The Novel Triterpenoid 2-Cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) Potently Enhances Apoptosis Induced by Tumor Necrosis Factor in Human Leukemia Cells*

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Tumor necrosis factor (TNF) is a potent activator of the nuclear factor-κB (NF-κB) pathway that leads to up-regulation of anti-apoptotic proteins. Hence, TNF induces apoptosis in the presence of inhibitors of protein or RNA synthesis. We report that a novel triterpenoid, 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) inhibits NF-κB-mediated gene expression at a step after translocation of activated NF-κB to the nucleus. This effect appears specific for the NF-κB pathway as CDDO does not inhibit gene expression induced by the phorbol ester 12-0-tetradecanoylphorbol-13-acetate (TPA). CDDO in combination with TNF caused a dramatic increase in apoptosis in ML-1 leukemia cells that was associated with activation of caspase-8, cleavage of Bid, translocation of Bax, cytochrome c release, and caspase-3 activation. Experiments with caspase inhibitors demonstrated that caspase-8 was an initiator of this pathway. TNF also induced a transient activation of c-Jun N-terminal kinase (JNK), which upon addition of CDDO was converted to a sustained activation. The activation of JNK was also dependent on caspase-8. Sustained activation of JNK is frequently anti-apoptotic, yet inhibition of JNK did not prevent Bax translocation or cytochrome c release, demonstrating its lack of involvement in CDDO/TNF-induced apoptosis. Apoptosis was acutely induced by CDDO/TNF in every leukemia cell line tested including those that overexpress Bcl-xL, suggesting that the mitochondrial pathway is not required for apoptosis by this combination. These results suggest that the apoptotic potency of the CDDO/TNF combination occurs through selective inhibition of NF-κB-dependent anti-apoptotic proteins, bypassing potential mitochondrial resistance mechanisms, and thus may provide a basis for the development of novel approaches to the treatment of leukemia.

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The abbreviations used are: TNF, tumor necrosis factor; Act D, actinomycin D; CDDO, 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid; CHX, cycloheximide; ERK, extracellular signal-regulated kinase; IκBα, inhibitor of NF-κB; JNK, c-Jun N-terminal kinase; NF-κB, nuclear factor-κB; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; z, benzyloxycarbonyl; fmk, fluoromethylketone; TPA, 12-0-tetradecanoylphorbol-13-acetate; COX-2, cyclooxygenase-2; iNOS, inducible nitric-oxide synthase; RelA, rel family member p65; MKK4, MAP kinase kinase 4; MEKK1, MAP/ERK kinase kinase 1.
overexpressing Bcl-xL. Hence, this combination may represent an effective therapeutic strategy in the treatment of human leukemia.

**MATERIALS AND METHODS**

**Reagents**—Stock solutions of CDDO (10 mM) were prepared in dimethyl sulfoxide (MeSO) and stored at −20 °C. TNF, CHX, and actinomycin D (act D) were purchased from Sigma and prepared in MeSO at stock concentrations of 10 μg/ml, 15 μg/ml, and 1 mg/ml, respectively. The general caspase inhibitor zVAD-fmk and the caspase-8 selective inhibitor zEtFD-fmk (Enzyme Systems, Livermore, CA) were dissolved in MeSO at stock concentrations of 20 mM and 10 mM, respectively, and then stored at −20 °C. SP600125 (Biomol, Plymouth Meeting, PA) was dissolved in MeSO at a stock concentration of 5 mM and stored at −20 °C. Antibodies were obtained from the following sources: IκBα (9242) polyclonal, phospho-IκBα (9246) monoclonal, and phospho–JNK (9251) polyclonal, Cell Signaling (Beverly, MA); JNK1 (SC-474) (also detects JNK2 polyclonal and p65α/β (SC-109), Santa Cruz Biotechnology (Santa Cruz, CA); cytochrome c (clone TH2.2/C12) monoclonal, BD PharMingen (San Diego, CA); Bax (clone 2D12) monoclonal, Zymed Laboratories Inc. (San Francisco, CA); caspase-8 (AAP-118) polyclonal, StressGen Biotechnologies Corporation (Victoria, BC, Canada); p21WAF1 (clone EA10) monoclonal, Oncogene Research Products (Boston, MA); the D4-GDI polyclonal antibody was developed in this laboratory (19); the Mcl-1 monoclonal antibody and the Bid monoclonal antibody were generously provided by Dr. R. Craig (Dartmouth Medical School, Hanover, NH) and Dr. X. Wang (HHMI, Dallas, TX), respectively. Unless otherwise specified, all other reagents were purchased from Sigma.

