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An IκBα Inhibitor Causes Leukemia Cell Death through a p38 MAP Kinase-dependent, NF-κB-independent Mechanism

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ABSTRACT

Treatment of U937 cells with an IκBα phosphorylation inhibitor, Bay 11-7085, induced a rapid phosphorylation of p38 mitogen-activated protein (MAP) kinase, significant apoptosis, extensive necrosis, and a weak phosphorylation of MAP kinase kinase. Bay 11-7085 had no effect on the basal levels of phosphorylated IκBα but completely inhibited phorbol 12-myristate 13-acetate-induced phosphorylation of IκBα. Although Bay 11-7085 prevented phorbol 12-myristate 13-acetate-induced NF-κB nuclear translocation, SN50, a specific inhibitor of nuclear translocation and function of NF-κB, did not induce any significant nuclear/DNA fragmentation, caspase 3 activation, or cell death. The p38 MAP kinase-specific inhibitor, SB203580, completely inhibited the phosphorylation of p38 MAP kinase and significantly decreased Bay 11-7085-induced apoptosis. In contrast, the MAP kinase kinase-specific inhibitor PD98059 had no effect on Bay 11-7085-induced apoptosis. Caspase-specific inhibitor, z-Val-Ala-Asp-fluoromethyl ketone prevented Bay 11-7085-induced activation of caspase 3 but was not able to block Bay 11-7085-induced phosphorylation of p38 MAP kinase. These data suggest that Bay 11-7085 induces apoptosis through a p38 MAP kinase-dependent, NF-κB-independent mechanism.

INTRODUCTION

The transcription factor NF-κB has been identified as a critical component of several signal transduction pathways (1). One important function of NF-κB is its ability to protect cells from apoptosis (2–4). NF-κB is a heterodimer comprising p50 and p65 subunits. It is sequestered in the cytoplasm by association with a binding protein named IκBα, which masks the nuclear localization signal of NF-κB (5). It has been demonstrated that a variety of external or internal signals modify IκBα/NF-κB complexes by the phosphorylation on serine residues of IκBα and subsequent degradation of the IκBα (6–8). Recently, Bay 11-7085 has been synthesized as a specific inhibitor of IκBα phosphorylation. In endothelial cells, this agent prevented NF-κB activation, resulting in decreased nuclear p65 (9). However, it is not clear whether Bay 11-7085 plays the same role in other cell types or what particular cell functions are affected by Bay 11-7085.

The human monocytic leukemia cell line, U937, was derived from a patient with generalized "histiocytic" lymphoma (10). This cell line is a well-established model for studying induction of apoptosis (11–13) and differentiation (14). TF-1 (15) is a factor-dependent human erythroleukemia cell line. PMA[2] can induce these two cell lines into macrophage-like differentiation (14, 15). In this study, we present evidence that Bay 11-7085, at low concentrations (3–10 μM), induces apoptosis, whereas high concentrations (≥20 μM) of this agent cause extensive necrosis, either in the absence or presence of PMA. Bay 11-7085-induced apoptosis occurs through a NF-κB-independent, p38 MAP kinase-dependent mechanism.

MATERIALS AND METHODS

Cell Culture. Human U937 and TF-1 myeloid leukemia cells were grown in suspension in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in the presence (for TF-1) or absence (for U937) of granulocyte macrophage colony-stimulating factor (5 ng/ml).

Antibodies and Reagents. PMA was obtained from Sigma Chemical Co. (St. Louis, MO). Antibodies against phosphorylated IκBα, phosphorylated p38 MAP kinase, phosphorylated MEK1/2, phosphorylated ATF-2, and activated caspase 3 were obtained from New England Biolabs (Beverly, MA). The polyclonal antibodies to total IκBα, IκBβ, MEK1/2, and p38 MAP kinase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-NF-κB antibodies and the Annexin V staining reagents were obtained from BD PharMingen (San Diego, CA). The ApoAlert DNA fragmentation assay kit was purchased from Clontech (Palo Alto, CA). Bay 11-7085 was obtained from Alexis Biochemicals (San Diego, CA). The caspase inhibitor (z-VAD-fmk), MEK inhibitor (PD98059), and p38 inhibitor (SB203580) were purchased from Calbiochem (La Jolla, CA), New England Biolabs, and Alexis Biochemicals, respectively. TNF-α and granulocyte macrophage colony-stimulating factor were bought from R&D systems (Minneapolis, MN).

Preparation of Bay 11-7085 and Cell Treatment. Bay 11-7085 was dissolved in DMSO (Sigma Chemical Co.) as a 40-mM stock solution according to the manufacturer's instruction. Cells were pretreated with Bay 11-7085 for 1 h at 37°C and then treated with or without PMA for the times required. In some experiments, cells were pretreated with z-VAD-fmk, SB203580, or PD98059 for 1 h before the addition of Bay 11-7085. Control cells were treated with the same amount of DMSO. For detection of phosphorylated MEK, cells were starved in serum-free medium overnight before treatment with Bay 11-7085.

Determination of Cell Death and Necrosis. Cell death was determined by trypan blue dye. One volume of trypan blue dye (0.4%) was added to one volume of cell suspension (2×10^6/ml) and incubated for 15 min at room temperature. Thereafter, an aliquot of the cell-trypan blue mixture was loaded in a hemocytometer, and 200 cells were counted under a light microscope to determine the percentage of nonviable cells, which are identifiable because they take up the blue dye.

Cell cytology was examined after cytospins were prepared and stained with Wright-Giemsa. The criteria used to determine necrosis was the loss of membrane integrity and cytoplasmic structure and extensive shrinkage of cells.

