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Cell Cycle Arrest and Cytochrome *c*-mediated Apoptotic Induction in A549 Human Lung Cancer Cells by MCS-C2, an Analog of Sangivamycin

Kang, Jeonghwa[1†], Dong-Keun Lee[2†], and Chul-Hoon Lee[1*]

[1]*Department of Medical Genetics, College of Medicine, Hanyang University, Seoul 133-791, Korea*
[2]*Department of Biological Sciences, College of Science, Konkuk University, Seoul 143-701, Korea*

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In the course of screening for novel modulators of cell cycle progression and apoptosis as anticancer drug candidates, we generated an analog of sangivamycin, MCS-C2, which was elucidated as 4-amino-6-bromo-7-cyclopentyl-7H-pyrrolo[2,3-*d*]pyrimidine-5-carboxamide. In the present study, we evaluated the molecular mechanisms of MCS-C2-induced cell cycle arrest and apoptosis in A549 human lung cancer cells. To investigate the effects of MCS-C2 on cell cycle progression in A549 cells, we measured the DNA content of A549 cells treated with 5 μM MCS-C2 using flow cytometry. The analysis revealed an appreciable G_2 phase arrest in treated cells. This event was associated with significant upregulation of p53 and $p21^{Cip1}$. In addition, the TUNEL assay was used to examine apoptotic induction in treated cells, and the effects of MCS-C2 on the expression of apoptosis-associated proteins were examined by Western blot. Apoptotic induction in MCS-C2-treated A549 cells was associated with cytochrome *c* release from mitochondria, which in turn resulted in the activation of caspase-9 and -3 and the cleavage of poly(ADP-ribose) polymerase (PARP). Based on these results, we conclude that MCS-C2 is a candidate therapeutic agent for the treatment of human lung cancer *via* upregulation and activation of p53.

Keywords: Sangivamycin analog, cell cycle arrest, apoptotic induction, A549 cells

Deregulated cell cycle progression and impaired apoptotic induction, the primary characteristics of cancer cells, are due to an imbalance between cell proliferation and death. Accordingly, controlling cell cycle progression is an important goal in the treatment of diseases characterized by deregulated cell proliferation, such as a cancer. Apoptosis is a major form of cell death characterized by a series of tightly regulated processes that involve the activation of a cascade of molecular events. The key regulators of cell cycle progression and apoptotic induction could be important molecular targets for therapeutic intervention. Specifically, inhibition of cell cycle regulation may be particularly useful in the treatment of diseases caused by uncontrolled cell proliferation. Cyclin-dependent kinases (CDK), in cooperation with various cyclins and cyclin-dependent kinase inhibitors (CKI), such as $p21^{Cip1}$ and $p27^{Kip1}$, are the primary regulators of cell cycle progression [8]. On the other hand, cells undergoing apoptosis have been found to have an elevated level of cytochrome *c* in the cytosol and a corresponding decrease in the mitochondria [16]. The release of mitochondrial cytochrome *c* activates caspase-3 [10], which is responsible for the proteolytic degradation of poly(ADP-ribose) polymerase (PARP) that occurs at the onset of apoptosis [6, 15].

Over the last few decades, much attention has been focused on natural products and small molecules as potential sources of novel anticancer drugs [3, 4, 7, 11]. The identification of candidate chemotherapeutic agents using mechanism-based studies holds great promise for devising more specific and effective treatments for cancer-related diseases. In the course of screening for novel modulators of cell cycle progression and apoptotic induction as anticancer drug candidates, as previously published, we generated MCS-C2, an analog of sangivamycin. The structure of MCS-C2 was elucidated based on the interpretation of NMR spectra, and the detailed mechanisms of apoptotic induction in MCS-C2-treated HeLa and LNCaP cells have been evaluated [5, 12]. Interestingly, sangivamycin showed no inhibitory activity toward human cytomegalovirus or herpes simplex virus type 1, nor did it affect the growth of murine leukemia cells [14]. In the current study, we report

*Corresponding author
Phone: +82-2-2220-0673; Fax: +82-2-2298-4857;
E-mail: chhlee@hanyang.ac.kr

†These authors contributed equally to this work.

for the first time the molecular mechanisms of MCS-C2-induced apoptosis as well as G_2-phase cell cycle arrest in A549 human non-small cell lung cancer cells.

