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Interactions of genistein with AMPK and its effect on PEPCK-C expression in HepG2 cells: a biocomputational study

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Abstract

AMP activated protein kinase (AMPK) suppresses hepatic gluconeogenesis via cytosolic phosphoenolpyruvate carboxykinase (PEPCK-C), which is beneficial to diabetic patients {particularly type 2 diabetes mellitus (T2DM)}. Activation of AMPK by flavonoids, including genistein, is often associated with their antidiabetic effect in different diabetic animals; however, the detailed mechanisms of AMPK regulation by genistein are yet be understood. Hence, in this study, biocomputational studies were performed to understand the possible interactions of genistein with AMPK. Further, the role of AMPK activation on regulation of PEPCK-C expression in HepG2 cells upon genistein treatment was also monitored using western blot and other molecular techniques. Computational studies showed that genistein closely resembled with the known activator of AMPK, A-769662, in terms of its interaction with the protein. Among the downstream targets of AMPK, the MAPK signalling pathway was found to be one of the possible pathways to be involved in regulation of PEPCK-C in these cells. Our results indicated that genistein activated AMPK in HepG2 cells, which in turn downregulated PEPCK-C probably through the MAPK signalling pathway. Hence, we herein concluded that the use of genistein might be beneficial to the T2DM patients by maintaining their glucose homeostasis possibly through modulation of hepatic gluconeogenesis.

Keywords: *T2DM; AMPK; PEPCK-C; Molecular docking; Genistein*

Introduction

In India, about 75 million people are suffering from diabetes till today in comparison to 425 million adults worldwide (www.diabetesatlas.org) and 1 in 2 people yet to be diagnosed, out of which 90% are type 2 diabetes mellitus (T2DM) patients. Hyperglycaemia via gluconeogenesis pathway in T2DM patients is one of the major factors for treating T2DM patients [1], and this pathway is primarily regulated by phosphoenolpyruvate carboxykinase (PEPCK) [2,3]. AMP activated protein kinase (AMPK) plays a significant role in cellular metabolism [4,5], and is activated by high AMP/ATP ratio [6,7]. AMPK activity is regulated covalently by Thr^{172} phosphorylation of α catalytic subunit by the upstream protein kinases, predominantly by liver kinase B1 (LKB1) and calmodulin dependent protein kinase kinases (CaMKK) [8].

AMPK is involved with various diseases (like diabetes, obesity, and cancers), and is activated by several anti-diabetic drugs such as metformin and rosiglitazone [9]. Notably, the activity of AMPK is decreased in animals models with defect metabolism and the decreased AMPK activity is also associated with T2DM or obesity in humans [10-11]. Activation of AMPK lowers hepatic glucose production (HGP) via gluconeogenesis, by lowering PEPCK and G6Pase genes, both *in vivo* and *in vitro* studies [12-14]. AMPK also enhances glucose transporter type 4 expression in muscle [15].

Recently, it has been shown that AMPK is one of the targets in the treatment of T2DM using some natural compounds [16]. Genistein is shown to modulate AMPK- and MAPK- signalling pathways for regulation of PEPCK-C in hepatic cell line and animal model [17,18]. But, the interactions of genistein with AMPK are not understood yet; hence, in this study, a biocomputational approach was followed to find out the plausible interactions of genistein with AMPK and its downstream effects on PEPCK-C regulation in the HepG2 cells.

Methods

Chemicals and antibodies

DMEM (D6429), antibiotic solution (A5955), RIPA buffer (R0278), protease inhibitor cocktail (P8340), RNA isolation kit (RTN70), insulin (13536), genistein (G6649), PD98059 (P215), and LY294002 (L9908) were procured from Sigma-Aldrich, U. S. A. cDNA synthesis kit (18080051) was obtained from Thermo Fisher Scientific, U. S. A. Western blotting detection reagent (RPN2232) was purchased from GE Health Care, U. K., while PVDF membranes (1620175) from Bio Rad, U. S. A.

Antibodies against Akt (2920), p-Akt (4060), Foxo1 (2880), p-Foxo1 (9461), p-GSK3β (9322), p-ERK½ (4370), β-actin (8457), anti-rabbit IgG-HRP (7074) and anti-mouse IgG-HRP (7076) were procured from Cell Signaling Technology, U.S.A. Anti-PEPCK-C (sc-74823) and anti-rabbit IgG-HRP (sc-2004) were obtained from SantaCruz Biotechnology, U. S. A. pRL-SV40 vector and luciferase reporter assay kit were from Promega, U. K. Other reagents were of the highest quality and were obtained from Sisco Research Laboratories, India.

AMPK model and docking simulations of genistein

The interactions of genistein with AMPK were analysed by molecular docking simulations on Discovery Studio 4.1 software (Biovia) using mammalian AMPK (PDB ID 4CFH) as the receptor molecule. All bound waters and ligand were removed from the protein. To evaluate the model, quality assessment methods are necessary to check the quality of the downloaded model. In our study, the quality of the model was verified in DISCOVERY STUDIO 4.1 by the method previously explained [19], which was found to be suitable for molecular docking. It was subjected to loop refinement and energy minimization in DISCOVERY STUDIO 4.1. The structure was classified into highly populated to forbidden regions by the Ramachandran plot. Further, VADAR program was used to validate the model to check and compare side chain geometries and rotomer conformations of the prepared model with its template structure [20]. "Macromolecules (Prepare protein)" program of DISCOVERY STUDIO 4.1 was used to refine the model as described by Dutta et al. [19].

