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# Quercetin accumulates in nuclear structures and triggers specific gene expression in epithelial cells☆

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**Abstract**

Quercetin is a flavonol modifying a number of cell processes in different cell lines. Here, we present evidence that nonconjugated quercetin enters cells possibly via organic anion transporter polypeptides and quickly accumulates in the nucleus where it concentrates at distinct foci. Furthermore, it induces major transcriptional events with a high number of transcripts being modified over time and about 2200 transcripts being continuously influenced by the agent. The latter transcripts are related to cell cycle and adhesion, xenobiotic metabolism, immune-related factors and transcription. In addition, quercetin up-regulates the expression of estrogen receptors α and β. The overall outcome on cell fate is reflected by an inhibition of cell proliferation, cell cycle arrest in the G1 phase and reduction of the cells' migratory potential due to actin cytoskeleton disorganization. Finally, we report that the flavonol modifies the transcription and/or activity of numerous transcription factors. In conclusion, our data support the idea that quercetin may actively accumulate in discrete cell structures and exert more than just antioxidant actions on epithelial cells by regulating mechanisms related to gene transcription.


*Keywords:* Cell adhesion; Cell cycle; Transcriptional analysis; Cell lines (HepG2; T47D); Quercetin

## 1. Introduction

Polyphenols (more than 8000 identified molecules containing a phenolic scaffold) constitute a large family of plant-derived compounds, incorporated in animals through dietary absorption of vegetal foods. Besides their powerful antioxidant properties, they exert a large number of biological actions, depending on their absorption and metabolism [1,2]. One of the most widely represented polyphenol in the human diet is the flavonol quercetin [3,4]. It is present in different fruits and vegetables, and its daily consumption, in a balanced diet, varies between 3 and 38 mg/day, resulting in circulating concentrations of 0.3–7.6 μM of the nonconjugated form [4,5]. After absorption, the major part of quercetin undergoes glucuronidation, methylation or sulfation in the liver (critically discussed in Refs. [3] and [6]) before its release to the circulation. Interestingly, a number of studies have shown that conjugated quercetin quickly enters the cell where it regains its active, nonconjugated form [7].

Classically, the main attributed activity of quercetin was related to its antioxidant effect. However, recent data are indicative of additional effects by direct interaction with plasma membranes [8] and accumulation to the nucleus [9] and mitochondria [10], affecting a number of cell functions. Indeed, quercetin interacts with steroid and aryl-hydrocarbon receptors in breast and prostate cancer cell lines [11,12], decreases cell proliferation and modulates several signal transduction pathways involving MEK/ERK and Nrf2/keap1 [13]. Rodent studies additionally suggest that dietary administration of quercetin may prevent chemically induced colon carcinogenesis, while epidemiological studies indicate that its reasonable food intake may be associated with the prevention of lung cancer [13].

The intracellular transport of the agent and its action in the nucleus have not been examined in detail until now. In the present work, we explore the kinetics and potential mechanisms involved in quercetin cellular internalization and nuclear accumulation. Additionally, through a kinetic transcriptome analysis, we investigated the effects of the flavonol on the transcriptional activity of the cell. We

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have used for this purpose the HepG2 hepatocellular carcinoma cell line, which retains the majority of normal liver functions, in view of previously reported effects of the agent on hepatocellular carcinoma cells [14–17]. We further verified some of our findings in breast adenocarcinoma T47D cells, providing evidence about an extended effect of the flavonol in epithelial cells.

## 2. Methods and material

### 2.1. Chemicals and cell cultures

The hepatocellular carcinoma cell line HepG2 was cultured in RPMI with 10% fetal bovine serum. Cell cultures were routinely maintained at 37°C and 5% $CO_2$. All biochemicals were obtained from Sigma (St. Louis, MO, USA). All culture materials were from Invitrogen (Carlsbad, CA, USA). Native quercetin (3,3′,4′,5,7-pentahydroxyflavone) was prepared from total red wine polyphenol extract by semipreparative high-performance liquid chromatography (HPLC). Its purity (>99.5%) was confirmed by analytical HPLC and proton nuclear magnetic resonance. Quercetin powder was conserved in a dark bottle at −20°C under nitrogen. Stock solutions of quercetin were made in absolute ethanol and kept at −20°C in the dark; subsequent solutions were freshly prepared in culture medium. Control experiments verified that ethanol concentrations (0.0003%–0.003%) did not modify the viability of HepG2 cells.

