Protein expression profiling identifies molecular targets of quercetin as a major dietary flavonoid in human colon cancer cells

Uwe Wenzel, Angelika Herzog, Sabine Kuntz and Hannelore Daniel

Department of Food and Nutrition, Molecular Nutrition Unit, Technical University of Munich, Freising, Germany

A high dietary intake of plant foods is thought to contribute to the prevention of colorectal cancers in humans and flavonoids as part of such a diet are considered to contribute to those protective effects. Quercetin is a major dietary flavonoid consumed with a diet rich in onions, tea, and apples. We used HT-29 human colon cancer cells and investigated the effects of quercetin on proliferation, apoptosis, and differentiation as processes shown to be disregulated during cancer development. To identify the cellular targets of quercetin action, two-dimensional gel electrophoresis was performed and proteins altered in expression level after quercetin exposure of cells were identified by mass spectrometry of peptide fragments generated by tryptic digestion. Quercetin inhibited the proliferation of HT-29 cells with an IC_{50}-value of 81.2 $\pm$ 6.6 μM. Cell differentiation based on surface expression of alkaline phosphatase was enhanced 4-fold and the activity of the pro-apoptotic effector caspase-3 increased 3-fold. Those effects were associated with the regulation of heat-shock proteins and annexins shown to both play a crucial role in the process of apoptosis. Cytoskeletal caspase substrates were found as regulated as well and various proteins involved in intermediary metabolism and in gene regulation showed altered steady-state expression levels upon quercetin treatment of cells. In conclusion, quercetin alters the levels of a variety of proteins involved in growth, differentiation, and apoptosis of colon cancer cells. Their identification as molecular targets of quercetin may explain the anti-cancer activities of this flavonoid.

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1 Introduction

Flavonoids are a class of polyphenolic plant compounds with over 6000 individual structures known [1]. They are recognized as dietary constituents with a variety of biological effects from which some have been associated with protective activity in the development of degenerative diseases, such as atherosclerosis or cancer [2–4]. Although a popular belief is that dietary polyphenols are anti-carcinogens because they possess antioxidative activity [1], direct evidence in support of this relationship is lacking. Based on their multifunctional structures, flavonoids may influence cancer development by a variety of other mechanisms including scavenging effects on activated carcinogens and mutagens [5, 6], actions on proteins that control cell cycle progression [7], or alterations of gene expression [8]. Quercetin (3,3',4',5,7-pentahydroxyflavone) is a major dietary flavonoid occuring mainly in apples, onions, and tea [9], and has been shown to inhibit expression of the cell-cycle promoting tumor marker p21-RAS in human colon cancer cell lines and in primary colorectal tumors [10]. In animals developing aberrant crypt foci by the application of a chemical carcinogen, a diet supplemented with 2% quercetin markedly reduced the development of the initial stages of colorectal cancer development [11].

In spite of these promising actions of quercetin as an anti-cancer agent, the underlying molecular mechanisms by which this flavonoid interferes with the processes of colon cancer development are not completely resolved. More-

Correspondence: Dr. Uwe Wenzel, Department of Food and Nutrition, Molecular Nutrition Unit, Hochfeldweg 2, D-85350 Freising-Weihenstephan, Germany
E-mail: uwenzel@wzw.tum.de
Fax: +49-8161-71-3999

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over, there are hints that under certain experimental conditions and especially when quercetin is applied at high concentrations adverse effects can occur as well. This is evidenced by the fact that in the presence of copper ions quercetin can act as a pro-oxidant and increase the number of DNA strand breaks [12]. Moreover, quercetin when administered in the absence of a chemical carcinogen by the oral route, was shown to induce aberrant crypt foci [12].

To assess the functions of quercetin on parameters known to be disregulated in human colonic cancers, we employed HT-29 human colon cancer cells and determined quercetin-mediated responses on proliferation, differentiation, and apoptosis. For a comprehensive analysis of the molecular targets of quercetin action in cells, a proteomics approach was used for identifying proteins altered in steady-state levels after exposure of HT-29 cells to quercetin for 24 h or 48 h. Proteome analysis included a prefractionation of soluble and lipophilic proteins isolated from cells prior to two-dimensional gel electrophoresis (2-D PAGE) and MALDI-TOF-MS based protein identification.

2 Materials and methods

2.1 Cell culture

The human colon cancer cell line HT-29 was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and was used at passage 150. Cells were cultured in 75 cm^2 tissue culture flasks (Renner, Dannstadt, Germany) in RPMI 1640 supplemented with 10% fetal calf serum (FCS) and 2 mM glutamine (Invitrogen, Karlsruhe, Germany). Antibiotics added were 100 units/mL penicillin and 100 μg/mL streptomycin (Invitrogen). The cultures were maintained in a humidified atmosphere of 5% CO_2 at 37°C. Cells were passaged at preconfluent densities by the use of a solution containing 0.05% trypsin and 0.5 mM EDTA (Invitrogen).

2.2 Cell proliferation

Proliferation of cells was determined as described previously [13]. In brief, HT-29 cells were seeded at a density of 5×10^3 per well onto 24-well cell culture plates (Renner) and allowed to adhere for 24 h. Thereafter, medium was replaced by fresh culture medium with or without (control) quercetin (Sigma, Deisenhofen, Germany) and cells were allowed to grow for another 72 h. Total cell counts were determined by SYTOX-Green (Bioprobes, Leiden, The Netherlands) that becomes fluorescent after DNA binding. Therefore, cells were lysed by 1% Triton X-100 in isotonic NaCl and cell numbers were determined based on a calibration curve. The calibration curve was measured using cell numbers between 1×10^3 and 1.5×10^5 cells that have been adjusted after determination of cell numbers in a Neubauer chamber and fluorescence of the corresponding cell numbers was measured at 538 nm after excitation at 485 nm using a fluorescence multiwell-plate reader (Fluoroskan Ascent, Labsystems, Bornheim-Hersel, Germany).

2.3 Differentiation assay

After having reached 40% confluency on 25 cm^2-culture flasks (Renner), HT-29 cells were incubated for 72 h in the presence or absence (control) of 150 μM quercetin. Cell numbers were determined after cells had been washed twice with PBS and trypsinized and the harvested cells were pelleted for 10 min at $1500 \times g$. The pellets were resuspended in 550 μL 1 M diethanolamine buffer, pH 9.8, with 0.5 mM $MgCl_2$ and homogenized. 500 μL of homogenate was mixed with 500 μL of 0.1 mM fluorescein-diphosphate (Bioprobes) in diethanolamine buffer and alkaline phosphatase activity as a marker for differentiation was determined by the release of fluorescein using the multiwell plate reader with excitation at 485 nm and emission at 538 nm.

2.4 Determination of apoptosis markers

Caspase-3-like activity was assessed as described by Nicholson *et al.* [14] and served as an early apoptosis marker. In brief, HT-29 cells were seeded at a density of 5×10^5 per well onto 6-well plates and allowed to adhere for 24 h. Cells were then incubated for 24 h in the absence (control) or presence of 150 μM quercetin. Subsequently, cells were trypsinized, cell numbers were determined and then the cells were centrifuged at $2500 \times g$ for 10 min. Cytosolic extracts were prepared by adding 750 μL of a buffer containing 2 mM EDTA, 0.1% CHAPS, 5 mM DTT, 1 mM PMSF, 10 μg/mL pepstatin A, 20 μg/mL leupeptin, 10 μg/mL aprotinin, and 10 mM HEPES/KOH, pH 7.4, to each pellet and homogenizing by 10 strokes. The homogenate was centrifugated at $100\,000 \times g$ at 4°C for 30 min and the cytosolic supernatant was incubated with the fluorogenic caspase-3 tetrapeptide-substrate Ac-DEVD-AMC (Calbiochem, Bad Soden, Germany) at a final concentration of 20 μM. Cleavage of the caspase-3 substrate was followed by determination of emission at 460 nm after excitation at 390 nm using the fluorescence microplate reader.

