Supplementary material and methods

Chemicals

Reagents, including curcumin (80.1% purity), corn oil, etoposide, fetal bovine serum (FBS), red blood cell lysing buffer with ammonium chloride, phosphate-buffered saline (PBS), polyethylene glycol, polysorbate 80, benzyl alcohol, citric acid, ethanol, formaldehyde, dimethyl sulfoxide (DMSO), propidium iodide, RNase A, DyNAmo SYBR Green master-MIX, sodium dodecyl sulphate (SDS), NaF, phenylmethylsulfonyl fluoride (PMFS), Na3VO4, protease and phosphatase inhibitor cocktails and all chemicals used in determination of GSH were obtained from Sigma–Aldrich Co. (St Louis, MO, USA). StemSpan™ Serum-Free Expansion Medium (SFEM) and StemSpan expansion supplement™ CC100 were purchased from Stemcell Technologies Inc. (Canada). RPMI 1640 medium was purchased from American Type Cell Culture (UK). Annexin-V-allophycocyanin (APC), 7-aminoactinomycin D (7-AAD), hydroxyethyl piperazineethanesulfonic acid (HEPES) binding buffer and cycletest PLUS DNA reagent kit were obtained from BD Biosciences Pharmingen (San Diego, CA, USA). (PE)-conjugated goat anti-mouse antibody specific against μ chain was acquired from Jackson Immuno Research Laboratories (West Grove, PA, USA). Primary rabbit monoclonal anti-γH2AX (Ser 139) and secondary anti-rabbit IgG (H+L) F(ab′)2 fragment of goat antibody conjugated with Alexa Fluor 647 were obtained from Cell Signaling, Inc. (Dallas, TX, USA) Mouse monoclonal NFkBp65 – phycoerythrin conjugated IgG1 antibody, a polyclonal rabbit primary anti-NFκB antibody (sc-372 #) and an anti-β-actin (C4) horseradish peroxidase (HRP) antibody (sc-47778 HRP #) were acquired from Santa Cruz Biotechnology, Inc. (IL, USA). 5-(and-6′)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) and Hank’s balanced salt solution (HBSS) were purchased from Life Science Technology. Cycletest PLUS DNA reagent kit was purchased from Becton Dickinson (San Jose, CA, USA). Gradisol G was from Polfa (Kutno, Poland). Sodium dodecyl sulfate-polyacrylamide gel and polyvinylidene difluoride membranes were obtained from Merck-Millipore. Polyvinylidene fluoride (PVDF) and the protein standard of molecular weights (Precision Plus Protein – Dual Xtra Standards) were purchased from Bio-Rad Laboratories. An enhanced chemiluminescence reagent (Western Bright™ Quantum Chemiluminescent HRP Substrate Kit) was purchased from Advansta Corporation. Restore Stripping Buffer was from Pierce, Rockford (IL, USA).

Isolation of granulocytes from human venous/peripheral blood
Blood samples were centrifuged in a density gradient using Gradisol G. Cells were centrifuged at $350 \times g$ for 20 minutes. Mononuclear cells were banded at the interface between two phases, and the remaining cell elements (granulocytes and erythrocytes) were centrifuged at $250 \times g$ for 10 minutes. The resulting cell pellet was washed three times with phosphate buffered saline (PBS) solution without $Ca^{2+}/Mg^{2+}$ at $250 \times g$ for 10 minutes. After the last wash, the supernatant was decanted and the remaining red blood cells were lysed using distilled water (150 million cells/1 ml of distilled water) for 0.5 minute. Then, two time concentrated PBS was added to the cell pellet and adjusted to a volume of 1 mL followed by a centrifugation at $250 \times g$ for 10 minutes at 4°C. The granulocytes were suspended in 0.5 mL of RPMI medium.

