Combretastatin-A4 Prodrug Induces Mitotic Catastrophe in Chronic Lymphocytic Leukemia Cell Line Independent of Caspase Activation and Poly(ADP-ribose) Polymerase Cleavage[1]

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ABSTRACT

We have previously reported that combretastatin-A4 prodrug (CA4P), an antitubulin/antiangiogenic agent isolated from the South African willow tree *Combretum caffrum*, induced cell death primarily through mitotic catastrophe in a panel of human B-lymphoid tumors. In this study, we investigated the molecular aspects of the mitotic catastrophe and whether or not it shares the same pathways of apoptosis. For this we studied the effect of CA4P on selected markers of apoptosis [caspases 9 and 3, poly(ADP-ribose) polymerase (PARP), bcl-2, and bax] and G_2-M protein regulators (p53, MDM2, 14-3-3σ, GADD45, cdc2, cdc25, chk1, wee1, p21, and cyclin B1). The chronic lymphocytic leukemia cell line WSU-CLL was used for this purpose. Western blot analysis showed that 24 h of CA4P (5 nM) exposure induces caspase 9 activation and PARP cleavage. However, the addition of Z-Val-Ala-Asp-fluoromethylketone (a general caspase inhibitor) or Z-Leu-Glu(OMe)-His-Asp(OMe)-CH_2F (a caspase 9 inhibitor) before CA4P treatment did not block cell death. No change in bcl-2 or bax protein expression was observed. Exposure of WSU-CLL cells to 4 and 5 nM CA4P was associated with overproduction of total p53 and no dramatic change in MDM2, 14-3-3σ, GADD45, the cyclin-dependent kinase cdc2, its inhibitory phosphorylation, the cdc2-inhibitory kinase (wee1), chk1, or cdc25 hyperphosphorylation. The overaccumulation of p21 and cyclin B1 protein was obvious at 24 h. Furthermore, CA4P treatment showed an increase in the expression of a marker of mitosis (mitotic protein monoclonal-2 antibody) and an overaccumulation of the cyclin B in the nucleus. Our findings suggest that CA4P induces mitotic catastrophe and arrest of WSU-CLL cells mostly in the M phase independent of p53 and independent of chk1 and cdc2 phosphorylation pathways. Apoptosis is a secondary mechanism of death in a small proportion of cells through activation of caspase 9 and PARP cleavage. The two mechanisms of cell death, *i.e.*, mitotic catastrophe and apoptosis, are independent of each other in our model.

INTRODUCTION

We have shown previously that CA4P[3] induces cell cycle arrest in G_2-M and morphological features of mitotic catastrophe in B-cell tumors (1). Combretastatin-A4 is a natural product derived from the bark of a South African willow tree, *Combretum caffrum* (2). It induces mitotic arrest by inhibiting microtubule assembly and has high affinity for tubulin binding (2, 3). It also has selective toxicity for proliferating endothelial cells *in vitro* and induces vascular shut down in tumor models *in vivo* (4, 5).

CLL is the most common form of leukemia in the United States, accounting for approximately 30% of all of the cases (6), and it results from clonal expansion of small B-lymphocytes. Despite the use of several agents such as chlorambucil and cyclophosphamide, various combination chemotherapies such COP (cyclophosphamide, vincristine, and prednisone) or CHOP (cyclophosphamide, vincristine, prednisone, and doxorubicin), or new therapeutic modalities such as nucleoside analogues and bone marrow transplantation, no curative therapy was found for CLL.

Mitotic catastrophe is one response of cancer cells to DNA damage (7). Until now, it was defined mostly by the morphology of cells (giant, multinucleated); however, the mechanism by which cells undergo mitotic catastrophe after DNA damage remains unclear. Some authors suggest that mitotic catastrophe shares some common pathways with apoptosis, whereas others believe it is independent of apoptosis. Cells in mitotic catastrophe have mitotic spindle formed, mitotic-specific phosphoproteins present, and cyclin B/cdc2 complex strongly expressed, whereas these characteristics were not found in cells undergoing apoptosis (8). Others have demonstrated that spontaneous pre-

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[3] The abbreviations used are: CA4P, combretastatin-A4 prodrug; PARP, poly(ADP-ribose) polymerase; MPM-2, mitotic protein monoclonal-2; CLL, chronic lymphocytic leukemia; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; PI, propidium iodide; Z-VAD-fmk, Z-Val-Ala-Asp-fluoromethylketone; Z-LEHD-fmk, Z-Leu-Glu(OMe)-His-Asp(OMe)-CH_2F.

