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EXPERIMENTAL STUDIES

GLYCYRRHIZIC ACID: NOVEL POTENTIAL PROTEIN TARGETS

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To date, a large body of data has been accumulated on the biological activity of a low-toxic natural glycoside, glycyrrhizic acid (GA), but the mechanism of its action at the molecular level has not been fully studied. Expanding knowledge about the spectrum of cellular protein targets of GA contributes to understanding new features of pharmacodynamics. The aim of the work was the experimental identification of a tissue-specific spectrum of protein molecules interacting with GA in a model system. Samples of an intact rat liver tissue lysate were incubated with GA covalently immobilized on EAH-Sepharose 4B, followed by elution of affinity-isolated protein molecules and their trypsinolysis. Using mass spectrometric analysis, 88 potential protein targets of GA were identified. According to the results of gel chromatographic separation of the rat liver lysate and semi-quantitative analysis of proteins, GA influenced Aldh6a1, Decr1, and Sod1 in fractions. Molecular docking in the Flare™ program used to model protein complexes with GA, resulted in selection of 5 proteins (Acox2, Acr1c9, Maoa, Mat1a, Nalcn), which formed complexes with GA with the most favorable ΔG and Rank score parameters. More than half (57%) of the affinity-isolated proteins are involved in the processes of basic cellular metabolism and biotransformation of endogenous and exogenous compounds. Data on the associations of potential protein targets of GA with diseases and different types of biological activity of GA have been systematized and compared.

Keywords: glycyrrhizic acid; cytochrome P450; glycyrrhizic acid binding protein; mass spectrometry; liver tissue lysate; affinity purification

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INTRODUCTION

Glycyrrhizic acid (GA) is a naturally occurring triterpene glycoside with a molecular weight of 822.9 g/mol (glycyrrhizinic acid, PubChem CID14982), isolated from licorice roots (*Glycyrrhiza glabra*). GA has antitumor [1], anti-inflammatory, antiviral, hepatoprotective activities [2] and is relatively low-toxic: it causes moderate and weak side effects [3–5]. GA is included in drugs as a pharmaceutical substance. For example, in Russia, GA is a component of the drug Phosphogliv®, which is used to treat patients with fatty degeneration of the liver of non-alcoholic etiology [6]. According to the EU Clinical Trials Register [7], other GA-based drugs, SNMC® (EudraCT No. 2004-000773-60) and Glycyron® (EudraCT No. 2008-008512-51) have been studied for their efficacy and safety in the treatment of patients with chronic hepatitis C [8] and gingivitis (EudraCT No. 2008-008512-51), respectively.

In recent years, new data on the spectrum of biological activity of GA based on its direct interactions with cellular proteins have been obtained *in vitro* and *in vivo* experiments. It is known that the effect of GA on the JAK2/STAT3 [9] and Hippo/YAP signaling pathways modulates various processes at the molecular level [10]. Zhang et al. found that GA binding to the N-terminal domain of the synaptic protein alpha-synuclein (SNCA), prevented its aggregation and thereby exerted a neuroprotective effect [11]. According to Wang et al., GA interacts with aflatoxin B1 aldehyde reductase 2 (AKR7A2), exhibiting antioxidant activity in liver stellate cells [12]. Ni et al. summarized data on the existence of at least ten protein targets of GA (CD274, CCR2, STAT3, ICAM1, TLR9, MAPK1, MPO, NOS2, PLAG2G1B, and mTOR) [13]. We suggest that the list of protein targets of GA may be much wider and include both individual proteins and protein complexes, because GA and compounds of similar structure are characterized by multiple types of pharmacological activity [13].

The aim of this work was the experimental identification of tissue-specific protein molecules interacting with GA in a model system.

To achieve this goal, we have used affinity isolation of proteins that form stable complexes with GA, immobilized on an inert carrier, from an intact total rat liver tissue lysate.

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MATERIALS AND METHODS

Materials

A water-soluble preparation of disodium salt of glycyrrhizic acid $C_{42}H_{60}Na_2O_{16}$ (Mafco Worldwide Corporation, USA) was used in the work.

Tissue samples were obtained from male Wistar rats (*Rattus norvegicus*) (n=3) aged 5 months. The animals were kept in natural light and had free access to standard food and water. The weight of the animals was 200–240 g. After decapitation under ether anesthesia, the liver samples were washed with 0.9% NaCl and frozen in liquid nitrogen.

Preparation of Intact Liver Lysate

Approximately equal fragments of liver samples from each animal were pooled into a mixed sample in order to eliminate their individual characteristics. The pooled liver samples were initially crushed using a glass mortar and pestle in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) containing a cocktail of protease inhibitors (GE Healthcare, USA). Then, the lysate was additionally suspended using a Silent Crusher S rotary homogenizer (Heidolph, Germany).

