Cell Cycle Arrest by Hybrid Liposomes for Human Lung Carcinoma Cells

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Abstract

Hybrid liposomes (HL), composed of L-α-dimyristoylphosphatidylcholine and polyoxyethylene(23) dodecyl ethers, were simply prepared by the sonication method. In this study, we investigated the effects of HL on cell cycle and apoptosis in human non-small cell lung cancer cells. Induction of cell cycle arrest at the G_0/G_1 phase and apoptosis by HL were observed in human non-small cell lung cancer cells (A549, H460 and H23). HL treatment also resulted in the induction of cyclin-dependent kinases inhibitor p21[WAF1/CIP1] and p27[KIP1] and a decrease in the protein expressions of cyclins D1 and E. It is noteworthy that the treatment of A549 cells with HL inhibited phosphorylation of Akt in a time- and dose-dependent manner. Furthermore, HL treatment inhibited the filopodia formation in A549 cells. These results suggest that HL-induced cell cycle arrest at the G_0/G_1 phase could be associated by the up-regulation of cdk inhibitor p21 and p27 through blocking Akt signaling.

Introduction

Non-small cell lung carcinoma (NSCLC), including adenocarcinoma, squamous-cell lung carcinoma, and large-cell lung carcinoma, is the most common form of lung cancer and accounts for the most deaths of any cancer worldwide. This cancer is relatively insensitive to chemotherapy such as cisplatin, paclitaxel and gemcitabine compared to small cell lung carcinoma (SCLC) [1,2]. In recent years, molecular targeted therapeutics have attracted much attention as an efficient therapy for NSCLC on the basis of molecular level studies on human cells [3]. Targeted molecules include specific proteins expressed in NSCLC such as epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF) receptor and anaplastic lymphoma kinase (ALK), and so on. However, the treatment options for NSCLC with EGFR mutations and ALK rearrangements are very limited [3,4]. Therefore, novel treatment strategies directed against NSCLC are needed.

We have produced hybrid liposomes (HL) that can be simply prepared by sonicating a mixture of vesicular and micellar molecules in buffer solutions [5]. HL, composed of L-α-dimyristoylphosphatidylcholine (DMPC) and polyoxyethylene (23) dodecyl ethers ($C_{12}(EO)_{23}$), are effective for inhibiting the growth of various cancer cells *in vitro* [6-9], *in vivo* [10,11] and clinical applications [12]. Recently, we have reported that HL composed of 90 mol% DMPC and 10 mol% $C_{12}(EO)_{23}$ induced apoptosis in various cancer cells including NSCLC (A549, H460, H23 and H520) cells [9,13-15]. Furthermore, HL-induced apoptosis in A549 cells was caused by the activation of caspases-3, -8, -9 and the reduction of mitochondrial membrane potential [15]. Significantly, the HL distinguished between cancer cells and normal cells which had higher and lower membrane fluidities respectively, and fused and accumulated preferentially into the membranes of cancer cells for NSCLC cells [9], colorectal cancer cells [13] and hepatocellular carcinoma cells [15]. More recently, we have reported that HL inhibits the growth of colorectal cancer (HCT116) cells through the induction of cell cycle arrest at G_0/G_1 phase along with apoptosis [16]. However, the research for the effects of HL on the cell cycle and their regulatory molecules for NSCLC is very limited.

In this study, we investigated the effects of HL composed of 90 mol% DMPC and 10 mol% $C_{12}(EO)_{23}$ on the cell cycle and apoptosis of NSCLC (A549, H460 and H23) cells *in vitro,* and found the induction of cell cycle arrest at G_0/G_1 phase through blocking Akt signaling along with apoptosis.

Material and Methods

Preparation of hybrid liposomes (HL)

HL composed of 90 mol% DMPC and 10 mol% $C_{12}(EO)_{23}$ was prepared by the following methods. Briefly, DMPC (NOF, Tokyo, Japan) and $C_{12}(EO)_{23}$ (Sigma Chemical, St Louis, MO, USA) were mixed in 5% glucose solution and sonicated with a bath type sonicator (VS-N300, VELVO-CLEAR, Tokyo, Japan) at 45°C under a nitrogen atmosphere with 300 W, followed by filtration with a 0.20 μm filter.

