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Research Paper Biotechnology

Genistein substantiate and fortifyoxidant potential of a cell by interacting with Bcl-2: an anti-apoptotic protein in rat model.

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ABSTRACT

Genistein, an isoflavonoid isknown for its antioxidant property. It is known that the presence of Bcl-2 is important for the antioxidant action of genistein. The possible mechanism of action of the genistein is by its interaction with Bcl-2, widely known for its characteristic to transport the GSH from the cytosol to the nucleus,protecting cells against mitochondrial oxidative stress and subsequent apoptosis.Here, we have validated the antioxidative effect of genistein on rat model (rattusnorvegicus) where oxidative stress was induced by D-GalN treatment. Genistein significantly attenuated the effects of D-GalN and levels of marker enzymes like AST, ALT and BCL-2 which were reestablished in the animals pretreated with it. We also introduce a computationally comprehensiveprotein 3-D model of Bcl-2 of rattusnorvegicus by using CPH model, SAVES and SWISS-MODEL workspace (http://www.cbs.dtu.dk/services/CPHmodels,http://nihserver.mbi.ucla.edu/SAVES/,http://swissmodel.expasy.org/qmean/cgi/index.cgi)and it was validated withvarious computational tools like ERRAT, PROCHECK, VERIFY 3D etc. Wealso present docking study to check the interaction between the Bcl-2 and genistein with the help of web based serverpatch dock, an online tool for Ligand Protein Docking (http://bioinfo3d.cs.tau.ac.il/PatchDock).Docking score between Bcl-2 and genistein was found to be165.38 kcal/mol, which validates the interaction between them. Therefore, it is proposed that the genistein works on to the Bcl-2 to increase the uptake of GSH from cytoplasm to nucleus.

KEYWORDS : Genistein, Bcl-2, GSH and D-GalN.

INTRODUCTION

Genistein, an isoflavone is found in low concentrations insoybeans and elevated amounts in certain soy-derived food. The glucoside from the aglycone is much more abundant in the unprocessed soybean, it has strong anti-proliferative andapoptotic potential [1,2]and it is also known to have antioxidant property [3].The possible mechanism may be by prevention of DNA mutation, reduction in cancer cell proliferation, inhibition of angiogenesis and induction of differentiation. It is known that presence of Bcl-2 an anti-apoptotic protein is important for the antioxidant action of genistein[4] as it was unable to show its action in BCL-2 knockdown in human malignant neuroblastoma SK-N-DZ cells[5].Bcl2has been extensively studied as a target for drug designing, therapeutics, as an anticancer agent and also as an important antioxidant molecule. It regulates an essential pool of mitochondrial GSH and this regulation may depend upon its direct interaction with GSH via the BH3 groove [6] therefore protects cells against mitochondrial oxidative stress and subsequent apoptosis. It is the candidate GSH-recruiting protein, in the nuclear envelope of mammalian cells, which promotes cell survival [7] as GSH acts as free radical scavenging molecule.Although Bcl-2 proteins are long recognized as targets for drug-discovery to treat apoptosis-related human pathologies (from cancer to neurodegenerative diseases), their precise mechanism of action is still to be discovered. Here we have tested the effect of the genistein on rats where oxidative stress was induced with D-GalN and it was found that the levels of certain marker enzymes of AST, ALT and GSH. A 3-D model of Bcl2 of *rattusnorvegicus* is validated and docked with the GSH molecule to validate the interaction between them.

MATERIAL AND METHODS

2.1 ANIMAL MODEL

2.1.1Chemicals and reagents

All the chemicals and reagents were procured from Sigma Aldrich Pvt. Ltd. India.

2.1.2 Animals and diet

30 male wistar rats(*rattusnorvegicus*), were procured from animal house,of age 5-7 weeks, weighing 250g. The rats were randomly divided into six groups (group 1 to group 6), after one week of feeding. All the groups were then housed individually in a temperature-controlled environment with 12-hour light-dark cycle and were allowed a free access to standard laboratory food (Rat chow) and water *ad libitum*.JamiaHamdard Animal Ethics Committee provided its approval to all the experimental protocols and procedures. This work was conducted under the project license, issued by JamiaHamdard.

2.1.3 D-Galactosamine induced hepatotoxicity and Genistein pre-treatment.

Rats were kept on fast overnight, provided they had a free access to water *ad libitum*. Group-I and II served as control and received normal saline. D-GalN (700mg/kgBW) was given intra-peritoneally to Group III and Group IV rats, whereas, Group V and Group VI were given a pre-treatment of Genistein (5mg/kgBW/day) via oral gavage for 8 days, which was followed by a single dose of D-GalN (9^{th}day). The rats were euthanized at 24h and 48h treatment of D-GalN. Blood was collected from the abdominal aorta at each time point. The livers were dissected out and stored at -70°C for further analysis.

