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Role of *Panax ginseng* and ginsenosides in regulating cholesterol homeostasis

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ABSTRACT

Hypercholesterolemia is harmful for human health since it may favor atherosclerosis and increase the risk of cardiovascular disease. To investigate the effect of a *Panax ginseng* extract and of some major components thereof (namely, ginsenosides Rb1 and Rb2) on cholesterol homeostasis in vitro, we quantitated total and free cholesterol levels and monitored the changes in the levels of key mediators of cholesterol synthesis, efflux and clearance. Treatments with ginsenosides and the extract reduced intracellular cholesterol levels by modulating the SREBP-2-HMGCR and LXR-IDOL signaling pathways. In addition, we observed an upregulation of the expression of the membrane transporters ABCA1 and ABCG1 and of cholesterol 7-hydroxylase suggesting the stimulation of processes for cholesterol excretion and cholesterol conversion into bile acids. Furthermore, both ginsenosides targeted HMGCR and inhibited its activity via a statin-like mechanism. Globally, our findings aid in deciphering the mechanisms of action of a major class of ginseng components in regulating lipid metabolism.

1. Introduction

Cholesterol is a main constituent of cell membranes and a precursor of steroid hormones, vitamin D, and bile acids. High cholesterol levels adversely affect human health and closely correlate with atherosclerosis and increased cardiovascular risk (CDV risk). Therefore, controlling cholesterol blood levels and cholesterol homeostasis is a major strategy to reduce the risk of such diseases and to maintain normal cellular and tissue functions (Afonso et al., 2018). Cholesterol synthesis occurs in the cytoplasm and endoplasmic reticulum via a series of enzymatic reactions known as the mevalonate pathway. Several enzymes are involved in this process, but the rate-limiting step in the synthesis of cholesterol is catalyzed by HMG-CoA reductase (HMGCR) that performs the NADPH-mediated conversion of 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) to mevalonate (Afonso et al., 2018; Goldstein, DeBose-Boyd, & Brown, 2006). HMGCR expression and, consequently, cholesterol concentration are controlled at transcriptional level. The sterol-regulatory element binding protein (SREBP)-2 is the master regulator of cholesterol biosynthesis since it controls the expression of numerous genes involved in cholesterol synthesis and uptake, including *HMGCR* and *LDLR* (low density lipoprotein receptor) (Bengoechea-Alonso & Ericsson, 2007). It has been demonstrated that the activation of SREBP-2 is finely regulated and depends upon intracellular cholesterol levels. In details, low cholesterol amounts activate SREBP-2 which in turn promotes HMGCR expression to increase cholesterol biosynthesis whereas accumulation of intracellular cholesterol inhibits the SREBP-2-dependent expression of HMGCR (Brown, Radhakrishnan, & Goldstein, 2018; Goldstein et al., 2006). The liver X receptor (LXR), the inducible degrader of the low-density lipoprotein receptor (IDOL) and LDLr are part of another feedback mechanism, independent of and complementary to the SREBP pathway, that controls cholesterol homeostasis (Zhang, Reue, Fong, Young, & Tontonoz, 2012). In fact, LXRs in response to high cellular sterol levels induce the expression of IDOL, an E3 ubiquitin ligase that mediates the ubiquitination and degradation of LDLr thus limiting further uptake of LDL-cholesterol from plasma (Zelcer, Hong, Boyadjian, & Tontonoz, 2009; Zhang et al., 2012). LXRs also modulate cholesterol homeostasis through the induction of the expression of several genes involved in cholesterol efflux, including

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ABCA1 and *ABCG1* (Gelissen et al., 2006). ABCA1 and ABCG1 are members of the ABC transporter superfamily, respectively a whole transporter and a homodimeric half-transporter, and they act synergistically to remove cholesterol from cells (Oram & Vaughan, 2006). Cholesterol homeostasis regulation also occurs through catabolic and secretion pathways that primarily include bile acid synthesis. In particular, cholesterol 7α-hydroxylase (CYP7A1), a microsomal cytochrome p450 enzyme, catalyzes the first and rate-limiting step in the classic or neutral pathway of bile acid synthesis to convert cholesterol into 7α-hydroxycholesterol (Lorbek, Lewinska, & Rozman, 2012).

A common approach to lower blood cholesterol levels is to target the enzyme HMGCR and statins are considered the drug of choice for the treatment of hypercholesterolemia acting as competitive inhibitors of HMGCR. However, statin-based treatment is associated with severe side effects in different patients and the finding of alternative and safer approaches has attracted increasing attention in recent years (Rossini et al., 2022).

Panax ginseng is one of the most used species of ginseng, also known as Asian or Korean ginseng. The main active components of P. ginseng are ginsenosides, a class of triterpenoid saponins. Numerous ginsenosides have been identified in P. ginseng, with Rg1, Rb1, Rb2, Re, Rd, Rc, being the most abundant species (J. H. Kim, 2018). Single ginsenosides and ginseng extracts have been widely characterized for their biological activity against inflammation, apoptosis, oxidative stress and mitochondrial dysfunction, and also beneficial effects on cardiovascular diseases have been documented (de Oliveira Zanuso et al., 2022; Fernandez-Moriano, Gonzalez-Burgos, Iglesias, Lozano, & Gomez-Serranillos, 2017; Xue et al., 2021). The ginsenoside Rg3 and a hydrolyzed ginseng extract were demonstrated to reduce lipid accumulation in HepG2 cells by way of AMPK activation (Han, Sung, & Lee, 2017; Lee, Lee, Kim, Kim, & Kim, 2012). A Korean red ginseng extract showed anti-atherosclerosis and anti-inflammatory effects in western diet-induced atherosclerosis in LDL receptor gene deleted-mice (Im et al., 2014). Another study reported that ginsenoside Rb1 promoted the autophagic pathway and inhibited apoptosis regulating Bcl-2 family proteins to protect against atherosclerosis (Zhou et al., 2018). The ginsenoside Rb2 was able to lower lipid levels in 3T3-L1 adipocytes by favoring SREBP and leptin mRNA expression (E. J. Kim et al., 2009).

