Quercetin Induces Tumor-Selective Apoptosis through Downregulation of Mcl-1 and Activation of Bax

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Abstract

Purpose: To investigate the in vivo antitumor efficacy of quercetin in U937 xenografts and the functional roles of Mcl-1 and Bax in quercetin-induced apoptosis in human leukemia.

Experimental Design: Leukemia cells were treated with quercetin, after which apoptosis, Mcl-1 expression, and Bax activation and translocation were evaluated. The efficacy of quercetin as well as Mcl-1 expression and Bax activation were investigated in xenografts of U937 cells.

Results: Administration of quercetin caused pronounced apoptosis in both transformed and primary leukemia cells but not in normal blood peripheral mononuclear cells. Quercetin-induced apoptosis was accompanied by Mcl-1 downregulation and Bax conformational change and mitochondrial translocation that triggered cytochrome c release. Knockdown of Bax by siRNA reversed quercetin-induced apoptosis and abrogated the activation of caspase and apoptosis. Ectopic expression of Mcl-1 attenuated quercetin-mediated Bax activation, translocation, and cell death. Conversely, interruption of Mcl-1 by siRNA enhanced Bax activation and translocation, as well as lethality induced by quercetin. However, the absence of Bax had no effect on quercetin-mediated Mcl-1 downregulation. Furthermore, in vivo administration of quercetin attenuated tumor growth in U937 xenografts. The TUNEL-positive apoptotic cells in tumor sections increased in quercetin-treated mice as compared with controls. Mcl-1 downregulation and Bax activation were also observed in xenografts.

Conclusions: These data suggest that quercetin may be useful for the treatment of leukemia by preferentially inducing apoptosis in leukemia versus normal hematopoietic cells through a process involving Mcl-1 downregulation, which, in turn, potentiates Bax activation and mitochondrial translocation, culminating in apoptosis.

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The natural product quercetin (3,5,7,3',4'-pentahydroxyflavone) is a widely distributed flavonoid that is present in many fruits and vegetables (e.g., onions and apples). Previous research has shown that quercetin induces apoptosis in a variety of tumors (1–3), including leukemia (4). However, the molecular mechanisms of quercetin-induced apoptosis are unclear. There is no available information concerning the in vivo efficacy of quercetin against leukemia.

Apoptosis involves 2 distinct pathways, one engaging death receptor-initiated extrinsic pathway and the other involving mitochondria-mediated intrinsic pathway (5). The intrinsic pathway involves the release of proapoptotic proteins (e.g., cytochrome c) into the cytosol, formation of the apoptosome, and activation of caspase-9 (6, 7), which subsequently cleaves and activates procaspase-3 (8). The mitochondrial pathway is mainly regulated by Bcl-2 family proteins (9–11). Antiapoptotic Bcl-2 family proteins (e.g., Bcl-2, Bcl-xL, and Mcl-1) antagonize membrane permeabilization and prevent the release of cytochrome c from mitochondria (12). Proapoptotic Bcl-2 family proteins can be further divided into 2 subgroups. The multidomain proapoptotic proteins (e.g., Bax and Bak) participate in the formation of mitochondrial pore through which cytochrome c releases (13–16). The BH3-only proteins (e.g., Bim and Bid) are required for the activation of multidomain proapoptotic proteins through association of antiapoptotic Bcl-2 proteins (17, 18). It has been reported that quercetin-mediated cell apoptosis involves mitochondria-mediated caspase activation (1, 4, 19–22).

Notably, Mcl-1 is a highly expressed antiapoptotic protein (23) implicated in malignant hematopoietic survival (23, 24). It has been shown that depletion of Mcl-1, using antisense oligonucleotides, rapidly triggers apoptosis in U937 cells (25). In contrast, selective expression of...
Mcl-1 in hematopoietic tissues of transgenic mice promotes the survival of hematopoietic cells and enhances the outgrowth of myeloid cell lines (26). Furthermore, overexpression of Mcl-1 protects cells from apoptosis induced by a variety of agents, including UV, etoposide, staurosporine, actinomycin D, and others (27–30). Two groups (4, 31) have indicated a decrease of Mcl-1 level in quercetin-treated cells. In addition, our results indicate that the natural product quercetin mediates Mcl-1 downregulation and activates Bax; this phenomenon stems from a novel mechanism involving against hematologic malignancies.

Translational Relevance

The hematologic malignancies are the types of cancer that arise from malignant transformation of cells derived from peripheral blood, lymphatic system, and bone marrow. Because of the increase in the morbidity and mortality of human leukemia in recent years, chemoprevention or intervention toward the control of human leukemia is highly desirable. The present study has provided evidences that the natural product quercetin induces human leukemia cell death through downregulation of Mcl-1 and activation of Bax. The data presented here suggest that the 2 Bcl-2 family proteins Mcl-1 and Bax may represent attractive targets for quercetin-induced apoptosis in human leukemia cells. In vivo data confirmed the antitumor efficacy of quercetin via induction of apoptosis by targeting Mcl-1 and Bax in xenografts of leukemia cells. The results of this study could have implications for the incorporation of agents such as quercetin into the chemoprevention or therapeutic intervention against hematologic malignancies.

