Quercetin and Cisplatin combined treatment altered cell cycle and mitogen activated protein kinase expressions in malignant mesothelioma cells

Asuman Demiroglu-Zergeroglu1*, Emel Ergene2, Nurettin Ayvali1, Victor Kuete3* and Hulya Sivas2

Abstract

Background: Malignant mesothelioma is a locally aggressive and highly lethal neoplasm of pleural, peritoneal and pericardial mesothelial cells without successful therapy. Previously, we reported that Quercetin in combination with Cisplatin inhibits cell proliferation and activates caspase-9 and -3 enzymes in different malignant mesothelioma cell lines. Moreover, Quercetin + Cisplatin lead to accumulation of both SPC111 and SPC212 cell lines in S phase.

Methods: In present work, 84 genes involved in cell growth and proliferation have analysed by using RT2-PCR array system and protein profile of mitogen activated protein kinase (MAPK) family proteins investigated by western blots.

Results: Our results showed that Quercetin and Quercetin + Cisplatin modulated gene expression of cyclins, cyclin dependent kinases and cyclin dependent kinases inhibitors. In addition genes involved in JNK, p38 and MAPK/ERK pathways were up regulated. Moreover, while p38 and JNK phosphorylations were increased, ERK phosphorylations were decreased after using Quercetin + Cisplatin.

Conclusion: This research has clarified our previous results and detailed mechanism of anti-carcinogenic potential of Quercetin alone and in combination with Cisplatin on malignant mesothelioma cells.

Keywords: Cell cycle, Cisplatin, JNK, MAPK, Mesothelioma, Quercetin, p38

Background

Malignant mesothelioma (MM) is a neoplastic proliferation that develops from pleural, peritoneal or rarely pericardial mesothelial cells [1]. Long time exposure to asbestos and/or erionite and genetic predisposition are known to cause MM [2]. Platinum drug ‘Cisplatin’ (CIS) is conventionally employed against MM [1]. Indeed, the combination therapies with CIS & Pemetrexed or Raltitrexed are currently used to treat MM patients [3]. However, it is required to improve alternative strategies or drugs since resistance of cancer cells to CIS therapy [4]. Quercetin (QU) is a plant derived flavonoid exhibiting anti-proliferative, growth suppressing and apoptotic effects in several cell lines [5–7]. QU induced anti-proliferative effect associated with alterations in the signal transduction pathways including MAPKs, PI3K/AKT and EGFR [7–10]. The enhanced anti-proliferative effect of QU with CIS was reported in cervix, leukaemia and hepatocellular carcinoma cells [11–13]. Additionally, we formerly reported that QU + CIS treatment of different MM cell lines (SPC111 and SPC212) caused a decrease in proliferation and an increase in apoptosis [14]. However, SPC212 cells were found to be much more sensitive to QU + CIS. Therefore, in the present work, 84 genes involved in cell proliferation and growth are evaluated in QU, CIS and/or QU + CIS treated SPC212 cells.
Methods

Compounds and reagents
QU and CIS (Sigma-Aldrich) stock solutions were prepared in dimethylsulfoxide (DMSO, cell culture tested; Sigma-Aldrich) and stored at −20 °C. The chemicals were diluted 100X in fresh media before each experiment. The anti-proliferative activity of single and combined chemical treatments were assessed in a monolayer culture condition by plating cells in 100 mm petri dishes.

Cell line and culture
A human MM cell line SPC212 was used as a model system. SPC212 was derived from a tumour with mixed histology of female patient, which was obtained as a gift from The Institute of Histology and General Embryology, University of Fribourg, Switzerland. Cells were cultured in RPMI, supplemented with 10 % FBS, L-glutamine (2 mM), 2 % NaHCO₃ (Sigma-Aldrich), and 1 % penicillin/streptomycin (Invitrogen-Gibco) in a humidified atmosphere containing 5 % CO2 at 37 °C. Cells were treated with single and/or combined concentrations of QU (50 μM) and CIS (5 μg/mL) according to our previous findings from MTS assay [14].