In this work, we analyzed the role of the ginsenosides Rb1 and Rb2 and of a *Panax ginseng* root extract previously prepared and characterized by our group (Manuscript accepted for publication in European Food Research and Technology journal) in regulating cholesterol homeostasis. In details, we dissected through in silico and in vitro approaches the interaction with HMGCR and evaluated the effects on the SREBP-2 and LXR-IDOL signaling pathways in HepG2 cells.

2. Materials and Methods

2.1. Reagents and chemicals

Media, reagents and plastics for cell cultures were purchased from Corning (Tewksbury, MA, USA). Ginsenosides Rb1 (purity ≥98%, C54H92O23, molecular weight 1109.3, CAS No 41753-43-9) and Rb2 (purity ≥97%, C53H90O22, molecular weight 1079.3, CAS No 11021-13-9) were purchased from Extrasynthese (Genay, France). The whole roots of *Panax ginseng* (*Panax ginseng* Meyer) coming from Jilin province, China, was purchased by A. Minardi & Figli s.r.l. (Bagnacavallo, RA, Italy). Description of the dried extract preparation and details on the composition are defined in Angeloni et al. (Manuscript submitted for publication). Human 3-hydroxy-3-methylglutaryl-CoA reductase was obtained from Sigma-Aldrich (Milano, Italy). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) used in cytotoxicity assays was from Merck Spa (Milan, Italy). Membranes for western blot analyses were purchased from Millipore (Milan, Italy). Immunodetection was performed with the enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, Milan, Italy). Antibodies used to detect HMGCR, SREBP-2, ABCA1, LDLR, IDOL, LXRα/β and CYP7A1 in Western blotting assays were obtained from Santa Cruz Biotechnology (Heidelberg, Germany). The antibody used to detect ABCG1 was obtained from Thermo Fisher Scientific (Italy).

2.2. Biosensor binding studies

The binding of Rb1 and Rb2 to HMGCR was explored using a general biosensor-based approach that exploits the interaction between a surface-blocked protein and the soluble molecules of interest. The sensing surface was thoroughly washed with PBS, pH 7.4, before being functionalized with human HMG-CoA reductase via EDC/NHS coupling chemistry (Edwards, Lowe, & Leatherbarrow, 1997). PBS was used to remove non-bound protein molecules, then activated carboxylic groups that were still available for binding were blocked with 1 M ethanolamine, pH 8.5. Finally, the ginsenosides were independently added at increasing concentrations in the range 1–11 μM, each time assessing baseline recovery between independent bindings. As previously reported, raw data were analyzed using both mono-exponential and bi-exponential models (Cuccioloni et al., 2011).

2.3. Bioinformatic analysis

The most probable binding site for Rb1 and Rb2 onto HMGCR was identified by molecular docking using the X-ray crystal structure of human HMGCR [pdb entry: 3CCT (Sarver et al., 2008)] and the 3D chemical structures of the two ginsenosides of interest (obtained from Pubchem (S. Kim et al., 2021) and energy minimized with Avogadro (Hanwell et al., 2012)). Hydrogen atoms were added to the protein prior to any analysis. Autodock 4.2, a software performing a Lamarckian genetic algorithm to explore the binding possibilities of a ligand in a binding pocket (Morris et al., 2009), was used with a grid around the catalytic pocket of HMGCR (grid spacing: 0.375 Å; root-mean-square (rms) tolerance: 0.8 Å; maximum energy evaluations: 2,500,000; other parameters were set to default values (Mozzicafreddo, Cuccioloni, Cecarini, Eleuteri, & Angeletti, 2009).

2.4. Chromatographic assay of HMGCR activity

HMGCR residual activity was determined after a 20-min preincubation of the reductase (0.4 μM) with increasing levels of the ginsenosides (0–35 μM), essentially as previously reported (Mozzicafreddo, Cuccioloni, Eleuteri, & Angeletti, 2010). In brief, each preformed ginsenoside-HMGCR complex was added to 1.55 μM HMG-CoA and 2.68 mM NADPH independently and incubated for 60 min at 37 °C. The resulting mixture was separated using an AKTA HPLC system (GE Healthcare) equipped with a Phenomenex Luna C18 reverse-phase (RP)-HPLC column set to 26 ± 0.1 °C.

2.5. Cell treatment and viability assay

HepG2 cells were grown in DMEM supplemented with 10% foetal bovine serum (FBS), antibiotic and antimycotic, at 37 °C with 95% air and 5% CO_2. The MTT assay was used to evaluate viability of treated cells. In details, after 24 h exposure to different concentrations of the ginseng extract and single ginsenosides Rb1 and Rb2, cells were washed in PBS (pH 7.5), the MTT (final concentration 0.5 mg/mL) was added to the medium without FBS and incubated at 37 °C for 2 h. Then, the medium was replaced with DMSO (100 μL). Three independent experiments each with six replicates were performed. The optical density was measured at 550 nm in a microtiter plate reader. For cell treatments, ginsenosides Rb1 and Rb2 (50 μM final concentration) and the ginseng root extract (100 μg/mL – 200 μg/mL – 400 μg/mL final concentrations) were dissolved in DMSO, vortexed and sonicated at 50 °C for 30 min to ensure complete solubilization. Control cells were treated with DMSO. Experiments were performed in triplicate. After 24 h, the medium was

removed, cells were washed and harvested in 4 mL of PBS and centrifuged at 1600×*g* for 5 min. For the time-course experiments, HepG2 cells were treated with 400 μg/mL of the extract and with 50 μM of ginsenosides Rb1 and Rb2 for 0, 1, 2, 4, 8 and 24 h. Pellets were resuspended in RIPA lysis buffer (containing protease inhibitors) and passed at least ten times through a 29-gauge needle. Obtained lysates were centrifuged at 12,000×*g* for 15 min and the supernatants stored at −80 °C until use. Protein concentration was measured by the Bradford assay using bovine serum albumin (BSA) as standard (Bradford, 1976).