Materials and Methods

General

Phosphate-buffered saline (PBS), RPMI 1640 medium, and fetal bovine serum (FBS) were purchased from Gibco, Ltd. (Grand Island, NY, U.S.A.). The A549 human lung carcinoma cell line was obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Cells were cultured in RPMI 1640 containing 10% heat-inactivated FBS and 1% penicillin at 37°C in a humidified atmosphere of 5% CO_2. MCS-C2 was synthesized and supplied by C&C Research Laboratories (Kyunggi-Do, Korea).

Cell Viability Test (MTT Assay)

A549 cells (1×10^5 cells/ml) were seeded in a 96-well culture dish and treated with various concentrations (0–100 μM) of MCS-C2 (Fig. 1A) for 24 and 48 h. Viability was assessed by the conventional colorimetric method based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Promega, Madison, WI, U.S.A.), and the viable cell number was measured spectrophotometrically at 570 nm using an enzyme-linked immunosorbent assay reader (Molecular Devices, Sunnyvale, CA, U.S.A.).

Flow Cytometric Analysis of Cell Cycle Progression

A549 cells (1×10^5 cells/ml) were treated with 5 μM MCS-C2, and then incubated in RPMI 1640 with 10% FBS for 24 h. The cells were washed twice with ice-cold PBS, harvested, fixed with ice-cold PBS in 70% ethanol, and stored at 4°C. For flow cytometry, the cells were incubated with 0.1 mg/ml RNase A for 30 min at 37°C and stained with 50 μg/ml propidium iodide (PI) for 30 min on ice, and then fluorescence was measured using a FACSCalibur flow cytometer (Becton Dickinson, San Diego, CA, U.S.A.) with CellQuest software.

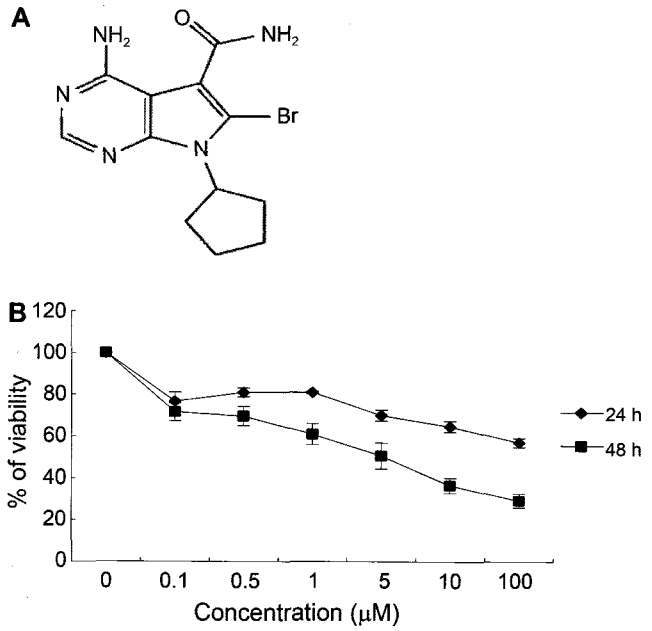

Fig. 1. Chemical structure of MCS-C2, a novel analog of the pyrrolo[2,3-*d*]pyrimidine nucleoside sangivamycin (**A**), and effects of MCS-C2 on growth of A549 cells (**B**).
A549 cells were treated with various concentrations of MCS-C2 for 24 and 48 h, and viability was determined by MTT assay. Results represent the mean ± SD of three independent experiments.

TUNEL Assay

The detection of apoptotic cells by the terminal deoxyribonucleotidyl transferase (TdT)-mediated dUTP-digoxigenin nick end labeling (TUNEL) assay was carried out according to the manufacturer's protocol (Apoptosis Detection System, Fluorescein; Promega). A549 cells (1×10^5 cells/ml) were treated with 5 μM MCS-C2, and then incubated in RPMI 1640 with 10% FBS for 48 h. The cells were then washed in PBS and fixed in 1% formaldehyde solution for 20 min on ice. Subsequently, the cells were washed again in PBS and resuspended in an equilibration buffer for 5 min at room temperature, and then incubated in TdT reaction buffer (50 μl) for 1 h at 37°C. After termination of the TdT reaction, the cells were incubated in 1 ml of PBS containing 25 μg/ml PI and 250 μg/ml RNase A for 30 min at 37°C. Fluorescein-12-dUTP-labeled DNA was quantitated using a FACSCalibur flow cytometer with CellQuest software.