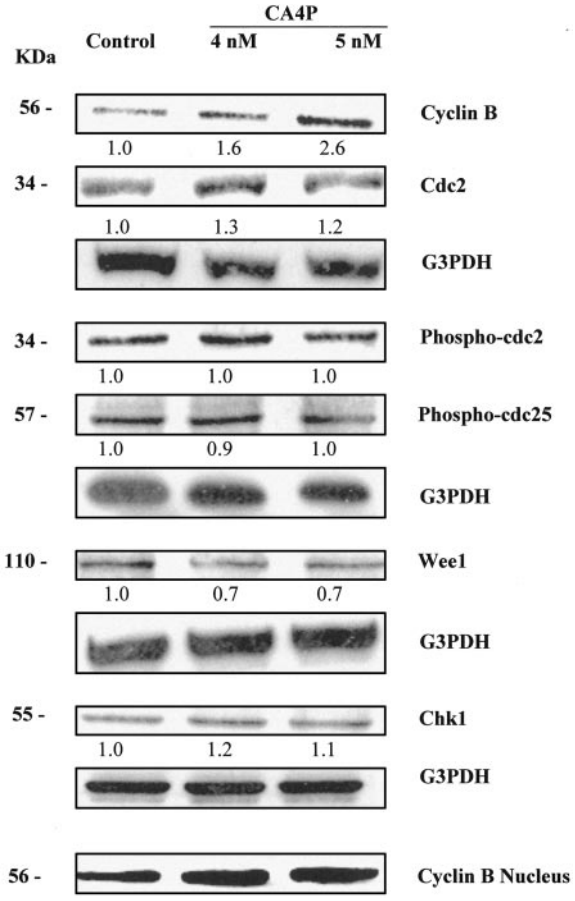

Fig. 1 Western blots of cyclin B1, cdc2, cdc2P, cdc25P, wee1, and chk1 in WSU-CLL cells after 24 h of CA4P exposure. Cells were treated for 24 h with CA4P (4 and 5 nM). Twenty μg of proteins were separated on 12% SDS-PAGE and probed with the different antibodies. *Numbers* at the *bottom* represent the protein levels relative to G3PDH as determined by densitometric scanning. The figure shown is representative of three independent assays.

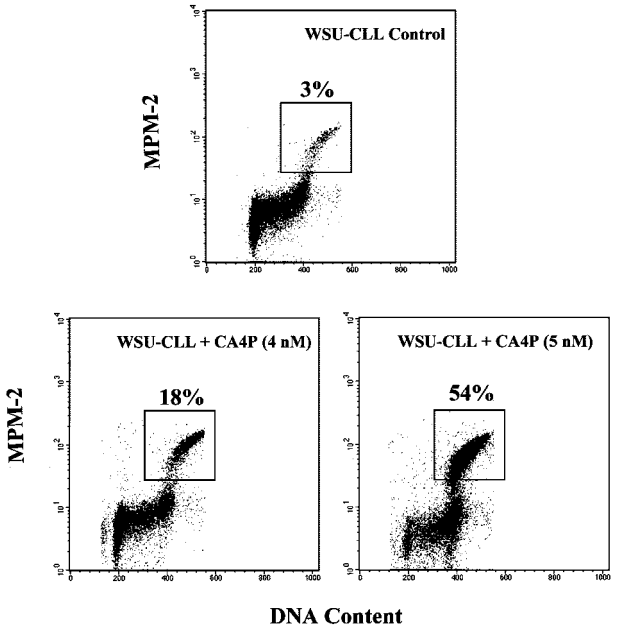

Fig. 2 Flow cytometry analysis of MPM-2 expression in untreated and CA4P-treated WSU-CLL cells. Cells (2×10^5 cells/ml) were untreated or treated with 4 and 5 nM CA4P. After 24 h of treatment, cells were fixed with 70% ethanol, stained with MPM-2 and PI, and analyzed by FACScan. Cells in the *square* stained positive for MPM-2.

mature chromosome condensation and mitotic catastrophe are independent of apoptosis in HeLa S3 cells exposed to X-irradiation (9). In contrast, other authors suggest that mitotic catastrophe is a late event of telomere cleavage that leads to drug-induced apoptosis (10).

