Role of Mitochondria in Quercetin-enhanced Chemotherapeutic Response in Human Non-small Cell Lung Carcinoma H-520 Cells

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Abstract. *Dietary phytochemicals have been shown to be chemopreventive against various types of cancer. This study was designed to investigate the enhancement of the chemoresponse to cisplatin by quercetin in human lung cancer H-520 cells and to elucidate the role of mitochondria in the induction of apoptosis. Apoptosis was detected by flow cytometry. The protein expressions of Bcl-X_L, Bcl-2, Bax, cytochrome c and AIF were studied by Western blotting. The transcription of antioxidant enzymes was quantitated by RT-PCR. The findings suggested that priming H-520 cells with quercetin increased the cisplatin-induced apoptosis by 30.2%. This was accompanied by down-regulation of Bcl-X_L and Bcl-2 and up-regulation of Bax. Both cytochrome c and AIF were implicated in the apoptotic process. There was no significant change in the transcription level of antioxidant enzymes in quercetin-mediated apoptosis. Based on these findings, it can be concluded that quercetin might act as an effective chemosensitizer in the chemotherapy of lung cancer by regulating the expression of various apoptosis-related genes.*

Lung cancer remains a major cause of mortality worldwide despite advances in surgery, radiotherapy and chemotherapy. Most patients present at an advanced stage of the disease. The treatment of advanced non-small cell lung carcinoma (NSCLC) remains an important area of research and there is now evidence that these patients can benefit from single-agent chemotherapy with drugs such as cisplatin, vinorelbine, gemcitabine, paclitaxel, pemetrexed and docetaxel. For more than two decades, the most effective systemic chemotherapy for NSCLC has been cisplatin-based combination treatment. Unfortunately, the outcome of cisplatin therapy on NSCLC seems to have reached a plateau. Therefore, the biological mechanisms of the action of cisplatin need to be understood in order to overcome this treatment plateau. Moreover, the development of resistance is a hurdle with the use of this drug (1).

In some cases, combination chemotherapy may have additional advantages, but the potential toxicity poses a major limitation to combination chemotherapy (2). Because optimal agents or combinations have not been defined and because of potential side-effects and associated chemoresistance in the case of chemotherapy (3), novel strategies and therapeutics are urgently needed. The flavonoids are a class of polyphenolic compounds widely distributed in the plant kingdom, which display a variety of biological activities, including chemoprevention and tumor growth inhibition. Quercetin has been shown to inhibit melanoma growth and may constitute a valuable tool in the combination therapy of metastatic melanoma and other cancers (4, 5). Because of its pharmacological safety, quercetin can be used as a chemopreventive and also in combination with chemotherapy to treat cancer, as it exerts antiproliferative and pro-apoptotic effects (6).

Materials and Methods

Cell culture and treatments. Human NSCLC cells NCI-H-520 were grown in DMEM medium supplemented with 10% fetal calf serum, containing antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin), in a humidified atmosphere of 5% CO_2 in air at 37˚C. Logarithmically-growing cells were used for all experiments. The cells were treated with standardized doses of quercetin (40 μM) and cisplatin (5 μg/ml) alone for 24 hours. In another set of experiments, the cells were primed with quercetin for 24 hours prior to treatment with cisplatin for 24 hours.

Measurement of cell viability. Growth inhibition of the H-520 cells was determined by the colorimetric MTT cell viability assay, as described earlier (7). In brief, the cells were grown in 96-well plate and treated with the respective agents for 24 hours in the presence

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or absence of anticancer drugs. At the end of the incubation period, 100 μl of 5.0 mg/ml MTT was added to each well and the plates were incubated for 4 hours at 37˚C. The colored formazan product was then dissolved in 100 μl of DMSO. The plates were read using the microtiter plate reader at a wavelength of 570 nm to determine the percentage of dead (or growth-inhibited) cells. The experiments were performed in triplicate for each treatment and repeated three times.

Isolation of nuclei, mitochondria and cytosol. Cells were sonicated in buffer containing 10 mM Tris-HCl pH 7.5, 10 mM NaCl and 1.5 mM $MgCl_2$, 175 mM sucrose and 12.5 mM EDTA and the cell extract was centrifuged at 1000 xg for 10 minutes to pellet the nuclei. The supernatant thus obtained was centrifuged at 18,000 xg for 30 minutes to pellet the mitochondria. The mitochondria were purified as previously described (8). The resulting supernatant was termed the cytosolic fraction. The pellets were lysed and protein was estimated in all the three fractions by Bradford's method (9). The purity of the fractions was confirmed by assaying the marker enzymes succinate dehydrogenase for mitochondria, lactate dehydrogenase for the cytosol and NMN adenylyl transferase for the nuclear fraction.

Western blotting. The whole cells, nuclei or the mitochondrial fractions were lysed in ice-cold lysis buffer (50 mM Tris, 150 mM NaCl, 2.5 mM EDTA, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40 and 0.02% sodium azide) containing protease inhibitors, as described previously (10). After incubation for 30 minutes on ice, the samples were centrifuged at 10,000 xg for 30 minutes. The protein content in the lysed extracts was determined using Bradford's method. Equal amounts of protein (80 μg/lane) were loaded onto 10-12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The primary antibodies against Bcl-X_L, Bcl-2, Bax, cytochrome c and AIF were obtained from Santa Cruz Biotechnology, CA, USA. The secondary antibody used was the appropriate alkaline phosphatase conjugated anti-mouse, anti-rabbit or anti-goat IgG (Promega, Madison, WI, USA). The proteins were detected using the BCIP-NBT substrate. Densitometric scanning was performed using the Alpha Imager 2200. Positive and negative controls were run with each antibody.

