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PAPER

Atorvastatin and clopidogrel interfere with photosensitization *in vitro*†

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Photodynamic therapy (PDT) has been used to eliminate undesired cells by using a combination of photosensitizers and light illumination to generate reactive oxygen species. There is great interest in applying PDT to atherosclerosis; preferential destruction of pro-inflammatory macrophages in atheromata might attenuate plaque growth or rupture-prone vulnerability. Here, we report on a previously unknown interaction between cardiovascular drugs that are commonly prescribed for atherosclerosis patients and the cytolytic effects of photodynamic therapy using Cathepsin B activatable photosensitizer L-SR15 on murine macrophage Raw 264.7 cells in culture. Atorvastatin and clopidogrel significantly interfered with *in vitro* photosensitization effect while aspirin did this to a lesser extent; these drugs did not change the efficiency of cellular uptake of L-SR15 after *in vitro* photosensitization. A photosensitization interference effect of atorvastatin and clopidogrel was also observed when using a conventional photosensitizer free Ce6 or NCI-H1299 cancer cells. Considering the clinical implications of PDT, our study merits further investigation in clinical settings as well as in animal models.

Introduction

Photodynamic therapy (PDT) has been applied to treat patients with dermatologic or oncologic diseases, such as acne, actinic keratoses, Bowen's disease, basal cell carcinoma, and Barrett's esophagus.[1] The principle of PDT is to kill unwanted cells by using a combination of photosensitizers and light illumination to generate highly reactive oxygen species (ROS) that locally destroys cells over short diffusion distances.[2]

Selective destruction of pathologic target cells within organs affected by disease is a highly desirable goal in the clinical setting, as it leaves normal cells and tissues intact. In this context, PDT, which can induce preferential destruction of unwanted cells such as cancer cells or pro-inflammatory macrophages using target-specific or protease-activatable photosensitizers, has great promise as a therapeutic modality.[1–10] There is great interest in applying this technique to atherosclerosis, where macrophages in atheromata mediate chronic and acute inflammatory cascades responsible for both plaque growth and maintenance, and eventual plaque destabilization and rupture.[11] The photosensitizers used in PDT are very lipophilic, and thus naturally seek the lipid-rich atherosclerotic plaque environment, where they have been detected.[3,4] It was also recently demonstrated that photosensitizers could accumulate in macrophages of atheromas, resulting in PDT-mediated stabilization of atherosclerotic plaques and reduction of plaque inflammation in arteries using animal models.[5–7]

In investigating PDT as a new mode of therapy for atherosclerosis, we discovered a previously unknown interaction; commonly prescribed cardiovascular drugs protected macrophages in culture from the expected cell killing effects of photosensitization. The drugs investigated were commonly prescribed ones for atherosclerotic patients: atorvastatin, a 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor to lower cholesterol levels, and clopidogrel, a platelet adenosine diphosphate (ADP) receptor antagonist to inhibit platelet aggregation. Both of these drugs have been reported to have antioxidant effects,[12] a likely mechanism for the reduced efficacy of photosensitization that we observed.

In this paper we study and quantify the effects of these drugs on the effectiveness of photosensitization in macrophage cell cultures.

Experimental

Synthesis of L-SR15, Cathepsin B activatable photosensitizer

L-SR15 photosensitizer was synthesized as described previously.[13] In brief, the biodegradable PEGylated poly-L-lysine backbone

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was used to induce aggregation and self-quenching of chlorin e6 (Ce6) molecules. Conjugation of Ce6 (Frontier Scientific, Logan, UT) to lysine residues on the backbone was performed using a coupling reagent 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl. The conjugates were purified using gel-filtration chromatography using Bio-Gel P-10 gel (Bio-Rad, Hercules, CA). In the absence of Cathepsin B, the photosensitizer is inactive, but the presence of the enzyme leads to backbone cleavage and activation by de-quenching of Ce6 molecules. In the activated state after cleavage, light illumination will lead to ROS generation and photosensitization, but activation requires prior proteolytic cleavage.[14] A previous report demonstrated efficient proteolytic cleavage of the L-SR15 in cells in which cathepsin B is abundant,[13] which was re-confirmed by our pilot experiments (data not shown).

