

Acetyl-11-Keto- β -Boswellic Acid Induces Apoptosis in HL-60 and CCRF-CEM Cells and Inhibits Topoisomerase I

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ABSTRACT

Antiproliferative action of different pentacyclic triterpenes has repeatedly been reported, and some lipoxygenase inhibitors have been shown to induce cell death in various cell systems. Acetyl-11-keto- β -boswellic acid (AKBA) is a pentacyclic triterpene that inhibits 5-lipoxygenase in a selective, enzyme-directed, nonredox, and noncompetitive manner. To investigate a possible effect of AKBA on leukemic cell growth, proliferation of HL-60 and CCRF-CEM cells was assayed in the presence of AKBA and a structural analog without effect on 5-lipoxygenase, amyrin. Cell counts and [3 H]thymidine incorporation were significantly reduced in a dose-dependent manner in the presence of AKBA ($IC_{50} = 30 \mu M$) but not amyrin. An additive effect of AKBA with the crosslinking of the CD95 receptor was also observed. Flow cytometric analysis of propidium iodide-stained

cells indicated that the cells underwent apoptosis. This was confirmed by flow cytometric detection of sub- G_1 peaks in AKBA-treated cells and by DNA laddering. However, because HL-60 and CCRF-CEM do not express 5-lipoxygenase mRNA constitutively, a mechanism distinct from inhibition of 5-lipoxygenase must account for the effect of AKBA. In a DNA relaxation assay with $\phi X174$ RF DNA, AKBA inhibited topoisomerase I from calf thymus at concentrations of $\geq 10 \mu M$. A semiquantitative cDNA polymerase chain reaction approach was used to estimate the relative level of expression of topoisomerases in both cell lines. The data suggest that induction of apoptosis in HL-60 and CCRF-CEM by AKBA may be due to inhibition of topoisomerase I in these cells.

Programmed cell death (PCD) is a feature of major importance not only in normal animal development but also in the turnover and renewal of many different cell populations in the adult body (Ellis et al., 1991; Raff et al., 1993). Cells dying by PCD undergo typical morphological changes, which are easily distinguishable from necrosis caused by accidental cell death (Wyllie et al., 1984; Walker et al., 1988).

PCD of lymphocytes is crucial in regulating immune responses and maintaining self-tolerance (Cohen et al., 1992). PCD is also induced in virus-infected or malignant cells by effector cells of the immune system, and inhibition of apoptosis by the bcl-2 gene product, for example, has been implicated in the development of cancer (Podack and Kupfer, 1991; Korsmeyer, 1992). Consequently, cytostatic agents influencing mechanisms of PCD have been described (Solary et al., 1994). Their clinical use has been limited by severe side effects (Rougier and Bugat, 1996); therefore, cytostatic drugs inducing PCD with low toxicity should be promising research tools.

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ABBREVIATIONS: AKBA, acetyl-11-keto- β -boswellic acid; CPT, camptothecin; DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; HETE, hydroxy-6,8,11,14-eicosatetraenoic acid; HPETE, hydroperoxy-6,8,11,14-eicosatetraenoic acid; PCD, programmed cell death; PCR, polymerase chain reaction; PI, propidium iodide; PMNL, polymorphonuclear leukocyte; PGB₂, prostaglandin B₂.

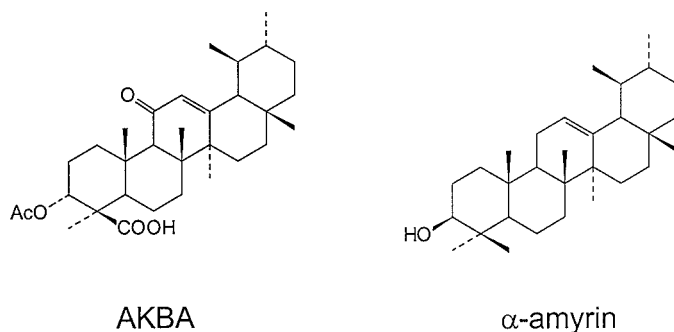


Fig. 1. Chemical structures of AKBA and α -amyrin.

Frankincense extracts have been used in the traditional medicine of India and other countries since ancient times for the treatment of several diseases, including inflammatory disorders and cancer (Martinez et al., 1989). Today, the frankincense extract *salai guggal* is used in India for the treatment of rheumatic diseases with a minimum of side effects. A clinical trial on ulcerative colitis with an extract of

Boswellia serrata gum resin demonstrated effects comparable to those of standard medication (Gupta et al., 1997). The main compounds of frankincense, the gum resin of *B. serrata* and *B. carterii*, boswellic acids, were identified as inhibitors of 5-lipoxygenase (Ammon et al., 1991) and human leukocyte elastase (Safayhi et al., 1997) and were shown to be direct, nonredox inhibitors of mammalian 5-lipoxygenases (Safayhi et al., 1992). Structure-activity relationships for acetyl-11-

keto- β -boswellic acid (AKBA) have been elucidated (Sailer et al., 1996). AKBA is a pentacyclic triterpene of the ursane type and inhibits 5-lipoxygenase in rat polymorphonuclear leukocytes (PMNLs) with $IC_{50} = 1.5 \mu\text{M}$. Recently, induction of apoptosis by 5-lipoxygenase inhibitors in chronic myelogenous leukemia cells (Anderson et al., 1995) by pentacyclic triterpenes in melanoma (Pisha et al., 1995) and different other cell lines, including leukemia, has been reported (for a review, see Mahato et al., 1992). In vitro, not clearly defined frankincense extracts showed inhibitory action on topoisom-

