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The Tubulin-Binding Agent Combretastatin A-4-Phosphate Arrests Endothelial Cells in Mitosis and Induces Mitotic Cell Death

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The tubulin-binding agent combretastatin A-4-phosphate (CA-4-P), rapidly disrupts the vascular network of tumors leading to secondary tumor cell death. *In vitro*, CA-4-P destabilizes microtubules and causes endothelial cell death. In this study we analyze the mechanisms by which CA-4-P induces the death of proliferating endothelial cells. We demonstrate that at ≥7.5 nmol/L, CA-4-P damages mitotic spindles, arrests cells at metaphase, and leads to the death of mitotic cells with characteristic G_2/M DNA content. Mitotic arrest was associated with elevated levels of cyclin B1 protein and $p34^{cdc2}$ activity. Inhibition of $p34^{cdc2}$ activity by purvalanol A caused mitotic-arrested cells to rapidly exit mitosis, suggesting that sustained $p34^{cdc2}$ activity was responsible for metaphase arrest. Pharmacological prevention of entry into mitosis protected cells from undergoing cell death, further establishing the link between mitosis and cell death induction by CA-4-P. CA-4-P-mediated cell death shared characteristics of apoptosis but was independent of caspase activation suggesting the involvement of a non-caspase pathway(s). These data suggest that induction of apoptosis in endothelial cells by CA-4-P is associated with prolonged mitotic arrest. Therefore, by activating cell death pathways, CA-4-P, in addition to being an effective anti-vascular agent, may also interfere with regrowth of blood vessels in the tumor. *(Am J Pathol 2004, 165:1401–1411)*

Tumor cells depend on the presence of a functional blood vessel network for their growth, survival, and metastatic spread. Although anti-angiogenic agents aim to interfere with the dynamic processes involved in new blood vessel development, anti-vascular agents target the already existing tumor blood vessels leading to the catastrophic disruption of blood flow and, consequently, tumor cell death.[1,2] Nevertheless, there is likely to be a considerable overlap in the specific vascular effects of anti-angiogenic and anti-vascular agents. Combretastatin A-4 (CA-4), a tubulin-binding agent isolated from the South African bush *Combretum caffrum*, belongs to a new family of small molecular weight drugs that are structurally related to colchicine but have potent anti-vascular properties at well-tolerated doses.[3,4] CA-4-phosphate (CA-4-P), a more soluble derivative of CA-4, causes rapid and selective damage to the tumor vasculature in both animal models and in man.[5,6] The mechanism of action of CA-4-P is not clearly understood, although morphological changes in endothelial cells and a rapid rise in tumor vascular permeability, mediated via disruption and reorganization of the endothelial cell microtubule and actin cytoskeletons, respectively, are implicated in the blood flow reduction observed in tumors within minutes of drug exposure.[7–9]

Disruption of interphase microtubules can account for the rapid anti-vascular effects of CA-4-P described above. However, microtubule-binding agents also disrupt mitotic spindles and are classically known for their anti-mitotic properties. For instance, the taxols and the vinca alkaloids are potent anti-mitotic drugs clinically used for the treatment of cancer.[10] Although these agents do have rapid anti-vascular effects, they are only observed at close to their maximum tolerated doses.[11–13] Several lines of evidence suggest that these anti-mitotics also act by inhibiting angiogenesis, possibly because of inhibitory effects on endothelial cell proliferation and induction of apoptosis.[14–16] CA-4-P itself, inhibits endothelial cell proliferation and induces apoptosis in proliferating endothelial cells *in vitro*.[17,18] *In vivo* it is active against

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angiogenesis associated with proliferative retinopathy[19] but there is only limited data regarding possible anti-angiogenic activity in tumors.[20]

Disruption of the mitotic spindle activates the spindle assembly checkpoint and as a result arrests cells in mitosis.[21] Mitotic arrest caused by various microtubule-interacting drugs was found to precede tumor cell apoptosis and the hypothesis that mitotic arrest was the cause of apoptosis has gained widespread support.[22–24] Eukaryotic cell entry into mitosis and subsequent exit into G_1 requires the sequential entry of cyclin B1 into the nucleus during prophase where it activates $p34^{cdc2}$ kinase, followed by subsequent degradation of cyclin B1 and thus down-regulation of $p34^{cdc2}$ activity during anaphase that allows the cell to exit from mitosis.[25,26] A sustained activation of $p34^{cdc2}$ is associated with mitotic arrest in response to various microtubule-targeting agents and is thought to be a trigger for apoptotic cell death induction in some cell systems.[24,27,28] Other investigators, however, are more in support of the idea that anti-microtubule agents induce apoptosis via mechanisms that are independent of their capacity to arrest cells in mitosis.[29,30]

We have previously demonstrated that CA-4-P induces a rapid form of primarily necrotic cell death in post-confluent non-proliferating endothelial cells that could contribute to the disruption of the tumor vascular function observed *in vivo*.[8] In the present report we analyze the mechanisms involved in the induction of cell death by CA-4-P in proliferating human endothelial cells, which has significance for potential downstream anti-angiogenic effects of the drug. Here we investigate the involvement of the cell cycle in the mechanism by which endothelial cells die after CA-4-P treatment and the potential link between entry into mitosis and induction of cell death by apoptosis. Understanding these mechanisms is important for exploiting the potential of this drug for the treatment of angioproliferative diseases including cancer.

Materials and Methods

Materials

CA-4-P was obtained from OXiGENE, Inc. (Watertown, MA). Anti-β-tubulin monoclonal antibody (clone TUB.2.1), fibronectin, heparin, mammalian protease inhibitor cocktail, and cell culture media were from Sigma (Poole, UK). Fetal calf serum was from Helena Biosciences (Tyne and Wear, UK). Mouse anti-cyclin B1 antibody was purchased from BD Biosciences Pharmigen (Oxford, UK). Mouse anti-poly-(ADP)-ribose polymerase (PARP) and anti-phopsho-histone H1 antibodies were obtained from Upstate (Botolph Claydon, UK). Purvalanol A and Z-VAD-FMK were from Calbiochem (Beeston, UK).

Cell Culture

Human umbilical vein endothelial cells from pooled donors (TCS CellWorks, Botolph Clayton, UK) were grown on gelatin-coated culture dishes in M199 supplemented with 20% fetal calf serum, 4 mmol/L L-glutamine, 20 μg/ml endothelial cell growth supplement (First Link, Birmingham, UK), and 80 μg/ml heparin. Cells were plated at 10^4 cells/cm^2 and were used 24 to 48 hours after plating. Only cells between the first and fourth passages were used for experiments.

Determination of Proliferation/Cell Viability

Cells were plated onto gelatin-coated dishes at a density of 2×10^4 cells/cm^2 and allowed to adhere for 24 hours after which they were treated with CA-4-P in fresh medium. At the end of the incubation period (24 to 48 hours), both adherent and non-adherent cells were harvested, pooled, and counted in a hemocytometer. Cell viability was evaluated by the trypan blue exclusion assay.

Analysis of DNA by Flow Cytometry

Cells were plated and treated with CA-4-P as described for the proliferation assay above. Adherent cells collected by trypsinization were pooled with floating cells, washed in phosphate-buffered saline (PBS), and fixed with ice-cold 70% ethanol. Fixed samples were extensively washed in PBS, treated with RNase A (20 μg/ml, 30 minutes, 37°C), and resuspended in propidium iodide (PI) (20 μg/ml). Stained cells were analyzed on a FACS flow cytometer (Becton Dickinson). The low-level gate was set at 10% of the value of the G_1 peak and the percentages of cells within the G_1 and G_2/M phases of the cell cycle were determined by analysis with CellQuest software (Becton Dickinson).