Affinity-Based Isolation of Proteins from Liver Tissue Lysate

Covalent GA immobilization by carboxyl groups was performed on pre-activated EAH-Sepharose 4B (Cytiva, USA) containing special linkers on its surface with free amino groups at the ends. All procedures were performed according to the manufacturer's protocol. The prepared sorbent suspension with immobilized GA (affinity sorbent) was placed in a volume of 300 µl into a special filter column with a diameter of 10 mm and the sorbent was washed with phosphate-buffered saline (5 aliquots of 500 µl each). After that, 4 ml of the intact rat liver tissue lysate, diluted with phosphate-buffered saline fivefold, were passed through. Then the sorbent suspension was washed with phosphate-buffered saline (5 aliquots of 500 µl each) to remove the protein material of the lysate weakly bound to the sorbent. After that, the proteins that formed stable complexes with the immobilized GA were eluted by passing 1 ml of 2% formic acid (4 aliquots of 250 µl each).

Similar procedures were performed with the control sorbent (without immobilized GA). The protein content was assayed using the Bradford reagent: optical density of the samples containing liver proteins was measured at 595 nm on a QE65000 spectrophotometer with a DH-2000 light source (Ocean Optics, USA). The concentration of total protein in the samples was determined using a calibration graph. Samples containing the protein material eluted from the control and affinity sorbents were then used for subsequent mass spectrometric (MS) identification.

Gel Chromatographic Fractionation of Liver Tissue Lysate Proteins

Gel chromatographic fractionation of the protein material of rat liver tissue lysate by molecular weight was performed using an AKTA Purifier 10 chromatograph (Cytiva) under the control of the UNICORN v5.31 program. A GA solution in a working buffer containing 10 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM EDTA, 1 mM dithiothreitol, 0.5% NaN_3, and 0.05% Tween-20 was added to 2 ml of the lysate (~40 mg of total protein) to a final GA concentration of 50 µM (experiment), and the lysate sample was incubated for 1 h at 4°C. The control lysate sample was treated similarly (but without adding the GA solution). The final GA concentration chosen for lysate incubation is approximately 2.5 times higher than that in blood plasma *in vivo*, as found in pharmacokinetic studies on animals [14]; however, it is at least one order of magnitude lower than the concentrations required for GA micelle and aggregate formation in acidic or slightly acidic media *in vitro* [15, 16]. The lysate samples were then centrifuged for 10 min at 12,000 *g* and 4°C. The supernatant was fractionated on a HiLoad 16/600 column with Superdex 200 prep grade (Cytiva), pre-equilibrated with the running buffer at a flow rate of 800 µl/min and a temperature of 15°C. Ten lysate fractions (30 kDa, 45 kDa, 60 kDa, 75 kDa, 105 kDa, 200 kDa, 265 kDa, 360 kDa, and 490 kDa) were collected for subsequent MS identification of proteins. Total protein concentration in the collected fractions was determined at 280 nm using a QE65000 spectrophotometer with the DH-2000 light source (Ocean Optics) and a calibration curve; the measured total protein concentrations ranged from 0.2 mg/ml to 1 mg/ml.

MS Identification of Proteins

LC-MS/MS analysis was performed on a micrOTOF-QII mass spectrometer (Bruker Daltonik, Germany) equipped with a CaptiveSpray ionization source (Bruker Daltonik) and coupled with a nanoElute nano-HPLC system (Bruker Daltonik). A total amount of 30 µg protein was subjected to tryptic digestion according to a modified FASP protocol [17] for subsequent MS identification. All samples were desalted using C18 SPE discs (Merck, USA) in StageTip tips according to the protocol [18]. Each sample was then resuspended and injected in a volume from 1 µl to 6 µl (depending on the observed peptide signal) onto an Acclaim™ PepMap™ C18 reversed-phase HPLC trap column (5 µm, 0.3 mm × 5 mm, Thermo Fisher Scientific, USA) using phase A (0.1% formic acid (Merck Millipore, USA) in water) at a pressure of 400 bar. Peptides were separated on an Aurora Ultimate CSI C18 analytical HPLC column (1.7 µm, 120 Å, 75 µm × 250 mm, IonOpticks, Australia) at a flow rate of 300 nl/min and a temperature of 50°C. Elution was performed using mobile phase A

[water:formic acid (100:0.1, v/v)] and mobile phase B [acetonitrile:formic acid (100:0.1, v/v)]. The elution gradient was as follows: 2–3% B over 0–1 min, to 17% B over 57 min, to 25% B over 78 min, to 34% B over 92 min, to 85% B over 93 min. The phase B concentration was then maintained at 85% for a further 7 min, after which it was reduced to the initial 2% for the next chromatogram.

MS analysis was performed in positive ionization mode with the following parameters: dry gas heated to 150°C with a flow rate of 3 l/min, capillary voltage of 1500 V. Full scanning MS were recorded in the range from 150 *m/z* to 2200 *m/z* with a data acquisition rate of 2 Hz. MS/MS analysis was performed in an automated mode with a fixed cycle time of 3 s for precursor ions, dynamic acquisition of MS/MS spectra with a resolution of 8 Hz to 32 Hz and for ions with charge states from 2+ to 4+. Raw data were analyzed using Bruker compass DataAnalysis 5.1 software (Bruker Daltonik) with default settings for proteomic analysis. The combined peak list was exported in a Mascot Generic Format (*.mgf). Identification was performed using the Mascot search engine version 2.3.0 (Matrix Science, UK) against the SwissProt database (version from May 20, 2020) with a restriction on the *Rattus* taxonomy. The search parameters were as follows: the cleavage enzyme was trypsin, the permissible number of missed cleavage points was set to 1, deamidation (Asn, Gln) and oxidation (Met) were variable modifications, the peptide mass tolerance was 40 ppm, the mass tolerance for MS/MS was set to 0.2 Da, and the preferred charge of peptides varied from 2+ to 4+ (according to the instrument parameters).

