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Original Article

Titanium dioxide and silica nanoparticles inhibit the efflux of adriamycin in multi-drug resistant L1210/ADM cells

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ABSTRACT — Various nanomaterials are used as food additives, medicines, and cosmetics; however, their biological effects are not completely understood. Since the cell membrane is the first point of contact between nanomaterials and cells, we investigated whether amorphous silica particles and titanium dioxide nanoparticles ($nTiO_2$) interact with the transporters present in the cell membrane. In this study, we prepared an adriamycin (ADM) resistant variant of the mouse lymphocytic leukemia cell line L1210 (L1210/ADM). Since our L1210/ADM cells were confirmed to excrete ADM in a P-glycoprotein (P-gp)-dependent manner, we investigated whether nano- and micro-sized amorphous silica particles (nSP and mSP) and $nTiO_2$ inhibited ADM excretion from L1210/ADM cells. Both nSP and $nTiO_2$ inhibited ADM efflux in a dose-dependent manner; however, mSP did not inhibit ADM efflux even at the highest dose (0.25 mg/mL). These results suggest that nSP and $nTiO_2$ interfere with P-gp, which is involved in ADM transport, and L1210/ADM cells are suitable for safety screening tests of nanomaterials.

Key words: Nanomaterials, Silica, Titanium dioxide, *Abcb1a*, P-glycoprotein

INTRODUCTION

With recent advances in science and technology, it has become possible to control and process the structure of materials at the nanometer level, and this has come to be known as "nanotechnology." Products produced using nanotechnology are collectively called "nanomaterials" and are ultra-fine substances with one of the three dimensions of 1 to 100 nm (ASTEM E2456-06, 2006). Nanomaterials have a high surface-to-volume ratio and surface reactivity (Oberdörster, 2010; Napierska *et al.*, 2010). Therefore, various nanomaterials are expected to exhibit different physicochemical properties owing to their quantum size effects. Nanomaterials are widely used as additives in medicines, cosmetics, and food. The exponentially increasing number of potential nanomaterials and possible differences in properties between the same types of nanomaterials make the use of slow and expensive *in vivo* toxicity testing impractical (Petersen *et al.*, 2022; Nel *et al.*, 2013a, 2013b; Shatkin *et al.*, 2016).

Numerous *in vitro* and *in vivo* studies have consistently demonstrated that nanoparticles induce intracellular reactive oxygen species generation, resulting in oxidative stress in cells and leading to any of the apoptosis, necrosis, and autophagy-mediated cell death mechanisms (Fu *et al.*, 2014; Khanna *et al.*, 2015).

If a nanomaterial is biologically toxic, it is necessary to clarify whether it acts on the cell surface or after being

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taken into the cell. Nanomaterials come in contact with cells through the cell membrane, where protein molecules, such as receptors and transporters, are expressed. We conducted hypothesis-oriented research to determine whether nanomaterials affect the molecules involved in substance transport in cell membranes (channels, transporters, etc.). This study investigated whether silica and titanium dioxide (TiO_2) nanomaterials interact with P-glycoprotein (P-gp) transporters present in the exposure route.

MATERIALS AND METHODS

Silica and TiO_2 particles

Several kinds of amorphous silica particle, Sicastar® were obtained from Micromod Partikeltechnologie GmbH (Rostock, Germany). NEO-STEM™ TSR50 was purchased from BITERIALS Co. Ltd. (Seoul, Korea). TiO_2 particles, MT-150 AW and MT-700B were obtained from TAYCA CORPORATION (Osaka, Japan). Each particle was suspended in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS(-)) to 100 mg/mL just before the experiment, and it was thoroughly stirred with a vortex mixer and diluted to a predetermined concentration. The silica and TiO_2 particles used in this study are listed in Table 1.