2.6. Cholesterol levels

Total (TC) and free cholesterol (FC) in HepG2 cells treated with ginsenosides Rb1 and Rb2 and the ginseng root extract were quantified with the AmplexRed Cholesterol Assay kit (Waltham, MA, USA) with values normalized to total cellular protein quantified via Bradford assay. The TC content was determined by measuring the cholesterol concentration in the presence of the enzyme cholesterol esterase. To measure FC, cholesterol esterase was omitted from the assay. Upon treatments, cells were trypsinized, washed with PBS, and centrifuged at 8000×*g* for 5 min. Briefly, the pellets were suspended in RIPA lysis buffer and lysed with a 29-gauge syringe. The working solution was freshly prepared in reaction buffer and contained Amplex® Red reagent (300 μM), horseradish peroxidase (2 U/mL), cholesterol oxidase (2 U/mL), and cholesterol esterase (0.2 U/mL). A cholesterol standard curve was created by diluting the provided cholesterol reference standard (5.17 mM) in reaction buffer. 50 μL of cell lysates diluted in reaction buffer and 50 μL of the working solution were placed on a 96-well plate and incubated at 37 °C. Fluorescence measurements were recorded after 30 min on a SpectraMax Gemini XPS microplate reader (λ_{exc}= 540 nm, λ_{em}= 590 nm). Each sample was set up in triplicate within the assay, and three independent experiments were performed.

2.7. Western blotting

Proteins were resolved by SDS–PAGE (8–12% acrylamide) and then transferred onto PVDF membranes. Membranes were incubated with primary monoclonal antibodies to detect HMGCR, SREBP-2, LDLR, IDOL, LXRα/β, CYP7A1, ABCA1 and ABCG1 and then with the specific peroxidase-conjugated secondary antibodies. ECL Western blotting detection reagents were used to detect proteins at the ChemiDoc MP system. Molecular weight markers (12–225 kDa, GE Healthcare) were loaded in each gel. Membranes were stripped and re-probed with an anti-GAPDH monoclonal antibody to ensure for equal protein loading. Stripping buffer contained 200 mM glycine, 0.1% SDS, and 1% Tween 20. Protein bands were quantified using ImageJ 1.52a software (NIH, Bethesda, MD, USA) and normalized to GAPDH.

2.8. Statistical analysis

Data are presented as mean values with standard deviations. Statistical analysis was performed with one-way ANOVA, followed by the Bonferroni post hoc test using SigmaStat Version 3.1 software (SPSS, Chicago, IL, USA), and a p-value less than 0.05 was considered to be statistically significant.

3. Results

3.1. Binding of Rb1 and Rb2 to human HMGCR

First, the interaction between Rb1 and Rb2 and human HMGCR was explored and quantitatively characterized according to a biosensor-

Table 1
Comparison of kinetic and equilibrium constants (k_{ass}, k_{diss} and K_d), computed equilibrium constants ($K_{d,p}$) and inhibition parameters (IC_{50} and K_i) for the interaction between Rb1/Rb2 and HMGCR.

Ginsenoside	k_{ass} ($M^{-1}s^{-1}$)	k_{diss} (s^{-1})	K_d (μM)	$K_{d,p}$ (μM)	IC_{50} (μM)	K_i (μM)
Rb1	163592 ± 9050	0.294 ± 0.012	1.8 ± 0.2	1.1	10.2 ± 2.3	3.1 ± 0.8
Rb2	13495 ± 1950	0.034 ± 0.002	2.5 ± 0.3	0.9	17.3 ± 4. 8	5.3 ± 1.7

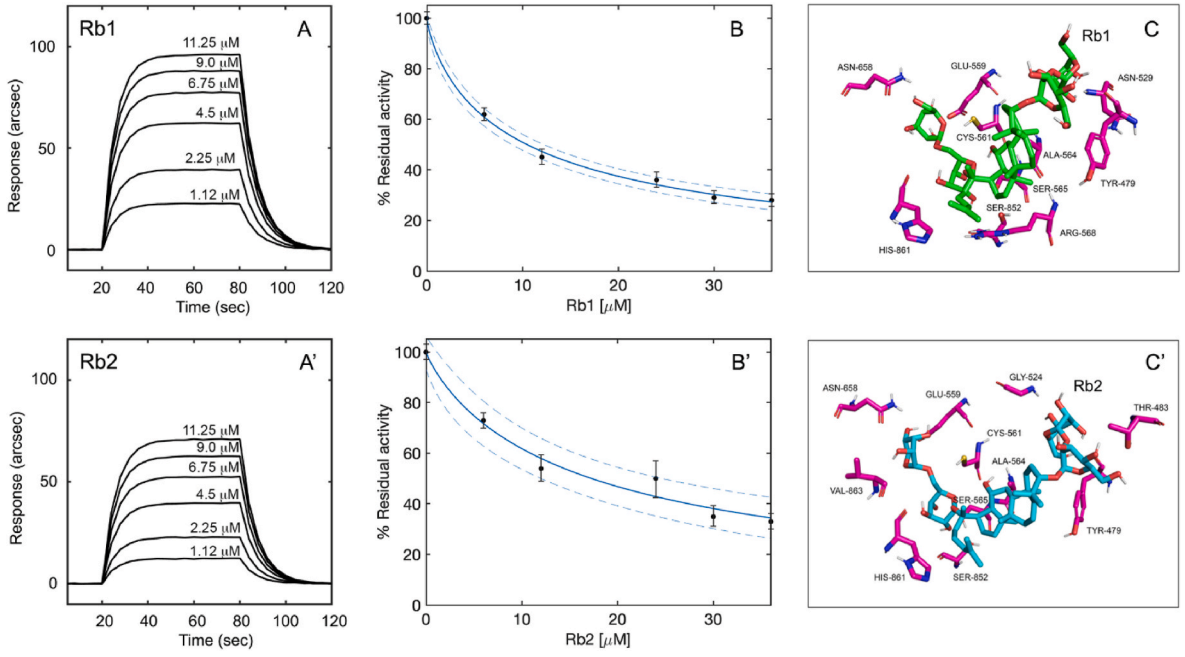

Fig. 1. Characterization of the ginsenosides Rb1 and Rb2 and HGMCR interaction. Representative sensor traces obtained upon binding of different concentrations of Rb1 (Panel A) and Rb2 (Panel A′) to surface-blocked HMGCR. Residual activity plots of HMGCR in the presence of increasing Rb1 (Panel B) and Rb2 (Panel B′) concentrations. Visualization of the HMGCR residues involved in the formation of the complexes with ginsenosides Rb1 (Panel C) and Rb2 (Panel C′) as derived from molecular docking analysis.

based assay (Fig. 1, panels A and A', Table 1). Both interactions were found to be reversible, with an affinity (in terms of equilibrium dissociation constant, K_d) in the sub-micromolar range under physiological-like conditions.

