Research Article

Multitarget Effects of Quercetin in Leukemia

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Abstract

This study proposes to investigate quercetin antitumor efficacy in vitro and in vivo, using the P39 cell line as a model. The experimental design comprised leukemic cells or xenografts of P39 cells, treated in vitro or in vivo, respectively, with quercetin; apoptosis, cell-cycle and autophagy activation were then evaluated. Quercetin caused pronounced apoptosis in P39 leukemia cells, followed by Bcl-2, Bcl-xL, Mcl-1 downregulation, Bax upregulation, and mitochondrial translocation, triggering cytochrome c release and caspases activation. Quercetin also induced the expression of FasL protein. Furthermore, our results demonstrated an antioxidant activity of quercetin. Quercetin treatment resulted in an increased cell arrest in G1 phase of the cell cycle, with pronounced decrease in CDK2, CDK6, cyclin D, cyclin E, and cyclin A proteins, decreased Rb phosphorylation and increased p21 and p27 expression. Quercetin induced autophagosome formation in the P39 cell line. Autophagy inhibition induced by quercetin with chloroquine triggered apoptosis but did not alter quercetin modulation in the G1 phase. P39 cell treatment with a combination of quercetin and selective inhibitors of ERK1/2 and/or JNK (PD184352 or SP600125, respectively), significantly decreased cells in G1 phase, this treatment, however, did not change the apoptotic cell number. Furthermore, in vivo administration of quercetin significantly reduced tumor volume in P39 xenografts and confirmed in vitro results regarding apoptosis, autophagy, and cell-cycle arrest. The antitumor activity of quercetin both in vitro and in vivo revealed in this study, point to quercetin as an attractive antitumor agent for hematologic malignancies. Cancer Prev Res; 7(12); 1240–50. ©2014 AACR.

Introduction

Acute myelogenous leukemia (AML) is a devastating disease with high mortality and morbidity (1). Myelodysplastic syndromes (MDS) constitute a heterogeneous group of clonal hematopoietic disorders primarily affecting the elderly and characterized by ineffective hematopoiesis, refractory cytopenia, and increased risk of developing AML (2). MDS and AML cells present multiple defects, an obstacle for the efficient eradication of neoplastic cells by the existing drugs. Less than 50% of patients successfully recover after intensive chemotherapy and bone marrow transplantation and elderly patients are not candidates for aggressive therapies rendering urgent the need for new drugs (3).

Flavonoids have been considered to possess promising chemopreventive and chemotherapeutic properties with multiple target interaction and multiple pathway regulation against various human cancers. Quercetin, a naturally occurring flavonol, is one of the most abundant flavonoids universally present in fruits and vegetables (4) and has been shown to possess multiple properties capable of reducing cell growth in cancer cells (5–7). The growth-suppressive effects of quercetin in leukemic cells have been previously described; the molecular mechanisms underlying quercetin-mediated cellular responses in AML cells, however, remain poorly defined.

Herein, we aimed to clarify certain pathways involved in quercetin antitumor activity. Thus, we performed in vitro and in vivo studies using the myeloid P39 cell line as a model and designed the study to investigate the effects of quercetin on signaling pathways of apoptosis, cell cycle and autophagy in the cell line and in a tumor xenograft model.

Materials and Methods

Reagents and antibodies

Quercetin (>98% pure), acridine orange (AO) salt, MTT, chloroquine, SP600125 and PD184352, TBHP, DCFDA, and rhodamine 123 reagents were obtained from Sigma.
Chemical Co. The FITC–Annexin V apoptosis detection Kit I from BD Pharmingen. Cyclin D, cyclin E, cyclin A, Cdk4, Cdk2, Cdk6, p21, p27, Bcl-xL, Bax, Bcl-2, cytochrome c, Mcl-1, Fas, FasL, actin, and GAPDH from Santa Cruz Biotechnology. Rb, pRBSS807/S811, Beclin-1, PI3K class III, mTOR, and p-mTORSer2448 from Cell Signaling Technology. pERKThr185/Tyr187, pJNK Tyr183/185, ERK1/2, and JNK from Invitrogen. Atg5-Atg12, Atg7, LC3I/II, and VDAC from Abcam Inc. Anti-rabbit, anti-mouse, and anti-goat peroxidase–conjugated antibodies from KPL, Inc.