It is widely known that chemotherapeutic drugs induce DNA damage and cell cycle arrest, which can, in case of prolongation, induce cell death through different mechanisms. It is also well established that cell cycle checkpoints are crucial in DNA repair and that the G_2-M transition is regulated by the mitosis-promoting factor, cyclin B, and cdc2 kinase. The activation of the cdc2 requires binding with cyclin B and can be negatively regulated by phosphorylation by wee1/myt1 kinases and positively regulated by dephosphorylation by cdc25, which can be inhibited by its phosphorylation by chk1 and chk2. Also, it has been reported that p53 plays a major role in the G_2 checkpoint with activation of downstream molecules such as 14-3-3σ, GADD45, and p21 (11, 12) and that it can lead to increased activation of normal endogenous MDM2 (13).

Cysteine proteases (caspases) play an important role in apoptosis induced by DNA damage through the proteolysis of specific targets. Among the targets is the PARP. Caspases are synthesized as proenzymes and activated by internal cleavage. A proapoptotic signal activates initiator caspase, which activates effector caspases (14). Different apoptosis signals induce different initiator caspases. One possible pathway is the activation of death receptor complexes that activate the initiator caspase 8 (15), whereas caspase 9 is induced in response to cytotoxic agents (14). Here we show that CA4P induces activation of caspase 9 and PARP cleavage, with apoptosis seen in only a small number of cells compared with total cell death. Moreover, treatment of WSU-CLL cells with the specific caspase 9 inhibitor (Z-LEHD-fmk) that also inhibited PARP cleavage or the general caspase inhibitor (Z-VAD-fmk) did not block cell death, confirming that the mitotic catastrophe and cell death induced by CA4P are independent of caspase 9 activation and PARP cleavage.

MATERIALS AND METHODS

Cell Culture. The human CLL cell line WSU-CLL was established in our laboratory at the Wayne State University School of Medicine (16). The cell line was grown in RPMI 1640 supplemented with 10% heat-inactivated FCS, 1% L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin at 37°C in an atmosphere of 5% CO_2. All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Drug. CA4P, a tubulin-binding drug isolated from the South African tree *C. caffrum* (2), was dissolved in PBS at 10^{-5} M and then diluted in culture media.

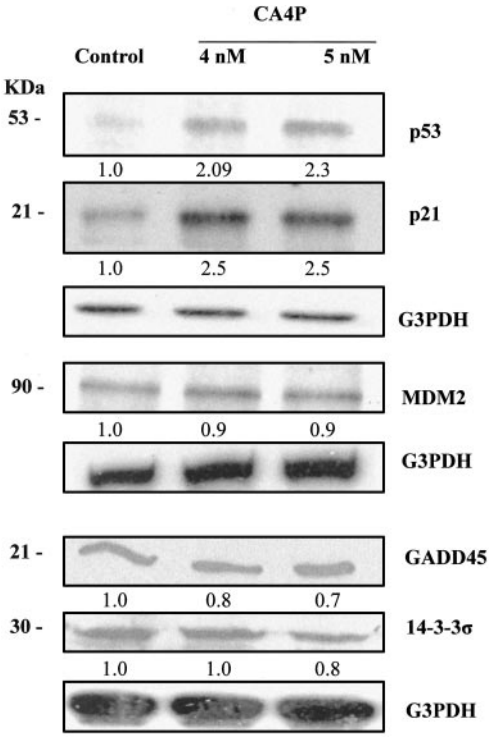

Fig. 3 Effect of CA4P on p53, MDM2, p21, 14-3-3σ, and GADD45 expression in untreated and CA4P-treated WSU-CLL cells. WSU-CLL cells were untreated or treated with 4 and 5 nM CA4P for 24 h. Twenty μg of proteins were separated on 12% SDS-PAGE and probed with p53, MDM2, p21, 14-3-3σ, and GADD45 antibody. *Numbers* at the *bottom* represent the protein levels relative to G3PDH as determined by densitometric scanning. The figure shown is representative of three independent assays.

