Reduction of rat prostate weight by combined quercetin–finasteride treatment is associated with cell cycle deregulation

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Abstract

Benign prostate hyperplasia and prostate cancer are major public health problems. We report herein that daily treatment of male rats with 50, 100 or 150 mg quercetin per kg body weight resulted in serum concentrations of quercetin equivalent to 25·3 μM, 43·3 μM and 54·3 μM respectively. Concomitantly, serum testosterone levels were increased by 1·79-, 1·83- and 3·48-fold, while serum dihydrotestosterone (DHT) levels were 125%, 92% and 73% of the control. A slight increase in prostate weight coupled with dilated prostate lumens full of secretory materials were observed. Finasteride alone caused a significant decrease in serum DHT level and prostate weight. Co-administration of quercetin with finasteride prevented the finasteride-induced decrease in serum DHT levels but significantly enhanced the reduction in wet prostate weight, which was reduced by 26·9% in finasteride-treated animals to 31·8%, 40·0% and 48·2% after fina-steride given together with the three doses of quercetin. The combined treatment altered cell cycle-regulated proteins in a wide spectrum. The expressions of cyclin D1, CDK-4, cdc-2 and phospho-cdc-2 at tyrosine 15, phospho-MEK1/2, phospho-MAP kinase, phospho-pRb at serine 780 and serine 807/811 were significantly inhibited, while the levels of p15, p21 and p27 were increased. In conclusion, quercetin–finasteride treatments caused wide cell cycle deregulation in rat prostates, which, in turn, decreased the proliferation rate, changed the secretion activities of epithelial cells and resulted in a marked reduction in wet prostate weight. The results suggest that quercetin synergizes with finasteride to reduce the wet prostate weight through a cell cycle-related pathway, which may be androgen independent.

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Introduction

Benign prostate hyperplasia (BPH) and prostate cancer are major public health problems (Stoner 1994). The aim of palliative treatment of BPH or prostate cancer is to downregulate the levels of circulating androgen or to block the transcription, activation and function of the androgen receptor, or both. Finasteride acts as a competitive and specific inhibitor of 5α-reductase, resulting in suppression of serum and intraprostatic dihydrotestosterone (DHT) concentrations to castrated levels, with subsequent reduction in prostatic size (The Finasteride Study Group 1993, Rittmaster 1994). Finasteride is approved for treatment of symptomatic BPH (Feigl *et al.* 1995). The only clinically significant side effects of finasteride are related to sexual function such as decreased libido and impotence (Stoner 1994).

Androgen plays a critical role in the growth, maintenance and function of the normal prostate gland (Cunha *et al.* 1987). Androgen-deprivation therapy causes marked and characteristic changes in normal prostate and in prostate cancer (Murphy *et al.* 1991, Ferguson *et al.* 1994).

The biological function of androgen in the prostate is mediated by the androgen receptor (AR). Activation of the AR leads to complex proliferative, apoptotic and angiogenic events, which are mediated by interaction with a series of co-activators and a smaller subset of co-repressors (Lu *et al.* 1999, Gregory *et al.* 2001, Petre *et al.* 2002). The mitotic signal of androgens is thought to target ultimately the cell cycle machinery (Yamamoto *et al.* 2000). Androgen stimulates the expression of the cell cycle genes CDK-1, CDK-2 and CDK-4 and increases the levels of cyclin A and cyclin B1 mRNAs (Lu *et al.* 1997, Gregory *et al.* 2001). The cyclin-dependent kinase (CDK) kinase activities can be further increased by repressing the expression of CDK inhibitor p16 gene (Lu *et al.* 1997), overexpressing phosphatase cdc25B (Ngan *et al.* 2003) or binding to cyclin E (Akita *et al.* 2001). Cyclin E has been reported to associate with AR to potentiate its activity in prostate cancer (Yamamoto *et al.* 2000). Although studies have also established the functional link of AR with cyclin D1 (Petre *et al.* 2002) and p21 (Lu *et al.* 1999), the multiple roles they play in cell proliferation, differentiation, and apoptosis are still intriguing. Cyclin D1 may

play a mitogenic role (CDK-4-dependent) and an anti-mitogenic role (dependent on regulation of the AF-1 domain) that can collectively control the rate of androgen-dependent cellular proliferation (Petre *et al.* 2002).

On the other hand, epidemiological studies have shown that the consumption of vegetables, fruit and tea is associated with a low risk of cancer (Steinmetz & Potter 1991, Yang & Wang 1993). Quercetin, one of the most common flavonoid glycones, has a wide range of biological activities including inhibition of protein kinase C (Agullo *et al.* 1997), tyrosine kinase (Akiyama *et al.* 1987), phosphatidylinositide 3-kinase (PI-3 kinase) (Agullo *et al.* 1997) and DNA topoisomerase II (Constantinou & Huberman 1995). Importantly, quercetin has antiproliferative activity *in vitro* against several cancer cells by inhibiting the expression of cyclin A (Yoshida *et al.* 1992), cyclin B1 (Choi *et al.* 2001), cyclin D1 (Kaneuchi *et al.* 2003), cdc2 (Choi *et al.* 2001, Yoshida *et al.* 1992) and CDK-4 (Bhatia *et al.* 2001). Treatment with quercetin arrests cell cycle progression either at the G1/S phase (Bhatia *et al.* 2001) or at the G2/M transitional boundary (Choi *et al.* 2001). *In vivo* synergy of quercetin with cisplatin against a lung cancer xenograft has been reported (Hofmann *et al.* 1988). Quercetin also inhibits cell invasion and induces apoptosis through a pathway involving heat shock proteins (Wei *et al.* 1994). Although the mechanisms of the antiproliferation effects of quercetin remain to be illustrated, there is evidence suggesting that the action of quercetin is probably mediated by interaction with the type II estrogen binding sites (Ranelletti *et al.* 1992) or the aryl hydrocarbon receptor (Ashida *et al.* 2000). These activities of quercetin make it a promising candidate for the treatment and prevention of various cancers including prostate cancer.

The cell cycle-regulated proteins are considered to be the common downstream effectors mediating the effects of finasteride and quercetin in the prostate gland. In this study, we report that quercetin when co-administered with finasteride prevented finasteride-induced changes in serum DHT levels, but caused reduction in wet prostate weight. The combined effects of quercetin and finasteride on wet prostate weight were associated with their ability to modulate the expression of cell cycle-regulated proteins. The expressions of cyclin D1, CDK-4, cdc-2 and phospho-cdc-2 at tyrosine 15, phospho-mitogen extracellular kinase 1/2 (MEK1/2), phospho-MAP kinase (MAPK), phospho-pRb at serine 780 and serine 807/811 were significantly down-regulated while the levels of p15, p21 and p27 were increased. The combined treatment also significantly reduced the ratio of hyperphosphorylated pRb to total pRb.

Materials and Methods

Reagents

Rabbit anti-phospho MEK1/2 (Ser217/221), mouse anti-phospho p44/42 MAP kinase (Thr202/Tyr204), rabbit anti-cdc-2, rabbit anti-phosphorylated cdc-2 (Tyr15), mouse anti-retinoblastoma (pRb), rabbit anti-phosphorylated pRb (Ser780), rabbit anti-phosphorylated pRb (Ser795), rabbit anti-phosphorylated pRb (Ser807/811) and rabbit anti-phospho-Akt-1 (Ser473) antibodies were purchased from New England BioLabs (Beverly, MA, USA). Rabbit anti-ERK-1, rabbit anti-p85 subunit of PI-3 kinase, mouse anti-α-tubulin, rabbit anti-cyclin A, rabbit anti-epidermal growth factor receptor (EGFR), rabbit anti-Raf, rabbit anti-pRaf, rabbit anti-cyclin B1, mouse anti-cyclin E, mouse anti-cyclin D1, mouse anti-CDK-2, rabbit anti-CDK-4, mouse anti-CDK-6, mouse anti-p21, mouse anti-p27 and mouse anti-p15 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse anti-Ki-67 was from NeoMarkers Inc. (Fremont, CA, USA). Anti-α-tubulin antibody was used at a final concentration of 0·5 μg/ml. Other antibodies were diluted into Tris–buffered saline Tween 20 (TBST) solution at a final concentration of 1 μg/ml, as recommended by the manufacturers.

