Physcion induces hemolysis and premature phosphatidylserine externalization in human erythrocytes

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Summary:

The prevalence of cancer-associated anemia (CIA) is high, and the mechanisms governing its development remain poorly understood. Eryptosis, the suicidal cell death of red blood cells (RBCs), may account for CIA as it is triggered by clinically approved chemotherapeutics including cisplatin and paclitaxel. Physcion (PSN), an anthraquinone extracted from rhubarb and other plants, has shown great promise as an anticancer agent. However, the potential toxicity of PSN to RBCs remains elusive. RBCs were isolated from heparinized blood, and incubated with 10-100 µM of PSN for 24 h at 37°C. Hemolysis was photometrically calculated from hemoglobin concentration in the medium at 405 nm, while flow cytometry was employed to investigate cardinal markers of eryptosis. Phosphatidylserine (PS) exposure was detected by Annexin-V-FITC, intracellular calcium by Fluo4/AM, cellular volume from forward scatter (FSC), and oxidative stress by 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA). PSN induced overt hemolysis at 50 and 100 µM which was not mediated through calcium influx, protein kinase C, casein kinase 1α, or receptor-interacting protein 1. Moreover, PSN caused significant increase in Annexin-V-FITC and Fluo4 fluorescence with no appreciable influence on FSC or DCF values. Accordingly, PSN stimulates premature eryptosis characterized by PS externalization and intracellular calcium overload without cell shrinkage or oxidative damage. In conclusion, this report shows, for the first time, that PSN is cytotoxic to RBCs by inducing hemolysis and programmed cell death which may limit its success as a chemotherapeutic agent.

Keywords: Physcion; anticancer; anemia; eryptosis; calcium.
Introduction:

Physcion (PSN), also known as parietin, rheochrysidin, and 1,8-dihydroxy-3-methoxy-6-methylantracene-9,10-dione, is a quinone extracted from many terrestrial and marine sources, including rhubarb (*Rheum sp.*), Polygoni Multiflori Radix (*Fallopia sp.*), Reynoutria japonica Houtt (*Reynoutria sp.*), Rumex japonicas Houtt (*Rumex sp.*), Rumex dentatus (*Rumex sp.*), and Osmunda japonica Thunb (*Osmunda sp.*). The wide bioactive spectrum of PSN includes antitumor, antimicrobial, and anti-inflammatory properties. PSN ameliorates cytokine release by mast cells and splenocytes, and attenuates atopic dermatitis and sepsis *in vivo*. In particular, PSN has previously been shown to be cytotoxic to a variety of tumors including lymphoma, leukemia, nasopharyngeal, hepatocellular, breast, gastric, colorectal, and cervical cancer cells.

The encouraging results from preclinical studies have paved the way for PSN to be considered as a potential natural alternative for cancer treatment. However, little is known about the safety of PSN to human erythrocytes (red blood cells, RBCs). This is relevant as chemotherapy-induced anemia remains a major obstacle in cancer management. Although the pathophysiology behind it remains elusive, anemia may develop as a consequence to bone marrow suppression, direct RBC hemolysis, or premature eryptosis. Eryptotic cells are rapidly cleared from the circulation by phagocytes that recognize and bind to exposed phosphatidylserine (PS) on the RBC membrane, effectively decreasing oxygen transport throughout the body.

Here, we provide an appraisal of an important safety aspect of PSN as a candidate drug by dissecting the cardinal parameters of hemolysis and eryptosis. Our findings show that PSN induces hemolysis and breakdown of membrane phospholipid asymmetry through calcium accumulation. Moreover, neither PSN-induced hemolysis nor eryptosis required calcium influx from the extracellular space or was associated with cell shrinkage or oxidative damage.
Materials and Methods:

**RBC collection**

This study was approved by the Ethics Committee/IRB of the College of Medicine, King Saud University (Project No. E-20-4544), and all donors provided informed consents. RBCs were separated from blood obtained in lithium heparin vacutainer tubes, and suspended in phosphate-buffered saline (PBS).

