Curcumin Inhibits Prosurvival Pathways in Chronic Lymphocytic Leukemia B Cells and May Overcome Their Stromal Protection in Combination with EGCG

Asish K. Ghosh, Neil E. Kay, Charla R. Secreto, and Tait D. Shanafelt

Abstract

Purpose: Chronic lymphocytic leukemia (CLL) is incurable with current chemotherapy treatments. Curcumin (diferuloylmethane), an active ingredient in the spice turmeric, inhibits tumor metastasis, invasion, and angiogenesis in tumor cell lines. We evaluated the effects of curcumin on the viability of primary CLL B cells and its ability to overcome stromal mediated protection.

Experimental Design: The *in vitro* effect of curcumin on primary CLL B cells was evaluated using fluorescence activated cell sorter analysis and Western blotting. For some experiments, CLL B cells were cocultured with human stromal cells to evaluate the effects of curcumin on leukemia cells cultured in their microenvironment. Finally, the effect of curcumin in combination with the green tea extract epigallocatechin-3 gallate (EGCG) was evaluated.

Results: Curcumin induced apoptosis in CLL B cells in a dose-dependent (5-20 μmol/L) manner and inhibited constitutively active prosurvival pathways, including signal transducers and activators of transcription 3 (STAT3), AKT, and nuclear factor κB. Moreover, curcumin suppressed expression of the anti-apoptotic proteins Mcl-1 and X-linked inhibitor of apoptosis protein (XIAP), and up-regulated the pro-apoptotic protein BIM. Coculture of CLL B cells with stromal cells resulted in elevated levels of STAT3, increased expression of Mcl-1 and XIAP, and decreased sensitivity to curcumin. When curcumin was administered simultaneously with EGCG, antagonism was observed for most patient samples. In contrast, sequential administration of these agents led to substantial increases in CLL B-cell death and could overcome stromal protection.

Conclusions: Curcumin treatment was able to overcome stromal protection of CLL B cells on *in vitro* testing and to synergize with EGCG when administered in a sequential fashion. Additional evaluation of curcumin as a potential therapeutic agent for treatment of CLL seems warranted.

B-cell chronic lymphocytic leukemia (CLL) is the most common leukemia in North America and, with the possible exception of allogeneic stem cell transplant, is incurable with current treatments (1). CLL B cells are largely noncycling (2), and their accumulation is primarily secondary to decreased apoptosis rather than increased proliferation (3). Notably, the apoptotic resistance of CLL B cells to chemotherapeutic agents and monoclonal antibodies is, in part, related to increased levels of anti-apoptotic proteins Mcl-1, Bcl-2, and X-linked inhibitor of apoptosis protein (XIAP; refs. 1, 3–7). CLL B cells have also been found to have a constitutively active signal transducers and activators of transcription 3 (STAT3) pathway (8, 9), possess an autocrine vascular endothelial growth factor signaling loop (10), and express constitutively activated nuclear factor κB (NFκB; ref. 11) and phosphatidylinositol-3 kinase. Importantly, selective inhibition of phosphatidylinositol-3 kinase induces apoptosis of CLL B cells (12, 13).

Previous work by our laboratory and others suggests that autocrine survival signals and interactions between the CLL B cell and its microenvironment influence apoptotic resistance and sensitivity to chemotherapeutic agents (8–10, 14). In the marrow, physical contact between stromal elements and leukemic cells promotes CLL B-cell survival (15–18), an effect mediated in part through integrins (e.g., very late antigen-4; ref. 18) on the surface of CLL B cells and their interaction with various ligands (e.g., vascular cell adhesion molecule-1, fibronectin, iC3b) expressed on marrow stromal cells (19–22). To add to the complexity of environmental/stromal protection, several other interactions (mediated by contact and soluble factors) between CLL B cells and their microenvironment have been shown to promote survival, proliferation, and up-regulation of anti-apoptotic proteins (20, 22–25).

There is now great interest in identifying pharmacological agents that are able to modulate these interactions that impact survival pathways in the hope of identifying potential novel therapies for treatment of CLL. Naturally occurring compounds are a potential source of agents that could modulate these

Authors' Affiliation: Division of Hematology, Mayo Clinic, Rochester, Minnesota
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Requests for reprints: Tait D. Shanafelt, Mayo Clinic, 200 First Street Southwest, Rochester, MN 55905. Phone: 507-284-1961; Fax: 507-266-9277; E-mail: shanafelt.tait@mayo.edu.

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Translational Relevance

The authors evaluate the effects of the spice curcumin on primary chronic lymphocytic leukemia cells *in vitro*. These experiments confirm that curcumin induces cell death in these leukemic cells and begins to dissect the mechanisms of these effects and how they are modulated by leukemia cell – stromal cell interactions. Given the tolerability of curcumin in initial human testing, the results of these studies provide insight for the design of potential clinical testing of this agent in chronic lymphocytic leukemia.

survival signals and interrupt stromal nurturing. We have previously shown that the green tea extract, epigallocatechin-3 gallate (EGCG), inhibits vascular endothelial growth factor receptor activation and induces apoptosis in primary CLL B cells (10). This agent has now entered clinical testing in patients with early-stage CLL (26, 27).

Another natural compound, curcumin (diferuloylmethane), one of the active ingredients in the spice turmeric, has emerged as an attractive therapeutic agent that combines clinical tolerability with intriguing pharmacological properties, including antitumor, anti-inflammatory, and antioxidant properties (28–30). Curcumin has recently been shown to inhibit tumor metastasis, invasion, and angiogenesis (29–32). Curcumin induces apoptosis in a variety of cancer cell lines and down-regulates expression of cell proliferation (cyclooxygenase-2, cyclin D1, and c-myc), as well as anti-apoptotic (inhibitor of apoptosis protein 1, inhibitor of apoptosis protein 2, XIAP, Bcl-2, Bcl-xL, Bfl-1/A1, tumor necrosis factor receptor–associated factor–1, cellular FLICE (FADD-like interluekin-1β-converting enzyme)-inhibitory protein (FLIP) and metastatic gene products (vascular endothelial growth factor, matrix metalloproteinase-9, ICAM-1) through suppression of IκBα kinase and Akt activation (29). Importantly, Everett et al. (33) recently reported that curcumin can induce apoptosis in CLL B cells and suggested this effect may relate to inhibition of constitutively activated NFκB.