Western Blot Analysis

A549 cells (1×10^5 cells/ml) were treated with 5 μM MCS-C2, incubated in RPMI 1640 with 10% FBS, and then harvested. To prepare a whole-cell extract, the cells were washed with PBS and suspended in a protein lysis buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 1 mM sodium *o*-vanadate, 100 μg/ml phenylmethylsulfonyl fluoride, and proteinase inhibitors). The protein content was determined with a Bio-Rad protein assay reagent using bovine serum albumin as the standard. Extracted proteins (30 μg) were separated using 10–14% SDS–PAGE and transferred to a polyvinylidene fluoride membrane (Millipore Corp., Bedford, MA, U.S.A.). The membranes were blocked with 5% (w/v) non-fat dry milk and then incubated with the appropriate antibodies in TBS (10 mM Tris-HCl and 150 mM NaCl, pH 7.6) containing 0.1% Tween-20, with gentle shaking for 24 h at 4°C. The secondary antibodies were peroxidase-conjugated goat anti-mouse antibody and goat anti-rabbit antibody. Signals were detected using an ECL Western blotting kit (GE Healthcare, Buckinghamshire, U.K.).

Statistical Analysis

Data are reported as the mean ± standard deviation of three independent experiments and were evaluated by the Student's *t*-test. Values of $p<0.05$ were considered to be statistically significant. Results were obtained from the average of three experiments.

Results and Discussion

MCS-C2-induced Inhibition of A549 Cell Growth

To determine whether MCS-C2 induces cell growth inhibition in a human lung cancer cell line, the viability of MCS-C2-treated A549 cells was assessed using the MTT assay. As shown in Fig. 1B, the growth of cells treated

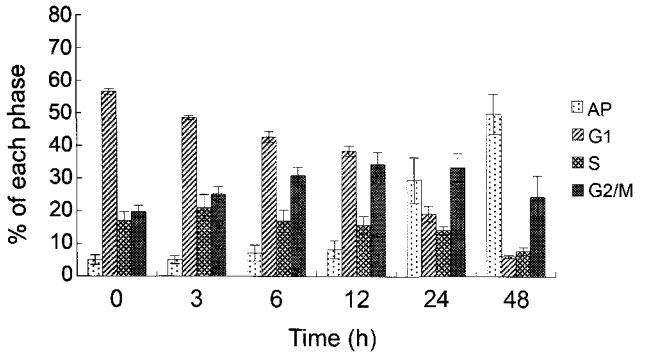

Fig. 2. Quantification of cell cycle arrest by flow cytometry in A549 cells.
A549 cells were treated with 5 μM MCS-C2 for the indicated time. The cells were then stained with PI and their nuclei analyzed for their DNA content by flow cytometry using CellQuest software. Results represent the mean ± SD of three independent experiments.

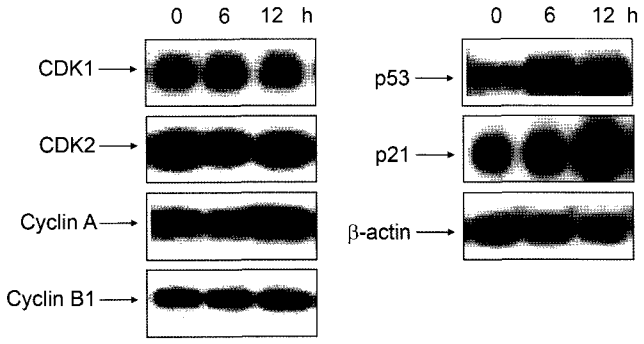

Fig. 3. Effects of MCS-C2 on G_2-phase regulatory proteins in cultured A549 cells.
A549 cells were treated with 5 μM MCS-C2, lysed, elecrophoretically separated on a 12% polyacrylamide gel, and then immunoblotted with antibodies against target proteins.

with 0.1–100 μM MCS-C2 for 24 and 48 h was inhibited in a dose-dependent manner. The IC_{50} value for growth inhibition for 48 h was 5.36 μM. Therefore, 5 μM MCS-C2 was used in subsequent experiments.