Immunofluorescence Microscopy

Cells were cultured on Permanox Lab-Tek chamber slides coated with 10 μg/ml human fibronectin (Invitrogen, Paisley, UK). For immunofluorescence visualization of microtubules and localization of cyclin B and phosphorylated histone H1, cells were fixed in 3.7% formaldehyde in PBS, permeabilized with 0.1% Triton X-100, and incubated with primary antibodies followed by sequential incubations with biotin-labeled anti-mouse/anti-rabbit IgG and fluorescein isothiocyanate-labeled avidin D (Vector Laboratories, Peterborough, UK). For simultaneous visualization of actin, 5 U/ml Texas Red-conjugated phalloidin (Molecular Probes, Leiden, The Netherlands) was added together with the avidin. Slides were mounted in Vectashield with 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories) to visualize nuclei. Fluorescence or phase contrast images were acquired with a Nikon Eclipse TE200 inverted microscope and a cooled charge-couple device camera (Cohu, San Diego, CA) and processed using Adobe Photoshop software.

Cell Death Enzyme-Linked Immunosorbent Assay (ELISA)

To assay apoptotic cell death, an ELISA-based photometric assay kit was used (Cell Death Detection ELISA^{PLUS};

Roche Diagnositics, Lews, UK) that measures cytoplasmic DNA-histone nucleosome complexes generated during apoptotic DNA fragmentation. Briefly cells were plated in 24-well cluster plates at a density of 10^4 cells/cm^2 and allowed to adhere overnight before exposure to drug in fresh medium. At specified times, cluster plates were centrifuged for 10 minutes at 200 $\times$ *g* and after removal of supernatants cells were lysed directly in the wells in 200 μl of lysis buffer supplied in the kit. Cytoplasmic fractions were analyzed for presence of nucleosomes according to the manufacturer's instructions. The increase in optical density over that obtained by control untreated cells is represented as fold increase in released nucleosomes.

Annexin V Staining of Apoptotic Cells

To determine whether exposure of phosphatidylserine occurred to the outer leaf membrane of cells, an indication of apoptosis, unfixed cells were analyzed using the Annexin-V-FLUOS staining kit (Roche Diagnostics). Briefly, cells in chamber slides were exposed to CA-4-P for specified time periods. The culture medium was then removed and any nonadherent cells were collected by centrifugation and replaced into their corresponding slide wells. Cells were covered with a solution containing annexin V and PI as recommended by the manufacturer and analyzed under an inverted fluorescence microscope.

Western Blotting Analysis

Cell lysates were prepared as described before.[31] Equal amounts of protein (Pierce BCA microassay) were separated on Novex Tris-glycine gels (Invitrogen), transferred to nitrocellulose membranes, and immunoreactive bands were visualized by enhanced chemiluminescence (Amersham-Pharmacia Biotech, Chalfont St. Giles, UK).

Statistical Analysis

Quantitative experimental data were analyzed by using a standard analysis of variance followed by the Tukey-Kramer honest significance difference test for multiple comparisons (JMP Statistics for the Apple Macintosh). A value of $P < 0.05$ was considered significant.

Results

CA-4-P Arrests Endothelial Cells in G_2/M and Inhibits Mitosis

The capacity of CA-4-P to modulate endothelial cell cycle progression was evaluated using asynchronous proliferating endothelial cultures, continuously exposed to the drug which were then stained with PI and analyzed by flow cytometry. Treatment with 100 nmol/L CA-4-P for 24 hours led to an accumulation of cells in G_2/M (65%) (Figure 1B) compared to control cells (20%) (Figure 1A). Under these conditions, numerous rounded loosely adherent, mitotic in appearance, cells accumulated in the cultures (Figure 1, compare F with E) and these cells had a characteristic G_2/M DNA content (Figure 1D) in contrast to the remaining strongly adherent cells that were distributed throughout the cell cycle (Figure 1C). Staining of cell nuclei of CA-4-P-treated cultures with DAPI revealed characteristically condensed mitotic metaphase chromosomes that often appeared damaged and fragmented

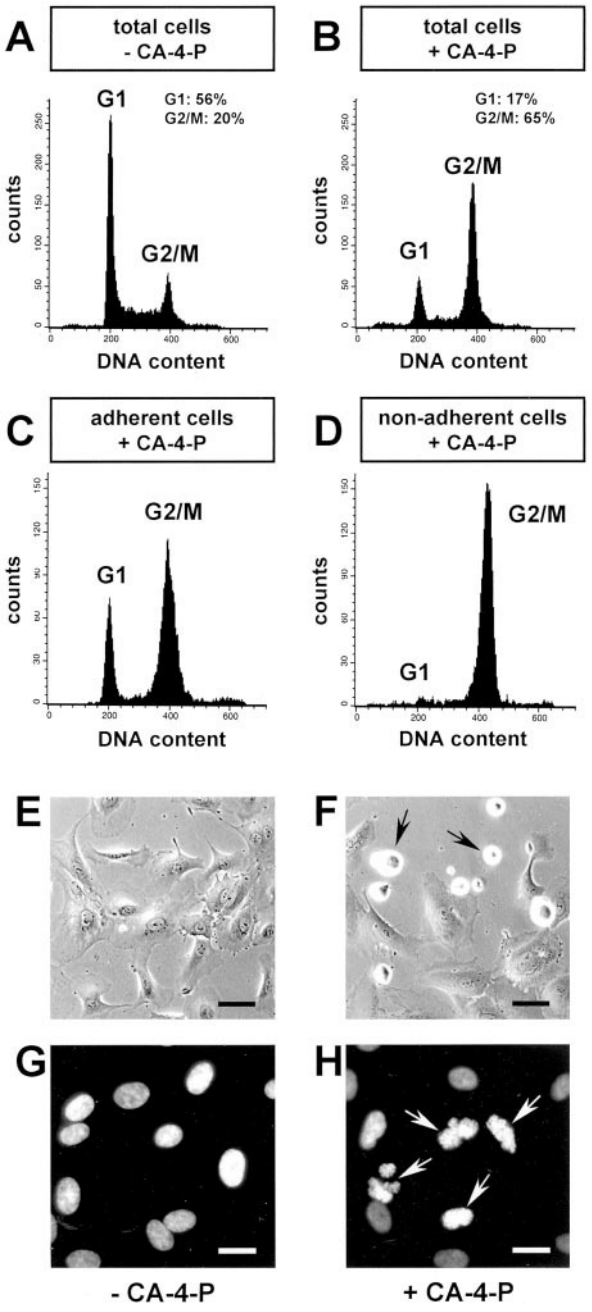


Figure 1. CA-4-P arrests proliferating human endothelial cells in mitosis. **A–D:** DNA content of endothelial cells was analyzed by PI staining and flow cytometry. Exponentially growing cells were left untreated (**A**) or treated with 100 nmol/L CA-4-P for 24 hours (**B**). Cells from treated cultures were separated into adherent (**C**) and nonadherent (**D**) subfractions before analysis. **E** and **F:** Phase contrast images of untreated (**E**) and CA-4-P-treated (**F**) (100 nmol/L, 24 hours) cells. **G** and **H:** DAPI-stained nuclei from untreated (**G**) and CA-4-P-treated cells (**H**). Scale bars: 20 μm (**G**, **H**); 50 μm (**E**, **F**).