Flow cytometry. Apoptosis was measured by flow cytometry, as described previously (11). In brief, the cells were washed twice with PBS and fixed in 70% ethanol overnight. The cells were then washed twice with PBS to remove ethanol and then incubated in propidium iodide (20 μg/ml) for 1 hour. Flow cytometry was performed using an EPICS XL-MCL flow cytometer (Coulter Electronics, Miami, FL, USA) software. Win MDI 2.8 software was used to generate histograms, which were then used to determine the cell cycle phase distribution after debris exclusion. The sub G_1-G_0 cell fraction was considered as representative of apoptotic cells.

Reverse transcriptase mediated-PCR. The mRNA levels of various enzymes related to oxidative stress were analyzed by RT-PCR. RNA was isolated using TRI-Reagent from Sigma, St. Louis, MO, USA, and precipitated by isopropanol, as described earlier (12). The precipitated RNA was dissolved in DEPC-water. The purity of the isolated RNA was checked by running it on formaldehyde gel and quantitated by taking absorbance at 260 nm and 280 nm. cDNA was made, using reverse transcriptase from Stratagene, TX, USA, and used as a template for PCR using primers specific for manganese-superoxide dismutase, copper-zinc superoxide dismutase, glutathione peroxidase and catalase mRNA.

Manganese superoxide dismutase: Forward primer: 5' GGCCT GATTATCTAAAAGCTATTTGG 3', reverse primer: 5' CGAT CGTGGTTTACTTTTTGCA'

Copper-zinc superoxide dismutase: Forward primer: 5' TGGTG GTCCATGAAAAAGCA 3', reverse primer: 5' CCAGCGTTTC CTGTCTTTGTACT 3'

Glutathione peroxidase: Forward primer: 5' TGTGCCCCTAC GGAGGTAC 3', reverse primer: 5' AGCTGGGCCCTTGAGA CAG 3'

Catalase: Forward primer: 5' AGAGGAAACGTCTGTGT GAGAACA 5', reverse primer: 5' TGACCGCTTTCTTCTG GATGA 3'

Bcl-2: Forward primer: 5' GCCGGTTCAGGTACTCAGTCA 3', reverse primer: 5' CATGTGTGTGGAGAGCGTCAA3'

Caspase-3 assay. Caspase-3 activity was measured by the direct assay of enzyme activity in the cell lysates using the synthetic fluorogenic substrate N-acetyl Asp-Glu-Val-Asp-7-amino-4-methyl coumarin (Ac-DEVD-AMC). The cells were washed with PBS, pH 7.5, and lysed in 10 mM Tris-HCl, pH 7.5, 130 mM NaCl, 1% Triton X-100, 10 mM $Na_4P_2O_7$ and 10 mM Na_2HPO_4 on ice ($10x10^6$ cells/ml of lysis buffer). One hundred μl of cell lysate were added to the reaction buffer (20 mM HEPES, pH 7.2; 10% glycerol; 2 mM DTT; 250 μM Ac-DEVD-AMC) and incubated at 37˚C for one hour. The amounts of fluorogenic moiety released were measured using a spectrofluorimeter, with excitation at 380 nm and emission at 420-460 nm (13).

Statistical analysis. Each experiment was repeated three times. All data are expressed as mean±SE. The Student's *t*-test was used to determine the significance between the control and various experimental groups. A difference was considered statistically significant at $p<0.05$.

Results

Quercetin enhances the apoptosis-inducing effects of cisplatin. To study the effect of quercetin pretreatment on cisplatin - induced cell death, NCI-H-520 cells were treated with quercetin (40 μM) for 24 hours, followed by cisplatin (5 μg/ml) for another 24 hours. Quercetin augmented the cisplatin-mediated apoptosis, as seen by the morphology of the cells (Figure 1). The untreated control cells showed 3.0% apoptosis (Figure 2a). Quercetin by itself caused 18.4% apoptosis (Figure 2b), whereas cisplatin by itself induced 22.2% apoptosis (Figure 2c). Pretreatment with quercetin increased the cisplatin-induced apoptosis to 52.4% (Figure 2d).