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Nuclear fragmentation and chromatin condensation served as late markers of apoptosis and were determined by staining of DNA with Hoechst 33258 (Sigma). HT-29 cells (3×10^4) were incubated with or without 150 μM quercetin for 24 h. Thereafter, cells were washed with PBS, allowed to air-dry for 30 min and then fixed with 2% paraformaldehyde prior to staining with 1 μg/mL Hoechst 33258 and visualization under an inverted fluorescence microscope (Leica DMIL, Wetzlar, Germany) equipped with an bandpass excitation filter of 340–380 nm and a longpass emission filter of 425 nm.

2.5 2-D PAGE

2.5.1 Sample preparation

For sample preparation, cells at 70% confluency were incubated for 24 h or 48 h with medium alone (control) or in addition with 150 μM quercetin. Subsequently, cells were washed three times with ice-cold 350 mM sucrose, containing proteinase-inhibitor CompleteMini (Roche, Mannheim, Germany) and then scraped off with a cell scraper (Renner). Cells of two flasks were combined in 6 mL ice-cold sucrose solution and subsequently centrifuged for 7 min at $2500 \times g$. The supernatant was discarded and 200 μL of lysis buffer (7 M urea, 2 M thiourea, 2% CHAPS, 1% DTT; all from Roth, Karlsruhe, Germany), 0.8% Pharmalyte (Amersham Biosciences, Freiburg, Germany), and protease-inhibitor were added to the pellet. Homogenization of the cells was achieved by ultrasonication (10 strokes, low amplitude) on ice. The lysed cells were centrifuged for 30 min at $100\,000 \times g$ at 4°C and the supernant containing the solubilized proteins was used directly or stored at −80°C. Separation of soluble and membrane proteins were accomplished by the use of ProteoPrep™ Universal Extraction Kit (Sigma) according to the manufacturer's insctructions.

2.5.2 2-D PAGE conditions

2-D PAGE, IEF in the first dimension and SDS-PAGE in the second dimension, was performed as described by Görg *et al.* [15] with slight modifications. In brief, IEF was performed on 18 cm IPG strips, pH 3–10 (Amersham Biosciences) using an Amersham IPGphor unit. Each strip was rehydrated for 12 h with 340 μL of rehydratation buffer (8 M urea, 0.5% CHAPS, 15 mM DTT, 0.5% IPG buffer). 500 μg of protein-suspension was then loaded onto the strip by cuploading. IEF was performed under the following conditions: 500 V (10 min, gradient), 4000 V (1.5 h, gradient), 8000 V (25 000 Vh, Step-n-hold). Subsequent to IEF, strips were incubated for 15 min in equilibration-buffer 1 (1.5 M Tris-HCl, pH 8.8, 6 M urea, 30% glycerol (87% v/v, 2% SDS) containing 1% DTT and then for another 15 min in equilibration buffer 2 (1.5 M Tris-HCl, pH 8.8, 6 M urea, 26% glycerol (2% SDS) containing 4% iodoacetamide before loading onto SDS-PAGE gels. One mm thick 12.5% SDS-polyacrylamide gels were casted according to Laemmli [16] and separation was performed by an Amersham Biosciences Ettan-Dalt II System with 4 mA per gel for 1 h and thereafter with 12 mA per gel.

2.5.3 Protein staining and analysis

For protein staining, gels were fixed in 40% ethanol and 10% acetic acid for 8 h and then exposed overnight to a Coomassie solution containing 10% $(NH_4)_2SO_4$, 2% phosphoric acid, 25% methanol (all from Roth, Germany), and 0.625% Coomassie Brilliant Blue G-250 (Serva, Heidelberg, Germany). Destaining was done in *aqua bidest* until the background was completely clear. Analysis of the Coomassie-stained gels was accomplished using the ImageMaster software (Amersham Biosciences) including background subtraction and volume normalization after the gels have been scanned by an ImageScanner (Amersham Biosciences). Gels were matched to a reference gel (virtual gel containing all spots detected on any gel) and then average gels derived from cells with or without quercetin treatment were generated. Average gels from at least four independent experiments were compared to each other and spots differing at least twofold in intensity were picked for further analysis by MALDI-TOF-MS.

2.6 MALDI-TOF-MS

Coomassie-stained spots were picked with a 2 mm or 3 mm "SKIN-Picker". The destaining of the spots occured with alternating washing procedures in pure acetonitrile and 50 mM NH_4HCO_3 (Sigma) until blue colour was removed. After drying in a SpeedVac the gel spots were exposed for 1 h at 4°C to 5 μL of 0.02 μg/μL sequencing-grade modified trypsin (Promega, Mannheim, Germany) and then the trypsin-supernatant was removed and the gel spots were washed twice with *aqua bidest*. For digestion, the spots were incubated for 20 h at 37°C with 5 μL of 50 mM NH_4HCO_3. The peptide fragments were extracted by incubating each spot in 10 μL of 0.1% trifluoroacetic acid (Sigma) in 50% acetonitrile for 6 h at 37°C. Supernatants were stored and a second extraction was performed under identical conditions overnight. The supernants derived from a spot were collected and dried in a SpeedVac. Mass

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analysis was performed according to the method of Bruker Daltonics (Leipzig, Germany) using the Autoflex mass spectrometer of Bruker Daltonics. The dried protein sample was resuspended in 4 μL of 1% trifluoroacetic acid and spotted onto the target using the thin layer affinity HCCA AnchorChip™ preparation of Bruker Daltonics. Proteins were identified by the use of the Mascot Server 1.9 (Bruker Daltonics) based on mass searches within human sequences only. The search parameters allowed for the carboxyamidomethylation of cysteine and one missing cleavage. The criteria for positive identification of proteins were set as follows: (i) a minimum score of 63, (ii) a mass accurancy of ±0.1%, (iii) at least a twofold analysis from two independent gels, and (iv) that the protein exhibits a significant difference in the number of matched peptides to the next potential hit.

2.7 Statistical analysis

Significances of difference between groups were determined by a nonpaired Student's *t*-test (GraphPadPrism). For each variable four independent experiments were carried out. Data are given as the mean ± SEM.

3 Results

3.1 Quercetin inhibits proliferation of HT-29 cells by promoting apoptosis and differentiation

Cancer cells in general display high proliferation rates, a low grade of differentiation, and a blunted response to apoptotic signals. We here show that quercetin affects these parameters. Proliferation was dose-dependently inhibited by quercetin with an EC_{50}-value of 81.2 ± 5.5 μM (Fig. 1a). The activity of alkaline phosphatase, a marker for differentiation, was enhanced more than 3-fold at maximum (Fig. 1b) and a half-maximal stimulation of differentiation was achieved at a quercetin concentration of 74.4 ± 8.2 μM (Fig. 1b). The proliferation and differentiation curves derived from quercetin treatment show that the maximal effects of the compound can be obtained at a concentration of around 150 μM. Caspase-3-like activity was determined as a function of incubation time, and showed a maximal 3-fold stimulation by 150 μM quercetin at 24 h and 36 h (Fig. 1c). Full execution of apoptosis after quercetin treatment could be determined by nuclear fragmentation and chromatin condensation in the HT-29 cells (Fig. 1d).