Materials and Methods

Cells

Human leukemia U937, Jurkat, and HL-60 cells were obtained from American Type Culture Collection and cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 1-glutamine, and antibiotics. U937 cells stably overexpressing Mcl-1, and their empty vector counterpart (pCEP) were kindly provided by Dr. Ruth Craig (Dartmouth Medical School). HL-60 cells stably overexpressing Bcl-2 (HL-60/Bcl-2) and Bcl-xL (HL-60/Bcl-xL) were kindly provided by Dr. Ming Ding (The National Institute for Occupational Safety and Health). Mononuclear cells were isolated from peripheral blood or bone marrow of leukemia patients or healthy donors were purchased from AllCells, LLC. Mononuclear cells were suspended in RPMI 1640 medium containing 10% fetal calf serum at 8 × 10^7/ml for treatment. Bax<sup>+/−</sup> and Bax<sup>−/−</sup> human colon cancer HCT116 cells were kindly provided by Dr. Bert Vogelstein (Johns Hopkins University) and Kenneth W. Kinzler (Howard Hughes Medical Institute) and sustained in MyCoys's 5A medium containing 10% FBS and antibiotics.

Chemicals and reagents

Quercetin (>99% pure) was purchased from Sigma Chemical Co., dissolved in DMSO, aliquoted, and stored at −20°C. The pan-caspase inhibitor Z-VAD-FMK was purchased from EMD Biosciences.

Assessment of apoptosis

The extent of apoptosis in leukemia cells was evaluated by flow cytometric analysis using FITC-conjugated Annexin V/propidium iodide (PI; BD PharMingen) staining as per the manufacturer’s instructions as previously described (40). Both early apoptotic (Annexin V–positive, PI-negative) and late apoptotic (Annexin V–positive and PI-positive) cells were included in cell death determinations.

Western blot assay

Cells were lysed and sonicated in 1× NuPAGE LDS sample buffer (Invitrogen). The protein concentration was measured using Coomassie Protein Assay Reagent (Pierce), and 30 µg of sample proteins were separated by SDS-PAGE and incubated with antibodies. The blots were then reprobed with an antibody against β-actin (Santa Cruz Biotechnology) or Cox IV (Cell Signaling) to ensure loading of proteins. Primary antibodies used were as follows: cytochrome c (Santa Cruz Biotechnology); cleaved caspase-3, cleaved caspase-9, and Bcl-xL (Cell Signaling); Mcl-1 and Bax (N-20) (BD PharMingen); PARP (Biomol Research Laboratories); and Bcl-2 (DAKO). Secondary antibodies conjugated to horseradish peroxidase were obtained from Kirkegaard and Perry Laboratories, Inc.
Subcellular fractionation
For cytosol fractionation, cells were lysed in lysis buffer (75 mmol/L of NaCl, 8 mmol/M of Na₂HPO₄, 1 mmol/M of NaH₂PO₄, 1 mmol/L of EDTA, and 350 μg/mL of digitonin) and needle strokes. After sequential centrifugation (1,000 × g to pellet nuclei, 10,000 × g to pellet membrane fraction, and 100,000 × g), the supernatant (S-100, cytosolic fraction) were collected and subjected to immunoblot. For mitochondrial and cytosol fractionations, cells (50 × 10⁶) were fractionated by mitochondrial fractionation kit (Active Motif) as per manufacturer’s instructions.

Analysis of Bax conformational change
To analyze the conformational change of Bax by flow cytometry, cells were fixed and permeabilized using FIX & PERM cell permeabilization reagents (Caltag Lab) as per manufacturer’s instructions. Cells were incubated with FITC-conjugated anti-Bax (clone 6A7; Santa Cruz Biotechnology) and then analyzed by flow cytometry. The results for each condition were calibrated by values for cells stained with mouse IgG (Santa Cruz Biotechnology) as the primary antibody. Values for untreated controls were arbitrarily set to 100%. For analysis of Bax conformational change by immunoprecipitation (IP), cells were lysed in CHAPS lysis buffer and then buffered and lysed in CHAPS lysis buffer and 500 μg of total lysates were immunoprecipitated using anti-Bax 6A7 antibody. Resulting immune complexes were analyzed by immunoblotting with anti-Bax antiserum (N-20).

Immunofluorescence
To analyze cellular distribution of Bax cells were fixed, permeabilized and probed with FITC-conjugated anti-Bax (Santa Cruz Biotechnology). Mitochondria were stained with 300 nmol/L of MitoTracker (Molecular Probes). Cells were plated on 35-mm collagen-coated, glass-bottomed dishes (MatTek Corporation) and visualized using an inverted Leica TCS SP5 laser scanning confocal microscope under a 60 × oil immersion objective.

RNA interference
U937 cells (1.5 × 10⁶) were transiently transfected with 10 nmol/L of siRNA specific for Mcl-1 or Bax (Santa Cruz Biotechnology), using Amaza Nucleofector device (program V-001) with Kit V (Amaza Biotechnology), as per manufacturer’s instructions. After 24 hours of transfection, cells were treated and analyzed for protein expression, apoptosis, or viability.