Cell culture

The mouse lymphocytic leukemia cell line L1210 was obtained from Riken Cell Bank (Tsukuba, Japan). L1210 cells were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 5% fetal bovine serum (Gibco, Grand Island, NY, USA), 100 U/mL penicillin - 0.1 mg/mL streptomycin (Sigma-Aldrich) at 37ºC in a humidified incubator containing 5% CO_2. Adriamycin (ADM) resistant variant of L1210 (L1210/ADM) was obtained in our laboratory by culturing these cells in gradually increasing doses of ADM up to 1.0 µmol/L after 8 months. L1210/ADM was maintained in the above culture medium containing 0.5-1.0 µmol/L ADM and incubated by the same above conditions.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from the L1210 and L1210/ADM cells using ISOGEN II (Nippon Gene, Tokyo, Japan). cDNA was synthesized from the total RNA samples using the Reverse Transcriptase M-MLV (TaKaRa Bio, Shiga, Japan) and oligo(dT) primers. PCR was performed using Emerald Amp PCR Master Mix (Takara) with the gene-specific primers under the following conditions: 30 cycles of 98ºC for 10 sec, 60ºC for 30 sec, and 72ºC for 30 sec. The gene specific primer sequences were as follows: forward, 5′-CAGTGGAACAGCGGTTTC-CAGGAGC-3′ and reverse, 5′- GGGTGTTGAGCTC-CCCAACGTC-3′ for the mouse *Abcb1a*; forward, 5′-TTCCCGCAGTGGCTCTTGAAGC-3′ and reverse, 5′-CACACCAGCACCAATCCCGGT-3′ for the mouse *Abcb1b*; forward, 5′-ACCACAGTCCATGCCATCAC-3′ and reverse, 5′-TCCACCACCCTGTTGCTGTA-3′ for the mouse *Gapdh*.

Western blot analysis

Protein samples from the cells were extracted in radioimmunoprecipitation assay (RIPA) buffer containing a Protease Inhibitor Cocktail (Sigma-Aldrich), followed by SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred onto a PVDF membrane and probed with antibodies against P-glycoprotein (1:500) ([C219], GTX23364, GeneTex, Irvine, CA, USA) and actin (1:1000) (Sigma-Aldrich). The signals were detected using an ECL plus (GE Healthcare, Uppsala, Sweden) and ImageQuant™ 400 imaging system (GE Healthcare).

Table 1. Silica and TiO_2 particles used in this study.

Name	Mean primarydiameter	Supplier	Surface modification	Abbreviation
[Sillica]				
sicastar® plain	70 nm	micromod	none	nSP70
	300 nm	micromod	none	nSP300
	1 µm	micromod	none	mSP1
	3 µm	micromod	none	mSP3
sicastar® COOH	70 nm	micromod	-COOH	nSP70-C
sicastar® NH_2	300 nm	micromod	-NH_2	nSP70-N
NEO-STEM™ TSR50	50 nm	BITERIALS	none	nSP50-RTIC
[TiO_2]				
MT-150AW	15 nm	TAYCA	none	-
MT-700B	80 nm	TAYCA	none	-

ADM efflux assay

L1210 and L1210/ADM cells were seeded into 24-well plates at a density of 1×10^5 cells/mL. After 21 hr of culture, 5 µmol/L of ADM hydrochloride (Sigma-Aldrich) was added to each well. In addition, 100 µmol/L verapamil hydrochloride (Sigma-Aldrich) was used as an inhibitor of P-gp. The cells were collected and washed once with ice-cold PBS(-). The residual ADM in the cells was extracted with 0.3 mol/L HCl and 1% SDS in 50% ethanol and the ADM concentration was determined by spectrofluorometer (Ex = 485 nm, Em = 590 nm) (GEMINI EM, Molecular Devices, San Jose, CA, USA).

Cytotoxicity test

The L1210/ADM cells were seeded into 24 well plates at a density of 1×10^5 cells/mL and treated with the test particles for 24 hr. To avoid misinterpretation and inappropriate results due to interference between the chemical reagents used in the cytotoxicity assay kit and the particles (Hartung and Sabbioni, 2011), live and dead cells were counted using the trypan blue exclusion method. Briefly, one part of the cell suspension was mixed with a trypan blue solution (1:1, v/v), and cell viability was evaluated by light microscopy (Maser *et al.*, 2015).

ADM adsorption assay

Test particles (Silica or $nTiO_2$) and 5 µmol/L ADM were added to PBS(-) and incubated for 3 hr at 37ºC. The samples were centrifuged for 40 min at 14,000 × g, and the ADM concentrations in the supernatants were determined using a spectrofluorometer (Ex = 485 nm, Em = 590 nm).

ADM efflux inhibition assay

L1210 and L1210/ADM cells were seeded into 24-well plates at a density of 1×10^5 cells/mL. After 24 hr of culturing, the cells were treated with silica or TiO_2 particles for 24 hr. In the last 3 hr of treatment, 5 µmol/L of ADM was added to each well. Then, the cells were collected and washed once with ice-cold PBS(-). The residual ADM in the cells was extracted with 0.3 mol/L HCl and 1% SDS in 50% ethanol and the ADM concentration was determined using a spectrofluorometer (Ex = 485 nm, Em = 590 nm).