The amino acids present in the active site of AMPK were analysed using NCBI conserved domain tool and CASTp server [21,22]. For molecular docking, the active site was identified using "Define and edit binding tool" (DISCOVERY STUDIO 4.1) and the ligands, genistein, A-769662 and AICAR, were

retrieved from PubChem database and saved as .smi files [23]. The "Small molecules Prepare ligand" tool of DISCOVERY STUDIO 4.1 was used to optimise the ligand, like addition of missing "H" atoms. Energy minimization of the ligands was also performed using [Minimization-Quick Minimization (Dreiding forcefield)] tool of DISCOVERY STUDIO 4.1.

Molecular docking was done strictly following the method described in Dutta et el.[36]. The predicted interactions of these molecules for AMPK were determined using "View Interactions-Ligand Interactions" tool of DISCOVERY STUDIO 4.1.

HepG2 cells culture

HepG2 cells were cultured in DMEM following the standard procedure as described by Dkhar et al [35]. Effect of genistein on expression of PEPCK-C mRNA was studied by taking one million HepG2 cells, serum-starved, and then treated with genistein (30 µM) for various intervals of time. In order to elucidate the various signal pathway(s) involved in effect of genistein, various inhibitors like PD98059 (for AMPK pathway) or LY294002 (for PKB/Akt pathway) 2 h prior to the genistein treatment.

Luciferase assay

For luciferase assay, the construct [PEPCK-C (-686/+83)] was transfected into the HepG2 cells by electroporation (Gene Pulser, model no. 165-2660, Bio-Rad) along with pRL-SV40 vector. Transfection was carried out in duplicates with 20 µg/mL of PEPCK-C construct and 1×10^5 cells per cuvette (0.2 cm) in 100 µL electroporation medium (Invitrogen). Cells from each cuvette were collected and plated into respective plates. After 8 h, the cells were treated with genistein (30 µM) in serum-free DMEM for 24 h.

Promega dual luciferase® reporter assay system was used for luciferase activity using a FB12 Tube Luminometer (Model no. 11010102, Berthold Detection Systems). Briefly, after treatment HepG2 cells were washed two times with 1x PBS, and lysed in 250 µL of 1x passive lysis buffer for 15 min in ice. Then, 20 µL of lysate was added to 100 µL of luciferase assay reagent II and the luciferase activity was measured for 10 sec the FB12 Tube Luminometer. Further, the reaction was normalized to Renilla upon addition of 100 µL stop and glo® reagent. The fold of change in the treatments in comparison to the controls were calculated and plotted.

Total RNA and semiquantitative PCR

Standard procedure as described by Dkhar et al. [18] was followed in order to assess the effect of genistein on PEPCK-C mRNA expression. In brief, total RNA was isolated and reversed transcribed to cDNA, and PEPCK-C mRNA transcripts was determined by semiquantitative PCR using the specific primers for PEPCK-C (FP: GTTTGACGCACAAGGTCATTTAAGG; RP: CACAAGGTTCCCCATCCTCTGAG). PEPCK-C was amplified in a 25 µL reaction mixture (1x PCR buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs, 10 pmol of primer, 1 U of Taq DNA polymerase and 30-50 ng of cDNA) using an automated thermal cycler (Veriti-4375786, Applied Biosystems). The cycling conditions are; 95 °C for 5 min, and 35 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, and 72 °C for 10 min. The amplicons were analysed on 1.5% (w/v) agarose gel.

Western blot

Upon genistein treatment, the key proteins in the signalling pathways were analysed by western blot as per the modified procedure outlined by Dkhar et al. [18], originally described by Laemmli [24]. In brief, the proteins from the cells were isolated in RIPA buffer, and quantified using Bradford's reagent [25]; 30-40 µg of isolated protein was resolved on 10% SDS-PAGE and transferred onto PVDF membrane. Then, the PVDF membranes were blocked with 5% (w/v) nonfat dried milk in TBST for 1 h at room temperature, and incubated with the respective 1° antibodies (Akt, phospho-Akt, Foxo1, phospho-Foxo1, phospho-GSK3β, phospho-ERK½ and PEPCK-C,) at 4 °C for 16 h. The membranes were washed in TBST and then incubated with the HRP-conjugated 2° antibodies for 2 h; the immunoreactive bands were detected using ECL and quantified. β-actin was used for the loading control.

Statistical analysis

Data were collected and presented as mean ± SEM (n=3), and data were analysed using one-way ANOVA and student's t-test for comparative analyses between the controls and the treatments. A *p* value more than or equals to 0.05 was considered statistically significant.