**Cell culture and treatment**

The myeloid cell line P39, derived from patient with an MDS-chronic myelomonocytic leukemia (CMML), was kindly provided by Eva Hellstrom-Lindberg, Karolinska Institute, Stockholm (which was established by Prof. Masami Nagai, National Cancer Center Hospital, Tsukiji 5-chome, Chuo-ku, Tokyo, Japan). This cell line is well established and widely discussed in the literature (8–11). P39 cells were cultured in RPMI-1640 medium containing 10% FBS, in a 37°C humidified atmosphere containing 5% CO₂. Quercetin was dissolved in DMSO, at a final concentration of 0.1% (v/v) in RPMI. For all subsequent experiments, P39 cells were treated with chloroquine (20 μmol/L), SP600125 (20 μmol/L), and PD184352 (20 μmol/L) or quercetin at final concentrations of 10, 50, or 100 μmol/L for 12, 24, or 48 hours. Control cells were treated with vehicle alone.

As P39 could be contaminated with HL-60 cells (12, 13), we karyotyped both cell lines. All P39 cells analyzed exhibited an XY chromosome karyotype as expected (P39 cell line was from a male patient, whereas HL-60 cell line was derived from a female patient). In addition, karyotyping of P39 cells analyzed showed +del(6)(q15), -16 and -17 chromosomal abnormalities in all metaphases, as previously described (14).

**Cell viability assay**

MTT assay was performed as previously described (15). Briefly, 5 × 10⁴ viable P39 cells were seeded into a 96-well in RPMI 10% FBS supplemented medium and incubated with different quercetin concentrations (10, 50, or 100 μmol/L). After 12, 24, and 48 hours, 10 μg/well of MTT (5 mg/mL) was added and incubated for 4 hours, then 100 μL of 0.1 N HCl in anhydrous isopropanol was added to each well to solubilize the formazan. Absorbance was measured at 570 nm, using an automated plate reader.

**Autophagy detection with AO staining**

In AO-stained cells, the cytoplasm and nuclei fluorescence were bright green and dim red, whereas acidic compartment fluorescence were bright red. Red fluorescence intensity is proportional to acidity degree (16). Green (510–530 nm) and red (650 nm) fluorescence emission from 1 × 10⁴ cells was measured with a flow cytometer using CellQuest software. Following treatment, cells were stained with 1 μg/mL AO in PBS for 15 minutes, washed 2 × with PBS, and analyzed on FACSCalibur.

**Caspases activity assay**

Caspase-3, -8, and -9 activities were measured by flow cytometry, using the Caspase Detection Kit (Calbiochem, Merck KgaA). Following quercetin treatment, P39 cells were incubated with 1 μL of FITC–DEVD–FMK or RED–IETD–FMK or RED–LEHD–FMK for 1 hour in a 37°C incubator with 5% CO₂. Cells were washed and resuspended with wash buffer. Caspase activity was analyzed by flow cytometry.

**Detection of apoptosis by flow cytometry**

P39 cells were seeded on 12-well plates and treated with different quercetin concentrations (10, 50, or 100 μmol/L). After 24 hours, the cells were washed twice with ice-cold PBS and resuspended in binding buffer containing 1 μg/mL propidium iodide (PI) and 1 μg/mL FITC-labeled Annexin-V. All specimens were analyzed on FACSCalibur after incubation for 15 minutes at room temperature in a light-protected area. Ten thousand events were acquired for each sample.

**Cell-cycle analysis**

Cells were fixed in 70% ethanol, for at least 2 hours at 4°C, and stained with 20 μg/mL PI containing 10 μg/mL RNase A for 30 minutes at room temperature. Fluorescence cell analysis was performed with a FACSCalibur Becton-Dickinson. Resulting DNA distributions were analyzed by Modfit (Verify Software House Inc.) for cell proportions during the cell-cycle phases.