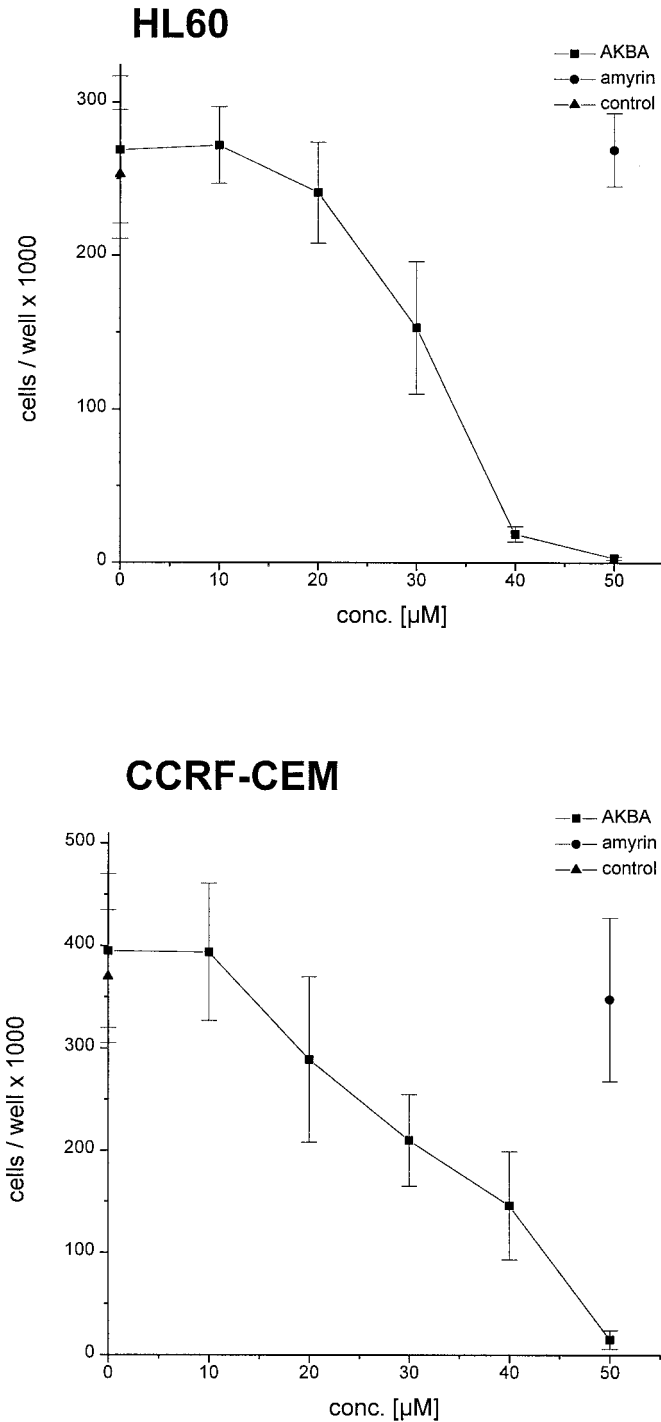


Fig. 2. Effect of AKBA on cell survival. Cells were grown for 48 h in the presence of AKBA, amyirin, or 0.5% DMSO alone. Untreated cells served as control. Cells were counted in a Neubauer hemocytometer, and cell survival was determined by trypan exclusion test ($n = 3$).