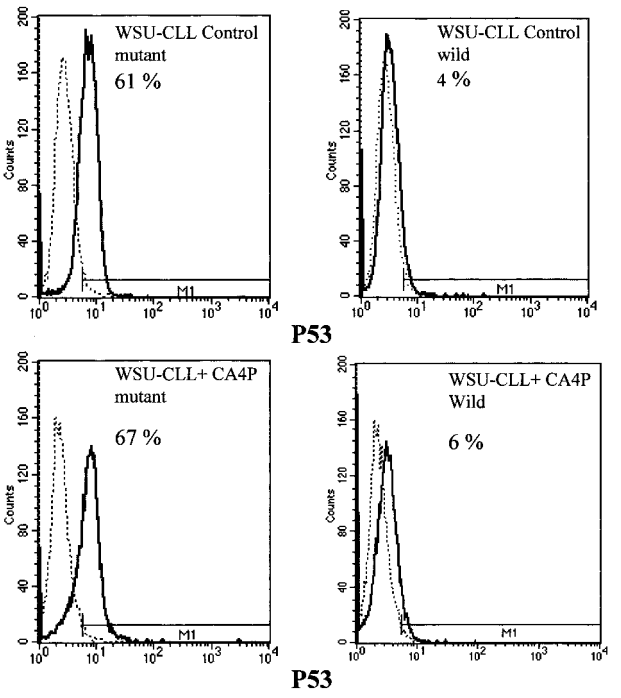

Fig. 4 Flow cytometry analysis of mutant and wild-type forms of p53 protein expression in WSU-CLL cells. Cells from untreated and 24-h CA4P-treated cultures were fixed with paraformaldehyde, permeabilized with ethanol, stained with monoclonal antibodies recognizing the mutant or wild-type p53, and analyzed by FACScan.

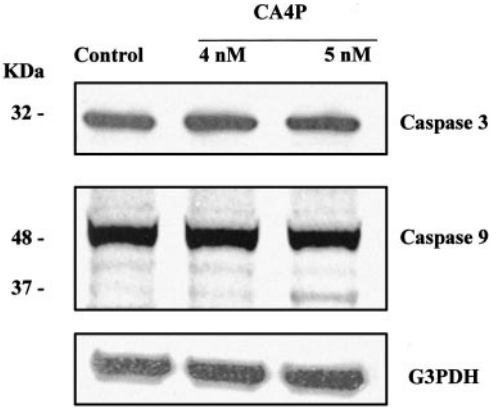

Fig. 5 Effect of CA4P on caspases 3 and 9. WSU-CLL cells were untreated or treated with 4 and 5 nM CA4P for 24 h. Twenty μg of proteins were separated on 12% SDS-PAGE and probed with caspase 3 and 9 antibody. Activation of caspase 9 is characterized by the increase in the intensity of the M_r 37,000 band. The figure shown is representative of three independent assays.

***In Vitro* Assays.** WSU-CLL cells were seeded at 2×10^5 cells/ml in a 24-well plate and treated with DMSO (control) or with CA4P at 4 or 5 nM or treated for 1 h with 150 μM Z-LEHD-fmk (caspase 9 inhibitor; dissolved in DMSO; Calbiochem) or 100–200 μM Z-VAD-fmk (general caspase inhibitor; dissolved in DMSO; Calbiochem) before CA4P addition. Cell viability was determined for 24 and 48 h using trypan blue (0.4%) exclusion (Life Technologies, Inc.).

Western Blot Analysis. Control- and CA4P-treated WSU-CLL cells were washed with PBS and lysed at 4°C for 30 min in lysis buffer (0.5% Triton X-100, 300 mM NaCl, 50 mM Tris-Cl, and 1 mM phenylmethylsulfonyl fluoride). Insoluble material was removed by centrifugation at 4°C for 15 min at 14,000 × *g*, and protein concentrations were determined using the Micro BCA protein assay (Pierce Chemical Co., Rockford, IL). Cellular proteins (20–40 μg/lane) were resolved on SDS-12–15% polyacrylamide gels, transferred to Hybond C-extra membranes (Amersham Life Science, Arlington Heights, IL) blocked with 5% fat-free milk and probed with specific primary antibodies, and then incubated with horseradish peroxidase-conjugated antimouse IgG or antigoat IgG or antirabbit IgG secondary antibodies (Santa Cruz Biotechnology). The primary antibodies used were mouse anti-cyclin B1, mouse anti-cdc2, mouse anti-p21, mouse anti-p53 (DO-1), mouse anti-bax, mouse anti-bcl2, mouse anti-MDM2, mouse anti-chk1, mouse anti-GADD45, goat anti-14-3-3σ, rabbit anti-wee1, rabbit anti-caspase 9 (Santa Cruz Biotechnology), mouse anti-phospho-cdc25 (ser216), mouse anti-phospho-cdc2 (Cell Signaling Technology), mouse anti-caspase 3, and mouse anti-PARP (Transduction Laboratories). In addition, the glycolysis-specific enzyme G3PDH was used as a loading control. The protein