Therapeutic evaluation of quercetin in xenograft model
The in vivo evaluation of quercetin was carried out using xenograft model of human U937 cells. Aethmic nude mice (NU/Nu, 6–8 weeks old; Charles River) were housed in a specific pathogen-free room within the animal facilities at the University of Kentucky, Chandler Medical Center. Animals were allowed to acclimatize to their new environment for 1 week prior to use. All animals were handled according to the Institutional Animal Care and Use (IACUC), University of Kentucky. U937 cells (6 × 10⁶) were resuspended in serum-free RPMI 1640 medium with Matrigel basement membrane matrix (BD Biosciences) at a 1:1 ratio (total volume = 100 μL) and then were subcutaneously injected into the flanks of nude mice. From the second day of injection, mice were randomly assigned to 3 treatment groups (n = 6 for each group) and administered intraperitoneally with quercetin (0, 20, and 40 mg/kg of body weight) in 150 μL of DMSO/0.9% physiologic saline (1:0.5) daily for consecutive 15 days. Body weight and tumor mass were measured every 5 days. Tumor volumes was determined by a caliper and calculated according to the following formula: (width² × length)/2. All animals were sacrificed immediately if tumor volume reached an approximate volume of 1,500 mm³ at day 16.

Detection of apoptosis by TUNEL, detection of Mcl-1 expression and Bax activation in tumor tissue sections
Tumor tissue sections of formalin-fixed, paraffin-embedded specimens were dewaxed in xylene and rehydrated in a graded series of ethanol. Apoptosis was detected using the TUNEL in situ apoptosis detection kit (DeadEnd Fluorometric TUNEL System; Promega). Briefly, tumor samples were incubated with proteinase K (2 mg/mL) and the TUNEL staining was done according to the manufacturer’s instructions. The percentage of TUNEL-positive cells relative to total cells was calculated for each sample under a fluorescent microscope, counting at least 200 cells. Mcl-1 expression in tumor samples was analyzed by immunofluorescence, using a primary anti-Mcl-1 antibody (Abcam) following FITC-conjugated anti-mouse secondary antibody (Invitrogen). Tumor samples were homogenized and lysed in CHAPS lysis buffer and then buffered and immunoprecipitated using anti-Bax 6A7 antibody. Resulting immune complexes were analyzed by immunoblotting with an anti-Bax antiserum (N-20).

Statistical analysis
The values were presented as means ± SD. Two-way analysis of variance (ANOVA) and Student’s t test were used for statistical analysis. P < 0.05 was considered significantly different.

Results
Exposure to quercetin led to pronounced apoptosis in U937 cells, associated with a decrease in Mcl-1 expression
A dose–response analysis of U937 cells revealed a moderate increase in apoptosis 9 hours after exposure to quercetin at concentrations 30 μmol/L or greater (Figs. 1A and 3A). A time-course study of cells exposed to 40 μmol/L of quercetin showed a marked increase in apoptosis as early as 2 hours after drug exposure and reached near-maximal levels after 9 hours (Fig. 1B). Expression of antiapoptotic Bcl-2 family proteins in U937 cells was monitored following treatment with
Quercetin. As shown in Figure 1C, a marked dose-dependent decrease of Mcl-1 expression was observed in quercetin-treated cells. Exposure of U937 cells to 40 μmol/L of quercetin at varying intervals resulted in a rapid downregulation of Mcl-1 that was detectable as early as 2 hours, and by 4 hours of treatment expression was almost absent (Fig. 1D). In contrast, the other 2 important antiapoptotic Bcl-2 family proteins Bcl-2 or Bcl-xL expression remained unaffected. Together, these findings show that quercetin induces rapid and dose-dependent downregulation of Mcl-1 in U937 cells.

Quercetin induced Bax conformational change and mitochondrial translocation in U937 cells
Following death stimuli, Bax undergoes an N-terminal conformational change, which can be detected by means of an antibody specifically recognizing the active protein conformer (6A7). Although no change in the overall protein levels of Bax was noted upon quercetin treatment (Fig. 2A), an increase in the expression of the active, conformationally changed form of Bax was observed in quercetin (40 μmol/L) exposed cells. The Bax conformational change appeared as early as 3 hours after addition of quercetin and increased progressively over the ensuing 9 hours. Flow cytometric analysis (Fig. 2B, flow data) also showed a 40% increase in the number of Bax-positive cells (control was set to 100%; quercetin treatment group was 140%) following incubation with quercetin (40 μmol/L) for 9 hours.

We further examined the intracellular Bax localization by immunoblot in mitochondrial and cytosolic protein samples from U937 cells treated with 0, 10, 20, 30, and 40 μmol/L of quercetin for 6 and 12 hours. Representative Western blots are shown in Figure 2C and 2D. The data show a decrease in Bax expression in the mitochondrial fraction and an increase in the cytosolic fraction, consistent with mitochondrial translocation and subsequent apoptosis induction.