**Measurement of mitochondrial membrane potential (ΔΨm)**

Rhodamine 123 efflux was analyzed using a FACSCalibur flow cytometer. Cells (5 × 10⁶) were incubated in PBS containing 200 μg/mL Rhodamine 123, for 20 minutes at 37°C. Thereafter, cells were washed twice with PBS and Rhodamine 123 fluorescence was detected using the FL1-H channel.

**Measurement of cytochrome c release**

To determine the cytochrome c release from the mitochondria compartment, P39 cells were treated with different quercetin concentrations for 24 hours. The cytosolic and the mitochondrial fraction were fractionated according to the manufacturer, using the Mitochondrial/Cytosol fractionation Kit MIT1000 (Millipore Corporation), for subsequent immunoblot analyses.

**ROS measurement**

Intracellular reactive oxygen species (ROS) generation was measured by flow cytometry following staining with 25 μmol/L of 2′,7′-dichlorofluorescin diacetate (DCFDA). P39 cells after ROS induction with tert-butyldihydroperoxide (TBHP; 100 μmol/L) were treated with different...
quercetin concentrations (10, 50, and 100 μmol/L). Then cells were collected, the fluorescence was analyzed using flow cytometer.

**Western blot analysis**

Total cell protein was extracted in RIPA buffer. Protein concentrations were quantified by the Bio-Rad Protein Assay Kit. Equal protein amounts were loaded on 8% to 15% SDS polyacrylamide gels and electrophoretically transferred to nitrocellulose membrane. Nonspecific binding sites were blocked by incubation with a buffer containing Tris (10 mmol/L, pH 7.4), NaCl (150 mmol/L), Tween 20 (0.1%), and fat-free dry milk (5%). Membranes were incubated overnight with a specific primary antibody, at 4°C, followed by horseradish peroxidase–conjugated secondary antibody, at room temperature for 1 hour. Immunoreactivities were visualized by ECL Western Blot Analysis System (Amersham Pharmacia Biotech).

**Human tumor xenograft model**

Female (NOD.CB17-Prkdc<sup>cs<sup>mut</sup> </sup>/J) lineage 6- to 8-week-old animals, from The Jackson Laboratory, bred at the Animal Facility Centre at the University of Campinas, under specific pathogen-free conditions, were matched for bodyweight before use. Animal experiments were performed followed institutional protocols and guidelines of the Institutional Animal Care and Use Committee. Mice were inoculated, s.c., in the dorsal region, on day 0 with 0.1 mL of P39 cell suspension (1 × 10<sup>7</sup> cells/mice). Every 7 days tumor volumes were evaluated according to the formula: tumor volume (mm<sup>3</sup>) = (length × width<sup>2</sup>) / 2. Quercetin treatment was initiated after tumors reached 100 to 200 mm<sup>3</sup>, and was administered once every 4 days by i.p injection at 120 mg/kg body weight. The control group received equal amounts of vehicle solution, as previously described (17). Mice were sacrificed after 21 days, tumors were then removed, minced, and homogenized in protein extraction buffer or immediately fixed in formalin for IHC.

**Immunohistochemistry**

Active caspase-3, p21, and LC3I/II were performed on paraffin-embedded sections using conventional immunohistochemical techniques. Briefly, a 4 μmol/L tumor section was dewaxed and rehydrated. Antigen retrieval was performed by pretreatment of slides in citrate buffer (pH 6.0) in a microwave oven for 12 minutes, sections were then incubated overnight with monoclonal antibodies of interest at 4°C. The reaction was detected with the streptavidin–biotin–peroxidase complex and stained with diaminobenzidine. Counterstaining was performed with Meyer’s hematoxylin.

**Statistical analysis**

For comparisons, an appropriate Student t test or ANOVA was performed. All statistical analyses were performed using Prism version 5.0a software (GraphPad Software). Group comparisons were considered significant for *, P < 0.05 and **, P < 0.0001.

**Results**

**Quercetin induces cytotoxicity and apoptosis in the P39 cell line**

Cell viability of quercetin-treated P39 cell lines was determined by MIT assay. Figure 1A shows that quercetin inhibited proliferation of P39 cells in a dose- and time-dependent manner. Quercetin significantly reduced cell viability at 50 and 100 μmol/L after 12, 24, and 48 hours of treatment. Accordingly, we cultured cells for 24 hours at 10, 50, and 100 μmol/L for the following experiments.

