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# Modulatory effects of quercetin on proliferation and differentiation of the human colorectal cell line Caco-2

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**Abstract**

The effect of the dietary flavonoid quercetin was investigated on proliferation and differentiation of the human colon cancer cell line Caco-2. Confluent Caco-2 monolayers exposed to quercetin showed a biphasic effect on cell proliferation and a decrease in cell differentiation ($0.001 < P < 0.05$). During differentiation Caco-2 cells formed 5 phase II metabolites, of which the amount of 4′-*O*-methyl-quercetin-3′-*O*-glucuronide correlated with the differentiation grade ($r = 0.99$, $P < 0.003$). The increment of cell proliferation at low quercetin concentrations and the decrease in cell differentiation are effects opposite to what would be expected for a functional food ingredient with anti-carcinogenic potential.


*Keywords:* Quercetin; Proliferation; Differentiation; Metabolism; Colorectal cancer; Caco-2

## 1. Introduction

When considered globally, colorectal cancer (CRC) takes the second place in cancer related deaths with an estimate of 492,000 people per year and is third when considering the yearly incidence of 945,000 people [1]. Under physiological conditions a limited number of stem cells are continuously dividing in the base of colonic crypts and are the source of epithelial cells [2]. Following division, daughter cells migrate upwards and undergo differentiation in the upper two-thirds of the crypt and finally undergo apoptosis when moving towards the top of the crypt, within 4–8 days [3]. In mammalian cells, these continuous processes of proliferation, differentiation and apoptosis are under strict regulation, but can be disrupted as a result of successive genetic mutations [4]. These 'genetic hits' occur in both tumour suppressor genes as well as oncogenes and finally give rise to colorectal carcinomas through

*Abbreviations used:* ALP, alkaline phosphatase; BrdU, bromo-2′-deoxyuridine; CRC, colorectal cancer; HPLC, high performance liquid chromatography; TEER, trans epithelial electrical resistance.

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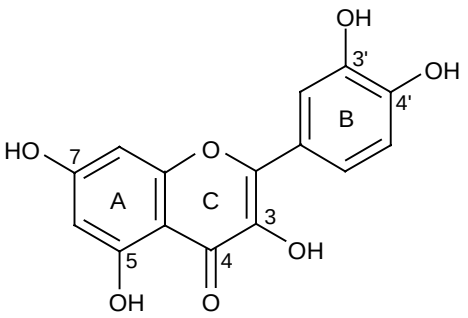


Fig. 1. Structural formula and carbon atom numbering of quercetin.

a gradual series of well-characterised histopathological changes, also known as the adenoma-carcinoma sequence [5].

Risk factors suggested in CRC aetiology include genetic predisposition and dietary intake, such as high consumption of fat and low intake of fibres and flavonoids [6–8]. One of the flavonoids highly present in human diet is quercetin (Fig. 1), which in nature is glycosylated and can be found in several fruits and vegetables. Major dietary sources of quercetin are amongst others onions, broccoli, leek and blueberries [9]. Following ingestion, natural quercetin derivatives can be deconjugated in the small intestine by cellular ß-glycosidase [10] or in the colon, by ß-glycosidase produced by colonic bacteria [11]. In humans, quercetin aglycone shows relatively poor bioavailability [12], probably as a result of rapid metabolic conversion, thereby forming glucuronidated, methylated and sulphated metabolites with different biological activities [13,14]. Quercetin has been proposed to exert anti-carcinogenic properties both in vitro and in vivo [15,16]. A possible mechanism involved is scavenging of free radicals. This electron donating capacity is exerted by its phenolic hydroxyl groups, especially by the two neighbouring hydroxyl groups present on the B-ring (Fig. 1), also known as the catechol moiety. Other proposed mechanisms of action include induction of apoptosis and inhibition of cell proliferation, protein kinase C and lipoxygenase (reviewed in Ref. [17]). Nowadays, quercetin is commercially available and claimed to be a beneficial food supplement. However, data regarding the benefits are relatively scarce and conflicting [18,19].

Aspects of cellular processes also involved in CRC, including proliferation and differentiation, can be studied in vitro using the Caco-2 model system, which is derived from a human colon adenocarcinoma [3]. Upon seeding, Caco-2 cells are able to proliferate towards confluency, thereby forming a monolayer including tight junctions, which maintain cell–cell contact and are essential in cellular polarization and differentiation [20]. When reaching confluency, Caco-2 cells show contact inhibition mediated cell cycle arrest and undergo spontaneous differentiation as a function of time. Differentiating Caco-2 cells express apical brush border enzymes, including sucrase isomaltase [21] and a placental-type of alkaline phosphatase (ALP) [22], which are positively correlated with the differentiation status of enterocytes. Another feature of differentiated Caco-2 cells is the resemblance with normal small intestinal cells, showing microvilli in a high density as well as drug absorption, metabolism and excretion [23].

