Evidence for interactions between homocysteine and genistein: insights into stroke risk and potential treatment

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Abstract

Elevated plasma homocysteine (2-amino-4-sulfanylbutanoic acid) level is a risk factor for stroke. Moreover, it has been suggested that high levels of homocysteine in the acute phase of an ischemic stroke can predict mortality, especially in stroke patients with the large-vessel atherosclerosis subtype. In clinical studies, supplementation with genistein (5, 7-dihydroxy-3- (4-hydroxyphenyl)-4*H*-1-benzopyran-4-one) decreased plasma homocysteine levels considerably. Therefore, genistein could be

considered as a potential drug for prevention and/or treatment of stroke. However, the mechanism of the effect of genistein on homocysteine level remains to be elucidated. In this report, direct functional interactions between homocysteine and genistein are demonstrated in *in vitro* experimental systems for determination of methylenetetrahydrofolate reductase (MetF) and glutathione peroxidase (GPx) activities, reconstructed with purified compounds, and in a simple *in vivo* system, based on measurement of growth rate of *Vibrio harveyi* and *Bacillus subtilis* cultures. Results of molecular modelling indicated that homocysteine can directly interact with genistein. Therefore, genistein-mediated decrease in plasma levels of homocysteine, and alleviation of biochemical and physiological effects of one of these compounds by another, might be ascribed to formation of homocysteine-genistein complexes in which biological activities of these molecules are abolished or alleviated.

Keywords

Homocysteine
Genistein
Methylenetetrahydrofolate reductase

Vibrio harveyi
Stroke

Introduction

Elevated plasma homocysteine (2-amino-4-sulfanylbutanoic acid) level is a risk factor for stroke (for a review, see Petras et al. 2014). In the literature, there are many reports supporting such a statement, like those published recently (Chen et al. 2015, ~~Han et al. 2015~~Han et al. 1999, Smith et al. 2016, Wang et al. 2015). Moreover, it has been suggested that high levels of homocysteine in the acute phase of an ischemic stroke can predict mortality, especially in stroke patients with the large-vessel atherosclerosis subtype (Shi et al. 2015).

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Genistein (5, 7-dihydroxy-3- (4-hydroxyphenyl) -4*H*-1-benzopyran-4-one), a natural isoflavone, has been suggested to protect central nervous system against oxidative stress and neuroinflammation (for a review, see Banecka-Majkutewicz et al. 2012). In vitro studies demonstrated that genistein can reverse homocysteine-induced changes in levels of some proteins involved in metabolism and detoxification (Fuchs et al. 2005). Moreover, homocysteine-dependent apoptosis of endothelial

cells was inhibited by genistein (Fuchs et al. 2006), and this isoflavone could protect such cells from homocysteine-caused inflammatory injury (Han et al. 2015). In clinical studies, 4-week supplementation of 150 mg genistein per day decreased plasma homocysteine levels considerably (Chen et al. 2005). Although such effects of genistein could not be detected when this isoflavone was administered for 6 months at the dose of 54 mg/day (D'Anna et al. 2005), the same dose used for 24 months resulted in lowering of the homocysteine concentration (Marini et al. 2010).

Already known biochemical actions of genistein include interactions with estrogen receptors (Martin et al. 1978; Wang et al. 1996), inhibition of tyrosine kinase activities (Akiyama et al. 1987; Moskot et al. 2014; Moskot et al. 2015), and inhibition of topoisomerase II function (Markovits et al. 1989; Salti et al. 2000). However, these activities cannot explain the mechanism of either genistein-mediated alleviation of homocystein-induced changes in cell cultures or genistein-dependent decrease in plasma homocysteine levels in patients.

One of possible targets for genistein might be methylenetetrahydrofolate reductase (MTHFR) (EC 1.5.1.20), an enzyme that catalyzes conversion of

5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which serves as a methyl donor in the remethylation of homocysteine to methionine (Bailey and Gregory 1999). This enzyme is conserved in the evolution, and its bacterial variant (MetF) can be used as a model in studies on the human protein (MTHFR). In fact, MetF and MTHFR are very similar both structurally (Guenther et al. 1999) and functionally (Jakóbkiewicz-Banecka et al. 2005), and specific mutations in the *Escherichia coli metF* gene correspond to common polymorphisms in the human gene (Guenther et al. 1999). However, recent studies indicated that genistein inhibited activity of MetF rather than stimulated it (Grabowski et al. 2015). Thus, such a function of genistein cannot explain its effect on the decrease of homocysteine levels. In this light, the aim of this work was to test if genistein is able to directly interact with homocysteine.