We next examined whether quercetin-induced the cell death was due to apoptosis (Fig. 1B–F), by using Annexin V–FITC/PI for cell staining. Quercetin treatment at 50 and 100 μmol/L significantly increased the apoptotic cell percentage, comprising 33.9% ± 6.52% and 43.9% ± 7.29%, respectively, after 24 hours (Fig. 1B). To determine the involvement of the mitochondria-mediated pathway in quercetin-induced apoptosis, we assessed changes in ΔΨ<sub>mi</sub> after quercetin treatment using flow cytometry. We found that quercetin treatment induced cell percentage decrease: Low Rhod23 fluorescence intensity was exhibited in 96.7% ± 1.97% of untreated cells and in 78.5% ± 2.43% and 48.7% ± 6.7% of cells treated at 50 and 100 μmol/L concentrations, respectively (Fig. 1C), implying that quercetin treatment resulted in ΔΨ<sub>mi</sub> rapid dissipation in a dose-dependent manner.

We then examined changes in apoptosis-related molecules protein levels. Figure 1D shows that a marked dose-dependent decrease in Bcl-2, Bcl-xL, and Mcl-1 expression was observed in quercetin-treated cells. Contrarily, Bax expression was noticeably increased in response to quercetin treatment. Cytochrome c was effectively released from mitochondria into the cytosol in a dose-dependent manner (Fig. 1E). Caspase activity in response to quercetin treatment was determined by flow-cytometry analysis. We found that P39 cell treatment with increasing quercetin concentrations (10, 50, and 100 μmol/L) induced a marked increase in the cell percentage of cleaved caspase-3, -8, and -9 (Fig. 1F). ROS levels in cells were induced by TBHP and treated with different quercetin concentrations (10, 50, and 100 μmol/L). After TBHP induction, we observed an increased fluorescence intensity (353% ± 87.65%) when compared with control cells. Figure 1G shows that all quercetin concentrations decreased P39 cells ROS levels, 301% to 24.86% to 10 μmol/L, 217% to 50 μmol/L, and 140% to 27.07% to 100 μmol/L, respectively (Fig. 1G), confirming the antioxidant activity of quercetin. We also evaluated whether the death receptor Fas/FasL system was involved in quercetin-induced apoptosis of P39 cells. After 24 hours, quercetin-treated cells exhibited increased FasL expression; quercetin, however, did not modify Fas receptor expression (Fig. 1H).
Figure 1. Quercetin induces cytotoxicity and apoptosis of the P38 cell line. A, cell viability of P38 cells treated with increasing doses of quercetin for 12, 24, or 48 hours measured by MTT assay. P38 cells were treated with 10, 50, and 100 μmol/L of quercetin for 24 hours. After treatment the cells were stained with Annexin V/FITC (B) or with Rhodamine 123 (C). The percentage of apoptotic cells and the membrane potential (ΔΨm), respectively, were determined by flow cytometry. BcL-2, BcL-xL, Bax and Mcl-1 expression (D) and cytochrome c release (E) were determined by Western blotting analysis. Caspase-3, -8, and -9 activation (F). G, measurement of ROS was determined by flow-cytometry analysis following staining with 25 μmol/L of DCFDA. The intracellular ROS production was induced by TBHP (100 μmol/L). H, Fas and FasL protein expression. GAPDH, voltage-dependent anion channel/porin (VDAC) and actin were used as internal control of sample loading. All data were representative of three independent experiments; *, P < 0.05; **, P < 0.001; #, P < 0.001 versus TBHP.
Quercetin blocks cell-cycle and ERK activation and JNK signaling pathways participate in the G1 arrest of P39 cells

To investigate cell-cycle progression P39 cells were cultured in serum-free conditioned medium for 24 hours and then recovered serum refeeding (18). The cells were treated with increasing quercetin concentrations (10, 50, and 100 μmol/L) for 24 hours, and the analysis of the cell-cycle distribution was performed. Figure 2A shows 49.57% ± 1.95% of untreated cells in G1 phase, 38.48% ± 1.51% in S phase, and 12.58% ± 0.88% in G2–M phase. Cells treated with 50 and 100 μmol/L quercetin concentrations markedly increased the number of cells in G1 phase (69.66% ± 1.92% and 70.84% ± 4%, respectively; Fig. 2A).