**Measurement of cytotoxicity and apoptosis**
The cells ($0.4 \times 10^6$) were incubated with curcumin (20 μM) and/or etoposide (10 μM) for 24 hours. After incubation, the cells were washed ($230 \times g$, 5 minutes). Cytotoxicity was determined using PI staining according to instructions developed and optimized by R&D Systems Flow Cytometry Laboratory. The cells were resuspended in 100 μl of PBS and 10 μL of PI staining solution (10 μg/ml) was added and the cells were incubated for 1 minute in the dark. Granulocytes were treated with Eto at the concentration of 100 μM, because the lower concentration of Eto (10 μM) does not exert a significant cytotoxic effect in these cells. In order to apoptosis detection, cells were resuspended in hydroxyethyl piperazineethanesulfonic acid (HEPES) binding buffer. The cells were incubated with 2 μl of Annexin V-allophycocyanin (APC) and 2 μl of propidium iodide (PI, 50 μg/ml) per 50 μl of HEPES for 15 minutes in the dark. Then, they were suspended in 400 μL HEPES and analyzed in an LSR II flow cytometer equipped with red and blue lasers, using 633 nm excitation for APC (660/20 BP filter) and 488 nm excitation for PI (575/26 BP filter). The percentages of early apoptotic (Annexin V-APC-positive/PI-negative), necrotic (Annexin-APC-negative/PI-positive) and late apoptotic cells (Annexin-APC-positive/PI-positive) were analyzed using FACSDiva software (Becton Dickinson). Doublets were excluded from analysis using gating on width versus area of the forward scatter (FSC) and side scatter (SSC). Analysis was performed on at least 20,000 events.

**Cell staining and analysis for γH2AX, cell cycle, and NFκB**
The cells were fixed on ice with 1% methanol-free formaldehyde solution in PBS for 15 minutes. The cells were then washed two times ($372 \times g$, 5 minutes) with 1% BSA in PBS.
Next, cells were permeabilized with ice-cold 70% ethanol in deionized water, which was added dropwise to the cells with stirring. The cells were kept in ethanol for 24 hours at -20°C and then washed two times with 1% BSA in PBS. After washing, the cells were incubated for 10 minutes in a solution containing 1% BSA and 0.2% Triton X-100 in PBS (BSA-T-PBS) to block non-specific staining. Cells (0.4 × 10^6) were stained with rabbit monoclonal anti-γH2AX (Ser 139) antibody dissolved in BSA-T-PBS, for 2 hours at room temperature. An isotype control was the rabbit monoclonal IgG XP antibody. The cells were then washed with BSA-T-PBS (300 × g, 5 minutes) and incubated with the secondary anti-rabbit IgG (H+L) F(ab')2 fragment of goat antibody conjugated with Alexa Fluor 647, diluted in BSA-T-PBS for 30 minutes at room temperature in the dark. Subsequently, the cells were washed with BSA-T-PBS (300 × g, 5 minutes) and stained with a DNA staining solution containing 5 µg/ml PI and 0.1% RNAse A in PBS, for 30 minutes at room temperature in dark. Then, the cells were analyzed with a LSRII flow cytometer.

In order to detect the level of NFkB, HL-60 cells were washed with PBS (230 × g) for 5 minutes. The nuclei were isolated using cycltest PLUS DNA reagent kit (Becton Dickinson). Briefly, the cells were washed two time with citrate buffer (300 × g) for 5 minutes. Next, the pelets were incubated with 250 µM of solution A containing trypsin in a spermine tetrahydrochloride detergent buffer. Then, 200 µM of solution B was added containing trypsin inhibitor and ribonuclease A in citrate stabilizing buffer with spermine tetrahydrochloride for 10 minutes. Mouse monoclonal anti-NFkBp65–PE IgG1 antibody or normal mouse IgG1-PE antibodies was added for 20 minutes. Staining was performed according the manufacturer’s instruction.

Red and blue lasers were used for the analysis of the cells, using 633 nm excitation for Alexa Fluor 647 (660/20 BP filter) and 488 nm excitation for PI/PE (575/26 BP filter). Cell doublets and debris were excluded from analysis using PI-width versus PI-area. Analysis was performed on at least 20,000 single cells. Isotype control and control cells were used to establish the gating strategy of γH2AX or NFkB-positive cells.

