Growth Inhibition by Novel Liposomes Including Trehalose Surfactant Against Hepatocarcinoma Cells Along with Apoptosis

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Abstract. *Novel liposomes composed of L-α-dimyristoylphosphatidylcholine (DMPC) and trehalose surfactant (DMTreCn) were produced by the method of sonication in buffer solution. The thickness of fixed aqueous layer of DMTreCn was larger than that of DMPC liposomes and increased in a dose-dependent manner. The remarkable inhibitory effects of DMTreCn on the growth of human hepatocellular carcinoma (HCC) (Hep-G2 and HuH-7) cells were obtained along with apoptosis, without affecting the growth of normal cells. DMTreCn induced apoptosis of Hep-G2 and HuH-7 cells through the activation of caspase-3, 8 and 9. Release of cytochrome c from mitochondria and activation of Bcl-2 family protein (BAX) were recorded, indicating that DMTreCn induced apoptosis of Hep-G2 and HuH-7 cells through mitochondrial pathway via BAX. It is noteworthy that the remarkable inhibitory effects of DMTreCn on the growth of human HCC cells were obtained along with apoptosis for the first time.*

Hepatocellular carcinoma (HCC) is the sixth most common malignancy worldwide and is the third most common cause of cancer-related death (1-3). Surgical resection is frequently limited due to metastases, cirrhosis, and other pathological changes in the liver parenchyma. Drug-resistant genes, including multidrug-resistant gene 1, are frequently overexpressed in HCC. Sorafenib is an oral multikinase inhibitor that acts by inhibiting tumor growth and has shown promising activity against HCC. However, many clinical studies have indicated several limitations to the application of sorafenib as a single agent (4, 5). Therefore, novel agents without side-effects in the treatment against HCC are desirable.

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Liposomes are closed vesicles that are formed when phospholipids are dispersed in water at relatively low concentrations. Liposomes have contributed significantly to delivery, for example, of antitumor agents, hormones and in immunomodulation (6, 7).

We have produced hybrid liposomes (HL) composed of vesicular and micellar molecules, which can be prepared simply by sonication of those molecules in a buffer solution (8). Changing the constituents and compositional ratios of HL can control the physical properties of HL, such as size, shape, the temperature of phase transition, and membrane fluidity.

HL composed of L-α-dimyristoylphosphatidylcholine (DMPC) and polyoxyethylenedodecyl ether, without using any drugs, have inhibited the proliferation of various type of tumor cell along with apoptosis *in vitro* (9-11) and *in vivo* (12, 13). The mechanistic details of apoptosis of tumor cells (10) and the correlation between antitumor effects and membrane fluidity of HL (14) and membrane fluidity of plasma membranes of tumor cells (15) have been clarified. Successful clinical chemotherapy with drug-free HL to patients with lymphoma has been reported without having any adverse effects after the approval of the Bioethics Committee (16). Furthermore, therapeutic effects of cationic liposomes against renal cell carcinoma have been obtained without any side-effects, both *in vitro* and *in vivo* (17).

Saccharides play important roles in adhering to cells, transmitting information, and recognizing molecules on the cell membranes through receptors, including lectin (18). For example, molecular recognition through lactose was found *in vivo* (19). The hydration of saccharides with hydrogen bonds provides stability. The hydration of sugar derivatives was discussed in relation to the hydration of the parent sugars (20). The preparation and characterization of glycol-liposomes have been reported (21). Specific inhibitory effects of three-component liposomes composed of L-α-dimyristoylphosphatidylcholine (DMPC), Tween 20, and sugar surfactants on the growth of glioma or hepatoma cells have been obtained *in vitro* (22, 23).

In this study, two-component liposomes composed of DMPC and trehalose surfactant (TreCn; n=14 and 16) (DMTreCn) were produced and the inhibitory effects of

DMTre were examined on the growth of HCC cells. Furthermore, induction of apoptosis of HCC cells by DMTreCn was examined.

Materials and Methods

Preparation of DMTreCn. DMTreCn (n=14 and 16) was prepared by using sonication (VELVO VS-N300, 300W) of a mixture containing DMPC (NOF Co. Ltd., Tokyo, Japan) and α-D-glycopyranosyl-α-D-glycopyranoside monomyristate (TreC14; Dojindo Ltd., Kumamoto, Japan) or α-D-glycopyranosyl-α-D-glycopyranoside monopalmitate (TreC16), in a 5% glucose solution at 45˚C at 300W, followed by filtration with a 0.45 μm filter.

Measurement of thickness of fixed aqueous layer. The thickness of fixed aqueous layer of DMTreCn was measured from the zeta potential (ζ) (24). DMTreCn was prepared in 5% glucose solution containing different concentrations of NaCl (20, 50, 100, 150, 200 mM). The values of ζ of the sample solutions were measured by laser Doppler photometry using an electrophoretic light scattering spectrophotometer (ELS-8000; Otsuka Electronics, Tokyo, Japan) with a He-Ne laser as a light source (633 nm, 10 mW) at the scattering angle (U: $m^2Volt^{-1}s^{-1}$) applying the Smoluchowski equation ($\zeta=4\pi\eta U/\varepsilon$), where η (Pa. s) and ε ($N/Volt^2$) are the viscosity and permittivity of solvent, respectively. ζ Values were measured at 37˚C. ζ is defined as the electrostatic potential at the position of the slipping plane Δ (nm), which occurs just outside the fixed aqueous layer of DMTre. Then, ζ is expressed as $\ln(\zeta)=\ln A -\Delta\kappa$, where κ is Debye–Hückel parameter ($=3.3\sqrt{c}$, c: M for NaCl). If the ζ values are measured at different concentrations of NaCl and plotted against κ, the slope gives the position of the slipping plane or the thickness of the fixed aqueous layer in nanometers.

Fluorescence depolarization method. Membrane fluidity of DMTreCn was evaluated on the basis of fluorescence depolarization method with a fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) (Nacalai Tesque, Kyoto, Japan). Fluorescence depolarization is caused by the molecular motion of the fluorescence probe, which reflects the microviscosity of the surrounding region (25). The results obtained by fluorescence measurements correspond closely with data from nuclear magnetic resonance (NMR) and differential scanning calorimetry (DSC) measurements in terms of the mobility of molecules in microenvironments (26). The fluorescence polarization of DPH was measured using a fluorescence spectrophotometer (F-2000, Hitachi, Tokyo, Japan) as described previously (14).

Cell culture. HCC cell lines (HepG2 and HuH-7) were obtained from Riken Bioresource Center Cell Bank (Tsukuba, Japan). HepG2 and HuH-7 cells were cultured in minimum essential medium (MEM) with 1% nonessential amino acids (NEAA; Gibco BRL, New York, USA) and Dulbecco's modified egle's medium (DMEM), respectively, supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific Inc., MA, USA), 100 units/ml penicillin and 100 μg/ml streptomycin (Meiji Seika Pharma Co., Ltd. Tokyo, Japan). The cells were cultured in a 5% CO_2 humidified incubator at 37˚C. Fibroblast cell line (WI-38), derived from normal embryonic lung tissue, were obtained from American Type Culture Collection (VA, USA). WI-38 cells were maintained in MEM with 10% FBS, 1.0 mM sodium pyruvate (Invitrogen, CA, USA), and 0.1 mM NEAA. The cells were cultured at 37˚C in humidified atmosphere containing 5% CO_2.