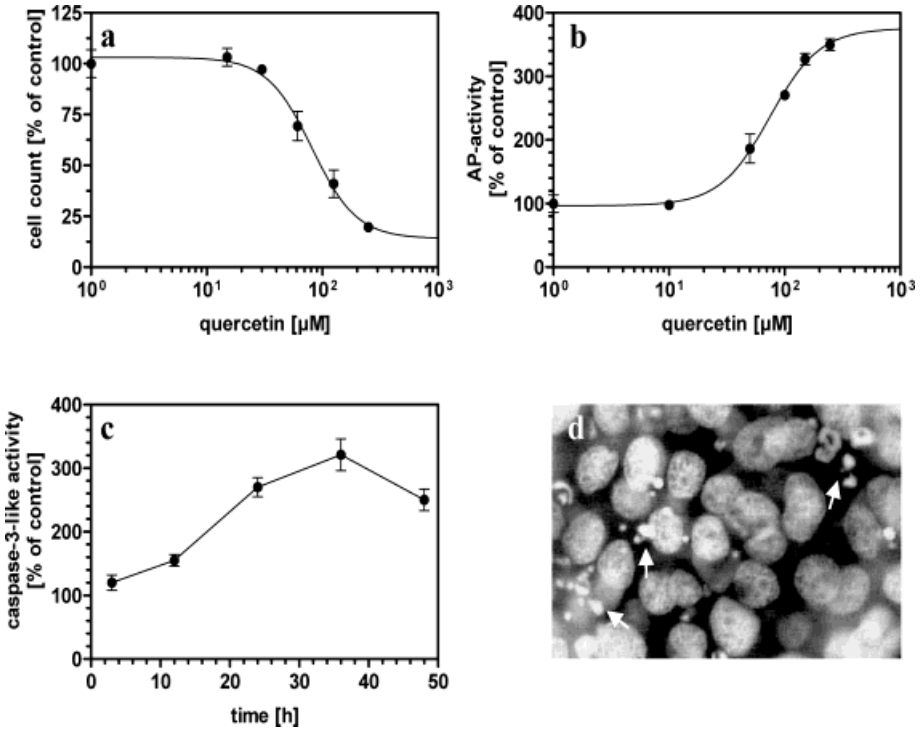

Figure 1. Effects of quercetin on proliferation, differentiation, and apoptosis in HT-29 cells. (a) Proliferation was measured over 72 h in the absence (control) or presence of quercetin at various concentrations. Cell numbers were determined subsequently using SYTOX-Green nucleic acid stain. (b) Alkaline phosphatase (AP)-activity, as determined by cleavage of fluorescein-diphosphate, served as a marker for differentiation and was determined after 72 h of incubation in the absence (control) or presence of 150 μM quercetin. (c) Caspase-3-like activity at various time-points of incubation with 150 μM quercetin was determined by measuring cleavage of Ac-DEVD-AMC. (d) Nuclear fragmentation served as a marker of the late apoptosis phase and was determined by Hoechst 33258 staining. Fragmented DNA (arrows) was visualized using an inverted fluorescence microscope.

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3.2 Proteins altered in their steady-state level by treatment of HT-29 cells with quercetin for 24 h

In order to assess the mechanisms of actions of quercetin in a comprehensive manner and to identify potential markers of unwanted side effects we determined changes in the proteome of HT-29 cells after 24 h of exposure to quercetin. To enrich proteins with low abundance, a soluble protein fraction and the membrane protein fraction were obtained by prefractionation and submitted to 2-D PAGE separately. More than 600 protein spots were resolved *via* 2-D PAGE of unfractionated HT-29 cells of which 20 spots were found to differ at least twofold based on density between protein preparations from cells treated with 150 μM quercetin or control cells (Fig. 2). Fifteen of the protein spots with changed steady-state levels could be identified by MALDI-TOF-MS analysis (Table 1, Addendum). Amongst those proteins we identified apoptosis regulators such as HSTCP1 (Table 1, Addendum), proteins that are proteolytically cleaved during apoptosis execution such as lamin B1, but also proteins involved in metabolism, detoxification or gene regulation (Table 1, Addendum).

Prefractionation of HT-29 proteins according to their solubility enabled to identify another 41 proteins with altered steady-state level after quercetin treatment of cells (Fig. 3). Twenty-one proteins were found to be altered in the cytosolic fraction and another 20 proteins in the membrane protein fraction. Altogether, out of the 41 proteins affected, 28 could be defined by MALDI-TOF-MS analysis (Table 1, Addendum). Amongst those, several chaperones known to play a crucial role in stress-response (Table 1) as well as additional cytoskeletal proteins or proteins involved in metabolism, detoxification and gene regulation were identified (Table 1, Addendum).