Immunocytochemistry

L1210 and L1210/ADM cells were seeded into 96-well plates at a density of 1×10^5 cells/mL. After 24 hr of culture, cells were treated with nSP50-RITC for 24 hr. The cells were fixed with methanol and labelled with a P glycoprotein antibody (1:500) (ab3366, Abcam, Cambridge, UK) and an Alexa Fluor™ 488 conjugated goat anti-rabbit IgG secondary antibody (1:1000) (Invitrogen, San Diego, CA, USA). Microscopic observations were performed using a laser confocal microscope (FV1000D, Olympus, Tokyo, Japan).

Statistical analysis

In the ADM efflux assay, statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test ($p < 0.05$ and 0.01, respectively).

In the cytotoxicity and ADM efflux inhibition assays, statistical analyses were performed using one-way ANOVA followed by Dunnett's multiple comparison test ($p < 0.05$ and 0.01, respectively).

RESULTS AND DISCUSSION

RT-PCR revealed that *Abcb1b* mRNA was expressed in both L1210 and L1210/ADM cells, whereas *Abcb1a* mRNA was expressed only in L1210/ADM cells (Fig. 1A). Western blot analysis revealed that signals putatively derived from *Abcb1a* were detected between 140 and 200 kDa (Fig. 1B). Signals detected between 140 and 200 kDa were considered P-gp. This relatively large molecular weight variance reflects that during the maturation process, P-gp may exist in cells in various molecular forms (Bohácová *et al.*, 2006). In the ADM efflux assay, when L1210/ADM cells were treated with 5 µmol/L ADM for 3 hr, the amount of residual intracellular ADM was significantly lower than that of L1210 cells, and significantly higher after treatment with 100 µmol/L verapamil (Fig. 2). No difference was observed in the amount of residual intracellular ADM between L1210 and L1210/ADM cells when treated with 100 µmol/L verapamil. These results suggest that L1210/ADM cells express *Abcb1a* mRNA and its gene product P-gp and have a higher ability to excrete ADM than L1210 cells.

Bohácová *et al.*, who generated ADR-resistant L1210 cells using a method similar to ours, suggested that L1210 cells under the selection pressure of ADM expressed common mechanisms of multi-drug resistance based predominantly on P-gp overexpression (Bohácová *et al.*, 2006).

To determine the cytotoxicity of the silica and TiO_2 particles, L1210/ADM cells were treated with various concentrations of silica or TiO_2 particles for 24 hr. None of the tested particles affected the viability of L1210/ADM cells (Fig. 3). In the ADM adsorption assay, the silica and TiO_2 particles without surface modification did

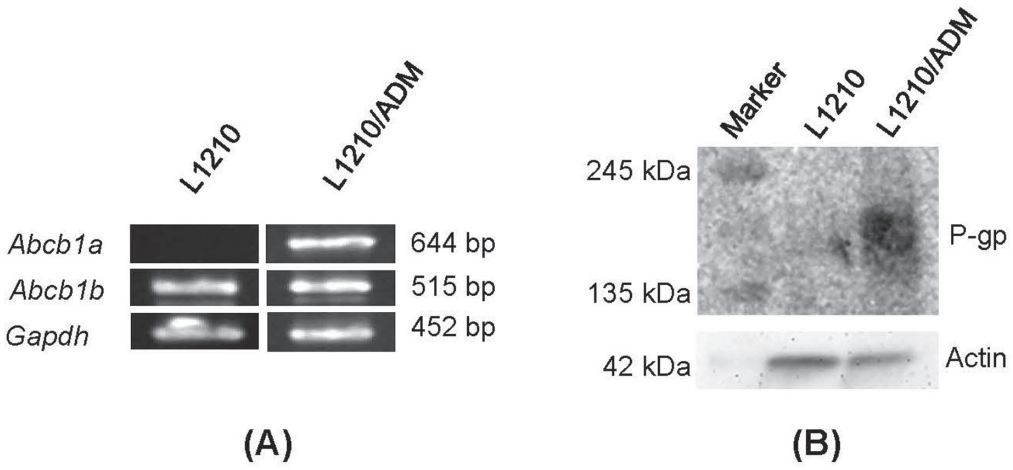

Fig. 1. Characteristics of L1210/ADM cells. (A) RT-PCR analysis of *Abcb1a*, *Abcb1b*, and *Gapdh* mRNA expression in L1210 and L1210/ADM cells. Total RNA samples were extracted from L1210 and L1210/ADM cells, followed by RT-PCR analysis for *Abcb1a*, *Abcb1b*, and *Gapdh* mRNA. (B) Western blot analysis of P-gp and Actin expression in L1210 and L1210/ADM cells. Protein samples were prepared from L1210 cells and L1210/ADM cells and subjected to western blotting to detect P-gp and actin protein.

