

# Preclinical Assessment of Curcumin as a Potential Therapy for B-CLL

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Curcumin, the principle component of the spice turmeric, has been used as an anti-inflammatory medication in India and China for centuries. Recent studies, predominantly using actively dividing cell lines, have suggested that this compound could be used as a chemopreventative or therapeutic agent for epithelial tumors. As curcumin has been reported to inhibit the NIK/IKK complex, an activity that would be expected to induce apoptosis in B cell malignancies, we sought to determine whether curcumin induces apoptosis *in vitro* in primary chronic lymphocytic leukemia (B-CLL) cells. Primary leukemic cells were incubated with varying dosages of curcumin, followed by assessment for apoptosis. The role of PPAR $\gamma$  or NF- $\kappa$ B signaling in curcumin-induced apoptosis was examined by cotreatment with a PPAR $\gamma$  antagonist or EMSA of nuclear NF- $\kappa$ B complexes. We also examined whether a clinically achievable concentration of curcumin (1  $\mu$ M) would augment the apoptotic effects of fludarabine, dexamethasone, vincristine or the PDE4 inhibitor rolipram. In B-CLL cells from 14 patients, curcumin-induced apoptosis with a mean EC<sub>50</sub> of 5.5  $\mu$ M. In contrast, the EC<sub>50</sub> for whole mononuclear cells from a healthy donor was 21.8  $\mu$ M. In a 48 hr wash-out time course, curcumin-induced apoptosis was time-dependent, with a substantial reduction in apoptosis observed when curcumin was removed after 5 hr. Curcumin treatment reduced basal nuclear NF- $\kappa$ B levels and 1  $\mu$ M curcumin augmented both vinca alkaloid and PDE4 inhibitor-induced apoptosis in B-CLL cells. Our studies suggest that curcumin may augment the efficacy of established or experimental therapies for B-CLL. *Am. J. Hematol.* 82:000–000, 2006. © 2006 Wiley-Liss, Inc.

**Key words:** curcumin; CLL; vincristine; PDE4 inhibitor

## INTRODUCTION

As an active ingredient of turmeric, a spice that has been consumed for centuries, curcumin (diferuloylmethane) is an attractive therapeutic agent that combines clinical tolerability with intriguing pharmacologic properties. In a phase I trial in which curcumin was examined as a potential chemopreventive agent for patients with premalignant skin, cervical, oral, bladder or esophageal lesions, as much as 8 gm of oral curcumin per day was tolerated for 3 months. Peak serum concentrations of 1.77  $\mu$ M at 1–2 hr were reported at this dose [1].

Curcumin induces apoptosis in a variety of cell lines derived from neoplastic cells and the mechanism by which this occurs has been an active area of research. In studies of the myeloid cell line ML-1a, Singh and Aggarwal reported that curcumin inhibited I $\kappa$ B $\alpha$  phosphorylation, degradation, and NF- $\kappa$ B translocation induced by a diverse range of stimuli

including TNF, hydrogen peroxide, and phorbol esters [2]. Subsequent studies confirmed curcumin-induced inhibition of NF- $\kappa$ B translocation in multiple cell types, including colon [3], gastric [4], pancreatic [5], and squamous epithelial tumor cell lines [6] as well as B-cell lymphoma and multiple myeloma cell lines [7,8]. Although the precise mechanism by which NF- $\kappa$ B translocation is inhibited remains unclear, Plummer et al. demonstrated that curcumin

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reduced NF- $\kappa$ B translocation in HEK293 cells transfected with NIK, IKK $\alpha$ , or IKK $\beta$  kinases [3]. These authors suggested that curcumin-induced effects on NF- $\kappa$ B translocation could be due to a direct effect on the activity of one of these kinases. An alternate target for curcumin, proposed by Chen and Xu, is the nuclear coreceptor PPAR $\gamma$ . Curcumin treatment of hepatic stellate cells transactivates PPAR $\gamma$  reporter constructs and such transactivation can be blocked by the PPAR $\gamma$  inhibitor PD68235 [9,10].

Our laboratory has an interest in identifying novel therapies for chronic lymphocytic leukemia (B-CLL), a common malignancy of CD5, CD19, CD23-positive B cells for which there is currently no cure. Given that B-CLL cells, like other B lineage cells, are sensitive to PPAR $\gamma$  agonist-induced apoptosis and express constitutive nuclear NF- $\kappa$ B, we wished to determine whether curcumin would induce apoptosis in primary leukemic B-CLL cells in vitro [11]. Our prior studies examining PDE4 inhibitors, a class of drugs that induce apoptosis in B-CLL by a PKA-mediated mechanism, have documented important differences between such primary leukemic cells and lymphoid cell lines, suggesting that a re-examination of curcumin-induced apoptosis in primary malignant lymphoid cells is of some importance [12]. We find that curcumin induces apoptosis in B-CLL cells with fourfold lower mean EC<sub>50</sub> (5.5  $\mu$ M) than that determined for normal mononuclear cells and that curcumin augments vincristine and PDE4 inhibitor-induced apoptosis at concentrations (1  $\mu$ M) that have been achieved safely in patients.

## MATERIALS AND METHODS

### Reagents

The following reagents were obtained from commercial sources: Rolipram and curcumin (Sigma-Aldrich), TOO70907 (Cayman Chemical), vincristine (Sicor), fludarabine (Berlex) and dexamethasone (American Pharmaceutical Partners).