Quercetin down-regulates Bcl-X_L and Bcl-2 and up-regulates Bax. Since the anti-apoptotic mitochondrial-related proteins

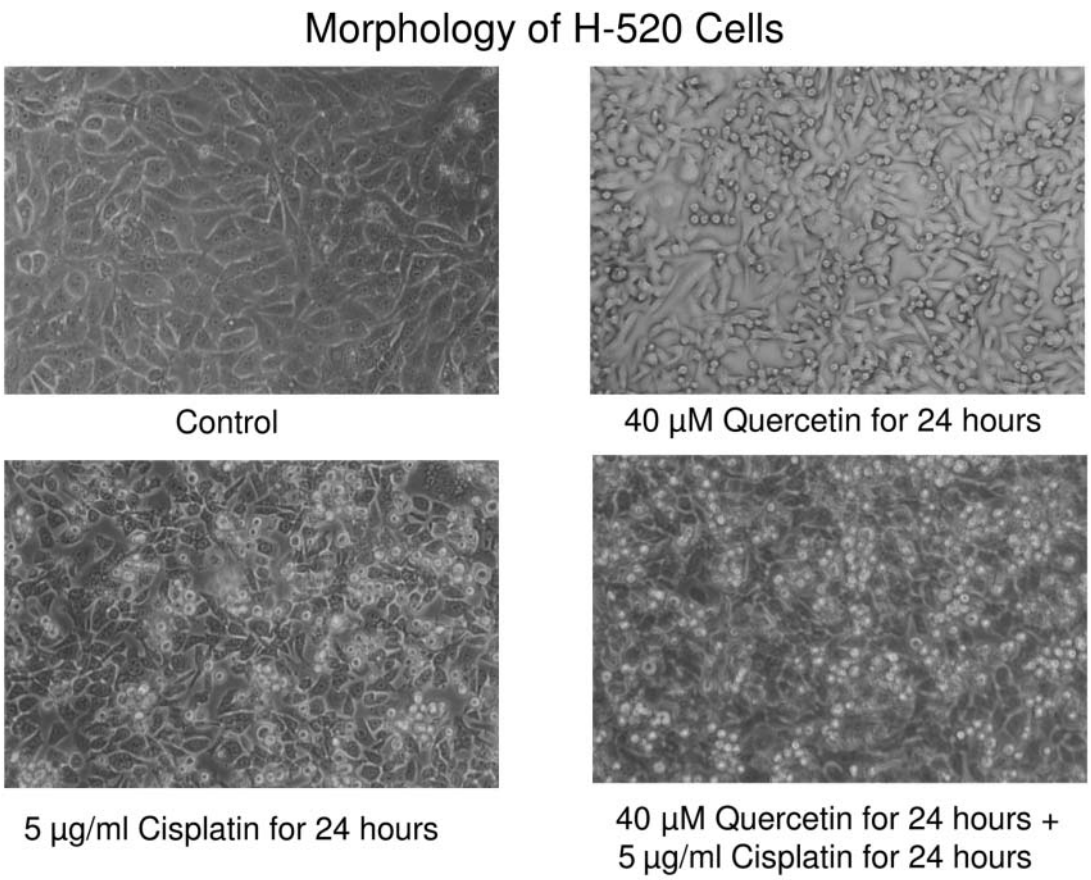

Figure 1. *Effect of priming with quercetin on cisplatin-induced apoptosis in NCI-H520 cells, as seen by morphology.*