In the present study, therefore, we have further assessed the impact of curcumin on CLL B-cell viability and dissected the mechanism of curcumin-mediated cytotoxic effects on CLL B cells. Specifically, we evaluated the effect of curcumin on prosurvival pathways constitutively activated in CLL B cells, including NFκB, STAT3, and AKT. We have also examined the cytotoxic effect of curcumin on CLL B cells in the presence of stromal cells. Finally, we have evaluated the effect of curcumin in combination with EGCG and the ability of combination therapy to overcome stromal protection.

Materials and Methods

Patient selection and CLL sample processing. Blood was obtained from CLL patients who had provided written informed consent under a protocol approved by the Mayo Clinic institutional review board according to the regulations of the Declaration of Helsinki or from healthy volunteers. All CLL patients had a confirmed diagnosis using the National Cancer Institute Working Group definition (34). Patients in this cohort were from all Rai stages and had not been treated before blood processing for this study within the last 2 y. CLL cells were isolated from heparinized venous blood by density gradient centrifugation. When assessed by flow cytometry (FACScan, Becton Dickinson), the isolated cells were predominantly CLL B cells (>90% $CD5^+/CD19^+$). Lymphocytes from healthy volunteers ($n = 5$) were separated by density gradient centrifugation. Freshly isolated CLL B cells or peripheral blood mononuclear cell from normal individuals were cultured in serum-free AIM-V medium at 37°C in an atmosphere containing 95% air and 5% CO_2.

Reagents. Immunologic reagents that recognize the following antigens were purchased from the indicated suppliers: mouse monoclonal antibodies to Bcl-2, Bcl-xL, XIAP, Bcl2-antagonist of cell death (BAD), and Bcl-associated X protein (BAX), and rabbit polyclonal antibody to BH3 interacting domain death agonist (BID) from BD Pharmingen/Transduction Laboratories; antibodies to actin and survivin from Novus Biologicals; antibody to Mcl-1 from Chemicon; antibody to poly (ADP-ribose) polymerase (PARP) from BIOMOL; antibodies to caspase-3, caspase-9, phospho-BAD (Ser-136), phospho-AKT (Ser-473), phospho-IκBα, phospho-STAT3 (Ser-727), AKT, and STAT3 from Cell Signaling Technology; and antibodies to IκBα and BIM from Santa Cruz. The following reagents were purchased from the indicated suppliers: curcumin (>94% purified, Sigma); FITC-conjugated annexin V (Invitrogen); propidium iodide (Becton Dickinson); pan-caspase inhibitor Z-VAD-fmk (BD Pharmingen); and AIM-V medium (Gibco). Epigallocatechin (EGCG) was a gift from Dr. Y. Hara (Mitsui Norin, Japan).

Apoptosis assay. Primary CLL B cells (1.0×10^6 cells/mL) were treated with either vehicle (DMSO) or curcumin for 24 to 48 h at increasing doses (5-20 μmol/L) in serum-free AIM-V medium. Cells were washed with PBS, stained with annexin V-FITC and propidium iodide, and analyzed for apoptosis by flow cytometry (FACScan).

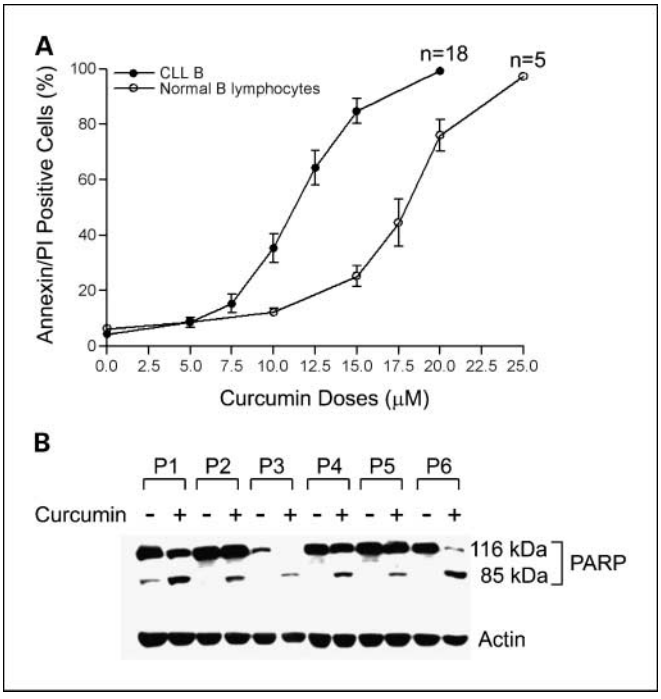

Fig. 1. Curcumin induces apoptosis in primary CLL B cells in a dose-dependent manner. *A,* primary peripheral blood mononuclear cell (≥90% $CD5^+/CD19^+$ lymphocytes) isolated from CLL patients ($n = 18$) were treated with increasing doses of curcumin for 24 h. Cells were harvested, stained with annexin/propidium iodide, and analyzed by flow cytometry for the induction of cell death. Similarly, peripheral blood mononuclear cell from normal individuals ($n = 5$) were treated with curcumin and induction of cell death in $CD19^+$ B lymphocytes was analyzed by flow cytometry (annexin/propidium iodide positivity). Mean values were plotted with SE bars. *B,* curcumin induced cell death involves PARP cleavage. Lysates from CLL B cells ($n = 6$) treated with curcumin or DMSO were analyzed for PARP cleavage by Western blot. P, patient. Curcumin-treated cells displayed cleavage of the native PARP (116 kDa) into its signature 85-kDa polypeptide fragment.