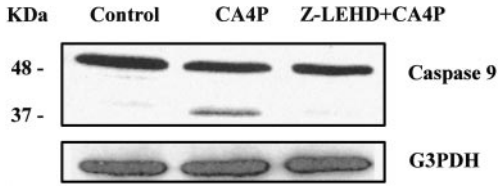

Fig. 6 Z-LEHD-fmk inhibits caspase 9 activation. WSU-CLL cells (2×10^5 cells/ml) were treated with DMSO (control) or 5 nM CA4P or with 150 μM Z-LEHD-fmk 1 h before the addition of CA4P. After lysis, protein extracts were subjected to SDS-PAGE followed by immunoblot analysis with antibody for caspase 9. The figure shown is representative of three independent assays.

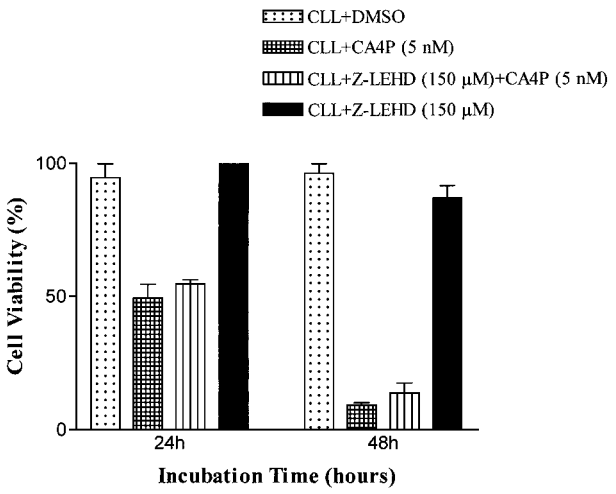

Fig. 7 Effect of the caspase 9 inhibitor (Z-LEHD-fmk) on cell death induced by CA4P. WSU-CLL cells (2×10^5) were treated with DMSO (control), CA4P (5 nM), or Z-LEHD-fmk (150 μM) followed after 1 h by CA4P (5 nM) or Z-LEHD-fmk (150 μM) and incubated for 24 and 48 h. Cell viability was determined by trypan blue exclusion. Experiments were performed in triplicate, and values represent the mean ± SD.

levels were visualized by peroxidase reaction using the enhanced chemiluminescence kit (Amersham Life Science).

Nuclear Protein Extraction. Cells from control- and CA4P-treated cultures were washed in PBS. Cell pellets were resuspended in lysis buffer [10 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.05% Triton X-100, and proteinase inhibitors] and homogenized with a Dounce homogenizer. The lysate was centrifuged at 10,000 × *g* for 15 min at 4°C. The supernatant containing the cytosolic extract was removed, and the pellet was resuspended in an equivalent volume of 10 mM Tris-HCl (pH 7.5) and 5 mM $MgCl_2$, followed by the addition of one nuclei pellet volume of 1 M NaCl, 10 mM Tris-HCl (pH 7.5), and 4 mM $MgCl_2$. The lysing nuclei were left on ice for 30 min and then centrifuged at 10,000 × *g* for 15 min at 4°C. The supernatant was removed, and protein concentrations and Western blot for cyclin B were determined as described above.

Analysis of p53 by Flow Cytometry. Cells (5×10^5) from untreated and 24-h CA4P-treated cultures were fixed in 2% paraformaldehyde for 30 min and permeabilized with chilled 80% ethanol for 30 min. Cells were then incubated for 30 min with 5 μl of monoclonal antibody recognizing mutant p53 (Pab 240; Santa Cruz Biotechnology) or 10 μl of monoclonal antibody recognizing wild-type p53 (Ab-5; Oncogene). As a negative control, cells were incubated with mouse IgG. Cells were washed with PBS-BSA, incubated for 30 min with FITC-conjugated goat antimouse IgG $F(ab')_2$ (DAKO), and analyzed on a FACScan (Becton Dickinson, Mountain View, CA).

