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Induction of Apoptosis by Dexrazoxane (ICRF-187) through Caspases in the Absence of c-*jun* Expression and c-Jun NH_2-Terminal Kinase 1 (JNK1) Activation in VM-26-Resistant CEM Cells

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ABSTRACT. Dexrazoxane (ICRF-187) is an inhibitor of the catalytic activity of DNA topoisomerase II (topo II) that does not stabilize DNA–topo II covalent complexes. Here, we examined cytotoxic signaling by ICRF-187 in human leukemic CEM cells and a teniposide (VM-26)-resistant subline, CEM/VM-1. Treatment of CEM and CEM/VM-1 cells with ICRF-187 induced apoptotic cell death characterized by internucleosomal DNA fragmentation, nuclear condensation, and induction of at least caspase-3- and -7-like protease activities (but not caspase 1). Treatment of these cells with Z-Asp-2,6-dichlorobenzoyloxymethyl-ketone, a potent inhibitor of apoptosis, inhibited ICRF-187-induced DEVD-specific caspase activity and apoptosis in a concentration-dependent manner. ICRF-187-induced apoptosis in CEM cells was associated with transient induction of c-*jun* and activation of c-Jun NH_2-terminal kinase 1 (JNK1). However, CEM/VM-1 cells, which were 3-fold more sensitive than CEM cells to ICRF-187 due to a decrease in topo II activity, exhibited ICRF-187-induced apoptosis in the absence of c-*jun* induction and JNK1 activation. These results indicate that catalytic inhibition of topo II by ICRF-187 leads to apoptosis through at least a caspase-3- and -7-like protease-dependent mechanism and suggest that c-*jun* and JNK1 are not required in ICRF-187-induced apoptosis in CEM cells. BIOCHEM PHARMACOL **58**;8:1247–1257, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. topoisomerase II; dioxopiperazine; c-Jun; JNK1; caspases; apoptosis

DNA topo II† is a critical intracellular target for a number of clinically effective antineoplastic agents including etoposide (VP-16), teniposide (VM-26), doxorubicin, and amsacrine (*m*-AMSA). These agents convert topo II into a DNA-damaging enzyme by blocking the DNA religation step, normally catalyzed by topo II, in DNA–topo II covalent complexes [1]. A second emerging group of agents that inhibit the catalytic activity of topo II without stabilizing DNA–topo II covalent complexes include aclarubicin [2], bis (2,6-dioxo) piperazines (ICRF-187, ICRF-193) [3, 4], and merbarone [5]. Aclarubicin prevents topo II from binding to DNA [2], and bisdioxopiperazine derivatives stabilize the enzyme in the form of a closed protein clamp by inhibiting its ATPase activity [6]. Although the well-known action of this second class of topo II-targeting agents is G_2/M arrest and subsequent apoptosis [7–9], the molecular mechanisms of cell killing by apoptosis are less understood.

Apoptosis, or programmed cell death, is a genetically regulated process occurring naturally in response to a variety of biological signals, and results in numerous cellular changes, such as plasma membrane blebbing, cell shrinkage, nuclear condensation, chromosomal DNA fragmentation, and formation of apoptotic bodies [10]. Despite the diversity of apoptosis-inducing stimuli, the initiation and execution stages of apoptosis are common, and involve activation of a cascade of the caspase family of aspartate-specific cysteine proteases that normally cleave interleukin-1, termed interleukin-1β-converting enzyme or ICE [11]. ICE-related proteases have been cloned in mammalian cells and are classified into three groups of caspase families: caspases-1-, -2-, and -3-like [12]. Caspases are activated during apoptosis by proteolytic cleavage [13]. Caspase-3, previously called CPP32/Yama/Apopain, shares a closer homology with Ced-3, the death protease of *Caenorhabditis elegans*, and is activated in many cell types during apoptosis. Once activated, caspases cleave a variety of proteins [14].

JNK (also known as SAPK, for stress-activated protein kinase) is a member of the MAP kinase (mitogen-activated

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† *Abbreviations*: topo II, topoisomerase II; ICE, interleukin 1β-converting enzyme; Ced-3, *Caenorhabditis elegans* cell death protein; AMC, amino-4-methyl-coumarin; DAPI, 4,6-diamino-2-phenylindole; AC-DEVD-MCA, acetyl-Asp-Glu-Val-Asp-4-methyl-coumaryl-7-amide; AC-YVAD-MCA, acetyl-Tyr-Val-Ala-Asp-4-methyl-coumaryl-7-amide; JNK, c-Jun NH_2-terminal kinase; and Z-Asp, Z-Asp-2,6-dichlorobenzoyloxy-methyl-ketone.

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protein kinase) group that includes the extracellular signal-regulated protein kinase (ERK1, 2) and p38 (also known as Mkp2/CBSP) [15]. JNK is activated by phosphorylation at Thr183 and Tyr185 by a dual specificity kinase, MKK4 (mitogen-activated protein kinase kinase) [16–18]. Once activated, JNK phosphorylates the transcription factor c-Jun at Ser63 and Ser73 within its N-terminal transactivation domain [19, 20], and augments its transcriptional activity [21–23]. In addition to phosphorylating c-Jun, JNK has also been shown to phosphorylate the transcription factors ATF2, Elk1 [24–26], and p53 [27]. Several reports have indicated that the JNK pathway may be involved in apoptosis, but its role in apoptosis remains controversial [28–35].

Here, we investigated the biochemical mechanisms of the cytotoxic signaling of ICRF-187 in CEM human leukemic cells and in a VM-26-resistant subline, CEM/VM-1, that is 2- to 3-fold more sensitive than CEM cells to ICRF-187. Also, we examined whether caspase, c-Jun, and JNK1 signaling are involved in ICRF-187-induced cell death. Our results are the subject of this paper.

