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เควอซิทินเสริมฤทธิ์เฟนฟอร์มินในการยับยั้งการเจริญเติบโตของเซลล์มะเร็งท่อน้ำดี

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Quercetin Enhances Phenformin in Inhibition of Cholangiocarcinoma Cell Growth

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หลักการและวัตถุประสงค์: Phenformin และสารฟลาโวนอยด์ quercetin และ myricetin มีฤทธิ์ในการยับยั้งการเจริญเติบโตของเซลล์มะเร็งหลายชนิด งานวิจัยนี้ศึกษาความสามารถในการยับยั้งการเจริญเติบโตของเซลล์มะเร็งท่อน้ำดี เมื่อเซลล์ได้รับ phenformin ร่วมกับ quercetin และ myricetin

วิธีการศึกษา: งานวิจัยใช้เซลล์มะเร็งท่อน้ำดี KKU-M156 ศึกษาความเป็นพิษต่อเซลล์ด้วยเทคนิค sulforhodamine-B assay (SRB) และวิเคราะห์ความต่างศักย์ของเมมเบรนไมโทคอนเดรีย (Mitochondrial membrane potential; $\Delta\Psi m$) ด้วยเทคนิค JC-1 assay

ผลการศึกษา: Phenformin สามารถยับยั้งการเจริญเติบโตของเซลล์ KKU-M156 และเมื่อให้ร่วมกับ quercetin แต่ไม่ใช่ myricetin สามารถเสริมฤทธิ์ phenformin ในการยับยั้งการเจริญเติบโตของเซลล์มะเร็ง Phenformin และ quercetin เมื่อให้เดี่ยว ๆหรือให้ร่วมกันสามารถทำให้สูญเสียศักย์ไฟฟ้าระหว่างเมมเบรนของไมโทคอนเดรีย

สรุป: Quercetin สามารถเพิ่มฤทธิ์ phenformin ในการยับยั้งการเจริญของเซลล์มะเร็งท่อน้ำดี โดยกลไกที่เสริมเพิ่มการบาดเจ็บต่อไมโทคอนเดรียนำไปสู่การตายของเซลล์ ผลการศึกษานี้แสดงในเบื้องต้นว่า phenformin ร่วมกับ quercetin มีศักยภาพที่จะเพิ่มสรรถนะการรักษามะเร็งท่อน้ำดี

Background and Objective: Phenformin, and flavonoid compounds, quercetin and myricetin are effective against many types of cancers. This study evaluated the treatments of phenformin in combination with quercetin or myricetin in the inhibition of cholangiocarcinoma (CCA) cells.

Methods: Cholangiocarcinoma cells, KKU-156, were used in this study. Cell cytotoxicity was evaluated by sulforhodamine-B assay (SRB). Mitochondrial transmembrane potential ($\Delta\Psi m$) was analyzed by JC-1 assay.

Results: Phenformin significantly inhibited proliferation of cancer cells. Phenformin in combination with quercetin, but not myricetin, could increase the cytotoxicity of cancer cells. Phenformin, quercetin or in the combination induced the loss of mitochondrial transmembrane potential.

Conclusions: Quercetin increased phenformin in inhibition of cancer cell growth. The mechanism may be by the increase of induction of mitochondrial damage leading to cell death. This finding suggests that phenformin in combination with quercetin has potential to increase efficacy of chemotherapy of cholangiocarcinoma.

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Keywords: Phenformin, quercetin, myricetin, cholangiocarcinoma, mitochondrial transmembrane potential

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Introduction

Cholangiocarcinoma (CCA) is an aggressive malignancy, arising from biliary epithelium of the bile duct. The prognosis of CCA is very poor due to very difficult in diagnosis and resistance to chemotherapy.[1] The most effective treatment for CCA requires a complete surgical resection of the tumor. However, 5-year survival rate is still low and recurrence of cancer is still common.[2] Moreover, combination of gemcitabine and cisplatin, the current standard chemotherapy in CCA, could offer only a small survival benefit.[3] New therapeutic strategies are required to improve survival and quality of life in CCA patients.

