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Effect of glycyrrhizin and its derivatives on integrity of human red blood cells

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ABSTRACT

Introduction: The first and most prevailing cells that glycyrrhizin (GL) and glycyrrhetinic acid (GA) encounter are red blood cells (RBCs). However, what follows this event is poorly understood. This study aims to evaluate the effect of GL and its derivatives on the integrity of human RBCs.

Methods: The integrity of human RBC was assessed under normal isotonic conditions and following osmotic and nystatin-induced colloid-osmotic stress by measuring the amount of hemoglobin released. The pore size was determined by the osmotic protection method.

Results: GL was found to be virtually non-hemolytic. However, removal of the carbohydrate moiety of GL imparted significant RBC lytic activity to the cis-(beta-) but not to the trans-(alpha-) isoform of GA. The hemisuccinate radical at position C3 (carbenoxolone) greatly diminished the hemolytic property of GA. The RBC lysis occurred by colloid-osmotic mechanism due to the formation of hydrophilic pores with the radius of ~2.3 nm. At the sublytic doses, the two stereo-isoforms displayed opposite effects on the osmo-resistivity of human RBC: osmoprotection for alpha-GA and osmotic sensibilization for beta-GA. Similar osmotic sensibilization was also observed for GL and carbenoxolone. The two stereo-isoforms exhibited different but not opposite weakening effects on the resistivity of the RBC to the colloid-osmotic stress induced by nystatin, a pore-former. The weakening effect was found intermediate for GL and absent for carbenoxolone.

Conclusion: Upon intestinal digestion and absorption, depending on the structure and dosage, the GL hydrolysis products interact with RBC with both beneficial and detrimental consequences.

Implication for health policy/practice/research/medical education:
This paper provides experimental evidence of the RBCs lysis in the presence of GL hydrolysis products, as well as for protection or sensibilization towards osmotic and colloid-osmic stress at the sub-lytic doses. These results have implications for the pharmacodynamics of GL and therapeutic strategies when using licorice-derived pharmaceuticals.

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Introduction

Licorice (*Glycyrrhiza glabra*) is a medicinal plant widely used in the geographically diverse traditional ethnomedicine since antiquity (1-4). Among a number of phytochemicals found in licorice, glycyrrhizic acid or glycyrrhizin (GL) was found to be a major component of the root extracts, reproducing most of the therapeutic effects of the plant (5-9). When tested in various disease models, both *in vitro* and *in vivo*, GL exhibited impressive pharmacological activities, such as anti-inflammatory effects for bacterial and viral infections, including severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (7,8), the inhibition of carcinogenesis and induction of apoptosis in cancer cells (3,9-11), cardioprotection (12), and some others.

GL is a triterpene glycoside; upon oral consumption, glucuronidase of the intestinal microbiota hydrolyzes it to produce D-glucuronic acid and glycyrrhetinic acid (GA), an aglycone (13-15). Both GL and its aglycone derivative exist in two stereoisomeric forms differing in orientation

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of proton at C18: α-GA with a *trans*- junction and β-GA with a *cis*-junction of the D/E rings (16) (Figure 1). The two forms have different physical and chemical properties and pharmacological effects (9,17). Thus, α-GA exerts stronger anti-inflammatory action (18) and inhibition of 11β-hydroxysteroid dehydrogenase 1 (19). In contrast, exceedingly higher activity of β-GA over the one of α-GA was observed in (i) inhibition of hepatotoxicity (20) and mutagenicity in bacteria *S. typhimurium* and DMBA-induced tumorigenesis in mice (21), (ii) in BACE1 inhibition assay (22), (iii) in the relaxation of preconstricted rings of rabbit superior mesenteric artery (23), (iv) in the suppression of the swelling-induced release of glutamate and taurine (24), (v) in the inhibition of human cardiac sodium channel Nav1.5 and its LQT-3 variant (25), and (vi) in the blockage of Kv1.3 currents in human Jurkat T cells (26). Gap junctions are known to be blocked by α-GA (27), as well as by carbenoxolone (CBX), a hemisuccinate derivative of β-GA (28).

