# Antileukemia effects of xanthohumol in Bcr/Abl-transformed cells involve nuclear factor-κB and p53 modulation

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## Abstract

The oncogenic Bcr-Abl tyrosine kinase activates various signaling pathways including phosphoinositide 3-kinase/Akt and nuclear factor-κB that mediate proliferation, transformation, and apoptosis resistance in Bcr-Abl(+) myeloid leukemia cells. The hop flavonoid xanthohumol inhibits tumor growth by targeting the nuclear factor-κB and Akt pathways and angiogenesis. Here, we show that xanthohumol has *in vitro* activity against Bcr-Abl(+) cells and clinical samples and retained its cytotoxicity when imatinib mesylate–resistant K562 cells were examined. Xanthohumol inhibition of K562 cell viability was associated with induction of apoptosis, increased p21 and p53 expression, and decreased survivin levels. We show that xanthohumol strongly inhibited Bcr-Abl expression at both mRNA and protein levels and show that xanthohumol caused elevation of intracellular reactive oxygen species and that the antioxidant *N*-acetylcysteine blunted xanthohumol-induced events. Further, we observed that xanthohumol inhibits leukemia cell invasion, metalloprotease production, and adhesion to endothelial cells, potentially preventing *in vivo* life-threatening complications of leukostasis and tissue infiltration by leukemic cells. As structural mutations and/or gene amplification in Bcr-Abl can circumvent an otherwise potent anticancer drug such as imatinib, targeting Bcr-Abl expression as well as its kinase activity could be a novel additional therapeutic approach for the treatment of Bcr-Abl(+) myeloid leukemia. [Mol Cancer Ther 2008;7(9):2692 – 702]

## Introduction

The Bcr/Abl oncogene results from a translocation (9;22) that fuses sequences from the BCR gene with the ABL gene. The discovery that Bcr/Abl is required for the pathogenesis of chronic myelogenous leukemia (CML) and that the tyrosine kinase activity of ABL is essential for Bcr/Abl-mediated transformation made the ABL kinase an attractive target for clinical intervention (1). Imatinib mesylate (Gleevec, STI-571) is a selective tyrosine kinase inhibitor that has been proven to be a powerful agent for leukemias caused by Bcr/Abl, but the emergence of imatinib resistance underscores the need for additional therapies (2). Targeting signaling pathways activated by Bcr/Abl is a promising approach for drug development. Of these, activation of phosphoinositide 3-kinase (3) and nuclear factor-κB (NF-κB; ref. 4) has emerged as essential signaling mechanisms in Bcr/Abl leukemogenesis. Bcr/Abl has also been implicated as a possible regulator of CML angiogenesis associated with elevated vascular endothelial growth factor (VEGF) expression levels (5). Xanthohumol, the principal flavonoid of the hop plant (*Humulus lupulus* L.), has been suggested to have potential cancer chemopreventive activities by inhibiting human breast (MCF-7), colon (HT-29), ovarian cancer (A-2780; ref. 6), and B-chronic lymphocytic leukemia cell proliferation *in vitro* (7). We have shown that xanthohumol has antiangiogenic properties *in vitro* and *in vivo* where it inhibited proliferation of endothelial and Kaposi's sarcoma–derived tumor cells *in vitro*, prevented angiogenesis in the Matrigel sponge model, and reduced Kaposi's sarcoma xenograft growth *in vivo*. In addition, we recently showed that xanthohumol targets cell growth and angiogenesis in hematologic malignancies (8). The antiangiogenic effects of xanthohumol correlated with a block of NF-κB activation and decreased phosphorylation of Akt (8, 9).

Based on our reported antiendothelial effects of xanthohumol and on the observation that endothelial and hematopoietic cells are mutually correlated in their development and growth, here we investigated the effects of xanthohumol on Bcr/Abl-expressing leukemia cells. Our results identify new signaling pathways upstream and downstream of Bcr/Abl that are targeted by xanthohumol and suggest it may be of therapeutic utility in patients with CML. Moreover, targeting both tumor cells and endothelial cells with agents possessing cytotoxic and antiangiogenic

activities may lead to synergistic antitumor effects interrupting a reciprocal stimulatory loop between leukemia and endothelial cells.

## Materials and Methods

### Reagents

Xanthohumol was purchased from Alexis Biochemicals, imatinib mesylate was kindly provided by Novartis. VEGF protein released into the media by leukemic cells was measured using a commercial human ELISA kit (Bender MedSystems).

### Cells

K562 and U937 CML and human umbilical vein endothelial cells were obtained from the American Type Culture Collection. Human umbilical vein endothelial cells were cultured on gelatin-coated plates (1% in PBS) in M199 containing 10% heat-inactivated fetal bovine serum, 100 μg/mL heparin, 10 ng/mL acidic fibroblast growth factor, 10 ng/mL basic fibroblast growth factor, 10 ng/mL epidermal growth factor, and 10 μg/mL hydrocortisone. K562 and U937 were grown in RPMI containing 10% heat-inactivated FCS. Mononuclear cells were isolated from peripheral blood or bone marrow of patients at diagnosis of chronic phase after informed consent was obtained. Cells were purified on Ficoll-Hypaque gradients. CD34^{+} cell-enriched populations were selected using immunomagnetic column separation (Miltenyi Biotech) and then cultured in RPMI in the presence of 20% heat-inactivated fetal bovine serum and 20 ng/mL human granulocyte/macrophage colony-stimulating factor. Colony-forming assays were conducted on normal bone marrow progenitors (10) to evaluate xanthohumol cytotoxicity. Imatinib-resistant K562 cells (K562-RI) were generated by exposure to increasing concentrations of imatinib beginning with 0.2 μmol/L and ending at 10 μmol/L. This process took 6 months and resulting populations were 100% viable. To obtain xanthohumol-resistant K562 cells (K562-RXN), xanthohumol was used at a starting concentration of 0.1 μmol/L. Surviving cells were collected by centrifugation, grown in regular medium to recover, and then progressively treated with a 2-fold higher concentration of xanthohumol up to a concentration of 2 μmol/L. The selection for resistance took 10 months. A denaturing high-performance liquid chromatography–based assay was employed for detection of ABL mutations (11). Experiments with resistant cells were carried out under the continuous presence of the compounds.

