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Report

Effects of Combretastatin A-4 prodrug against a panel of malignant human B-lymphoid cell lines

Sanaa M Nabha,[1] Nathan R Wall,[1] Ramzi M Mohammad,[1] George R Pettit[2] and Ayad M Al-Katib[1]

[1]Division of Hematology and Oncology, Karmanos Cancer Institute, School of Medicine, Wayne State University, Detroit, MI 48201, USA. [2]Cancer Research Institute and Department of Chemistry, Arizona State University, Tempe, AZ 85287-1604, USA.

Combretastatin A-4 (CA-4) is one of a family of compounds isolated from the South African willow tree *Combretum caffrum*. CA-4 was found to be active against murine melanoma and a variety of other human solid tumors. For the first time, we report the effect of CA-4 against a panel of malignant human B-lymphoid cell lines [early pre-B acute lymphoblastic leukemia (Reh), diffuse large cell lymphoma (WSU-DLCL$_2$), chronic lymphocytic leukemia (WSU-CLL) and Waldenstrom's macroglobulinemia (WSU-WM)]. Our results indicate, using the prodrug form of CA-4, a concentration-dependent growth inhibition in all tested cell lines, although WSU-DLCL$_2$ was more sensitive. Exposure to 4 nM CA-4 for 96 h induced 77% growth inhibition in Reh, 86% in WSU-CLL and 92% in WSU-WM. When used against the WSU-DLCL$_2$ cell line, this same concentration of CA-4 was completely toxic. Morphological examination showed CA-4 induced the formation of giant, multinucleated cells, a phenomenon commonly found in mitotic catastrophe. Only minimal numbers of cells showing characteristics of apoptosis were detected. In WSU-DLCL$_2$ cells, CA-4 (3 nM) induced the highest apoptosis (5%) after 48 h, while the percentage of dead cells was approximately 47%. Exposure of Reh, WSU-CLL, WSU-WM and WSU-DLCL$_2$ cells for 24 h to 5 nM CA-4 induced 19, 28, 57 and 75% G$_2$/M arrest, as determined by flow cytometry, respectively. Based on these preliminary studies, we believe that mitotic catastrophe is the predominant mechanism by which CA-4 induces cell death rather than apoptosis. Further studies to elucidate the mechanisms of CA-4 activity *in vitro* and *in vivo* are currently under investigation in our laboratory. [© 2000 Lippincott Williams & Wilkins.]

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Correspondence to AM Al-Katib, Professor of Medicine, Division of Hematology and Oncology, Wayne State University School of Medicine, 3990 John R (4 Brush South), Harper Hospital, Detroit, MI 48201, USA.
Tel: (+1) 313 745-8217; Fax: (+1) 313 993-0307;
E-mail: alkatiba@karmanos.org

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Introduction

Over 54 000 cases of non-Hodgkin's lymphoma (NHL) occur annually and almost half of these are considered to be aggressive NHL.[1] At the present time, the majority of patients with newly diagnosed aggressive NHL receive an anthracycline-containing induction regimen such as CHOP (cyclophosphamide, doxorubicin, vincristine and prednisone). However, CHOP induction therapy cures approximately 40% of aggressive NHL patients, underscoring the need to develop more effective treatment strategies.[2] B-CLL is the most common form of leukemia in the USA, accounting for approximately 25%[3] of all the cases. There is no curative therapy for chronic lymphocytic leukemia (CLL). Acute lymphoblastic leukemia (ALL) is the most common malignancy found in children, and accounts for one-fourth of all childhood cancers and approximately 75% of all cases of childhood leukemia. Around 2000 new cases per year of ALL are diagnosed in the USA.[4] Waldenstrom's macroglobulinemia (WM) is also a B cell tumor, similar to CLL, which remains incurable. We have focused our efforts on enhancing the therapeutic results in B cell tumors by investigating a variety of new compounds that have been developed through the NCI's program to explore natural products as a source of anti-cancer agents. One such agent that has shown promise against a variety of tumors and thus warranted investigation in NHL and leukemia is combretastatin A-4 (CA-4).