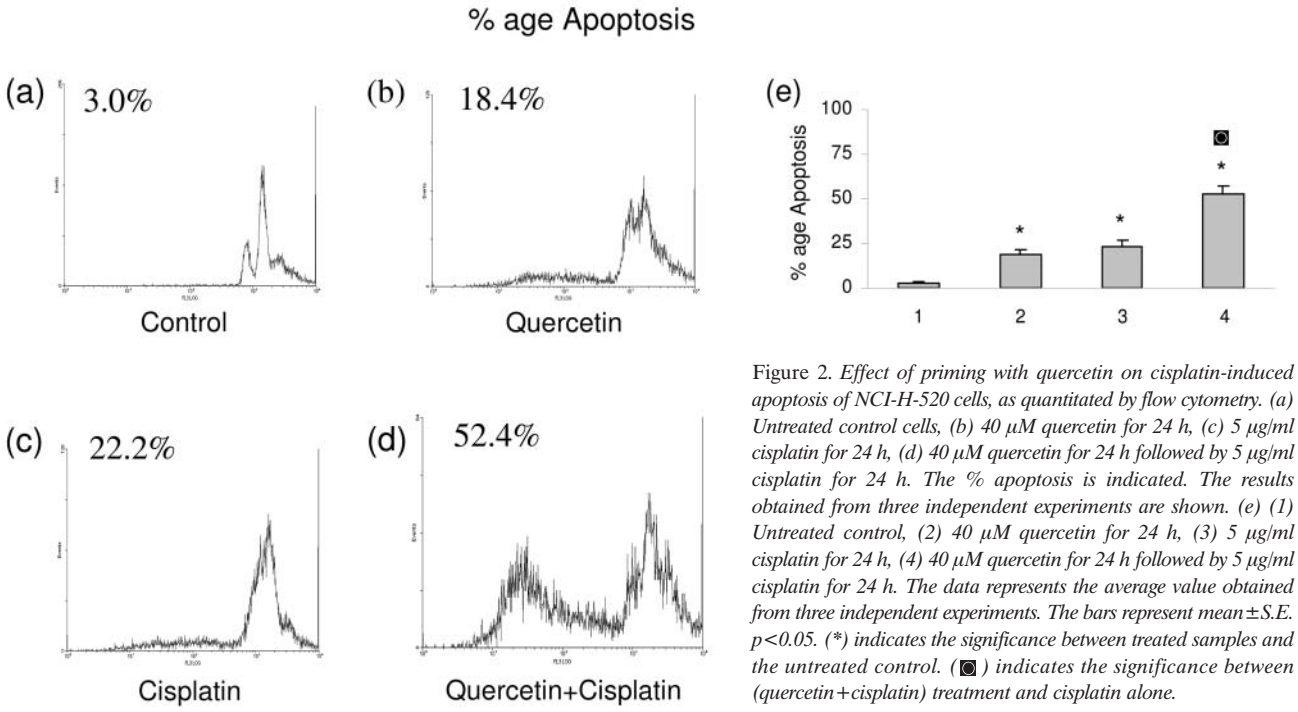

Figure 2. *Effect of priming with quercetin on cisplatin-induced apoptosis of NCI-H520 cells, as quantitated by flow cytometry. (a) Untreated control cells, (b) 40 µM quercetin for 24 h, (c) 5 µg/ml cisplatin for 24 h, (d) 40 µM quercetin for 24 h followed by 5 µg/ml cisplatin for 24 h. The % apoptosis is indicated. The results obtained from three independent experiments are shown. (e) (1) Untreated control, (2) 40 µM quercetin for 24 h, (3) 5 µg/ml cisplatin for 24 h, (4) 40 µM quercetin for 24 h followed by 5 µg/ml cisplatin for 24 h. The data represents the average value obtained from three independent experiments. The bars represent mean±S.E. $p<0.05$. (*) indicates the significance between treated samples and the untreated control. (■) indicates the significance between (quercetin+cisplatin) treatment and cisplatin alone.*

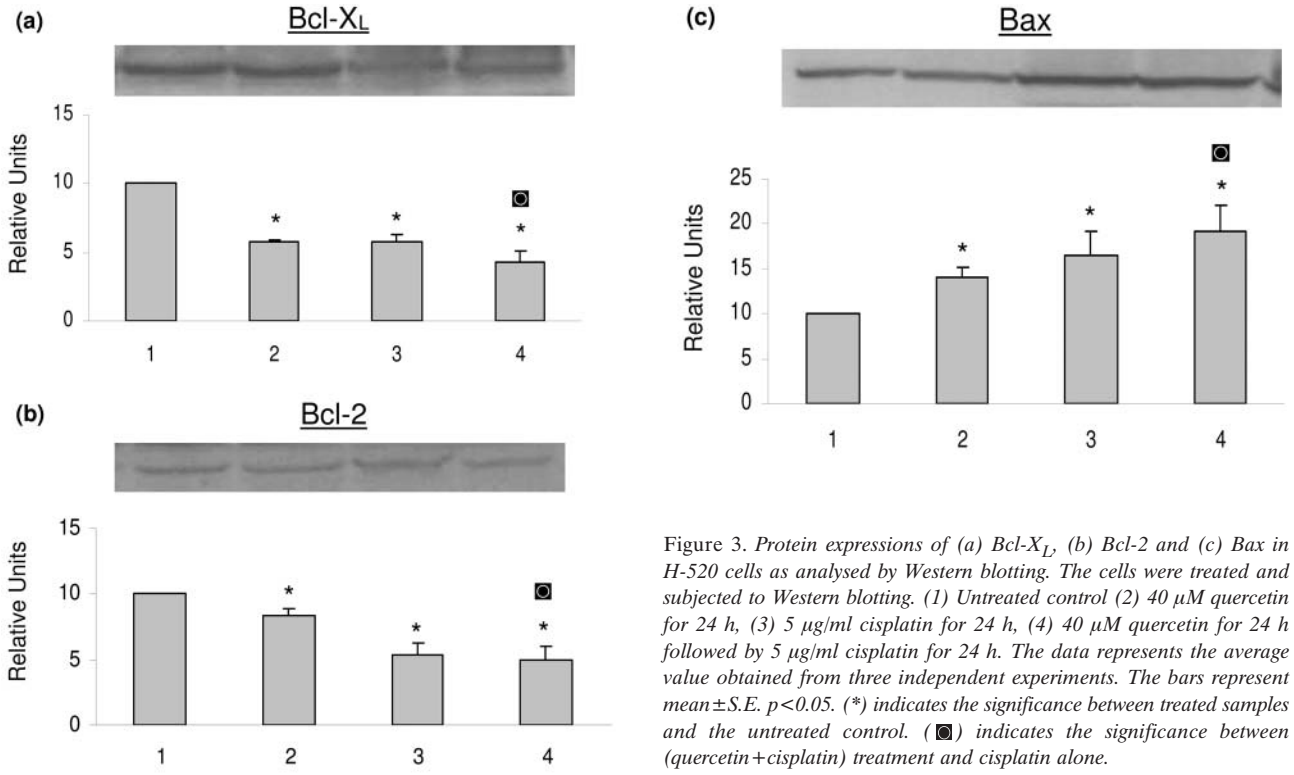

Figure 3. *Protein expressions of (a) Bcl-X_L, (b) Bcl-2 and (c) Bax in H-520 cells as analysed by Western blotting. The cells were treated and subjected to Western blotting. (1) Untreated control (2) 40 μM quercetin for 24 h, (3) 5 μg/ml cisplatin for 24 h, (4) 40 μM quercetin for 24 h followed by 5 μg/ml cisplatin for 24 h. The data represents the average value obtained from three independent experiments. The bars represent mean±S.E. $p<0.05$. (*) indicates the significance between treated samples and the untreated control. (■) indicates the significance between (quercetin+cisplatin) treatment and cisplatin alone.*