### Cell Proliferation, Apoptosis, Invasion, Gelatin Zymography, and Adhesion Assay

The number of viable cells was measured by the MTT test at different times and with a different number of starting cells (2,000-10,000 per well) depending on the duration of the experiment. The concentration of drug that reduced cell proliferation by 50% ($IC_{50}$) compared with controls was calculated by nonlinear regression fit of the mean data obtained in triplicate experiments. To measure any enrichment of cytoplasmic histone-associated DNA fragments after xanthohumol or imatinib treatments, a commercially available kit was employed (Cell Death Detection ELISA kit; Roche) and 10,000 cells were used in all experiments. Chemoinvasion was carried out in BioCoat Matrigel invasion chambers (BD Biosciences) following the manufacturer's instructions. Supernatants from NIH3T3 cells were used as chemoattractants in 24-well plates, and the inserts were placed in wells and incubated at 37°C for 6 h. In parallel experiments, trypan blue exclusion was carried out to test cell viability. After the incubation period, the invasive cells that migrated into the lower chamber were fixed, stained, and counted under a light microscope. Supernatants of cells from the invasion assay were centrifuged to remove particulates, the protein content was measured by the Bradford method (Bio-Rad), and gelatin zymography was done on identical sample amounts as described previously (12). Gels were then stained in 0.1% Coomassie brilliant blue followed by destaining. The enzyme-digested regions were observed as white bands against a blue background. Adhesion to endothelial cells was carried out as described (13).

### Detection of Intracellular Reactive Oxygen Species

K562 cells ($10^5$ per well) were treated with 5 μmol/L xanthohumol for different lengths of time in the absence or presence of the reactive oxygen species (ROS) scavenger *N*-acetylcysteine (NAC) at 10 mmol/L. Thirty minutes before the end of the treatment, the cells were incubated with 50 μmol/L dichlorofluorescein diacetate for detection of ROS. The cells were then washed twice with phenol-free HBSS and analyzed with a microplate fluorometer with excitation set at 488 nm and emission at 530 nm.

### Real-time Reverse Transcription-PCR

Real-time reverse transcription-PCR for estimation of p21, survivin, p210-Bcr/Abl, and p53 mRNAs were carried out as described previously (14) by using the following primers: *p21* sense 5′-GGACAGCAGAGGAAGAC and antisense 5′-GGCGTTTGGAGTGGTAGAAA; *survivin* sense 5′-ACTGAGAACGAGCCAGACTT and antisense 5′CGGACGAATGCTTTTTATGTTC; *Bcr-Abl* sense 5′-TCAGAAGCTTCTCCCTGACA and antisense 5′-TCCACTGGCCACAAAATCATA; and *p53* sense 5′-CCAGCCAAAGAAGAAACCAC and antisense 5′-CTCATTCAGCTCTCGGAAC. The relative expression of each gene was assessed in comparison with the housekeeping gene *glyceraldehyde-3-phosphate dehydrogenase* amplified with the following primers: sense 5′-GAAGGTGAAGGTCGGAGT and antisense 5′-CATGGGTGGAATCATATTGGAA. cDNAs were amplified for 50 cycles using iQ Supermix (Bio-Rad) containing the intercalating agent SYBR Green in a two-step amplification scheme (95°C, 15 s and 60°C, 30 s). Fluorescence was measured during the annealing step on a Bio-Rad iCycler iQ instrument. Blank controls that did not contain cDNA were run in parallel. All samples were run in triplicate. Following amplification, melting curves with 80 steps of 15 s and a 0.5°C temperature increase per step were done to control for amplicon identity. Relative expression values with SE and statistical comparison (unpaired two-tailed *t* test) were obtained using Qgene software (15).

### Protein Extraction and Western Blot Analysis

To test for activation of the NF-κB pathway, K562 cells were serum starved for 16 h and then treated with 5 μmol/L xanthohumol for 6 h in serum-free medium in the absence or presence of 10 mmol/L NAC. Thirty minutes before the end of incubation, cells were stimulated with tumor necrosis factor-α (TNF-α; 10 ng/mL). For nuclear and cytoplasmic protein extracts, the pellets of control and treated cells were resuspended in cytoplasmic lysis buffer and incubated on ice for 10 min, vortexed, and centrifuged at 12,000 rpm for 2 min at 4°C. Supernatants containing the cytoplasmic proteins were kept separately and the nuclei in the pellets were resuspended in nuclear lysis buffer for 20 min on ice and centrifuged at 12,000 rpm for 2 min at 4°C. To obtain whole-cell lysates, cell pellets were lysed in radioimmunoprecipitation assay buffer containing protease inhibitors. Protein concentration was determined by using the DC Protein Assay kit (Bio-Rad). Equal amounts of samples were resolved by SDS-PAGE, transferred to nitrocellulose, and probed at 4°C overnight with the following anti-human antibodies (Cell Signaling Technology): rabbit polyclonal anti-phospho-p65 (Ser$^{539}$), anti-phospho-IκB (Ser$^{32}$), and anti-phospho-IKKα (Ser$^{180}$)/IKKβ (Ser$^{181}$). NF-κB activity was further analyzed using a commercially available ELISA kit (TransAM; Active Motif) following the manufacturer's instructions. Survivin, p21, Bcr-Abl, and p53 analyses were carried out on extracts (9) obtained from control or treated cells grown in complete medium and filters were probed with the following anti-human antibodies (Santa Cruz Biotechnology): mouse monoclonal anti-survivin and rabbit polyclonal anti-p21, anti-Bcr, and anti-p53 antibodies. Protein complexes containing Bcr-Abl were immunoprecipitated with anti-Bcr antibody and protein A/G plus-agarose (Santa Cruz). The immunoprecipitates were washed three times in radioimmunoprecipitation assay buffer and eluted with SDS sample loading buffer and Western blot analyses were done using a mouse monoclonal anti-phospho-tyrosine antibody (Santa Cruz). After washing, the blots were incubated for 1 h at room temperature with horseradish peroxidase–conjugated secondary antibodies (Amersham) and specific complexes were revealed by enhanced chemiluminescence solution (Amersham). An anti- glyceraldehyde-3-phosphate dehydrogenase antibody conjugated to horseradish peroxidase (Novus Biologicals) or a mouse monoclonal anti-β-tubulin antibody (Sigma) was used as loading control for all samples.