**Cell Culture**—ML-1 (kindly provided by Dr. R. Craig Dartmouth Medical School, Hanover, NH), HL-60 (American Type Culture Collection, Manassas, VA), HL-60/Bcl-xL (kindly provided by Dr. K. Bhalla, USF, Tampa, FL), U937, U937/Bcl-xL (kindly provided by Dr. S. Grant, MCV, Richmond, VA), THP-1 (kindly provided by Dr. B. Perez, DHMC, Lebanon, NH) and Jurkat cells were passaged in RPMI 1640 plus 10% fetal calf serum and incubated at 37 °C in 5% CO2/95% humidified air. Cells were treated according to the schedules described in the results. In studies utilizing CDDO, cells were treated for 1 h with CDDO prior to addition of TNF.

**Preparation of Nuclear Extracts**—The preparation of nuclear and cytosolic extracts is a modification of a previously reported procedure (20). Briefly, cells (5 × 10^6) were washed in ice-cold phosphate-buffered saline pH 7.2, resuspended in 250 μl buffer A (10 mM HEPES, pH 7.9, 0.1 mM EDTA, 0.1 mM EGTA, 10 mM KCl, 1.0 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) plus 1% Nonidet P-40 and incubated on ice for 15 min. Samples were centrifuged (15,000 × g for 2 min), and the supernatant (cytosolic fraction) was reserved. The pellet (nuclear fraction) was washed in Buffer A plus 1% Nonidet P-40 and resuspended in boiling Laemmli sample buffer.

**Preparation of Mitochondrial and Cytosolic Fractions**—Samples were obtained using the digitonin permeabilization method (21). Briefly, cells were permeabilized on ice with 8.75 μg of digitonin/10^6 cells in 33 μl of buffer containing 75 mM NaCl, 1 mM Na_2HPO_4, 8 mM
FIG. 3. CDDO inhibits IκBα resynthesis. A, cells were treated with 1 μM CDDO for 1 h followed by the addition of 10 ng/ml TNF for varying times. Proteins were separated by SDS-PAGE and then immunoblotted with antibodies against phospho and total forms of both IκBα and JNK. B, cells were incubated with 10 ng/ml TNF from 0.5–3 h, or cells were pretreated with 1 μM CDDO for 1 h followed by the addition of TNF for 0.5–3 h. Proteins in cell lysates were separated by SDS-PAGE and then immunoblotted with antibodies against phospho and total forms of IκBα. C, cells were treated with 10 ng/ml TNF for 15 min, or cells were treated with either 5 μg/ml CHX or 1 μM CDDO for 1 h followed by the addition of 10 ng/ml TNF for 15 min. Cells were washed, cytosolic and nuclear fractions were harvested, proteins were separated by SDS-PAGE and immunoblotted with antibodies against p65RelA or IκBα.

Na₂HPO₄, and 250 mM sucrose. Cells were incubated for 30 s in ice-cold buffer followed by centrifugation for 1 min at 14,600 × g. The supernatant was then harvested as the cytosolic fraction, and the pellet was boiled for 5 min, sonicated and stored at −20 °C until assayed. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12%), with the exception of blots probed for cytochrome c and Bax where 15% SDS-PAGE gels were used, and transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA). Membranes were subsequently blocked in 5% nonfat milk/Tris-buffered saline (pH 7.4) and 0.05% Tween-20. They were then incubated with appropriate antibody overnight at 4 °C. Membranes were washed in Tris-buffered saline (pH 7.4) and 0.05% Tween-20. They were then incubated for 45 min with either goat anti-rabbit or goat anti-mouse antibody conjugated to horseradish peroxidase (Bio-Rad). Proteins were visualized by enhanced chemiluminescence (Amersham Biosciences).

RESULTS

TNF Activates the NF-κB and JNK Signal Transduction Pathways—We initially characterized the response of ML-1 human myelocytic leukemia cells to TNF. Since TNF is known to activate NF-κB signaling we performed a time course with TNF and measured IκBα phosphorylation and degradation, a requisite sequence of events in the activation of NF-κB (22). In agreement with a previous report (23), ML-1 cells exhibited rapid IκBα phosphorylation and degradation in response to TNF (Fig. 1A). This response was followed by IκBα resynthesis and phosphorylation, both of which depend on activation of the NF-κB signaling pathway (Fig. 1A). In addition to NF-κB activation, TNF transiently stimulates JNK signaling (24). We observed TNF to induce a rapid but transient increase in JNK activity with peak activation occurring at 10 min and then declining by 30 min as assessed with a phospho-specific antibody that recognizes the activated form of JNK (Fig. 1A). The kinetics of JNK dephosphorylation were strongly associated with the kinetics of IκBα resynthesis. This finding is in agreement with recent reports suggesting the involvement of NF-κB-dependent gene transcription in the suppression of JNK activation (17, 18).

