in vivo *19*: 69-76 (2005)

Synergistic Antiproliferative Action of the Flavonols Quercetin and Kaempferol in Cultured Human Cancer Cell Lines

MARGARET LEIGH ACKLAND[1], SIMONE VAN DE WAARSENBURG[1] and ROD JONES[2]

[1]*Centre for Cellular and Molecular Biology, Deakin University, Burwood, Victoria;*
[2]*Department of Primary Industry, Knoxfield, Victoria 3156, Australia*

Abstract. *The consumption of vegetables containing the flavonols quercetin and kaempferol reduces the risk of cancer. We utilized human gut (HuTu-80 and Caco-2) and breast cancer cells (PMC42) to show the synergistic effect of quercetin and kaempferol in reducing cell proliferation. A trend in reduction of total cell counts was seen following a single exposure, a 4-day exposure or a 14-day exposure to quercetin and kaempferol. Combined treatments with quercetin and kaempferol were more effective than the additive effects of each flavonol. The reduction in cell proliferation was associated with decreased expression of nuclear proliferation antigen Ki67 and decreased total protein levels in treated cells relative to controls. In conclusion, the synergistic antiproliferative effect of quercetin and kaempferol demonstrated in cultured human cells has broad implications for understanding the influence of dietary nutrients in vivo, where anticancer effects may be a result of nutrients which act in concert.*

There is strong epidemiological evidence linking frequent consumption of fruit and vegetables with a reduced risk of a number of cancers (1, 2). Recent research has identified a wide range of phytochemicals that may be implicated in cancer prevention, including flavonols (3). Flavonols are widespread in vegetables and much research has been directed to the potential health attributes of quercetin in particular. For example, epidemiological studies showed an inverse association between flavonoid intake and incidence of lung cancer (4, 5).

The major flavonols found in commonly consumed vegetables are quercetin, followed by kaempferol (6), with onions and tea being the major sources of quercetin in the Western diet (7). Kaempferol is not as widely spread as quercetin, but is found in many commonly consumed vegetables, such as broccoli, kale and endive (6).

Correspondence to: Margaret Leigh Ackland, Centre for Cellular and Molecular Biology, Deakin University, Burwood, Victoria, Australia. e-mail: leigha@deakin.edu.au

Key Words: Anticancer effects, flavonols, human cells.

Quercetin has been found in the plasma of non-supplemented humans at a concentration of between 0.5 - 13 μM (8, 9). It appears that quercetin glycosides are cleaved during absorption in the large intestine, but intact glycosides can be absorbed in the small intestine (10); after absorption, the aglycone is rapidly conjugated to form glucuronides (11). There is further evidence that the flavonol glucoronides are deconjugated by β-glucuronidase during cellular absorption in the liver, meaning quercetin and possibly kaempferol aglycones may be taken up at the cellular level (12). Therefore, *in vitro* addition of quercetin and kaempferol aglycones to human cancer cell lines at levels normally found in plasma after a meal closely mimics the *in vivo* condition.

Recent data has demonstrated a number of potential roles for quercetin in cancer prevention. The chemopreventative effects of quercetin have been shown in mice treated with azomethane, where the number of focal areas of dysplasias in colonic mucosa was reduced in animals on diets supplemented with quercetin (13). Quercetin reduced colonic hyperproliferation and dysplasia in mice treated with azomethanol to induce colonic neoplasia (14) and it also inhibited the number of mammary tumors in rats challenged with N-nitrosomethylurea (15).

The mechanisms by which flavonoids exert their antitumor effects are not entirely clear, but inhibition of cell proliferation has been reported on a number of occasions. Antiproliferative effects of quercetin have been reported in cultured human tumor cells of the prostate (16), cultured MCF-7 human breast cancer cells (17), squamous carcinoma cells of the tongue (18), ascites tumor cells (19) and human gastric cancer cells (20). Quercetin had a cytotoxic effect on two actively proliferating human colonic cancer cell lines, HT29 and Caco-2 (21), and inhibited growth and expression of cancer-associated p21-ras proteins (22, 23). Quercetin has also been found to affect intracellular signaling pathways (24, 25). Kaempferol, a closely related but less studied flavonol, reduced proliferation in human melanoma cells (26) and human prostate cancer cells (16) and inhibited cell growth of

human prostate cancer cells through inhibition of tyrosine phosphorylation (24).

It is unlikely that the observed reduction in cancer incidence *in vivo* is due to the action of single compounds; it is more likely to be due to one or several synergies generated by a number of compounds ingested at the same time. Quercetin and catechin were reported to synergistically inhibit platelet aggregation (27), while quercetin and resveratrol synergistically interacted with ethanol to inhibit the inducible nitric oxide synthase pathway (28). In broccoli, two glucosinolate derivatives synergistically enhanced the Phase II enzyme systems, while Vitamin D acted with interleukins to inhibit proliferation in MCF-7 breast cancer cells (29).

In the current study, we demonstrated synergistic and additive responses between the two most commonly ingested flavonols, quercetin and kaempferol, on cell proliferation in the human intestinal lines Caco-2 and HuTu-80 and in the PMC42 breast carcinoma cell line.