Dynamic light scattering method

The size of HL was measured with an electrophoretic light scattering spectrophotometer (ELS-8000, Otsuka Electronics, Japan). The size of HL was shown in the supplementary Figure S1. Using a He-Ne laser as light source, a 633 nm laser line by 10 mW power was applied with a scattering angle 90°. The hydrodynamic diameter (d_{hy}) of HL was calculated by Stokes-Einstein equation ($d_{hy}=kT/(3\pi\eta D)$, where k is Boltzmann's constant, T is the absolute temperature, η is the viscosity of solvent and D is the diffusion coefficient).

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Cell culture

Human non small cell lung cancer (NSCLC) cell lines were used in lung adenocarcinoma (A549 and H23) and large cell carcinoma (H460). A549 cells were obtained from the RIKEN cell bank (Ibaraki, Japan). H23 and H460 cells were obtained from the American Type Cell Culture (ATCC, Manassas, VA, USA). A549 cells were cultured in D-MEM medium (Life Technologies, Carlsbad, CA, USA) and H23 and H460 cells were cultured in RPMI-1640 medium (Life Technologies) containing penicillin (100 units/ml), streptomycin (50 μg/ml) and 10% fetal bovine serum (FBS, Thermo Fisher Scientific, Waltham, MA, USA) in a 5% CO_2 humidified incubator at 37°C.

Cell cycle analysis by using flow cytometry

Cells were seeded at a density of 4.0×10^4 cells per well in 6 well plates (Sumitomo Bakelite, Tokyo, Japan) and incubated in a humidified atmosphere of 5% CO_2 at 37°C. After 24 hours, HL was added into each well and the plates were incubated for 6, 24 or 48 hours. Subsequently, cells were centrifuged and resuspended in Ca^{2+}/Mg^{2+}-free phosphate buffered saline (PBS (-)) containing 40 μg/ml propidium iodide (PI, Molecular Probes, Eugene, OR, USA), 1 mg/ml RNase and 0.1% Triton X-100 in a dark room. The DNA contents and percentage of cells in each phase of cell cycle were analyzed by a flow cytometer (Epics XL system II, Beckman Coulter, Brea, CA, USA) [16].

Caspase activity

The active caspase-3 activity was measured using PhiPhiLuxG1D2 (OncoImmunin, Gaitherssburg, MD, USA) according to the manufacturer's instructions. Cells (2.0×10^4 cells/ml) were treated with 400 μM HL for 24 hours, incubated with 10 μM PhiPhiLux-G1D2 substrate for 30 min, and then the caspase-3 activity was visualized by a confocal laser scanning microscope (TCS-SP, Leica, Mannheim, Germany).

Enzyme immunometric assay

Cells were seeded at a density of 4.0×10^4 cells per well in 6 well plates and incubated for 24 hours. Then, HL was added at 200 μM and the plates were incubated for 48 hours. After the treatment with trypsin, the cells were centrifuged at 1200 rpm for 5 min, washed with PBS (-), and resuspended in cell lysis buffer solution containing 50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1% sodium deoxycholate and 0.1% SDS. Then, p21[WAF1/CIP1], p27[KIP1], or p53 in the cell lysates was assayed using a TiterZyme® human p21 Enzyme Immunometric Assay Kit (Assay Designs, Ann Arbor, MI, USA), a TiterZyme® human Total p27[Kip1] Enzyme Immunometric Assay Kit (Assay Designs), or a Human p53 ELISA Kit (Abcam, Cambridge, MA, USA) according to the manufacturer's recommendations [16,17].

Cell cycle-regulatory proteins by flow cytometry

Cyclin D1 and E proteins expression were detected by immunocytochemistry using a flow cytometer [18,19]. Cells were treated with 200 μM HL for 48 hours. Briefly, cells were washed with PBS(-), trypsinized, centrifuged and fixed in fixation buffer (75% ethanol in PBS(-)) for 2 hours at -20°C. Cells were washed with a washing buffer (0.1% NaN_3 and 2% FBS in PBS(-)) and permeabilized with cold permeabilization buffer (0.25% Triton-X 100 in washing buffer) for 5 min on ice. After that, cells were washed twice with a washing buffer and stained with FITC conjugated anti-cyclin D1 (1 μg/10^6 cells, DCS-6, SANTA CRUZ, Santa Cruz, CA, USA) or FITC conjugated anti-cyclin E (1 μg/10^6 cells, HE12, SANTA CRUZ) for 30

min on ice in the dark. Mouse anti IgG_{2a} or IgG_{2b} served as the isotype control. Cells were washed and measured by a flow cytometer.

