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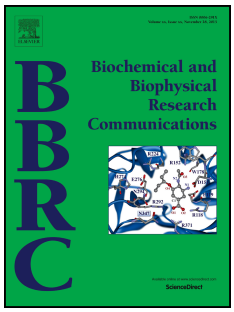

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Genistein inhibits activities of methylenetetrahydrofolate reductase and lactate dehydrogenase, enzymes which use NADH as a substrate

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Abstract

Genistein (5, 7-dihydroxy-3- (4-hydroxyphenyl)-4*H*-1-benzopyran-4-one) is a natural isoflavone revealing many biological activities. Thus, it is considered as a therapeutic compound in as various disorders as cancer, infections and genetic diseases. Here, we demonstrate for the first time that genistein inhibits activities of bacterial methylenetetrahydrofolate reductase (MetF) and lactate dehydrogenase (LDH). Both enzymes use NADH as a substrate, and results of biochemical as well as molecular modelling studies with MetF suggest that genistein may interfere with binding of this dinucleotide to the enzyme. These results have implications for our understanding of biological functions of genistein and its effects on cellular metabolism.

Highlights:

- genistein inhibits activities of methylenetetrahydrofolate reductase and lactate dehydrogenase
- molecular docking confirms that genistein may interfere with the binding site of NADH
- the inhibition is increased when genistein is preincubated with the enzyme and the dinucleotide

Key words: genistein; methylenetetrahydrofolate reductase; lactate dehydrogenase; NADH

INTRODUCTION

5, 7-Dihydroxy-3- (4-hydroxyphenyl)-4*H*-1-benzopyran-4-one, commonly known as genistein, is a natural isoflavone, occurring mostly in leguminous plants [1]. This compound, preliminarily identified as a phytoestrogen, has been subsequently demonstrated to possess surprisingly high spectrum of biological activities. Among them, genistein was found to restore the metabolic balance of bone formation and resorption [2], to alleviate metabolic problems in obesity and type 2 diabetes, mostly due to its anti-oxidant and anti-inflammatory features [3], to protect central nervous system against oxidative stress and neuroinflammation [4], to cause cancer cell growth arrest and apoptosis and to inhibit angiogenesis and metastasis [5], to halt the growth of some bacteria, including human pathogens [6], and to inhibit viral infection [7]. Therefore, phytoestrogenic, anti-inflammatory, antiangiogenesis, antiproliferative, antioxidant, immunomodulatory, pain relief, antibacterial, antiviral and joint protection properties of genistein led to many proposals of the use this isoflavone in treatment of various disorders, including cancer as well as metabolic, inflammatory, infectious, neurological and even genetic diseases [8;9;10;11;12].

The molecular mechanisms of genistein actions are connected mainly to its binding to estrogen receptors [13; 14], inhibition of tyrosine kinase activities resulting in either enhancement or impairment of expression of hundreds of genes [15;16], and direct interaction with topoisomerase II causing modulation of its functions [17; 18]. On the other hand, some clinical studies indicated that genistein may influence the plasma levels of homocysteine [19; 20; 21], an amino acid that is considered to be a risk factor in cardiovascular diseases and stroke [4]. Since such effects of the tested isoflavone on homocysteine levels could be hardly

explained by already known mechanisms of its action, we were searching for possible explanation of this phenomenon. Therefore, we aimed to test if genistein can influence activities of enzymes involved in the homocysteine metabolism. One of main enzymes of homocysteine metabolism is methylenetetrahydrofolate reductase (MTHFR), (EC 1.5.1.20), catalysing conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which serves as a methyl donor in the remethylation of homocysteine to methionine [22]. This enzyme has been conserved during evolution to such extent that specific mutations in the gene coding for a bacterial homolog (MetF) of the human methylenetetrahydrofolate reductase (MTHFR) correspond to common polymorphisms in the human gene [23]. Moreover, products of the wild-type and mutated *Escherichia coli* (*metF*) and human (*MTHFR*) genes are very similar both structurally [23] and functionally [24]. Therefore, in our studies, we have employed the *E. coli* MetF protein as a model.