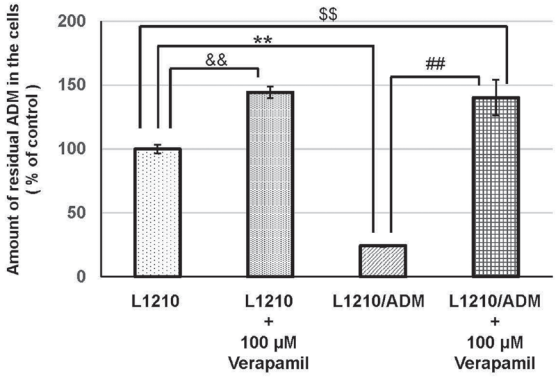

Fig. 2. Difference in ADM efflux ability between L1210 and L1210/ADM cells. L1210 and L1210/ADM cells were seeded into 24-well plates at a density of 1×10^5 cells/mL. After 21 hr culture, 5 μmol/L of ADM was added to each well. In addition, 100 μmol/L verapamil was used as an inhibitor of P-gp. The residual ADM in the cells was extracted with 0.3 mol/L HCl and 1% SDS in 50% ethanol and the ADM concentration was determined by spectrofluorometer (Ex = 485 nm, Em = 590 nm) Data were normalized to the protein concentration of each sample. Data are shown as the mean ± S.D.**Significantly different from the L1210 group, $p < 0.01$.##Significantly different from the L1210/ADM group, $p < 0.01$.$^{\$\$}$Significantly different from the L1210 group, $p < 0.01$.

Table 2. ADM concentration in the supernatant after co-incubation of ADM with particles.

Particles	Concentration (μg/mL)		
	25	75	250
nSP70	100.0 ± 1.9	96.1 ± 1.9	104.3 ± 5.3
nSP300	95.9 ± 3.3	96.9 ± 2.9	99.5 ± 4.4
mSP1	103.1 ± 2.5	104.0 ± 5.9	102.1 ± 3.8
mSP3	104.0 ± 6.8	102.9 ± 9.1	103.3 ± 4.9
MT-150AW	101.8 ± 7.3	107.5 ± 13.9	99.2 ± 8.7
MT-700B	102.8 ± 6.5	108.5 ± 18.3	97.3 ± 6.8

not absorb ADM in PBS(-) (Table 2). The cytotoxicity test and ADM adsorption results exclude the possibility of ADM accumulation due to intracellular ATP depletion and ADM adsorption.

In the ADM efflux inhibition assay, nSP70, nSP300, mSP1, and mSP3 did not affect the ADM efflux from L1210 cells (Fig. S1). The amount of ADM remaining in the L1210/ADM cells increased with increasing doses of nSP70 and nSP300 (Fig. 4A). Furthermore, surface-modified nSP70-C and nSP70-N enhanced the inhibition of ADM efflux from L1210/ADM cells. In contrast, mSP1 and mSP3 did not affect the ADM efflux from L1210/ADM cells at concentrations up to 250 ng/mL. Both MT-150AW and MT-700B significantly inhibited ADM efflux at concentrations > 75 ng/mL (Fig. 4B).

Laser confocal microscopy revealed that P-gp signals

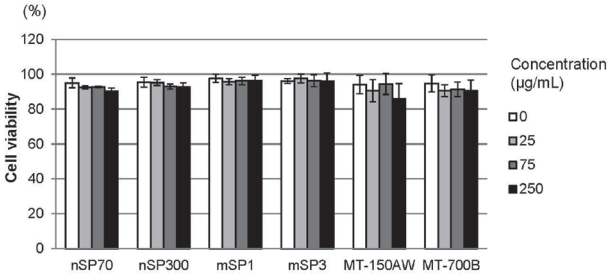

Fig. 3. Viability of the particle-treated L1210/ADM cells. L1210/ADM cells were seeded into 24 well plates at a density of 1×10^5 cells/mL and treated with test particles for 24 hr. Live and dead cells were counted by trypan blue exclusion method. Data are shown as the mean ± S.D.

