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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-pentahydroxy-flavone) 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 2×10^4 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 (1×10^6 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].

2.11. Real-time polymerase chain reaction

DNAse treatment and reverse transcription were performed on total RNA as previously described [28]. Primers were selected from qPrimer Depot (qPrimerDepot-http://primerdepot.nci.nih.gov) (Supplemental Table 1) and synthesized by VBC Biotech (Vienna, Austria). Real-time polymerase chain reaction (PCR) with SBGR was performed with DyNAmo SYBR Green qPCR Kit with ROX (Finnzymes, Oy, Finland) according to the manufacturer's instructions, using the ABI Prism 7000 Sequence Detection System (Applied Biosystems). The reaction conditions for real-time PCR were 95°C for 3 min followed by 40 cycles of 95°C for 15 s then 60°C for 60 s. The changes were normalized according to cyclophilin A RNA expression. Each set of primers was tested with at least three different RNA samples treated independently.

2.12. Western blot

Cells, exposed or not to quercetin (3 μM), were washed twice with ice-cold PBS and suspended in cold lysis buffer containing 1% Nonidet P-40, 20 mM Tris (pH 7.4) and 137 mM NaCl supplemented with protease inhibitors. Equal amounts of total protein extracts were suspended in Laemmli's sample buffer and separated by SDS–polyacrylamide gel electrophoresis. Proteins were transferred onto nitrocellulose membranes and blotted with anti-cyclin-D1 (1/100, RM-9104, Thermo-Scientific, Fremont, CA, USA) or anti-β-actin (1/1000, Sigma-Aldrich, Saint Louis, MO, USA). Afterwards, membranes were washed and incubated for 1 h at room temperature with anti-rabbit HRP conjugated secondary antibody (1/5000, Pierce). The ECL system (Pierce, SuperSignal West Pico Chemiluminescent Substrate) was used for revelation of specific bands.

2.13. Statistical analysis

Statistical analysis of results other than those from Genechip and TFactS data was performed with PASW v 19.0 (IBM, SPSS Inc., Chicago, IL, USA) with Student's *t* test or one-way analysis of variance where applicable.

3. Results

3.1. Internalization and localization of quercetin in HepG2 cells

In order to study the mechanisms regulating quercetin internalization and intracellular distribution, we have used the previously reported intrinsic fluorescence of the agent observed in viable cells [9].

3.1.1. Internalization

When HepG2 cells were incubated with quercetin, a time-dependent rapid accumulation of intracellular fluorescence was observed (Fig. 1A), indicative of an active uptake mechanism. This mechanism relays on temperature (Fig. 1A, C), suggesting an energy-dependent process. This was further verified by incubating HepG2 cells with the nonspecific ATPases and phosphatases inhibitor sodium orthovanadate (Na_3VO_4, Fig. 1A, C), resulting in a significant inhibition of quercetin uptake. Quercetin intracellular accumulation is only observed in viable nonpermeabilized cells, suggesting interaction with an active cellular membrane component. These data confirm previous observations suggesting that, in addition to a passive lipophilic polyphenol diffusion [29], an active mechanism of quercetin transport also occurs [10,30].

We have further verified that intracellular increase of fluorescence corresponds to quercetin accumulation. Acidified methanol extraction of HepG2 cells, incubated with quercetin for 2 min–24 h, resulted in a relative quercetin concentration rapidly decreasing over time down to 60% in favor of an *O*-methylated metabolite (Supplemental Figure 1); neither oxidative derivatives nor acetyl- or glycoconjugates were detected. Thereafter, the concentration of native and metabolized quercetin remained stable throughout 24 h.

3.1.2. Detection of membrane transporter systems participating in quercetin internalization

Active transport into cells necessitates the implication of specific membrane transporters. We therefore studied the involvement of a number of transporter families present on HepG2 cells through competition of quercetin intracellular accumulation by the co-incubation of cells with known transporter substrates. Different sugars (glucose, dextrose or sucrose) do not modify quercetin uptake (not shown), suggesting that glucose transporters are not involved in unconjugated quercetin internalization. In contrast, organic anions (verapamil, taurocholic acid) reduced and delayed quercetin internalization (Fig. 1B). Organic anions enter the cell through two distinct families of transporters: Na^+-taurocholate cotransporting polypeptide and organic anion transporter polypeptides (OATPs). Several members of the OATP family have been suggested to belong to the GSH-X exchangers' family [31–33], while contradictory reports have also been published [34,35]. In HepG2 cells, only OATP isoforms are expressed [31]. In order to study if quercetin internalization is dependent on mechanisms that have been related to OATPs, we incubated cells with 2 mM H_2O_2 [33], a concentration not influencing significantly cell viability or mitochondrial function but drastically reducing intracellular GSH (Supplemental Figure 2). This resulted in a reduced quercetin fluorescence (Fig. 1B), suggesting that at least a GSH-dependent OATP may participate in quercetin influx.

3.1.3. Intracellular localization

In HepG2 cells, internalized quercetin fluorescence was detected in the cytoplasm but was predominantly present in the nucleus (Figs. 1A and 2A). Cytoplasmic distribution was weak and short lasting, not allowing further analysis. However, quercetin was only partially colocalized with actin filaments (Fig. 2B), in contrast to previously described data [36]. In order to explore nuclear staining and to study whether nuclear pore transporters are involved in quercetin intranuclear accumulation, we used paclitaxel, an agent modifying nuclear envelope organization and integrity and impeding chaperoned transport through nuclear pore complexes [26]. Paclitaxel-treated cells presented high-grade nuclear atypia (multinucleated or lobulated nuclei, Fig. 2A) and presented nuclear envelope ruptures (Fig. 2A arrowheads). However, quercetin nuclear accumulation was not altered by paclitaxel, suggesting that the flavonol nuclear transport did not depend on nuclear pore functionality.

