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A novel quinazolinone derivative induces cytochrome *c* interdependent apoptosis and autophagy in human leukemia MOLT-4 cells



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ARTICLE INFO

Article history:

Received 22 May 2014

Received in revised form 8 July 2014

Accepted 29 July 2014

Available online 12 August 2014

Keywords:

2,3-Dihydro-2-(quinoline-5-yl)
quinazolin-4(1*H*)-one structure [DQQ]

Autophagy

Apoptosis

Cytochrome *c*

Caspase

ABSTRACT

Crosstalk between apoptosis and autophagy is budding as one of the novel strategies in the cancer therapeutics. The present study tinted toward the interdependence of autophagy and apoptosis induce by a novel quinazolinone derivative 2,3-dihydro-2-(quinoline-5-yl) quinazolin-4(1*H*)-one structure [DQQ] in human leukemia MOLT-4 cells. DQQ induces cytochrome *c* arbitrated apoptosis and autophagy in MOLT-4 cells. Apoptosis induces by DQQ was confirmed through a battery of assay e.g. cellular and nuclear microscopy, annexin-V assay, cell cycle analysis, loss of mitochondrial membrane potential and immune-expression of cytochrome *c*, caspases and PARP. Furthermore, acridine orange staining, LC3 immunofluorescence and western blotting of key autophagy proteins revealed the autophagic potential of DQQ. A universal caspase inhibitor, Z-VAD-FMK and cytochrome *c* silencing, strongly inhibited the DQQ induce autophagy and apoptosis. Beclin1 silencing through siRNA partially reversed the cell death, which was not as significant as by cytochrome *c* silencing. Although, it partially reversed the PARP cleavage induced by DQQ, indicating the role of autophagy in the regulation of apoptosis. The present study first time portrays the negative feedback potential of cytochrome *c* regulated autophagy and the importance of quinazolinone derivative in discovery of novel anticancer therapeutics.

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Abbreviations: AO, acridine orange; ATG, autophagy related gene; LC3, microtubule-associated protein light chain 3; MTT, 3-(4,5-Dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide; PARP-1, poly(ADP-ribose) polymerase-1; PI, propidium iodide; Rh-123, rhodamine-123; ZVAD(zVAD fmk), benzyloxycarbonyl-Val-Ala-Asp fluoro-methyl-ketone; MMP, mitochondrial membrane potential.

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¹ IIIM communication No. IIIM/1648/2014.

<http://dx.doi.org/10.1016/j.toxrep.2014.07.018>

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1. Introduction

Cancer is the leading cause of death in the developed as well as developing world and it is one of the most threatening health disorders worldwide. An estimate of 7.6 million deaths was caused due to cancer worldwide accounting 13% of total deaths in 2008 and leukemia is one of the leading causes of cancer deaths among the young males [1,2]. According to the latest report, there is a significant decline in mortality induced by leukemia over past 10 years and despite of significant turn down in death rates, leukemia still is a big problem [1]. Therefore, there is an unmet need to discover and develop novel anticancer agents. In this regard, we have testified autophagic and apoptotic potential of a novel quinazolinone derivative, 2,3-dihydro-2-(quinoline-5-yl)quinazolin-4(1H)-one [DQQ] in human leukemia MOLT-4 cells. Quinazolinone ring, a well known structural element of many natural products and synthetic agents, have been established as a useful privileged scaffold for library design and drug discovery applications [3]. These compounds do not only have a wide application as organic congeners, but have remarkable biological and pharmacological activities [4,5]. Many quinazolines have been approved by FDA for different diseases such as prazosin used to treat high blood pressure, gefitinib and erlotinib are tyrosine kinase inhibitors that specifically target EGFR and are used to treat non small cell lung cancer, pancreatic cancer and several other types of cancers [5]. In addition, 2,3-dihydroquinazolinones have proven to act as potent tubulin inhibitors with impressive anti proliferative activity against several human cancer cell lines. Although different derivatives of quinazolinone have been reported for their anticancer activities in different cancers, but there was no report against any type of leukemia. Therefore, we have for the first time evaluated DQQ anticancer potential in human leukemia cells and explore its autophagic and apoptotic potential.

Apoptosis and autophagy are type one and two programmed cell death, respectively. They have a complex relation with each other. Several chemotherapeutic agents induce autophagy and apoptosis, which is a hallmark of all cancers. Many studies suggested autophagy as survival phenomenon and have reported to inhibit the process of apoptosis [6,7], on the other hand, there were several reports which reveal that autophagy activate and accelerate apoptosis [8,9]. Autophagy and apoptosis are interdependent and inhibition of important autophagic genes such as beclin-1, ATG5, ATG7 and ATG10 leads the cell to apoptotic cell death [10], in contrast the addition of inhibitors that block the fusion of autophagosome with lysosomes, manifested mixed type of morphological features of autophagy and apoptosis [6]. Caspase-3 has been found to have a role in controlling both apoptosis and autophagy and its inhibition associated with reversal of both autophagic and apoptotic cell death [11]. The specific inhibition of the proapoptotic function of cytochrome *c*, a key regulator of mitochondria-mediated apoptosis, enhanced autophagy following chemotherapeutic treatment [12]. Apoptosis and autophagy potential of quinazolinone ring members have been investigated in different cell lines [13,14], and in most of the cases

the autophagy induced by different quinazoline derivatives are of the protective nature [15]. Our study, for the first time explore the interdependence of autophagy and apoptosis induced by 2,3-dihydro-2-(quinoline-5-yl)quinazolin-4(1H)-one [DQQ] and negative feedback potential of cytochrome *c* regulated autophagy in human leukemia MOLT-4 cells.

2. Materials and methods

2.1. Cell culture, growth conditions and treatments

Human acute lymphoblastic leukemia cells MOLT-4 and K-562 were obtained from European Collection of Cell Cultures (ECACC). Cells were grown in RPMI-1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS), penicillin (100 units/ml), streptomycin (100 µg/ml), L-glutamine (0.3 mg/ml), sodium pyruvate (550 mg/ml), and NaHCO₃ (2 mg/ml). Cells were grown in a CO₂ incubator (Thermocon Electron Corporation, USA) at 37 °C in an atmosphere of 95% air and 5% CO₂ with 98% humidity. Cells treated with DQQ and other inhibitors were dissolved in DMSO while the untreated cells received the vehicle (DMSO <0.2%).

2.2. Reagents and chemicals

RPMI-1640, DMEM, EMEM, propidium iodide (PI), 3-(4,5,-dimethylthiazole-2-yl)-2,5 diphenyltetrazolium bromide (MTT), 2,7-dichlorofluoresceine diacetate (DCFH-DA), MG-132, Hoechst-33258, protease inhibitor cocktail, RNase, rhodamine-123 (Rh-123), streptomycin, fetal bovine serum, phenyl methane sulfonyl fluoride (PMSF), L-glutamine, pyruvic acid, NAC, sMIT and bovine serum albumin were purchased from Sigma-Aldrich (Bangalore, India). ApoAlert caspases-8 and -3 fluorescent assay kits, primary antibodies of cytochrome *c* and Beclin1 were purchased from B.D Biosciences (San Jose, CA). Pan specific caspase inhibitor Z-VAD-fmk, AnnexinV-FITC apoptosis detection kit, primary antibodies to Bcl-2, Bax, caspase-3, caspase-8, PARP-1, β-actin and siRNA transfection reagent were from Santa Cruz Biotechnology (Santa Cruz, CA). Other remaining antibodies were purchased from Cell signaling technology (Danvers, MA). Electrophoresis reagents, protein marker and protein estimation kit were from Bio-Rad Laboratories (Hercules, CA). Hyper film and ECL plus reagents were purchased from Amersham Biosciences, UK. All other bio-chemicals and reagents used in studies were AR grade and purchased from Sigma Aldrich, India.

