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Morin fosters apoptosis in experimental hepatocellular carcinogenesis model

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ABSTRACT

Here we investigated the *in vivo* effect of morin (500 ppm in diet) in fostering apoptosis in diethylnitrosamine (DEN) (200 mg/kg bodyweight) mediated experimental hepatocellular carcinogenesis model. We analyzed the expression of cytosolic protein Akt and their important apoptotic downstream targets like caspase-9, Bcl-2, Bax, GSK-3β *in vivo*, by immunoblot analysis. *In silico* docking studies indicated that morin could serve as a better inhibitor than the classical PI3K inhibitor LY294002. The results obtained from *in vivo* studies confirm this. We also demonstrate here that morin's interaction with a defined set of amino acids of PI3K p110γ catalytic subunit resulted in the down-regulation of p-Akt^{Ser473}, p-Akt^{Thr308} and total Akt causing the attenuation of its downstream targets in DEN-induced hepatocellular carcinoma. Further, morin caused the up-regulation of tumor suppressor PTEN, an important negative regulator of Akt, thus initiating apoptosis. Supplementation of morin to experimental animals modulated Bcl-2/Bax ratio causing the release of cyt C and up-regulation of caspase-3 and -9. Morin was also found to prevent the Akt-mediated suppression of GSK-3β possibly causing cell cycle arrest at the G1/S phase. These observations were supported by the DNA fragmentation and transmission electron microscopy results, which showed the occurrence of apoptosis. In conclusion, our findings demonstrate that morin begets apoptosis in DEN-induced hepatocellular carcinoma.

1. Introduction

Hepatocellular carcinoma (HCC) is the most frequently diagnosed malignant neoplasm of the liver. HCC is the second leading cause of cancer-related mortality in males in developing countries [1]. Hepatitis viral infections, chronic alcohol consumption and mycotoxins represent the chief etiological factors of HCC [2]. Recent breakthroughs suggest the association of phosphoinositide-3-kinase (PI3K)/Akt pathway, in HCC tumorigenesis [3–5]. PI3K activation initiates a signal transduction cascade that promotes cancer cell growth, survival and metabolism [3,6]. Based on sequence homology and substrate preference PI3Ks are divided into three classes—Class I, II and III. Class I PI3Ks, catalyze the generation of second messenger phosphatidyl-inositol-3,4,5-trisphosphate (PIP3) from phosphatidyl-inositol-4,5-bisphosphate (PIP2). Class I PI3Ks are heterodimers composed of a regulatory subunit and a catalytic subunit (Fig. 1). The Class I PI3K catalytic subunits (p110) comprise the α, β, γ and δ isoforms, which associate with one of the five different regulatory subunits (p85/p101) encoded by separate genes [5,6]. Class I PI3Ks are the ones extensively studied in cancer and are found to be associated with HCC [7]. Both Class II and III PI3K are not yet fully understood, but the initial information suggests they are differentiated from Class I by their structure and function [8].

Akt (serine/threonine kinase) is one of the key downstream targets of PI3K [9]. Activation of Akt signalling and impaired expression of phosphatase and tensin homolog (PTEN) (a negative regulator of Akt) has been reported in 40–60% of human HCC [10]. Upon activation, Akt inactivates several downstream targets including Bcl2 family members, caspase-9 and glycogen synthase kinase-3 beta (GSK-3β), thereby blocking apoptosis [11]. Components of the PI3K-Akt pathway present promising targets for therapeutic intervention for two reasons. First, abrogation of this pathway could simultaneously inhibit the proliferation of tumor cells and sensitize them toward apoptosis [12]. Second, many components in the PI3K-Akt pathway are kinases, one of the most "drugable" classes of intracellular targets and are ideal for the development of small molecule inhibitors [13].

Current scientific interest in the management of cancer is directed towards the utilization of naturally occurring compounds for chemotherapeutics [14]. A successful anticancer drug should kill or incapacitate cancer cells without causing excessive damage to normal cells. Most promising compounds in this

Abbreviations: DEN, diethylnitrosamine; HCC, hepatocellular carcinoma; ppm, parts per million; LY294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; TBS-T, Tris-buffered saline with 0.2% Tween 20; EDTA, ethylenediaminetetraacetate; HL-60, human promyelocytic leukemia cell line; LNCaP, lymph node carcinoma of the prostate.

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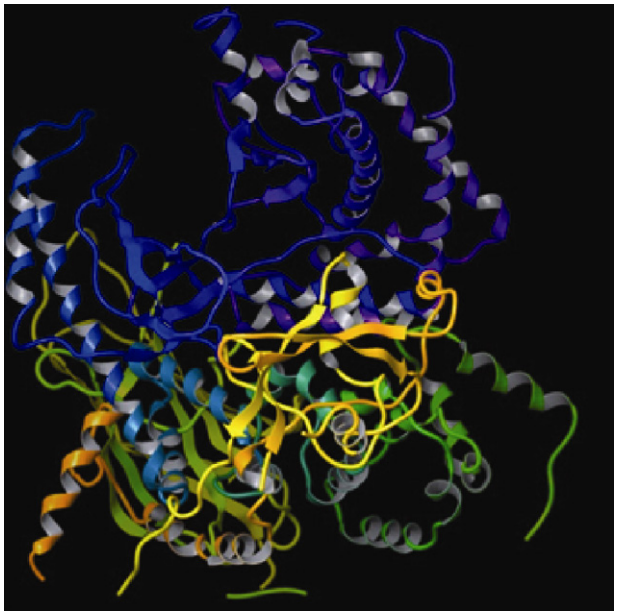