**Chemicals and reagents**

PSN from *Radix et Rhizoma Rhei* (purity ≥98.0%) was purchased from Solarbio Life Science (Beijing, China). A 10 mM stock solution of PSN was prepared by dissolving 5 mg in 1.76 mL of dimethylsulfoxide (DMSO). PBS, Hank's balanced salt solution (HBSS), calcium-free HBSS, Annexin-V-FITC, Fluo4/AM, 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA), casein kinase 1α (CK1α) inhibitor D4476, protein kinase C (PKC) inhibitor staurosporin (StSp), receptor-interacting protein 1 (RIP1) inhibitor necrostatin-2 (Nec-2), ROSUp, ATP Content Assay Kit, Catalase (CAT) Activity Assay Kit, Superoxide Dismutase (SOD) Activity Assay Kit, Glutathione Peroxidase (GPX) Activity Assay Kit, and 6-Phosphate Dehydrogenase (G6PD) Activity Assay Kit were obtained from Solarbio. All dyes and inhibitors were dissolved in DMSO to prepare stock solutions of 100 mM. RBCs, making up a 5% hematocrit, were treated with the vehicle control (0.1% DMSO) or 10-100 µM of PSN for 24 h at 37°C, and then the supernatant, live cells, or hemolysates were used for analysis.

**Hemolysis**

Hemoglobin leakage was photometrically determined at λ_{max} of 405 nm, and the percentage of hemolyzed cells was calculated based on absorbance values of cells suspended distilled water (100% hemolysis), as shown below:

\[
\text{Hemolysis} (\%) = \frac{\text{Abs. experimental cells} - \text{Abs. blank}}{\text{Abs. positive control} - \text{Abs. blank}} \times 100
\]

**Phosphatidylserine exposure**
A homogeneous 50 µL aliquot of control and treated cells was washed and resuspended in 150 µL of 1% annexin staining solution and incubated at room temperature in the dark for 10 min. Analysis of FITC fluorescence intensity was then performed by exciting the cells at 488 nm and capturing emitted light at 520 nm using FACSCanto™ II (BD, New Jersey, USA).

**Adenosine triphosphate (ATP)**

Intracellular ATP levels were determined using Solarbio’s ATP content assay kit. In brief, RBC lysates were deproteinized and subjected to a coupled reaction of ATP-dependent hexokinase and G6PD. The absorbance of NADPH, produced by the second reaction, at 340 nm, is proportional to intracellular ATP content.

**Intracellular calcium**

A homogeneous 50 µL aliquot of control and treated cells was washed and resuspended in 150 µL of 5 µM Fluo4 staining solution and incubated at 37°C away from light for 30 min. Following repeated washing to remove unbound dye, cells were analyzed for calcium content at 488/520 nm Ex/Em spectra.

**Oxidative stress**

A homogeneous 50 µL aliquot of control and treated cells was washed and resuspended in 150 µL of 10 µM H₂DCFDA staining solution and incubated at 37°C away from light for 30 min. Cells were washed thrice, and DCF fluorescence was determined at λ_{Ex} = 488 nm and λ_{Em} = 520 nm.

**Antioxidant enzymes**

CAT, SOD, GPX, and G6PD were measured using Solarbio’s colorimetric enzyme assay kits. CAT activity was estimated from hydrogen peroxide decomposition (λ_{max} = 240 nm). SOD activity was inversely proportional to xanthine-derived superoxide formation of purple formazan detected at 560 nm. GPX activity was inversely related to the yellow chromophore at 412 nm indicative of reduced glutathione reaction with (5-5'-dithiobis [2-nitrobenzoic acid]) (DTNB). G6PD activity was determined by monitoring NADPH formation at 340 nm.
**Statistical analysis**