Bivariate Analysis of MPM-2 Expression *versus* DNA Content by Flow Cytometry. Cells (1×10^6) from untreated and CA4P (4 and 5 nM)-treated cultures were washed in PBS and fixed with 70% ethanol for 30 min at 4°C. Cells were washed with PBS and incubated with MPM-2 antibody (6 μg/ml; Upstate Biotechnology) for 1 h at 40°C. Cells were then washed with PBS and incubated with FITC-conjugated goat antimouse IgG $F(ab')_2$ (Dako) for 1 h at room temperature. After washing with PBS, cells were stained with PI (10 μg/ml; Sigma) containing RNase (53 units/ml; Sigma) and analyzed on a FACScan (Becton Dickinson).

RESULTS

Effect of CA4P on the Regulators of the G_2-M Phase

As shown in Fig. 1, CA4P caused a concentration-dependent increase in cyclin B. No major changes were observed in the expression of cdc2, phospho-cdc2, phospho-cdc25, wee1, and chk1. Because mitotic catastrophe is known to be associated with an overaccumulation of cyclin B in the nucleus (17), we determined the expression of cyclin B in the nucleus. Effectively, 24 h of exposure to CA4P (at 4 and 5 nM) was associated with an increase of cyclin B in the nucleus (Fig. 1). The presence of cyclin B in the nucleus indicates that cells were able to exit the G_2 phase, enter the M phase, and be arrested there. Thus, we investigated the reactivity of the MPM-2 antibody in WSU-CLL cells before and after exposure to CA4P (4 and 5 nM). MPM-2 is an antibody that recognizes a group of phosphorylated form proteins that are phosphorylated only in mitosis (18, 19); therefore, the MPM-2-positive cells that possess 4 N DNA content will be indicative of cells in mitosis. As shown in Fig. 2, compared with the untreated cells that showed 3% positivity for MPM-2, 24 h of 4 and 5 nM CA4P treatment induced 18% and 54% positivity for MPM-2, respectively. The dual staining with PI shows that MPM-2-positive cells have 4 N DNA indicative of M phase.

Effect of CA4P on p53, p21, MDM2, 14-3-3σ, and GADD45

Exposing WSU-CLL cells to either 4 or 5 nM CA4P caused an obvious increase in total p53 and p21 expression (Fig. 3). However, no major change was observed in the expression of MDM2, 14-3-3σ, and GADD45 (Fig. 3). Next, we sought to determine the status of p53 in our WSU-CLL cells. This was accomplished by flow cytometry using antibodies specific to mutant and wild-type forms of p53. Such analysis showed that WSU-CLL possesses mutant-type p53 (Fig. 4), whereas wild-type p53 was not detected. Twenty-four h of treatment with CA4P at 5 nM showed no significant increase in mutant or wild-type forms of p53, in contradiction to the Western blot. However, an increase was shown in total p53 by flow cytometry

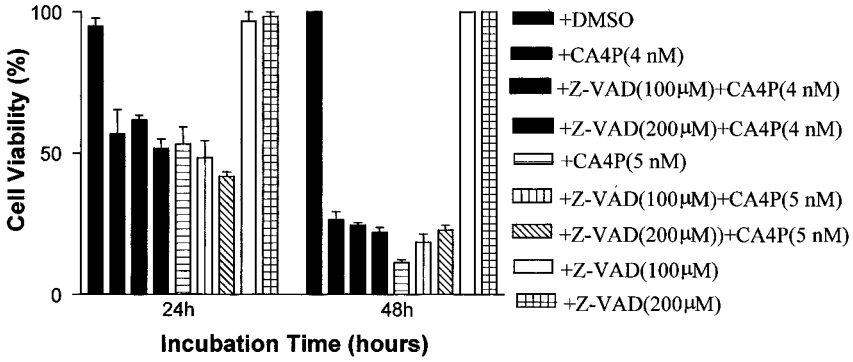

Fig. 8 Effect of general caspase inhibitor (Z-VAD-fmk) on cell death induced by CA4P. WSU-CLL cells (2×10^5) were treated with DMSO (control), CA4P (4 and 5 nM), or Z-VAD-fmk (100 and 200 μM) followed after 1 h by CA4P (4 and 5 nM) or Z-VAD-fmk (100 and 200 μM) and incubated for 24 and 48 h. Cell viability was determined by trypan blue exclusion. Experiments were performed in triplicate, and values represent the mean ± SD.