Fig. 1. Structure of PI3K (blue colored region represents the catalytic C-terminal end) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.).

regard are flavonoids which induce apoptosis in cancer cells by modulation of cell signaling [15–18]. Morin (3, −5, −7, −2′, −4′-pentahydroxyflavone) (Fig. 2) is one such dietary flavonoid that is present in almond [19], red wine [20] and Osage orange [14], reported to cause apoptosis in a time- and dose-dependent manner in cell lines such as HL-60 and LNCaP [20,21]. Morin has been shown to inhibit the growth of COLO205 cells [22], azoxymethane-induced aberrant crypt foci in rats [23] and exhibit chemopreventive effects on chemically induced rat tongue carcinogenesis [24]. We previously demonstrated that morin possesses anti-inflammatory and anti-carcinogenic properties favoring abrogation of diethylnitrosamine (DEN)-induced HCC [25,26]. However, very little is known about the molecular mechanisms of morin-induced apoptosis in HCC. In this study, we have used an experimental HCC model to elucidate the mechanisms underlying the morin-induced apoptosis in DEN-induced HCC.

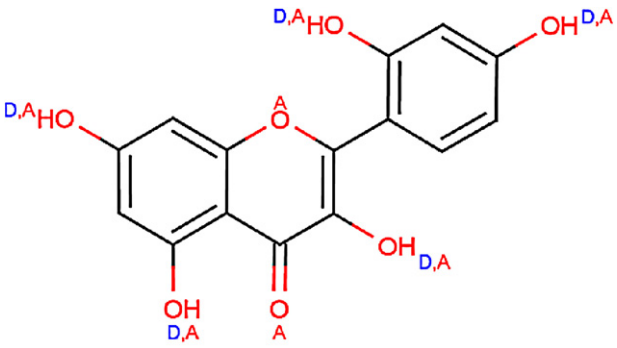


Fig. 2. Marvin chem analysis of structure of morin: morin contains 5 hydrogen donor sites (D) and 8 acceptor sites (A) as represented here.

2. Materials and methods

2.1. In silico analysis

2.1.1. Physico-chemical studies

Marvin chem (Version 4.1, ChemAxon) calculator plug-ins were used to calculate diverse structural properties of morin like the log *P* (partition coefficient) value, molecular weight and number of hydrogen donor and acceptor sites.

2.1.2. Sequence analysis

The protein sequence for PI3K p110γ catalytic subunit in human [gi: 21237725; NP_002640.2], rat [gi|62650582; XP_234053.3] and mouse [gi|30048086; gb|AAH51246.1] were retrieved from the NCBI (National Centre for Biotechnology Information) database. Pairwise alignments between them were performed using BLASTP (Basic Local Alignment Search Tool) [27]. Multiple sequence alignment (MSA) was performed using Clustal X [28]. Selection of crystal structure for PI3K catalytic subunit p110γ human was performed using a protein BLAST against the PDB (Protein Data Bank) database.

2.1.3. Docking studies

Based on the PDB-BLAST results we chose the crystal structure of human PI3K p110γ subunit (PDB code: 1E8Z) for the docking calculations. The protein structure was optimized, visualized and analyzed using the default settings with the protein preparation wizard in Maestro (Version 8.5, Schrödinger) after removing the ligand (Staurosporine) present in the active site of 1E8Z. Chemical structure of morin and LY294002 (2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one) were manually prepared in LigPrep (Schrödinger). Morin and LY294002 were individually, docked with protein 1E8Z using Glide (Version 5.0, Schrödinger). The docking runs were performed in Standard Precision (SP) mode. Only the top five energy-ranked ligand poses were retained and energy minimised using OPLS force field for each studied compound. The top-scoring conformer for each ligand was identified on the basis of the E-model score whilst the subsequent comparison between the two ligands was performed taking into account their corresponding G-score values (this is a recommended procedure in Glide).

