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Identification of Quercetin as a Potential Band 3 Protein Antioxidant Using Ektacytometry and in Silico Molecular Docking Technology

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Abstract

The purpose of this study was to analyze the protective action of natural flavonoid to the reactive oxygen stress on the Band 3 protein by ektacytometry and computational docking technology. For this, quercetin, a major active ingredient of the plum and onions were used as ligand for molecular interaction. The 3D crystallographic structure of molecular target Band 3 was obtained from PDB database (PDB ID: 4YZF). 4KU(2,2'-ethane-1,2-diylbis{5-[(sulfanylmethyl)amino]benzenesulfonic acid}), a Band 3 protein ligand was taken as the standard for comparative docking analysis. Quercetin significantly blocked H_2O_2-induced oxidative stress on the red blood cell membrane deformability. In addition, quercetin showed optimum binding affinity with a molecular target Band 3 with the binding energy of −7.46 kcal/mol as compared to the standard 4KU(−7.11 kcal/mol). These results strongly indicated that quercetin could be one of the potential ligand to protect reactive oxygen species mediated erythrocyte membrane damage and related blood circulation diseases.

INTRODUCTION

Computer aided drug discovery(CADD) and bioinformatic technologies have become very important resource to identify the potential targets for various ligands. In silico drug screening technology offers the advantage of identifying lead compounds from several potentially useful hit compounds. These molecular docking technologies offer very efficient and fast methods to do so[1,2]. Researchers in the pharmacology field have employed the new drug development technologies successfully to determine potentially useful binding sites and used the results to improve, indentify, and develop drugs that fit better into the binding pocket in the target proteins[2].

The main role of erythrocytes is to mediate the oxygen–carbon dioxide exchange between tissues and lungs. Since erythrocytes are easily exposed to oxidative stresses, H_2O_2, ascorbic acid, and azocompounds are frequently used as good models to investigate the effect of the reactive oxygen species(ROS) on the red blood cell membrane. The change of the Red blood cell(RBC) membrane rigidity is mainly due to a reduction in mobility of the proteins embedded in the phospholipidic bilayer. One of the most studied integral membrane proteins is Band 3 protein(Fig. 1b)[3]. It is responsible for gas exchange, ion balance across cell membrane, osmotic and mechanical properties of the erythrocyte, and cell shape maintaining. Many morphological and anemic disorders in the erythrocytes are caused by oxidative stress effect on the Band 3 protein[4]. Therefore, it could be a important issue to find novel protective molecules for maintaining Band 3 structure against oxidative stress.

Quercetin(Fig. 1a) is a famous antioxidant. It is found in citrus fruit(plum), buckwheat and onions, and preliminary studies proved its potential therapeutic qualities and cancer protection[5]. But, the pharmacological effect of quercetin on the Band 3 protein has not been well studied. Using ektacytometry and molecular docking technique, we investigated the potential effect of quercetin as a novel protector for the H_2O_2-induced oxidative stress on Band 3 protein and red blood cell deformability. In this study, the structural 3D model of the quercetin in Band 3 protein active site has been performed, which may expedite further development of more natural Band 3-protectors to control reactive oxygen species induced blood circulation diseases.

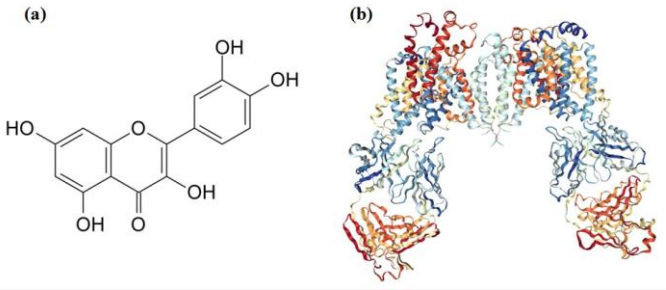

Figure 1: (a) Chemical structure of the quercetin. (b) Three-dimensional structure of the erythrocyte Band 3 protein (PDB ID = 4YZF)

MATERIALS AND METHODS

Red blood cell deformability

All chemicals were purchased from Sigma Aldrich Korea. Blood was obtained from healthy volunteers who provided

informed consent. 16 healthy adults, ages 20–29 years, volunteered their blood samples. In each person, 6μl of blood was collected from the finger tip using lancet. Red blood cell deformability was measured by a slit flow ektacytometer based upon analysis of RBC laser diffraction images at various levels of shear stress (RheoscanD slit-flow ektacytometer, RheoMeditech Inc., South Korea) using cells suspended at about 1% hematocrit in a viscous, isotonic solution of 360 kDa polyvinylpyrrolidone. RBC deformation at shear stresses between 1 and 20 Pa was quantified by calculating an elongation index (EI) equal to (L−W)/(L+W) where L is the length and W is the width of the deformed cell; at a constant shear stress, EI increases with cell deformability[6]. The significance level of the erythrocyte deformability among the blood samples were set at *$p \leq 0.05$ versus H_2O_2 alone. All data were represented as the means ±SEM (Standard Error of the Means).