(data not shown) using the same antibody that was used for the Western blot. This result was confirmed by immunohistochemistry (data not shown).

Effect of CA4P on Selected Markers of Apoptosis

Caspases 9 and 3 and PARP. Using an immunoblotting technique, our results show that caspase 9 is activated by CA4P treatment. Fig. 5 shows that the level of the cleaved subunit (P37) of caspase 9 increased after 24 h in 5 nM CA4P. However, no activation of caspase 3 (loss of the procaspase 3 or appearance of the active form) was noticed. The treatment of WSU-CLL cells with 150 μM Z-LEHD-fmk, a caspase 9 inhibitor, 1 h before the addition of CA4P did prevent caspase 9 cleavage (Fig. 6). Cell death seen after 24 and 48 h of CA4P treatment alone was not prevented by the addition of 150 μM Z-LEHD-fmk (Fig. 7). To verify that caspases other than caspases 3 and 9 are not involved in the cell death induced by CA4P, we treated the WSU-CLL cells with 100 or 200 μM Z-VAD-fmk, a general caspase inhibitor, 1 h before CA4P treatment (Fig. 8). As shown, Z-VAD-fmk did not inhibit cell death in CA4P-treated cultures (Fig. 8). Morphological examination of cells showed that Z-VAD-fmk inhibited only the small percentage of apoptosis that was induced by CA4P but did not inhibit mitotic catastrophe (Fig. 9).

Proteolytic cleavage by caspases of PARP from a M_r 116,000 polypeptide to a M_r 85,000 fragment is a final common pathway of drug-induced apoptosis. Five nM CA4P induced cleavage of the M_r 116,000 polypeptide to the M_r 85,000 fragment after 24 h in WSU-CLL (Fig. 10). The M_r 116,000 protein almost disappeared. However, treatment with Z-LEHD-fmk inhibited this cleavage.

bax and bcl-2. There was no change in the expression of bax or bcl-2 protein when cells were treated with 4 or 5 nM CA4P (data not shown).

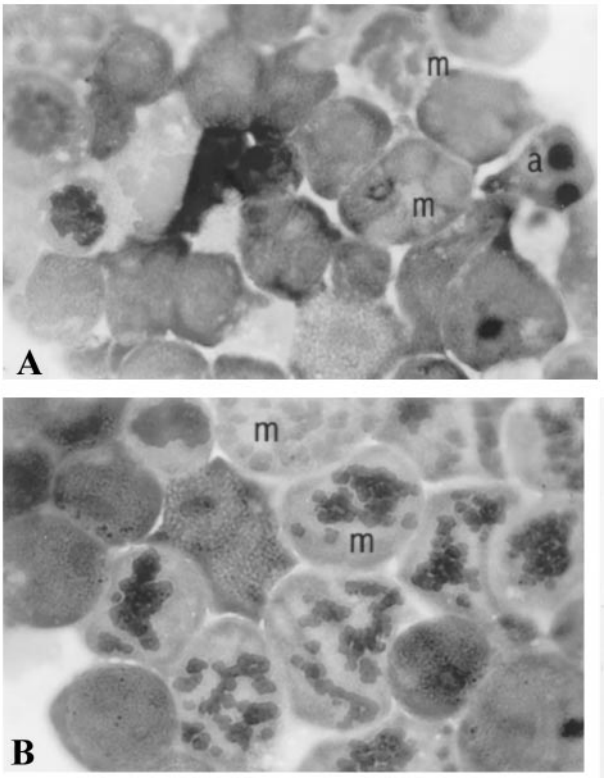

Fig. 9 Mitotic catastrophe induction in WSU-CLL cells. Photographs show the characteristic features of WSU-CLL cells treated with CA4P (×1000). *A*, WSU-CLL cells treated for 24 h with 5 nM CA4P. *B*, WSU-CLL cells treated for 1 h with Z-VAD-fmk before the addition of 5 nM CA4P. Note the giant, multinucleated cells characteristic of mitotic catastrophe (*m* in *A* and *B*) and apoptotic cells (*a* in *A*).