Cardiovascular drugs

Atorvastatin and clopidogrel (gifts from Samjin Pharmaceutical Co., Seoul, Korea), were dissolved in dimethylsulfoxide (DMSO) and diluted with cell culture medium. We chose a range of concentrations of 0.1–1 μM for atorvastatin and 0.5–8 μM concentrations for clopidogrel, based on reported maximum plasma concentrations (2.9 mg L^{-1} for clopidogrel metabolite and 0.25 mg L^{-1} for atorvastatin) at clinical doses of 75 mg of clopidogrel and 80 mg of atorvastatin.[15,16]

Acetylsalicyclic acid (aspirin) is a non-steroidal anti-inflammatory drug and one of the most frequently used non-prescription drugs with analgesic, antipyretic, anti-inflammatory, and antiplatelet effects.[17] Direct anti-oxidant activity of aspirin has not been reported to our knowledge. Therefore, we used aspirin as a control agent. It was purchased (Sigma, St Louis, USA) and dissolved in ethanol and diluted with cell culture medium. Based on the maximum blood concentration of total salicylates in blood (18.9 mg L^{-1} at 600 mg dose of buffered aspirin),[18] a concentration range of 7–60 μM of aspirin was used.

In vitro photosensitization (*in vitro* PDT)

In vitro photosensitization was carried out using murine macrophage Raw 264.7 cells grown in phenol red free DMEM plus 10% fetal bovine serum, 100 units ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin. Cells were grown in a humidified incubator with 5% CO_2 at 37 °C to reach approximately 70% confluency. Cells were incubated with either 1 or 4 μM Ce6 concentrations of the L-SR15 for 6 h, washed twice with PBS, and changed to fresh medium. Cells were illuminated for 40 s using a 670-nm diode laser, with a light spot diameter of 6 mm, to give light doses of 0.1, 3, and 8 J cm^{-2}. In typical experiments, we used 4 μM Ce6 concentrations of the L-SR15 and 8 J cm^{-2} illumination at a dose rate of 200 mW cm^{-2}. After illumination, cells were further incubated in a humidified chamber for 16 h. At least three independent experiments were performed for each photosensitization experiment.

In vitro photosensitization in the presence of cardiovascular drugs

Raw 264.7 cells were treated with various concentrations of atorvastatin, clopidogrel, and aspirin according to experimental conditions. Final concentrations of DMSO or ethanol in the cell culture media treated did not exceed 0.05%. Equal amounts of DMSO or ethanol were added where no chemicals were added. Cells were incubated with 4 μM Ce6 concentrations of the L-SR15 with or without cardiovascular drugs for 6 h, washed twice with PBS, changed to fresh medium, exposed to light at 8 J cm^{-2}, and further incubated for 16 h.

Effects of cardiovascular drugs were also analyzed using a conventional photosensitizer, free Ce6. Raw 264.7 cells were incubated in culture media containing one of vehicle, 0.5 μM atorvastatin, 2 μM clopidogrel, or 20 μM aspirin for 4 h. Raw 264.7 cells were then rinsed twice, incubated in serum free medium containing 4 μM free Ce6 for 2 h, rinsed twice, exposed to light at 8 J cm^{-2}, and further incubated.

Since PDT has been used to treat cancer patients,[1] we also used a lung cancer cell line (NCI-H1299, a gift from Dr S. K. Kim, National Cancer Center, Goyang, Korea) to analyze the effects of aspirin, atorvastatin, or clopidogrel on phototoxicity in cancer cells. NCI-H1299 cells were incubated with 4 μM Ce6 concentrations of the L-SR15 with or without cardiovascular drugs for 6 h, washed twice with PBS, changed to fresh medium, exposed to light at 8 J cm^{-2}, and further incubated for 16 h.