2.1.4 ENZYMATIC ANALYSIS

2.1.4.1Serum transferases

Plasma alanine transaminase (ALT) and aspartate aminotransferase (AST) activities were measured spectrophotometrically as indicators of hepatocellular disintegration and necrosis [8,9].

2.1.4.2Hepatic lipid peroxidation and glutathione content

Total glutathione content was determined by yeast glutathione reductase, 5, 5-dithiobis (2-nitrobenzoic acid), and NADPH, at 412nm in liver homogenates after precipitation with 1% picric acid. Oxidised level of glutathione (GSSG) was determined by the same method in presence of 2-vinylpyridine, and reduced glutathione was the difference between total glutathione and GSS [10].

2.1.4.3Expression level of BCL-2

The expression of BCL-2 waschecked using RT PCR technique in rats given D-Gal andD –Gal and genistein.(Fig.1)

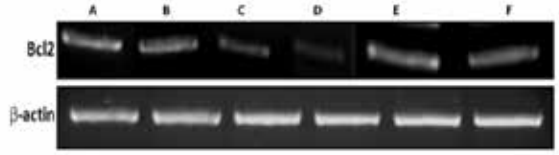

Fig.1.Gene expression analysis of apoptotic and anti-apoptotic (Bcl-2) genes after D-GalN treatment and Genistein pre-treatment as measured by RT-PCR. Comparisons are made between (A)Control rats after 24 hrs,(B)Control rats after 48 h, (C) D-GalN 24h, (D) D-GalN 48h (E) Genistein pretreatment and D-GalN 24h, (F)Genistein pretreatment and D-GalN 48h

BIOINFOMATIC APPROACH

2.2.1 HOMOLOGY MODELING

2.2.1.1Template selection and confirmation of absence ofBcl-2 isoformin Rattusnorvegicusby Sensitive Sequence Similarity Searches:

The sequence in FASTA format for Human Bcl-2,isoform 2from human,was isolated from RSCP PDB [11] as template and compared this against a complete Non-Redundant Protein Database using NCBI PSI-BLAST to ensure that this protein matching eukaryotic protein exclusively in the first step truly had no Bcl-2 in *Rattusnorvegicus*.

2.2.1.2Visualization and Model Validation:

The protein structure was predicted by homology modeling using different servers; CPH model, SAVES and SWISS-MODEL workspace (http://www.cbs.dtu.dk/services/CPHmodels,http://nih server.mbi.ucla.edu/SAVES/,http://swissmodel.expasy.org/qmean/cgi/index.cgi).The program CPH models 3.0 server [12] was used to build the model as PDB file of Bcl-2 in *Rattusnorvegicus* according to the homology modeling method.The PDB of Bcl-2 in *Rattusnorvegicus* was visualized by using PyMOL version 1.3(http://pymol. sourceforge.net/) [13].The structure which was obtained from homology modeling was validated by SAVES. The steriochemical quality of the model was verified with program PROCHECK [14] in order to select best model.3D-profiling of the residue was done by VERIFY 3D Structure evaluation Server[15,16]. ERRAT was used for verifying protein structure for evaluating the progress of crystallographic model building and refinement [17].

2.2.2 DOCKING

2.2.3Ligand preparation ofgenistein

The ligand file was obtained from the pubchem database in sdf format (3D) and this file was converted into a pdb file by help of the online server: Online SMILES Translator and Structure File Generator (*http://cactus.nci.nih.gov/translate/)* and Open Babel: An open chemical toolbox. Docking of genistein and Bcl-2 was done by the help of patch dock an online tool for Ligand Protein Docking (http://bioinfo3d.cs.tau.ac.il/PatchDock) [18] was used to dock the ligand with the validated 3-D model of Bcl-2.

Result

3.1*Enzymatic analysis*

It was observed in our study that the levels of Bcl-2 and GSH decreased in case of oxidative stress induced by the D-GalN, however these levels were restored in genistein pretreated rat model. Besides, the levels of AST and ALT increased in D-Gal inducedoxidative stress and were retained in the rats which were pretreated with the genistein. (Table1) (Fig 1.)

