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Inhibitory Effects of Trehalose Liposomes against Breast Cancer Cells Leading to Apoptosis *in vitro*

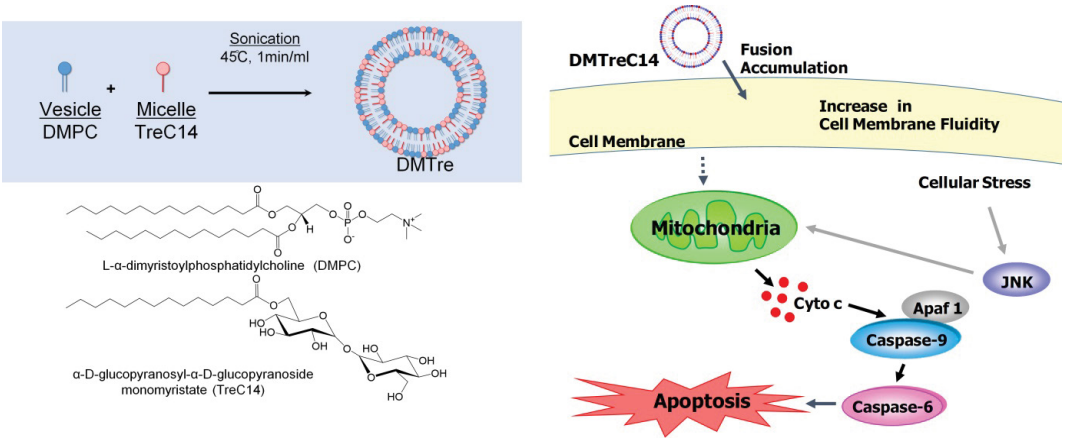
Keiji Kuwabara, Hideaki Ichihara and Yoko Matsumoto*

Division of Applied Life Science, Graduate School of Engineering, Sojo University, 4-22-1 Ikeda, Nishi-ku, Kumamoto 860-0082, Japan

Abstract

In this study, the effect of trehalose liposomes (DMTreC14) constituted using α-D-glycopyranosyl-α-D-glucopyranoside monomyristate (TreC14) and L-α-dimyristoylphosphatidylcholine, on the growth of the human breast cancer cell-line, MCF-7 was examined. It was observed that DMTreC14 had an inhibitory effect on the growth of MCF-7 cells and caused apoptosis. This was attributed to DMTreC14 induced decrease in mitochondrial membrane potential followed by the release of cytochrome-c and activation of caspase-6 and caspase-9 in MCF-7 cells. Furthermore, it was observed that there was an increased accumulation of DMTreC14 in the cell membrane of MCF-7 cells, followed by an increase in the fluidity of the cell membrane. In addition, activation of c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) was also observed upon treatment with DMTreC14.

Graphical Abstract



Keywords: Trehalose liposomes; Nanomedicine; Breast cancer; Apoptosis; Migration

Abbreviation: DMPC: L-α-Dimyristoylphosphatidylcholine; DMTreC14: Trehalose Liposomes Composed of DMPC and TreC14; TreC14: α-D-Glycopyranosyl-α-D-Glucopyranoside Monomyristate

Introduction

Breast cancer is one of main causes of cancer-related death in women in the world. Combination chemotherapy is the most common treatment employed for patients with breast cancer. However, combination chemotherapy treatments have severe side effects due to poor target specificity [1,2].

Sugar plays an important role in cell recognition, cell response to stimulus, and many other biological processes. Trehalose is disaccharide comprising of two α-glucopyranose rings bound by a α, α-1, 1-glycosidic linkage. It is present in diverse organisms including bacteria, yeast, fungi, and insects [3]. It has been reported that trehalose has the capacity to displace water molecules present on liposomes having a high hydration power [4].

Inhibitory effects of trehalose liposomes composed of phosphatidylcholine and trehalose surfactants, on colon cancer, gastric cancer, hepatic cancer, leukemia, and lung carcinoma cells have been reported [5-9]. However, the effect of treatment with trehalose containing liposomes, on the growth of breast cancer cells has not yet been investigated.