### Leukemic Cell Isolation and Culture

After Institutional Review Board (IRB)-approved informed consent, peripheral blood was drawn in heparinized tubes from patients with flow cytometry-verified B-CLL or T-CLL, or from healthy donors, as indicated in the text. Patients with active infections or other serious medical conditions were not included in this study. Mononuclear cells were obtained by centrifugation over Histopaque 1077 (Sigma). As leukemic cells made up over 90% of such cell preparations, the cells were utilized for further studies without further purification. Cells were cultured in RPMI 1640 media (Biowhittaker, Walkersville, MD)

supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine, 100 U/mL penicillin and 100 U/mL streptomycin (Sigma) at 37°C and 5% CO<sub>2</sub> in air.

### IgV<sub>H</sub> Mutation Analysis

Analysis of IgG heavy chain variable region mutation rates was carried out as described by Crespo et al. [13]. Total RNA was isolated from B-CLL cells using Ultraspec (Biotech Laboratories). cDNA was synthesized from 5  $\mu$ g of RNA with Maloney-murine leukemia virus reverse transcriptase (Invitrogen, Life Technologies). cDNA was amplified with a set of six 5' heavy-chain variable region family-specific primers originally described by Campbell et al. along with 3' primers complementary to the IgM or IgG constant region described by Fais et al. [14,15]. After concentration and purification by Centricon ultrafiltration (Millipore, Billerica, MA) or extraction from agarose gel (QIAEX II, Qiagen, Valencia, CA), PCR products were sequenced directly using the same 3' primers.

### Apoptosis Assays

The experiments shown in Table I and Figs. 3 and 5 were carried out by Hoechst 33342 analysis. One million cells were incubated in triplicate in 48 well plates with or without drug treatment for 48 hr in 1 mL of culture media. Cells were transferred to polypropylene Falcon FACS tubes, incubated for 15 min at 37°C with Hoechst 33342 at a final concentration of 0.25  $\mu$ g/mL [16,17]. Cells were then stored on ice until analysis on a MoFlo cytometer using a 450 nm bandpass filter. In the experiments shown in Figs. 1 and 2, apoptosis was detected as membrane depolarization with dihexyloxacarbocyanine (DiOC<sub>6</sub>(3)) from Molecular Probes (Eugene, OR) as previously described [18]. Cells were cultured with or without drugs as described earlier, transferred to polystyrene Falcon FACS tubes and incubated for 30 min with DiOC<sub>6</sub>(3) at a final concentration of 20 nM. FACS analysis was performed with a FACScan (Becton Dickinson, Franklin Lakes, NJ). In triplicate samples measured by both techniques, the mean apoptosis observed by Hoechst 33342 or DiOC<sub>6</sub>(3) analysis did not differ by more than 10%. Data were analyzed using FlowJo software (Tree Star, Ashland, OR) by gating for the apoptotic population.

### Electrophoretic Mobility Shift Analysis

Nuclear extracts from B-CLL cells were isolated and analyzed using the technique of Mizuno and Rothstein [19]. Nuclei were isolated by hypotonic lysis in a high salt buffer containing 430 mM NaCl and protease inhibitors. Radiolabeled NF- $\kappa$ B or NF-Y

**TABLE 1. Assessment of In Vitro Curcumin Sensitivity in Leukemic Cells From 14 B-CLL Patients**

Patient no.	VH status	Rai Stage	WBC/AbsL ( $\times 10^9/L$ )	Therapy	Curc EC <sub>50</sub> ( $\mu M$ )
1	Mut (V4–34)	I	53/48	None	1
2	Unmut (V4–34)	IV	136/129	CVP, P, R	7
3	Unmut (V1–69)	I	11/7	R	3
4	Unmut (V5–51)	I	36/33	None	10
5	ND	0–I	36/31	Ch	5
6	Unmut (V3–74)	III	76/54	F	6
7	ND	0	16/9	None	10
8	Unmut (V6–01)	0	63/56	None	1
9	ND	IV	83/59	F	5
10	Unmut (V3–33)	I	133/117	None	10
11	Mut (V3–48)	IV	36/34	Ch, Ig	3
12	Unmut (V3–7)	IV	425/410	None	5
13	Mut (V3–53)	0	14/10	None	4
14	ND	III	54/44	None	7

The EC<sub>50</sub> for curcumin-induced apoptosis was determined by a FACS-based assay following culture for 48 hr with from 0.3 to 30  $\mu M$  curcumin. Prior therapy is indicated as follows: chlorambucil (Ch), cyclophosphamide (C), fludarabine (F), intravenous immunoglobulin (Ig), prednisone (P), rituximab (R), vincristine (V). The total (WBC) and absolute lymphocyte count (AbsL) at the time that the leukemic specimens were obtained is shown. Rai stages were also determined as of the time that the specimens were obtained. The presence (Mut) or absence (Unmut) of somatic hypermutation in the leukemic clone's heavy chain variable region was determined by PCR cloning and sequencing using a 98% cutoff. Where determined, the heavy chain variable region identified is shown. ND: not done.

oligonucleotides (Santa Cruz Biotechnology) were incubated for 30 min with 1  $\mu g$  of nuclear protein and 0.5  $\mu g$  of poly (dI–dC). For supershift analysis, antisera raised against the N terminus of human p65 or against human c-Rel were preincubated with nuclear extracts for 1 hr on ice. These antisera were generous gifts from Dr. Nancy Rice (NCI).