To investigate the mechanisms involved in G1 cell-cycle arrest induced by quercetin, expression of cell-cycle regulatory proteins were examined. Western blot analysis showed that 24-hour quercetin treatment resulted in a pronounced decrease in protein levels of CDK2, CDK6, cyclin D, cyclin E, and cyclin A in a dose-dependent manner (Fig. 2B and C). P39 cells treated with 10, 50, and 100 μmol/L quercetin for 24 hours resulted in a dose-dependent Rb phosphorylation loss (Fig. 2D). These results indicated that the quercetin-mediated G1 phase cell-cycle arrest in the P39 cell line was associated with decreased Rb phosphorylation.

We next examined p21 and p27 levels by Western blot analysis, which showed increased p21 and p27 levels after 24-hour quercetin treatment (Fig. 2D), suggesting that quercetin increase of p21 and p27 protein levels is one of the possible mechanisms for inhibiting CDK (cyclin-dependent kinase)–cyclin kinase activity.

Several recent reports have suggested that MAPKs such as ERK1/2 and JNK MAPK exert their roles in response to various apoptotic stimuli (19, 20). Figure 2E shows that quercetin promoted pronounced ERK1/2 and JNK phosphorylation in a dose-dependent manner after 24-hour treatment. We further studied that the contribution of ERK1/2 and JNK pathways toward growth inhibition and apoptotic responses induced by quercetin was further studied using the corresponding selective inhibitors PD184352 and SP600125. When P39 cells were pretreated with 1 hour with PD184352 (20 μmol/L) or SP600125 (20 μmol/L) and incubated with quercetin for 24 hours, no differences in apoptotic cell percentages were found (Fig. 2F). Using the same selective inhibitors, we assessed cell-cycle distribution (Fig. 2G). After 24 hours, P39 cell exposure to a combination of quercetin and PD184352 or SP600125, we noted significant decrease in the accumulation of G1 phase cells (41.47% ± 9.74% or 43.8% ± 10.43%) compared with cells exposed to quercetin alone (70.61% ± 6.51%). These data support that ERK and JNK activation plays an important role in quercetin-induced G1 phase arrest on P39 cells.

Quercetin induces autophagy in P39 cells

To determine whether quercetin induces autophagy in P39 cells, we conducted AO staining to identify acidic vesicular organelle formation. Figure 2H shows quercetin-treated P39 cells with a high intracellular AO, expressed by increased red fluorescence in relation to control cells (P39 basal acid compartment).

To further characterize quercetin-induced autophagy in P39 cells, we assessed a key hallmark of autophagy: The conversion of LC3-I to LC3-II (21). Figure 2I shows that quercetin increased LC3-II expression in a dose-dependent manner. In addition, we detected specific autophagy markers, essential for autophagosome formation (16). Western blot analysis revealed a dose-dependent increase of P13K, Beclin-1, Atg5-Atg12, and Atg7 in P39 cells after 24-hour quercetin treatment (Fig. 2J). Taken together, these data demonstrate that quercetin induces P39 cell autophagy.

Akt–mTOR signaling is considered a key negative regulator of autophagy (22). Figure 2J shows that quercetin treatment resulted in a notable inhibition of both Akt and mTOR phosphorylation after 24 hours.

To determine whether autophagy serves as a survival mechanism for P39 cells, we evaluated the impact of autophagy on quercetin-mediated cytotoxicity by pharmacologically inhibiting autophagy. Cells were treated with chloroquine (endosomal acidification inhibitor), quercetin alone, or combined. Figure 2K shows that chloroquine enhanced quercetin-induced suppression of P39 cell growth in a dose-dependent manner. Accordingly, quercetin-induced apoptotic cell death increase in the presence of chloroquine, determined by flow cytometry following Annexin V–FITC and PI dual labeling. The role of autophagy inhibition in quercetin-mediated cell death was further investigated by assessing cell-cycle analysis. Autophagy inhibition did not alter the modulation of quercetin in G1 phase (Fig. 2L). Collectively, our results suggest that quercetin-induced autophagy plays a protective role against apoptotic cell death in P39 cells.

