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Flavonolignans inhibit ADP induced blood platelets activation and aggregation in whole blood

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ABSTRACT

Flavonolignans are a group of active chemical compounds presented in the silymarin – a standardized extract obtained from fruits and seeds of Milk thistle (*Silybum marianum* L. Gaernt.). Since the 70 s of the last century, flavonolignans have been regarded to the official medicine as a substances having hepatoprotective properties. However many researches performed in recent years have demonstrated that flavonolignans posses many other healthy properties including modulation of variety cell-signaling pathways.

The aim of our study was to examine the effects of three major flavonolignans (silybin, silychristin and silydianin) on ADP-induced blood platelet activation using the flow cytometry analysis as well as determine the mechanism of this interaction by bioinformatic ligand docking method.

We observed that all tested flavonolignans in dose-dependent manner inhibit formation of blood platelet aggregates and microparticles as well as decrease expression of P-selectin and activation of integrin $\alpha_{IIb}\beta_3$. Our computer-generated models confirm the flow cytometry analysis. We observed that all tested flavonolignans have conformations which are able to bind to the extracellular domain of P2Y12 receptor and probably block interaction with ADP.

Our studies may help in the development of a new potential anti-platelet agent, which might be an alternative to the current using drugs.

1. Introduction

Flavonolignans are a group of active chemical compounds presented in the silymarin – standardized extract obtained from fruits and seeds of Milk thistle (*Silybum marianum* L. Gaernt.). Milk thistle is an annual to biannual plant of the *Asteraceae* family, flowering in July–August (with reddish-purple flowers). Native habitats of *S. marianum* are southern Europe, southern Russia, Asia Minor, northern Africa and is naturalized in North and South America as well in South Australia regions [1]. Milk thistle has been used for thousands of years as a remedy for a variety of ailments. In the ancient Greece, this plant was administrating to cure a liver dysfunctions [2]. In the 1970s silymarin which represents 1.5–3% of the dry weight of *S. marianum* seeds has been regarded to official medicine as substance having an hepatoprotective properties and since this time has been used for liver treatment [3]. However many studies have demonstrated that flavonolignans presented in silymarin have many other healthy properties. Have been shown that these compounds posses strong antioxidant properties as well as modulate of variety cell-signaling pathways. Additionally, flavonolignans were also tested as a potential anticancer and chemopreventive agents [4]. Flavonolignans structurally are composed of a flavonoid unit (taxifolin) and a phenylpropane unit (coniferyl alcohol), linked by an oxeran ring [5,6]. This type of the connection is presents at the formation of lignans and thus comes the name of this group of compounds [7]. The main representatives of flavonolignans are silybin, isosilybin, silychristin, isosilychristin silydianin and silimonin [4].

The blood platelets, the smallest morphotic elements of the blood are crucial for the coagulation physiology to maintain hemostatic balance and are involved in various pathological conditions such as atherosclerosis and thrombosis. Due to a large number of specific membrane receptors blood platelets are high reactive cells, readily activated by many physiological and unphysiological agonists [8]. Blood platelets play a huge role in both primary and secondary hemostasis. In primary hemostasis activated platelets adhere to the vascular injury site which leads to platelets aggregation and formation of the platelet plug that seals the breach in the vessel wall and prevents excess of blood loss [9,10]. Next, activated blood platelets express on their surface the negatively charged phospholipids which provide a catalytic surface for the major interactions of the coagulation cascade and clot formation in secondary hemostasis [11–14].

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The uncontrolled platelet's activation is one of the most important risk factor of the cardiovascular system disturbance associated with the occurrence of thromboembolic complications [15]. Platelets are able to forming pathogenic, occlusive intracoronary thrombi which are responsible for acute ischemic events [16] leading to ischemic acute coronary syndromes, stroke as well deep vein thrombosis. These pathological states are one of the major reasons of deaths or chronic conditions that limit the quality of life and generate high costs of therapy and care. For these reasons on whole world researches are trying to find novel anti-platelet agents that would have a significant effect on the maintenance of the proper hemostatic stability.

The aim of our study was to examination the effects of three major flavonolignans (silybin, silychristin and silydianin) on blood platelets activation by ADP as well as determine the mechanism of this interaction by bioinformatic ligand docking method.

2. Materials and methods

2.1. Reagents

Dimethyl sulfoxide (DMSO), Tris, flavonolignans: silybin, silychristin and silydianin (structures presented in Fig. 1) were obtained from Sigma-Aldrich Chemical Co (St. Louis, MO, USA). Flow cytometry reagents: anti-CD61/PerCp, anti-CD61/PE, anti-CD61/FITC, anti-CD62/PE, PAC-1/FITC, isotype controls and CellFix were obtained in Becton Dickinson (San Diego, CA, USA). ADP was purchased in Chrono-Log (Havertown, PA USA). All other chemicals were reagent grade or highest quality available products.

2.2. Blood samples preparation

The study on the human blood samples was performed under the guidelines of the Helsinki Declaration for Human Research and approved by the Committee on the Ethics of Research in Human Experimentation at the University of Lodz (KBBN-UŁ/II/17/2011). Blood samples collected from 12 different healthy donors were purchased from the Regional Center for Transfusion Medicine in Lodz (Poland). All samples were taken in the morning in the fasting status. All donors were checked by a medical doctor and had no cardiovascular disorders, allergy, lipid or carbohydrate metabolism disorders and were untreated with any drugs [17].