### Data Analysis

Data from the apoptosis assay were fitted to a standard four-parameter logistic model (Hill Equation) with curve-fitting software (Prism 4, Graphpad Software, San Diego, CA). As data were expressed as a percentage of apoptotic cells, the maximal effect was fixed at 100%. Nonlinear regression analysis was used to derive the 50% effective curcumin concentration (EC<sub>50</sub>) and the steepness of the dose-response curve (Hill slope). The goodness of fit for each patient was assessed by the  $R^2$  value and the standard error of the log EC<sub>50</sub> value. For the data derived from experiments in which curcumin was combined with other pharmacologic agents (see Fig. 5), paired  $t$  tests were performed comparing combined therapy with either drug alone.

## RESULTS

In dose titration studies using drug concentrations ranging from 0.3 to 30  $\mu M$ , incubation with curcumin for 48 hr induced apoptosis in a dose-related fashion in primary B-CLL cells. When curcumin dose was plotted logarithmically, the apoptosis data from such studies fit a sigmoidal curve, suggesting that apoptosis

was the result of binding of curcumin to a saturable receptor. Of note, however, when a sixteen point dose titration study was performed, a modest increase in B-CLL apoptosis was noted at concentrations below the EC<sub>50</sub>, suggesting that curcumin might target more than one receptor in B-CLL cells (Fig. 1A).

A time course with 10  $\mu M$  curcumin demonstrated that induction of apoptosis in B-CLL cells is optimal after 28–48 hr of exposure (Fig. 1B). As therapeutic use of curcumin is likely to achieve intermittent peaks rather than steady-state levels in serum, we exposed B-CLL cells to curcumin for varying periods of time, followed by washing and completion of 48 total hours of culture. Control samples at each time point were also washed so as to adjust for any loss of cells undergoing basal apoptosis. The results from these studies demonstrated that substantial induction of apoptosis in cultured primary leukemic cells required prolonged exposure to curcumin, as washing cells after 5 hr of exposure followed by 43 further hours of culture resulted in substantially lower levels of apoptosis (Fig. 1C).

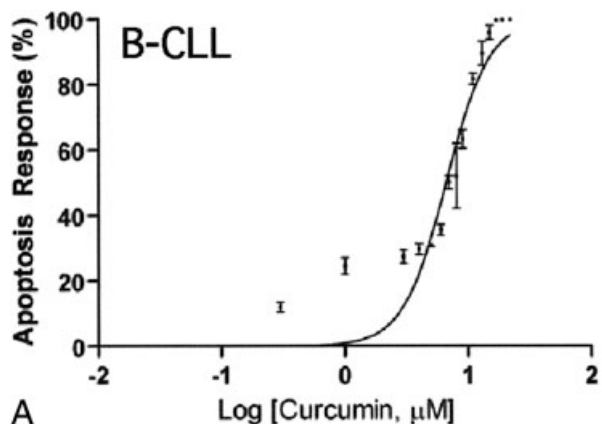
### Contrasting B-CLL and Normal Mononuclear Cell Curcumin Sensitivity

Leukemic cells from a total of 14 B-CLL patients were then assessed for sensitivity to curcumin following 48 hr exposure to a graded series of drug concentrations. The EC<sub>50</sub> for curcumin-induced apoptosis for the group as a whole was 5.5  $\mu M$  (range 1–10  $\mu M$ ). Although the observed EC<sub>50</sub>s appeared to be normally distributed, a sigmoidal dose response to curcumin was consistently observed. The sensitivity of

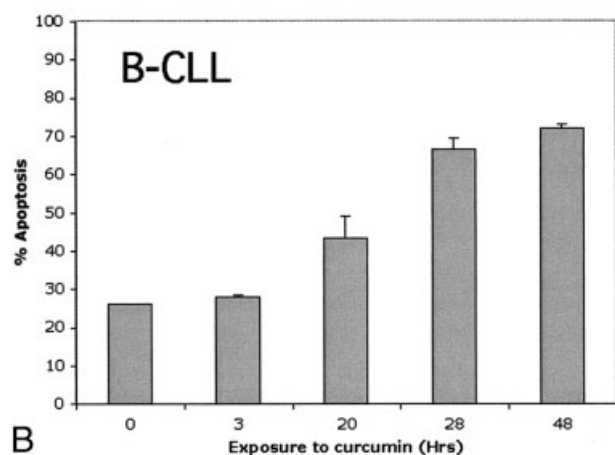
B-CLL cells to curcumin did not correlate with the Rai stage, absolute lymphocyte count, or prior therapy of the patients from whom they were obtained (Table I). As the absence of somatic hypermutation is predictive of more rapid progression in B-CLL, we sequenced the clonal heavy chain variable regions of 10 of the leukemic cell samples assessed in this study [20]. Using a cutoff of less than 98% sequence identity as discriminator for “mutated” VH

regions, we found no clear association of variable region hypermutation with in vitro sensitivity to curcumin within this small sample group.