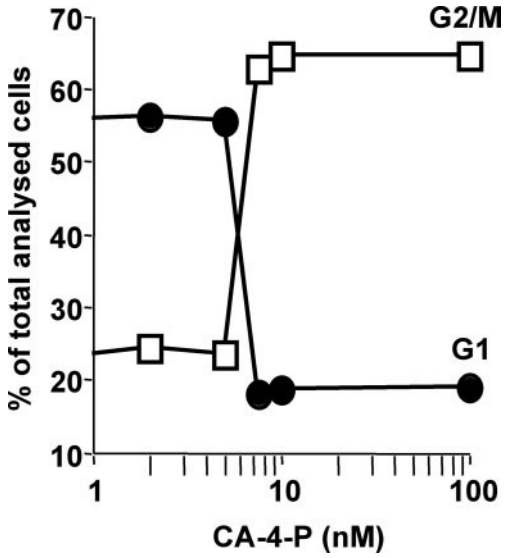


Figure 2. Arrest in mitosis occurs over a threshold concentration of CA-4-P. Asynchronous proliferating cells were treated with the indicated amounts of CA-4-P for 24 hours. The distribution between G_1 and G_2/M phases of the cell cycle was established by flow cytometry. Values represent percentages of cells in each phase of the cell cycle (percentage of total numbers of analyzed cells for each treatment group). Each percentage represents a mean value ± SEM of three determinations derived from one representative experiment. SEM was less than 5% for all points and error bars are not visible.

and unevenly displayed in a significant proportion of the cells (Figure 1, compare H with G).

Mitotic arrest was initially detectable at ~8 hours of continuous CA-4-P exposure, with 32% of the cells in G_2/M compared to 23% in parallel untreated cultures (data not shown) and was maximal at 24 hours (Figure 2). Mitotic arrest was evident over a threshold concentration of 7.5 nmol/L CA-4-P and greater, (Figure 2). Immunofluorescence analysis showed that at ≥7.5 nmol/L CA-4-P, interphase microtubules were still evident although severely damaged (Figure 3, compare D and E with A to C) whereas mitotic spindle assembly was absent (Figure 3, compare I and J with F). At lower tested concentrations (2.5 and 5 nmol/L CA-4-P), mitotic spindles were detectable but these appeared less regular and smaller in size than those formed in the absence of the drug (Figure 3, G and H). In these conditions cells retained the capacity to exit mitosis, as demonstrated by an increase in cell numbers during an observation period of 24 hours (data not shown) while nuclear damage characterized by multilobular deformed nuclei and micronuclei was evident in a significant proportion of cells (Figure 4).

Cyclin B1 levels of expression and $p34^{cdc2}$ activity rise through prophase and metaphase and subsequently, as cells progress into anaphase, cyclin B1 is degraded leading to inactivation of $p34^{cdc2}$.[25,26] Accordingly, immunostaining experiments confirmed this pattern of cyclin B1 expression in endothelial cells undergoing mitosis (Figure 5, A and B; and Figure 6). In cultures exposed to ≥7.5 nmol/L CA-4-P, sustained increased levels of cyclin B1 were detected, specifically localized to mitotic-arrested cells (Figure 5, C and D). Histone H1 is a substrate for $p34^{cdc2}$ protein kinase.[32] Using an antibody to phosphorylated histone H1, normal mitotic cells (Figure 5, E

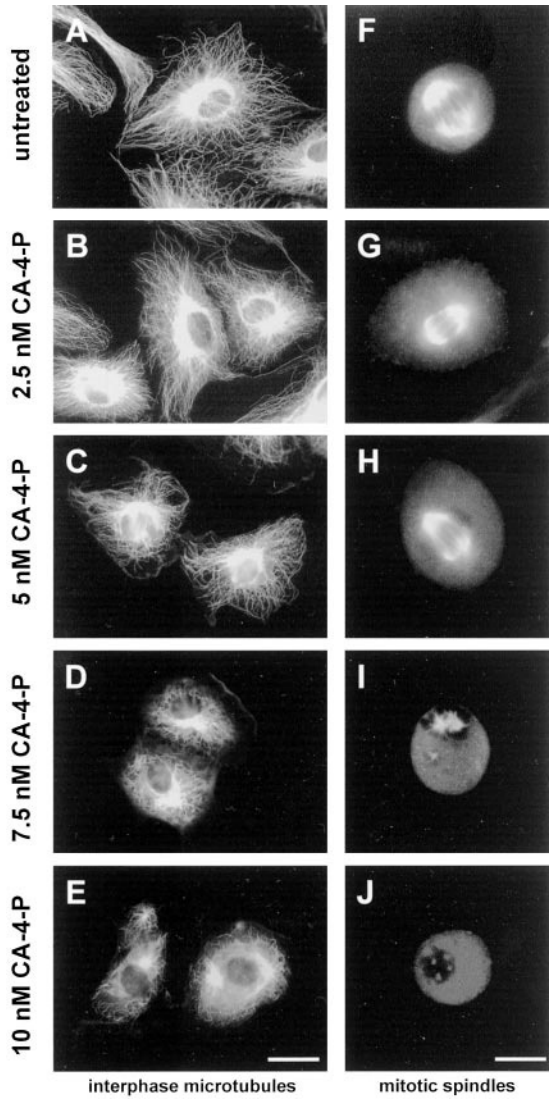


Figure 3. Mitotic spindles and interphase microtubules are sensitive to disruption by CA-4-P. Proliferating endothelial cells were treated with indicated concentrations of CA-4-P for 24 hours and then were fixed and immunostained with an antibody to β-tubulin. **A–E:** Interphase microtubules; **F-J:** mitotic spindles. Scale bars: 30 μm (**A–E**); 15 μm (**F–J**).

and F) and mitotic-arrested cells (Figure 5, G and H) were shown to contain high levels of phosphorylated histone, an indication of sustained $p34^{cdc2}$ activity. Taken together these data show that in response to concentrations of CA-4-P above a certain threshold, (≥7.5 nmol/L) a significant proportion of cells become specifically arrested in mitosis and fail to progress through the cell cycle.

A sustained activation of $p34^{cdc2}$ is associated with mitotic arrest in response to microtubule-targeting agents.[24,28] To investigate further the role of CA-4-P and $p34^{cdc2}$ activity in preventing cells from exiting mitosis at the spindle checkpoint, experiments were performed in which cells were treated with CA-4-P for 14 hours so that a significant proportion of cells accumulated and arrested in mitosis. Then, purvalanol A, an inhibitor of $p34^{cdc2}$ activity,[33] was added to the cells and 30 minutes and 1 hour later cells were fixed and stained for cyclin B1,