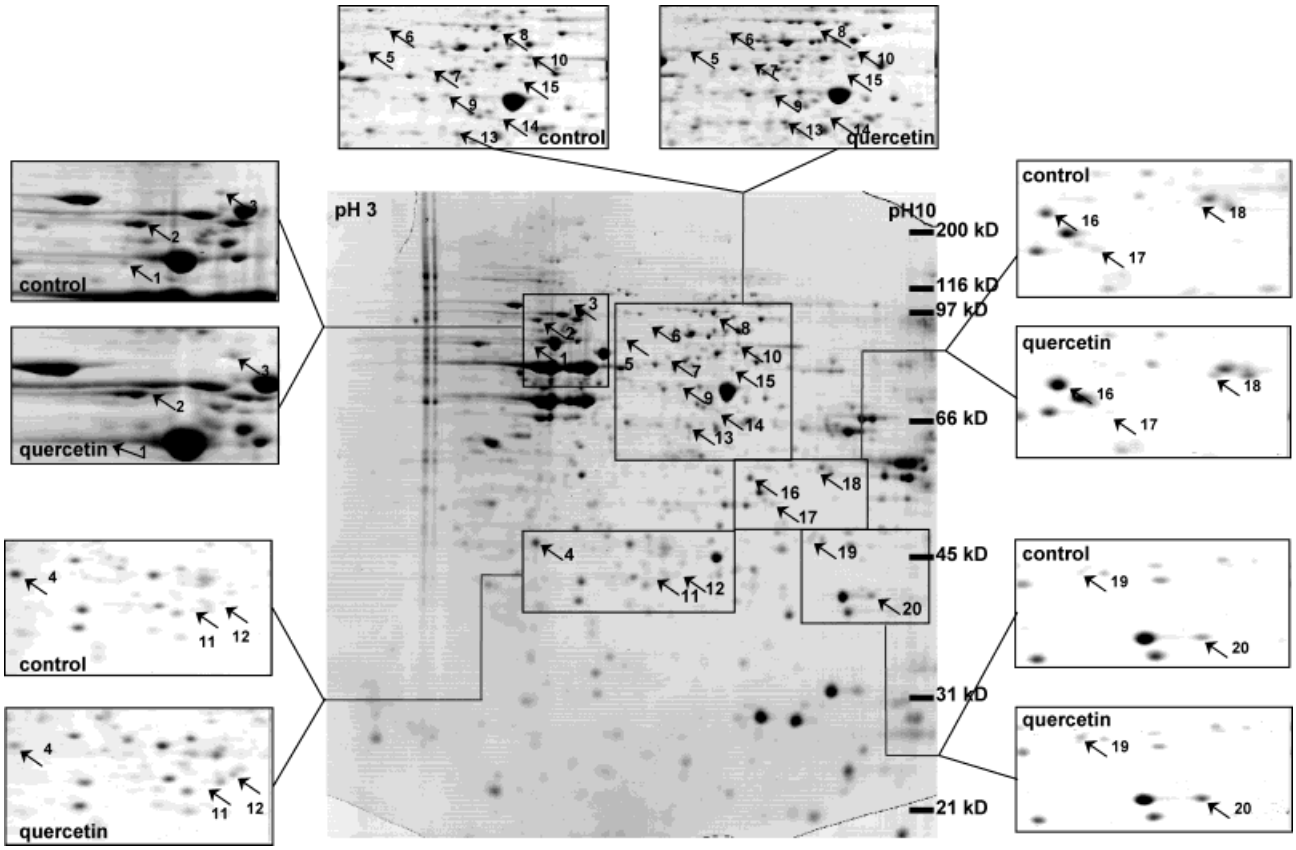

Figure 2. 2-D PAGE of proteins from HT-29 cells grown for 24 h in the absence (control) or presence of 150 μM quercetin. Proteins were separated on a pH 3–10 IPG-strip in the first dimension and on a 12.5% SDS-polyacrylamide gel in the second dimension. A typical Coomassie-stained gel derived from HT-29 control cells is shown in the middle section. Enlarged areas of gels from the control cells or from cells treated with 150 μM quercetin are shown around the representative control gel.

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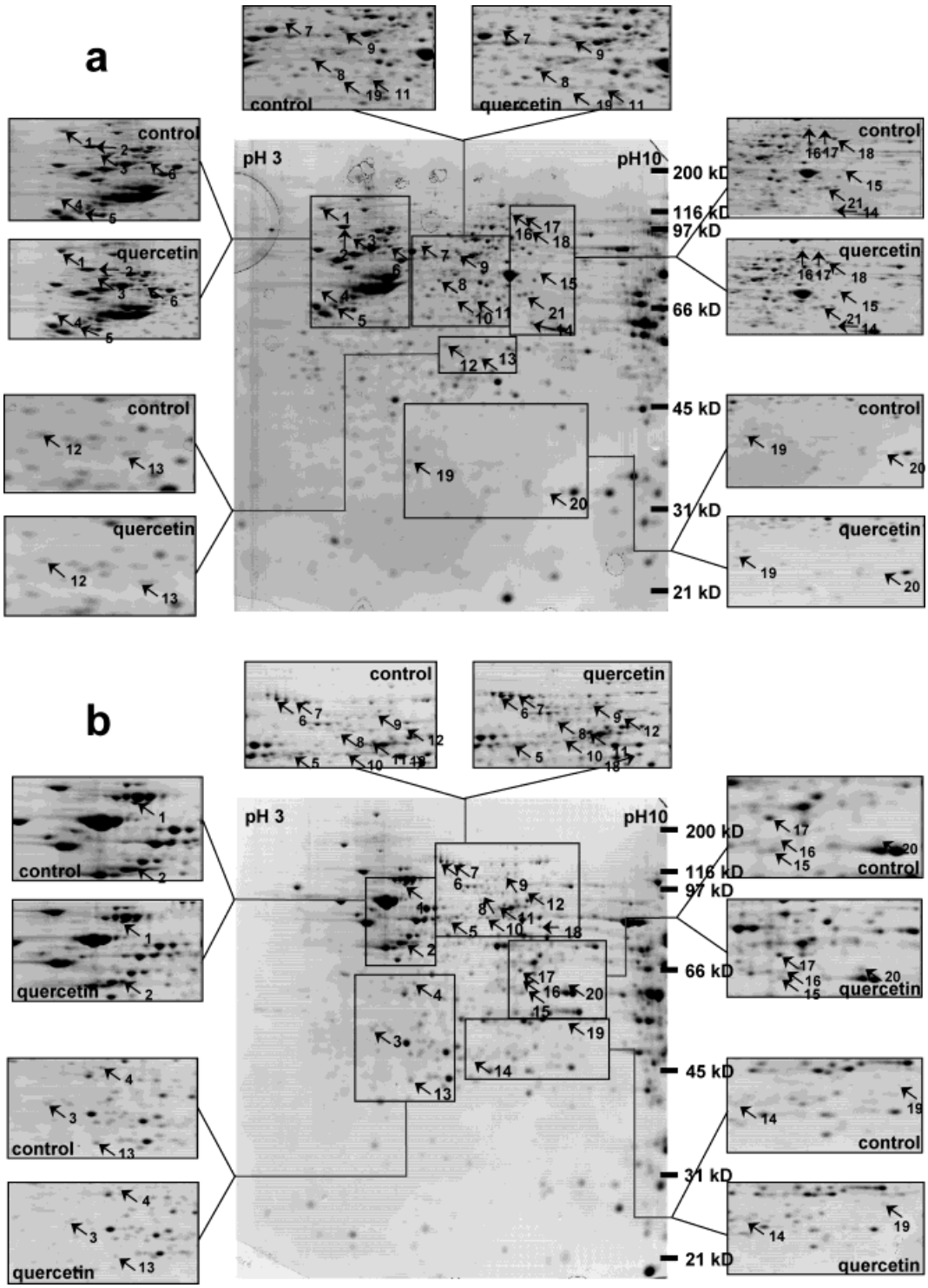

Figure 3. 2-D PAGE of (a) cytosolic and (b) lipophilic proteins from HT-29 cells. Cells were incubated for 24 h either with 150 μM quercetin or with medium alone (control). Subsequently, proteins were separated according to their solubility by the use of ProteoPrep™ Universal Extraction Kit and run on a pH 3–10 gel in the first dimension and on a 12.5% SDS-polyacrylamide gel in the second dimension. Staining of the protein spots was accomplished by Coomassie Brilliant Blue G-250. The mid panels show typical control gels (a) for the cytosplasmatic fraction and (b) the lipophilic proteins containing fraction. Enlargements around the mid panels show identical sections of gels from control cells or quercetin-treated cells.

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Proteins from the same protein classes – but not necessarily the same protein entities as those identified as regulated at 24 h – were found as altered also after 48 h of quercetin exposure of HT-29 cells (Fig. 4). Stathmin and triosephosphate isomerase, that both serve as valid tumor markers, were reduced in steady-state level 3- to 4-fold by quercetin (Table 2, Addendum), but not yet at 24 h (Fig. 2). Others, such as the detoxifying enzyme glutathione-*S*-transferase, showed similar increased expression levels at both treatment periods (Tables 1 and 2, Addendum). Proteins that showed completely different expression patterns for the two time points were also found. HSTCP1, a protein similar in structure to the heat-shock protein 60 and shown to prevent the activation of caspase-3 by decreasing cytochrome *c* release from mitochondria [17] showed a 2-fold decrease after 24 h, but an almost almost 6-fold increase in protein density after 48 h of quercetin treatment (Tables 1 and 2, Addendum).