Table 1. Assessment of *in vitro* curcumin sensitivity in leukemic cells from B-CLL patients

Patient no.	Rai stage	WBC/ALC ($\times 10^9$/L)	CD38*	ZAP-70 †	IGH status	FISH	Curcumin IC_{50} (μmol/L) range
1	IV	163.9/144.15	Negative	Negative	UM	13q-	12.5
2	IV	30/16.91	Positive	ND	UM	ND	10.0
3	I	25.5/14.53	Negative	Negative	ND	11q-	10-12.5
4	I	55.4/51.69	Negative	Negative	M	13q-	10-12.5
5	I	32.5/26.32	Positive	Positive	UM	13q-	12.5-15.0
6	IV	79.1/66.44	Negative	Positive	ND	17p-	15.0
7	II	100.8/97.77	Negative	Positive	UM	11q-	15-20
8	I	61.4/58.33	Negative	Negative	M	Normal	12.5-15.0
9	II	45.9/44.06	Positive	Positive	UM	Trisomy 12	10-12.5
10	I	29.8/27.11	ND	Positive	UM	13q-	12.5
11	IV	56.2/50.58	Negative	Negative	ND	Trisomy 12	10-12.5
12	I	40.5/38.47	Positive	Positive	UM	6q-	10-12.5
13	I	42.8/33.81	Negative	Negative	M	13q-	12.5-15.0
14	II	23.9/12.66	Positive	Negative	ND	Trisomy 12	10-12.5
15	I	43.1/32.32	Positive	ND	UM	Trisomy 12	5-7.5
16	0	46.2/42.04	ND	Positive	UM	Normal	10-12.5
17	III	19.4/17.04	Positive	Positive	UM	13q-	7.5-10.0
18	I	31.1/26.74	Negative	Negative	ND	Normal	7.5-10.0

Abbreviations: ALC, absolute lymphocyte count; ZAP-70, Zeta-associated protein 70; IGH, Immunoglobin heavy chain mutation status; UM, unmutated; M, mutated; ND, not done; FISH, fluorescence *in situ* hybridization.
*Cutoff value, ≥30%.
† Cutoff value, ≥20%.

Similarly, freshly isolated normal peripheral blood mononuclear cell (1.0×10^6/mL) were treated with curcumin at various doses (5-25 μmol/L) for 24 to 48 h, and we then analyzed cell death by staining with CD19-Allophycocyanin (APC), annexin-FITC, and propidium iodide (Sigma) on a FACSCalibur Instrument (Becton Dickinson) using Cell Quest software. Cells staining with annexin V-FITC and/or propidium iodide were considered positive for cell death.

Treatment of CLL B cells with curcumin in the presence of stromal cells. For stromal experiments, the human bone marrow stromal cell line HS-5 (35) was grown and maintained in DMEM (Biosource) containing 10% fetal bovine serum (Biosource), as described previously (14). HS-5 cells were cultured in 12-well tissue-culture plates at a cell density of 1.0×10^5/well overnight, washed twice with serum-free AIM-V medium, and then incubated with primary CLL B cells at a cell density of 1×10^6 cells/well. CLL B cells were cultured by either direct contact with HS-5 cells or indirectly exposed to HS-5 cells via transwells (pore size, 0.45 μm) with serum-free AIM-V medium for 24 h before then being cultured with increasing doses of curcumin (10, 15, and 20 μmol/L) or DMSO. For comparison, CLL B cells cultured without stromal cells were treated similarly with curcumin or DMSO. After 24 h, CLL B cells were harvested, washed in PBS, and stained with APC-conjugated antibody to CD19, annexin V-FITC, and propidium iodide. Apoptosis in CD19 positive lymphocytes was then analyzed on a FACSCalibur Instrument.

Assessment of combination treatment with curcumin and EGCG on CLL B-cell death. CLL B cells were treated with various doses of curcumin (2.5-15 μmol/L) or EGCG (25-150 μmol/L) individually or in combination using a constant ratio (1:10) for 24 h. Cells were harvested, stained with annexin/propidium iodide, and viability analyzed by flow cytometry. After concentration-effect curves were generated for each agent, data were analyzed using the CalcuSyn software program (Biosoft), which uses the method of Chou and Talalay (36), to determine whether combination treatment yields greater effects than expected from summation alone. A combination index of 1 indicates an additive effect, a combination index above 1 indicates an antagonistic effect, and a combination index below 1 indicates a synergistic effect (37). In other experiments, CLL B cells were treated with DMSO, curcumin (10 μmol/L), EGCG (100 μmol/L), or sequentially with both drugs. For sequential treatment experiments, 1.0×10^6 CLL B cells/mL were treated with DMSO, curcumin (10 μmol/L) alone, EGCG (100 μmol/L) alone, or both agents for 24 h; washed; and cultured for another 24 h in media alone or with the addition of the second agent (EGCG or curcumin), as indicated. Cells were then harvested and stained with annexin-FITC/propidium iodide to analyze cell death on FACScan flow cytometer (Becton Dickinson). Similarly, sequential treatment of the primary CLL B cells was also done in coculture with the stromal cells in experiments to determine if combination therapy could reduce the survival impact of stromal cells.

Immunoblotting. For immunoblot experiments, primary CLL B cells were treated with DMSO or curcumin (20 μmol/L) for 36 to 48 h and then lysed in lysis buffer containing 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 2 mmol/L EGTA, 1% NP40, 10 mmol/L NaF, 1 mmol/L Na_3V0_4, and a cocktail of protease inhibitors. Following incubation on ice for 20 mins, cell lysates were passed through 27 G needle and sonicated briefly (30-40 s). The whole cell extract was collected after a centrifugation at 16,000 × *g* for 15 mins. Protein content was determined, and equal amounts of proteins were loaded on SDS-polyacrylamide gels after digesting in Laemmli SDS-sample buffer. Separated proteins were transferred onto 0.45-μm nitrocellulose papers (BioRad) and immunostained with specific antibodies. Protein bands were detected using an enhanced chemiluminescence detection kit (Pierce).

Statistical analysis. The percent kill and percentage viable cells were evaluated across CLL patients and were summarized graphically and quantitatively. These percentages were analyzed graphically for each dose level independently, as well as across dose levels graphically. Means and SEs were calculated using Excel (Microsoft Corporation). Graphical analyses were done using Sigma plot software. See the previous section on combination treatment for discussion and mathematical analysis of additive, synergistic, or antagonistic effects of combination therapy using the CalcuSyn software program.