MATERIALS AND METHODS

Proteins and small molecules

The MetF protein was purified as described previously [25]. Lactate dehydrogenase (LDH) was purchased from Sigma – Aldrich. Genistein, NADH, menadione and buffer ingredients were obtained from Sigma - Aldrich

MetF activity test

MetF activity assay was performed as described previously [25], 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^{o}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.

Effect of genistein on the MetF activity

MetF activity assay in the presence of genistein was performed according to the standard assay (described above), but genistein was added to the reaction mixture to final concentrations from 0 to 500 μM. In control experiments, DMSO (a solvent used for preparation of genistein stock solution) was added to the reaction mixture in the amount equivalent to that used in the assay with 500 μM genistein. In order to investigate the mechanism of the enzyme activity inhibition by genistein, the reaction mixture was titrated with increasing concentrations of this isoflavone, from 0 to 400 μM. The concentrations of NADH were between 0 and 600 μM. Enzyme reaction kinetics was determined according to Michaelis–Menten and Lineweaver-Burk equations and plots.

Influence of reaction initiation factor on genistein-mediated inhibition of MetF activity

Three variants of the test have been developed. First variant assumed preincubation of genistein, MetF and menadione for 5 min, then NADH was added. Second variant assumed preincubation of genistein, NADH and menadione for 5 min, then enzyme was added. Third variant assumed preincubation of genistein, MetF, and NADH for 5 min. Reaction was started by the addition of menadione. All tests were performed in reaction buffer (50 mM phosphate buffer pH 7.2 with 0.3 mM EDTA).

Effects of genistein on the activity of lactate dehydrogenase

The commercially available enzyme (lactate dehydrogenase, LDH) was used. LDH activity was measured by estimation of the decrease in absorbance of NADH consumed during the reaction. The reaction mixture consisted of the 0.1 μM enzyme, genstein, NADH, 1 mM pyruvate acid and 50 mM phosphate buffer containing 10% glycerol and 0.3 mM EDTA and genistein in concentration 0 to 500 μM. The reaction was conducted at 37^{o}C. Absorbance was monitored at 340 nm. In order to investigate the mechanism of the enzyme activity inhibition by genistein, reaction mixture was titrated with increasing concentrations of genistein. The experiment was performed with concentrations of genistein from 0 to 400 μM, while the concentrations of NADH were between 0 and 1200 μM. Enzyme reaction kinetics was determined according to Michaelis–Menten and Lineweaver-Burk equations and plots.

Molecular modeling

The crystal structure of 1ZP3 of *Escherichia coli* methylenetetrahydrofolate reductase/FAD complex, which was previously deposited [26] in the Protein Data Bank (PDB) [27] was used in our docking experiments. The model of genistein molecule for the docking procedure was constructed using the Avogadro [http://avogadro.openmolecules.net/] molecular editing software. We used also the FAD molecule model, which was already present in the crystal structure of 1ZPT3. The starting geometries of the ligand molecule models were optimized using the built-in Avogadro minimization algorithm based on the MMFF94 force field employing the Steepest Descent Algorithm with 500 steps of minimization. AutoDock Vina [28] was used to perform the molecular docking experiments with the default optimization parameters offered by the program. The ADT [29; 30] program from the MGLTools removed non polar hydrogen atoms at the docking simulation preparation phase. The methylenetetrahydrofolate reductase protein model was treated as a rigid body in

all docking simulations. For each of the docking experiments, a rectangular grid was constructed with default value of 1Å grid spacing. It surrounded the enzyme active site. The sizes of the rectangular grids allowed free movement of the ligands and, for each of the complex models, were 48Å×46Å×90Å, and the grid centre coordinates were x = -33.804Å, y = -15.506Å, z = -25.276Å. The genistein-NADH complex was obtained by using Autodock Vina software. Autodock Vina [28] docking procedure was used to obtain 2500 sets of low-energy genistein-NADH complexes. Each set consisted of 20 docked low-energy complex configurations. For such complex, 2500 independent docking runs were performed, obtaining 20 low-energy complexes each time.