Effects of MCS-C2 on Cell Cycle Progression in A549 Cells

To investigate the effects of MCS-C2 on cell cycle progression in A549 cells, we measured the DNA content of cells treated with 5 μM MCS-C2 using flow cytometry, which revealed an appreciable arrest in the G_2 phase. As shown in Fig. 2, the A549 cell population in the G_2 (G2/M) phase and sub-G_1 [apoptotic population (AP)] phase gradually increased from 19.9% at 0 h to 34.4% at 12 h, and from 5.0% at 0 h to 49.9% at 48 h after exposure to 5 μM MCS-C2, respectively.

Effects of MCS-C2 on Expression of Cell Cycle-related Proteins

To elucidate the detailed mechanism of A549 cell cycle inhibition, we used Western blotting to determine the correlation between MCS-C2-induced G_2-phase arrest and the expression patterns of the cell cycle regulators cyclins A and B1, CDK1 and 2, and the CKI p21, as well as the level of phosphorylation of p53 after MCS-C2 treatment. As shown in Fig. 3, induction of G_2-phase arrest was associated with the significant upregulation p53 and $p21^{Cip1}$, which play key roles in regulating the entry of cells into mitosis at the G2/M transition checkpoint. However, the expressions of the CDKs and cyclins as well as hyperphosphorylated pRb (data not shown) were not affected.

The upregulation of p53 may explain the increased cellular level of $p21^{Cip1}$, which is one of the downstream targets of p53; these two proteins exhibited almost identical patterns of elevated levels in a time-dependent manner in MCS-C2-treated A549 cells (Fig. 3).

Effects of MCS-C2 on Apoptotic Induction in A549 Cells

To investigate apoptotic induction in MCS-C2-treated A549 cells, a TUNEL assay was used to examine DNA fragmentation in nuclei. As shown in Fig. 4, apoptotic induction was observed in cells treated with 5 μM MCS-C2 for 48 h. In addition, to determine whether apoptosis-related proteins were involved in the mediation of MCS-C2-induced cell death, we examined caspase activation and cleavage of PARP by Western blot. As shown in Fig. 5, MCS-C2 induced the proteolytic cleavage of inactive procaspase-9 and -3 into active caspase-9 and -3, respectively. Furthermore, one of the substrates for effector caspases during apoptosis is PARP, an enzyme that is

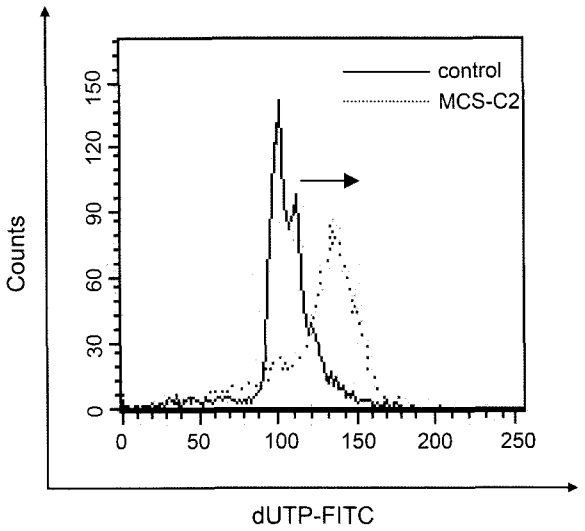

Fig. 4. Apoptotic induction by MCS-C2 in A549 cells, as determined by TUNEL assay.
The A549 cells were treated with 5 μM MCS-C2 for 48 h, and then stained with d-UTP-fluorescein isothiocyanate and PI and analyzed using flow cytometry.