Whole blood samples were preincubated with flavonolignans (silybin, silychristin and silydianin) at the concentration range of 10–100 μM by 30 min at 37 °C. All tested compounds were initially dissolved in 20% DMSO to the preliminary concentration of 20 mM. Other solutions of used compounds were also performed in 20% DMSO (prepared in 50 mM TBS, pH 7.4). The final concentration of DMSO in all samples was 0.1%. In control samples the same volume of solvent (20% DMSO prepared in 50 mM TBS, pH 7.4) was added and probes were warmed 30 min at 37 °C.

2.3. Flow cytometry studies of platelet reactivity in whole blood samples

The fresh whole blood samples (control or preincubated with flavonolignans) were activated by 20 μM ADP (10 min, 37 °C). Additional one sample was not activated. Next, samples were fixed in 1% Cellfix concentration at 37 °C (10 μl of blood + 90 μl of Cellfix solution). After 1 h of fixation samples were stained with specific antibodies: anti-CD61/PerCP, anti-CD62P/PE and PAC-1/FITC (3 μl of each antibodies + 10 μl of sample) and left behind for 30 min in a dark in RT. For compensation procedure set of probes with all used fluorochromes was prepared (10 μl of not activated sample was stained by 3 μl of anti-CD61/PerCP or anti-CD61/PE or anti-CD61/FITC) additional one unstained sample was prepared. Blood platelets in whole blood samples were detected according to PerCP fluorescence. Fluorescence of 10,000 platelets (CD61/PerCP-possitive objects) was measured using LSR II Flow Cytometer (Becton Dickinson, San Diego, CA, USA). The specific fluorescence fractions from platelets were obtained after subtraction of nonspecific fluorescence in the control samples (labeled with anti-CD61/PerCP and proper isotype control). In CD61/PerCP-possitive objects PE and FITC fluorescences were detected and reported values were presented as fractions of CD62P or PAC-1 positive platelets and represent the expression of a given target antigen. Additionally, the fraction of platelet-derived microparticles as well as platelet aggregates in whole blood samples were measured in experiments of P-selectin expression and PAC-1 binding to platelets.

Based on the size and granularity forward light scatter (FSC) vs. side light scatter plots (SSC) characteristic we determined in CD61/PerCP positive objects formation of platelet aggregates as well as release of platelet-derived microparticles. Using reference beads we estimated FSC gates, CD61PerCP positive objects with FSC lower than 10^3 were characterized as a microparticles while objects with FSC higher than $10^{4,4}$ were considered as a aggregates. Gates for PE and FITC fluorescences were estimated based on fluorescence of unstained probes. In case of PE the objects with fluorescences level higher than $10^{3,2}$ were characterized as platelets with surface expression of P-selectin, while in case of FITC with fluorescences level higher than $10^{2,2}$ were characterized as platelets with PAC-1 antibody binded. The percentage values of CD62P positive platelets and PAC-1 positive platelets were calculated relative to the total number of platelets (CD61 positive cells) present in each sample. All data analysis were performed in FACSDiva Version 6.1.2.

2.4. Ligand docking

The ligand dockings for the selected flavonolignans were calculated *in silico* with Autodock Vina 1.0, an algorithm released by Scripps Research Institute [18] (http://vina.scripps.edu/) according to previous used procedure [19–21]. Protein coordinates were found on the site of the RSCB Protein Data Bank (http://www.rcsb.org). All three-dimensional chemical structures of flavonolignans and clopidogrel active metabolite were downloaded from PubChem (http://pubchem.ncbi.nlm.nih.gov/) and converted to pdb files using Avogadro 1.1.0, an open-source molecular builder and visualization tool (http://avogadro.openmolecules.net/) [22]. Molecular modeling was performed using human P2Y purinoreceptor 12 PDB structure 4PXZ [23] as a receptor. Receptor preparation for docking procedure was performed in Swiss-PdbViewer (http://spdbv.vital-it.ch/). The nonbonded atoms, present in the crystal structure were removed. Next, the receptor structure was adapted in Auto Dock Tools v 1.5.6rc1 (http://autodock.scripps.edu) [24]. In first stage missing hydrogen atoms were added, after that both partial atomic Gasteiger charges [25] and Kollman [26] united atom charges were calculated and assigned. Non-polar hydrogens were merged, and rotatable bonds were assigned, keeping all the amide bonds as non-rotatable. The receptor file was saved as pdbqt format, which is pdb plus "q" charges and "t" AutoDock type. Vina was run with settings: center_x 18.334; center_y 0.877; center_z 50.548; size_x 126; size_y 126; −size_z 126. The binding places were computed and the binding affinity of the ligand to the receptor was counted in kcal/mol. Docking procedure was repeated 12 times. Obtained affinities were presented as mean ± SD.

The analysis and visualization of the three-dimensional structure of the protein with the bound ligand was performed with Python Molecular Viewer of Auto Dock Tools v 1.5.6rc1 (http://autodock.scripps.edu) [24] and Swiss-PdbViewer (http://spdbv.vital-it.ch/) [27]. Validation of the docking protocol was performed using 2MeSADP compound, which was docked to the same place