Regardless of the comparable values in K_d, the analysis of kinetic parameters revealed significant differences in the recognition phase and in the kinetic stability of the two complexes: in fact, the interaction between Rb1 and HMGCR was characterized by 10-fold faster association and dissociation kinetics compared with Rb2 (these differences being mostly "thermodynamically hidden" by the ratio k_{diss}/k_{ass}).

3.2. Effect of Rb1 and Rb2 on the HMGCR activity

Considering the results of the binding between ginsenosides and HMGCR, we investigated the ability of compounds Rb1 and Rb2 to modulate the activity of the reductase using the chromatographic method described in the Materials and Methods section. Both compounds inhibited enzyme functionality in a concentration dependent manner, with Rb1 being slightly more effective than Rb2 (Fig. 1, panels B and B'). The inhibition parameters obtained from in vitro activity assays on isolated human HMGCR are shown in Table 1.

3.3. Analysis of ginsenosides-HMGCR binding mode

Molecular docking studies helped rationalizing the binding modes of ginsenosides Rb1 and Rb2 to HMGCR (Fig. 1, panels C and C', SF1 and SF2). In line with the observed inhibition, for both molecules the best scoring models evidenced the preferential binding at the catalytic pocket of HMGCR (in a region largely overlapping with the NADPH binding site) with a moderate binding affinity in the μM range, comparable to biosensor data (Table 1). Structurally, ginsenosides were predicted to form a variable number of H-bonds and hydrophobic interactions with a number of conserved aminoacids.

3.4. Effects of treatments on cell viability

HepG2 cells were treated with increasing concentrations of the ginsenosides Rb1 and Rb2 (from 0 to 100 μM) and of the ginseng extract (from 0 to 800 μg/mL) and cell viability was evaluated after 24 h using the MTT assay (Fig. 2). Notably, no visible signs of toxicity were detected in HepG2 cells treated with ginsenoside Rb2, whereas there was a slight decline in cell viability upon exposure to the highest concentration (800 μg/mL) of the ginseng root extract. As for ginsenoside Rb1, a slight dose-dependent increase in cell number was measured with concentrations ≥10 μM.

3.5. Effects of treatments on cholesterol levels

The AmplexRed Cholesterol Assay kit was used to measure the amount of TC and FC in HepG2 cells after 24 h of treatment with increasing concentrations of ginseng root extract and 50 μM of ginsenosides Rb1 and Rb2. Fig. 3, panel A, shows that treatments with 200 and 400 μg/mL ginseng root extract similarly decreased both TC and FC cholesterol. This decrease was more pronounced in cells treated with the ginsenosides Rb1 and Rb2 (Fig. 3, panel A). Besides, time-course experiments revealed that treatment of HepG2 cells with 400 μg/mL ginseng root extract and both ginsenosides (50 μM) for 0, 1, 2, 4, 8, 24 h decreased TC and FC levels in a time-dependent manner starting at 8 h (Fig. 3, panel B). No changes in cholesterol levels were observed in DMSO-treated cells (control) throughout the 24 h time-course.

3.6. Effects of treatments on SREBP-2 signaling pathway

To gain insight into the mechanisms that favor the observed cholesterol decrease in HepG2 cells and to evaluate the role of HMGCR, we first explored the expression of proteins that are part of the SREBP-2 signaling pathway. SREBP-2, a member of the sterol regulatory element-binding proteins family, mainly regulates the expression of genes involved in cholesterol metabolism including *HMGCR and LDLR*. Data shown in Fig. 4 indicate that, compared to the control group, there is a 1.2-fold reduction in SREBP-2 expression in cells treated with the highest concentration of the ginseng root extract ($p<0.05$). Ginsenosides Rb1 and Rb2 were even more effective in reducing the expression of the transcription factor in treated cells (2.4- and 2.14-fold decrease, respectively, $p<0.01$). In line with the reduced levels of the SREBP-2 protein, there was also a decline in the expression of LDLr, that ultimately increases the clearance of LDL-cholesterol from the bloodstream, and HMGCR, the rate-limiting enzyme of the mevalonate pathway of cholesterol biosynthesis. In both cases, the decrease was significant in HepG2 cells treated with the highest ginseng root extract concentration (400 μg/mL) and even more evident upon treatments with ginsenosides