**Western blot procedure**

First, the cell samples were homogenized in 2% SDS homogenizing buffer containing 20 mM NaF, 1 mM PMFS, 1 mM Na3VO4, protease and phosphatase inhibitor cocktails. The homogenates were boiled at 95°C for 10 minutes, and next were centrifuged at 10,000 rpm for 10 minutes at 4°C and supernatants were collected. The protein concentrations in the supernatant were determined using the BCA protein assay with bovine serum albumin, BSA
as a standard, and then normalized. The normalized samples were then mixed with sample loading buffer (125 mM Tris–HCl, pH 6.8; 4% SDS, 20% glycerol, 10% 14.3 M mercaptoethanol, 2 mM EDTA, bromophenol blue) in a ratio of 1:1 and subsequently boiled for 5 minutes. For Western blot analysis, protein samples were electrophoresed on 12% sodium dodecyl sulfate-polyacrylamide gel and then transferred onto polyvinylidene difluoride membranes (Immobilon-P; 0.45 µm). The gel was run at 100 V. Proteins were transferred onto a PVDF membrane using a MiniTrans-Blot Cell (Bio-Rad) and transfer buffer (0.30% Tris–Base, 1.44% glycine, 20% absolute methanol) at 110 V at low temperature (with ice) for 60 minutes. The protein standard of molecular weights was used (Precision Plus Protein – Dual Xtra Standards). The PVDF membrane was blocked for 1 hour at room temperature with a blocking solution. The blocking buffer for the membranes contained 5% milk in Tris-buffered saline, TBS (20 mM/l of Tris–HCl, pH 7.5, 150 mM/l NaCl) with 0.1% Tween 20 (200 µl/100 ml TBS). Then the membranes were incubated overnight with a polyclonal rabbit 1:500 dilution of a primary anti-NFkBp65 antibody (sc-372#) and 1:1,000 dilution of an anti-β-actin (C4) HRP antibody (sc-47778 HRP #). The protein bands were visualized using an enhanced chemiluminescence reagent (WesternBright™ Quantum Chemiluminescent HRP Substrate Kit). The protein amount was normalized to β-actin level in the same sample. The quantitative analysis of specific bands was performed with the G-Box Syngene using Genesys densitometry software (GeneTools version 4.03; Synoptic Ltd; Cambridge, England).

In order to control for protein loading, each blot was stripped using Restore Stripping Buffer and re-probed for β-actin HRP (1:1,000).

**ROS measurements**

Reactive oxygen species (ROS) were detected in the cells using 5-(and-6′)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) staining. Acetate groups of CM-H2DCFDA are cleaved by intracellular esterases to non-fluorescent dichlorofluorescein (DCFH) which reacts with intracellular antioxidants like glutathione and other thiols. DCFH undergoes rapid oxidation under the influence of ROS to a fluorescent dye with Ex/Em of 502/523 nm. Briefly, HL-60 cells were incubated with curcumin (10 or 20 µM) and/or etoposide (10 µM) for 24 hours. After incubation, the cells were washed with Hank’s balanced salt solution (HBSS) and incubated with 10 µM CM-H2DCFDA in 1 mL HBSS for 30 minutes in the dark. Then, they were washed with cold HBSS and suspended in 400 µL of this buffer and quickly analyzed.
Determination of intracellular glutathione (GSH) in HL-60 cells

The HL-60 cells (3×10⁶/ml) were harvested, washed in PBS, and lysed, and the proteins precipitated with 3% PCA at 0°C for 10 minutes. After centrifugation at 11,000 × g, 25 µl of the supernatants was added to 96-well microtiter plate wells containing 15 µl of 0.5 M Na₃PO₄, 125 µl 0.1 M NaH₂PO₄, 5 mM EDTA, and 25 µl10 mM 5,5′-dithiobis-2-nitrobenzoic acid (DTNB). The final pH was 7.5. Absorbance of the chromophore was measured at 412 nm after standing at room temperature for 5 minutes and the concentration of GSH calculated with molar absorption coefficient of 13,600 M⁻¹ cm⁻¹.

Liquid chromatography/mass spectrometry

The chromatographic separation was performed on an Agilent HPLC 1100 series system (Agilent, Waldbronn, Germany), which was equipped with a degasser, a binary pump, an auto-sampler and a thermo-stated column compartment. The samples were separated on a Supelco Discovery C18 column (250 mm ×4.0 mm ID, 5 mm particle size). The column was thermostated at 40°C. The mobile phase was composed of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) using the following gradient program: 0–2 minutes, isocratic gradient 30% (A); 2–6 minutes, linear gradient 30%–95% (A); 6–11 minutes, linear gradient 95%–30.0% (A); 11–16 minutes, isocratic gradient 30% (A). The flow rate was 0.4 ml/min; the injection volume was 30 µl.