Results

Plausible interactions of genistein and AMPK

For possible interactions of genistein with AMPK, the mammalian AMPK protein crystal structure (4CFH) was obtained from Protein Data Bank (PDB) website and refined for docking studies in DISCOVERY STUDIO 4.1 (Fig. 1A). The amino acids of the conserved sites were designated using DISCOVERY STUDIO 4.1 for molecular docking. Ramachandran plot of the model showed that 98% amino acids were in the most favourable area, 1.2% amino acids in the allowed areas, and 0.8% in the disallowed area (Fig. 1B). The AMPK model was loaded to the CASTp server for selection of active sites. Out of 29 binding sites obtained from CASTp server, the 29th site was found to have the largest area. It was also found from multiple sequence alignment and secondary structure prediction that the residues in 29th site (Ser 16, Met 17, Ala 18, Trp 19, His 20, Leu 21, Gly 22, Arg 24, Pro 29, Asn 36, Pro 37, Tyr 38, Gln 58, Leu 59, Tyr 60, Gln 61, Val 62, Tyr 68, Asp 70, Phe 71, Arg 72, Ser 73, Asp 75) were conserved in the active site of the model. Dorsomorphin (Compound-C), being known inhibitor of AMPK, was used as a control ligand to perform molecular docking with AMPK. The generated model was compared with the AMPK-Compound C complex (3AQV). Various parameters of VADAR for quantitative and qualitative protein structures assessment like hydrogen bonds, dihedral angles, accessible surface area, volume, stereo packing quality index, 3D profile quality index were found to be similar to the expected and the observed values (Table 1). The small RSMD value among the models confirmed the structural similarity between the two models. Further, the atomic co-ordinates and interactions of the AMPK-Compound C complex (PDB ID 3AQV) and their docked structure were compared and found to be similar (Table 2). After validating the model using various parameters {Ramachandran and hydrophobicity plot (Fig. 1B), VADAR statistics etc. (Table 1)}, the model generated using DISCOVERY STUDIO 4.1 was further selected for docking studies.

The atomic interactions of genistein with AMPK model showed alike network of interactions for ATP. It was revealed that Glu 94, Tyr 95 and Val 96 residues of ATP binding site were involved in hydrogen bond formation with genistein. One unfavourable acceptor/donor clash was observed between Asp 157 and the ligand (Fig. 2A). The bonds between the predicted interacting residues of AMPK with A-769662 were either hydrogen, alkyl hydrophobic or mixed pi-alkyl bonds. It was also observed that the interacting residues of AMPK with A-769662 lied near the ATP binding sites (Fig. 2B). In the case of AICAR, Arg 141, Lys 214, Ser 305 and Asp 308 residues of AMPK were found to form hydrogen bonds (Fig. 2C). When the top poses were superimposed with A-769662 (Fig. 2D), the surface structures of both these compounds showed similar pocket (ATP binding site) in the binding site of AMPK. However, when the known activator compound of AMPK, AICAR, was overlaid with top poses of genistein, it showed different binding pockets (Fig. 2E).

Genistein and PEPCK-C expression

To validate biocomputational data, HepG2 cells were cultured in DMEM (Fig. S1) and treated with genistein and other modulators. The cytotoxicity of genistein was determined as described by Dkhar et al. [18], and it was found to be non toxic at the experimental concentration used in this study.

For the time-dependent effect of genistein on the expression of PEPCK-C, genistein 30 µM treated HepG2 cells were harvested at various time, and PEPCK-C mRNA levels were determined by semi-quantitative RT-PCR. It was observed that, genistein decreased the PEPCK-C mRNA levels gradually from 16 h onward in HepG2 cells as demonstrated by semi-quantitative PCR (Fig. S2).

PEPCK-C promoter activity in HepG2 cells

Upon genistein treatment, the transcriptional activity of PEPCK-C gene was also analysed by luciferase reporter assay. In brief, the cells were transfected with the pGL3 basic vector carrying the PEPCK-C promoter insert (-686/+83) (Fig. 3A), and co-transfected with pRL-SV40, which served as the control. After 8h, the cells were treated with genistein (30 µM) in serum-free media for 24h. Result demonstrated that genistein significantly decreased PEPCK-C promoter activity (~0.3-fold) as compared to the untreated HepG2 cells (Fig. 3B).

Interactions of MAPK pathway and PKB/Akt pathway for the expression of PEPCK-C

The cross-talk between PKB/Akt pathway and MAPK pathway for regulation of PEPCK-C expression by genistein was studied by using PD89059, the MEK inhibitor. To accomplish this, the cells were pre-treated with PD98059, 2 h prior to genistein treatment. The cells were collected in RIPA buffer and lysate was collected for western blot analysis. Results demonstrated that treatment with PD89059 did not induce any detectable in the key proteins of the MAPK and PKB/Akt pathways in HepG2 cells incubated with this inhibitor with respect to the untreated HepG2 cells (Figs. 4A-C).

To further explore the possible interaction of PKB/Akt and MAPK pathways for regulation of PEPCK-C expression by genistein, the effect of the PI3K inhibitor, LY294002, on genistein-induced ERK½ activation was studied. In brief, the cells were treated with LY294002 and then with genistein; insulin treatment served as a positive control. The results showed that genistein alone increased 3.5 folds of p-ERK½ levels; the presence of LY294002, however, could not block the event (Fig. 5A). Further, the changes in the p-ERK½ status were also supplemented corresponding decline in PEPCK-C expression. While insulin reduced the PEPCK-C protein level by ~0.6-fold; PEPCK-C expression was unaltered with LY294002 treatment (Fig. 5B). These results, therefore, excluded the possibility of any cross-talk between the MAPK pathway and PKB/Akt pathway PEPCK-C expression by genistein.