2.3. Synthesis of 2,3-dihydro-2-(quinoline-5-yl)quinazolin-4(1H)-one (DQQ)

2,3-Dihydro-2-(quinoline-5-yl)quinazolin-4(1H)-one (DQQ) was synthesized as described earlier [16] (Fig. 1A). Anthranilamide (1, 1 eq) and quinoline-4-carbaldehyde (2, 1 eq) was dissolved in acetonitrile (5 ml) followed by amberlist-15 (50 mol%). The reaction mixture was stirred at room temperature, filters, concentrates and purified by column chromatography. DQQ is a light yellow solid with 85% yield; *mp*: 253–255 °C; ¹H NMR (400 MHz, DMSO-d₆); δ

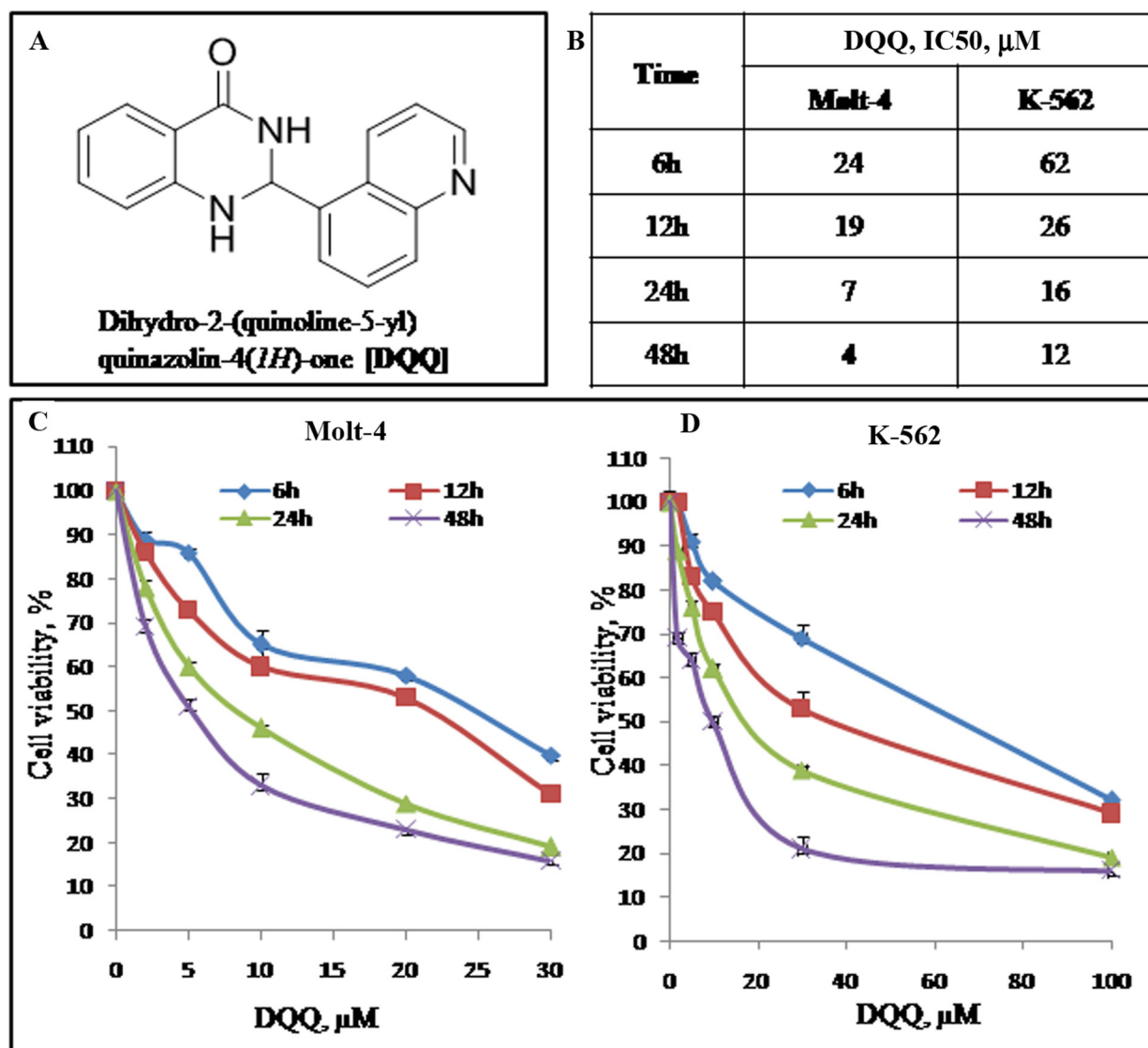


Fig. 1. DQQ inhibited cell proliferation in human leukemia MOLT-4 and K562 cells. (A) Chemical structure and IUPAC name of 2,3-dihydro-2-(quinoline-5-yl)quinazolin-4(1H)-one (DQQ). (B) and (C) Cell proliferation assay. MOLT-4 and K562 cells were seeded in 96 well plates and treated with various concentrations of DQQ for 6h, 12h, 24h and 48h. MTT dye was added 3h before the termination of the experiment. Cell viability was calculated as described in Section 2. Data are mean \pm SD ($n=8$ wells).

(ppm), 9.30, (d, $J=8.4$ Hz, 1H), 8.95 (s, 1H), 8.38 (s, 1H), 8.08, (m, 1H), 7.80 (m, 3H), 7.59 (m, 1H), 7.29 (t, $J=7.2$ Hz, 1H) 7.12 (s, 1H), 6.77 (m, 2H), 6.49 (s, 1H); ^{13}C NMR (100 MHz, DMSO- d_6); δ (ppm), 163.9, 150.2, 148.3, 148.2, 135.7, 133.3, 133.1, 130.3, 128.6, 127.4, 125.8, 120.9, 117.4, 115.0, 114.5, 79.1, 65.8; IR: (KBr-pellet) 3399, 3294, 2920, 2850, 1647, 1610, 1502, 1462, 1383, 1296, 1156, 1073, 795, 762, 708 cm^{-1} ; MS (Q-TOF): m/z 276 [M+1] $^+$, 298 [M+Na] $^+$; HRMS: m/z 276.1130 calcd for $\text{C}_{17}\text{H}_{14}\text{N}_3\text{O} + \text{H}^+$ (276.1137).

2.4. Cell proliferation assay

MTT assay was done to determine the viability of the cells and was done as described previously [17]. Briefly, 6×10^3 cells were seeded in 96 well plates and were treated

with different concentrations of DQQ for 48 h. 20 μl of MTT dye (2.5 mg/ml) was added 3 h before the termination of the experiment. The plates were centrifuged at $400 \times g$ for 15 min and formulated MTT formazan crystals were dissolved in 150 μl of DMSO, absorbance was measured at 570 nm with reference wavelength 620 nm.

2.5. Phase contrast microscopy

Morphological changes in cell were studied by phase contrast microscopy. MOLT-4 cells were incubated in twelve well plates and treated with different concentration of DQQ (2–10 μM) for 24 h, after that cells were subjected to photography on an inverted microscope attached to the DP-12 camera (1X70, Olympus).

2.6. Hoechst 33258 nuclear staining

Cells were treated with different concentrations of DQQ (2–10 μM) for 24 h and washed twice with PBS at $400 \times g$ for 5 min. Cells were then stained with 1 ml of staining solution (10 $\mu\text{g/ml}$, Hoechst 33258, 0.01 M citric acid and 0.45 M disodium phosphate containing 0.05% Tween-20) and stained for 30 min in the dark at room temperature. After staining the cells were resuspended in 50 μl of mounting fluid (PBS:glycerol, 1:1) and 10 μl mounting suspension was observed for nuclear morphology under inverted fluorescence microscope using UV excitation (Olympus 1X70, magnification 30X) [18].

2.7. Flow cytometric analysis of apoptosis and necrosis

MOLT-4 cells (1×10^6) were treated with 2 μM , 5 μM and 10 μM concentrations of DQQ for 24 h. Cells were double stained with annexin-V/PI by using kit manufacturer's protocol (no. sc4252, Santa Cruz Biotechnology, USA). The cells were scanned for fluorescence intensity in FL-1 (FITC) and FL-2 (PI) channels. The fraction of cell populations in different quadrants was analyzed using quadrant statistics. The FACS analysis of apoptosis and necrosis was done as described earlier [19].

2.8. Cell cycle analysis

Cell cycle phase distribution was studied by propidium iodide fluorescence. MOLT-4 cells (1×10^6) were incubated with different concentrations of DQQ (2–10 μM) for 24 h. The cells were then washed twice with ice-cold PBS, harvested, fixed with ice-cold PBS in 70% ethanol and stored at 4 °C overnight. After fixation, these cells were incubated with RNase-A (0.1 mg/ml) at 37 °C for 90 min, stained with propidium iodide (100 $\mu\text{g/ml}$) for 30 min on ice in dark, and then measured for DNA content using BD FACSCalibur flow cytometer (Becton Dickinson, USA). Resulting DNA distributions were analyzed by Modfit software (Verity Software House Inc., Topsham, ME) for the proportions of cells in apoptosis, G1, S, and G2/M phases of the cell cycle [20].

