Comparative In Vitro Toxicity Study of Docetaxel and Nanoxel, a Docetaxel-Loaded Micellar Formulation Using Cultured and Blood Cells

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Abstract
Nanoxel-PM™ (Nanoxel) is a docetaxel-loaded methoxy-poly(ethylene glycol)-block-poly(D,L-lactide) (mPEG-PDLLA). This newly developed and marketed nanoformulation exhibits an improved pharmacokinetic profile, efficacy, and safety. Although the safety of Nanoxel to docetaxel as well as its bioequivalence must be clinically confirmed, all biological activities have not been examined in in vitro or in vivo studies. Here, the toxicity in a cultured cell system and the effects on blood cells were tested with Nanoxel and docetaxel. The in vitro cytotoxicity of Nanoxel was found to be comparable to or slightly lower than that of docetaxel depending on the concentrations tested or the cell types. Neither docetaxel nor Nanoxel induced erythrocytes hemolysis and produced reactive oxygen species up to 100 µM. However, Nanoxel was able to enhance the aggregatory response of platelets to collagen, whereas docetaxel attenuated such aggregation in a range of 50-100 µM, while thrombin-induced aggregation was not affected by either of them. Docetaxel or Nanoxel did not alter basal level of Ca²⁺ and 5-hydroxytryptamine-evoked Ca²⁺ transient in vascular smooth muscle cells. These results suggest that the mPEG-PDLLA micellar formulation alters the toxicological properties of docetaxel, and that extra cautions are needed when evaluating the safety of nanomedicine.

Key words: Docetaxel, Nanoxel, Nanomedicine, Cytotoxicity, Platelet aggregation

INTRODUCTION

Docetaxel is a highly potent anticancer drug belonging to the taxane family (1). It exerts anti-proliferative activity through promoting the assembly of tubulin into stable microtubules and stabilizing the polymers against depolymerization, thereby blocking microtubule dynamics (2). Docetaxel has been used to treat a wide range of tumors, including advanced and metastatic cancer, non-small cell lung cancer, and advanced gastric cancer (3). As is the case with most chemotherapeutic agents, docetaxel also causes diverse adverse effects like neutropenia, leukopenia, neurological toxicity, alopecia, and asthenia in the majority of patients, which is a serious obstacle to its clinical use (4).

A good deal of effort has gone toward the development of novel delivery systems to improve therapeutic efficacy and reduce adverse reactions. To this end, nanotechnology has been applied to delivery systems, which, in general, enhances efficacy and potency by increasing surface area, solubility, and dissolution rate, and through the rapid onset of therapeutic action. Indeed, several drug delivery systems have been applied for docetaxel thus far, including docetaxel-loaded nanoparticles (5), self-emulsified docetaxel (6), malic acid-esterified docetaxel (7), and PEGylated liposomes (8) and N-palmitoyl chitosan-anchored liposome (9) for docetaxel.

Nanoxel-PM™ (Nanoxel) is a docetaxel-loaded methoxy-poly(ethylene glycol)-block-poly(D,L-lactide) (mPEG-PDLLA) micellar formulation (10). PDLLA was chosen as a hydrophobic core-forming material and mPEG segments were combined to form amphiphilic diblock copolymers. Mannitol was additionally used as a cryoprotectant. This formulation was employed so as to facilitate the solu-
bilitation of docetaxel, and thus does not require surfactants like Cremophor EL and polysorbate 80, which may cause hypersensitivity reactions and fluid retention. Accordingly, Nanoxel was reported to be superior to the conventional formulation of docetaxel in terms of efficacy, pharmacokinetic properties, and toxicity (10). Although its general safety has been shown and commercial marketing has been approved by the FDA, all the biological activities of Nanoxel were not compared with the active ingredient docetaxel.

In this study, the in vitro cytotoxicity of Nanoxel was tested in parallel with docetaxel. In addition, the effects on the circulatory system, platelet aggregation, cancer were used for the cytotoxicity study. In order to test the effects on the circulatory system, platelet aggregation, the hemolysis of erythrocytes, and calcium signaling in VSMCs were examined. In addition, the reactive oxygen species (ROS)-generating capacity was assessed as a general toxicological property of nanomaterial.