When GL and its derivatives are absorbed from the intestine into the bloodstream, the first and most prevailing cells they encounter are red blood cells (RBCs). However, the interaction of these molecules with erythrocytes remains poorly understood. Therefore, the main objective of the present study was to investigate what happens when GL and its derivatives interact with human RBCs under normal isosmotic conditions, as well as upon osmotic and colloid-osmotic stress.

Materials and Methods

Drugs and reagents

GL, α-GA, β-GA, and CBX were provided by Sigma-Aldrich (St. Louis, MO, USA), nystatin from Wako (Osaka, Japan), and HEPES from Dojindo (Kumamoto, Japan). GL and its derivatives were added from concentrated stock solutions in dimethyl sulfoxide (DMSO). The final concentration of DMSO did not exceed 0.1%, and at these concentrations the solvent did not significantly affect the findings.

Solutions

The normal Ringer solution contained (mM): 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 11 HEPES, 5 glucose (pH 7.4, adjusted with NaOH, 290 mOsm/kg-H_2O). The H-buffer contained (mM): 5 KCl, 10 HEPES, 2 $CaCl_2$, 1 $MgCl_2$, 5 glucose, pH 7.4 (40 mOsm/kg-H_2O). Hypotonic solutions were prepared by mixing the Ringer solution with H-buffer at different ratios to yield the indicated osmolarities.

Cells

The RBC preparation and integrity assays were performed as described earlier (29). Briefly, the blood samples were collected from the median cubital vein of healthy donors by venipuncture using disposable sterile syringes. The blood was diluted 10 times with the normal Ringer solution supplemented with heparin (10 units) and washed three times in normal Ringer solution by centrifugation at 1000 g for 10 minutes. The buffy coat was removed after the first centrifugation. Four hundred µL of 4% erythrocyte suspension in isotonic or hypotonic solutions, with or without GA and its derivatives and/or nystatin, was incubated for various periods of time at 37°C. The cell suspension was then centrifuged at 1000 g for 10 minutes, and the RBC lysis was determined by photometric measurement of the hemoglobin release at 540 nm. The RBC lysis was expressed as a percentage of the total hemoglobin release, which was determined by treatment of cell suspension with 1% Triton X-100 and

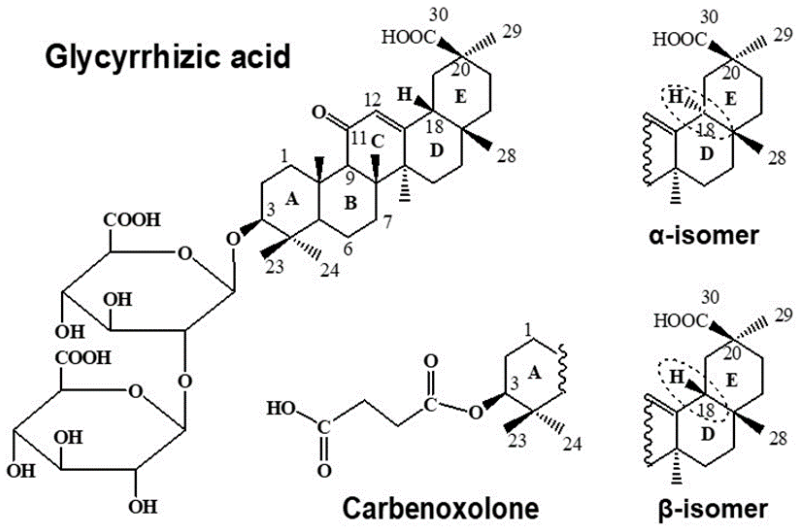

Figure 1. General structure of glycyrrhizin and its derivatives. The α- and β-glycyrrhetinic acids lack the sugar moiety and differ in orientation of the proton at the 18th carbon (C18) because of different junction modes (*trans* and *cis*, respectively) of the D/E rings, as indicated. Carbenoxolone is a hemisuccinate derivative of β-glycyrrhetinic acid at C3 of ring A.

with no other drugs added. The spontaneous RBC lysis was about 1% on the 60 minutes incubation at 37°C. The polyethylene glycols (PEG) were used at the following concentrations generating extra osmotic pressure of 40 mOsm/kg-H_2O: PEG1500 (25 mM), PEG2000 (25 mM), PEG3000 (20 mM), PEG4000 (15 mM), and PEG6000 (12 mM). The osmolality of solutions was measured with a freezing-point depression osmometer OM 802 (Vogel, Germany). The hydrodynamic radii (R_h) were taken from other reports (30,31).