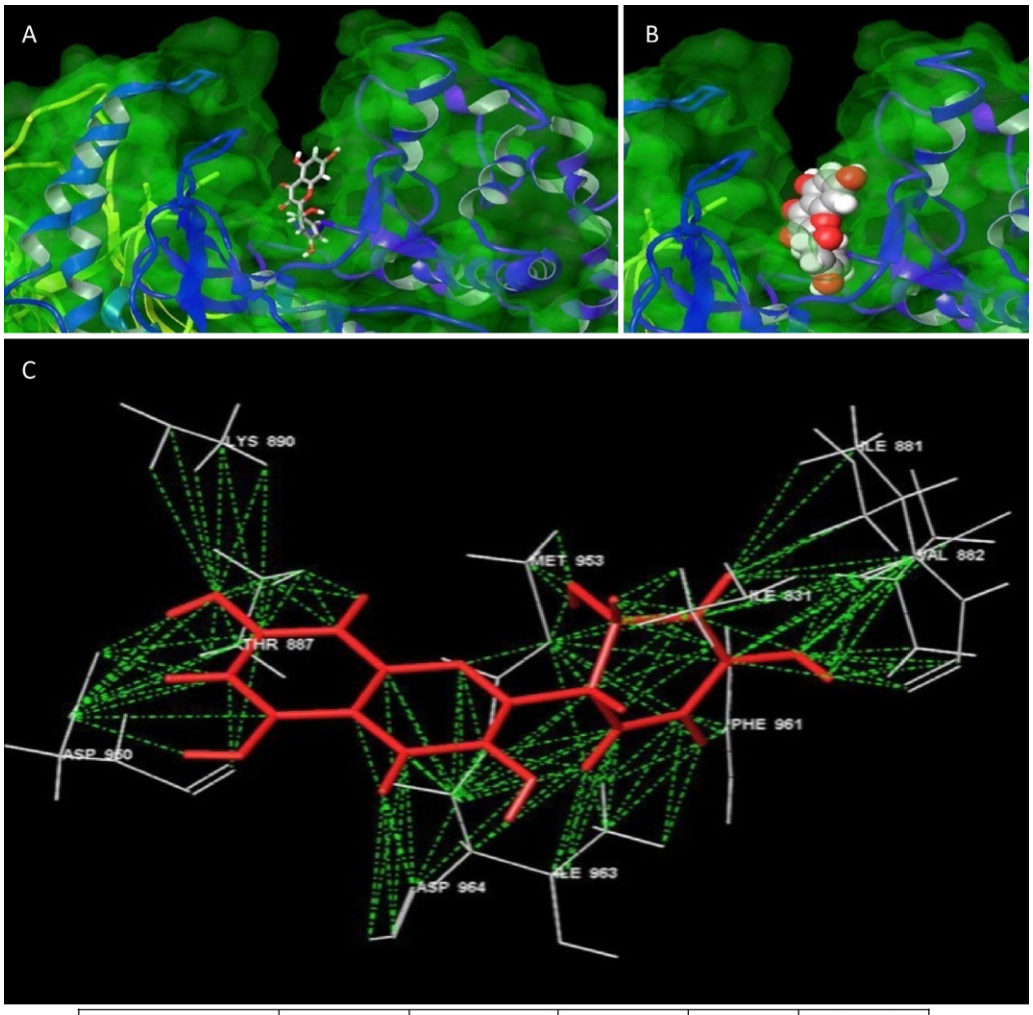
2.2. In vivo analysis

2.2.1. Experimental hepatocarcinogenesis model using diethylnitrosamine (DEN)

The experimental hepatocarcinogenesis was initiated by using DEN (Sigma, USA). DEN is the most important environmental carcinogen among nitrosamines and primarily induces tumors of liver [29,30]. The presence of nitrosamines and their precursors in human environment, together with the possibility of their endogenous formation in human body from ingested secondary amines and nitrites, have led to the suggestions of their potential involvement in HCC [31]. It is now widely used as a standard experimental model for HCC [25,26].

2.2.2. Experimental animals and design

Morin was purchased from Sigma (USA). The experimental animals were divided into four groups, each group containing six animals, analyzed for a total experimental period of 16 weeks as follows: Group 1, normal control rats fed with standard rat chow and pure drinking water. In the preliminary studies performed, the optimal dosage for its hepatoprotective efficacy was found to be 500 ppm [25]. In Group 2 rats, 500 ppm of morin alone was administered with diet for 16 weeks served as drug-control. Group 3 rats were induced with DEN (200 mg/kg bodyweight)



D

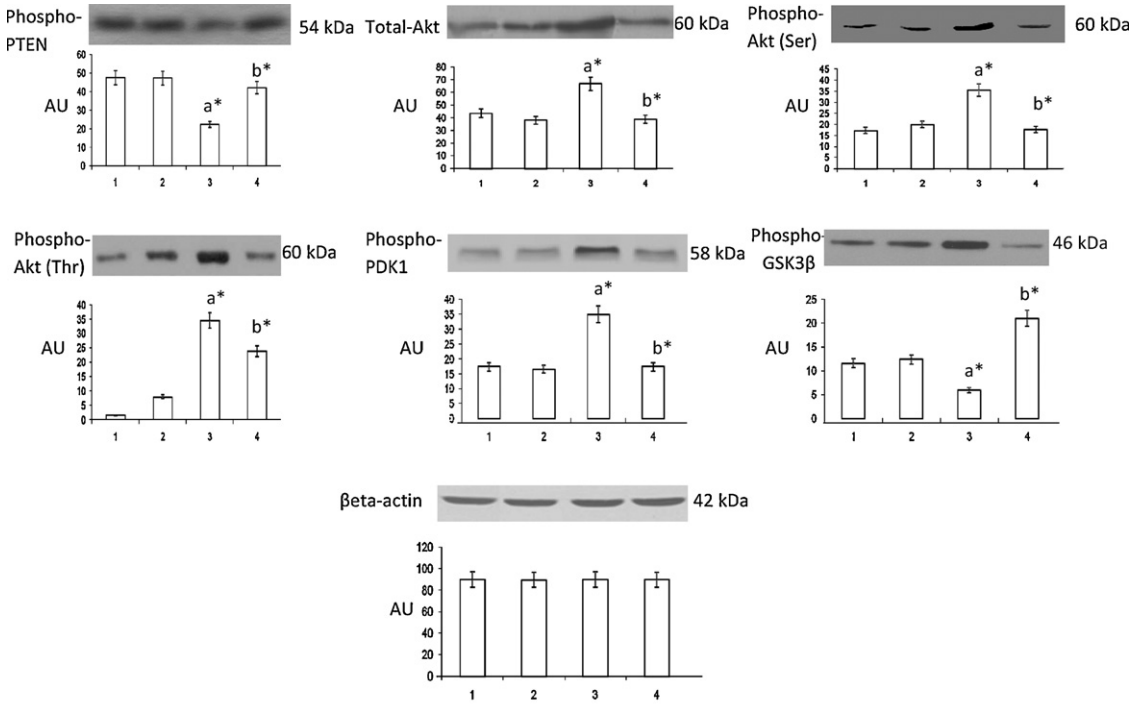
Compound	G-Score	E-Model	Energy	Hbnd	Good vdw
Morin	-7.22	-75.60	-44.20	5	226
LY294002	-7.45	-62.40	-42.20	1	162