2.9. Measurement of mitochondrial membrane potential (ψ_m)

MOLT-4 cells were seeded in 12 well plates and incubated with different concentration of DQQ (2–10 μM) for 24 h. Rhodamine-123 is a fluorescent probe used in estimation of mitochondrial membrane potential (ψ_m). Rhodamine-123 dye (200 nM) was added 30 min before termination of the experiment. Cells were collected at $400 \times g$, washed once with PBS and mitochondrial membrane potential was measured in the FL-1 channel of flow cytometer (FACS Calibur, Becton Dickinson, USA) [21].

2.10. Caspase assay

Cells were treated with indicated concentrations of DQQ for 24 h. Cells were collected, washed with PBS twice and lysed in cell lysis buffer. Caspase-8 and -3 activities in the

cell lysates were determined fluorimetrically by using BD ApoAlert caspase fluorescent assay kits [22].

2.11. Microscopic detection of autophagy through acridine orange staining

Induction of autophagy was analyzed by staining cells with acridine orange as described earlier [11]. Briefly, 0.5×10^6 cells were seeded in 6 well plates and treated with the indicated doses of DQQ for 24 h. Cells were incubated with 1 $\mu\text{g/ml}$ acridine orange for 15 min prior to the termination of the experiment and were washed with PBS before analysis on a fluorescence microscope (Olympus 1X70).

2.12. Immunofluorescence staining with LC3 for autophagy detection

Immunofluorescence for LC3 was done as described previously [11]. Briefly, 0.5×10^5 cells were treated with 2 μM , 5 μM and 10 μM concentrations of DQQ for 24 h, and collected at $400 g$. The pellets were resuspended in incomplete medium and were subjected to poly-L-lysine (0.01% sol, Sigma) coated cover slips for 10 min at room temperature. Poly-L-lysine was aspirated and cover slips were allowed to dry completely. Cells were fixed in 4% paraformaldehyde for 15 min, washed thrice for 5 min with PBS, permeabilized with 0.2% Triton X-100, washed again and finally blocked with 5% goat serum albumin for 20 min. After blocking cells were incubated with LC3B antibody for 1 h followed by washing with PBS and incubation with secondary antibody (anti-rabbit Alexa Fluor-488) for 45 min. Cells were washed again and incubated with DAPI (1 $\mu\text{g/ml}$) for 5 min. Autophagy/apoptosis induction was analyzed by using fluorescence microscope (1X81, Olympus); photographs were taken by using DP-71 camera.

2.13. Preparation of cytosolic and mitochondrial lysates

MOLT-4 cells (3×10^6) were treated with 2 μM , 5 μM and 10 μM concentrations of DQQ for 24 h. Cytosolic fractions were prepared by selective plasma membrane permeabilization with digitonin [23]. Briefly, 2×10^6 cells were lysed for 1–2 min in lysis buffer containing 75 mM NaCl, 8 mM Na_2HPO_4 , 1 mM NaH_2PO_4 , 1 mM EDTA, 350 $\mu\text{g/ml}$ digitonin and 1% (v/v) eukaryotic protease inhibitor cocktail. The lysates were centrifuged at $12,000 \times g$ for 1 min, and the supernatant collected as cytosolic fraction. Residual pellet was lysed with buffer composed of 150 mM NaCl, 50 mM Tris (pH 8.0), 5 mM EDTA, 1% (vol/vol) Nonidet p-40, 1 mM phenylmethylsulfonyl fluoride, 20 $\mu\text{g/ml}$ aprotinin, and 25 $\mu\text{g/ml}$ leupeptin for 30 min at 4 °C. After centrifugation at $12,000 \times g$ for 10 min at 4 °C, cell lysates were transferred to fresh tubes and stored as mitochondrial fraction.

2.14. Western blot analysis

Equal amount of protein (30–70 μg) were subjected to SDS-PAGE and then electro transferred to PVDF membrane for 100 min at 4 °C at 100 V. Nonspecific binding was blocked by incubation with 5% non-fat milk or 3% BSA in

tris-buffered saline containing 0.1% Tween-20 (TBST), for 1 h at room temperature. The membranes were incubated with respective primary antibodies for 4 h and washed twice with TBST. After that, blots were incubated with horseradish peroxidase conjugated secondary antibodies for 1 h and washed three times with TBST. Blots were incubated with ECL plus reagent and signal captured by using hyperfilm (GE Healthcare) [24].

2.15. Small interfering RNA (siRNA)

Human cytochrome *c* and beclin 1 specific siRNA were transfected into MOLT-4 cells by using manufacturer protocol. Briefly, 2×10^5 MOLT-4 cells were seeded in six well plates and incubated in transfection media containing equal amounts of transfection reagent and siRNA for 8 h. Complete media was added to cells different experiments were performed within 72 h of transfection. Knocking down of the expression of the respective proteins was checked by western blotting.

2.16. mTOR kinase assay

mTOR inhibition of DQQ was found out by using K-LISA™ mTOR kit from Calbiochem (no. CBA055). It is an ELISA-based assay that utilizes a p70S6K-GST fusion protein as a specific mTOR substrate. The assay was carried out according to the manufacturer's protocol. Briefly, 100 μ l of recombinant p70S6K-GST fusion protein was pre-incubated at room temperature in the glutathione coated 96-well plate for 1 h after that a mixture of 49 μ l of ice-chilled mTOR kinase and 1 μ l of test compounds or DMSO was added. The reaction was initiated by the addition of 50 μ l of mTOR kinase assay buffer containing 100 μ M ATP and 1 μ M DTT. The plate was treated first with 100 μ l of anti-p70S6K-T389 for 1 h and then with 100 μ l of HRP-conjugated antibody for 1 h to detect the T389-phosphorylated p70S6K. Absorbance was measured at 450 nm and 595 nm using microplate spectrophotometer. The IC₅₀ values were calculated by analysis non linear regression with variable slope by using GraphPad Prism-5 software.

2.17. Statistical analysis

Data expressed as mean \pm SD or a representative of one of three similar experiments unless otherwise indicated. Comparisons were made between control and treated groups or the entire intra group using one way and two ways ANOVA with post Bonferroni test through GraphPad Prism 5.00.288 statistical analysis software by GraphPad Software, Inc. *p*-values < 0.01 were considered significant when compared to untreated control or respective DQQ treated cells.

3. Results

3.1. DQQ inhibited cell proliferation in MOLT4 and K562 cells

Cells treated with different doses of DQQ for different time frames, displayed inhibited viability in a dose and time

dependent manner (Fig. 1B and C). The IC₅₀ of DQQ against K562 and MOLT-4 was determined at different time points which come out to be 24 μ M, 19 μ M, 7 μ M and 4 μ M in 6 h, 12 h, 24 h and 48 h, respectively, in MOLT-4 cells (Fig. 1B and C), while in case of K562 cells the IC₅₀ values were 62 μ M, 36 μ M, 16 μ M and 12 μ M in 6 h, 12 h, 24 h and 48 h, respectively (Fig. 1B and C). The IC₅₀ values of DQQ in K562 cells were comparatively higher than observed in MOLT-4 cells. Thus, the MOLT-4 cell line was taken for further mechanistic studies.

3.2. DQQ induced apoptosis in MOLT-4 cells

Apoptosis was one of the modes of leukemic cell death induced by DQQ, which was further confirmed by a battery of apoptosis assays Hoechst and annexin-V staining, cell cycle and mitochondrial potential analysis. Phase contrast and nuclear microscopy results revealed that DQQ substantially induced apoptosis in MOLT-4 cells in a dose dependent manner (Fig. 2A and B). Nuclei of untreated MOLT-4 cells appeared round in shape, while treatment with DQQ resulted in nuclear condensation and the formation of apoptotic bodies. The morphological changes were accompanied by an increase in the number of scattered apoptotic bodies, indicated by white arrows (Fig. 2B). AnnexinV/PI staining is widely used to distinguish between apoptosis and necrotic population. The results of AnnexinV/PI staining suggested that the cell death induced by DQQ was of apoptotic nature as the amount of population positive for PI was negligible. The percentage of apoptotic population was significantly higher (10–20 times) in DQQ treated MOLT-4 cells as compared to untreated control (Fig. 2C). Apoptosis was further confirmed by cell cycle analysis using propidium iodide staining. Measurement of DNA content makes it possible to identify apoptotic cells and cell cycle phase specificity. The results revealed that DQQ substantially induced 3–10 times increase in hypo-diploid sub-G₀ DNA fraction (apoptotic, $< 2n$ DNA) in cell cycle phase distribution (Fig. 2D). The sub-G₀ fraction (apoptotic) was 7% in control cells, which increased up to 69% after 10 μ M concentrations of DQQ treatment in MOLT-4 cells (Fig. 2D). The early event which was associated with DQQ induced apoptosis was found to be loss of mitochondrial membrane potential ($\Delta\psi_m$). Mitochondrial membrane potential loss is one of the important and commonly occurring events in apoptosis. DQQ significantly enhanced the same in a dose-dependent manner in MOLT-4 cells (Fig. 2E). The MMP loss was increased from 6% to 63% in untreated and DQQ treated MOLT-4 cells, respectively (Fig. 2E).