Molecular Docking

Molecular docking of GA into the protein structure was performed in the Flare™ 8.0.0 program (Cresset Group, UK). The 3D structures of proteins for molecular modeling were taken from the Protein Data Bank (PDB): monoamine oxidase A (PDB ID: 1O5W, resolution 3.20 Å) [19], 3-alpha-hydroxysterol dehydrogenase (PDB ID: 1AFS, resolution 2.50 Å) [20], S-adenosylmethionine synthase isoform 1 (PDB ID: 1QM4, resolution 2.66 Å) [21], and non-selective sodium channel NALCN (PDB ID: 7CU3, resolution 2.65 Å) [22]. For the remaining proteins with unknown 3D structures, the spatial 3D models predicted by AlphaFold2 were used (AlphaFold Protein Structure Database, developed by Google DeepMind and EMBL-EBI, https://alphafold.ebi.ac.uk/). 3D protein models were selected based on the very high reliability score (pLDDT score >90). The Flare™ program was used to extract the ligand and other small molecules from the 3D protein structures and models, and to perform energy minimization (300 steps). Next, the Flare™ program was used to construct the spatial structure of GA using the SMILES code (PubChem CID14982). Molecular docking of GA into the 3D protein structures was performed in the Normal mode. Prediction of GA binding sites was performed over the entire surface of the 3D protein structure, including the contact zone of subunits for oligomeric proteins. The models of protein/GA complexes were ranked based on the Rank score < -7 and ΔG < -10. Amino acid residues forming links between different protein subunits were determined using the online server PDBePIS v1.52 (Protein interfaces, surfaces and assemblies' service PISA at the European Bioinformatics Institute) [23]. The contact zone of the A and B subunits of monoamine oxidase A was analyzed using the 3D structure (PDB ID: 1O5W) [19].

Functional Enrichment Analysis

The WebGestalt web platform [24] was used to analyze the protein representation in the KEGG (Kyoto Encyclopedia of Genes and Genomes, https://www.kegg.jp/kegg/pathway.html) and Reactome (https://reactome.org) metabolic and signaling pathways with default settings (the *Rattus norvegicus* species). The proteins isolated from rat liver tissue lysates were annotated by known associations with diseases of human orthologs using the online server “Diseases” [25]. Disease terms were selected based on a confidence score of 3 or higher.

RESULTS AND DISCUSSION

Identification of Potential Protein Targets of GA

The application of the approach based on the isolation of proteins interacting with GA is aimed at elucidating the entire set of potential proteins that form stable bonds with GA; this is important for characterizing significant targets in the context of studying the molecular basis of multiple biological activities of GA. Table 1 shows the data on the MS identification of 88 potential protein targets isolated from the intact rat liver tissue lysate on a sorbent with covalently immobilized GA. The results were corrected for proteins non-specifically bound to the control sorbent (without GA immobilization). The term “intact” here indicates that the lysate samples were not treated with mild detergents and weak acids for preliminary dissociation of wild-type protein complexes. The median molecular mass (MM) of the protein was 56.6 kDa, with minimum and maximum values of 7.5 kDa and 303.2 kDa, respectively. According to the Uniprot database [26], oligomeric state is known for 29 out of 88 proteins, mostly homodimers. Analysis of known physical interactions between the isolated proteins (after excluding cytochromes P450 and UDP-glucuronyl transferases) using the online servers STRINGdb [27] and GeneMania [28] showed a negative result, except for interactions between Actr1a and Actr2,