MTT assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) assays were carried out to determine the cytotoxic effects of *in vitro* photosensitization. MTT solution (final concentration 0.4 mg ml^{-1}) was added to cells grown in phenol red free medium, and cells were further incubated for 3 h. Medium was removed, and the converted formazan dye was solubilized with acidic isopropanol (10 mM HCl in absolute isopropanol). Absorbance at 570 nm with background subtraction at 650 nm was measured.

Annexin V-FITC staining assay

The effects of aspirin, atorvastatin, or clopidogrel on *in vitro* photosensitization were also analyzed using Annexin V-FITC staining assay (Abcam, Cambridge, UK) to detect apoptotic or necrotic cells. Cells grown on a slide chamber Lab-Tek II (Nunc, Rochester, USA) were incubated in medium containing 4 μM L-SR15 with or without cardiovascular drugs for 6 h, washed twice, and illuminated as described above. Cells were further incubated for 1 h after illumination, washed with PBS, incubated with the Annexin V binding buffer containing Annexin V-FITC for 5 min, washed with PBS twice, fixed with 4% paraformaldehyde for 10 min, and washed with PBS twice. DAPI was used to stain nuclei of cells. Cells were then analyzed using a fluorescence microscope (final magnification 100×).

Effects of cardiovascular drugs on the intracellular uptake of the L-SR15

Raw 264.7 cells were incubated in the culture medium containing 4 μM L-SR15 plus one of vehicle (0.05% DMSO), 0.5 μM atorvastatin, 2 μM clopidogrel, or 20 μM aspirin for 6 h. Cells were rinsed twice with PBS and treated with lysis buffer (0.1 N NaOH and 0.1% SDS) without light illumination. Intracellular concentration of Ce6 was determined using a fluorometer (Malvern, Worcestershire, UK) at 650 nm (ex) and 670 nm (em).

Statistics

Data are presented as mean ± standard deviation (SD). One-way analysis of variance with Dunnett's post-hoc tests were used for comparison of continuous variables between groups. A value of $p < 0.05$ was considered significant.

Results and discussion

In vitro photosensitization using the Cathepsin B activatable photosensitizer L-SR15 induces cell death of macrophages in a dose-dependent manner

Since PDT is based on the localization and activation of photosensitizers in a target tissue of interest, optimal concentrations of the photosensitizer and illumination dose of light were determined. As shown in the Fig. 1, illumination of light (nonionizing and nonthermal electromagnetic energy) between 0.1 and 8 J cm^{-2} showed little, if any, cytotoxic effect in the absence of the L-SR15. Illumination at the 0.1 J cm^{-2} even in the presence of the photosensitizer at both 1 and 4 μM Ce6 concentrations did not show significant cytotoxic effect. However, illumination of light at 3 J cm^{-2} gave noticeable cytotoxic effects at both 1 μM and 4 μM Ce6 concentrations of the photosensitizer. Illumination at 8 J cm^{-2} showed further cytotoxic effects, 46 ± 17% ($p < 0.01$) at 1 μM and 82 ± 6% ($p < 0.001$) at 4 μM Ce6 concentrations of L-SR15 (Fig. 1). These results demonstrate that *in vitro* photosensitization using the L-SR15 induces cell death of macrophages in a dose-dependent manner depending on both light power and photosensitizer concentration.