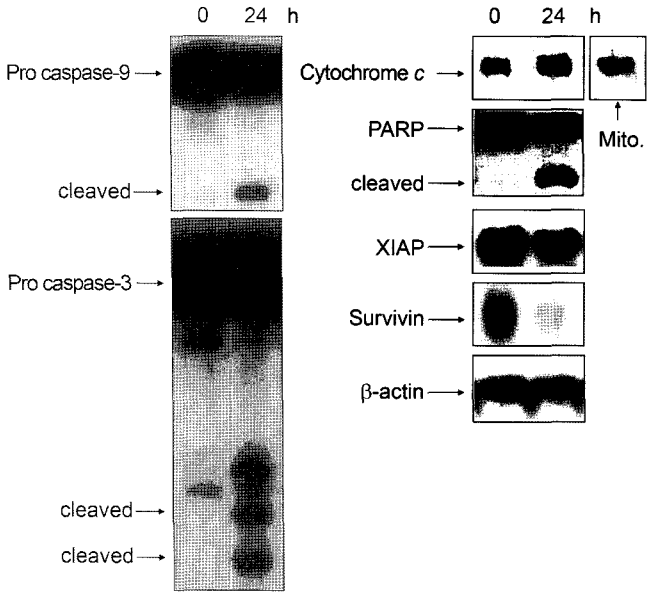

Fig. 5. Effects of MCS-C2 on apoptosis regulatory proteins in cultured A549 cells.
A549 cells were treated with 5 μM MCS-C2, lysed, elecrophoretically separated on a 10–14% polyacrylamide gel, and then immunoblotted with antibodies against target proteins. Mito.: mitochondrial fraction

involved in DNA repair, genome surveillance, and maintenance of genomic integrity in response to environmental stress. Therefore, the cleavage of PARP was used as an indicator of MCS-C2-induced apoptosis, which became obvious after 24 h of treatment. In contrast, the anti-apoptotic factors X-linked inhibitor-of-apoptosis protein (XIAP) and survivin were significantly downregulated in MCS-C2-treated A549 cells (Fig. 5).

To examine the mitochondria-mediated intrinsic pathway involved in MCS-C2-induced apoptosis, we analyzed the release of cytochrome *c* from mitochondria to the cytosol by Western blot. As shown in Fig. 5, a significant release of cytochrome *c* was observed in A549 cells treated with 5 μM MCS-C2 for 24 h.

Apoptosis, which plays an important role in the development and tissue homeostasis in eukaryotes, is a major form of cell death characterized by a series of tightly regulated processes that involve the activation of a cascade leading to cell death. Induction of apoptosis is, therefore, a highly desirable goal of preventive strategies for cancer control [13]. In the present study, we demonstrated that the apoptotic induction in A549 cells treated with 5 μM MCS-C2 is mediated by cytochrome *c* release through the mitochondria-dependent intrinsic pathways. However, in contrast to previous work, thus far we have not demonstrated a significant change in the levels of pro-apoptotic and anti-apoptotic Bcl-2 proteins in MCS-C2-treated A549 cells (data not shown). Therefore, further studies on the protein–protein interactions of pro- and anti-apoptotic proteins and their translocation to the mitochondria are needed to confirm the detailed molecular mechanism of MCS-C2-induced apoptosis in A549 cells.

Serine/threonine protein kinases play pivotal roles in the cellular signalling that controls the proliferation and differentiation of eukaryotic cells [9]. The CDKs, which belong to this protein kinase family, are key regulators of cell cycle progression in cooperation with various endogenous cyclins and CKIs including $p21^{Cip1}$, $p27^{Kip1}$, and $p16^{INK4}$ [2, 8]. Furthermore, it has been reported that small bioactive molecules that cause DNA damage, such as MCS-C2, act through posttranslational modifications of p53 and activate its downstream targets in various human cancer cells. For example, it has been shown that phosphorylation of p53 at Ser-15 is induced through the activation of an ataxia telangiectasia-mutated (ATM) signaling pathway, which includes the upregulation of *ATM* [1]. This specific modification, in turn, results in acetylation of p53. These posttranslational modifications are directly responsible for $p21^{Cip1}$ expression owing to the binding affinity of acetylated p53 for the $p21^{Cip1}$ promoter.

In the present study, we demonstrated that MCS-C2 induces G_2-phase arrest in A549 cells, using flow cytometry. As shown in Fig. 2, this event was associated with significant upregulation of $p21^{Cip1}$, which plays a key role in regulating the entry of cells into mitosis at the G2/M transition checkpoint. Furthermore, a p53 upregulation could explain the increased cellular level of $p21^{Cip1}$, one of its downstream targets, since their patterns of time-dependent increase in protein levels in A549 cells were almost identical.

In conclusion, we showed that MCS-C2 is a candidate as a novel anticancer agent. Therefore, further research, including an *in vivo* study using a xenograft animal model and *p53* knockout using the small interfering RNA technique, should be performed in the future.

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