4 Discussion

Several epidemiological studies indicate an inverse association between the intake of flavonols and the risk of cardiovascular disease and cancer [18, 19]. Quercetin, as a major plant flavonol occurs predominantly in apples, onions, and tea [9], and is consumed in amounts up to 100 mg per day [20]. Based on the high dietary intake quercetin was claimed as one of the major molecular entities mediating the protective effects of a flavanol-rich diet as derived from epidemiological studies. Many of the proposed protectective effects of quercetin have been attributed to its potent antioxidative activities [21]. Several *in vitro* studies have shown that quercetin protects DNA against oxidative damage [22–24] and protection against H_2O_2-induced DNA strand breaks was demonstrated in lymphocytes from diabetic patients consumed a diet rich in quercetin for 2 weeks in a crossover design [25]. In contrast, in the absence of an oxidative challenge high con-

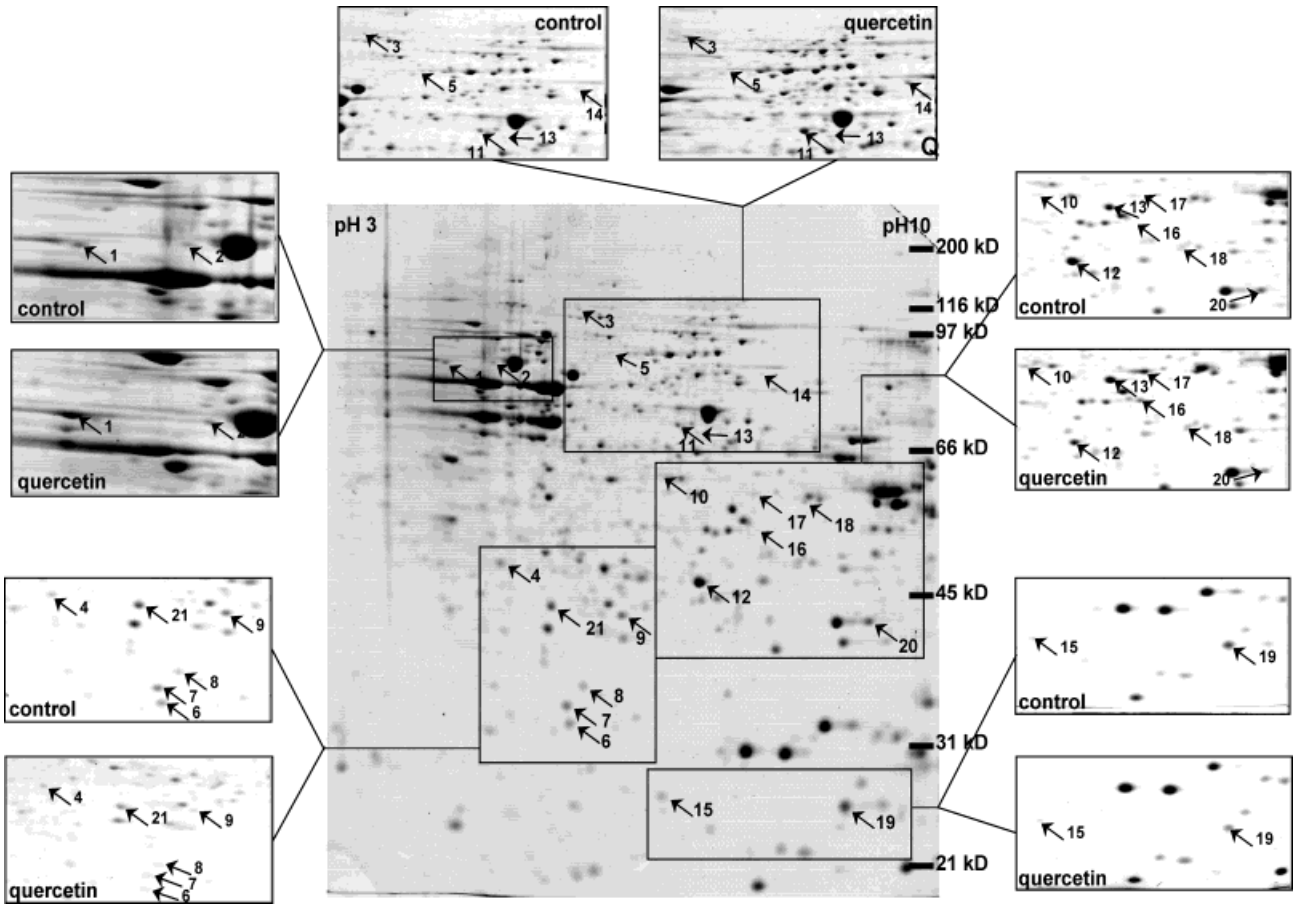

Figure 4. Effects of quercetin-exposure for 48 h on the proteome of HT-29 cells. Proteins from HT-29 cells were isolated after incubation in the presence of 150 μM quercetin or medium alone (control). Separation of proteins was performed by 2-D PAGE and proteins were stained by Coomassie Brilliant Blue G-250. Enlargements around the control gel in the center part show identical sections of gels from control cells and quercetin-treated cells, respectively.

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centrations of quercetin increased DNA strand breaks in various cells including lymphocytes [26]. This emphasizes that the biological activities of quercetin can vary drastically depending on the concentration provided and the milieu in which it acts. Similar divergent effects of quercetin were found in a chemoprevention study in which mice were treated with azoxymethan [12]. Whereas quercetin reduced the development of colon cancers in the mice exposed to the chemical carcinogen, it enhanced the initial hyperproliferative stages of tumor formation when administered alone [12].

To explore the underlying mechanisms by which quercetin can interfere with colon cancer development, we investigated its effects on proliferation, differentiation, and the programmed cell death in the human colon cancer cell line HT-29. Cells were used in all experiments at preconfluent densities to allow cells to respond to treatment either with differentiation or apoptosis and to simulate the response of proliferating cells of the crypt zone *in vivo*. At a concentration of 150 μM, quercetin completely blocked cell growth associated with an enhanced surface expression of alkaline phosphatase as an indicator of an increased differentiation state and associated with the induction of apoptosis as shown by a prominent increase in caspase-3-like activitiy. For a comprehensive analysis of the protein entities that respond with alterations in expression level to quercetin exposure of HT-29, proteins isolated from cells were separated by 2-D gel electrophoresis followed by peptide mass fingerprinting *via* MALDI-TOF analysis for identification of the proteins.

Major changes of the steady-state concentration of proteins involved in apoptosis execution were observed upon quercetin exposure to cells. The anti-apoptotic heat shock proteins HSTCP1 and hsp70-1, showed markedly reduced levels after 24 h of treatment. Hsp70-1 is considered as a promising target in treatment of cancers and in particular in those, resistant to classic caspase-mediated mechanisms [27, 28]. Moreover, a substrate of hsp-70, GrpE [29], also showed reduced levels, indicating a strong blockade of the anti-apoptotic pathway in which these proteins are integrated. Whereas HSTCP1 declined after 24 h, it showed an almost 6-fold higher steady-state level after 48 h demonstrating the functional stress response in cells after apoptosis has been initiated. Apoptosis initiation by quercetin leads to the activation of caspases and the subsequent cleavage of substrates including various cytoskeletal proteins [30]. Lamins are cytoskeletal target proteins and their concentration was reduced both at 24 h and 48 h of quercetin exposure. Moreover, ninein, responsible for the release of short microtubules from the centrosome in migrating cells [31], was completely absent at 24 h and markedly reduced at 48 h, suggesting that the migration capability of the HT-29 cells is impaired. The quercetin-dependent increase in annexin II, a protein that was proven to inhibit cell migration [32], would also reduce or block the cells ability to migrate.