RESULTS

The *E. coli* methylenetetrahydrofolate reductase (MetF) protein has been purified, and its enzymatic activity was estimated. We found that genistein inhibits the catalyzed reaction in the dose-response manner (Fig. 1A). Effects of genistein were observed at concentration as low as 25 μM, and at 500 μM of genistein, the enzyme activity was almost completely inhibited. DMSO, a solvent of genistein, had no significant influence on the MetF activity. K_m values of the reactions were calculated as 175, 240, 250, 270 and 300 μM NADH for 0, 50, 100, 200 and 400 μM of genistein, respectively. V_{max} values were 0.105, 0.054, 0.048, 0.029, 0.013 M NADH/min, respectively. The Lineweaver-Burk equation identified a mixed type of inhibition (Fig. 1B).

To determine a possible mechanism for genistein-mediated inhibition of MetF, the enzymatic activity was measured in reactions preceded by incubation of genistein with different reaction components. In the case of pre-incubation of genistein with NADH or MetF,

we observed an inhibitory effect on the enzyme. However, when all three components (genistein, NADH, MetF) were preincubated, this effect was even more pronounced (Fig. 2). This suggested the existence of independent interactions: genistein-NADH and genistein-MetF.

To test the hypothesis about direct interactions of genistein with NADH and MetF, we have conducted molecular docking experiments. Fig. 3A shows the configuration of the MetF-genistein complex in the presence of FAD in the protein binding site. Fig. 3B depicts all protein residues interacting directly with the genistein molecule. Three hydrogen bonds formed between genistein and the backbone nitrogen of Leu277, backbone oxygen of Glu28 and amide oxygen in the side chain of Gln183, should be noted. There are also some other enzyme residues in the vicinity of genistein molecule: Phe29, Phe30, Thr59, Asp120, Thr227, Tyr275 and FAD cofactor. We observed a similar configuration of the methylenetetrahydrofolate reductase/NADH+FAD complex obtained by docking NADH molecule into the binding pocket present in 1ZPT enzyme/FAD complex. The RMSD value between the crystal structure of NADH and the docked molecule, calculated over all heavy atoms, was equal to 0.20Å, the binding enthalpy of NADH was equal to -7.7 kcal/mol for this complex. As shown in Fig. 3A, there are multiple hydrogen bonds formed by NAD and amide bond in the side chain of Gln183. In addition, docking experiment was performed to show interaction between genistein and NADH. The genistein molecule intercalates between two parallel NADH rings. This configuration of the genistein-NADH complex is characterized by the lowest obtained Gibbs interaction free energy value equal to -4.0 kcal/mol. Importantly, our results of molecular modelling are compatible with the MetF-NADH crystal structure, reported previously [26].

Based on the above results, we aimed to test whether methylenetetrahydrofolate reductase is a specific enzyme that genistein can interact with, or various enzymes using

NADH as a substrate may be inhibited by this isoflavone. Therefore, we have estimated activity of another NADH-dependent enzyme, lactate dehydrogenase, in the presence and absence of genistein. We found that the reaction catalyzed by this enzyme was efficiently inhibited by genistein (Fig. 4A). K_m values of the reactions were calculated as 120, 140, 150, 160 and 175 μM NADH for 0, 50, 100, 200 and 400 μM of genistein, respectively. V_{max} values were 0.106, 0.093, 0.087, 0.080, 0.070 M NADH/min, respectively. The Lineweaver-Burk equation identified a mixed type of inhibition, similarly to the results obtained for MetF (Fig. 4B).

DISCUSSION

Although genistein is known for its multiple effects on cells and organisms, including action as a phytoestrogen, inhibition of inflammatory processes, impairment of angiogenesis, negative regulation of cancer cell proliferation, function as an antioxidant, and inhibition of bacterial and viral development [7;2;4;1;5] only a few kinds of molecular targets for this isoflavone have been documented. First, it can bind to estrogen receptors, influencing reactions dependent on this hormone [13;14]. Second, it inhibits tyrosine kinase activity of certain transmembrane receptors, thus, impairing signal transduction pathways which regulate expression of many genes involved in the control of various metabolic processes [15;16]. Third, it interferes with functions of topoisomerase II which leads to DNA defects and perturbations in genetic material replication [17;18]. Here, we demonstrate that there are newly discovered targets of genistein. This isoflavone inhibited activities of two enzymes, methylenetetrahydrofolate reductase and lactate dehydrogenase. Both these enzymes use NADH as a substrate.