CA-4 is one of a family of compounds that has been isolated from the South African willow tree *Combretum caffrum*.[5] CA-4 has been shown to have tubulin binding abilities[5,6] like the vinca alkaloids vincristine

and vinblastine, which are potent anticancer agents currently in use clinically.[7–9] CA-4 has a high affinity for tubulin at or near the colchicine (another tubulin-binding agent) binding site, causing the destabilization of the tubulin polymers of the cytoskeleton.[5,6] This destabilization results in the disruption of the cell's ability to successfully complete cell division. CA-4 was found active against the P388 and murine B-16 melanoma cell lines.[10] It also has selective toxicity against proliferating endothelial cells *in vitro* and induced vascular shut down in tumor models *in vivo*.[11,12]

Here, for the first time, we report on the antitumor effects of CA-4 against a panel of cell lines representing four different stages of the B cell maturation pathway: early pre-B ALL, diffuse large cell lymphoma, CLL and WM. Interestingly, exposure of these four cell lines to CA-4 at low concentrations resulted in the formation of giant, multinucleated cells characteristic of early mitotic catastrophe.[13] However, when CA-4 concentrations were increased, a concentration-dependent chromosome condensation, massive nuclear fragmentation and mitotic catastrophe resulted. Only small numbers of apoptotic cells were detected.

Materials and methods

Cell culture

The human early pre-B acute lymphoblastic leukemia cell line Reh was obtained from ATCC (Rockville, MD). The Reh cell line was established from a 15-year-old girl and was characterized as being at the early pre-B stage.[14] The human CLL cell line (WSU-CLL), the human diffuse large cell lymphoma cell line (WSU-$DLCL_2$) and the human WM cell line (WSU-WM) were established in our laboratory at Wayne State University School of Medicine.[15–17] All cell lines were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 1% L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin at 37°C in an atmosphere of 5% CO_2. All chemicals were obtained from Sigma (St Louis, MO.).

Drug

The prodrug form of CA-4, a tubulin-binding drug, isolated from the South African tree *C. caffrum*,[5] was dissolved in phosphate-buffered saline (PBS) at 10^{-6} M and then diluted in culture media. CA-4 was obtained from Dr George R Pettit (Cancer Research Institute and Department of Chemistry, Arizona State University, Tempe, AZ).

Cell growth analysis

Cells were seeded in 24-well culture plates (Costar, Cambridge, MA) at a concentration of 5×10^4 viable cells/ml. Untreated and CA-4-treated cultures at different concentrations (0.5, 1, 2, 3, 4 and 5 nM) were set in triplicate. Cultures were incubated at 37°C, and 5% CO_2 in a humidified incubator. Cell viability and total cell number were determined daily for 4 days using Trypan blue (0.4%) exclusion (Gibco, Grand Island, NY).

Morphological analysis

Cytocentrifuge smears from untreated and CA-4-treated cultures were prepared using the Cytospin 2 centrifuge (Shandon Southern Instruments, Sewickley, PA) daily for 2 days. The smears were air-dried, stained with tetrachrome for 5 min and analyzed using light microscopy. Three hundred cells were counted 3 times and statistically analyzed. Features of apoptosis that were looked for included cell shrinkage, nuclear chromatin condensation, formation of membrane blebs and apoptotic bodies. Features of cell mitotic catastrophe included the appearance of spontaneous premature chromosome condensation and multiple nuclear fragments which are readily distinguished from apoptotic nuclear fragments. Features of cell death included cell swelling, nuclear expansion and gross cytolysis.

Cell cycle analysis

Cells (10^6) from untreated and 24, 48, 72 and 96 h CA-4-treated cell lines were fixed in absolute alcohol for 30 min at 4°C. After treatment with RNase for 40 min at 37°C, cells were stained with propidium iodide (50 μg/ml) and analyzed on a FACScan (Becton Dickinson, Mountain View, CA). Data on 20 000 cells was acquired and processed using Lysys II software (Becton Dickinson) (Molecular and Cellular Imaging and Analytical Cytometry Core Facility of the Barbara Ann Karmanos Cancer Institute at Wayne State University School of Medicine).

Annexin-V

A calcium-dependent phospholipid-binding protein with a high affinity for phosphatidylserine (PS), annexin-V, was used to detect the early stage apoptosis.[18] Briefly, 2×10^5 cells/ml were grown in RPMI containing 10% FCS. Cells were treated with CA-4 at concentrations of 3, 4 and 5 nM for Reh, WSU-CLL and WSU-WM, and of 2, 3 and 5 nM for WSU-$DLCL_2$, and then harvested and processed after 6, 24 and 48 h.