Table 1. Potential GA-binding proteins isolated from the rat liver tissue lysate

Protein code	Uniprot name	Mascot score	Uniprot ID	MM*, Da	Oligomeric state**
Ywhab	14-3-3 protein beta/alpha	50	P35213	28037	Homodimer
Ywhaq	14-3-3 protein theta	50	P68255	27761	Homodimer
Htr6	5-hydroxytryptamine receptor 6	40	P31388	46892	n/d***
Acox2	Peroxisomal acyl-coenzyme A oxidase 2	68	P97562	76750	n/d
Acsl6	Long-chain-fatty-acid-CoA ligase 6	36	P33124	78130	n/d
Actr1a	Alpha-centractin	30	P85515	42587	n/d
Aldh3a2	Aldehyde dehydrogenase family 3 member A2	58	P30839	54047	n/d
Aldh2	Aldehyde dehydrogenase, mitochondrial	165	P11884	56453	Homotetramer
Maoa	Amine oxidase [flavin-containing] A	36	P21396	59470	Homodimer
Aox2	Aldehyde oxidase 2	33	A0A096P6M6	147777	Homodimer
Actr2	Actin-related protein 2	30	Q5M7U6	44705	n/d
Arsb	Arylsulfatase B	31	P50430	58922	Homodimer
Asgr1	Asialoglycoprotein receptor 1	44	P02706	32828	n/d
Chd6	Chromodomain-helicase-DNA-binding protein 6	33	D3ZA12	303258	n/d
Cyp1a1	Cytochrome P450 1A1	40	P22443	59356	n/d
Cyp1a2	Cytochrome P450 1A2	40	P04799	58222	n/d
Cyp2c55	Cytochrome P450 2C55	33	P33273	55954	n/d
Cyp27a1	Sterol 26-hydroxylase, mitochondrial	45	P17178	60695	n/d
Cyp2a1	Cytochrome P450 2A1	73	P11711	55959	n/d
Cyp2a3	Cytochrome P450 2A3	31	P20812	56474	n/d
Cyp2b3	Cytochrome P450 2B3	51	P13107	56348	n/d
Cyp2c13	Cytochrome P450 2C13, male-specific	43	P20814	55824	n/d
Cyp2c23	Cytochrome P450 2C23	44	P24470	56397	n/d
Cyp2d3	Cytochrome P450 2D3	44	P12938	56605	n/d
Cyp2d10	Cytochrome P450 2D10	128	P12939	57039	n/d
Cyp2d26	Cytochrome P450 2D26	99	P10634	56648	n/d
Cyp3a1	Cytochrome P450 3A1	32	P04800	57880	n/d
Cyp3a2	Cytochrome P450 3A2	32	P05183	57694	n/d
Cyp4a14	Cytochrome P450 4A14	91	P20817	58195	n/d
Ddb1	DNA damage-binding protein 1	51	Q9ESW0	126781	n/d
Decr1	2,4-dienoyl-CoA reductase, mitochondrial	38	Q64591	36110	Homotetramer
Hsd17b12	Very-long-chain 3-oxoacyl-CoA reductase	33	Q6P7R8	34819	n/d
Hsd17b2	Estradiol 17-beta-dehydrogenase 2	48	Q62730	41939	Homodimer
Hsd17b8	Estradiol 17-beta-dehydrogenase 8	32	Q6MGB5	26774	Homotetramer
Hsd11b1	Corticosteroid 11-beta-dehydrogenase isozyme 1	90	P16232	31863	Homodimer
Dhrs4	Dehydrogenase/reductase SDR family member 4	51	Q8VID1	29803	Homotetramer
Akr1c9	3-alpha-hydroxysteroid dehydrogenase	35	P23457	37004	Monomer
Ecsit	Evolutionarily conserved signaling intermediate in Toll pathway, mitochondrial	40	Q5XIC2	49588	n/d
Ftcd	Formimidoyltransferase-cyclodeaminase	64	O88618	58877	Homooctamer
Gng7	Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-7	34	P43425	7519	Trimer
Slc25a18	Mitochondrial glutamate carrier 2	54	Q505J6	34148	n/d
Gna13	Guanine nucleotide-binding protein subunit alpha-13	42	Q6Q7Y5	43984	n/d
Gnas	Guanine nucleotide-binding protein G(s) subunit alpha isoforms XLas	42	Q792G6	122810	n/d
Gorab	RAB6-interacting golgin	30	B1H222	41606	n/d
Grb2	Growth factor receptor-bound protein 2	38	P62994	25190	Homodimer

Table 1. Potential GA-binding proteins isolated from the rat liver tissue lysate (continued)