Phospho-Akt (p-Akt) protein expression was detected by immunocytochemistry using flow cytometer. Cells were treated with HL for 3 or 24 hours. Briefly, cells were washed with PBS(-), trypsinized, centrifuged and fixed in fixation buffer (4% paraformaldehyde in PBS(-)) for 10 min at 37°C. Cells were centrifuged at 1500 rpm × 5 min and permeabilized with cold 90% methanol in PBS(-) for 30 min on ice. After that, cells were washed twice with an incubation buffer (0.5% bovine serum albumin in PBS(-)) and blocked in the incubation buffer for 10 min at room temperature. Cells were stained with Alexa Fluor 488 conjugated anti-phospho-Akt (2 μl/10^6 cells, D9E, Cell Signaling Technology, Danvers, MA, USA) for 1 hour at room temperature in the dark. Rabbit monoclonal antibody IgG was served as the isotype control. Cells were washed and measured by a flow cytometer. The percentage of relative protein expression was calculated using the formula: (mean-fluorescence intensity of HL-treated cells)/(that of untreated cells) × 100%.

Total internal reflection fluorescence microscopy

To assess the filopodia formation of A549 cells, the cells were analyzed by total internal reflection fluorescence (TIRF) microscopy [20]. The cells (2.0×10^4 cells/mL) were seeded in glass bottom dishes (Mat Tek, Flint, MI, USA) and incubated for 24 hours. Subsequently, the cells were treated with 200 μM HL for 3 hours. The cells were washed with PBS (−) and fixed with a 10% formaldehyde solution for 10 min at room temperature. After washing with PBS (−), the cells were permeabilized with 0.1% Triton X-100 for 10 min at room temperature. After that, the cells were incubated with 0.3 units/mL of rhodamine-labeled phalloidin (Molecular Probes) for 30 min. The stained cells were observed with a TIRF microscope system (IX71, Olympus, Tokyo, Japan) equipped with an air-cooled CCD camera (EM-CCD C9100-13, Hamamatsu Photonics, Hamamatsu, Japan).

Results and Discussion

First, we examined the cell cycle analysis of A549 cells treated with HL using flow cytometry. As shown in Figures 1A and 1B, HL-treated A549 cells were significantly blocked in the G_0/G_1 populations in a dose- and time-dependent manner. On the other hand, the sub-G_1 populations of A549 cells gradually increased in the higher concentration range ([DMPC]=300-500 μM) as shown in Figure 1C. Furthermore, the treatment with 400 μM HL induced the activation of caspase-3 (Figure 1D). In addition, similar results were obtained in H460 and H23 cells (Supplementary Figures S2 and S3). With respect to the concentration of HL having anti-growth activity, we have previously reported that HL inhibited the growth of NSCLC (A549, H460 and H23) cells at 50% inhibitory concentration ranging from 229 to 255 μM for DMPC concentration [13]. These results indicate that the growth inhibition by HL could be mediated by the induction of G_0/G_1 cell cycle arrest or apoptosis for NSCLC cells.

Progression through the cell cycle can be regulated by cyclins and their associated cyclin-dependent kinases (cdk). It is well known that G_0/G_1 arrest is associated with the up-regulation of cdk inhibitor p21[WAF1/CIP1] and p27[KIP1] and the down-regulation of cyclin D1 and cyclin E, which play key roles in regulating the entry of cells at the G_1/S transition check point [21,22]. We have already reported that HL-induced G_0/G_1 arrest in human colorectal cancer (HCT116) cells was mediated through an increase in p21 [16]. Thus, we examined the effects of HL on several cell cycle regulatory proteins, including p21,

p27, cyclin D1 and cyclin E protein. The expression of p21 and p27 protein was significantly increased after the treatment with 200 μM HL (Figures 2A and 2B). On the other hand, the expression of cyclin D1 and cyclin E protein was significantly decreased after the treatment with 200 μM HL (Figures 3A and 3B). In addition, similar results were obtained in HCT116 cells (Supplementary Figures S4 and S5). These results strongly suggest that HL induced G_0/G_1 arrest in NSCLC cells via the up-regulation of cdk inhibitor p21 and p27.