Materials and methods

Proteins and small molecules

The MetF protein was purified as described previously (~~Shepard~~Sheppard et al. 1999). Homocysteine, genistein, NADH, menadione, glutathione peroxidase, glutathione reductase,

NADPH, reduced glutathione, hydrogen peroxide, tert-butyl hydroperoxide and buffer ingredients were purchased from Sigma – Aldrich.

MetF activity test

MetF activity assay was performed as described previously (~~Shepard~~Sheppard et al. 1999), by measuring a decrease in absorbance of NADH, consumed during the reaction. The reaction mixture consisted of 50 mM phosphate buffer containing 10% glycerol and 0.3 mM EDTA, 400 µM NADH, and 1.4 mM menadione (vitamin K3 is used as an artificial substrate for MetF). The activity of MetF was determined by measurement of the kinetics of the reaction at 37 °C. The reaction mixture was prepared without the enzyme, and incubated for 5 min. Following reaction initiation by the addition of the 0.3 µM enzyme, the measurement was carried out for 30 min, by monitoring the absorbance at a wavelength of 340 nm. When indicted, the reaction was supplemented with genistein and/or homocysteine, added to indicated concentrations. In control experiments, DMSO (a solvent used for preparation of stock solutions) was added instead of the tested compound(s).

Glutathione peroxidase (GPx) activity test

Glutathione peroxidase (GPx) activity assay was performed as described previously (Lawrence and Burk 1976), by measuring a decrease in absorbance of NADPH, consumed during the reaction. The reaction mixture consisted of 50 mM phosphate buffer (pH 7.0), 0.2 mM glutathione (reduced), glutathione reductase (1 U/ml), 0.5 mM EDTA, 0.1 mM NADPH, and glutathione peroxidase (5 mU/ml). The mixture was pre-incubated for 10 min. The reaction was started by the addition of hydroperoxide. The activity of GPx was determined by measurement of the kinetics of the reaction; the decrease of absorbance at 340 nm was determined. When indicted, the reaction was supplemented with genistein and/or homocysteine, added to indicated concentrations. In control experiments, DMSO in water solution was added instead of the tested compound(s).

Effect of genistein and/or homocysteine on *Vibrio harveyi* and *Bacilus subtilis* growth

Vibrio harveyi strain BB7 (Belas et al. 1982) was grown at 30 °C in BOSS liquid medium (Klein et al. 1998) to early exponential phase. *Bacillus subtilis* strain 168 was grown at 30 °C in LB liquid medium. Genistein and/or homocysteine was/were added to indicated concentrations, and A_{600} of the

culture was measured at indicated times. In control experiments, DMSO (a solvent used for preparation of stock solutions) was added instead of the tested compound(s).

Isothermal titration calorimetry (ITC)

ITC experiments were performed at 298.15 K using an AutoITC isothermal titration calorimeter (MicroCal, Northampton, USA) with a 1.4491-mL sample and the reference cells. The reference cell contained distilled water. All reagents were dissolved directly into water/DMSO solution. The experiment consisted of injecting 10.02 μL (29 injections, 2 μL for the first injection only) of 10 mM solution of homocysteine into the reaction cell which contained 1 mM solution of genistein. All the solutions were degassed prior to titration. The titrant was injected at 5-min intervals to ensure that the titration peak returned to the baseline before the next injection. Each injection lasted 20 s. For homogeneous mixing in the cell, the stirrer speed was kept constant at 300 rpm. Calibration of the AutoITC calorimeter was carried out electrically by using electrically generated heat pulses. The CaCl2 - EDTA titration was performed to check the apparatus and the results (n - stoichiometry, K, ΔH) were compared with those

obtained for the same samples (test kit) at MicroCal.