## Results and Discussion

### Xanthohumol Modifies Human Leukemia Cell Viability

*In vitro*, xanthohumol showed a dose-dependent antileukemic activity at micromolar concentrations (Fig. 1A, *left*) with IC$_{50}$ of 10 and 5.4 μmol/L at 48 and 72 h, respectively. At 24 h, a notable apoptotic activity of xanthohumol on K562 cells was observed (Fig. 1A, *right*). To assess the safety of xanthohumol on human bone marrow progenitors, we tested the compound in an *in vitro* colony-forming unit assay. At concentrations up to 5 μmol/L, xanthohumol did not inhibit growth of bone marrow progenitors isolated from healthy volunteers, whereas smaller colonies were obtained at higher concentrations (data not shown). This is consistent with the previously observed lack of apoptosis induction in normal endothelial cells by xanthohumol (9). As CML blast-crisis patients frequently progress on imatinib therapy, we tested the efficacy of xanthohumol following two different approaches: (*a*) by using CML samples from patients at the moment of diagnosis and (*b*) by generating imatinib-resistant K562 cells. As shown in Fig. 1B (*left*), 48 h exposure to xanthohumol impairs fresh leukemia viability in a significant and dose-dependent manner similar to that obtained in the presence of 1 μmol/L imatinib. Apoptosis induction after 24 h exposure to 5 μmol/L xanthohumol was already evident alone or in combination compared with the limited apoptosis obtained with 1 μmol/L imatinib alone (Fig. 1B, *right*). To examine potential activity of xanthohumol on acquired imatinib resistance observed in clinical practice, K562 cells were cultured in the presence of increasing concentrations of imatinib (starting at 0.2 μmol/L) up to 10 μmol/L to generate an imatinib-resistant cell line (K562-RI). Several mutations have been reported in association with the resistant phenotype and a denaturing high-performance liquid chromatography–based assay showed that the K562-RI cells harbored the E255K mutation. As shown in Fig. 1C, K562-RI cells grew faster (*left*) and with less spontaneous apoptosis (*right*) compared with the parental population. When K562-RI-resistant cells were cultured for 48 h in the presence of xanthohumol (Fig. 1D, *left*), a significant and dose-dependent decrease in their growth rate was observed. Despite resistance to imatinib, these cells displayed enhanced sensitivity to 5 μmol/L xanthohumol-induced apoptosis compared with parental cells (Fig. 1D, *right*), raising the possibility that some imatinib-resistant cells might actually be hypersensitive to xanthohumol *in vivo*. Identical results were obtained using resistant cells cultured in drug-free medium before all experimental procedures (data not shown). To assess whether Bcr/Abl(+) cells could develop resistance also to xanthohumol, K562 cells were cultured in the presence of increasing concentrations of the molecule. After several attempts, we selected cells resistant to 2 μmol/L xanthohumol that could be maintained only if were periodically cultured in the absence of the compound to recover with a slow proliferation rate. Xanthohumol-resistant K562 cells (K562-RXN) had no Bcr/Abl mutations and showed a decreased viability (Fig. 1C, *left*), compared with parental cells, that was associated with an elevated spontaneous apoptosis rate (Fig. 1C, *right*), suggesting that K562 cells are more inclined to become resistant to imatinib rather than to xanthohumol. When K562-RXN cells were cultured in the presence of imatinib at 0.1 μmol/L, almost 100% of the cells underwent apoptosis in 24 h, preventing cell growth (data not shown).

### Xanthohumol Inhibits Leukemic Cell Invasion through Extracellular Matrix and Adhesion to Endothelial Cells

Important features of CML are the presence of increased numbers of circulating progenitors and extramedullary
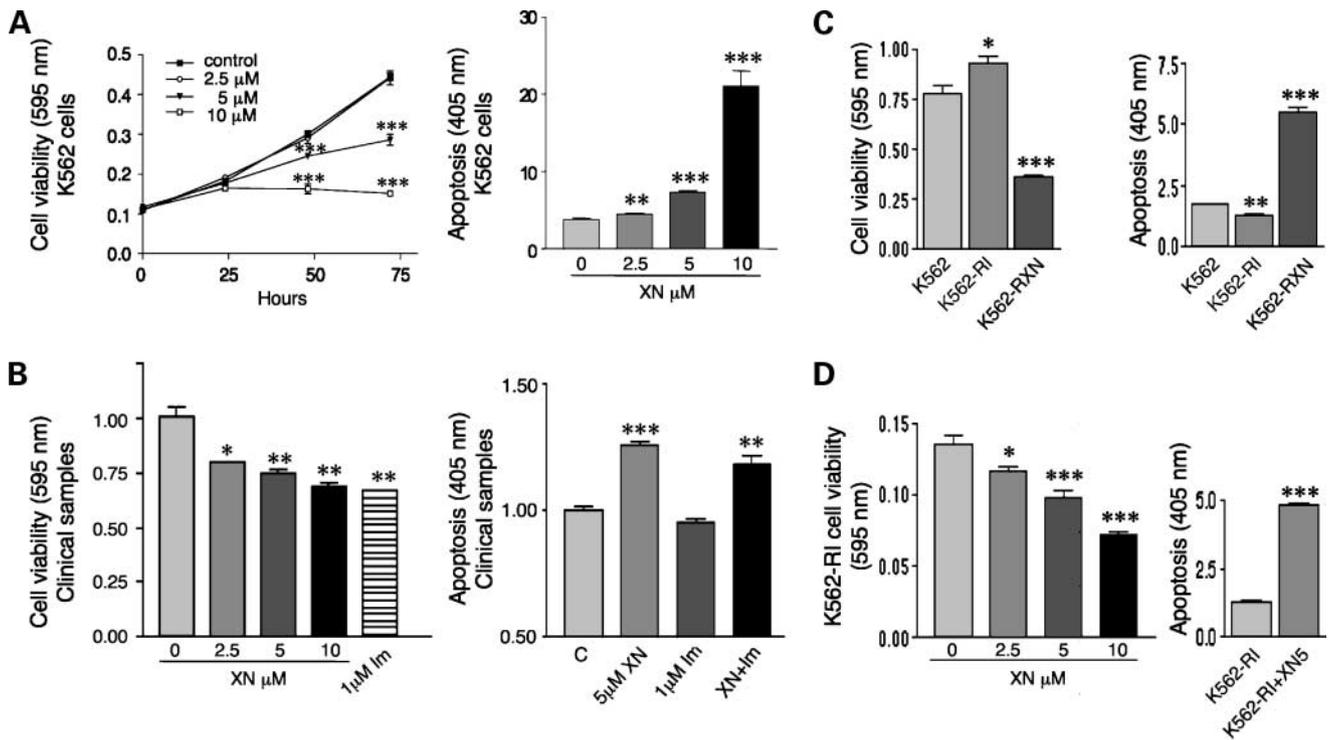
**Figure 1.** *In vitro* effects of xanthohumol on K562 and CML cells. **A,** effects of xanthohumol on K562 cells viability and apoptosis. *Left,* xanthohumol at 5 and 10 μmol/L significantly (***, $P < 0.001$ with respect to controls; two-tailed $t$ test) reduced cell viability after 48 h. *Right,* after 24 h of treatment, xanthohumol induced significant levels (**, $P < 0.01$; ***, $P < 0.001$, with respect to controls; two-tailed $t$ test) of apoptosis in a dose-dependent manner. **B,** *left,* effects of 48 h exposure to xanthohumol on growth of mononuclear cells from CML patients at the time of diagnosis (6 samples). Xanthohumol at 5 and 10 μmol/L significantly reduced cell proliferation similar to 1 μmol/L imatinib (*Im*; *, $P < 0.05$; **, $P < 0.01$, with respect to untreated controls). Mean of experiments carried out twice on all samples. Values are normalized against controls set at 1. **B,** *right,* 5 μmol/L xanthohumol significantly (***, $P < 0.001$) induced apoptosis in fresh leukemia samples after 24 h, whereas 1 μmol/L imatinib did not. One result from four patient samples analyzed in triplicate and generating similar results. **C,** 10,000 K562 cells and their imatinib (10 μmol/L, K562-RI) or xanthohumol (2 μmol/L, K562-RXN) resistant derivatives were plated; 24 h after seeding, K562-RI showed significant increased growth rate (*left*; *, $P < 0.05$) associated with decreased spontaneous apoptosis (*right*; **, $P < 0.01$). Conversely, K562-RXN cells grew slower (*left*; ***, $P < 0.001$) with a high rate of spontaneous apoptosis (*right*; ***, $P < 0.001$). When K562-RI cells (2,000 per well) were incubated for 48 h in the presence of increasing xanthohumol concentrations, a significant and dose-dependent growth inhibition was observed (**D,** *left*) and 5 μmol/L strongly induced apoptosis after 24 h (**D,** *right*; ***, $P < 0.001$).