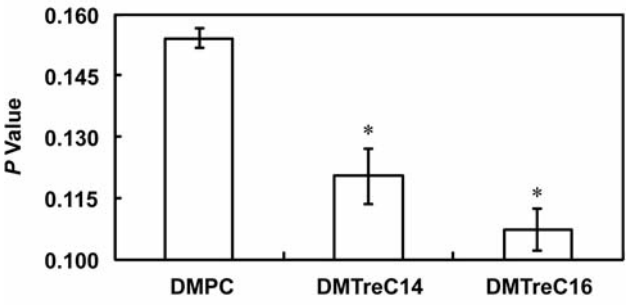

Figure 1. *Fluorescence polarization (P) value of two-component liposomes composed of L-α-dimyristoylphosphatidylcholine (DMPC) and trehalose surfactant (DMTreCn). Data presented are the mean±S.D. *Significant difference from the DMPC liposomes, $p<0.05$. DMPC=1.0×10^{-4} M. Trehalose surfactant (TreCn)=2.3×10^{-4} M.*

Assessment of growth inhibition. The inhibitory effects of DMTreCn on the growth of Hep-G2 and HuH-7 cells were examined on the basis of the 2-methoxy- 4-nitrophenyl-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-1) assay (27) (Cell Counting Kit-1; Dojindo Laboratories, Kumamoto, Japan). Cells (5.0×10^4 cells/ml) were seeded in 96-well plates and cultured in a 5% CO_2 humidified incubator at 37˚C for 24 h. DMTreCn (DMPC=1.0×10^{-4} M, TreCn=0.1-2.3×10^{-4} M) was added then cells were cultured for 48 h. WST-1 solution was added and cells incubated for 3 h. The absorbance at 450 nm was measured by spectrophotometer (E_{max}; Molecular Devices Co., CA, USA). The inhibitory effects of DMTreCn on the growth of Hep-G2 and HuH-7 cells were evaluated by ($A_{mean}/A_{control}$) ×100, where A_{mean} and $A_{control}$ denote the absorbance of water-soluble formazan, in the presence and absence of DMTre, respectively.

Fusion and accumulation of DMTreCn into cell membrane. The fusion and accumulation into the cell membrane of DMTreCn including a fluorescence probe (1-palmitoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)-amino]dodecanoyl]-sn-glycero- 3-phosphocholine (NBDPC; Avanti Polar Lipids, Inc., Alabama, USA) was performed using confocal laser microscopy (TCS-SP; Leica Microsystems, Berlin, Germany). Cells (3.5×10^5 cells/ml) were cultured in a 5% CO_2 humidified incubator at 37˚C for 24 h. The cells were treated with DMTreCn (DMPC=1.0×10^{-4} M, TreCn=2.3×10^{-4} M) including fluorescence-labeled lipid (NBDPC=5.0×10^{-6} M) for 4 h and were observed using confocal laser microscopy with a 488 nm Ar laser line (detection at 505-555 nm).

Flow cytometry. Cells treated with DMTreCn were centrifuged at 1,000 × *g* for 5 min and washed with Hanks' balanced salt solution, then fixed in chilled ethanol. The cells were washed again, treated with RNase (0.25 mg/ml) and then stained with propidium iodide (PI; 0.5 mg/ml) that has 493 nm excitation and 635 nm emission wavelength. The samples were analyzed using a flow cytometer (Epics XL system; Beckman Coulter, CA, USA) with single excitation at 488 nm of 15 mW argon laser. The PI signals were detected by FL3 sensor of 605-635 nm. Apoptotic DNA rates were calculated apoptotic DNA content/DNA content ×100.

Terminal deoxynucleotidyl transferse-mediated dUTP-biotin nick-end labeling (TUNEL) method. DNA strand breaks of HCC cells during apoptosis were detected by TUNEL method with an *in situ* cell death

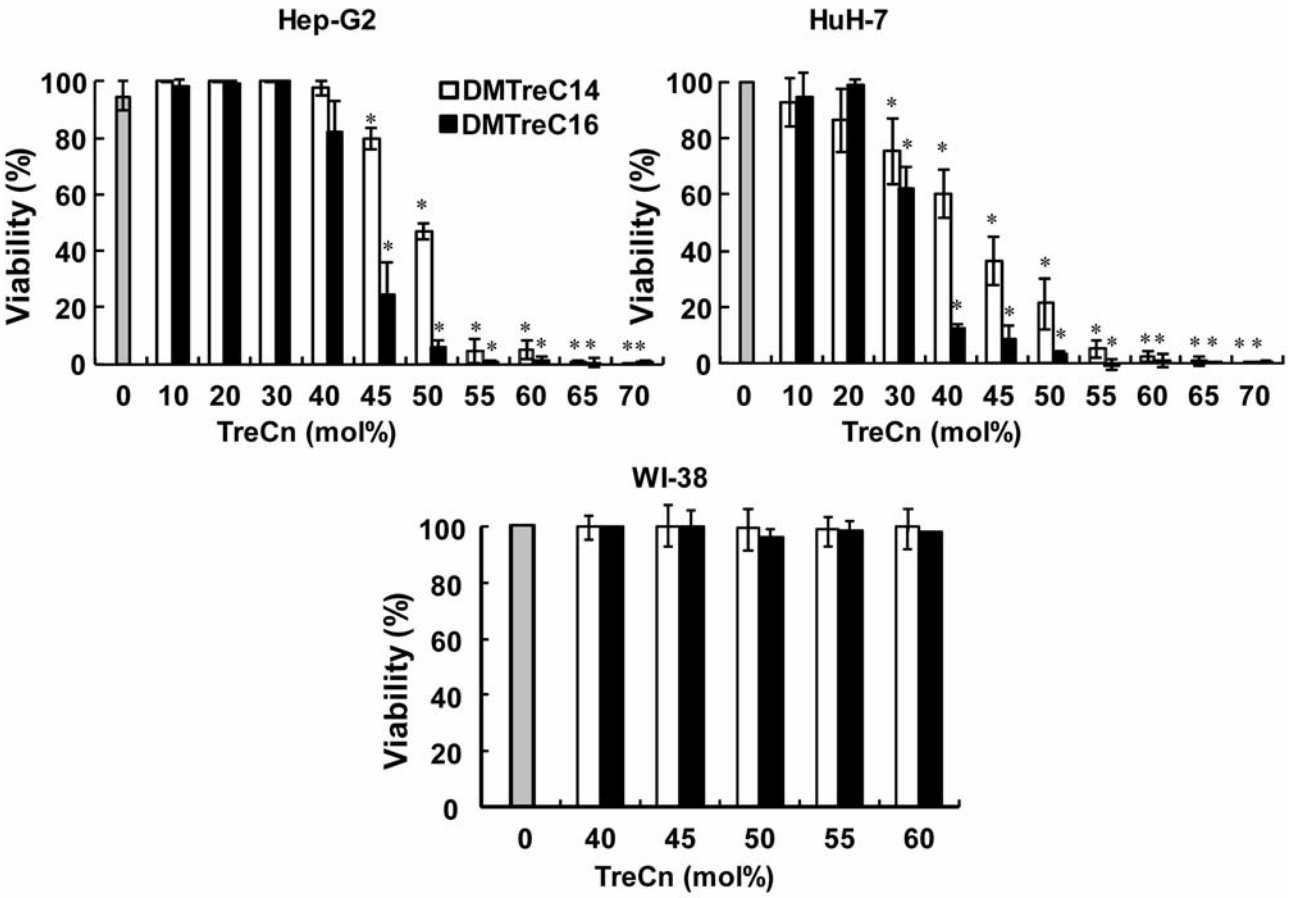

Figure 2. *Inhibitory effects of two-component liposomes composed of DMPC and trehalose surfactant (DMTreCn) on the growth of Hep-G2 and HuH-7 cells. DMTreCn inhibited the growth of hepatocarcinoma (Hep-G2 and HuH-7) cells in a dose-dependent manner without affecting the growth of normal (WI-38) cells, although DMPC liposomes had no inhibition against those tumor cells. Data presented are the mean±S.D. *Significant difference from the DMPC liposomes, $p<0.05$. DMPC=1.0×10^{-4} M. TreCn=0.1-2.3×10^{-4} M.*

detection kit (Roche Ltd., Basel, Switzerland). Cells were seeded in glass bottom dishes and incubated for 24 h. Subsequently, the cells were treated with DMTreCn for 48 h. Then the cells were washed with phosphate buffered saline [PBS (–)], fixed with 4% paraformaldehyde solution for 20 min at room temperature, and labeled with fluorescein isothiocyanate (FITC)-conjugated dUTP and terminal deoxynucleotidyltransferase. The nuclei of HCC cells were also stained with a fluorescent dye TO-PRO-3 (Molecular Probes, Inc., ORE, USA). The stained cells were observed using a confocal laser scanning microscope (TCS-SP; Leica Microsystems, Wetzlar, Germany) with a 488 nm Ar laser line for TUNEL (detection, 515-565 nm) and a 633 nm He/Ne laser line for TO-PRO-3 (detection; 640-700 nm), respectively.