The results of docking simulations suggest that genistein molecule forms complexes with MetF, replacing any molecule accompanying the FAD cofactor. The configurations of the lowest-energy MetF-genistein complexes depend on the presence of other molecules in the enzyme active site. For example, the NAD molecule occupies the same binding cleft as genistein in the enzyme active site, and genistein interacts also with the same residues as NAD does. Since molecular modelling studies indicated interactions of genistein with the active centre of MetF, it is likely that the inhibitory properties are due to competition with NADH for the enzyme binding. Moreover, our molecular modelling tests indicated that genistein can form complexes with NADH.

The question appears whether inhibitory effects of genistein on enzymatic activities of methylenetetrahydrofolate reductase and lactate dehydrogenase can have a physiological significance. Clinical trials on humans and experiments on animals indicated that genistein is biocompatible, with no significant adverse effects, even in long-term (several months) use of its high doses. Examples of such studies include 1-year treatment of children with genistein at the dose of 150 mg/kg/day [31], 9-month treatment of mice at the dose of 160 mg/kg/day [32], and 1-year treatment of dogs at the dose of 500 mg/kg/day [33]. On the other hand, some bacterial species are sensitive to as low genistein concentrations as 10-100 μM [34], which correspond to doses about 10-100 times lower than those mentioned above in human and animal studies. Intriguingly, *E. coli* was found to be resistant to genistein at concentrations up to 100 μM [34]. Nevertheless, those results are not contradictory to those presented in this report. First, in the *in vitro* experiments, MetF revealed still considerable activity in the presence of genistein at μM (Fig. 1), thus, such residual activity might be enough to ensure bacterial growth despite partial inhibition by this isoflavone. Second, permeability of the cell envelope for genistein may be different in different bacterial species, thus, intracellular concentrations of this compounds can vary between them. In fact, it was

demonstrated previously that in *Vibrio harveryi* (a bacterium extremely sensitive to genistein), the cell envelope permeability for crystal violet (a model molecule in such studies, which is of similar size to that of genistein) was significantly higher than that in *Salmonella enterica* serovar Tiphimurium (a bacterium closely related to *E. coli*) [35].

Definitely, it appears that some bacteria are significantly more sensitive to genistein than normal human cells, as no cytotoxicity could be observed in the latter ones at doses up to 100 μM in *in vitro* studies [36;37]. This is in contrast to cancer cells for which genistein was cytotoxic at concentrations of 50 μM or lower [38;39]. Therefore, it is possible that genistein-mediated impairment of activities of methylenetetrahydrofolate reductase and/or lactate dehydrogenase might contribute to antibacterial and anticancer properties of this isoflavone. Nevertheless, since normal human and animal cells, as well as whole organisms, remain physiologically unaffected upon the treatment with genistein at doses deleterious for bacteria, viruses and cancer cells, it is still reasonable to consider this compound as a drug for various diseases.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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FIGURE LEGENDS

Figure 1. Panel A MetF activity in the presence of increasing concentrations of genistein. In the experiment shown on panel A, 0.3 μM enzyme was used for each reaction. The activity measured in the control experiment (without genistein) was assumed to be 100% and other values reflect this value. The presented results are mean values from three independent experiments. Error bars represent standard deviation (SD). Panel B represents the kinetics of MetF-catalyzed reaction as a Lineweaver-Burk plot.