Fig. 1. Quercetin (Quer) markedly induces apoptosis in U937 human leukemia cells in dose- and time-dependent manners and is associated with downregulation of Mcl-1. A, U937 cells were treated with 0, 10, 20, 30, and 40 μmol/L of quercetin for 9 hours. B, the cells were treated with 40 μmol/L of quercetin for 0, 1, 2, 4, 6, 9, 12, and 24 hours. For A and B, after treatment the cells were stained with Annexin V/PI, and the percentage of apoptotic cells was determined using flow cytometry. C, the cells were treated with 0, 10, 20, 30, and 40 μmol/L of quercetin for 6 and 12 hours. D, the cells were treated with 40 μmol/L of quercetin for 0, 1, 2, 4, 6, 9, 12, and 24 hours. After treatment in C and D, immunoblot analysis was done to monitor expression of Mcl-1, Bcl-2, and Bcl-xL. Blots were subsequently stripped and reprobed with antibody against β-actin to ensure equivalent loading.
fractions. As shown in Figure 2C, Bax was found predominantly in cytosolic fraction in untreated cells. Incubation with quercetin induced a redistribution of Bax from cytosolic to the mitochondrial compartment. These results were corroborated by visualization of immunostaining with FITC-conjugated Bax and confocal imaging (Fig. 2D). In untreated cells, Bax was sparsely distributed in the cytosol, whereas upon quercetin treatment, Bax exhibited a more punctuate pattern and had an overlap with mitochondria that was stained with MitoTracker Red CMXRos, indicating a clear shift of the cellular localization of Bax from cytosol to mitochondria. These data suggest that quercetin treatment leads to a significant Bax conformational change, accompanied by its translocation to mitochondria. Besides Bax, we also investigated the overall expression of other proapoptotic Bcl-2 proteins, Bak and Bim, which did not change following quercetin treatment (data not shown). These findings suggest that activation of Bax may contribute to the induction of apoptosis in cells exposed to quercetin.
Fig. 3. Quercetin (Quer) downregulates Mcl-1 and promotes Bax activation in multiple human leukemia cell lines and primary leukemia blasts but not in normal human peripheral blood mononuclear cells. A, U937, Jurkat, and HL-60 cells were exposed to 0, 20, 40, and 60 \(\mu\)mol/L of quercetin for 9 hours, after which the percentage of apoptotic cells (Annexin V/PI staining) was determined by flow cytometry. Untreated U937, HL-60, and Jurkat cells were lysed and subjected to immunoblot analysis to detect basic protein levels of Mcl-1 (inset). B, Jurkat and HL-60 cells were treated with 40 \(\mu\)mol/L of quercetin for 0, 2, 4, 8, and 12 hours, after which immunoblot analysis was done to monitor Mcl-1 expression. For Western blot analysis, blots were subsequently stripped and reprobed with an antibody against \(\beta\)-actin to ensure equivalent loading. Alternatively, cells were treated with 40 \(\mu\)mol/L of quercetin for 9 hours, and flow cytometry analysis was done to detect the percentage of cells with active Bax (FITC-Bax 6A7 positive). Untreated control was set up as 100%. C, mononuclear cells were isolated from the BM (bone marrow) or PM (peripheral blood) of 5 patients with leukemia (designated as 1–5), including 2 AML, 1 MM, and 2 CLL patients. Cells were then incubated with 0, 20, 40, and 60 \(\mu\)mol/L of quercetin for 9 hours. At the end of this period, the percentage of apoptotic cells (Annexin V/PI staining) was determined by flow cytometry. The blasts were incubated with 40 \(\mu\)mol/L of quercetin and then lysed for immunoblot using Mcl-1 primary antibody (patients 2 and 5; inset, left) or analyzed by flow cytometry by using FITC-Bax 6A7 antibody (patient 5; inset, right). For Western blot analysis, blots were subsequently stripped and reprobed with an antibody against \(\beta\)-actin to ensure equivalent loading. For flow cytometry analysis, untreated control was set up as 100%. D, mononuclear cells were isolated from the PM of 2 healthy donors. Cells were then incubated with 0, 20, 40, 60, and 80 \(\mu\)mol/L of quercetin for 9 hours. At the end of this period, the percentage of apoptotic cells (Annexin V/PI staining) was determined by flow cytometry. The blasts were incubated with 40 and 80 \(\mu\)mol/L of quercetin and then lysed for immunoblot by using Mcl-1 primary antibody (donor 1; inset, left) or analyzed by flow cytometry by using FITC-Bax 6A7 antibody (donor 1; inset, right). For Western blot analysis, blots were subsequently stripped and reprobed with an antibody against \(\beta\)-actin to ensure equivalent loading. For flow cytometry analysis, untreated control was set up as 100%.

Quercetin induced lethality in association with Mcl-1 downregulation and Bax activation in multiple leukemia cell lines as well as primary human leukemia cells but not in normal human peripheral blood mononuclear cell

To determine whether quercetin-mediated lethality observed in U937 cells also occur in other cell lines, parallel studies were done in Jurkat and HL-60 cells. As shown in Figure 3A, 9-hour exposure to quercetin resulted in a dose-dependent cell death in both cell lines. Markedly, a rapid decline in Mcl-1 protein levels (Fig. 3B, immunoblot data) and an obvious increase in active Bax (Fig. 3B, flow data) were observed in Jurkat and HL-60 cells. Moreover, these leukemia cells exhibited different susceptibilities to quercetin-mediated lethality (Fig. 3A). U937 cells, which were the most sensitive of the 3 cell lines, exhibited relatively high basic Mcl-1 expression (Fig. 3A, immunoblot data), most rapid degradation of Mcl-1 (Figs. 1D and 3B), and an obvious increase in active Bax.
and most active Bax cells (Figs. 2B and Fig. 3B, flow data), suggesting that levels and turnover rate of Mcl-1 as well as extent of Bax activation may be related to the sensitivity of leukemia cells to quercetin.