Cytology Examination of Nuclear Fragmentation and Flow Cytometric Analysis of DNA Fragmentation. During log-phase growth, cells treated with Bay 11-7085 or PMA or both were collected, and cytospins were prepared by centrifuging aliquots of harvested cell suspensions at 500 rpm for 4 min, after which the cells were stained with Wright-Giemsa. Nuclear fragmentation (cytological evidence of apoptosis) and cell differentiation (14, 15) were determined by examining morphological alterations in both the cytoplasm and nucleus under a light microscope at ×1000.

DNA fragmentation was assayed using the ApoAlert DNA fragmentation assay kit. The assay is based on TdT-mediated dUTP nick end labeling. TdT catalyzes incorporation of fluorescein-dUTP at the free 3′-hydroxyl

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[2] The abbreviations used are: PMA, phorbol 12-myristate 13-acetate; MAP, mitogen-activated protein; MEK, MAP kinase kinase; z-VAD-fmk, z-Val-Ala-Asp-fluoromethyl ketone; TNF, tumor necrosis factor; TdT, terminal deoxynucleotidyl transferase; PI, propidium iodide; PS, phosphatidylserine.

ends of fragmented DNA. Fluorescein-labeled DNA can be detected via flow cytometry. In brief, cells were incubated in six-well plates in the presence or absence of Bay 11-7085 for 24 h, after which the cells were removed from the plates and resuspended in 50 μl of TdT incubation buffer containing a nucleotide mix. TdT-mediated incorporation of labeled dUTP into DNA fragments was performed by incubating the tube at 37°C in a water bath for 60 min. Thereafter, 0.5 ml of PI/RNase A solution was added, and DNA fragmentation was determined by flow cytometry following the manufacturer's instruction.

Preparation of Whole Cell Lysates and Nuclear Extracts. Cell lysates and nuclear extracts were prepared as described previously (16). In brief, cells were collected by centrifugation, lysed in 1× lysis buffer, and protein concentrations of lysates were then measured. To prepare cytoplasmic and nuclear extracts, U937 cells (1×10^7) were washed twice with PBS and once with PBS containing 1 mM Na_3VO_4 and 5 mM NaF. Subsequently, the cells were washed with 2 ml of 1× hypotonic buffer [20 mM HEPES (pH 7.9), 1 mM EDTA, and 1 mM EGTA], and lysed in 1× hypotonic buffer supplemented with 0.2% NP40. Thereafter, the supernatants (cytoplasmic extracts) were transferred to a fresh tube, and the nuclear pellets were collected by centrifugation at 15,000 × g for 10 min and resuspended in 50–100 μl of 1× high-salt buffer [420 mM NaCl, 20 mM HEPES (pH 7.9), 1 mM EDTA, 1 mM EGTA, and 20% Glycerol; Ref. 17], after which they were incubated at 4°C for 30 min under constant rotation. Subsequently, the nuclear extracts were collected by centrifugation and stored at −80°C.

Western Blotting Assays. Lysates containing equal amounts of protein were separated on a 10–15% SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane. The proteins in the membrane were then immunoblotted with antibodies to NF-κB, IκBα, IκBβ, or caspase 3 for 1 h at room temperature or overnight at 4°C. The first antibody-containing solution was then removed and the blot was washed three times in 1× TBST buffer [20 mM Tris, 137 mM NaCl (pH 7.6), and 0.1% Tween 20]. To detect the antibody reaction, the blot was incubated with an HRP-conjugated secondary antibody at room temperature for 1 h, and the product was detected by chemiluminescence (New England BioLabs), as recommended by the manufacturer.

p38 MAP Kinase Activity *in Vitro*. U937 cells were chilled on ice, washed with cold PBS, and lysed in cold lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium PP_i 1 mM β-glycerophosphate, 1 mM PMSF, 1 mM sodium orthovanadate, and 1 μg/ml leupeptin). Cell lysates were centrifuged at 13,000 × g for 10 min at 4°C, and the supernatant fluid fractions were transferred to fresh tubes. Protein concentrations of the lysates were determined by colorimetric assay using the DC protein assay kit, as described in the "Preparation of Cell Lysates and Nuclear Extracts" section. Lysates contained 200 μg of total protein in 500 μl and were immunoprecipitated by incubating at 4°C overnight with anti-phosphorylated p38 MAP kinase (Thr180/Tyr182) monoclonal antibody. Subsequently, protein A-Sepharose was added. The immunoprecipitates were washed three times with cell lysis buffer and twice with kinase buffer (25 mM Tris, 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM sodium orthovanade, and 10 mM $MgCl_2$). The kinase activity assay was performed by incubating the immunoprecipitates in 50 μl of kinase buffer supplemented with 200 μM ATP and 2 μg of ATF-2 fusion protein (New England Biolabs). After 30 min at 30°C, the reaction was stopped by adding 30 μl of 3× SDS sample buffer and loaded onto 12% SDS-PAGE. Phosphorylation of ATF-2 was analyzed by Western blotting using an anti-phosphorylated ATF-2 (Thr71) antibody.

Annexin V Staining. Cells treated with or without Bay 11-7085 were collected and resuspended in 1× binding buffer [0.01 M HEPES/NaOH (pH 7.4), 0.14 mM NaCl, and 2.5 mM $CaCl_2$] at a concentration of 1×10^6 cells/ml. Subsequently, 100 μl of the cell suspension was transferred to a 5-ml tube and Annexin V (5 μl) and PI (10 μl) were added. The cells were incubated at room temperature for 15 min, after which 400 μl of 1× binding buffer was added, and apoptosis, as judged by Annexin V staining, was analyzed by flow cytometry.

Statistical Analysis. All results were expressed as the mean ± SD of data obtained from three to four separate experiments. The statistical significance of differences between group means was determined using Student's *t* test.