MATERIALS AND METHODS

Cells and Growth Inhibition Assay

Human leukemic CEM cells and the VM-26-resistant subline CEM/VM-1 have been described previously [36, 37]. The CEM/VM-1 cells are ~50-fold resistant to VM-26 and collaterally sensitive to ICRF-187 by ~2- to 3-fold. Unsynchronized cells were grown in SMEM (Minimum Essential Medium Eagle) (BioWhittaker) supplemented with 10% (v/v) fetal bovine serum (Sigma Chemical Co.), and 2 mM l-glutamine (Life Technologies), at 37° in a humidified chamber of 5% CO_2/95% air. Drug cytotoxicity was assayed in a 48-hr growth inhibition assay as described previously [38]. In brief, the percentage of counts of the drug-treated cells to those of the control cells was determined, and the concentration of drug required to inhibit growth of cells by 50% (IC_{50}) for each drug was calculated. Fold-resistance was calculated by dividing the IC_{50} of the CEM/VM-1 cell line by the IC_{50} of that drug in the CEM parent line. Experiments were done at least three times, and averages of IC_{50} in resistant cell lines were compared with that of the parental line. ICRF-187 was provided by Dr. Ellen Friche (Riggs Hospital, Copenhagen) and by Pharmacia and Upjohn Co.

Nuclear Staining and Cell Viability Assays

After 24 hr of treatment with ICRF-187, CEM and CEM/VM-1 cells were collected by centrifugation at 3000 *g* for 5 min, washed twice with ice-cold PBS, fixed in a solution of methanol:acetic acid (3:1) for 30 min, stained with 1 μg of DAPI/mL for 10 min, and washed with PBS. The nuclear morphology of cells was observed by fluorescence microscopy with a 40X objective. At least 200 cells were scored in three randomly chosen fields for the incidence of apoptosis. The percentage of apoptotic cells was calculated as a ratio of the number of apoptotic cells with fragmented nuclei and condensed chromatin to the total number of cells. The negative images of the stained cells are shown in the figures. Cell viability was determined by the trypan blue dye exclusion assay and on the basis of morphology; cell death was always due to the induction of apoptosis.

DNA Fragmentation Assay

At the indicated times of treatment, 2×10^6 cells were harvested, washed twice with ice-cold PBS, and lysed in 400 μL of lysis buffer [10 mM Tris–HCl, pH 7.4, 20 mM EDTA, pH 8.0, 0.2% (w/v) Triton X-100] on ice for 30 min. After centrifugation, the DNA, released into the cytosol due to DNA fragmentation and contained in the supernatant, was extracted, precipitated, and analyzed by electrophoresis on 1% (w/v) agarose gels as described previously [39].

Northern Blot Analysis

Total cellular RNA was isolated from 10^7 cells by guanidinium thiocyanate-phenol-chloroform extraction [40]. Total RNA (20 μg) was separated on a 7% (v/v) formaldehyde-1.2% (w/v) agarose gel in 1X MOPS buffer [4.2 g of MOPS (morpholine propane sulfonic acid), 2.67 mL of 3 M sodium acetate, 2 mL of 0.5 M EDTA (pH 8), 0.72 mL of 10 N NaOH], and transferred to Hybond membranes (Amersham) by using 0.125 M ammonium acetate. The prehybridization and hybridization were performed as described previously [41]. The c-*jun* cDNA probe (1 kb *Pst*I–*Eco*RI) was provided by Dr. M. Roussel (St. Jude Children's Research Hospital, Memphis). The GAPDH cDNA probe (1.3 kb *Pst*I–*Pst*I) was provided by Dr. A. Jacquemin-Sablon (Institute Gustave Roussy, Villejuif).

Western Blot Analysis

Protein samples were subjected to SDS–PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with 5% (w/v) non-fat dry milk in Tris-buffered saline containing 0.05% (w/v) Tween 20 and incubated with primary antibodies: rabbit anti-Jun from New England Biolabs, Inc., and rabbit anti-JNK1 (C-17) from Santa Cruz Biotechnology, Inc.; the secondary anti-rabbit antibody was from Amersham. Phosphorylation of c-Jun and JNK was detected with anti-phospho-c-Jun Ser63/73 antibody and anti-phospho-JNK Thr183/Tyr185 antibody essentially following the immunoblotting protocol provided by the manufacturer (New England Biolabs, Inc.). Proteins were visualized using the ECL system (Amersham). The intensity of autoradiograms was quantified using a Bio-Rad GS-700 imaging densitometer (Bio-Rad). Appropriate controls for the linearity of the autoradiography were performed in all the experiments. The blots were

TABLE 1. IC_{50} Values of CEM and CEM/VM-1 cells to topoisomerase II inhibitors, resistance, and cross-resistance ratios

	Drug					
	VM-26		Merbarone		ICRF-187	
Cell line	IC_{50} (μM)*	R†	IC_{50} (μM)	R	IC_{50} (μM)	R
CEM	0.03 ± 0.04‡	1	21.5 ± 4	1	53 ± 7	1
CEM/VM-1	1.50 ± 0.3	50	53.9 ± 10	3	21.5 ± 3	0.4

*IC_{50} = 50% inhibitory concentration in a 48-hr growth inhibition assay.
†R = resistance ratio, IC_{50} of CEM/VM-1 cells divided by that of the CEM cells.
‡Values represent means ± SD of 3 separate experiments done in duplicate.

exposed to X-ray film in such a way that the intensity of the bands was in the linear range.

ICE-Like Protease Assay

At the indicated times of ICRF-187 treatment, 2×10^6 cells were washed twice with ice-cold PBS and once in buffer A (50 mM Tris–HCl, pH 7.4, 50 mM β-glycerophosphate, 1 mM EGTA, 5 mM $MgCl_2$, 1 mM dichlorodiphenyltrichloroethane, 1 mM phenylmethylsulfonyl fluoride, 10 μg pepstatin A/mL, 10 μg aprotinin/mL, and 10 μg leupeptin/mL). Cells were then lysed for 15 min on ice in buffer A plus 100 μg digitonin/mL [42]. After 10 min of centrifugation, caspase activity in the supernatant (cytosol) was determined in 50-μL reactions using the caspase-1-specific substrate tetrapeptide reporter AC-YVAD-MCA (Peninsula Laboratories) or the caspase-3- and -7-specific substrate AC-DEVD-MCA (Peptides International) [43]. The aspartate-based ICE inhibitor Z-Asp was purchased from Bachem Inc. Briefly, 10 μg of protein extract was incubated with 100 μM substrate peptide in 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.5, 10% (w/v) sucrose, 10 mM dichlorodiphenyltrichloroethane, 0.1% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate. Fluorescence was measured after 2 hr at 37° with excitation at 360 nm and emission at 460 nm using a fluorescence spectrophotometer (PerSeptive Biosystems, Inc.).