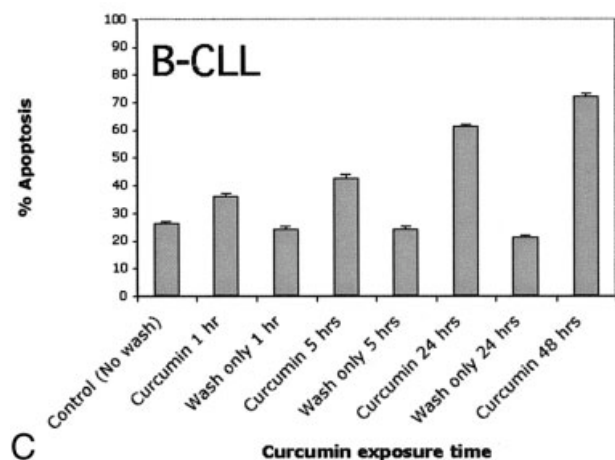
If curcumin is to be a useful therapeutic agent in B-CLL, it would be preferable that drug levels achieved during therapy did not induce apoptosis in patients’ nonmalignant mononuclear cells. To determine if this is the case, normal circulating human mononuclear cells were isolated, followed by incubation for 48 hr with varying concentrations of curcumin. Normal mononuclear cells were distinctly less sensitive to curcumin than B-CLL cells, with an  $EC_{50}$  of  $\sim 21.8 \mu\text{M}$ , a value more than twice that observed in any of the B-CLL patients studied (Fig. 2A). A similar  $EC_{50}$  ( $20 \mu\text{M}$ ) was observed in normal human T cells following purification by a negative selection technique (Fig. 2B). In contrast, primary leukemic cells from a patient with CD4+ T-CLL demonstrated modest increments in apoptosis at curcumin concentrations as low as  $1 \mu\text{M}$  (Fig. 2C).



A



B

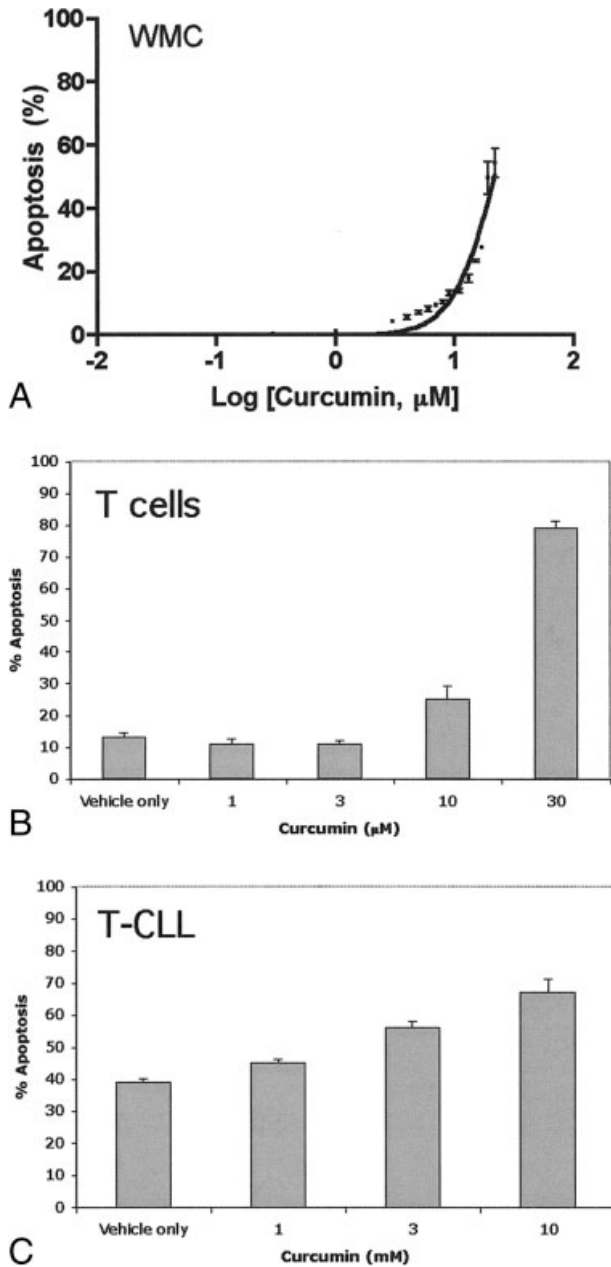


C

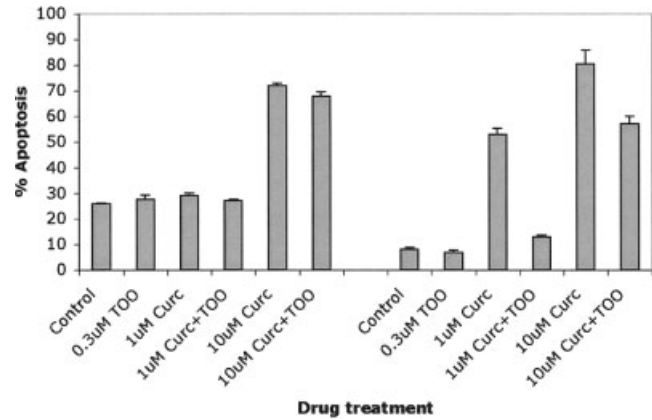
#### PPAR $\gamma$ Receptor and NF- $\kappa\text{B}$ Studies

PPAR $\gamma$  receptor is expressed in normal and malignant human B cells and PPAR $\gamma$  agonists have been reported to induce apoptosis in such B lineage cells [21,22]. We confirmed such studies using the thiazolidinedione PPAR $\gamma$  agonist ciglitazone (data not shown). As curcumin-induced apoptosis has been linked to its activity as a PPAR $\gamma$  receptor agonist, we examined whether TOO70907, a nonthiazolidinedione PPAR $\gamma$  receptor antagonist, could inhibit curcumin-induced apoptosis in B-CLL cells [23]. Prior studies have reported an apparent TOO70907 binding affinity of 1 nM and demon-