Animals

Animals were maintained and treated according to the guidelines of the Local Animal Care Committee. Ten-week-old male Sprague-Dawley rats, provided by the Animal Holding Unit, National University of Singapore, were divided into 8 groups ($n=8$). To test the effect of quercetin on rat prostate, rats were daily gavaged with quercetin (Sigma, St Louis, MO, USA), dissolved in 5% dimethyl sulfoxide (DMSO) aqueous solution, at a dose of 50, 100 or 150 mg per kg body weight (BW). To block the conversion of testosterone to DHT, one group of rats was treated daily with finasteride 1 mg/kg BW (5 mg/tablet, Merck Frost, Quebec, Canada) by gavage. We have previously reported that at this dose the weight of the prostate was reduced to 80% of the control, while the DHT level was decreased to 39·5% and the testosterone level was increased 2·4-fold as compared with the control (Huynh *et al.* 1998). To investigate the combined effects of quercetin and finasteride on prostate weight and serum androgen levels, rats were treated daily with 1 mg finasteride/kg BW plus 50, 100 or 150 mg quercetin/kg BW. Control rats received the same dose of vehicles. To determine the concentration of quercetin in the serum, the rats were immobilized in a restraint device at 4 h post drug administration and the blood was collected with a 23-gauge needle after the tail vein was slightly enlarged by mopping with 70% alcohol. After 10 days of treatment, animals were weighed and killed, and wet prostate, pancreas, liver, kidney, testis and serum were collected. After weighing, a portion of the prostate, together with other tissues, was fixed in 10% buffered formalin for paraffin embedding; the remaining tissue was immediately frozen in liquid nitrogen for further analysis.

Table 1 Effects of quercetin (Q; 50, 100, 150 mg/kg), finasteride (F; 1 mg/kg) and finastride plus quercetin (FQ) on the body weight, wet prostate weight, serum testosterone and dihydrotestosterone (DHT) levels of rats (means ± s.e.m., $n=8$)

	Body weight (g)	Wet prostate weight (mg)	Serum testosterone (ng/ml)	Serum DHT (pg/ml)
Treatment				
Control	343·7 ± 7·6	455·0 ± 15·5[a]	1·79 ± 0·29[a]	227·9 ± 19·9[a,b]
Q50	334·1 ± 6·7	495·7 ± 25·1[a]	3·21 ± 0·67[b]	284·4 ± 25·3[a]
Q100	353·9 ± 5·5	509·8 ± 22·3[a]	3·28 ± 0·87[b]	210·5 ± 27·4[a,b]
Q150	338·7 ± 7·2	474·6 ± 21·4[a]	6·23 ± 1·14[c]	166·5 ± 35·1[b]
F1	343·2 ± 3·7	332·4 ± 19·5[b]	5·65 ± 0·92[c]	118·4 ± 24·3[c]
F1Q50	343·4 ± 3·2	310·3 ± 18·7[b,c]	3·43 ± 0·72[b]	275·2 ± 39·4[a]
F1Q100	348·7 ± 5·6	273·2 ± 17·2[c]	5·16 ± 0·49[c]	205·9 ± 34·2[a,b]
F1Q150	336·0 ± 5·8	235·5 ± 14·8[c]	4·85 ± 0·98[c]	172·1 ± 23·5[b]

Numbers within columns with different superscript letters are significantly different from one another at $P<0·05$ (ANOVA).

Immunohistochemistry and histology

Fixed prostate, pancreas, liver, kidney and testis were routinely processed in a tissue processor and embedded in paraffin. Sections of 5 µm were cut and subjected to immunohistochemical study using the ImmunoCruz Staining System (Lab Vision Corporation, Fremont, CA, USA). Briefly, the slides were deparaffinized, rehydrated gradually through graded alcohols, and incubated in 3% H_2O_2 for 20 min to block endogenous peroxidase activity. The antigens were retrieved by boiling the slides in 10 mM citrate buffer (pH 6·0) for 15 min. After blocking unspecific background with 5% skim milk for 20 min at room temperature, the slides were incubated with mouse anti-Ki-67 antibody overnight at 4 °C. The slides were then incubated with the appropriate biotinylated secondary antibody, followed by peroxidase-conjugated streptavidin complex and diaminobenzidene. The sections were finally counterstained with hematoxylin. The slides were examined under an Olympus Provis microscope (BX60, Olympus Optical Corp., Toyota, Japan) and images were captured digitally with the Olympus DP11 camera (Olympus Optical Corp.). The Ki-67 labeling index was expressed as the percentage of clearly labeled Ki-67 reactive nuclei in total epithelial cells in randomly selected fields at a magnification of × 200 ($n=6$).

Quantitation of apoptosis

Sections of 5 µm were used to quantitate apoptosis in prostate tissues. Fragmented DNA was labeled using the ApoAlert DNA fragmentation assay (Clontech Laboratories, Palo Alto, CA, USA) which is based on the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) as described (Nickerson *et al.* 1998). Labeling indices were obtained by counting the number of labeled cells among at least 100 epithelial cells per region and were expressed as percentage values.

Serum androgen measurement

Serum testosterone and DHT levels were determined using their respective enzyme-linked immunosorbent assay (ELISA) kits (IBL Immuno-Biological Laboratories, Hamburg, Germany) as described by the manufacturer. Briefly, an unknown amount of antigen present in the sample and a fixed amount of enzyme-labeled antigen competed for the binding sites of the antibodies coated onto the wells. After incubation, the wells were washed to stop the competition reaction and the tetramethyl-benzidine (TMB) substrate solution was added. The measured optical density of the standards was used to construct a calibration curve against which the concentrations of the unknown samples were calculated. The concentration of antigen present in the samples was inversely proportional to the optical density measured at a wavelength of 450 nm.

Determination of the concentration of total quercetin metabolites in serum

After ingestion of quercetin, the major circulating metabolites in rat blood are glucurono-sulfo conjugates of isorhamnetin and of quercetin (Morand *et al.* 1998). Quercetin, quercetin glycosides, quercetin glucuronides and quercetin sulfates were hydrolyzed to their aglycones by incubating 180 µl rat serum, acidified to pH 4·9 with 20 µl 0·58 M acetic acid solution, for 30 min at 37 °C in the presence of 10 µl enzyme mixture (5×10^6 U/l β-glucuronidase and $2·5 \times 10^5$ U/l sulfatase). The aglycones were then extracted with 500 µl methanol/HCl (200 mM) followed by centrifugation (14 000 *g* for 10 min) (Morand *et al.* 2000). The concentrations of aglycones were determined by a reversed-phase high performance liquid chromatography (RP-HPLC) method in a chromatograph (Waters 2695 Separation Module, Milford, MA, USA) equipped with a Photodiode Array
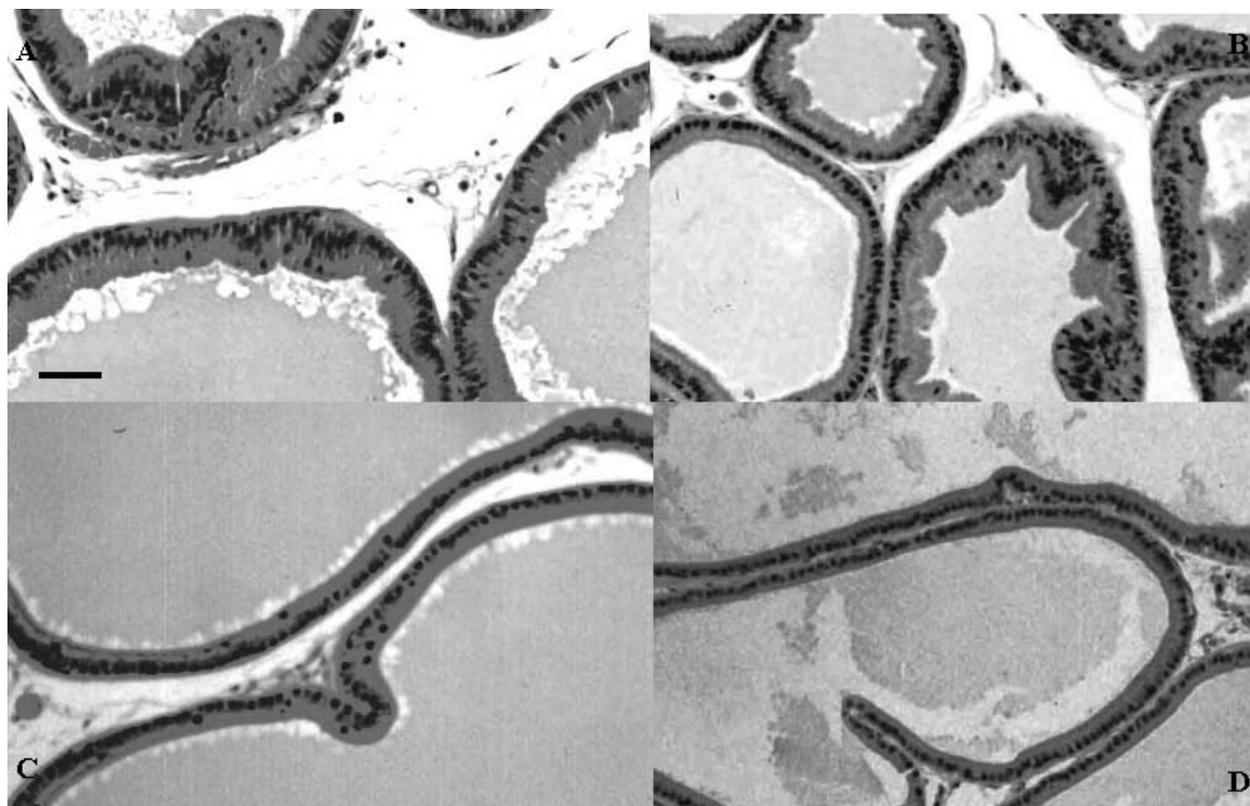
Figure 1 Effects of quercetin, finasteride and finasteride plus quercetin on prostate gland morphology. Rats were treated for 10 days with (A) vehicle, (B) finasteride (1 mg/kg body weight daily), (C) quercetin (150 mg/kg body weight daily), or (D) finasteride plus quercetin. Hematoxylin-eosin stain was used; scale bar=10 μm.