hematopoiesis that promotes adhesion of leukemia cells to vascular endothelium and generates conditions that favor leukostasis and tissue infiltration (16). Kinase-dependent and kinase-independent mechanisms contribute to the abnormal adhesion and migration of CML progenitors (17). Leukemic blast cells secrete matrix metalloproteinase-2, a marker for dissemination in myeloproliferative malignancies (18). Untreated K562 cells invaded through Matrigel in response to fibroblast complete medium (NIH3T3), whereas few cells invaded in the absence of a chemoattractant (serum-free medium; Fig. 2A). Addition of xanthohumol during the assay inhibited K562 cell invasion at concentrations as low as 2.5 μmol/L (Fig. 2A). Trypan blue exclusion under these conditions showed no decreased cell viability compared with controls (data not shown). Consistent with decreased cell invasiveness, zymographic evaluation of supernatants from the invasion assays showed that xanthohumol dose-dependently inhibited matrix metalloproteinase-2 activity (Fig. 2A, *inset*), leaving unaltered its synthesis as evaluated by real-

time reverse transcription-PCR and Western blotting (data not shown). Similar results were obtained when leukemia cells from patients at the time of diagnosis were allowed to invade in the presence of xanthohumol (Fig. 2B), which reduced invasion to background levels.

Leukemic cells have the ability to generate conditions that promote their own adhesion to vascular endothelium, a property that may have important implications for the pathophysiology of leukostasis and tissue infiltration (16). Leukemia cells exposed to xanthohumol for 6 h and labeled with the fluorescent dye calcein AM showed a dose-dependent and significant reduction in adherence to confluent endothelial cell monolayers (Fig. 2C). The repressive effects of xanthohumol on endothelial cell activation (9) may further contribute to decrease local inflammatory reactions (19).

### Xanthohumol Inhibits NF-κB Activity

The Bcr-Abl kinase signals to downstream survival pathways including phosphoinositide 3-kinase and NF-κB (20, 21). Although the phosphoinositide 3-kinase pathway
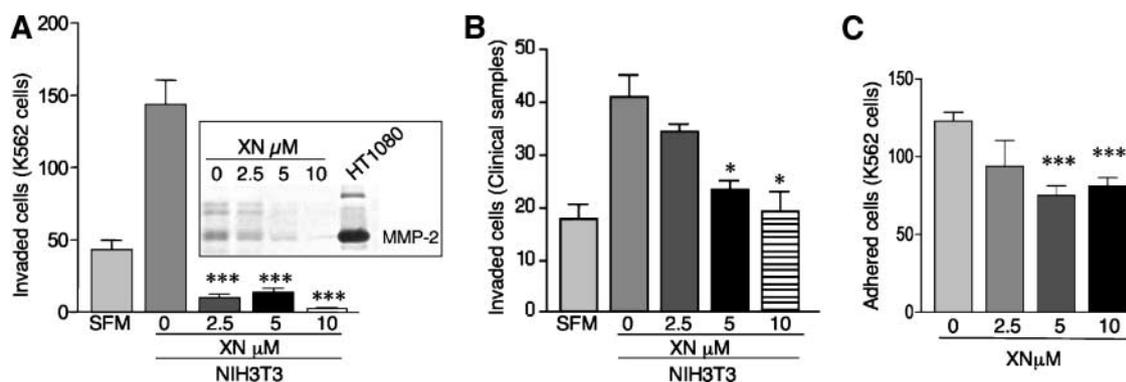
**Figure 2.** Inhibition of K562 and CML cell invasion and adhesion by xanthohumol is associated with decreased metalloprotease activity. **A,** inhibition of K562 cell invasion by 6 h xanthohumol treatment (***, $P < 0.001$, with respect to controls; two-tailed $t$ test). *Inset,* zymography detection of secreted gelatinase activity in medium from the invasion assay indicates that xanthohumol dose-dependently inhibits matrix metalloproteinase-2 activity. When CML cells from patients (three samples) were allowed to invade in the presence of xanthohumol (**B;** only one representative experiment is shown), the results were less evident although still significant (*, $P < 0.05$). Serum-free medium (*SFM*) and supernatants of NIH3T3 fibroblasts were used as negative and positive controls, respectively. **C,** myeloblast-human umbilical vein endothelial cell adhesion assay. Calcein AM–labeled K562 cells ($5 \times 10^5$ per well) exposed to xanthohumol for 6 h showed a significant decreased adhesion to human umbilical vein endothelial cells (***, $P < 0.001$, with respect to controls; two-tailed $t$ test). Mean ± SE.

is reported to be important for Bcr-Abl transformation and leukemia cell proliferation, we did not notice any change in phospho-Akt levels following either xanthohumol or imatinib alone or in combination (data not shown). Activation of the NF-κB pathway in Bcr-Abl(+) cells results in modulation of genes that regulate apoptosis, proliferation, and invasion. Based on the results shown above, we tested whether the biological activities of xanthohumol are mediated through NF-κB modulation, as observed in endothelial cells (9) and hematologic malignancies (8).
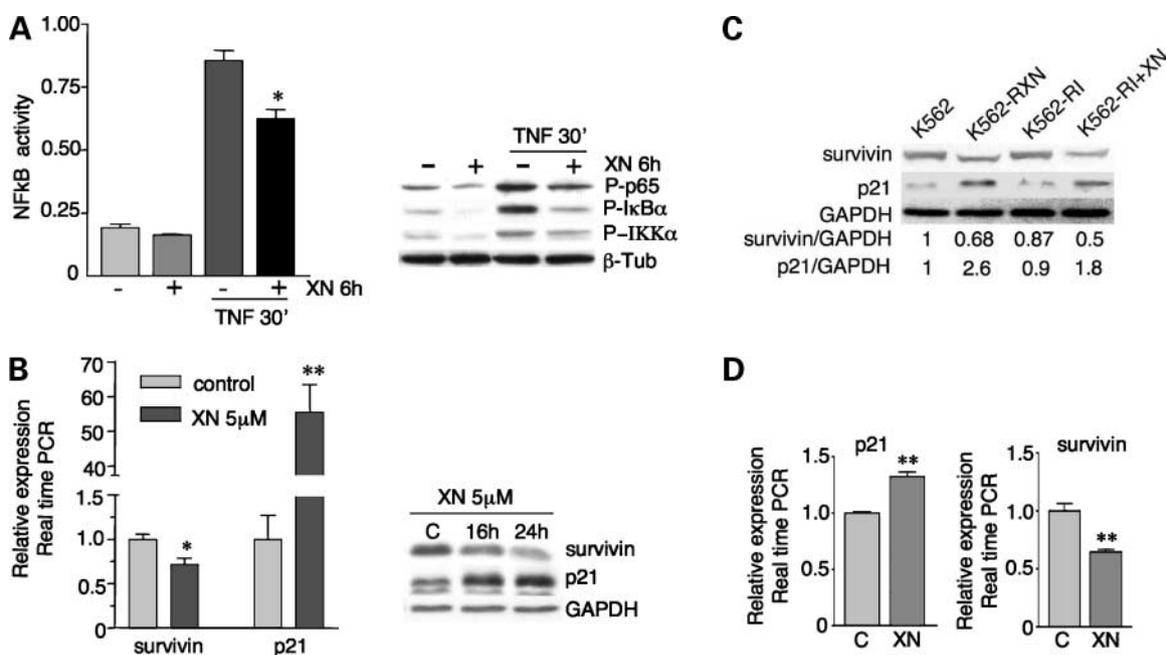


