Introduction

Telomeres are distinct structures located at the ends of linear chromosomes, which play crucial roles in protecting chromosomes from DNA degradation, in DNA repair mechanisms, and in fusion (1, 2). Telomeres shortening or uncapped telomeres activate the DNA damage response, resulting in cellular senescence and apoptosis (2). To counter these effects, an enzyme called telomerase, a well-known enzyme that elongates telomeric DNA. Evidence indicated that telomerase has been detected with higher activity in a variety of human cancer tissues (3). Thus, targeting telomerase in comparison with most other cancer targets are more universal, critical, and specific for cancer cells.

Compounds that can stabilize the G-quadruplex structures and accelerate the shortening of telomere length have been shown to inhibit the activity of telomerase (4). A series of substituted anthraquinone compounds have been shown to inhibit telomerase activity possibly through targeting the G-quadruplex structures. NSC746364 is a novel 2,7-diamidoanthraquinone derivative that has been found to have cytotoxic effects on various cancer cell lines. However, the detailed pharmacological mechanisms are still unknown. As human telomerase has been considered as a novel and potentially highly specific target for antitumor drug design (5–7), various approaches have been developed, including inhibition of telomerase enzyme activity and telomere-disrupting agents. GRN163L, the oligonucleotide template antagonist of human telomerase RNA component (hTR) sequence (8), is currently in clinical trials for chronic lymphocytic leukemia. In addition to inhibiting telomerase activity in cancer cells, the development of G-quadruplex stabilizers has emerged as a highly promising approach.
Human telomeric DNA consists of tandem repeats of the sequence (TTAGGG)n and can form G-quadruplex DNA secondary structures, which is known to inhibit the activity of telomerase (12). As a consequence, compounds that can stabilize the telomeric DNA G-quadruplex have been considered as potentially valuable antitumor drugs. Several compounds that target the G-quadruplex and inhibit telomerase activity have been identified, including porphyrins (4, 13–14), anthraquinones (15), and PIPER (N,N′-bis[2-(1-piperidino)ethyl]-3,4,9,10-perylenetetracarboxylic diimide) (16).

Thus, developing compounds that can stabilize the G-quadruplex DNA secondary structure has been considered as potentially therapeutic intervention to telomerase-positive cancer cells. Wide arrays of ligands that target the telomeric G-quadruplex have been developed and identified (9). Disrupting telomere DNA maintenance can be sensed as DNA damage, which in turn rapidly activated signal transducers that responded to DNA damage. These serine-threonine kinases, such as ATM (ataxia telangiectasia mutated protein or Sc and Sp Tel1), ATR (ATM and p-Chk1 protein or Sc Mec1 and Sp Rad3) (17), and DNA-PK, senses DNA damage and later on, stalls replication forks, causes cell cycle arrest, and rapidly activates the apoptotic pathways.

Anthraquinone-containing extracts from different plants such as senna, cascara, aloe, frangula, and rhubarb have been found to have a wide variety of pharmacological activities, including anti-inflammatory, antimicrobial, and antitumor activities (18, 19). These widely distributed compounds in the plant kingdom and their synthetic derivatives currently take a prominent position in anticancer drug development. For example, mitoxantrone and amantantrone are synthetic 1,4-bis[(aminoalkyl) amino]anthraquinones that have been developed to treat many malignancies (20, 21). Although the molecular mechanism of the antitumor activity of anthraquinone is complicated, studies have indicated that its intercalative interaction with DNA may play a major role (22–24). From the study on the structure–activity relationships of anthraquinones, the first anthraquinone derivative that has been shown to interact with quadruplex structures and inhibit telomerase was a symmetric 2,6-disubstituted aminoalkylamido anthraquinone molecule (15).

NSC746364 emerged from a series of synthetic symmetrical 2,7-diamidoanthraquione derivatives (25) that elicit potent antitumor activity against a full panel of different types of human tumor cell lines, including non–small-cell lung cancer (NSCLC), colon cancer, breast cancer, ovarian cancer, leukemia, renal cancer, melanoma, prostate cancer, and central nervous system cancer (26). Although NSC746364 has shown to inhibit telomerase and exerts cytotoxic effect on a variety of cancer cells, the underlying molecular regulations are still unclear. Therefore, the purpose of the present study was to investigate and clarify its exact molecular mechanisms for inhibiting cell growth of NSCLC.

Materials and Methods

Preparation of NSC746364

The synthesis and chemical characterization of NSC746364 have been described previously (25, 27). NSC746364 was dissolved in DMSO (Sigma-Aldrich, St. Louis, MO, USA), to make a 100 mM stock solution. All stock solutions were aliquoted and stored as −20°C and protect from light.