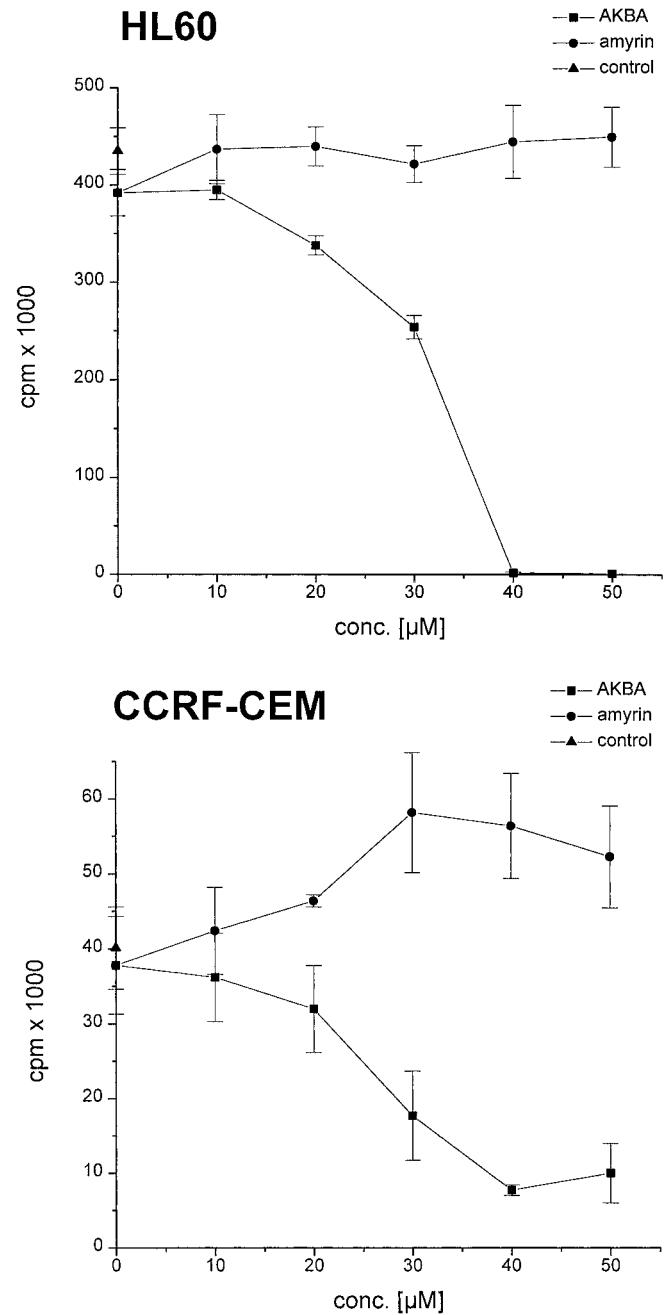


Fig. 3. Effect of AKBA on $[^3\text{H}]$ thymidine incorporation. Cells (1×10^5) were plated onto 96-well flat-bottom culture plates and then incubated with AKBA or amyirin for 4 h and for an additional 18 h after the addition of 37 kBq $[^3\text{H}]$ thymidine per well. Untreated cells served as control. Cells were harvested on glass fiber filters, and $[^3\text{H}]$ thymidine incorporation was assayed by liquid scintillation spectrometry ($n = 3$).

erase II (Wang et al., 1991). Our study aim was to investigate the effects of AKBA and its structural analog amyrin (Fig. 1) on two human leukemic cell lines, HL-60 and CCRF-CEM. AKBA was found to inhibit cell growth and to induce apoptosis in both cell lines. Because CCRF-CEM cells do not express 5-lipoxygenase mRNA constitutively (Jakobsson et al., 1992), another mechanism must account for the observed effects. This may also be true for HL-60 cells, which are not capable of producing 5-lipoxygenase metabolites unless differentiated with agents like dimethyl sulfoxide (DMSO), retinoic acid, or vitamin D₃ (Kargman and Rouzer, 1989; Brungs et al., 1994). Because we could demonstrate that AKBA inhibits a mammalian topoisomerase I, we suggest that this mechanism could be responsible for the apoptosis-inducing effect of AKBA.

Materials and Methods

Chemicals. AKBA was obtained from the gum resin of *B. carterii* (olibanum) by extraction into ether, precipitation with barium hydroxide, acetylation to mixed anhydrides with acetic anhydride, cleavage of the mixed anhydrides, and crystallization of the mixture of acetyl-boswellic acids from methanol (Winterstein and Stein, 1932). AKBA was isolated from this mixture by C18 reversed-phase high performance liquid chromatography and characterized by infrared, ¹H NMR, and mass spectroscopy and by its melting points as described elsewhere (Safayhi et al., 1992). Amyrin was purchased from Roth (Karlsruhe, Germany), and camptothecin (CPT) was from Sigma (Munich, Germany). Solutions were prepared in DMSO and diluted with RPMI 1640; the final concentration of DMSO in all assays was 0.5%. Anti-CD95 monoclonal antibody (clone CH-11) was obtained from Coulter Immunotech (Hamburg, Germany), and [³H]thymidine was purchased from Amersham Buchler (Braunschweig, Germany).

Cell Culture. HL-60 and CCRF-CEM cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine (all from Seromed, Berlin, Germany).

5-Lipoxygenase Assay and Measurement of 5-Lipoxygenase Metabolites. The formation of 5-lipoxygenase products was assayed as described elsewhere (Sailer et al., 1996). In brief, 1×10^7 cells were suspended in phosphate-buffered saline and stimulated at 37°C for 10 min by ionophore A23187 and Ca⁺⁺. Glycogen-elicited rat PMNLs (Sailer et al., 1996) were used as control; HL-60 or CCRF-CEM cells were cultured in the presence of 0.5% DMSO for 48 h before testing. The incubation was terminated by cooling to 4°C and

acidification to pH 3 with formic acid. For quantification of 5-lipoxygenase products, 170 ng of prostaglandin B₂ (PGB₂) was added to each sample as internal standard. Extraction of eicosanoids, separation by reversed-phase high performance liquid chromatography, and ultraviolet detection were carried out as described in detail previously (Ammon et al., 1991). Detection wavelength was set to 280 nm for PGB₂ and leukotriene B₄ isomers or to 235 nm for hydroxy-6,8,11,14-eicosatetraenoic acid (HETE) and hydroperoxy-6,8,11,14-eicosatetraenoic acid (HPETE).

[³H]Thymidine Incorporation. For [³H]thymidine-incorporation, 1×10^5 cells/100 µl were plated onto 96-well flat-bottom culture plates. After incubation for 4 h with AKBA or amyrin, respectively, [³H]thymidine was added (37 kBq), and cells were incubated for an additional 18 h. The culture plates were frozen at -20°C. For measurement of [³H]thymidine incorporation, cells were thawed and harvested on glass fiber filters. The filters were submerged in scintillation cocktail, and radioactivity was measured in a scintillation counter.

Flow Cytometry. Flow cytometric analysis was performed on a fluorescent activated cell scan analysis (FACScan) flow cytometer (Becton-Dickinson, San José, CA). Cells were stained with propidium iodide (PI), gates were set for forward and sideward scatter, and 4000 cells were acquired. Data were analyzed using Lysis II (Becton Dickinson) software.