Further attempts were made to determine whether quercetin could also trigger cell death in primary human leukemia blasts. Parallel experiments were done on primary mononuclear cells isolated from blood or bone marrow of 5 leukemia patients [2 acute myeloid leukemia (AML), 1 multiple myeloma (MM), and 2 chronic lymphocytic leukemia (CLL)]. Treatment with quercetin (0–60 μmol/L) for 9 hours resulted in a dose-dependent increase in cell death in mononuclear cells of all human leukemia types (Fig. 3C). A marked decrease in Mcl-1 expression (Fig. 3C, immunoblot data) and increase in active Bax cells (Fig. 3C, flow data) were also observed in leukemia blasts. These findings indicate that quercetin induces cell lethality in primary leukemia blasts in association with Mcl-1 downregulation and Bax activation, analogous to findings in continuously cultured human leukemia cell lines.

In contrast, quercetin exerted little toxicity toward normal human peripheral blood mononuclear cells (Fig. 3D). Neither Mcl-1 expression or Bax activation was changed upon quercetin treatment.

**Treatment with quercetin resulted in a marked induction of mitochondrial injury and caspase activation in human leukemia cells but not in normal human peripheral blood mononuclear cells**

It has been reported that quercetin-induced apoptosis is related to mitochondria-mediated caspase activation (21). We investigated mitochondria alterations as well as caspase activation in response to quercetin treatment. For JC-1 staining, if mitochondria membrane potential (ΔΨm) decreases, the fluorescence will change from red to green. As shown in Supplementary Figure 1A, there was an obvious shift of fluorescence from red to green after quercetin exposure, which indicates the occurrence of the mitochondrial membrane depolarization following quercetin exposure. Moreover, administration with quercetin triggered a pronounced increase in the release of cytochrome c and Smac/Diablo into the cytosolic fraction (S-100), which was noted after 4-hour treatment and became more apparent at later exposure intervals (8 and 12 hours; Supplementary Fig. 1B). Furthermore, the caspase cascade was activated, as determined by the detection of the active form of caspase-9, -3, and -7, and the proteolytic cleavage of PARP, an endogenous substrate of caspases (Supplementary Fig. 1C). These events were readily apparent after 8 hours of treatment. The findings show that mitochondrial injury and caspase activation were detected later than Mcl-1 degradation and Bax activation, indicating the possibility of a primary role for Mcl-1 downregulation and Bax activation in quercetin-mediated cell death.

To confirm the role of caspase cascade, U937 cells were treated with 40 μmol/L of quercetin in the presence of pan-caspase inhibitor Z-VAD-FMK at 20 μmol/L. Addition of Z-VAD-FMK significantly diminished the extent of cell death (data not shown) and abrogated quercetin-induced caspase activation and PARP cleavage (Supplementary Fig. 1D), showing the caspase dependence of the cell death phenomena. However, Z-VAD-FMK was ineffective in preventing downregulation of Mcl-1 protein, suggesting that Mcl-1 reduction is not a consequence of caspase process.

In contrast, quercetin had little effects on the mitochondria membrane depolarization in normal human peripheral blood mononuclear cells (Supplementary Fig. 1E), nor did it induce PARP cleavage (Supplementary Fig. 1F).

**Overexpression of Mcl-1 substantially attenuated quercetin-mediated mitochondrial injury, caspase activation, and apoptosis**

If downregulation of Mcl-1 is responsible for the subsequent induction of apoptosis, then maintenance of Mcl-1 levels is expected to prevent apoptosis. To test this hypothesis, studies were conducted by employing U937 cells stably overexpressing Mcl-1 (30). Enforced Mcl-1 expression decreased lethal effects of quercetin (Fig. 4A), whereas empty vector control U937/pCEP cells were approximately as sensitive as parental cells. As shown in Figure 4B, treatment with quercetin diminished Mcl-1 expression in U937/pCEP cells but just had a little inhibition on Mcl-1 expression in their U937/Mcl-1 counterparts. Bcl-2 and Bcl-xL expression in U937/Mcl-1 was roughly equivalent to those in U937/pCEP cells. Moreover, enforced expression of Mcl-1 blocked quercetin-mediated mitochondrial membrane depolarization (Supplementary Fig. 2A) and cytochrome c release (Supplementary Fig. 2B). In addition, exposure to quercetin failed to promote caspase-9, -3, and -7 cleavages and PARP degradation (Supplementary Fig. 2C) in Mcl-1 overexpressed U937 cells. To determine whether overexpression of Bcl-2 and Bcl-xL could compensate the reduction of Mcl-1 at the mitochondrial outer membrane and prevent apoptosis (41), we compared the sensitivity to quercetin of the HL-60/Bcl-2 and HL-60/Bcl-xL cell lines with the parental HL-60 cell line. Overexpressing Bcl-2 or Bcl-xL did not either protect cells from quercetin-mediated lethality (Supplementary Fig. 2D) or protect Mcl-1 and PARP from degradation (data not shown). These data indicate that it is the downregulation of Mcl-1 expression that may play a specific role in quercetin-induced selective apoptosis in leukemia.