3.3. DQQ induced apoptosis via both extrinsic and intrinsic pathways

We investigate the pathway of apoptosis induced by DQQ in MOLT-4 cells by monitoring the level of different mitochondrial proteins and caspases. Upregulation of Bax and down regulation of Bcl-2 have long been associated with the activation of apoptosis. DQQ inhibit the mitochondrial anti-apoptotic protein Bcl-2 and induce the

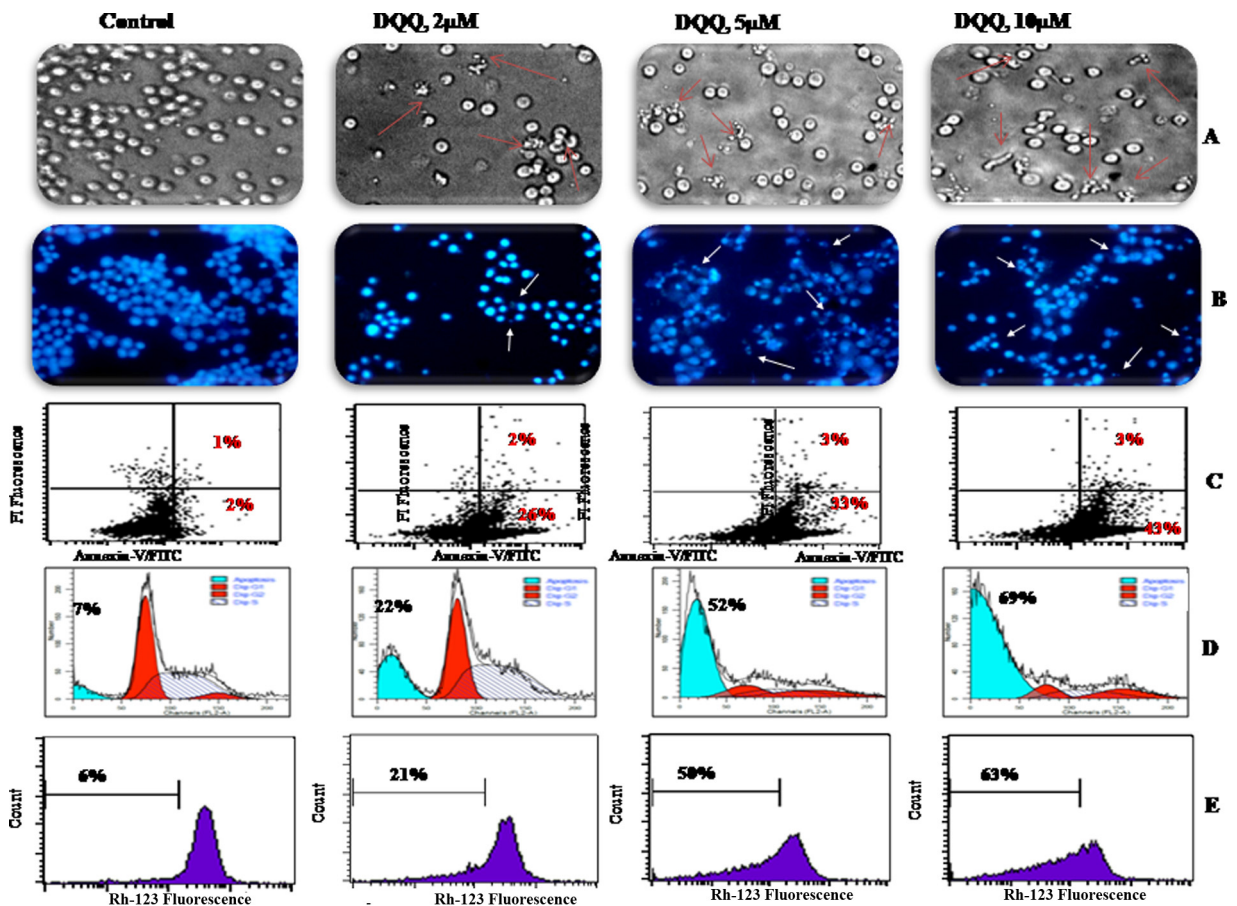


Fig. 2. Effect of DQQ on the apoptosis and early events associated with it. (A) and (B) Effect of HMC on cellular and nuclear morphology. MOLT-4 cells were seeded in a 12 well plate, treated with indicated concentrations of DQQ for 24 h and visualized for cellular and nuclear morphology as described in Section 2. Condensed nuclei and the apoptotic bodies are indicated by white arrows. Data are representative of one of three similar experiments. (C) Flow cytometric analysis of apoptosis and necrosis induced by DQQ using Annexin V-FITC and PI double staining. Other conditions were described in Section 2 and data is representative of one of three similar experiments. (D) Cell cycle phase distribution of DQQ treated MOLT-4 cell lines were analyzed by staining of cells with PI for 30 min and DNA fluorescence were determined flowcytometrically (FACS Calibur, BD Biosciences) as described in Section 2. Data were analyzed by Modfit software (Verity Software House Inc., Topsham, ME) for the proportions of different cell cycle phases. The fraction of cells from apoptosis, G1, S and G2 phases analyzed from FL2-A vs cell counts are shown in %. Data are representative of one of three similar experiments. (E) DQQ induced loss of mitochondrial membrane potential ($\Delta\psi_m$). MOLT-4 cells were incubated with the indicated doses of DQQ for 24 h. Thereafter, cells were stained with Rhodamine-123 (200 nM), added 40 min before experiment termination and analyzed in FL-1 channel of flow cytometer. Data are representative of one of three similar experiments.

translocation of Bax from cytosol to mitochondria and simultaneously released cytochrome *c* from mitochondria to cytosol, which was associated with mitochondrial membrane potential loss (Figs. 2E and 3A). DQQ drastically reduce the Bcl-2/Bax ratio in MOLT-4 cells from 10 to 0.2 levels (Fig. 3C). The Bcl-2/Bax ratio has also been found to play key role in the activation of caspase-3 [25]. Caspase activation is one of the basic events in the process of apoptosis. DQQ significantly induce caspase-3 and -8 levels (4 times) in MOLT-4 cells in a dose-dependant manner (Fig. 3B). The caspase activation was further confirmed by western blotting against procaspase-3 and procaspase-8 (Fig. 3A). DQQ significantly alter mitochondrial apoptotic proteins and caspase-8 level that interlinks both the apoptotic pathway and finally lead to caspase-3 activation and PARP-1 cleavage (Fig. 3A–C). The above data suggest that

DQQ induced apoptosis in MOLT-4 cells via both extrinsic and intrinsic pathways.

3.4. DQQ inhibited AKT/mTOR signaling pathway

The role of AKT/mTOR has long been contemplated in the regulation of autophagy and apoptosis. This pathway has been reported as a negative regulator of both apoptosis and autophagy [26]. Therefore, it was evident to see the effect of DQQ on the proteins of AKT/mTOR pathway. Western blot analysis of different proteins of this pathway revealed that DQQ significantly hampered the expression of pAKT, pmTOR and its substrate pP70S6K in MOLT-4 cells (Fig. 3A). The most significant inhibitory effect was on pmTOR followed by its substrate p70S6K (Fig. 3A). The mTOR kinase IC₅₀ value of DQQ was found to be 6 nM in a

cell free Elisa assay (Fig. 3D). DQQ was found to be a strong mTOR inhibitor and its expression almost negligible, even at low concentration (2 μ M).