The cells were then washed in PBS and resuspended in binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Annexin-V and propidium iodide were then added to a final concentration of 100 ng/ml (according to the manufacturer's specifications; PharMingen, San Diego, CA), and cells were incubated in the dark for 10 min, washed again in PBS and resuspended in 300 μl of binding buffer. PI (10 μl) was added to each sample before flow cytometric analysis. In each sample, 20 000 cells were counted.

Results

Cell growth analysis

CA-4 showed a concentration-dependent growth inhibition effect in all four cell lines tested (Figure 1). CA-4 treatment of the Reh (Figure 1A), WSU-CLL (Figure 1B) and WSU-WM (Figure 1C) cell lines was shown to cause no significant decrease in cell growth at 0.5, 1 and 2 nM after 4 days. However, in comparison with the control cells, CA-4 at 3 nM induced 40% cell growth inhibition in Reh, WSU-CLL and WSU-WM, while 4 nM CA-4 induced 77, 86 and 92% growth inhibition in Reh, WSU-CLL and WSU-WM, respectively. CA-4 at 5 nM was toxic to all cell lines tested. In WSU-$DLCL_2$ (Figure 1D), CA-4 induced significant growth inhibition at 1 and 2 nM (35 and 59%, respectively, compared to control), while a cytotoxic effect occurred at 3, 4 and 5 nM. The WSU-$DLCL_2$ lymphoma cell line is, therefore, more sensitive than Reh, WSU-CLL or WSU-WM.

Morphological changes

Reh, WSU-CLL and WSU-WM cell lines exposed to CA-4 at 4 nM exhibited 6.7, 9.3 and 21%, mitotic arrest, respectively, and 1.7, 2.7 and 2% apoptosis, respectively, after 48 h of treatment (Table 1). At

Table 1. Morphological examination of cells after 24 and 48 h of treatment with CA-4 at the concentration of 4 nM for Reh, WSU-CLL and WSU-WM, and 3 nM for WSU-$DLCL_2$ (results are given in percentages)

Time (h)	Viable	Mitotic	Apoptotic	Dead
Reh				
24	85±4	10±2	1±1	6±1.4
48	83±4.6	6.7±2.9	1.7±1.2	9±4.6
WSU-CLL				
24	90±2.6	8.3±0.6	0±0	1.7±2.1
48	85.3±5.5	9.3±4.9	2.7±1.5	2.7±1.2
WSU-WM				
24	78.5±5.8	16.3±4.9	0.3±0.6	4.3±0.6
48	69.7±1.5	21.0±4.6	2.0±1.0	7.3±2.5
WSU-$DLCL_2$				
24	85.3±5.8	7±1.7	1±1	6.3±1.5
48	23±3.5	25.3±7.6	5.0±2.6	46.7±6.7