Cell culture

A549 cells, a human lung adenocarcinoma epithelial cell line, were obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were grown in Dulbecco’s modified Eagles’s medium (DMEM; Gibco, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS, Gibco) and 100 units/mL penicillin G, and 100 µg/mL streptomycin sulfates. The culture medium was replaced every two days and cells were passaged once every week. Cells that became at least 80% confluent were starved for 24 h in FBS-free DMEM followed by a treatment with the indicated concentration of NSC746364 in DMEM containing 10% FBS for the indicated time.

Cell viability analysis (MTT assay)

MTT assay was performed to measure the cytotoxicity of NSC746364 on A549 lung cancer cells. Cells were seeded in 24-well plates with 2 × 104 cells/well in DMEM supplemented with 10% FBS. After 24 h, cells were washed with phosphate-buffered saline (PBS) and then exposed to either DMSO alone or different concentrations (5, 10, and 20 µM) of NSC746364. After 24 and 48 h, the number of viable cells was determined. Briefly, MTT (0.5 mg/mL in DMEM containing 10% FBS) was added to each well (400 µL per well), and the plate was incubated at 37°C for 4 h. Cells were then spun at 300 g for 5 min, and the medium was carefully aspirated. A 400 µL aliquot of DMSO was added, and the absorbance at 595 nm was measured for each well in an ELISA reader (Anthos, 2001; BioTek Instruments, Inc., Winooski, VT, USA).

Cell cycle analysis

The DNA content of the treated cells was assessed using flow cytometry following propidium iodide (PI) staining. Cells were seeded in 6-cm petri dishes (Greiner Bio-one, Frickenhausen, Germany) with 2 × 105 cells/dish.
in DMEM supplemented with 10% FBS. After 24 h, cells were washed with phosphate-buffered saline (PBS) and starved for 24 h in FBS-free DMEM followed by exposing to either DMSO alone or serial dilutions (5, 10, and 20 μM) of NSC746364 for 24 or 48 h. After the treatment, cells were harvested with trypsin-EDTA (Gibco), washed twice with 10 mL ice-cold PBS, fixed in 70% ethanol, and kept at −20°C prior to FACS analysis. For DNA content analysis, cells were centrifuged and resuspended in 0.4 mL of DNA staining solution (0.4 mg/dL PI, 1% Triton X-100, 0.1 mg/mL RNase A in PBS). The cell suspension was stored at 4°C and protected from light for a minimum of 30 min and analyzed within 2 h. Cells were analyzed using a FACSscan flow cytometer (Becton Dickinson, San Jose, CA, USA). The percentages of hypodiploid (apoptotic, sub-G1) events and percentages of cells in the G0/G1, S, and G2/M phases were determined using the DNA analysis software ModFitLT, version 2.0 (Verity Software, Topsham, ME, USA).

**Western blot**

A549 cells cultured in petri dishes were incubated with 5, 10, and 20 μM of NSC746364 in DMEM containing 10% FBS for 24 h. For the time course experiments, cells were treated with 20 μM of NSC746364 in DMEM containing 10% FBS for 15, 30, and 60 min. Cells were then lysed in protein extraction buffer (PRO-PREP Solution; Intron Biotechnology, Seongnam, Korea), followed by incubation at 95°C for 5 min. Samples were separated using SDS-PAGE, transferred to PVDF membranes, blocked with 5% nonfat dry milk in TBST (Tris-base saline containing 0.05% Tween-20) for 1 h, and then probed with the primary antibodies against cyclin B1 (#4138; Cell Signaling Technology Inc., Danvers, MA, USA), Cdc2 (#9112; Cell Signaling Technology), phosphorylated-Cdc2 (#9114, Cell Signaling Technology), phosphorylated-ATM (ab36810; Abcam, Cambridge, UK), phosphorylated-ATR (sc-109912; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), phosphorylated-Chk1 (#2341, Cell Signaling Technology), Chk1 (#2345, Cell Signaling Technology), phosphorylated-Chk2 (#2661, Cell Signaling Technology), Chk2 (#2662, Cell Signaling Technology), caspase-3 (#AB1899; Chemicon International, Inc.) or β-actin (sc-47778, Santa Cruz Biotechnology Inc.) overnight at 4°C. The blots were then incubated with horseradish peroxidase–linked secondary antibody for 1 h followed by development with the ECL reagent, and chemiluminescence signals were detected by an LAS-4000 luminescence/fluorescence imaging system (Fujifilm Corporation, Tokyo). The intensities were quantified by densitometric analysis software Multi Guage version 2.0 (Fujifilm). The analyzed data are expressed as the ratio of the band intensity of the target protein to that of β-actin.