Mechanisms of action evoked by nutrients with anti-carcinogenic properties are amongst others inhibition of cell proliferation and stimulation of cell differentiation as has been demonstrated with the flavonoid 2-phenyl-4H-1-benzopyran-4-one in the colorectal cell line HT-29 [24], fish oil in the HT-29 and Caco-2 cell lines [25] and the short chain fatty acid butyrate in Caco-2 cells [26,27]. Based on proposed mechanisms involved in inhibition of carcinogenesis, the hypothesis of the present study was that quercetin should decrease cell proliferation and enhance cell differentiation in the Caco-2 cell model. Therefore, the aim of this study was to investigate the effects of physiological relevant concentrations of quercetin on proliferation and differentiation of Caco-2 cells. Furthermore, in order to assess whether the effect on cell proliferation and differentiation can be ascribed to quercetin and/or its metabolites and whether the extent and type of quercetin metabolism is dependent on the differentiation status of Caco-2 cells, quercetin phase II metabolites were identified and quantified as a function of time.

## 2. Material and methods

### 2.1. Chemicals and materials

Quercetin dihydrate and sodium ascorbate were purchased from Sigma-Aldrich (Zwijndrecht,

The Netherlands). Dulbecco's modified eagle medium with L-glutamine, 25 mM HEPES and 4.5 g/l D-glucose as well as supplements (100×MEM non-essential amino acids, gentamicin, L-glutamine (100×) and phosphate buffered saline (PBS) were purchased from Invitrogen™ Life Technologies (Breda, The Netherlands). Fetal calf serum was ordered from Cambrex (Verviers, Belgium). Flasks with a 75 $cm^2$ growth area, flat-bottomed 96-well plates, low protein binding membrane filters CA 0.22 μm and transwell® polycarbonate membrane inserts (12 wells/plate) were purchased from Corning Life Sciences (Cambridge, UK). PicoGreen® dsDNA quantitation kit was obtained from Molecular Probes (Leiden, The Netherlands). Dimethyl sulfoxide (DMSO) [Acros Organics] was purchased from Boom B.V. (Meppel, The Netherlands). The cell proliferation ELISA Bromo-2′-deoxyuridine (BrdU) colorimetric kit was ordered from Roche Diagnostics (Almere, The Netherlands). HPLC-grade acetonitrile and trifluoroacetic acid were purchased from Lab-Scan Ltd. (Dublin, Ireland) and Baker (Deventer, The Netherlands), respectively.

### 2.2. Quercetin stability in culture medium

Since quercetin has been reported to be unstable in an aqueous environment [18], its stability was tested in the presence of different ascorbate concentrations. To this end, 40 μM quercetin aglycone originating from a 200×DMSO stock solution was incubated at 37 °C in 5% $CO_2$ and 95% humidity in combination with 0, 0.1, 1 or 10 mM of filter-sterilised sodium ascorbate in culture medium, in the absence of cells. The culture medium as needed for the rest of the experiment, further to be referred to as DMEM culture medium, consisted of Dulbecco's Modified Eagle Medium supplemented with 10% (v/v) heat inactivated mycoplasma tested fetal calf serum, 2 mM L-glutamine (6 mM final concentration), 1% (v/v) MEM non-essential amino acids and 50 μg/ml gentamicin. Samples were taken in triplicate at $t=0$, 24 and 48 h post-incubation, frozen in liquid nitrogen and kept at −80 °C until further analysis. For precipitation of FCS in DMEM culture medium, samples were first diluted two-fold with acetonitrile, vortexed vigorously and centrifuged at 13,000 rpm during 10 min. Finally, high performance liquid chromatography (HPLC) analysis of quercetin and its metabolites in the supernatant was performed according to a previously described method [28].

### 2.3. Cell culture

Caco-2 cells, originating from a human colorectal adenocarcinoma, were obtained from American Type Culture Collection (Manassas, VA, USA). For subculturing, near-confluent monolayers were rinsed using PBS with 0.022% (w/v) EDTA, followed by treatment with 0.05% (w/v) trypsin in PBS with 0.022% (w/v) EDTA. Caco-2 cells of passage 40 and 41 were subcultured in T75 flasks in a 1:10 split ratio at 37 °C in 5% $CO_2$ and 95% humidity, with DMEM culture medium.

### 2.4. Cell proliferation

Cell proliferation was measured as a marker for cytotoxicity induced by quercetin. Since 1 mM ascorbate was found to be non-toxic for Caco-2 cells and showed an acceptable grade of stability in culture medium, this ascorbate concentration was chosen as the optimal concentration for stabilisation of quercetin in subsequent experiments. Caco-2 cells of passage 42 were seeded in duplicate in flat-bottomed 96-well plates at a density of 10,000 cells/well. After formation of a confluent monolayer on day 3 post-seeding, Caco-2 cells were exposed to 0–80 μM quercetin stabilised by 1 mM ascorbate. As controls, cells were exposed to 1 mM ascorbate in DMEM culture medium, including the solvent i.e. 0.5% (v/v) DMSO. Culture medium of all experimental conditions was completely changed every 24 h and cell proliferation was measured every 24 h during a 96 h time period, using the Cell Proliferation ELISA, BrdU colorimetric kit. First, 96-wells plates were incubated with 10 μM BrdU to allow incorporation of this pyrimidine analogue instead of thymidine into the DNA of proliferating Caco-2 cells. Following a DNA denaturation step, for improvement of interaction with the BrdU antibody during the next step, cells were subsequently incubated with peroxidase (POD) labelled anti-BrdU. Finally, POD-labelled immune complexes were coloured by incubation with tetramethyl-benzidine and absorbances were measured

with a Versamax tunable microplate reader at 370 nm, with a reference wavelength of 492 nm.