Materials and Methods

Cells. The immortalised human breast cell line, PMC42, was cultured in RPMI (ThermoTrace, Melbourne, Australia) supplemented with 10% fetal bovine serum (ThermoTrace) and penicillin and streptomyocin. The human gut cancer cell lines, HuTu-80 and Caco-2, were cultured in RPMI and DMM with 1% non-essential amino acids (ThermoTrace), respectively. Both media were supplemented with 10% fetal calf serum (ThermoTrace) and penicillin and streptomyocin. Cells were maintained as monolayer cultures in 5% CO_2 at 37˚C. At confluency cells were detached by incubating with 0.025% trypsin/EDTA.

Chemicals. Quercetin (3,3',4',5,7-Pentahydroxyflavone) and kaempferol (3,5,7-Trihydroxy-2-[4-hydroxyphenyl]-4H-1-benzopyran-4-one) were supplied by Sigma-Aldrich, Sydney, Australia. Stock solutions of 100 μM quercetin and kaempferol were prepared in respective media and diluted to concentrations of 1 μM, 5 μM and 10 μM.

Treatment protocol. Cells were seeded at 10^6 cells in either cell culture flasks or onto glass coverslips. Cells were treated once, daily for 4 days or daily for 14 days with quercetin and kaempferol, and then processed for cell counting, protein estimation, immunofluorescence or collected as cell pellets for Western blot analysis.

Trypan blue exclusion assay. A 200 μl aliquot of trypsinised cells was mixed with an equal volume of trypan blue (Sigma) and cells were then counted using a haemocytometer The mean and standard deviation was determined from 7 to 8 separate counts for each treatment.

Total protein measurement. Cell pellets were homogenised in 1% sodium dodecyl sulphate (SDS) in 10mM Tris-HCl, pH 7.5, then sonicated for complete cell disruption. The homogenate was centrifuged at 18,000xg at 4˚C for 10 min, supernatant collected and mixed with protease inhibitor (Complete Mini Protease Inhibitor Cocktail Tablets, Roche Diagnostics, Castle Hill, NSW, Australia). The total protein content was measured using the DC Protein Assay Kit (Bio-Rad Laboratories, California, USA) calibrated against bovine serum albumin (BSA) standards.

Western blot analyses. Between 30-60 μg of total protein was separated by SDS/PAGE and transferred to nitrocellulose membranes (Pall Gelman, Ann Arbor, Michigan, USA). Membranes were blocked in 1% (w/v) casein in Tris-buffered saline (TBS) overnight at 4˚C. Diluted primary antibodies were applied and incubated for 2h at room temperature. Monoclonal Ki67 (Dako Co.) antigen was diluted 1/750. Membranes were rinsed and exposed to a 1/2000 dilution of HRP-conjugated secondary antibody (Silenus, Melbourne, Australia) for 1h at RT. After washing off excess secondary antibody, membranes were rinsed twice in TBS with 0.1% tween 20. Proteins were detected by enhanced chemiluminescence (POD Chemiluminescence Blotting Substrate, Roche Diagnostics). To monitor protein loading, membranes were stripped in Re-Blot solution (Chemicon International, Temecula, California, USA) and re-probed with monoclonal β-actin) primary antibody (Sigma) diluted 1/3000. A solution consisting of 1% casein in TBS was used for washing as well as the antibody diluent.

Immunofluorescence. Treated cells grown on glass coverslips were used. Cells were fixed in 4% (w/v) paraformaldehyde in PBS for 10 min and permeabilised with 0.1% (v/v) Triton X-100 (Sigma) in PBS for 10min. Cells were blocked in 1% BSA in PBS for 1h at RT and incubated overnight at 4˚C with the primary antibody (Ki67 diluted 1/100). Fluorescent-tagged secondary antibody (Alexa 488 or 568 Molecular Probes, Eugene, Oregon, USA) diluted 1/2000 was applied for 2h. After washing off excess secondary antibodies, cells were mounted in Antifade Reagent (Bio-Rad).

Statistics. The results are presented as means ±SEM of four experiments, each performed in triplicate. A Student's *t*-test was used to evalulate the statistical significance of the difference between experimental and control groups.

Results

Single treatment. Following a single exposure to quercetin or kaempferol at 1 μM, 5 μM or 10 μM or combined quercetin and kaempferol (1 μM, 5 μM and 10 μM) for 72 h, significant reductions in HuTu-80 cell counts were seen with all treatments, relative to untreated cultures (Figure 1a), with most effective treatments being 5 μM quercetin, which resulted in a 29% reduction in cell number. Proliferation was more significantly reduced in Caco2 cells by a single dose of quercetin and kaempferol (at 1 μM, 5 μM and 10 μM) and all combined quercetin and kaempferol concentrations (1 μM, 5 μM and 10 μM; Figure 1b). The maximum reduction in total cell count was seen following treatment of cells with a combination of 10 μM quercetin and kaempferol, where cell numbers were reduced by 74%. In PMC42 breast cancer cells, significant reductions in cell counts relative to untreated cultures were seen after 72 h with 5 μM quercetin, 10 μM kaempferol, or a combination of 10 μM quercetin and 10 μM kaempferol (Figure 1c). The