detector (Waters 2996) and a Waters Symmetry column (C_{18}, 5 μm, $3 \cdot 9 \times 150$ mm). The mobile phase consisted of 60% methanol and 40% phosphoric acid solution ($0 \cdot 5\%$). The injection volume, the flow rate and the detection wavelength were 20 μl, $0 \cdot 7$ ml/min and 370 nm respectively. The total quercetin metabolites in serum were determined by summing the concentrations of the aglycones, i.e. isorhamnetin, quercetin and tamarixetin, which were determined against their respective standard curves.

Western blot analysis

To determine the changes in the expression levels of cell cycle-regulated proteins, the frozen rat prostate was homogenized in lysis buffer (1 mM $CaCl_2$, 1 mM $MgCl_2$, 1% NP-40, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μM phenylmethylsulfonyl fluoride, and 100 μM $NaVO_4$). One hundred micrograms proteins were subjected to Western blot analysis as described (Huynh *et al.* 1995). Blots were incubated with the indicated primary antibodies followed by horseradish peroxidase–conjugated donkey antimouse or antirabbit secondary antibody (1:7500). Blots were visualized with a chemiluminescent detection system (Amersham Pharmacia Biotech UK Ltd, UK) as described

by the manufacturer. For quantitative analysis, the density of bands corresponding to the protein blotting with the antibodies under study was calculated with the Quality One software (Bio-Rad Laboratories, Hercules, CA, USA) and was normalized to that of α-tubulin.

Statistical analysis

Comparison between the different groups was performed by one-way ANOVA (SPSS 10, SPSS Inc. Chicago, IL, USA) with the Student's unpaired *t*-test applied for paired comparisons of means. *P* values of $0 \cdot 05$ or less were considered significant, while values of $0 \cdot 01$ or less were considered very significant.

Results

Effects of finasteride and quercetin on the rat body weight and wet prostate weight

Treatment of rats with 50, 100 or 150 mg quercetin/kg for 10 days resulted in an insignificant increase in wet prostate weight regardless of the doses used (Table 1). Finasteride

at a dose of 1 mg/kg body weight caused a significant 26·9% reduction in wet prostate weight as compared with controls ($P < 0.001$) (Table 1). Co-administration of finasteride with 50, 100 and 150 mg quercetin/kg BW decreased the wet prostate weight by 31·8%, 40·0% and 48·2% respectively. There was a linear relationship between the wet prostate weight and the quercetin dose in the co-treated groups ($R^2 = 0.991$), with the most significant effect being observed when finasteride was administered together with 150 mg quercetin ($P < 0.001$). There was, however, no statistically significant change in the body weight of rats.

Effects of finasteride and quercetin on the histology of rat prostate, pancreas, liver, kidney and testis

As shown in Fig. 1A, the alveoli of control prostates were lined with a layer of tall columnar epithelial cells with a high cytoplasm/nuclear ratio. The luminal epithelial cells showed a marked reduction in cytoplasm and their secretory activity became diminished after finasteride treatment for 10 days (Fig. 1B). Compared with the control prostate gland, the lumens of prostate glands derived from finasteride-treated rats were markedly reduced in size (Fig. 1B compared with Fig. 1A). Quercetin treatment, on the other hand, caused a dramatic dilation of the prostate lumen. The lumens were filled with secretory materials (Fig. 1C), indicating that quercetin enhanced the secretory activity of epithelial cells. The increase in luminal volume was accounted for by a significant ($P < 0.01$) decrease in the average cell number per unit area, reaching 63% of control rats (Table 2). Co-administration of finasteride and quercetin, however, led to additive effects on the thickness of the prostate epithelium. The cytoplasm was greatly reduced and the epithelial layer became very thin (Fig. 1D). No marked change in the cell morphology or histology was observed in the slides of pancreas, liver, kidney and testis (data not shown).

Effects of finasteride and quercetin on serum androgen levels

Quercetin and finasteride, when administered as a single agent for 10 days, significantly increased serum testosterone levels ($P < 0.001$) after 10 days of treatment (Table 1). As expected, finasteride significantly decreased serum DHT levels to half that of the control rats (118 vs 228 pg/ml, $P < 0.01$) (Table 1). Quercetin treatment, however, resulted in a biphasic change in serum DHT concentration, which was slightly increased at a dose of 50 mg/kg BW, but decreased at a dose of 150 mg/kg BW. Similar effects were seen when quercetin was given together with finasteride. It was noteworthy that the intake of quercetin dampened the effects of finasteride on the levels of both serum testosterone and DHT. Compared

Table 2 Effects of quercetin (Q; 50, 100, 150 mg/kg), finasteride (F; 1 mg/kg) and finastride plus quercetin (FQ) on the prostate epithelial thickness, lumen diameter, cell numbers per selected field and Ki-67 indices (means ± s.e.m., $n \geq 8$)

	Control	Q50	Q100	Q150	F1	F1Q50	F1Q100	F1Q150
Epithelial thickness (µm)	6·1 ± 1·5[a]	5·1 ± 1·2[b]	4·5 ± 1·3[b]	4·7 ± 1·1[b]	4·7 ± 1·4[b]	5·0 ± 1·3[b]	4·1 ± 1·1[b]	4·0 ± 1·2[b]
Lumen diameter (µm)	66·8 ± 11·3[a]	85·5 ± 13·1[a,b]	95·7 ± 21·0[b]	114·4 ± 27·6[b]	35·9 ± 13·4[c]	72·3 ± 15·8[a]	79·8 ± 16·6[a,b]	97·6 ± 19·4[a,b]
Cell number	1330 ± 230[a]	1319 ± 194[a]	1077 ± 261[a,b]	845 ± 170[b]	1539 ± 345[a]	1133 ± 206[a,b]	1011 ± 196[a,b]	1068 ± 134[a,b]
Ki-67 index (%)	1·50 ± 0·31[a]	1·67 ± 0·28[a]	2·59 ± 0·47[b]	2·66 ± 0·68[b]	0·19 ± 0·13[c]	0·12 ± 0·09[c]	0[d]	0·02 ± 0·01[d]