**Figure 3.** Effects of xanthohumol on NF-κB activation, survivin, and p21 expression. **A,** ELISA analysis (*left*) showed that 6 h treatment with 5 μmol/L xanthohumol reduces the amount of active NF-κB in TNF-α (10 ng/mL) K562-stimulated cells. Mean ± SE (*, $P < 0.05$, with respect to control; two-tailed $t$ test). Western blot analysis (*right*) showed that xanthohumol represses nuclear phosphorylated p65 as well as cytosolic IκBα and IKKα levels in K562 cells after stimulation with TNF-α and under basal conditions. **B,** real-time reverse transcription-PCR (*left*; RNA from 16 h treated cells) and Western blot analyses (*right*) for survivin and p21 expression. Xanthohumol modulates these regulators of cell cycle progression and apoptosis by modulating NF-κB activity. **C,** Western blotting and densitometric analyses of basal levels of p21 and survivin in K562-RXN and K562-RI cells well correlate to their proliferation rate; consistent with growth arrest and apoptosis induction after 16 h exposure to 5 μmol/L xanthohumol, K562-RI cells showed p21 induction and survivin repression. **D,** clinical samples exposed for 16 h to 5 μmol/L xanthohumol showed the same pattern of p21 and survivin modulation observed in treated K562 cells (**, $P < 0.01$). Mean of experiments carried out twice on three samples and normalized against controls set at 1.
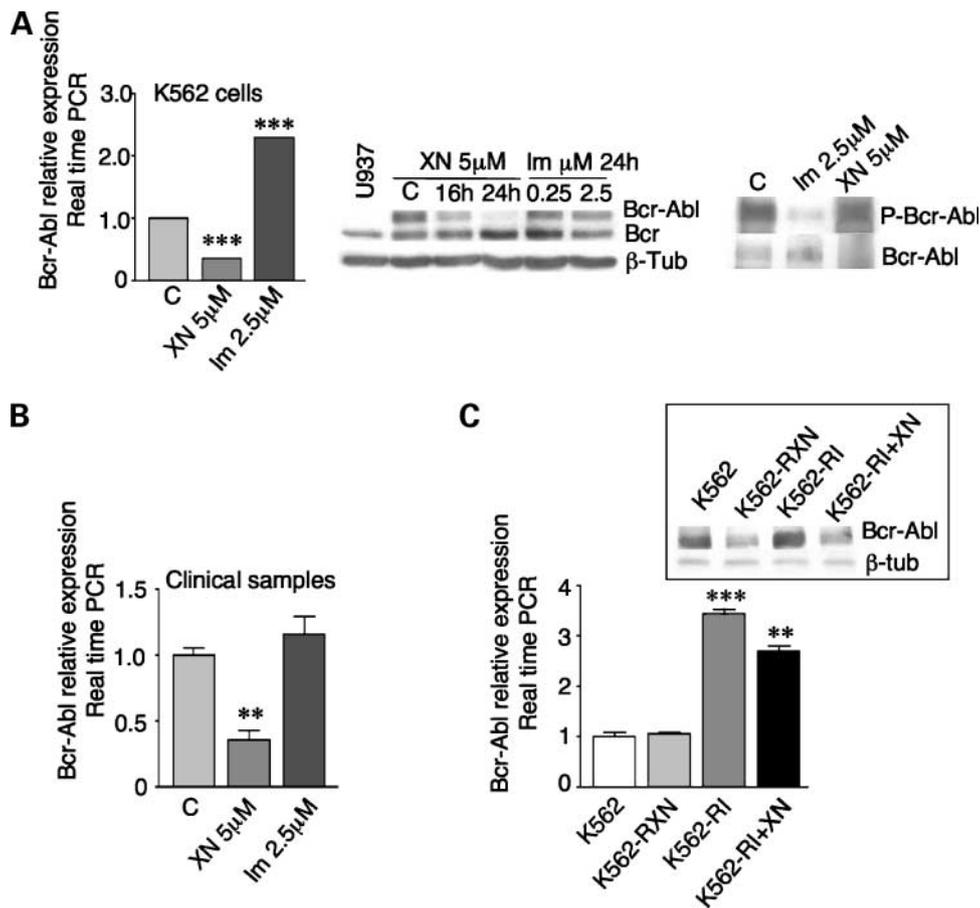
**Figure 4.** Effects of xanthohumol on Bcr-Abl expression. **A,** real-time reverse transcription-PCR (*left*) and Western blot analyses (*middle*) for Bcr-Abl expression in K562 cells. Sixteen hours of exposure to 5 μmol/L xanthohumol significantly decreased both Bcr-Abl mRNA and protein levels. Under the same conditions, the kinase inhibitor imatinib had no effect on protein levels, although it strongly induced Bcr-Abl mRNA; U937 cells as negative control for Bcr-Abl expression. Immunoprecipitation of lysates (*right*) with anti-Bcr antibodies followed by Western blot analyses for phosphorylated Bcr-Abl (*P-Bcr-Abl*) revealed no modulation in Bcr-Abl kinase activity from samples treated with xanthohumol for 3 h compared with the inhibition in imatinib-treated samples (*top*). As a control for loading, the membrane was reprobed with an anti-Bcr antibody, further confirming the almost complete disappearance of the Bcr-Abl variant (*bottom*) as soon as after 3 h of exposure to xanthohumol. Bcr-Abl mRNA down-regulation by xanthohumol, but not by imatinib, was confirmed in clinical samples exposed to 5 μmol/L for 16 h (**B**; **, $P < 0.01$). Mean of experiments carried out twice on three samples and normalized against controls set at 1. **C,** real-time reverse transcription-PCR and Western blot analyses (*inset*) for Bcr-Abl expression in K562-resistant cells. Compared with parental cells, K562-RI showed high Bcr-Abl levels, which were promptly reduced on exposure to 5 μmol/L xanthohumol for 16 h. As expected, K562-RXN cells showed reduced Bcr-Abl protein content.