Data analysis

The dose-response data were approximated using a Hill equation of the following form:

$$L = L_{min} + (L_{max} - L_{min}) / (1 + (C/C_{50\%})^h) \quad (1)$$

where L is the RBC lysis (%), L_{min} and L_{max} are the minimal and maximal values of L, respectively; C is the concentration of the substance (GL, its derivatives or nystatin) in μM; $C_{50\%}$ is the concentration of the substance rendering a half-maximal lytic or inhibitory effect (μM) and h is the Hill coefficient.

The osmotic resistivity data were approximated using an equation of the following form:

$$L = L_{min} + (L_{max} - L_{min})/(1 + (\Pi/\Pi_{50\%})^s) \quad (2)$$

where L is the RBC lysis (%); L_{min} and L_{max} are the minimal and maximal values of L, respectively; Π is the solution osmolality; $\Pi_{50\%}$ is osmolality of the solution inducing a half-maximal cell lysis (mOsm/kg-H_2O) and s is a steepness parameter.

The data were analyzed using Origin 8 software (OriginLab Corporation, Northampton, MA, USA). The pooled data are given as means ± SEM of n observations. Comparisons between the two experimental groups were made using the unpaired Student's t test. Differences were considered statistically significant at $P < 0.05$.

Results

In our experimental conditions, the spontaneous RBC lysis was at the level of 1.1 ± 0.5% (n = 6). GL, even when applied at the maximally used concentration of 500 μM, did not cause appreciably greater RBC lysis with an average value of 1.46 ± 0.12% (n = 6) (Figure 2A, B: red squares and bar). However, the aglycones of GL exhibited more profound lytic effects on human RBC in a manner dependent on the orientation of the E/D ring junction (Figure 1). Specifically, the *cis*-form (β-GA) exhibited a high lytic activity with a half-maximal effect at $C_{50\%}$ = 192.2 ± 4.7 μM and a Hill coefficient of 8.5 ± 0.8 (Figure 2A, B: blue down triangles and bar). In contrast, the *trans*-form (α-GA) was considerably weaker at lysing the RBC with cell lysis level of only 8.6 ± 0.6 % (n=6) at the highest tested dose of 500 μM (Figure 2A, B: green up triangles and bar). As compared to β-GA, carbenoxolone, the hemi-succinyl derivative of β-GA, was a very weak hemolyser, too (Figure 2A, B: purple circles and bar), suggesting that hydrophilic substitution at the C3 of the ring A interfered with the RBC lysis activity of β-GA.

To elucidate the mechanism of the RBC lytic activity of β-GA, we employed the osmotic protection test based on the assumption that impermeable nonelectrolytes, when applied extracellularly at concentrations sufficient to balance the oncotic pressure of hemoglobin, produce a