Data are represented as means \( \pm \) S.E.M. In each experiment, autologous cells served as control, and multiple means were analyzed by one-way ANOVA followed by Tukey’s or Dunnett’s *post-hoc* test. In all cases, a cutoff *P* value of <0.05 was used for statistical significance.
Results:

PSN induces hemolysis

Naked hemoglobin contributes greatly to vascular lesions and organ damage associated with hemolytic episodes. To determine the hemolytic potential of PSN, cells were incubated with the vehicle control (0.1% DMSO) or 10-100 µM for 24 h at 37°C, and hemoglobin concentration in the supernatant was then measured. As previously reported\(^{14}\), Fig. 2A shows that PSN, compared to control values, did not elicit significant hemolysis at 5 µM (5.62% vs. 6.90%) or 10 µM (5.62% vs. 8.48%). However, statistically significant hemolysis was observed at 25 µM (5.62% vs. 60.63%), 50 µM (5.62% vs. 62.25%) 75 (5.62% vs. 64.0%), and 100 µM (5.62% vs. 59.84%).

Calcium is a key regulator of cell survival. Therefore, we evaluated the ability of PSN to induce hemolysis following calcium removal from the medium. To this end, control and experimental cells were incubated with and without 50 µM PSN in HBSS and calcium-free HBSS. As depicted in Fig. 2B, although PSN significantly increased the percentage of hemolyzed cells compared to the control (4.26% vs. 16.11%) in presence of extracellular calcium, it was, nevertheless, unaffected by calcium exclusion (3.92% vs. 19.29%). Moreover, we scanned different signaling pathways that play a major role in mediating eryptosis. As the results show in Fig. 2C, PSN-induced hemolysis (3.85% vs. 12.64%) was not significantly ameliorated in presence of 1 µM of StSp (12.64% vs. 17.58%), 20 µM of D4476 (12.64% vs. 14.66%), or 100 µM of Nec-2 (12.64% vs. 14.85%). Altogether, PSN induces hemolysis independently of calcium availability, PKC, CK1α, or RIP1.

PSN causes PS exposure

Cell membrane scrambling results in externalization of PS normally facing toward the cytoplasm. To examine whether or not PSN induces eryptosis, cells were exposed to the vehicle control or to 10-100 µM of PSN for 24 h at 37°C and stained with Annexin-V-FITC as described earlier to label exposed PS. As seen in Fig. 3D, PSN at 10 µM did not significantly increased the percentage of Annexin-V-binding cells in comparison to control values (3.78% vs. 4.80%). However, significantly increased percentages of eryptotic cells were observed following exposure to 50 µM (3.78% vs. 10.51%) and 100 µM (3.78%
Furthermore, as shown in Fig. 3E, ATP levels in control samples were not significantly reduced at 10, 50, or 100 µM of PSN (2.35 mM vs. 2.27, 2.19, and 2.24 mM). Therefore, PSN-induced stimulation of cell membrane scrambling with significant PS exposure is apparently independent of ATP depletion.

**PSN increases cytosolic calcium**

Maintenance of membrane asymmetry is under the control of calcium ion activity. Thus, we were prompted to examine the potential influence of PSN on intracellular calcium levels. To this end, control and experimental cells were labeled with cell-permeable calcium indicator Fluo4/AM for 30 min at 37°C. Compared to control cells, Fig. 4D indicates that while no appreciable increase in Fluo4 fluorescence was observed at 10 µM of PSN (202.16 a.u. vs. 280.0 a.u.), cells treated with 50 µM (202.16 a.u. vs. 311.16 a.u.) and 100 µM (202.16 a.u. vs. 303.0 a.u.) exhibited significantly enhanced Fluo4 fluorescence intensity. Next, in order to investigate the contribution of extracellular calcium to the detected cytosolic calcium increase, control and experimental cells were incubated with and without extracellular calcium and assayed for intracellular calcium content. Figure 4E shows that removal of calcium from the extracellular space did not significantly decrease Fluo4 fluorescence in cells treated with 50 µM of PSN compared to control specimens (292.83 a.u. to 380.33 a.u. vs. 274.0 a.u. to 391.66 a.u.). Congruently, the percentage of PSN-treated cells with elevated Fluo4 intensity was similarly unaffected in comparison to control values upon elimination of extracellular calcium (2.76% to 4.21% vs. 2.0% to 3.71%). Collectively, PSN promotes intracellular calcium accumulation independently of extracellular calcium influx.