Molecular modeling

Autodock Vina (Petras et al. 2014) docking procedure was used to obtain 2500 sets of low-energy genistein/homocysteine complexes. Each set consisted of 20 docked low-energy complex configurations. For that complex 2500 independent docking runs were performed obtaining 20 low-energy complexes each time. That number comes from limitations of the docking procedure. All preparation of the starting molecule models was conducted in mol2 format using Avogadro program and Autodock Tools was applied subsequently for conversion into Autodock specific pdbqt format. The docking simulations started from random configurations of the molecules. In analyzed case each complex consisted of two heteroaromatic molecules out of which one was regarded as rigid “receptor” while the other (the “ligand”) can change its conformation as long as the aromaticity rules allow it. The genistein was treated as rigid “receptor” molecule during every docking experiment. The default Autodock Vina solvation model was used. After applying search procedure, all 50,000 resulting genistein/homocysteine complexes were ranked according to their Gibbs

interaction free energy. The scoring function of the Autodock Vina software did not distinguish well between similar lowest-energy configurations of the studied complexes assigning many of them the same, lowest-energy value of Gibbs interaction free energy and therefore the population of the lowest-energy configurations is not uniform. The set of all received lowest-energy conformations was saved and clustered with 0.5 Å threshold value into families of similar poses using Gromacs tools g_cluster.

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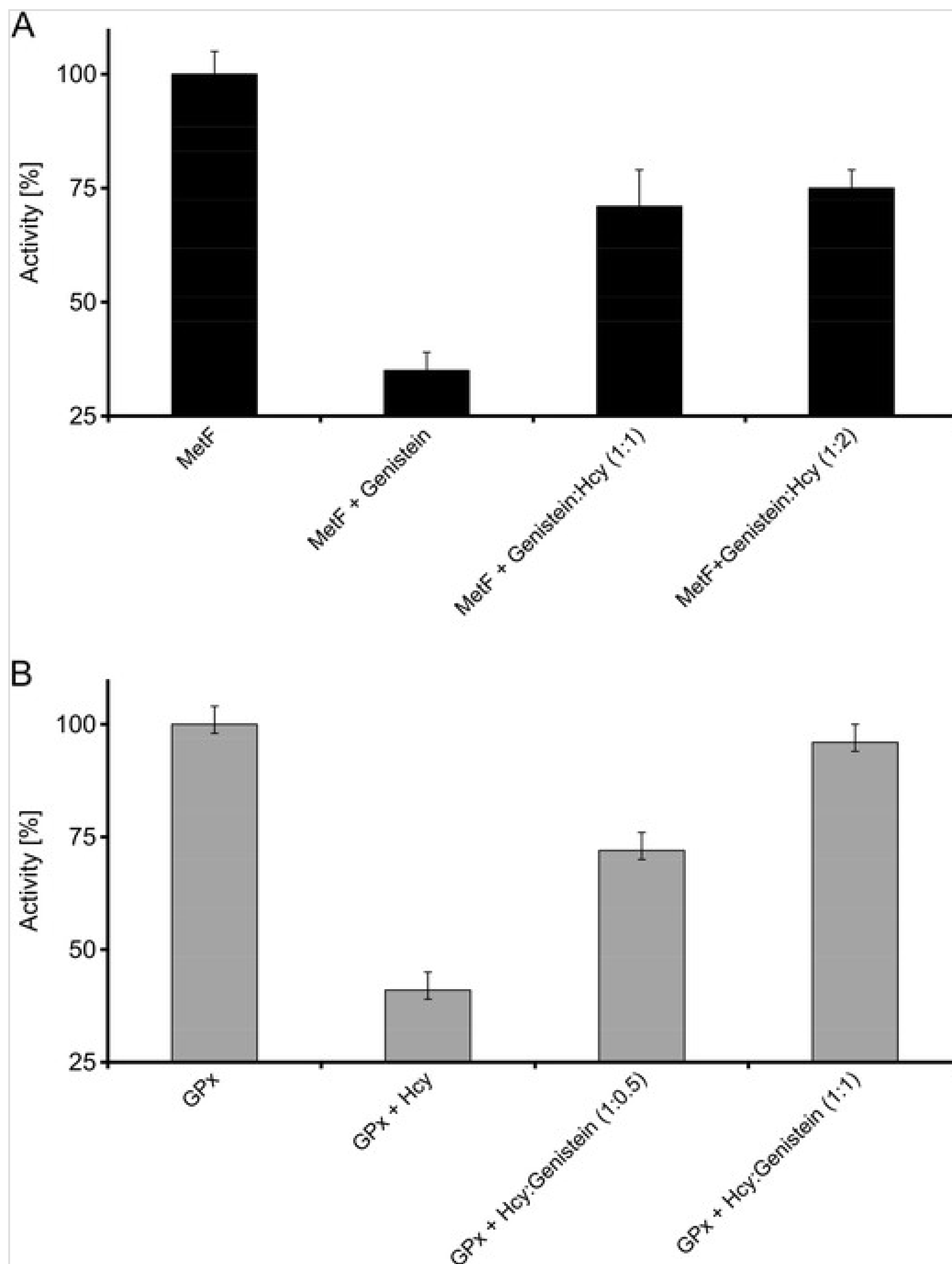
Results