RNA isolation and RT²-PCR
Total RNA was extracted from 2 × 10⁶ cultured SPC212 cells exposed to 50 μM QU, 5 μg/mL CIS and in combination or only 0.01 DMSO as a solvent control for 48 h using RNeasy Mini Kit and treated with RNase-free DNase (SABiosciences, Qiagen). cDNAs were kept on ice and then immediately used for RT-PCR array or were stored at −20 °C until using the further processes. The RT²-PCR assays were performed on Stratagene Mx3005P using the MAP Kinase Signalling Pathway RT² profiler PCR Array (#PAHS-061Z; SABiosciences) and RT² SYBR Green Master Mix (SABiosciences, Qiagen) according to the manufacturer’s protocol. Before the total RNA extractions, cell lysate was homogenized by using QIA shredder homogenizer (SABiosciences, Qiagen) and then reverse transcription of 2 μg of total RNA into cDNA was performed using the RT² First Strand Kit (SABiosciences, Qiagen). cDNAs were kept on ice and then immediately used for RT-PCR array or were stored at −20 °C until using the further processes. The RT²-PCR assays were performed on Stratagene Mx3005P using the MAP Kinase Signalling Pathway RT² profiler PCR Array (#PAHS-061Z; SABiosciences) and RT² SYBR Green Master Mix (SABiosciences, Qiagen) according to the manufacturer’s protocol. Thermal profile was set as segment 1 for denaturation (1 cycle): at 95 °C for 10 min; segment 2 for annealing/extension (40 cycle): at 95 °C for 15 s, at 60 °C for 1 min; segment 3 for melting curve (1 cycle): at 95 °C for 1 min, at 55 °C for 30 s, at 95 °C for 30 s. The mRNA expression levels of 84 MAP Kinase Signalling Pathway-related genes in the QU and in combination treated cells were compared to control cells, and then evaluated according to the ΔΔCt method using Data Analysis web-based software (www.SABiosciences.com/parraydataanalysis.php). Data were normalized to housekeeping genes included in a RT²PCR Array plate (ACTB; β-actin and RPLP0; ribosomal protein large P0). Several experiments were performed and only the one with p-values above 0.90 for all genes after analysis using Data Analysis web-base software was considered.

SDS PAGE/Western blotting
Cells were cultured as 2 × 10⁶ cells per 100-mm-dish incubated for 24 h, and then serum starvation is performed for the next 24 h. Later, medium was replaced with serum-free medium and cells were incubated for 12, 24 and 48 h with or without chemicals, as 5 μg/mL CIS and 50 μM QU in combination. Then, whole-cells were obtained from PBS suspension and passed through syringes for 15 times to explode cells. Lysates were collected within 200 μl of lysis buffer and 1 % protease phosphatase inhibitor cocktail (Thermo Scientific). Protein concentrations of samples were determined by Pierce BCA Protein Assay Kit. 15 μg of total protein from each sample were separated on a 12 % SDS-PAGE and transferred to PVDF membranes (Hybond-P Amersham, GE Healthcare). Membranes were blocked 1 h in TBS-T containing 5 % non-fat dried milk that followed by application of antibodies for p-ERK and ERK (Santa Cruz Bio), p-p38, p-JNK, JNK and Actin (Cell signalling) and then respective secondary antibodies conjugated with horseradish peroxidase. Thermo, Pierce ECL kit was used to develop membranes to detect chemiluminescence which is detected via ChemiDoc XRS (Bio-Rad) imaging system.

Results

The effects of QU + CIS on cell cycle and MAPK pathway genes
To investigate whether QU + CIS changed gene expressions of SPC212 cells, we exposed cells with QU + CIS (50 μM + 5 μg/mL) and QU (50 μM). The RT²-PCR array revealed that compared to untreated cells expression of cyclin dependent kinase inhibitor (CDI) genes [CDKN1A (p21), CDKN1B (p27), CDKN1C (p57), CDKN2A (p16) and CDKN2B (p15)] were up regulated in QU + CIS and QU treated cells respectively.

Interestingly, cyclins [CCNA (cyclin A1), CCNA2 (cyclin A2), CCNB1 (cyclin B1), CCNB2 (cyclin B2), CCND1 (cyclin D1), CCND2 (cyclin D1), CCND3 (cyclin D3) and CCNE1 (cyclin E1)] and cyclin dependent kinase2 (CDK2) gene expressions were also elevated greater than two-fold in QU + CIS treated cells (Fig. 1).

We next examined the alteration of JNK/SAPKs and p38MAPK pathway genes after drug applications. As seen in Fig. 2, treatment with QU + CIS resulted in increased expression of DLK, MAP3K4 (MEKK4), MAP2K4 (MEK4), CDC42 and MAPK8IP2/JIP1 (interacts with JNK1) genes on two-fold or more. Moreover, transcription factors (TFs) activated through JNK pathway including ATF2 (Creb-2), NFATC4 (NFAT3) and
CREBBP (transcriptional co-activator) were also up-regulated comparing to untreated cells. Although any up-regulation in the expression of genes involved in JNK pathway, arise gene expressions of p38 pathway (MAP2K6/MEK6 and MAPK11/p38β) were observed in QU treated cells.