Sub-G₁ peaks were detected as follows: cells were incubated with either 0.5% DMSO as control, 5 µM camptothecin, or 50 µM AKBA. After 4 h, the cells were washed with phosphate-buffered saline and fixed with ethanol 70%. After staining cells with PI (50 µg/ml) for 30 min, 10,000 cells were acquired. DNA histograms were calculated for FL2 = PI.

cDNA Polymerase Chain Reaction. tRNA was extracted from 2×10^7 cells using the Optiprep kit (Biometra, Goettingen, Germany). cDNA polymerase chain reaction (PCR), selection of oligonucleotide sequences, and separation of PCR products by polyacrylamide gel electrophoresis were performed as described previously (Beck et al., 1995). Primers for topoisomerase I were 5'-CAGACG-GAAGCTCGGAAACAC-3' (sense) and 5'-CAAGTAACCTTGTTAT-CATGCC-3' (antisense). Amplimers were identified by digestion with restriction endonucleases. After staining of gels with ethidium bromide, signals were directly digitalized by the CS-1 videoimager (Cybertech, Berlin, Germany). Signal intensities of topoisomerases I, IIα, and IIβ were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression using WINCAM densitometric software (Cybertech, Berlin, Germany). Mean values and standard deviations were calculated from the results of three independent experiments.

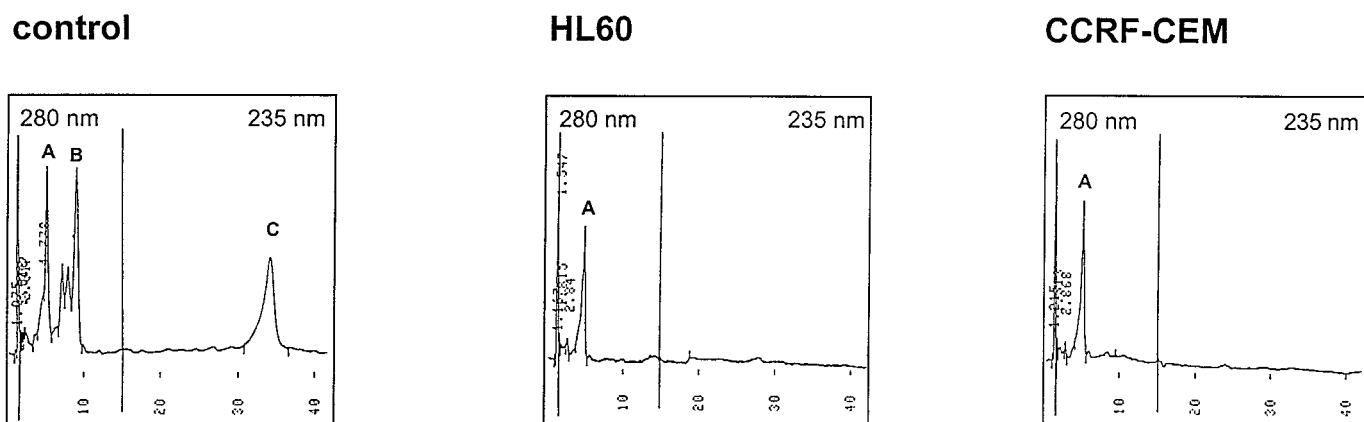


Fig. 4. Determination of 5-lipoxygenase metabolites. Cells (rat PMNLs as positive control, HL-60, or CCRF-CEM) (10^7) were stimulated with ionophore A23187 and Ca⁺⁺, and 5-lipoxygenase products were extracted and analysed by reversed-phase high performance liquid chromatography (A, PGB₂; B, leukotriene B₄ isomers; C, 5-HETE).

Extraction of Fragmented DNA from Apoptotic Cells. Approximately 5×10^6 cells were lysed by the addition of lysis buffer (0.1% Triton X-100, 5 mM Tris-HCl, pH 8.0, 20 mM ethylenediaminetetraacetic acid). After the addition of 2.5% polyethylene glycol 8000 and 1 M NaCl (final concentrations), samples were kept on ice for 10 min and then centrifuged at 16,000g for 10 min at room temperature. The supernatants were precipitated with ethanol and used without quantification for polyacrylamide gel electrophoresis to show DNA laddering.

Relaxation of Supercoiled ϕ X174RF DNA. Supercoiled ϕ X174RF DNA (0.5 μ g) was incubated with 1 U of topoisomerase I from calf thymus (all from GIBCO BRL, Eggenstein, Germany) for 30

min at 37°C with 0.5% DMSO as control or in the presence of test compounds. The reaction was terminated by the addition of sodium dodecyl sulfate (0.5% final). After proteinase K (0.15 μ g/ μ l) digestion for 30 min, DNA topoisomers were separated by agarose gel electrophoresis and stained with ethidium bromide. The lowest inhibitory concentration of the compounds was estimated visually.

Data. The results of independent observations (n = number of individual experiments) are shown as mean \pm S.D. values.

Results

Effect of AKBA on Cell Proliferation. HL-60 and CCRF-CEM cells were grown for 48 h in the presence of AKBA, the structural analog amyrin, 0.5% DMSO, or RPMI 1640 supplemented with 10% fetal calf serum as control. Cells were counted, and cell survival was determined by trypan blue exclusion test. AKBA inhibited cell proliferation in both cell lines with $IC_{50} = 30 \mu$ M, whereas amyrin had no