TNF Induces Apoptosis in the Presence of Protein or RNA Synthesis Inhibitors—To assess the capacity of ML-1 cells to undergo apoptosis in response to TNF, cells were exposed to TNF in combination with either CHX or act D for 3 h. As an index of apoptotic activity, lysates were immunoblotted for p21WAF1, Mcl-1, and phospho-IκBα.

FIG. 4. Lack of effect of CDDO on TPA-induced gene expression. Cells were treated with 1 μM CDDO and (A) 7.5 μg/ml CHX or (B) 1 μg/ml act D for 1 h followed by either 100 nM TPA or 10 ng/ml TNF for 3 h. Proteins were separated by SDS-PAGE and then immunoblotted with antibodies against p21WAF1, Mcl-1, and phospho-IκBα.
ment with microtubule-interfering agents (14). The correlation of sustained JNK activation and cell death induced by CHX/TNF or act D/TNF is consistent with the findings of others (24).

CHX or act D inhibited TNF-induced IκBα resynthesis (Fig. 1B). The inhibition of IκBα resynthesis correlated with sustained phospho-JNK levels and D4-GDI cleavage suggesting that the synthesis of NF-κB-dependent proteins may be important to maintaining the anti-apoptotic phenotype in TNF-treated cells. Taken together, these results show that apoptosis induced by TNF in combination with CHX or act D is characterized by a sustained activation of JNK and an inhibition of IκBα resynthesis.

TNF Induces Apoptosis in the Presence of CDDO—TNF induces COX-2 expression in a NF-κB-dependent manner (26), whereas CDDO has been reported to inhibit cytokine-induced COX-2 mRNA and protein (6). This suggests that CDDO may negatively regulate NF-κB signaling. Since NF-κB has been implicated in the protection of TNF-treated cells from apoptosis (27), we tested whether CDDO would inhibit NF-κB signaling and increase apoptosis in TNF-treated ML-1 cells. Cells were treated with CDDO alone or in combination with TNF for 3 h and then assayed for JNK phosphorylation and apoptosis as determined by D4-GDI cleavage. CDDO concentrations of as much as 1 μM did not activate JNK and caused no cytotoxicity as assessed by D4-GDI cleavage (Fig. 2). However, JNK activation and apoptosis were detected at a CDDO concentration of 10 μM, the highest dose tested. In contrast, CDDO and TNF co-treatment resulted in a marked increase in JNK phosphorylation and apoptosis at lower concentrations of CDDO (1 μM) (Fig. 2). ERK activation was also measured and found to be unaffected by 1 μM CDDO treatment (data not shown). These data indicate that whereas short term exposure to CDDO or TNF separately are not acutely toxic, their use in combination results in a dramatic increase in the incidence of apoptosis.

CDDO Inhibits TNF-mediated IκBα Resynthesis—To investigate where CDDO inhibits the NF-κB signal transduction cascade, we measured TNF-induced IκBα phosphorylation and degradation over a 10-min timeframe in the presence or absence of CDDO. CDDO had no effect on TNF-induced phosphorylation or degradation of IκBα, suggesting that CDDO may be inhibiting NF-κB signaling at a level downstream of IκBα degradation (Fig. 3A). The impact of CDDO on TNF-induced JNK activation was also measured. CDDO did not affect the kinetics of JNK phosphorylation by TNF over this short time period (Fig. 3A).