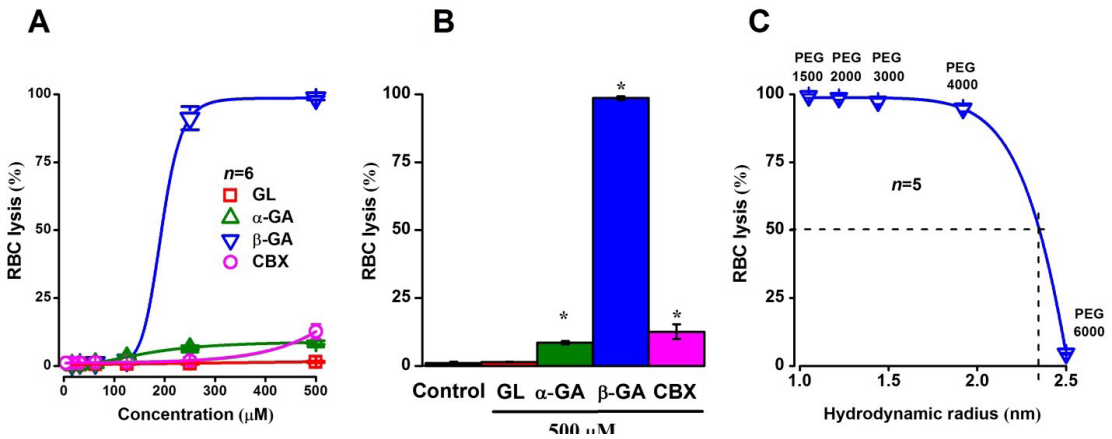

Figure 2. Effects of glycyrrhizic acid (GL) and its derivatives on the integrity of human red blood cells. (A) Dose-response of the steady-state level of the red blood cells (RBC) lysis measured after 60 min incubation of cells with the indicated concentrations of GL, α-glycyrrhetinic acid (α-GA), β-glycyrrhetinic acid (β-GA), and carbenoxolone (CBX); the solid line for β-GA is a fit to the Equation (1) with parameters given in the text. (B) The steady-state RBC lysis in the presence of GL, α-GA, β-GA, and CBX at the maximally used concentration of 500 μM. (C) Effects of polyethylene glycols (PEG) with molecular weights ranging from 1,500 to 6,000 Da (indicated as numbers next to the symbols) on the steady-state level of RBC lysis measured after 60 min incubation of the cells with 400 μM β-GA. The polyethylene glycols used in concentrations generating extra 40 mOsm/kg-H_2O (see Methods section for details). *Statistically different from control at $P < 0.05$ (Student's t test).

protective effect by restoring double-Donnan equilibrium, which was degraded upon the permeabilization of the erythrocyte plasma membrane to small organic and inorganic osmolytes. In our experiments, the osmotic protection started from PEG4000 (R_h=1.92 nm) and was complete in the presence of PEG6000 (R_h=2.5 nm) (Figure 2C). The fact that PEG6000 was able to cancel the RBC lysis in the presence of β-GA at a dose twice as high as its $C_{50\%}$ value suggests the colloid-osmotic mechanism of the RBC lysis in which the β-GA molecules permeabilize the cell plasma membrane by forming water-filled pores permeable to small osmolytes but not to PEG6000. The half-maximal protection was observed at R_h=2.3 nm (dashed lines in Figure 2C). This value can be considered as an estimate of the effective size of the β-GA-formed pores.

Next, we tested whether glycyrrhizic acid and its derivatives affect the sensitivity of human RBC toward osmotic and colloid-osmotic stress. In control experiments, when the extracellular osmolality was decreased, the human RBC started to lyse beginning from around 110 mOsm/kg-H_2O and reached the 100%-lysis at 40 mOsm/kg-H_2O with half-maximal lysis observed at $\Pi_{50\%}$ = 87.1 ± 1.9 mOsm/kg-H_2O (n = 5, Figure 3A: Control). In the presence of GL, we observed a significant shift of the osmosensitivity curve in a rightward direction (Figure 3A, red squares) in a dose-dependent manner (Figure 3E: red squares), suggesting a smaller magnitude of the hypotonic challenge is sufficient to produce the similar degree of the RBC lysis. Therefore, GL produced an osmotic sensibilization effect on human erythrocytes. In contrast to GL, in the presence of α-GA at the concentration of 25 μM, we observed a small but significant decrease of $\Pi_{50\%}$ (Figure 3B, E, green-up triangles). This means that lower extracellular osmotic pressure (and thus, a larger osmotic gradient between the extra- and intracellular space) is necessary to produce a comparable degree of cell lysis. Therefore, α-GA at 25 μM produced osmo-protective but not the sensibilizing effect on human RBC. However, it should be noted that the osmoprotection by α-GA gradually disappeared when the concentration was further increased up to 100 μM (Figure 3E, green up triangles), possibly due to a weak cytolytic action of this compound (Figure 2A, B: green up triangles).

In contrast to α-GA but similar to GL, β-GA and CBX increased $\Pi_{50\%}$ in a dose-dependent manner (Figure 3C, D: blue and purple symbols, respectively), which is an indication of sensibilization of the cells towards the osmotic stress. The magnitude of the sensibilizing effect was roughly similar for GL, β-GA, and CBX (Figure 3E). It is important to note that in these experiments, the two stereo-isoforms displayed opposite effects on the osmotic resistivity of human RBC: Osmo-protection for *trans*-isomer, α-GA, and osmotic sensibilization for the *cis*-isomer, β-GA.