### 2.5. Cell differentiation

To asses the effect of quercetin on cell differentiation, near-confluent cells were subcultured at a split ratio of 1:10. To enable differentiation, cells of passage number 41 were seeded in triplicate at a density of ≈112,000 cells/cm$^2$ on polycarbonate membrane transwell® inserts (12 wells/plate) with a membrane diameter of 12 mm (growth area 1.13 cm$^2$) and a 0.4 μm pore size. Upon reaching confluency on day 2 post-seeding (i.e. experimental day 0), the apical side of the Caco-2 monolayers was exposed to 0.5 ml of 40 μM quercetin stabilised by 1 mM filter-sterilised sodium ascorbate (40 μM quercetin). In addition, Caco-2 cells were exposed to DMEM culture medium with (control) and without (untreated) 1 mM sodium ascorbate, for analysis of possible ascorbate mediated effects on cell differentiation and of spontaneous differentiation, respectively. Both, untreated and control cells received culture medium with the quercetin solvent i.e. 0.5% (v/v) DMSO. Basolateral compartments were filled with 2.0 ml culture medium only. Caco-2 cells were exposed to quercetin in the apical compartment only, in order to mimic luminal exposure of the in vivo gut. Apical and basolateral culture medium of all conditions was completely changed every 24 h. Caco-2 cells were allowed to undergo differentiation and cells were harvested on days 0 (i.e. prior to quercetin exposure), 3, 5, 7 and 10 post-confluency. First, monolayers were rinsed two times with ice-cold PBS-only. Subsequently, membranes were cut out, taken up in 1 ml ice-cold PBS in cryovials, snap frozen in liquid nitrogen and finally stored at −80 °C until further analysis of ALP activity, double stranded DNA contents and protein amount.

### 2.6. Alkaline phosphatase activity

Activity of alkaline phosphatase (ALP), a marker for Caco-2 cell differentiation [20], was measured in triplicate on days 0, 3, 5, 7 and 10 post-confluency. First, cells on membranes were lysed by sonification for 3×20 s in ice-cold PBS. Subsequently, ALP activity was determined on a BM/Hitachi 911 using a colorimetric assay in which the release of p-nitrophenol out of the substrate p-nitrophenol phosphate is proportional with ALP activity [29].

### 2.7. Trans epithelial resistance

Trans epithelial electrical resistance (TEER) was measured as an additional marker for cell differentiation [20,30]. As the temperature is one of the factors influencing the TEER, culture medium in transwell® inserts was allowed to cool down to room temperature (20±1 °C), thereby avoiding temperature fluctuations in day-to-day measurement. TEER was measured in triplicate on days 0, 3, 5, 7 and 10 post-confluency, using a Millicel-ERS Volt Ohm meter (Millipore, Amsterdam, The Netherlands). TEER values were calculated according to the following equation: TEER = R × filter area (Ωcm$^2$).

### 2.8. Picogreen® dsDNA quantification

To correct ALP for the amount of cells present in each well, double stranded DNA (dsDNA)—as a marker for cell number—was measured. First, 100 μl 200× diluted Picogreen® dsDNA quantification reagent was added to 100 μl of cell lysate in a flat-bottomed 96-well plate. Following a 5 min incubation step, fluorescence of Picogreen® was determined at a wavelength of 530 nm after excitation at 485 nm. dsDNA was quantified according to a calibration curve of the λ-dsDNA standard in TE-buffer (10 mM Tris, 1 mM EDTA, pH 7.5).

### 2.9. Protein amount

Since ALP activity of differentiating Caco-2 cells has also been reported as ALP units/mg protein [3], protein amount of cell homogenates was measured according to Bradford [31].

### 2.10. Quercetin metabolism

In order to assess whether metabolism of quercetin is associated with the differentiation grade of Caco-2 cells, culture medium was sampled on days 1, 3, 5, 7 and 10 post-confluency from of the apical and basolateral compartments, just before changing culture medium. Thus, culture medium samples

obtained on above mentioned time points reflect quercetin metabolism over the last 24 h, as quercetin in culture medium was completely changed every 24 h. Samples were snap-frozen in liquid nitrogen and stored at −80 °C, until further analysis. Quercetin and its metabolites were detected on HPLC, with detection between 220 and 445 nm using a Waters 996 photodiode array detector. Quercetin phase II metabolites were characterised based on retention time, UV–vis (nm), LC-MS, and $^1$H NMR characteristics as described previously and quantified assuming similar absorption coefficients of quercetin and its metabolites [32].