Fig. 3. Docking analysis and the interaction of morin with human PI3K. (A) Backbone structure of morin in the active site p110 gamma subunit of PI3K [1E8Z]. (B) Ball and stick model of morin in the active site p110 gamma subunit of PI3K [1E8Z]. (C) Interaction of morin with defined set of amino acids within the 2 Å vicinity of the active site. (D) Comparative docking parameters values for morin and LY294002 when docked with PI3K p110 gamma subunit autonomously.

alone in drinking water for 16 weeks [25,26]. Group 4 rats were administered DEN (200 mg/kg body weight) in drinking water for the first 10 weeks followed by post-treatment with morin (500 ppm) in diet for the remaining 6 weeks [26]. At the end of 16 weeks, experimental rats ($n = 6$ per group) were anaesthetized with sodium-pentothal after overnight fasting and euthanized and the liver tissue was processed for further experiments. Animal experiments were carried out in strict accordance with the guidelines set by the institutional ethical committee for the use of small animals in biomedical research at University of Madras, Chennai, India (IAEC No: 01/027/06).

2.2.3. Immunoblot analysis

A portion of the liver tissue (100 mg) was homogenized in 0.1 M Tris buffer, pH 7.4 and centrifuged at 3000 × *g* for 10 min at 4 °C. The supernatant was subjected to immunoblotting and the expression patterns of cytosolic p-PTEN, p-PDK1, total Akt, p-Akt^{ser473}, p-Akt^{Thr308}, p-GSK-3β (Santa Cruz Biotechnology, USA); caspase-3, caspase-9, cyt C, Bcl-2 and Bax (BD Pharmingen) were detected. 50 μg of supernatant protein was loaded onto 10–15% polyacrylamide gels and separated by SDS-PAGE. After electrophoresis proteins were transferred to nitrocellulose membrane. The membranes were then blocked in 10% nonfat milk in Tris-buffered saline containing 0.2% Tween 20 (TBS-T) at room temperature for 3 h and probed with their corresponding primary antibodies diluted in TBS-T (1:500 dilution of each) and incubated overnight at 4 °C, with gentle shaking. The membranes were then washed with TBS-T gently and subsequently incubated with appropriate secondary antibodies (anti-rabbit and anti-mouse IgG) linked to horseradish peroxidase at 1:4000 dilution. The bands were visualized with enhanced chemiluminescence detection system (Amersham Biosciences, USA). β-Actin served as loading



Lane 1: Control (Group 1); Lane 2: Drug control (Group 2);
Lane 3: DEN-Induced (Group 3); Lane 4: DEN+Morin (Group 4);
A- Representative immunoblots and their corresponding densitometry.
AU represents arbitrary units.
Values are expressed as mean ± SD (n=6).
'*' symbol represents statistical significance at $p < 0.05$.
Comparisons are made as
a- Group 1 (Control) Vs Group 3 (DEN-induced);
b- Group 3 (DEN-induced) Vs Group 4 (DEN+Morin).

Fig. 4. Analysis of expression of proteins by immunoblotting. Lane 1: control (Group 1); Lane 2: drug-control (Group 2); Lane 3: DEN-induced (Group 3); Lane 4: DEN + morin (Group 4); (A) representative immunoblots and their corresponding densitometry. AU represents arbitrary units. Values are expressed as mean ± S.D. ($n = 6$). *Statistical significance at $p < 0.05$. Comparisons are made as a—Group 1 (control) vs Group 3 (DEN-induced); b—Group 3 (DEN-induced) vs Group 4 (DEN + morin).

control of protein. Intensity of bands was digitized by gel scanner and densitometry analysis was done using UN-SCAN-IT (Version 6.1).

2.2.4. DNA fragmentation

Liver tissue (0.5 g) was suspended in 3.0 ml of 1% SDS, 1 mM EDTA, 10 mM Tris–HCl (pH 7.4) and homogenized in a Teflon-coated homogenizer for 30 s. The homogenate was incubated at 37 °C with Ribonuclease A (Sigma, USA) at a concentration of 200 μg/ml for 1 h, followed by treatment overnight with proteinase K (500 μg/ml) (Sigma, USA) at 55 °C. After subsequent phenol chloroform extraction, the DNA was subjected to agarose gel (1.5%) electrophoresis for 2 h at 50 V in a TAE buffer (40 mM Tris–acetate, 1 mM EDTA) containing 0.5 μg/ml ethidium bromide (Sigma, USA) and visualized under UV light.

2.2.5. Transmission electron microscopy (TEM)

The liver samples were fixed in Karnovsky's fixative immediately after euthanization of rats for 6–8 h at 4 °C. These were post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer for 2 h at 4 °C, dehydrated in ascending grades of acetone, infiltrated and embedded in araldite CY212 and polymerized at 60 °C for 72 h. Thin (60–70 nm) sections were cut with an ultra-microtome. The sections were mounted on copper grids and stained with uranyl acetate and lead citrate and observed under a transmission electron microscope.

2.2.6. Statistical analysis

Data were analyzed by "Analysis of Variance" (ANOVA) and groups were compared by least significant difference [LSD] test using SPSS/10 software. $P < 0.05$ was considered as significant. All the results were expressed as mean ± S.D.