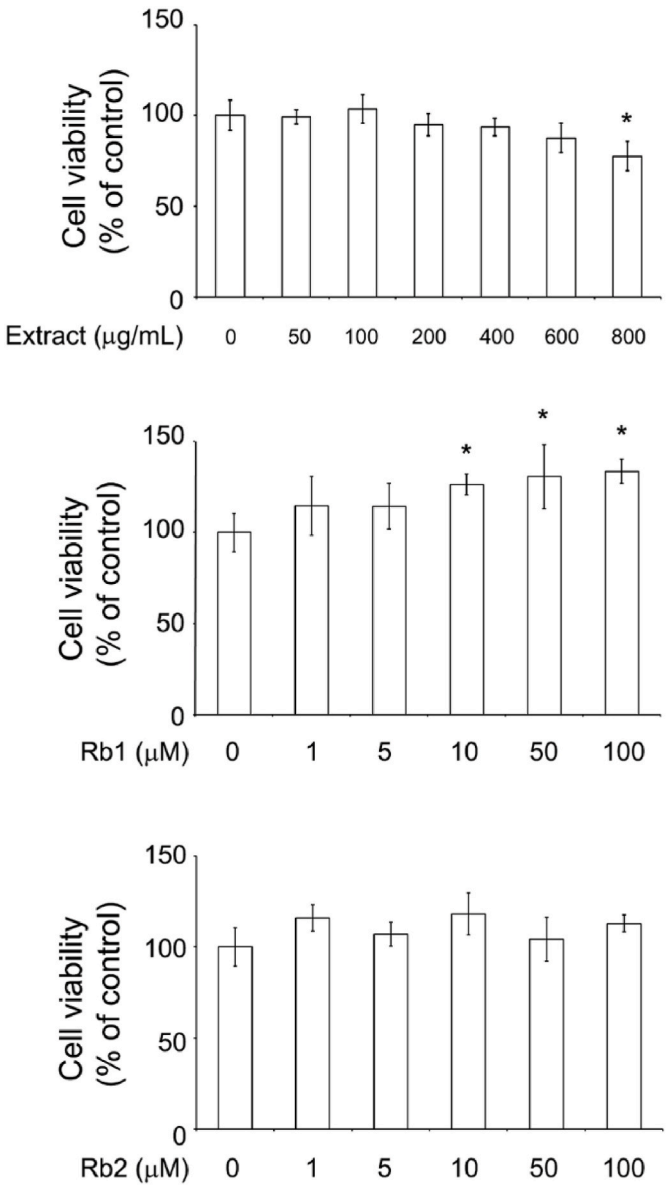

Fig. 2. Effects of treatments on HepG2 cell viability. Cells were treated with increasing concentrations of the ginseng root extract and ginsenosides Rb1 and Rb2 and the MTT assay was performed to check for cell viability. Results are expressed as percentage compared to control cells treated with DMSO and are obtained from three independent experiments each with six replicates. Asterisks indicate data points statistically significant with respect to the control (*$p< 0.05$).

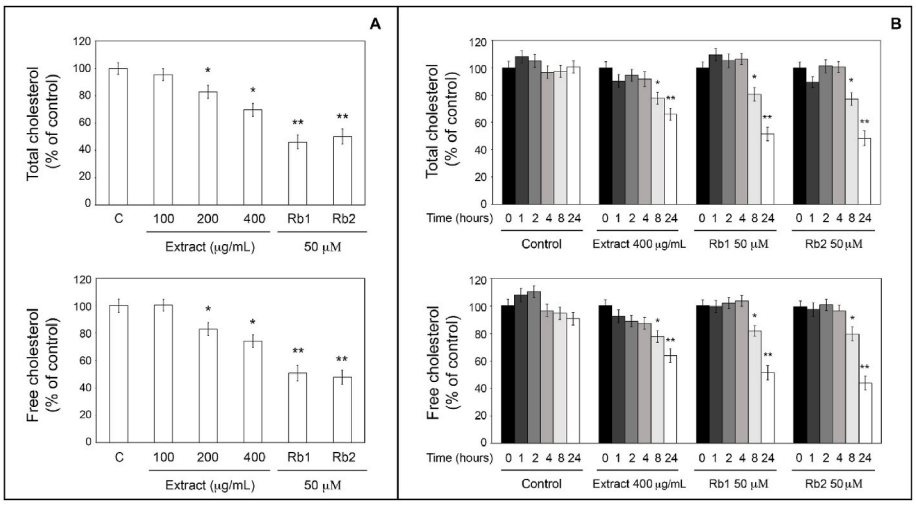

Fig. 3. Effects of treatments on cellular cholesterol levels. Panel A. TC and FC measured in HepG2 cells upon 24 h treatment with 0–400 μg/mL of ginseng extract and with 50 μM of ginsenosides Rb1 and Rb2. Results are expressed as percentage compared to control cells (*p< 0.05, **p< 0.01). Panel B. Time-course experiments (0–24 h) to monitor TC and FC changes upon treatment with 400 μg/mL of the extract and 50 μM of Rb1 and Rb2. Cholesterol was measured using the AmplexRed Cholesterol Assay kit as described in the Materials and methods section. Experiments were performed in triplicate. For each treatment, results are expressed as percentage compared to cholesterol levels at 0 h exposure (*p< 0.05, **p< 0.01).