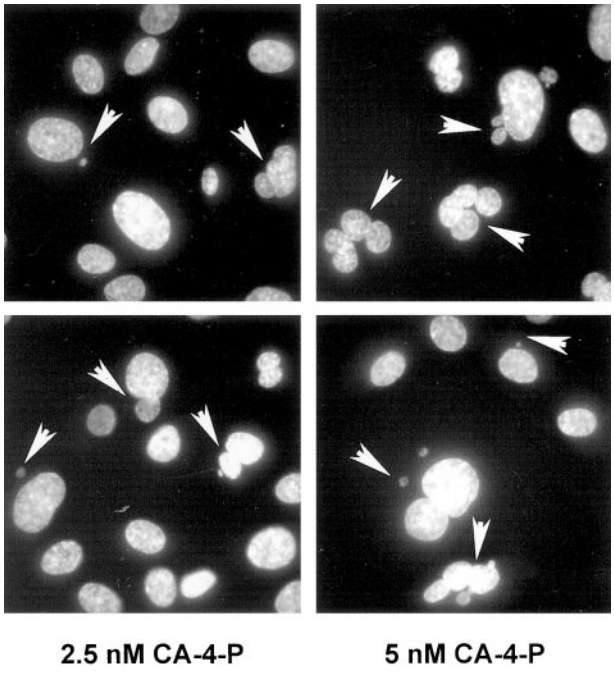


Figure 4. Low doses of CA-4-P result in nuclear damage in the absence of mitotic arrest. Proliferating endothelial cells were treated with either 2.5 nmol/L or 5 nmol/L CA-4-P for 24 hours and cells were fixed and nuclei stained with DAPI. Representative images of cells showing multilobed nuclei or nuclei associated with micronuclei are indicated by the **arrows**.

actin filaments, and nuclear DNA. Within 30 minutes to 1 hour of purvalanol treatment, previously rounded mitotic cells, typically lacking actin filamentous structures (Figure 6A), adhered back to the substratum, spread, and started forming actin filaments (Figure 6, B and C). In these cells cyclin B1 levels were down-regulated (Figure 6, compare D with E and F) and chromosomal DNA distributed into several multilobed irregular DNA-containing bodies within 1 hour of treatment (Figure 6, compare G with I). In proliferating cultures maintained in the absence of CA-4-P, mitotic cells exhibited the characteristic pattern of cyclin B1 expression described above (see a cell in prophase/metaphase in Figure 6; J, L, and N). Proliferating cultures exposed to purvalanol A for 60 minutes were characterized by an absence of cells in the early stages of mitosis (prophase, metaphase) with occasional cells in anaphase being present (Figure 6; K, M, O). These data confirm that sustained $p34^{cdc2}$ activity is responsible for maintaining endothelial cells arrested in metaphase in response to CA-4-P and suggest that purvalanol A accelerates the progression of cells through mitosis.

CA-4-P Induces Cell Death in Mitotic-Arrested Endothelial Cells

Apoptosis can occur at any stage in the cell cycle via activation of complex signal transduction pathways.[34] For microtubule-interacting agents, apoptosis is often but not inevitably associated with mitotic block.[22,23,29] An ELISA-based cell death assay that detects cytoplasmic DNA-histone complexes generated during apoptotic

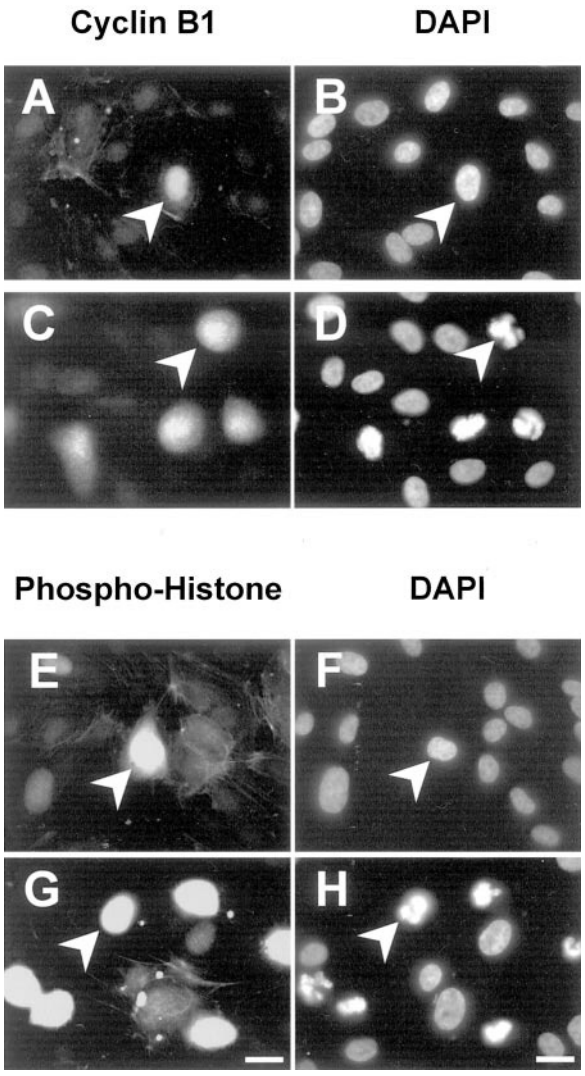


Figure 5. Mitotic-arrested endothelial cells express sustained elevated levels of cyclin B1 and $p34^{cdc2}$ protein kinase activity. Proliferating endothelial cells, either controls (**A** and **B** and **E** and **F**) or treated with 100 nmol/L CA-4-P for 24 hours (**C** and **D** and **G** and **H**) were fixed and stained with either an anti-cyclin B1 antibody (**A** and **C**) or anti-phospho-histone H1 antibody (**E** and **G**), which indicates $p34^{cdc2}$ activity. **B** and **D** and **F** and **H** are identical cells as in **A** and **C** and **E** and **G**, respectively, counterstained with DAPI to visualize nuclei. **Arrows** in **A** and **B** and **E** and **F** indicate cells in prophase showing elevated levels of expression of cyclin B2 and phosphorylated histone, respectively. **Arrows** in **C** and **D** and **G** and **H** indicate CA-4-P mitotic-arrested cells showing elevated levels of cyclin B1 and $p34^{cdc2}$ activity, respectively. Scale bars, 35 μm.

DNA fragmentation into nucleosomes revealed that CA-4-P induced apoptosis that was detectable after 8 hours of continuous exposure of the cells to the drug (Figure 7A), a time that correlated with accumulation of mitotic-arrested cells (see previous Results section).

Nucleosomal fragments were present in the loosely adherent mitotic cells, but were absent from the strongly adherent fraction of the cell population (Figure 7B). This, taken together with data in Figure 1D, suggests that cell death by apoptotic DNA degradation is primarily restricted to mitotic cells. It is important to point here that DNA degradation was not detectable as a sub-G_1 peak in the flow cytometric histograms (Figure 1B). However, this does not constitute proof of no apoptosis.[35] Apoptosis

