53 and rabbit polyclonal anti-GADD45α, anti-cyclin A, anti-cyclin B1, anti-CDC2, anti-ERK 1, and anti-p38 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse monoclonal anti-phospho-SAPK/JNK (Thr 183/Tyr 185) and rabbit polyclonal anti-β-tubulin, anti-CDC25C, anti-phospho-Akt (Ser 473), anti-Akt, anti-phospho-p38 (Thr 180/Tyr 182), anti-SAPK/JNK, anti-phospho-mTOR (Ser 2448), anti-mTOR, anti-phospho-p70S6K (Thr 389), anti-p70S6K, anti-phospho-4EBP1 (Thr 37/46), and anti-4EBP1 were purchased from Cell Signaling Technology (Danvers, MA, USA). Alexa Fluor 488-labeled chicken anti-rabbit IgG was purchased from Invitrogen (Carlsbad, CA, USA). DAPI was purchased from Vector Laboratories (Burlingame, CA, USA). The test compounds, oxazole-containing macrolides (Fig. 1), were isolated from the marine sponge *C. corticata* as described previously (Shin et al., 2004) and dissolved in 100% dimethyl sulfoxide (DMSO).

2.2. Cell culture

Human lung cancer cells (A549), colon cancer cells (HCT-116), breast cancer cells (MDA-MB-231), liver cancer cells (SK-HEP-1), and stomach cancer cells (SNU-601) cells were provided by the Korean Cell Line Bank (Seoul, Korea). The cells were cultured in medium (DMEM for MDA-MB-231 and SK-HEP-1 cells; RPMI 1640 for A549, HCT-116, and SNU-601 cells) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics-antimycotics (PSF; 100 units/mL penicillin G sodium, 100 μg/mL streptomycin, and 250 ng/mL amphotericin B). The cells were incubated at 37 °C and 5% CO_2 in a humidified atmosphere. Cells were maintained in 75 cm^2 culture flask and subcultured at various ratios according to their doubling times (22–30 h). Paclitaxel-resistant A549 cells (A549-PA) were developed by our group from parental A549 cells through continuous exposure to gradually increasing concentrations of paclitaxel and maintained in RPMI 1640 medium containing 100 nM paclitaxel (Kim et al., 2012).

2.3. Cell proliferation assay

Cells (5–7.5 × 10^4 cells/ml) were treated with various concentrations of compounds (total volume of 200 μl/well) in 96-well culture plates for 72 h. After treatment, the cells were fixed with 10% TCA solution, and cell viability was determined with a sulforhodamine B (SRB) assay (Chung et al., 2011a). The results were expressed as percentages relative to solvent-treated control incubations, and IC_{50} values were calculated using non-linear regression analysis (percent survival versus concentration).

2.4. Microscopy and immunocytochemistry

The morphology of cells was observed using an Olympus CKX41 microscope (Tokyo, Japan) at 100× magnification. For immunocytochemistry, cells were grown on cover slips in dishes. After treatment, the cells were fixed with 4% paraformaldehyde (in PBS) for 15 min, and the fixed cells were permeabilized with 0.1% Triton X-100 (PBS) for 5 min. After blocking with 1% BSA (in PBS) for 30 min at room temperature, the cells were incubated with primary antibody at 4 °C overnight. Following the overnight incubation, cells were incubated with FITC-conjugated secondary antibody for 2 h at room temperature. DAPI (0.5 μg/ml) was used to counterstain the nuclei. The images were acquired using a Zeiss ApoTome microscope (Carl Zeiss, Jena, Germany).

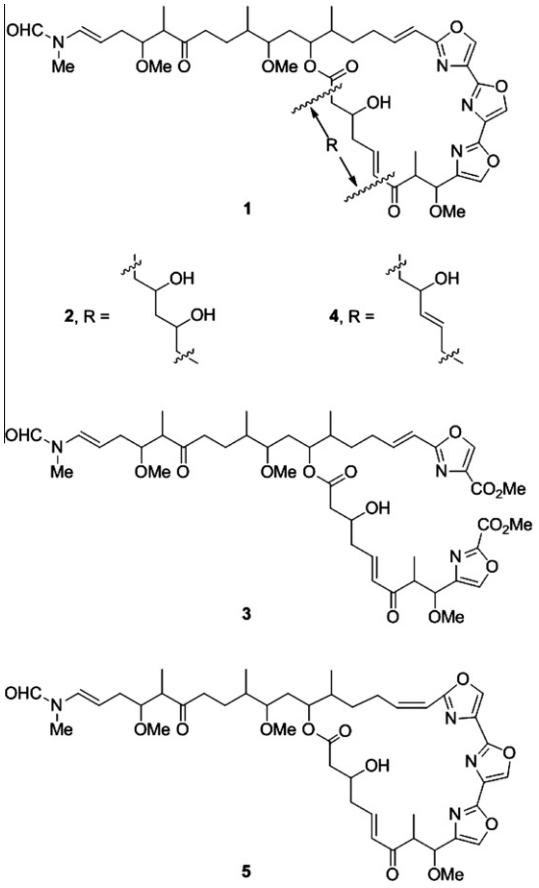

Fig. 1. Chemical structures of oxazole-containing macrolides: halichondramide (**1**); jaspisamide A (**2**); halishigamide D (**3**); neohalichondramide (**4**); and (19*Z*)-halichondramide (**5**).

2.5. Cell cycle analysis

A549 cells were plated at a density of 1 × 10^6 cells per 100-mm culture dish and incubated for 24 h. Fresh media containing various concentrations of test sample were added to culture dishes. Following a 24 h incubation, the cells were harvested (trypsinization and centrifugation) and fixed with 70% ethanol overnight at 4 °C. Fixed cells were washed with PBS and incubated with a staining solution containing RNase A (50 μg/ml) and propidium iodide (50 μg/ml) in PBS for 30 min at room temperature. The cellular DNA content was analyzed with a FACSCalibur® flow cytometer (BD Biosciences, San Jose, CA, USA). At least 10,000 cells were used for each analysis, and the distribution of cells in each phase of the cell cycle was displayed as histograms.

2.6. Western blot analysis

Cells were seeded into 100-mm dishes at a density of 1 × 10^6 cells/dish. After 6 or 24 h of incubation, cells were treated with various concentrations of the test compound for the indicated times.