In this study, we investigated the effect of DMTreC14 formulated using α-D-glycopyranosyl-α-D-glucopiranoside monomyristate (TreC14) and L-α-dimyristoylphosphatidylcholine (DMPC) on the growth of breast cancer (MCF-7) cells. Furthermore, the pathways underlying DMTreC14 induced MCF-7 cell growth inhibition, were also examined.

***Corresponding author:** Yoko Matsumoto, Division of Applied Life Science, Graduate School of Engineering, Sojo University, 4-22-1 Ikeda, Nishi-ku, Kumamoto 860-0082, Japan, Tel: +81 96 326 3965; E-mail: mailto:matumoto@life.sojo-u.ac.jp

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Materials and Methods

Preparation of DMTreC14

DMTreC14 were prepared using a sonicator (VS-N300; VELVO, Japan) after the mixing DMPC (purity>99%; NOF Co. Ltd., Japan) and TreC14 (Dojindo Ltd., Japan) in 5% glucose solution at 45°C with 300 W, and then filtered using a 0.45 μm filter.

Dynamic light scattering measurements

The diameter (d_{hy}) of DMTre was measured by a light scattering spectrometer (ELSZ-0, Otsuka Electronics, Japan) using He-Ne laser (633 nm) at scattering angle=90°.

Cell culture

Human breast cancer (MCF-7) cell lines were purchased from ATCC (VA, USA). MCF-7 cells were cultured in RPMI-1640 medium (Gibco, MD, USA) including 10% fetal bovine serum (FBS, HyClone Laboratories Inc., UT, USA), penicillin (100 unit/ml) and streptomycin (50 μg/ml) in incubator humidified at atmosphere of 5% CO2 and at 37°C.

Assessment of 50% inhibitory concentration of DMTreC14

The 50% inhibitory concentration (IC_{50}) of DMTreC14 on the growth of MCF-7 cells was determined on the basis of WST-8 [2-methoxy-4-nitrophenyl-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium, monosodium salt] assay (Dojindo Laboratories, Japan). MCF-7 cells (8.0 × 104 cells/ml) were seeded in 96-well plates and cultured in a 5% CO_2 humidified incubator at 37°C for 24 h. Cells were cultured for a further 48 h after adding DMTreC14 (0.1-1 mM on the basis of DMPC concentration). WST-8 solution was added and the cells were incubated for 3 h. The absorbance at a wavelength of 450 nm was measured by spectrophotometer (Emax; Molecular Devices Co., CA, USA). The inhibitory effects of DMTreC14 on the growth of MCF-7 cells were evaluated by Amean/Acontrol, where Amean and Acontrol denote the absorbance of water-soluble formazan, in the presence and absence of DMTreC14, respectively.

Analysis of apoptosis by DMTreC14 using flow cytometer

MCF-7 cells were seeded at a density of 8.0×10^4 cells incubated in an incubator supplying 5% CO_2 at 37°C for 24 hours. DMTreC14 was added to the cells and the dishes were incubated for 48 h. Cells were centrifuged and suspended in PBS (-) containing 1 mg/ml RNase, 0.1% Triton X-100 and 40 μg/ml propidium iodide (PI, Molecular Probes, Eugene, OR, USA) in a dark box. The percentage of apoptotic cells was analyzed using a flow cytometer (Epics XL system II, Beckman Coulter, Brea, CA, USA).

Assessment of apoptotic induction using terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) method

Estimation of apoptotic was performed using the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) method. Towards this, an In Situ Cell Death Detection Kit (Roche Diagnostics K.K., Japan) was used. DMTreC14 ([DMPC]=100 μM, TreC14]=233 μM) were added to a suspension of MCF-7 cells (8.0×10^4 cells/ml); the cells were then incubated for 48 h. Cells were fixed using 4% paraformaldehyde, and then processed for TUNEL assay as per the the manufacturer's instructions. Imaging was performed on a confocal laser microscope (TCS-SP; Leica Microsystems, Germany) having a 488 nm Argon laser line (detection at 515-565 nm).