Activation of caspases. Cells were seeded at a density of 2.0×10^4 cells per well in 6-well plates and incubated for 24 h. DMTreCn was added to each well and the plates were incubated for a further 48 h. The cells were centrifuged at 1,000 × *g* for 5 min and resuspended in 50 μl of chilled cell lysis buffer. Each substrate solution (PhiPhiLux-G1D2, CaspaLux 8-L1D2, CaspaLux 9-M1D2 for caspase-3, caspase-8, and caspase-9, respectively; OncoImmunin Inc., Gaithersburg, MD, USA) was added to the cell pellets and cells were incubated for 1 h. After washing twice with 1 ml of ice-cold PBS(–), the cells were resuspended in 1 ml fresh PBS(–). Activation of caspases was determined and analyzed using a flow cytometer according to the manufacturer's instructions. The stained cells were observed using a confocal laser microscope with a single excitation 488 nm of 15 mW Ar laser.

Mitochondrial membrane potential assay. Mitochondrial membrane potential of HCC cells was determined by measuring the retention of the fluorescent dye 3,3'-dihexyloxacarbocyanine [DiOC6(3)] in the cellular mitochondria. Cells were seeded in 6-well plates and incubated for 24 h. Subsequently, the cells were treated with DMTreCn (DMPC=1.0×10^{-4} M, TreCn=2.3×10^{-4} M) and then incubated with 40 nM DiOC6(3) for 30 min. The stained cells were collected by centrifugation and washed with PBS(–). The quantification of the cells retaining DiOC6(3) in the mitochondria was performed with a flow cytometer.

Cytochrome c release assay. Lysates of cells treated with DMTreCn were washed with 1 ml wash buffer and resuspended with 250 μl blocking buffer. Antibody to cytochrome *c* was added to each tube

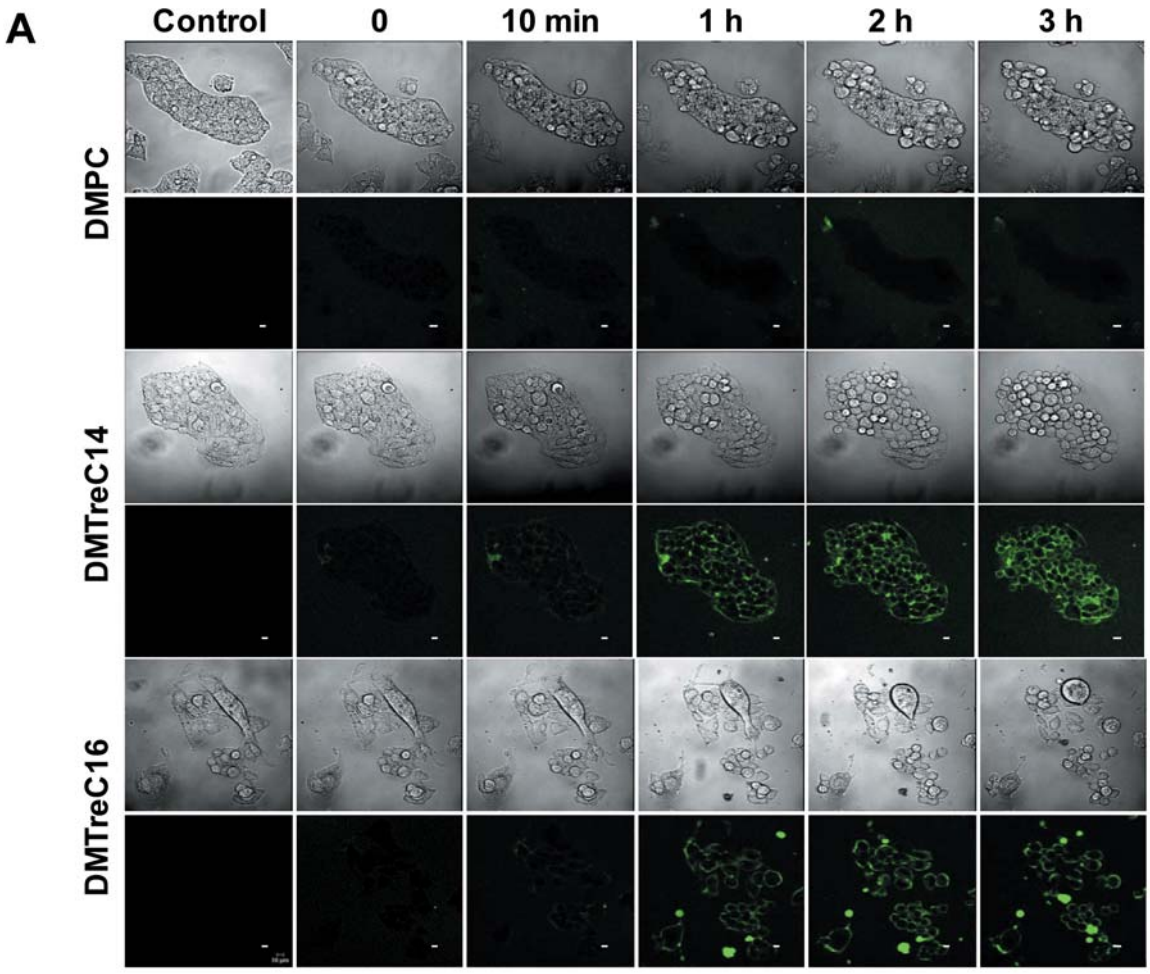

Figure 3. *Continued*

and gently mixed for 1 h. The cells were washed with wash buffer and 500 μl anti-IgG isothiocyanate (FITC) was added. Releasing cytochrome *c* of mitochondria was analyzed by using flow cytometry.

Bcl-2 family protein (BAX) assay. Cells were seeded at a density of 2.0×10^4 cells per well in 6-well plates and incubated for 24 h. DMTreCn was added to each well and the plates were incubated for 48 h. The cells were centrifuged at 1,000 × *g* for 5 min, fixed with a 4% paraformaldehyde solution and permeabilized with 70% ethanol for 30 min. The cell lysates were washed with 1 ml wash buffer and gently resuspended. Antibody to BAX (50 μl) was added to each tube and gently mixed for 30 min. The cells were washed with washing buffer and 30 μl anti-IgG FITC was added. The BAX protein of the cells was analyzed using a confocal laser microscope with a single excitation 488 nm of 15 mW Ar laser.

Statistical analysis. Results are presented as the mean±S.D. Data were statistically analyzed using the Student's *t*-test. A *p*-value of less than 0.05 was considered to represent a statistically significant difference.

Results

Physical properties of DMTreCn. The thickness of the fixed aqueous layer of DMTreCn was measured from the zeta potential: Values were 1.90±0.13 nm for DMPC liposomes, 2.50±0.36 nm for DMPC/50 mol% TreC14, 3.54±0.42 nm for DMPC/60 mol% TreC14, 4.24±0.97 nm for DMPC/70 mol% TreC14, respectively. The values for DMTreC14 were larger than that of DMPC liposomes. It is noteworthy that the thickness of the fixed aqueous layer of DMTreC14 increased in a dose-dependent manner. In the case of TreC16, clear solutions of DMTreC16 in 5% glucose containing NaCl were not obtained.