3.5. DQQ induced autophagy in MOLT-4 cells

The autophagy induction in cells treated with DQQ was analyzed by acridine orange staining. The results of acridine orange staining revealed that it induced formation of acidic vacuolar organelles (AVO) in MOLT-4 cells, while the number of AVO was negligible in control cells. The number of AVO increased with increasing doses of DQQ (Fig. 4A). Furthermore, western blot analysis of key proteins of autophagy such as beclin1, ATG7, ATG5 and LC3-II revealed that DQQ significantly increased their expression in a dose dependent manner (Fig. 4A). The autophagy induction was further confirmed by LC3 immunofluorescence. The results indicated that DQQ treatment induced dose dependent increase in LC3 fluorescence in MOLT-4 cells (Fig. 4B).

3.6. DQQ induce caspase dependent apoptosis and autophagy

As DQQ induced activation of caspase in MOLT-4 cells and caspase have a significant role in the induction of both autophagy and apoptosis [11]. We found that addition of pan specific caspase inhibitor Z-VAD-fmk to DQQ treated MOLT-4 cells significantly reversed the inhibition of cell viability effect (Fig. 5A). The viability was reversed from 55% to 87% and from 41% to 60% in Z-V-FMK pretreated samples treated with 5 μ M and 10 μ M of DQQ, respectively (Fig. 5A). Furthermore, effect of Z-V-FMK pretreatment was observed in the expression of important proteins of autophagy and apoptosis. The expression of beclin1, ATG7, caspase 3 and PARP and was reversed in Z-V-FMK pretreated samples (Fig. 5B). All these data suggested that DQQ induce caspase arbitrated apoptosis and autophagy in MOLT-4 cells.

3.7. DQQ induce cytochrome c mediated autophagy and apoptosis

Earlier experiments suggested that DQQ induced translocation of cytochrome c and hence activation of apoptosis. Role of cytochrome c in apoptosis induction and autophagy inhibition was very well known [12]. Contradictory to existing reports, we were first time reporting the negative feedback regulation of cytochrome c mediated induction of autophagy. The cell viability data revealed a dramatic effect of cytochrome c silencing on reversal of cell death induced by DQQ. The viability was reversed from 60% to 98% in untreated and DQQ treated (5 μ M) MOLT-4 cells, transfected with cytochrome c siRNA, respectively (Fig. 6A). A similar kind of reversal was observed in cells transfected with cytochrome c siRNA and treated with 10 μ M of DQQ (Fig. 6A). Furthermore, the expression of autophagic protein LC3-II was reversed in the cytochrome c silenced cell, suggesting the undeviating proportional role of cytochrome c on autophagy induction (Fig. 6B). The effect of cytochrome c silencing on MMP loss was also assessed

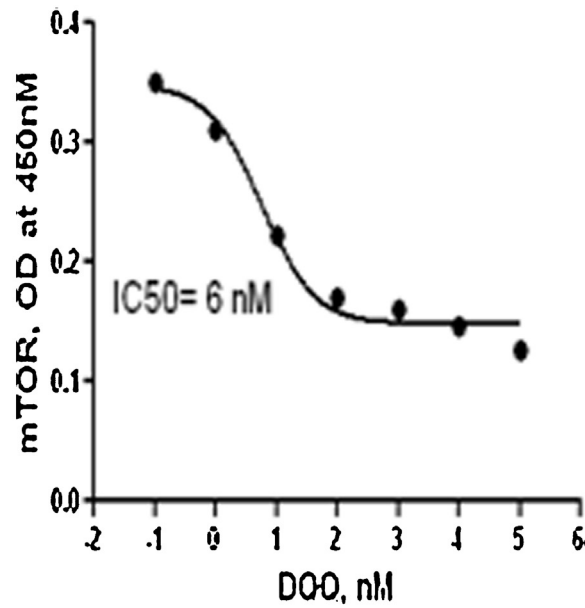
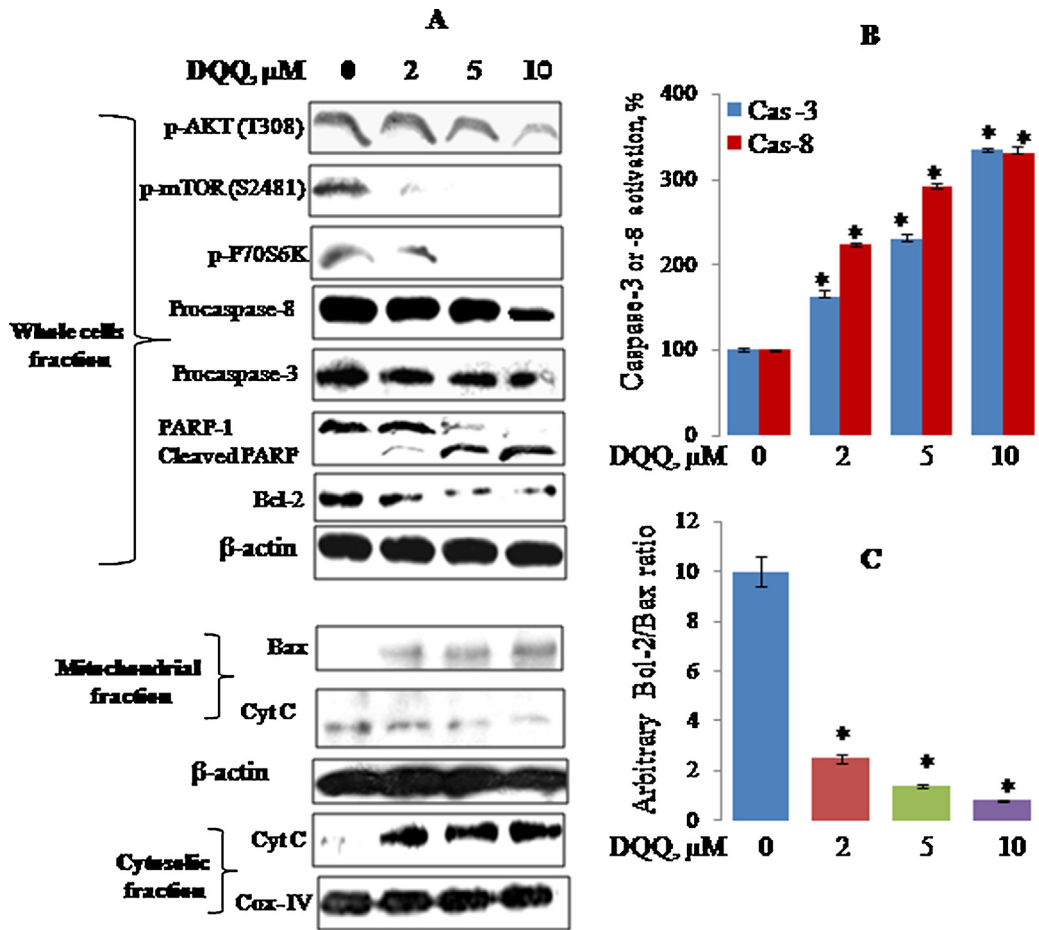
and results of the same revealed that cytochrome c silencing reversed the MMP loss induced by DQQ (Fig. 6C). The MMP loss was reversed from 58% to 14% and from 66% to 37% in cells treated with 5 μ M and 10 μ M of DQQ, respectively (Fig. 6C). The autophagy inhibition by cytochrome c silencing was also confirmed by acridine orange staining. The results of acridine orange staining showed that autophagy induced by DQQ in normal MOLT-4 cells was significantly reversed in MOLT-4 cells transfected with cytochrome c siRNA (Fig. 6D). Collectively, all these data suggested that cytochrome c is required for both DQQ induced apoptosis and autophagy in MOLT-4 cells.

3.8. Beclin1 silencing partially reversed the cell death induced by DQQ

The results of the previous experiments showed that apoptosis inhibition through Z-V-FMK and cytochrome c silencing also reversed the autophagy induced by DQQ. So, it was evident to check the effect of autophagy inhibition on cell viability and apoptosis. Beclin1 silencing through siRNA partially reversed the effect of DQQ on cell viability inhibition, which was not as much significant as by cytochrome c inhibition (Fig. 7A). The Cell viability inhibition was reversed from 60% to 82% and from 45% to 65% in beclin1 silenced cells treated with 5 μ M and 10 μ M of DQQ, respectively (Fig. 7A). Furthermore, the PARP-1 cleavage was also partially reversed in beclin1 silenced MOLT-4 cells treated with similar concentrations of DQQ (Fig. 7B). Acridine orange staining revealed that autophagy induced by DQQ was dramatically reversed in beclin 1 silenced sample (Fig. 7C). The results indicated the partial role of beclin1 on apoptosis and cell death induced by DQQ in MOLT-4 cells.