Quercetin reduces P39 tumor growth

In vitro data described earlier prompted us to further test quercetin anticancer efficacy in an in vivo model, mice subcutaneously xenografted with P39 cells, as described in Materials and Methods. Figure 3A shows that quercetin treatment significantly decreased tumor volume compared with the control group. Mice treated with 120 mg/kg of quercetin resulted in approximately a 30% inhibition of tumor growth compared with controls at day 21. Overall, tumor growth in xenograft mice was reduced in the quercetin group compared with control mice (Fig. 3B).

MAPK and Akt pathways coordinate and regulate signaling mechanisms for cell proliferation, differentiation, survival, and death. Western blot analysis demonstrated alterations in levels of key players of MAPK and Akt pathways in P39 xenografts after quercetin treatment Fig. 3C and D. Activation of the death signaling molecules such as phosphorylated forms of ERK (p-ERK) and JNK MAPK (p-JNK) indicates the therapeutic efficacy of
Quercetin treatment in P39 xenografts. Mice treated with 120 mg/kg of quercetin resulted in significant downregulation of phospho-Akt (Fig. 3E) and phospho-mTOR (Fig. 3F), suggesting that quercetin treatment operates through the same signaling pathway both in vitro and in vivo.
Quercetin modulates apoptosis, autophagy, and cell cycle in P39 xenografts

As quercetin treatment inhibited tumor growth, we decided to determine apoptosis, autophagy, and cell-cycle status in tumor tissue of quercetin-treated animals. Figure 4 shows quercetin-induced apoptosis activation and significant decreased expression of Bcl2, Bcl-XL, and Mcl-1 expression followed by increased Bax expression (Fig. 4A–D). Cleaved caspase-3 staining was positive in 8.10% +/- 2.13% cells in the control group, whereas the quercetin-treated group presented 39.68% +/- 6.9% positive cells (Fig. 4E and F). Mice treated with 120 mg/kg of quercetin resulted in marked increase of protein expression related to autophagy as Beclin-1, PI3K, Atg5–Atg12, and Atg7. To detect LC3 protein processing after quercetin treatment, IHC was used to analyze protein levels of cleaved LC3 in tumor sections. Contrasting with controls (12.30% +/- 2.62%), cleaved LC3-positive cells increased significantly in the quercetin-treated group (41.66% +/- 6.67%; Fig. 5A–F). Cell-cycle analyses revealed that quercetin treatment in the xenograft model led to decreased expression of phosphorylated Rb and cyclin D and cyclin E proteins. However, quercetin treatment resulted in pronounced induction of p21 (54.54% +/- 8.10%) compared with control (12% +/- 2.61%; Fig. 6A–I). These data suggest that quercetin treatment modulates apoptosis, autophagy, and cell cycle both in vitro and in vivo.

Discussion

Quercetin is ubiquitously found in plants and plant food sources and several beneficial health effects have been associated with the dietary intake of this bioflavonoid (4). Various studies indicate a possible use of quercetin for cancer treatment through its interaction with multiple cancer-related pathways (23). Herein, we used a P39 cell line, derived from an MDS-CMML patient, a model. Quercetin treatment resulted in reduction of P39 cell viability and
decreased tumor volume of P39 subcutaneously xenografted in NOD/SCID mice. These effects were accompanied by modulation of apoptotic cell death and stimulation of an intrinsic apoptosis pathway. P39 cell exposure leads proapoptotic protein Bax upregulation and suppression of antiapoptotic proteins Bcl-2, Bcl-xl, and Mcl-1. Mitochondrial dysfunction such as loss of mitochondrial membrane potential has been reported as an early and central event that occurs after apoptosis induction (24). Our observation of a reduction in Rho123 fluorescence intensity and release of cytochrome c from mitochondria to the cytoplasm suggested that intracellular events related to P39 cell death caused by quercetin treatment involved mitochondrial dysfunction and changes in mitochondrial membrane potential.