Inactive NF-κB consists of a heterotrimer composed by the p50 and p65 subunits together with the protein IκBα. The phosphorylation, ubiquitination, and degradation of IκBα releases the p50-p65 heterodimer, which then translocates to the nucleus to induce specific gene expression. ELISA analysis showed that pretreatment of K562 cells with 5 μmol/L xanthohumol significantly suppressed TNF-α-induced NF-κB activation (Fig. 3A, *left*). Western blot analysis (Fig. 3A, *right*) confirmed that xanthohumol reduced nuclear phosphorylated p65, as well as cytosolic phosphorylated IκBα, in both resting K562 cells and following TNF-α stimulation. IKK is required for TNF-induced phosphorylation of IκBα (22); we found that xanthohumol suppressed basal and TNF activated IKK. These data show that xanthohumol interferes with signaling pathways leading to NF-κB activation.

NF-κB regulates expression of the antiapoptotic protein survivin (23); further, Bcr-Abl elevates survivin expression, whereas survivin disruption sensitizes Bcr-Abl(+) cells to apoptosis induced by imatinib and chemotherapy agents (24). As xanthohumol induced growth arrest in the presence of apoptosis and modulated NF-κB activation, we evaluated whether it could also affect survivin expression in leukemia cells. Short exposure of K562 cells to 5 μmol/L xanthohumol decreased survivin expression at the mRNA (Fig. 3B, *left*) and protein (Fig. 3B, *right*) levels. Another protein, p21, which induces cell growth arrest by cyclin-dependent kinase inhibition (25), was strongly increased after 16 and 24 h of xanthohumol treatment (Fig. 3B). Conversely, we observed that survivin and p21 expression remained unaltered on K562 cells exposure to imatinib (data not shown), confirming the different

mechanisms of action of the two compounds. Analysis of survivin and p21 basal levels in imatinib- and xanthohumol-resistant cells revealed interesting changes, which correlated with the observations reported above: decreased survivin and increased p21 levels, indicative of slow proliferating cells, were detected in K562-RXN cells, whereas the higher proliferation rate of K562-RI cells was associated with augmented survivin and low p21 levels (Fig. 3C). Interestingly, but not unexpected, the decreased growth rate and increased apoptosis observed in K562-RI cells exposed to xanthohumol (Fig. 1C) correlated with a xanthohumol-modified protein profile where survivin was reduced and p21 was up-regulated (Fig. 3C). These data further confirm that imatinib-resistant cells still retain xanthohumol responsiveness. To test whether xanthohumol could modify the expression of p21 and survivin in an *in vivo* context, cells from clinical samples were exposed 16 h to 5 μmol/L xanthohumol and RNAs were extracted. Real-time PCR analyses confirmed that both genes were modulated in a similar manner (Fig. 3D).

### Xanthohumol Down-regulates Bcr-Abl and Controls Proliferation/Apoptosis-Related Genes

Agents that lower the Bcr-Abl levels have been shown to enhance the apoptotic effects of imatinib on CML cells (26–28). We examined whether xanthohumol-induced apoptosis was associated with Bcr-Abl reduction. K562 cells express high levels of Bcr-Abl compared with the Bcr-Abl(-) U937 cell line; 5 μmol/L xanthohumol rapidly (beginning at 3 h; data not shown) decreased Bcr-Abl expression at the mRNA (Fig. 4A, *left*) and protein (Fig. 4A, *middle*) levels. Under the same conditions, the tyrosine kinase inhibitor imatinib had no effect, leaving unaltered the Bcr-Abl protein level, although strongly increasing its mRNA. Immunoprecipitation of lysates from control and xanthohumol treated cells with anti-Bcr antibodies, followed by Western blot analyses for tyrosine phosphor-

ylation, revealed that xanthohumol did not modulate Bcr-Abl kinase activity (Fig. 4A, *right*), whereas, as expected, imatinib treatment did. Real-time reverse transcription-PCR on mRNAs from clinical samples showed that exposure to xanthohumol (5 μmol/L for 16 h), but not to imatinib, decreased Bcr-Abl expression (Fig. 4B), thus confirming that the molecule has similar mechanisms of action both in cell lines and in clinical samples.

Differential levels of Bcr-Abl oncoprotein expressed by CML cells may reflect the extent and duration of response to imatinib, whereas high levels of Bcr-Abl in advanced phases of the disease may explain the development of resistance (29). Accordingly, we noticed that imatinib-resistant cells expressed a significantly higher amount of Bcr-Abl mRNA than parental or xanthohumol-resistant cells (Fig. 4C) and a short exposure to xanthohumol (5 μmol/L for 16 h) was sufficient to significantly decrease Bcr-Abl expression, further confirming that imatinib-resistant cells retain xanthohumol responsiveness. Western blot analyses confirmed these data showing, in addition, low Bcr-Abl content in K562-RXN cells (Fig. 4C, *inset*). As stated above, Bcr-Abl elevates survivin expression and survivin disruption sensitizes Bcr-Abl(+) cells to apoptosis (24); consistent with this, Bcr-Abl modulation in xanthohumol-treated cells, as well as in K562-RI and K562-RXN cells, strongly correlated with survivin expression, cell growth, and apoptosis.

Several reports indicate that there is a substantial cross-talk between the Bcr-Abl and p53 signaling networks (30–32) and loss of p53 impedes the antileukemic response to Bcr-Abl inhibition (33). To ascertain whether p53 has a role in CML responsiveness to xanthohumol, K562 cells (Fig. 5A) and clinical samples (Fig. 5B) were exposed to 5 μmol/L xanthohumol for increasing times and then analyzed for p53 expression. In K562 cells and clinical samples, a rapid and transient mRNA induction was
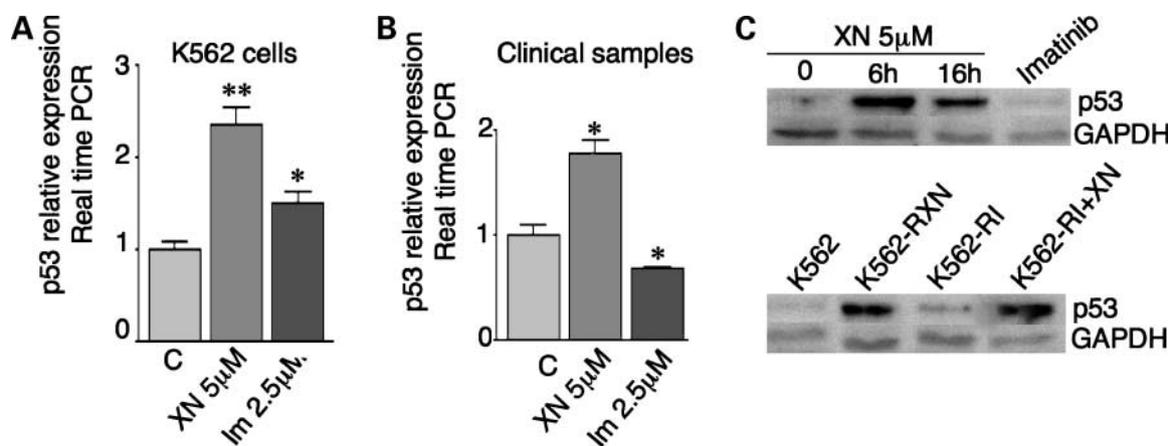