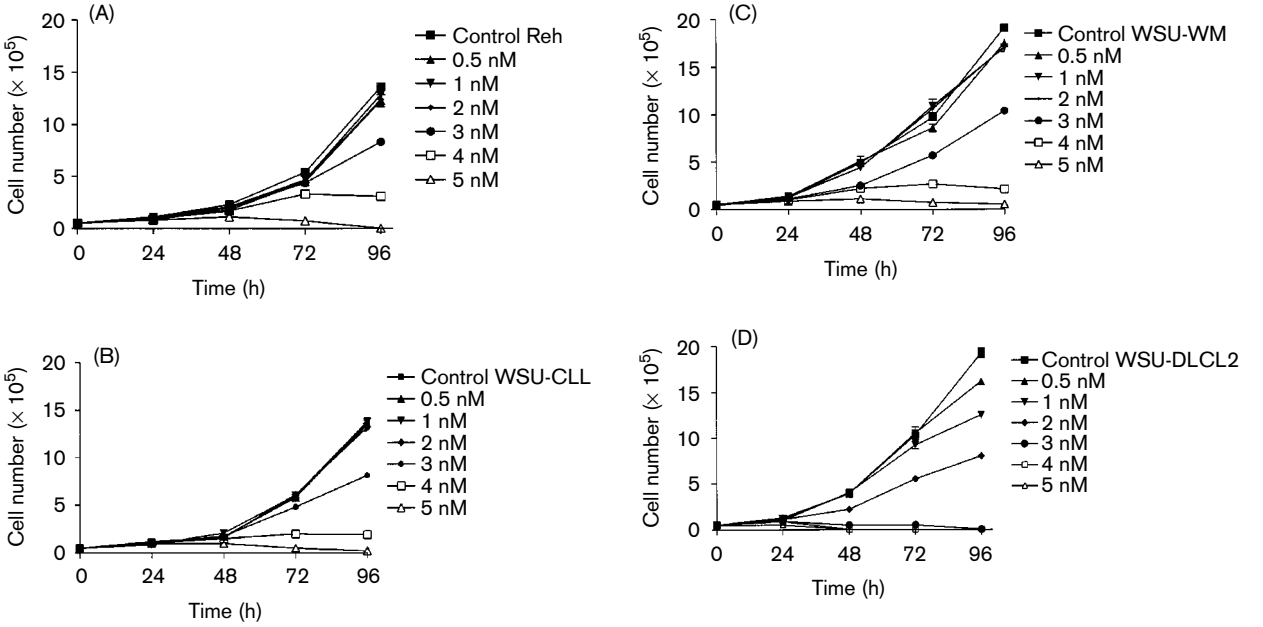

Figure 1. Effects of CA-4 on the growth of four human B cell lines. Cells (5×10^4/ml) were grown in RPMI 1640 supplemented with 10% FBS and CA-4 at different concentrations for 4 days. (A) Reh, (B) WSU-CLL, (C) WSU-WM and (D) WSU-$DLCL_2$.