Discussion

AMPK modulates PEPCK-C and G6Pase expression in liver, thereby supresses hepatic gluconeogenesis [26]. Activation of AMPK by flavonoids, a class of secondary plant metabolites, has been associated with their antidiabetic property in HepG2 cells and C2C12 cells [27-28] and in diabetic mice [29]. The effect of different isoflavones such as genistein, formonetin, prunetin, and daidzein on AMPK expression in various cell lines has been studied [18,30-33]. Since the mechanism of genistein on AMPK activation is not known yet, the compound was docked into the AMPK active site. Further, PEPCK-C expression in the HepG2 cells was studied upon genistein treatment, and it was speculated that PEPCK-C expression might be inhibited by genistein through AMPK activation.

An attempt was made to establish the possible interactions between genistein and AMPK, and using the known modulators of AMPK, *in silico* molecular docking was carried out. The human AMPK is a heterotrimer protein with α catalytic unit (552 aas), a β regulatory unit (270 aas), and another regulatory γ unit (331 aas), respectively [34-35]. AMP binding to AMPK triggers phosphorylation of Thr 172 by LKB1 [8,36,37], and binding of both AMP and ADP causes phosphorylation of Thr 172 by CaMKKβ [8]; however, this mechanism has been challenged by Gowans et al. [36]. Nucleotides like AMP mainly protect Thr 172 from dephosphorylation by phosphatases [36,38,39]. The AICAR, one of the activators of AMPK, is phosphorylated inside the cell into ZMP (an analogue of AMP) and bind to the regulatory γ unit of AMPK for allosteric activation [40]. Another small molecule (A-769662) activates the enzyme in a similar manner to AMP, which binds to the α catalytic domain and β regulatory domain of AMPK, and shows its potency in nanomolar range [41,42]. Further, biophysical characterisation of AMPK confirm that AMP and A-769662 activate AMPK in distinct manners [43]. Int his study, we reported that there were different binding pockets for AICAR and A-769662 on AMPK. Importantly, our study showed that genistein and A-769662 shared similar binding pockets on the AMPK catalytic site. Overall, these results suggested that genistein closely resembled A-769662 in terms of interactions with AMPK. Analyses of the genistein-AMPK complex revealed that Glu 94, Try 95, Val 96 residues of ATP binding site were involved in hydrogen bond formation with genistein.

Transcriptional regulation of PEPCK-C is a very crucial step in regulating PEPCK-C expression. In this study, genistein decreased PEPCK-C promoter activity (~0.3-fold) as compared to the untreated HepG2 cells. Hence, possible molecular mechanisms for PEPCK-C expression were further studied. The regulation of energy metabolism involves signalling network, and the interaction of PKB/Akt and MAPK pathway plays a significant role in diabetes [44]. Activated ERK impairs insulin signal transduction and function in T2DM patients [44,45], and also MAPKs induce negative modulators of insulin

sensitivity [46,47]. By contrast, MEK activation has insulinotropic effects without altering insulin sensitivity *in vivo* [48]. Similarly, enhanced ERK activity in young mice has non-modulatory effects on Akt activation and action of insulin [49]. Conversely, inhibition of PKB/Akt pathway prevents MAPK pathway activation in some cell types [50]. Hence, the cross talk between MAPK and PKB/Akt pathways can regulate metabolic activities in T2DM patients. In this study, in order to determine the possible interaction between PKB/Akt and MAPK pathways, HepG2 cells were treated with the inhibitors of the pathways. However, no cross-talks between the pathways were observed in the cells in regulating the expression of PEPCK-C by genistein. From biocomputational analysis, genistein and A-769662 showed similar binding pocket in the binding site of AMPK, while AICAR showed different binding pocket. This could be the reason why differential signal mechanisms were observed when the cells were treated with AICAR and genistein with regard to PKB/Akt pathway; AICAR activated PKB/Akt pathway, whereas genistein did not [35]. This indicates that the AMPK activation by genistein could have followed different pathway, unlike AICAR, possibly MAPK signalling pathway in order to regulate the expression of PEPCK-C in these cells.

In conclusion, for obtaining the possible interactions of genistein with AMPK, genistein was docked into the active site of the mammalian AMPK protein crystal structure (4CFH) and compared with other complexes of AMPK. The atomic interactions of genistein with AMPK model showed alike network of interactions for ATP, and genistein closely resembled with the known activator of AMPK, A-769662, in terms of its interaction with the AMPK protein. To validate the biocomputational data, HepG2 cells were treated with genistein, and expression of PEPPCK-C in HepG2 were monitored; it was found that genistein decreased the PEPCK-C mRNA levels gradually after 12 h of incubation, and PEPCK-C protein level was also decreased. The luciferase results demonstrated that genistein decreased PEPCK-C promoter activity (~0.3-fold) as compared to the untreated HepG2 cells. Among the downstream targets of AMPK, the MAPK signalling pathway was found to be one of the possible pathways to be involved in regulation of PEPCK-C in these cells. Our results indicated that genistein activated AMPK in HepG2 cells, which in turn downregulated PEPCK-C probably through the MAPK signalling pathway. The results presented herein excluded the possibility of any cross-talk between the MAPK pathway and PKB/Akt pathway for PEPCK-C expression by genistein in HeG2 cells. Hence, we herein conclude that the use of genistein may be beneficial to the T2DM patients by maintaining their glucose homeostasis possibly through modulation of hepatic gluconeogenesis. It may be concluded that genistein might be used as an alternative or complementary treatment for T2DM. Possibly, AMPK may be a possible agent in prevention of T2DM by regulating PEPCK-C expression.