Nuclear quercetin presented a homogeneous specific background staining, with spots of higher intensity. Immunofluorescence for fibrillarin, a nucleolar small nuclear ribonucleoprotein, revealed a partial colocalization (Fig. 2B), suggesting that quercetin might (partially) concentrate at nucleoli. This was further verified by treatment of cells with actinomycin D, a transcriptional inhibitor and nucleolus-disrupting agent, which also disrupted quercetin spotted nuclear distribution, resulting in a speckled profile (Fig. 2B). Supporting evidence for flavonol specific nuclear and nucleolar accumulation were also derived from the study of quercetin distribution throughout cell cycle, as the compound was excluded from mitotic, transcriptionally inactive, chromatin (Supplemental Figure 3).

It is of interest that this intracellular, and especially nuclear/nucleolar, accumulation of quercetin is not a unique characteristic of HepG2 cells, but is also found in the breast cancer cell line T47D, with almost identical results (Supplemental Figure 4).

3.2. Effect of quercetin on transcription

As shown, quercetin accumulates in nucleoplasmic structures within minutes, after its introduction to the extracellular medium; however, previous results show that no direct interaction of the flavonol with DNA was evidenced [9]. We have therefore assayed a possible effect of the polyphenol on transcription by assaying the whole transcriptome of HepG2 cells after 2, 4, 12 and 24 h of quercetin treatment. The agent induces significant changes in numerous transcripts (Fig. 3A). Out of ~55 000 assayed transcripts, about 14 000–17 000 were modified by quercetin at each time point.