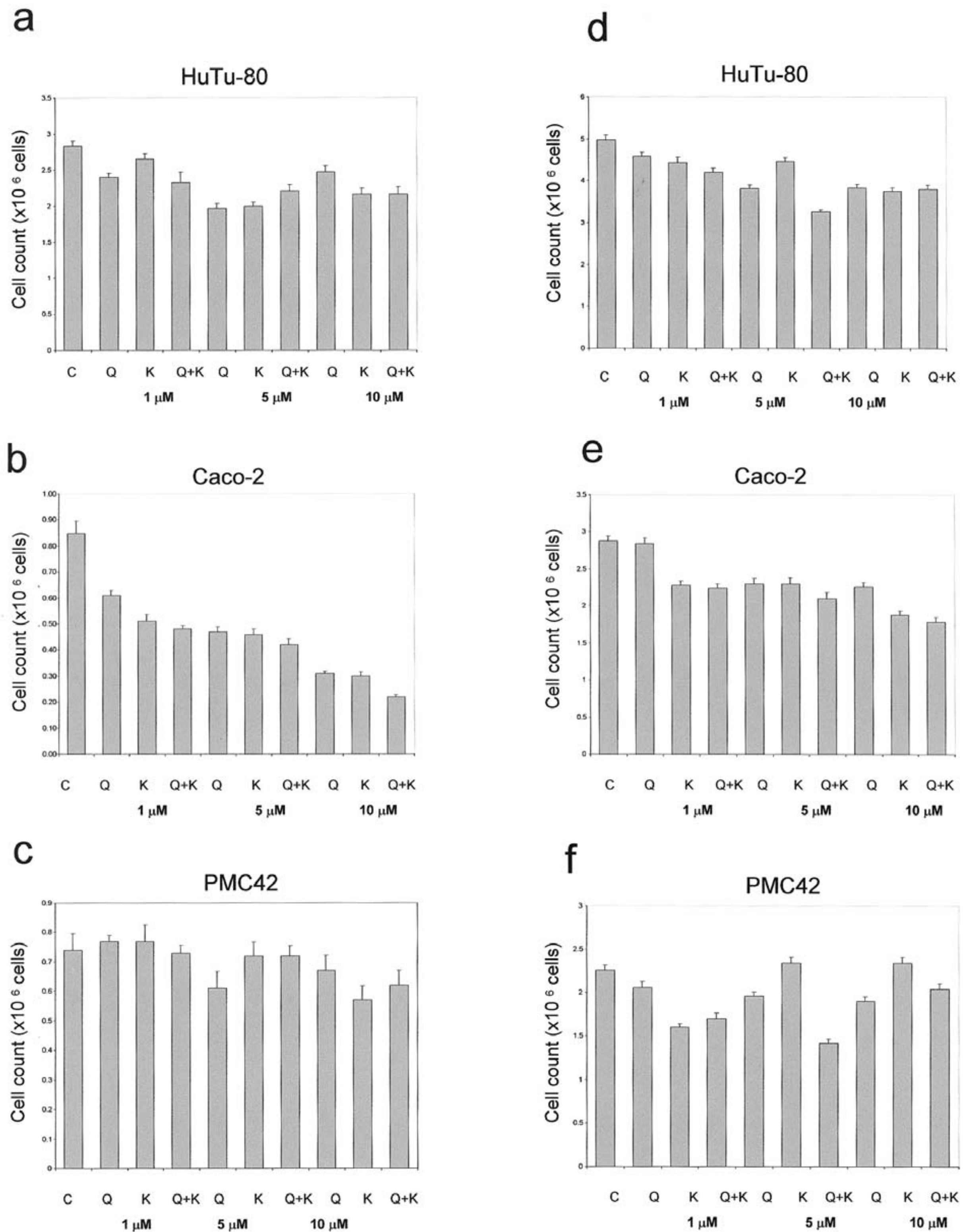

Figure 1. *Effect of a single exposure to quercetin (Q), kaempferol (K) and combined quercetin and kaempferol (Q+K) on cell proliferation relative to control cells (C) in HuTu-80 human gut cells (a), Caco-2 human gut cells (b) and PMC42 human breast cells (c).*
Effect of a 4-day exposure to quercetin (Q), kaempferol (K) and combined quercetin and kaempferol (Q+K) on cell proliferation relative to control cells (C) in HuTu-80 human gut cells (HuTu-80) (d), Caco-2 human gut cells (e) and PMC42 human breast cells (f).