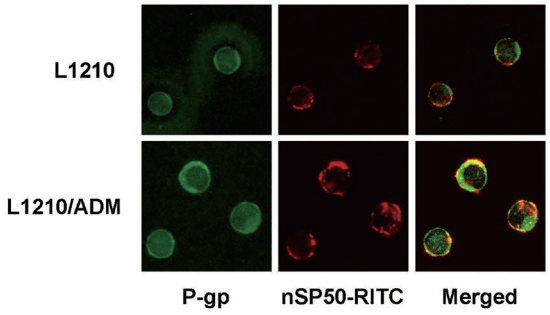

Fig. 5. Localization of fluorescent-caged nSP50-RITC and P-gp in L1210 and L1210/ADM cells. L1210 and L1210/ADM cells were seeded into 96-well plates at a density of 1×10^5 cells/mL. After 24 hr culture, cells were treated with nSP50-RITC for 24 hr. The cells were fixed with methanol and labelled with P glycoprotein antibody and an Alexa Fluor™ 488 conjugated goat anti-rabbit IgG secondary antibody. Microscopic observations were performed using a laser confocal microscope.

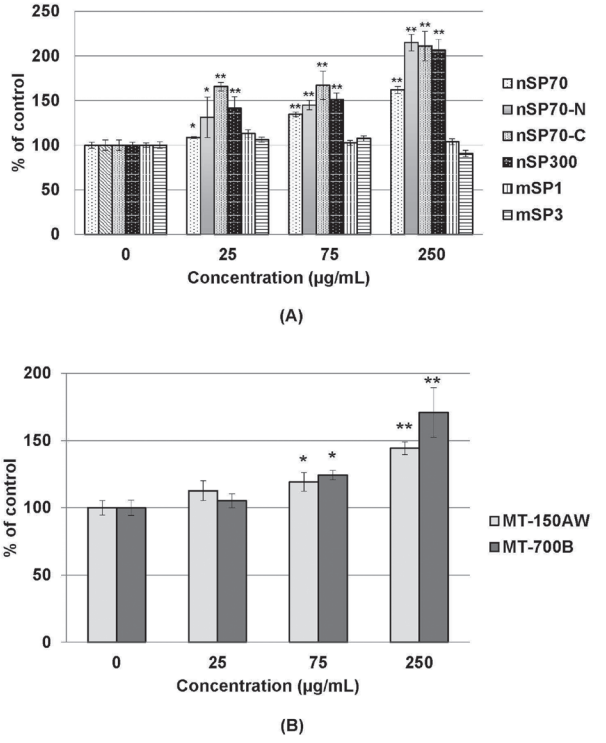

Fig. 4. Inhibition of ADM efflux from L1210/ADM cells by silica particles (A) and TiO_2 particles (B) treatment.L1210/ADM cells were seeded in 24-well plates at a density of 1×10^5 cells/mL. After 24 hr culture, cells were treated with silica or TiO_2 particles for 24 hr. In the last 3 hr of the treatment, 5 μmol/L of ADM was added to each well. Then, the cells were collected and washed once with ice-cold PBS(-). The residual ADM in the cells was extracted with 0.3 mol/L HCl and 1% SDS in 50% ethanol and the ADM concentration was determined by spectrofluorometer (Ex = 485 nm, Em = 590 nm). Data were normalized to the protein concentration of each sample. Data are shown as the mean ± S.D.*, ** Significantly different from the 0 μg/mL group, $*p < 0.05$, $**p < 0.01$.

(green) and nSP50-RITC (red) appeared to colocalize around the cell membrane of L1210/ADM cells (Fig. 5).

Our results showed that L1210/ADM cells express P-gp and that silica and TiO_2 nanoparticles inhibit ADM efflux via P-gp.

P-gp is involved in the multi-drug resistance of cancer cells (Juliano and Ling, 1976). P-gp transports substrates, including anthracycline anticancer drugs, digoxin, tacrolimus, and steroids, and are mainly expressed in the small intestine, kidney, liver, and brain (Fromm, 2003; Lin and Yamazaki, 2003). As both nSP and $nTiO_2$ are used as food additives (Murugadoss *et al.*, 2017; Baranowska-Wójcik *et al.*, 2020), it is noteworthy that they inhibit the transporter function of P-gp expressed in the small intestine. The inhibition of P-gp transporters by excessive nSP or $nTiO_2$ exposure may lead to the accumulation of xenobiotics, which are substrates for P-gp.

These results suggest that nSP and $nTiO_2$ interfere with P-gp, which is involved in ADM transport, and L1210/ADM cells are suitable for a safety screening tests of nanomaterials.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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