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Induction of G_2/M cell cycle arrest and apoptosis by a benz[*f*]indole-4,9-dione analog in cultured human lung (A549) cancer cells

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Abstract—A synthetic benz[*f*]indole-4,9-dione analog, 2-amino-3-ethoxycarbonyl-*N*-methylbenz[*f*]indole-4,9-dione (SME-6), showed a potent growth inhibition of a panel of human cancer cell lines. The mechanism of action study revealed that the growth inhibitory effect of SME-6 was highly related to the induction of G_2/M cell cycle arrest and apoptosis in human lung cancer cells (A549). These data may provide new information for understanding the mechanisms by benz[*f*]indole-4,9-diones-mediated antitumor activity.
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Heterocyclic quinones with nitrogen atoms have been reported to exhibit antitumor and antibacterial activities.[1–3] In our continuous efforts to develop novel antitumor agents we extended to synthesize benz[*f*]indole-4,9-dione analogs, heterocyclic pyrrole ring derivatives attached to 1,4-naphthoquinone based on the cytotoxic potential of 1,4-naphthoquinones.[4] Benz[*f*]indole-4,9-dione analogs showed potential cell growth inhibitory activity against human cancer cells in our previous study.[5] One probable mechanism was suggested the catalytic inhibition of topoisomerase activity.[5] However, the exact mechanism of action of these compounds remains still unclear.

Many anticancer agents evoked the cytotoxicity against cancer cell by apoptotic process.[6,7] Apoptosis, programmed cell death, is a physiological process that can play a pivotal role in the control of tumor cell proliferation and cell death, hence regulating cell number.[8] During apoptosis, various characteristic responses occur, including changes in the plasma membrane, such as loss of membrane asymmetry and attachment, cell shrinkage, chromatin condensation, chromosomal DNA fragmentation and the formation of membrane-bound apoptotic bodies that are phagocytozed by neighboring cells.[9] The regulation of cell cycle progress and expression of checkpoint-related proteins are also highly involved in apoptotic cell death or proliferation. Since the mechanism by which benz[*f*]indole-4,9-dione analogs exert cell growth inhibition remains unknown, we currently report the cell growth-inhibitory potential, the induction of cell cycle arrest and apoptosis of a benz-[*f*]indole-4,9-dione analog, 2-amino-3-ethoxycarbonyl-*N*-methylbenz[*f*]indole-4,9-dione (SME-6, Fig. 1), in cultured human lung (A549) cancer cells, as an additional important mechanism of action.

Primarily, the cell growth-inhibitory effect of 2-amino-3-ethoxycarbonyl-*N*-methylbenz[*f*]indole-4,9-dione (SME-6) was determined in three cultured human cancer cell

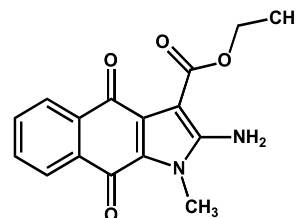

Figure 1. Chemical structure of 2-amino-3-ethoxycarbonyl-*N*-methylbenz[*f*]indole-4,9-dione (SME-6).

Keywords: Benz[*f*]indole-4,9-dione; G_2/M cell cycle arrest; Apoptosis.
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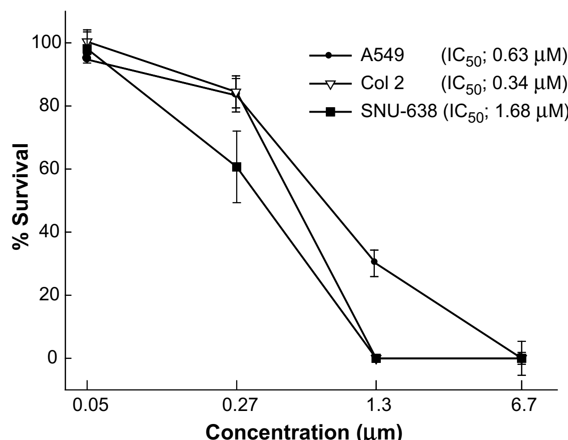

