A Naphthoquinone Derivative Can Induce Anemia through Phosphatidylserine Exposure-Mediated Erythrophagocytosis

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ABSTRACT
A naphthoquinone derivative, β-lapachone (βL; 3,4-dihydro-2,2-dimethyl-2H-naphtho[1,2-b]pyran-5,6-dione), is receiving huge attention for its potent therapeutic effects against various diseases. However, during the preclinical safety evaluation, repeated oral treatment of βL in rats induced anemia, i.e., a significantly decreased erythrocyte count. In this study, in an effort to elucidate the mechanism underlying the βL-induced anemia, we investigated the effects of βL on erythrocytes with freshly isolated human erythrocytes in vitro and rat in vivo. βL did not induce erythrocyte hemolysis, indicating that direct hemotoxicity was not involved in βL-associated anemia. Meanwhile, phosphatidylserine (PS) exposure along with spherocytic shape change and microvesicle generation, important factors in the facilitation of erythrophagocytosis, were increased significantly by βL. The PS exposure on erythrocytes was from βL-induced reactive oxygen species generation and subsequent depletion of reduced glutathione and protein thiol, which culminated in the modified activities of phospholipid translocases, i.e., inhibition of flip-flop and activation of scramblase. It is important to note that coincubation of macrophage with βL-treated erythrocyte in vitro showed increased erythrophagocytosis, demonstrating that the removal of erythrocyte by macrophage can be facilitated by βL-induced PS exposure. In good accordance with these in vitro results, after oral administration of βL in rats, increased PS exposure and depletion of glutathione were observed along with enhanced splenic sequestration of erythrocytes. In conclusion, these results suggest that βL-induced anemia might be mediated through the PS exposure and subsequent erythrophagocytosis, providing novel insight into the drug-induced anemia.

A naturally occurring naphthoquinone, β-lapachone (βL; 3,4-dihydro-2,2-dimethyl-2H-naphtho[1,2-b]pyran-5,6-dione) from the lapacho (Tabebuia avellanedae) tree is known to have various pharmacological activities, including antiviral, antiprotozoal, and anticancer effects (Dong et al., 2009; Menna-Barreto et al., 2009; Trachootham et al., 2009). These biological activities of βL were suggested to be from the inhibition of DNA topoisomerase activity or the impairment of DNA repair through reactive oxygen species (ROS) generation via NAD(P)H:quinine oxidoreductase 1 (Reinicke et al., 2005; Bey et al., 2007). Reduction of βL by NAD(P)H:quinine oxidoreductase 1 generates unstable hydroquinone and ROS, inducing cytotoxicity against tumor cells through sulfhydryl-mediated reaction and nonspecific enzyme inhibition (Oliveira-Brett et al., 2002). Incidentally, it is well known that ROS generation and sulfhydryl modification can induce cytotoxicity and tissue injuries, but few studies have been focused on the potential toxicity of βL.

Recently, antiobesity and antidiabetic effects of βL have been newly discovered (Hwang et al., 2009; Kim et al., 2009), and preclinical development of βL as a new drug candidate has been initiated. However, during a routine repeat dose-toxicity study, a significant reduction in erythrocyte count, anemia, was observed in the rats treated with βL, raising a concern over its toxicity against erythrocytes. There was no obvious indication of bone marrow toxicity or other nonspecific toxicity in the general histopathology or blood biochemistry examination, signifying that a more complicated and specific mechanism might be involved in the βL-induced anemia.

ABBREVIATIONS: βL, β-lapachone; ROS, reactive oxygen species; GSH, glutathione; PS, phosphatidylserine; MV, microvesicle; A23187, calcimycin, C_{27}H_{37}N_{3}O_{6}; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); FITC, fluorescein isothiocyanate; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; CFDA, 5-(and-6)-carboxyfluorescein diacetate; DMEM, Dulbecco’s modified Eagle’s medium; DCF, dichlorofluorescein; 5-HPQ, 5-hydroxyprimaque.