Membrane fluidity of DMTreCn (n=14 and 16) (DMPC/70 mol% TreCn) was evaluated from fluorescence polarization of a fluorescent probe on the basis of the fluorescence depolarization method. The results are shown in Figure 1. The polarization value of DMTreCn was less

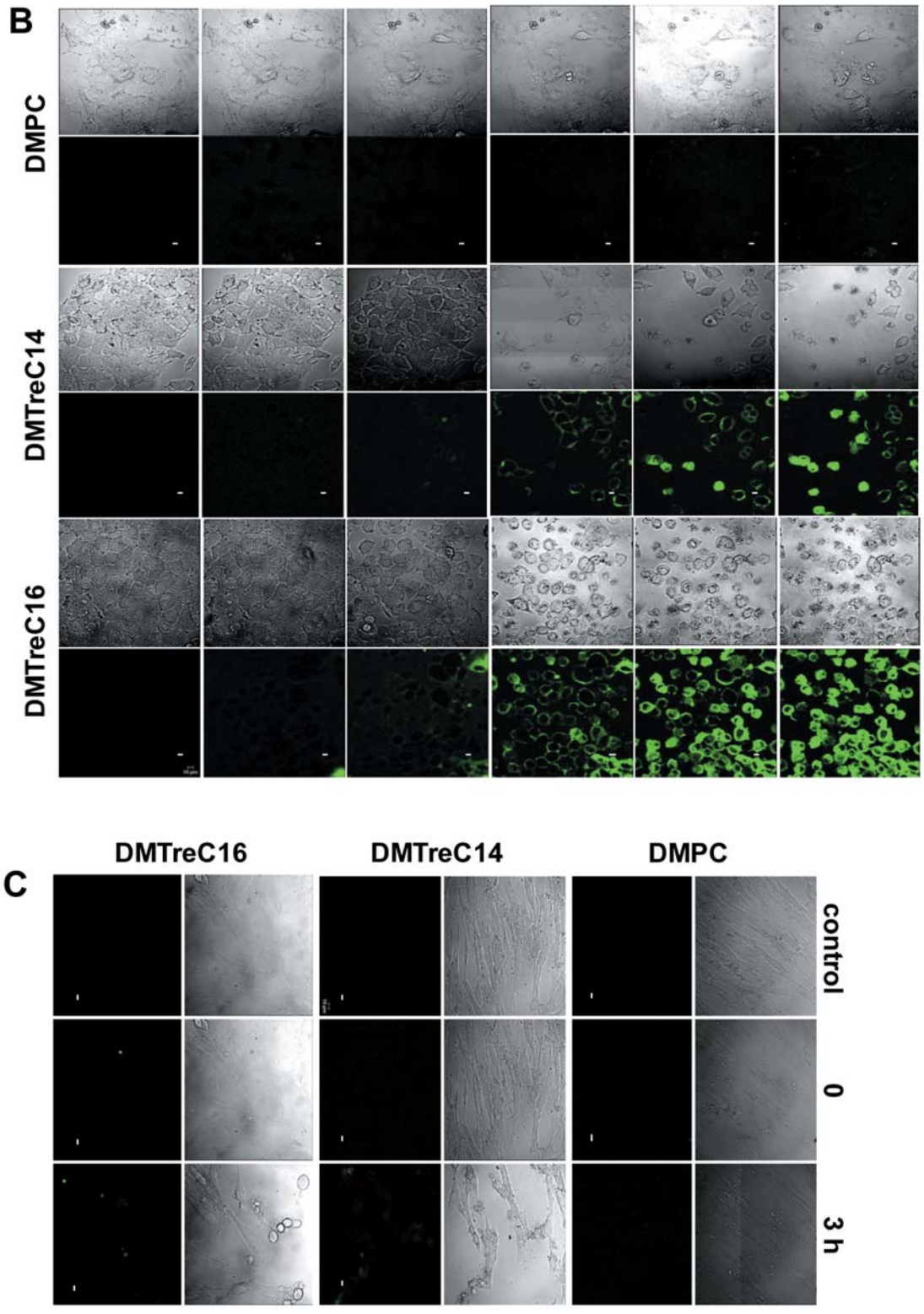

Figure 3. *Fluorescence micrographs of Hep-G2 (A), HuH-7 (B), and WI-38 (C) cells after treatment with L-α-dimyristoylphosphatidylcholine (DMPC) liposomes and two-component liposomes composed of DMPC and trehalose surfactant (DMTreCn) with fluorescence probe using confocal microscope. Accumulation of DMTreCn with fluorescence probe into Hep-G2 and HuH-7 cell membranes was observed. DMPC=1.0×10^{-4} M. TreCn=2.3×10^{-4} M.*

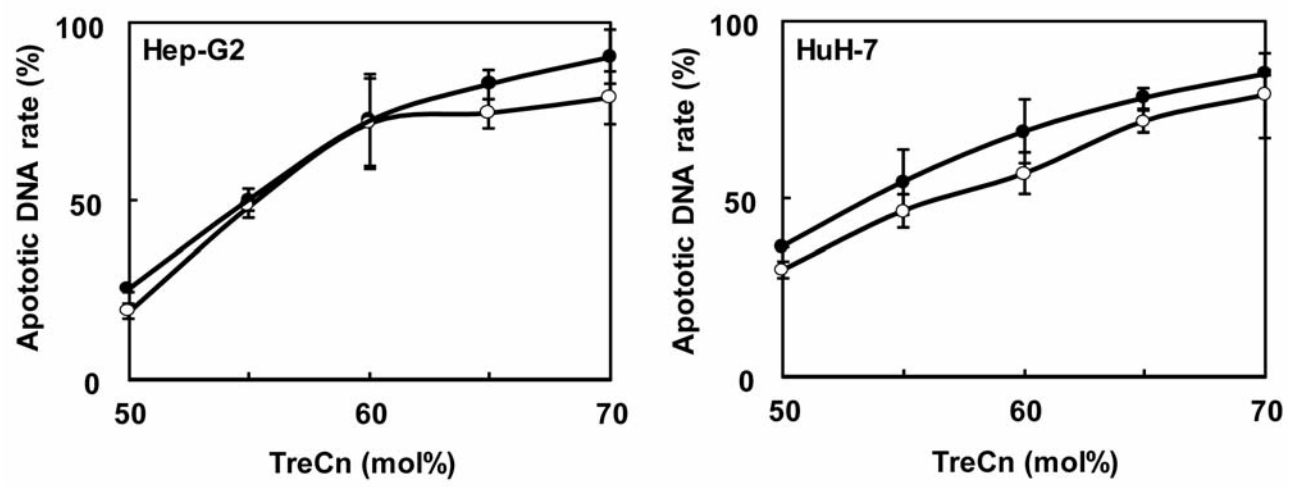

Figure 4. *Apoptotic DNA rate of Hep-G2 and HuH-7 cells increased after treatment with two-component liposomes composed of DMPC and α-D-glycopyranosyl -α-D-glycopyranoside monomyristate (DMTreC14) ○ and two-component liposomes composed of DMPC and α-D-glycopyranosyl-α-D-glycopyranoside monopalmitate (DMTreC16) ● as the dose of DMTreCn increased and reached a high apoptotic DNA rate (80-90%). Data presented are the mean±S.D. DMPC=1.0×10^{-4} M. TreCn=1.0-2.3×10^{-4} M.*

than that of DMPC liposomes, indicating that membrane fluidity of DMTreCn was greater than that of DMPC liposomes. Interestingly, the polarization values decreased in the sequence, DMTreC14>DMTreC16, indicating that membrane fluidity of DMTreC16 was greater than that of DMTreC14.