Unlike the hypotonicity-induced RBC lysis, the colloid-osmotic lysis is occurred in isotonic conditions and is driven by the oncotic gradient, which is unbalanced under the single-Donnan conditions. A classic example of the colloid-osmotic lysis is the hemolysis induced by nystatin, a pore-forming polyene antibiotic. We used the effective concentration of nystatin causing 50% hemolysis ($C_{50\%}$) as an indicator of the cellular resistivity to the colloid-osmotic lysis.

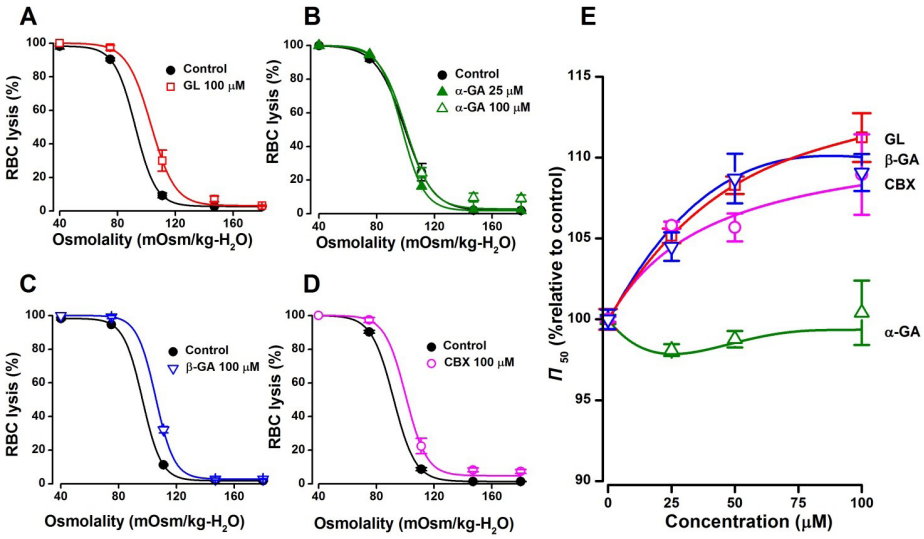

Figure 3. Effects of glycyrrhizic acid (GL), α-glycyrrhetinic acid (α-GA), β-glycyrrhetinic acid (β-GA), and carbenoxolone (CBX) on the osmotic resistivity of human red blood cells (RBC). (A, B, C, and D) The steady-state RBC lysis was measured after 60 min incubation of cells in the hypotonic Ringer solutions in the presence of GL and its derivatives at the indicated concentrations (n=3–4). (E) The osmolality of half-maximal lysis ($\Pi_{50\%}$) in the presence of GL, α-GA, β-GA, and CBX applied at the concentrations as indicated. The $\Pi_{50\%}$ values were obtained by fitting the averaged osmotic resistance data to Equation (2) and normalized to the value obtained for the control cells with no drugs added (Control in A). The error bars in E are generated by fitting the algorithm of the Origin 8 software.

In our experimental conditions, the effective half-maximal concentration of nystatin causing 50% hemolysis was $C_{50\%}$ = 101.6 ± 2.4 μM (n = 5, Figure 3A: Control). Addition of GL to the extracellular medium resulted in a clear and dose-dependent shift in the concentration-dependence curve towards the lower concentrations of nystatin (Figure 4A, E: red squares). The finding indicates that the cells in the presence of GL became less capable of resisting the colloid-osmotic stress. Under these experimental conditions, α-GA produced moderate cell sensibilization to nystatin, similar to GL (Figure 4B, E: green up triangles), whereas the sensibilizing effect of β-GA was much greater (Figure 4C, E: blue down triangles). Meanwhile, CBX did not significantly alter the sensitivity of human RBC to the polyene (Figure 4A, E: purple circles).