Measurements of caspase activity with flow cytometer

MCF-7 (8.0×10^4) cells treated with DMTreC14 ([DMPC]=100 μM, [TreC14] = 233 μM) were incubated with the substrate solutions for the identification of various caspases (PhiPhiLux-G1D2, CaspaLux 6-J1D2, CaspaLux 8-L1D2, CaspaLux 9-M1D2 for caspase-3, caspase-6, caspase-8, and caspase-9, respectively; OncoImmunin Inc., Gaithersburg, MD, USA) for 60 min at 37°C. Caspase activity was measured using a flow cytometer.

Mitochondrial membrane potential

DMTreC14 treated MCF-7 cells were incubated at 37°C for 30 min with 40 nM of 3, 3-dihexyloxacarbocyanine iodide [$DiOC_6(3)$] (Molecular Probes, OR, USA) in order to evaluate the mitochondrial transmembrane potential ($\Delta\psi m$). These cells were then centrifuged, suspended in 500 μL of PBS and were used for flow cytometric analysis.

Assessment of release of cytochrome-c by flow cytometer

Cytochrome-c from mitochondria was measured using the FlowCellect Cytochrome-c Kit (EMD Millipore Co., MA, USA). MCF-7 cells (8.0×10^4) were treated with DMTreC14 ([DMPC]=100 μM, [TreC14]=233 μM). Twenty-four hours following incubation with DMTreC14, the cells were stained using an anti-cytochrome-c FITC antibody. Caspase activity was determined and analyzed using a flow cytometer.

Assessment of expression values of p-JNK by flow cytometer

MCF-7 cells were seeded at a concentration of 8.0×10^4 and incubated for 24 hours. These cells were then treated with DMTreC14 (100 μM) for 24 hours, following which, the cells were fixed using 10% formaldehyde for 60 min. These fixed cells were treated with a mixture of 95% methanol and 2.5% acetic acid at -20°C for 20 minutes. These cells then were stained using either the p-JNK mouse monoclonal antibody Alexa Fluor 488 conjugate (Santa Cruz Biotechnology, Inc., Cambridge, UK) (5 μg/ml) or the normal mouse IgG antibody Alexa Fluor 488 conjugate (Santa Cruz Biotechnology, Inc., Cambridge, UK) as an isotype control (5 μg/ml) at 4°C for 1 h. p-JNK (G-7) is a mouse monoclonal antibody against a sequence containing phosphorylated JNK of human origin. Staining was then measured on a flow cytometer.

Fusion and accumulation of DMTreC14 into the cell membrane

The fusion and accumulation of DMTreC14 along with 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, Alabama, USA), into the cell membranes of MCF-7 cells was tracked using confocal laser microscopy (TCS-SP; Leica Microsystems, Germany). MCF-7 cells seeded at a concentration of 2.0×10^5 cells/ml were incubated at 37°C in a humidified incubator provided with 5% CO_2 for 24 h. The cells were treated with DMTreC14/NBDPC ([DMPC]=0.087 mM, [TreC14]=0.233 mM, [NBDPC]=0.013 μM) for 0.5-60 minutes. Images were acquired using a confocal laser microscope ((488 nm Argon laser line (detection at 505-555 nm)) and a flow cytometer ((15 mW 488 nm air-cooling Argon laser and FL1 sensor (505-545 nm)).

Measurement of cellular membrane fluidity

Membrane fluidity measurements were made using the fluorescence depolarization method on a spectrophotofluorometer (F-7100; Hitachi, Japan). For the fluorescence polarization studies, MCF-7 cells were labeled using DPH (1, 6-diphenyl-1, 3, 5-hexatriene, Nacalai

Tesque, Japan). The fluorescence depolarization (P) value of DPH was measured in cells treated with DMTreC14.