### 2.11. Statistics

Overall analysis of Caco-2 proliferation modulated by different quercetin concentrations, time of quercetin incubation, as well as the interaction between these two parameters was performed using the 2-way ANOVA, incorporating incubation time, quercetin concentration, as well as the interaction between incubation time and quercetin concentration. Analysis of the effect of different quercetin concentrations on cell proliferation within each time point, as well as comparison of the effect of quercetin treatment on cell differentiation was performed using the Student's *t*-test. Correlations between cell differentiation data obtained by several methods, as well as the correlation between the differentiation grade and quercetin metabolite status were calculated with the Pearson's correlation coefficient. Differences were considered significant when $P < 0.05$.

## 3. Results

### 3.1. Quercetin stability

Stability of quercetin was assessed over a 48-h time period in the presence of 0, 0.1, 1 and 10 mM ascorbate. Fig. 2 depicts the stability of 40 μM quercetin in DMEM culture medium, in combination with three ascorbate concentrations. After 24 h quercetin could not be detected in incubations with 0 or 0.1 mM ascorbate. Addition of 1 or 10 mM ascorbate on the other hand, resulted in stabilisation of quercetin, showing 83% of the initial quercetin concentration for both conditions after 24 h, declining to 23 and 73%, respectively, after 48 h.

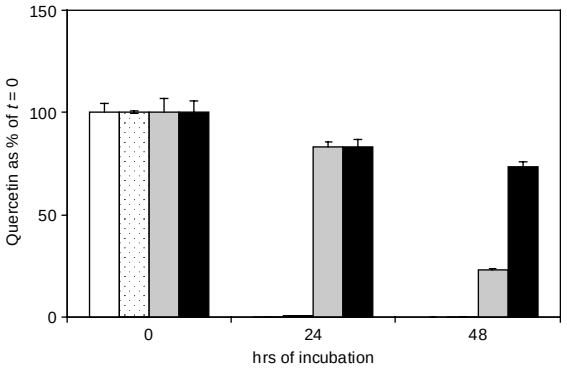


Fig. 2. Stability of 40 μM quercetin in culture medium in the absence (open bar) or presence of 0.1 mM (dotted bar), 1 mM (grey bars) and 10 mM (black bars) of ascorbate, presented as percentage of $t = 0$. Values are given as mean ± SD ($n = 3$).

Based on these data, 1 mM ascorbate was used to stabilise quercetin and culture medium was completely changed every 24 h.

### 3.2. Cell proliferation

The proliferation rate of Caco-2 cells was assessed during consecutive 24 h time intervals, for a 96 h time period following initial exposure to quercetin (Fig. 3(A–D)). Incubation with 1 mM ascorbate did not affect Caco-2 monolayer integrity, regardless of the quercetin concentration. When data of all four time points were considered, a significant inhibition on cell proliferation was observed with increasing quercetin concentrations ($P < 0.0001$) and with an increasing time of quercetin incubation ($P < 0.0001$). In addition, cell proliferation was inhibited significantly by the interaction between incubation time and quercetin concentration ($P < 0.002$). When looking at different time points individually, quercetin at 40 and 80 μM inhibited cell proliferation throughout the experiment, with the exception of 40 μM at $t =$ 24 h (Fig. 3A). Strikingly, a biphasic effect on cell proliferation could be seen at $t = 96$ h (Fig. 3D), showing an increment of cell proliferation at quercetin concentrations up to 20 μM, followed by a decrease at higher concentrations.