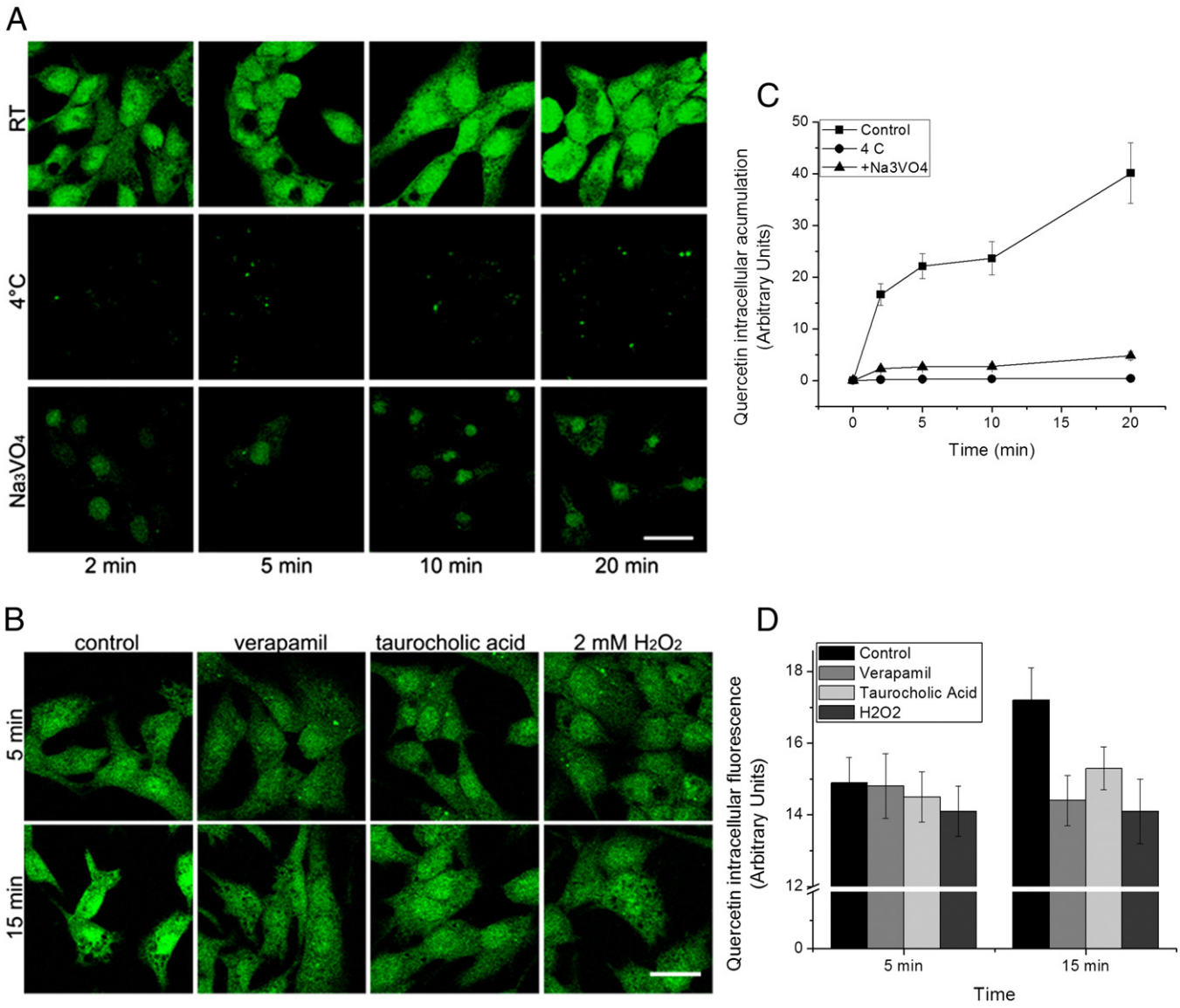


Fig. 1. Quercetin influx in HepG2 cells is mediated by an active transport mechanism. (A) Time course of quercetin uptake (3 μM in PBS) by HepG2 cells. The flavonol is traced by its intrinsic fluorescence, as previously described [9]. Cells were incubated at room temperature, at 4°C or in the presence of the nonspecific ATPases/phosphatases inhibitor sodium orthovanadate (scale bar=25 μm). (B) Coincubation of HepG2 cells with quercetin and OATP substrates (verapamil or taurocholic acid) (all at 300 μM) inhibits its influx. Similarly, GSH depletion with 2 mM H_2O_2 for 1 h (which does not induce any change in cell viability or mitochondrial potential, see Supplemental Figure 1, but decreases drastically intracellular GSH) results in reduced quercetin uptake, implicating OATP transporters in flavonol import (scale bar=25 μm). (C and D) Quantification of data presented, respectively, in A and B. Ten noncontiguous images were quantified per experiment (repeated three times) with the Image J program (Rasband, W.S., ImageJ, US National Institutes of Health, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/, 1997-2009). Means±S.E.M. are shown.

However, the number of up- or down-regulated transcripts varied significantly over time: at 2 and 4 h, almost 60% and 45% of modified genes are up-regulated; thereafter, a second wave of up-regulation follows, leading to 58% of up-regulated genes at 24 h. This second wave probably results from a combination of direct and indirect modification of gene transcription by quercetin. A total of 2175 transcripts (Fig. 3B), corresponding to 1459 known genes, were modified by quercetin over the 24-h incubation period (Supplemental Table 2) and were further analyzed, assuming that they represent the "signature" of quercetin continuous action on the genome. Our data show the following:

1. Analysis of common transcripts, modified by quercetin over time, revealed a number of significantly modified elements:
   a. *Transcripts related to cell–cell adhesion and cellular motility regulation based on Gene Ontology terminology*. Gene ontology analysis revealed that a significant number of the transcripts were related to cell adhesion/cellular motility. Quantitative reverse transcriptase (qRT)-PCR assay of some of the modified genes related to this function (Supplemental Figure 5A-D) showed that contactin associated protein-like 4 (CNTNAP4) and WNT1 inducible signaling pathway protein 1 (WISP-1), directly related to cell invasion and motility [37–40], were up-regulated; in contrast, integrin-α9 (ITGA9) and protein tyrosine kinase 7 (PTK7), elements inversely related to cell progression/motility [41–43], were found to be decreased by both methods (transcriptome and qRT-PCR).
   b. *Pathways related to cell cycle regulation.* These included (a) cell cycle checkpoints, (b) G1/S DNA damage checkpoints, (c) p53-dependent G1/S DNA damage checkpoint and (d) Rb tumor suppressor/checkpoint signaling in response to DNA damage. We have found by qRT-PCR a more than sevenfold increase in