3. Results

3.1. In silico analysis

Marvin chem analysis of the physico-chemical properties of morin revealed that it possessed low molecular weight of 302 Da, log P value of 0.35, 5 hydrogen donor sites and 8 acceptor sites (Fig. 2). BLASTP analysis between human and rat PI3K p110γ; human and mouse PI3K p110γ revealed 96% similarity between them (supplementary material). MSA (Clustal X) performed among human, rat and mouse PI3K revealed >90% similarity of conserved

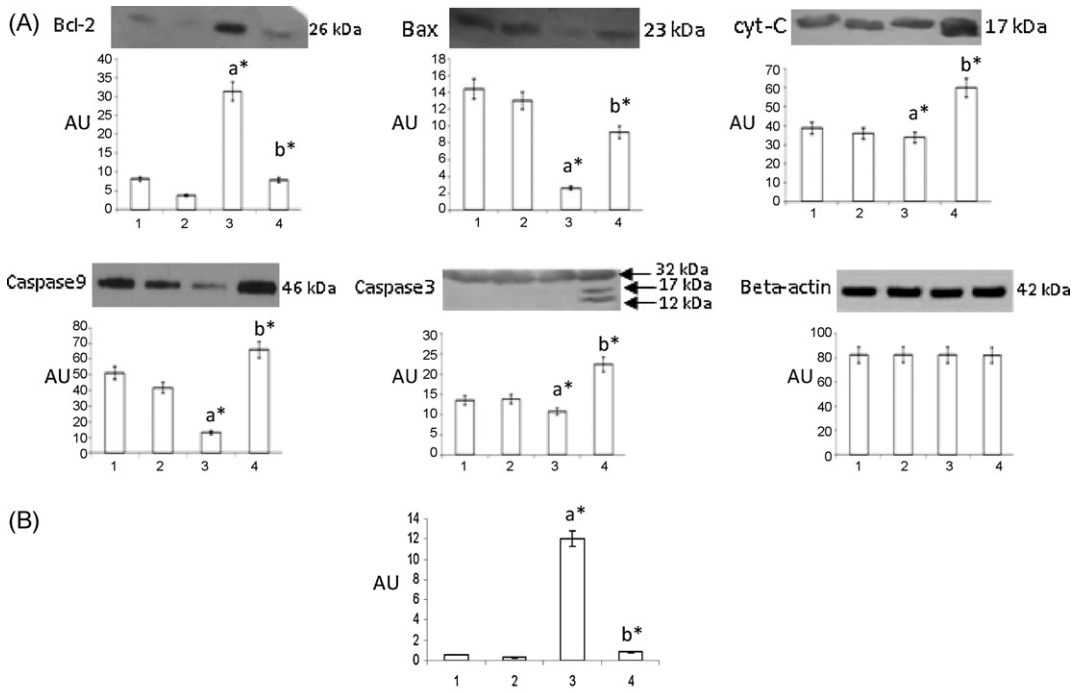


Fig. 5. Analysis of expression of proteins by immunoblotting. Lane 1: control (Group 1); Lane 2: drug-control (Group 2); Lane 3: DEN-induced (Group 3); Lane 4: DEN + morin (Group 4); (A) representative immunoblots and their corresponding densitometry; (B) densitometric representation of Bcl2/Bax ratio. AU represents arbitrary units. Values are expressed as mean ± S.D. ($n = 6$). *Statistical significance at $p < 0.05$. Comparisons are made as a—Group 1 (control) vs Group 3 (DEN-induced); b—Group 3 (DEN-induced) vs Group 4 (DEN + morin).

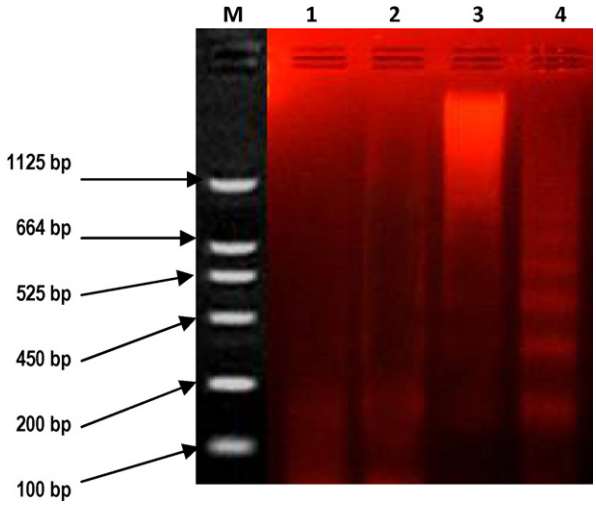


Fig. 6. DNA fragmentation analysis. M: marker lane; Lane 1: control (Group 1); Lane 2: drug-control (Group 2); Lane 3: DEN-induced (Group 3); Lane 4: DEN + morin (Group 4).

amino acids between them and the presence of identical amino acids at their C-terminal catalytic end (supplementary material). We chose PDB: 1E8Z as the best available suitable human crystal structure for performing PI3K docking studies. The catalytic domain of human PI3K(1E8Z) has two lobes, a smaller N-terminal lobe consisting of a five stranded β sheet flanked by three α helices and a larger, primarily helical C-terminal lobe. Based on the comparison of interaction studies of PI3K catalytic subunit p110γ with morin/LY294002, we concluded that morin could serve as a better inhibitor than classical PI3K inhibitor (LY294002) (Fig. 3).