**MATERIALS AND METHODS**

**Reagents.** Docetaxel and Nanoxel were purchased from Tokyo Chemical Industry (Tokyo, Japan) and Samyang Biopharmaceuticals (Seongnam, Korea), respectively. Thrombin, 5-hydroxytryptamine (5-HT), menadione, and butylated hydroxyanisole (BHA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Collagen was acquired from Chrono-log (Havertown, PA, USA) and 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-1) was obtained from Seoul Clinical Genomics (Seoul, Korea). Fura-2/AM and 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red) were purchased from Molecular Probes (Eugene, OR, USA). Premix WST-1 Cell Proliferation Assay System was acquired from Takara Bio (Shiga, Japan). All other chemicals used were of the highest purity available and purchased from standard suppliers.

**Animals.** All animal experiments were conducted in accordance with protocols approved by the Ethics Committee of Animal Service Center at Dongguk University. Male Sprague-Dawley rats were purchased at five to six weeks of age from Duehan Biolink (Eumseong, Korea). The rats were housed in the laboratory animal facility and acclimated for a week prior to experimentation. The laboratory animal facility was maintained at constant temperature and humidity with a 12 hr light/dark cycle. Diet and water were provided ad libitum.

**Cells and cell culture.** VSMCs were isolated from rat thoracic aortas through enzymatic digestion as previously described (11). Briefly, isolated aortas were cut open longitudinally and cleaned of connective tissue, fat, and endothelium. Aortas were then digested with collagenase and elastase in order to remove the adventitia and dissociate the VSMCs. Individual cells were plated in a culture dish and grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were passaged by trypsinization, and the passages between three and eight were used for experimentation.

The mouse brain endothelial cell line bEnd.3, human monocytic cell line THP-1, and human breast cancer cell line MCF-7 were purchased from American Type Culture Collection (Manassas, VA, USA). Cells were culture in DMEM (for bEnd.3 and MCF-7 cell lines) or Roswell Park Memorial Institute (RPMI) 1640 (for THP-1 cell line) supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were maintained at 37°C and 5% CO₂ in a humidified chamber incubator and subcultured when they reached 80-90% confluence.

**Measurement of cell lysis and viability.** Cell lysis and viability were assessed by lactate dehydrogenase (LDH) leakage assay and formazan formation assay, as previously described (12). Briefly, cells were plated on multiwell plates at densities of 1 × 10⁴ cells/well on a 96-well plate and grown overnight until they reached 80-90% confluence. Cells were incubated with docetaxel or Nanoxel for 24 hr before culture media were collected and centrifuged at 12,000 g for 2 min. The resulting supernatant was used for LDH activity assay. Briefly, 50 µL of the supernatant was added to 200 µL of working reagent containing 0.2 mM NADH and 2.5 mM sodium pyruvate in the individual wells of a 96-well plate. The decrease in absorbance at 340 nm was measured for 20 min with the SpectraMax M3 microplate reader (Molecular Devices, San Jose, CA, USA). Relative LDH activity was calculated from the slope of the graph of decreasing absorbance. LDH activities measured from 1% Triton X-100-treated cells were regarded as 100% lysis.

Following 24 hr incubation with testing materials, the remaining cells were quantified in order to determine cell viability in the formazan formation assay. Treated cells were incubated with Premix WST-1 for an additional 2 hr. Media were then collected and centrifuged at 16,000 g for 2 min. Cell viability was determined by measuring the absorbance at 450 nm with a reference wavelength of 630 nm using the SpectraMax M3 microplate reader.