**Fig. 1. Curcumin induces apoptosis in B-CLL cells in a dose and time-dependent manner. Panel 1A:** B-CLL cells were treated with increasing curcumin doses as indicated for 48 hr followed by FACS-based assessment for apoptosis with DiOC $_6$ (3) staining. Data were normalized to account for basal apoptosis. The means and standard errors of each curcumin dose are indicated. The data were fitted to a four-point logistical equation (Hill equation) as indicated by the solid line.  $EC_{50}$  was  $6.6 \mu\text{M}$  (95% confidence intervals 6.1 to  $7.2 \mu\text{M}$ ).  $R^2$  of curve fit = 0.91,  $N = 3$ . **Panel 1B:** B-CLL cells were cultured for a total of 48 hr, with addition of curcumin for the last 3, 20, 28, or 48 hr, as indicated. Apoptosis was assessed by DiOC $_6$ (3) FACS analysis. **Panel 1C:** B-CLL cells were cultured for a total of 48 hr. Curcumin was added at the beginning of the culture for the indicated period, followed by washing. As a control for the effects of washing, parallel cultures of untreated cells were also washed at the same time point, as indicated. Apoptosis was assessed by DiOC $_6$ (3) FACS analysis. The SEM of triplicate samples is shown.



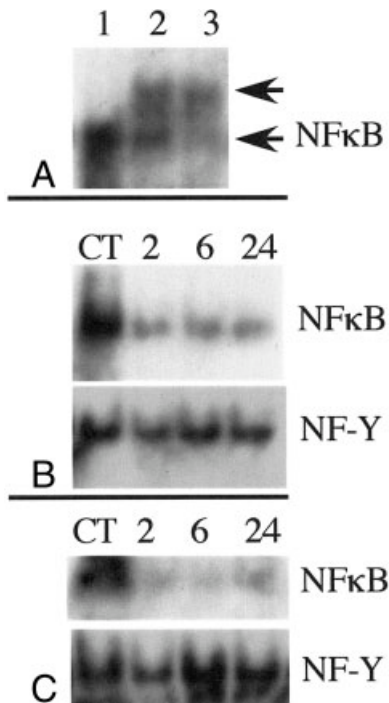
**Fig. 2.** T-CLL cells, but not normal peripheral blood T cells, are sensitive to apoptosis induced by low concentrations of curcumin. Panel 2A: Whole mononuclear cells (WMC) obtained from a healthy donor were treated with increasing curcumin doses as indicated for 48 hr followed by FACS-based assessment for apoptosis with DiOC<sub>6</sub>(3) staining. Data were normalized to account for basal apoptosis. The means and standard errors of each curcumin dose are indicated. The data were fitted to a four-point logistical equation (Hill equation) as indicated by the solid line. EC<sub>50</sub> was 21.8  $\mu\text{M}$  (95% confidence interval 20.4 to 23.3  $\mu\text{M}$ ). R<sup>2</sup> of curve fit = 0.91, N = 3. Panel 2B: Peripheral blood T cells, isolated by magnetic bead negative selection from peripheral blood of a healthy donor, were incubated for 48 hr with varying concentrations of curcumin, followed by assessment for apoptosis. Panel 2C: Leukemic cells from a patient with a CD4+ chronic T cell leukemia (T-CLL) were analyzed as in Panel B.



**Fig. 3.** The PPAR $\gamma$  antagonist TOO reduces curcumin-induced apoptosis in leukemic cells from only a subset of B-CLL patients. B-CLL cells were cultured for 48 hr with vehicle alone or 1 or 10  $\mu\text{M}$  curcumin, with or without the addition of the PPAR $\gamma$  antagonist TOO at 0.3  $\mu\text{M}$ , followed by assessment for apoptosis. Results from representative leukemic samples insensitive (left) or sensitive (right) to inhibition of curcumin-induced apoptosis are shown.

stated that this compound blocks both cell based reporter gene expression and adipocyte differentiation [23]. TOO70907 itself induced apoptosis in B-CLL cells at concentrations of 1  $\mu\text{M}$  or higher (data not shown). However, at 0.3  $\mu\text{M}$ , TOO70907 had no significant pro or antiapoptotic activity itself. In two of five patients examined, addition of 0.3  $\mu\text{M}$  TOO70907 partially protected B-CLL cells from apoptosis induced by treatment for 48 hr with either 1 or 10  $\mu\text{M}$  curcumin (Fig. 3, patient #10 and data not shown). In contrast, in three other patients, addition of TOO70907 had no significant protective effect (Fig. 3, patient #10 and data not shown). These studies suggest that curcumin is likely to have significant PPAR $\gamma$  receptor-independent pro-apoptotic activity.

Curcumin has also been reported to inhibit IKK, resulting in a reduction in NF- $\kappa\text{B}$  signaling. As CLL cells constitutively express nuclear NF- $\kappa\text{B}$  and basal signaling by this transcription factor appears to play an important antiapoptotic role in these leukemic cells, we next sought to determine whether curcumin treatment of B-CLL cells altered levels of basal nuclear NF- $\kappa\text{B}$ . As previously reported, incubation of nuclear extracts of B-CLL cells with a <sup>32</sup>P-labeled oligonucleotide containing a consensus NF- $\kappa\text{B}$ -binding sequence revealed constitutive nuclear  $\kappa\text{B}$  binding activity [11]. The nature of this basal  $\kappa\text{B}$ -binding activity was further explored through supershift analysis. The B-CLL sample nuclear  $\kappa\text{B}$ -binding complex was “supershifted” by incubation with antibodies to either p65 or cRel, suggesting the presence of constitutive nuclear p50/p65 and p50/