Inhibitory effects of DMTreCn on the growth of Hep-G2 and HuH-7 cells. The inhibitory effects of DMTreCn (n=14 and 16) on the growth of human hepatocarcinoma (Hep-G2 and HuH-7) cells were examined. The results are shown in Figure 2. DMTreC14 and DMTreC16 inhibited the growth of two tumor cell lines (Hep-G2 and HuH-7) in a dose-dependent manner, without affecting the growth of normal (WI-38) cells, though DMPC liposomes showed no inhibition.

Fusion and accumulation of DMTreCn. The fusion and accumulation of DMTreCn (n=14 and 16) including fluorescence–labeled lipid (NBDPC) toward Hep-G2 and HuH-7 cells was examined using confocal laser microscopy. The results are shown in Figure 3. An increase in the accumulation of DMTreC14 and DMTreC16 into Hep-G2 and HuH-7 cell membranes was observed after 3 h. Moreover, fusion and accumulation of DMTreC14 and DMTreC16 in normal (WI-38) cells was examined. The results are shown in Figure 3C. No accumulation of DMTreC14 and DMTreC16 into normal cell membranes was observed. These results suggest that membranes of normal and tumor cells uptake DMTreC14 and DMTreC16 differently.

Induction of apoptosis for Hep-G2 and HuH-7 cells by DMTreCn. The induction of apoptosis of Hep-G2 and HuH-7 cells by DMTreC14 and DMTreC16 was examined using flow cytometric analysis. The results are shown in Figure 4. Apoptotic DNA increased after treatment with DMTreC14 and DMTreC16 as the dose of DMTreCn increased and reached a high apoptotic rate (80-90%), indicating that DMTreCn induced apoptosis of Hep-G2 and HuH-7 cells.

Fluorescence micrographs of Hep-G2 and HuH-7 cells after treatment with DMTreC14 and DMTreC16 on the basis of TUNEL method are shown in Figure 5. Two tumor cells were dyed green or yellow after the treatment with DMTreC14 and DMTreC16, indicating that apoptosis was induced by DMTreC14 and DMTreC16. These observations show that the inhibitory effects of DMTreCn were accompanied by the induction of apoptosis of Hep-G2 and HuH-7 cells.

Activation of caspases in Hep-G2 and HuH-7 cells by DMTreCn. Activation of caspases is a central process in the execution of apoptosis. To investigate the apoptotic pathways induced by DMTreC14 and DMTreC16, flow cytometric analysis and confocal laser microscopy were used. The results are shown in Figure 6. It is noteworthy that the fluorescence intensity increased and tumor cells dyed green after treatment with DMTreC14 and DMTreC16, indicating that DMTrCne induced apoptosis of Hep-G2 and HuH-7 cells through the activation of caspases.

Mitochondrial pathway by DMTreCn. Mitochondrial pathway in apoptosis of Hep-G2 and HuH-7 cells induced by DMTreC14 and DMTreC16 were examined using flow

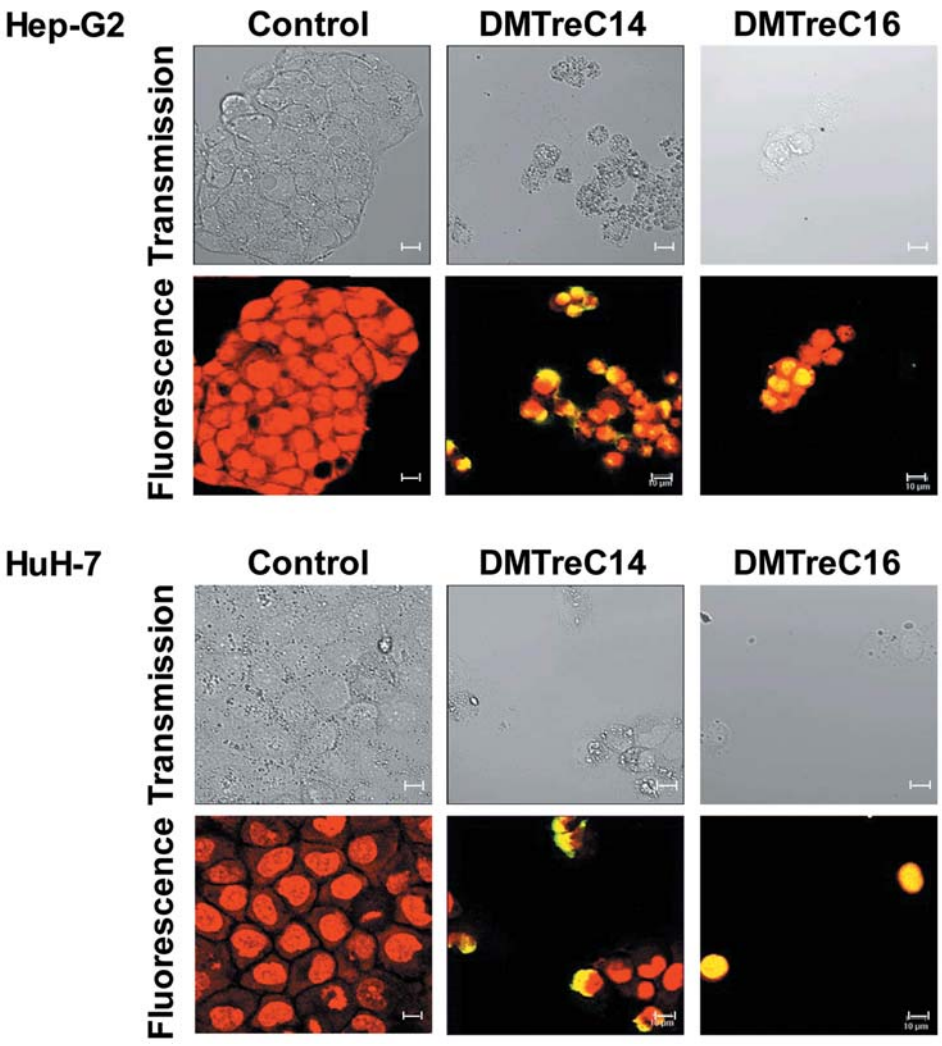

Figure 5. *Fluorescence micrographs of Hep-G2 and HuH-7 cells treated with DMTreC14 and DMTreC16 using TUNEL method. Green and yellow fluorescence indicating fragmentation of DNA by induction of apoptosis was observed.*

cytometric analysis. The results are shown in Figure 7. The mitochondrial transmembrane potential of HuH-7 cells decreased after treatment with DMTreC14 and DMTreC16, although that of Hep-G2 cells was the same as that of the control cells. However, as shown in Figure 8, fluorescence intensity of Hep-G2 and HuH-7 cells after the treatment with DMTreC14 and DMTreC16 decreased, indicating that cytochrome *c* of mitochondria was released and caspase-9 activated by DMTreC14 and DMTreC16.

Furthermore, BAX assay for Hep-G2 and HuH-7 cells after treatment with DMTreC14 and DMTreC16 was examined. The results are shown in Figure 9. Hep-G2 and HuH-7 cells dyed green after treatment with DMTreC14 and DMTreC16, indicating that the cytochrome c release was related to the activation of BAX (28).

Discussion

Chemotherapy with sorafenib is effective for patients with HCC (4). However, many clinical studies have indicated several limitations to the application of sorafenib (4). Although they kill tumor cells, chemotherapy and radiation therapy damage normal cells. Therefore, novel therapeutic agents without side-effects are desirable.