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Table 1. VADAR (Volume Area Dihedral Angle Reporter) report on quantitative protein structure evaluation.

Statistic	Observed	Expected
Mean helix Phi (ϕ)	-38.6 (sd=36.4)	-65.3 (sd=11.9)
Mean helix Psi(ψ)	-18.3 (sd=20.2)	-39.4 (sd=25.5)
residue in phipsi (ϕ,ψ) core	358 (45%)	713 (90%)
residue in phipsi (ϕ,ψ) allowed	44 (5%)	55 (7%)
residue in phipsi (ϕ,ψ) generous	3 (0%)	8 (1%)
residue in phipsi (ϕ,ψ) outside	387 (48%)	0 (0%)
residue in omega (Ω) allowed	8 (1%)	24 (3%)
residue in omega (Ω) generous	3 (0%)	0 (0%)
residue in omega (Ω) outside	396 (50%)	8 (1%)
Packing defects	245	55
Free energy of folding	-781.24	-768.06
residue 95% buried	217	381
buried charges	25	0
Total Accessible Surface Area (ASA)	37688.4 Å^2	25828.8 Å^2
ASA of backbone	4364.7 Å^2	-
ASA of sidechains	33323.7 Å^2	-
% side ASA hydrophobic	27.04	-
Mean h-bond distance	2.3 (sd=0.4)	2.2 (sd=0.4)
Mean h-bond energy	-1.5 (sd=1.1)	-2.0 (sd=0.8)

Table 2. Comparison of atomic co-ordinates of compound C with AMPK complex (PDB ID 3AQV) and AMPK model showing the networking amino acids , types of bonds, and distance between the atoms.

AMPK complex with PDB code	Ligands	Network-ing amino acids	Types of bonds	Distance between atoms (Å)	AMPK complex	Ligands	Networking amino acids	Types of bonds	Distance between atoms (Å)
Human AMPK α2 (T172D) complexed with compound C (PDB ID 3AQV)	Dorsomorphin (Compound C)	L22	Hydrophobic	4.5	AMPK model (PDB ID 4 CFH)	Dorsomorphin (Compound C)	L22	Hydrophobic	4.8
		V30	Hydrophobic	4.4			V30	Hydrophobic	4.4
		A43	Hydrophobic	4.6			A43	Hydrophobic	3.8
			Hydrophobic	4.9				--	--
		K45	Hydrophobic	5.4			K45	Hydrophobic	4.7
		M93	Pi-sulfur	5.4			M93	Hydrophobic	5.0
		E94	Hydrogen bond	3.2			E94	Hydrogen bond	3.2
		Y95	Hydrophobic	5.1			Y95	--	--
			Hydrophobic	5.2				Hydrophobic	5.2
			H-donor	3.0				H-donor	3.0
		V96	Hydrophobic	5.1			V96	Hydrophobic	5.1
			Hydrogen bond	3.0				Hydrogen bond	3.0
		K107	Hydrophobic	4.7			K107	Hydrophobic	5.4
		L146	Hydrophobic	4.8			L146	Hydrophobic	4.5
			Hydrophobic	4.8				--	--
		A156	Hydrophobic	3.8			A156	Hydrophobic	4.0
		M146	Hydrophobic	5.0			M146	Hydrophobic	5.4

Legend to Figures

Fig. 1 A. Biological assembly image of active form of mammalian AMPK (PDB ID 4CFH) downloaded from Protein Data Bank website (https://www.rcsb.org). B. Ramachandran plot for the model was analysed using RAMPAGE (Ramachandran Plot Assessment Program). The plot showed 98% residues in the most favoured regions.