**Preparation of washed platelets (WP) and platelet aggregation studies.** WP were prepared as previously described (13). Briefly, blood was collected from the abdominal aorta of rats anesthetized with ether using acid-citrate-dextrose (ACD; 85 mM trisodium citrate, 66.6 mM citric acid, 10 mM sodium heparin, 100 mg/mL sodium ethylenediaminetetraacetic acid (EDTA), and 1% human serum albumin) and centrifuged at 1600 g for 10 min to yield platelet-poor plasma. RBCs were removed by centrifugation at 1600 g for 10 min. Washed platelets were resuspended in 100 volumes of Tyrode’s buffer. Platelet aggregation was induced by stimulating washed platelets with a range of concentrations of ADP (1-100 µM). Aggregation was monitored using a Chronolog (Havertown, PA, USA) aggregometer and recorded as percent aggregation relative to a 1% Triton X-100 treated sample.
acid, and 111 mM glucose) as an anticoagulant (ACD : blood = 1 : 6). After centrifugation at 400 g for 15 min, platelet-rich plasma was obtained from the supernatant. Platelets were centrifuged again at 500 g for 10 min, then washed once with washing buffer solution (138 mM NaCl, 2.8 mM KCl, 0.8 mM MgCl$_2$, 0.8 mM Na$_2$HPO$_4$, 10 mM HEPES, 0.55 mM glucose, 22 mM trisodium citrate, 0.35% bovine serum albumin; pH 6.5) by suspending and centrifuging. WP were prepared by resuspending the platelet pellets in suspension buffer solution (138 mM NaCl, 2.8 mM KCl, 0.8 mM MgCl$_2$, 0.8 mM Na$_2$HPO$_4$, 10 mM HEPES, 5.6 mM glucose, 1 mM CaCl$_2$, 0.3% bovine serum albumin; pH 7.4). The number of cells was adjusted to 2 × 10$^8$ cells/mL.

Platelet aggregation experiments were performed using a four-channel aggregometer (Chrono-log). WP were treated with docetaxel or Nanoxel for 5 min, and then aggregation was induced by either 2.5 µg/mL collagen or 0.12 U/mL thrombin, which is the concentration inducing approximately 50% aggregation.

**Preparation of erythrocyte suspension and measurement of hemolysis.** Erythrocyte suspension was prepared and hemolysis was assessed as previously described (14). Briefly, blood was withdrawn from the abdominal aorta of ether-anesthetized rats and subjected to centrifugation at 1,000 g for 10 min. The buffy coat was then removed, and remaining erythrocytes were washed twice with phosphate-buffered saline (PBS; 6 mM sodium phosphate, 140 mM NaCl; pH 6.5) by suspending and centrifuging. The erythrocyte suspension was treated with 1% Triton X-100. Erythrocytes were incubated with docetaxel or Nanoxel at 37°C, and the extent of hemolysis was assessed using the SpectraMax M3 microplate reader. Percent hemolysis was expressed as the ratio of the light absorbance of test sample compared to a standard sample completely hemolyzed with 1% Triton X-100.

**ROS measurement in VSMCs.** ROS were quantified with the colorimetric probe WST-1 and the fluorescent probes Amplex Red. VSMCs were treated with docetaxel or Nanoxel in the presence of 500 nM WST-1 or 10 µM Amplex Red and 0.1 U/mL HRP for WST-1 or Amplex Red assays, respectively. The absorbance at 450 nm or fluorescence intensity at Ex/Em = 560/585 nm was measured at 12- or 3-min intervals for 60 or 12 min, respectively, using the SpectraMax M3 microplate reader. All assays were performed at 37°C.

**Ca$^{2+}$ measurement in VSMCs.** Intracellular Ca$^{2+}$ was measured using the fluorometric method employing a fura-2 and digital imaging, as previously described (15). Briefly, VSMCs grown on coverslips were incubated in physiological salt solution (PSS; 140 mM NaCl, 5 mM KCl, 5 mM NaHCO$_3$, 1.8 mM CaCl$_2$, 1.4 mM MgCl$_2$, 1.2 mM NaH$_2$PO$_4$, 10.5 mM glucose, and 10 mM HEPES; pH 7.4) containing 1 µM fura-2/AM, 1% bovine serum albumin and 1 µM docetaxel or Nanoxel for 60 min. Coverslips were mounted in a superfusion chamber on the microscope stage and continuously superfused with PSS at a rate of 2 mL/min. Ca$^{2+}$ transient was evoked with 10 µM 5-HT. All experiments were conducted at 33°C. Cells were imaged with a Nikon Eclipse Ti-U inverted microscope equipped with a S Fluor 40× objective lens (Nikon, Tokyo, Japan) and an Evolve 512 electron-multiplying charge-coupled device camera (Photometrics, Tucson, AZ, USA). Illumination was provided by a Sutter DG-4 filter changer (Sutter Instruments, Novato, CA, USA). The excitation and emission wavelengths used for fura-2 were 340/380 and 535 nm, respectively. Images were acquired and analyzed using a Meta Imaging System (Molecular Devices).