Numbers within columns with different superscript letters are significantly different from one another at $P < 0.05$ as determined by one-way ANOVA.
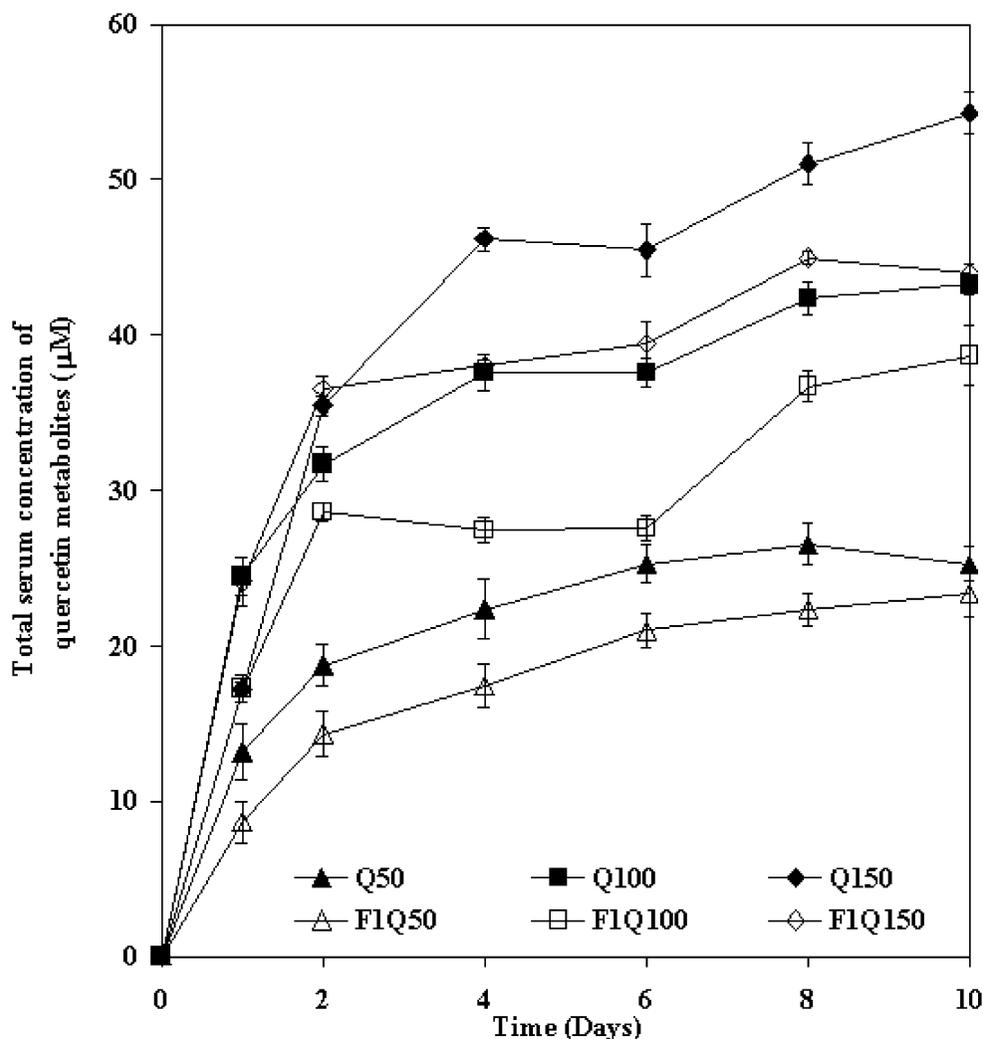
Figure 2 Total serum concentrations of quercetin metabolites 4 h after ingestion of quercetin in the presence or absence of finasteride (mean ± S.E.M., *n*=8). Rats were treated as described in Table 1. The concentrations of quercetin metabolites at a given time were determined by the RP-HPLC method as described in Materials and Methods and were plotted against time. Q, quercetin; F, finasteride.

with finasteride alone, the combined treatment increased serum DHT levels by 2·32-, 1·74- and 1·45-fold at doses of 50, 100 and 150 mg quercetin/kg BW respectively.

Concentration of quercetin metabolites in serum

To determine the total serum concentrations of quercetin and its metabolites, serum-derived from quercetin-treated rats was treated with β-glucuronidase and sulfatase which reduced quercetin metabolites to their aglycones i.e. isorhamnetin, quercetin and tamarixetin. The levels of these aglycones were measured by the RP-HPLC method. As shown in Fig. 2, a dose-dependent elevation in serum quercetin metabolites was observed. The levels of serum quercetin metabolites reached a plateau on the sixth

day. The serum concentration of quercetin metabolites was 25·3 µM, 43·3 µM and 54·3 µM after 10 days of treatment with 50 mg, 100 mg and 150 mg quercetin/kg BW respectively. Co-administration of quercetin with finasteride caused a significant decline in serum quercetin metabolite concentrations ($P < 0.01$) (Fig. 2). This effect was observed for all the three tested quercetin regimens.

Effects of quercetin and finasteride on prostate apoptosis and proliferation

The TUNEL assay and Ki-67 indices were performed on prostate sections to determine whether the apoptosis or cell cycle arrest of prostate epithelial cells contributed to the reduction in wet prostate weight caused by combined
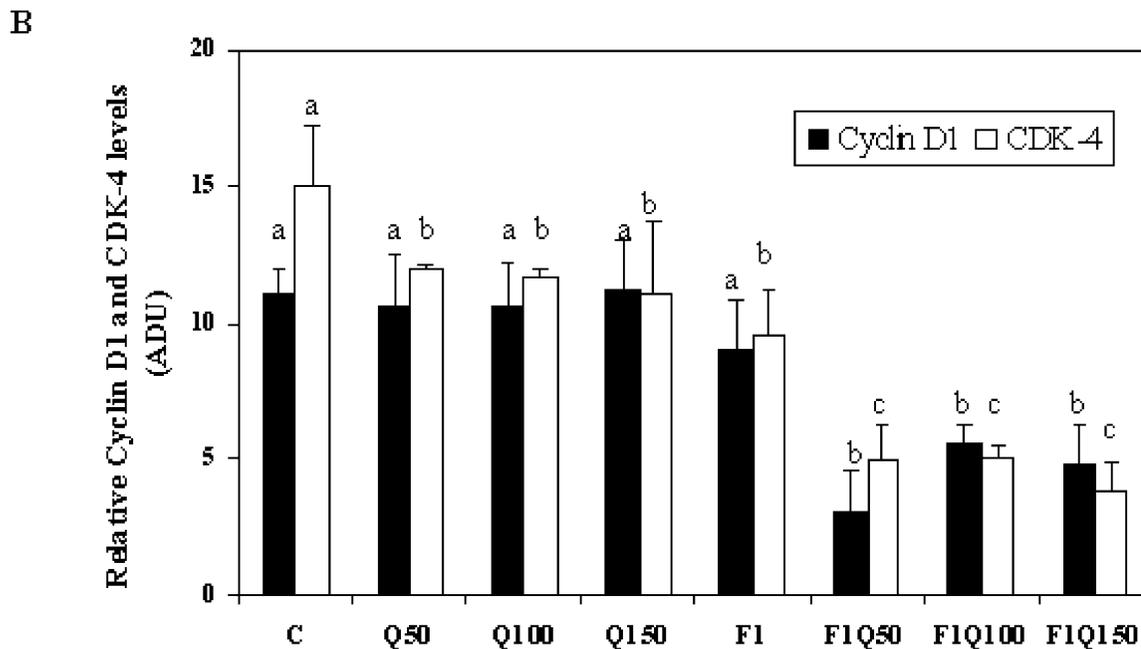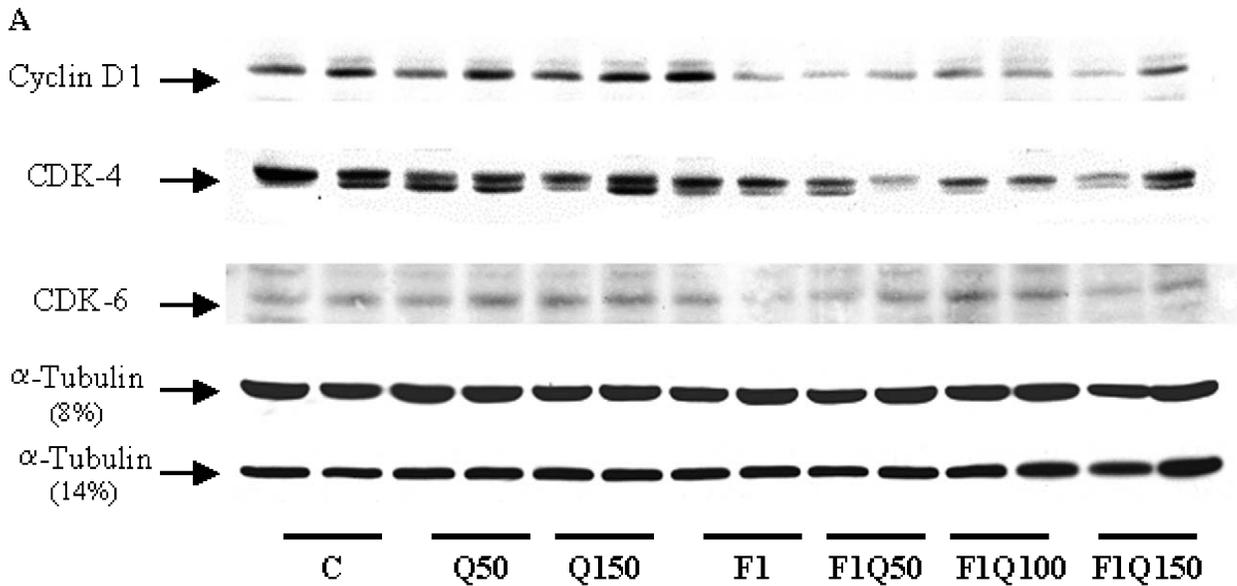
A