RESULTS

Growth Inhibitory Effects of Topo II Inhibitors

The stable VM-26-resistant CEM cell line (CEM/VM-1), selected in this laboratory by continuous exposure to increasing concentrations of VM-26 followed by cloning, has been characterized previously [36, 37]. These cells were ~50-fold resistant to VM-26 compared with the parental CEM cells and 3-fold cross-resistant to merbarone (Table 1). However, these cells were ~2- to 3-fold more sensitive than the CEM cells to ICRF-187 (Table 1). The resistance to merbarone and the sensitivity to ICRF-187 of CEM/VM-1 may be explained by the differences in the mechanism of action on topo II between these catalytic inhibitors [44]. The ~2- to 3-fold sensitivity of CEM/VM-1 cells to ICRF-187 correlates with the ~2- to 3-fold decrease in topo II activity due to alteration in the phosphorylation of the enzyme in these cells when compared with the parental CEM cells [45]. Similar results also have been observed in etoposide (VP-16) resistant human leukemia K562 cells [46]. Together, these results are consistent with the idea that decreased topo II levels and activity result in increased sensitivity to bisdioxopiperazine derivatives [47].

Induction of Apoptosis by ICRF-187 in CEM and CEM/VM-1 Cells

To characterize the cell death induced by ICRF-187, we examined the morphology of CEM and CEM/VM-1 cells with a fluorescent DNA-binding agent, DAPI. Within 24 hr after ICRF-187 treatment of CEM (500 μM) and CEM/VM-1 (215 μM) cells, ~30% of the cells exhibited condensed and fragmented nuclei (Fig. 1A). Internucleosomal DNA cleavage, a hallmark of apoptosis, was evident by 18 hr (Fig. 1B). ICRF-187 induced apoptosis in a concentration-dependent manner in both cell lines as measured at 24 hr (Fig. 1C). The 215 and 500 μM concentrations of ICRF-187 may seem high, but these levels correspond to 10 times the concentration of the drug that inhibits 50% of the growth of these cells in 48 hr, and they only kill, as indicated above, ~30% of CEM and CEM/VM-1 cells in 24 hr (Fig. 1C). These results indicate that ICRF-187 induced apoptotic cell death in CEM and CEM/VM-1 cells and are consistent with previous reports indicating that the bisdioxopiperazine derivatives ICRF-154 and MST-16 induce apoptosis characterized by internucleosomal DNA fragmentation in thymocytes and lymphoma cells [8, 9].

Induction of Caspase Activities by ICRF-187

Activation of caspases plays a critical role in the execution stage of apoptosis [13, 14]. Therefore, we asked whether caspases are activated during ICRF-187-induced apoptosis in CEM and CEM/VM-1 cells. Cytosolic extracts from these cells treated with ICRF-187 were incubated with the fluorogenic peptide substrates Ac-YVAD-MCA and Ac-DEVD-MCA. The first substrate detects caspase-1-like activity, whereas the second detects caspase-3- and -7-like protease activity [43]. Treatment of CEM and CEM/VM-1 cells with ICRF-187 was accompanied by an ~5- to 6-fold