**Statistical analyses.** Means and standard errors (SE) of means were calculated for all experimental groups. Data were subjected to one-way or two-way analysis of variance followed by Dunn’s test in order to determine the significance of the differences relative to controls. Statistical analysis was performed using SigmaPlot software version 13.0 (Systat Software, San Jose, CA, USA). P values of <0.05 were considered to be statistically significant.

**RESULT**

**Cytotoxicity of docetaxel and Nanoxel.** The cytotoxic potentials of docetaxel and Nanoxel were evaluated in VSMCs, bEnd.3 cells, THP-1 cells, and MCF-7 cells. Cell lysis and viability were measured by LDH leakage assay and WST-1 assay, respectively. Both docetaxel and Nanoxel exhibited moderate but statistically significant cytotoxicity, and such cytotoxicity was concentration-dependent in a range of 1 nM-100 µM (Fig. 1). The extent of cytotoxicity differed depending on cell types; VSMCs and bEnd.3 were more vulnerable to docetaxel and Nanoxel than were THP-1 and MCF-7. The cytotoxicity of Nanoxel was comparable to or less cytotoxic than that of docetaxel, depending on cell types. The difference of cytotoxicity between them was prominent in the relatively high concentrations of 10 and 100 µM (Fig. 1).

**Hemolytic activity of docetaxel and Nanoxel.** Docetaxel and Nanoxel were tested for their hemolytic activities using erythrocyte suspension prepared from rat whole blood. The erythrocyte suspension was treated with 10-100 µM docetaxel or Nanoxel and incubated for 30-180 min. Docetaxel or Nanoxel failed to cause hemolysis up to 180

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min (Fig. 2). For a positive control, erythrocytes were treated with 10 mM BHA, which successfully induced hemolysis under this experimental condition (Fig. 2, left panel).

**Effect of docetaxel and Nanoxel on platelet aggregation.** Docetaxel or Nanoxel treatment alone did not induce platelet aggregation (data not shown). In order to test the effect on platelet aggregation, WP were preincubated with docetaxel or Nanoxel for 5 min and aggregation was induced by the application of collagen or thrombin. Docetaxel could inhibit platelet aggregation induced by collagen at higher than 50 µM, and it was not effective at lower concentrations (Fig. 3A). In contrast, Nanoxel enhanced platelet aggregation at 50 and 75 µM, while the higher concentration of 100 µM did not affect aggregation. Platelet aggregation by thrombin was not significantly affected by pretreatment of docetaxel or Nanoxel (Fig. 3B).

**ROS generating capability of docetaxel and Nanoxel in VSMCs.** ROS generation by docetaxel and Nanoxel was measured using WST-1 and Amplex Red in VSMCs. WST-1 is a specific probe for superoxide anion and Amplex Red selectively detects hydrogen peroxide (16). Neither docetaxel nor Nanoxel were capable of generating superoxide or hydrogen peroxide in a range of 0.1-100 µM (Fig. 4). Menadione was used as a positive control and it successfully produced superoxide and hydrogen peroxide at 50 µM.

**Effects of docetaxel and Nanoxel on the Ca²⁺ signaling in VSMCs.** Intracellular Ca²⁺ in vascular smooth muscle is a major determinant of vasomotor function, and thus the impact of docetaxel and Nanoxel on Ca²⁺ signaling was tested in VSMCs. Neither docetaxel nor Nanoxel alone were found to affect the basal level of intracellular Ca²⁺ (Fig. 5B). Then, the impact on intracellular Ca²⁺ elevation was examined with a vasoconstrictor 5-HT. When cells were stimulated with 10 µM 5-HT, typical Ca²⁺ tran-
transient was evoked (Fig. 5A, upper panels). However, such Ca\(^{2+}\) transient was not altered by pretreatment with 1 \(\mu\text{M}\) docetaxel or Nanoxel for 60 min (Fig. 5A, lower panel). There was no statistical difference in the amplitude of Ca\(^{2+}\) peak (Fig. 5B).