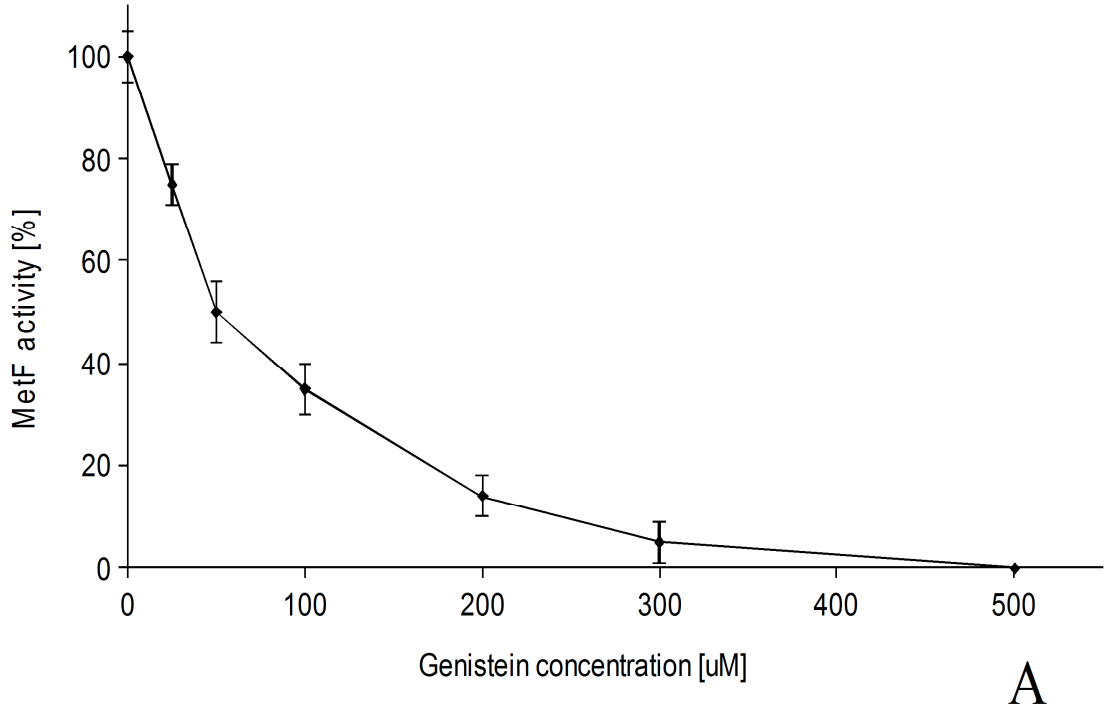
Figure 2. MetF activity in reactions initiated by preincubation of various compounds. The reaction was performed without genistein (control experiment) or with genistein, but with pre-incubation of the pre-mix consisting of either genistein and MetF; genisten and NADH; or genistein, MetF and NADH, as described in the Materials and Methods. The activity measured in the control experiment (without genistein) was assumed to be 100%. The presented results are mean values from three independent experiments. Error bars represent standard deviation (SD). Statistically significant differences were found between all pairs of results ($p<0.05$ in t-test).

Figure 3. Results of the molecular docking experiment. Panel A: the cartoon showing MetF residues directly involved in interaction with NADH. The hydrogen bonds are also shown as the dashed lines with the distance between heavy atoms. Panel B: the cartoon showing protein