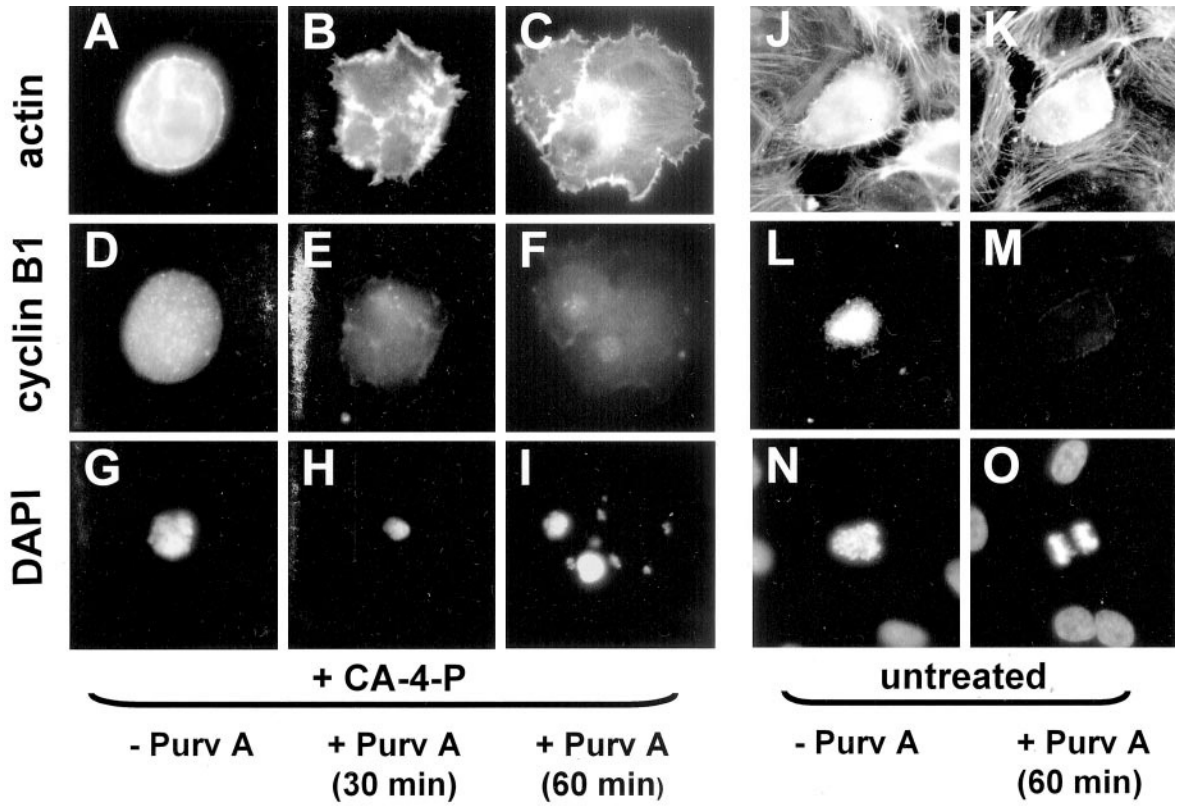


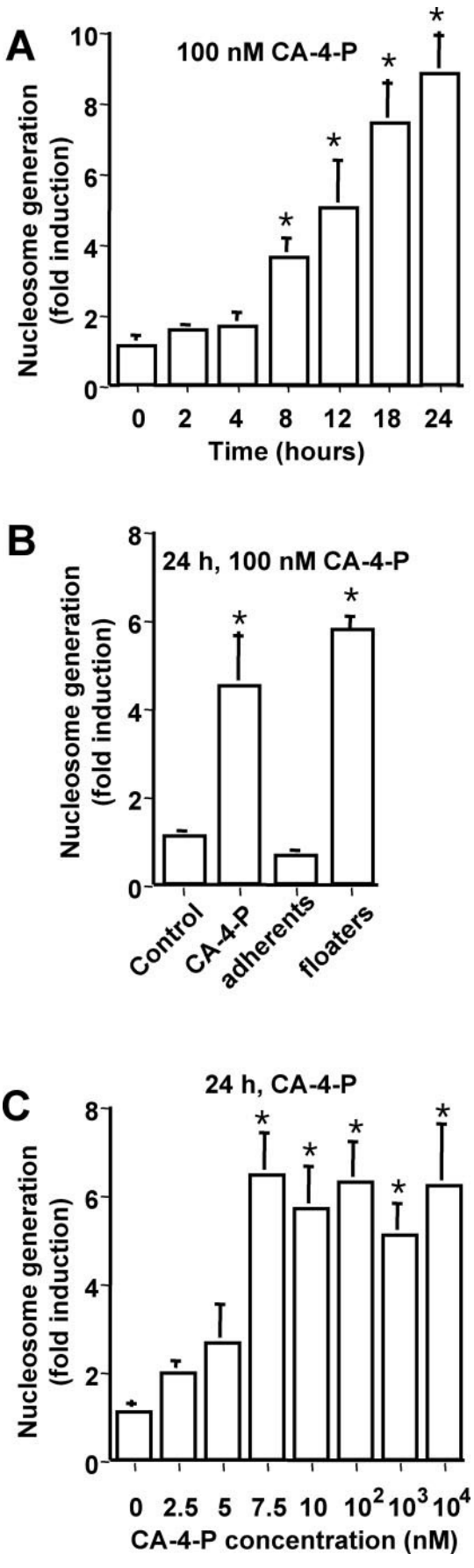
Figure 6. Sustained $p34^{cdc2}$ activity is responsible for CA-4-P-mediated arrest in mitosis. Proliferating cells were treated with 100 nmol/L CA-4-P for 14 hours and then 10 μmol/L purvalanol-A was added to the medium. At 30 minutes and 60 minutes after purvalanol-A addition, cells were fixed and double stained for actin (**A–C**) and cyclin B1 (**D–F**). Nuclei (**G–I**) were counterstained with DAPI. Proliferating untreated cells and cells treated with purvalanol A for 60 minutes were also stained for actin (**J**, **K**), cyclin B1 (**L**, **M**), and DNA (**N**, **O**), respectively. Scale bar, 25 μm.

detection by this flow cytometric method relies on the fact that small fragments of DNA are eluted after washing of alcohol-fixed apoptotic cells with buffer. After staining with a quantitative DNA-binding dye such as PI, cells that have lost DNA will take up less stain and will appear on the left of the G_1 peak. To be seen in the sub-G_1 area, a cell must have lost enough DNA to appear there and if cells enter apoptosis from the S or G_2/M phases, they are less likely to appear in the sub-G_1 area. Therefore, absence of a sub-G_1 peak in cells treated with CA-4-P is consistent with apoptotic cells derived from phases of the cell cycle other than G_1.

Dose analysis revealed that a wide range of concentrations of CA-4-P (7.5 to 10^4 nmol/L) led to significant nucleosome generation (Figure 7C), consistent with the mitotic arrest data (Figure 2). Nucleosome generation was also evident, but to a lesser extent, when lower concentrations of CA-4-P (2.5 or 5 nmol/L) were used that did not induce mitotic arrest. It should be noted that at these lower concentrations, CA-4-P did cause spindle malformation (Figure 3) and nuclear DNA damage (Figure 4) that could account for apoptotic degradation in the absence of mitotic arrest.

To investigate further the involvement of mitotic-arrested cells in the initiation of apoptotic cell death, parallel experiments were performed in which live cells were stained *in situ* with fluorescein isothiocyanate-annexin V to determine exposure of phosphatidylserine to the outer cell membrane. No annexin V-positive staining was evident in cultures exposed to CA-4-P for ≤6 hours (data not shown). However, annexin V staining was evident starting at 8 hours of exposure to CA-4-P, which led to the accumulation of mitotic cells in the culture. In these cells, annexin V fluorescence co-localized with the mitotic cells and was absent from all adherent spread-out cells (Figure 8A).

We then investigated whether caspases were involved in endothelial cell death mediated by CA-4-P, by monitoring cleavage of the DNA repair enzyme PARP. Treatment with 100 nmol/L CA-4-P resulted in cleavage of PARP that was detected in total endothelial cell extracts by Western blotting (Figure 8B). Analysis of the mitotic and adherent subfractions revealed that PARP cleavage was restricted to the mitotic fraction of the population whereas PARP remained intact in adherent non-mitotic cells (Figure 8C). To determine whether cell death and apoptotic nucleosome generation was because of the activation of caspases, cells were simultaneously treated with CA-4-P and the general caspase inhibitor Z-VAD-fmk (100 μmol/L). Z-VAD-fmk did not inhibit either the entry of cells into mitosis (data not shown) or the levels of nucleosome generation induced by CA-4-P (Figure 8D) suggesting that mitotic cell death was because of non-caspase-dependent mechanisms.