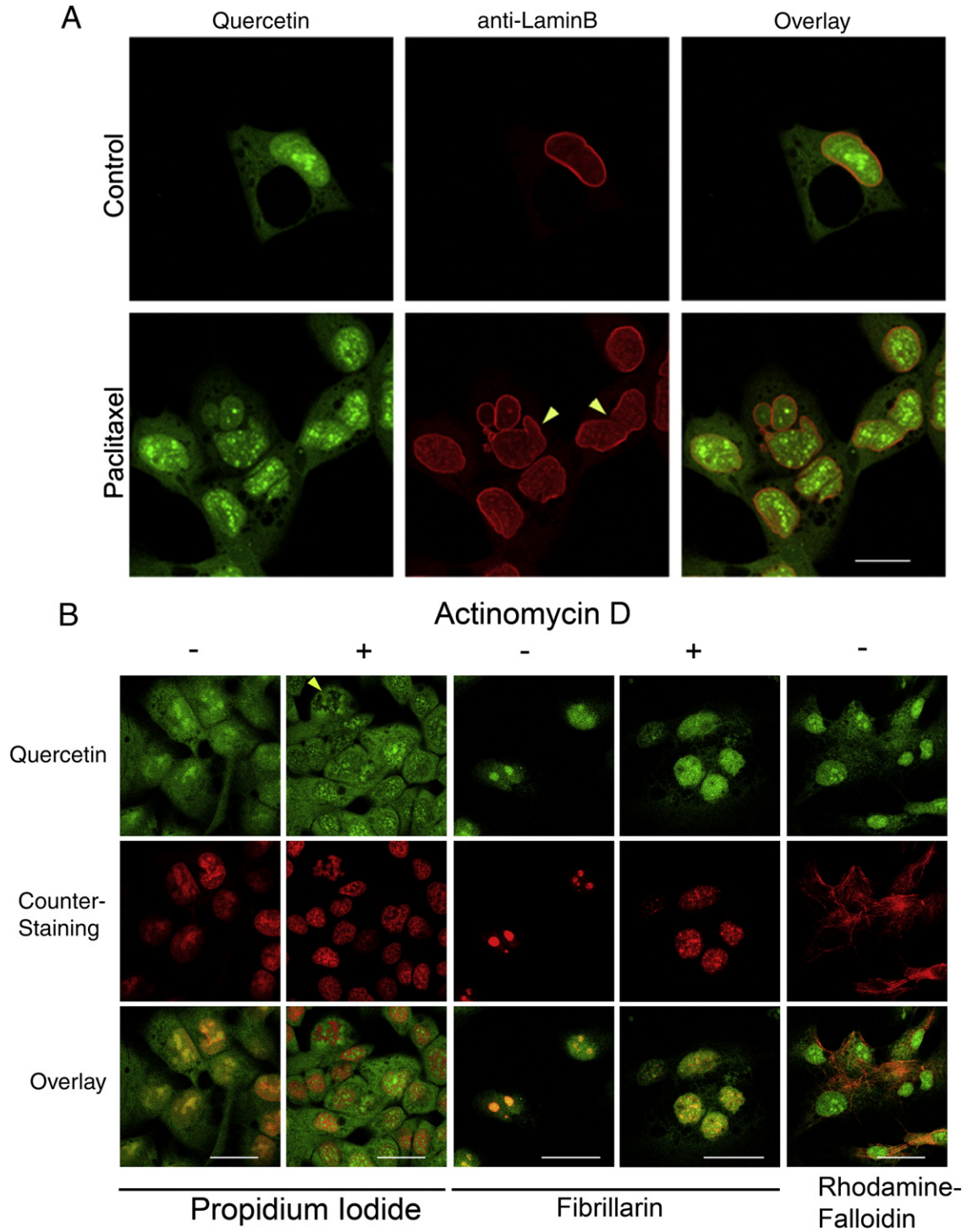


Fig. 2. Quercetin subcellular localization. (A) HepG2 cells were treated for 30 min with quercetin, fixed and counterstained with an anti-lamin B antibody, staining the nuclear envelope. Quercetin concentrates in specific structures within the nucleus. Pretreatment of cells with paclitaxel, a taxane which modifies nucleic membrane permeability and nuclear pore complex (NPC) localization, thus inhibiting cytoplasmic–nucleic transport [26], does not modify its nuclear localization in spite of an apparent disruption of the NPC (arrowheads). (B) HepG2 cells were treated for 30 min with quercetin (3 μM in PBS, first row, green), postfixed and thereafter counterstained (red, middle row) with propidium iodide, an anti-fibrillarin antibody, and rhodamine–phalloidin under standard conditions or after overnight treatment with 1 μM actinomycin D. Colocalization is depicted as a yellow signal in the lower row. Quercetin is mainly accumulating in cell nuclei, especially at nucleolar areas, and does not colocalize with either actin or condensed mitotic chromatin (arrowhead). Nucleolar dissociation with actinomycin D results in speckled profile, overlapping with fibrillarin spots (scale bar=25 μm).

the expression of cyclin-dependent kinase inhibitor 1A (p21, Cip1, Supplemental Figure 5I), a common determinant of all the above pathways, which could result in an increased degradation of cyclin-D1, explaining data presented below.

c. *Pathways relayed to xenobiotic metabolism and hydroxylations.* Interestingly, the effect of quercetin on CYP enzymes is bimodal: at early time points, the flavonol enhanced, while after longer incubation times, it decreased CYP transcription, equally verified

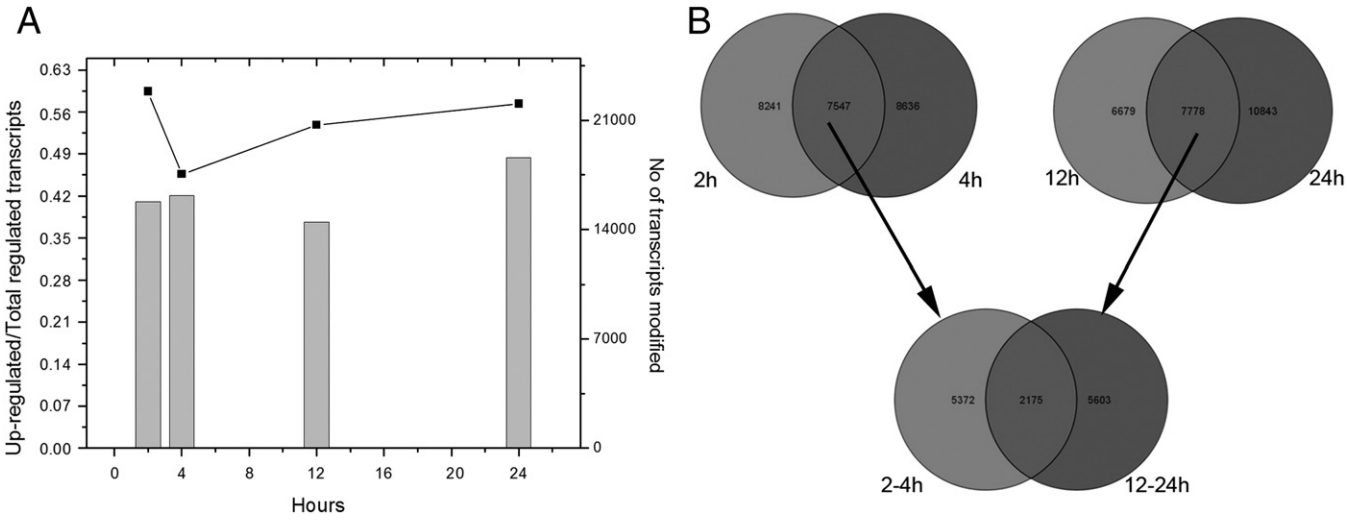


Fig. 3. Time-related whole transcriptome analysis of quercetin in HepG2 cells. HepG2 cells were cultured for 2, 4, 12 or 24 h with 3 μM quercetin. Whole RNA was isolated, reverse transcribed and hybridized on an Affymetrix HGU133A plus 2 chip. (A) The number of significantly modified transcripts is presented (bars) as compared to control cells incubated with vehicle for the same time periods. Line shows the fraction of up-regulated over total modified genes for the indicated periods. (B) Venn diagrams of modified transcripts are shown for the different incubation times. As shown, 7547 transcripts were comodified at 2 and 4 h, and 7778 transcripts were commonly modified after 12 and 24 h of incubation with quercetin. The lower Venn diagram, analyzing these two sets, identifies a subset of 2175 transcripts modified throughout the study (2–24 h) by quercetin. The complete set of these transcripts is presented in Supplemental Table 1.