Cyclin D 1 ➔

CDK-4 ➔

CDK-6 ➔

α-Tubulin ➔
(8%)

α-Tubulin ➔
(14%)

C   Q50   Q150   F1   F1Q50   F1Q100   F1Q150

B

Figure 3 Effects of quercetin (Q), finasteride (F) and finasteride plus quercetin (FQ) on the expression of cyclin D1 and CDK4/CDK-6 in the prostate gland. Treatments were the same as those described in Table 1. Tissue lysates (100 μg protein/lane) were subject to Western blot analysis as described in Materials and Methods. Blots were incubated with mouse anti-cyclin D1, rabbit anti-CDK-4, mouse anti-CDK-6 and mouse anti-α-tubulin antibodies. Representative blots are shown in (A). Densitometric scanning of the cyclin D1 and CDK-4 bands after being normalized to the levels of α-tubulin is shown in (B). Data are expressed as the mean of 8 samples ± S.E.M. Bars with different letters are significantly different from one another at $P < 0.05$ as determined by one-way ANOVA. ADU, arbitrary densitometric units. (C), control.

quercetin–finasteride treatment. Apoptosis was increased from $0.3 \pm 0.2\%$ in the control group to $1.1 \pm 0.7\%$ in finasteride–treated groups. The apoptotic rate in quercetin–finasteride treatments was $1.3 \pm 0.8\%$. The

results indicated no significant changes in apoptosis between finasteride and quercetin–finasteride treatments. The Ki–67 index of prostate epithelial cells in the control group was 1.5% and that was significantly decreased by
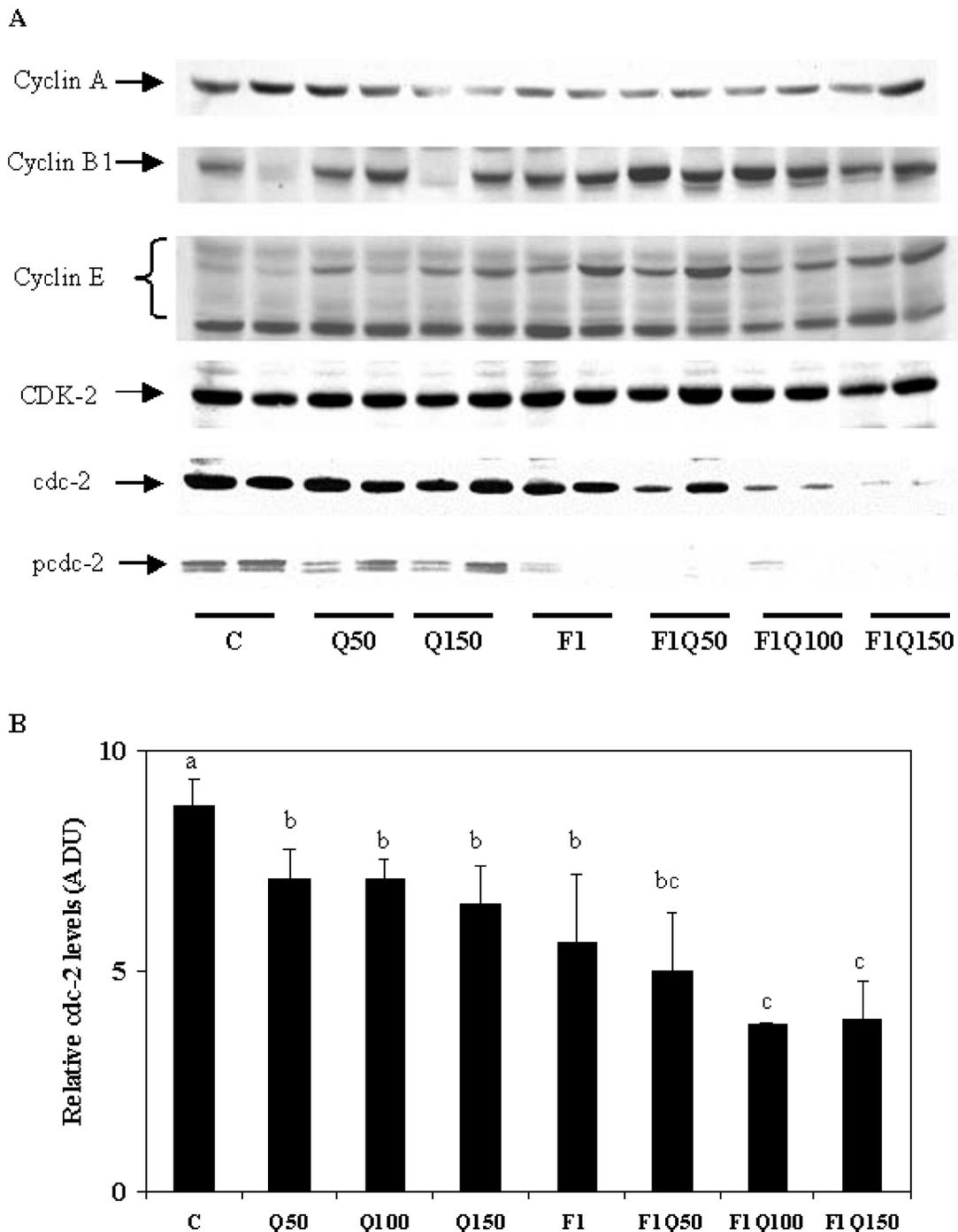
Figure 4 Effects of quercetin (Q), finasteride (F) and finasteride plus quercetin (FQ) on the expression of cyclin A, cyclin B1, cyclin E, CDK-2 and cdc-2 in the prostate gland. Rats were treated as described in Table 1. Tissue lysates (100 µg protein/lane) were subject to Western blot analysis as described in Materials and Methods. Blots were incubated with rabbit anti-cyclin A, rabbit anti-cyclin B1, mouse anti-cyclin E, mouse anti-CDK-2, rabbit anti-cdc-2 and rabbit anti-phosphorylated (p) cdc-2 (Tyr15) antibodies. Representative blots are shown in (A). Densitometric scanning of the cyclin cdc-2 bands after being normalized to the levels of α-tubulin is shown in (B). Data are expressed as the mean of 8 samples ± S.E.M. Bars with different letters are significantly different from one another at $P < 0.05$ as determined by one-way ANOVA. ADU, arbitrary densitometric units. (C), control.

finasteride or combined treatments ($P < 0.05$) (Table 2). On the other hand, quercetin significantly increased the Ki-67 index at doses of 100 and 150 mg/kg BW.

Effects of quercetin and finasteride on cyclin D/CDK-4/6

The major rate limiting step in the mammalian cell cycle is the transition of G1 to S phase, which is initiated by the activation of cyclin D and its association to CDK-4 and CDK-6 (Coqueret 2002); therefore the *in vivo* effects of quercetin and finasteride on cyclin D1 and CDK-4/6 were examined. As shown in Fig. 3, quercetin alone had no effect on the levels of cyclin D1. Finasteride, at a dose of 1 mg/kg BW, reduced the cyclin D1 and CDK-4 levels by 19% and 27% respectively. Co-administration of quercetin and finasteride resulted in an approximately 50% to 70% reduction in the expression of cyclin D1 and CDK-4 respectively ($P < 0.05$). Quercetin and finasteride when given alone or in combination did not alter the expression of CDK-6.

Effects of quercetin and finasteride on other cyclins and CDKs

In order for the cells to enter into the S phase of the cell cycle, activation of cyclin E is required. Activated cyclin E binds to and activates CDK-2 (Sherr 1996). Cyclins A and B are then combined with CDK-2 and cdc-2 to induce cell cycle progression. Because cyclin E is required for CDK-2 activation and cyclins A and B are required for cell cycle progression through S and into M phase (Sherr 1996), the levels of cyclin A, cyclin B1, cyclin E, CDK-2, cdc-2 and phosphorylated (p) cdc-2 were determined. As shown in Fig. 4, cyclin E appeared as two bands at approximately 50 kDa and 42 kDa. Quercetin or fina-steride alone caused a slight increase in the 50 kDa band without significantly affecting the total cyclin E levels. Co-administration of quercetin and finasteride resulted in a decrease in the 42 kDa band while elevating the 50 kDa band. Cyclin A expression was downregulated to 23·4% and 40·5% of the control by finasteride and the 150 mg/kg dose of quercetin respectively. No further decrease in cyclin A levels was observed when quercetin and fina-steride were given together. The levels of CDK-2, the common catalytic partner of cyclin E and cyclin A, did not differ among all treatment groups. For the cdc-2 kinase–cyclin B complex, while the cyclin B1 levels were increased by 1·8-fold, the cdc-2 expression was decreased by approximately 50% by finasteride as compared with controls (Fig. 4). Quercetin alone produced only a moderate effect on cdc-2 expression. Finasteride and quercetin acted synergistically to reduce both cdc-2 and its phosphorylation at tyrosine 15.