As mentioned previously, TNF causes the phosphorylation and degradation of IκBα followed by NF-κB-dependent resynthesis of IκBα. Thus, we tested whether CDDO affects IκBα resynthesis. IκBα was degraded and then resynthesized in response to TNF (Fig. 3B). However, when cells were exposed to TNF in combination with CDDO no resynthesis of IκBα was observed (Fig. 3B).

p65RelA is a member of the NF-κB family and heterodimerizes with other family members and binds to IκBα in an inactive complex in unstimulated cells. Upon incubation with TNF,
IκBα is phosphorylated and degraded by a ubiquitination-dependent process allowing NF-κB translocation into the nucleus where it can modulate gene expression. Because of our finding that resynthesis of the NF-κB-dependent protein IκBα was inhibited by CDDO, we next tested whether CDDO might be interfering with the translocation of NF-κB from the cytosol to the nucleus. ML-1 cells were pretreated with CHX or CDDO for 1 h followed by TNF for 15 min. Nuclear and cytosolic fractions were prepared and immunoblotted for p65RelA expression. TNF treatment caused the translocation of p65RelA from the cytosol to the nucleus, whereas treatment with either CDDO or CHX alone had no affect on p65RelA translocation (Fig. 3C). In cells treated with CDDO or CHX in combination with TNF, there was no inhibition of p65RelA translocation. We also measured IκBα protein levels, which should be predominantly cytosolic. Indeed, we found IκBα to be exclusively cytosolic and, as expected, was degraded in all samples treated with TNF. Taken together these results indicate that CDDO inhibits IκBα resynthesis at a level downstream of p65RelA accumulation in the nucleus.

CDDO Does Not Inhibit TPA-induced Protein Induction—The finding that CDDO did not inhibit TNF-induced p65RelA translocation into the nucleus suggested that CDDO might be having an effect at the level of NF-κB binding to DNA or on subsequent transcriptional activity. Alternatively, CDDO could be acting in a nonselective manner thereby suppressing the synthesis of all proteins. We measured the capacity of CDDO to inhibit protein expression induced by the phorbol ester TPA. Cells were treated with either CDDO or CHX for 1 h followed by TPA or TNF for 3 h. Lysates were prepared, and proteins were separated by SDS-PAGE and then immunoblotted with antibodies against phospho-JNK, total JNK, caspase-8, Bid, and D4-GDI. Proteins from supernatant fractions were prepared using a digitonin lysis procedure and immunoblotted with a monoclonal antibody directed to cytochrome c.
in general but instead exerts an inhibitory effect on protein synthesis that is selective in nature. Presumably, the target of CDDO is at the level of transcription because 1 \( \mu \text{M} \) CDDO has been previously shown to inhibit cytokine-induced iNOS and Cox-2 mRNA (6). However, TPA can also cause mRNA stabilization in addition to enhancing transcription (28). To confirm that TPA was indeed inducing transcription in this model, cells were incubated with act D. We found that whereas CDDO pretreatment had no effect on TPA-induced p21\(^{WAF1}\) and Mcl-1 levels, act D completely blocked the induction of these proteins (Fig. 4B). These results are consistent with the idea that CDDO is a selective inhibitor of transcription.

**CDDO/TNF-induced Apoptosis Involves Caspase-8 Activation**—CDDO at a concentration of 5 \( \mu \text{M} \) has been shown to induce apoptosis after 24 h in cell culture through a caspase-8-dependent mechanism (8). Therefore, we examined whether nontoxic concentrations of CDDO that sensitize ML-1 cells to apoptosis in the presence of TNF also activated caspase-8. ML-1 cells were treated with TNF in the presence or absence of CDDO, and cells were scored for apoptosis. Whereas CDDO or TNF treatment alone displayed no apoptosis after 3 h, the combination of CDDO and TNF caused a rapid induction of apoptosis in 100% of the cell population by 3 h (Fig. 5A). We also observed the conversion of TNF-induced JNK phosphorylation from a weak transient signal to a strong sustained activation (Fig. 5B). This increase in JNK phosphorylation preceded the cleavage of D4-GDI, the activation of caspase-8, and the cleavage of Bid, a pro-apoptotic Bcl-2 family member required for receptor-mediated release of cytochrome c from mitochondria (Fig. 5B) (3).
Whereas these results implicate caspase-8 in the pathway of CDDO/TNF-induced apoptosis, it is possible that caspase-8 is being cleaved and activated by caspase-3 in an amplification loop. This sequence of events occurs in chemical-mediated apoptosis in which Bax translocates to mitochondria, cytochrome c is released into the cytosol, caspase-3 is activated, and consequently caspase-8 becomes activated (31). Therefore, we examined the biochemical events of CDDO/TNF-induced apoptosis using the broad spectrum caspase inhibitor zVAD-fmk. Treatment of ML-1 cells with CDDO/TNF caused the activation of caspase-8, cleavage of Bid, translocation of Bax from the cytosol to the mitochondria, and the release of cytochrome c from the mitochondria to the cytosol (Fig. 6). As shown above, JNK phosphorylation and D4-GDI cleavage also occurred. In cells treated with zVAD-fmk, caspase-8 cleavage, Bid cleavage, Bax translocation, and cytochrome c release were all inhibited suggesting that caspase-8 activation was the upstream signal for apoptosis induced by CDDO/TNF. We also observed that zVAD-fmk blocked CDDO/TNF-induced JNK phosphorylation. These results contrast to chemical-induced apoptosis in which zVAD-fmk does not inhibit JNK activation (14), Bax translocation, or the release of cytochrome c from mitochondria (31).