Results

***Curcumin induces apoptosis in primary CLL B cells* in vitro.** Primary CLL B cells in serum-free AIM-V medium were treated

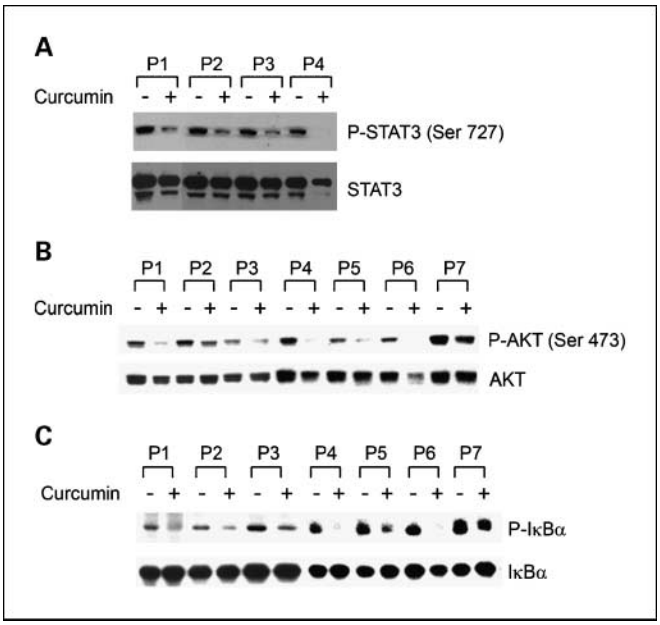

Fig. 2. Curcumin inhibits prosurvival signaling pathways active in CLL B cells. Curcumin-treated CLL B cells were analyzed by Western blot using phospho-specific antibodies to assess the phosphorylation profile of the prosurvival signaling molecules known to be constitutively elevated in CLL, including STAT3 ($n = 4$; *A*), AKT ($n = 7$; *B*), and IκBα ($n = 7$; *C*). Curcumin treatment decreased phosphorylation levels of STAT3, AKT, and IκBα in primary CLL B cells. Total STAT3, AKT, and IκBα were used as loading controls for the respective experiments. Representative figures of at least 10 CLL B patients' samples.

with increasing doses of curcumin for 24 hours. At the indicated time, cells were harvested, stained with annexin V/propidium iodide, and analyzed by flow cytometry for viability. Curcumin treatment induced apoptosis in CLL B cells in a dose-dependent manner (Fig. 1A). The mean IC_{50} dose of curcumin at 24 hours was between 10 and 12.5 μmol/L. Under the same experimental conditions, the average IC_{50} dose for the normal CD19+ B lymphocytes was higher at between 17.5 and 20 μmol/L. The sensitivity of CLL B cells to curcumin did not correlate with the Rai stage using the IC_{50} data and seemed to be independent of prognostic parameters (Table 1).

To examine whether curcumin-induced apoptosis involved PARP cleavage, we analyzed lysates obtained from curcumin-treated CLL B cells by Western blot. PARP was cleaved from the native form (116 kDa) into its 85-kDa signature polypeptide after treatment with curcumin (Fig. 1B). Activation of upstream effector or initiator caspases (e.g., caspase-3 and caspase-9) was not observed with curcumin treatment (data not shown), suggesting that curcumin-induced apoptosis is not dependent on the caspase pathway. To confirm curcumin-induced apoptosis was caspase independent; CLL B cells were treated with curcumin in the presence or absence of the pan-caspase inhibitor zVAD-fmk. Treatment of CLL B cells with zVAD-fmk (100 μmol/L) failed to protect them from curcumin-induced apoptosis (data not shown).

Curcumin inhibits prosurvival pathways in CLL B cells. A number of prosurvival signaling pathways are known to be constitutively activated in CLL B cells, including STAT3, phosphatidylinositol-3 kinase, and NFκB (reviewed in refs. 13, 30). Therefore, to examine the effect of curcumin on these survival pathways, we analyzed lysates from primary CLL B cells following treatment with curcumin by Western blot (Fig. 2). Curcumin treatment decreased phosphorylation of both STAT3 (Fig. 2A) and AKT (Fig. 2B), a downstream target of activated phosphatidylinositol-3 kinase. In most cell types, NFκB is present constitutively in the cytosol in a latent inactive form, wherein it is retained through its interaction with inhibitory IκB (inhibitor of NFκB) proteins, masking its nuclear localization sequence. A variety of stimuli induce phosphorylation of IκB at N-terminal serine residues by the IκB kinase complex, followed by ubiquitination and degradation of IκB by the proteosome (38). Its degradation leads to activation of NFκB complex with subsequent translocation to the nucleus, wherein it can induce transcription of its target genes. However, CLL B cells express constitutively activated NFκB (11), and consistent with the previous report (33), we also found curcumin inhibited phosphorylation of IκBα in CLL B cells (Fig. 2C), suggesting NFκB inhibition.

Curcumin modulates expression of anti- and pro-apoptotic proteins in CLL B cells. Several anti-apoptotic proteins including Mcl-1, XIAP, and Bcl-2 are elevated in CLL B cells and contribute to apoptotic resistance. Because Mcl-1 is the downstream target of STAT3 and NFκB regulates the expression of XIAP, survivin, and Bcl-2 (39, 40), we analyzed the lysates of CLL B cells treated with curcumin for the expression of XIAP (Fig. 3A), Mcl-1, and Bcl-2 (Fig. 3B) by Western blot. We found that curcumin did decrease the expression of XIAP and Mcl-1 in

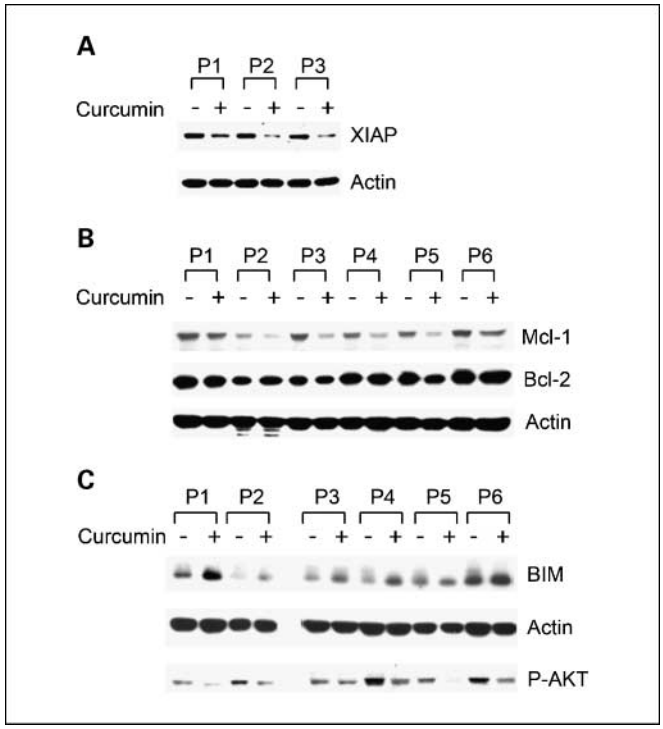

Fig. 3. Curcumin modulates the expression of certain pro- and anti-apoptotic proteins in CLL B cells. Lysates of CLL B cells isolated from various patients as indicated treated with curcumin were analyzed to assess the effect of curcumin on the anti-apoptotic proteins XIAP ($n = 3$), Mcl-1 ($n = 6$), and Bcl-2 ($n = 6$), as well as the pro-apoptotic protein BIM ($n = 6$) using specific antibodies. Actin was used as the loading control. Curcumin treatment of CLL B cells suppressed the expression of XIAP (*A*) and Mcl-1 (*B, top row*), but not Bcl-2 (*B, middle row*). Curcumin treatment of CLL B cells also resulted in up-regulation of the pro-apoptotic protein BIM expression (*C, top row*). AKT is the upstream negative regulator of BIM expression. Inhibition of AKT phosphorylation by curcumin (*C, bottom row*) is also shown.