Wound healing assay

MCF-7 cells were seeded at a concentration of 3.5×10^5 cells/ml and were cultured in medium containing10% FBS. A wound was created on the monolayers of MCF-7 cells using a sterile pipette tip. These wounded MCF-7 cell monolayers were treated with two concentrations of DMTreC14 i.e., 20 μM and 40 μM. The wounded monolayers were photographed using a light microscope (EVOS fl, Life Technologies, CA, USA) following 24 h incubation (within the doubling time (29 h) of MCF-7 cells as per the ATCC protocol). The areas of migration were measured using ImageJ (Version 1.46 r, National Institutes of Health, MD, USA).

Statistical analysis

Data are given as mean ± S.E. Statistical comparisons were made using Student's t-test. $P<0.05$ was considered to represent a statistically significant difference.

Results and Discussion

Physical properties of DMTreC14

We examined the morphology of DMPC and TreC14 containing DMTreC14 using dynamic light scattering measurements. DMTreC14 having a hydrodynamic diameter <100 nm were preserved for a duration of 4 weeks. In contrast, liposomes containing DMPC were unstable and precipitated 14 days from the day of preparation.

Growth suppression leading to induction of apoptosis in MCF-7 cells by DMTreC14

We investigated the effect of DMTreC14 treatment on the growth of MCF-7 cells using the WST-8 cell proliferation assay. It was observed that DMTreC14 suppressed the growth of MCF-7 cells in a dose dependent manner, and exhibited an IC_{50} (50% inhibitory concentration) of 50 μM in MCF-7 cells (Figure 1A). The apoptotic DNA rate of DMTreC14 treated MCF-7 cells was evaluated using flow cytometer. The apoptotic DNA rate increased after treatment with DMTreC14 in a dose dependent manner. The apoptotic DNA rate reached a high apoptotic rate (90%) (Figure 1B). The induction of apoptosis in DMTreC14 treated MCF-7 cells was investigated using a fluorochrome-based TUNEL assay. DMTreC14 treated MCF-7 cells fluoresced green, indicating an accumulation of double stranded DNA breaks, and by extension, induction of apoptosis following DMTreC14 addition; control and DMPC liposomes did not exhibit green fluorescence (Figure 1C). These results indicate that as DMTreC14 induces apoptosis in MCF-7 cells, it can also suppress the proliferation of MCF-7 cells.

DMTreC14 induces activation of the apoptotic pathway in MCF-7 cells

We investigated the effect of DMTreC14 on the apoptotic pathways in MCF-7 cells. The activation of caspases-3, -6, -8, and -9 was investigated in DMTreC14 treated MCF-7 cells. Results revealed increased activation of caspase-6 and caspase-9 (Figure 2A). Activation of caspase-3 was not observed as MCF-7 cells do not express caspase-3. Therefore, it was concluded that DMTreC14 induced apoptosis in MCF-7 cells occurs via the activation of caspases-9 and -6. Next, we examined the DMTreC14 induced mitochondrial events associated with apoptosis. Towards this, mitochondrial transmembrane potential of DMTreC14 treated MCF-7 cells was analyzed using DiOC6(3) (3, 3'-Dihexyloxacarbocyanine Iodide), a lipophilic dye exhibiting a selectivity towards live mitochondrial cells. Following the addition of DMTreC14, a decrease in the mitochondrial transmembrane potential of MCF-7 cells was observed (Figure 2B). Release of cytochrome-c from the mitochondrion is a key step in apoptosis. We examined the effect of DMTreC14 treatment on the cytochrome-c release from the mitochondria by staining with FITC anti-Cytochrome-c antibody using flow cytometry. It was observed that DMTreC14 treated MCF-7 cells exhibited cytochrome-c release from the mitochondria (Figure 2C). This