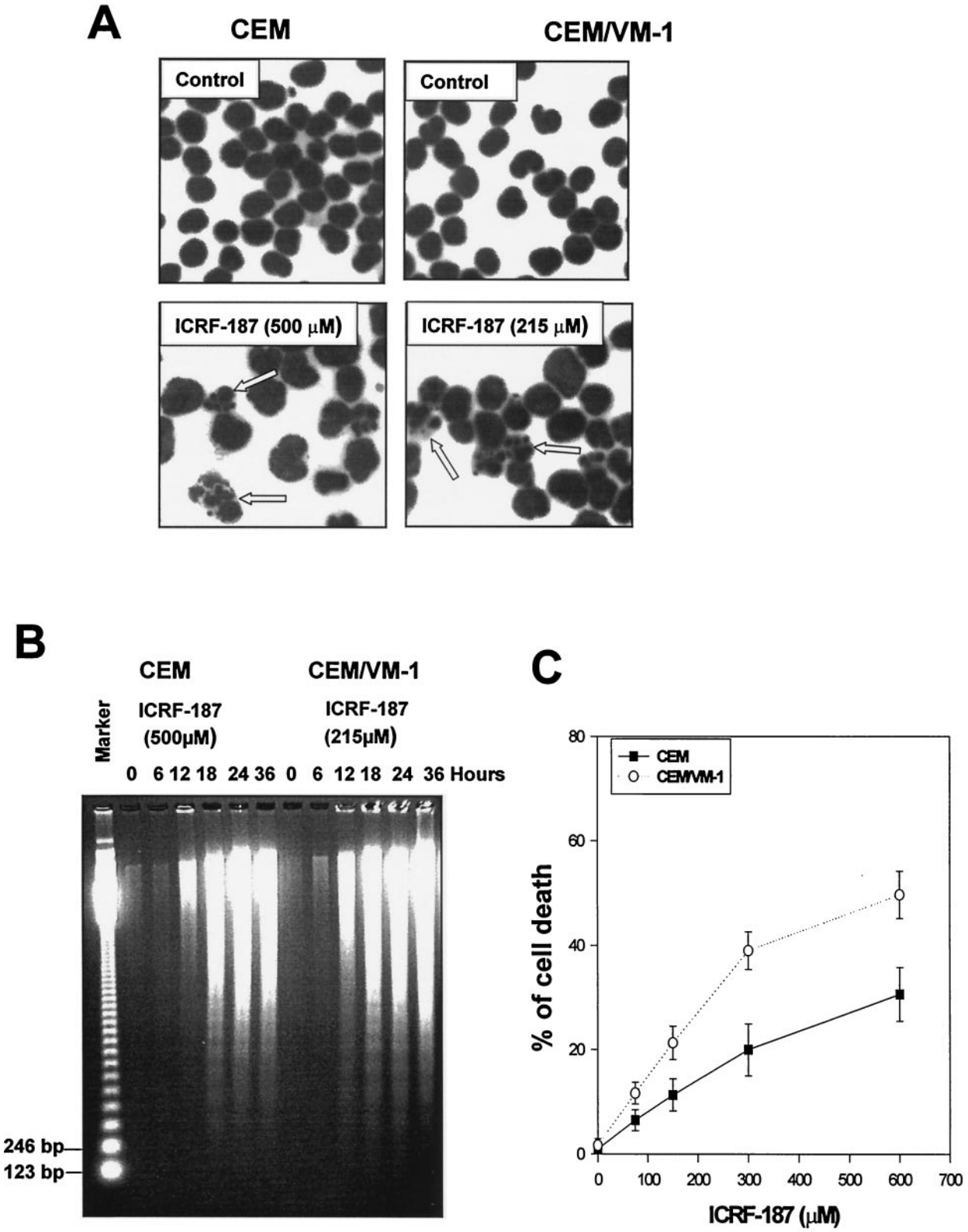

FIG. 1. Induction of apoptosis by ICRF-187 in CEM and CEM/VM-1 cells. (A) Nuclear condensation. CEM and CEM/VM-1 cells were treated with either DMSO as control or 10 times the IC_{50} concentrations of ICRF-187. Cells were harvested, washed with ice-cold PBS, fixed in methanol:acetic acid (3:1), and stained with DAPI, as described in Materials and Methods. The arrows indicate examples of apoptotic cells with condensed or fragmented nuclei. (B) Internucleosomal DNA cleavage. Cells (~2- to ~3 × 10^6) were treated with ICRF-187 for the indicated times. Cellular DNA was extracted and analyzed by 1% (w/v) agarose gel electrophoresis as described in Materials and Methods. Marker: 123-bp ladder marker. (C) Percentage of cell death following treatment of cells with increasing concentration of ICRF-187 for 24 hr. Values are means ± SD of three independent experiments.

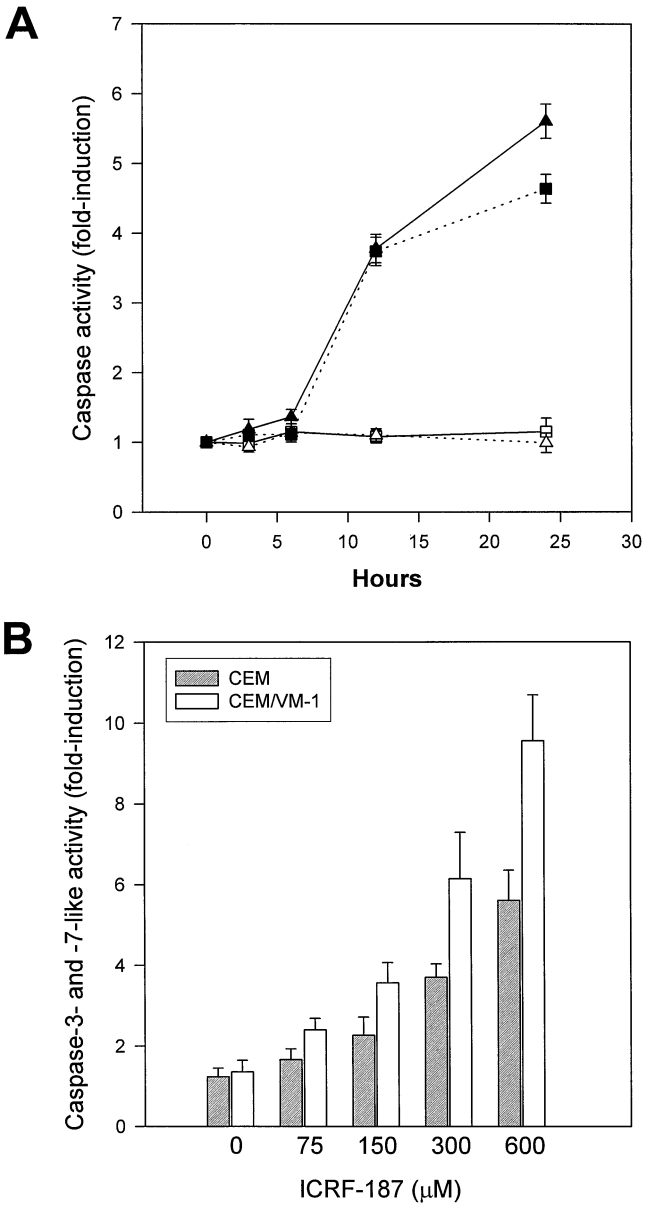

FIG. 2. Caspase activity in CEM and CEM/VM-1 cells following ICRF-187 treatment. (A) Activation of caspase-1- (open symbols) and caspase-3- and -7-like protease (closed symbols) following treatment of CEM (□; ■) and CEM/VM-1 (△; ▲) cells with 500 and 215 μM ICRF-187, respectively. (B) Concentration–response activation of caspase-3- and -7-like protease activity following ICRF-187 treatment at 24 hr. Values are means ± SD for three independent experiments, expressed relative to untreated controls.

increase in caspase activity that cleaved Ac-DEVD-MCA within 12–24 hr (Fig. 2A). No increase in caspase activity that cleaved Ac-YVAD-MCA was observed during ICRF-187 treatment (Fig. 2A). Ac-DEVD-MCA cleavage also increased in a concentration-dependent manner (Fig. 2B). These results indicate that at least caspase-3- and -7-like proteases, but not caspase-1, are activated during ICRF-187-induced apoptosis in both cell lines.

Inhibition of ICRF-187-Induced Apoptosis by Z-Asp

The above results indicated that at least caspase-3- and -7-like proteases are activated during ICRF-induced apoptosis. To confirm that this is indeed the case, we examined the effect of Z-Asp, an aspartate-based ICE inhibitor that prevents apoptosis caused by various types of cytotoxic agents [48, 49], on apoptosis induced by ICRF-187 in CEM and CEM/VM-1 cells. As shown in Fig. 3, Z-Asp was able to inhibit ICRF-187-induced DEVD-specific caspase activity and cell death in a concentration-dependent manner in both cell lines. These results provide strong support to the above data, suggesting that at least caspase-3- and -7-like proteases are key mediators of the apoptotic-signaling pathway induced by ICRF-187 in both drug-sensitive and -resistant CEM cells.