Expression of the p53 tumor suppresser protein plays an important role in either cell cycle arrest or apoptosis by various drugs [23,24].
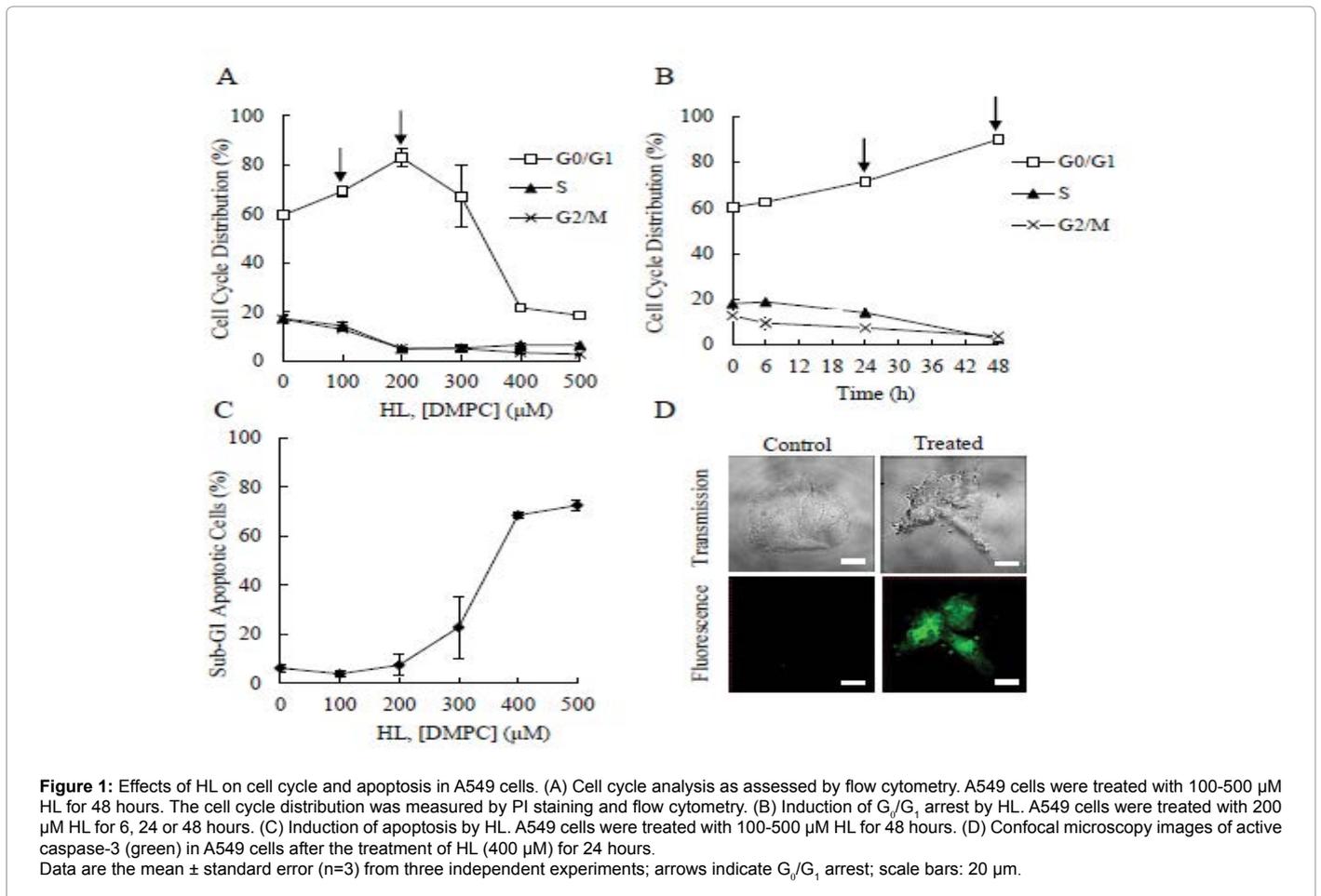
Figure 1: Effects of HL on cell cycle and apoptosis in A549 cells. (A) Cell cycle analysis as assessed by flow cytometry. A549 cells were treated with 100-500 μM HL for 48 hours. The cell cycle distribution was measured by PI staining and flow cytometry. (B) Induction of G_0/G_1 arrest by HL. A549 cells were treated with 200 μM HL for 6, 24 or 48 hours. (C) Induction of apoptosis by HL. A549 cells were treated with 100-500 μM HL for 48 hours. (D) Confocal microscopy images of active caspase-3 (green) in A549 cells after the treatment of HL (400 μM) for 24 hours.
Data are the mean ± standard error (n=3) from three independent experiments; arrows indicate G_0/G_1 arrest; scale bars: 20 μm.
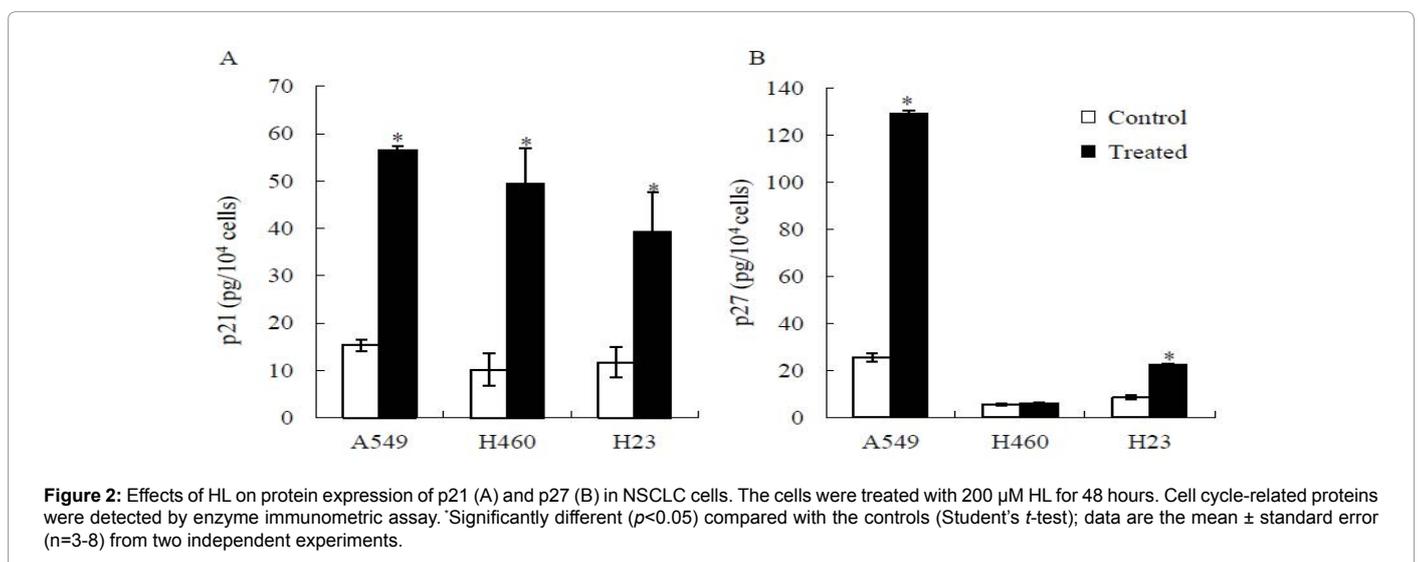