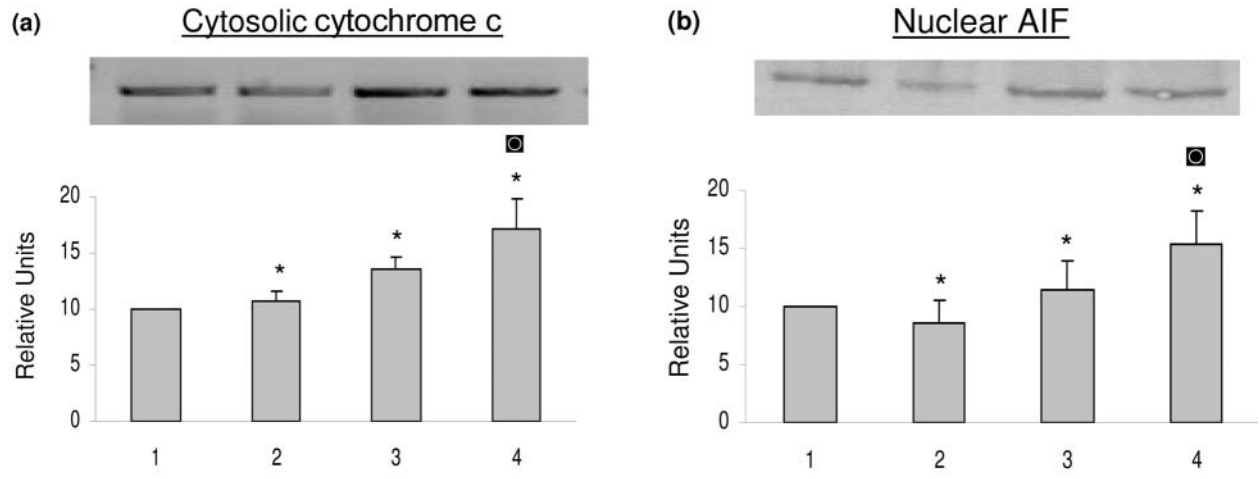

Figure 4. *Effect of quercetin priming on the translocation of (a) cytochrome c and (b) apoptosis-inducing factor (AIF) in H-520 cells as analyzed by Western blotting. The cells were treated with the respective priming agents and drugs as described and the cytosolic fraction was subjected to Western blotting to quantitate cytochrome c. The nuclear fraction was analyzed for AIF protein. (1) Untreated control, (2) 40 μM quercetin for 24 h, (3) 5 μg/ml cisplatin for 24 h (4) 40 μM quercetin for 24 h followed by 5 μg/ml cisplatin for 24 h. The data represents the average value obtained from three independent experiments. The bars represent mean±S.E. $p<0.05$. (*) indicates the significance between treated samples and the untreated control. (■) indicates the significance between (quercetin+cisplatin) treatment and cisplatin alone.*

Bcl-X_L and Bcl-2 and pro-apoptotic protein Bax are important regulators of apoptosis, their expressions were analyzed. Bcl-X_L protein expression was decreased by 42% for both quercetin and cisplatin. In cisplatin primed with quercetin cells, the decrease was 57% as compared to the untreated control cells (Figure 3a). The Bcl-2 levels decreased by 16% with quercetin alone, 46% with cisplatin alone, while cisplatin primed with quercetin decreased