**Cellular nuclear staining**

A549 cells were seeded in a 6-well plate and incubated with various concentrations of NSC746364 in DMEM medium containing 10% FBS for 24 h. After 24-h incubation, cells were washed three times with PBS, fixed with 3% formaldehyde for 15–20 min, followed by treatment with 0.1% Triton X-100 for 15 min, and then stained with 1 μg/mL of 4′,6-diamidino-2-phenylindole (DAPI) for 1 min. Washing cells two times with PBS. Cells were observed by using a fluorescence microscope and then all pictures were taken at 40-fold magnification.

**Statistical analysis**

Data are expressed as the mean ± S.E.M. Statistical analysis was conducted using the ANOVA test. A P-value < 0.05 was considered significant.

**Results**

**NSC746364 suppressed cell growth of A549 cells**

The effect of NSC746364 on cell proliferation was assessed using the MTT proliferation assay. To test the effect of NSC746364 on the proliferation of A549 cells, the cells were treated with different concentrations of NSC746364. After 24- and 48-h (Fig. 1) incubation, the cell viability was determined by using the MTT assay. As shown in Fig. 1, treatment with NSC746364 significantly inhibited the cell viability in a dose- and time-dependent manner.
Fig. 2. Effect of NSC746364 on the cell cycle distribution of A549 cells. A549 cells were treated with various concentrations of NSC746364 for 24 h (A and B) and 48 h (C and D). *P < 0.05, **P < 0.01, ***P < 0.001, as compared to the control (0 μM). *P < 0.05, as compared to 5 μM NSC746364.
NSC746364 arrested cell cycle at G2/M phase

The cell cycle distribution of A549 cells was examined by flow cytometry of cells treated with various concentrations of NSC746364 for 24 h (Fig. 2: A and B) and 48 h (Fig. 2: C and D). The experimental results showed that NSC746364 modulated cell cycle progression through inducing cells to accumulate at the G2/M phase with concurrent decrease of cells at the G0/G1 and S phases.

NSC746364 modulated cell cycle regulatory proteins

The expression profile of numerous dominant regulatory proteins of the G2/M phase, cyclin B1, Cdc2, Chk1, and Chk2, were determined by western blotting in A549 cells treated with NSC746364. Our results indicated that NSC746364 down-regulated the protein level of cyclin B1 and p-Cdc2 in a dose-dependent manner, but the expression level of Cdc2 protein was not affected (Fig. 3). Additionally, NSC746364 also markedly increased the phosphorylation levels of Chk1 and ATR but not those of Chk2 and ATM (Fig. 4: A and B). The obvious increase of phosphorylated Chk1 and phosphorylated ATR could be detected in the very early stage (15 min after NSC746364 treatment).

NSC746364 activated ATM/ATR-mediated DNA damage-sensing pathways

Our results suggested that NSC746364 may induce the decrease of A549 cell viability by causing ATR-Chk1 cascade–mediated cell cycle arrest of the G2/M phase. Thus, to further confirm the mechanism that underlies the antitumor effects of NSC746364 via ATM/ATR-mediated DNA damage-sensing pathways, A549 cells were pre-incubated with either 1.1 or 10 mM caffeine, an inhibitor of ATM and ATR kinases (28). The A549 cells were pre-treated with 1.1 or 10 mM caffeine for 30 min and then co-incubated with or without 20 μM NSC746364 for 15 min. The results obtained showed that phosphorylated ATR was markedly reduced in NSC746364-treated A549 cells by the addition of caffeine at the concentration of 10 mM (Fig. 5A). Cells pre-treated with caffeine showed marked attenuation of the effect of NSC746364 on p-ATR (Fig. 5A) and cell cycle arrest (Fig. 5B) and caffeine also significantly reversed the cell viability as compared to the NSC746364 alone group (Fig. 5C).

NSC746364 induced apoptosis of A549 cells

Our results also showed that NSC746364 induced programmed cell death by activating caspase-3 (Fig. 6A).
The increasing intensity of DAPI staining demonstrates the dose-dependent apoptotic effects of NSC746364 on A549 cells (Fig. 6B).

**Discussion**

In the present study, we have shown that NSC746364 can inhibit the growth of A549 cells. One of the major mechanisms by which NSC746364 mediates its effects against lung cancers seems to be through activation of DNA-damage signaling pathways, which in turn stalls the cell cycle at the G2/M phase, subsequently leading to cellular apoptosis.