**Fig. 4.** Curcumin treatment of B-CLL cells markedly reduces basal levels of nuclear NF- $\kappa$ B while having no effect on basal levels of nuclear NF-Y. Panel 4A: Electrophoretic mobility shift analysis (EMSA) of nuclear extracts of B-CLL cells incubated with a labeled oligonucleotide containing the consensus binding site for NF- $\kappa$ B. In lane 1, a constitutive shifted complex in resting B-CLL cells is shown. This broad complex was "supershifted" by incubation of the nuclear extract/oligonucleotide mix with antisera to either p65 (lane 2) or cRel (lane 3). Panels 4B and 4C: B-CLL cells from two patients were incubated for 24 hr with media alone or with 10  $\mu$ M curcumin. Curcumin was added for the final 2, 6 or the entire 24 hr of tissue culture, as indicated. Nuclear extracts were isolated and incubated with labeled oligonucleotides containing either the consensus binding sites for NF- $\kappa$ B or NF-Y, as indicated, followed by EMSA. The sample examined in panel 4A for supershifting is derived from the same leukemic sample as the study shown in panel 4B.

cRel heterodimers (Fig. 4A). In four of five B-CLL patients tested, treatment with curcumin for 2, 6, or 24 hr resulted in a marked reduction in nuclear NF- $\kappa$ B (Fig. 4B,C and data not shown). To examine the possibility that curcumin induced nonspecific nuclear changes that affect the activity of all transcription factors, the same nuclear extracts were also incubated with an oligonucleotide containing the consensus binding sequence for NF-Y, another transcription factor known to be present in the nuclei of B lineage cells. Results from these studies revealed NF-Y binding that was unchanged by curcumin treatment (Fig. 4B,C) and support the hypothesis that curcumin-induced apoptosis in B-CLL is mediated through a reduction in basal NF- $\kappa$ B signaling.

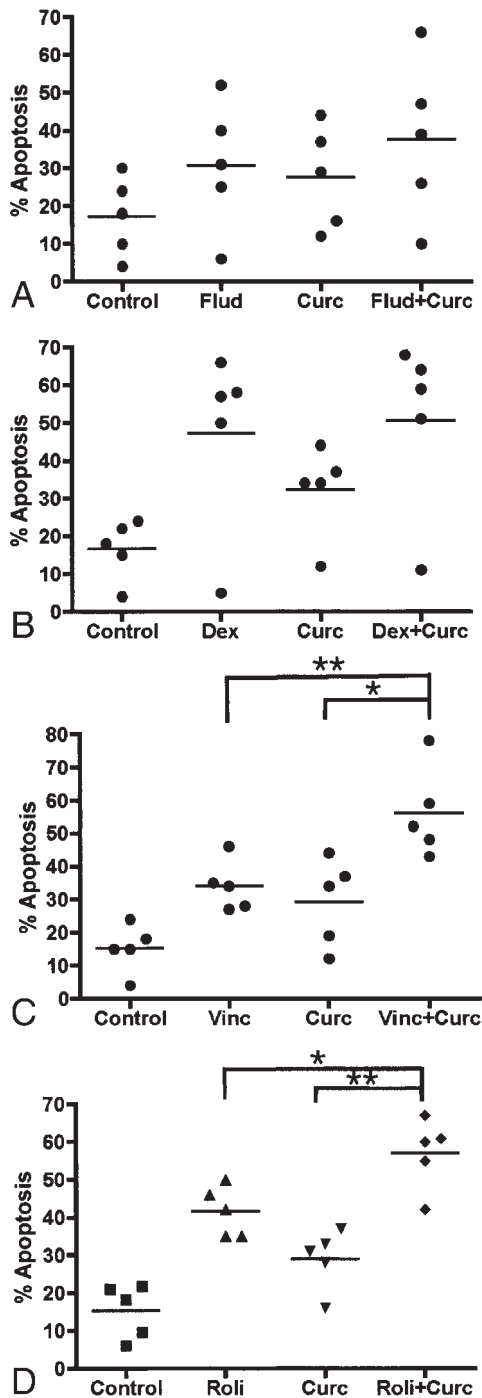
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### Curcumin Augments Vinca Alkaloid and PDE4 Inhibitor-induced Apoptosis

As a prior phase 1 study has demonstrated that a serum level of 1  $\mu$ M curcumin is well tolerated in patients with a variety of premalignant conditions, we were interested in determining whether at this concentration curcumin synergized with other pharmaceutical agents known to induce apoptosis in B-CLL [1]. For each of the four agents studied, fludarabine, dexamethasone, vincristine and rolipram, a dose titration was performed to identify a concentration of drug that resulted in augmented but submaximal apoptosis when used alone (data not shown). Curcumin (1  $\mu$ M) had no effect (three patients) or a subadditive effect (two patients) when tested in leukemic cells from five patients combined with 1–10  $\mu$ M fludarabine (Fig. 5A). Similarly, curcumin either had no effect (three patients) or a subadditive effect (two patients) on apoptosis induced by 100 nM dexamethasone (Fig. 5B). Similar results were obtained at 10 nM and 1  $\mu$ M dexamethasone (data not shown). In contrast, curcumin had a supra-additive effect in four of five B-CLL patients tested when added to 10 nM vincristine ( $P < 0.05$  by paired *t* test, Fig. 5C). As prior studies have demonstrated sensitivity of B-CLL to PDE4 inhibitors, a class of compounds that increase B-CLL cAMP levels and activate PKA, we also examined the effects of combining curcumin with the prototypic PDE4 inhibitor rolipram. In five of five B-CLL patients tested, curcumin augmented apoptosis induced by 10  $\mu$ M rolipram in either an additive (one patient) or supra-additive (four patients) fashion ( $P < .05$  by paired *t* test, Fig. 5D). These studies suggest that clinically well-tolerated levels of curcumin might augment the efficacy of treatment of B-CLL with either vinca alkaloids or PDE4 inhibitors.