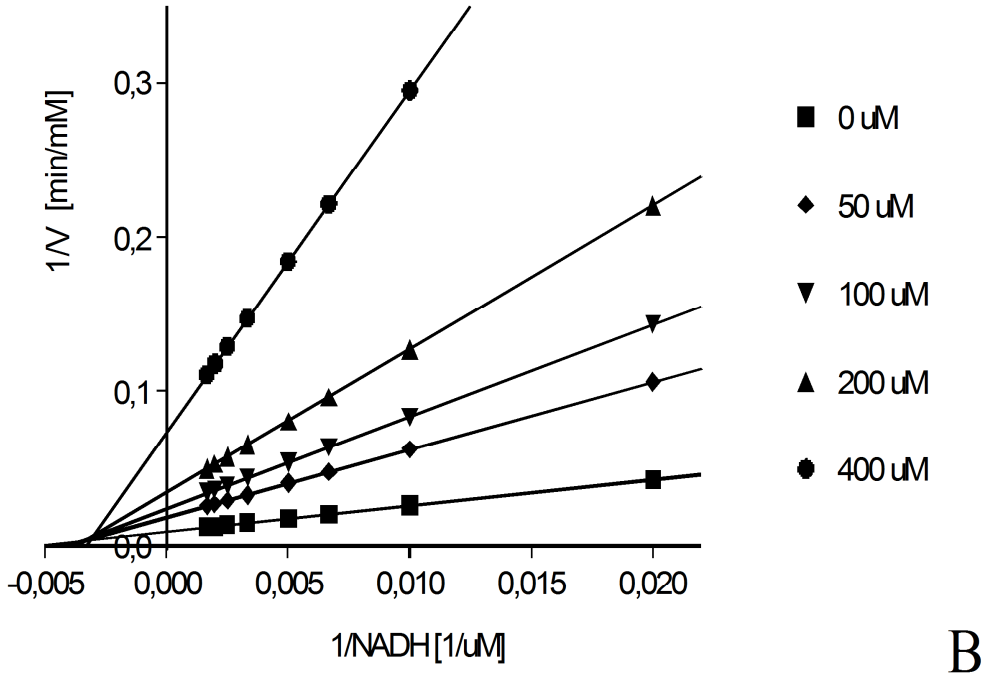
residues directly involved in interaction with genistein (denoted as Lig1). The hydrogen bonds are also shown as the dashed lines with the distance between heavy atoms. All cartoon representations of ligands and their binding sites were prepared using LigPlot+ software.

Figure 4. Panel A LDH activity in the presence of increasing concentrations of genistein. In the experiment shown on panel A, 0.1 μM enzyme was used for each reaction. The activity measured in the control experiment (without genistein) was assumed to be 100% and other values reflect this value. The presented results are mean values from three independent experiments. Error bars represent standard deviation (SD). Panel B represents the kinetics of LDH-catalyzed reaction as a Lineweaver-Burk plot.

Figure 1


MetF activity [%]
100
80
60
40
20
0
0
100
200
300
400
500
Genistein concentration [uM]

A


1/V [min/mM]
0,3
0,2
0,1
0,0
-0,005
0,000
0,005
0,010
0,015
0,020
1/NADH [1/uM]
0 uM
50 uM
100 uM
200 uM
400 uM