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Because of the frequent exposure to oxygen-rich environment, erythrocytes often become an easy target for ROS (Banerjee and Kuypers, 2004) and ROS-generating drugs (Beutler, 1969), especially in individuals with decreased glutathione (GSH) levels resulting from the genetic glucose-6-phosphate dehydrogenase deficiency (Fanello et al., 2008), chemotherapy, or other pathophysiological conditions. Depletion of cellular GSH and protein thiol makes erythrocytes more susceptible to ROS, causing various abnormal conditions, including hemolysis (Beutler, 1969), shape change (Lucantoni et al., 2006), and phosphatidylserine (PS) exposure (Banerjee and Kuypers, 2004). Damaged erythrocytes are engulfed and destroyed by tissue macrophages of spleen and liver, a process called erythrophagocytosis, inducing severe anemia as observed in sickle cell anemia or other disease states (Kuypers et al., 1998; de Jong et al., 2001; Lang et al., 2007).

PS exposure, an apoptotic marker on the cell surface is considered as a key signal to tissue macrophages for erythrophagocytosis (Kobayashi et al., 2007; Mercer and Helenius, 2008). The PS exposure on erythrocytes can be induced by intracellular calcium increase and the depletion of ATP and thiol-regulated modification of phospholipid translocase activity (Banerjee and Kuypers, 2004; de Jong and Kuypers, 2006; Barber et al., 2009). PS-exposing erythrocytes frequently accompany apoptotic features such as spherocytic shape change and PS-bearing microvesicle (MV) generation. More importantly, through the various PS receptors or PS exposure and MV generation without hemolysis in human erythrocytes was easily removed by macrophages through erythrophagocytosis (Zhou, 2007; Dasgupta et al., 2008).

In the current study, we discovered that βL could induce PS exposure and MV generation without hemolysis in human and rat erythrocytes. It is important to note that βL-treated erythrocytes were easily removed by macrophages through erythrophagocytosis. Its underlying mechanism was elucidated, and the relevance of its finding to in vivo was examined in a repeat dose study with rats in an effort to provide novel insight into the role of PS exposure in drug-induced anemia.

**Materials and Methods**

**Materials**

βL was chemically synthesized by Mazence Inc. (Suwon, Korea). 1-Palmitoyl-2-(6-((7-nitro-2-1,3-benzoxadiazol-4-yl)amino)[3-carboxy]yl]-sn-glycero-3-phosphoserine and 1-octeyl-2-(6-((7-nitro-2,1,3-benzoxadiazol-4-yl)amino)[3-hexanoyl]sn-glycero-3-phosphocholine were purchased from Avanti Polar Lipids (Alabaster, AL). Glutaraldehyde solution, osmium tetroxide, purified human thrombin, and 5-(and-6)-carboxyfluorescein diacetate (CFDA) were obtained from Invitrogen. Dulbecco’s modified eagle medium (DMEM), Dulbecco’s phosphate-buffered saline, penicillin/streptomycin, and fetal bovine serum were purchased from Invitrogen. Dulbecco’s modified eagle medium (DMEM), Dulbecco’s phosphate-buffered saline, penicillin/streptomycin, and fetal bovine serum were purchased from Invitrogen.

**Measurement of GSH and Protein Thiol Levels**

GSH level in erythrocytes was measured according to the method described by Amer et al. (2004). In brief, vehicle- or βL-treated
erythrocytes were washed and incubated at 37°C for 3 min, with 40 μM mercury orange. Cells were diluted in Ringer's solution and analyzed with an FACSCalibur flow cytometer (BD Biosciences).

Protein thiol concentrations were measured by a modified assay based on a previously described colorimetric method (Di Monte et al., 1984). One milliliter of βL-treated erythrocytes was centrifuged at 7000g for 1 min, and the pellet was resuspended with lysis buffer (5 mM sodium phosphate, pH 8.0) and incubated on ice for 30 min. Total lysate was resuspended with 5% perchloric acid in a ratio of 2:5 and then centrifuged at 7000g for 2 min. The pellet was solubilized in 1 ml of Tris-EDTA buffer (0.5 mM Tris-HCl and 5 mM EDTA, pH 7.6) containing 1% SDS. DTNB (250 μM) was added to the samples, and the change of the absorbance was measured at 412 nm. The amount of protein thiol was calculated on the basis of a glutathione calibration curve and divided by the protein content, which was measured by protein assay kit (Bio-Rad Laboratories, Hercules, CA).