S. No	AST(U/L)	ALT(U/L)	GSH(mg/g)
Group-I	70.51 ± 4.31	66.83 ± 5.93	48.31 ±1.99
Group-II	75.55±4.08	68.87±3.98	50.91±3.21
Group-III	580.07 ± 12.89^{a*}	505.37 ±14.63^{a*}	24.04 ± 2.04^{a*}
Group-IV	648.45±14.49^{a,b*}	567.27±16.49^{a,b*}	18.09 ± 2.07^{a,b*}
Group-V	77.61 ± 5.77^{c*}	77.12 ± 6.02^{c*}	46.18 ± 3.36^{c*}
Group-VI	89.72± 4.76^{c*}	83.93 ± 6.30^{c*}	45.79 ± 2.33^{c*}

Table 1

Effect of Genistein and D-Galactosamine on the activities of serum AST, ALT and GSH. P <0.001,

a = significant difference compared to control group, b = significant difference compared to D-Galactosamine group. Group 1 is control sacrificed after 24hrs of saline adminstration,Group2 is control sacrificed after 48hrs of saline administration, Group 3 and Group4 are D-Gal induced oxidative stress and Group 5 and 6 are genistein pre-treated groups followed by D-Gal induced oxidative stress

3.2Isolation of Template selection and confirmation of absent Bcl-2 isoform in humans:

The Bcl-2 isoform from humans (accession ID: 1GJH) was retrieved from RSCP PBD and was compared against complete Non-Redundant Protein Database using The NCBI PSI-BLAST and Bcl-2 absent in PBD used as subject in *Rattusnorvegicus*.(NCBI Reference Sequence: NP_058689.1)

3.3Visualization and Model Validation:

The three dimensional structure of Bcl-2 was generated by using PyMOL program (Fig 2).Further, to verify the predicted structure, validation was carried out with PROCHECK program. Ramchandran plot of non-glycine and non-proline residue in the structure showed that 94.9% of the total amino acids were presented in most favored regions and the other 5.1% of amino acids were presented in allowed regions including disallowed region with 0.0%. VERIFY_3D shows residues had an averaged 3D-1D score greater than 0.2 indicating that the environment profile of the model is good. ERRAT2 shows 100.00 overall quality factors indicating good resolution structure. Moreover, quality of the model can be compared to reference structure of high resolution obtained from X-Ray crystallography analysis through Z score and "0" is the average Z score for good model. The Z score of Bcl-2 is -0.55 showing the possibility to be a better model.

3.4Docking

Docking study between the validated 3-D model of Bcl-2 of *Rattusnorvegicus*with genistein was done (figure 4) and docking score (free energy of binding, inhibition constant) i.e dispersion/repulsion (vdW), hydrogen bonding(Hbond) and desolvation energy, electrostatic energy, total intermolecular energy etc. were found to be -165.38 kcal/mol. Fig2,Fig3

Discussion

It was observed in our study that the levels of Bcl-2 decreased in case of oxidative stress induced by the D-GalN,however these levels were restored in genistein pretreated rat model. Besides, the levels of GSH decreased in D-Gal inducedoxidative stress and was retained in the rats which were pretreated with the genistein. Further, the docking study between the validated 3-D models of Bcl-2 of *Rattus*norvegicuswith genistein revealed a delta G of -9 which means that Genistein has high affinity in binding with Bcl-2. The exact mechanism of action of genistein has yet not been elucidated; therefore, it is proposed that the genistein binds with the BCL2 to increaseGSH recruitment in the nucleus which is in well accordance with the previous reports on genistein, wherein it was unable to show its action in Bcl-2 gene knockdown in human malignant neuroblastoma SK-N-DZ cells [5]. It was observed that Bcl-2 overexpression sensitizes MCF-7 cells to genistein by multiple mechanisms [19]. Reports on theoverexpression of the *Bc-l2* gene protected MCF-7/ADR cells from this apoptotic cell death induced by oxidative stress have well been documented [20].

Conclusion

Results from the*in vivo* study suggested the role of genisteinas an antioxidant, evident from the biochemical parameters (enzymatic analysis) where in the levels of GSH and Bcl-2 have been restored after rats were exposed toD-GalN induced oxidative stress. Besides, the levels of marker enzymes of oxidative stress like AST and ALT were also restored in rats which were pretreated with genistein. In this study, we

proposed a valid and stable 3D model of Bcl-2 in *Rattusnorvegicus* whose structure is not present in PDB (Protein Data Bank). The results obtained by docking the ligand genistein with target protein,enforce the evidence that ligand interacts with the target Bcl-2 with high affinity and may act as trigger for recruitment of GSH in nucleus via Bcl-2.

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