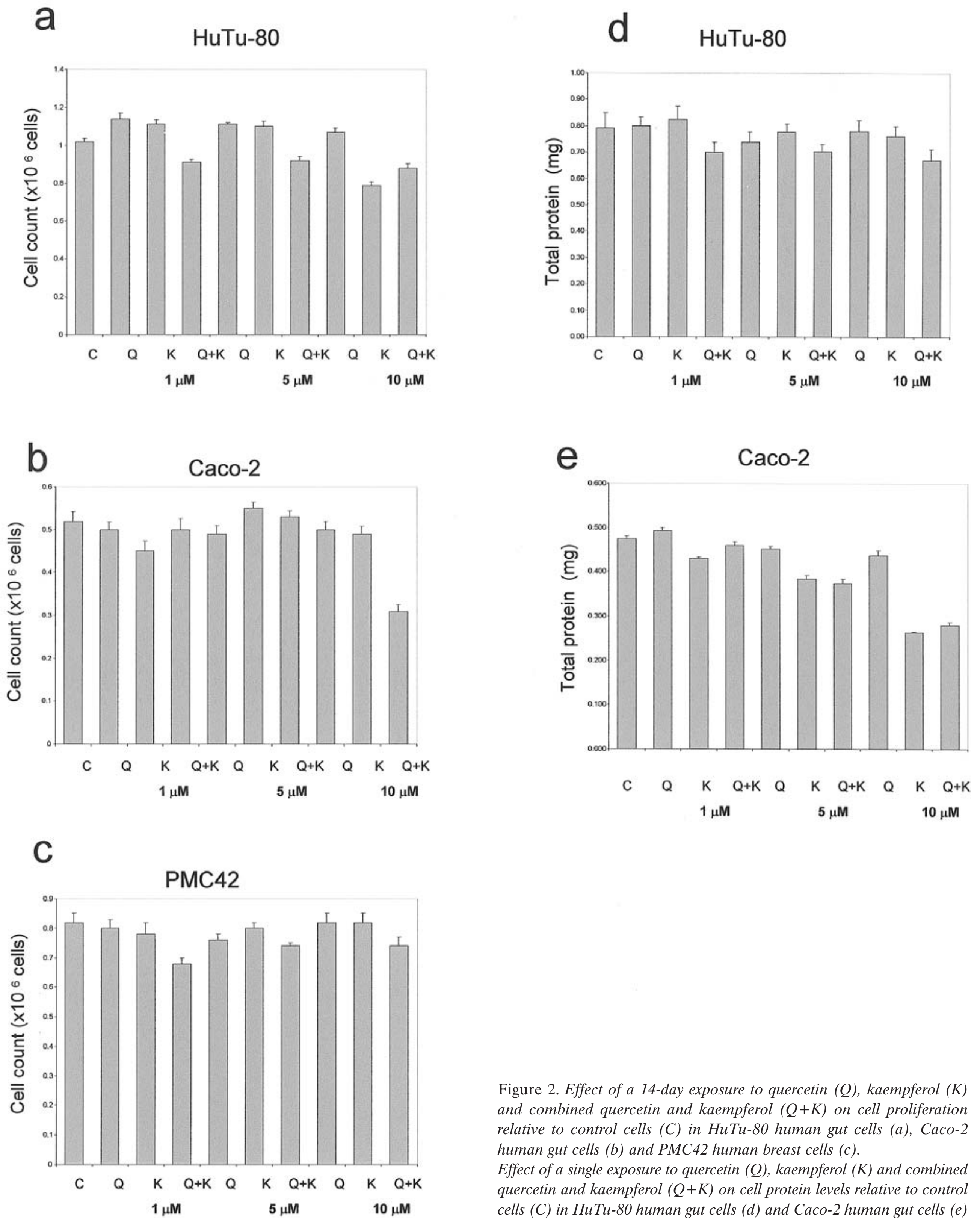

Figure 2. *Effect of a 14-day exposure to quercetin (Q), kaempferol (K) and combined quercetin and kaempferol (Q+K) on cell proliferation relative to control cells (C) in HuTu-80 human gut cells (a), Caco-2 human gut cells (b) and PMC42 human breast cells (c).*
Effect of a single exposure to quercetin (Q), kaempferol (K) and combined quercetin and kaempferol (Q+K) on cell protein levels relative to control cells (C) in HuTu-80 human gut cells (d) and Caco-2 human gut cells (e)