Effects of quercetin and finasteride on cyclin-dependent kinase inhibitors (CKIs)

Because the activity of CDKs can be negatively regulated by CKIs (Vidal & Koff 2000, Lee & Yang 2001), the

effects of quercetin, finasteride and their combination on the levels of p15, p16, p21 and p27 were investigated. As shown in Fig. 5, although the expression of p15 was significantly down-regulated by finasteride and quercetin alone, the levels of p15 were significantly elevated when finasteride was given together with the two higher doses of quercetin (100 and 150 mg/kg BW) ($P < 0.05$). In contrast, quercetin alone caused a significant increase in p21 levels ($P < 0.05$). Although finasteride alone made no contribution to the expression of p21, co-administration of quercetin with finasteride caused a significant increase in the level of p21 ($P < 0.05$). Similarly, the combined treatment also augmented the expression of p27. No difference in the expression of p16 was observed (data not shown).

Effects of quercetin and finasteride on pRb and its phosphorylation

It has been reported that pRb negatively regulates the cellular G1/S transition of the proliferative cell cycle (Adams 2001) and cyclin D1/CDK-4 preferentially phos-phorylates pRb at serine 780 (Kitagawa *et al.* 1996) and serine 795 (Pan *et al.* 1998, Grafstrom *et al.* 1999). The abundance of hypophosphorylated pRb and its phos-phorylation on serine 780, serine 795 and serine 807/811 in quercetin-, finasteride- and quercetin–finasteride-treated prostates was examined using pRb and phospho-specific antibodies. As shown in Fig. 6, both quercetin and finasteride and finasteride–quercetin caused a slight increase in total pRb. When the blots were stripped and re-blotted with anti-phospho pRb (Ser780), anti-phospho pRb (Ser795) or anti-phospho pRb (Ser807/811), a significant increase in phosphorylated pRb at serine 780 and serine 807/811 was observed in quercetin- or finasteride-treated samples ($P < 0.01$). Co-treatment of finasteride with quercetin, on the other hand, caused a significant reduction in pRb phosphorylation at serine 780 and serine 807/811. Phosphorylation of pRb at serine 795 was not affected by any of the treatments (data not shown). The relative ratio of total pRb phosphorylation to the total pRb hypophosphorylation was significantly higher in quercetin- and finasteride-treated groups. This ratio was reversed when quercetin was co-administered with finasteride (Fig. 6).

Effects of quercetin and finasteride on the MAPK and PI-3 kinase pathways

It has been demonstrated that growth factors and steroid hormones promote cell cycle progression via the MAP kinase and PI-3 kinase pathways, both of which contribute to the expression and stability of cyclin D1 (Roovers & Assoian 2000, Coqueret 2002) which is known to target pRb. It was reasonable to suppose that downregulation of cyclin D1 following quercetin/finasteride combined
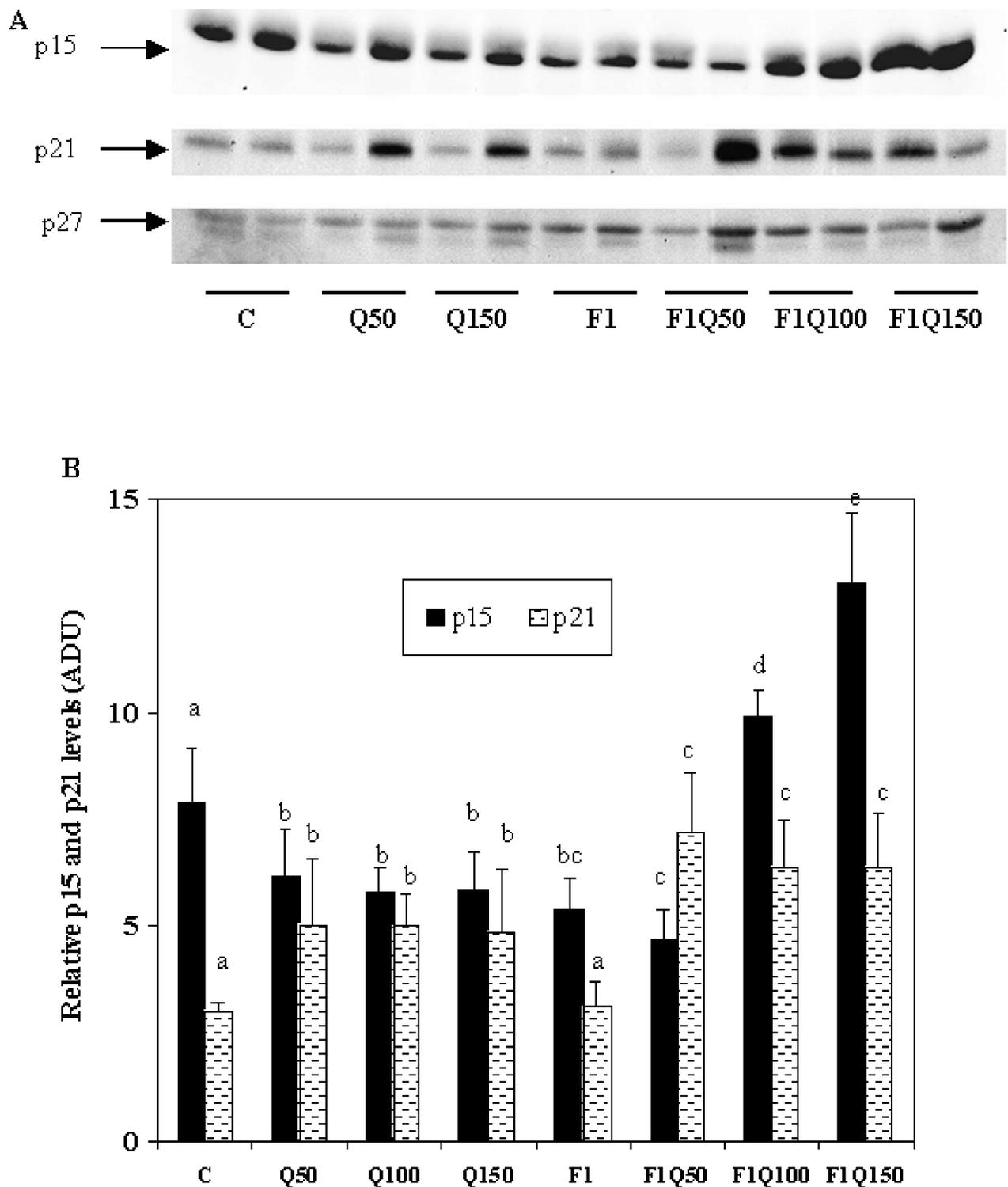
Figure 5 Effects of quercetin (Q), finasteride (F) and finasteride plus quercetin (FQ) on the expression of p15, p21 and p27 in the prostate gland. Rats were treated as described in Table 1. Tissue lysates (100 μg protein/lane) were subject to Western blot analysis as described in Materials and Methods. Blots were incubated with mouse anti-p15, mouse anti-p21 and mouse anti-p27 antibodies. Representative blots are shown in (A). Densitometric scanning of p15 and p21 bands after being normalized to the levels of α-tubulin is shown in (B). Data are expressed as the mean of 8 samples ± S.E.M. Bars with different letters are significantly different from one another at $P < 0.05$ as determined by one-way ANOVA. ADU, arbitrary densitometric units. (C), control.
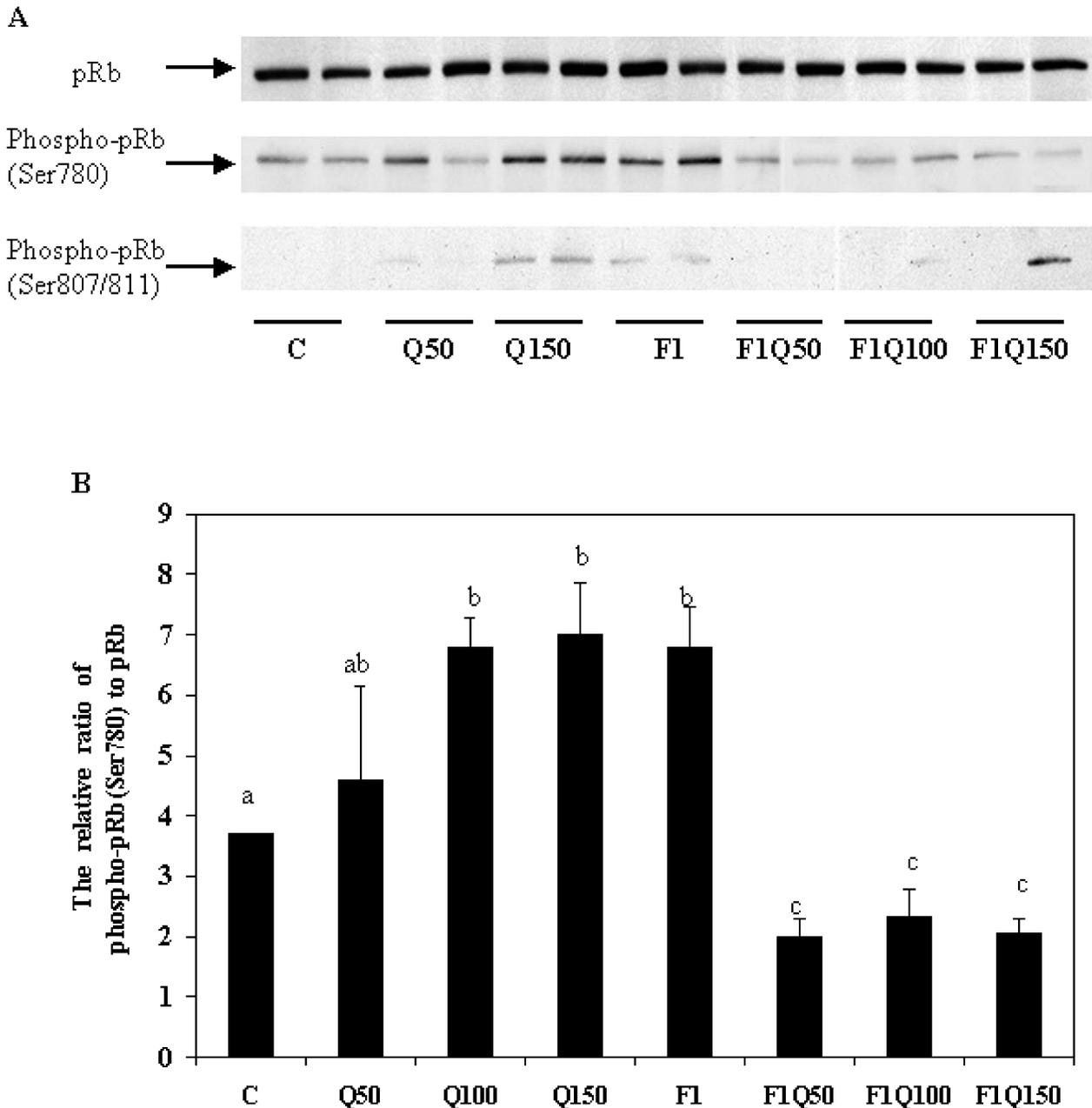
A