Protein code	Uniprot name	Mascot score	Uniprot ID	MM*, Da	Oligomeric state**
Hsp90ab1	Heat shock protein HSP 90-beta	550	P34058	83229	Monomer
Hspa2	Heat shock-related 70 kDa protein 2	54	P14659	69599	n/d
Ephx1	Epoxide hydrolase 1	157	P07687	52548	n/d
Prodh2	Hydroxyproline dehydrogenase	30	Q2V057	50970	n/d
Lgals3bp	Galectin-3-binding protein	34	O70513	63701	Homodimer
Lima1	LIM domain and actin-binding protein 1	40	F1LR10	83746	n/d
L3mbtl2	Lethal(3)malignant brain tumor-like protein 2	40	Q3MIF2	78916	n/d
Lonp1	Lon protease homolog, mitochondrial	42	Q924S5	105726	Homohexamer
Aspg	60 kDa lysophospholipase	35	P30919	60756	Homotetramer
Lrif1	Ligand-dependent nuclear receptor-interacting factor 1	30	Q499M7	82618	n/d
Mccc1	Methylcrotonoyl-CoA carboxylase subunit alpha, mitochondrial	40	Q5I0C3	79279	Dodecamer
Mettl7b	Methyltransferase-like protein 7B	30	Q562C4	27886	n/d
Mat1a	S-adenosylmethionine synthase isoform type-1	30	P13444	43670	Homotetramer
Aldh6a1	Methylmalonate-semialdehyde dehydrogenase [acylating], mitochondrial	33	Q02253	57771	Homotetramer
Mogs	Mannosyl-oligosaccharide glucosidase	33	O88941	91814	n/d
Pmpcb	Mitochondrial-processing peptidase subunit beta	33	Q03346	54230	Homodimer
Mvp	Major vault protein	54	Q62667	95739	n/d
Myl6	Myosin light polypeptide 6	66	Q64119	16964	n/d
Nalcn	Sodium leak channel non-selective protein	32	Q6Q760	200360	n/d
Nde1	Nuclear distribution protein nudE homolog 1	32	Q9ES39	38504	n/d
Nsdhl	Sterol-4-alpha-carboxylate 3-dehydrogenase, decarboxylating	43	Q5PPL3	40386	Homodimer
Phyh	Phytanoyl-CoA dioxygenase, peroxisomal	38	P57093	38563	n/d
Pigr	Polymeric immunoglobulin receptor	54	P15083	84745	n/d
Rack1	Receptor of activated protein C kinase 1	35	P63245	35055	Monomer
Rdh7	Retinol dehydrogenase 7	43	P55006	35713	n/d
Reep6	Receptor expression-enhancing protein 6	43	Q5XI60	23298	n/d
Rpn1	Dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 1	33	P07153	68262	n/d
Slc27a5	Bile acyl-CoA synthetase	69	Q9ES38	76217	n/d
Sfxn1	Sideroflexin-1	37	Q63965	35523	n/d
Sod1	Superoxide dismutase [Cu-Zn]	31	P07632	15902	Homodimer
Ssr1	Translocon-associated protein subunit alpha	32	Q7TPJ0	35607	Homotetramer
Sts	Steryl-sulfatase	40	P15589	62638	Homodimer
Stxbp5	Syntaxin-binding protein 5	30	Q9WU70	127579	n/d
Tkt	Transketolase	74	P50137	67601	Homodimer
Trip12	E3 ubiquitin-protein ligase TRIP12	40	F1LP64	223788	n/d
Ugt1a1	UDP-glucuronosyltransferase 1-1	43	Q64550	59624	Homodimer
Ugt1a3	UDP-glucuronosyltransferase 1-3	43	Q64637	60101	Homodimer
Ugt2b	UDP-glucuronosyltransferase 2B2	118	Q62789	60946	n/d
Ugt2b7	UDP-glucuronosyltransferase 2B7	90	Q62789	60050	n/d
Ugt2b15	UDP-glucuronosyltransferase 2B15	47	P36511	61020	n/d
Ugt2b37	UDP-glucuronosyltransferase 2B37	61	P19488	60553	n/d
Hdlbp	Vigilin	34	Q9Z1A6	141496	n/d
Eci2	Enoyl-CoA delta isomerase 2, mitochondrial	36	Q5XIC0	42994	n/d

*MM, molecular mass; **according to the Uniprot database; ***n/d, no data.

as well as Ssr1 and Rpn1. Thus, potential protein targets of GA differ in MM, and one third of them are capable of homodimerization. However, based on existing data on protein-protein interactions (PPI), they do not form a single network.

Gel Chromatography of a Protein Material of the Tissue Lysate

Using gel chromatography, a sample with protein material of tissue lysate can be separated into multiple fractions, each containing individual proteins and protein complexes with close MM values. Analysis of the protein composition of fractions logically complements the data of affinity isolation of GA protein targets in terms of their participation in protein complex formation (monomeric proteins, homo-/heterodimers, higher-order protein complexes). Preincubation of liver tissue lysate samples in the presence of a pharmacologically active concentration of GA before gel chromatography helps to evaluate the effect of GA on the distribution of GA protein targets in tissue lysate fractions.

Table 2 shows examples of 12 proteins detected in lysate fractions in the control (incubation of the lysate sample without GA) and in the experimental sample (incubation of the lysate sample with GA). The relative representation of the proteins identified in the lysate fractions by the emPAI changes slightly (≤2 times) between the control and the experimental samples with the exception of the Aldh6a1, Decr1, and Sod1 proteins. In the case of these proteins changes were more pronounced (>2 times). Although Aldh6a1 is a homotetramer (MM 232 kDa), in the experiment with GA its relative representation increases in the 60 kDa fraction, which corresponds to the MM of its monomer, and may be associated with the effect of GA on the oligomeric state of Aldh6a1. The 2,4-dieno-CoA reductase protein (Decr1) is a homotetramer, and in the presence of GA the equilibrium in the Decr1 monomer-dimer system may shift towards an increase in the dimer. Decreased relative abundance of the Sod homodimer [Cu-Zn] (Sod1, MM ~32 kDa) was detected in the fraction with MM 30 kDa in the presence of GA. Thus, using gel chromatography data we have identified at least three proteins whose complex formation profile may change in the presence of GA.