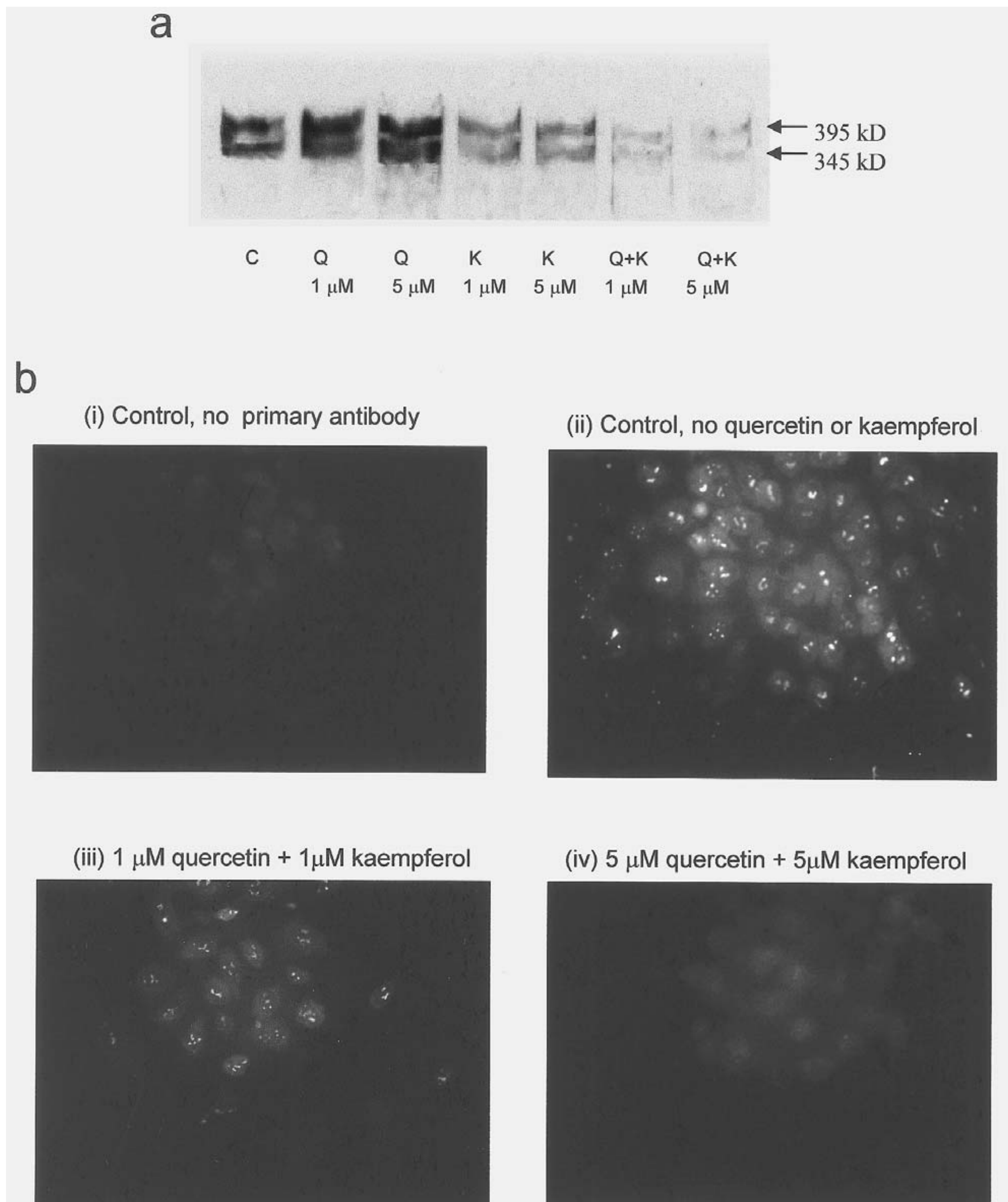

Figure 3. *(a).Western blot analysis to detect nuclear proliferation antigen, Ki67, in HuTu-80 human gut cells following a 14-day exposure to quercetin (Q), kaempferol (K) and combined quercetin and kaempferol (Q+K) relative to control cells (C).*
(b).Indirect immunofluorescence to detect nuclear proliferation antigen in HuTu-80 human gut cells treated with combined quercetin (1 μM) and kaempferol (1 μM) for 14 days but processed in the absence of antibody (i). Control cells not treated with quercetin or kaempferol for 14 days (ii). Cells exposed to combined quercetin (1 μM) and kaempferol (1 μM) for 14 days (iii). Cells treated with medium containing quercetin (5 μM) and kaempferol (5 μM) for 14 days (iv).

most effective treatment was kaempferol at 10 μM, which resulted in an 18% reduction in cell number compared with control cells.

Four-day treatment. Total cell counts in HuTu-80 were significantly reduced following all treatments. The combination of quercetin and kaempferol at 5 μM was most effective at reducing cell proliferation with a decrease in cell numbers of 34% (Figure 1d). In Caco-2 cells, a significant reduction in total cell counts was seen following exposure to quercetin and kaempferol at 5 μM or 10 μM, or combined quercetin and kaempferol (1 μM, 5 μM and 10 μM; Figure 1e). The maximum reduction in cell count was seen in cells treated with a combination of 10 μM quercetin and 10 μM kaempferol, which resulted in a reduction in cell number of 38%. In the breast line PMC42, significant reductions in total cell counts were seen following all treatments, except kaempferol at 1 or 5 μM (Figure 1f). The maximum reduction of 39% in total cell counts was seen with a combination of 5 μM quercetin and 5 μM kaempferol. This equates to approximately 4 times greater inhibition in cell number compared to the sum of 5 μM quercetin or 5 μM kaempferol applied alone, indicating a possible synergistic response.