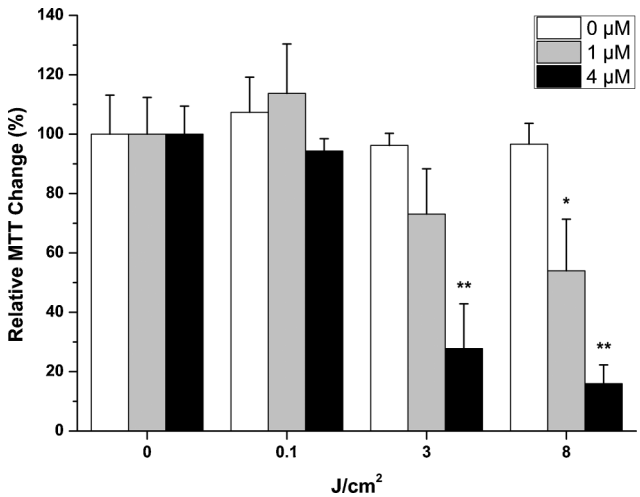

Fig. 1 Cytotoxic effect of *in vitro* photosensitization in a dose-dependent manner. Raw 264.7 cells were incubated in media containing 0 μM, 1 μM, or 4 μM Ce6 of L-SR15 photosensitizer for 6 h, rinsed and illuminated with a 670-nm diode laser for 40 s, and analyzed using an MTT assay. Optical measurement of cytotoxicity without illumination was calculated as 100%. $n = 4$. *$p < 0.01$ *vs.* 0 J cm^{-2} at 1 μM Ce6. **$p < 0.001$ *vs.* 0 J cm^{-2} at 4 μM Ce6 of L-SR15.

Atorvastatin and clopidogrel interfere with *in vitro* photosensitization effect

Given the fact that the mechanism of PDT is to induce cell death by generation of ROS, this study was designed to address whether statin and clopidogrel, widely-prescribed cardiovascular drugs reported to have antioxidant effect, can interfere with the efficiency of photosensitization. First, we confirmed that incubation of cells with the drug compounds at pharmacological concentrations did not change cell viability (supplemental Fig. S1†). When *in vitro* photosensitization was applied to cells however, atorvastatin could interfere with the cytotoxic effect of the photosensitizer L-SR15 at submicromolar concentrations (Fig. 2A). Clopidogrel also efficiently inhibited cytotoxic effect of *in vitro* photosensitization from submicromolar concentrations (Fig. 2B). The efficiency of aspirin to interfere with the cytotoxicity of *in vitro* photosensitization was lower than that of clopidogrel and atorvastatin, and did not reach significance.

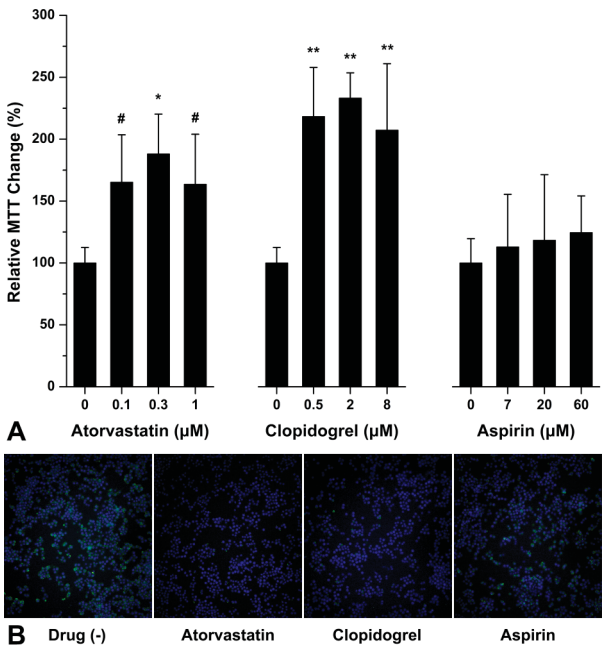

Fig. 2 Atorvastatin and clopidogrel interfere with cytotoxicity of *in vitro* photosensitization. (A) Raw 264.7 Cells were incubated in the media containing L-SR15 with or without various concentrations of clinical drugs for 6 h. Cells were illuminated and analyzed using an MTT assay. Optical measurement of cytotoxicity without drugs was calculated as 100%. $n = 3$. *$p < 0.05$ and #$p = 0.07$ *vs.* lane 1 (without atorvastatin). **$p < 0.01$ *vs.* lane 1 (without clopidogrel). (B) After photosensitization, cells were incubated with Annexin V-FITC for 5 min and analyzed under a fluorescence microscope (magnification 100×, 50 ms exposure). Annexin-positive FITC signal is less abundant in the cells treated with atorvastin or clopidogrel, confirming a protective role for these compounds against *in vitro* photosensitization.