CLL B cells, whereas Bcl-2 and survivin (data not shown) expression levels remained unaltered. Together, these results suggest that curcumin treatment reduces XIAP and Mcl-1 levels in CLL B cells possibly through the inhibition of the upstream prosurvival signaling pathways STAT3 and NFκB.

One major way by which AKT mediates cell survival is through the phosphorylation and inactivation of Forkhead box class O proteins, a family of transcription factors regulating cell proliferation, survival, and stress responses (41). Recently, it has been found that Forkhead box class O 3a is constitutively phosphorylated on its AKT target site Thr-32 in B-CLL cells (42), suggesting constitutive inactivation of Forkhead box class O 3a. One key Forkhead box class O target gene is BIM, a BH3 domain protein that is capable of inducing apoptosis (43, 44). Given the inhibitory effects of curcumin on AKT phosphorylation, we then examined BIM expression in CLL B cells after treatment with curcumin. Curcumin treatment increased expression of BIM in CLL B cells, a finding which correlated with AKT inhibition (Fig. 3C). However, we did not observe up-regulation of other pro-apoptotic proteins of the Bcl-2 family, including Bid, Bad, or Bax, in CLL B cells following curcumin treatment (data not shown). Together, these data suggest that curcumin inhibits the expression of constitutively elevated anti-apoptotic proteins (XIAP and Mcl-1) and specifically up-regulates the pro-apoptotic protein BIM in CLL B cells.

Curcumin overcomes stromal protection of CLL B cells. Previously, we have shown that stromal cells protect CLL B cells from spontaneous and drug-induced apoptosis through soluble and contact-mediated interactions (14). To begin to dissect the mechanism of stromal cell–mediated protection of CLL B cells, we analyzed prosurvival signaling pathways and anti-apoptotic protein levels in CLL B cells after coculture of CLL B cells with the human stromal cell line HS-5 (separated by transwells). After a 48-hour coculture with HS-5 in transwells, CLL B cells were lysed and analyzed for the activation of STAT3, AKT, and NFκB by Western blot. We found that coculture with stromal cells increased phosphorylation of STAT3 (Ser-727; Fig. 4A), but not NFκB or AKT (data not shown), in CLL B cells. Interestingly, this increase in STAT3 phosphorylation seemed to be due to an increase in the level of total STAT3 protein rather than simply an effect on phosphorylation. Coculture of CLL B cells with stromal cells also increased expression of Mcl-1 (Fig. 4B) and XIAP (Fig. 4C, *top row*), although Bcl-2 levels remained unaltered (Fig. 4C, *middle row*). Similar results were found when CLL B cells were cocultured in direct physical contact with the stromal cells (data not shown). Together, these results suggest that soluble factors secreted in the coculture system of CLL B and HS-5 human stromal cells, as well as direct contact between the latter two cell types, induce up-regulation and activation of STAT3 and increased levels of XIAP and Mcl-1 in CLL B cells, which are likely responsible, at least in part, for stromal cell–mediated protection.

We next examined whether curcumin was able to overcome this stromal protection of CLL B cells. For this, CLL B cells were cultured either alone or with human stromal cells (HS-5 or primary bone marrow stroma; ref. 14) in either direct contact or in a transwell system. After 24 hours of coculture, curcumin was added at various increasing concentrations (0, 10, 15, and 20 μmol/L), and cells were cultured for an additional 24 hours. CLL B cells were then harvested and analyzed for apoptosis by staining with annexin/propidium iodide. Coculture with HS-5 stromal cells provided substantial protection of CLL B cells against apoptosis at lower doses of curcumin (10-15 μmol/L); however, a higher dose (20 μmol/L) of curcumin was able to overcome stromal protection (Fig. 4D). We also observed similar results when primary human bone marrow cells (14) were used as the source of stromal cells for these experiments (Fig. 4E).

Combination treatment with curcumin and the dietary polyphenol EGCG increases death in CLL B cells. We previously showed that the dietary polyphenol EGCG induces apoptosis in CLL B cells *in vitro* (10), and this agent is currently in phase II testing as a treatment for CLL (27). To evaluate the combined effect of these two dietary products with favorable toxicity profiles in initial human (45) testing, we treated primary CLL B cells (n = 10) with either curcumin (2.5-15 μmol/L) or EGCG (25-150 μmol/L) alone or in combination at a constant ratio (1:10). Following 24-hour treatment, cells were harvested and induction of cell death was assessed using annexin/propidium iodide staining. On average, the combination of curcumin and EGCG seemed to increase apoptosis more than treatment with the individual drug alone (Fig. 5A).

To more accurately determine the effects (additive, synergistic, or antagonistic) of combination therapy on the individual sample, these results were evaluated mathematically using the method of Chou and Talalay (36). Although synergy (i.e., combination index, <1.0) was observed in 4 of 10 samples, combination therapy was actually antagonistic (i.e., combination index, >1.0) in the remaining six cases (Fig. 5B). Therefore, based on these results suggesting both the agents had single-agent activity but that they are antagonistic in most patients when administered simultaneously, we evaluated the effect of sequential administration on induction of CLL B-cell death. For these experiments, CLL B cells were cultured with sublethal doses of either curcumin (10 μmol/L), EGCG (100 μmol/L), or both drugs together for 24 hours. Cells were then washed and immediately cultured for another 24 hours in either media alone or with the second agent (EGCG or curcumin) for an additional 24 hours. Cells were harvested and induction of apoptosis was assessed using annexin/propidium iodide staining, as analyzed by flow cytometry. Consistent with the suggestion of antagonism indicated by the method of Chou and Talalay (ref. 36; Fig. 5B), simultaneous culture had a less than additive effect. In contrast, sequential exposure to EGCG and curcumin seemed to have more than an additive effect (Fig. 5C). Remarkably, the sequence of exposure also seemed to impact leukemic cell death, wherein exposure to EGCG followed by curcumin induced more apoptosis than the reverse sequence. This observation was consistent in samples from all five patients studied. Thus, these results suggest that pretreatment with either drug can sensitize the CLL B cells to the second drug (EGCG or curcumin) when administered in a sequential fashion and that sequential administration led to dramatically more leukemic cell death than simultaneous administration.