Fourteen-day treatment. Total cell counts in HuTu-80 80 were significantly reduced following treatment with a combination of quercetin and kaempferol at 1, 5 or 10 μM, or 10 μM kaempferol alone (Figure 2a). The most effective treatment was 10 μM kaempferol, which resulted in a 21% reduction in cell number. In CaCo-2 cells, a significant reduction in total cell counts (42%) was only seen following exposure to the combination of 10 μM quercetin and 10 μM kaempferol (Figure 2b). Again, the combined treatment resulted in an inhibition that was approximately four times higher than the sum of the individual treatments applied alone, indicating a possible synergistic response. In the breast line PMC42, significant reductions in cell counts relative to the control were seen only following treatment with combinations of 1, 5 or 10 μM quercetin and kaempferol (Figure 2c). Most effective was the 1 μM combination with a reduction of 17% in cell numbers.

Total protein. Significant reductions in total protein concentrations relative to untreated cells were found after a single exposure of HuTu-80 cells to 1, 5 or 10 μM quercetin and kaempferol combined (Figure 2d). Exposure to quercetin or kaempferol applied alone did not significantly affect total protein levels indicating that only combinations were effective, possibly as a synergistic response. More significant reductions in total protein concentrations relative to untreated cells were found after a single exposure of Caco-2 cells to 5 μM or 10 μM kaempferol or a combination of 5 μM or 10 μM quercetin and kaempferol (Figure 2e). The most effective treatment was 10 μM kaempferol and 10 μM quercetin and kaempferol combined, which resulted in 45 and 41% reduction in total protein, respectively.

A Western blot analysis to detect the relative amounts of Ki67 nuclear proliferation antigen in HuTu-80 cells treated with quercetin and kaempferol for 14 days relative to the untreated controls showed bands of the expected size of 395 and 345kD. In a representative result from 4 analyses, a reduction of 74% in band intensity was seen in cells treated with a combination of 1 μM quercetin and 1 μM kaempferol relative to the untreated cells. A reduction of 78% was seen in extracts from cells treated with a combination of 5 μM quercetin and 5 μM kaempferol, relative to untreated cells (Figure 3a). Immunofluorescence showed that after 14 days a reduced label was present in HuTu-80 cells exposed to a combination of 1 μM quercetin and 1 μM kaempferol and a combination of 5 μM quercetin and 5 μM kaempferol (Figure 3b).

Discussion

While there is an ever-increasing volume of literature presenting data on the effects that food constituents ("phytochemicals") have on the etiology of certain diseases, the overwhelming majority of these studies concentrate on the efficacy of single compounds. Humans ingest a wide variety of foods and the interactions between the phytochemicals within these foods may go some way to explaining the discrepancies observed between the epidemiological data linking fruit and vegetable consumption with disease prevention (1) and the disappointing results from intervention trials using single phytochemicals or vitamins [*e.g.* Vitamin E (30); beta-carotene and vitamin A (31)]. Our data shows that a combination of the flavonols, quercetin and kaempferol is more effective in reducing cell proliferation than either quercetin or kaempferol applied alone. Both these flavonols are normally contained in various vegetables (major source of quercetin is in onions and tea; kaempferol in broccoli (7) and so our data provide *in vitro* support for the epidemiological evidence that eating a wide range of vegetables offers the best protection against a variety of cancers. Our results indicate that the antiproliferative action of quercetin and/or kaempferol varies between different cancer cell lines. Proliferation in the colon cancer line CaCo-2 was more significantly inhibited by all treatments compared to HuTu-80 and the breast cancer line PMC42 (Figures 1-2). Combined quercetin/kaempferol treatments were also the most effective when applied for 4 days in all cell lines. The PMC42 breast carcinoma line showed less of a decrease overall, possibly due to its enhanced capacity to metabolise toxic substances (32).The

combined treatments appeared to instigate a synergistic effect in CaCo-2 cells after a 14-day application (Figure 2). In this case a synergistic effect was seen when the inhibitory effect of the combined 10 μM quercetin/kaempferol treatment was > 4-fold higher than the additive effect *i.e.* the inhibition caused by 10 μM quercetin added alone plus the inhibition caused by 10 μM kaempferol added alone. There is also a possible synergistic effect of the 1 μM and 5 μM quercetin/kaempferol treatment applied for 4 days on PMC42; again the combined effect at 5 μM was 4-fold higher than the additive effect of 5 μM quercetin and kaempferol added alone (Figure 2). There was, however, less synergistic effect with combined treatments in PMC42 after 14 days relative to the HuTu-80 and Caco-2 cells, possibly due to increased metabolism of the flavonols in PMC42 cells.

Total protein estimates on HuTu-80 and Caco-2 following a single exposure to quercetin and kaempferol caused a reduction in total protein levels, but at a lower rate relative to cell counts. This result is consistent with an effect in blocking the cell cycle after protein synthesis and prior to the mitotic phase. Previous studies showed that kaempferol blocked cell cycle progression in PC-3 human prostate cancer cells (16) and in melanoma cells (26). In HuTu-80 cells, the antiproliferative effects of quercetin and kaempferol were confirmed by a reduction in Ki67 nuclear proliferation antigen, seen by Western blot analysis and immunofluorescence. A similar reduction in Ki67 was found in Caco-2 and, to a lesser extent, in PMC42 breast cancer cells (data not shown).