The effects of aspirin, atorvastatin, or clopidogrel on *in vitro* photosensitization were also clearly visualized from the Annexin V-FITC staining assay (Fig. 2B). It demonstrated that illumination of cells with light in the presence of L-SR15 induced efficient cell death (Annexin V-stained cells: 87.7 ± 1.58%), which was consistent with the results in Fig. 1. The efficiency of atorvastatin (Annexin V-stained cells: 6.3 ± 0.91%) and clopidogrel (Annexin V-stained cells: 10.1 ± 6.57%) to interfere with the phototoxicity was higher than that of aspirin (Annexin V-stained cells: 51.2 ± 7.73%). These results were consistent with the results carried out by the MTT assay (Fig. 2A).

Effects of drug–light time interval or combinations of drugs on *in vitro* photosensitization

The effects of L-SR15 and clinical drugs on Raw cell survival after *in vitro* photosensitization were analyzed at a different time interval between the treatment of cardiovascular drugs and illumination of light. Photosensitization interference effect of atorvastatin and clopidogrel was not observed when the drug compounds were added to the medium 12 h before light illumination (Fig. 3). This result may be due to the excretion of the treated drugs out of the cells or metabolism inside the cells during further incubation time.

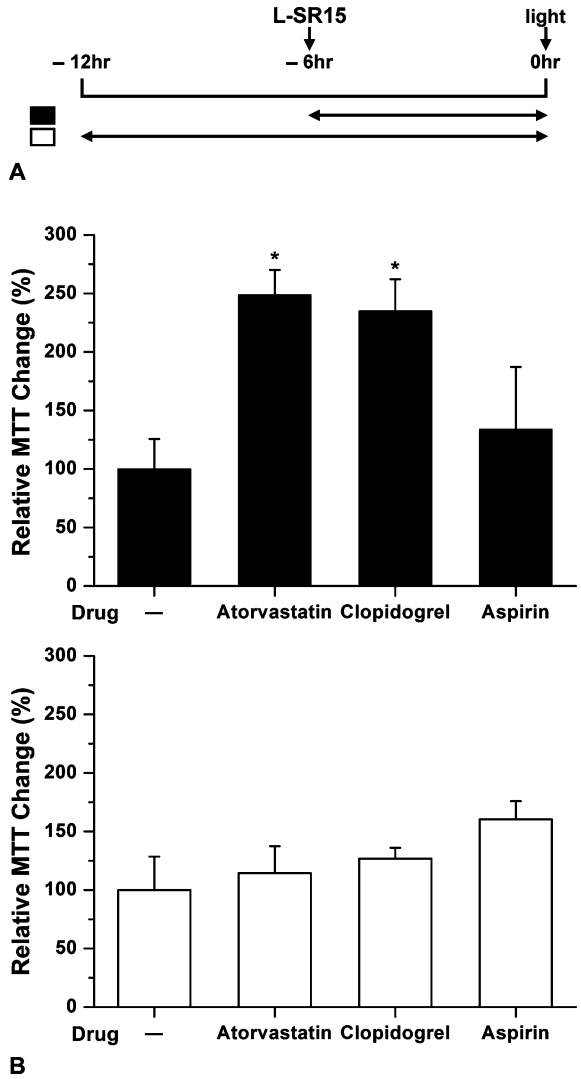

Fig. 3 Effects of changes in drug–light interval (DLI) on efficiency of *in vitro* photosensitization. The drug compounds (0.5 μM atorvastatin, 2 μM clopidogrel, and 20 μM aspirin) and 4 μM L-SR15 photosensitizer were added to medium 6 h before illumination (closed bars). The drug compounds were added to medium 12 h before illumination and L-SR15 was added 6 h before illumination (open bars). Optical measurement of cytotoxicity without drug compounds was calculated as 100%. $n = 4$. $^*p <$ 0.01 *vs.* lane 1 (without drugs) in 6 h DLI.