DISCUSSION

In this study, we provide molecular evidence that mitotic catastrophe is the major mode by which CA4P induces cell death in the WSU-CLL model. These findings support our previously published morphological observations (1). Mitotic catastrophe in our model is independent of apoptosis, which affected only a small percentage of cells. Recognition of mitotic catastrophe and elucidation of the molecular pathways that trigger it are important in developing new anticancer drugs.

We have shown that CA4P treatment induced mitotic catastrophe after arresting the WSU-CLL cells in mitosis, based on the overexpression of cyclin B in the nucleus, the positive staining of MPM-2, and the unchanged expression of cdc2 or its regulators (wee 1, chk1, and cdc25; Fig. 1). Other authors have also found that mitotic catastrophe was associated with nuclear

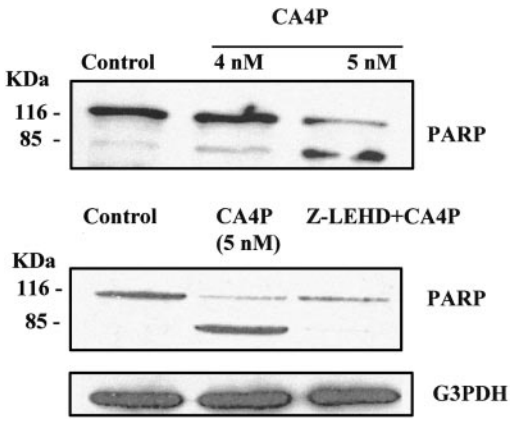

Fig. 10 Effect of CA4P and Z-LEHD-fmk followed by CA4P on PARP cleavage. WSU-CLL cells (2×10^5 cells/ml) were treated with DMSO (control), CA4P (4 and 5 nM), or Z-LEHD-fmk (150 μM) followed after 1 h by CA4P (5 nM) and incubated for 24 h. After lysis, protein extracts were subjected to SDS-PAGE followed by immunoblot analysis with antibody for PARP. The figure shown is representative of three independent assays.

accumulation of cyclin B (11, 17). We have also shown a lack of involvement of p53 in this mitotic arrest and mitotic catastrophe, based on the induction of p53 by CA4P without concomitant effects on the 14-3-3σ protein, MDM-2, and GADD45 that have been reported in other systems to change in abundance after p53 induction (11, 13). This conclusion is also supported by the mutant, nonfunctional status of p53 in WSU-CLL cells. p21 did increase in the cells after CA4P treatment; however, given the status of p53, this seems to be p53 ndependent. Moustakas *et al.* (20) have shown that in mammalian cells, p21 can be activated by factors other than p53, and recently, Yoshikawa *et al.* (21) showed that p21 can be induced and that mitotic catastrophe can occur in colorectal carcinoma cells treated with 5-fluorouracil independently of p53. However, our results and the fact that the cells are arrested in the M phase suggest that the induction of p21 failed to inhibit the mitotic complex cyclin B-cdc2 and to maintain a G_2 checkpoint.

We have shown that the cell death is due primarily to mitotic catastrophe and not apoptosis, based on the fact that CA4P did not affect the expression of bax and bcl-2, although CA4P treatment did induce caspase 9 activation and PARP cleavage, probably due to the small percentage of cell death induced by apoptosis. However, treatment of the cells with caspase inhibitors before the addition of CA4P did inhibit apoptosis and PARP cleavage but did not inhibit mitotic catastrophe and cell death (Figs. 7 and 8).

Little is known about mitotic catastrophe and whether or not it shares common pathways with apoptosis. Our studies indicated that these two mechanisms are independent of each other. Similar results were reported by other authors. Ruth *et al.* (22) have shown that apoptosis is not induced by the same pathway as mitotic catastrophe in radiated cells. Yoshikawa *et al.* (21) have also shown that fluorouracil treatment of colorectal cells induces mitotic catastrophe independent of apoptosis. Mitotic catastrophe has also been shown to be independent of bcl-2 and apoptosis after etoposide treatment of human epithelial cells (23). However, other authors have indicated that the two mechanisms precede each other. Multani *et al.* (10) suggested that apoptosis is an early event in the mitotic catastrophe process. Whether our findings are unique to the WSU-CLL cell line or reflect a major mechanism of CA4P activity is not known and deserves further investigation.

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