Block of Entry into Mitosis Attenuates CA-4-P-Induced Cell Death

The link between mitotic arrest and induction of endothelial cell death by CA-4-P was established by preventing cell entry into mitosis using the pharmacological inhibitor, purvalanol A. This inhibitor of $p34^{cdc2}$ kinase was previously shown to exhibit anti-mitotic properties by inducing a G_2/M block in exponentially growing fibroblasts and various tumor cell lines.[33,36] In agreement with such reports, proliferating endothelial cells treated with 10 μmol/L purvalanol A for 24 hours accumulated in G_2/M (compare Figure 9A with Figure 1A) with no detectable reduction in cell viability (data not shown). In a recent report, we demonstrated that concentrations of ≥100 nmol/L CA-4-P induced rapid membrane blebbing and necrosis in proliferating endothelial cells if p42/p44 MAPK were inhibited.[8] Purvalanol A is also a potent inhibitor of p42/p44 MAPK[36] and in combination with ≥100 nmol/L CA-4-P was indeed found to induce rapid membrane blebbing and necrosis (data not shown). Therefore, in the next experiments 10 nmol/L CA-4-P was specifically chosen for its efficacy at mediating mitotic arrest and apoptosis without inducing membrane blebbing and necrosis in the presence of inhibitors of p42/p44 MAPK such as PD98059 and purvalanol A (data not shown). Purvalanol A prevented a further progression of cells through the cell cycle in response to simultaneous treatment with CA-4-P (Figure 9, compare B with A) and inhibited any significant accumulation of mitotic cells (Figure 9B, inset). This decrease in frequency of mitotic cells correlated with reduction in the levels of expression of cyclin B1, which rose in cells arrested by CA-4-P (Figure 9C). Cyclin B1, although expressed in both G_2 and M, is generally higher in M-phase cells. Furthermore, phosphorylated histone H1, which is a characteristic of mitotic cells, was also absent in cells treated with purvalanol A. Taken together, these data strongly suggest that purvalanol A prevents endothelial cells from entering mitosis by mediating their arrest in the G_2 phase of the cell cycle.

The effects of purvalanol A on nucleosome generation and endothelial survival were then investigated. As shown in Figure 9D, nucleosome generation induced by CA-4-P was significantly inhibited if cells were simultaneously exposed to purvalanol A. Figure 9E shows that whereas control untreated cells continued to divide throughout a period of 24 to 48 hours, CA-4-P induced a significant reduction in the number of viable cells at 24 hours of continuous exposure that declined even further by 48 hours. In the presence of purvalanol A alone, cell

Figure 7. CA-4-P induces apoptotic nucleosome generation in proliferating endothelial cells. Proliferating endothelial cells were exposed to CA-4-P and cytoplasmic nucleosomes were detected by the Cell Death Detection ELISAplus kit. Nucleosome generation is expressed as fold induction over control untreated cells. **A:** Cells exposed to 100 nmol/L CA-4-P for indicated times; *, values significantly different from parallel control untreated cells. **B:** Cells exposed to 100 nmol/L CA-4-P for 24 hours and then nucleosomes were assayed in floating and adherent cells; *, values significantly different from control and adherent cells. **C:** Cells were exposed to indicated CA-4-P concentrations for 24 hours; *, values significantly different from parallel control untreated cells.

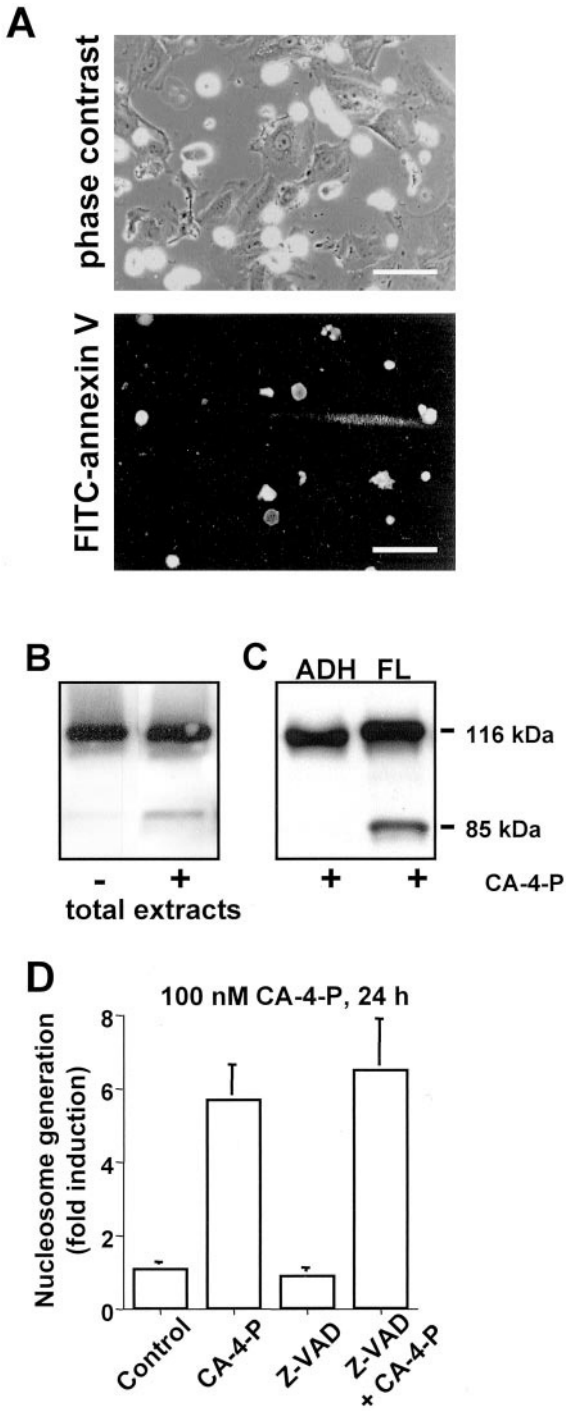


Figure 8. CA-4-P mitotic-arrested cells are positive for apoptotic markers but caspases do not participate in the apoptotic cell death induction. **A:** Cells grown in chamber slides were treated with 100 nmol/L CA-4-P for 9 hours and stained with annexin V and PI without prior fixation. Only mitotic cells stain positive for annexin V and these were negative for PI staining (not shown). **B:** Total extracts derived from cells treated with 100 nmol/L CA-4-P for 18 hours were analyzed by Western blotting using an antibody to PARP that detects intact (116 kd) and caspase-cleaved (85 kd) PARP. **C:** Cells treated with CA-4-P as in **A** and adherent and floating mitotic cells were separated out before protein extraction and Western blotting analysis for PARP cleavage. **D:** Cells were treated with 100 nmol/L CA-4-P for 24 hours in the presence or absence of caspase inhibitor Z-VAD-fms (100 μmol/L) before extraction of cytoplasmic nucleosomes and analysis by Cell Death Detection ELISA. Scale bars, 75 μm (**A**).

numbers remained similar to those of control cells at the start of the experiment demonstrating that purvalanol A prevented their proliferation. In the presence of CA-4-P purvalanol A prevented any further significant reduction in viable endothelial cell numbers to below those obtained by purvalanol alone.