Other proteins that might contribute to the proposed anticancer activities of quercetin, include annexin I that promotes apoptotic cell engulfment [33] and which was found to be upregulated by quercetin at 48 h. The human DEAD box protein found in increased levels in retinoblastoma and neuroblastoma [34] as well as stathmin, expressed at high levels in a wide variety of human cancers [35], showed reduced levels in quercetin-treated cells. Triosephosphate isomerase, suggested as a marker protein in lung adenocarcinoma [36] and nm23-H4 that is overexpressed in colorectal cancers [37], also responded with lower expression levels to quercetin at 24 h or 48 h.

As a novel finding we observed major changes in levels of various proteins of the intermediary metabolism in cells exposed for 24 h to quercetin. This included enzymes of the Krebs-cycle or those that deliver products to it. NADH-dehydrogenase as a respiratory chain protein decreased in quantity after 24 h of quercetin exposure, whereas acyl-CoA dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase were 3- and 4-fold increased, respectively. These results suggest that quercetin causes severe alterations of metabolism that could lead to a reduced oxidative phosphorylation of substrates and an enhanced energy generation *via* fatty acid oxidation. At 48 h of incubation with quercetin, even the flux through the glycolytic pathway could be reduced, as deduced from reduced amounts of triosephosphate isomerase, whereas the sustained acyl-CoA dehydrogenase level was still preserved.

Although most of the findings on the changes in protein levels are in agreement with the pro-apoptotic and antiproliferative effects of quercetin, indications for some adverse effects can be obtained as well. For example, glutathione-*S*-transferase levels were found to be reduced significantly after 24 h and 48 h. This enzyme of phase-II xenobiotic metabolism has been described to play a crucial role in carcinogen detoxification [38] and its induction has been demonstrated to reduce the risk of colon cancer development in humans with intrinsic low glutathione-*S*-transferase activity [39]. The reduced glutathione-*S*-transferase level found here upon quercetin treatment may represent a response to a reduced prooxidant state of the HT-29 cells. The expected reduced cellular detoxification capacity by a lowered glutathione-*S*-transferase after quercetin exposure seems in HT-29 cells – at least partially – be compensated by an increased expression level of thioredoxin peroxidase found at both time points. The activity of thioredoxin peroxidase pro-

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vides a cellular stress-protection, although it has been shown that this does not prevent apoptosis to occur [40].

Other putative cancer promoting activities of quercetin can be predicted from the complete disappearance of the stress-activated MAP kinase p38-delta, as this protein is specifically required for enterocytic anoikis [41]. Anoikis describes apoptotic processes initiated by cell detachment from the extracellular matrix. The lowered MAP kinase p38 delta expression would consequently suppress anoikis and this could be even more severely impaired by the quercetin-mediated 5-fold increase in the steady-state level of rho-activated serine/threonine-specific kinase which is known to be of central importance in signaling pathways that promote metastasis [42]. The predicted promotion of metastatic events may be counterbalanced by the simultaneous disappearance of the laminin receptor in quercetin-treated cells. The laminin receptor is thought to be involved in tumor cell migration and metastasis formation, and shows generally an enhanced expression in human colorectal carcinoma [43]. It is interesting to note that quercetin might also be able to mark the transformed cells for the immune system as we detected an increased amount of the gp96 protein. This is recognized as a tumor rejection antigen and serves as an effective vaccine shown to elicit anti-tumor immune responses in animals and in man [44]. The dual effects that quercetin may have on pathways that either promote or suppress metastasis requires further experimental exploration but these findings support previous observations of quercetin action in cell and animal models in which both, tumor-promoting and tumor-inhibiting effects, have been reported [45, 46]. Since quercetin is unstable in solution we can not *a priori* exclude that some of changes in the proteome observed after quercetin exposure of cells occur by metabolites of quercetin and are not caused by the original structure similar to the situation in an intact organism [47].

In conclusion, proteome analysis performed in HT-29 cells exposed to the dietary flavonoid quercetin identified numerous molecular targets that can be linked to its proposed anti-cancer activities in colonic tissue. Regarding the adverse effects of quercetin observed in some cancer model systems, our studies do provide some clues for its bimodal activity on the molecular level.

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Addendum

Table 1. Proteins with changed steady-state levels in HT-29 cells exposed for 24 h to quercetin

Spot No.	Protein description	Protein amount (quercetin/ control)	Residues of identified peptides	% Coverage	Accession No.
Annexins					
14	Annexin II[a)]	2.93 ± 0.71*	1–9, 10–27, 28–46, 47–62, 49–62, 68–76, 104–114, 115–134, 119–134, 152–167, 157–167, 157–168, 178–195, 179–195, 212–219, 233–245, 313–323, 313–328, 324–338	54	BAA05730
Chaperones					
1	Endoplasmin precursor GP 96 (heat shock familiy 90)[a)]	2.93 ± 0.72*	23–46, 31–46, 55–63, 96–114, 122–135, 364–374, 375–383, 395–413, 473–482, 491–509, 721–733	18	P14625
2	BIP[a)]	2.20 ± 0.14**	32–42, 64–78, 80–95, 84–95, 105–120, 121–134, 146–163, 147–163, 168–179, 180–195, 180–196, 280–288, 289–306, 307–318,309–318, 336–349, 359–368, 418–428, 430–446, 457–474, 475–492, 506–514, 515–522, 541–555, 584–599, 584–601, 604–615	48	AJ271729
5	HSTCP1	0.38 ± 0.16*	19–33, 64–73, 110–122, 112–122, 131–145, 190–199, 234–243, 248–264, 299–309, 299–310, 469–480, 469–481, 500–510, 513–526, 516–526, 533–541	31	XP_116321
5	GrpE[a)]	0.38 ± 0.07*	26–34, 33–50, 35–50, 67–77, 80–89, 81–89, 92–100, 128–138, 139–153, 187–196, 187–200	43	AAH24242
10	HSP70-1[a)] chaperone	0.38 ± 0.06**	4–25, 57–71, 57–72, 113–126, 129–155, 160–171, 172–187, 221–236, 237–246, 237–247, 300–311, 302–311, 326–342, 329–342, 349–357, 349–361, 362–384, 424–447, 510–517, 525–533(m.c.1), 540–550, 629–641	41	NP_005336
20	Peptidyl prolyl isomerase[a)], cyclophilin A	Only control	2–19, 20–28, 20–31, 56–69, 77–91, 83–91, 119–131, 132–144, 134–144	51	AAM63961