Specific inhibitory effects of three-component liposomes composed of DMPC, Tween20, and sucrose surfactants on the growth of glioma were reported (22), and liposomes composed of DMPC, Tween20, and lactose surfactants were effective in inhibiting hepatoma cell growth (23). Three-component liposomes have shown remarkable inhibitory effects compared with DMPC liposomes and DMPC/Tween20 liposomes on the

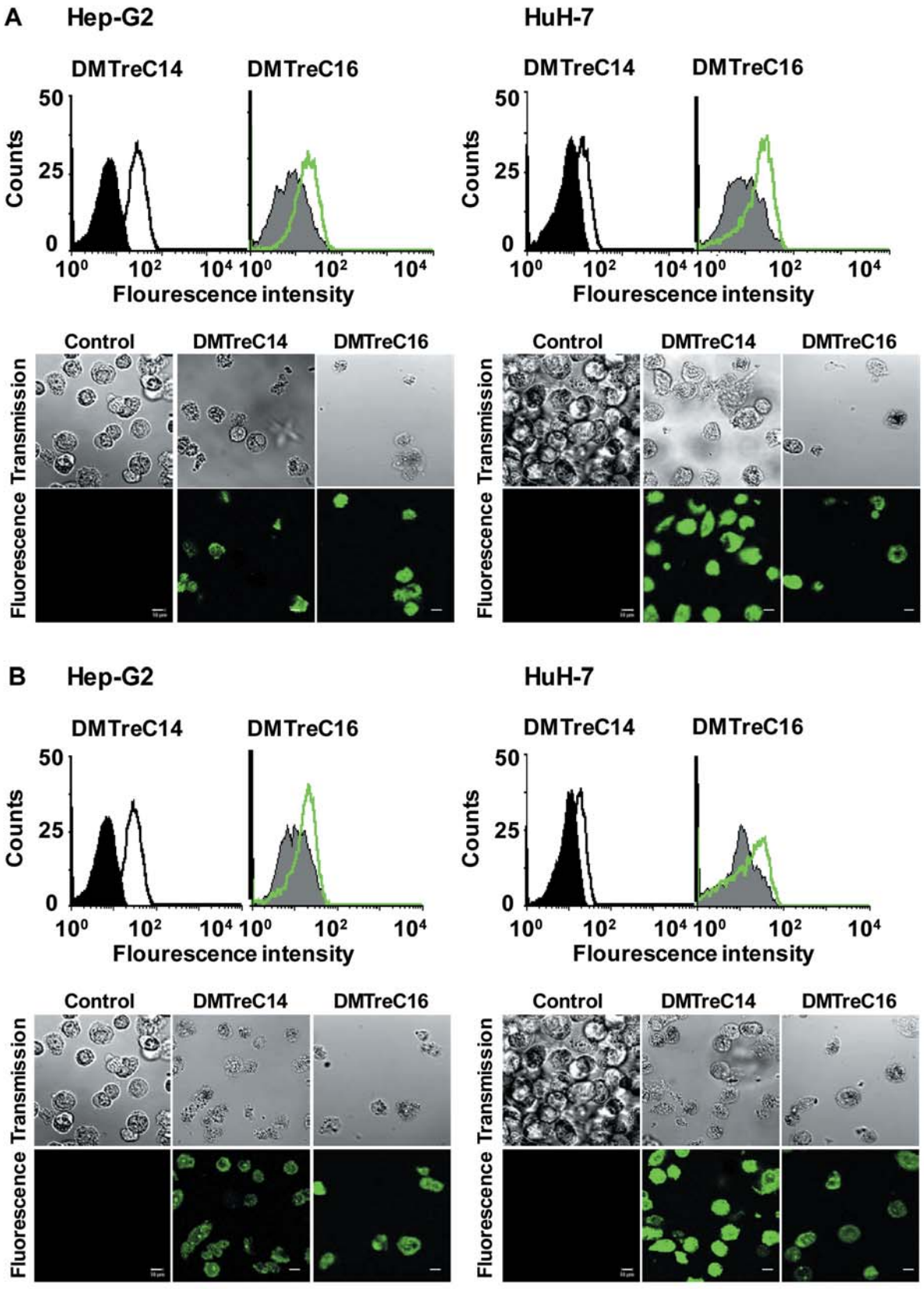

Figure 6. *Continued*

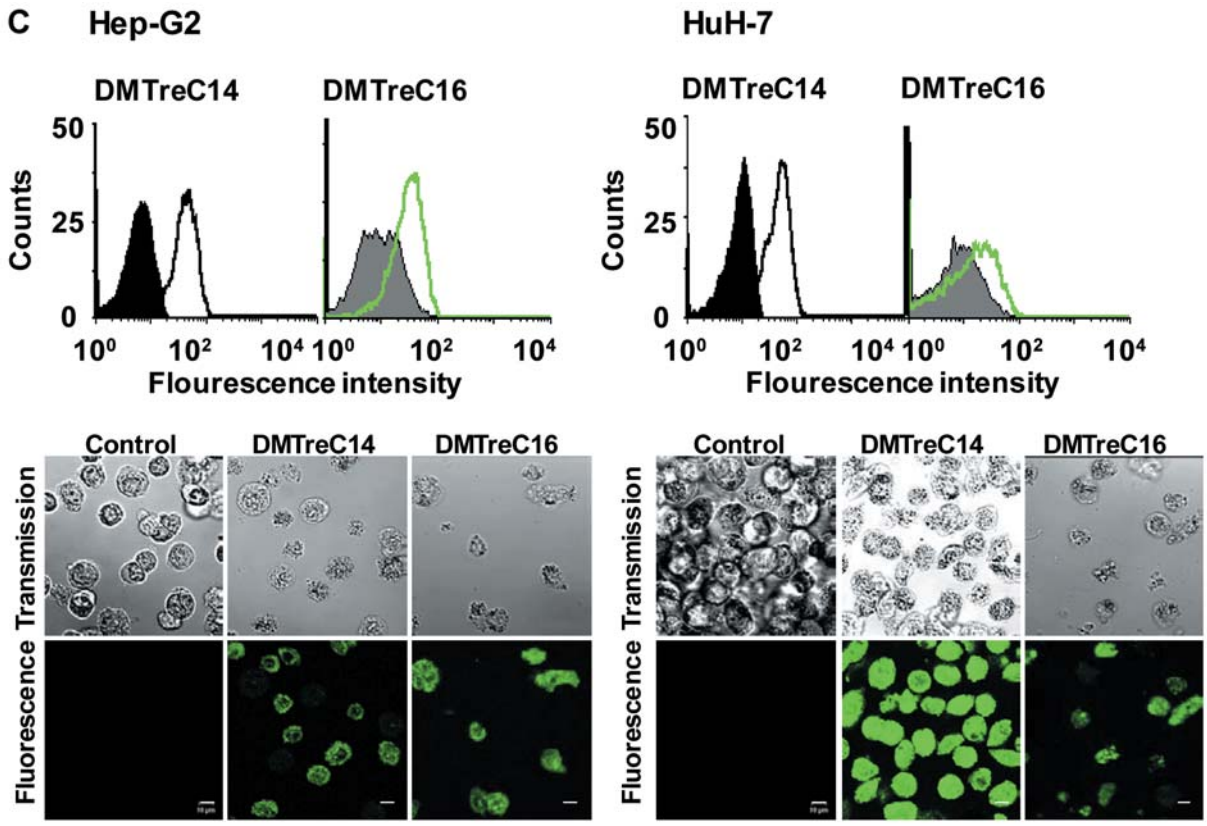

Figure 6. *Activation of caspase-3 (A), caspase-8 (B), and caspase-9 (C) by DMTreCn in Hep-G2 and HuH-7 cells were observed using flow cytometry and confocal microscopy. Scale bar 10 μm. Control (shaded histogram), two-component liposomes composed of DMPC and trehalose surfactant (DMTreCn) (unshaded histogram). L-α-dimyristoylphosphatidylcholine (DMPC)=1.0×10^{-4} M. TreCn =2.3×10^{-4} M.*