Discussion

This study shows that CA-4-P induces mitotic arrest in endothelial cells that plays an important role in the onset of cell death via mechanisms strongly characteristic of apoptosis. Evidence that CA-4-P primarily kills proliferating cells in mitosis comes from experiments that demonstrated that only cells in G_2/M were significantly positive for any of the apoptotic markers evaluated, namely nucleosome formation, annexin V staining, and cleavage of PARP. This conclusion was further reinforced by experiments in which block of entry into mitosis by purvalanol A, markedly protected against CA-4-P-mediated cell death.

There is compelling evidence that tubulin-binding agents such as paclitaxel and the vinca alkaloids kill cancer cells primarily by apoptosis.[10,37] Because mitotic arrest caused by such agents was frequently found to precede apoptosis, a hypothesis that arrest of the cell cycle at mitosis is the primary stimulus for apoptosis has been widely accepted. For example, apoptosis induced by paclitaxel was found to either occur directly after a mitotic arrest or after an aberrant mitotic exit into a G_1-like multinucleate state.[22,23,38] Nevertheless, some investigators have provided evidence against the involvement of mitotic arrest in apoptosis induction by microtubule-binding agents by demonstrating apoptotic events in other phases of the cell cycle.[29,30] The link between mitotic arrest or damage of the mitotic machinery and CA-4-P-mediated endothelial cell death is clear from the results of the current study. However, what is not clear is whether arrest in mitosis is the actual trigger for cell death. Purvalanol A, used here to block entry into mitosis, could also be inhibiting other signal kinases that are upstream of pro-apoptotic pathways.[39] Endothelial mitotic cell death could therefore result from the culmination of upstream events that may involve activation or expression of pro-apoptotic genes by the action of CA-4-P on pre-mitotic cells. It would be of particular interest to investigate further the mechanisms involved in the apoptotic actions of CA-4-P by studying gene expression using apoptotic gene arrays. Identification of apoptosis-related genes that are positively or negatively regulated by CA-4-P would provide further insight into the mechanisms by which this compound kills proliferating endothelial cells.

Several signaling pathways are activated by CA-4-P in endothelial cells[4] and these could contribute toward cell death. For instance, we have recently demonstrated that CA-4-P activates the GTPase Rho[8] that is implicated in proapoptotic pathways[40] and could therefore signal to apoptosis in this system. However, the fact that a wide range of CA-4-P concentrations (7.5 nmol/L to 10 μmol/L) arrested cells in mitosis and induced endothelial cell death to a similar extent (Figure 7) suggests that the

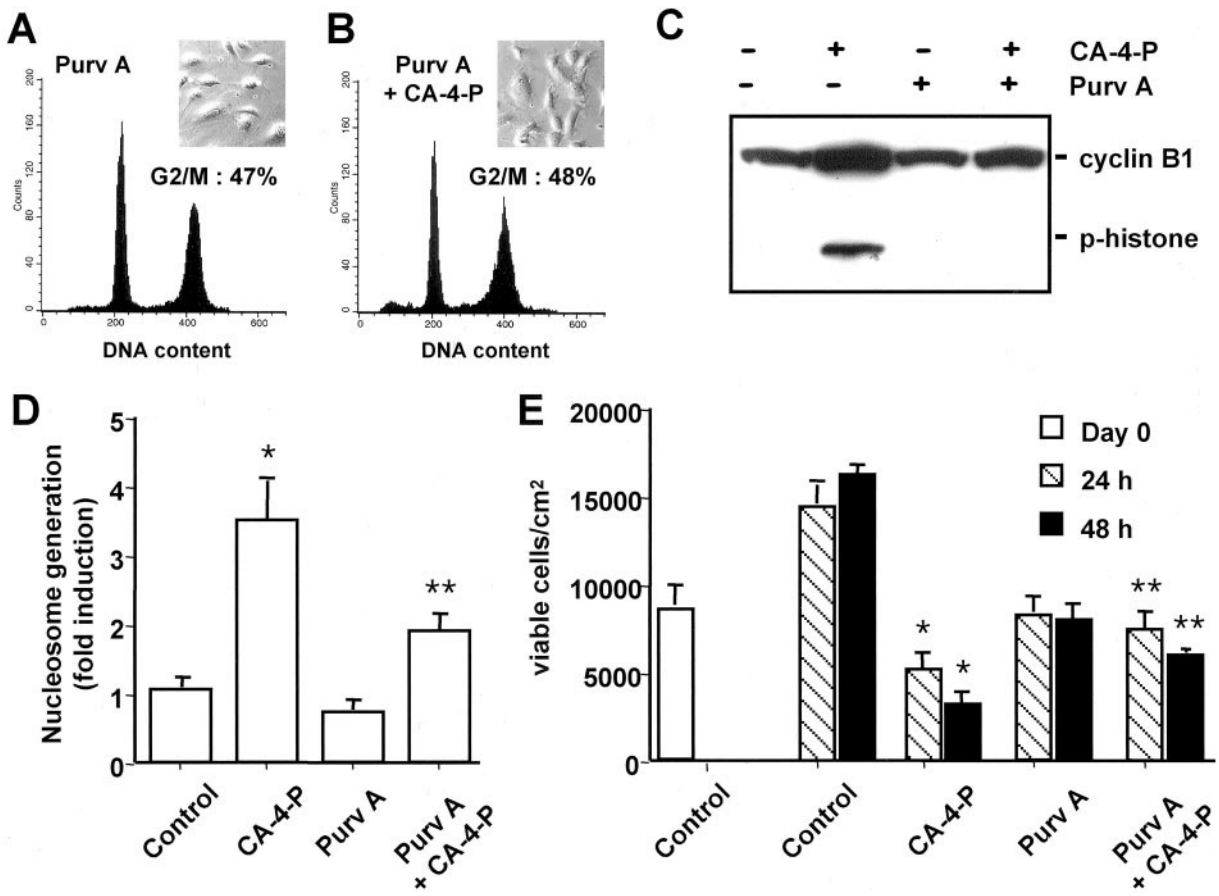


Figure 9. Purvalanol A arrests endothelial cells in G_2, prevents CA-4-P-mediated entry into mitosis, and protects against CA-4-P-mediated cell death. **A** and **B:** DNA content of endothelial cells was analyzed by PI staining and flow cytometry. Exponentially growing cells were treated with 10 μmol/L purvalanol A (**A**) or 10 nmol/L CA-4-P plus 10 μmol/L purvalanol A for 24 hours (**B**). **Insets** show phase contrast images. **C:** Cells were either left untreated or treated with purvalanol A ± CA-4-P as in **A** and **B** and protein extracts were analyzed by Western blotting for cyclin B1 expression and phosphorylation of histone H1. **D:** Cells were treated with purvalanol A ± CA-4-P as in **C** after which cytoplasmic nucleosomes were quantified by Cell Death Detection ELISA. The assay was performed in triplicate and the results represent the mean values ± SEM from one independent representative experiment. *, CA-4-P values significantly different from controls; **, purvalanol A + CA-4-P values that are significantly different from CA-4-P alone. **E:** Exponentially growing cells were treated as in **C**. At 24 and 48 hours after treatment cells were harvested, adherent and floating cells pooled, and viability was evaluated by trypan blue exclusion assay. The assay was performed in triplicate, and the results represent the mean values ± SEM from one independent representative experiment. *, CA-4-P values that are significantly different from controls; **, denotes purvalanol A + CA-4-P values that do not significantly vary from values for purvalanol A alone.

death-inducing stimulus is associated primarily with mitotic arrest and not some other signal generated by interaction with interphase microtubules.