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Table 1. Continued

Spot No.	Protein description	Protein amount (quercetin/ control)	Residues of identified peptides	% Coverage	Accession No.
Cytoskeletal Proteins					
1	Ninein	Only control	1514–1523, 1627–1638, 1643–1651, 1654–1673, 1697–1706, 1707–1719, 1745–1753, 1750–1763, 1764–1772, 1796–1805, 1845–1858, 1881–1891, 1883–1891, 1915–1921, 1930–1941, 2001–2010, 2034–2046, 2034–2047	8	Q9UH61
2	Lamin B1	Only control	1–13, 14–25, 51–66, 51–70, 79–89, 109–122, 111–122, 134–144, 197–208, 241–249, 258–270, 299–311, 320–329, 350–366, 367–377, 367–378, 379–386, 457–472, 532–541	24	NP_005564
3	Lamin B1[a)]	0.42 ± 0.21	1–9, 51–66, 79–89, 79–90, 102–110, 109–122, 111–122, 124–135, 134–144, 145–156, 157–166, 222–233, 258–270, 367–377, 379–386	34	NP_005564
6	Mitofilin[b)]	2.11 ± 0.43*	171–195, 196–211, 258–269, 276–284, 331–343, 331–344, 354–366, 372–385, 396–410, 517–525, 527–545, 527–547, 546–564, 548–564, 582–600, 601–623, 624–632, 690–708, 709–718, 719–726	27	Q16891
7	Myosin heavy chain 12[b)]	2.33 ± 0.33*	515–526, 570–581, 587–597, 641–659, 776–783, 792–801, 802–810, 839–849, 942–949, 985–994, 1007–1015, 1008–1017, 1051–1058, 1198–1208, 1218–1226, 1287–1301	11	A59254
9	Desmoplakin I[a)]	3.22 ± 0.88*	852–860, 852–862, 871–879, 880–889, 882–889, 890–897, 901–911, 974–981, 982–991, 1016–1024, 1043–1050, 1075–1084, 1094–1102, 1109–1116, 1157–1169, 1181–1190, 1232–1240, 1241–1250, 1265–1272, 1220–1231, 1251–1258, 1251–1264, 1336–1343, 1389–1397, 1399–1408, 1418–1433, 1472–1482, 1501–1511, 1512–1521, 1522–1531, 1532–1541, 1560–1568, 1588–1595, 1659–1674, 1820–1832, 1870–1881	7	A38194
8	Myosin-IXA[b)]	2.33 ± 0.33*	666–681, 702–710, 711–720, 740–754, 776–790, 811–820, 832–842, 845–854, 877–884, 923–934, 1005–1021, 1022–1028, 1029–1036, 1037–1043, 1218–1228, 1229–1237, 1254–1265	10	AAH34228
19	Lamin A[a)]	0.43 ± 0.04*	1–8, 12–25, 29–41, 51–60, 61–72, 79–89, 79–90, 124–133, 134–144, 145–156, 156–166, 181–189, 182–190, 197–208, 209–216, 226–235, 250–261	24	P02545

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Table 1. Continued

Spot No.	Protein description	Protein amount (quercetin/ control)	Residues of identified peptides	% Coverage	Accession No.
Metabolism					
3	NADH dehydrogenase (ubiquinone)	0.39 ± 0.09***	63–68, 185–200, 292–299, 300–308, 409–420, 420–428, 421–428, 471–483, 502–511, 608–617, 693–702	14	S17854
5	Putative acetyl-transferase[b)]	0.38 ± 0.15**	185–201, 263–276, 339–357, 358–366, 448–454, 497–506, 550–556, 606–613, 688–696, 697–714, 757–765	22	Q9BXJ9
6	Succinate dehydrogenase	0.36 ± 0.04***	233–246, 251–261, 262–282, 313–325, 362–379, 424–451, 452–465, 466–480, 518–527, 590–598, 601–615, 616–623, 637–647, 648–662	40	Q99643
14	Acyl-CoA-dehydrogenase	3.91 ± 0.50***	24–35, 36–45, 184–201, 184–202, 203–214, 215–225, 226–237, 261–272, 362–374	26	NP_000009
15	Glutamate dehydrogenase	0.27 ± 0.08***	51–60, 69–76, 77–90, 108–123, 124–136, 125–136, 152–162, 172–183, 172–187, 204–211, 212–231, 303–318, 353–363, 364–386, 400–420, 400–420 , 445–453, 454–460, 461–476, 497–516, 504–516	42	NP_005262
16	Glyceraldehyde-3-phos-phate-dehydrogenase	3.08 ± 0.04***	62–80, 67–80, 67–84, 67–84, 87–107, 108–117, 118–139, 119–139, 119–139, 119–139, 146–162, 201–215, 235–248, 310–323	44	G3P2_HUMAN
17	Aconitate hydratase, iron-dependent	0.50 ± 0.09**	2–10, 58–68, 59–68, 69–84, 87–95, 118–138, 145–160, 234–245, 367–378, 371–378, 412–424, 430–437, 438–457–522–534, 574–587, 578–587, 592–605, 634–648, 657–671, 702–717, 731–739	31	T52543
	Detoxification				
10	Catalase	0.42 ± 0.05***	19–37, 23–46, 38–46, 98–105, 156–168, 243–262, 252–262, 263–272, 354–362, 430–443, 431–443, 444–455, 457–467, 458–467, 499–521, 506–521	28	P04040
13	Glutathion-*S*-transferase[b)]	0.41 ± 0.14**	1–12, 2–12, 2–14, 15–30, 20–30, 31–45, 56–71, 76–101, 83–101, 104–121, 122–141, 190–209, 192–210	69	A37378
20	Thioredoxin peroxidase	2.67 ± 0.39***	8–16, 111–120, 129–136, 141–151, 159–168, 169–190	44	NP_006397
Kinases/ Phosphatases					
6	Pyruvate kinase[a)] M1 isoenzyme	0.50 ± 0.02*	32–42, 43–55, 73–88, 92–114, 136–150, 151–161, 173–185, 188–205, 188–206, 206–223, 207–223, 278–293, 319–335, 367–375, 383–398, 400–421, 467–474, 475–488	45	P14618
12	MAP kinase p38delta[a)]	Only control[a)]	2–21, 22–37, 25–37, 25–37, 57–72, 57–72, 103–118, 119–128, 119–133, 146–167, 168–180, 213–226	51	O15264

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Table 1. Continued

Spot No.	Protein description	Protein amount (quercetin/ control)	Residues of identified peptides	% Coverage	Accession No.
20	Serin/threonin specific protein kinase[b)]	5.06±0.39***	528–539, 529–539, 589–596, 591–601, 602–61, 667–678, 710–718, 710–719, 730–739, 820–830, 820–833, 877–885, 878–885, 1022–1029	11	S69211
Gene regulation					
4	Ribosomal prot RS 40 k (Laminin receptor)[a)]	Only control	54–63, 64–80, 86–102, 90–102, 103–117, 121–128, 129–155, 156–166	32	A31233
9	Proliferation associated 2G4	0.47 ± 0.11**	15–23, 17–23, 30–48, 33–48, 94–107, 121–135, 260–275, 276–282, 353–368	46	AAH07561
13	RNA binding motif protein 4	0.50 ± 0.04***	1–11, 4–11, 36–45, 60–71, 80–92, 112–120, 121–129, 141–149, 152–164, 183–198, 314–341, 350–358	38	AAH00307
15	HSPC017[b)]	2.45 ± 0.30*	77–91, 92–101, 128–141, 130–141, 142–149, 150–165, 224–235, 243–253, 254–264, 265–280, 269–280, 286–297, 336–347	37	AAD26998
16	DEAD box protein RB human[a)]	0.50 ± 0.05	85–92, 93–108, 127–140, 218–228, 234–246, 314–324, 325–343, 395–410, 446–452, 467–484, 500–508, 531–543, 562–571, 572–590, 620–628	26	A47743
17	KIAA1349[b)], zinc finger protein	2.02 ± 0.23*	143–154, 160–170, 227–239, 305–313, 361–369, 399–416, 492–500, 516–528, 520–528, 529–537, 540–547, 577–590, 591–603, 604–612, 660–670	22	Q9P2J8
19	Polyb, romo-1[b)]	4.03 ± 0.27***	301–317, 417–425, 417–434, 426–434, 426–436, 491–511, 525–534, 541–552, 542–552, 580–595, 680–690	12	NP_060783
Other Proteins					
8	Weakly similar to hyaluronan-mediated motility receptor	0.48 ± 0.09*	44–57, 84–92, 130–143, 130–144, 144–154, 228–235, 292–301, 349–357, 379–390, 391–400, 401–408, 410–420, 423–431	28	BAB55389
11	D50929[a)]	Only control	416–438, 547–559, 573–579, 673–681, 695–702, 733–740, 778–787, 788–794, 811–817, 890–900, 947–959, 981–990	9	BAA09488
13	NIT protein 2[a)]	2.13 ± 0.55	27–39, 45–58, 59–73, 61–73, 123–134, 135–157, 201–221, 210–221, 315–328	12	NP_002155
15	Platelet activating factor acetyl hydrolase IB α (LIS1)[a)]	2.85 ± 0.74	20–31, 20–32, 33–45, 71–87, 92–100, 133–143, 147–162, 163–174, 175–185, 228–237, 238–258, 302–315, 303–315, 360–367, 374–389, 390–404	45	LIS_HUMAN
16	Unknown protein for MGC8882[b)]	2.11 ± 0.44	209–219, 272–279, 307–318, 333–344, 338–346, 367–376, 368–376, 368–382, 386–391, 392–402, 412–422, 471–479, 497–504, 523–538, 593–599, 605–615, 640–649, 706–717	22	Q96ED9