B

Figure 6 Effects of quercetin (Q), finasteride (F) and finasteride plus quercetin (FQ) on the expression of pRb and phosphorylated pRb in the prostate gland. Rats were treated as described in Table 1. Tissue lysates (100 μg protein/lane) were subject to Western blot analysis as described in Materials and Methods. Blots were incubated with mouse anti-pRb, rabbit anti-phosphorylated pRb (Ser780) and rabbit anti-phosphorylated pRb (Ser807/811) antibodies. Representative blots are shown in (A). The relative ratio (in arbitrary densitometric units) of the densitometric scanning of hyperphosphorylated pRb (Ser780) bands to pRb bands after being normalized to the levels of α-tubulin is shown in (B). Data are expressed as the mean of 8 samples ± S.E.M. Bars with different letters are significantly different from one another at $P < 0.05$ as determined by one-way ANOVA. (C), control.

treatment might be due to inhibition of MAPK or PI-3 kinase or both. As shown in Fig. 7, quercetin or finasteride alone had a minimal effect on activation of MAPK and p85 subunit of PI-3 kinase as determined by the levels of phosphorylated MAPK and phosphorylated Akt-1

respectively. Co-treatment of quercetin and finasteride led to a significant decrease in phosphorylated MEK1/2, phosphorylated MAPK but not phosphorylated Akt-1, suggesting that quercetin/finasteride preferentially inhibits the activation of MAPK but not PI-3 kinase. The p44 MAPK
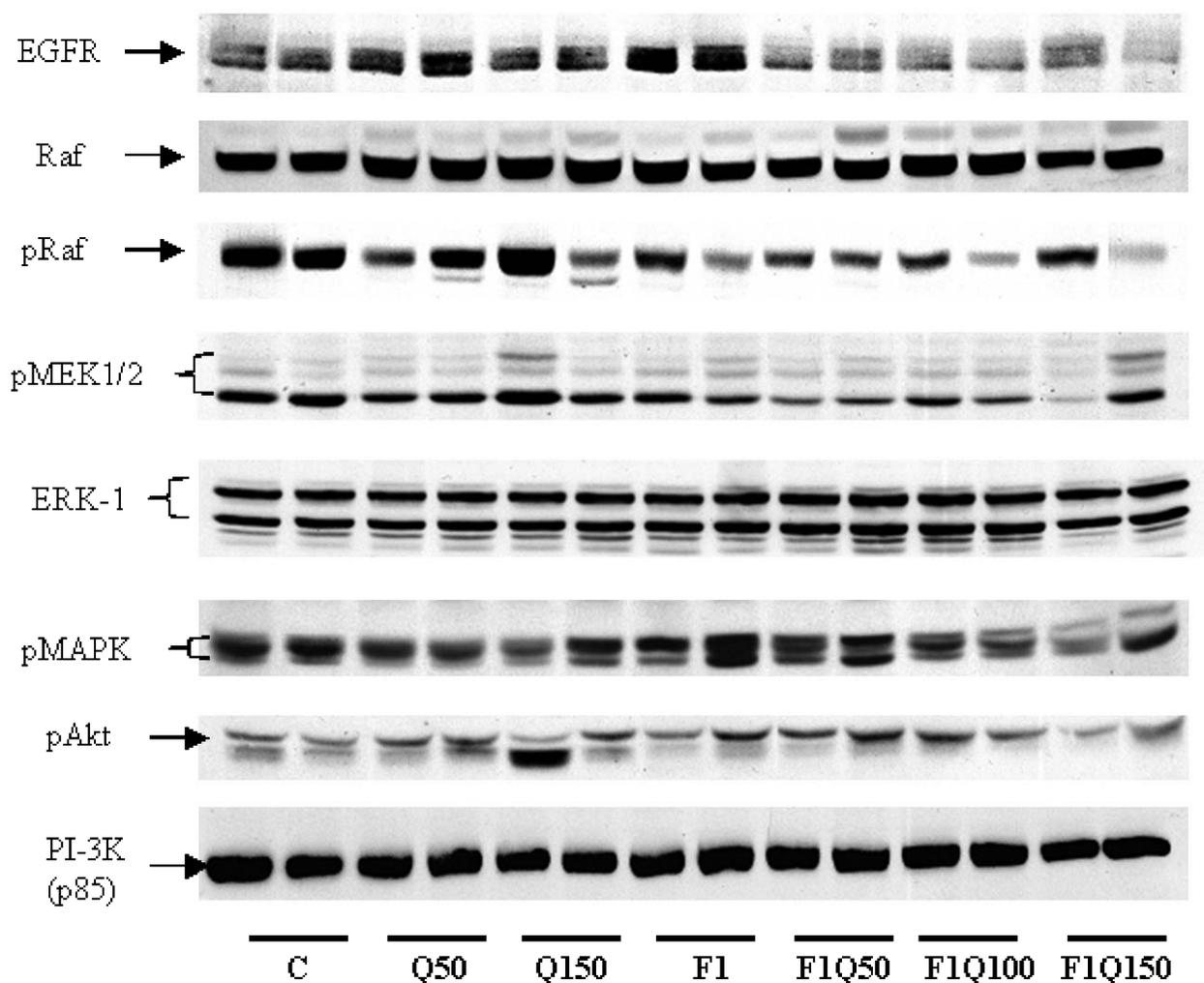
Figure 7 Effects of quercetin (Q), finasteride (F) and finasteride plus quercetin (FQ) on the expression of EGFR, Raf, pRaf, pMEK1/2, ERK-1, pMAPK, pAkt and PI-3 kinase (PI-3K) in the prostate gland. Rats were treated as described in Table 1. Tissue lysates (100 μg protein/lane) were subject to Western blot analysis as described in Materials and Methods. Blots were incubated with rabbit anti-EGFR, rabbit anti-Raf, rabbit anti-pRaf, rabbit anti-phospho MEK1/2 (Ser217/221), mouse anti-ERK-1, mouse anti-phospho p44/42 MAP kinase (Thr202/Tyr204), rabbit anti-phospho-Akt-1 (Ser473) and rabbit anti-p85 subunit of PI-3 kinase antibodies. Representative blots are shown. (C), control.

band showed a gradually faster mobility with an increase in quercetin dose together with finasteride. The upstream proteins of MAPK, e.g. EGFR and pRaf, were also significantly downregulated by the combined treatments (Fig. 7).