4. Discussion

Apoptosis and autophagy are referred to as programmed cell death type 1 and type 2, respectively. These are two important processes that control the turnover of organelles and proteins within the organism. Many stressors and chemical agents have been found to sequentially elicit autophagy and apoptosis within the same cell [27]. Autophagy and apoptosis have been shown to have a complex relation with each other, as under certain circumstances autophagy protects the cell from death by adapting certain mechanism and thus inhibit apoptotic cell death. However, in certain cases it can lead the cell to death and constitute the alternate death pathway [27]. In some case autophagy may lead to apoptosis and both acts together to induce programmed cell death [28]. In this study, we have tried to study the crosstalk between autophagy and apoptosis. We for the first time report the cytochrome c mediated induction of autophagy in MOLT-4 cells. Our preliminary experiments showed that a novel quinazolinone derivative 2,3-dihydro-2-(quinoline-5-yl) quinazolin-4(1H)-one [DQQ], substantially induced cell death in MOLT-4 cells. Furthermore, the mechanistic studies discovered that the cell death induced by DQQ in MOLT-4 cells was autophagic as well as apoptotic in nature. The apoptosis and autophagy induction was confirmed by an array of experiments like



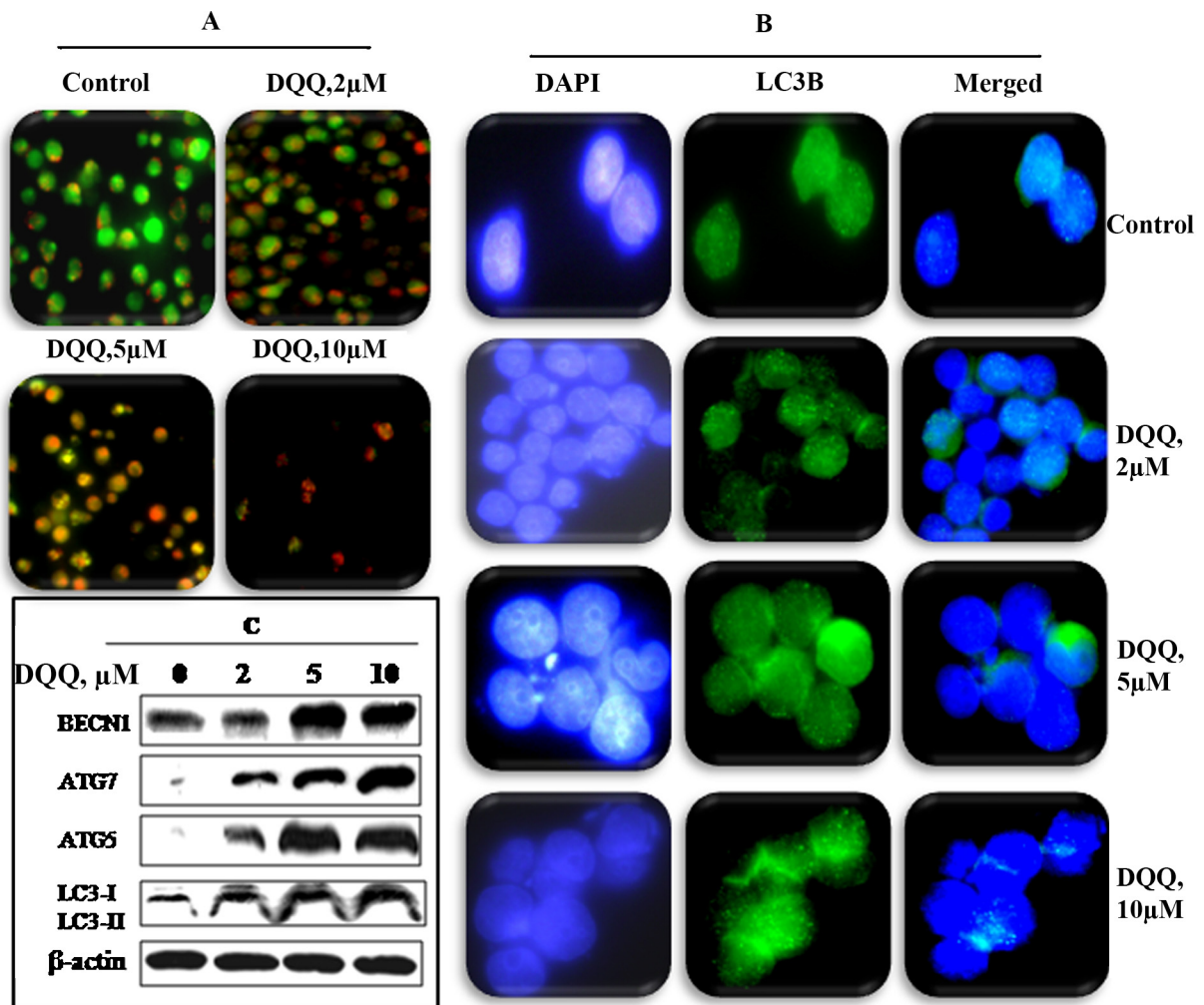


Fig. 4. DQQ induced autophagy in MOLT-4 cells. (A) Autophagy detection through acridine orange staining. Cells were seeded in a six well plate and treated with the indicated doses of DQQ for 24 h. Acridine orange (AO) was added 15 min before the termination of the experiment. After completion of treatment period cells were collected washed once and observed under fluorescence microscope. (B) LC3 immunofluorescence staining for autophagy detection. Cells were seeded in a 12 well plate and treated with different concentrations of DQQ for 24 h. Cells were processed for immunofluorescence against LC3 as described in Section 2. DAPI was used as nuclear staining dye and both the images were merged on same magnification. (C) Effect of DQQ on the key autophagic proteins. MOLT-4 cells were treated with DQQ at the indicated concentrations for 24 h time period, lysates were prepared using RIPA buffer, equal amount of proteins (50–70 µg) were separated by SDS-PAGE and western blot as described in Section 2. β-Actin was used as an internal control and data were representative of one of three similar experiments.

cellular and nuclear microscopy, Annexin-V binding, loss of MMP, cell cycle analysis, immunofluorescence and immunoexpression of key apoptotic and autophagic proteins. DNA damage is considered as the sign of apoptosis [29], DQQ potentially induced DNA damage, which was confirmed through Hoechst staining. The DNA damage was

further confirmed by cell cycle analysis using PI staining and DQQ potentially induced G0/G1 phase of cell cycle, which was directly correlated to apoptosis [30]. Furthermore, DQQ mediated apoptosis induction was confirmed by annexin V/PI staining and the results of the same suggested dose dependent increase in apoptosis.

Fig. 3. Influence of DQQ on the expression of important proteins involved in the initiation of apoptosis and AKT/mTOR signaling. (A) MOLT-4 cells were treated with the indicated doses of DQQ for 24 h. A protein lysates were prepared and electrophoresis as described in Section 2. COX IV and β-actin were used as internal controls to represent the same amount of proteins applied for SDS-PAGE. Specific antibodies were used for detection of the indicated proteins in respective cell fractions. Data are representative of one of three similar experiments. (B) DQQ induces caspase-8 and -3 levels in MOLT-4 cells. The caspases activities were determined by fluorimetric method in the cell lysates by using BD ApoAlert caspase fluorescent assay kits. Data are mean ± S.D. from three similar experiments and *p* values: * <0.01 was considered to be significant when compared with untreated control. (C) Influence of DQQ on the Bcl-2 and bax of MOLT-4 cells. The relative density of each band was measured using image j software. Data are mean ± SD of three similar experiments; statistical analysis was done by using bonferroni method and *p* value <0.01 was considered to be significant. (D) DQQ inhibits mTOR kinase. mTOR inhibition by DQQ was found out as per the manufacturer's protocol provided in K-LISA™ mTOR kit from Calbiochem (no. CBA055). The IC50 values were calculated by analysis non linear regression with variable slope by using GraphPad Prism-5 software.