Figure 2: Effects of HL on protein expression of p21 (A) and p27 (B) in NSCLC cells. The cells were treated with 200 μM HL for 48 hours. Cell cycle-related proteins were detected by enzyme immunometric assay. *Significantly different ($p<0.05$) compared with the controls (Student's t-test); data are the mean ± standard error (n=3-8) from two independent experiments.
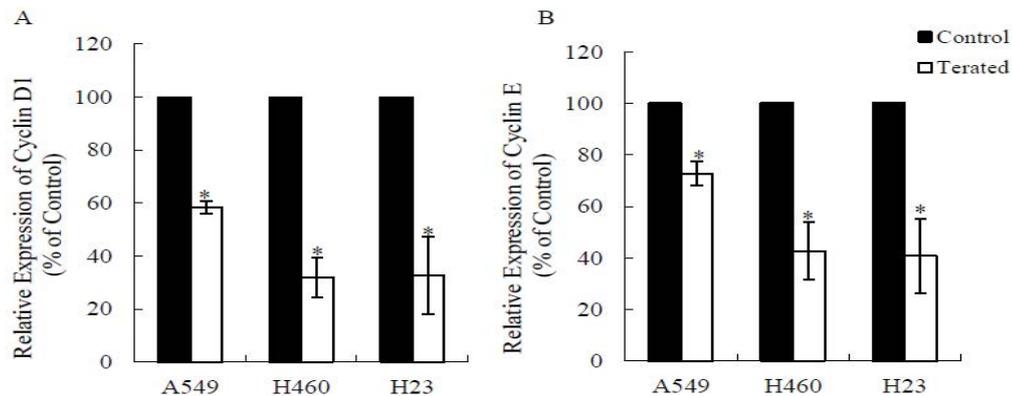
Figure 3: Effects of HL on protein expression of cyclin D1 (A) and cyclin E (B) in NSCLC cells. Cells were incubated with 200 µM HL for 48 hours. Then, cell cycle-related proteins were detected by flow cytometry.ˣSignificantly different (*p*<0.05) compared with the controls (Student's *t*-test); data are the mean ± standard error (n=3) from three independent experiments.
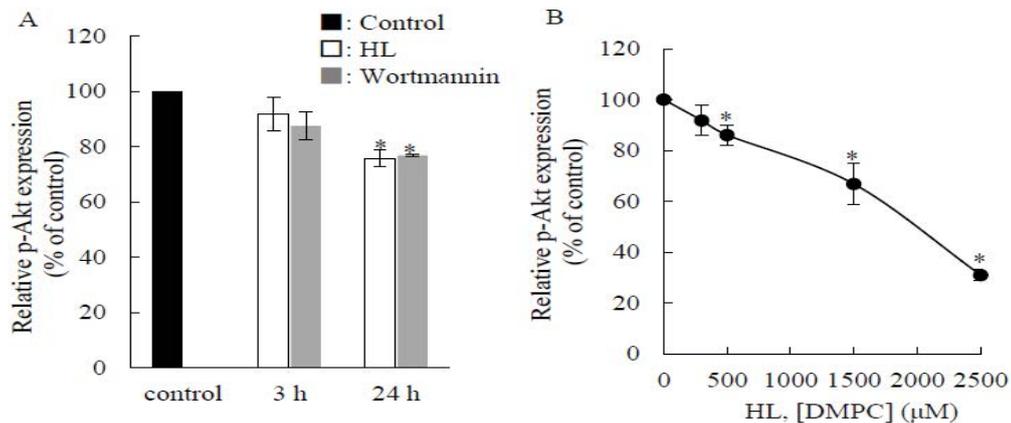