RESULTS

Bay 11-7085 Causes Apoptosis and Necrosis in Proliferating U937 Cells

To study the role of IκBα phosphorylation in regulation of cell growth and in cell signaling pathways, Bay 11-7085 was added to U937 cells in log-phase growth, after which cell viability was examined. Surprisingly, as little as 3 μM of the agent caused ~18% cell death, and 10 μM Bay 11-7085 resulted in a significant reduction in cell viability of 32% after 24 h (Fig. 1). Maximal cell killing of 95% was observed at concentrations of 20 μM Bay 11-7085 treatment. Bay 11-7085-induced cell death is not limited to U937 cells. The same concentrations of Bay 11-7085 induced similar patterns of cell death in the TF-1 leukemia cell line (Fig. 1) and its factor-independent subline, TF-1a (18, data not shown).

Next, we carried out a number of experiments to determine characteristics of the cell death induced by Bay 11-7085. U937 cells treated with or without Bay 11-7085 were stained with Wright-Giemsa, and cell morphology was examined under a light microscope (Fig. 2*A*). Normal U937 cells have a relatively homogeneous morphology consisting of blast-like cells of 15–20 μM in diameter. Their nuclear:cytoplasmic ratio is large, with nuclei occupying >80% of the cell cross-section. Addition of Bay 11-7085 at concentrations of 3–10 μM caused nuclear fragmentation, evidenced by appearance of multiple nuclear fragments in nondividing cells. The nuclear fragmentation was dose dependent, with the most nuclear fragments being detected at a concentration of 10 μM of Bay 11-7085. The number of nuclear fragments in a cell varied from 3 to 40, depending on the concentrations of Bay 11-7085, and varied from cell to cell. To confirm that the observed nuclear fragments were indicative of DNA fragmentation, the ApoAlert TdT assay was performed. As seen from Fig. 2*B*, normal cells have low baseline dUTP-FITC fluorescence. As low as 3 μM of Bay 11-7085 caused an ~20% increase in FITC-labeled DNA fragments. The increase of free DNA fragments was proportional to the concentration of Bay 11-7085, with a maximum of dUTP-labeled DNA fragmentation being observed at a concentration of 10 μM, at which a 55% increase in dUTP-labeled DNA fragmentation was detected. However, an additional increase of Bay 11-7085 (20 μM) caused little nuclear or DNA fragmentation (data not shown), but brought about extensive necrosis with cell body shrinkage and the

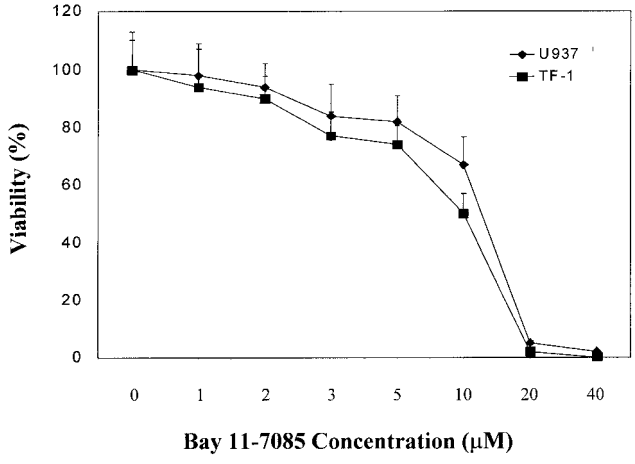

Fig. 1. Bay 11-7085 induces cell death. U937 and TF-1 cells in log phase treated with or without Bay 11-7085 for 24 h were incubated with trypan blue for 15 min at room temperature. A drop of the cell suspension was loaded on a hemocytometer, and stained (*blue*) and unstained cells were counted under a light microscope. Cell viability was calculated as described in "Materials and Methods." Values are means ± SD of three independent experiments.