The use of metformin in diabetes patients has been reportedly associated with reduced risk of CCA in a case-control study.[4] Moreover, metformin and its congener, phenformin, have demonstrated antineoplastic *in vitro* and *in vivo*.[5,6] Phenformin is a more potent antidiabetic agent and more lipophilic than metformin and does not require transporter to get enter into the cells.[7] The molecular targets of phenformin is similar to metformin in that they stimulate AMP-activated protein kinase (AMPK) and suppress mTOR signaling pathway which lead to inhibit growth through mitochondrial pathway.[8,9]

Quercetin and myricetin are flavonol compounds from the flavonoid group. Both compounds are commonly found in apples, grapes, broccoli and nuts. Quercetin and myricetin have been demonstrated antitumor activity in various cancers including CCA, breast and bladder cancers.[10,11] Two compounds show several effects on cancer cells including the inhibition of glucose transport and glucose utilization.[12] This effect may be complementary with effects of phenformin, since phenformin suppresses anabolic processes by decreased glucose utilization via activation of AMPK for the antitumor effect.[13] Present study was to investigate whether these flavonols could enhance antitumor activity of phenformin.

Material and Methods

Chemicals and reagents

Phenformin hydrochloride, quercetin, and myricetin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ham's F12 nutrient mixture and 0.25% trypsin-EDTA were obtained from Gibco® Life Technologies (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from HiMedia Laboratories (Mumbai, India). Sulforhodamine B (SRB) obtained from Sigma-Aldrich (St. Louis, MO, USA). JC-1 mitochondrial transmembrane potential assay kit was obtained from Cayman Chemical (MI, USA). Cyclosporine was purchased from Novartis Pharmaceutical (Basel, Switzerland).

Cell culture

Human CCA cell line, KKU-M156, were cultured in Ham's F12 medium supplemented with sodium bicarbonate, 10 mM N-2-hydroxyethyl-piperazine-N'-2-ethane-sulfonic acid (HEPES; pH 7.3), gentamicin sulfate (100 µg/L), penicillin G (100 U/L), and 10% (v/v) FBS. Cells were maintained under an atmosphere of 5% CO_2 in air at 37°C. The cells were sub-cultured every 2 days using 0.25% trypsin-EDTA.

Cell viability assay

Sulforhodamine B (SRB) was used to determine cell viability. KKU-M156 cells were seeded onto 96-well plate with a density of $7.5x10^3$ cells/well and allowed overnight for adherence. CCA cells were treated with various concentrations of phenformin, 20 µM quercetin, 25 µM myricetin, or the combinations for 24 h. The media was removed and the cultured cells were fixed with cold 10% trichloroacetic acid for 1 h at 4°C and stained with 0.4% SRB in 1% acetic acid 30 minute at room temperature. The excess dye was removed with 1% acetic acid and protein-bound dye was solubilized with 10 mM Tris for determination of absorbance using a microplate reader at a wavelength of 570 nm.

Mitochondrial membrane potential assay

Determination of the mitochondrial transmembrane potential ($\Delta\Psi$m) was performed using 5,5',6,'-tetrachloro-1,1',3,3'tetraethylbenzimidazolyl carbocyanine iodide (JC-1) assay, as previously described.[14] KKU-M156 cells were seeded onto 96 black-well plate with density of $2x10^5$ cells/well and incubated overnight for adherence. The media was then replaced with fresh media containing phenformin, quercetin 20 μM, and cyclosporine 50 nM and incubated for 6 h. The culture media was replaced with 5 μM JC-1 staining solution in serum free media and incubated at 37°C for 45 min. The cells were washed with JC-1 buffer and measured fluorescent signals using a fluorescent microplate reader. The excitation and emission wavelengths were set at 485/535 nm (cut-off 515 nm) for measurement of J-monomers and at 535/595 nm (cut-off 590) for J-aggregates.