Sequential treatment with EGCG and curcumin overcomes stromal protection of CLL B cells. Building on these experiments that showed sequential administration was superior to concurrent treatment when cells were cultured in media alone, we next evaluated the ability of sequential treatment to overcome stromal-mediated protection. For these experiments, freshly isolated CLL B cells were treated with the EGCG and

curcumin concurrently or sequentially (as described above) in either media alone or in direct contact with HS-5 human stromal cells. These experiments showed that sequential therapy was superior to concurrent therapy and that the EGCG then curcumin sequence was superior to the reverse sequence (Fig. 5D) in overcoming stromal-mediated survival of CLL B cells.

Discussion

In this study, we show that curcumin is a potent cytotoxic agent for primary CLL B cells that inhibits specific prosurvival pathways known to be relevant to CLL B-cell biology. Importantly, our data show that curcumin differs from most other therapeutic agents currently under study in lymphoid malignancies. First, curcumin-induced apoptosis in CLL B cells is not dependent on caspase activation. Second, curcumin specifically down-regulated Mcl-1 and XIAP without discernible effects on levels of Bcl-2 or survivin, a finding that is relatively uncommon among most therapeutics used to treat lymphoid malignancy (46). Third, the biological effects of curcumin on CLL B cells seems to be explained, at least in part, by inhibition of the prosurvival pathways known to be constitutively active in CLL B cells, including STAT3, AKT, and NFκB. Although coculture of CLL B cells with stromal cells selectively up-regulates STAT3 protein and its phosphorylation status, curcumin was still able to overcome the stromal-induced protection at higher doses or when administered with EGCG in a sequential fashion. Finally, it is known that curcumin is well tolerated and showed minimal toxicity at up to 8 g/d (~115 mg/kg/d) in a phase I clinical trial (47). Collectively, these data indicate that the use of curcumin in the treatment of CLL may be worthy of study.

Curcumin has been shown to inhibit neoplastic initiation, promotion, and progression in several cancers wherein numerous mechanisms have been proposed to account for