Molecular docking analysis

The three-dimensional structure of Band 3 protein (PDB: 4YZF) was downloaded from the RCSB protein Data Bank[7]. The chemical structure of quercetin was obtained from PubChem compound database(Pubchem CID = 5280343). It was prepared by ChemBioDraw and MOL SDF format of this ligand was converted to PDBQT file using PyRx tool to generate atomic coordinates[8]. For docking analysis, PDB coordinates of the target protein and quercetin molecule were optimized by Discovery Studio version 4.5 software. These coordinates had minimum energy and stable conformation. The active sites are the coordinates of the ligand in the original target protein grids, and these active binding sites of target protein were analyzed also using the Discovery Studio version 4.5 and NX-QuickPharm program(Neuronex Inc., South Korea)[9]. A computational ligand-target docking approach was used to analyze structural complexes of the Band 3(target) with quercetin(ligand) in order to understand the structural basis of this protein target specificity. Docking was carried out by PyRx, AutoDock Vina, and NX-QuickPharm option based on scoring functions. The energy of interaction of quercetin with the Band 3 protein is assigned "grid point." At each step of the simulation, the energy of interaction of ligand and protein was evaluated using atomic affinity potentials computed on a grid. The significance level of the quercetin binding affinity on the Band 3 was set at *, $p \leq 0.05$ versus 4KU. All data were represented as the means ±SEM (Standard Error of the Means).

RESULTS

Effect of quercetin on the H_2O_2-induced oxidative stress on the erythrocyte.

In many previous studies, oxidative stress induced by H_2O_2 significantly decreased the deformability of erythrocytes[10], and erythrocyte deformability could be used as an index to reflect oxidative damage. As shown in Fig. 2 and 3, H_2O_2 (100μM) dramatically reduced elongation index(EI) of the red blood cell membrane compare to the control group. Quercetin(10μM) pretreatment(5min before), on the other hand, effectively protected H_2O_2-induced oxidative stress on the erythrocytes. Quercetin alone did not affect the deformability of the erythrocytes (Fig. 2, Fig. 3c and 3g). Interestingly, at shear stress 3 Pa, H_2O_2 treated group(red circle line) showed the big difference of the RBC membrane shape than the standard(green circle line) (Fig. 3b) and quercetin group significantly inhibited H_2O_2's stress effect (Fig. 3d). These results clearly depict that quercetin has RBC membrane protective effect on the H_2O_2-induced oxidative stress.

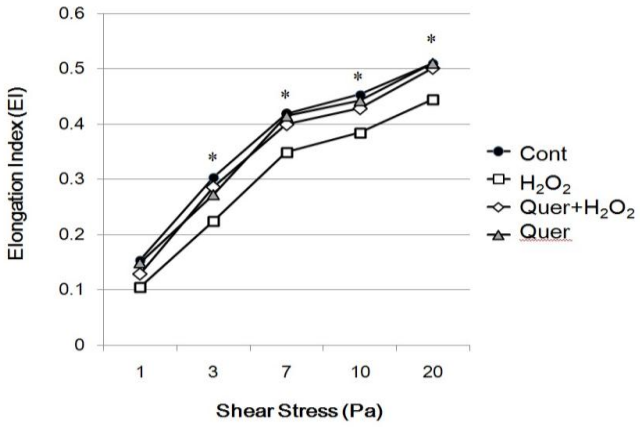

Figure 2: Plot of the erythrocyte elongation index(EI) changes in a shear stress dependent manner. The results are the mean S.E.M. of four independent volunteers. *, P< 0.05 value mean quercetin + H_2O_2 (diamond symbol) treated groups compared with H_2O_2 alone (white box symbol).