Measurement of Phospholipid Translocation
Phospholipid translocation was measured according to the methods described previously (Hilarius et al., 2004). In brief, erythrocytes (5 × 10^7 cells/ml) were incubated with βL and then loaded with 0.5 μM 1-palmitoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]caproyl]-sn-glycero-3-phosphoserine or 1-oleoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-sn-glycero-3-phosphocholine. Aliquots from the cell suspension were removed at the indicated time intervals and placed on ice-cold Ringer's solution for 10 min in the presence or absence of 1% BSA. The amount of internalized probe was determined by comparing the fluorescence intensity associated with the cells before and after back-extraction. Samples were analyzed with a FACSCalibur flow cytometer (BD Biosciences).

Measurement of Intracellular ATP and Calcium Level
The measurement of ATP and calcium levels was simply modified from the methods described previously by Chung et al. (2007). Samples for detection of intracellular ATP level were adapted to luciferin/luciferase assay in a Luminoskan microplate luminometer (Thermo Fisher Scientific, Waltham, MA) using an ATP assay kit (Sigma-Aldrich). For detecting intracellular calcium increase, erythrocytes were loaded with 3 μM fluo-4 acetoxymethyl ester for 1 h at 37°C in the dark. After βL was incubated for indicated time, samples were analyzed with a FACSCalibur flow cytometer (BD Biosciences).

Measurement of in Vitro Erythrophagocytosis
Mouse macrophage cell line RAW 264.7 cells were purchased from American Type Culture Collection (Manassas, VA) and maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin at 37°C under 5% CO2 atmosphere. RAW 264.7 cells were seeded (1 × 10^6 cells/well) and then observed with phase-contrast microscopy (model IX70; Olympus, Tokyo, Japan). Chemical treated erythrocytes were washed and re-suspended in DMEM and then cocultured with macrophages at 37°C under 5% CO2 atmosphere. For flow cytometric analysis, chemically treated erythrocytes were loaded with 10 μM CFDA for 30 min before cocultivation. After the cocultivation, macrophages were harvested and washed for several times to remove unbound erythrocytes. Samples were analyzed with an FACSCalibur flow cytometer (BD Biosciences). Data from 50,000 events were collected and analyzed using CellQuest Pro software (BD Biosciences).

In Vivo Experiments
Detection of PS Exposure after βL Administration in Rats.
All of the protocols used in vivo experiments were approved by the Ethics Committee of Animal Service Centre at Seoul National University. Male Sprague-Dawley rats (Samtako Co., Osan, Korea) weighing 200 to 250 g were used in all experiments. Before the experiments, animals were acclimated for 1 week. Food and water were provided ad libitum. At the time of experiment, rats were randomly grouped for control (saline, containing 1% sodium lauryl sulfate), 25, 75, and 200 mg/kg βL doses. Four hours after βL was administered per mouth, blood was collected from the abdominal aorta using 3.8% trisodium citrate as anticoagulant. An aliquot of blood sample was diluted 200-fold with the following buffer (10 mM HEPES-Na, 136 mM NaCl, 2.7 mM KCl, 2.0 mM MgCl2, 1.0 mM Na2HPO4, 5.0 mM dextrose, 5 mg/ml BSA, and 2.5 mM CaCl2, pH 7.4) and was stained with annexin V-FTTC for 15 min in the dark. Exposure was measured as described above.

Hematoxylin and Eosin Staining and Prussian Blue Staining of Organs.
Liver and spleen were obtained surgically after blood collection, weighed, and fixed with 10% formalin. The tissue specimens were cut into 4-μm-thick sections and stained with hematoxylin and eosin and ferrocyanide. After counterstaining with nuclear fast red solution, the ferrocyanide-stained slides were dehydrated and mounted. The slides were observed with bright-field microscope (CX41; Olympus).

Statistical Analysis
The means and standard errors of means were calculated for all treatment groups. The data were subjected to one-way analysis of variance followed by Duncan’s multiple range test or Student’s t test to determine which means were significantly different from the control. In all cases, a p value of <0.05 was used to determine significant differences.

Results
During the preclinical development, a significant reduction in erythrocyte counts was observed in the rats treated with βL for 2 weeks (Fig. 1A). However, in freshly isolated human erythrocytes, even the high concentration of βL did not induce direct hemolysis (Fig. 1B). In contrast, PS exposure, an important pathological change of erythrocytes, was significantly enhanced by βL treatment in concentration- and time-dependent manners (Fig. 1, C and D).