B

47

48

49 **Figure 2**

50

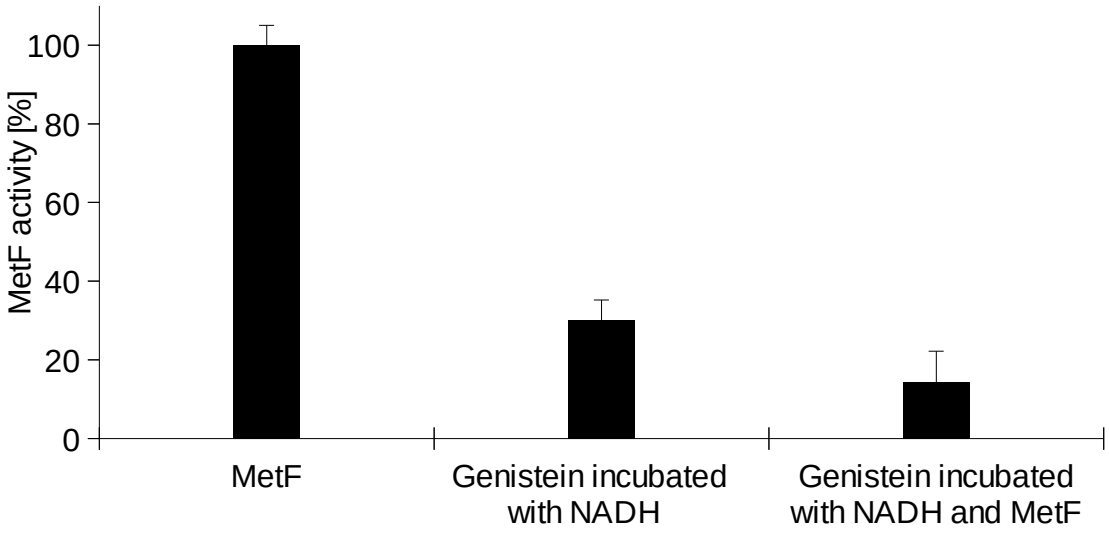
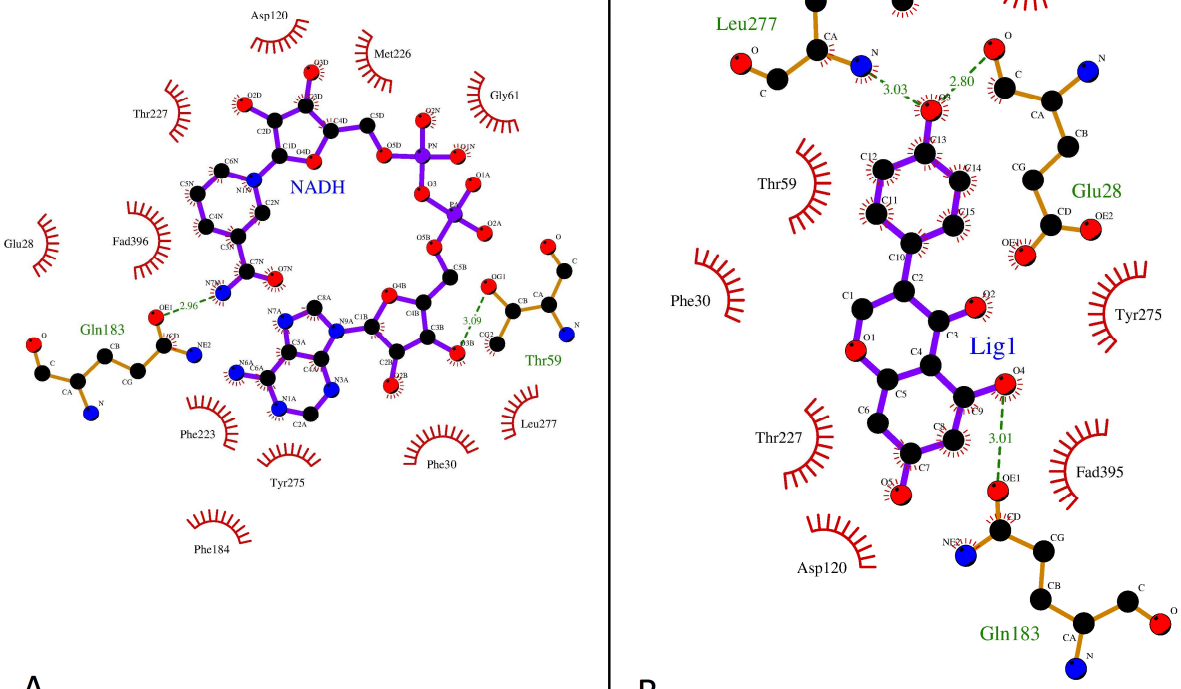
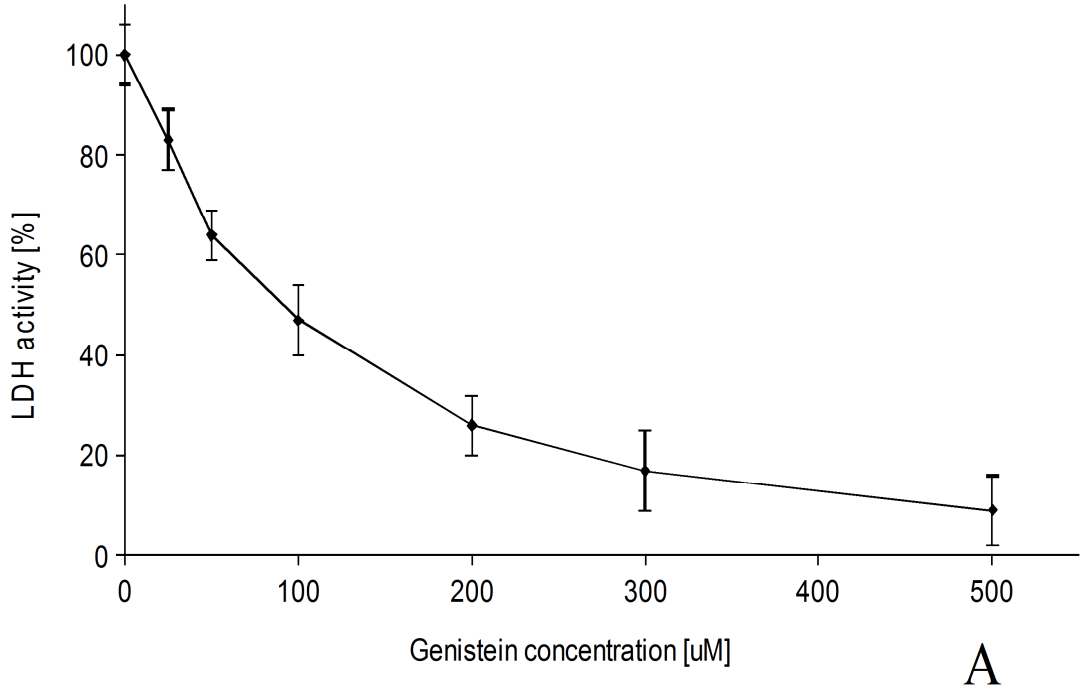