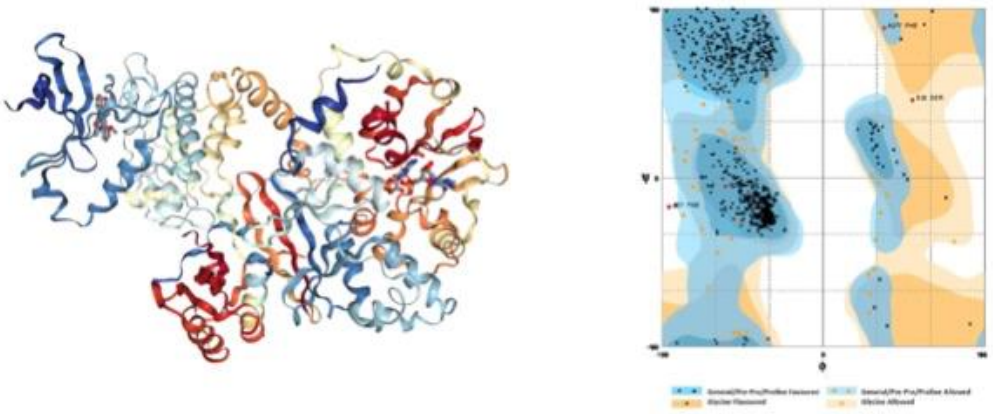
Fig. 2 Likely interactions of genistein (A), A-769662 (B) and AICAR (C) with the AMPK model (PDB ID 4CFH). The protein backbone is rendered as a grey ribbon, and dotted lines represent the hydrogen bonds. The catalytic residues and the ligand are shown as thin and thick stick models, respectively. N atoms are in blue colour, O atoms in red, C atoms in green, and H atoms in grey. DISCOVERY STUDIO 4.1 was used to generate the soft surface structures (corresponding to volume of binding site). D. Molecular overlay of the top poses of genistein-AMPK complex and A-769662-AMPK complex showing similar binding pocket. E. Molecular overlay of the top poses of genistein-AMPK complex and AICAR-AMPK complex showing different binding pockets in the catalytic site of the enzyme.

Fig. 3 Effect of genistein on PEPCK-C promoter activity. A. *Homo sapiens* PEPCK-C promoter sequence (-1500 to +298) was obtained from The Eukaryotic Promoter Database (https://epd.vital-it.ch/) and specific primers (in bold) were designed to amplify the region encompassing -686/+83. B. HepG2 cells were transfected with the reporter vector cloned with promoter fragment of PEPCK-C (-686/+83). Reporter activity was measured 24 h after genistein treatment and data were represented as mean±standard error of mean (n=3) of fold change in luciferase activity with respect to the untreated cells. *significant at *p* value <0.05.

Fig. 4 Effect of PD98059 on PKB/Akt pathway protein. HepG2 cells were treated with PD98059 for 2 h prior to treatment with genistein. Proteins were isolated and used for western blot. Three independent experiments were performed and a representative result was shown. A. Effect of PD98059 on the phosphorylation status of Akt, Foxo1 and GSK3β. Densitometric quantifications of p-Foxo1/t-Foxo1 (B) and p-GSK3β/β-actin (C).

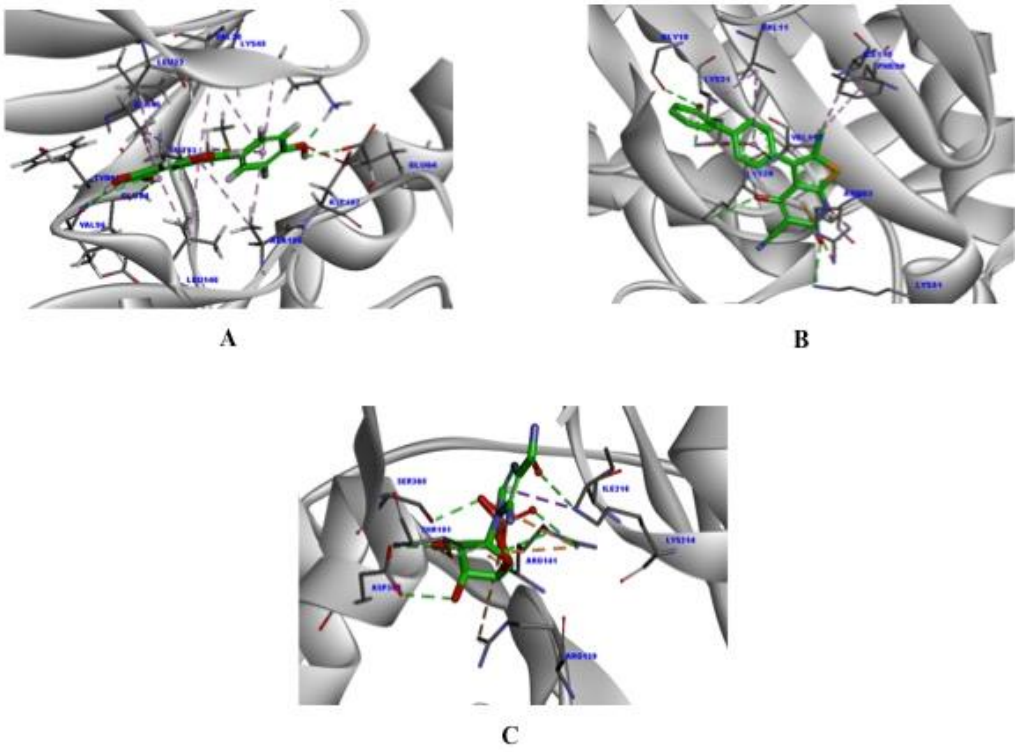
Fig. 5 Effect of LY294002 on ERK½ phosphorylation and PEPCK-C expression. HepG2 cells were treated with various modulators (genistein, phosphatidylinositol 3-kinase inhibitor LY294002, and insulin) for 24 h, proteins were isolated and used for western blot to observe the effect on ERK½ phosphorylation and PEPCK-C expression. Three independent experiments were performed and a representative result was shown. **significant at *p*-value <0.01 and ***significant at *p*-value <0.001. A. Effect of LY294002 on the phosphorylation status of ERK½, and PEPCK-C (negative image) protein levels. Densitometric quantification of PEPCK-C/β-actin (B).