Along with the PS exposure, βL caused the release of erythrocyte membrane-derived MVs in concentration- and time-dependent manners (Fig. 2, A–C). In line with the MV generation, βL-treated erythrocytes displayed echinocytes (Fig. 2D, arrow), cells with protruded surface, and MV-shed spherocytes (Fig. 2D, arrowhead), whereas vehicle-treated erythrocytes showed normal discocytes. As shown in Fig. 2E, most of βL-generated MVs (99%) also expressed PS on their surfaces, which can be also engulfed by macrophages (Willekens et al., 2005).

βL-induced cytotoxicity is mainly mediated by oxidative stress through ROS generation (Reinicke et al., 2005). To investigate whether ROS can be induced by βL, erythrocytes were incubated with βL and ROS was measured by DCF fluorescence and luminol-enhanced chemiluminescence. As shown in Fig. 3, A and B, βL increased ROS generation in a concentration-dependent manner. In accordance with the ROS generation, βL caused the depletion of GSH (Fig. 3C) and protein thiol (Fig. 3D), which is critical in the maintenance of cellular homeostasis when cells are exposed to the oxidative stress. To clarify the role of cellular ROS and protein thiol in βL-induced erythrocyte PS exposure, antioxidants and thiol donor were pretreated and PS exposure was measured. Both antioxidants and thiol donor could reverse βL-induced PS exposure (Fig. 3E), suggesting that the thiol...
depletion from βL-induced ROS generation play an important role in the disturbance of PS asymmetry.

The depletion of intracellular protein thiol can activate scramblase and inhibit flippase that induces and maintains PS on the outer membrane, respectively (Tyurina et al., 2007). Consistently, after βL treatment, the scramblase was activated, as measured by increased inward translocation of C6-NBD PC (Fig. 4A), and the activity of flippase was inhibited as determined by decreased inward translocation of C6-NBD PS (Fig. 4B). In addition to the depletion of protein thiol, flippase can also be affected by the depletion of intracellular ATP. As a result, βL was shown to induce ATP depletion in a concentration-dependent manner (Fig. 4C). It is interesting to note that intracellular calcium level, another important mediator of scramblase activation and flippase inhibition, was not increased in βL-treated erythrocytes (Fig. 4D).

PS exposure on erythrocytes can be a signal for the recognition and subsequent erythrophagocytosis by tissue macrophages. First, we confirmed that the βL-induced PS exposure was similar in rat erythrocytes to human erythrocytes (data not shown). To investigate the erythrophagocytosis, βL-exposed rat erythrocytes were cocultivated with murine macrophage cell line RAW 264.7 cells for 18 h. As shown in Fig. 5A,
H9252 L-exposed erythrocytes were bound and engulfed by macrophages. The erythrophagocytosis by H9252 L was further confirmed by significantly increased fluorescent macrophages resulting from phagocytosis of CFDA-loaded erythrocytes (Fig. 5B), which was in a good correlation with H9252 L-induced PS exposure (Fig. 1C).

The effects of H9252 L on PS exposure and splenic sequestration were examined in rats in vivo. Four hours after the oral administration of 0, 75, or 200 mg/kg H9252 L (5 ml/kg), blood samples were collected to determine PS exposure and GSH levels on erythrocytes. H9252 L indeed induced PS exposure and GSH depletion in vivo (Fig. 6A), whereas erythrocyte integrity, as assessed by osmotic fragility-induced hemolysis was not affected (data not shown), in a good accordance with in vitro data. To explore the long-term consequences of the H9252 L-induced erythrocyte PS exposure, rats were administered with H9252 L once daily for 2 weeks; the time when anemia was
observed. The relative organ weights of both liver and spleen were increased significantly (Fig. 6B, top), and more importantly, the spleen of βL-treated rats was accumulated with iron as determined by increased blue-staining with Prussian blue (Fig. 6B, bottom), suggesting that βL accelerated erythrocyte clearance indeed through erythropagocytosis.