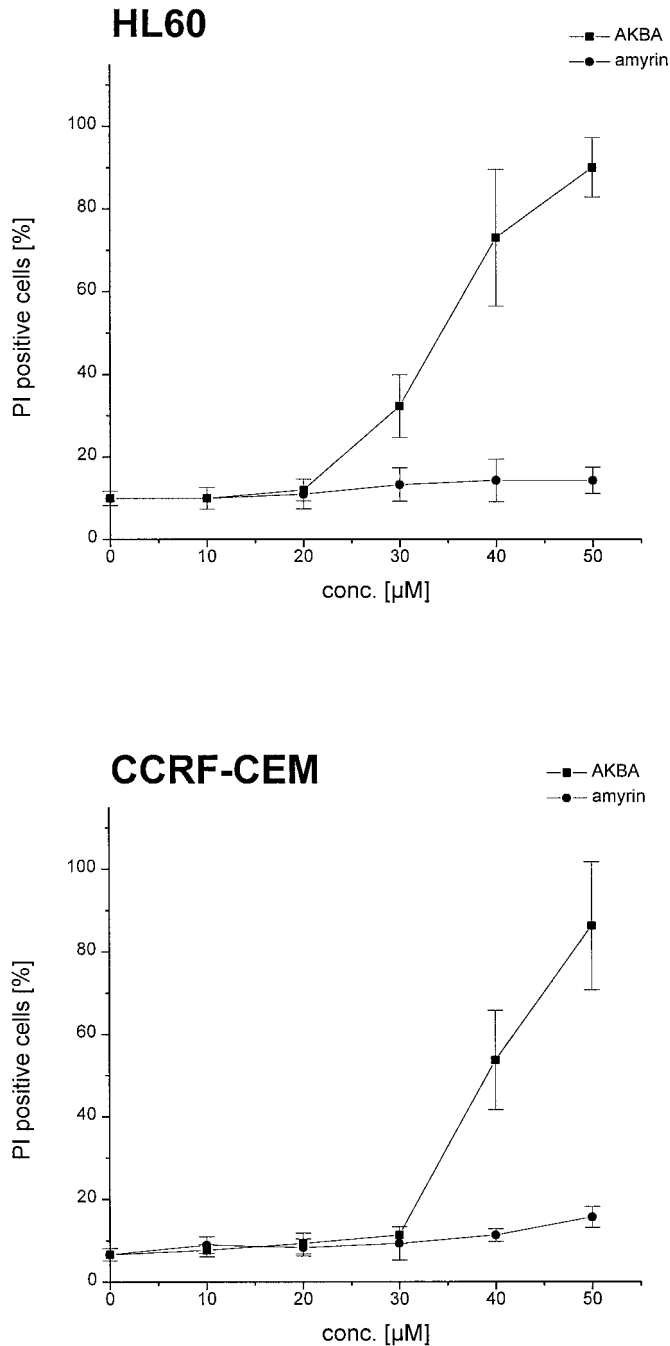


Fig. 5. PI staining of HL-60 and CCRF-CEM cells. After incubation with either AKBA or amyrin for 24 h, cells were stained with 1 μ g/ml PI and analyzed in a flow cytometer. Gates were set for forward and sideward scatter, and 4000 cells were acquired ($n = 3$).

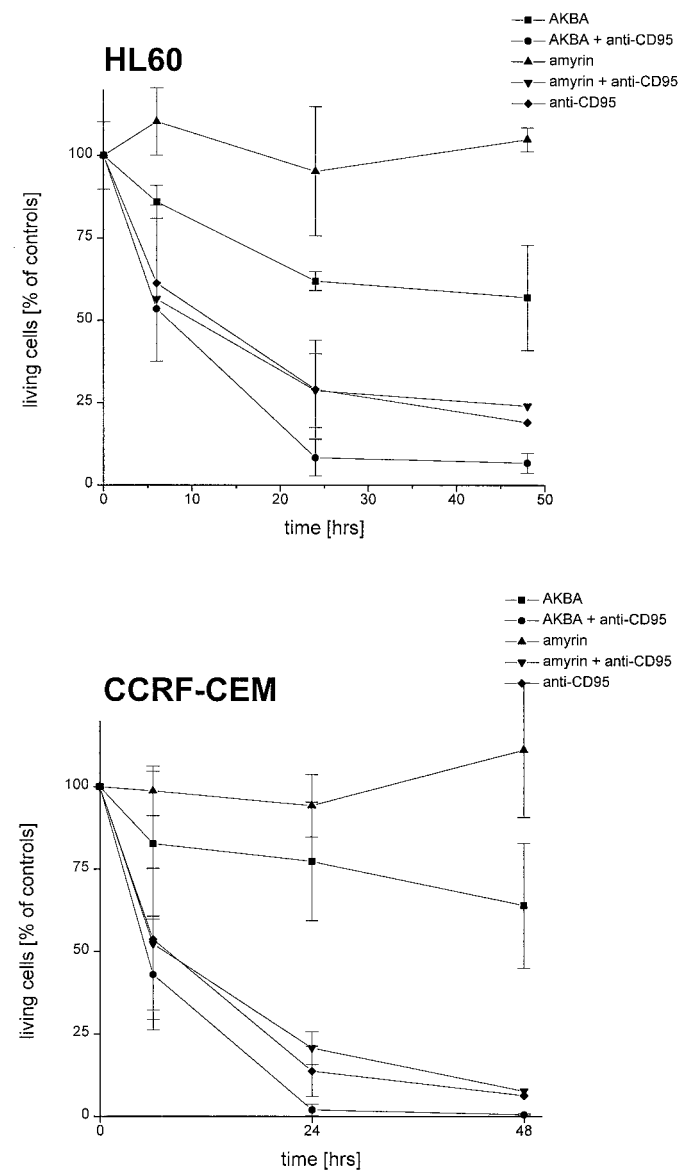


Fig. 6. Coincubation with anti-CD95 monoclonal antibody. Cells were grown in the presence of either AKBA or amyrin with or without anti-CD95 monoclonal antibody or anti-CD95 monoclonal antibody alone for up to 48 h and analyzed by flow cytometry ($n = 3$).

effect at the highest dose (50 μM ; Fig. 2). DMSO (0.5%) had no effect on cell count compared with untreated cells. These results were confirmed by [^3H]thymidine-incorporation experiments (Fig. 3).