Synergistic effects have been reported for a number of dietary constituents. For example, quercetin and catechin contained in red wine synergistically inhibited platelet aggregation and hydrogen peroxide formation (9, 27). There appears to be a further synergy between quercetin, resveratrol and ethanol contained in red wine and the inhibition of inducible nitric oxide (33). In cancer cells, Vitamin D acted synergistically with interleukins to inhibit proliferation in the breast cancer line MCF-7 (29), while two isothiocyanates from broccoli synergistically enhanced Phase II enzymes, such as quinone reductase and GSH-transferase (34).

Our study shows the antiproliferative effects of quercetin and kaempferol at concentrations between 1 to 10 μM. These concentrations are compatible with those found under normal physiological conditions (0.5 to 13 μM) (8, 9). This is in contrast to other studies where relatively high levels of quercetin and kaempferol were used, for example, up to 100 μM quercetin and kaempferol in human PC-3 prostatic cells (16), 100 μM quercetin in human tongue squamous carcinoma cells (18), between 10 and 70 μM quercetin in human gastric cell lines (20), 15-120 μM quercetin in HT-29 and Caco-2 cells (21), 50 μM kaempferol in Caco-2 and HT29 (35) and between 30 and 100 μM quercetin in COLO320 DM human colon cancer cells (22). However, quercetin at 7.23 μM was shown to be effective in inhibiting xanthine oxidase (36).

In conclusion, we show a synergistic effect of quercetin and kaempferol at physiological concentrations between 1 and 10 of 5 μM, in reducing cell proliferation in two human gut cell lines HuTu-80 and Caco-2 and the PMC42 human breast cell line. The greatest reduction in cell proliferation was seen in Caco-2 cells following a single dose of combined quercetin/kaempferol at 10 μM. Four- and 14-day treatments were also effective in reducing proliferation of Caco-2, HuTu-80 and PMC42 cells. The reduction in cell proliferation was associated with decreased expression of nuclear proliferation antigen Ki67 and decreased total protein levels in treated cells relative to controls. The synergistic effect of quercetin and kaempferol demonstrated in cultured cells has broad implications for understanding the anticancer effects of dietary nutrients *in vivo*, where individual nutrients may act in concert to reduce carcinogenesis.

References

1 Steinmetz KA and Potter JD: Vegetables, fruit and cancer. 1. Epidemiology. Cancer Causes Control *2*: 325-357, 1991.

2 Cox BD, Whichelow MJ and Prevost AT: Seasonal consumption of salad vegetables and fresh fruit in relation to the development of cardiovascular disease and cancer. Public Health Nutr *3(1)*: 19-29, 2000.

3 Reddy L, Odhav B and Bhoola KD: Natural products for cancer prevention: a global perspective. Pharmacol Ther *99(1)*: 1-13, 2003.

4 Knekt P, Jarvinen R, Seppanen R *et al*: Dietary flavonoids and the risk of lung cancer and other malignant neoplasms. Am J Epidemiol *146*: 223-30, 1997.

5 Stefani ED, Boffetta P, Deneo-Pellegrini H *et al*: Dietary antioxidants and lung cancer risk: a case-control study in Uruguay. Nutr Cancer *34*: 100-110, 1999.

6 Hertog MGL, Hollman PCH and Katan MJ: Content of potentially anticarcinogenic flavonoids of 28 vegetables and 9 fruits commonly consumed in the Netherlands. J Agric Food Chem *40*: 2379-2383, 1992.

7 Hollman PCH and Katan MB: Dietary flavonoids: intake, health effects and bioavailability. Food Chem Toxicol *37*: 937-942, 1999.

8 Paganga G and Rice-Evans CA: The identification of flavonoids as glycosides in human plasma. FEBS Lett *401*: 78-82, 1997.

9 Violi F, Pignatelli P and Pulcinelli FM: Synergism among flavonoids in inhibiting platelet aggregation and H_2O_2 production. Circulation *105*: e53, 2002.

10 Murota K and Terao J: Antioxidative flavonoid quercetin: implication of its intestinal absorption and metabolism. Arch Biochem Biophys *417*: 12-7, 2003.

11 Day AJ, Bao Y, Morgan MRA and Williamson G: Conjugtion position of quercetin glucuronides and effect on biological activity. Free Radical Biol Med *29*: 1234-1243, 2000.

12 O'Leary KA, Day AJ, Needs PW *et al*: Flavonoid glucuronides are substrates for human liver β-glucuronidase. FEBS Lett *503*: 103-106, 2001.