by qRT-PCR for CYP2C8 and CYP11A1 (Supplemental Figure 5E–F). Furthermore, mitochondrial sirtuins 4 (ADP-ribosyltransferase) and 5 (deacetylase of cytochrome *c*, a protein with a central function in oxidative metabolism [44]) are also significantly modified by quercetin (Supplemental Figure 5G–H).

d. *Immune-related pathways* were also modified by quercetin, linking the flavonol to immune-related factors and especially B- and T-cell receptors. These pathways, together with the modified expression of a number of cytokine (interferon, interleukins), chemokines and CD molecules (see Supplemental Table 2), may

Transcription Factor	P.value	Quercetin 2h	4h	12h	24h
CREB1	0.00E+00				
ETS1	0.00E+00				
GLI	0.00E+00				
SPI1	1.00E-05				
TCF/BETA-CATENIN	1.00E-05				
STAT	2.00E-05				
STAT1	5.64E-03				
STAT2					
STAT3	3.90E-04				
STAT4					
STAT5	2.55E-02				
EGR1	4.00E-05				
AP1	1.10E-04				
SP1	1.10E-04				
TFAP2A	1.50E-04				
FOXO	2.40E-04				
FOXO1	5.47E-03				
FOXO2					
FOXO3	3.54E-02				
FOXO4					
FOXO5					
FOXO6					
PPARG	6.20E-04				
CEBPA	6.50E-04				
ATF1	1.04E-03				
HNF4A	1.09E-03				
NFIA	1.70E-03				
MYC	2.09E-03				

Transcription Factor	P.value	Quercetin 2h	4h	12h	24h
CEBPD	2.11E-03				
NR2E1	2.12E-03				
E2F4	2.52E-03				
POU2F1	2.80E-03				
ATF2	2.87E-03				
NFAT1	3.51E-03				
NFKB	3.54E-03				
E2F1	4.10E-03				
ETS2	4.10E-03				
ID1	4.16E-03				
FLI1	6.23E-03				
NR3C1	6.81E-03				
FOS	8.14E-03				
SIX3	1.00E-02				
ATF4	1.16E-02				
SP3	1.38E-02				
YY1	1.38E-02				
JUNB	1.55E-02				
JUND	3.15E-02				
PPARA	1.55E-02				
CDX2	1.81E-02				
E2F	1.83E-02				
NFAT2	2.28E-02				
REST	2.84E-02				
NFIC	3.19E-02				
SREBP	3.42E-02				
ETV4	4.55E-02				

-2< >2

Fig. 4. Heat map of transcripts of transcription factors mRNA, which were found to be involved in the significant modification of common genes by quercetin over 24 h of incubation (see Material and Methods for further details).

explain previous data on the immunomodulatory effects of the agent (see Ref [45] for a recent review).

e. Interestingly, quercetin not only interacts with estrogen receptors (both ERα and β) as reported previously [11,12], but also up-regulates their transcription (Supplemental Table 2 and Supplemental Figure 5J).

2. The effect of quercetin on translation regulator activity (GO0045182) function suggests that this agent could modulate (directly or indirectly) a number of transcription factor biosynthesis or function.

Reverse *in silico* analysis of regulated genes [27] identified a number of transcription factors potentially involved in quercetin transcriptional effects (Supplemental Table 3). A thorough inspection of our list, concerning modified transcription factor genes (Fig. 4), revealed that quercetin had a significant effect on several other transcription factors during the time-course study. We have therefore concluded that quercetin action occurs both by an early enhancement of the transcription of a number of nuclear factors as well as by their activation.

3.3. Quercetin actions on cell fate

3.3.1. Effect of quercetin on HepG2 proliferation and cell cycle

Transcriptional pathway analysis of quercetin-treated cells pointed out pathways related to cell cycle arrest and cell proliferation. To further verify this element, we treated cells with the agent and assessed its effect on cell growth. Quercetin significantly inhibited the proliferation of HepG2 cells, with an IC_{50} of 1.03×10^{-7} M and a maximum inhibition of 69% (Fig. 5A) after 6 days. Further analysis of this inhibitory response showed that it was due to cell-cycle arrest at G1 (58.8%±4.3% vs. 48.9%±3.8%, quercetin vs. control, $P<.05$) (Fig. 5B). Analysis of potential elements that could explain the effect of quercetin on cell cycle revealed that the agent decreases significantly cyclin D1 levels after a 24-h incubation (Fig. 5C–D), a result that could be attributed to the increase in the expression of cyclin-dependent kinase inhibitor 1A (Supplemental Figure 5I).