Induction of c-jun Gene Expression by ICRF-187 in CEM but Not in CEM/VM-1 Cells

Earlier work from this laboratory [41] demonstrated an association between c-*jun* proto-oncogene expression and apoptosis induced by the cleavable complex DNA–topo II stabilizing drug VM-26 in both CEM and CEM/VM-1 cells and most recently by merbarone in CEM cells [39]. We asked here whether ICRF-187 did the same. As shown in Fig. 4, treatment of CEM cells with 500 μM ICRF-187 induced a transient mRNA expression of c-*jun*, with maximum induction at 12 hr, followed by down-regulation by 24 hr and decrease to the base level at 48 hr (data not shown). The induction of c-*jun* in CEM cells also was increased in a concentration-dependent manner (Fig. 4C). By contrast, c-*jun* expression was not observed in CEM/VM-1 cells during ICRF-187 treatment over the same time period (Fig. 4, A and B). Importantly, concentrations of ICRF-187 up to 600 μM had little or no effect on the induction of c-*jun* expression. ICRF-187 also induced *jun*B and *jun*D in CEM cells but not in CEM/VM-1 (data not shown). It is worth noting that the lack of c-*jun* induction in CEM/VM-1 cells is not related to an alteration in the c-*jun* gene, since it can be induced by VM-26 in these cells [41]. This is seen more clearly in the right side of the panels in Fig. 4A, in which CEM and CEM/VM-1 were treated with equitoxic concentrations of VM-26.

Consistent with c-*jun* gene expression, ICRF-187 induced an increase in c-Jun protein and phosphorylation at Ser63 in a time- and concentration-dependent manner in CEM but not in CEM/VM-1 cells (Fig. 5). However, the increase in c-Jun protein concentration may mask the increase in its phosphorylation. The phosphorylated form of c-Jun in Fig. 5A, which migrates with a molecular mass of 39 kDa [indicated by star symbol (*)] to distinguish it from the nonspecific bands, was induced by ICRF-187. These bands correspond to the phosphorylated form of c-Jun induced by VM-26 treatment, seen in both cell lines (Fig. 5A). These results support the mRNA induction of c-*jun* in CEM but not in CEM/VM-1 cells and suggest that

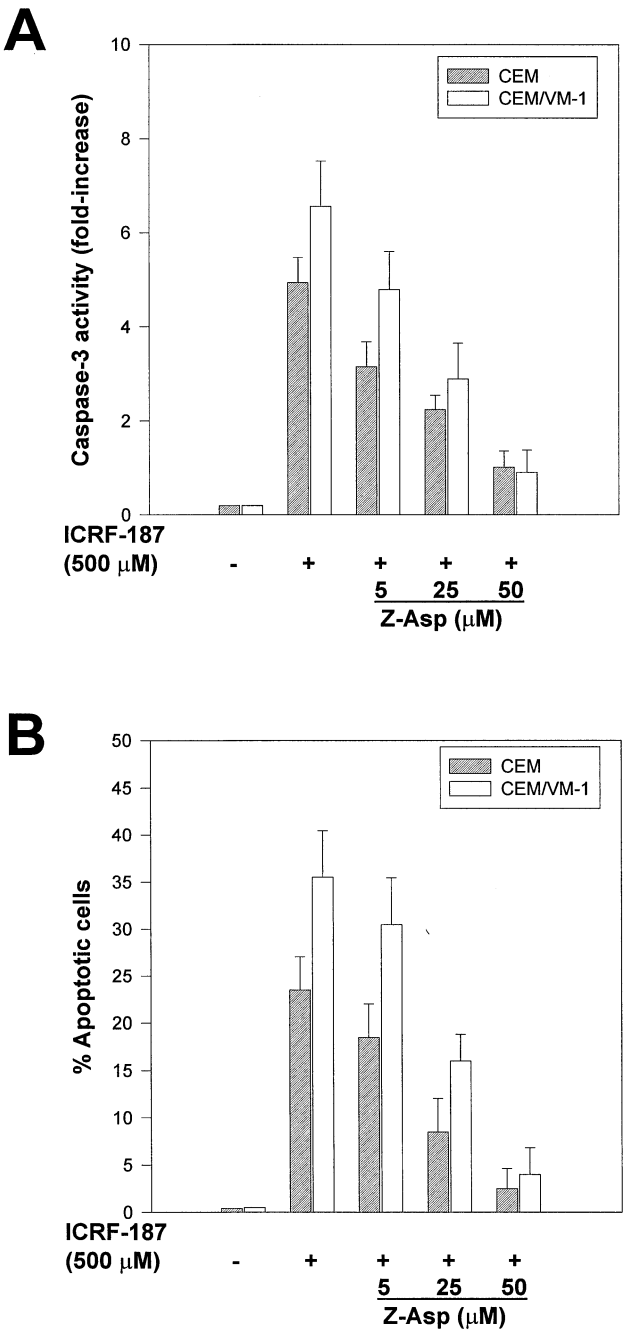

FIG. 3. Effects of Z-Asp on caspase cleavage of DEVD and apoptosis induced by ICRF-187 in CEM and CEM/VM-1 cells. (A) Abrogation of ICRF-187-induced caspase activities by Z-Asp. Cells were treated with ICRF-187 (500 μM), Z-Asp (50 μM), or with increasing concentrations of Z-Asp followed by ICRF-187 treatment. Caspase activities were assayed as described under Materials and Methods. (B) Inhibition of ICRF-187-induced apoptosis by Z-Asp. At 24 hr, 2 × 10^6 of treated and untreated cells were harvested, and the percentage of apoptotic cells (with condensed or fragmented nucleus) was determined by DAPI staining as described in Materials and Methods. Values are means ± SD for three independent experiments. The percentage of apoptotic cells was calculated as described in Materials and Methods.

apoptosis induced by ICRF-187 in CEM/VM-1 cells is not mediated through c-*jun* expression.