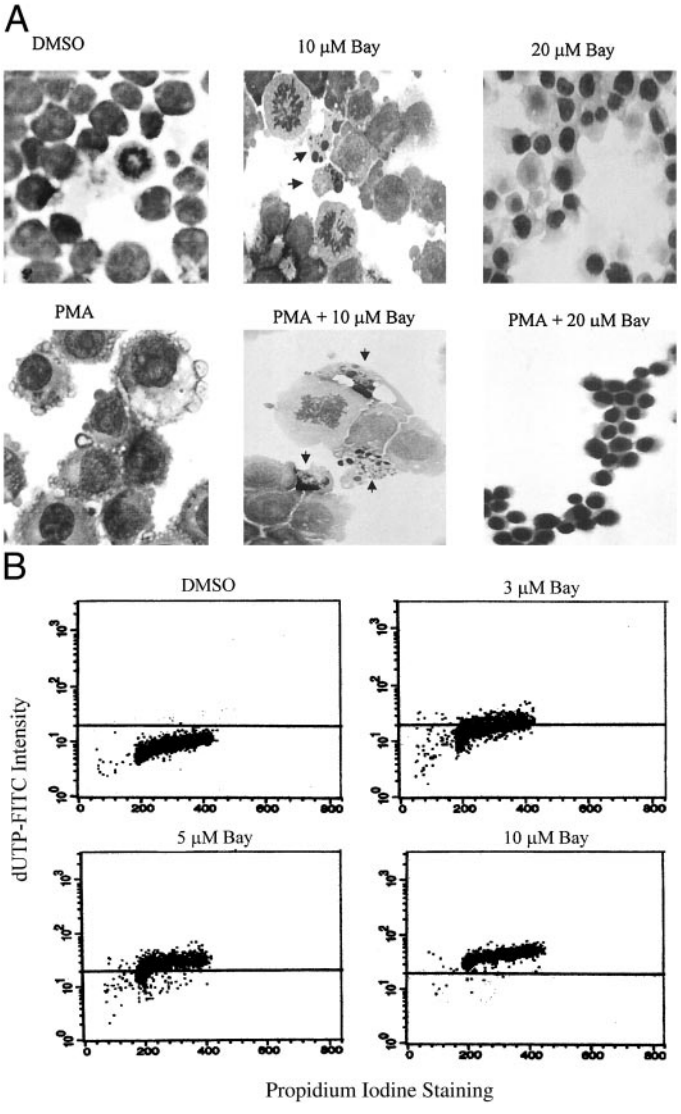

Fig. 2. Bay 11-7085 induces DNA fragmentation. *A*, U937 cells (2×10^6 in 2 ml) were treated *in vitro* with increasing concentrations of Bay 11-7085 for 24 h (*top*) or pretreated with or without Bay 11-7085 for 30 min, and then PMA (10^{-6} M) was added for another 24 h (*bottom*). Thereafter, the cells were collected, and cytospins were prepared as described in "Materials and Methods." The cells were evaluated for nuclear fragmentation and cell morphology after staining with Wright-Giemsa. *B*, U937 cells (2×10^6 in 4 ml) were seeded in six-well plates until they were in log phase of growth. The cells were then treated *in vitro* with increasing concentrations of Bay 11-7085 for 24 h, after which the cells were harvested by centrifugation and washed in PBS. The TdT-labeled DNA fragmentation assay was performed, and DNA fragmentation was analyzed by flow cytometry following the instructions of the manufacturer, as described in "Materials and Methods." *Horizontal axis*, total DNA content; *vertical axis*, dUTP-FITC-labeled DNA fragments. *Bay*, Bay 11-7085. The experiments in *A* and *B* were repeated three times, with similar results.

loss of membrane integrity (Fig. 2*A*). Under the microscope, these cells decreased to approximately one-third of the pretreatment cell size. DMSO (the vehicle for Bay 11-7085) did not induce nuclear/DNA fragmentation or cell death. These results are consistent with the cell viability measured by trypan blue staining, in which 20 μM of Bay 11-7085 caused >95% U937 and TF-1 cell death (Fig. 1).

Bay 11-7085-induced Apoptosis Is NF-κB-independent

Because phosphorylation of IκBα has been shown to be the primary mechanism by which NF-κB is activated (6–8) and cells are protected from apoptotic death, we next investigated whether Bay 11-7085-induced apoptosis is a result of inhibition of NF-κB activation. We show here that proliferating U937 cells express very low levels of phosphorylated IκBα, despite a substantial amount of total IκBα (Fig. 3). Addition of Bay 11-7085 (1–10 μM) did not affect the basal levels of the phosphorylated IκBα or the total amount of IκBα proteins, as detected by Western blotting with antibodies against phosphorylated IκBα and total IκBα, respectively. These results show that Bay 11-7085 is not able to influence the basal levels of phosphorylated IκBα, and the apoptosis induced by Bay 11-7085 seems not to be related to an alteration of IκBα phosphorylation. Next, we investigated the effect of Bay 11-7085 on PMA-induced IκBα phosphorylation and NF-κB activation. PMA (10^{-6} M) rapidly (2 min) induced a marked increase (5-fold) in phosphorylated IκBα. However, this phosphorylation was very brief. By 5 min, the levels of phosphorylation were significantly decreased and returned to basal levels. The low level of phosphorylated IκBα lasted for ~1–2 h, followed by a dramatic increase of phosphorylated IκBα, with a ~8-fold increase by 4 h (Fig. 3). These up-regulated levels were maintained up to 24 h (data not shown). Treatment of U937 cells with PMA also caused a rapid and transient down-regulation of total IκBα, which is apparent at 5 min and lasted ~1–2 h. However, a shorter (2 min) treatment had no significant effect on the level of total IκBα, which is consistent with previous reports (7). In contrast, the levels of IκBβ were not significantly affected by PMA, with only a slight decrease being

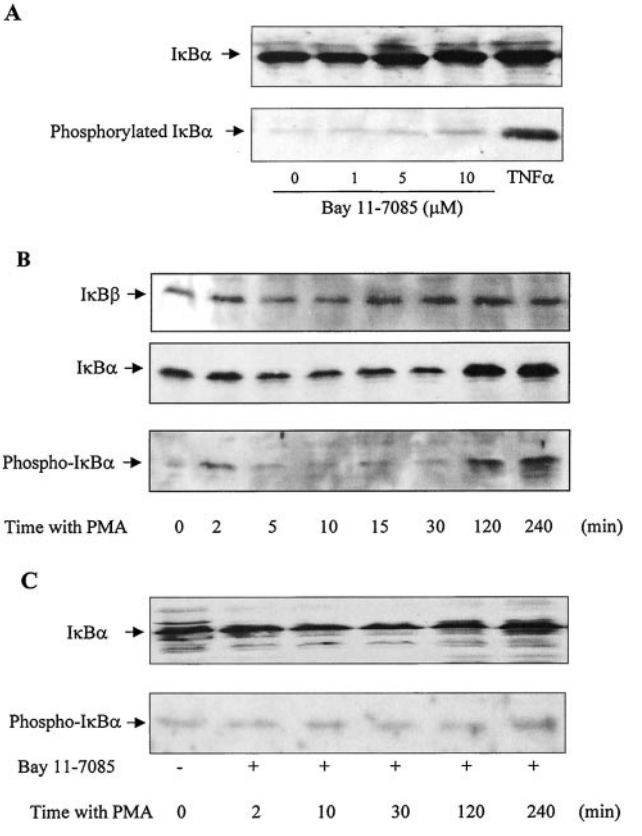

Fig. 3. Effect of Bay 11-7085 on the expression of IκBα and IκBβ in U937 cells treated with or without PMA. *A*, U937 cells treated with or without Bay 11-7085 (10 μM) for 4 h were lysed in lysis buffer. Aliquots of lysates were subjected to 12% SDS-PAGE for analysis of the expression of total and phosphorylated IκBα by Western blotting. Lysates from cells treated with TNFα (20 ng/ml) were used as a positive control. *B*, U937 cells treated with PMA (10^{-6} M) for the times indicated in the absence of Bay 11-7085 were collected by centrifugation and lysed in lysis buffer. Aliquots of the lysates were analyzed by Western blotting with an anti-IκBα, anti-IκBβ, or anti-phosphorylated IκBα antibody. *C*, U937 cells pretreated with or without Bay 11-7085 for 30 min with subsequent addition of PMA (10^{-6} M) for the times indicated were collected and lysed in lysis buffer. Aliquots of the lysates were analyzed by Western blotting and probed with an anti-IκBα or anti-phosphorylated IκBα antibody. Three independent experiments in *A*, *B*, and *C* were repeated, with identical results.