Since many patients are prescribed combined medication regimens, we also determined if aspirin, clopidogrel, and atorvastatin give synergistic or additive effect on interference of *in vitro* photosensitization. As shown in Fig. 4, any combination set of aspirin, clopidogrel and atorvastain did not even show additive effect on the interference of *in vitro* photosensitization. The efficiency to inhibit the cytotoxic effect of *in vitro* photosensitization seems to be determined by either statin or clopidogrel, but the combination of statin and clopidogrel did not show further inhibition of *in vitro* photosensitization.

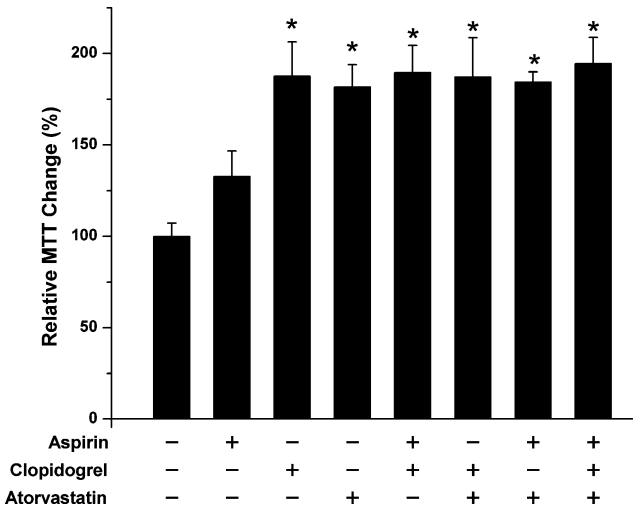

Fig. 4 Atorvastatin and clopidogrel do not show additive or synergistic effect on interference with *in vitro* photosensitization. Concentrations of clinical drugs were 20 μM aspirin, 2 μM clopidogrel, and 0.5 μM atorvastatin. Optical measurement of an MTT assay without drugs was calculated as 100%. $n = 4$. $^*p < 0.01$ *vs.* lane 1. *P* values between lanes 3 and 4, 5, 6, 7, or 8 are bigger than 0.1.

Atorvastatin and clopidogrel did not affect the cellular uptake of the photosensitizer L-SR15

In order to determine mechanisms by which cardiovascular drugs interfere with *in vitro* photosensitization, we analyzed if cardiovascular drugs change the cellular uptake efficiency of the L-SR15. Incubation of cells in the presence of cardiovascular drugs did not noticeably change cellular uptake efficiency of the L-SR15 (Fig. 5A). We also confirmed that the cardiovascular drugs did not affect the cleavage activity of Cathepsin B enzyme (data not shown). Thus, the decreased efficacy of *in vitro* photosensitization

Fig. 5 Atorvastatin and clopidogrel do not change the efficiency of cellular uptake of L-SR15. Raw 264.7 cells were incubated in the presence of 4 μM Ce6 of L-SR15 with 0.5 μM atorvastatin, 2 μM clopidogrel, or 20 μM aspirin for 6 h. Cells were rinsed and treated with lysis buffer. Cellular concentration of Ce6 was determined using a fluorometer at 650 nm (excitation) and 670 nm (emission). Fluorescence intensity without drugs was calculated as 100%. Results are presented as mean ± SD; $n = 4$.

by the drug compounds appears to have resulted from bona fide anti-oxidant property of the compounds.