Discussion

Epidemiological studies have suggested an association between the low risk of hormone-dependent cancers including prostate cancer and the intake of some phyto-chemicals, a rich source of which are fruits and vegetables (Denis *et al.* 1999). These phytochemicals, depending on their concentrations, showed a mixed agonist/antagonist activity on steroid hormone systems. Quercetin, genistein and resveratrol (Lu & Serrero 1999, Maggiolini *et al.* 2001) could act as an agonist at doses below 1 μM, and as an antagonist at higher doses in MCF-7 cells. This was also the case for quercetin and genistein in LNCaP cells (Denis *et al.* 1999). Furthermore, the cell cycle effect of quercetin was shown to be dose dependent (Choi *et al.* 2001, Kaneuchi *et al.* 2003). Most of the previous studies were focused on the *in vitro* effects of quercetin using cell lines. The *in vivo* effects of this compound remained unknown. Because absorption efficiency and rate of clearance of quercetin *in vivo* was far more complicated than in the *in vitro* cell culture experiments, we conducted these experiments to examine the *in vivo* effects of quercetin

alone or in combination with finasteride on wet prostate weight.

The AR–dependent and –independent effects of phytoestrogens such as quercetin, genistein (Denis *et al.* 1999, Maggiolini *et al.* 2001) and resveratrol (Lu & Serrero 1999) on prostate cancer cell lines have been widely reported. In addition, resveratrol showed both a stimulatory and an inhibitory effect on the AR–positive LNCaP cell line, but only an inhibitory effect on the AR–negative DU145 prostate cancer cell line (Kuwajerwala *et al.* 2002). Compared with finasteride alone, the finasteride–quercetin combined treatment increased serum DHT levels (Table 1). Despite the strong potency of DHT in the prostate, quercetin–finasteride treatments registered a dramatic decrease in wet prostate weight in a dose-dependent manner. This implies that quercetin might synergize with finasteride to activate or block an androgen-independent pathway(s) to regulate the growth and activity of prostate epithelial cells.

In this study, we demonstrated that combined quercetin–finasteride treatments reduced the phosphorylation of Raf, MEK1/2 and MAPK. A wide variety of hormones, growth factors and differentiation factors employ the MAPK pathway. We tested the expression levels of various tyrosine receptor kinases and growth factor receptors and found that EGFR was significantly down-regulated by the combined treatments. The decrease in EGFR might account, at least in part, for the observed decreases in the expression of the phospho-MEK1/2 and phospho-MAPK pathways in the prostates treated with the quercetin–finasteride combination. It is widely accepted that mitogens promote cell cycle progression via the MAPK and PI-3 kinase pathways, both contributing to the expression and stability of cyclin D1 (Roovers & Assoian 2000, Coqueret 2002). Therefore, it was possible that the reduction in MAPK activity resulted in the inhibition of cyclin D1 expression in the combined treatments. Down-regulation of cyclin D1 and its catalytic partner, CDK-4, would prevent the formation of cyclin D/CDK-4 complexes. In this way, the phosphorylation of retinoblastoma protein (pRb) by the cyclin D/CDK-4 complex could be reduced allowing more hypophosphorylated pRb to bind E2F transcription factors and thereby negate E2F activity (Coqueret 2002, Yam *et al.* 2002). Inhibition of cdc-2 expression by quercetin–finasteride treatment would prevent the formation of the cyclin A/cdc-2/CDK2 or the cyclin B/cdc-2/CDK-2 complex, which play an important role in both S and M phase (Yam *et al.* 2002). The undetectable levels of phospho-cdc-2 at Tyr15 might be due to the low levels of cdc-2 in quercetin–finasteride treatment. Among all the cyclins and CDKs examined, only cyclin B1 was found to be up-regulated by the combined treatment. It was noteworthy that there was a minor band which migrated faster than the main cyclin B1 band. Although the status of this band remains to be determined, it could be the dephos-

phorylated form of cyclin B1. It has been reported that phosphorylation of cyclin B1 on five sites was required for cyclin B1 to accumulate in the nucleus and to activate cyclin B–cdc-2 kinase (Peter *et al.* 2002). Furthermore, among the five phosphorylation sites, phosphorylation of Ser94 and Ser96 could not progress without the activity of MAPK (Walsh *et al.* 2003). Considering the electrophoretical mobility pattern of phospho-MAPK, whose mobility was significantly increased with the increased dose of quercetin in the combined treatments, we postulated that although the level of cyclin B1 was increased, it might not be activated due to the inhibition of MAPK activity by the combined treatments. The low molecular weight (LMW) forms of cyclin E, which are predominantly derived from proteolytic processing of the full length cyclin E (50 kDa), are hyperactive and can stimulate the cell to progress through the cell cycle more effectively than does full-length cyclin E (Porter *et al.* 2001). The inhibition on the generation of LMW isoforms would negatively regulate the cellular G1/S transition in the combined groups.

The cell cycle progression was further inhibited by the up-regulation of CKI levels in the combined treatments. The induction of p21, p15 and p27 might be important mechanisms for G_0/G1 and G2/M arrest in mammalian cells. The p21 protein is able to bind cdc2-cyclin B1, cyclin A/CDK-2 and cyclin E/CDK-2 complexes and inhibit their activity (Lu *et al.* 1999). The inhibitory function of p15 in cell cycle progression was to compete with D-type cyclins for binding to the CDK subunits (Vidal & Koff 2000, Lee & Yang 2001). In addition, phosphorylation of pRb at serine 780 was significantly reduced by quercetin–finasteride treatment. Hypophosphorylated pRb sequestered the transcriptional activity of the E2Fs (Adams 2001). Because cyclin D1/CDK-4 preferentially phosphorylates serine 780 (Kitagawa *et al.* 1996) and p15 modulates pRb phosphorylation (Stone *et al.* 1995), up-regulation of p15 or inhibition of cyclin D1 and CDK-4 expression following finasteride–quercetin treatment might, at least in part, be responsible for the inhibition of pRb phosphorylation at serine 780.

In this study, we demonstrated that finasteride–quercetin treatments down-regulated the expression of phospho-MEK1/2 and phospho-MAPK, and decreased the levels of cyclin D1, cdc-2, phosphorylated cdc-2, CDK-2 and CDK-4 and phosphorylated pRb. The combined treatments also led to up-regulation of p15, p21 and p27 and inhibition of pRb phosphorylation at serine 780 and serine 807/811. The deregulation of cell cycle-regulated proteins would change the physiological state of prostate epithelial cells and, therefore, alter the secretion activities of the prostate. In accordance with the phosphorylation levels of pRb, quercetin increased the prostatic secretion activity while quercetin–finasteride treatments inhibited it. Consistent with the altered expression of cell cycle-regulated proteins, the epithelial cell layer of the

prostate became thinner in the combined treatments and the cellular proliferation rate was also dramatically reduced. Thus, the reduction in wet prostate weight following quercetin–finasteride treatments was ascribed to the combination of their effects on epithelial secretion and cellular proliferation, both of which might result from cell cycle deregulation. The combined effects of quercetin and finasteride on cellular proliferation and cell cycle deregulation suggested that quercetin plus a low dose of finasteride may be useful in the treatment of BPH and prostate cancer. In this way, side effects of finasteride related to sexual dysfunction could be reduced or eliminated while its therapeutic goals were retained or even enhanced.

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