**Knockdown of Mcl-1 via RNA interference enhanced quercetin-mediated cell death**

To further confirm the functional role of Mcl-1 in quercetin-mediated apoptosis, RNA interference of Mcl-1 was employed to U937 cells. A large reduction in Mcl-1 protein levels was detected by immunoblot analysis after U937 cells were transfected with Mcl-1 siRNA (Fig. 4D). Although residual Mcl-1 expression was very low after Mcl-1 siRNA transfection, a further decline could be discerned after quercetin exposure (Fig. 4D), which sensitized U937 cells to quercetin lethality (P < 0.01, compared with those untreated cells transfected with Mcl-1 siRNA; see Fig. 4C).
The siRNA transfection had no effect on Bcl-xL protein levels, but it decreased Bcl-2 expression in the presence of quercetin. Taken together, these findings suggest that the reduction in Mcl-1 levels following quercetin exposure may be a critical factor contributing to the induction of apoptosis.

**Knockdown of Bax substantially diminished the lethality of quercetin and knockout of Bax completely abrogated quercetin-induced cell death**

Our study showed that Bax activation occurred in quercetin-treated cells. We therefore tested the role of Bax in the lethality by quercetin. RNA interference was used to knockdown Bax in U937 cells prior to treatment with quercetin. Transfection with Bax siRNA yielded a sharp reduction in Bax protein level (Fig. 5B). Apoptosis induced by quercetin was significantly reduced from 59% to 21% by knockdown of Bax (Fig. 5A). The Mcl-1 protein level was unperturbed by Bax siRNA transfection compared with control siRNA treated cells, and exposure to quercetin resulted in equivalent decrease in Mcl-1 expression (Fig. 5B), arguing against the possibility that quercetin downregulates Mcl-1 through Bax. We further used HCT116 Bax+/− and HCT 116 Bax−/− cells to investigate the role of Bax in quercetin-induced cell death. Firstly, we compared the cell viability upon quercetin exposure and found that administration of quercetin could still induce cell death dramatically in Bax+/− cells but failed to induce the lethality in Bax−/− cells (Supplementary Fig. 3A). Also, we observed that PARP cleavage occurred in Bax−/− cells but not in Bax+/− cells during...
treatment with quercetin (Supplementary Fig. 3B). The level of Mcl-1 and changes of Mcl-1 expression were similar between Bax\(^+\)/C0 and Bax/C0/C0 cell types. Bcl-2 and Bcl-xL levels were roughly equivalent in these 2 cell types in the absence or presence of quercetin (Supplementary Fig. 3B).

As Supplementary Fig. 3C showed, in the absence of quercetin, Mcl-1 siRNA decreased cell survival in Bax\(^+\)/C0 cells but had no effect on cells lacking Bax, suggesting that the presence of Bax is required for Mcl-1 to exert its antiapoptotic activity. Collectively, these results indicate that apoptosis induced by quercetin is dependent upon the presence and activation of Bax.

**Mcl-1 inhibited conformational change and translocation of Bax without direct interaction**

Recent studies suggest that Mcl-1 antiapoptotic activity may be related to its inactivation of Bax (42), which prompted our investigation of the regulation of Bax by Mcl-1. The effects of Bax by Mcl-1 were addressed using cells that are either overexpressing or knocking down Mcl-1.

**Fig. 5.** A and B, downregulation of Bax successfully protects cells from cell death induced by quercetin (Quer). U937 cells were transfected with either Bax siRNA or control siRNA for 24 hours, after which cells were treated with 40 \(\mu\)mol/L of quercetin for additional 9 hours, after which (A) apoptosis was determined using the Annexin V/PI assay by flow cytometry. * values for Bax siRNA-treated cells were significantly decreased compared with those for control siRNA-treated cells after treatment with quercetin by ANOVA; \(P<0.05\). (B) total cellular extracts were prepared and subjected to immunoblot analysis using antibodies against Bax, Mcl-1, and PARP. CF = cleaved fragment. Blots were subsequently stripped and reprobed with an antibody against \(\beta\)-actin to ensure equivalent loading. C–E, Mcl-1 inhibits Bax conformational change and translocation. C, wild-type (WT) U937, U937 stably overexpressing Mcl-1, and U937 transfected with Mcl-1 siRNA cells were treated with 40 \(\mu\)mol/L of quercetin for 9 hours, after which the percentage of 6A7 Bax-positive cells (WT U937–untreated cells were set up as 100%) was determined by flow cytometry. * values for U937/Mcl-1 cells were significantly decreased compared with those for WT U937 cells after treatment with quercetin by ANOVA; \(P<0.05\). * values for Mcl-1 siRNA-treated cells were significantly increased compared with those for WT U937 cells after treatment with quercetin by ANOVA; \(P<0.05\). U937/Mcl-1 and its empty vector control (U937/pCEP) cells (D) and U937 transfected with Mcl-1 siRNA or control siRNA cells (E) were treated with 0 and 40 \(\mu\)mol/L of quercetin, after which cytosolic (designated as C) and mitochondrial (designated as M) fractions were prepared and subjected to immunoblot analysis by using an anti-Bax antibody. For Western blot analysis, blots were subsequently stripped and reprobed with an antibody against \(\beta\)-actin (cytosolic fraction) or Cox IV (mitochondrial fraction) to ensure equivalent loading.
Mcl-1. Wild-type U937 cells treated with quercetin displayed a rapid increase in conformationally changed Bax (Fig. 5C). In contrast, overexpression of Mcl-1 partially reduced Bax conformational change after exposure to quercetin (Fig. 5C). Knockdown of Mcl-1 expression by siRNA exhibited an increase in Bax conformational change upon quercetin treatment compared with wild-type cells. Taken together, these results provide clear evidences that Mcl-1 is a critical determinant of quercetin-mediated Bax conformational change.