Figure 2. The cell growth-inhibitory activity of SME-6 in human cancer cells. Cells were treated with the indicated concentrations of SME-6 for 72h and the cells were quantified by staining with SRB. Values were averaged expressed as a percentage relative to the untreated control. Values indicate the mean ± SD in triplicate tests.

lines A549 (lung), Col2 (colon), and SNU-638 (stomach) by a colorimetric SRB protein dye staining method.[10] As shown in Figure 2, after 72h incubation, SME-6 exhibited the potential decrease of growth in a concentration-dependent manner against cancer cells tested, and the IC_{50} was ranged in the 0.3–1.5μM. This value was comparable with the positive control antitumor agents ellipticine and doxorubicin in the range of IC_{50} with 0.1–0.5μg/mL.

Based on the potential growth inhibitory activity of SME-6 against several human cancer cells we primarily selected and explored to study the mechanism of action against human lung cancer cells because lung cancer is the top of cancer death in Korea. A549 human lung cancer cells were treated in the absence or presence of SME-6 for 24 or 48h and the distribution of cells in various compartments of the cell cycle was analyzed by flow cytometry as described previously.[10] When treated with the compound (3.0μM), cells were accumulated at the G_2/M phase and then subsequently increased the sub-G_0 peaks in a time-dependent manner, indicative of typical apoptotic phenomena as shown in Figure 3. The cells treated with 3.0μM of SME-6 accumulated the sub-G_0 phase of cell cycle up to 24% by 48h. These results indicate that SME-6 possibly evokes the growth arrest at the M/G_1 phase transition period and thus accumulates the G_2/M phase of cell cycle, and then subsequently induces the apoptotic process.

To further explore whether the growth inhibitory potential by SME-6 is related to the apoptotic process, the morphological changes were primarily examined by utilizing the A549 cells[11] with various concentrations of the compound for 24h. As illustrated in Figure 4, cells that were exposed to SME-6 show the distinct morphological features of cell growth arrest (low density of cell number) and enlarged differentiated-type characteristics in a concentration-dependent manner compared to the polygonal features of DMSO-treated normal control cells. Typical morphological changes of apoptosis with membrane blebbing formation were also shown under

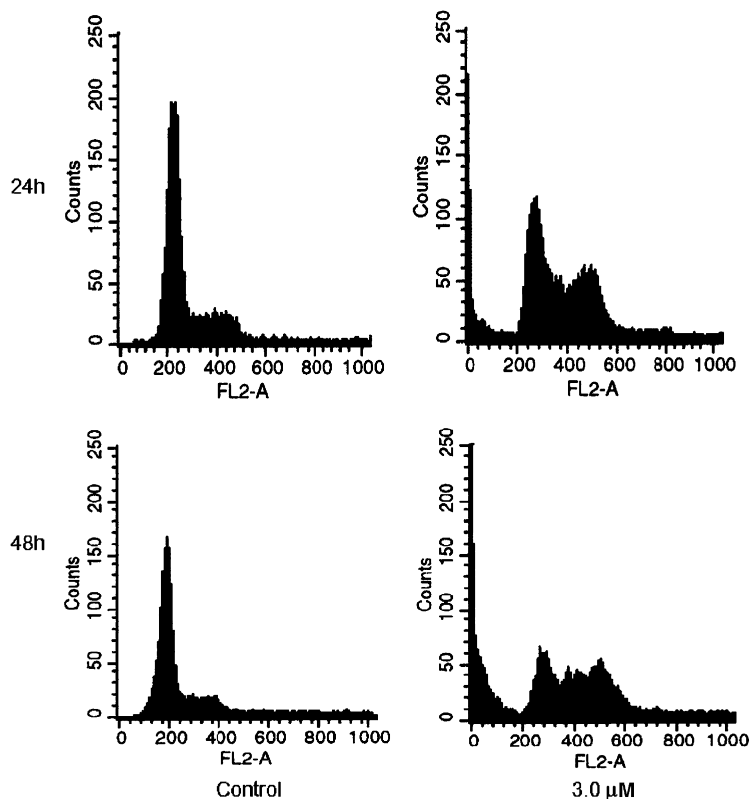

Figure 3. The effect of SME-6 on cell cycle progression in A549 cells. Cells were treated with SME-6 (3.0μM) for 24 or 48h and then the cell cycle was analyzed by flow cytometry analysis.