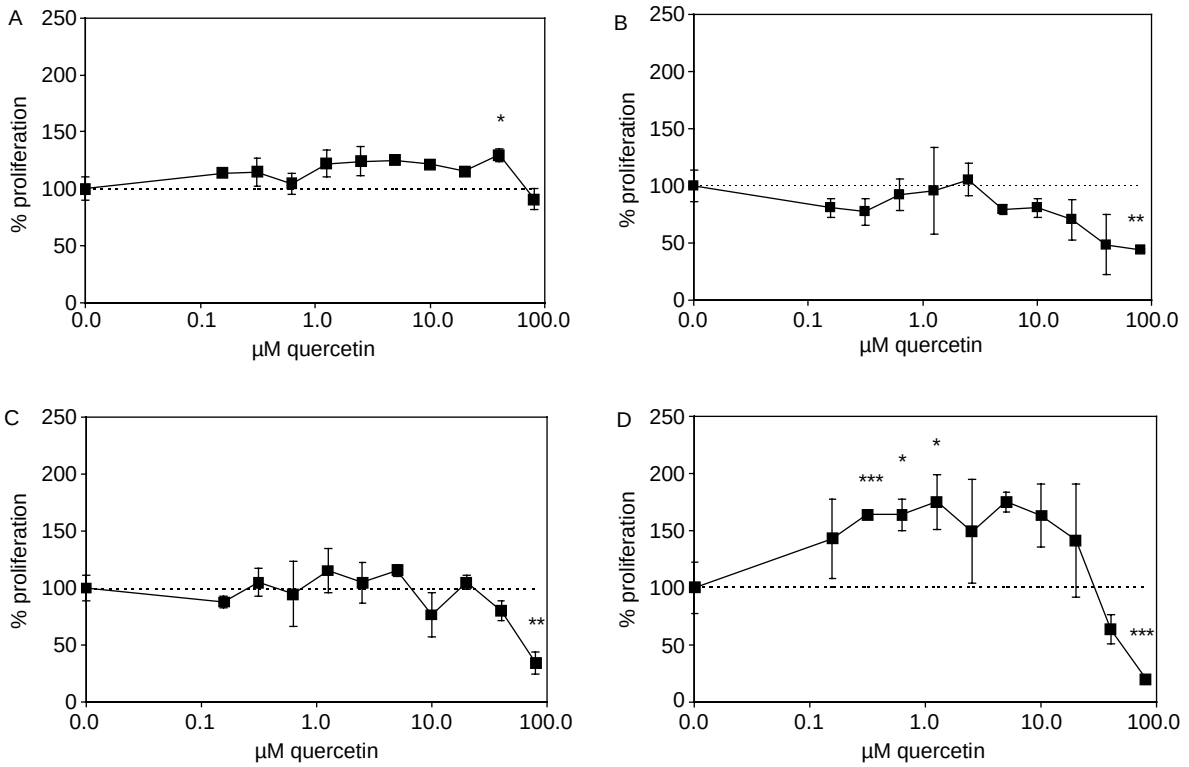


Fig. 3. Cell proliferation of Caco-2 cells incubated with 1 mM ascorbate stabilised quercetin, at $t=24$ (panel A), $t=48$ (panel B), $t=72$ (panel C) and $t=96$ h (panel D) after initial exposure. For each time point separately, proliferation rate is presented relative to corresponding control cells treated with 1 mM ascorbate in combination with 0.5% DMSO (i.e. 100% proliferation). Values are given as mean ± SD ($n=2$). *$P<0.05$, **$P<0.01$ and ***$P<0.001$.

### 3.3. Cell differentiation

Since alkaline phosphatase is one of the brush border membrane associated enzymes present on differentiated Caco-2 cells [20], activity of this enzyme was measured as a marker for cell differentiation. Previously, ALP activity of differentiating Caco-2 cells has been reported to be corrected for the number of cells present in culture, by measurement of milligram protein [3]. Besides assessment of protein amount, in the present study dsDNA was measured as an alternative marker for cell number, since it could not be excluded that Caco-2 protein expression might differ between treatments and the amount of dsDNA per cell is likely to be more constant than protein expression. Absolute amounts of mg protein and ALP activity showed high correlations for quercetin ($r=0.95$, $P<0.05$), ascorbate ($r=0.93$, $P<0.02$) and DMEM ($r=0.98$, $P<0.003$) treated cells. In addition, corrected ALP activity expressed as ALP/μg dsDNA and ALP/mg protein correlated significantly with one another for all experimental conditions [$0.96<r<0.99$; $0.001<P<0.05$; protein data not shown]. From day 3 onwards quercetin treated cells showed a significantly lower increase in ALP activity per microgram dsDNA, reaching a maximum of only 58% of ALP activity observed for ascorbate treated controls on day 10 post-confluency (Fig. 4). In the course of the experiment, ALP activity of ascorbate treated control cells showed a similar increasing trend as untreated cells.

Trans epithelial resistance, measured as an additional marker for cell differentiation [20,30], showed comparable differentiation grades relative to ALP units/μg dsDNA ($0.93<r<0.99$; $0.0006<P<0.05$) and to ALP units/mg protein ($0.89<r<0.97$;

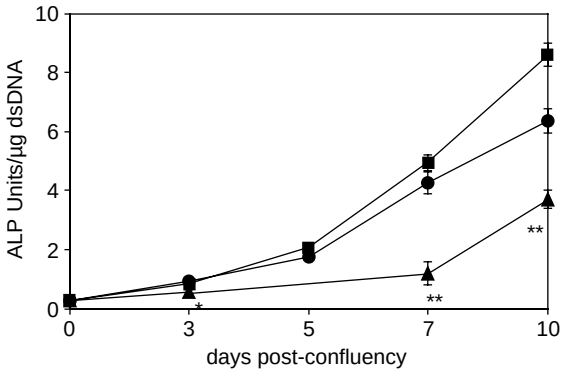


Fig. 4. Time dependent ALP activity in vehicle exposed cells (—■—: 0.5% DMSO), control cells (—●—: 1 mM ascorbate + vehicle) and treated cells (—▲—: 40 μM quercetin stabilised by 1 mM ascorbate). Values are given as mean ± SD ($n = 3$). Data on day 5 for quercetin series not determined. *$P < 0.01$ and **$P < 0.001$ when compared to ascorbate controls.

$0.007 < P < 0.05$) for different treatments (data not shown).