We next tested whether the caspase-8 selective inhibitor zIETD-fmk could inhibit CDDO/TNF-induced apoptosis. A 3-h incubation with CDDO/TNF resulted in rapid and potent apoptosis of the cell population, whereas cells pretreated with zIETD-fmk were rescued from CDDO/TNF-induced apoptosis in a dose-dependent manner (Fig. 7A). Analogous to zVAD-fmk, zIETD-fmk blocked CDDO/TNF-induced caspase-8 cleavage, JNK activation, cytochrome c release, Bax translocation, and the caspase-dependent cleavage of Bid and D4-GDI (Fig. 7B). These data suggest that the synergistic apoptosis observed with CDDO/TNF utilizes caspase-8 as an upstream initiating caspase. Furthermore, these results demonstrate that the sustained activation of JNK is downstream of caspase-8 activation.

The JNK Inhibitor SP600125 Does Not Inhibit Apoptosis Induced by CDDO/TNF—To test the role of JNK activation in CDDO/TNF-induced apoptosis, we used the novel JNK-selective inhibitor SP600125 (32). This compound suppresses the JNK signaling pathway through inhibition of JNK, and less potently, MKK4. Cells were treated with SP600125 and CDDO for 1 h followed by TNF for 3 h. The phosphorylation of c-Jun, an index of JNK signaling activity, was inhibited by the lowest concentration of SP600125 tested, whereas higher concentrations slightly reduced the phosphorylation and activation of JNK (Fig. 8). However, SP600125 had almost no effect on inhibiting CDDO/TNF-induced caspase-8 activation, D4-GDI and Bid cleavage, or the cytoplasmic loss of Bax and appearance of cytochrome c (Fig. 8). This is in contrast to the caspase-8 inhibitor zIETD-fmk, which completely suppressed Bax translocation and cytochrome c release into the cytoplasm (Fig. 8B). Interestingly, chromatin condensation studies revealed that 5 μM SP600125 afforded no significant protection against apoptosis even though it completely blocked JNK activity (Fig. 8A). Higher concentrations caused a dose-dependent protection against CDDO/TNF-induced apoptosis, but this protection was transient as all cells were apoptotic by 6 h (data not shown).

CDDO/TNF Potently Induces Apoptosis in a Variety of Leukemia Cell Lines—We also investigated the effect of CDDO and TNF on other leukemia cell lines. ML-1, HL-60, U937, Jurkat, and THP-1 cells were treated with TNF in the presence or absence of a 1-h CDDO pretreatment, and apoptosis was measured after 3, 6, or 24 h (Fig. 9). Additionally, we tested two cell lines that have been transfected to stably express the potent anti-apoptotic protein Bcl-xL, as well as K562 cells that have high levels of endogenous Bcl-xL. All cell lines with the exception of K562 exhibited some apoptosis after treatment for 24 h with CDDO alone. TNF treatment resulted in only a modest amount of apoptosis after 24 h in U937, U937/Bcl-xL, Jurkat, ML-1, and THP-1 cell lines with no apoptosis observed in the remaining cell lines. All cell lines except for Jurkat and K562 cells were acutely sensitive to the combination of CDDO/TNF, with nearly 100% apoptosis occurring in the cell population after 3 h. However, Jurkat and K562 cells did display enhanced apoptotic sensitivity after 24 h of treatment. These data suggest that CDDO/TNF is a potent apoptotic combination and that it has the capacity to override the anti-apoptotic effects of Bcl-xL, possibly through bypassing the mitochondrial pathway.

DISCUSSION

The development of novel therapeutic strategies for the treatment of cancer is needed to improve upon existing chemotherapy regimens. CDDO represents such an approach because it has demonstrated efficacy in a variety of cell lines with effects ranging from differentiation to apoptosis (6, 8, 9). Interestingly, whereas CDDO has been reported to induce apoptosis at high concentrations after prolonged exposures, we demonstrate here that the kinetics and apoptotic potency of CDDO is markedly enhanced when combined with TNF. We have investigated the mechanism of this enhanced apoptosis at several levels.