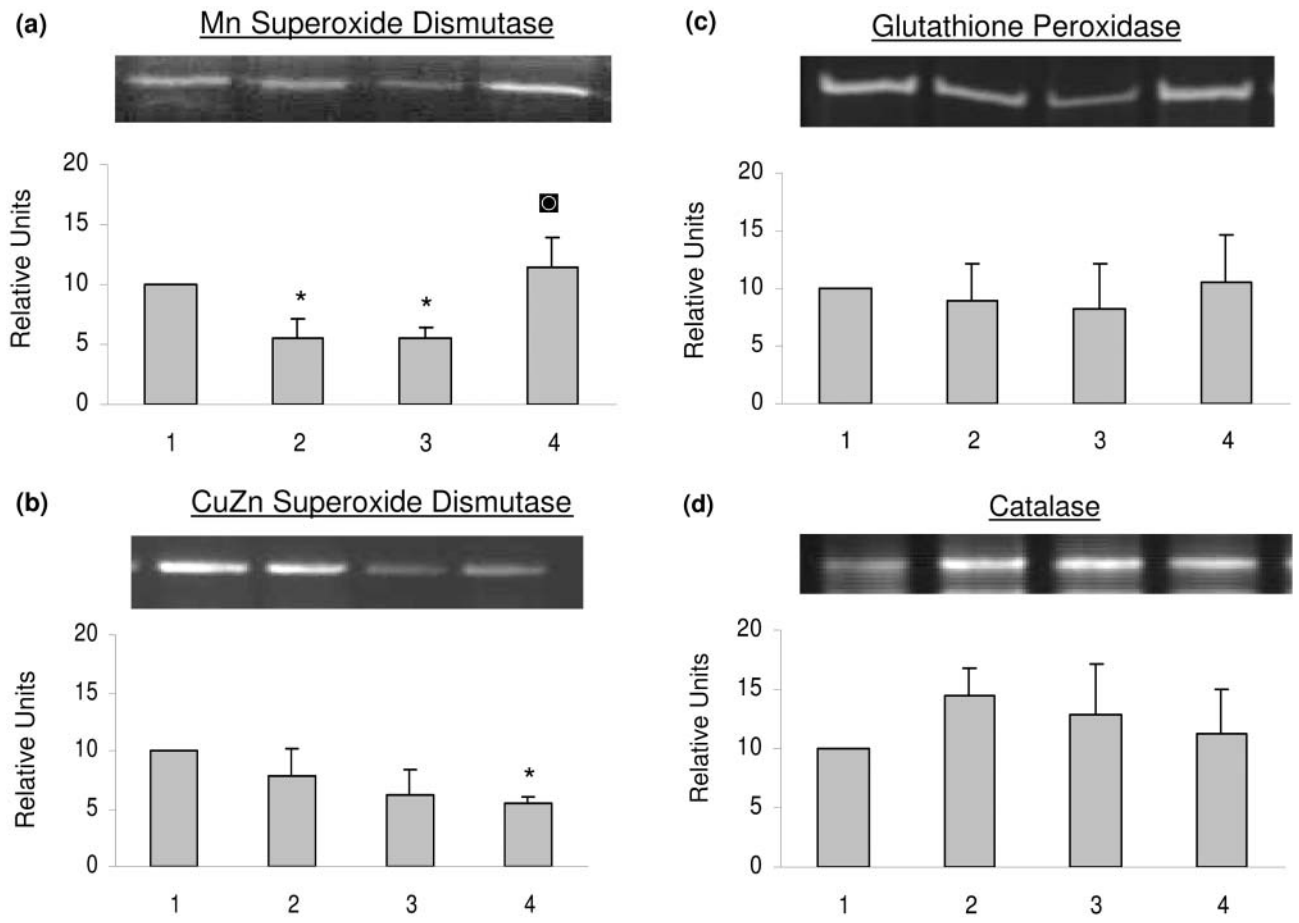

Figure 5. *Effect of quercetin priming on the expression of antioxidative enzymes in H-520 cells as analyzed by RT-PCR. The cells were treated with the respective priming agents and drugs as described. RNA was isolated and the various antioxidative enzymes were quantitated by RT-PCR. (1) Untreated control, (2) 40 μM quercetin for 24 h, (3) 5 μg/ml cisplatin for 24 h, (4) 40 μM quercetin for 24 h followed by 5 μg/ml cisplatin for 24 h. The data represents the average value obtained from three independent experiments. The bars represent mean±S.E. $p<0.05$. (*) indicates the significance between treated samples and the untreated control. (■) indicates the significance between (quercetin+cisplatin) treatment and cisplatin alone.*

Bcl-2 by 50% as compared to the untreated control cells (Figure 3b). The Bax protein expression increased by 44% with quercetin, 66% with cisplatin alone and by 92% with cisplatin primed with quercetin, as compared to the control cells (Figure 3c).

Quercetin augments the release of cytochrome c from the mitochondria. The release of cytochrome c from the mitochondria is associated with the activation of the apoptotic cascade. The cytosolic cytochrome c increased by 8% with quercetin alone, 36% with cisplatin alone and 71% when cisplatin was primed with quercetin (Figure 4a).

Pretreatment with quercetin increases the nuclear AIF levels. Translocation of the mitochondrial protein AIF to the nucleus is also associated with apoptosis; therefore, the AIF protein expression in the nuclear fraction was analyzed. Treatment with quercetin decreased the nuclear AIF levels by 15%, cisplatin increased the nuclear AIF by 18%, whereas cisplatin treatment primed with quercetin increased the level of nuclear AIF by 57%, as compared to the controls (Figure 4b).

Non-involvement of antioxidant enzymes in quercetin-primed H-520 cells. Oxidative stress, induced by reactive oxygen intermediates, often causes cell death *via* apoptosis, which is regulated by many functional genes and their protein products. Therefore, various antioxidant enzymes were analyzed at the transcription level. Mn superoxide dismutase decreased by 45% both with quercetin and cisplatin treatment, while in the cells primed with quercetin it showed an increase of 14% as compared to the control, which was not significant. The increase in Mn superoxide dismutase with quercetin plus cisplatin treatment, as compared to cisplatin alone, was 59%. Cu-Zn superoxide dismutase, glutathione

peroxidase and catalase did not show any significant changes, either with quercetin or cisplatin alone or with their combination, suggesting their non-involvement in the apoptotic process (Figure 5).

Quercetin priming increases the caspase-3 activity. The activation of the effector caspase, caspase-3, is the final step in the mitochondrial pathway of apoptosis. Quercetin increased caspase-3 activity by 3.72-fold and cisplatin by 5.91-fold, as compared to the untreated control cells. Whereas in the cells pretreated with quercetin followed by cisplatin, the caspase-3 activity increased by 10.78-fold as compared to the untreated control cells (Figure 6).