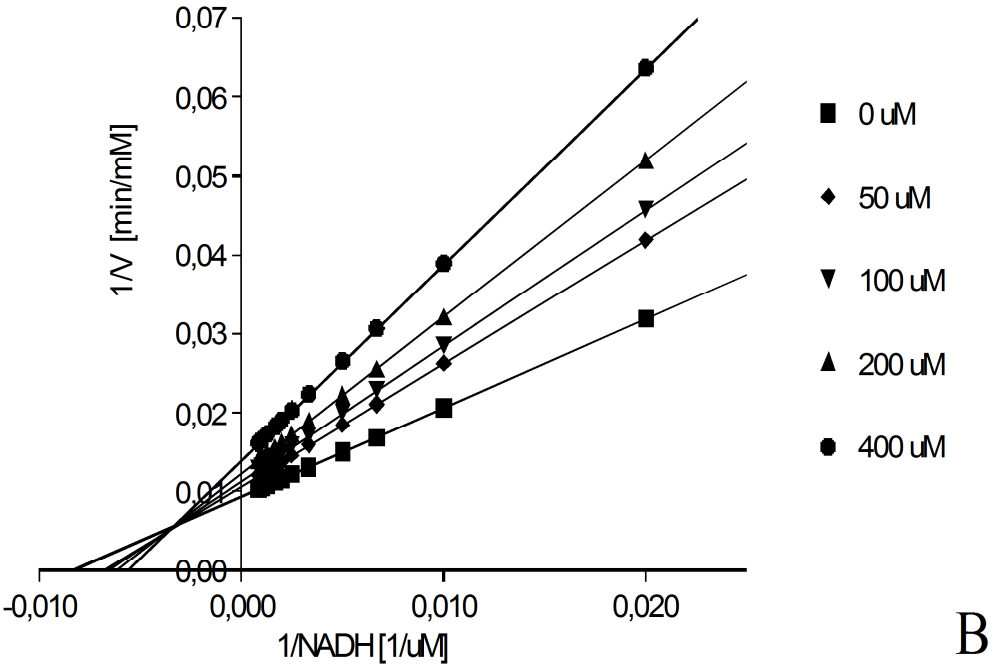
Figure 3


Asp120
Met226
Gly61
Thr227
NADH
Glu28
Fad396
Gln183
2.96
3.09
Thr59
Phe223
Leu277
Phe30
Tyr275
Phe184
A
Phe29
Leu277
3.03
2.80
Thr59
Glu28
Phe30
Tyr275
Lig1
Thr227
3.01
Fad395
Asp120
Gln183
B

68 **Figure 4**



A



B

69

70