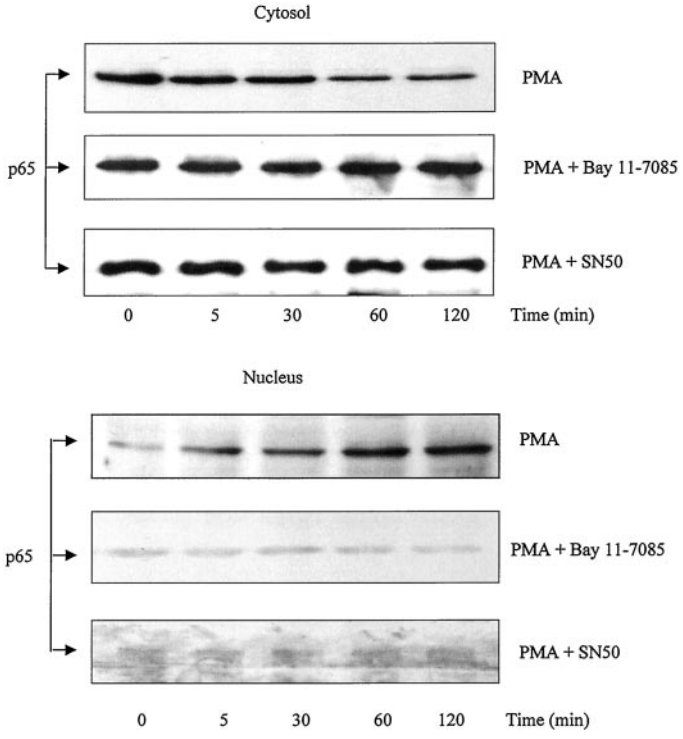

Fig. 4. Bay 11-7085-induced apoptosis is independent of the NF-κB pathway. U937 cells pretreated with Bay 11-7085 (10 μM) or SN50 (100 μM) for 1 h with subsequent addition of PMA (10^{-6} M) for 24 h were collected by centrifugation and lysed in lysis buffer. Cytoplasmic and nuclear extracts were prepared as described in "Materials and Methods." Subsequently, the expression of p65 was detected by Western blot with anti-p65 antibody.

detected during the period of 5–10 min after initiating Bay 11-7085 treatment. U937 cells pretreated with Bay 11-7085 (10 μM) showed complete inhibition of PMA-induced IκBα phosphorylation. Parallel to these changes in the levels of IκBα phosphorylation, PMA also induced macrophage-like differentiation of U937 cells. As shown in Fig. 2, proliferating U937 cells have a relatively homogeneous morphology. U937 cells treated with PMA (10^{-6} M) for 24 h, by comparison, possess a distinctly macrophage-like morphology. Small increases in nuclear size are observed. However, the cytoplasmic portion is greatly increased, with a cross-sectional dimension of 20–35 μM. Nuclear chromatin is more condensed, and no nucleoli are observed. Under the microscope, it is apparent that U937 cells treated with Bay 11-7085 in the presence of PMA showed significant nuclear fragmentation, but there was no effect on PMA-induced differentiation. The degree of nuclear fragmentation is similar to that observed in U937 cells treated with Bay 11-7085 alone, with maximal nuclear fragmentation (~30%) being detected in response to 10 μM of Bay 11-7085. When 20 μM or more Bay11–7085 was added to cells, extensive necrosis was induced, with cell body shrinkage, loss of cell membrane integrity, and loss of cytoplasmic structure being observed.

The NF-κB complex is composed of two proteins of molecular weight M_r 50,000 and 65,000, referred to as p50 and p65, respectively. It has been suggested that p50 is a repressor rather than an activator of κB-dependent transcription *in vitro* (19, 20). Because the p50/p65 combination leads to marked transcriptional activation, whereas p50 alone does not, the transactivation activity of NF-κB seems to be provided primarily by the p65 subunit or at least to require the p65 subunit (21). Therefore, p65 nuclear translocation was investigated in response to PMA and Bay 11-7085. As seen in Fig. 4, in the cells without PMA treatment, p65 was present in the cytosol but not in the nucleus. After addition of PMA, the levels of cytosolic p65 were markedly down-regulated, with a maximal reduction of p65 being observed at 1 h. This decrease of cytosolic p65 was prevented by simultaneous exposure of the cells to Bay 11-7085. In contrast, Bay 11-7085 (10 μM) completely blocked PMA-induced p65 nuclear translocation. On the basis of these data, we thought initially that the effect of Bay 11-7085 on cell apoptosis would be through inhibition of activation of the NF-κB pathway in the cells treated with PMA. To test this possibility, a specific inhibitor of NF-κB translocation into the nucleus, SN50, was added to U937 cells, and the expression of p65 in both cytosol and nucleus was examined by Western blotting. SN50 completely blocked p65 nuclear translocation without inducing nuclear fragmentation, cell death (cell viability was between 95–100%), caspase 3 activation, or phosphatidylserine translocation (by Annexin V staining) at any concentration up to 100 μM (data not shown).