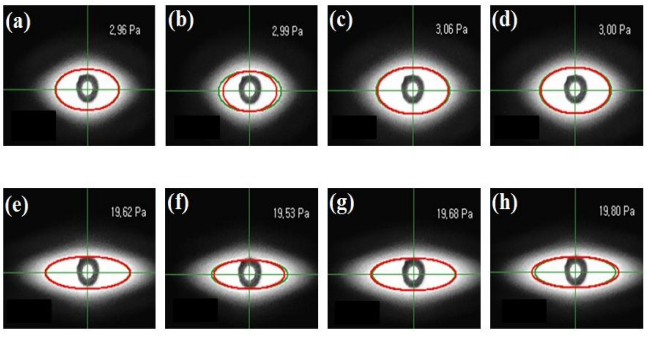

Figure 3: Experimentally obtained RBC membrane RheoscanD image patterns at shear stress ~3 Pa (upper panel) and at shear stress ~20 pa (lower panel). Control blood sample (a and e), H_2O_2-treated blood sample (b and f), quercetin pretreated sample with H_2O_2 (d and h), and quercetin alone (c and g), respectively. Green circle line means standard and red circle line means detected RBC membrane deformability.

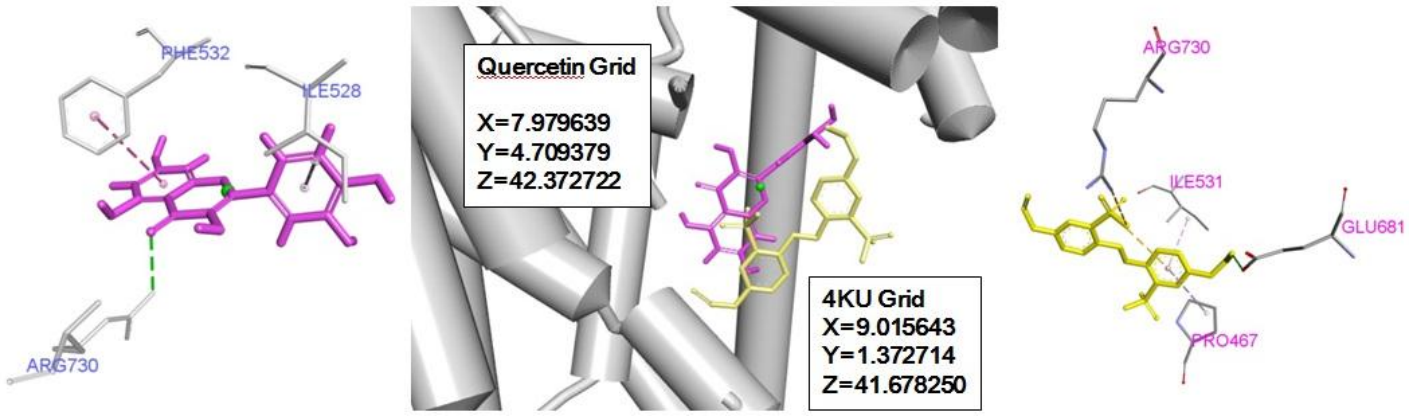

Figure 4: Three dimensional molecular docking pattern and binding position of the 4KU (yellow color, Grids X=9.015643/Y=1.372714/Z=41.678250) and the quercetin (purple color, Grids X=7.979639/Y=4.709379/Z=42.372722) on the Band 3 protein (grey color) active site.

Quercetin binds in high affinity to the Band 3 active site

PyRx Autodock 4 docking analysis was applied to investigate the molecular binding interactions of quercetin and 4KU molecules, respectively with Band 3 (Fig. 4) and to elucidate the possible molecular mechanism. As shown in Table 1, quercetin interacted with 3 amino acid residues (Conventional hydrogen bond: ARG 730, Pi-Alkyl interactions: ILE 528 Pi-Pi T shaped interaction: PHE 532) (Fig. 4 and Table 1) and 4KU also interacted with 3 amino acid residues (Conventional hydrogen bond: GLU 681, Pi-Alkyl interactions: ILE 531 and PRO 467) (Fig. 4B and Table 1). The shortest bond length between the quercetin and the Band 3 was 3.03Å (Table 1). The longest bond length between the 4KU and the Band 3 was 5.19Å (Table 1).

Table 1: Molecular interactions and interacting residues of the Band 3 protein with 4KU and quercetin.

Ligands	Amino Acids	Bond Length (Å)	Types of Interaction
4KU	ILE 531 PRO 467 GLU 681	5.13 5.19 3.57	Pi-Alkyl Pi-Alkyl Hydrogen bond
Quercetin	PHE 532 ARG 730 ILE 528	5.07 3.03 4.10	Pi-Pi T shaped Hydrogen bond Pi-Alkyl

The average molecular binding affinity (docking energy) scores of the 4KU and quercetin on the Band 3 target were -7.11 kcal/mol (4KU, SEM ±0.35) and -7.46 kcal/mol (quercetin, SEM ±0.23) (Table 2).