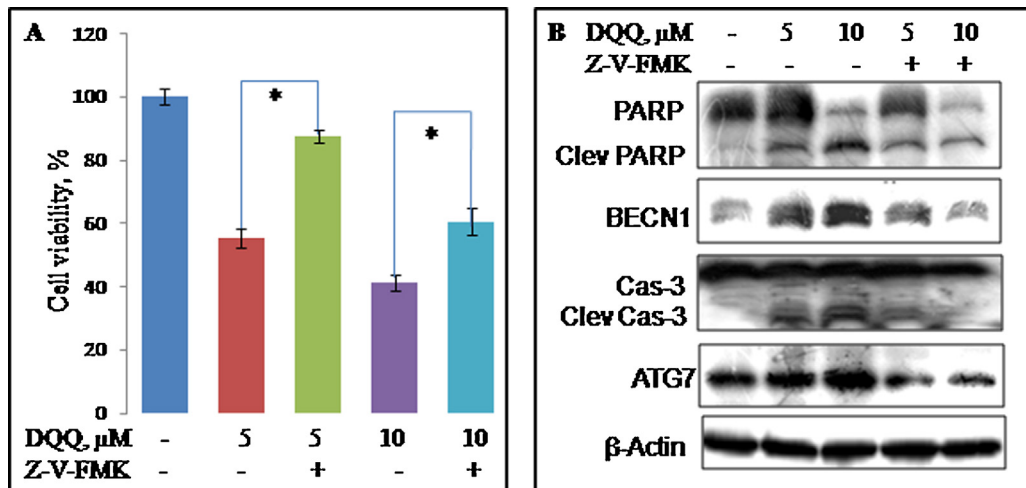


Fig. 5. Effect of caspase inhibitors on DQQ induces apoptosis and autophagy. (A) MOLT-4 cells were seeded in 96 well plates and pretreated with pan specific caspase inhibitor, Z-VAD-FMK (30 μM) 30 min before the addition of DQQ. MTT dye was added 3 h before the termination of the experiment. Cell viability was calculated as described in Section 2. Data are mean \pm S.D. from three similar experiments and p values: <0.001 was considered to be significant when compared with DQQ. (B) DQQ induces caspase dependent apoptosis and autophagy. MOLT-4 cells were treated indicated concentrations of DQQ for the 24 h time period in the presence and absence of caspases inhibitors (30 μM) Z-VAD-FMK. Cells were collected and lysed in RIPA buffer and equal amount of protein samples were loaded on SDS PAGE for western blot analysis as described in Section 2. β -Actin was used as an internal control and equal amount of proteins applied for SDS-PAGE. Specific antibodies were used for detection of the indicated proteins. Data are representative of one of three similar experiments.

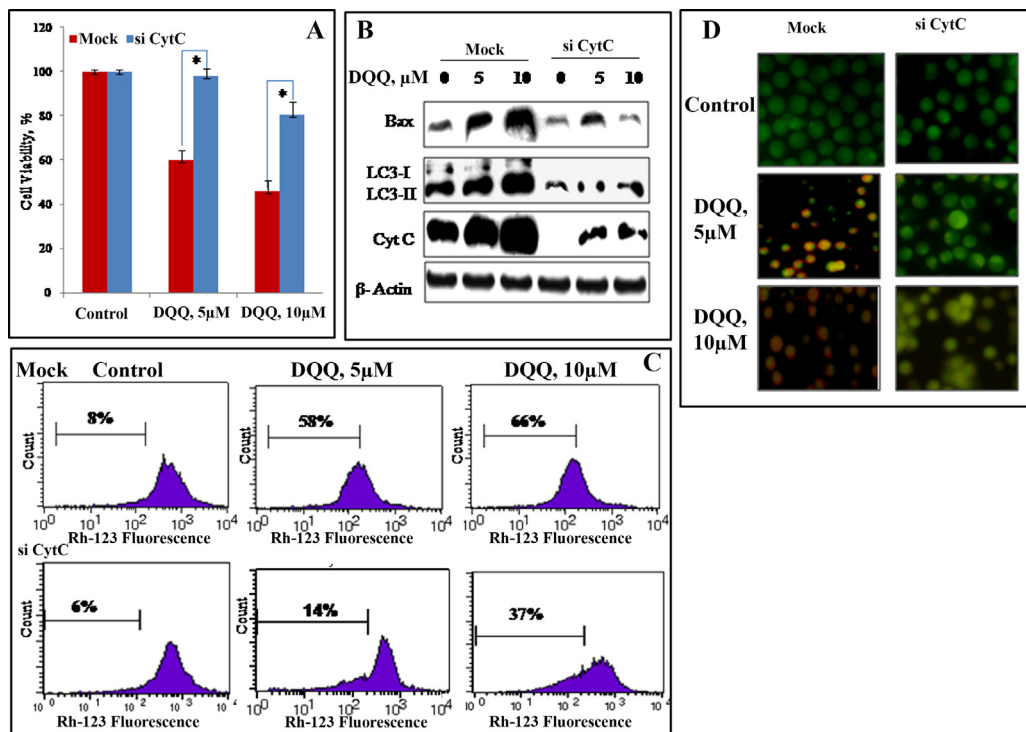


Fig. 6. siRNA mediated silencing of cytochrome c reversed the apoptosis as well as autophagy induced by DQQ in MOLT-4 cells. (A) Cells were transfected with cytochrome c siRNA as described in Section 2. Cells were seeded in 96 well plates and treated with different concentrations of DQQ for 48 h. Cells were incubated with MTT solution and optical density of formazan crystals was measured as described in Section 2. Data are mean \pm S.D. from three similar experiments and p values: <0.001 was considered to be significant when compared with DQQ. (B) Effect of cytochrome c silencing on DQQ induce apoptosis and autophagy. Transfected and non transfected cells were seeded in six well plates and incubated with the indicated doses of DQQ for 24 h. Cells were lysed in RIPA buffer and immunoblotting of the indicated proteins were done as described in Section 2. Data are representative of one of three similar experiments. (C) Effect of cytochrome c silencing on DQQ induce loss of mitochondrial membrane potential. Normal and cytochrome c siRNA transfected MOLT-4 cells were treated with different doses of DQQ for 24 h. Cells were stained with Rhodamine-123 (200 nM), added 40 min before experiment termination and analyzed in FL-1 channel of flow cytometer. Data are representative of one of three similar experiments. (D) Cytochrome c siRNA reversed the autophagy induced by DQQ. Cells were transfected with cytochrome c siRNA as described in Section 2. Transfected and non transfected cells were treated with indicated doses of DQQ. Acridine orange was added 15 min prior to termination of the experiment and fluorescence was observed as described in Section 2.