Bay 11-7085-induced Apoptosis Is p38 MAP Kinase-dependent

Activation of MAP Kinases. Earlier observations indicated that Bay 11-7085 caused activation of p38 MAP kinase, which has been linked to its anti-inflammatory function (9). To investigate a possible role of p38 MAP kinase in the regulation of Bay 11-7085-induced apoptosis, we measured the phosphorylation/activation of this kinase in the cells treated with or without Bay 11-7085. As shown in Fig. 5, Bay 11-7085 rapidly induced phosphorylation of p38 MAP kinase. The phosphorylation was persistent, with early detection at 10 min after initiating Bay 11-7085 treatment. Peak activation occurred at 2–3 h and remained elevated even 24 h after the treatment relative to vehicle (DMSO)-treated control cells. Bay 11-7085 had no effect on levels of total p38 MAP kinase protein, because the levels of total proteins detected by anti-p38 MAP kinase antibody remained constant at all time points tested up to 36 h. Bay 11-7085 also induced MEK phosphorylation, although this activation is relatively weak as com-

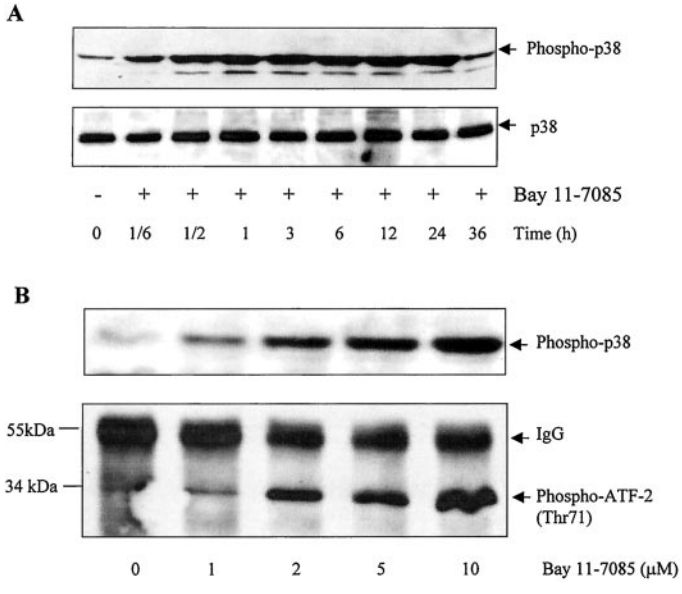

Fig. 5. Effect of Bay 11-7085 on the expression and activity of MAP kinases. *A*, cells were treated for different times with Bay 11-7085 (10 μM) and the expression of phosphorylated p38 MAP kinase was detected by immunoblot using an antibody specific against phospho-p38 MAP kinase. As a control, blot also was probed with an antibody against total (phosphorylated plus nonphosphorylated) p38 MAP kinase. Experiments were repeated three times, with similar results. *B*, cell lysates were precipitated with anti-phosphorylated p38 MAP kinase antibody. The resulting immunoprecipitates were then incubated with an ATF-2 fusion protein in the presence of ATP and kinase buffer. Subsequently, phosphorylation of ATF-2 was analyzed by Western blotting using an anti-phosphorylated ATF-2 antibody. Identical results were obtained in two separate experiments. *Top*, expression of phosphorylated p38 MAP kinase in response to 0–10 μM of Bay 11-7085 treatment; *Bottom*, p38 MAP kinase activity, as measured by the degree of ATF-2 phosphorylation in response to the same concentrations of Bay 11-7085.

pared with phosphorylation levels of p38 MAP kinase (data not shown).

Activated p38 MAP Kinase Induces Phosphorylation of ATF-2. Next, we asked whether the Bay 11-7085 induced phosphorylated p38 MAP kinase was still active functionally. For this purpose, a p38 MAP kinase assay was performed *in vitro*. We selected ATF-2 as a substrate of p38 MAP kinase because this transcription factor has been reported to be involved in the apoptosis process (22, 23). With this method, an antibody specific for phosphorylated p38 MAP kinase (Thr180/Tyr182) was used to immunoprecipitate phosphorylated p38 MAP kinase from cells treated with Bay 11-7085. The resulting immunoprecipitate was then incubated with an ATF-2 fusion protein for 30 min at 30°C in the presence of ATP and kinase buffer. Subsequently, phosphorylation of ATF-2 was measured by Western blotting using an antibody specific for Thr71-phosphorylation-specific ATF-2. The immunoprecipitates obtained with the anti-phosphorylated-p38 MAP kinase antibody from the cells treated with Bay 11-7085 at concentrations of 2–10 μM Bay 11-7085 contained significant kinase activity for ATF-2 phosphorylation, with a 30-fold increase in the kinase activity being detected at a concentration of 10 μM as compared with the cells treated with DMSO only (Fig. 5).