Mass spectrometric analyses were accomplished on an Applied Biosystems MDSciex (Concord, Ontario, Canada) API 2000 triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) interface. ESI ionization was performed in the positive ionization mode. High purity nitrogen used as a sheath gas, was generated with a nitrogen generator. All experiments were carried out in the positive ion mode. The ion source parameters were as follows: ion spray voltage (IS): 5,000 V; nebulizer gas (gas 1): 50 psi; turbo gas (gas 2): 40 psi; temperature of the heated nebulizer (TEM): 500°C; curtain gas (CUR): 10 psi. Nitrogen (99.9%) was used as the curtain and collision gas. The ion path parameters for curcumin and its derivatives were as follows: focusing potential (FP): 200 V; entrance potential (EP): 10 V; collision cell exit potential (CXP): 15 V, respectively. The quantization analysis was performed using the MRM mode and tandem LC/MS. The following pairs of ions were monitored with the following values of m/z: 369.3/177.3 for CUR; 339.4/177.3 for DMC; 309.5/147.2 for BDMC; 373.3/177.2 for THC, and 449.4/177.2 for COS (Curcumin-O-sulfate). Data were analyzed by using the Analyst software 1.4 (Perlan Technologies).
Detection of apoptosis/necrosis in leukemic and normal cells in BNML rats

The isolated cells were counted in a Bürker haemocytometer and erythrocytes were lysed with red blood cell-lysing buffer containing 0.83% ammonium chloride in 0.01 M Tris buffer (pH 7.5). A splenic cell suspension (1×106 cells) was incubated for 45 minutes on ice in 100 µL of PBS containing 5% FBS and 10% mouse primary monoclonal anti-RM124 antibody. Next, the cells were washed in PBS (230× g, 10 minutes, 4°C), incubated with secondary goat anti-mouse monoclonal antibody conjugated to R-PE for 30 minutes and washed in PBS (230× g, 10 minutes, 4°C) and then with fluorescein isothiocyanate (FITC)-conjugated mouse anti-rat CD45R monoclonal antibody for 20 minutes to evaluate the nonspecific binding of the secondary antibody to B-lymphocytes. Subsequently, the cells were stained with annexin V-APC (An-APC) and 7AAD according to the instructions provided by the manufacturer, and incubated in the dark for 15 minutes. All steps were performed on ice. The cells were analyzed using a LSRII flow cytometer and FACSDiva software (BD Biosciences Immunocytometry Systems, San Jose, CA, USA).

Survivin mRNA expression in sorted BNML cells and B lymphocytes

Ten million spleen cells stained with antibodies as described above were sorted by MoFlo cell sorter (Beckman Coulter Inc.). Leukemic cells were gated as RM124(+)CD45R(-) and B lymphocytes as RM124(-)CD45R(+). Additionally, leukemic cells were defined by high FSC/SSC values to distinguish them from non-specific binding on B-cell surface (Figure 1). IgM-RPE isotype control was used to confirm specificity of RM124 antibody.

Expression of mRNA was investigated using real-time PCR. Total RNA was isolated from sorted leukemic, B lymphocytes and HL-60 cells by acid guanidinium thiocyanate–phenol–chloroform extraction. Reverse transcription with oligo-dT primers was performed on 2 µg of total RNA, for 1 hour at 42°C, using MMLV reverse transcriptase, according to the vendor’s instruction. Real-time polymerase chain reactions using a qRT-PCR DyNAmo HS SYBR Green qPCR Kit, were performed on samples containing 50 ng cDNA, 0.5 µl of primers, 7.5 µl of DyNAmo SYBR Green master-MIX, and nuclease-free water added to 15 µl of total volume. The reaction was carried out using the following protocol: 95°C for 10 minutes, followed by 40 three-step cycles of 95°C for 30 seconds, 60°C for 60 seconds, 72°C for 45 seconds, and then 72°C for 5 minutes as a final elongation. The primer was specific for survivin (5’ CTG CCC TAC CGA GAA TGA GCC TG 3’ and 5’ GCC AGG GGA GTG CTT CCT ATG C 3’) and for housekeeping EF2 (5’ CTG CGT GTC ACT GAT GGA GCA C 3’ and 5’ AGG CTC CAG TTG CAG TTC CAG C 3’) genes.