Statins are frequently prescribed to patients with or at the risk of cardiovascular diseases to lower low density lipoprotein cholesterol. In addition, statins have been reported to have vascular protective effects against endothelial dysfunction by reducing the cellular level of ROS.[12] The anti-oxidant activity by statins is mediated by inhibiting Rho GTPase and Rac1-mediated NADPH oxidase pathways.[12] Clopidogrel blocks activation of the glycoprotein IIb/IIIa pathway in the platelet and inhibits platelet aggregation.[13] In addition to the anti-platelet effect, a recent study demonstrated that clopidogrel could down-regulate ROS production as well as cell adhesion between platelets and polymorphonuclear leukocytes.[19]

Atorvastatin and clopidogrel interfere with *in vitro* photosensitization when using a conventional photosensitizer or a cancer cell line

Consistent with the results obtained using the activatable photosensitizer L-SR15, atorvastatin and clopidogrel interfered with *in vitro* photosensitization using a conventional photosensitizer, free Ce6 (Fig. 6A). In addition, consistent with the results obtained using the Raw 264.7 macrophage cell line, atorvastatin and clopidogrel interfered with the cytotoxicity effect of *in vitro* photosensitization on the lung cancer cell line NCI-H1299 (Fig. 6B).

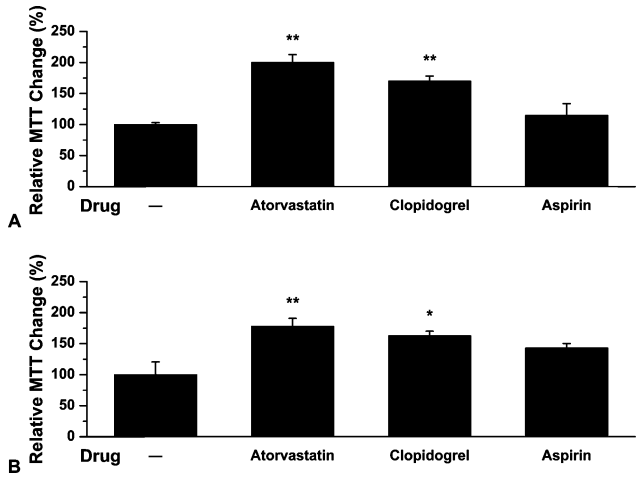

Fig. 6 Atorvastatin and clopidogrel interfere with *in vitro* photosensitization (A) in Raw 264.7 cells when using a conventional photosensitizer free Ce6 and (B) in NCI-H1299 cancer cells when using the L-SR15 photosensitizer. (A) Raw 264.7 cells were incubated in media containing one of vehicle (DMSO), 0.5 μM atorvastatin, 2 μM clopidogrel, or 20 μM aspirin for 4 h. Cells were rinsed and incubated in medium containing 4 μM Ce6 for 2 h, rinsed twice, and exposed to light illumination. MTT assay was conducted and optical measurement of cytotoxicity without drugs was calculated as 100%. $n = 4$. $^{**}p < 0.001$ *vs.* lane 1 (without drugs). (B) NCI-H1299 cells were treated with L-SR15 and one of vehicle (DMSO), 0.5 μM atorvastatin, 2 μM clopidogrel, or 20 μM aspirin for 6 h. Cells were illuminated and analyzed using an MTT assay. Optical measurement of cytotoxicity without drugs was calculated as 100%. $n = 3$. $^{*}p < 0.01$ or $^{**}p < 0.001$ *vs.* lane 1 (without drugs).

Conclusions

We have found a potentially significant therapeutic interaction between commonly prescribed cardiovascular drugs and the cytolytic effects of photodynamic therapy on macrophages in cell culture. Atorvastatin and clopidogrel interfered with *in vitro* photosensitization effect, while aspirin did this to a lesser extent.

Although our study was carried out using a cell culture system, the *in vitro* results raise an important concern about the use of these cardiovascular drugs while receiving PDT. In addition, results of this study suggest that optimal incubation time of the drug compounds and photosensitizer for efficient PDT needs to be determined for each photosensitizer used for PDT at clinical settings. Either clopidogrel or aspirin can be used for secondary prevention of cardiovascular or cerebrovascular events.[20] If an anti-platelet drug cannot be discontinued in patients at high risk for recurrent stroke or myocardial infarction, aspirin might be a better alternative to clopidogrel during PDT since aspirin had a relatively weak interference with the cytotoxic effects of PDT. Considering the clinical implications of cardiovascular drug interference with PDT, we believe that our study merits further investigation in clinical settings as well as in animal models.

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