Statistical analysis

All values were presented as mean ± SEM from three experiments using GraphPad Prism version 6.0 software (GraphPad Software, San Diego, CA, USA). Statistical differences between treatment and control groups were analyzed by one-way analysis of variance (ANOVA) with post-hoc test by SigmaStat version 3.11 software (Systat Software, San Jose, CA, USA). Statistical significance was preset at $p<0.05$.

Results

Antitumor activity of phenformin and flavonol compounds

The structures of phenformin, quercetin and myricetin are shown in Fig.1A. Phenformin was tested for cytotoxicity in KKU-156 cells. It was shown that phenformin induced cytotoxicity in concentration-response relation with median inhibitory concentration (IC50) of 1363±427 μM (Fig.2A). When phenformin was used in combination with quercetin or myricetin at varied concentrations, the cytotoxicity was significantly increased in co-treatment with quercetin, when compared with phenformin alone. But the combination of phenformin and myricetin did not increase cytotoxicity (Fig. 2B and C). Quercetin alone at the same concentration used in the combinations induced cytotoxicity by 27.5±3.6%, while myricetin showed cytotoxic effect by 14.8±2.4%. It is apparent that quercetin has an additive cytotoxic effect to phenformin.

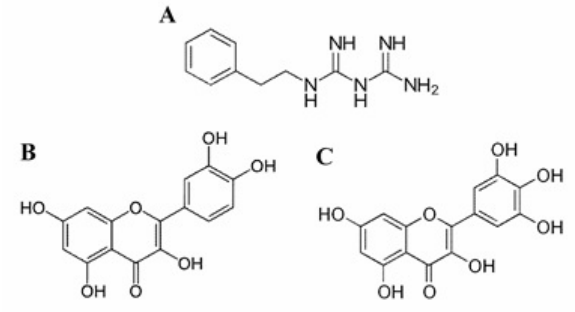

Figure 1 The structure of (A) phenformin, (B) quercetin, and (C) myricetin

Phenformin and quercetin induced mitochondrial dysfunction

As mitochondria are the powerhouse of energy production by oxidative phosphorylation in mitochondrial matrix and play central role in cell death, disturbance in mitochondrial function may lead to cell dysfunction and cell death.[15] Phenformin induced the loss of mitochondrial transmembrane potential ($\Delta\Psi$m), as determined by JC-1 assay. In healthy mitochondria, JC-1 is taken up into the mitochondria and forming J-aggregates which emit the red-orange fluorescent signal. The damage mitochondria cannot accumulate JC-1, then JC-1 exists as J-monomer form in cytosol and emits the green fluorescent signal. The ratio of red and green fluorescent signals is an indicative of the status of $\Delta\Psi$m. It was apparent that phenformin induced decline of the ratio of J-aggregates/J-monomers in concentration response manner (Fig.3A and B). The loss of $\Delta\Psi$m, was even exaggerated in the presence of quercetin. It is noted that quercetin itself also induced the loss of $\Delta\Psi$m,.

To examine whether the change in $\Delta\Psi$m, was due to the opening of the mitochondrial permeability transition pore (MPTP), which plays critical role in maintenance of mitochondrial transmembrane potential. Cyclosporine A, a MPTP inhibitor, was used in the study. Cyclosporine could partial prevent the loss of $\Delta\Psi$m, induced by phenformin or quercetin or the combinations of phenformin and quercetin (Fig. 4 and Fig.5A, B). This indicates that the depolarization of mitochondria is due to the opening of MPTP.