It was demonstrated recently that genistein inhibits activity of the MetF enzyme in the purified in vitro system (Grabowski et al. 2015). To test possible functional interactions between genistein and homocysteine, we have used this experimental model, and measured MetF activity in the presence of genistein and/or homocysteine. While genistein significantly decreased activity of MetF in the purified in vitro system, alleviation of this genistein-mediated inhibition of the enzyme function was observed in the presence of homocysteine (Fig. 1a).

Fig. 1

Aalleviation of genistein-mediated effects by homocysteine (**a**), and of homocysteine-mediated effects by genistein (**b**), in in vitro experimental systems. In panel A, the MetF enzyme was added to final concentration 0.3 µM in each experiment. Genistein was added to 100 µM where indicated, and homocysteine was added to either 100 µM or 200 µM, giving genistein:homocysteine molar ratio 1:1 or 1:2, respectively. The presented results are mean values from 3 experiments with error bars indicating SD. In panel B, the relative GPx enzyme activities were determined. Homocysteine was added to 50 µM where indicated, and genistein was added to either 50 µM or 25 µM, giving homocysteine:genistein molar ratio 1:1 or 1:0.5, respectively. The presented results are mean values from 3 experiments with error bars indicating SD



On the other hand, it has been shown that homocysteine inhibits activity of glutathione peroxidase (GPx) in vitro if present at high concentrations (50–500 μM), especially when the level of glutathione is low (Durmaz and Dikmen 2007). We have demonstrated that homocysteine-

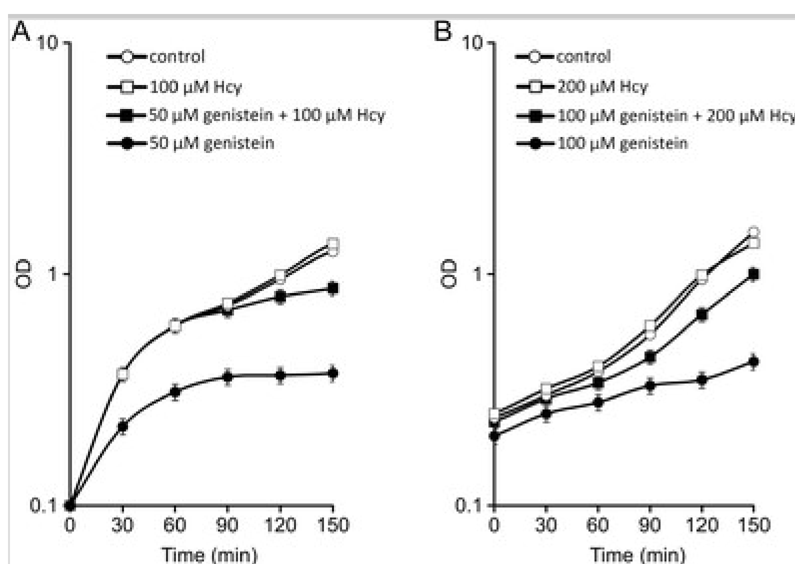
mediated inhibition of glutathione peroxidase activity can be alleviated by genistein (Fig. 1b).