Similarly, genes involved in classical MAPK pathway were explored after treatment either with QU or QU + CIS (Fig. 3). Genes including ARAF, GRB-2, MAPK3/ERK1 and KSR-1, FOS, ELK-1, E2F1 and EGR1 were up-regulated two-fold or more in single QU treatments. Unlike, expression of EGFR, BRAF, MOS, MAP2K1/MEK1, LAMTOR3/MP1, FOS and ETS2 genes were raised in combined treatments. In addition, genes activated in response to cell protection and/or survival including HSPA5/HSP70 and CHICK/IKKa were up regulated in combine treatments. Interestingly, only two genes were down regulated after exposure to QU + CIS including MAPK interacting kinase (MNK1) and tumour suppressor protein, TP53 (p53).

The effects of QU + CIS on MAPK pathway proteins

Finally, expression and activation levels of ERK, JNK and p38 proteins were evaluated after treatments in 48 h. As shown in Fig. 4, phosphorylation level of ERKs (p44 and p42) was decreased, but JNKs (p54 and p46) and p38 were increased in first 24 h and then, decreased (Fig. 4).
Discussion

Development of effective inhibitors for proliferating cancerous cells is one of the most important issues in cancer treatment. Since development of drug resistance, cancer cells have been subjected to exposure chemicals especially in combinations [3]. It is well-known that the progression of cell cycle is regulated by Cylins, CDKs and CDIs in mammalian cells. Two distinct families of CDIs are classifying as p21/p27 and p16/p18. While the p21 family members are potent inhibitors to almost all CDK enzymes, the p16 family members are specific inhibitors to CDK4 and CDK6 only [15]. Earlier, we revealed that QU + CIS application caused accumulation of high percent of cells in S phase at 48 h [14]. In here, up regulated gene expression of CDIs (CDKN1A, CDKN1B, CDKN2A and CDKN2B after QU + CIS exposure supports our previous result and identifies which genes act on cell cycle arrest mechanism of SPC212 cells. Unexpectedly, raised expression CDK2, G1/S, S and G2/M cyclin genes propose resistance of cells to CIS. CDK2 is known to play transition from G1 to S phase through S phase to G2 phase [16]. Since CDK2 is associated with CIS resistance [17], it is an important candidate target for cancer therapy. Therefore, it may recommend integrating specific CDK2 inhibitor into QU + CIS mix for future work. Single QU treatments in SPC212 cells caused up regulation of CDKN1C, MEK6 and MAPK11 gene expressions. Recently, it is reported that environmental stress transiently activates p38MAPK, which in turn, phosphorylates p57 leading an increased affinity to CDK2-CyclinE/A activity and subsequent G1 arrest [18]. Thus, this can be also the case in QU treated SPC212 cells. Besides, raised MAPK gene expressions may indicate maintenance of this effect.

Tanida et al. demonstrated that CIS induced toxic effects via MAPKs [19]. Zhang et al. and others [20–22] reported that ELK-1, Rac and CDC42 activated p38MAPK. In addition, Fan et al. and others [23–26] showed that DLK and MEK also activated both p38 and JNK pathways. Similarly, along with our results we assume that CDC42, DLK, MEKK4, MEK4 and JNK interacting protein (JIP1) up regulations implies involvement of p38 and JNK pathways in QU + CIS exposed SPC212 cells. Besides, up regulations of TF genes including ATF-2, NFAT and CREBBP (co-activator) encouraged

![Fig. 3](image-url) mRNA levels of MAPK and survival pathway members. Two-fold or more differences compared to control cells were evaluated by RT²-PCR array. Group 1: DMSO Control versus Quercetin; Group 2: DMSOcontrol versus Quercetin + Cisplatin

![Fig. 4](image-url) Phosphorylation and total protein levels of MAPKs. Cells were treated with 5 μg/ml CIS, 50 μM QU or both for a series of time and their proteins were analysed by Western Blotting as described in material methods. The experiments were repeated at least three times with similar results.
contribution of p38 and JNK pathways. Moreover, as it is demonstrated in Fig. 4, increased phosphorylation of JNK and p38 proteins indicated activation of both pathways in response to combined treatments. Thornton et al. [27] reported that p38 negatively regulates cell cycle by several mechanisms through down regulation of cyclins, up regulation of CDI and modulation of p53. Our results propose that QU and QU + CIS mediate S phase arrest through up regulation of CDI, which may be associated with gene and protein up regulations of p38 in SPC212 cells. Beyond cell cycle arrest, activation of p38 and JNK pathways are also often correlated with stress-related apoptosis [28]. Initially we found that exposure to cells with QU + CIS caused enzymatic Caspase-3 and caspase-9 activations [14]. Therefore, another possibility that QU + CIS generated apoptosis of SPC212 cells as a result of activation of JNK and p38 pathways.