growth of tumor cells. The inhibitory effects of two-component liposomes composed of DMPC and DMTreC14 on the growth of human colon carcinoma and gastric carcinoma *in vitro* were reported (29). The thickness of the fixed aqueous layer of DMTreCn was larger than that of DMPC liposomes and increased in a dose-dependent manner. The degree of construction of water molecules in tumor tissue was shown to be less than that in normal tissue using the spin-lattice relaxation time for protons in water molecules, suggesting that the motions of water at the tumor cell surface might be in a more active and disordered state than normal cells (30). Therefore, inhibitory effects of DMTreCn could be related to hydration in tumor cells. Membranes of tumor cells are more fluid than those of normal cells (31, 32). DMTre selectively fused and accumulated into Hep-G2 and HuH-7 cells but not into normal (WI-38) ones, suggesting that DMTreCn uptake differs between carcinoma and normal cell membrane. Correlations between antitumor effects and membrane fluidity of two-component liposomes were reported (14). Inhibitory effects of DMTreC16 on the growth of hepatoma cells were larger than that of DMTreC14 without affecting normal cells. Interestingly, the membrane fluidity of DMTre16 was also larger than that of DMTreC14.

The induction of apoptosis and their pathways of apoptosis by two-component liposomes composed of DMPC and polyoxyethylenedodecyl ether have been clarified for tumor cells (10). That is, the apoptotic signal first passes through the mitochondria, caspase-9 and caspase-3, then through APO-1/CD95 (FAS), caspase-8 and caspase-3 after the fusion and accumulation into tumor cello membrane, and then reaches the nucleous. DMTreCn activated caspases 3, 8, and 9 and the apoptotic signal passed through mitochondria. Mitochondria transmembrane potential of HuH-7 cells decreased after treatment with DMTreC14 and DMTreC16, although that of HepG-2 cells was the same as that for controls. However, the release of cytochrome c of mitochondria was observed for Hep-G2 and HuH-7 cells after treatment with DMTreC14 and DMTreC16. Inhibitory effects of DMTreC14 and DMTreC16 on the growth of Hep-G2 and HuH-7 cells were obtained for the first time without affecting normal cells and apoptotic signal transduction by DMTreC14 and DMTreC16 was clarified.

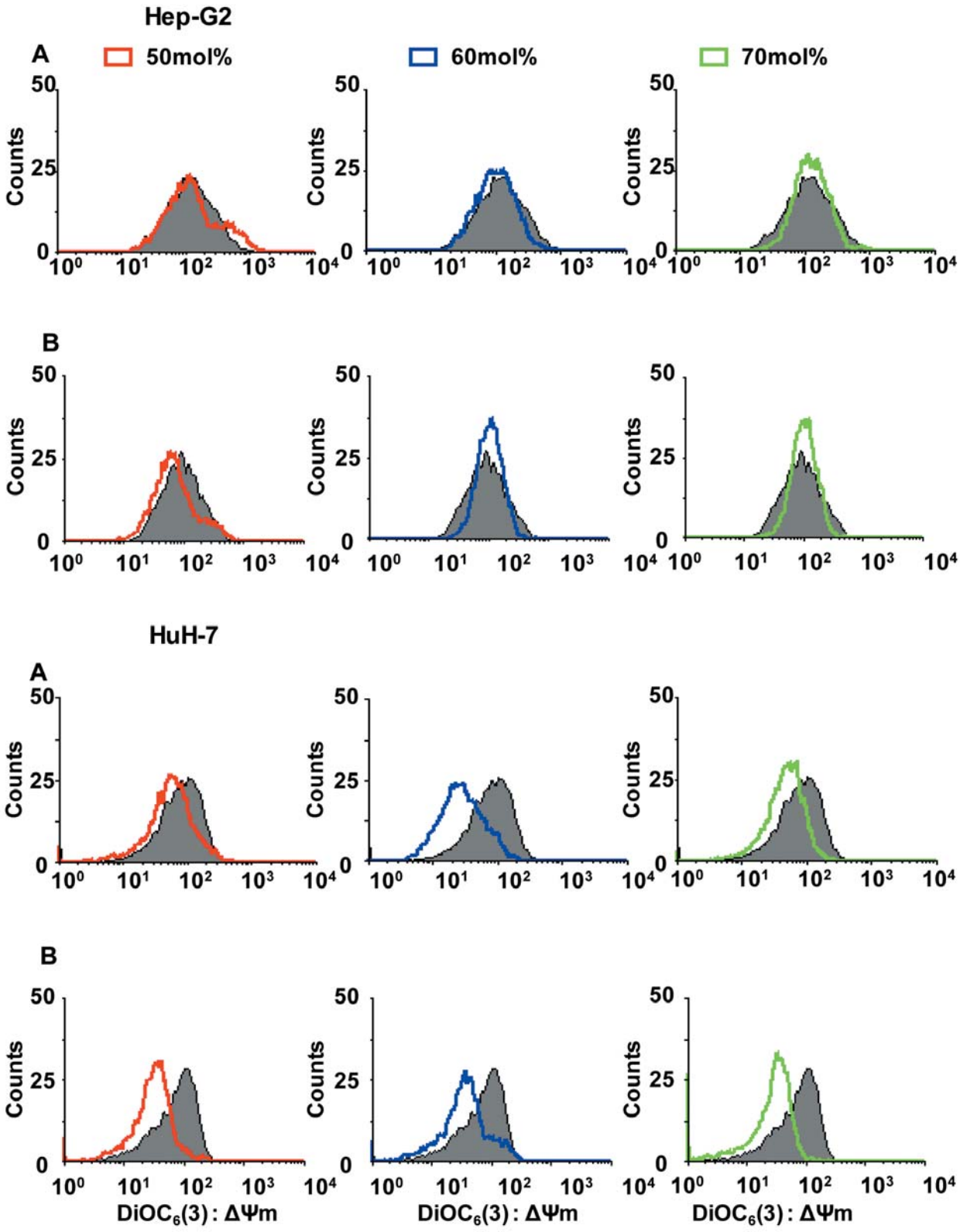

Figure 7. *Mitochondrial transmembrane potential of HuH-7 cells was decreased after treatment with DMTreC14 (A) and DMTreC16 (B), although the one of Hep-G2 cells was the same as the one for the controls. Control (shaded histogram), DMTreCn (unshaded histogram). DMPC=1.0×10^{-4} M. TreCn=2.3×10^{-4} M.*