Determination of 5-Lipoxygenase Activity in HL-60 and CCRF-CEM Cells. Rat PMNLs or HL-60 or CCRF-CEM cells (10^7) were stimulated with ionophore A23187 and calcium. Leukotriene B₄ isomers and 5-H(P)ETE could be detected in rat PMNL used as positive control but not in HL-60 and CCRF-CEM cells after cultivation in the presence of 0.5% DMSO for 48 h and stimulation with calcium/ionophore (Fig. 4). From this experiment, it can be concluded that cultivation of HL-60 or CCRF-CEM cells in medium with 0.5% DMSO does not lead to the induction of 5-lipoxygenase activity after 48 h.

Induction of Apoptosis by AKBA. AKBA-treated and control cells were stained with PI and analyzed by flow cytometry. Although amyirin was without any effect on PI staining, a concentration of AKBA exceeding 30 μM did result in an increasing number of PI-positive cells (Fig. 5).

Morphological changes like shrinkage and membrane blebbing could easily be seen by light microscopy in AKBA-treated cells. Flow cytometric analysis of cells treated with AKBA revealed an increase in granularity and a decrease in cell volume (data not shown). Cells treated with amyirin did not exert any of these features. These results suggested an apoptotic cell death induced by AKBA and cells therefore were analyzed for signs of apoptosis. Coincubation with AKBA 30 μM and an apoptosis-inducing CD95 IgM monoclonal antibody showed an additive effect in both cell lines (Fig.

6). A CD95-IgG antibody, which binds to the CD95 receptor without inducing apoptosis, could not prevent the effect of AKBA when the cells were preincubated with this antibody (data not shown). This indicated that the effect of AKBA must be downstream of the CD95 receptor.

DNA Fragmentation. DNA fragmentation was analyzed in HL-60 and CCRF-CEM cells treated with either 5 μM CPT, 50 μM AKBA, or 0.5% DMSO as control for 4 h. As detected by flow cytometry, the cells showed an increase in hypodiploid DNA (sub-G₁ peaks) when treated with CPT or AKBA (Fig. 7). Cleavage of DNA by endonucleases to 180-bp fragments could be detected by extraction of DNA and agarose gel electrophoresis. Again, in cells incubated with the known topoisomerase I inhibitor CPT (5 μM) or AKBA (50 μM), DNA fragmentation could be detected (Fig. 8).

Inhibition of Topoisomerase I. Because inhibitors of topoisomerases are known to induce apoptosis in various cell lines (Solary et al., 1994), one may speculate whether the inhibition of one or more topoisomerases is the mechanism by which AKBA induces apoptosis in HL-60 and CCRF-CEM cells. Because these cells do not express 5-lipoxygenase mRNA constitutively (Jakobsson et al., 1992) and, as we demonstrated, do not produce 5-lipoxygenase metabolites, inhibition of this enzyme cannot be responsible for the observed effects. We therefore tested for topoisomerase I inhibitory activity of AKBA. Supercoiled ϕX174RF DNA is relaxed by topoisomerase I from calf thymus at 37°C to topoisomers that can be separated by agarose gel electrophoresis. Incubation of supercoiled ϕX174RF DNA with topoisomerase I and AKBA showed inhibition of topoisomerase I at concen-