### 2.2. Detection of quercetin autofluorescence

HepG2 cells were plated in poly-L-lysine-coated coverslips in six-well plates ($10^5$ cells/well), incubated with quercetin (3 μM, the median of the reported plasma concentration of the agent [4,5]), quickly washed with phosphate-buffered saline (PBS) and fixed with 4% formaldehyde for 5 min. Subsequently, coverslips were put upside-down on glass slides, with a drop of Mowiol antifading reagent. Specimens were observed with Leica TCS SP confocal scanner system (Heidelberg, Germany) using a 40× oil immersion objective and zoom software options. Windows were set to 488 $nm_{ex}$/500–540 $nm_{em}$, as described previously [9]. Quantification of fluorescence was made by measuring fluorescence in at least 10 different noncontiguous photographed fields by the use of the Image J (NIH, Bethesda, MD, USA) program.

### 2.3. HPLC analysis of quercetin metabolites

Cells were incubated for 30 min with 3 μM quercetin in PBS, quickly washed, extracted with acid methanol (0.2% acetic acid), scrapped, sonicated and centrifuged. The supernatant was dried *in vacuo*, rediluted in 25 μl of acetonitrile and analyzed with HPLC–electron spray ionization (ESI)–mass spectrometry (MS) in a Waters 1525 binary HPLC pump system with a Waters oven (Waters, Milford, MA, USA) and online DAD-FLD-ESI/MS detection (Thermo Electron, Waltham, MA, USA). UV signal was recorded from a Waters 2996 photodiode array detector, and data obtained by a Waters 2475 multi λ fluorescence detector were further monitored with Empower software. The outlet flow was fully directed to a Thermo Finnigan LCQ Advantage ion trap mass spectrometer with an ESI source. For HPLC analysis, an Atlantis RP18 column (5 μm, 250×4.6 mm internal diameter) with a guard column (2 cm) was used. Elution was performed at 30°C with a 500-μl/min flow rate and in isocratic solvent system of water/acetonitrile/acetic acid (42:58:2, v/v/v). The mass spectrometer was operated in either negative or positive mode at 4.5 kV voltage source, −38.0 V (+9.0 V) capillary voltage, −40 V (−10 V) tube lens offset and 200°C capillary temperature [9].

### 2.4. Cell viability and growth assay

HepG2 cells were plated at a density of $2\times10^4$ cells/ml in 24-well plates. They were grown for a total of 6 days, with a change of the medium containing fresh quercetin on day 3. The 6-day test period was chosen in order to be able to assay the effect of quercetin on at least two cell cycles. Growth and viability were measured by the tetrazolium salt assay [18]. Initial experiments showed that although MTT color development is proportional to mitochondrial activity, this method of cell growth estimation was not affected by quercetin [11,12].

For the detection of cell viability in the case of $H_2O_2$ treatment (as the agent could interfere with the mitochondrion and, in such a case, MTT method might not be appropriate), cell viability was estimated with the viable cell staining with crystal violet. Briefly, cells were washed with PBS and stained with 0.1% crystal violet/methanol (20% v/v) for 3 min. Cells were then washed again with PBS, left to dry and lysed with 1% sodium dodecyl sulfate (SDS), and the optical density was measured at 600 nm.

### 2.5. Measurement of glutathione content

Glutathione content was measured using the dithiobis(2-nitrobenzoic acid)-glutathione reductase method as described previously [19]. Briefly, cells were treated with $H_2O_2$ for 1 h, trypsinized, washed with PBS and resuspended in a 2.25% 5-sulfosalicylic acid solution. After three freeze–thaw cycles in liquid nitrogen/37°C, the lysate was centrifuged (14 000*g*, 20 min, 4°C) and the supernatant was used for the assay, while the precipitated protein was collected with 0.2 M NaOH containing 0.1% SDS and used for protein determination. Two microlitres of each sample were diluted in 48 μl of a buffer containing 30 mM sodium phosphate and 0.3 mM EDTA (pH 7.5) and added to 100 μl of the reaction mixture (30 mM sodium phosphate, 0.3 mM EDTA, 0.15 mM DNTB, 0.2 mM NADPH and 1 U/ml GSH reductase). Absorbance was measured at 405 nm in a microplate reader. Samples from three different wells were assayed in triplicates.