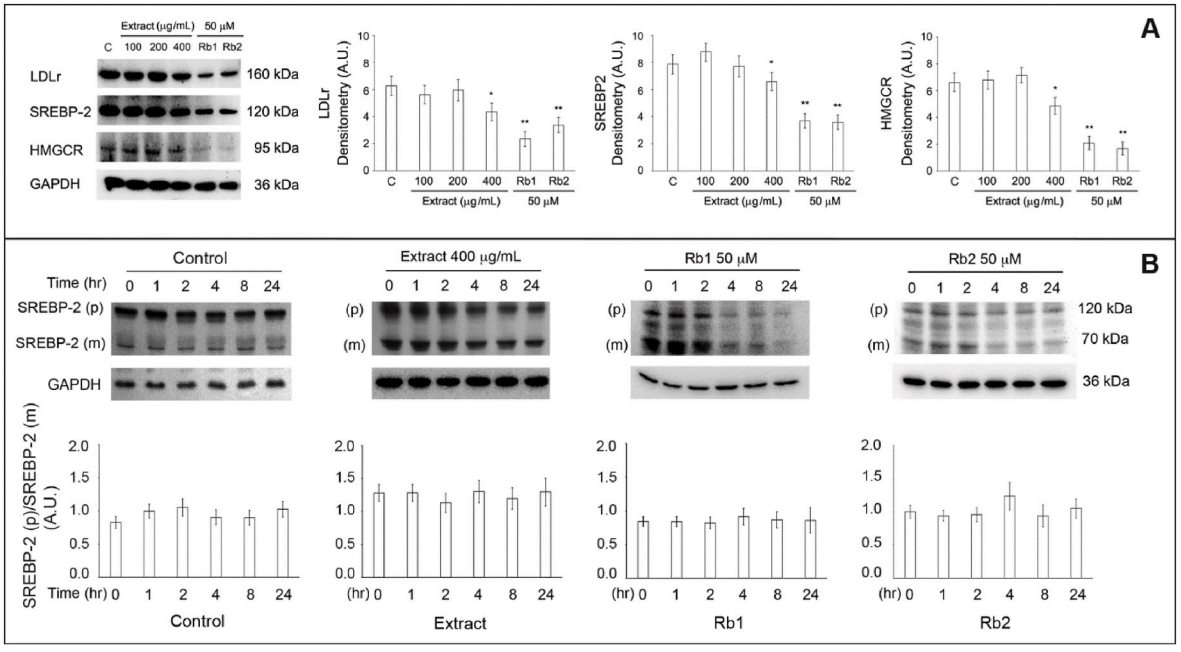

Fig. 4. Effects of treatments on SREBP-2 signaling pathway. Panel A. SREBP-2, LDLr and HMGCR expression levels detected in HepG cells treated for 24 h. Equal protein loading was verified by using an anti-GAPDH antibody and normalized expressions are reported as arbitrary units (A.U.). Experiments were performed in triplicate. Asterisks indicate data points statistically significant with respect to the control (*p< 0.05, **p< 0.01). Panel B. SREBP-2 (precursor and mature form) levels in HepG2 cells treated for the indicated time. Data were normalized to respective GAPDH and then to untreated cells and are reported as the ratio of SREBP-2 (p) and SREBP-2 (m) intensities. (A.U.) Arbitrary Units. Experiments were performed in triplicate. Asterisks indicate data points statistically significant with respect to 0 h exposure (*p< 0.05, **p< 0.01).

Rb1 and Rb2 (Fig. 4, panel A).

Time-course experiments were performed to evaluate the sequence of events in the SREBP-2 pathway. Cells were exposed to 400 μg/mL of the extract or 50 μM Rb1/Rb2 and measurements of SREBP-2 precursor and mature forms were performed throughout a 24 h time-course. No changes in the levels of both SREBP-2 forms were detected in DMSO-treated cells (control cells) during the 24 h time-course whereas treatments reduced precursor and mature SREBP-2 in parallel from 4 h after exposure thus preceding the decrease in cholesterol concentration (Fig. 4, panel B).

3.7. Effects of treatment on LXRs-IDOL signaling pathway

Cholesterol metabolism is also regulated by LXRs by promoting cholesterol efflux and suppressing de novo synthesis and uptake. The liver X receptors α and β (LXRα/β) control the expression of the inducible degrader of LDL receptor (IDOL) that promotes LDLr ubiquitination and its degradation (Zelcer et al., 2009). The data collected show an increase in the levels of LXRs upon exposure to the ginseng root extract which is statistically significant at a concentration 400 μg/mL. Similarly, HepG2 cells treated with Rb1 and Rb2 exhibited a clear rise in LXRα/β levels

(1.4- and 1.8-fold increase, respectively) (Fig. 5, panel A). Accordingly, we obtained an increase in the expression of IDOL upon treatment with the ginseng root extract (400 μg/mL, 1.5-fold increase compared to control cells), as well as upon treatments with Rb1 and Rb2 (2.2- and 2.5-fold increase compared to control cells) (Fig. 5, panel A). This finding being in line with the observed reduction in LDLr expression. In addition, considering that LXR upregulates membrane transporters ABCA1 and ABCG1, two key players in cholesterol efflux, we investigated the expression of the two proteins upon 24 h treatment with the extract and ginsenosides. Results show an increase in the levels of ABCA1 (1.5, 1.7 and 1.8-fold increase compared to control cells upon exposure to 400 μg/mL of extract, Rb1 and Rb2, respectively) and ABCG1 (1.3, 1.7 and 1.7-fold increase compared to control cells upon exposure to 400 μg/mL of extract, Rb1 and Rb2, respectively), indicating that treatments also promoted cholesterol efflux from cells in a LXR-dependent manner (Fig. 5, panel B).

3.8. Effects of treatment on CYP7A1 expression levels

We then evaluated if the observed cholesterol reduction is also the consequence of an increased cholesterol excretion through its conversion to bile acids. CYP7A1 is the rate-limiting enzyme in the classic pathway of cholesterol conversion to bile acids that is limited to the liver (J. Y. Chiang, Kimmel, & Stroup, 2001; J. Y. L. Chiang & Ferrell, 2020; Gupta, Pandak, & Hylemon, 2002; Lehmann et al., 1997). We obtained an upregulation of CYP7A1 in HepG2 cells exposed to both single ginsenosides (1.6- and 1.3-fold increase compared to control cells, Rb1 and Rb2 respectively) and to the highest concentration of the extract (1.8-fold increase compared to control cells) (Fig. 5 panel C).

4. Discussion

Hypercholesterolemia is among the risk factors for cardiovascular disorders. Controlling the levels of circulating cholesterol through specific drugs or dietary recommendations is therefore advantageous to prevent atherosclerosis. Statins are the most common prescribed medication for high cholesterol levels, however, people who take *statins* may experience some *side effects* (*Newman* et al., *2019*). Ginseng has been extensively studied in humans and several biological effects have been well characterized. Ginseng products are often referred to as "adaptogens", which indicate plant-derived agents that can increase resistance to physical, chemical, and biological stress stabilizing homeostasis and metabolic functions (Ratan et al., 2021). *Panax ginseng* Myer is a common herb used in East Asian countries especially in Korea, China, and Japan. Ginsenosides are the most important components and most of the research on *Panax ginseng* focuses on these molecules. Ginsenosides Rb1 and Rb2 are among the most abundant ginsenosides in ginseng roots. In vitro and in vivo studies have demonstrated properties for both ginsenosides against aging, oxidation, inflammation, obesity, hyperglycemia, and diabetes (Miao et al., 2022; Zhou et al., 2019). Cellular cholesterol metabolism is tightly regulated by several signaling pathways and its biosynthesis occurs through the mevalonate pathway and is stimulated when sterol levels are low. The key enzyme in this metabolic process is HMGCR (Buhaescu & Izzedine, 2007). No data are currently available on the direct interaction between ginsenosides and the reductase. Therefore, we first characterized through different approaches the interaction between this enzyme and ginsenosides Rb1 and Rb2 and observed that both molecules bound HMGCR reversibly with comparable affinity in the low micromolar range and inhibited the enzyme activity competitively with NADPH.