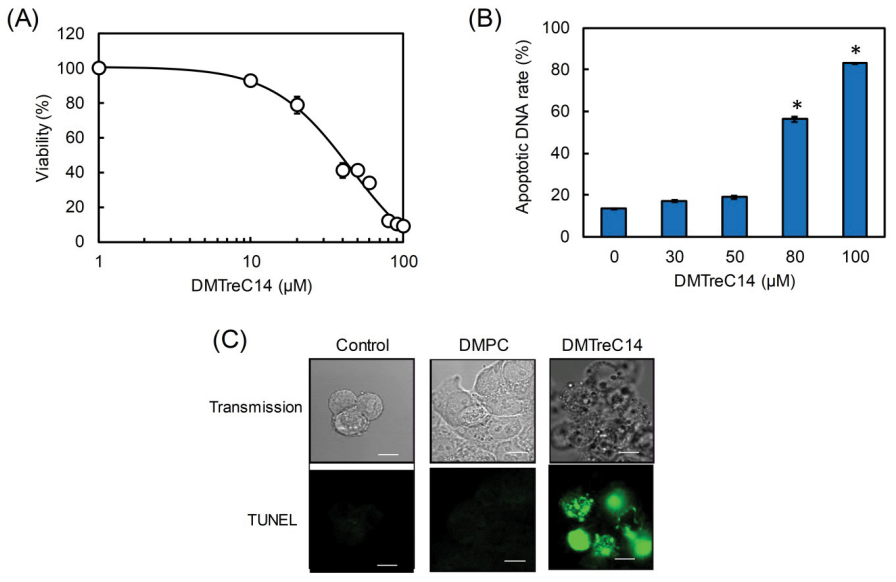

Figure 1: Growth suppression of DMTreC14 leading to apoptosis for MCF-7 cells. (A) Viability of MCF-7 cells treated with DMTreC14. Data represent the mean ± S.E (B) Apoptotic DNA rate of MCF-7 cells treated with DMTreC14. Data represent the mean ± S.E. [DMPC]=30-100 μM, [TreC14]=69.9-233 μM. *p<0.05 (vs. Control) (C) Fluorescence micrographs of MCF-7 cells treated with DMTreC14 using the TUNEL method. [DMPC]=100 μM, [TreC14]=233 μM. Scale bars: 10 μm.

release of cytochrome-c was attributed to DMTreC14 induced decrease in the mitochondrial transmembrane potential. Furthermore, in order to examine the involvement of cellular and endoplasmic reticulum stress in DMTreC14 induced apoptosis of MCF-7 cells, we analyzed the activation of the stress-activated mitogen-activated protein kinase, JNK/SAPK by flow cytometry. It was observed that JNK activation had increased remarkably in MCF-7 cells treated with DMTreC14 (Figure 2D). These results indicate that DMTreC14 induced apoptosis of MCF-7 cells occurs due to the convergence of three different mechanisms: (1) activation of caspases-6 and -9, (2) triggering of mitochondrial events, and (3) activation of JNK by cellular stress.

Effect of DMTreC14 treatment on the cell membrane during apoptosis of MCF-7 cells

We investigated the fusion and accumulation of DMTreC14 in the cell membrane of MCF-7 cells using flow cytometer based tracking of fluorescent lipid labelled DMTreC14 (DMTreC14/NBDPC). Time-dependent increase in the fluorescence of DMTreC14/NBDPC the plasma membrane of MCF-7 cells was observed; this increase in the fluorescence of DMTreC14/NBDPC was greater than that was observed using DMPC liposomes, suggesting that DMTreC14 gets accumulated in the plasma membrane of the treated MCF-7 cells (Figure 3A). We also examined the fusion and accumulation of DMTreC14 in the cell membrane of MCF-7 cells using confocal microscopy. Similar to the results from the flow cytometry based assay, an increase in the accumulation of DMTreC14 (DMTreC14/NBDPC) was observed using confocal microscopy, though no accumulation of DMPC was detected (Figure 3B). Furthermore, using the fluorescence polarization (P) analysis, we examined the membrane fluidity of MCF-7 cells was examined. It was observed that the P value of DMTreC14 treated MCF-7 cells had decreased, indicative of an increase in the membrane fluidity of these MCF-7 cells (Figure 3C). Increase in membrane fluidity has been reported in apoptosis and some cancers such as leukemias, lymphomas, and some neural tumors [10,11]. These results suggest that the mechanism of action of DMTreC14 involves fusion with the cell membrane followed by enhancement of the membrane fluidity.