The consequences of telomere dysfunction caused by telomere-disrupting agents, such as G-quadruplex inhibitors, results in activation of DNA-damage—
Fig. 5. Blockage of ATM/ATR attenuated the effect of NSC746364 on cell cycle and cell viability. Cells were pre-treated with caffeine (10 mM) for 30 min, followed by incubation with or without NSC746364 (20 μM) for 15 min (A). Cell cycle phase distribution (B) and cell viability (C) were determined by flow cytometry and MTT assay, respectively. *P < 0.05, **P < 0.01, ***P < 0.001, as compared to the control. #P < 0.05, ##P < 0.01, compared to the NSC746364-treated group.
response signaling leading to activation of cell cycle checkpoints and apoptosis (29–33). NSC746364 is one of the novel 2,7-diamidoanthraquinone derivatives possessing a potential G-quadruplex targeting action (25). Our results demonstrate that NSC746364 can effectively inhibit cell growth (Fig. 1), arrest cell cycle at G2/M phase (Fig. 2), and induce cell apoptosis (Fig. 6). Our present data indicated that pretreatment of cells with caffeine markedly attenuated the effect of NSC746364 on cell cycle arrest (Fig. 5B) and also significantly increased the cell viability as compared to the NSC746364 alone group (Fig. 5C). The sub-G1 peaks on DNA histograms usually represent the apoptotic cells with degraded DNA (34, 35). However, the sub-G1 peak was just slightly elevated as we measured the DNA contents of cells after NSC746364 treatment (Fig. 2). All these results indicate that the anticancer effects of NSC746364 might be mainly exerted through inhibiting cell growth, with a minor portion of cells undergoing apoptosis.

The telomere-specific protein complex (named as shelterin) has been identified with six proteins, TRF2, POT1, RAP1, TIN2, and TPP1, which is essential for genome stability and cell survival. In addition, telomerase is also one of the mechanisms for maintaining telomere integrity. Disrupting telomere DNA maintenance by telomerase inhibitor or telomere-targeting agents can be
sensed as DNA damage, which in turn rapidly activated signal transducers leading to DNA damage. As shown in Figs. 3 and 4, NSC746364 activates ATM/ATR signaling pathways by phosphorylating ATR at Ser-428. However, it seems that NSC746364 dominantly increases ATR/Chk1 phosphorylation but not ATM/Chk2 phosphorylation. In Fig. 5, caffeine, an ATM and ATR kinase inhibitor (27), effectively abrogates the effects of NSC746364 on cell cycle arrest and cell viability. Recent studies showed that distinct shelterin components independently repress ATM and ATR signaling. Activation of ATM at telomeres is repressed by TRF2, while POT1 is required to prevent ATR activation (36–39). Thus, one of the possible mechanisms to regulate ATR activation by NSC746364 is through repression of the shelterin protein, POT1.

Although telomerase is the major mechanism for telomere elongation in most cells, the alternative lengthening of telomere (ALT) mechanism such as gene conversion has also been demonstrated to be involved in the telomere elongation and restructuring mechanisms for telomere-length maintenance (40). In the ALT mechanism, the longer telomeres can be a template of DNA polymerase–mediated telomere elongation after that was invaded by a short telomeric strand of a second chromosome (41). The telomeric G-quadruplex structure preferentially formatted at the farthest 3′ end of the telomeric single-strand overhang, which will remain a shorter single-strand tail that cannot be an effective template for the telomerase or the ALT-mediated telomere extension (42, 43). However, several proteins have been identified to be involved in the unfolding or destabilization of the telomeric G-quadruplex structure (44–46), which suggested that the coordination between telomeric G-quadruplex formation and its unfolding may determine the availability of telomere extension. Thereby, a G-quadruplex targeting agent, such as NSC746364, may provide broader effects than a telomerase inhibitor to prevent the telomere extension no matter whether they are caused by telomerase or ALT-mediated mechanisms. Additionally, the non-telomeric G-quadruplex motif has also been identified in the promoter region of genes involved directly in the gene regulation at the level of RNA transcription (47). These putative promoter G-quadruplex-forming regions are strongly associated with nuclease hypersensitivity sites (48), and it is noted that the putative G-quadruplex motifs are prevalent in proto-oncogenes and essentially absent in tumor-suppressor genes (49). Several proto-oncogenes, such as c-myc, VEGF, and HIF-1a, have been shown to possess the G-quadruplex motifs in the promoter region (50–52). Thereby, the RNA transcription of proto-oncogenes can be controlled by ligand-mediated G-quadruplex stabilization, whose effect can be further developed as a novel anticancer application.

Taken together, our study demonstrates that NSC746364 can suppress cell growth of A549 cells and its pharmacological mechanism may be through targeting ATR/Chk1 signaling pathways. Our results unmask the pharmacological mechanisms of NSC746364 in inhibiting A549 human lung cancer cells growth in vitro to shed light on the conjunctive roles of NSC746364 with some other pharmacological anticancer agents. Further studies using animal models or clinical evaluations need to be conducted to confirm the proposed theory in this aspect.

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Conflicts of Interest

The authors declare that there are no conflicts of interest.

References

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