**Figure 5.** Bcr-Abl inhibition is associated with p53 induction. Real-time reverse transcription-PCR for p53 expression in K562 cells (**A**) and clinical samples (**B**; three samples) exposed for 6 h to 5 μmol/L xanthohumol or 2.5 μmol/L imatinib. All amplifications were done in triplicate (*, $P < 0.05$; **, $P < 0.01$, with respect to controls set at 1; two-tailed *t* test). **C,** p53 immunoblotting analyses of nuclear lysates from xanthohumol- or imatinib-treated K562 cells (*top*) and resistant cells (*bottom*). Results strongly confirm real-time PCR data and further highlight p53 involvement in the antileukemic response to xanthohumol.
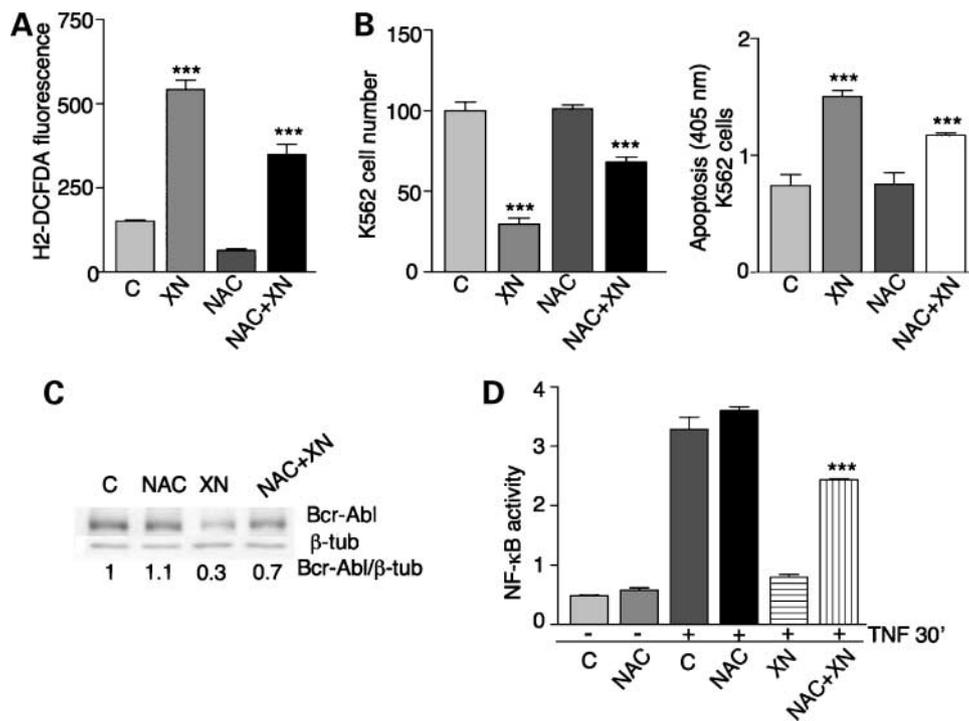
**Figure 6.** ROS generation by xanthohumol. NAC inhibits xanthohumol-induced ROS production and cytotoxicity in K562 cells. **A,** K562 cells treated with 5 μmol/L xanthohumol alone for 6 h or pretreated for 90 min with NAC at 10 mmol/L were stained with dichlorofluorescein diacetate to assess intracellular ROS production. The strong xanthohumol-induced ROS production is significantly inhibited by pretreatment with NAC. NAC alone produced a marginal effect on the basal ROS level (mean ± SE from quadruplicate samples; ***, $P < 0.001$). **B,** K562 cell growth inhibition (*left*) by 48 h exposure to 5 μmol/L xanthohumol is strongly inhibited by the presence of NAC at 10 mmol/L; under the same conditions, NAC also protected cells from apoptosis (*right*) induced after 24 h exposure to 5 μmol/L xanthohumol (mean ± SE from two independent experiments run in triplicate; ***, $P < 0.001$). **C,** Western blot and densitometric analyses for Bcr-Abl expression in K562 exposed for 16 h to xanthohumol or NAC alone or in combination: ROS inhibition by NAC attenuates the xanthohumol-induced Bcr-Abl down-regulation. **D,** ELISA analyses showed that 6 h treatment with NAC significantly attenuated the inhibitory activity of xanthohumol on NF-κB in TNF-α (10 ng/mL) K562-stimulated cells. NAC alone produced marginal effects. Mean ± SE (***, $P < 0.001$, two-tailed *t* test).

observed following treatment with xanthohumol that was maximal after 6 h treatment (Fig. 5A and B). Identical results were obtained on nuclear isolates from xanthohumol-treated K562 cells analyzed by Western blotting (Fig. 5C, *top*). Exposure to imatinib (2.5 μmol/L) did not substantially modify p53 mRNA (Fig. 5A and B) and protein (Fig. 5C, *top*) levels. One possible explanation is that xanthohumol, by inhibiting nuclear translocation of the survival protein NF-κB, also inhibits p53 targeting for degradation by the proteasome (34). In this context, because p53 binding *in vivo* to consensus sites in the p21 promoter has been described (35), the p53-induced growth arrest could also be mediated by its downstream target p21 (36, 37) and the strong increase in nuclear p53 protein observed on xanthohumol treatment could also contribute to up-regulation of p21 protein. Consistent with these observations, the augmented level of p21 detected in K562-RXN cells (Fig. 3C) under basal conditions is associated with an evident p53 up-regulation (Fig. 5C, *bottom*) detectable in the same sample. As reported by Brusa et al. (32), p53 overexpression was associated with a significant reduction of Bcr-Abl expression levels resulting, at least in part, from post-transcriptional events affecting the stability of p210 Bcr-Abl fusion protein.

Indeed, we found that the greater reduction of Bcr-Abl transcription by xanthohumol (Fig. 4A) was associated with the overexpression of p53 (Fig. 5C, *top*). Further, K562-RXN cells, which have basally high p53 (Fig. 5C, *bottom*) and very low Bcr-Abl levels (Fig. 4C), are strongly prone to undergo apoptosis in the presence of imatinib. Conversely, like that found in K562-RI cells, high Bcr-Abl levels (Fig. 4C) in the presence of low p53 (Fig. 5C, *bottom*) appear to be indicative of resistance to targeted therapy. This appears to be circumvented by xanthohumol treatment, which induces p53 up-regulation (Fig. 5C, *bottom*), Bcr-Abl down-regulation (Fig. 4C), and apoptosis (Fig. 1D). Another important feature becomes clear from our data: as the human survivin gene is negatively regulated by p53 (38), the observed p53 up-regulation following xanthohumol exposure may well explain survivin mRNA and protein decrease. Thus, p53 appears to contribute to the antileukemic effects of xanthohumol *in vitro* and might contribute to overcome imatinib resistance *in vivo*.