p38 MAP Kinase Inhibitor, but not MEK Inhibitor, Blocked Bay 11-7085-induced Apoptosis. The rapid activation of p38 MAP kinase in response to Bay 11-7085 treatment indicates that p38 MAP kinase might affect the triggering of early events of apoptosis. To test this possibility, we analyzed the effect of the p38 MAP kinase inhibitor SB203580 on caspase activity and Annexin V-binding protein. Both assays have been described as measuring early events of apoptosis. It has been reported that caspase 3 is a key protease that becomes activated during the early stages of apoptosis (24, 25). Activated caspase 3, which is found in cells undergoing apoptosis, consists of multiple subunits, including M_r 19,000, M_r 17,000, and possible other smaller subunits, depending on the activity of caspase 3 and the cell type. These subunits are derived from a M_r 32,000 proenzyme. To determine whether Bay 11-7085 induced caspase 3 activation, Western blot analysis was performed with an antiactivated caspase 3 antibody (Fig. 6*A*). U937 cells in the absence of Bay 11-7085 expressed very low levels of M_r 19,000 and M_r 17,000 caspase 3. A 3-h incubation with Bay 11-7085 up-regulated the expression of activated caspase 3, and the effect was dose dependent. As low as 2 μM of Bay 11-7085 promoted an ~2-fold increase in the levels of M_r 19,000 and M_r 17,000 forms, with a maximal expression (a 5.6-fold increase) of these proteins being detected at a concentration of 10 μM of Bay 11-7085. However, after an additional increase of the agent, the expression of activated caspase 3 was much less and was at basal levels with Bay 11-7085 concentrations of 20 and 40 μM. The specificity of caspase 3 activation was confirmed by the application of caspase inhibitor, z-VAD-fmk. Cells pretreated with z-VAD-fmk (80 μM) completely inhibited Bay 11-7085-induced activation of caspase 3. To determine whether p38 MAP kinase played a critical role in Bay 11-7085-induced apoptosis, U937 cells were pretreated with SB203580 at various concentrations, after which Bay 11-7085 were added and the expression of activated caspase 3 was determined. A concentration of 15 μM SB203580 almost completely inhibited activated caspase 3 (Fig. 6*A*, *middle*) and prevented the phosphorylation of p38 MAP kinase (Fig. 6*A*, *bottom*). The inhibition was dose dependent. Low concentrations ($<$15 μM) of SB203580 has less blocking effect, whereas higher concentrations of SB203580 induced some cytotoxic effect (data not shown). In contrast, PD98059 and z-VAD-fmk had no effect on Bay 11-7085-induced phosphorylation of p38 MAP kinase (Fig. 6*A*, *bottom*).

The membrane phospholipid, PS is a protein that translocates from the inner to the outer leaflet of the plasma membrane during apoptotic

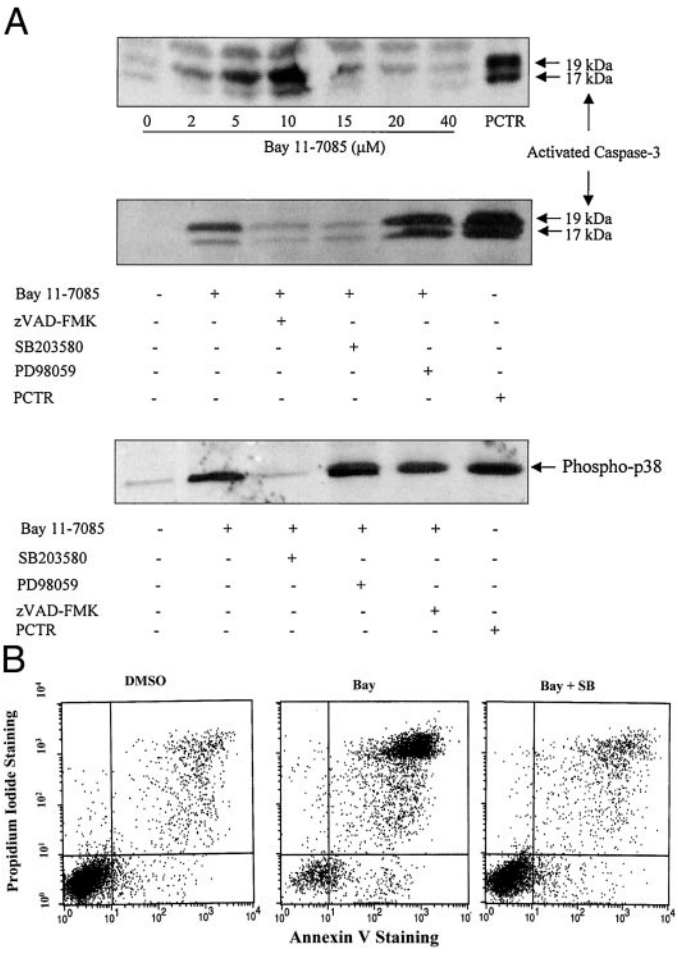

Fig. 6. Effect of kinase or caspase inhibitors on Bay 11-7085-induced apoptosis. *A*, cells were pretreated with SB203580 (15 μM), PD98059 (30 μM), or z-VAD-fmk (80 μM) with subsequent addition of Bay 11-7085 (10 μM) for another 3 h. As a negative control, growing cells were treated with DMSO alone at the same condition. Aliquots of the lysates were analyzed by Western blotting, using antibodies against phosphorylated active caspase 3 (*top* and *middle*) or p38 MAP kinase (*bottom*). *PCTR*, positive control from Jurkat cell extracts treated with cytochrome C (*top* and *middle*) and from anisomycin-treated C6 glioma cells (*bottom*), respectively. The experiments were repeated two times, with similar results. *B*, cells were pretreated with SB203580 or DMSO in 24-well plates for 30 min, after which Bay 11-7085 was added and incubated for 4 h before cells were collected. The cells were then resuspended in 1× binding buffer at a concentration of 1×10^6/ml. Subsequently, 100 μl of cell suspensions were incubated with 5 μl of Annexin V and 10 μl of PI for 15 min at room temperature, as recommended by the manufacturer. Thereafter, 400 μl of 1× binding buffer was added, and Annexin V- and PI-positive populations were analyzed by flow cytometry. *Bay*, Bay 11-7085.

cell death, thereby becoming exposed to the external cellular environment. Annexin V has a high affinity for PS, and binds to cells with exposed PS. As seen from Fig. 6*B*, the majority of U937 cells in the absence of an inducer of apoptosis were located in the *bottom left quarter* in the figure. Therefore, these cells are Annexin V- and PI staining-negative. Bay 11-7085 caused a significant shift of the U937 cells, with a majority of the population (70%) relocated in the *top right quarter*, in which cells are both Annexin V- and PI-positive. If U937 cells were pretreated with SB203580 for 1 h, and then Bay 11-7085 was added, the proapoptotic effect of Bay 11-7085 was prevented. These data strongly suggest that p38 MAP kinase triggers early apoptosis in Bay 11-7085-treated U937 cells. Necrosis induced by Bay 11-7085 was not affected by the inhibition of p38 MAP kinase, because 20 μM of Bay 11-7085 still induced extensive cell death an the loss of cell membrane integrity and cytoplasmic structure in the presence of these inhibitors.