Fig. 1



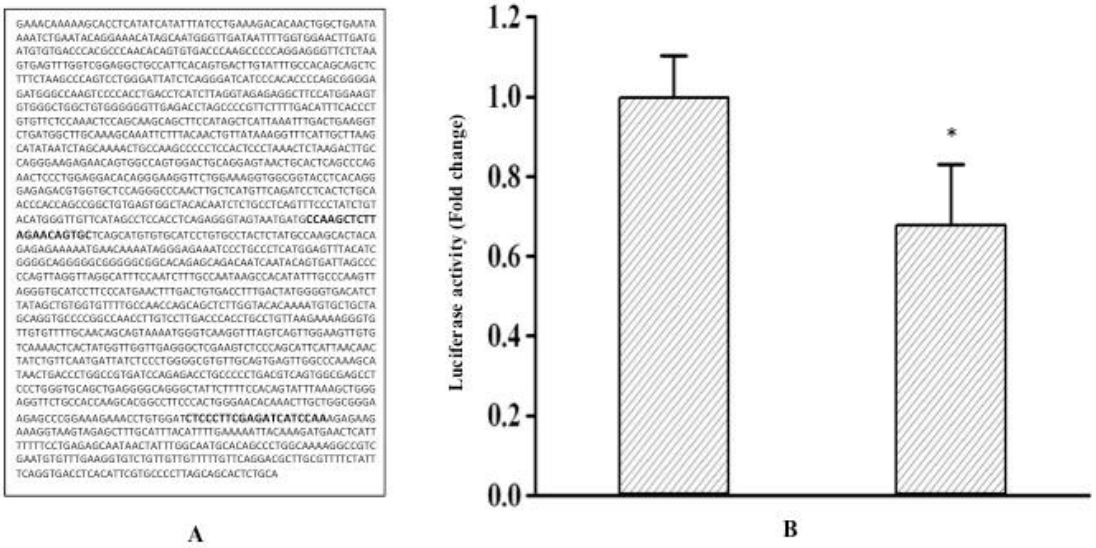
A B

Fig. 2



A B C D E

Fig. 3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TCAGGTGACCTCACATTCGTGCCCCTTAGCAGCACTCTGCA
Luciferase activity (Fold change)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*

A B

Fig. 4

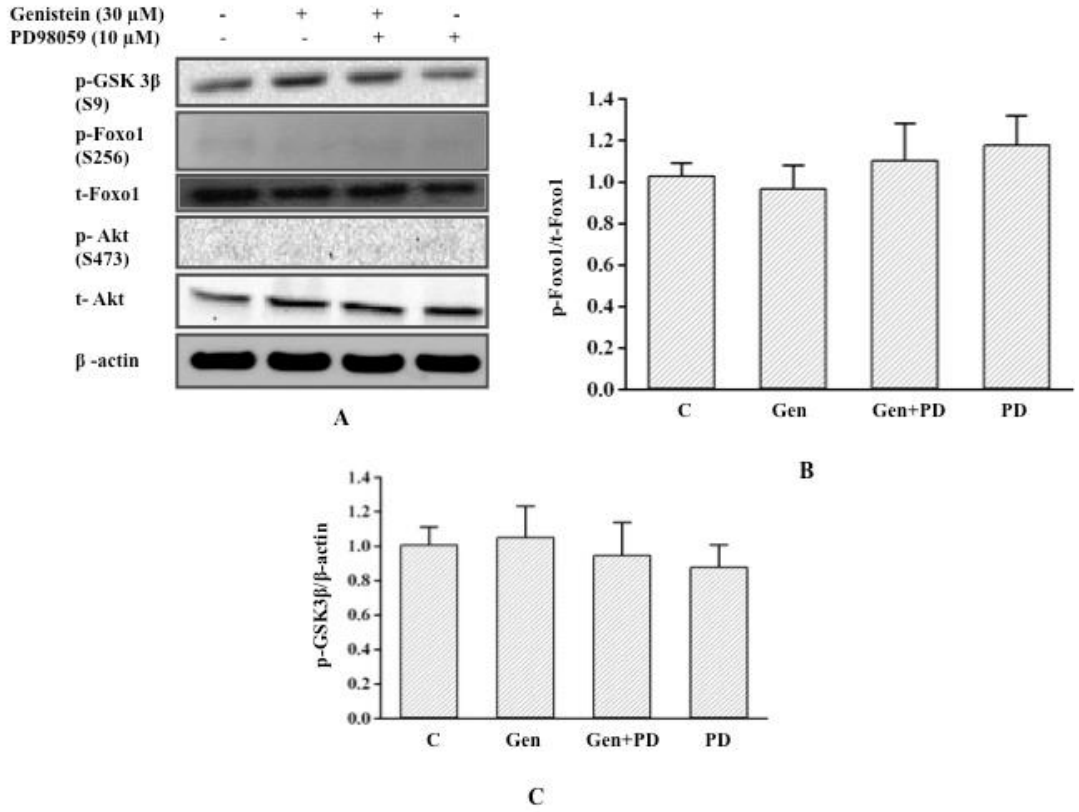
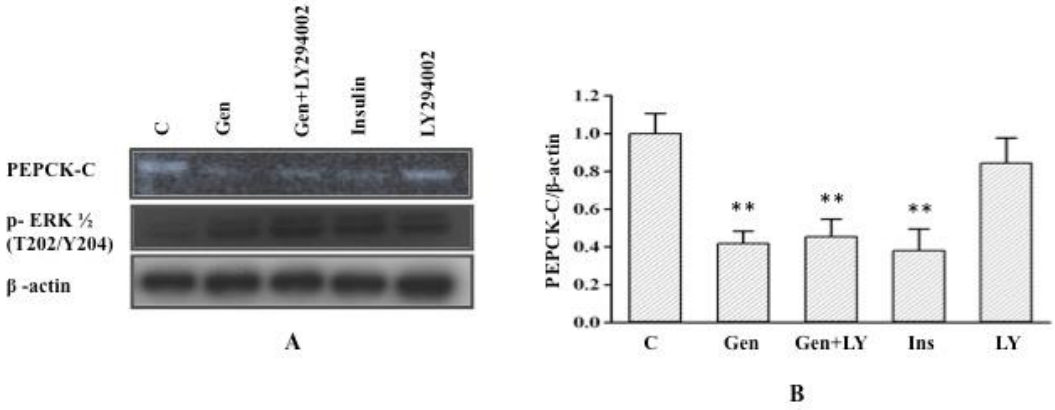