Inhibition for migration of MCF-7 cells by DMTreC14

We investigated the effects of DMTreC14 on the invasiveness of MCF-7 cells, using a wound healing assay.. Towards this, the effect of DMTreC14 at two different concentrations (20 μM and 40 μM) was investigated. It was observed that as compared to the control and DMPC, DMTreC14 treatment inhibited the migration of MCF-7 cells to a greater extent. This inhibition of the MCF-7 cell migration by DMTreC14 occurred in dose-dependent manner (Figure 4A). DMTreC14 was observed to inhibit the migration of MCF-7 cells to a greater extent as compared to the control (Figure 4B). The inhibition of cell migration by DMTreC14 suggests a possible anti-invasive effect of DMTreC14 in MCF-7 cells.

Conclusion

We have established for the first time that liposomes composed of trehalose (DMTreC14) have an inhibitory effect on the growth of human breast cancer (MCF-7) cells. This inhibition in growth was found to be due to DMTreC14 induced apoptosis following the fusion and accumulation of DMTreC14 into the cell membrane of MCF-7 cells, leading to increased membrane fluidity. With regard to the molecular mechanisms of DMTreC14 induced apoptosis in MCF-7 cells, it was identified that three phenomenon play a key role in this process: (1) DMTreC14 induced activation of caspase-6 and caspase-9, (2) DMTreC14 induced reduction of the mitochondrial membrane potential and subsequent release of cytochrome-c, and (3) DMTreC14 induced activation of the stress-activated protein kinase, JNK. Furthermore, it was also identified that DMTreC14 exerted anti-

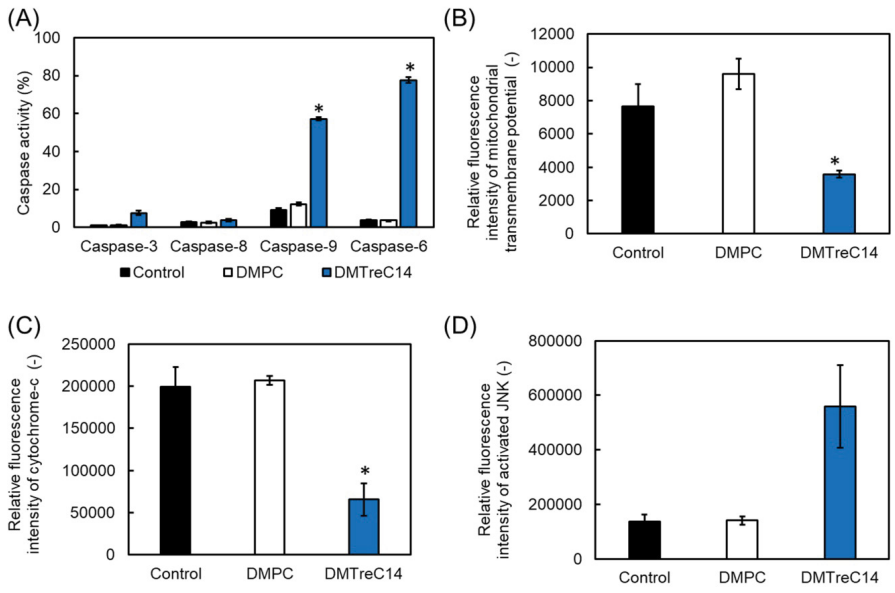

Figure 2: (A) Activation of caspase--6, and -9 in MCF-7 cells treated with DMTreC14 using fluorogenic apoptosis assay substrates by a flow cytometer. Data represent the mean ± S.E. [DMPC]=100 μM, [TreC14]=233 μM. *p<0.05 (vs. Control and DMPC) (B) Decrease in mitochondrial transmembrane potential of MCF-7 cells treated with DMTreC14. Data represent the mean ± S.E. [DMPC]=100 μM, [TreC14]=233 μM. *p < 0.05 (vs. Control and DMPC) (C) Release of cytochrome c from mitochondria of MCF-7 cells treated with DMTreC14. Data represent the mean ± S.E. [DMPC]=100 μM, [TreC14]=233 μM. *p<0.05 (vs. Control and DMPC) (D) Activation of JNK in MCF-7 cells treated with DMTreC14 of MCF-7 cells treated with DMTreC14. Data represent the mean ± S.E. [DMPC]=100 μM, [TreC14]=233 μM.