**DISCUSSION**

Several novel formulations of docetaxel have been introduced in the market and widely prescribed to patients (17). Nanoxel is one of the recently developed nanoformulations of docetaxel. Although the bioequivalence of Nanoxel to docetaxel and its safety must be clinically confirmed, all biological activities have not been examined in *in vitro* or *in vivo* studies. Here, the toxicity in a cultured cell system and the effects on blood cells such as erythrocytes and platelets were tested with Nanoxel and docetaxel in parallel. The *in vitro* cytotoxicity of Nanoxel was found to be...
comparable or slightly lower than that of docetaxel, depending on the concentration tested or the cell types (Fig. 1). Nanoxel or docetaxel did not exhibit hemolytic activity in erythrocytes (Fig. 2), failed to produce ROS in cultured cells (Fig. 4), and did not affect intracellular Ca$^{2+}$ signals (Fig. 5). However, their effects on platelets differed from each other; Nanoxel was able to enhance the aggregatory response of platelets to collagen, whereas docetaxel attenuated such aggregation. The mechanism underlying distinct platelet effects was not clarified in this study. However, this observation raises the possibility that the mPEG-PDLLA micellar formulation alters the toxicological properties of docetaxel. Accordingly, extra cautions will be needed to evaluate the safety of nanomedicine.

Both docetaxel and Nanoxel are generally administered by i.v. route, and thus their primary exposure targets are blood and the vascular system. Hence, the toxicity or safety in these tissues must be tested, and the impacts on blood and vascular cells were examined in this study. Neither docetaxel nor Nanoxel caused hemolysis of erythrocytes up to 100 $\mu$M of concentration and 3 hr of treatment time (Fig. 2). The in vitro hemolytic activity of docetaxel has been previously reported (18,19), but this is not in agreement with the results of this study. The reason for the discrepancy is unknown, but the testing material itself may be responsible for such a difference. For instance, previous studies used Tween 80 and ethanol as vehicles for docetaxel, which are from marketed docetaxel (18,19), while 0.1% dimethyl sulfoxide was used as a vehicle in this study. Although Tween 80 and ethanol are generally regarded to be biocompatible, the combination of Tween 80 and ethanol is well known for its nonspecific toxicity and may enhance the toxicity of docetaxel (20,21). Further study is needed regarding the hemolytic properties of docetaxel and Nanoxel.

Striking differences between docetaxel and Nanoxel were found in the platelet study (Fig. 3); docetaxel was able to inhibit platelet aggregation. Although the anti-aggregatory activity of docetaxel has never been studied, it may well suppress aggregation considering its mode of action, stabilizing microtubule structure, because cytoskeletal dynamics are also critical events in the process of platelet aggregation. However, such an anti-aggregatory effect was seen only in collagen-induced aggregation, not in aggregation by thrombin. If the impact on cytoskeleton is the only mechanism contributing to aggregation inhibitory activity, docetaxel would be effective against not only collagen but also thrombin. Therefore, mechanisms other than microtubule-stabilizing activity must be involved in the anti-aggregatory activity of docetaxel, but these remain elusive. In contrast to docetaxel, Nanoxel enhanced platelet aggregation by collagen without any effect on thrombin-induced aggregation. This difference must be ascribed to the difference in docetaxel formulation. Thus, it is
likely that the micellar formulation employed in the preparation of Nanoxel has a pro-aggregatory effect. The molecular mechanism underlying the platelet effect of Nanoxel formulation must be investigated further. This observation suggests the necessity for more detailed safety evaluation of nanoformulations.

ROS generating capability is one of the characteristics of nanomaterials due to the larger surface area and the increased probability of chemical reactions on surface (22). However, such a characteristic appears to be limited to inorganic nanoparticles. Liposomes or micelles with organic surfaces composed of lipids and polymers did not produce ROS. This was also the case with the mPEG-PDLLA micellar formulation of Nanoxel (Fig. 4). Presumably, ROS production is not the main concern of nanoformulations in terms of toxicity.

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CONFLICT OF INTEREST

The authors have no conflict of interest to disclose.

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