Considering these preliminary data, we further analyzed the effects of ginsenosides Rb1 and Rb2 on cholesterol metabolic pathways of

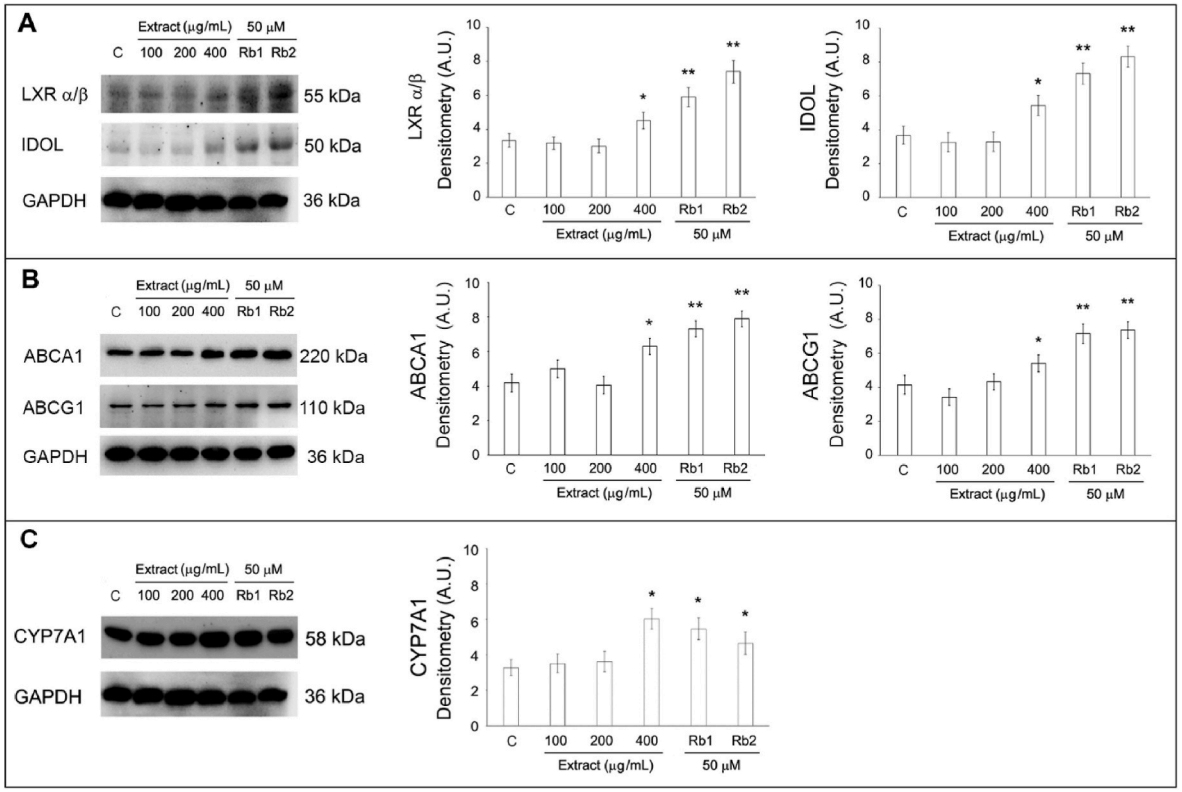

Fig. 5. Effects of treatments on the expression levels of LXRα/β, IDOL, ABCA1, ABCG1 and CYP7A1. LXRα/β, IDOL, ABCA1, ABCG1 and CYP7A1 expression levels were detected using specific primary antibodies. Equal protein loading was verified by using an anti-GAPDH antibody and normalized expressions are reported as arbitrary units (A.U.). Experiments were performed in triplicate. Asterisks indicate data points statistically significant with respect to the control (*$p<0.05$, **$p<0.01$).

HepG2 cells and compared them with a *P. ginseng* extract previously characterized by our group. The quantitation of the ginsenosides in the extract was performed by LC-MS (Manuscript accepted for publication in European Food Research and Technology journal). The dried extract contained a total ginsenoside concentration of 25.39 ± 0.32 mg/g, with 7.64 ± 0.03 mg/g of ginsenoside Rb1 and 3.28 ± 0.01 mg/g of ginsenoside Rb2, in agreement with findings from Xiu et al. and Wang et al. who reported that ginsenoside Rb1 is one of the main ginsenoside in ginseng roots (Wang, Zhang, Yang, Zhao, & Wang, 2016; Xiu et al., 2019). This extract and individual ginsenosides were shown to induce anti-inflammatory and antioxidant effects in THP1 LPS-stimulated cells (Manuscript accepted for publication in European Food Research and Technology journal).

Cells were first exposed to increasing concentrations of the extract/ginsenosides and no signs of toxicity were detected with only a slight increase in cell viability observed upon treatment with Rb1. Ginsenosides Rb1 and Rb2 and the root extract significantly reduced cellular levels of both total and free cholesterol starting at 8 h of exposure. Our data are in line with previous findings on ginseng ability to lower cholesterol levels. GINST, a hydrolyzed ginseng extract, was reported to inhibit cholesterol synthesis in HepG2 cells by decreasing HMGCR expression by means of AMPKα activation (Han et al., 2017). P. ginseng extracts were demonstrated to ameliorate blood lipid profile reducing total and LDL-cholesterol levels (Hernandez-Garcia, Granado-Serrano, Martin-Gari, Naudi, & Serrano, 2019). A recent article from Qui et al. demonstrated that ginsenosides Rb1, Rg1, Rg3, and CK decreased cholesterol content in U251 glioblastoma cells (Qiu et al., 2022). In order to explore the molecular events behind the reduced cholesterol levels and the involvement of HMGCR, we investigated the SREBP-2 signaling pathway. SREBP-2 is a transcription factor involved in a feedback mechanism for cholesterol metabolism regulation in response to intracellular sterol levels. It controls the expression of two proteins such as HMGCR and LDLr, that uptakes LDL-cholesterol form the blood (Islam, Hlushchenko, & Pfisterer, 2022). The ginseng root extract (400 μg/mL) induced a slight but significant decrease in the expression of SREBP-2 and this effect was more evident in cells treated with Rb1 and Rb2. In line with the downregulation of SREBP-2, we detected a decrease in the expression of HMGCR and LDLr, which are normally transcribed upon SREBP-2 activation. Also in this case, the expression of both proteins significantly decreased upon treatment with the highest dose of the root extract and with the ginsenosides Rb1 and Rb2. Time-course experiments performed to monitor the sequence of events in the SREBP-2 pathway revealed that the levels of the precursor and the mature form of SREBP-2 decrease in parallel from 4 h exposure thus preceding cholesterol reduction. The fact that the decrease in SREBP-2 (p) levels does not result from an increase in the conversion to SREBP-2 (m), which is the transcriptionally active form, is in line with the lower expression of SREBP-2 target genes in ginseng/ginsenoside-treated cells. Thus, treatments prevent cholesterol synthesis in HepG2 cells through the downregulation of SREBP-2 that subsequently diminishes the expression of its target genes, including HMGCR, involved in cholesterol synthetic pathways.