Figure 4. Quercetin induces apoptosis in P39 xenografts. NOD/SCID mice with P39 tumors were administered without or with quercetin (120 mg/kg) once every 4 days. A to D, after 3 weeks, tumors were harvested and relative levels of protein in the tumor lysates were determined by Western blotting. E, IHC analysis of cleaved caspase-3 in tumor sections. F, the percentage of cleaved caspase-3–positive nuclei of cells per field; four fields per tumor section. Counts were made using a ×40 objective connected to a light microscope (Olympus CBA, Olympus America). Values are means ± SD (mean of 6 mice/group); *, P < 0.05; **, P < 0.001.

Figure 5. Quercetin induces autophagy in P39 xenografts. NOD/SCID mice with P39 tumors were treated or not with quercetin (120 mg/kg), once every 4 days. A to D, after 3 weeks, tumors were harvested and relative levels of protein in tumor lysates were determined by Western blotting. E, IHC analysis of cleaved LC3 in tumor sections. F, the percentage of LC3-positive nuclei of cells per field; four fields per tumor section. Counts were made using a ×40 objective connected to a light microscope (Olympus CBA, Olympus America). Values are means ± SD (mean of 6 mice/group); *, P < 0.05; **, P < 0.001.
potential ($\Delta \psi_m$). However, we attempted to identify subsequent events in P39 cells that completed the apoptotic process. Low mitochondrial membrane potential is well known to cause the release of proapoptotic factors such as cytochrome c from mitochondria to cytosol (25), which mediates caspase-9 activation, thereby triggering a cascade of caspase activation (26). These findings are in agreement with our data in which quercetin treatment resulted in significant enhancement of cleaved caspase-9, -8, and -3 percentage. Enhancement in the active caspase-8 percentage and increased expression of FasL indicate that the extrinsic or death receptor pathway of apoptosis is also activated by quercetin in P39 cells.

Quercetin is considered an excellent free radical scavenger. The antioxidant and anti-inflammatory properties of quercetin have been associated with the amelioration of adverse side effects derived from cancer therapy, which is considered a good strategy to prevention and treatment of cancer (27). Our results show that quercetin is an antioxidant in P39 cells, possibly related to its capacity to interact with important antioxidant cellular defense systems, as the NRF2/Keap complex. This complex is connected with apoptotic pathways through regulation of proteins from the Bcl-2 family (28). Keap1 appears to bind the BH2 domain of Bcl-2 and competes with Bax for its Bcl-2–binding site, facilitating apoptosis (28).

Several pathways are common for apoptosis and autophagy (29). Mitochondria, an organelle of great interest in the regulation of programmed cell death, is also especially sensitive to autophagy, a physiologically regulated and evolutionarily conserved process that involves the sequestration of cytoplasmic contents into autophagosomes for traffic to lysosomes in which it is degraded and recycled (22, 30). Although the roles of autophagy in protein and organelle catabolism are well accepted, the involvement of this process in cancer chemotherapy is still controversial. Some anticancer agents could induce autophagy and enhance chemotherapeutic efficacy (31). However, some agents induce autophagic process as a protective mechanism allowing cells to escape from apoptosis (32). Our results demonstrated that quercetin triggered autophagy both in vitro and in vivo in P39 cells.

Figure 6. Quercetin induces arrest in G1 phase of cell cycle in P39 xenografts. NOD/SCID mice with P39 tumors were administered without or with quercetin (120 mg/kg), once every 4 days. A to G, after 3 weeks, tumors were harvested and relative levels of protein in the tumor lysates were determined by Western blotting. H, IHC analysis of p21 protein in tumor sections. I, the percentage of p21-positive nuclei of cells per field; four fields per tumor section. Counts were made using a ×40 objective connected to a light microscope (Olympus CBA, Olympus America) Values are means ± SD (mean of 6 mice/group); *, P < 0.05; **, P < 0.0001.
Further functional analyses showed that the inhibition of autophagy markedly increased quercetin-induced apoptosis, suggesting that quercetin-induced autophagy plays a protective role in P39 cells.