### 2.6. Mitochondrial membrane potential assay ($\Delta\Psi m$)

Mitochondrial membrane integrity was evaluated by staining with rhodamine 123 (R123, Molecular Probes, Invitrogen) as described previously [20]. This cationic fluorescent dye concentrates in the membrane of functional mitochondria because of the high negative electric potential across the mitochondrial inner membrane [21,22], being therefore proportional to the mitochondrial membrane potential ($\Delta\Psi m$). After 1-h treatment with $H_2O_2$ (0.02–-2 mM), cells were detached from culture flasks and diluted in PBS ($1\times10^6$ cells/ml). They were incubated with 1 μM R123 for 15 min at room temperature and assayed by flow cytometry with a Becton-Dickinson FACSArray apparatus (Becton-Dickinson, Franklin Lakes, NJ, USA). Data were analyzed with the CELLQuest (Becton-Dickinson) software.

### 2.7. In vitro scratch migration assay

*In vitro* scratch assay migration assay was performed as described previously [23]. Briefly, cells were seeded in six-well plates and allowed to adhere for 24 h. The cells were treated with 10 μg/ml mitomycin C (Sigma) for 3 h (in order to block the effect of cell proliferation [24]) and washed with PBS, and a 1-mm-wide scratch was made across the cell layer using a sterile pipette tip. Fresh, full medium containing quercetin ($10^{-6}$–$10^{-12}$ M) was added. Photographs were taken every 24 h at the same position of the scratch.

### 2.8. Immunofluorescence

Cells were grown on poly-L-lysine-coated coverslips. After treatment, they were fixed with 4% formaldehyde in PBS for 5 min, permeabilized with 0.5% Triton X-100 and incubated in blocking buffer (PBS, 0.5% Triton X-100 and 1% fish skin gelatin). Actin was stained with rhodamine–phalloidin (Invitrogen, 1/100). Lamin B was identified with an antibody against a synthetic peptide [25]. Fibrillarin was detected with anti-fibrillarin antibody (1/100, ab5821) and anti-rabbit IgG-Texas Red (1/250, ab6719, both from Abcam, Cambridge, UK). Immunofluorescence was performed as described previously [26]. Slides were mounted with Mowiol antifading medium and visualized with a confocal laser scanning module (Leica, Heidelberg, Germany) equipped with an Ar–Kr laser at 488 or 568 nm.

### 2.9. Cell cycle analysis

Cells were washed with PBS and 1% bovine serum albumin. Then, 3 ml of cold absolute ethanol was added, incubated at 4°C for 1 h, washed and provided with 1 ml of a 50-μg/ml propidium iodide (50 μg/ml) in sodium citrate and 50 μl of a 10-μg/ml RNase A solution. Cells were incubated for 3 h at 4°C, assayed by flow cytometry and analyzed with the CELLQuest (Becton-Dickinson) software. Experiments were repeated three times.

### 2.10. RNA extraction and microarray hybridization and analysis

HepG2 cell cultures were incubated with or without quercetin (3 μM) for 2–24 h after overnight serum starvation. Total RNA was isolated using Nucleospin II columns (Macherey-Nagel, Dttren, Germany) according to the manufacturer's instructions. RNA was labeled and hybridized according to the Affymetrix protocol (Affymetrix GeneChip Expression Analysis Technical Manual) by the Laboratoire Transcriptome of CHU Montpellier, France, using the HGU133A plus 2 chip, analyzing a total of 54 675 genes. Signals were detected by an Affymetrix microarray chip reader.

Normalization was performed with the raw data using Genespring GX V9.0 (Agilent, Foster City, CA, USA), and quotients normalized against vehicle (*q*, quercetin/vehicle) were calculated. Student's *t test* (significance set at $P<.05$) was used for the comparison of treated/untreated cells. The Gene Ontology (http://www.geneontology.org) structured controlled vocabularies (ontologies) were used (at $P<.1$) on the gene lists generated at each time point. Additionally, results were analyzed with pathways from the Biological Pathways Exchange (Biopax, http://www.biopax.org) using a $P<.05$ cutoff value for significance. Gene array data have been stored at the NIH Gene Expression Omnibus repository (accession number GSE15162).

For the reverse detection of activated transcription factors, we have used the web resource TFactS (www.tfacts.org). The program utilizes a catalog containing transcription factors associated with 2720 target genes and 6401 experimentally validated regulations and analyzes a set of regulated genes accordingly under a strict set of statistical tests. A result is considered as significant if a statistical significance is attained for the *t* test and at least one supplementary test [27].