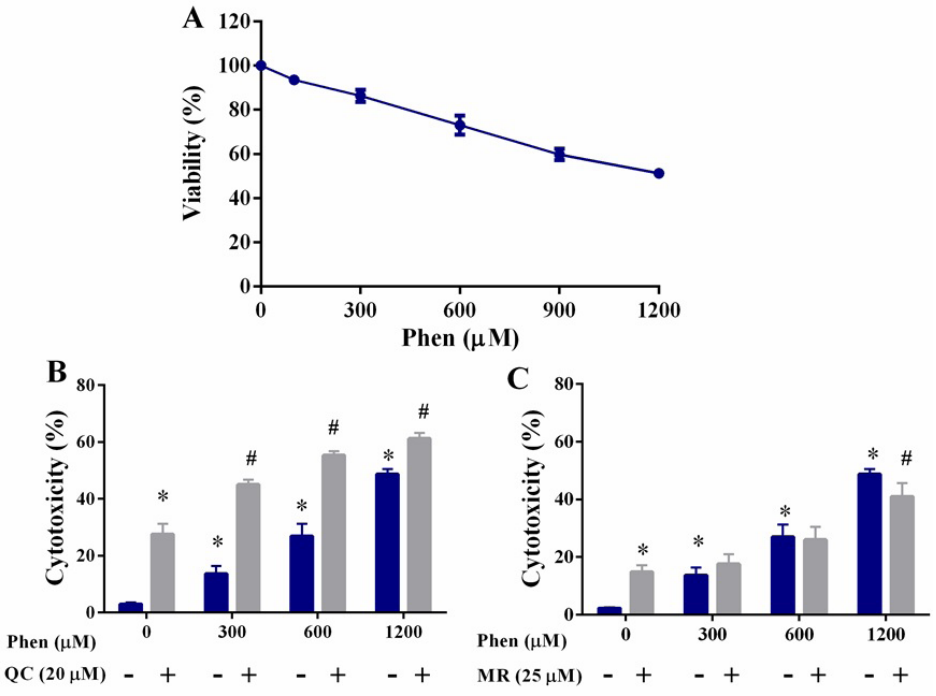

Figure 2 Antiproliferative effect of phenformin in CCA cells. KKU-M156 cells were treated with various concentrations of (A) phenformin (Phen), (B) Phen in combinations with quercetin (QC) or with myricetin (MR) and incubated for 24 h. After treatment, the cell viability was determined using the SRB assay. Each bar represents the mean ± SEM of three independent experiments. *p < 0.05 versus untreated control group, #p < 0.05 Phen alone versus the corresponding combination of Phen and QC.