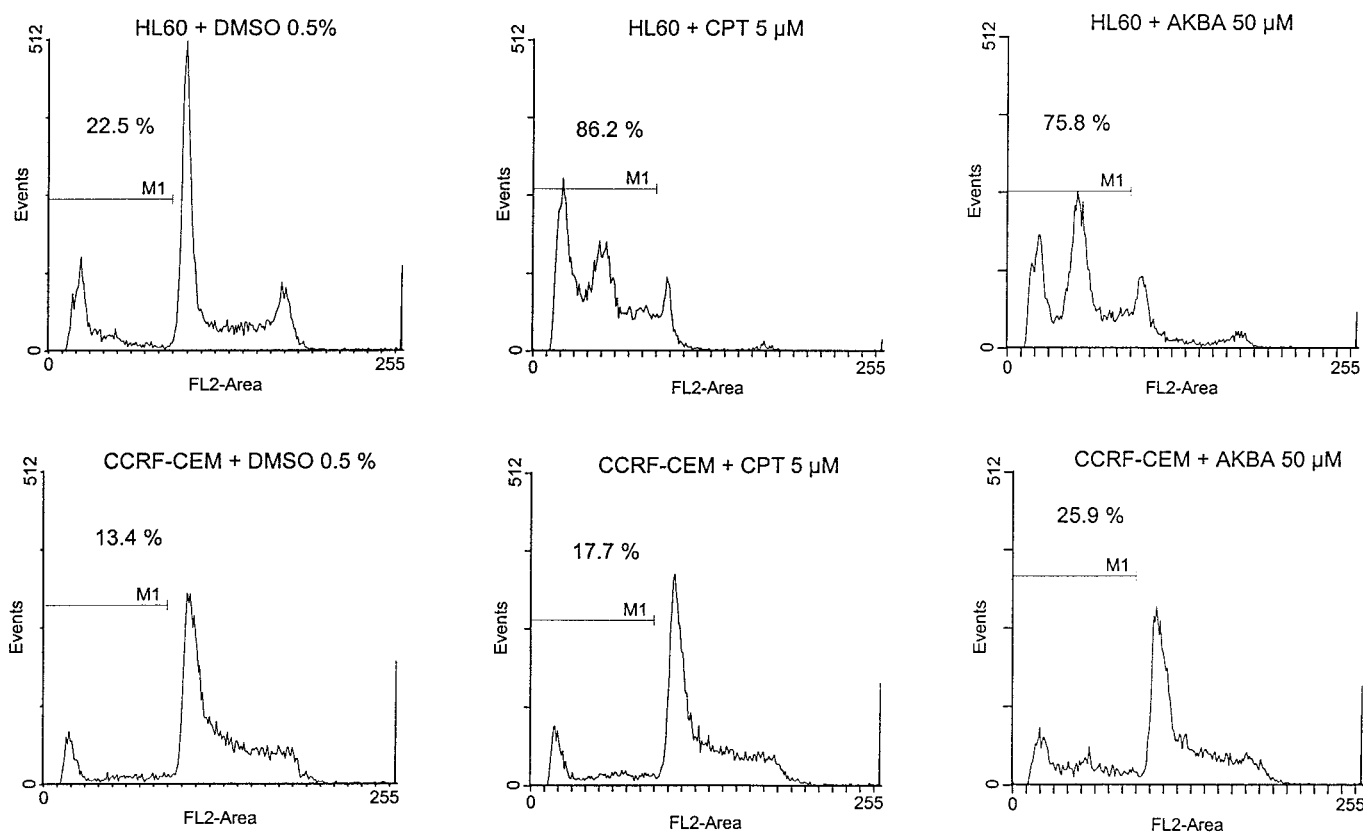


Fig. 7. DNA histograms. Cells were incubated as indicated with either 0.5% DMSO as control, 5 μM CPT, or 50 μM AKBA for 4 h. After staining with PI, 10,000 cells were acquired on a flow cytometer, and DNA histograms were calculated for PI = FL2. Percentage of apoptotic cells (M1) is indicated.

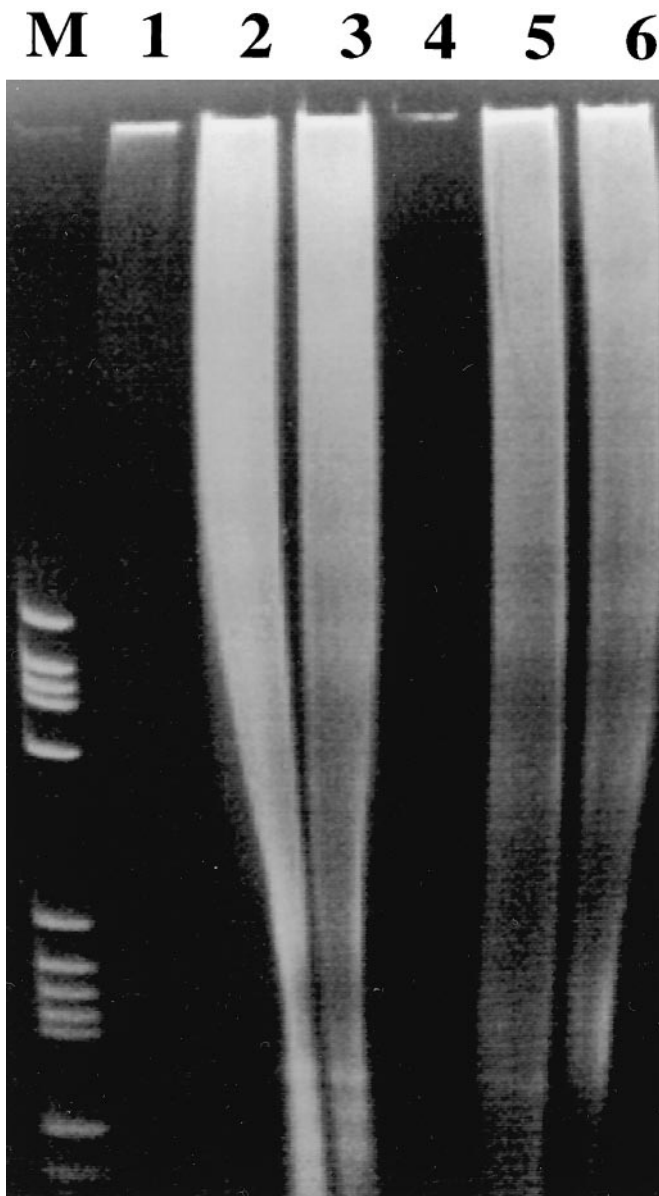


Fig. 8. DNA fragmentation in HL-60 and CCRF-CEM cells. Cells (5×10^6) were incubated with 0.5% DMSO as control (lane 1, HL-60; lane 4, CCRF-CEM), 5 μM CPT (lane 2, HL-60; lane 5, CCRF-CEM), or 50 μM AKBA (lane 3, HL-60; lane 6, CCRF-CEM) for 12 h (M = molecular weight marker). Cells were lysed, and high-molecular-weight DNA was precipitated by the addition of 2.5% polyethylene glycol 8000 and 1 M NaCl. After centrifugation, the remaining fragmented DNA was precipitated with 70% ethanol and electrophoresed on an 8% polyacrylamide gel.

trations of AKBA of $\geq 10 \mu\text{M}$. At a concentration of $< 10 \mu\text{M}$, AKBA was without effect on topoisomerase I activity (data not shown). Amyrin was without any effect up to 50 μM (Fig. 9).