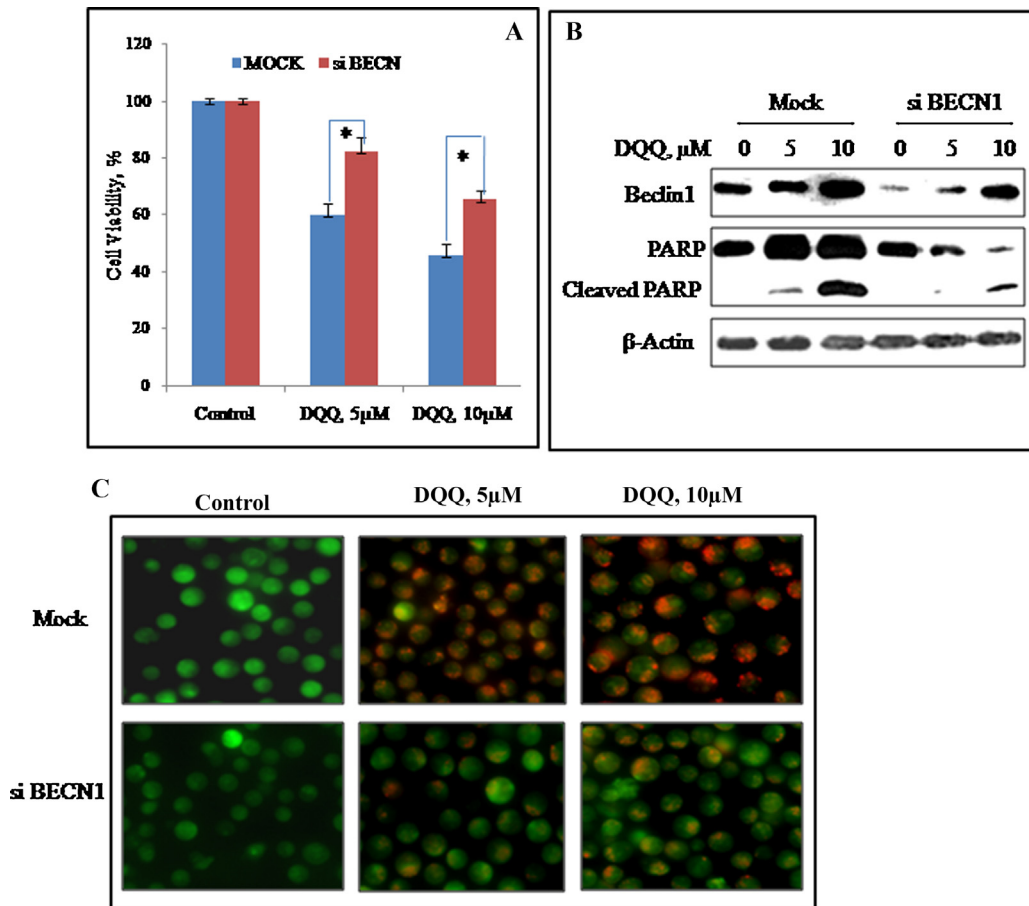


Fig. 7. *Beclin1* silencing partially reversed autophagy and apoptosis induced by DQQ. MOLT-4 cells were transfected with beclin1 siRNA as described in Section 2. Cells were seeded in 96 well plates and treated with different concentrations of DQQ for 48 h and cell viability was measured as described in Section 2. Data are mean \pm S.D. from three similar experiments and *p* values: * <0.001 was considered to be significant when compared with DQQ. (B) *Beclin1* siRNA transfected and non-transfected MOLT-4 cells were treated with 5 μ M and 10 μ M concentration of DQQ for 24 h. Cells were lysed in RIPA buffer western blot of the indicated proteins were done as described in Section 2. Data are representative of one of three similar experiments. (C) *Beclin1* knockdown reversed the autophagy as observed by acridine orange staining. siRNA transfected and non transfected cells were treated with indicated doses of DQQ. Acridine orange was added 15 min prior to termination of the experiment. Acridine orange fluorescence was measured under fluorescence microscope as described in Section 2.

Apoptosis can be triggered by various stimuli by extrinsic or intrinsic pathways. Extrinsic pathway involved the signal transduction from death receptors and caspase-8 while the intrinsic apoptotic pathway involves mitochondrial apoptotic proteins (Bcl-2, Cyt c, Bax), which are activated downstream of mitochondrial pro-apoptotic events [18]. The early event which was responsible for DQQ induces apoptosis, found to be loss of mitochondrial potential (Fig. 2E). DQQ significantly triggers mitochondrial potential loss in MOLT-4 cells that indicating the involvement of mitochondrial PTP in the release of pro apoptotic proteins. We further investigated the role of members of the Bcl-2 family of proteins from mitochondria. Bcl-2 family proteins play both pro-apoptotic and anti-apoptotic role in cancer cells. The normally Bcl-2 level is much higher in the cancer cell. We observed a drastic reduction in Bcl-2 expression after the treatment of MOLT-4 cells to DQQ that might be linked with the loss of mitochondrial membrane potential. Bcl-2 inhibition by DQQ alter the

symmetry of mitochondria, which causes the opening of the mitochondrial transformable pore and brings about Bax translocation from cytosol to mitochondria and consequent release of small molecules like cytochrome c from mitochondria to cytosol. DQQ drastically decreased the Bcl-2/Bax ratio in MOLT-4 cells in a concentration dependent manner in 24 h time period (Fig. 3C). Translocation of these proteins impairs mitochondrial functions and brings the cells to a “point of no return” to enter apoptosis via caspases activation. Caspase activation is solely regulated by the mitochondrial release of apoptotic protease activating factor-1 (Apaf-1) and cytochrome c, which is a part of the mitochondrial dependant apoptosis pathway [31]. Translocation of cytochrome c from mitochondria to cytosol is considered as one of the main events of mitochondrial dysfunction and subsequent apoptosis; it is also associated with immediate exposure of phosphatidylserine exposure. In light of earlier experiments that demonstrated induction of MMP loss and exposure of phosphatidylserine by

DQQ, we decided to check the cytochrome *c* translocation and found that it significantly induced cytochrome *c* release from mitochondria that activate caspase-3 (Fig. 3A and B). The elevated level of caspase-3 could utilize poly-ADP Ribose polymerase (PARP, 116 kDa), a DNA repair enzyme as its substrate. As a result a cleaved product (85 kDa) of PARP was observed in our study. Subsequently, DQQ treatments also induce caspase-8, which was part of the extrinsic apoptosis pathway (Fig. 3A and B). So these findings suggest that DQQ caused induction of apoptosis through both intrinsic and extrinsic apoptotic pathways in human leukemia MOLT-4 cells.

We have also found DQQ as a potent inducer of autophagy in MOLT-4 cells. The autophagy induction was confirmed by acridine orange staining, LC3 immunofluorescence and western blot analysis. PI3K/AKT signaling pathway is one of the important targets in cancer therapy and has been found to be negatively associated with both autophagy and apoptosis [26]. DQQ significantly inhibited the expression of major proteins of this pathway suggesting the role of PI3K/AKT pathway in autophagy and apoptosis induced by DQQ in MOLT-4 cells.

Caspases have been found to have a regulatory role on both apoptosis and autophagy [11] and DQQ was inducing caspases activation; this pretext led us to check the role of caspases in autophagy and apoptosis regulation. Interestingly, we have found that apoptotic and autophagic cell death induced by DQQ was caspase-dependent. A universal caspase inhibitor, Z-VAD-FMK, revert back the entire key event associated with DQQ mediated MOLT-4 cell death. Caspase inhibitor reversed cell growth inhibition and key protein expression of PARP-1, caspase-3, beclin1 and ATG7, which were induced by DQQ (Fig. 5A and B). These findings put forward the key role of caspases in the induction of apoptosis and autophagy. Therefore, we can say that DQQ induce caspase dependant autophagy and intrinsic and extrinsic apoptosis in human leukemic MOLT-4 cells.

Furthermore, cytochrome *c* inhibition through siRNA, very significantly blocked the activity of DQQ in terms of viability, apoptosis and autophagy (Fig. 6A–C). However, we did not get such type of significant reversal effect by silencing the MOLT-4 cells through beclin1 siRNA (Fig. 7A and B). The MOLT-4 cell viability reversal effect of DQQ via cytochrome *c* siRNA was much higher than the caspase inhibitor and beclin1 siRNA. Interestingly, our study first time portrays the negative feedback control role of cytochrome *c* in the activation of autophagy. Thousands of publications revealed the role of cytochrome *c* in apoptosis induction, but none has described its role in autophagy induction, although there are evidences suggesting the inhibitory role of cytochrome *c* on autophagy [12]. Furthermore, the crosstalk between autophagy and apoptosis was confirmed by silencing of beclin1 through siRNA. The results of the experiments revealed that beclin1 inhibition partially reversed the viability and the PARP-1 cleavage inhibition induced by DQQ; indicating the partial role of beclin1 in apoptosis. The experiment also confirmed the notion that autophagy and apoptosis induced by DQQ in MOLT-4 cells were interdependent. Much of the work has been done in the field of apoptosis and autophagy;

however the relation between the two is still controversial and unexplored to some extent.

In conclusion, the present study briefly describes the crosstalk between autophagy and apoptosis induced by a novel quinazolinone derivative, DQQ, in human leukemia MOLT-4 cells. It induces extrinsic and intrinsic apoptosis, confirmed by apoptotic bodies' formation, PS exposure, enhance sub-G0 population and induction of various apoptotic proteins like Bcl-2/Bax, PARP and caspase. We for the first time elucidated the negative feedback role of cytochrome *c* in autophagy induction. Hence, our discovery of this novel mechanism not only further insight the interdependent role of apoptosis and autophagy, but also disclose the clinical significance of agent like DQQ, that simultaneously induce apoptosis and autophagy.

Conflict of interest statement

All authors declare that there are no conflicts of interest in this study.

Transparency document

The [Transparency document](#) associated with this article can be found in the online version.

Acknowledgments

ASP, SKG and AK thanks Council of Scientific and Industrial Research (CSIR), New Delhi, India for their research fellowships. SK is a senior research fellows receiving financial support from UGC, New Delhi, India. We appreciate the technical assistance provided by Dr. Praduman R Sharma and Mr. Ashok Kumar during microscopy studies. This research was supported in part by a grant from the CSIR 12th five year project (BSC-0205).

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