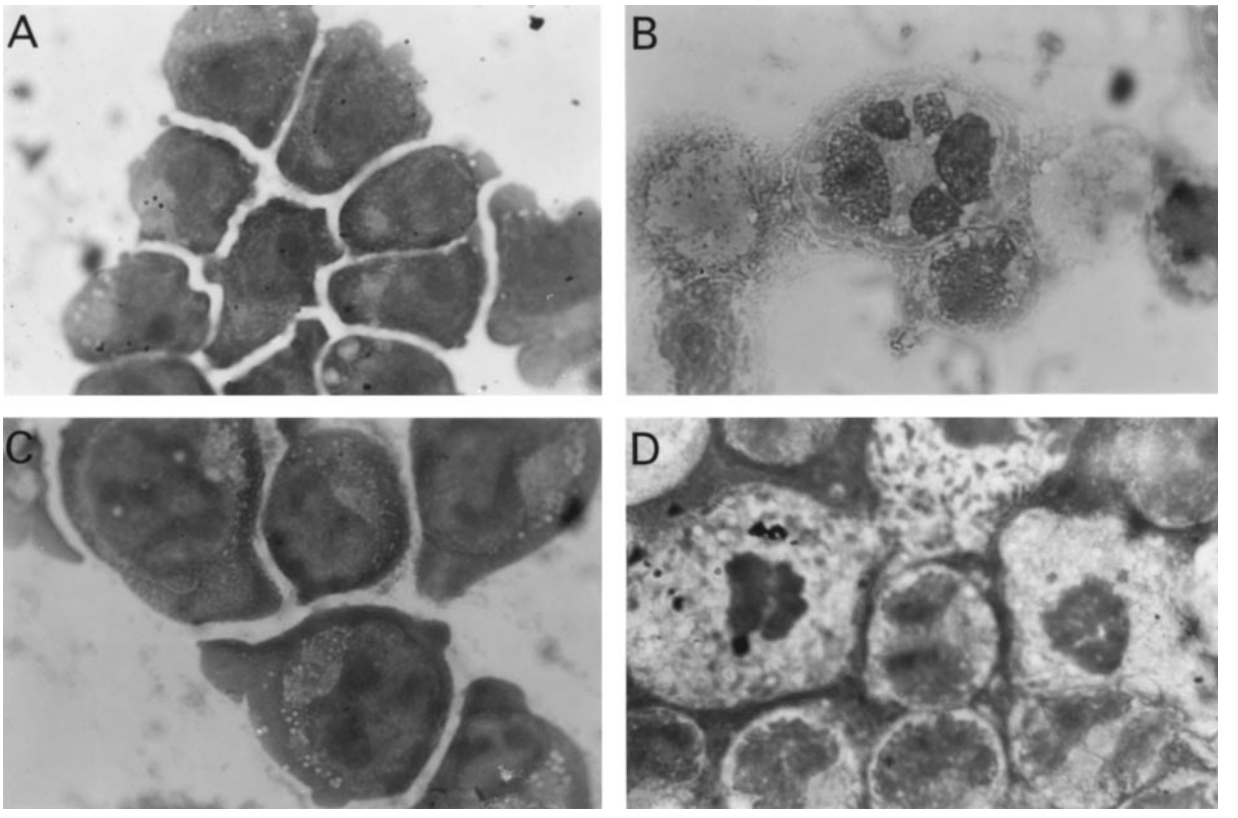

Figure 2. Mitotic catastrophe induction in WSU-CLL and WSU-$DLCL_2$ cells. Photographs showing the characteristic features of WSU-CLL and WSU-$DLCL_2$ cells ($\times$1000) untreated or treated with CA-4. (A) Control WSU-CLL cells. (B) WSU-CLL cells treated for 48 h with 4 nM CA-4. (C) Control WSU-$DLCL_2$ cells. (D) WSU-$DLCL_2$ cells treated for 24 h with 3 nM CA-4. Note the giant, multinucleated cells characteristic of mitotic catastrophe in (B) and (D). Photos are of cells representative of all cells in this study.

48 h WSU-$DLCL_2$ cells treated with 3 nM CA-4 exhibited 25% mitosis, 5% apoptosis and 47% were dead. Figure 2(B and D) represents the morphological appearance of WSU-CLL and WSU-$DLCL_2$ exposed to CA-4, and harvested after 24 h of treatment for WSU-$DLCL_2$ and 48 h for WSU-CLL. We note the abnormality in mitosis (or altered mitotic cell morphology). This phenomenon is characterized by a fragmentation of the nucleus without apparent entry into anaphase and telophase.

Annexin-V staining for apoptosis

The low number of cells in apoptosis was confirmed by Annexin-V–PI staining (Figure 3 and Table 2). This assay showed that 1.5% of Reh cells, 1.1% of WSU-CLL cells, 6.2% of WSU-WM cells and 16.2% of WSU-$DLCL_2$ cells bound annexin-V after 24 h of CA-4 treatment. CA-4 at 5 nM caused a dramatic increase in annexin-V binding in the WSU-WM (10.2%) and WSU-$DLCL_2$ (25%) cell lines.

Table 2. Percentage of apoptotic cells after treatment with CA-4: cells were stained with annexin V and PI after 6, 24 and 48 h or CA-4 exposure

Time (h)	Control	3 nM	4 nM	5 nM
Reh				
6	1.2	2.3	2.4	2.3
24	1.3	1.1	1.5	3.3
48	1.1	1.4	3.4	16
WSU-CLL				
6	0.6	0.8	1.1	1.1
24	0.8	0.9	1.1	2.9
48	1.1	1.2	3.3	12.4
WSU-WM				
6	2.9	2.3	3.0	3.3
24	4.9	5.8	6.2	10.2
48	3.5	7.0	11.1	15.9
WSU-$DLCL_2$				
6	4.1	3.8	4.1	3.6
24	4.8	5.9	8.1	25.0
48	4.7	7.7	16.2	4.7