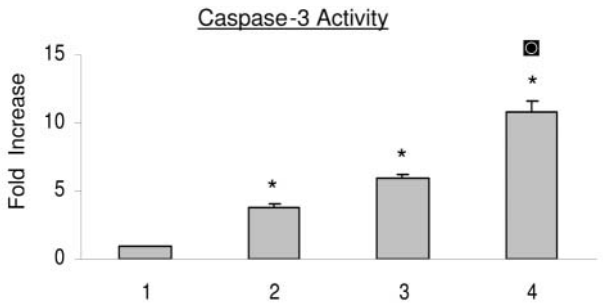

Figure 6. *Activation of caspase-3 as a result of quercetin priming in H-520 cells. The cells were treated with the respective priming agents and drugs as described. The activity of the enzyme caspase-3 was measured using Ac-DEVD-AMC as the substrate. (1) Untreated control, (2) 40 μM quercetin for 24 h, (3) 5 μg/ml cisplatin for 24 h, (4) 40 μM quercetin for 24 h followed by 5 μg/ml cisplatin for 24 h. The data represents the average value obtained from three independent experiments. The bars represent mean±S.E. $p<0.05$. (*) indicates the significance between treated samples and the untreated control. (■) indicates the significance between (quercetin+cisplatin) treatment and cisplatin alone.*

Discussion

There are many facets to cancer prevention and the possibility of using drugs or naturally-occurring compounds to prevent the initiation of, or to suppress, tumour growth is quite promising. However, to date, very few such agents have been used in the clinic with any success. An ideal chemopreventive agent would restore normal control to a preneoplastic or cancerous cell population by modifying aberrant signalling pathways or inducing apoptosis, or both, in cells beyond repair. The characteristics of such an agent include selectivity for damaged or transformed cells, good bioavailability and more than one mechanism of action to foil redundancy or crosstalk in signalling pathways (14).

As increased research effort is being targeted towards this area, the distinction between chemotherapeutic and chemopreventive agents is beginning to blur. Chemotherapeutic drugs are now being designed to target over- or under-active signalling molecules within cancer cells, a philosophy which is just as relevant in chemoprevention. The development of dietary agents is particularly attractive because of our long-standing exposure to them, their lack of toxicity and encouraging indications from epidemiology.

In vitro and *in vivo* studies have demonstrated that some dietary components, like quercetin, regulate the molecules in the cell signal transduction pathways including the NF-kappaB, Akt, MAPK, p53, AR and ER pathways (15). Tumour cells often evade apoptosis by expressing several anti-apoptotic proteins, the down-regulation and mutation of pro-apoptotic genes and alterations in signalling pathways, which give them survival advantage and, thereby, allow them to resist therapy-induced apoptosis. Most death pathways depend on input from several parts of the cell, the major one being the mitochondrion that is essential for the vast majority of death pathways. The mechanisms of mitochondrial involvement are beginning to be elucidated and may involve the participation of Bcl-2 family members, reactive oxygen species and caspases. As part of a central mechanism of amplification of the apoptotic signal, the mitochondria may be an appropriate target for therapeutic agents designed to modulate apoptosis.

The regulation of apoptosis is a complex process and involves a number of mitochondrial-associated proteins including Bcl-2 and Bcl-2-related family members such as Bcl-X_L, Bcl-X_S, Bad and Bax (16). Bcl-2 functions as a global inhibitor of apoptosis, most probably through multiple mechanisms. The Bcl-2 gene product has protected cells against apoptosis in a variety of experimental systems. Bcl-2, located on the outer membrane of the mitochondria, may prevent cell death by blocking the release of cytochrome c. Cells that overexpress Bcl-2 resist apoptosis induced by anticancer drugs (17). Suppression of Bcl-2 promotes apoptosis in response to a number of stimuli, including anticancer drugs (18).

In the present study, a 40-μM dose of quercetin induced significant apoptosis in H-520 cells and it enhanced cisplatin-mediated apoptosis by 30.2%. This was accompanied by a decrease in the expressions of Bcl-X_L and Bcl-2 and an increase in the Bax protein levels as compared to the control. Treatment with quercetin has been shown to result in a significant increase in the expression of Bax, while the levels of Bcl-2 protein are reduced by quercetin in A549 lung cancer cells (19). Bcl-2 and Bcl-X_L exert their anti-apoptotic effects, at least in part, by binding to Bax and related pro-apoptotic proteins. They also prevent Bax and the pro-apoptotic proteins from inducing the release of cytochrome c and the activation of caspase-9.

Translocation of cytochrome c from the mitochondria to the cytosol is the central process in the mitochondrial pathway of apoptosis, which leads to further downstream apoptotic events, culminating in activation of caspase-3. The

increase in cytosolic cytochrome c with quercetin priming suggests that cytochrome c is released into the cytosol and mediates further downstream events in cisplatin-mediated apoptosis. The significant elevation in caspase-3 activity further supports this involvement.

A mitochondrial flavoprotein, called the apoptosis-inducing factor (AIF), translocates to the nucleus when apoptosis is induced and recombinant AIF causes chromatin condensation in isolated nuclei and large- scale fragmentation of DNA, independent of caspase activation (20). AIF was significantly altered by the combination treatment with quercetin and cisplatin, implicating its role in the apoptotic process. The involvement of the mitochondria and the release of cytochrome c have been shown in quercetin-mediated apoptosis in leukaemia HL-60 cells (21).