Activation of JNK1 by ICRF-187 in CEM but Not in CEM/VM-1 Cells

JNK, also called stress-activated protein kinase (SAPK), has been shown to be activated in response to a variety of apoptotic stimuli [28–35], suggesting that JNK may regulate early molecular events leading to apoptosis. JNKs are activated by phosphorylation at Thr183 and Tyr185 by the dual specificity enzyme SEK/MKK4 [16–18]. Once activated, JNKs phosphorylate c-Jun at Ser63 and Ser73 within its NH_2-terminal activation domain, and augment its transcriptional activity and expression [19–23]. To better understand the basis for the lack of c-*jun* expression in CEM/VM-1 cells during ICRF-187 treatment, we examined JNK1 activation during ICRF-187 treatment in these cells. As shown in Fig. 6, equitoxic concentrations of ICRF-187 caused persistent JNK1 activation in a time- and concentration-dependent manner in CEM but not CEM/VM-1 cells. JNK1 activation in CEM cells was not due to an increase in JNK1 protein concentration. It is worth noting that the *JNK1* gene is not altered in CEM/VM-1 cells since treatment of these cells with VM-26 activated JNK1, as indicated in the right side panels for each cell line in Fig. 6A. These results suggest not only that the lack of c-*jun* expression in CEM/VM-1 cells is due to a lack of a cellular signal upstream of JNK1 but also that JNK1 and c-*jun* are not required in apoptosis induced by ICRF-187 in CEM/VM-1 cells.

DISCUSSION

Previous studies have indicated that the bisdioxopiperazine derivatives ICRF-154 and MST-16, which inhibit the catalytic activity of topo II without stabilizing DNA–topo II covalent complexes, induce apoptosis characterized by internucleosomal DNA fragmentation in thymocytes and lymphoma cells [8, 9]. However, the biochemical and molecular mechanisms by which these agents induce apoptosis remain unknown. In this study, we used human leukemic CEM cells and a VM-26-resistant subline, CEM/VM-1, that is ~2- to 3-fold more sensitive than CEM cells to ICRF-187 due to an ~2- to 3-fold decrease in topo II activity and phosphorylation, to study the biochemical mechanisms of apoptosis induced by the dioxopiperazine derivative ICRF-187. Consistent with previous observations, we found that ICRF-187 exerts its cytotoxic effect by induction of apoptosis in CEM and CEM/VM-1 cells alike. The apoptosis induced by ICRF-187 was characterized by nuclear condensation and internucleosomal DNA fragmentation. Further, treatment of these cells with ICRF-187 caused activation of at least caspase-3- and -7-like proteases, but not caspase-1. Treatment of CEM and CEM/VM-1 cells with Z-Asp, an aspartate-based ICE inhibitor that prevents apoptosis caused by various types of cytotoxic

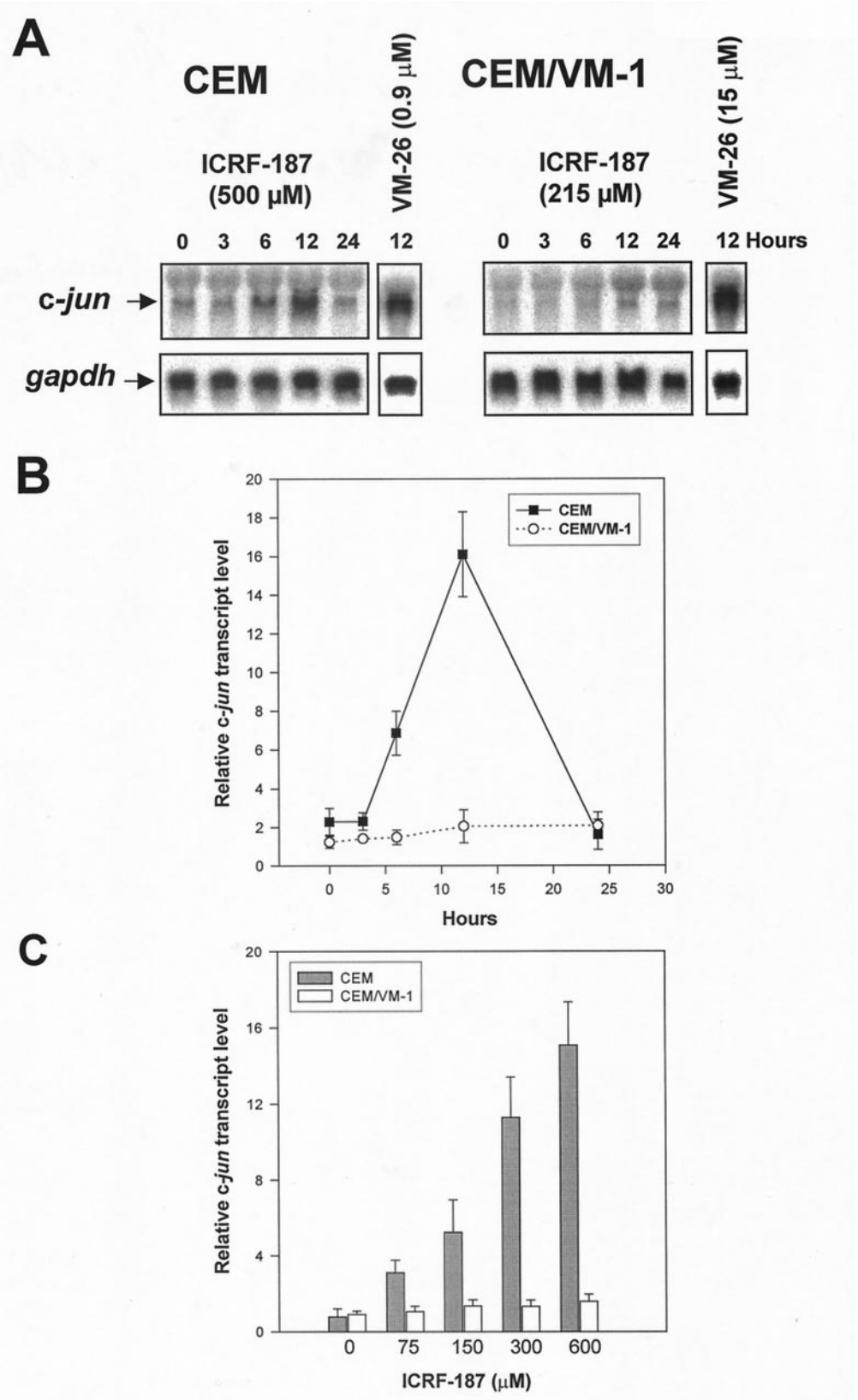

FIG. 4. Effects of ICRF-187 treatment on *c-jun* gene expression in CEM and CEM/VM-1 cells. (A) Expression of *c-jun* following ICRF-187 and VM-26 treatment. Total RNA (20 μg) was extracted at the indicated time of treatment, and hybridized to a ^{32}P-labeled cDNA probe as indicated in Materials and Methods. Shown are representative blots from three separate experiments. (B) Quantitation of *c-jun* levels following ICRF-187 treatment. Levels of *c-jun* transcripts were quantitated by densitometric scanning. (C) Concentration–response of *c-jun* induction at 12 hr after ICRF-187 treatment. Values are means ± SD for three independent experiments.