It has previously been shown that CDDO efficiently inhibits cytokine-inducible levels of COX-2, INOS mRNA, and protein (6). This suggested that CDDO may inhibit NF-κB signaling, and this is supported by the observations reported here. We found that CDDO had no effect on the initial phosphorylation and degradation of IκBα after treatment with TNF. Moreover, TNF-dependent translocation of p65RelA into the nucleus still occurred in the presence of CDDO. However, NF-κB-dependent resynthesis of IκBα was abolished by CDDO. These results suggest that the mechanism of action of CDDO is at a step downstream of NF-κB translocation into the nucleus. We also found that CDDO did not inhibit TPA-mediated protein induction, thereby demonstrating that the effect of CDDO is not as a general inhibitor of transcription in this model. A likely target for CDDO is therefore the NF-κB transcriptional machinery. Alternatively, CDDO could be exerting a destabilizing effect on specific mRNA transcripts.

Upon incubation of cells with TNF, a transient activation of JNK was observed. This activation is thought to occur via receptor-mediated activation of ASK1 (33). Recent reports have identified two NF-κB response genes that might be responsible for the rapid down-regulation of this JNK activation during incubation with TNF (17, 18). Consistent with the observation that CDDO inhibits NF-κB-mediated gene expression, we observed that this transient activation of JNK was converted to a sustained activation upon incubation with CDDO/TNF. However, as discussed further below, the sustained activation of JNK was prevented by caspase-8 inhibition. Reports have implicated caspases in the regulation of MEKK1 signaling. Although full length MEKK1 functions as an activating kinase for the NF-κB signaling pathway, this effect is disrupted by caspase-dependent cleavage of MEKK1 allowing cleaved MEKK1 to signal through the MEKK1/MKK4/JNK signaling pathway (34). We predict that MEKK1 cleavage by caspases is responsible for the sustained activation of JNK observed here. Considering that sustained activation of JNK is commonly considered a pro-apoptotic stimulus, we next investigated the mechanism of induction of apoptosis by CDDO/TNF. We found
that CDDO/TNF induced apoptosis via activation of caspase-8 and paralleled cleavage of the pro-apoptotic molecule Bid. This event was associated with the translocation of Bax to the mitochondria and the subsequent release of cytochrome c. Caspase-8 activation was shown to be the initiating event since the addition of zVAD-fmk (broad spectrum caspase inhibitor) or zIETD-fmk (selective caspase-8 inhibitor) prevented Bid translocation, cytochrome c release, and all subsequent apoptotic events.

Apoptosis induced through death receptors is considered to occur by one of two pathways depending upon the cell type (35). In a type 1 cell, caspase-8 directly activates downstream caspase-3, thereby obviating any need for a mitochondrial component. A type 2 cell is dependent on caspase-8-mediated cleavage of Bid which translocates to the mitochondria and, in concert with Bax or Bak, releases cytochrome c (3, 4). Our observations with CDDO/TNF suggest that the mitochondrial pathway is still functional in ML-1 cells. We therefore questioned whether the sustained activation of JNK was contributing to the mitochondrial pathway and thereby required for apoptosis. By selectively inhibiting JNK activity with SP600125 (32) we found that whereas use of this inhibitor effectively inhibited JNK activity, it did not prevent CDDO/TNF-induced apoptosis (Fig. 9). Furthermore, SP600125 did not prevent Bax translocation or cytochrome c release. It therefore appears that sustained JNK activation is dispensable for apoptosis induced by CDDO/TNF.

There currently appears to be two contrasting opinions regarding the role of JNK in death receptor-mediated apoptosis. In agreement with our results, experiments with JNK-deficient mouse embryo fibroblasts have shown that JNK is required for chemical- or UV radiation-induced apoptosis that involves the mitochondrial pathway, whereas JNK is dispensable during activation through the Fas death receptor (15). In contrast it has recently been shown that inhibition of JNK can protect cells from CHX/TNF (17, 18). However, we note that these latter experiments were performed in cells already genetically modified to be defective in NF-κB signaling, hence these cells may be compromised by the loss of other NF-κB response genes.

As we screened additional cell lines, we found that CDDO/TNF was a potent combination in many leukemic cell lines. Furthermore, it became evident that overexpression of anti-apoptotic Bcl-2 family members may be an effective therapy for the treatment of leukemia.

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