### 3.4. Quercetin metabolism

HPLC analysis was performed on apical and basolateral culture medium, in order to assess whether quercetin metabolising capability of Caco-2 cells changes with the differentiation grade and to determine whether changes in cell differentiation are associated with changes in the amounts of quercetin or with (one of) its metabolites. To enable mutual comparison, per experimental day quercetin and its metabolites originating from the apical and basolateral compartments are expressed as percentage of the sum of peak areas.

Apical and basolateral levels of quercetin aglycone remaining after 24 h of incubation, account for 48% and 39% of peak areas, respectively, on day 1 post-confluency, followed by a decrease in the course of the differentiation (Fig. 5A). Quercetin was metabolised into 5 (double) phase II conjugates which were characterised as 4′-*O*-methyl-quercetin-3′-*O*-glucuronide (Fig. 5B), 3′-*O*-methyl-quercetin (=isorhamnetin, Fig. 5C), 4′-*O*-methyl-quercetin-7-*O*-glucuronide (Fig. 5D), 3′-*O*-methyl-quercetin-4′-*O*-glucuronide (Fig. 5E) and 3′-*O*-methyl-quercetin-7-*O*-sulphate (Fig. 5F). Apical and basolateral amounts of 4′-*O*-methyl-quercetin-3′-*O*-glucuronide, as well as the sum of these amounts, were positively correlated with the differentiation grade of quercetin treated cells until day 7 ($r = 0.99$, $P < 0.003$), thereafter showing a slight decrease (Fig. 5(B)). Glucuronidated 4′-*O*-methylquercetin metabolites (Figs. 5B and D) were found from day 3 onwards, without having detected 4′-*O*-methyl-quercetin itself throughout the experiment.

## 4. Discussion

Quercetin is a flavonoid of the flavonol type and believed to be beneficial for human health based on amongst others its proposed ability to act as a scavenger of free radicals and its potency to inhibit cell proliferation of transformed epithelial cells [17,18]. In the present study, we have analysed the effect of quercetin on proliferation and differentiation of the human colon adenocarcinoma cell line Caco-2. In addition, we have investigated whether the differentiation grade of Caco-2 cells is related to quercetin or its metabolites and to the extent of quercetin metabolism.

These experiments have been performed with quercetin concentrations up to 80 μM, based on epidemiological and human absorption data. In the Zutphen Elderly Study, the intake of quercetin in terms of aglycone has been estimated to be 16.3 mg per day [33]. Furthermore, absorption studies with subjects who underwent colectomy, show 76% recovery of orally administered quercetin aglycone in their ileostomy bag [12], which can be considered as a model compartment for the colon. Combining these data leads to the conclusion that on average 12.4 mg out of the 16.3 mg quercetin intake can reach the colon. When making the assumption that human colon volumes may vary between 0.5 and 1 l depending on dietary intake, quercetin concentrations of 40–80 μM in the colon are likely. Quercetin is commercially available and therefore intake of quercetin supplements may occur [34]. Ingestion of a 500 mg quercetin supplement would lead to an increment of approximately 40 times the physiological colon concentration, up to 2–3 mM, without scientific support regarding human safety in this concentration range.

Employing physiological relevant concentrations under optimised culture conditions, the results of