Oxidative stress signals, induced by the formation of reactive oxygen species, are also considered as important activators of apoptosis. Quercetin-enhanced apoptosis appears to be mediated by reactive oxygen species, as evidenced by the non-involvement of the antioxidant enzymes.

Time-tested pharmacological safety and multiple molecular targets favourable to treat a disease such as cancer are in favour of quercetin being developed as a drug for the prevention and therapy of various cancers, either alone or in combination with standard drugs.

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References

1 Wang G, Reed E and Li QQ: Molecular basis of cellular response to cisplatin chemotherapy in non-small cell lung cancer. Oncol Rep *5*: 955-965, 2004.
2 Govindan R and Garfield DH: Treatment approaches in patients with advanced non-small cell lung cancer and poor performance status. Semin Oncol *31*: 27-31, 2004.
3 Seve P and Dumontet C: Chemoresistance in non-small cell lung cancer. Curr Med Chem Anti Canc Agents *5*: 73-88, 2005.
4 Caltagirone S, Rossi C, Poggi A, Ranelletti FO, Natali PG, Brunetti M, Aiello FB and Piantelli M: Flavonoids apigenin and quercetin inhibit melanoma growth and metastatic potential. Int J Cancer *87*: 595-600, 2000.
5 Schwarz D, Kisselev P and Roots I: CYP1A1 genotype-selective inhibition of benzo[α]pyrene activation by quercetin. Eur J Cancer *41*: 151-158, 2005.
6 Dragnev KH, Petty WJ, Ma Y, Rigas JR and Dmitrovsky E: Nonclassical retinoids and lung carcinogenesis. Clin Lung Cancer *6*: 237-244, 2005.
7 Kalemkerian GP and Ou X: Activity of fenretinide plus chemotherapeutic agents in small-cell lung cancer cell lines. Cancer Chemother Pharmacol *43*: 145-150, 1999.
8 Wang G-Q, Gastman BR, Wieckowski E, Goldstein LA, Gambotto A, Kim T-H, Fang B, Rabinovitz A, Yin X-M and Rabinowich H: A role for mitochondrial bak in apoptotic response to anticancer drugs. J Biol Chem *276*: 34307-34317, 2001.
9 Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem *72*: 248-254, 1976.
10 Sharma H, Sen S, Mathur M, Bahadur S and Singh N: Combined evaluation of expression of telomerase, survivin, and anti-apoptotic Bcl-2 family members in relation to loss of differentiation and apoptosis in human head and neck cancers. Head Neck *8*: 733-740, 2004.
11 Khanna N, Reddy VG, Tuteja N and Singh N: Differential gene expression in apoptosis: identification of ribosomal protein S29 as an apoptotic inducer. Biochem Biophys Res Commun *277*: 476-486, 2000.
12 Sharma H, Sen S, Muzio LL, Mariggio A and Singh N: Antisense-mediated downregulation of anti-apoptotic proteins induces apoptosis and sensitizes head and neck squamous cell carcinoma cells to chemotherapy. Cancer Biol Ther *4*: 720-727, 2005.
13 Sen S, Sharma H and Singh N: Curcumin enhances Vinorelbine mediated apoptosis in NSCLC cells by the mitochondrial pathway. Biochem Biophys Res Commun *331*: 1245-1252, 2005.
14 Manson MM, Farmer PB, Gescher A and Stewad WP: Innovative agents in cancer prevention. Rec Results Cancer Res *166*: 257-275, 2005.
15 Sarkar FH and Li Y: Cell signaling pathways altered by natural chemopreventive agents. Mutat Res *555*: 53-64, 2004.
16 Boise LH, Gonzalez-Garcia M, Postema CE, Ding L, Lindsten T, Turka LA, Mao X, Nunez G and Thompson CB: Bcl-x, a Bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell *74*: 597-608, 1993.
17 Jie Yang, Jones DP *et al*: Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science *275*: 1129-1132, 1997.
18 Fisher TC, Milner AE, Gregory CD, Jackman AL, Aherne GW, Hartley JA, Dive C and Hickman JA: Bcl-2 modulation of apoptosis induced by anticancer drugs: resistance to thymidylate stress is independent of classical resistance pathways. Cancer Res *53*: 3321-3326, 1993.
19 Nguyen TT, Tran E, Nguyen TH, Do PT, Huynh TH and Huynh H: The role of activated MEK-ERK pathway in quercetin-induced growth inhibition and apoptosis in A549 lung cancer cells. Carcinogenesis *25*: 647-659, 2004.
20 Susin SS, Zamzami N, Kroemer G *et al*: Bcl-2 inhibits the mitochondrial release of an apoptogenic protease. J Exp Med *184*: 1331-1341, 1996.
21 Wang IK, Lin-Shiau SY and Lin JK: Induction of apoptosis by apigenin and related flavonoids through cytochrome c release and activation of caspase-9 and caspase-3 in leukaemia HL-60 cells. Eur J Cancer *35*: 1517-1525, 1999.

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