### Involvement of ROS in Xanthohumol-Induced Cytotoxicity

Flavonoids are generally considered to be antioxidants, although there have also been observed pro-oxidant

properties of some flavonoids (luteolin and apigenin). To investigate the mechanisms of the xanthohumol-induced effects observed here, the effect of xanthohumol on ROS in K562 cells was examined. Interestingly, we found that xanthohumol treatment elevated ROS as detected by the increase of dichlorofluorescein diacetate fluorescence. This appeared to be an early event, doubling within 30 min of exposure to the drug (data not shown). ROS content in response to xanthohumol reached a maximum at 6 h of treatment (Fig. 6A) and declined at 24 h (data not shown). Xanthohumol treatment in the presence of the ROS scavenger NAC (10 mmol/L) significantly reduced the induction of ROS in K562 cells, having a marginal effect on the basal ROS levels (Fig. 6A). Involvement of ROS in inducing cytotoxicity in human leukemia cells has been widely reported and ROS-dependent apoptosis in K562 cells exposed to arsenic or adaphostin has been associated with a decline in Bcr/Abl protein levels (39, 40). Furthermore, DNA damage induced by increased ROS further activates p53, establishing an amplification loop (41). If xanthohumol-induced ROS were, at least in part, involved in its cytotoxicity and apoptosis induction and Bcr/Abl and p53 regulation, then modulation of ROS production by the antioxidant NAC should repress these events. Coexposure of K562 cells to 10 mmol/L NAC and xanthohumol indeed significantly protected the cells from 5 μmol/L xanthohumol-induced cell growth arrest and apoptosis (Fig. 6B) as well as antagonized Bcr/Abl down-regulation by xanthohumol (Fig. 6C), providing further support for the hypothesis that these changes occur downstream of ROS production. Another pathway that is under ROS-mediated control in some systems is that leading to activation of NF-κB (42); above a certain threshold, ROS may actually negatively affect this signaling through direct interference with the DNA-binding activity of NF-κB that, once in the nucleus, has to be kept in a reduced state to bind DNA. This could contribute to the reduced NF-κB-binding activity we observed in cells exposed to xanthohumol and stimulated with TNF-α (Figs. 3A and 6D). As a confirmation, the ROS scavenger NAC in the presence of xanthohumol strongly and significantly ameliorated NF-κB-binding activity in TNF-α-stimulated cells (Fig. 6D). Taken together, these data suggest that induction of ROS participates in the mechanisms of action of xanthohumol.

### Xanthohumol Reduces Bcr-Abl-Mediated VEGF Secretion: Possible Anti-CML Properties through an Angiogenesis-Dependent Mechanism

Leukemias are angiogenesis-dependent malignancies (43, 44). VEGF release by leukemic blasts may be an important stimulus for angiogenesis in the bone marrow, as overexpression of VEGF and increased angiogenesis are consistent findings in CML (43) and a prognostic significance of VEGF expression in chronic-phase CML has been proposed (45). Moreover, in the NOD/SCID mouse model, efficiency and speed of engraftment of myeloid and lymphoid human malignancies correlate with VEGF production (46). We have described previously that the antiangiogenic activity of xanthohumol was associated

with decreased NF-κB activity and VEGF secretion (8, 9). As Bcr-Abl is a possible angiogenesis regulator in CML (5) and imatinib-treated patients show reduced VEGF plasma concentrations (47), imatinib might produce some of its anti-CML properties *in vivo* through angiogenesis inhibition. Here, we tested whether the marked decrease in Bcr-Abl expression induced by xanthohumol could also affect VEGF secretion. K562 cells produce high amounts of VEGF and 16 h of exposure to 5 μmol/L xanthohumol significantly ($P < 0.05$) reduced VEGF levels to 60% of control values (from $280 \pm 9$ to $165 \pm 7$ pg/mL/$10^6$ cells). Similar reduced amounts were detected in control K562-RXN cells ($168 \pm 9$ pg/mL/$10^6$ cells) in agreement with the low levels of Bcr-Abl expression in these cells. ELISA quantification in fresh leukemia samples was below the detection threshold of the method (16 pg/mL). As the endothelium is quite sensitive to the dose of VEGF to which it is exposed, the 40% reduction in VEGF production observed may well be physiologically significant. Another intriguing aspect of VEGF-driven angiogenesis in hematologic neoplasias is the finding that VEGF-stimulated endothelial cells generate stem cell factor, granulocyte/macrophage colony-stimulating factor, and interleukin-6 (48). These cytokines, in turn, may act as growth factors for myeloid and lymphoid malignant cells, thus generating a paracrine machinery between hematopoietic malignant cells and newly generated endothelium. In this scenario, xanthohumol could inhibit the angiogenic process through decreasing VEGF secretion in leukemic cells as well as through inhibiting endothelial cell activities, causing the interruption of a reciprocal stimulatory loop between leukemic and endothelial cells.

Collectively, the *in vitro* findings presented here generate a rationale for investigation of clinical efficacy of molecules such as xanthohumol that are endowed with antitumor and antiangiogenic properties. Our results argue persuasively that imatinib-resistant samples, overexpressing and carrying Bcr/Abl mutations, will likely be highly susceptible to xanthohumol, making combination therapy an interesting alternative. Several studies have shown that neoplastic cells of hematopoietic origin are particularly susceptible to strategies in which cell cycle and survival signaling pathways are simultaneously interrupted. In particular, the data suggest that interference with the cytoprotective NF-κB pathway plays an important role in Bcr-Abl(+) cells where a functional relationship between NF-κB survival signaling and Bcr-Abl kinase activation has been well documented (49, 50). Clinical studies with imatinib indicate that Bcr-Abl is required to sustain the proliferative advantage of CML cells, but there is considerable evidence that leukemia cells may persist in CML patients responsive to imatinib treatment (10). The mechanisms underlying incomplete elimination of malignant cells are unclear, but it is possible that kinase inactivation of Bcr-Abl does not abrogate by itself proliferation and survival signals in physiologic conditions. A treatment strategy that eventually combines an agent interfering with the NF-κB and p53 pathways and lowering Bcr-Abl levels with an agent that

inhibits the Bcr-Abl tyrosine kinase activity could potentially improve therapies against Bcr-Abl(+) human leukemias that are either sensitive or resistant to chemotherapy. Moreover, the advantage is that xanthohumol targets the Bcr-Abl mRNA and protein through a mechanism distinct from that of imatinib, and its activity does not appear to be circumvented by Abl kinase mutations.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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## Antileukemia effects of xanthohumol in Bcr/Abl-transformed cells involve nuclear factor- κB and p53 modulation

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