Very limited data are available on the involvement of SREBP-2 on the cholesterol lowering activity of ginsenosides Rb1 and Rb2. Previous data indicated that steatotic hepatocyte L02 cells treated with ginsenoside Rb1 and other notoginseng components showed reduced HMGCR and SREBP-2 expression and a simultaneous upregulation of CYP7A1, an enzyme involved in bile acids synthesis in the liver (Z. Chen et al., 2016). A paper from Lee et al., focused on ginsenoside Rg3 and suggested its possible role in lowering CVD risk through the regulation of lipid homeostasis. In fact, ginsenoside Rg3 significantly reduced hepatic cholesterol and triglyceride levels, inhibited expression of SREBP-2 and HMGCR and increased AMPK activity (Lee et al., 2012).

Another feedback mechanism for the regulation of cholesterol biosynthesis is mediated by LXRα/β and IDOL. Levels of both proteins resulted upregulated in treated HepG2 cells thus correlating with the decrease in the amount of LDLr. In fact, LXRs control the activation of IDOL, a ligase that facilitates the removal of LDLr thus reducing their expression on the cell surface and the uptake of LDL/cholesterol particles (Zelcer et al., 2009; Zhang et al., 2012). LXRs are important modulators of inflammatory processes, innate and adaptive immunity (Bilotta, Petillo, Santoni, & Cippitelli, 2020). Previous studies demonstrated that the activation of LXRs can affect the proliferation and survival of different types of cancer cells which are characterized by reprogramed metabolic pathways and altered cholesterol homeostasis (Bilotta et al., 2020). Our data indicate that ginsenosides Rb1 and Rb2 and the ginseng extract can reduce LDLr levels acting at transcriptional level on SREBP-2 and favoring its lysosomal-mediated degradation through the LXRs-IDOL pathway. On this regard, additional work is needed to better evaluate the possible impact of LDLr downregulation in in vivo studies. Hepatic LDL receptors are important regulators of plasma LDL-cholesterol concentrations. Their decline could in fact prevent the intake of plasma-derived LDL-C thus altering the amount of cholesterol at systemic level. To date, several randomized clinical trials have shown dissimilar results. In fact, some studies support the positive influence on blood lipid levels of ginseng supplementation whereas other findings do not observe a significant effect. For example, a systematic review and meta-analysis of randomized clinical trials indicates that ginseng extract may induce an improvement in blood lipid profile mainly by a reduction in total and LDL-cholesterol levels (Hernandez-Garcia et al., 2019). Conversely, another systematic review and meta-analysis does not support the beneficial effects of ginseng supplementation on blood parameters, including triglycerides and cholesterol. However, the same work reports that subgroup analyses showed a significant lowering effect on blood lipid parameters upon higher ginseng doses and longer treatments (Ziaei et al., 2020). Therefore, additional studies performed optimizing ginseng doses and treatment duration are needed to definitely clarify the ginseng supplementation effects.

LXRs activation directly upregulates ABCA1 and ABCG1, membrane transporters responsible for cholesterol efflux (Gelissen et al., 2006). In the current work, we observed that treatments increased LXRs levels and stimulated ABCA1/ABCG1 expression presumably favoring cholesterol efflux and contributing to the observed decrease in cellular cholesterol. These outcomes further strengthen the role of LXRα in the cholesterol lowering activity of ginseng in HepG2 cells.

The liver plays an important role in cholesterol homeostasis through the elimination of excess circulating cholesterol in the form of bile acids. CYP7A1 is the rate-limiting enzyme responsible for the conversion of cholesterol to bile acids through the classic pathway that accounts for about 75% of bile acids production (J. Y. Chiang et al., 2001; J. Y. L. Chiang & Ferrell, 2020; Gupta et al., 2002; Lehmann et al., 1997). Kawase et al. demonstrated that a red ginseng root extract was able to upregulate CYP7A1 mRNA levels in hypercholesterolemic rats and in rat primary hepatocytes (Kawase et al., 2013). They also found that the ginsenosides Rg_1, Re, Rg_3, Ro, and Rg_2 increased CYP7A1 mRNA levels in vitro (Kawase et al., 2013). In agreement with these findings, we show that CYP7A1 was increased in response to ginsenosides Rb1 and Rb2 and ginseng extract treatments indicating the activation of cholesterol catabolism and excretion through the classic pathway for bile acid synthesis. Interestingly, targeting enzymes of bile acid synthesis to increase conversion of cholesterol may represent an anti-atherogenic approach. Indeed, transgenic expression of CYP7A1 in mice conferred resistance to atherosclerosis, prevented high-fat/high-cholesterol diet-induced obesity and decreased inflammation and the induction of the enzyme through diet-related strategies ameliorated atherosclerotic risk (Q. Chen, Wang, Ma, & Zhai, 2012; Del Bas et al., 2005; Li et al., 2010; Miyake et al., 2002).

Our study reports for the first time the ability of ginsenosides Rb1 and Rb2 to interact with HMGCR promoting the inhibition of its activity. We also demonstrate that single ginsenosides and a ginseng extract can efficiently lower cholesterol concentration in HepG2 cells through the

modulation of the SREBP-2-HMGCR and LXRs-IDOL signaling pathways. Moreover, the upregulated expression of the membrane transporters ABCA1 and ABCG1 and of cholesterol 7-hydroxylase suggests the stimulation of processes for cholesterol excretion and cholesterol conversion into bile acids. Together these findings further contribute to elucidate the biological effects and molecular mechanisms activated by ginseng components and responsible for the regulation of lipid metabolism.

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Author statement

The present article is not under consideration for publication elsewhere. The paper is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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