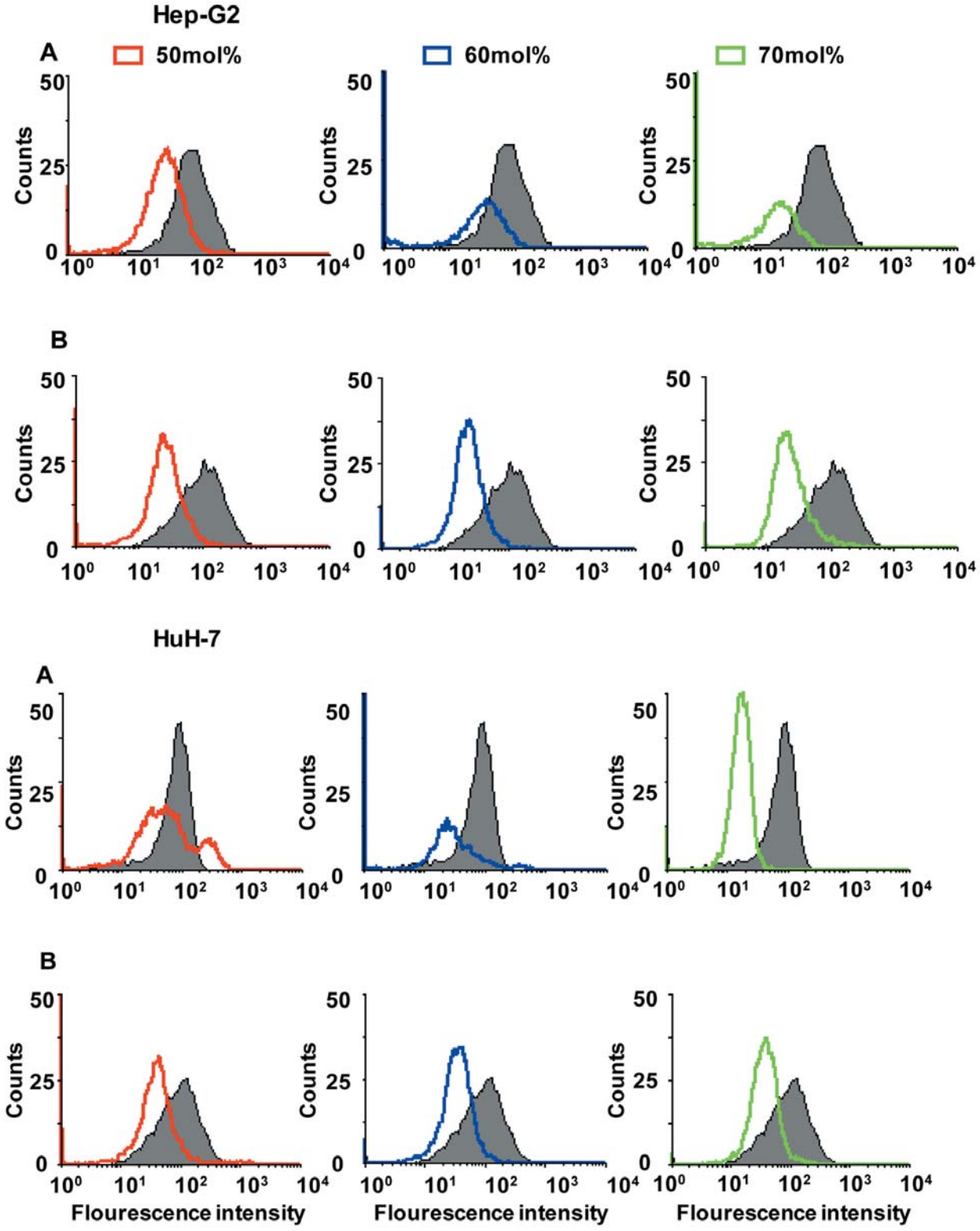

Figure 8. *Decrease in fluorescence intensity for Hep-G2 and HuH-7 cells after the treatment with DMTreC14 (A) and DMTreC16 (B) was observed using antibodies to cytochrome c and IgG FITC. Control (shaded histogram), DMTreCn (unshaded histogram). DMPC=1.0×10^{-4} M. TreCn=2.3×10^{-4} M.*

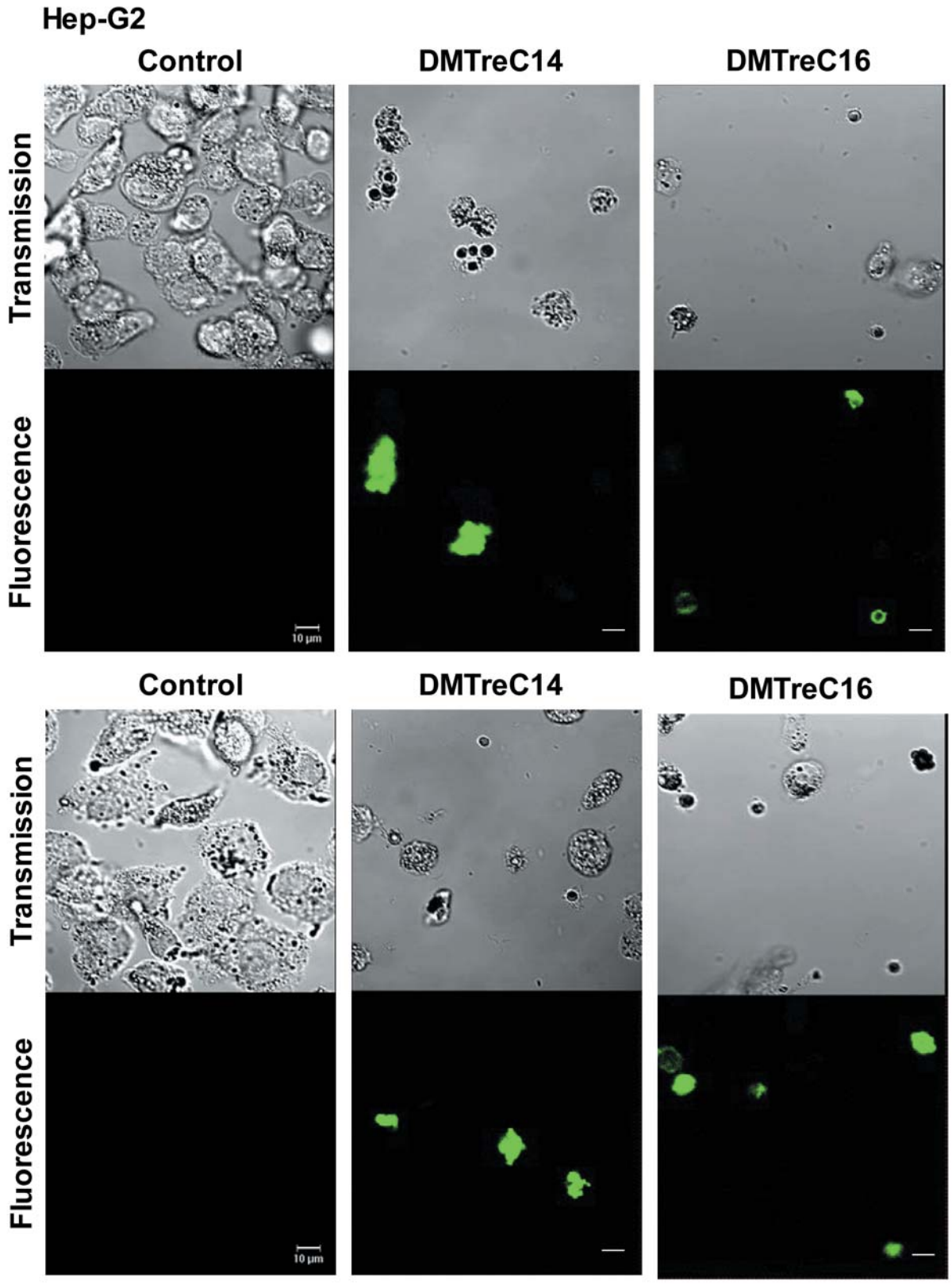

Figure 9. *Activation of Bcl-2 family protein (BAX) by two-component liposomes composed of DMPC and trehalose surfactant (DMTreCn) in Hep-G2 and HuH-7 cells was determined using antibodies against BAX and IgG FITC. DMPC=1.0×10^{-4} M. TreCn=2.3×10^{-4} M.*

In conclusion, novel liposomes composed of DMPC and trehalose surfactants (DMTreC14 and DMTreC16) were produced, and inhibitory effects of DMTreC14 and DMTreC16 on the growth of hepatocarcinoma cells leading to apoptosis without affecting normal cells were obtained for the first time. Specific fusion and accumulation of DMTreC14 and DMTreC16 into hepatocarcinoma cells was observed. DMTreCn induced apoptosis of hepatocarcinoma cells through the activation of caspase-8, -9 and -3 and release of cytochrome *c* from mitochondria *via* BAX. The results in this study indicate that DMTreCn could provide a novel therapy for tumors by targeting the cell membranes, explaining the biophysical characteristics of tumor cells.

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