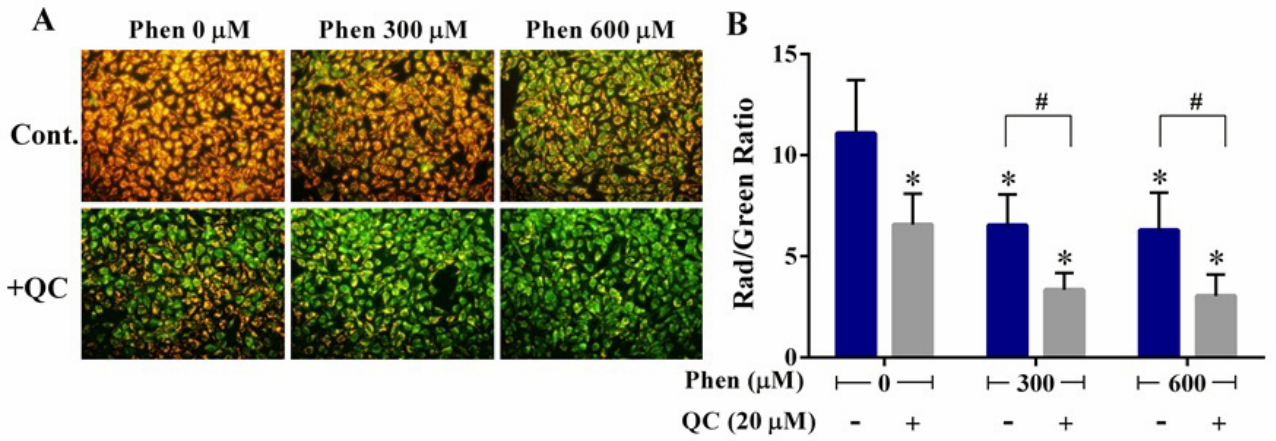

Figure 3 Mitochondrial transmembrane potential ($\Delta\Psi$m) in CCA cells treated with phenformin and quercetin. KKU-M156 cells were treated with phenformin (Phen) and combination with quercetin (QC) for 6 h. After treatment, $\Delta\Psi$m was determined by JC-1 assay. (A) Representatives of images of KKU-M156 treated with Phen and QC. (B) Bar graph of fluorescent signal of red/green ratio. Each bar represent the mean ± SEM from three independent experiments. *p < 0.05 versus untreated control group, #p < 0.05 Phen alone versus the combination of Phen and QC.

Discussion

Phenformin inhibits several cancer growth including some resistant cancer cells.[13,16] The mechanism of action is suggested to be mediated via AMPK-mTOR in the suppression of anabolic process and cause oxidative stress through mitochondrial pathway.[13,16] Quercetin and myricetin both potently inhibit glucose transport in rat adipocytes.[12] It was then suggested that interference of glucose uptake by these flavonols could enhance phenformin in suppression of tumor cell growth, since tumor cells generally requires high glucose for their proliferation and energy metabolism.[17] However, only quercetin shows enhanced cytotoxicity of phenformin in CCA cells. Differential effect between quercetin and myricetin may be that quercetin, but not myricetin, could affect glucose uptake in HepG2 cells.[18] It is

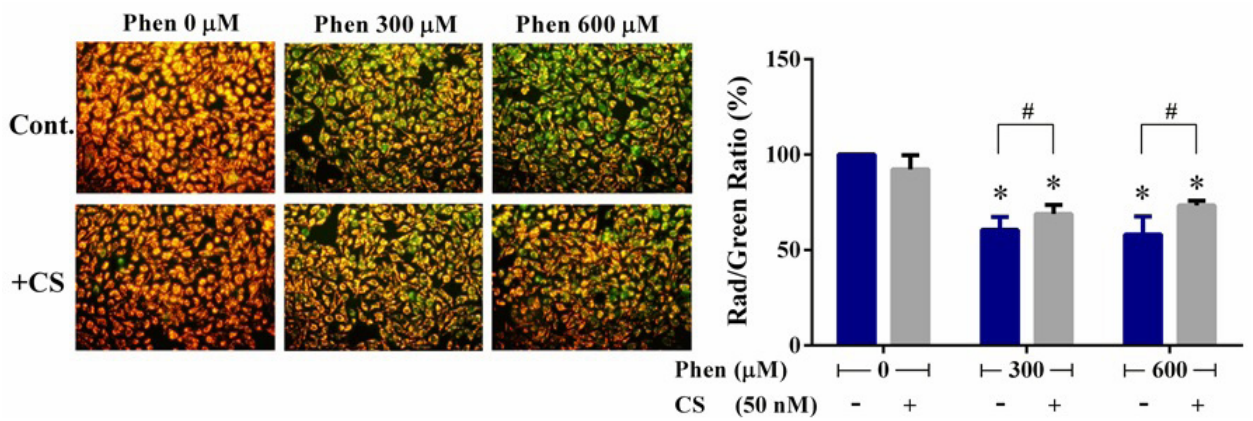

Figure 4 Mitochondrial transmembrane potential ($\Delta\Psi$m) in cells treated with phenformin and cyclosporine. KKU-M156 cells were treated with phenformin (Phen) and cyclosporine (CS) for 6 h. After treatment. $\Delta\Psi$m was determined using the JC-1 assay. (A) Representatives of images of KKU-M156 after treatment. (B) Bar graph of fluorescent signal of red/green ratio. Each bar represents the mean ± SEM from three independent experiments. *p < 0.05 versus untreated control group, #p < 0.05 Phen alone versus the combination of Phen and QS.