**Calcium overload is not associated with cell shrinkage**

In response to calcium accumulation, Gardos channels (calcium-sensitive potassium channels) are activated resulting in potassium chloride and water exit, and reduced cell size. Thus, alterations in FSC as an estimate of cellular volume were measured in control and PSN-treated cells. Interestingly, Fig. 5D reveals no significant decrease in FSC values in cells treated with PSN at 10 µM compared to control cells (70.06 a.u. vs. 70.19 a.u.), 50 µM (70.06 a.u. vs. 70.19 a.u.), or 100 µM (70.06 a.u. vs. 74.14 a.u.).
To confirm the lack of changes in cell volume, we, again, measured FSC values in control and experimental cells upon extracellular calcium deprivation. As the results show (Fig. 5E), average FSC values of control and PSN-treated cells were not appreciably altered in presence (85.38 a.u. to 85.61 a.u) or absence (83.98 a.u. to 85.35 a.u.) of extracellular calcium. Furthermore, Fig. 5F relates Fluo4 fluorescence to FSC intensity in control and treated cells, and shows that Fluo4 increase in treated cells was not accompanied by decrease in FSC values. Therefore, PSN-induced cytosolic calcium overload is not associated with changes in cell volume.

**PSN-induced eryptosis is not mediated through oxidative damage**

Reactive oxygen species (ROS) damage proteins, lipids, and carbohydrates, and leads to eryptosis. To examine whether PSN perturbs the redox status of RBCs, cells were tagged with H2DCFDA for 30 min at 37°C, and DCF fluorescence was subsequently recorded. As shown in Fig. 6B, compared to control cells, no statistically significant increase in DCF fluorescence was observed by PSN at 10 µM (169.83 a.u. to 198.33 a.u.), 50 µM (169.83 a.u. vs. 155.16 a.u.), or 100 µM (169.83 a.u. vs. 227.83 a.u.).

To further investigate the modulatory role of PSN on RBC redox status, key antioxidant enzymes were measured. In congruence with the apparent lack of oxidative damage observed in Fig. 6B, when compared to control values, neither CAT (99.97% vs. 99.94%, 107.73%, and 101.97%; Fig. 6C), SOD (99.99% vs. 103.52%, 98.76%, and 99.65%; Fig. 6D), GPX (100% vs. 101.13%, 99.98%, and 101.99%; Fig. 6E), or G6PD (100% vs. 128.96%, 126.29%, and 124.59%; Fig. 6F) displayed significantly altered activity levels at 10, 50, or 100 µM, respectively.

Next, we sought to examine the effect of PSN on DCF fluorescence in oxidized cells. To this end, RBCs were either treated with the vehicle control or 10-100 µM of PSN in presence and absence of 1 mg/mL of ROSUp for 1 h at 37°C, and ROS were then estimated. As shown in Fig. 6G, PSN at 50 µM significantly inhibited ROSUp-induced increase DCF fluorescence (253.0 a.u. vs. 125.5 a.u.).

Altogether, these data seem to suggest that PSN-induced eryptosis is not preceded by ROS accumulation. Moreover, although PSN does not appear to appreciably modulate the redox balance in RBCs, it rather seems to exhibit a protective effect against ROS inducers by as of yet unidentified mechanisms.
Discussion:

Quinones have been recognized for centuries as alternative therapies for a wide assortment of ailments. PSN is particularly proposed as a candidate for chemotherapy due to promising potential in vitro and in vivo. In this work, we show, for the first time, that antitumor concentrations of PSN are capable of inducing overt hemolysis and premature suicidal death of RBCs. Hemolysis and eryptosis may account for chemotherapy-related anemia associated with many drugs in current clinical use.  