The Akt–mTOR signaling pathway, often hyperactivated in cancer, is a key regulator of homeostasis, controlling essential pathways leading to cell growth, protein synthesis, and autophagy (33, 34). Quercetin has been described to interfere with both mTOR activity and activation of the PI3K/Akt signaling pathway, giving quercetin the advantage to function as a dual-specific mTOR/PI3K inhibitor. Herein, our data demonstrated that quercetin dephosphorylated Akt and mTOR in P39 cells and P39 xenografts, highlighting the functional importance related to the involvement of Akt–mTOR signaling in quercetin-mediated protective autophagy in P39 cells. Our results, however, showed that autophagy inhibition did not alter quercetin modulation in cell-cycle arrest. These findings suggest that the autophagy process is not associated with cell cycle in quercetin-treated P39 cells.

In this study, cell-cycle phase analyses revealed that cell-cycle arrest caused by quercetin treatment occurred at G₁ phase. This is further supported by decreased levels of CDK2, CDK6, cyclin D, cyclin E, cyclin A, and pRb accompanied by induction of p21 and p27. Accordingly, Mu and colleagues (35) demonstrated that in HepG2 human hepatoma cells, quercetin blocks cell-cycle progression at G₁ phase and exerts this effect through the p21, p27, and p53 increase. p21 is an important growth arrest mediator and a CDK activity regulator, inhibiting cell entry into G₂ phase in response to DNA damage and blocking the reentry of G₂ cells into S phase by blocking cyclin E–CDK2-mediated Rb phosphorylation (36). As Rb is a direct substrate of CDKs, the decrease in Rb phosphorylation here observed is consistent with quercetin inhibition of CDK expression and cell arrest in G₁ phase. There are multiple mechanisms, however, known to potentially inhibit Rb phosphorylation, including proteosomal degradation (37) and inhibition of upstream Ras/RAF/MAPK or PI3K/Akt pathways (38, 39).

The MAPK family is an essential part of the signal transduction machinery in signal transmissions from cell surface receptors and environmental stimulation, containing three major MAPK subfamilies: ERK, p38, and JNK (40, 41). ERK has a well-established role in regulating G₁–S phase progression in response to mitogenic stimulation and is critical for Ras-mediated transformation (42). JNK is known to interact with, phosphorylate, and increase the stability of the cell-cycle regulator, p21 WAF1/CIP1 (43). Thus, ERK and JNK are signaling elements in several types of cells through which quercetin may regulate cell growth (44, 45). We herein observed that quercetin exposure induced an increase in ERK1/2 and JNK phosphorylation in P39 cells and in P39 xenografts. The ERK- and JNK-dependent effect of quercetin observed in P39 cells was strengthened by our findings using specific inhibitors of ERK and JNK (PD184352 and SP600125, respectively), which caused significant decrease in cell accumulation in G₁ phase after quercetin treatment. These data imply that ERK and JNK signaling is tightly related to cell growth inhibition and death in quercetin-treated P39 cells.

Quercetin emerges as a molecule possessing multiple properties, all directed at ameliorating pathologic conditions associated with degenerative diseases. The ability of quercetin to interfere with different targets of cancer development qualifies this molecule as a multitarget inhibitor with synergistic effects, which lead to cancer cell elimination. Significant progress in molecular and cellular biology has resulted in better characterizing and understanding the molecular abnormalities of acute and chronic leukemia. This knowledge has provided new opportunities for developing innovative and more effective therapies. However, the available therapies for leukemias are only partially efficient, mainly owing to heterogeneity of the disease and to the fact that disease progression causes a major impact on the resistance to chemotherapy and relapse (46). Accordingly, drugs with single-target action are clearly inefficient in treating multifactor diseases. Single-target drugs may not always induce the desired effect upon the entire biologic system; one reason is that organisms can affect effectiveness through compensatory ways. Thus, scientists are not only looking toward the chemical industry but also toward traditional herbal medicines to find multitarget interventions.

In summary, our findings indicate that quercetin effectively induces cell death in human leukemia cells in vitro, as well as in leukemia xenografts and that phenomenon stems from a mechanism involving multilevels of cooperation between apoptosis, autophagy, and cell-cycle arrest. The antitumor activity of quercetin both in vitro and in vivo revealed in this study, point to quercetin as an attractive antitumor agent for hematologic malignancies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: V. Maso, A.K. Calgarotto, S.T.O. Saad
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