DISCUSSION

Bay 11-7085 has been synthesized as a specific inhibitor of IκBα phosphorylation. It was shown to inhibit cytokine-induced phosphorylation of IκBα, to suppress nuclear translocation of NF-κB, and to transactivate genes regulated by κB promoter elements in endothelial cells (9). In this study, we have found that Bay 11-7085 also induced apoptosis and extensive necrosis in several human leukemia cell lines. These effects of Bay 11-7085 were dose dependent. At low concentrations (≤10 μM), Bay 11-7085 induced apoptosis as indicated by the following observations: (*a*) addition of Bay 11-7085 to U937 cells treated with or without PMA induced nuclear and DNA fragmentation; (*b*) Bay 11-7085 caused PS translocation in the cell membrane and activation of caspase 3; and (*c*) Bay 11-7085 caused apoptotic cell death without affecting cell body size or PMA-induced macrophage-like differentiation. Whereas at a high concentration (≥20 μM), Bay 11-7085 caused extensive cell death by necrosis, significant shrinkage of the cells, loss of cell membrane integrity, and no significant activation of caspase 3, nuclear/DNA fragmentation, or cell differentiation was found. These effects of Bay 11-7085 are similar to the apoptotic and cytotoxic effects of TNFα on some cancer cells (26–28).

By using several approaches, we demonstrated that Bay 11-7085-induced apoptosis is NF-κB pathway-independent. First, in the absence of PMA, U937 cells only express a very low level of phosphorylated IκBα and NF-κB, which was expressed mainly in the cytoplasm. Addition of Bay 11-7085 had no effect on the low basal levels of phosphorylated IκBα or the expression of NF-κB. Second, although Bay 11-7085 inhibited PMA-induced phosphorylation of IκBα and NF-κB nuclear translocation, these changes were not necessarily linked to Bay 11-7085-induced apoptosis and cell death. Direct evidence resulted from the use of SN50, a specific NF-κB inhibitor, which prevents NF-κB nuclear translocation. SN50 pretreatment of U937 cells blocked PMA-induced NF-κB nuclear translocation without inducing any cell death, nuclear/DNA fragmentation, or caspase activation at any concentrations up to 100 μM.

During the process of the apoptosis, Bay 11-7085 rapidly induced phosphorylation of p38 MAP kinase, which remained elevated for up to 24 h, suggesting that p38 MAP kinase might play an important role in the control of the apoptosis. To test this hypothesis, a number of approaches were used. By examining the presence of p38 MAP kinase in nuclei and the functional activity of p38 MAP kinase in nuclear extracts, we demonstrated that p38 MAP kinase is located in both the cytoplasm and nucleus. After Bay 11-7085 treatment, p38 MAP kinase was phosphorylated rapidly, with a majority of the p38 MAP kinase shifting to the nucleus (data not shown). This suggests that at least some of nuclear transcription factors might be activated by p38 MAP kinase. To determine this, we selected ATF-2 as a substrate for p38 MAP kinase function, because ATF-2 has been reported to participate in the apoptosis process (22, 23). ATF-2 is a member of the ATF/CREB family. Our results showed that immunoprecipitates of p38 MAP kinase from cells treated with Bay 11-7085 phosphorylated ATF-2, and that the levels of phosphorylation of ATF-2 directly corresponded to the expression levels of phosphorylated p38 MAP kinase and paralleled the dose of Bay 11-7085 used to treat U937 cells (Fig. 5).

The role of p38 MAP kinase in the apoptosis was demonstrated further by using p38 MAP kinase, inhibitor SB203580. At a concentration of 15 μM, SB203580 completely inhibited the phosphorylation of p38 MAP kinase, caspase 3 activation, and PS translocation. Because PS translocation and caspase 3 activation appeared in the early stages in apoptosis, it suggest that p38 MAP kinase regulates early events of apoptosis, presumably upstream of the caspase pathway. Additional support for this opinion was by the fact that that caspase-specific inhibitor z-VAD-fmk blocked the activation of Bay 11-7085-induced caspase 3 but was not able to prevent phosphorylation of p38 MAP kinase. Although Bay 11-7085 also induced slightly MEK activation, the specific MEK inhibitor, PD98059, was not able to block Bay 11-7085-induced apoptosis or phosphorylation of p38 MAP kinase.

Although previous studies suggested that Bay 11-7085 is not cytotoxic to endothelial cells (9), we showed here that a high concentration of Bay 11-7085 induced extensive necrosis of these human leukemia cell lines. Trypan blue staining and microscope examination confirmed that >95% of the cells were dead at 24 h after initiating high-dose Bay 11-7085 (20 μM) treatment. All these effects occurred not only in U937 cells, but also in the other two human leukemia cell lines tested, TF-1 and TF-1a.

Taken together, Bay 11-7085 induces apoptosis at low concentrations (3–10 μM) and necrosis at higher concentrations (≥20 μM). The activation of p38 MAP kinase may play a critical role in the induction of apoptosis. However, the study reported here does not exclude the anti-inflammatory function of Bay 11-7085 via p38 MAP kinase activation reported previously (9). This agent may have potential for clinical investigation as an inducer of cancer cell death and for the study of apoptosis pathways and signals.

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