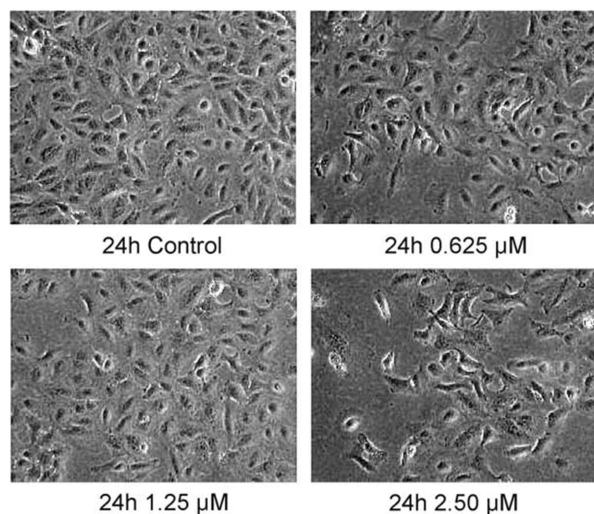

Figure 4. Morphological change in cultured A549 cells treated with SME-6. Morphological changes of A549 cells treated with DMSO alone (control) or various concentrations of SME-6 for 24h were observed under the phase-contrast microscope and photographed.

scanning electron microscopic analysis (Fig. 5).[12] The apoptotic morphological changes were also detected by nuclear staining[11] with the Hoechst 33258 dye under the fluorescent microscope exhibiting a relatively higher nuclear condensation or a fragmentation of cells that were treated with SME-6 compared to nontreated control cells (Fig. 6).

Further, in order to investigate whether the induction of G_2/M cell cycle and apoptosis mediated by SME-6 was related to the expression of regulatory proteins, p53 and cyclin B1 levels were determined by western blot analysis.[13] As shown in Figure 7, when the A549 cells were treated with various concentrations of the compound for 24h the levels of p53 protein expression were gradually increased in a concentration-dependent manner compared to the DMSO-treated control cells. With the treatment of 2.5μM SME-6, the p53 accumulation was started at 4h and then also time-dependent up to 24h, indicating that SME-6-mediated apoptosis is p53-dependent. Cyclins are considered as major checkpoints in the control of cell cycles. Especially, cyclin B1 is highly related to the G_2/M phase cell cycle. When treated with SME-6 cyclin B1 expression was also accumulated with the time- and concentration-dependently, suggesting that SME-6-mediated inhibition of cell growth is related to arrest of cell cycle at G_2/M phase.

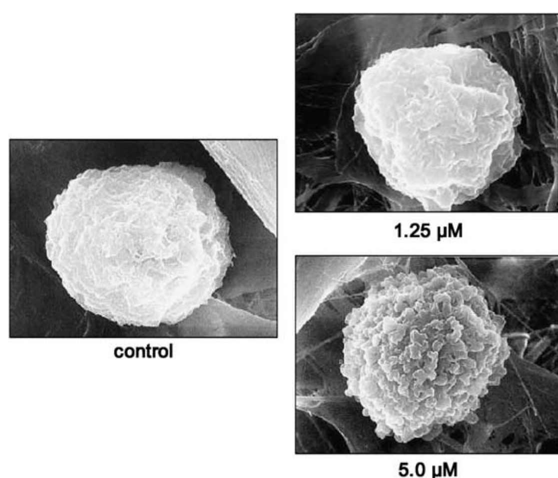

Figure 5. Photographs of scanning electron microscopic (SEM) observation in A549 cells treated with SME-6. Cells were treated with SME-6 for 24h and then observed under scanning electron microscope.