It is well documented that activated p53 triggers a number of signalling pathways leading to cell cycle arrest, DNA repair, apoptosis and senescence [29]. Surprisingly, p53 gene expression was down regulated in QU + CIS treated cells. Tafolla et al. [30] exposed that activation of JNK pathway lead to negative regulation of p53. Thus, we thought that negative regulation of p53 after QU + CIS treatment may related to JNK pathway activations in SPC212 cells. However, it is also possible that p38 and JNK signalling mediated apoptosis was not depended on p53 activity [31]. Indeed, analysed initial apoptotic gene expressions patterns signified that TP73 (p73) gene was up regulated in the combine treatments (data not shown). In addition, QU mediated mitochondrial apoptosis was reported in the absence of p53 but in the presence of TP63 (p63) and p73 [32] and CIS activated p73-dependent apoptosis was informed by Yoshida et al. [33]. Subsequently, our results indicate existence of a p53-independent apoptosis in response to QU + CIS treatments.

Mainly, activation of MAPK/ERK pathway is associated with proliferation and survival of cells. On the contrary, increasing evidence shows that MAPK activation contributes to apoptosis in some cell types. Conflicting results exists on the consequences of CIS initiated MAPK activations. Persons et al. [34] demonstrated that CIS induced MAPK1/2 activation protects cells from cytotoxicity of CIS. However, Wang et al. [35] indicated that CIS creates MAPK initiated apoptosis. Similar contradictory results also reported on QU’s activities. Some researchers showed that apoptotic contribution of QU requires inactivation of the MAPK pathway and others declare opposite effects [7]. Our gene analysis demonstrated that although some MAPK pathway genes were up regulated, the protein phosphorylations were down regulated in QU and QU + CIS treated cells. In combine treatments another up regulated expressions was on HSP70/HSPA5 and IKKa/CHUCK genes. HSP70 occasionally was expressed in response to unfolded protein stress [36] and IKKa/CHUCK was induced by DNA damages or activation of NF-kappa-β pathway [37]. These results propose that QU + CIS induced stress dependent survival signals leading to increased MAPK gene activation. At the same time, the cytostatic and/or apoptotic capacities of agents decreased post-transcriptional phosphorylation of SPC212 cells. In addition, we observed down regulation of MNK1 gene after QU + CIS treatments. Activation of MNKs (MNK1 and MNK2) was indicated in drug resistance and negative regulation of apoptosis via modulating transcription of anti-apoptotic genes [38, 39]. Down regulation of MNK1 gene may suggest subsequent down regulation of anti-apoptotic genes leading to apoptosis of SPC212 cells.

Conclusions

In conclusion, QU + CIS induced S phase arrest that is regulated by almost all CDIs in SPC212 cells. The suppressed cell proliferation arrested cell cycle and triggered cell death of SPC212 cells depends on three MAPK pathway activations. Although survival /resistance pathways activated by QU + CIS treatments, SPC212 cells were undergone to apoptosis which probably implies JNK and p38 MAPK pathway involvement.

Abbreviations
cDNA, complementary DNA; CIS, cisplatin; DMSO, dimethylsulfoxide; DNA, deoxyribonucleic acid; FBS, fetal bovine serum; MAPK, mitogen activated protein kinase; MM, malignant mesothelioma; QU, quercetin; RNA, ribonucleic acid; RPMI, Roswell Park Memorial Institute 1640 medium; RT-PCR, reverse transcriptase–real time polymerase chain reaction

Acknowledgements

Authors acknowledge the Turkish Scientific and Technological Research Council of Turkey (TUBITAK) and the Anadolu University for funding the work.

Funding

Part of this work was funded by grant TBAG 2336 103 T-145 from Turkish Scientific and Technological Research Council of Turkey (TUBITAK), Scientific Research Projects Commission of Anadolu University (grant 091027) also funded this work.

Availability of data and materials

The datasets supporting the conclusions of this article are presented in this main paper.

Authors’ contributions

EE, ADZ, NA, VK and HS carried out the study; EE HS ADZ and VK wrote the manuscript; HS and ADZ supervised the work; HS and ADZ designed the experiments. All authors read the manuscript and approved the final version.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable in this section.

Ethic approval and consent to participate

Not applicable in this section.
References

1. Jakobsen JN, Sørensen JB. Review on clinical trials of targeted treatments in malignant mesothelioma. Cancer Chemother Pharmacol. 2011;68:1–15.