3.2. *In vivo analysis*

3.2.1. *Morin alters the expression of p-PDK1, total Akt, p-Akt^{ser473}, p-Akt^{Thr308}, p-GSK-3β and p-PTEN*

Immunoblot analysis demonstrated no significant difference between control (Group 1) and drug-control (Group 2) expression levels. We observed an increased expression of total Akt, p-Akt^{ser473}, p-Akt^{Thr308}, p-PDK1 and p-GSK-3β; and decreased expression of tumor suppressor protein p-PTEN in the DEN-induced Group 3 animals (Fig. 4). In Group 4 animals, there was

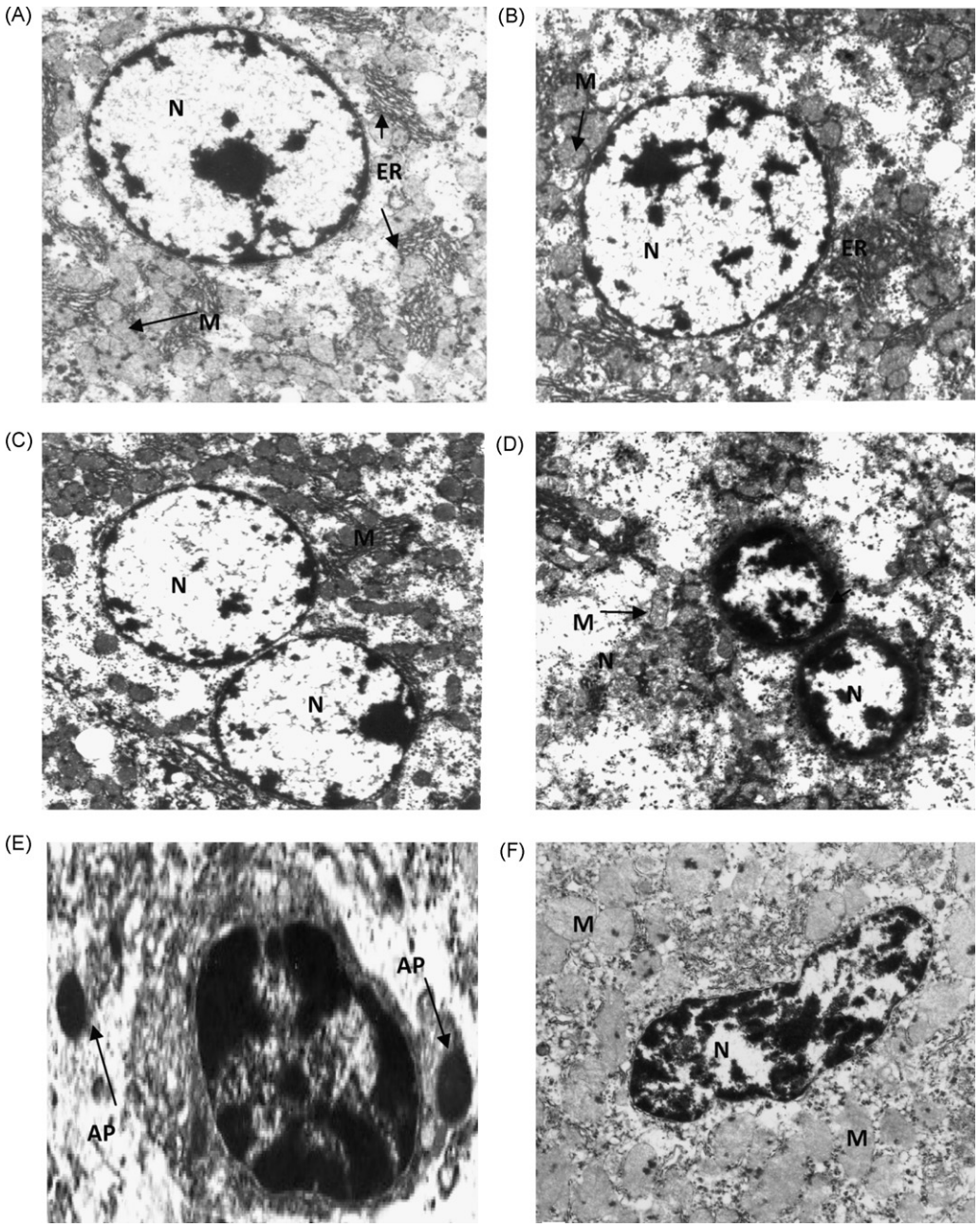


Fig. 7. Ultra-structural changes in the liver of control and experimental groups of rats. (A) Normal structure of the nucleus (N), mitochondria (M), endoplasmic reticulum (ER) and cytoplasm of liver cells of control animals (7000×). (B) Liver cell showing maintenance of normal architecture in morin administered rats (7000×). (C and D) Presence of multiple dysplastic nuclei close to each other with irregular cytoplasm in the liver cells of DEN administered rats (C—6000×, D—5000×). (E and F) Liver cell nuclei showing apoptotic bodies, chromatin condensation and mitochondrial swelling, features of apoptosis in DEN + morin post-treated rats (E—10,000×, F—8000×).

an augmented expression of p-PTEN and suppression of survival proteins like total Akt, p-Akts, PDK1 and GSK-3β thus favoring apoptosis (Fig. 4).