One of the strongest effects of genistein on living cells was observed previously when *V. harveyi* cultures were treated with this isoflavone. Namely, rapid and almost complete inhibition of bacterial growth has been demonstrated (Ulanowska et al. 2006; Ulanowska et al. 2007). Thus, we have employed such in vivo experimental system to test if homocysteine can also modulate genistein-mediated effects on living cells. As expected, genistein strongly inhibited *V. harveyi* growth (Fig. 2a), however, the alleviation of the genistein-caused bacterial growth inhibition was significant at the 2:1 homocysteine:genistein molar ratio (Fig. 2a). Analogous results were obtain in experiments with cultures of another bacterium, *B. subtilis*, which growth is also inhibited by genistein, and can be partially restored by homocysteine (Fig. 2b).

Fig. 2

Effects of genistein and/or homocysteine on *V. harveyi* (**a**) and *B. subtilis* (**b**) growth at 30 °C. Genistein and/or homocysteine were added at time 0. A_{600} of cultures was measured at indicated times. In panel A, bacteria were either untreated (*open circles*) or treated with: 50 µM genistein (*closed circles*), 100 µM homocysteine (*open squares*), or 50 µM

genistein and 100 µM homocysteine (*closed squares*). In panel B, bacteria were either untreated (*open circles*) or treated with: 100 µM genistein (*closed circles*), 200 µM homocysteine (*open squares*), or 100 µM genistein and 200 µM homocysteine (*closed squares*). In both panels, the presented results are mean values from 3 experiments with error bars indicating SD



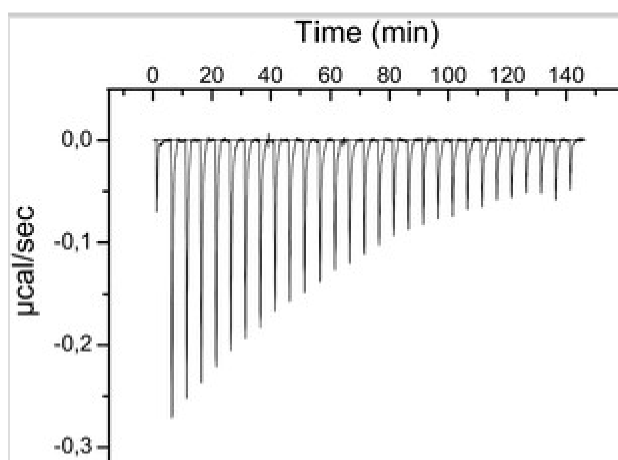
The results presented in Figs. 1 and 2 indicated that homocysteine can significantly alleviate effects of genistein in biological experimental systems, both in vitro and in vivo, and genistein can alleviate effects of homocysteine in another system. Therefore, we asked if these two compounds can interact directly, and performed isothermal titration calorimetry (ITC) and molecular modelling studies.

The stability constant (logKITC = 3.03 ± 0.08) and binding enthalpy (ΔITCH = −0.22 kcal/mol) of

interactions of homocysteine with genistein were obtained directly from ITC experiments by fitting isotherms (using nonlinear least-squares procedures) to a model that assumes a single set of identical binding sites. Then, the free energy of binding (ΔITCG = −4.14 kcal/mol) and entropy change (TΔITCS = +3.92 kcal/mol) were calculated using the standard thermodynamic relationships: ΔITCG = −RTlnKITC = ΔITCH - TΔITCS (Fig. 3).

Fig. 3

Isothermal titration calorimetry of the binding interaction between homocysteine and genistein at 298.15 K