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Table 1. Continued

Spot No.	Protein description	Protein amount (quercetin/ control)	Residues of identified peptides	% Coverage	Accession No.
17	GTP-binding protein homolog chicken	0.44 ± 0.11**	45–57, 48–57, 89–99, 89–100, 101–118, 107–118, 131–139, 140–155, 156–172, 176–183, 186–212, 213–225, 226–245, 246–257, 265–280, 309–317	45	B33928 human
19	Sequence 135 from patent EP 1067182[a)]	Only control	82–89, 90–104, 105–116, 126–135, 128–135, 128–146, 147–155, 156–168, 192–199, 203–211, 204–211, 204–216	45	CAC39749
21	KIAA1751[a)]	0.25 ± 0.08***	29–41, 42–48, 80–86, 144–150, 144–157, 151–160, 168–174, 193–199, 197–204, 205–210	9	BAB21842

The spot numbers are identical to those given in Figs. 1 and 2. Proteins altered in level at least twofold by treatment of cells with 150 μM quercetin were identified by MALDI-TOF-MS. The identified peptide fragments are shown in relation to the amino acid sequences of the identified protein

a) Proteins found to be altered in the cytosolic fraction

b) Proteins found to be altered in the second, lipophilic proteins containing fraction

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ *versus* control

Table 2. Proteins with changed steady-state levels in HT-29 cells exposed for 48 h to quercetin

Spot No.	Protein description	Protein amount (quercetin/ control)	Residues of identified peptides	% Coverage	Accession No.
Annexins					
10	Annexin I	2.12 ± 0.33*	9–25, 29–52, 58–71, 81–96, 81–97, 113–123, 128–143, 166–176, 185–203, 188–203, 204–212, 213–227, 214–227, 294–302	46	NP_000691
17	Annexin II	2.05 ± 0.48	1–9, 10–27, 28–46, 47–62, 49–62, 68–76, 104–114, 115–134, 119–134, 152–137, 157–167, 157–168, 178–195, 179–195, 212–219, 233–245, 313–323, 313–328, 324–338	54	BAA05730
	Chaperones				
5	HSTCP1, similar to HSP60, chaperonin	5.59 ± 0.98*	19–33, 110–122, 131–145, 146–153, 234–243, 248–259, 248–264, 299–310, 369–378, 391–400, 434–443, 469–480, 469–481, 485–496, 500–510, 516–526, 533–541	31	XP_116321
Detoxification					
20	Probable thioredoxin peroxidase	7.01 ± 1.19**	8–16, 17–27, 94–110, 110–120, 111–120, 129–136, 141–151, 169–190	44	A46711
21	Glutathione*S*-transferase	0.50 ± 0.09	1–12, 2–12, 2–14, 15–30, 20–30, 31–45, 56–69 71, 76–101, 83–101, 104–121, 122–141, 190–209, 192–210		A37378

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Table 2. Continued

Spot No.	Protein description	Protein amount (quercetin/ control)	Residues of identified peptides	% Coverage	Accession No.
Cytoskeletal proteins					
2	Ninein	0.44 ± 0.16	1514–1523, 1627–1638, 1643–1651, 1654–1673, 1697–1706, 1707–1719, 1745–1753, 1750–1763, 1764–1772, 1796–1805, 1845–1858, 1881–1891, 1883–1891, 1915–1921, 1930–1941, 2001–2010, 2034–2046, 2034–2047	8	Q9UH61
3	Mitofilin	Only control	85–102, 217–229, 276–284, 287–297, 305–313, 316–330, 344–353, 354–366, 354–370, 372–385, 428–436, 444–457, 458–467, 486–495, 548–564, 565–577, 582–600	33	Q16891
7	Lamin A	0.22 ± 0.02**	1–8, 12–25, 29–41, 51–60, 61–72, 79–89, 79–90, 124–133, 134–144, 145–156, 156–166, 181–189, 182–190, 197–208, 209–216, 226–235, 250–261	24	P02545
Metabolism					
1	Human thyroid binding protein	0.49 ± 0.15*	31–42, 32–42, 82–97, 121–130, 196–207, 214–230, 223–230, 231–247, 255–263, 301–308, 301–309, 309–316, 317–326, 327–338, 339–345, 376–386, 410–424, 437–444	47	AAA61169.1
6	Stathmin	0.21 ± 0.04**	14–26, 27–40, 29–40, 43–51, 52–59, 53–60, 62–69, 85–94, 104–111, 137–148	56	BAA5794
12	Triosephosphate-isomerase	0.32 ± 0.03***	5–13, 6–13, 18–32, 19–32, 33–52, 59–68, 69–84, 99–112, 100–112, 113–130, 156–174, 175–187, 194–205, 206–218, 219–247	75	TPIS_HUMAN
13	Acyl CoA dehydrogenase	4.27 ± 0.24***	24–35, 36–45, 184–201, 184–202, 203–214, 215–225, 226–237, 261–272, 362–374	26	NP_000009
	Kinases/phosphatases				
8	Nucleoside diphosphate kinase; Nm23-H4	0.42±0.10*	7–18, 19–27, 57–66, 89–105, 106–114, 115–128	46	O00746
Gene regulation					
11	Translation elongation factor EF-Tu	2.02 ± 0.17**	24–32, 57–73, 94–105, 95–105, 108–123, 210–227, 239–252, 253–271, 272–281, 286–297, 287–297, 316–327, 352–361	32	P43897
15	Ribosomal protein L13	0.38 ± 0.15*	6–17, 23–35, 60–75, 87–103, 104–120		BC009190.1
	Other proteins				
9	KIAA1751	0.45 ± 0.04**	29–41, 42–48, 80–86, 144–150, 144–157, 151–160, 168–174, 193–199, 197–204, 205–210	9	BAB21842
16	GTP-binding protein homolog chicken	2.60 ± 0.15***	45–57, 48–57, 89–99, 89–100, 101–118, 107–118, 131–139, 140–155, 156–172, 176–183, 186–212, 213–225, 226–245, 246–257, 265–280, 309–317	59	B33928 human

The spot numbers are identical to those given in Fig. 3. Protein levels altered at least twofold by the treatment of cells with 150 μM quercetin after 48 h of incubation were identified by MALDI-TOF-MS as described.
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ *versus* the control

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