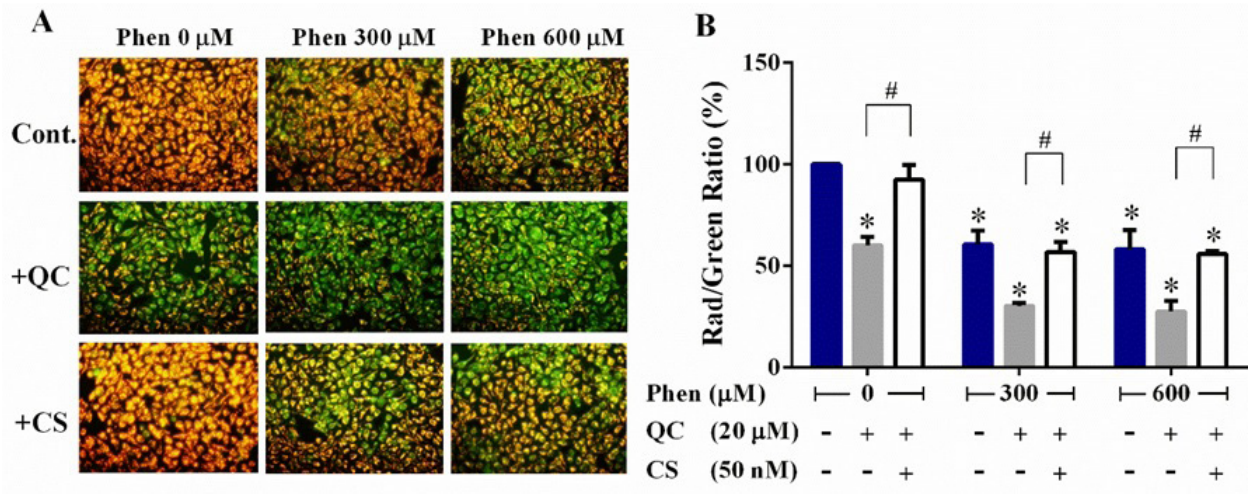

Figure 5 Mitochondrial transmembrane potential ($\Delta\Psi$m) in cells treated with phenformin, quercetin and cyclosporine. KKU-M156 cells were treated with phenformin (Phen), quercetin (QC), and cyclosporine (CS) for 6 h. After treatment, $\Delta\Psi$m was measured by the JC-1 assay. (A) Representatives image of KKU-M156 after treatment. (B) Bar graph of fluorescent signal of red/green ratio. Each bar represents the mean ± SEM from three independent experiments. *p < 0.05 versus untreated control group, #p < 0.05 combination of Phen and QC versus the combination of Phen, QC and QS.

probable that effects on glucose transport is cell type-dependent and needs further investigation. Moreover quercetin is among flavonoid derivatives which show to be a potent AMPK activators and could suppress mTOR pathway.[10]

The present study showed that only quercetin significantly enhances the cytotoxicity of phenformin. The inhibition of glucose transport may be one among many other effects which mediate antiproliferation of cancer cells. Phenformin causes the loss of $\Delta\Psi$m, which is due to the opening MPTP and that leads to the outer mitochondrial membrane permeabilization and consequence to cell death.[15] The effect via mitochondrial is validated by using cyclosporine, a MPTP inhibitor.[19] Cyclosporine can partially inhibit the loss of $\Delta\Psi$m. The effect of phenformin on mitochondrial function is shown to be similar to metformin which leads to oxidative stress and cell death.[20] Moreover, quercetin alone induces the loss of $\Delta\Psi$m, which the effect is validated by cyclosporine. The enhanced antitumor effect of quercetin may be by enhanced dysfunction of mitochondria with consequence of increased cell death.

It is concluded that quercetin increases phenformin-induced cell death by intensification of mitochondrial damage which leads to cell death. Quercetin in combination with phenformin increase efficacy of antitumor activity and could be a possible

effective strategy in cancer chemoprevention.

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