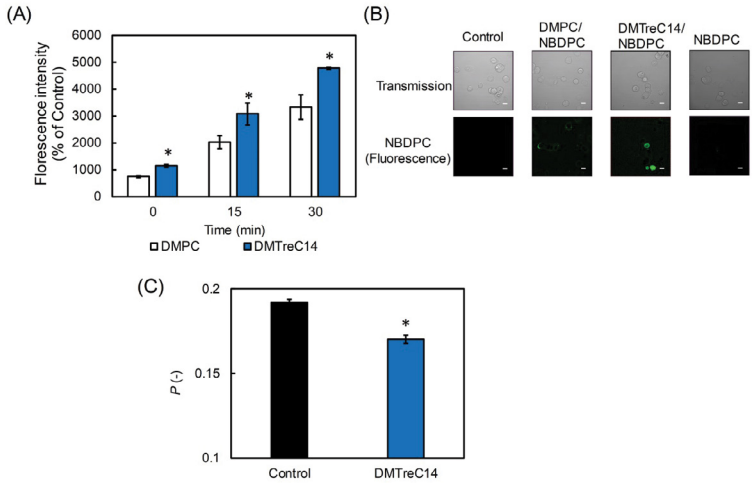

Figure 3: (A) Accumulation of DMTreC14 (DMTre/NBDPC) including fluorescence probe in MCF-7 cells using flow cytometer. Data represent the mean ± S.E. [DMPC]=100 μM, [TreC14]=233 μM. ,p<0.05 (vs. DMPC) (B) Fluorescence micrographs of accumulation of DMTreC14 (DMTre/NBDPC) including fluorescence probe in MCF-7 cells using a confocal laser microscope. Reaction time: 1 h. [DMPC]=100 μM, [TreC14]=233 μM (C) Increase in membrane fluidity of membrane of MCF-7 cells treated with DMTreC14. Data represent the mean ± S.E. [DMPC]=30 μM, [TreC14]=69.9 μM. ˚p<0.05 (vs. DMPC).

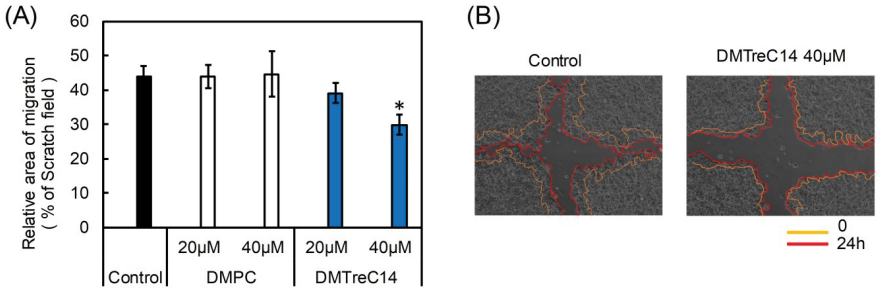

Figure 4: (A) Inhibition on relative area of the migration of MCF-7 cells by DMTreC14 for 24 h on the basis of scratch wound method (B) Inhibition on the migration of MCF-7 cells by DMTreC14 on the basis of scratch wound method. Yellow lines indicate initial wound area (0); red lines indicate migrating cells (24 h).

migration effects on MCF-7 cells. It is noteworthy that DMTreC14 has a remarkably high inhibitory effect on the growth of MCF-7 cells.

Acknowledgement

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Conflict of Interest

The authors declare that there is no conflict of interest.

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