2. Rudd RM. Malignant mesothelioma. Br Med Bull. 2010;93:105–23.

3. Favoni RE, Diaga A, Malastera P, Florio T. Preclinical studies identify novel targeted pharmacological strategies for treatment of human malignant pleural mesothelioma. Br J Pharmacol. 2012;166:53–52.

4. Girosio F, Scagletti G. Systematic treatment of malignant pleural mesothelioma. Fut Oncol. 2012;8:293–305.

5. Jaganathan SK, Mandal M. Antiproliferative effects of honey and its polyphenols. J Biomed Biotechnol. 2009;2009:830616.

6. Russo GL, Russo M, Spagnuolo C, Tedesco I, Bilotto S, Iannitti R, et al. The flavonoid quercetin in disease prevention and therapy: facts and fancies. Biochem Pharmacol. 2012;83:6–15.

7. Zhao JL, Zhao J, Jiao HJ. Synergistic growth-suppressive effects of quercetin on cisplatin-induced apoptosis of HL-60 and L1210 leukemia cells. Leuk Res. 2002;26:376–82.

8. Agullo G, Gamet-Payrastre L, Manenti S, Viala C, Remesy C, Chap H, et al. Quercetin: a pleiotropic kinase inhibitor against cancer. Cancer Treat Res. 2014;159:185–205.

9. Nguyen T, Tran E, Nguyen T, Do PT, Huynh TH, Huynh H. The role of MEK-ERK pathway in quercetin-inhibited growth inhibition and apoptosis in A549 lung cancer cells. Carcinogenesis. 2004;25:647–59.

10. Price PM, Yu F, Kaldis P, Aleem E, Nowak G, Safirstein R, et al. Dependence of Biochemistry, Faculty of Science, University of Dschang, P.O. Box 67, Dschang, Cameroon.

11. Tanida S, Mizoshita T, Ozeki K, Tsukamoto H, Kamiya T, Kataoka H, et al. Ablation of MEK4 kinase activity causes neurolulation and skeletal patterning defects in the mouse embryo. Mol Cell Biol. 2005;25:8948–59.

12. Whitmarsh AJ, Davis RJ. Role of mitogen-activated protein kinase kinase 4 in cancer. Oncogene. 2007;26:3172–84.

13. Demiroglu-Zergeroglu A, Basara-Cigerim B, Kilic E, Yanikkaya-Demirel G. The flavonoid quercetin in cancer prevention and management. Nutr Rev. 2010;68:418–28.

14. Yoshida K, Ozaki T, Furuya K, Nakanishi M, Kikuchi H, Yamamoto H, et al. ATM-dependent nuclear accumulation of IKK-alpha plays an important role in the regulation of p73-mediated apoptosis in response to Ad-e5FAS1 in A549 lung cancer cells. Mol Cancer. 2013;12:35.

15. Vargas AJ, Burd R. Hormesis and synergy: pathways and mechanisms of quercetin in cancer prevention and management. Nutr Rev. 2010;68:418–28.

16. Bottero V, Busuttil V, Loubat A, Magné N, Fischel JL, Milano G, et al. Activation of nuclear factor kappaB through the IKK complex by the topoisomerase poisons SN38 and doxorubicin: a brake to apoptosis in HeLa human carcinoma cells. J Biol Chem. 2002;277:53822–9.

17. Joaquin M, Gubern A, Posas F. A novel G1 checkpoint mediated by the p57 kinase (CDK) inhibitors block the phosphorylation of cyclin-dependent kinases by the CDK-activating kinase. J Biol Chem. 1995;270:18195–9.

18. Aprelikova O, Xiong Y, Liu ET. Both p16 and p21 families of cyclin-dependent kinase (CDK) inhibitors block the phosphorylation of cyclin-dependent kinases by the CDK-activating kinase. J Biol Chem. 1995;270:18195–9.

19. Fan G, Merritt SE, Kortenjann M, Shaw PE, Holzman LB. Dual leucine zipperbearing kinase (DLK) activates p46SAPK and p38mapk but not ERK2. J Biol Chem. 1996;271:24788–93.

20. Huntwork-Rodriguez S, Wang B, Watkins T, Ghosh AS, Pozniak CD, Bustos D, et al. JNK-mediated phosphorylation of DLK suppresses its ubiquitination to promote neuronal apoptosis. J Cell Biol. 2011;202:767–73.

21. Zhang HM, Li L, Papadopoulos N, Hodgson G, Evans E, Galbraith M, et al. Mitogen-induced recruitment of ERK and MSK to SRE promoter complexes by ternary complex factor Elk-1. Nucleic Acids Res. 2003;31:2594–607.