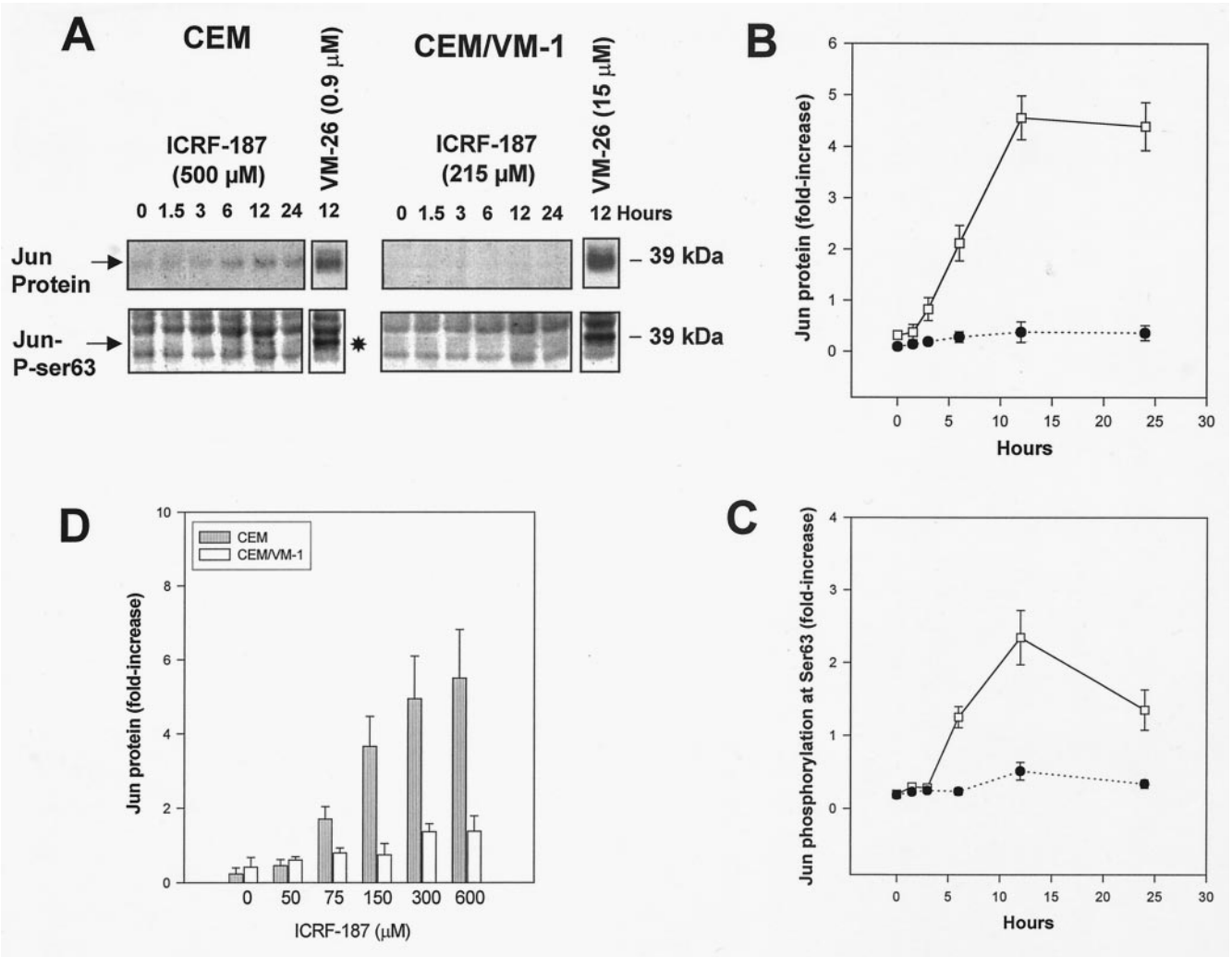

FIG. 5. Production of c-Jun protein and its phosphorylation at Ser63 following ICRF-187 treatment. (A) Whole cell extracts (250 μg) were prepared following ICRF-187 and VM-26 treatment and analyzed by western blot as described under Materials and Methods. Shown are representative blots of three separate experiments. (B and C) Quantification of c-Jun protein bands and c-Jun bands corresponding to the phosphorylated form of c-Jun, indicated by the star symbol (*), following treatment of CEM (□) and CEM/VM-1 (●) cells in panel A, relative to the value for the 0-hr time point. Each point represents the mean ± SD of three separate experiments. (D) Concentration–response of c-Jun protein increase at 12 hr following ICRF-187 treatment. Values represent the means ± SD of three independent experiments.

agents [48, 49], inhibited ICRF-187-induced DEVD-specific caspase activity (caspase-3- and -7-like proteases) and apoptosis, indicating for the first time that apoptosis induced by ICRF-187 involves at least a caspase-3- and -7-like protease-dependent mechanism. Work from this laboratory indicated that merbarone-induced apoptosis involves induction of caspase-3- but not caspase-1-like protease activity [39], as has been shown for VP-16-induced apoptosis [50]. Together, these data suggest that the catalytic and the DNA-damaging topo II inhibitors share at least the activation of caspase-3- and -7-like proteases. Further, our data suggest that DNA cleavage by DNA–topo II covalent complex stabilizing agents is not essential for induction of caspases and apoptosis.