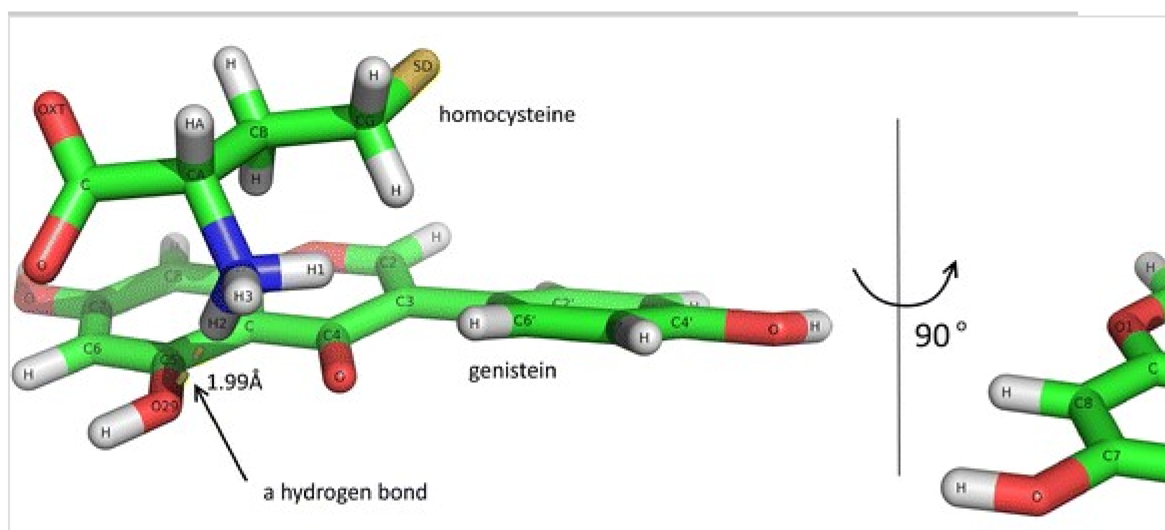


Calculation of the binding energy from docking simulations revealed the existence of interactions between homocysteine and genistein, and it is in agreement with the thermodynamic results obtained from ITC. The lowest–energy complex is shown in Fig. 4. All carbon atoms of the homocysteine

molecule are located in the plane parallel to the fused heterocyclic ring of genistein molecule. The interaction of these molecules is dominated by the Van der Waals forces. We observed also formation of the moderate hydrogen bond between hydrogen atom of the ammonium homocysteine group and the oxygen atom from the hydroxyl group at position 5 of the isoflavone ring with the distance between mentioned atoms equal to 1.99 Å. These results indicate that genistein and homocysteine molecules can interact directly.

Fig. 4

Results of the molecular docking experiment. The cartoon shows direct interactions between homocysteine and genistein. These interactions are characterized by the presence of Van der Waals forces and hydrogen bond between ammonium atom of homocysteine and oxygen atom from genistein (1.99 Å)



Discussion

Various studies indicated that elevated level of plasma homocysteine is a risk factor for stroke (reviewed by Petras et al. 2014). One of potential methods for decreasing homocysteine concentration in blood is administration of genistein, as demonstrated in clinical studies (Chen et al. 2005; Marini et al. 2010). However, the mechanism of genistein-mediated lowering the level of plasma homocysteine remained unknown.

In this report, we demonstrate that genistein directly interacts with homocysteine. This direct interaction was demonstrated in a physico-chemical experiment (isothermal titration calorimetry), and by molecular modelling. Functionality of genistein-homocysteine interactions was indicated in biochemical studies (homocysteine alleviated genistein-mediated inhibition of MetF, and genistein alleviated homocysteine-mediated inhibition of GPx) and in a biological experiment (inhibition of growth of *V. harveyi* and *B. subtilis* cultures).

Direct interactions between genistein and homocysteine provide possible explanation for effects observed in humans. Such interactions could lead to formation of complexes, resulting in

biological inactivity of both compounds that perhaps might by eliminated from the organism due to either excretion or filtration in the urinary system. In such a way, genistein could decrease the level of free homocysteine in plasma, the effect reported previously (Chen et al. 2005, Marini et al. 2010). Moreover, other effects of genistein on alleviation of homocysteine-mediated changes, like modulated levels of proteins involved in metabolism and detoxification (Fuchs et al. 2005), apoptosis of endothelial cells (Fuchs et al. 2006), and inflammatory injury (Han et al. 2015), might also be potentially explained by direct genistein-homocysteine interactions.

Apart from possible explanation of the mechanisms of genistein-mediated reduction of homocysteine-caused effects, the results presented in this report might have also a practical aspect. Since homocysteine has been demonstrated to be a risk factor for stroke (Petras et al. 2014), and high levels of homocysteine in the acute phase of an ischemic stroke can predict mortality, especially in stroke patients with the large-vessel atherosclerosis subtype (Shi et al. 2015), one might consider genistein as a potential therapeutic agent in either prevention of stroke or treatment of patients with

stroke, especially in cases of elevated plasma homocysteine levels.

Acknowledgments

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Compliance with ethical standards

Competing interests The authors declare that they have no competing interests.

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