Table 2: Binding affinity (Docking Energy) of the 4KU and quercetin to the Band 3 protein active site.

Ligands	Binding Affinity (Docking Energy : kcal/mol)	SEM
4KU	-7.11	±0.35 (n=9)
Quercetin	-7.46 *	±0.23 (n=9)

The numbers of the binding modes of both ligands to the Band 3 active site were nine (n=9), respectively. The average bond length of the quercetin (4.06Å) among the interacting amino acids in Band 3 protein active site was shorter than 4KU (4.63Å) (Table 1). Based on the present molecular docking data, quercetin appeared as a strong binder to the Band 3 target protein than the 4KU.

DISCUSSION

Many previous reports are supporting the hypothesis that dietary consumption of antioxidants including flavonoids effectively prevent the incidence of the ROS-related diseases, in particular, cardiovascular diseases[11,12]. RBC Deformability plays a critical role in blood circulation since they RBCs have to pass through capillaries whose diameter is smaller than their size. This is possible if the cells are sufficiently deformable. But if it is not so, as it takes place in some diseases, then the oxygen delivery to organs and tissues of the human body is violated. Impaired deformability of RBCs, which are often observed in diabetes mellitus, can be used to diagnose and monitor patients at a risk for diabetic vascular complications at earlier stage[13,14]. The aim of the present study was to evaluate the effect of quercetin, one of

the most abundant flavonoids in herbs and fruits, on H_2O_2-mediated impairment of erythrocyte deformability. Several previous studies have revealed that lipid peroxidation of RBC membrane resulting in the lowering of its deformability[15,16]. In contrast, Snyder, et al showed that spectrin-hemoglobin aggregation and the alterations in membrane function were completely prevented by prior exposure of the erythrocytes to carbon monoxide[17]. Bilto and Abdalla[18] also reported that quercetin increased the filterability of erythrocytes through 5 microns diameter pores. In this study, we used Rheoscan-D ektacytometry leading-edge technologies including microfluidics, laser-diffraction, image-processing. This technology only requires extremely small amount of blood (5~6μl) and short measuring time (30 sec) per test. Our experimental results imply that quercetin affects the physicochemical property of erythrocytes membranes. It was noteworthy that quercetin pretreatment significantly protected erythrocyte deformability when it was mixed with erythrocyte suspension with H_2O_2 (Fig. 2 and Fig. 3d, 3h). In accordance with the previous reports, our data also demonstrated that quercetin effectively protected erythrocyte deformability againt the ROS-mediated damage (Fig. 2 and Fig. 3).

The erythrocyte membrane skeleton consists of an extensive network of proteins composed principally of spectrin, which is complexed with actin, band 4.1, and band 4.9. Spectrin may also regulate cell shape and surface characteristics by its ability to attach to the integral membrane protein, Band 3[19,20]. Lopes, et al showed that the presence of higher fibrinogen concentrations, similar to those found in inflammatory conditions, erythrocyte deformability is increased only when Band 3 protein is dephosphorylated by the presence of syk inhibitor and at low shear stress[21]. Quercetin prevented peroxynitrite-induced inhibition of PTP activity and, more importantly, to inhibit the activity of the non-src kinase syk, a kinase that is upstream in the Band 3 tyrosine phosphorylation cascade[22]. Quercetin has a planner structure favorable to enter into cell membranes effectively and has strong affinity with liposomal membranes. Thus, it is reasonable to check whether the quercetin chemical structure is appropriate for interacting with Band 3 protein, as well as to exert antioxidant effect. The computational docking of Band 3 target with quercetin using Auto docking procedure revealed that all the lowest energy complexes of Band 3 are stabilized by intermolecular hydrogen bonds and aromatic ring stacking interactions (Table 1). The calculated final docked energies for quercetin is −7.46 kcal/mol and for 4KU (standard) is −7.11 kcal/mol (Table 2). Docking results revealed that this quercetin compound can enter the substrate-binding region of the Band 3 active site bettern than 4KU. The interacting bond lengths and patterns of the ligand among the Band 3 amino acids might be critical factors for regulating target protein activity.

CONCLUSION

These results demonstrated clearly that quercetin accurately interact with Band 3 protein target. Therefore, quercetin might play an important role in inhibiting ROS-mediated erythrocyte membrane damage. These data also suggest that computer aided drug design process using PyRx, Discovery Studio 4.5, and NX-QuickPharm tools is highly reliable and can be a good example for indentifying the action mechanism between the Band 3 and its interacting ligands.

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