### DISCUSSION

In this study, we have examined the potential therapeutic effects of curcumin on primary B-CLL cells. Unlike constitutively dividing lymphoid cell lines, primary B-CLL cells do not proliferate under standard tissue culture conditions. We find that curcumin induces apoptosis in B-CLL cells with a mean  $EC_{50}$  of 5.5  $\mu$ M (95% confidence interval of 3.3–9.7  $\mu$ M), fourfold lower than the  $EC_{50}$  observed in normal mononuclear cells (21.8  $\mu$ M). The sigmoidal dose response relationship we observed in apoptosis experiments carried out with increasing curcumin concentrations is consistent with the hypothesis that curcumin-induced apoptosis in B-CLL is the result of binding of this drug to a specific, saturable receptor.



**Fig. 5.** Effects of co-treatment with low dose curcumin on fludarabine, dexamethasone, vincristine or rolipram-induced apoptosis in B-CLL. B-CLL cells were incubated for 48 hr with either vehicle alone or 1  $\mu$ M curcumin combined with either fludarabine (1  $\mu$ M for four patients and 10  $\mu$ M for a fifth; panel 5A), dexamethasone (100 nM; panel 5B), vincristine (10 nM; panel 5C) or rolipram (10  $\mu$ M; panel 5D), followed by FACS analysis for apoptosis. All data points are the mean of triplicate samples. The SEM of triplicate samples was consistently less than 5%. Sample pairs indicated by one asterisk indicate a paired t test  $P < 0.05$ , while sample pairs indicated by two asterisks indicate a paired t test  $P < 0.01$ .

When curcumin was used as a single agent, washout studies demonstrate that 24–48 hr of in vitro exposure is required for maximal induction of apoptosis.

These results have implications for the design of trials examining the potential efficacy of curcumin in the systemic treatment of non-GI tract malignancies. As a result of rapid inactivation by hepatic and intestinal glucuronidation, large oral doses of curcumin are required to achieve even transient serum levels of 1  $\mu$ M. Thus, if these in vitro data prove to be predictive of dose response in vivo, effective use of curcumin as a single agent in B-CLL would appear to require a constant intravenous infusion in order to achieve a therapeutic dose of drug. Co-ingestion of piperine, the active ingredient of black pepper and a known inhibitor of glucuronidation, augments the bioavailability of curcumin by 2,000% in human volunteers [24]. An oral regimen combining these two compounds may allow clinical trials to test the efficacy of curcumin in human cancer, either alone or in combination with other chemotherapeutic agents. The therapeutic utility of such an approach would appear to be more plausible if trials are designed to evaluate the ability of curcumin to augment the efficacy of established chemotherapeutic agents such as vincristine. We find that curcumin augments the levels of apoptosis induced in B-CLL cells by either vincristine or the PDE4 inhibitor rolipram at a concentration (1  $\mu$ M) significantly lower than curcumin's  $EC_{50}$  as a single agent.

Numerous mechanisms have been proposed to account for the ability of curcumin to induce apoptosis in malignant cell lines. We have examined the potential relevance of two such proposed mechanisms that are particularly relevant to B-lineage cells: inhibition of NF- $\kappa$ B signaling and activation of PPAR $\gamma$  signaling. While we do not find consistent evidence that curcumin-induced apoptosis results from PPAR $\gamma$  signaling, we did observe that levels of B-CLL cell nuclear NF- $\kappa$ B were reduced by curcumin treatment in the majority of B-CLL samples tested. B-lineage cells, including B-CLL cells, are unusual in that constitutive, low levels of nuclear NF- $\kappa$ B binding activity are present in the absence of exogenous stimuli [11]. Although the explanation for constitutive NF- $\kappa$ B activation in B cells is not clear, it may result from basal signaling from surface immunoglobulin, as B cells rendered deficient in surface immunoglobulin undergo apoptosis [25]. The mechanism by which curcumin blocks NF- $\kappa$ B signaling appears to be through inhibition of I $\kappa$ B phosphorylation, but curcumin's specific target remains to be definitively identified.

Despite the use of a variety of therapeutic agents such as fludarabine, cyclophosphamide, rituximab,

and alemtuzumab that have increased the frequency of complete responses in B-CLL, the usual course of this illness remains that of persistent recurrences and repeated therapy. As a well-tolerated compound that has been ingested for centuries without apparent toxicity, curcumin deserves clinical assessment in the therapy of B-CLL.