13 Yang K, Lamprecht SA, Liu Y *et al*: Chemoprevention studies of the flavonoids quercetin and rutin in normal and azoxymethane-treated mouse colon. Carcinogenesis *21*: 1655-1660, 2000.
14 Deschner EE, Ruperto J, Wong G *et al*: Quercetin and rutin as inhibitors of azoxymethanol-induced colonic neoplasia. Carcinogenesis *12*: 1193-1196, 1991.
15 Verma AK, Johnson JA, Gould MN *et al*: Inhibition of 7,12 dimethylbenz(a)anthracene- and N-nitrosomethylurea-induced rat mammary cancer by dietary flavonol quercetin. Cancer Res *48*: 5754-5758, 1988.
16 Knowles LM, Zigrossi DA, Tauber RA *et al*: Flavonoids suppress androgen-independent human prostate tumor proliferation. Nutr Cancer *38*: 116-22, 2000.
17 So FV, Guthrie N, Chambers AF *et al*: Inhibition of proliferation of estrogen receptor positive MCF-7 human breast cancer cells by flavonoids in the presence and absence of excess estrogen. Cancer Lett *112*: 127-133, 1997.
18 Elattar TM and Virji AS: The inhibitory effect of curcumin, genistein, quercetin and cisplatin on the growth of oral cancer cells *in vitro*. Anticancer Res *20*: 1733-8, 2000.
19 Assaum J, Matsuzaki H, Kuroda M, Takeda Y, Kishi K and Hiraki Y: Effects of quercetin on the cell growth and the intracellular accumulation and retention of adriamycin. Anticancer Res *20*: 2477-2483, 2000.
20 Yoshida M, Sakai T, Hosokawa N *et al*: The effect of quercetin on cell cycle progression and growth of human gastric cancer cells. FEBS Lett *260*: 10-13, 1990.
21 Agullo G, Gamet L, Besson C *et al*: Quercetin exerts a preferential cytotoxic effect on active dividing colon carcinoma HT29 and Caco-2 cells. Cancer Lett *87*: 55-63, 1994.
22 Hosokawa N, Hosokawa Y, Sakai T *et al*: Inhibitory effect of quercetin on the synthesis of a possibly cell-cycle-related 17-kDa protein, in human colon cancer cells. Int J Cancer *45*: 1119-1124, 1990.
23 Ranelletti FO, Ricci R, Larocca LM *et al*: Growth-inhibitory effect of quercetin and presence of type-II estrogen-binding sites in human colon-cancer cell lines and primary colorectal tumors. Int J Cancer *50*: 486-492, 1992.
24 Lee SC, Kuan CY, Yang CC and Yang SD: Bioflavonoids commonly and potently induce tyrosine dephoshorylation/ inactivation of oncogenic proline-directed protein kinase FA in human prostate cancer cells. Anticancer Res *18*: 1117-1121, 1998.
25 Weber G, Shen F, Prajda N, Yeh YA, Yang H, Herenyiova M and Look KY: Increased signal transduction activity and down-regulation in human cancer cells. Anticancer Res *16*: 3271-3282, 1996.
26 Casagrande F and Darbon JM: Effects of structurally related flavonoids on cell cycle progression of human melanoma cells: regulation of cyclin-dependent kinases CDK2 and CDK1. Biochem Pharmacol *61*: 1205-15, 2001.
27 Pignatelli P, Pulcinelli FM, Celestini A *et al*: The flavonoids quercetin and catechin synergistically inhibit platelet function by antagonising the intracellular production of hydrogen peroxide. Am J Clin Nutr *72*: 1150-1155, 2000.
28 Chan MM, Mattiacci JA, Hwang HS *et al*: Synergy between ethanol and grape polyphenols, quercetin, and resveratrol, in the inhibition of the inducible nitric oxide synthase pathway. Biochem Pharmacol *60(10)*: 1539-48, 2000.
29 Koren R, Rocker D, Kotestiano O *et al*: Synergistic anticancer activity of 1,25-dihydroxyvitamin D(3) and immune cytokines: the involvement of reactive oxygen species. J Steroid Biochem Mol Biol *73(3-4)*: 105-12, 2000.
30 Lonn E, Yusef S, Hoogwerf B *et al*: Effects of Vitamin E on cardiovascular and microvascular outcomes in high-risk patients with diabetes: results of the HOPE study and the MICRO-HOPE substudy. Diabetes Care *25*: 1919-1927, 2002.
31 Omenn GS, Goodman GE, Thornquist MD *et al*: Effects of a combination of beta-carotene and Vitamin A on lung cancer and cardiovascular disease. N Engl J Med *334*: 1150-1155, 1996.
32 Michalczyk AA, Allen J, Blomeley RC *et al*: Constitutive expression of hZnT4 zinc transporter in human breast epithelial cells. Biochem J *364(Pt 1)*: 105-13, 2002.
33 Chan MM, Mattiacci JA, Hwang HS *et al*: Synergy between ethanol and grape polyphenols, quercetin and resveratrol in the inhibition of the inducible nitric oxide synthase pathway. Biochem Pharmacol *60*: 1539-48, 2000.
34 Nho CW and Jeffery E: The synergistic upregulation of phase II detoxification enzymes by glucosinolate breakdown products in cruciferous vegetables. Toxicol Appl Pharmacol *174*: 146-152, 2001.
35 Kuo SM: Antiproliferative potency of structurally distinct dietary flavonoids on human colon cancer cells. Cancer Lett *110*: 41-48, 1996.
36 Chang WS, Lee YJ Lu FJ and Chiang HC: Inhibitory effects of flavonoids on xanthine oxidase. Anticancer Res *13*: 2165-2170, 1993.

Received September 17, 2004
Accepted December 20, 2004