In Silico Verification of Interactions of Potential Protein Targets with GA

The main limitation of the applied experimental approach to the isolation of GA protein targets from intact tissue lysates is that both individual proteins and protein complexes can form stable complexes with GA immobilized on an inert sorbent. It would be more correct to define the 88 protein targets identified in the study (Table 1) as potential protein targets of GA (GA-binding proteins). The set of proteins isolated from the lysate is represented by proteins that directly interact with GA (direct partners) and indirect partners that were isolated from the tissue lysate as higher-order protein complexes "labeled" with a direct partner. Experimental verification of paired interactions of GA with each of the isolated proteins would be a valuable extention of this pilot stage of the study. Due to technical limitations in experimental verification, it is difficult to unambiguously classify the identified protein targets into direct (GA-binding proteins) and indirect GA partners. For this reason, we performed theoretical verification

Table 2. Potential GA-binding proteins identified in different molecular mass fractions of rat liver tissue lysate

Protein code	MM, Da*	Oligomeric state**	Lysate fraction, kDa	emPAI*** (C)	emPAI*** (E)
Ywhab	28037	Homodimer	75	0.12	0.25
Acox2	76750	Homodimer	105	0.09	0.04
Aldh2	56453	Homotetramer	105	0.66	0.57
Decr	36110	Homotetramer	75	0.19	0.55
Dhrs4	29803	Homotetramer	490	0.11	0.11
Akr1c9	37004	Monomer	30	0.41	0.29
Gng7	7519	Trimer	490	0.47	0.47
Lgals3bp	63701	Homodimer	490	0.11	0.05
Aldh6a1	57771	Homotetramer	60	0.25	0.74
Phyh	38563	n/d	30	0.18	0.18
Sod1	15902	Homodimer	30	2.88	0.79
Tkt	67601	Homodimer	75	0.05	0.10

*MM, molecular mass; **oligomer state according to the Uniprot database; ***emPAI – exponentially modified protein abundance index; n/d, no data; (C), control (lysate incubation without GA); (E), experiment (lysate incubation with GA).

of GA interactions with proteins using molecular docking in the Flare™ program, using 3D protein structure data from the PDB and predicted 3D models from the AlphaFold Protein Structure Database.

Parameters of the generated protein/GA complexes corresponding to the lowest ΔG and Rank score selection parameters are presented in Table 3. Visualization of the GA-interacting amino acid residues of the Acox2, Acr1c9, Maoa, Mat1a, and Nalcn proteins is given in the Supplementary Materials (Table S1). In the process of modeling protein/GA complexes, we also tested the possibility of GA binding to proteins that could be in different oligomeric states. For example, GA interacted with both monomers A and B of the Mat1a protein, and with the surface of monomer B in the A–B dimer of the Maoa protein. Using Maoa as an example, we have tested the hypothesis about possible GA binding in the contact zone (interface) of monomers A and B. Models of GA complexes with each of the Maoa monomers were selected based on the ΔG and Rank score parameters: -7.97 and -5.33 (monomer A) and -8.97 and -5.73 (monomer B), respectively. Since the ΔG and Rank score parameters characterizing GA interaction in the interface of Maoa monomers were significantly lower than the parameters of the GA interaction with the surface of the A–B dimer, the hypothesis was not confirmed *in silico*. Thus, based on the results of molecular docking, 5 proteins (Acox2, Acr1c9, Maoa, Mat1a, and Nalcn) were prioritized; these were potential direct partners of GA, which could theoretically act as a modulator of the activity of enzymes. For example, GA inhibits monoamine oxidase A and B (Maoa and Maob), which was shown by Bhattacharjee et al. [29].

Functional Annotation of Identified Potential Protein Targets of GA

More than one fifth (~22%) of all potential protein targets of GA identified in this study are involved in oxidation reactions and biotransformation of xenobiotics and drugs. Such proteins include isoenzymes of the multigenic cytochrome P450 family (Cyp1a1, 1a2, 2c55, 27a1, 2a1, 2a3, 2b3, 2c13, 2c23, 2d3, 2d10, 2d26, 3a1, 3a2, 4a14) and the UDP-glucouranosyltransferase family (Ugt1a1, 1a3, 2b, 2b7, 2b15, 2b37), which are presumably involved in the biotransformation of GA. In this context, GA can probably act as a substrate of cytochrome P450 in hepatocytes. However, it has been reported that GA may inhibit some cytochromes P450. For example, Wang et al. [30] demonstrated that GA acted as a competitive inhibitor of rat Cyp3a1, one of the GA target proteins identified in this study (Table 1). Another study demonstrated that GA inhibited activity of human Cyp3a4 [31].

Several potential protein targets of GA (~12%) include oxidoreductases (Aldh2, Aldh3a2, Aldh6a1, Aox2, Dhrs4, Hsd11bx, Nsdhl, and Rdh7), which catalyze oxidation and reduction reactions: transfer electrons or hydrogen atoms from one substrate to another. Functional enrichment analysis of a reduced set of proteins (excluding cytochromes P450 and UDP-glucuronyl transferases) has shown that 24 out of 88 potential protein targets of GA (23%) are involved in the metabolism of amino acids (histidine, alanine, tryptophan, arginine, and proline); degradation of valine, leucine, and isoleucine; degradation of fatty acids; biosynthesis of steroid hormones. Thus, 57% (22%+23%+12%) of the proteins isolated from the liver tissue lysate that interact with GA immobilized on the sorbent participate in cellular metabolism and biotransformation of endogenous and exogenous compounds.