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## REFERENCES

- Cheng AL, Hsu CH, Lin JK, et al. Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. *Anticancer Res* 2001;21:2895–2900.
- Singh S, Aggarwal BB. Activation of transcription factor NF- $\kappa$ B is suppressed by curcumin (diferuloylmethane) [corrected]. *J Biol Chem* 1995;270:24995–5000.
- Plummer SM, Holloway KA, Manson MM, et al. Inhibition of cyclo-oxygenase 2 expression in colon cells by the chemopreventive agent curcumin involves inhibition of NF- $\kappa$ B activation via the NIK/IKK signalling complex. *Oncogene* 1999;18:6013–6020.
- Foryst-Ludwig A, Neumann M, Schneider-Brachert W, Naumann M. Curcumin blocks NF- $\kappa$ B and the motogenic response in *Helicobacter pylori*-infected epithelial cells. *Biochem Biophys Res Commun* 2004;316:1065–1072.
- Li L, Aggarwal BB, Shishodia S, Abbruzzese J, Kurzrock R. Nuclear factor- $\kappa$ B and I $\kappa$ B kinase are constitutively active in human pancreatic cells, and their down-regulation by curcumin (diferuloylmethane) is associated with the suppression of proliferation and the induction of apoptosis. *Cancer* 2004;101:2351–2362.
- Aggarwal S, Takada Y, Singh S, Myers JN, Aggarwal BB. Inhibition of growth and survival of human head and neck squamous cell carcinoma cells by curcumin via modulation of nuclear factor- $\kappa$ B signaling. *Int J Cancer* 2004;111:679–692.
- Han SS, Chung ST, Robertson DA, Ranjan D, Bondada S. Curcumin causes the growth arrest and apoptosis of B cell lymphoma by downregulation of egr-1, c-myc, bcl-XL, NF- $\kappa$ B, and p53. *Clin Immunol* 1999;93:152–161.
- Bharti AC, Donato N, Aggarwal BB. Curcumin (diferuloylmethane) inhibits constitutive and IL-6-inducible STAT3 phosphorylation in human multiple myeloma cells. *J Immunol* 2003;171:3863–3871.
- Xu J, Fu Y, Chen A. Activation of peroxisome proliferator-activated receptor- $\gamma$  contributes to the inhibitory effects of curcumin on rat hepatic stellate cell growth. *Am J Physiol Gastrointest Liver Physiol* 2003;285:G20–G30.
- Chen A, Xu J. Activation of PPAR $\{\gamma\}$  by curcumin inhibits Moser cell growth and mediates suppression of gene expression of cyclin D1 and EGFR. *Am J Physiol Gastrointest Liver Physiol*. 2005;288:G447–G456.
- Furman RR, Asgary Z, Mascarenhas JO, Liou HC, Schattner EJ. Modulation of NF- $\kappa$ B activity and apoptosis in chronic lymphocytic leukemia B cells. *J Immunol* 2000;164:2200–2206.
- Tiwari S, Dong H, Kim EJ, Weintraub L, Epstein PM, Lerner A. Type 4 cAMP phosphodiesterase (PDE4) inhibitors augment glucocorticoid-mediated apoptosis in B cell chronic lymphocytic leukemia (B-CLL) in the absence of exogenous adenylyl cyclase stimulation. *Biochem Pharmacol* 2005;69:473–483.
- Crespo M, Bosch F, Villamor N, et al. ZAP-70 expression as a surrogate for immunoglobulin-variable-region mutations in chronic lymphocytic leukemia. *N Engl J Med* 2003;348:1764–1775.
- Campbell MJ, Zelenetz AD, Levy S, Levy R. Use of family specific leader region primers for PCR amplification of the human heavy chain variable region gene repertoire. *Mol Immunol* 1992;29:193–203.
- Fais F, Ghiotto F, Hashimoto S, et al. Chronic lymphocytic leukemia B cells express restricted sets of mutated and unmutated antigen receptors. *J Clin Invest* 1998;102:1515–1525.
- Kim DH, Lerner A. Type 4 cyclic adenosine monophosphate phosphodiesterase as a therapeutic target in chronic lymphocytic leukemia. *Blood* 1998;92:2484–2494.
- Hardin JA, Sherr DH, DeMaria M, Lopez PA. A simple fluorescence method for surface antigen phenotyping of lymphocytes undergoing DNA fragmentation. *J Immunol Methods* 1992;154:99–107.
- Moon E-Y, Lerner A. PDE4 inhibitors activate a mitochondrial apoptotic pathway in chronic lymphocytic leukemia cells that is regulated by protein phosphatase 2A. *Blood* 2003;101:4122–4130.
- Mizuno T, Rothstein TL. Cutting edge: CD40 engagement eliminates the need for Bruton's tyrosine kinase in B cell receptor signaling for NF- $\kappa$ B. *J Immunol* 2003;170:2806–2810.
- Hamblin TJ, Davis Z, Gardiner A, Oscier DG, Stevenson FK. Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. *Blood* 1999;94:1848–1854.
- Padilla J, Kaur K, Cao HJ, Smith TJ, Phipps RP. Peroxisome proliferator activator receptor- $\gamma$  agonists and 15-deoxy- $\delta$ (12,14) (12,14)-PGJ(2) induce apoptosis in normal and malignant B-lineage cells. *J Immunol* 2000;165:6941–6948.
- Padilla J, Leung E, Phipps RP. Human B lymphocytes and B lymphomas express PPAR- $\gamma$  and are killed by PPAR- $\gamma$  agonists. *Clin Immunol* 2002;103:22–33.
- Lee G, Elwood F, McNally J, et al. T0070907, a selective ligand for peroxisome proliferator-activated receptor  $\gamma$ , functions as an antagonist of biochemical and cellular activities. *J Biol Chem* 2002; 277:19649–19657.
- Shoba G, Joy D, Joseph T, Majeed M, Rajendran R, Srinivas PS. Influence of piperine on the pharmacokinetics of curcumin in animals and human volunteers. *Planta Med* 1998;64:353–356.
- Kraus M, Alimzhanov MB, Rajewsky N, Rajewsky K. Survival of resting mature B lymphocytes depends on BCR signaling via the Ig  $\alpha/\beta$  heterodimer. *Cell* 2004;117:787–800.