**Discussion**

In the present study, we demonstrated that βL could induce anemia indirectly by enhancing erythropagocytosis and subsequent splenic sequestration of erythrocytes through PS exposure without direct cytotoxicity on erythrocytes. βL-enhanced PS exposure was accomplished from the activation of scramblase and the inhibition of flippase by ROS generation and the depletion of GSH and protein thiol. The βL-treated erythrocytes also displayed abnormal shape and MV generation, which could further contribute to the erythropagocytosis by tissue macrophages. These in vitro observations were confirmed in rat in vivo as determined by increased splenic sequestrations of erythrocytes in βL-treated rats. This is the first study showing that drug-induced PS exposure in erythrocytes can induce increased erythrocyte clearance and ultimately anemia, to our best knowledge.

βL and its derivatives are receiving huge interest for potential therapeutic activities in various diseases, including cancer, obesity, and cardiovascular diseases (Dong et al., 2009; Hwang et al., 2009; Kim et al., 2009). Notably, ROS generating capability is suggested as a key mode of action for both therapeutic and adverse effects of βL (Reinicke et al., 2005; Bey et al., 2007). Considering the vulnerability of erythrocytes to oxidative stress, toxicity of βL to erythrocytes could be easily foreseeable. The recent finding of Miao et al. (2008) that a βL derivative could be rapidly sequestered into and metabolized in erythrocytes further supports the specific and selective toxicities of βL to erythrocytes. Besides βL, many ROS-generating drugs are being developed or marketed for therapeutic uses in cancer and other diseases (Davis et al., 2001; Fibach and Rachmilewitz, 2008; Trachootham et al., 2009). In conjunction with our findings with βL, we suggest that serious attention should be paid for the potential toxicity of the ROS-generating drugs to erythrocytes.

The maintenance of GSH level is vital for the protection of erythrocytes from ROS. Exemplifying this, in glucose-6-phosphate dehydrogenase deficiencies where sufficient NADPH is not provided for the maintenance of reduced GSH, erythrocytes are vulnerable to oxidative hemolysis by ROS-generating drugs, such as glibenclamide, doxorubicin, dapsone, and so on (Mason et al., 2007; Ganesan et al., 2009). Excessive ROS and resultant depletion of GSH could alter the activities of vital enzymes or proteins that need reduced cysteine residue and free thiol for their activity (Devaux, 1988; de Jong and Kuypers, 2006), bringing about erythrocyte damages. In the present study, we demonstrated that βL-induced ROS could inhibit flippase and activate scramblase, perturbing the membrane phospholipid asymmetry and inducing PS exposure.

PS exposure is a key signal for the erythrocyte clearance by tissue macrophages (Mandal et al., 2005; Tyurina et al., 2007; Fairn and Grinstein, 2008) and novel PS receptors on splenic macrophages are being identified such as Tim4 (T-cell immunoglobulin- and mucin domain-containing molecule) (Kobayashi et al., 2007; Miyanishi et al., 2007; Park et al., 2007) and PS-binding glycoprotein lactadherin (Dasgupta et al., 2008). Role of PS exposure is well established in the anemia associated with genetic or pathologic disease states, but its implications in chemical-induced anemia have not been fully understood. Recently, Lang et al. (2008) suggested the possible contribution of erythrocyte PS exposure and eryptosis, apoptotic features in erythrocyte in lead-induced anemia through in vitro experiments. However, the clear demonstration of their in vitro results in relevant in vivo model has not been provided yet. Our study showing that βL could induce anemia through increased PS exposure and erythropagocytosis in vivo provides an important piece of evidence for the contribution of PS exposure in chemical-induced anemia.

Recently, Bowman et al. (2005a) reported that 5-hydroxy-primaquine (5-HPQ), a metabolite of primaquine could enhance the clearance of rat erythrocyte by splenic macrophage. They found that 5-HPQ-induced ROS could generate
hemoglobin thyl radical and adduct with membrane protein and suggested that oxidative damage of membrane cytoskeleton and CD47 might increase erythropagocytosis in rat (Bowman et al., 2005b). However, they could not find PS exposure after 5-HPQ treatment in erythrocytes. The discrepancy from our findings might be explained by the difference in species, i.e., between rat and human in our study or the chemicals used. We confirmed that BL could induce PS exposure significantly in rat erythrocytes (data not shown), suggesting that the difference in chemicals is the most probable reason underlying the discrepancy from our study.

In conclusion, we demonstrated that BL-induced anaemia might be mediated by PS exposure and increased erythropagocytosis by spleen through in vitro and in vivo models. Most importantly, this study has provided an important clue for the role of PS exposure and erythropagocytosis in the drug-induced anaemia.

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