Recent reports have indicated that JNK1 positively regulates VP-16-induced apoptosis in human myeloid leukemic U937 cells by activating a Z-Asp-sensitive ICE/CED-3-like protease [51]. Furthermore, the abrogation of JNK1 activation by 2-deoxyglucose inhibits apoptosis induced by these agents in U937 cells [52]. However, JNK1 fails to correlate with apoptosis in other systems. In B cells, JNK1 appears to play a protective role [28], and in the case of detachment-induced apoptosis in MDCK cells neither JNK1 nor p38 activation is sufficient to induce apoptosis [32]. Since ICRF-187 induced apoptosis in CEM/VM-1 cells in the absence of c-*jun* and JNK1 induction and since VM-26 induced those genes in both cell lines, we suggest the presence of a unique ICRF-187 cellular signaling pathway that is upstream of JNK1 and c-*jun* and is not shared by VM-26. Moreover, our data suggest that this ICRF-187-sensitive pathway is altered in the mutant CEM/VM-1 cells, and that JNK1 and c-*jun* are not required for ICRF-187-induced apoptosis in those cells. In addition, our results showing activation of at least caspase-3- and -7-like

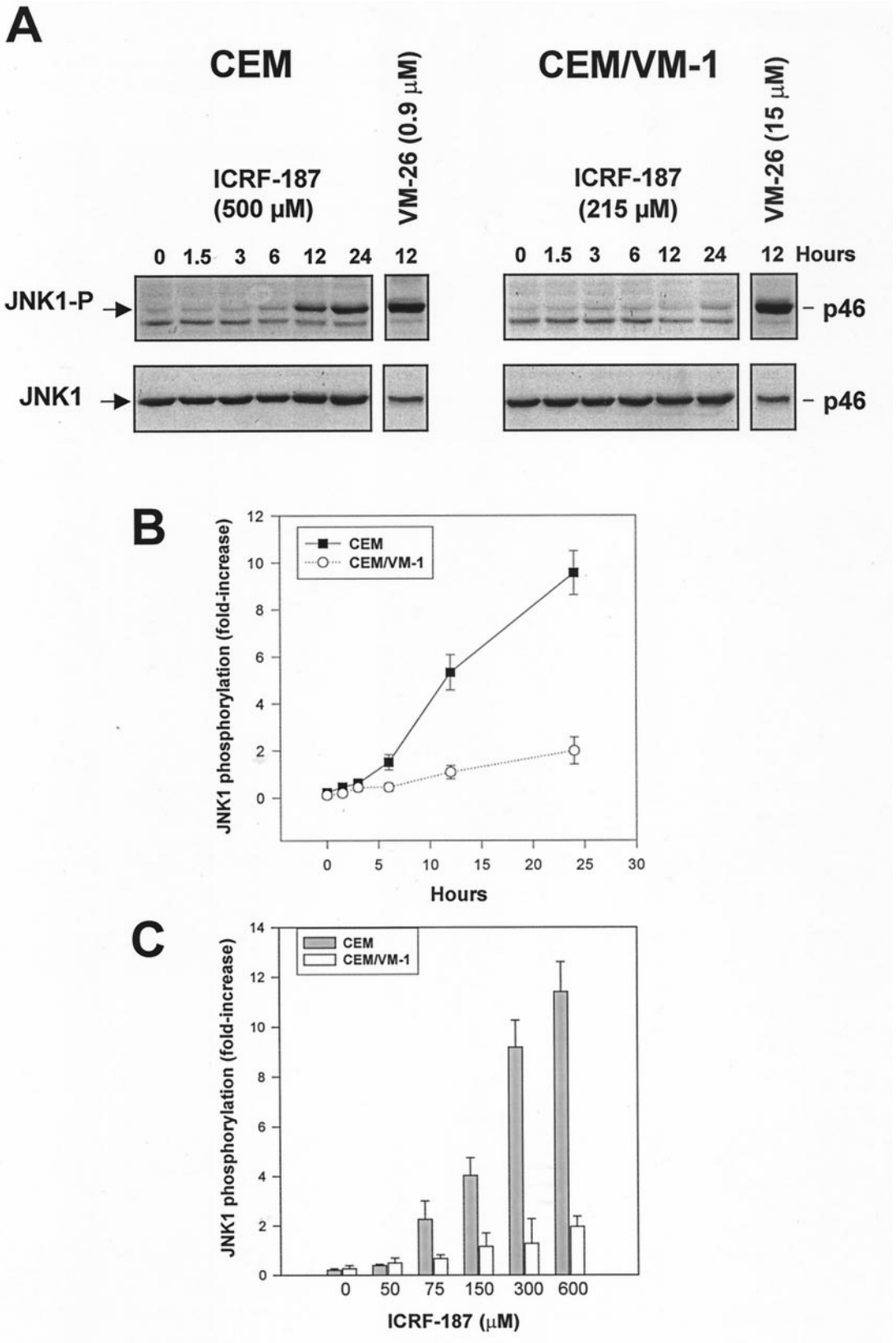

FIG. 6. Activation of JNK1 following ICRF-187 treatment in CEM and CEM/VM-1 cells. (A) Western blot analysis of JNK1 activation following ICRF-187 and VM-26 treatment. Activation of JNK1 was measured via phosphorylation at Thr183 and Tyr185 using a phospho-specific rabbit polyclonal antibody. See Materials and Methods for details. Shown are representative blots of three separate experiments. (B) Quantitation of JNK1 phosphorylation levels in panel A, relative to the value for the 0-hr time point. Each point represents the mean ± SD of three separate experiments. (C) Concentration–response of JNK1 activation by phosphorylation at 24 hr following ICRF-187 treatment. Values represent the means ± SD of three independent experiments.

proteases by ICRF-187 in the absence of c-*jun* and JNK1 induction in CEM/VM-1 cells suggest that JNK1 and c-*jun* are not upstream regulators of caspase-3-like protease in these cells.

In summary, the present data indicate that ICRF-187, a catalytic inhibitor of topo II that does not stabilize the DNA–topo II covalent complex, induces apoptosis in human leukemic CEM and CEM/VM-1 cells through activation of at least caspase-3- and -7-, but not caspase-1-like protease activity, and that apoptosis in CEM/VM-1 cells does not require c-*jun* and JNK1 signaling. Further studies with CEM and CEM/VM-1 cells transfected with cDNAs encoding JNK1 and c-Jun or their dominant negative forms, may help in exploring what role, if any, JNK1 and c-Jun signaling may play in the cellular response to ICRF-187.

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