To test whether Mcl-1 affects the translocation of Bax to mitochondria, U937 cells stably overexpressing Mcl-1 or vector alone were treated with quercetin and the mitochondria and cytosol were isolated. As shown in Figure 5D, Bax underwent a shift from cytosol to mitochondria in U937/pCEP cells whereas enforced expression of Mcl-1 prevented Bax translocation from cytosol to mitochondria following quercetin treatment. On the other hand, knockdown of Mcl-1 by siRNA enhances quercetin-induced Bax translocation to mitochondria (Fig. 5E). These data indicate that the Bax redistribution following quercetin treatment is regulated by Mcl-1.

One possible explanation for the inhibition of Bax by Mcl-1 is that Bax is sequestered in a Bax/Mcl-1 complex. To test this possibility, co-immunoprecipitation (co-IP) was carried out to determine the interaction between these 2 proteins. As shown in Supplementary Figure 3D, no direct interaction between Bax and Mcl-1 was detected in either

Fig. 6. Quercetin (Quer) inhibits tumor growth and induces apoptosis in the xenograft animal model. Six- to 8-week-old nude mice received subcutaneous transplants of $6 \times 10^6$ U937 cells. From the second day, mice were randomized into a control group (6 mice per group) and 2 treated groups (6 mice per group, quercetin 20 and 40 mg/kg). Intraperitoneal administration of quercetin and tumor volume assessment were conducted as described in "Materials and Methods." Representative animals with solid tumor volume are shown in (A), tumor volume measured on day 11 and day 16 is shown in (B), body weight is shown in (C), and representative immunohistochemical images for TUNEL staining and percentage of apoptotic cells (TUNEL-positive cells) in tumor tissue are shown in (D), representative IF images for Mcl-1 expression are shown in (E), and Bax activation using IP (anti-Bax 6A7) following immunoblotting (anti-Bax N-20) is shown in (F) in tumor samples. *, values of tumor volume for the quercetin treatment groups were significantly decreased compared with those for the nontreatment group by Student’s t test; $P < 0.05$. *, values of TUNEL-positive cells for the quercetin treatment groups were significantly increased compared with those for the nontreatment group by Student’s t test; $P < 0.05$. 
control or quercetin-treated conditions. It may therefore be concluded that Mcl-1 inhibits Bax activation through a mechanism(s) that does not require a direct interaction between these two proteins.

**Quercetin exhibited antitumor activity in xenografts of human leukemia U937 cells**

The *in vitro* data described earlier prompted us to further test the anticancer efficacy of quercetin in an *in vivo* model, that is, in mice xenografted subcutaneously with U937 cells. Treatment of mice with 20 and 40 mg/kg of quercetin resulted in 53% and 90% inhibition of tumor growth compared with controls in day 16 (Fig. 6A and B). As Fig. 6C showed, the body weights of the xenograft mice were not significantly variable between different treatment groups after 16 days. We further determined apoptotic cells in tumor tissue by TUNEL assay. Our data showed that TUNEL-positive apoptotic cells of tumor sections significantly increased in quercetin (20 and 40 mg/kg)-treated U937 xenograft mice as compared with the control group (TUNEL-positive cells: control 11.5 ± 1.03%; quercetin (20 mg/kg) treatment: 26.7 ± 2.03%; quercetin (40 mg/kg) treatment: 56.3 ± 4.02%; Fig. 6D). Moreover, Mcl-1 expression in tumor samples decreased upon quercetin treatment, as shown by immunofluorescence results (Fig. 6E). Bax activation was observed in quercetin-exposed xenografts (Fig. 6F). These data implicate a therapeutic value of quercetin in preventing or eradicating tumor growth in xenograft models of human hematologic malignancies.

**Discussion**

The present study is focused on the tumor-selective apoptosis induced by quercetin, a prospective anticancer drug that has been used in preclinical and small phase I clinical evaluation (43).