Circulating hemoglobin causes oxidative damage to vital organs, and aggravates renal disease. Moreover, hemoglobin degradation products interfere with immune function and exacerbate inflammatory conditions. PSN also induced significant loss of membrane asymmetry (Fig. 3) resulting in PS externalization. Cells exposing this phospholipid on their outer membrane leaflet are flagged for rapid elimination from circulating blood before complications arise. Eryptotic cells lose membrane elasticity and hence predispose to the development of thrombosis. Furthermore, these cells aggregate with endothelial cells through CXCL16, similarly giving rise to occlusive lesions. To prevent these detrimental consequences, eryptosis may be perceived as a defense mechanism through which senescent and damaged RBCs are removed. Nevertheless, excessive or premature eryptosis, as demonstrated by PSN in this report, may outweigh the capacity of bone marrow to upregulate erythropoiesis, and anemia occurs. Along those lines, further studies are indeed warranted to examine the potential effects of PSN on erythropoiesis.

Calcium signaling is the most commonly disrupted transduction pathway during eryptosis. In our study, PSN caused a profound elevation in cytosolic calcium activity (Fig. 4) which was apparently not mediated through calcium influx since calcium levels did not significantly decrease upon exclusion of extracellular calcium (Fig. 4E & F). A similar observation was also seen in the case of hemolysis (Fig. 2B). Calcium channel activity is accordingly not essential to PSN-induced RBC death. It can, therefore, be inferred that blocking calcium channels, as an adjuvant therapy alongside PSN, may not mitigate the toxic effects on RBCs.
When intracellular calcium activity increases, potassium channels, under the regulation of calcium, mediate potassium chloride efflux, membrane hyperpolarization, and water loss. The end result is significant cell shrinkage due to diminished cellular volume. However, our study revealed that despite elevated calcium activity (Fig. 4), no appreciable reduction in cell size followed (Fig. 5). This anomaly may be attributed to inhibition of Na\(^+\)-K\(^+\)-ATPase pump which could overrule calcium-induced potassium exit. Of note, other stimulators of eryptosis, including the closely related quinone emodin, amiodarone, and ellipticin, do not significantly modify cell volume.

Accumulation of ROS impairs the capacity of RBCs to carry oxygen and thereby triggers eryptosis. Oxidative stress may, likewise, result from enhanced calcium activity (Fig. 4). Moreover, PSN has been shown to induce overproduction of ROS in nucleated cells. Nonetheless, our data show that PSN-induced eryptosis is not mediated through oxidative stress (Fig. 6), unlike emodin, but similar to micafungin, triclosan, and N,N-diethyl-3-methylbenzamide. Therefore, counteracting eryptosis by antioxidants is unlikely to attenuate PSN-induced cell death.

This is also the case with hemolysis under inhibition of major signaling kinases including PKC, CK1\(\alpha\), and RIP1 (Fig. 1C). PKC isoforms are involved in a variety of cellular processes including growth, development, survival, and apoptosis. In erythrocytes, PKC inhibition has been shown to be protective against energy exhaustion as well as some toxic agents. CK1\(\alpha\) similarly mediates eryptosis secondary to energy exhaustion, oxidative stress, and calcium loading. Moreover, this kinase also participates in eryptosis elicited by various compounds as reported by us and others. RIP1 is a unique kinase that signals for both apoptosis and necroptosis, and is involved in triclosan-induced RBC death as we have previously shown. It seems that PSN may not require the activity of a distinct enzyme or that blocking a single mediator is overruled by others.

In conclusion, the current study reveals a novel role of PSN in stimulating premature and suicidal erythrocyte death that is associated with PS exposure and calcium overload (Fig. 7). This toxic effect predisposes to the development of anemia, and, therefore, the clinical application of PSN in chemotherapy may require further assessment.
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Conflict of Interest:

The authors declare no conflict of interest.