Figure 4: Inhibitory effects of HL on phosphorylation of Akt (Ser473) in A549 cells. (A) Cells were incubated with 300 µM HL for 3 or 24 hours. Wortmannin, an inhibitor of PI3K/Akt pathway, was used as a positive control. (B) Cells were incubated with 300-2500 µM HL for 3 hours. Cells were stained with Alexa Fluor 488 conjugated anti-phosho-Akt antibody and analyzed by flow cytometry.ˣSignificantly different (*p*<0.05) compared with the controls (Student's *t*-test); data are the mean ± standard error (n=3) from three independent experiments.

Expression of p21 is also regulated by both p53-dependent and -independent mechanisms [24]. We therefore examined the effects of HL on p53 expression in A549, H460, and HCT116 cells which have wild type p53, and in H23 cells which have mutant p53 [23]. However, treatment with 200 µM HL did not change or slightly decreased the p53 level (Supplementary Figure S6), suggesting that p21 induction by HL in NSCLC cells is p53-independent pathway.

Phosphorylated-Akt (p-Akt) kinases regulate the cell-cycle progress via down-regulation of p21 and p27 [19]. Akt signaling is also implicated in apoptotic mitochondrial pathways via up-regulation of pro-apoptotic proteins and down-regulation of anti-apoptotic proteins [19,20]. Therefore, we examined the inhibition of the expression of p-Akt in A549 cells by HL. Wortmannin, an inhibitor of phosphatidylinositol-3 kinase (PI3K)/Akt kinases, was used as a positive control (50 µM). The results are shown in Figures 4A and 4B. It is noteworthy that the treatment with HL significantly inhibited phosphorylation of Akt in a time- and dose-dependent manner. These results suggest that HL-induced G_0/G_1 arrest and apoptosis could be mediated by the inhibition of p-Akt expression in A549 cells.