Discussion

We have demonstrated here that GL itself is virtually non-hemolytic; however, the removal of the GL carbohydrate moiety imparted significant RBC lytic activity to the *cis*-(β-GA) but not to the *trans*- (α-GA) isoform of the molecule. This result is surprising because only a minor change in the molecular structure (opposite orientation of E/D rings junction in β- versus α-isoform of GA) resulted in a dramatic change in the cytolytic activity. The similar lower activity of the α-GA compared to the one of β-GA was observed for a number of other biological activities, as well (see Introduction).

The RBC lysis by β-GA occurred by a colloid-osmotic mechanism due to the formation of water-filled pores with a radius of ~2.3 nm, as evidenced by osmoprotection by extracellularly added polyethylene glycols to balance the oncotic pressure of hemoglobin (~40 mOsm/kg-H_2O (32)). This approach has previously been used to determine the size of the pores formed by sticholysin I from the sea anemone (33) and by polyene antibiotics amphotericin B and nystatin (34,35). The estimated size of the pore formed by β-GA was smaller than the one of the hemoglobin molecules having a radius of 2.8–3.1 nm (36). Thus, the observed release of hemoglobin occurs not through the β-GA-formed pores but results from the loss of cell integrity upon uncontrolled volume increase in the single-Donnan system.

CBX is a gap junction blocker, also inhibiting the 11β-hydroxysteroid dehydrogenase, and is used for the treatment of various ulcers (28). This molecule was generated by a hemisuccinate substitution at the C3 of ring A in the β-GA structure. Our findings suggest that this maneuver resulted in a greatly diminished hemolytic property. We assume that the presence of the carbohydrate in GL, the *trans*-orientation of E/D junction in α-GA, and hemisuccinate radical at the C3 of ring A, in CBX interfered with the formation of the water-filled pore