In view of the extensive evidence that Mcl-1 plays an important role in the survival of malignant hematopoietic cells (25, 44, 45), the development of anticancer compounds that can diminish Mcl-1 protein levels has been the focus of intense interest. Because Mcl-1 protein has a short half-life [(30 minutes (46, 47)], it is particularly susceptible to downregulation by agents. Here, we discovered that quercetin is efficient in killing tumor cells exhibiting relatively high levels of Mcl-1. The findings that overexpression of Mcl-1 diminished quercetin lethality highlight the central role of Mcl-1 downregulation. This interpretation is further supported by the results showing that knockdown of Mcl-1 could enhance quercetin-mediated apoptosis. It is noteworthy that overexpression of Bcl-2 or Bcl-xL failed to protect leukemia cells from quercetin-induced cell death, reflecting the important contribution of Mcl-1 downregulation to the lethality of this drug. Our unpublished data indicate that quercetin-induced manganese superoxide dismutase downregulation, Akt inactivation as well as a translational initiation factor eIF4E-mediated translational mechanism are responsible for this Mcl-1 reduction. Therefore, interventions disabling Mcl-1 may be an optimal way to kill leukemia cells. It should be noted that Mcl-1 overexpression did not completely block quercetin-induced cell death, suggesting that elimination of Mcl-1 may be necessary but not sufficient to trigger apoptosis, and other apoptosis-inducing actions might be required for achieving the full therapeutic effects.

In our study, although the overall Bax protein levels are not altered during quercetin-induced apoptosis, our data clearly show that Bax conformational change is one of the early steps in drug-induced apoptosis. Our data also show that an increase in Bax translocation leads to mitochondria-mediated caspase activation: mitochondria dysfunction promoted by Bax translocation leads to leakage of cytochrome c from mitochondria, which subsequently activates caspase-9 and -3. The importance of Bax in quercetin-treated lethality is confirmed by the observation that knockdown of Bax effectively protects cells from quercetin-mediated cell death and that cells lacking Bax displayed complete resistance to quercetin. These results suggest that the presence of Bax is essential for quercetin-treated lethality and that activation of Bax represents a highly potent apoptotic stimulus in the presence of quercetin.

Mcl-1 has previously been shown to inhibit Bax activation when overexpressed at high levels (36). In accord with this observation, our findings that enforced expression of Mcl-1 essentially diminishes quercetin-induced Bax activation strongly support that Mcl-1 plays an important role in regulating the function of Bax. Conversely, low levels of Mcl-1 enhance the formation of active Bax following exposure to quercetin. These findings are consistent with the results described by Nijhawan et al. (27) that Mcl-1 operates upstream of Bax and Bcl-xL translocation to the mitochondria in UV-treated HeLa cells. The inhibition of Bax by Mcl-1 in the absence of direct interactions between the 2 proteins could occur through several possible mechanisms. One would be that Mcl-1 acts through other multidomain proapoptotic Bcl-2 family proteins, such as active Bak, in an as yet to be defined way. However, in our case, Bak activation has not been observed. Therefore, this possibility can be excluded. A second possibility is that Mcl-1 inhibits Bax through a process involving the Bax “activator.” BHF3-only proteins, such as Bid or Bim, could be possible targets for Mcl-1 in this regard (48). Very recent studies from our group have uncovered a novel mechanism of quercetin-mediated Bax activation that involves Bid cleavage (unpublished data).

It has been reported that administration of quercetin eliminates colorectal cancer xenografts (49) as well as human breast cancer MDA-MB-435 cells xenografts (50). In our *in vitro* studies using a nude mice U937 xenograft model, tumor volumes were reduced compared with controls after quercetin treatment, indicating an antileukemia activity of this compound. To further validate the apoptotic mechanism found *in vitro*, we next examined the TUNEL staining, Mcl-1 expression, and Bax activation in tumor specimens obtained from control and quercetin-treated animals. The increase in TUNEL-positive cells and Bax activation as well as the decrease in Mcl-1 expression were...
detected in the quercetin-treated xenografts compared with the control group. To the best of our knowledge, this is the first report that describes an effective extrapolation of the in vitro apoptosis-inducing effects of quercetin on the leukemia cells to the in vivo situation.

In summary, the present findings indicate that quercetin effectively induces cell death in human leukemia cells, including primary leukemia blasts, as well as in leukemia xenografts. This effect occurs in association with the rapid downregulation of Mcl-1 and is accompanied by the conformational change and translocation of Bax, which are dependent on Mcl-1 reduction. The potent antileukemia activity of quercetin found both in vitro and in vivo in this study along with the novel mode of action make this compound an attractive anticancer agent for hematologic malignancies. In addition, this work also identifies Mcl-1 and Bax as potential biomarkers of quercetin activity that directly relate to its mechanism of action. Such biomarkers may be useful to show the mode of action of quercetin in vivo, using patient biopsy samples. Further efforts are warranted to elucidate the mechanism(s) by which quercetin inhibits Mcl-1, as well as to identify other possible factors that contribute to Bax activation during quercetin treatment. This study could provide a better understanding of how this compound exerts its antitumor activity in vivo and aid in developing this compound either alone or in combination with established chemotherapeutic agents to treat leukemia and potentially other hematologic malignancies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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