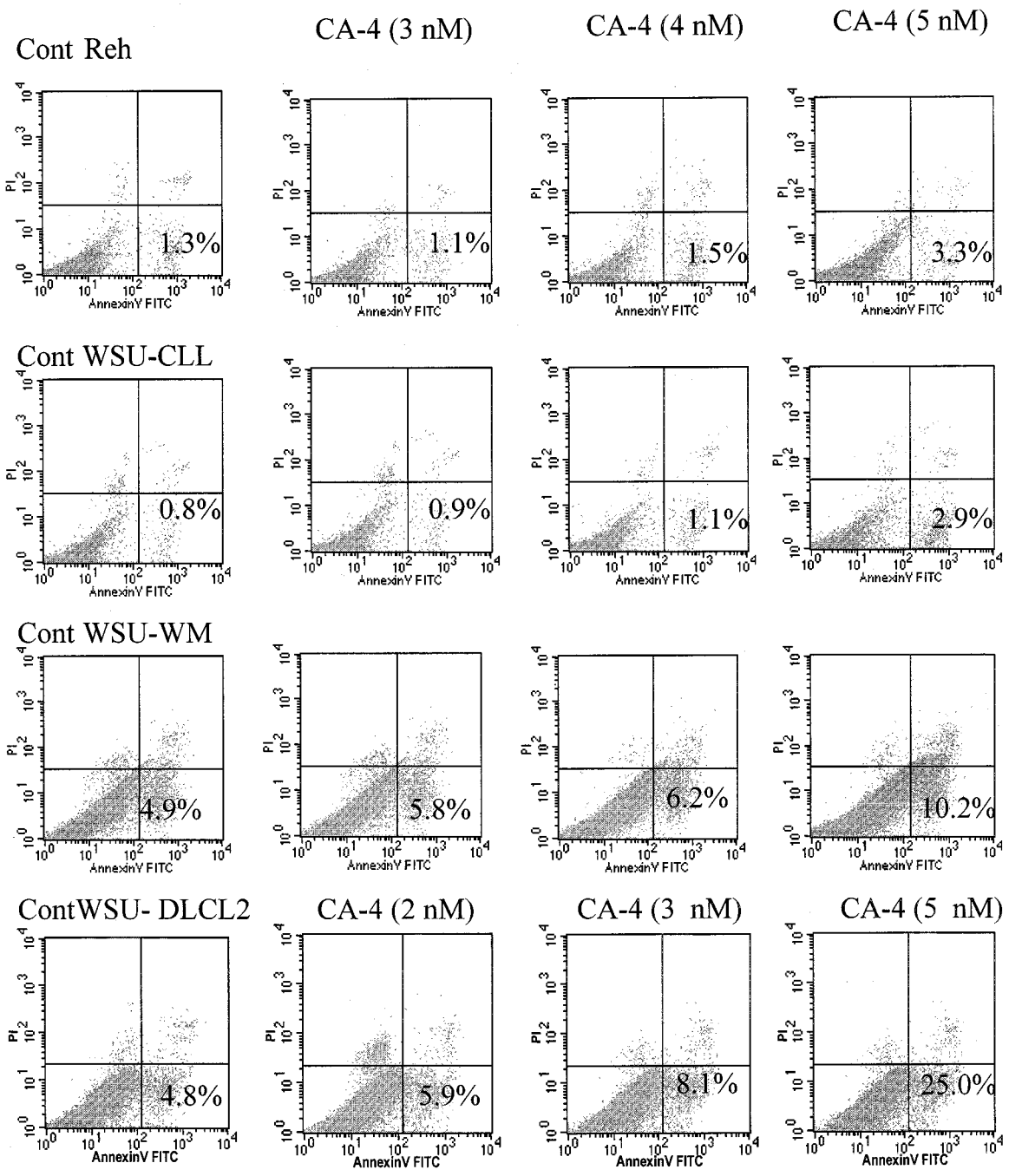

Figure 3. A representative figure showing the effect of CA-4 on the four cell lines after 24 h. CA-4-treated cells (5×10^4/ml) were stained with annexin-V and PI to determine percent apoptosis. Lower right quadrant is representative of percent of cells that had entered the early stage of apoptosis. Upper right quadrant is representative of percent of cells that had entered the late stage of apoptosis. Lower left quadrant is representative of percent of viable cells.