Expression of Topoisomerase I, Topoisomerase II α , and Topoisomerase II β mRNA. In all experiments shown, the effect of AKBA was more pronounced on HL-60 than on CCRF-CEM cells. Although this difference was not significant, we argued whether this difference could be attributable to topoisomerase expression. A semiquantitative cDNA PCR approach was used to analyze HL-60 and CCRF-CEM cells. Both cell lines express topoisomerase I, topoisomerase II α , and topoisomerase II β mRNA. When signals were digitalized

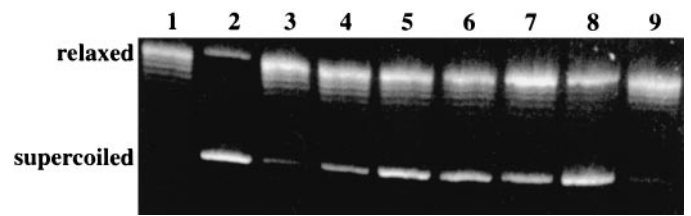


Fig. 9. Relaxation of supercoiled ϕX174RF DNA. The DNA was incubated with topoisomerase I and 0.5% DMSO (lane 1), without topoisomerase (lane 2), or 10, 20, 30, 40, and 50 μM AKBA (lanes 3 to 7), 50 μM CPT (lane 8), and 50 μM amyryn (lane 9).

after agarose gel electrophoresis of the PCR products and normalized to GAPDH gene expression, a lower expression of all three topoisomerases in CCRF-CEM cells compared with HL-60 cells can be seen (Tab. 1).

Discussion

Inhibitors of lipoxygenases have been shown to inhibit proliferation and to induce apoptosis in tumor cells (Anderson et al., 1995). In this study, we investigated the effects of the 5-lipoxygenase inhibitor AKBA on two human cancer cell lines, HL-60 and CCRF-CEM. AKBA, but not the structural analog amyryn, reduced viability and proliferation of cells with an IC_{50} value of 30 μM . In addition, DNA degradation was manifested by the appearance of sub- G_1 peaks in the DNA histogram and DNA laddering. Sub- G_1 peaks could also be observed to some extent in untreated HL-60 cells, which may be attributable to spontaneous apoptosis in a confluent cell population, yet no DNA laddering was detectable in these cells. Together with the typical morphological changes of the cells detected by light microscopy and flow cytometry, we conclude that the mode of cell death induced by AKBA is apoptosis.

When apoptosis was induced by an anti-CD95 antibody, coinubation of cells with AKBA revealed an additive effect. Preincubation with an anti-CD95 IgG monoclonal antibody binding to the CD95 receptor without inducing apoptosis could not prevent the cells from apoptosis when exposed to AKBA. Therefore, AKBA must induce apoptosis downstream of the CD95 receptor.

The IC_{50} of AKBA needed for growth inhibition and induction of apoptosis in HL-60 and CCRF-CEM cells is 20-fold higher than that for inhibition of 5-lipoxygenase in rat PMNL. This observation is in accordance with a report showing growth inhibition by other 5-lipoxygenase inhibitors (Anderson et al., 1995). HL-60 and CCRF-CEM do not express the 5-lipoxygenase mRNA constitutively (Jakobsson et al., 1992). Although HL-60 cells can exert 5-lipoxygenase activity after differentiation (Kargman and Rouzer, 1989;

TABLE 1

Relative gene expression of topoisomerases I, II α , and II β in CCRF-CEM cells compared with HL-60 cells

After cDNA PCR, signal intensities of topoisomerase amplicates were normalized to GAPDH gene expression. HL-60 was arbitrarily set to 100% ($n = 3$).

	Signal Intensity	
	HL-60	CCRF-CEM
	%	%
Topoisomerase I	100	74.7 \pm 16.1
Topoisomerase II α	100	91.7 \pm 20.3
Topoisomerase II β	100	86.3 \pm 28.9

Brungs et al., 1994), they are not able to form 5-lipoxygenase products when cultured in 0.5% DMSO for 48 h (Fig. 4). This suggests that a mechanism different from inhibition of 5-lipoxygenase must account for the effects of AKBA in these cells. Extracts from frankincense with terpenic compounds (Wang et al., 1991) are also known to inhibit topoisomerases. Furthermore, several flavonoids inhibit the DNA binding or the DNA religation step of eukaryotic topoisomerase I (Boege et al., 1996) and are also known as inhibitors of lipoxygenases. This emphasizes the hypothesis that the mechanism of AKBA in our two cell lines would be inhibition of topoisomerase I.

In a cell-free DNA relaxation assay with ϕ X174RF DNA and topoisomerase I from calf thymus, AKBA inhibited this enzyme at concentrations of $\geq 10 \mu\text{M}$, whereas amyirin, although of very similar chemical structure, was without any effect up to $50 \mu\text{M}$. Topoisomerase I inhibitors are known inducers of apoptosis (Solary et al., 1994), and CPT induced apoptosis in HL-60 and CCRF-CEM as did AKBA. Inhibition of topoisomerase I by AKBA may explain the observed apoptotic cell death.

This line of argumentation is further enhanced by the expression of the mRNA of topoisomerase I, topoisomerase II α , and topoisomerase II β in HL-60 and CCRF-CEM cells. All three enzymes were expressed in both cell lines, but expression of all three topoisomerases was slightly lower in CCRF-CEM compared with HL-60 cells, and the difference was most prominent for topoisomerase I. This observation might explain the more pronounced effect of AKBA on HL-60.

Taken together, the data show induction of apoptosis in leukemic cell lines induced by a natural compound, AKBA, which inhibits topoisomerase I in vitro. AKBA is a constituent of *B. serrata* gum resin, which is used in traditional medicine in India and Africa for the treatment of rheumatic diseases and of cancer. Boswellic acids did not show serious side effects when used in humans (Gupta et al., 1997), although in a single-dose study in two subjects, peak plasma concentrations of AKBA from 15 to $30 \mu\text{M}$ were observed after the oral administration of 800 mg of *salai guggal* (Mack, 1990). Thus, AKBA as a pharmacological active constituent of *B. serrata* extract seems to reach relevant plasma concentrations, which may result in the inhibition of topoisomerase I. Further in vivo studies should address this problem.

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