Microtubule targeting compounds activate the mitotic spindle checkpoint that monitors chromosome attachment to the mitotic spindle and delays chromosome segregation during anaphase until defects in the mitotic spindle apparatus are corrected.[21] In parallel, degradation of cyclin B1 is inhibited leading to a prolonged activation of cyclin B/p34^{cdc2} activity. A sustained p34^{cdc2} activity is a consequence of mitotic arrest of various tumor cell lines by drugs such as taxol[28,41] and also in normal endothelial cells in response to CA-4-P, as we demonstrate here. p34^{cdc2} activity is thought to act as a pro-apoptotic mediator in some systems. For example taxol-mediated death of breast cancer cells was shown to be inhibited by inhibitors of p34^{cdc2} activity or cyclin B anti-sense oligonucleotides.[41] Furthermore, a sustained p34^{cdc2} activity was found to be required for apoptosis induction by noscapine, an anti-microtubule drug, in mitotic-arrested murine mammary carcinoma cells.[24] Whether sustained p34^{cdc2} activity is the actual trigger for cell death induction by microtubule-targeting agents is not currently known. Certainly, sustained p34^{cdc2} activity is required for the continuous arrest of endothelial cells in metaphase, because treatment of such CA-4-P metaphase-arrested cells with p34^{cdc2} activity inhibitor purvalanol A, caused the majority of mitotic-arrested cells to exit mitosis. This indicates that a sustained p34^{cdc2} activity is a key component of the mitotic block and cells can proceed to exit mitosis even in the absence of spindle assembly, provided p34^{cdc2} activity is efficiently down-regulated.

The cellular context is likely to be important in determining the mechanisms by which cell death is induced by agents such as CA-4-P. Whereas studies on the apoptotic effects of microtubule targeting agents have been conducted primarily on tumor cell lines, very little is known about the way these compounds affect normal cells and endothelial cells in particular. Endothelial cells appear to have a functional spindle assembly checkpoint and CA-4-P was found to arrest these cells in metaphase. A recent study has demonstrated that a functional spin-

dle assembly checkpoint is associated with susceptibility to induction of apoptosis by anti-microtubule agents in some human lung cancer lines as opposed to lung cancer cell lines with defective spindle assembly checkpoints that were resistant to induction of apoptosis.[42] Defects in spindle assembly checkpoint have been linked to the pathogenesis of several human tumors and can result in the aberrant exit of cells from mitosis and subsequent generation of aneuploid cells.[43] A defective spindle assembly is likely to be the cause of frequently observed aberrant mitoses in response to microtubule-targeting agents.[10] Nevertheless such aberrant mitotic exit resulting in G_1-multinucleate cells can also be the trigger for apoptosis as described for paclitaxel and some tumor cell types.[23,38] Indeed, CA-4-P leads to the formation of multinucleate cells in several human and murine tumor cell lines (our unpublished observations) which suggests that differences in spindle assembly checkpoints operate in different cell types and may affect the subsequent fate of the cells in response to this compound.

Mitotic catastrophe has been described as the predominant form of cell death mediated by CA-4-P in human B-lymphoid tumors and some leukemia cell lines.[44] Mitotic catastrophe results from aberrant mitosis leading to the formation of giant non-viable multinucleated cells and is a form of cell death thought to share some common pathways with apoptosis.[45] CA-4-P-arrested mitotic endothelial cells appeared to remain arrested in metaphase from which they subsequently entered a cell death pathway without exiting mitosis. Cells treated with low concentrations of CA-4-P (2.5 to 5 nmol/L), appeared to exit mitosis but nuclei were characteristically deformed (Figure 4). Based on these morphological observations it is likely that endothelial cells treated with CA-4-P share at least some characteristics with cells undergoing mitotic catastrophe. Nucleosome generation, a characteristic of apoptotic cell death, was detected in these cells, which suggests that apoptotic mechanisms play an important role. Caspases however, do not appear to be necessary for CA-4-P-mediated mitotic endothelial cell death because the general caspase inhibitor of Z-VAD-fms failed to prevent either the entry of cells into mitosis or the subsequent generation of nucleosomes and cell death. Therefore, cell death induction by CA-4-P is caspase-independent, even though caspases appear to be co-activated in the process. This is in agreement with findings by Nabha and colleagues[44] who showed that inhibition of caspases, that were activated by CA-4-P treatment in leukemia cell lines, did not prevent the observed mitotic catastrophe and cell death. Caspase-independent cell death, while caspases were co-activated, has also been reported for other microtubule-binding agents such as paclitaxel and nocodazole which suggests that caspase activation may be a mere bystander effect of such agents.[42,46]

We have previously demonstrated that CA-4-P induces a rapid form of primarily necrotic type of cell death in predominantly postconfluent endothelial cells.[8] This form of cell death was observed at high doses of CA-4-P ($\geq$100 nmol/L) and was associated with the capacity of the drug to activate Rho kinase and induce a blebbing morphology, which was characterized by altered actin reorganization and reduced adhesion. The postconfluent nature of the cell cultures used in those experiments, which were characterized by a very low mitotic index, and the fact that high concentrations of the drug were required to induce necrotic cell death precluded that mitotic cells were specifically targeted.

The specific targeting of proliferating endothelial cells cannot explain the rapid tumor blood flow reduction seen as a result of drug administration *in vivo*. However specific targeting of proliferating endothelial cells is important for the efficacy of anti-angiogenic cancer therapies. Indeed, using several models of angiogenesis *in vitro* and *in vivo*, Ahmed and colleagues[20] recently demonstrated an anti-angiogenic component in the mechanism of action of CA-4-P. Here we demonstrate that spindle microtubules are very sensitive to treatment with CA-4-P. This appears to be the case for other microtubule-binding agents such as colchicine, vinblastine, and taxol that also cause mitotic arrest at concentrations below those required to depolymerize certain populations of cytoskeletal microtubules.[37,47] Nanomolar concentrations of CA-4-P were found to be effective at inhibiting endothelial spindle assembly *in vitro* which could imply that low doses could confer potent anti-proliferative and anti-angiogenic effects. Whether low-dose regiments of CA-4-P can inhibit tumor angiogenesis *in vivo* remains to be determined. An important limitation would be the relative short half life of the drug *in vivo*.[48] However, exposure to drug is undoubtedly higher in tumors than in normal tissues because of self-trapping as blood flow decreases. Cells in mitosis or about to enter mitosis at the time of CA-4-P administration would be most susceptible to these effects described here. Cells in asynchronous cell cultures began to accumulate in mitosis and showed signs of apoptosis at ~8 hours after CA-4-P treatment. This correlation between time of detectable arrest in mitosis and detectable apoptosis may suggest that apoptosis follows after a relatively short period of mitotic arrest. Efficient targeting of the mitotic endothelial cell fraction would, in principle, benefit from repeated dosing of the drug *in vivo*. Interestingly, CA-4-P was recently shown to inhibit angiogenesis in a mouse model of proliferative retinopathy after a schedule of daily dosing of the drug.[19] Further studies are necessary to establish whether repeated dose regimens of CA-4-P could be effective at inhibiting tumor blood vessel growth.

In conclusion the results of the current study identify a definitive link between mitotic arrest caused by the microtubule-targeting agent CA-4-P and cell death of endothelial cells. Further studies are warranted to better understand the underlying molecular mechanisms of mitotic arrest and the influence of the tumor microenvironment, to improve the susceptibility of the tumor microvasculature to microtubule-targeting agents such as CA-4-P.

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