3.2.2. Morin alters the expression of pro-/anti-apoptotic proteins

The expression levels of pro-apoptotic proteins Bax, caspase-3, caspase-9, cyt C and anti-apoptotic protein Bcl-2 of the experimental groups were analyzed by immunoblot (Fig. 5). We observed no significant difference between the control (Group 1) and drug-control (Group 2) expression levels. We observed an increased expression of Bcl-2 and decreased expression of Bax, caspase-3, caspase-9 and cyt C in the DEN-induced Group 3 animals. The vice versa expression levels were observed in Group 4 experimental animals. There was a significant increase in the ratio of Bcl-2/Bax in Group 3 DEN-induced experimental animals favoring anti-apoptotic factors, resulting in prevention of apoptosis. In case of Group 4 experimental rats, there was a significant decrease in the Bcl-2/Bax ratio. This clearly demonstrated the tilting of balance from cell proliferation to apoptosis mediated through the release of cyt C from mitochondria and activating caspases/apoptosis.

3.2.3. Morin induces DNA fragmentation

Fragmentation of cellular DNA at the internucleosomal linker regions has been observed in cells undergoing apoptosis. To determine if morin could induce apoptosis, we analyzed DNA frag-

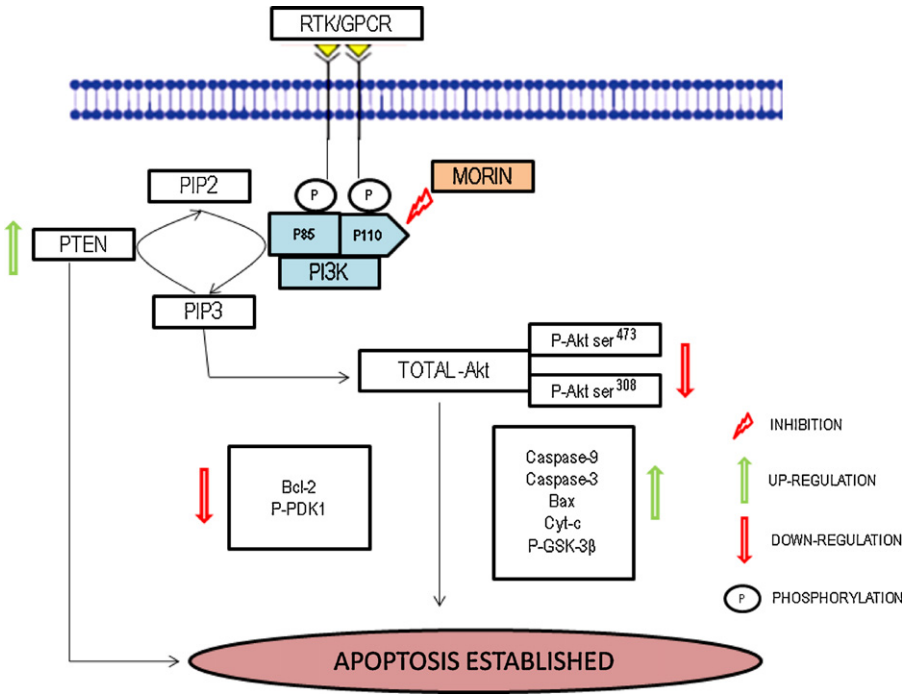


Fig. 8. Schematic representation of the mechanism of action of morin. Morin's inhibition of PI3K p110γ catalytic subunit, resulting in the down-regulation of downstream targets p-Akt^{ser473}, p-Akt^{Thr308}, PDK1 and Bcl-2; simultaneously up-regulating PTEN and pro-apoptotic members thereby fostering apoptosis in the tumours of DEN-induced HCC animals.

mentation in liver of control and experimental animals. Fig. 6 shows that there was no DNA fragmentation in control (Group 1), drug-control (Group 2) and cancer (Group 3) bearing animals. In Group 4 animals, we observed DNA fragmentation, a characteristic of apoptosis. This provides evidence that morin-induced apoptosis in HCC bearing animals.

3.2.4. Morin caused apoptotic changes at ultra-structural level

Fig. 7 illustrates the ultra-structural studies of the liver cells of Group 1 animals showing normal nuclei and cytoplasm. Morin alone treated Group 2 animals showed similar normal architecture to Group 1. In DEN-induced Group 3 animals, the presence of multiple nuclei close to each other with irregular cytoplasm demonstrates dysplasia. In Group 4, the occurrence of morphological characters of apoptosis such as cell shrinkage, membrane blebbing, apoptotic bodies, nuclear chromatin condensation and mitochondrial swelling were observed. This clearly confirmed the occurrence of apoptosis in Group 4 animals.

4. Discussion

Morin has a molecular weight less than 500 Da. It has not more than 5 hydrogen donor sites and 10 acceptor sites and a log *P* value under 5. This property indicates its drug likeliness/oral bioavailability as suggested by Lipinski's rule of five [32]. Also, morin is an isomer of quercetin, the lead compound on which LY294002 was designed, which has been extensively used both *in vivo* and *in vitro* for targeting PI3K in different cancers [33].

The sequence analysis of PI3K catalytic subunit p110γ between mammals suggested that >96% of amino acids were highly conserved (supplementary material) and especially identical amino acids were observed at the C-terminal active site region of PI3K p110γ. This aided us in further looking for the availability of crystal structure for PI3K catalytic subunit p110γ for humans in the PDB database for performing docking studies and 1E8Z was chosen as it was having the best resolution among resolved structures for human PI3K. The docking studies were further performed between (morin)1E8Z and compared with (LY294002)1E8Z.