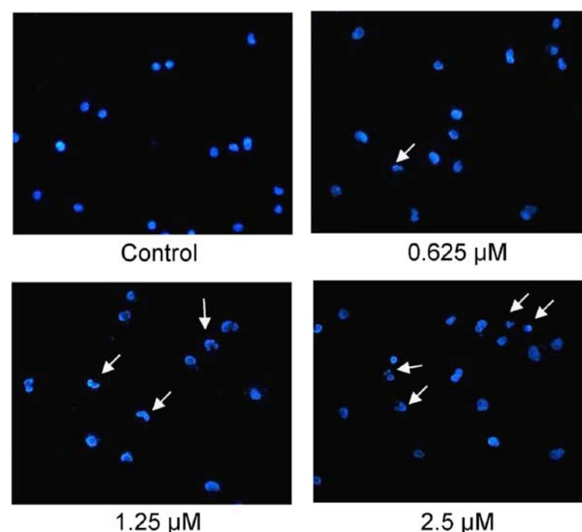

Figure 6. Nuclear staining examination of A549 cells treated with SME-6. A549 cells were treated with DMSO (control) or SME-6 for 24h, then stained with the Hoechst 33258 fluorescence dye, and finally observed under a fluorescent microscope. Arrows indicate cells in apoptosis with nuclear condensation and DNA fragmentation.

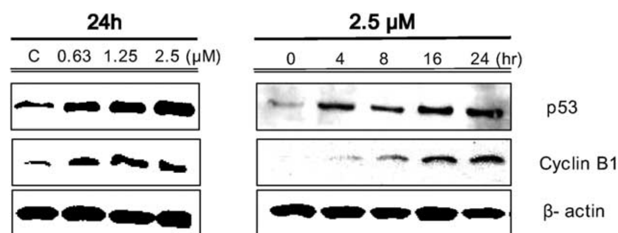

Figure 7. The effects of SME-6 on the expression of p53 and cyclin B1 in A549 cells.

In summary, the present study suggests that benz[*f*]-indole-4,9-diones-mediated growth inhibition of cancer cells might be highly related to the induction of apoptosis by means of cell cycle arrest at G_2/M phase and p53-depedency. These data may provide new information for understanding the mechanisms by benz[*f*]indole-4,9-diones-mediated antitumor activity.

Acknowledgements

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12. *Scanning electron microscope (SEM)*: Cultured A549 cells were treated with test compound for 24 h and then were harvested by centrifugation at 1000*g* for 10 min. Cells were fixed in 2% glutaraldehyde for 2 h, refixed in 1% buffered O_SO_4 for 2 h, dehydrated in graded alcohols. The sample was dried by the critical-point technique. After trimming, mounting and coating with gold–platinum, the specimens were observe under scanning electron microscope (JSM-5200, JEOL Co., Tokyo, Japan).
13. *Western blot analysis*: A549 cultures were plated in 10 cm dishes at 1×10^6 cells/dish. After 24 h incubation, the cells were treated with test compound as indicated times. After washing with PBS twice, 1 mL boiling 2*X* electrophoresis sample buffer (1*X* = 125 mM Tris–HCl buffer (pH 6.8), 2% SDS, 5% glycerol, 0.003% bromophenol blue, and 1% β-mercaptoethanol) was added to the cells, which were then scraped from the dish and boiled for 5 min. Each protein fraction was subjected to 7.5% SDS-PAGE. Proteins were transferred onto PVDF membranes by electroblotting and the membranes were treated for 1 h with blocking buffer (5% dry milk in PBST). The membranes were then incubated for 3 h at 37 °C with primary antibodies diluted in blocking buffer, washed with PBST, incubated for 2 h at 37 °C with horseradish–peroxidase conjugated antibodies in blocking buffer, washed with PBST, and developed using ECL reagent (Amersham Corp., Arlington Heights, IL, USA).