According to the ChEMBL portal [32], GA (CHEMBL441687) demonstrates inhibitory activity in the range of the IC_{50} concentrations from 0.4 nM to 822 μM) and binds to some proteins (with the equilibrium dissociation constant (K_D) from 87 μM to 150 μM). The values of these parameters are given for at least 11 proteins (Abcc2, Amy2, Gusb, Hmgb1, Hsd11b1, Hsd11b2, Ptpn, Pygm, Slc21a1, Slc21a4, and Slc21a7). Interestingly, Hsd11b1 (11-beta-hydroxysteroid dehydrogenase 1) was found in our experiments as a potential target of GA. Membrane transporter group proteins (Slc25a18 and Slc27a5) were isolated from the rat liver tissue lysate as potential targets of GA. As it has been mentioned above, three other members of the Slc21 family (Slc21a1, Slc21a4, and Slc21a7) are also potential targets of GA. According

Table 3. Main characteristics of protein/GA complexes according to the molecular docking simulations

Protein	Docking model No.	Free energy change, ΔG, kcal/mol	Rank score	Oligomeric state
Acox2, peroxisomal acyl-coenzyme A oxidase 2	0	-11.35	-7.83	Monomer
Akr1c9, Akr1c9, 3-alpha-hydroxysteroid dehydrogenase	1	-12.35	-9.31	Monomer
Maoa, monoamine oxidase A [flavin-containing]	0	-10.68	-7.51	Homodimer
Mat1a, S-adenosylmethionine synthase isoform type-1	0	-12.13	-8.69	Homotetramer
Nalcn, sodium leak channel non-selective protein	0	-13.76	-8.72	Monomer

Table 4. Associations between human diseases and potential GA-binding proteins, as well as biological activity of GA

Potential GA-binding proteins	Diseases, associated with GA-binding proteins	Biological activity of GA	References
Actr2, Aldh2, Asgr1, Aspg, Cyp1a1, Cyp1a2, Cyp27a1, Cyp2a3, Ddb1, Ephx1, Gnas, Grb2, Hsd17b2, Hsp90ab1, Lgals3bp, Lonp1, Maoa, Mat1a, Mvp, Phyh, Pigr, Rack1, Sod1, Tkt, Ugt1a1, Ugt2b7	Cancer	Antitumor	[34]
Sts	Lung cancer		[35]
Ugt2b15	Prostate cancer		[36, 37]
Gna13	Burkitt lymphoma		n/d
Aldh2, Asgr1, Cyp1a1, Cyp1a2, Cyp27a1, Cyp2a3, Ddb1, Grb2, Mat1a, Rack1, Sod1, Ugt1a1	Liver diseases	Hepatoprotective	[38, 39]
Cyp1a2, Cyp27a1, Grb2, Lgals3bp, Mat1a, Slc27a5	Nonalcoholic fatty liver disease		[40]
Lgals3bp, Slc27a5	Liver cirrhosis		[41]
Ugt1a1, Ugt1a3, Ugt2b7	Bilirubin metabolism disorder		n/d
Cyp1a1, Cyp1a2, Cyp27a1, Grb2, Lgals3bp, Pigr, Sod1	Autoimmune diseases	Immunomodulating	[42]
Asgr1, Ftcd	Autoimmune hepatitis		
Cyp1a2, Cyp27a1, Grb2, Hsd11b1, Slc27a5, Sod1	Type 2 diabetes mellitus	Antidiabetic	[43, 44]
Cyp1a1, Cyp1a2, Cyp27a1, Grb2 Sod1	Kidney diseases	Nephroprotective	[45, 46]
Aldh2, Cyp1a2, Myl6, Sod1	Ischemic heart disease	Cardioprotective	[47, 48]
Cyp1a2, Cyp27a1, Maoa, Sod1	Neurodegenerative disease	Neuroprotective	[49–52]
Htr6, Ywhaq	Alzheimer’s disease		[53]
Sod1, Ywhaq	Cognitive impairments		n/d
Maoa, Sod1, Ywhaq	Dementia		n/d
Cyp27a1, Hsd11b1, Phyh	Lipid metabolism disorders	Lipid metabolism correction	[54–56]
Maoa, Sod1	Parkinsonism	Antiparkinsonian	[57, 58]
Cyp1a1, Cyp27a1, Sod1	Atherosclerosis	Atheroprotective	[59, 60]
Aldh2, Cyp1a2, Maoa, Sod1	Cerebro-vascular diseases	Atheroprotective	n/d
Aspg, Grb2, Phyh	Lymphatic system diseases	Antiinflammatory	[42, 61]
Cyp27a1, Hsd11b1	Osteoporosis		[62, 63]
Aspg	Osteonecrosis		[64]
Pigr	Inflammatory bowel diseases		n/d
Aspg, Grb2	Thrombosis	Antithrombotic	[65–67]
Maoa, Nde1, Sod1, Ugt2b7	Epilepsy	n/d	n/d

n/d, no data.

to the DrugBank portal [33] (https://go.drugbank.com/), the following protein targets of GA have been described: Hsd11b1, Tnf, Casp3, Lpl, Akr1c4, Akr1c3, Akr1c2, and Abcb11. Akr1c9 protein (3-alpha-hydroxysteroid dehydrogenase) was isolated from the liver tissue lysate. Like a member of the aldo-keto reductases of family 1 (Akr1c4, Akr1c3, and Akr1c2), it is involved in the metabolism of steroids. Thus, our experimental data on the set of potential GA targets are partially consistent with the already known data on GA targets and have an element of novelty. Since the experiments used a model system based on intact rat liver tissue lysate, the identified set of potential protein targets of GA cannot yet be extrapolated to other tissues.