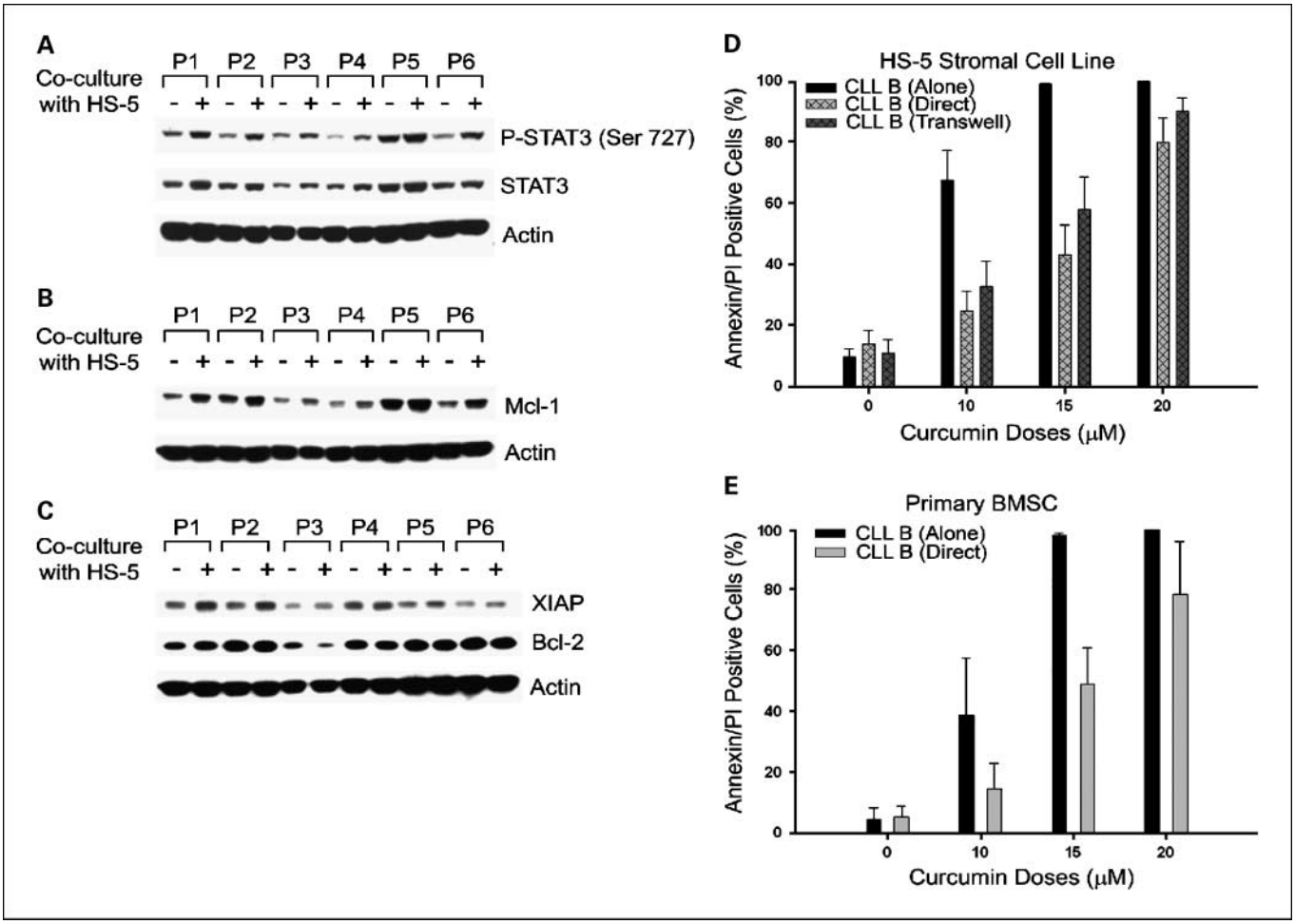

Fig. 4. Coculture of CLL B cells with HS-5 modulates STAT3 activation and apoptosis-regulatory proteins. *A,* primary CLL B cells cocultured with HS-5 human stromal cells in transwells for 48 h were harvested, and the cell lysates ($n = 6$) were analyzed for enhancement of STAT3 activation by Western blot using a specific antibody to phosphorylated STAT3 (Ser-727). Total STAT3 level was also analyzed by stripping the membrane using a specific antibody. Actin was used as the loading control. Up-regulation of total STAT3 and enhancement of STAT3 activation from the basal level was observed when CLL B cells were cocultured with stromal cells. *B* and *C,* CLL B-cell lysates described in (*A*) were also analyzed for the expression of Mcl-1, XIAP, and Bcl-2 by Western blot. Coculture with stromal cells resulted in increased expression of Mcl-1 (*B*) and XIAP (*C, top row*) in CLL B cells. Bcl-2 expression remained unaltered (*C, middle row*). *D, E,* modulation of curcumin induced apoptosis in CLL B cells when cocultured with stromal cells. Primary CLL B cells ($n = 9$) were cultured alone or together with HS-5 human stromal cells (*D*) or primary human bone marrow cells (*E*) in either transwells or direct cell contact for 24 h. Cells were then treated with the indicated doses of curcumin for 24 h. Cells were harvested and stained with CD19-APC and annexin-FITC/propidium iodide by flow cytometric analysis. Viability of $CD19^+$ lymphocytes was assessed and is represented by mean values with SE bars. Higher-dose curcumin (20 μmol/L) seemed to overcome the effects of stromal protection.