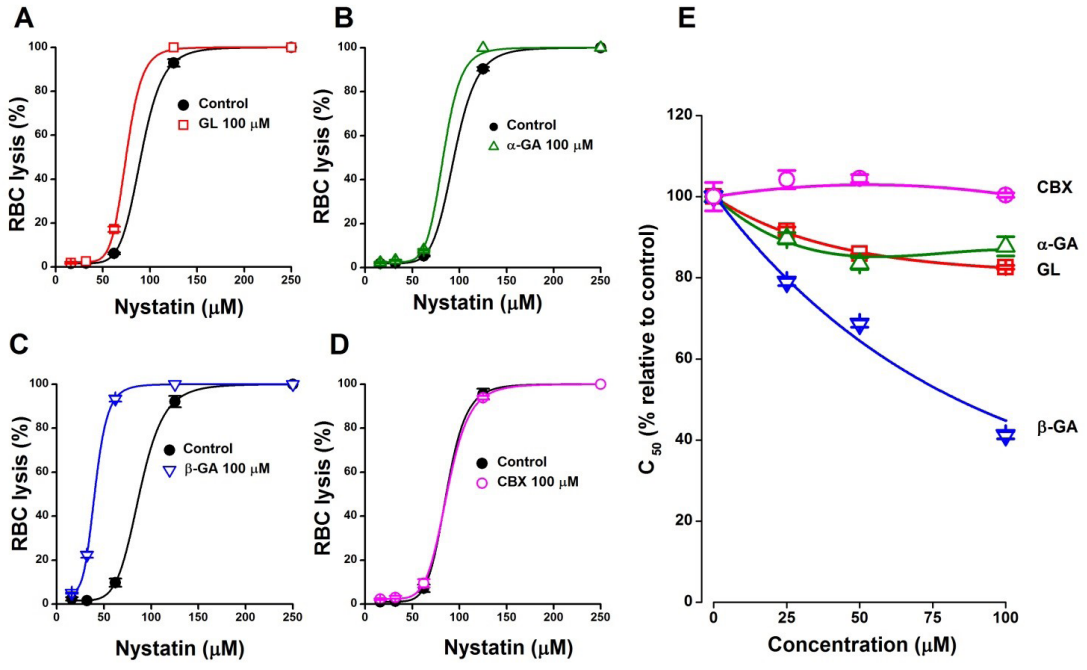

Figure 4. Effects of glycyrrhizic acid (GL), α-glycyrrhetinic acid (α-GA), β-glycyrrhetinic acid (β-GA), and carbenoxolone (CBX) on the sensitivity of human red blood cells (RBC) to colloid osmotic lysis induced by nystatin. (A, B, C, and D) The steady-state RBC lysis in normal Ringer solution was measured after 60 min incubation of cells in the presence of different concentrations of nystatin in the presence of GL, α-GA, β-GA, and CBX at the indicated concentrations (*n*=3–4). (E) The effective concentration of nystatin inducing 50%-lysis ($C_{50\%}$) in the presence of β-GA. The $C_{50\%}$ values were obtained by fitting the averaged dose-response data to Equation (2) and normalized to the value obtained for the control cells with no drugs added (Control in A). The error bars in E are generated by fitting algorithm of the Origin 8 software.

that was successfully accomplished only by the native β-GA structure. The Hill coefficient of 8.5 obtained for the hemolytic activity suggests that 8 or more molecules would be necessary to build up the β-GA pore.

The precise structure of this pore needs further clarification. Certainly, the concentration of half-maximal lysis of around 190 μM is considerably higher than the concentration of GA reachable by oral ingestion of GL (up to around ten μM in the peripheral circulation depending on the amount of drug ingested) (37). However, in the blood vessels surrounding the gastro-intestinal tract, the GA concentration could be higher than the one in the peripheral blood. Since the amount of circulating adenosine triphosphate (ATP) mainly results from the RBC lysis (38), even low level of GA-induced hemolysis is expected to significantly increase the circulating ATP levels and thus to affect the physiologically and pathophysiologically important purinergic signaling pathways in the whole organism (39).

At the sublytic doses, the two stereo-isoforms displayed opposite effects on the osmo-resistivity of human RBC: Osmoprotection for *trans*-isomer, α-GA, and osmotic sensibilization for the *cis*-isomer, β-GA. In contrast, the two stereo-isoforms exhibited different but not opposite weakening effects on the resistivity of the RBC towards the colloid-osmotic stress. By analogy to gossypol (29), we assume that the osmoprotective and osmotic sensibilization effects of GL and its derivatives could result from the intercalation of these molecules into the bilayer lipid matrix of the RBC plasma membrane (7). The osmotic sensibilization effect of GL, β-GA, and CBX could result from the unfavorable packaging of the *cis*-isomeric forms inside the plasmalemma, whereas the *trans*-orientation of E/D junction in α-GA could remove the steric obstacle for intercalation. The reported decrease in the elasticity of human erythrocytes in the presence of micromolar GL might be related to the weakening effect of this compound and its derivatives on the RBC (40).

Since the cells swell under the colloid-osmotic stress even in isosmotic conditions, we assume that the volume regulation mechanisms activated upon the RBC swelling in the single-Donnan situation are involved in the cellular resistivity towards the colloid-osmotic stress and are affected by the β-GA, as well as by GL and α-GA, but not by CBX. Consistent with this hypothesis, the β-GA, but not the α-GA was previously found to be able to suppress the VSOR-mediated release of glutamate and taurine from primary cultured astrocytes (24). Based on this hypothesis, we may assume that recently identified VSOR-inhibitors of plant origin, such as flavonoids (41) and tannins (42), could also be able to modulate the osmoresistivity of the RBC. In addition, GL and its derivatives are expected to have an impact on the cell death induction and protection in other cell types, in which VSOR plays a critical role (43-47).

Conclusion

The results of the present study suggest that upon intestinal digestion and absorption, the GL hydrolysis products interact with the RBCs with both beneficial and detrimental consequences depending on the molecular structure and dosage.

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Authors' contributions

DF, SR, OK, and RK designed and performed experiments. PM contributed to the project design, data analysis, drawing figures, and writing the manuscript. DF, PM, and RS wrote the paper. RS was the project leader, and finalized the paper. All authors approved the final manuscript for publication.

Conflict of interests

The authors declare no conflict of interests.

Ethical considerations

All procedures for human RBCs isolation were conducted in compliance with the Helsinki Declaration, following the ARRIVE guidelines (https://arriveguidelines.org/) and approved by the Bioethics Committee of the Institute of Biophysics and Biochemistry, National University of Uzbekistan (BEC/IBB-NUU/2019/01-2).

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