Actin cytoskeleton dynamics, such as lamellipodia and filopodia, affect the cell division cycle [21,22]. Filopodia formation in cells is also dependent on the activation of PI3K and Akt kinases [23-25]. Thus, we examined the effect of HL on a filopodia formation in A549 cells. The results are shown in Figure 5. The treatment with HL drastically inhibited the filopodia formation in A549 cells. A similar tendency was observed in A549 cells incubated in the serum-starved (non-FBS) medium for 24 hours. These results suggest that the inhibitory effects of HL on expression p-Akt in A549 cells could be related to blocking the actin cytoskeleton dynamics such as filopodia formation. With respect to cell cycle regulation, some researchers have reported that cell cycle arrest was closely associated with metabolic events in plasma membranes [21,22,26-29]. We have previously reported that HL affects the fluidity of plasma membranes [30], cellular lipid constituents [31-37] and lipid microdomain (lipid rafts [30] or caveolae structures [31]) in cancer cells, and trigger the growth-inhibition or apoptosis. Although the mechanistic details are not yet clear, it seems that HL could accumulate in plasma membranes of NSCLC cells [13], change the membrane characteristics related to cell cycle progression, and induce G_0/G_1 phase arrest or apoptosis.
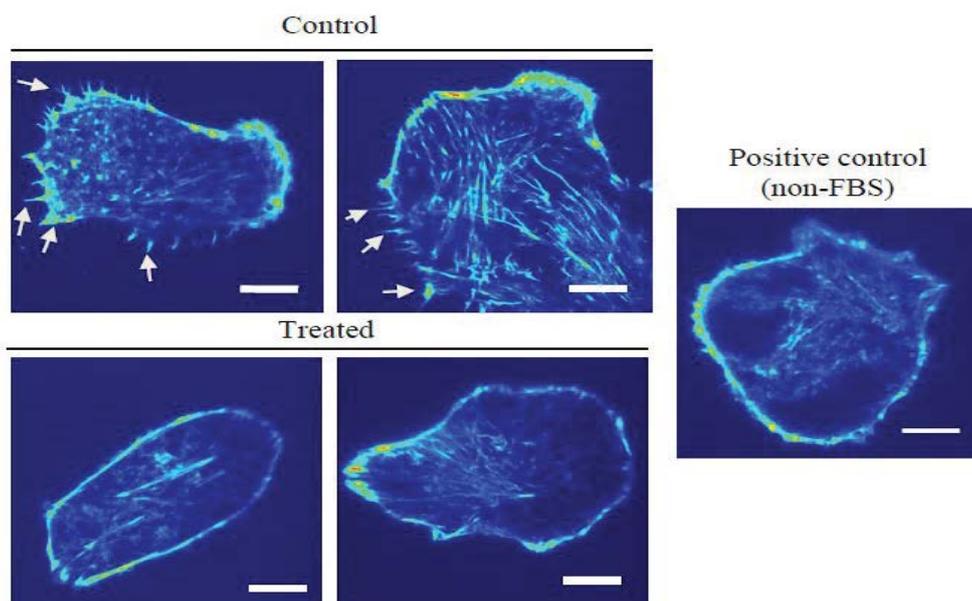
Figure 5: HL inhibited filopodia formation of A549 cells. A549 cells were treated with 200 μM HL for 3 hours. As positive control, A549 cells were incubated in the serum-starved (non-FBS) medium for 24 hours. Then, the cells were stained with rhodamine phalloidine and observed by TIRF microscope. Scale bars: 100 μm; allows indicate the filopodia formation of A549 cells.

Conclusion

The hybrid nanoparticle, HL composed of 90 mol% DMPC and 10 mol% $C_{12}(EO)_{23}$, exerted for the first time the activity against the growth of NSCLC cells by causing apoptosis and arresting cells in the G_0/G_1 phase of the cell cycle through the inhibition of Akt signaling. This study suggests that HL could be applied in novel nanomedicinal chemotherapy for NSCLC.

Acknowledgements

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