Morin was found to interact with a defined set of amino acids located within the 2 Å vicinity of the active site of 1E8Z (Fig. 5) similar to LY294002. They are Ile 831, Ile 881, Val 882, Thr 887, Lys 890, Met 953, Asp 960, Phe 961, Ile 963 and Asp 964. Intriguingly, majority of these amino acids are known to bind to the active site ATP binding pocket of PI3Kinase 110 gamma subunit as revealed by their interaction with other flavonoids like quercetin, myricetin; inhibitors like wortmannin, staurosporine and LY294002; and ATP [33]. This provides support for our finding. Similarly, morin was found to bind to the same ATP binding catalytic pocket of PI3K p110γ subunit, this could mean that it is a specific protein kinase inhibitor (Fig. 3). Furthermore, observations from our docking results were similar to those previously observed for LY294002 with PI3K p110γ subunit [33]. Both (morin)1E8Z and (LY294002)1E8Z docking G-scores were calculated (Fig. 3). Unlike LY294002 which involves covalent irreversible interactions, morin involved only weak hydrogen bond and van der Waals interactions, clearly revealing its reversible and non-toxic nature. Based on the G-scores and better weak interactions like hydrogen bonding and van der Waals forces, it was concluded that morin was an efficient non-toxic inhibitor than LY294002 suggesting its role as a specific protein kinase inhibitor of PI3K p110γ subunit. Technical limitations like the lack of high-quality specific monoclonal antibodies for PI3K p110γ subunit at the time of this study, prompted us to perform the *in silico* studies. Nevertheless, we confirm the effect of morin on downstream targets using *in vivo* analysis.

The *in vivo* protein expression studies downstream of the PI3K pathway further demonstrate that phosphorylation of Akt at residues Thr^{308} and Ser^{473} resulting in the hyperactivation of Akt fostering further phosphorylation of downstream signals via PDK1; simultaneously suppressing the expression of PTEN resulting in DEN-induced HCC. The cell survival pathway mediated by PI3K/Akt

was suppressed/down-regulated by morin (Lane 4 of total Akt, ser^{473} and Thr^{308} immunoblots of pAkt). This suggested the attenuation of the downstream signals and simultaneous up-regulation of the tumor suppressor protein PTEN by morin. Akt down-regulation thus was mediated by morin exclusively in Group 4 experimental animals resulting in the prevention of cell survival in HCC.

Akt is involved in cell cycle regulation by preventing GSK-3β mediated phosphorylation and degradation of Cyclin D1 thereby preventing cell cycle arrest [4]. In our present study, we observed that the down-regulation of PI3K/Akt in Group 4 experimental animals resulted in activation of GSK-3β leading to cell cycle arrest at G1/S phase, possibly mediated by Cyclin D1. The attenuation of cell survival pathway along with synchronized favoring of cell cycle arrest at the G1/S phase leads to suppression of DEN-induced hepatocellular carcinogenesis by morin.

Members of the Bcl2 family consisting of both pro-apoptotic and anti-apoptotic molecules maintain the balance of cell death and survival in a cell [34,35]. Some physiological apoptotic molecules are down-regulated or inactivated in HCC and the balance between death and survival is mainly disrupted due to over activation of anti-apoptotic signals [36]. In view of this, the expression of pro-apoptotic Bcl-2, Bax, cyt C, caspase-3 and -9 were studied by immunoblotting. Bcl-2/Bax ratio may alter the cyt C release and also play a key role in deciding whether the cell should switch towards proliferation or towards apoptosis [37,38]. The results of the present study revealed that morin up-regulated the expression of Bax and caused simultaneous down-regulation of Bcl-2, thereby decreasing Bcl-2/Bax ratio. This results in the release of cyt C from mitochondria, that leads to the activation of caspase-3 and -9 stimulating apoptosis in Group 4 experimental animals.

The induction of tumor cell death by apoptosis is a major goal of cancer therapy and the detection of apoptosis in tumor tissue has become an important diagnostic parameter [39]. In fact, formation of DNA fragments of oligonucleosomal size (180–200 bp) is a hallmark of apoptosis. Such release of small oligonucleosomal fragments is recognized as a characteristic DNA ladder on conventional agarose gels [40]. In our present study, we observed the occurrence of characteristic DNA ladder distinctively only in Group 4 animals. DNA laddering was absent in the all the other three groups. This confirmed the induction of apoptosis by morin only in Group 4 animals.

The ultra-structural studies were performed to further confirm the occurrence of apoptotic morphological changes at the cellular level. The control and the drug-control animals showed normal nuclei and cytoplasm. The animals induced with DEN showed the presence of dysplastic nuclei, loss of cell architecture and membrane damage with irregular cytoplasm. Morphological changes of apoptosis were observed in the liver cells of morin post-treated rats. Liver cell with shrunken nucleus, condensed chromatin, membrane blebbing and formation of apoptotic bodies were visualized in Group 4 rats. Thus, the results of the ultra-structural studies undoubtedly confirmed that morin has the ability to cause apoptosis.

From *in silico* interaction studies we conclude that morin could serve as a better inhibitor than the classical specific PI3K inhibitor LY294002. The interaction of morin to PI3K was found to be responsible for the *in vivo* down-regulation of total Akt, p-Akt^{ser473} and p-Akt^{Thr308}, thereby causing the attenuation of its downstream pro-apoptotic targets in DEN-induced HCC. In conclusion, our findings from *in silico* and *in vivo* studies indicate the mechanisms underlying the morin-induced apoptosis in DEN-induced HCC (Fig. 8).

Conflict of interest

There is no conflict of interest between the authors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cbi.2009.11.011.

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