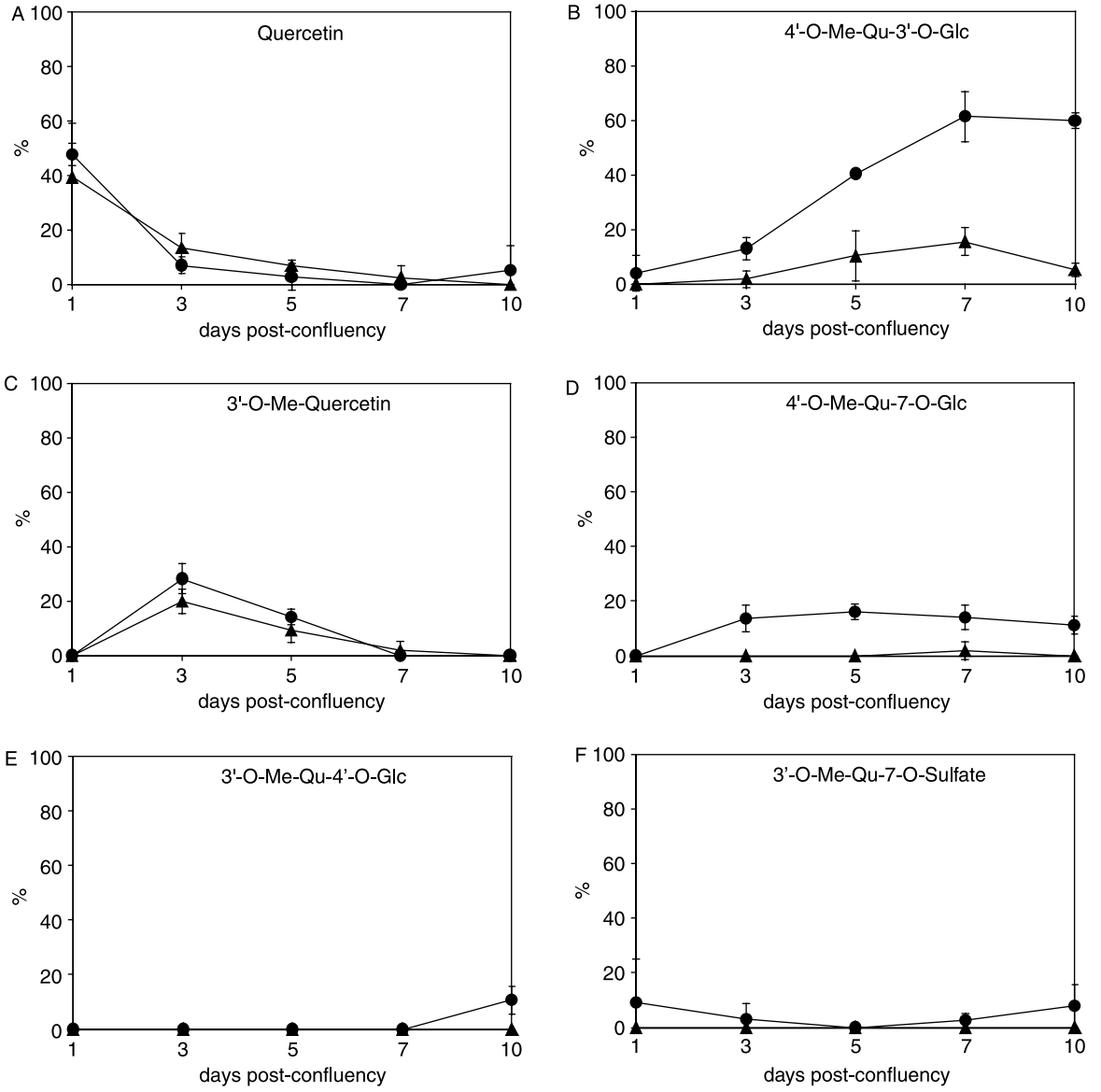


Fig. 5. Time course of quercetin and its metabolites originating from apical (—●—) and basolateral (—▲—) compartments, expressed as percentage of the sum of peak areas measured on each experimental day. Values are given as mean ± SD ($n = 3$).

the present study indicate that quercetin is an inhibitor of Caco-2 differentiation and modulates Caco-2 proliferation in a biphasic fashion. Furthermore, differentiating Caco-2 cells demonstrated glucuronidation, sulphation and methylation of quercetin aglycone. Quantification of these metabolites revealed that both apical and basolateral 4′-*O*-methyl-quercetin-3′-*O*-glucuronide correlated with the Caco-2 differentiation grade.

Based on mechanisms observed in human colon carcinoma cell lines exposed to nutrients with anti-carcinogenic properties, including flavonoids [24,35], fish and olive oil [25] and the short chain fatty acid butyrate [26,27], it was expected that quercetin would

stimulate differentiation and decrease proliferation of Caco-2 cells. Interestingly, our experiments showed effects opposite to what would be expected for a phytochemical with anti-carcinogenic potency. Previously, quercetin has been found to decrease proliferation [18,36–39], and enhance differentiation of human colorectal carcinoma cell lines [39]. The discrepancies in the effect of quercetin on cell differentiation observed between the present study and Wenzel et al. [39] might have been caused by usage of the HT-29 cell line in stead of Caco-2 cells. Moreover, comparison of their experimental protocol to that the present study suggests that initial flavonoid exposure in a pre-confluent (40% confluency) stage of cell cultures might have contributed to the discrepancy in differentiation outcome, as compared to post-confluent experiments described in the present paper. Thus, the confluency stage of cell layers at initial exposure might be crucial for differentiation experiments, especially for Caco-2 cells, which at a post-confluent stage spontaneously differentiate into cells with small intestinal enterocyte-like features [3]. However, the confluency stage of Caco-2 cell cultures is probably not of importance for the outcome of proliferation experiments, since initial quercetin exposure of both pre-confluent [36] or confluent [38] Caco-2 cells resulted in inhibited cell proliferation. Strikingly, above mentioned proliferation and differentiation experiments share a common experimental condition: all experiments were performed by exposing cells to quercetin for 2–3 days, without stabilisation of the flavonoid [18,36–39]. Therefore, it is not clear whether these results are indeed caused by quercetin itself. Since our experiments have been performed with stabilised quercetin, it can be concluded that the observed effects are not caused by the flavonoids degradation products. Moreover, correction of ALP activity for the amount of cells present in transwell® inserts, indicated that inhibition of Caco-2 differentiation is not due to a quercetin mediated decrease in cell number.