3.3.2. Effect of quercetin on cell migration and the actin cytoskeleton

Pathway analysis showed that quercetin modified the expression of cell-motility-related genes together with cell communication and cell adhesion functions. We have therefore examined whether the flavonol could affect scaffold proteins and cell migration. Short incubation of cells with quercetin results in a disorganization of the polymerized actin network (Fig. 6C), with loss of intracellular stress-like fibers, without any effect on actin synthesis (transcriptome data and results presented in Fig. 5C, in which a constant protein load was used). However, and in contrast to previous findings [36], only a partial colocalization of quercetin with actin filaments was observed in either HepG2 (Fig. 2B) or T47D cells (Supplemental Figure 4). Longer incubation with quercetin showed that migration of HepG2 cells was inhibited in a time- and dose-dependent manner (Fig. 6A–B).

4. Discussion

The increased interest in polyphenol research resulted from the discovery that, in addition to their potent antioxidant actions, they may also interfere with major cellular processes. Quercetin is a cardinal dietary flavonol found at high concentration in the majority of edible plants [3]. Thirty-six percent to fifty-three percent of ingested quercetin is found in the blood, whereas a substantial portion is eliminated from lungs as CO_2 [46]. Polyphenols' absorption and metabolism, including quercetin, have been extensively studied using Caco-2 (intestinal cancer cells) and HepG2 (hepatocellular-carcinoma-derived cells, which retain the majority of the normal functions of the human hepatocyte), as gut and liver represent the major sites of polyphenol absorption and metabolism in humans [3,6]. Caco-2 monolayers accumulate quercetin more efficiently than quercetin 3-glycoside, while they can hydrolyze the latter [47]. HepG2 cells also accumulated quercetin [48] and further polymerize or transform it to *O*-methylated (isorhamnetin) and oxidized (protocatechuic acid) derivatives. However, as shown here, the majority of internalized quercetin remains in its native form long enough to exert specific actions. Quercetin has been reported to interfere with a number of cellular mechanisms, including free radical scavenging, protein kinase and topoisomerase inhibition, deacetylase activation and regulation of gene expression through NFκB or AP-1 and xenobiotic or antioxidant response elements [49,50].

A major problem encountered in polyphenol-related bibliography is the effective concentration and the purity of agents utilized in different studies. In the present study, we aimed an approach of quercetin effects at concentrations compatible with the agent's physiological levels in the blood, in contrast to a series of previous studies that have used very high polyphenol concentrations [4,5]. In addition, we have used a highly purified preparation of quercetin in order to decipher its proper biological activity, as commercially available preparations of the flavonol may contain impurities or oxidized products (5%–10%, J. Vercauteren, unpublished data).

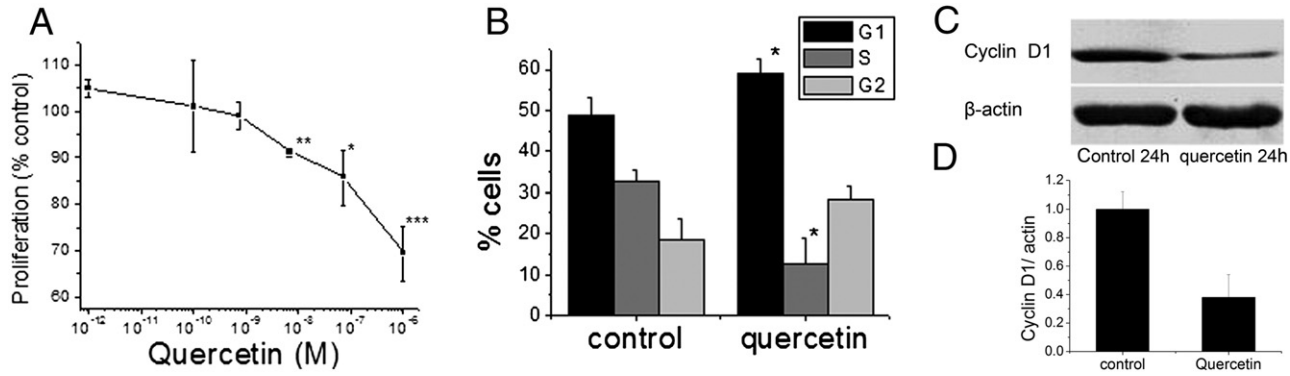
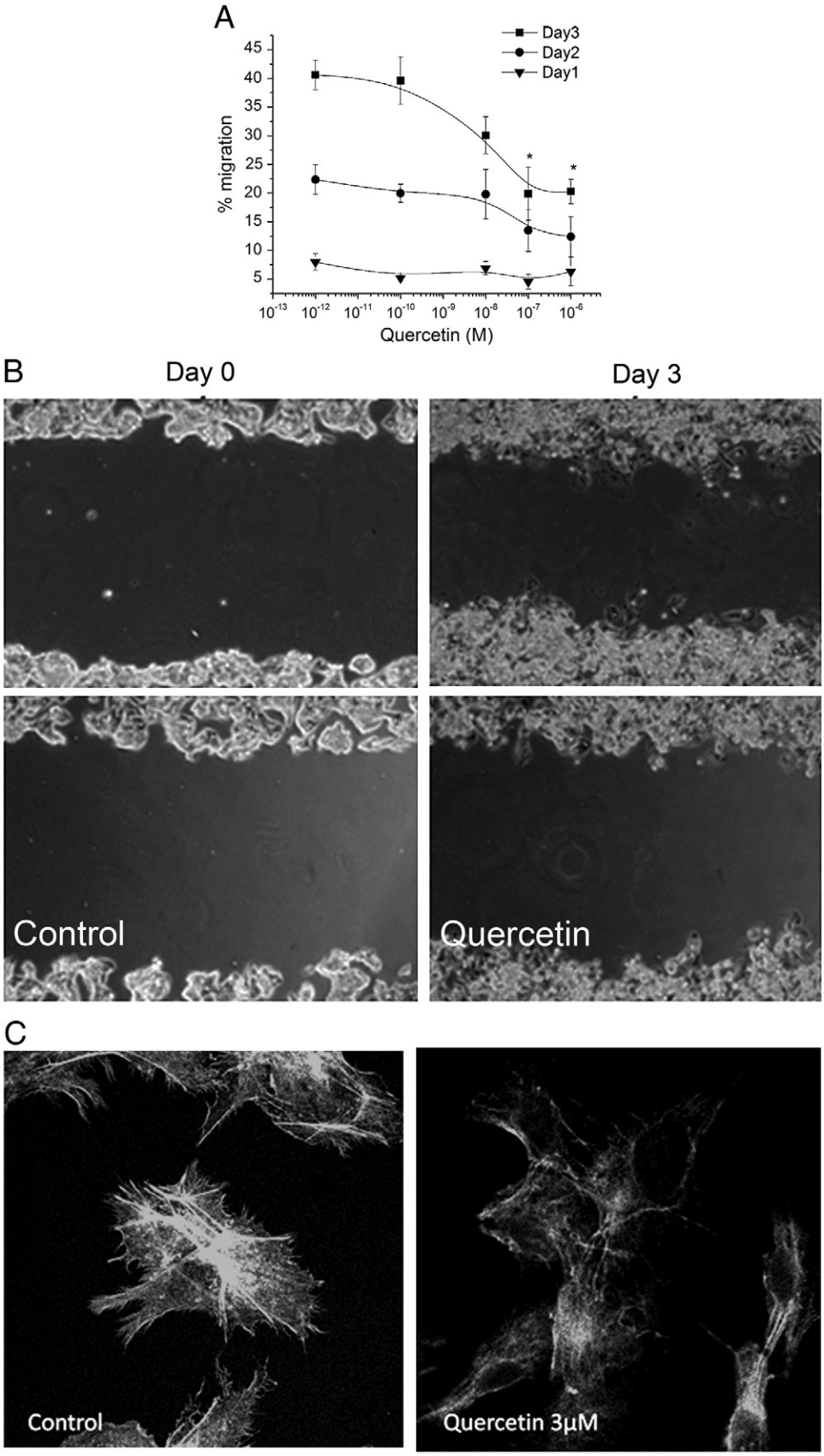