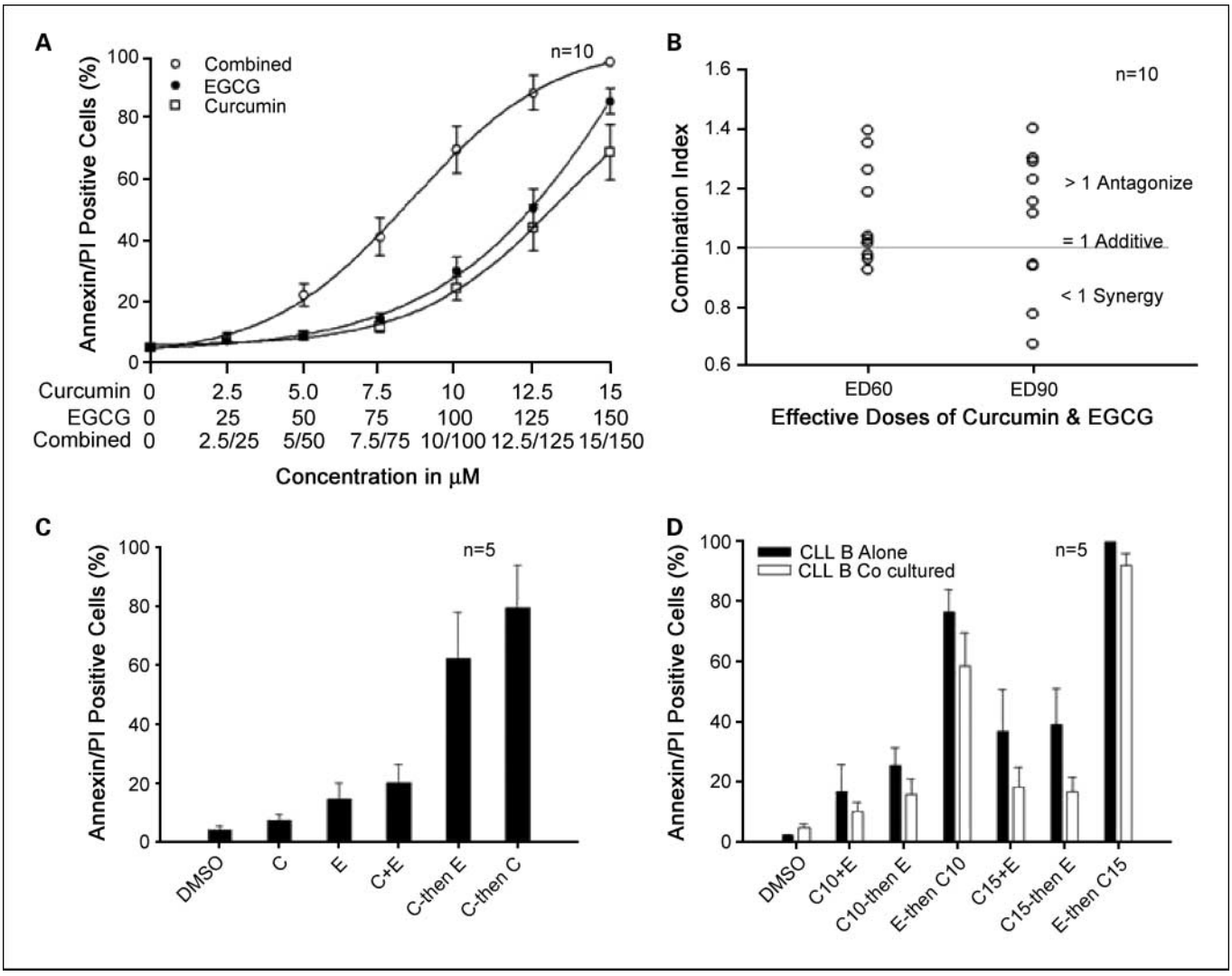

Fig. 5. Effect of combination treatment with curcumin and EGCG on primary CLL B-cell survival. *A,* primary CLL B cells ($n = 10$) were treated with increasing doses of curcumin or EGCG alone or in combination using a constant ratio (1:10). After 24 h of treatment, viability was assessed using annexin/propidium iodide staining. The mean value at each dose level is represented in the figure along with the SE. *B,* combination index values at effective dose 50 (50% cell death) and effective dose 90 (90% cell death) for curcumin and EGCG (constant ratio, 1:10) for CLL B cells from 10 patients were calculated using Calcusyn software. Values ‹1 imply synergy; values equal to 1 imply an additive effect; and values ›1 imply antagonism. The simultaneous administration of curcumin and EGCG led to antagonism in most patients tested. *C,* sequential treatment is superior to concurrent therapy. Based on the results in (*B*), suggesting that, although both agents have single-agent activity, they are antagonistic in most patients when administered simultaneously, we next evaluated the effect of sequential administration. CLL B cells were cultured ($n = 5$) with sublethal doses of curcumin (*C*, 10 μmol/L), EGCG (*E*, 100 μmol/L), or both drugs together (C + E) for 24 h. Cells were then washed and cultured for another 24 h in either media alone or with the second agent (*C* then *E*; *E* then *C*) for an additional 24 h using the same doses. Cells were harvested and apoptosis was assessed using annexin/propidium iodide staining, as analyzed by flow cytometry. The results show sequential administration (*C* then *E*; *E* then *C*) was dramatically superior to simultaneous administration (C + E) and that the *E* then *C* sequence seemed superior to the reverse. *D,* sequential treatment of CLL B cells with curcumin/EGCG overcomes stromal protection. CLL B cells ($n = 5$) were treated with curcumin (*C*) at 10 and 15 μmol/L, EGCG (*E*) at 100 μmol/L, or together (C10 + E and C15 + E), either cocultured in direct contact with HS-5 stromal cells or cultured alone for 24 h. Cells were then washed and cultured for another 24 h in either media alone or with the second agent (C10 then E; C15 then E; E then C10 and E then C15) for an additional 24 h. Cells were harvested and induction of apoptosis was assessed using annexin/propidium iodide staining, as analyzed by flow cytometry. The results show sequential administration (E then C) was dramatically superior to simultaneous administration (C + E) or the reverse sequence and that this approach can overcome stromal protection. Results are presented as mean values with SE bars.

the ability of curcumin to induce apoptosis in malignant cells. We have examined the potential relevance of those mechanisms that are more specific to CLL leukemic B biology: inhibition of STAT3, AKT, and NFκB signaling pathways (13, 48). We confirm the findings of Everett and colleagues (33) that curcumin reduces the constitutive phosphorylation level of IκBα, suggesting an inhibition of NFκB activity in CLL B cells. Our analysis shows curcumin induces a significant decrease in XIAP expression, an anti-apoptotic protein elevated in CLL and also a downstream target of the NFκB pathway. Curcumin treatment inhibited STAT3 activity and decreased the expression of Mcl-1, a downstream target of activated STAT3, in most CLL B cells. Curcumin treatment of CLL B cells also reduced the phosphorylation level of AKT. The serine/threonine kinase AKT has been considered an attractive target for cancer therapy and prevention (49). AKT kinase plays critical roles in mammalian cell survival and is constitutively active in various cancers, including CLL (48). Because curcumin inhibited AKT, we evaluated its effects on the pro-apoptotic protein BIM, a downstream target of AKT. Indeed, we found that curcumin

treatment of primary CLL B cells resulted in increased expression of BIM, which correlated well with inhibition of AKT (Fig. 3C). The precise mechanism by which curcumin inhibited these constitutively active prosurvival pathways in CLL B cells remains unclear.

Although numerous compounds have been shown to induce apoptosis when CLL B cells are cultured in isolation, in their tissue microenvironment, these cells experience a variety of nurturing signals through interactions with stromal cells. Modulators of CLL B-cell survival are complex and include both soluble factors and signals derived from direct cell-cell contact (20). Extensive work has indicated that stromal cells play an important role in progression of cancers and protect the malignant cells from apoptosis induced by various chemotherapeutic agents (20). For maximal efficacy, therapeutic strategies able to overcome stromal mediated protection are needed. To design and develop such strategies, it is necessary to understand precisely what signaling pathways are modulated through leukemic cell stromal cell interactions. Our work here further shows that soluble factors produced on coculture of marrow stroma and primary CLL B cells increase expression and enhance the phosphorylation status of STAT3. Moreover, soluble factors induced enhanced expression of the anti-apoptotic proteins Mcl-1 and XIAP, which could explain the increased resistance of CLL B cells to apoptosis in presence of stromal cells.

We did additional experiments to evaluate the effect of curcumin on CLL B cells when leukemic cells were allowed to interact with human stromal cells. Similar to other agents (20), we found that coculture with bone marrow stromal cells protects CLL B cells from curcumin-induced apoptosis. Although higher dose (20 μmol/L) curcumin was able to overcome the protection of CLL B cells by marrow stroma, it is not known if this level can be achieved *in vivo*.

We believe it is unlikely that a single agent, no matter how active, will be able to cure this disease. Therefore, to evaluate the effect of curcumin in combination with other agents, we explored the combination of curcumin with the green tea extract EGCG (10). This combination was selected because both are dietary products with favorable toxicity profiles in phase I trials (14, 27, 47), which may be an attractive combination for clinical testing in patients with early-stage disease. Although, on average, we found that the combination of curcumin and EGCG increased the apoptotic cell death of CLL B cells compared with either drug alone, mathematical modeling of the combined effects at the level of the individual patient showed antagonism in most patients (6 of 10 samples). This observation prompted us to examine the effect of sequential administration of curcumin and EGCG. Sequential exposure to these agents was superior to simultaneous treatment with a 3- to 4-fold increase in cell death compared with simultaneous administration. The sequence of administration also seemed to impact efficacy, wherein EGCG followed by curcumin induced more cell death than the reverse sequence. Sequential administration of these two agents was also able to overcome stromal mediated protection of CLL B cells at lower curcumin doses. These preclinical results strongly encourage us to use a sequential approach in designing subsequent clinical trials with these agents. In addition, these studies indicate how *in vitro* experiments can inform the design of clinical trials beyond simply a summary curve such as that in Fig. 5A. Subsequent assessment of association with clinical outcome will be of great interest.

In conclusion, the present study indicates that curcumin is cytotoxic to primary CLL B cells. This cytotoxic effect of curcumin is complex and is associated with the inhibition of prosurvival pathways and down-regulation of anti-apoptotic proteins Mcl-1 and XIAP, which are characteristics associated with leukemic cell resistance to chemotherapeutic agents (1, 3, 4, 6) and increased expression of the pro-apoptotic protein BIM. Importantly, curcumin treatment was able to overcome protection of CLL B cells by marrow stroma on *in vitro* testing and to synergize with EGCG when the agents were administered in a sequential fashion. Additional evaluation of curcumin as a potential therapeutic agent for the treatment of CLL seems warranted.

Disclosure of Potential Conflicts of Interest

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