Supplementary Materials

The online version of this article contains supplementary materials.
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**Figure 1: Molecular structure of PSN.** Characteristic quinone group dione (9,10) is visible, along with phenol and methoxy groups.
Figure 2: PSN causes hemolysis. (A) Arithmetic means ± SEM of PSN-induced hemolysis. (B) Arithmetic means ± SEM of PSN-induced hemolysis with and without extracellular calcium. (C) Arithmetic means ± SEM of cells treated with the vehicle, 50 µM of PSN, or a combination of 50 µM of PSN and 20 µM of D4476, 1 µM of StSp, or 100 µM of Nec-2. Cells were incubated for 24 h at 37°C under different experimental conditions and hemolysis was calculated from hemoglobin leakage. ns indicates not significant, while **(P<0.01) and ****(P<0.001) indicate significant difference from control (one-way ANOVA).
Figure 3: PSN induces eryptosis. Representative histograms of Annexin-V-FITC fluorescence of cells treated with the vehicle control (A) and with 100 uM PSN (B), and an overlay of the two peaks (C). Arithmetic means ± SEM of the percentage of cells with enhanced PS externalization (D) following staining of control and experimental cells with Annexin-V-FITC for 10 min at room temperature away from light. Intracellular ATP levels are shown under control and experimental conditions (E). ns indicates not significant, while **(P<0.01) indicate significant difference from control (one-way ANOVA).
**Figure 4: PSN promotes intracellular calcium accumulation.** Representative histograms of Fluo4 fluorescence of control (A), 100 uM PSN-treated cells (B), and an overlay of the two peaks (C). Also shown are arithmetic means ± SEM of Fluo4 MFI (D) and the percentage of cells with high Fluo4 fluorescence (E), identified by Fluo4/AM staining after incubation of control or 10-100 uM PSN-treated cells for 24 h at 37°C. ns indicates not significant, whereas ***(P<0.001)*** indicate significant difference from control (one-way ANOVA).
Figure 5: PSN does not influence cell volume. Representative histograms of FSC of control (A), 100 µM PSN-treated cells (B), and an overlay of the two peaks (C). Also shown are arithmetic means ± SEM of FSC geomean values of control and experimental cells in presence (D) and absence (E) of extracellular calcium (E). A dot-plot depicting the distribution of control (black) and experimental (brown) cells based on FSC values as a function of Fluo4 fluorescence. ns indicates not significant, whereas ***(P<0.001) indicates significant difference from control (one-way ANOVA).
Figure 6: Lack of oxidative stress in PSN-induced eryptosis. Representative histograms of DCF fluorescence of control, 100 µM PSN-treated cells, and an overlay of the two peaks (A). (B) Arithmetic means ± SEM of DCF fluorescence of control and PSN-treated cells. Arithmetic means ± SEM of the activity of antioxidant enzymes CAT (C), SOD (D), GPX (E), and G6PD (F). DCF fluorescence in presence and absence of 1 mg/mL of ROSUp with and without 10-100 µM of PSN (G). Control and experimental cells were incubated for 1 or 24 h at 37°C, stained with H₂DCFDA for 30 min at 37°C, and analyzed for DCF fluorescence at 520 nm. ns indicates not significant, while * (P<0.05) indicates significant difference from ROSUp-treated cells (one-way ANOVA).
Figure 7: A working model of PSN-induced premature RBC death. PSN causes premature hemolysis evident as significant increase in cell-free hemoglobin, and surviving cells undergo eryptotic transformation culminating in breakdown of membrane asymmetry with phosphatidylserine externalization to the outer membrane leaflet. PSN-induced cell death is mediated through dysregulation of cellular calcium homeostasis leading to cytosolic accumulation with no appreciable influence on cell volume or redox status.