Fig. 5. Quercetin induces a dose-dependent decrease of HepG2 cell growth by arresting cells in G1 phase of the cell cycle. (A) Dose-dependent inhibition of cell proliferation by quercetin. HepG2 cells were incubated with the indicated concentrations of quercetin for a total of 6 days, with one change of the medium containing quercetin (3 μM) at day 3. Mean±S.E.M. of three different experiments performed in triplicate. (B) Quercetin modifies cell cycle of HepG2 cells. Cells were incubated with 3 μM quercetin for 24 h. Figure presents the percentage of cells at each phase of the cell cycle. Mean±S.E.M. of three different experiments. *$P<.05$, at least. (C and D) Quercetin (3 μM) induces a significant decrease of cyclin D1 after a 24-h incubation of HepG2 cells. A typical immunoblot is presented in C, and its quantification is shown in D (*$P<.01$ compared to control at 24 h).

While the planar structure of quercetin could result in a passive penetration through the plasma membrane [10,51], the agent could also use molecular transporters present on cellular surfaces. Indeed, quercetin glucosides could use glucose endocytosis mechanisms [52]. Here we show that quercetin aglycone is transported in an energy-dependent manner and provide evidence about a possible GSH-



dependent OATPs implication in its internalization [31–33]. Our data are in line with a recent work presenting similar results on the HEK293 cell line stably expressing OATP1A2 and OATP2B1 [53]. However, it should be noted that not all OATPs function in a GSH-dependent manner [34,35]. Our findings suggest that a GSH-dependent mechanism is, to some extent, participating in quercetin internalization process. Interestingly, OATPs are also responsible for the transport of bile acids, suggesting that increased serum bilirubin, detected in advanced liver pathologies, could inhibit the intracellular translocation of antioxidants. Therefore, the increased levels of plasma total antioxidant capacity observed in primary biliary cirrhosis [54] could be explained, at least partially, by the inability of cells to internalize polyphenols.

In the cell, quercetin is homogeneously distributed into the cytosol, without a distinct association with proteins [9]; it further concentrates to mitochondria [10] and the nucleus, as shown here, through a mechanism not involving nuclear pore complexes. Similar nuclear accumulation has previously been described for GFP or dextrans [55,56]. In the cytosol, quercetin has been shown to interact with steroid hormone receptors [11,12], and as reported here, it also modifies their transcription, while it exerts a number of discrete actions related to cell growth and migration. In the nucleus, the agent concentrates in specific structures and exerts a massive control of transcription.

The transcriptional activity of quercetin takes place in two distinct waves: an early one peaking at 2 h and a late one exerted after 24 h. However, we have identified and concentrated on 2175 transcripts (corresponding to 1459 gene signatures) continuously modified by quercetin over 24 h at least by a factor of 2 and considered as the main transcriptional "signature" of the flavonol in HepG2 cells. These continuously modified genes are involved in pathways related to cell motility and cell cycle regulation, xenobiotic metabolism, immune-related factors, steroid receptors (up-regulation of ERα and β) and transcription *per se*.