Genistein (30 μM) - + + -
PD98059 (10 μM) - - + +
p-GSK 3β (S9)
p-Foxo1 (S256)
t-Foxo1
p- Akt (S473)
t- Akt
β -actin
A
p-Foxo1/t-Foxo1
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
C
Gen
Gen+PD
PD
B
p-GSK3β/β-actin
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
C
Gen
Gen+PD
PD
C

Fig. 5

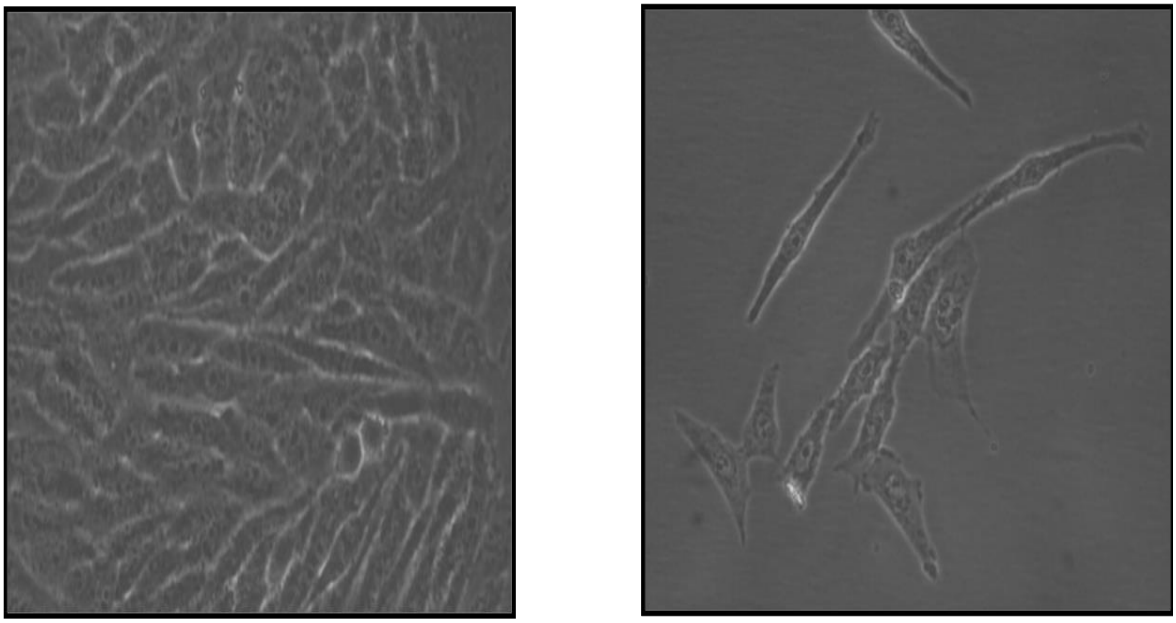

C
Gen
Gen+LY294002
Insulin
LY294002
PEPCK-C
p- ERK ½
(T202/Y204)
β -actin
A
PEPCK-C/β-actin
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
**
**
C
Gen
Gen+LY
Ins
LY
B

Legend to Supplementary Figures

Fig. S1 HepG2 cells (hepatocarcinoma cell line) were grown in DMEM supplemented with 10% FBS and 1x antibiotic mix. A. Confluent HepG2 cells. B. HepG2 cells 24 h after trypsinization.

Fig. S2 Time-dependent effect of genistein on PEPCK-C expression in HepG2 cells. HepG2 cells were treated with 30 µM genistein for different time intervals, RNAs were isolated, reverse transcribed, and cDNAs were used for semiquantitative RT-PCR analysis of PEPCK-C amplicons.

Fig. S1



A B

Fig. S2