Our data indicate that metabolism of quercetin is associated with the degree of cell differentiation. In the course of Caco-2 differentiation, the amount of quercetin aglycone remaining after 24 h of incubation showed a decrease in favour of formation of its metabolites, predominantly of the differentiation dependent 4′-O-methyl-quercetin-3′-O-glucuronide. In a previous report the activity of the lipoxygenase enzyme, which is elevated in CRC [40], has been found to be inhibited most efficiently by quercetin when compared to its metabolites, regardless of the type or position of conjugation [14]. Thus, in terms of anti-carcinogenic potency quercetin seems to be more effective than its metabolites, when considering this particular mechanism in carcinogenesis. As in the present Caco-2 model, concentrations of the 4′-*O*-methyl-quercetin-3′-*O*-glucuronide rose far above quercetin concentrations, this metabolite might have resulted into loss of quercetin's anti-carcinogenic effect. It should be noted that these experimental conditions cannot be extrapolated to the in vivo gut. Following quercetin administration in the apical compartment, which stands as a model for the gut lumen, the flavonoid was metabolised by phase II enzymes. Consequently, Caco-2 cells were exposed to a mixture of quercetin and its metabolites during a 24 h time period, until culture medium was changed. Obviously this condition is not likely to occur in vivo, as luminal quercetin and its metabolites will be excreted via faeces and/or eliminated from the gut lumen upon their absorption into plasma.

The presence of glucuronidated, methylated and sulphated quercetin conjugates in our experiment confirm the activity of UDP-glucuronosyltransferase (UDPGT), catechol-*O*-methyl transferase (COMT) and sulphotransferase, respectively, in Caco-2 cells [23,41]. Murota et al. have described a time dependent increment of a metabolite as 'conjugated quercetin', present in both apical and basolateral compartments [23]. This quercetin conjugate might reflect 4′-*O*-methyl-quercetin-3′-*O*-glucuronide, of which formation has been shown to be differentiation dependent in our study.

The types of quercetin conjugation observed in the present study, i.e. methylation, glucuronidation and sulphation have also been described for human plasma after supplementation with quercetin or its conjugate rutin (quercetin-3-*O*-*ß*-rutinoside) [13,42]. Rutin is deconjugated in the intestinal tract and leads to local release of quercetin [11], as mimicked in our experiment by apical quercetin exposure of Caco-2 cells in transwell® inserts.

In the present paper, quercetin has been found to inhibit Caco-2 cell proliferation at concentrations $\geqq 40$ μM in the course of time, but strikingly only at

$t = 96$ h cell proliferation was increased at quercetin concentrations up to 20 μM. This biphasic effect on cell proliferation has been described before in experiments with the estrogen receptor (ER) positive human colon carcinoma cell lines HCT-116 and HT-29 and the human breast cancer cell line MCF-7, exposed to stabilised quercetin [19]. Assuming that the ER positive cell line Caco-2 [43,44] expresses higher ER levels as a function of the differentiation grade, biphasic cell proliferation in our study might be ER controlled as has been suggested for genistein mediated biphasic proliferation in Caco-2 [45] and MCF-7 cells [46].

Parallel to the iso-flavonoid genistein [45], in our experiments, the ER-ligand and -agonist quercetin [47] at low concentrations probably acted as a phytoestrogen and stimulated cell proliferation through ER induced activation of intracellular protein tyrosine kinases. At high concentrations, however, quercetin leads to inhibition of cell proliferation, probably via inhibition of tyrosine kinases.

Since the mutagenic activity of quercetin has been reported in the late 1970's [48,49], the discussion whether quercetin is carcinogenic [19] or not [18,39] was stimulated. In vivo studies also show inconsistency: quercetin exerted both anti-carcinogenic [50] and pro-carcinogenic [51] activity in azoxymethane (AOM) induced colorectal cancer. Moreover, when administered alone, i.e. without AOM-induction of colorectal cancer, quercetin induced tumours in the ileal segment of the small intestine [52] and gave rise to putative preneoplastic lesions (focal areas of dysplasia) in the colon, in combination with increased colonic cell proliferation [53]. In line with the latter, enhanced cell proliferation was also found in the present in vitro model for colorectal cancer, but only at low quercetin concentrations. Together with the present findings, these observations indicate that the anti-carcinogenic effect of quercetin is still a matter of debate.

In conclusion, the present study indicates that the flavonoid quercetin modulates Caco-2 cell proliferation in a biphasic way and inhibits cell differentiation at physiological relevant concentrations. Biphasic modulation of cell proliferation occurs at a later time point and is therefore probably dependent on the differentiation grade of Caco-2 cells and/or the presence of quercetin metabolites. Furthermore, quercetin aglycone is subject to intestinal phase II enzymes and metabolised into 4′-*O*-methyl-quercetin-3′-*O*-glucuronide, 3′-*O*-methyl-quercetin (=isorhamnetin), 4′-*O*-methyl-quercetin-7-*O*-glucuronide, 3′-*O*-methyl-quercetin-4′-*O*-glucuronide and 3′-*O*-methyl-quercetin-7-*O*-sulphate.

In general, proposed anti-carcinogenic mechanisms are not fully applicable to Caco-2 cells exposed to quercetin, probably as a result of flavonoid metabolism. To further elucidate the modulating effects of quercetin on cell proliferation and differentiation, transcriptome analysis will be performed to analyse which differentially expressed genes are involved in these processes.

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