Only few studies have focused on the effect of quercetin in gene transcription, but none was conducted in hepatocyte-derived cells. They can be grouped in two main categories: (a) studies, like the one presented here, addressing the effect of quercetin in isolated cell populations, such as vascular endothelial [57], cardiomyocytes [58], cancer colorectal cells [59–61] or fibroblasts [62], and (b) studies exploring the effects of quercetin *in vivo* after prolonged administration in humans [63] or experimental animals [64,65]. In cancer cell lines or primary cell studies [57–61], using varying quercetin concentrations (100 nM to 50 μM) and with chips containing significantly lower number of transcripts (4–6000, with the exception of data included in Refs. [60] and [62]), a small number of genes were affected by the agent (59–200). A high number of modified genes, similar to that presented here, was reported in one study performed in CO115 colon cancer cells [60] under 100 μM of the flavonol. In the majority of studies, quercetin was reported to modify the expression of genes related to xenobiotic metabolism and detoxification, signal transduction, transport, cell cycle regulation and modification of transcription. However, a finding reported for the first time here is that, in HepG2 cells, quercetin modifies the transcription of a large number of transcription factors. Although the effect of the flavonol on EGR and STAT1 [61] was previously reported in Caco-2 cells, this is the first work providing evidence about a massive effect of the agent on transcription factors, an element bridging the gap between quercetin intracellular phenotypic actions and transcriptional events. In addition, three quercetin intervention studies dealt with some of the above-mentioned gene groups: two were conducted in rodents, examining enteric mucosa (one in rats, using 10-g/kg diet, for 11 weeks [65] and one in mice, with 5 or 50 mg/day of quercetin in tap water [64]). A modified expression of 207 and 100 transcripts, respectively, grouped in xenobiotic metabolism, nucleotide modification and metabolism. In one study, additional categories linked to inhibition of carcinogenesis, redox homeostasis, immune reaction, protein modification, translation and cell–cell interaction were identified [65]. In the same study, quercetin inhibited cell proliferation, as shown here and reported in previous studies [60,61]. Finally, in a recent study, transcriptome differences in human monocytes from volunteers fed with quercetin (50–150 mg/day) were explored [63]. A set of 788 genes was modified by a quercetin diet, related to hematopoiesis and immune system genes, nucleic acid metabolism and apoptosis.

The effect of quercetin on cell growth has been constantly found in all transcriptome studies and verified in different cell lines [14–17]. Interestingly, both in a previous study in Caco-2 cells [61] and in HepG2 cells, quercetin induced a G1 cell cycle arrest, probably related to cyclin D inhibition, due to the induction of cyclin-dependent kinase inhibitor 1A (p21, Cip1) [14,16,61,66–69]. The effect of quercetin on cell cycle has also been reported to be mediated by induction of Mutyh and inhibition of annexin 1 (ANXA1) in the colon of quercetin-fed rats [65], a result not verified in our study, suggesting species- or tissue-specific differential effects of the flavonol.

In the present study, we further report that quercetin decreases the migratory capacity of HepG2 cells and disrupts the actin cytoskeleton of HepG2 and T47D cells. This can be attributed to the alteration of a number of adhesion–migration genes (up-regulation of CNTNA4 and Wnt pathway-related genes and down-regulation of integrin A9 and PTK7 as shown here), confirming data reported in Caco-2 cells [61]. Few prior studies have assayed the effect of quercetin on adhesion molecules expression, including a model of melanoma lung metastasis [70] or pancreatic cancer cells [71] reporting a down-regulation of focal adhesion kinases, while in MCF7 breast cancer cells, modification of cell adhesion was related to an inhibition of MMP-9 [72]. Interestingly, the disrupting effect of quercetin on actin cytoskeleton was also reported in plants [73], suggesting a conserved role of quercetin as a regulator of cell adhesion mechanisms.

In conclusion, our data show that quercetin enters epithelial cells and concentrates to intracellular structures such as the mitochondrion and the nucleus. In the cytosol, the flavonol disrupts actin cytoskeleton and inhibits cellular proliferation and migration. In the nucleus, quercetin modifies the transcription of a large number of genes related to cell motility and cell cycle regulation, xenobiotic metabolism, immune-related factors and transcription, while it up-regulates the expression of estrogen receptors α and β. Finally, it modifies the transcription and/or activity of a large number of other transcription factors, an element further regulating its control on transcription. Our data support the idea that natural compounds like quercetin may actively accumulate in discrete cell structures and display a pleiotropic action in addition to their antioxidant effects on epithelial cells.

Fig. 6. Quercetin decreases the migration of HepG2 cells. Cells were seeded in six-well plates and allowed to adhere for 24 h. The cells were treated with 10 μg/ml mitomycin C for 3 h and washed with PBS, and then a 1-mm-wide scratch was made across the cell layer using a sterile pipette tip. Fresh medium containing quercetin (10^{-6}–10^{-12} M) was added. Photographs were taken every 24 h at the same position of the scratch, and results were analyzed using the Image J software (NIH, Bethesda, MD, USA). (A) Inhibition of the migratory potential of HepG2 cells pretreated with mitomycin C by different concentrations of quercetin at 24, 48 and 72 h. Mean±S.E.M. of four different experiments in duplicate (*P<.05). (B) Characteristic microphotographs of the effect of quercetin on HepG2 migration after 3 days of exposure. (C) Typical images of HepG2 cells untreated (control) and treated with quercetin (3 μM) for 30 min stained with rhodamine–phalloidin for the visualization of the actin cytoskeleton.

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