Table 4 presents the results of systematization of data on associations between the identified protein targets of GA with diseases, as well as with different types of biological activity of GA. Currently, 12 types of GA activity are known [34–67], each of which is well correlated with the corresponding group of diseases associated with one or more protein targets of GA (Table 1). These include such hub proteins as Aldh2, Cyp1a1, Cyp1a2, Cyp27a1, Hsd11b1, Grb2, Maoa, Slc27a5, Sod1, and Ywhaq, which are associated with several different groups of diseases and occupy central positions in metabolic or signaling pathways. Therefore, pharmacological action on these hub proteins, in particular the GA action, could be a useful option for correcting abnormal molecular processes under pathological conditions. Future studies will confirm or refute the activity of GA on specific proteins.

In general, GA is characterized by a favorable safety profile, but at the same time, it is known that the long-term use of GA-containing drugs can lead to the development of such side effects as hypertension [5, 68] and hypokalemia [5]. Interestingly, according to the online server "Diseases" [25], some of the potential protein targets of GA that we have identified in this study (Aldh2, Cyp1a1, Cyp1a2, Grb2, Hsd11b1, Maoa, and Sod1) are associated with hypertension.

The results of the functional annotation of the set of protein targets of GA and the data of molecular docking indicate that affinity-isolated proteins from the tissue lysate may be involved in the implementation of the biological effects of GA. Further experimental verification of binary interactions of GA with the identified protein targets will contribute to a more detailed understanding of the phenomenon of multiple biological activity of GA *in vivo*.

CONCLUSIONS

This study culminated in identification of 88 potential protein targets for GA. This significantly expands the existing knowledge about the possible action of this compound at the molecular level through the experimental identification of tissue-specific proteins. GA has been included in some drugs as a pharmaceutical substance; it is a promising low-toxic compound of natural origin with a set of useful biological activities. Continued studies of the pharmacodynamics of GA will help to predict therapeutic effects and create more selective drugs.

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COMPLIANCE WITH ETHICAL STANDARDS

The experiments were conducted in compliance with generally accepted standards of humane treatment of laboratory animals and in accordance with the Order of the Ministry of Health of the Russian Federation No. 199n of April 1, 2016 "On approval of the rules of good laboratory practice".

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

Supplementary materials are available in the electronic version at the journal site (pbmc.ibmc.msk.ru).

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ГЛИЦИРРИЗИНОВАЯ КИСЛОТА: НОВЫЕ ПОТЕНЦИАЛЬНЫЕ БЕЛКОВЫЕ МИШЕНИ

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К настоящему времени накоплен большой объём данных о биологической активности малотоксичного природного гликозида — глицирризиновой кислоты (ГК), однако механизм действия данного соединения на молекулярном уровне до конца не изучен. Расширение знаний о спектре клеточных белковых мишеней ГК способствует пониманию новых особенностей фармакодинамики. Целью работы была экспериментальная идентификация тканеспецифического спектра белковых молекул, взаимодействующих с глицирризиновой кислотой в модельной системе. Образцы интактного лизата ткани печени крысы инкубировали с ковалентно иммобилизованной ГК на EAH-сефарозе 4B с последующей элюцией аффинно выделенных белковых молекул и их трипсинолизом. С помощью масс-спектрометрического анализа были идентифицированы 88 потенциальных белковых мишеней ГК. Дополнительно, по результатам гель-хроматографического разделения лизата ГК влияла на полуколичественное распределение белков Aldh6a1, Decr1 и Sod1 во фракциях. Молекулярный докинг в программе Flare™ использовали для моделирования комплексов ГК и белков, по результатам которого выбрали 5 белков (Acox2, Acr1c9, Maoa, Mat1a, Nalcn) образующих с ГК комплексы с наиболее благоприятными параметрами ΔG и Rank score. Больше половины (57%) аффинно выделенных белков участвуют в процессах базового клеточного метаболизма и биотрансформации эндогенных и экзогенных соединений. Систематизированы и сопоставлены данные об ассоциациях потенциальных белковых мишеней ГК с заболеваниями и разными типами биологической активности ГК.

Полный текст статьи на русском языке доступен на сайте журнала (http://pbmc.ibmc.msk.ru).

Ключевые слова: глицирризиновая кислота; цитохром P450; белок, связывающий глицирризиновую кислоту; масс-спектрометрия; лизат ткани печени; аффинная очистка

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