Effect of CA-4 on cell cycle

CA-4 induced a dose-dependent increase in G_2/M in all cell lines tested. In comparison to the control cells, both Reh and WSU-CLL cells, after 5 nM of CA-4 exposure, showed an increase in the number of cells in G_2/M (Figure 4A and B). The percentage of cells in G_2/M increased from 10% (untreated) to 19% (treated) in Reh and 8% (untreated) to 28% (treated) in WSU-CLL. In contrast, WSU-WM cells showed a dramatic increase (25, 38 and 57%, respectively) in G_2/M after 24 h of exposure to 3, 4 and 5 nM of CA-4 as compared to

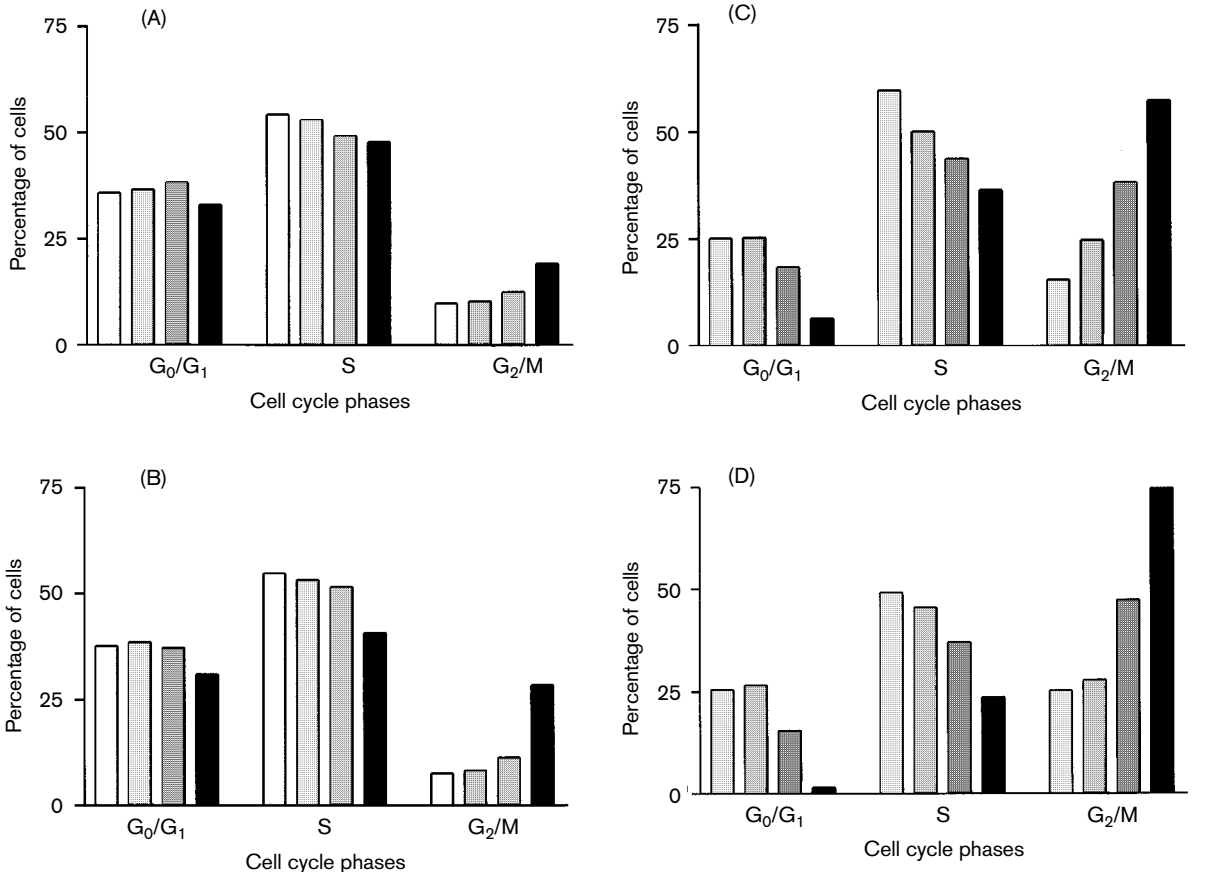

Figure 4. Effects of CA-4 on cell cycle after 24 h of exposure to CA-4 of B cell lines. Cells (5×10^4/ml) were grown in RPMI 1640 supplemented with 10% FBS and CA-4 at various concentrations (from lightest to darkest columns: control, 3, 4 and 5 nM for Reh, WSU-CLL and WSU-WM; control, 2, 3 and 5 nM for WSU-$DLCL_2$). (A) Reh, (B) WSU-CLL, (C) WSU-WM and (D) WSU-$DLCL_2$.

control cells (15.4%) (Figure 4C). A similar but more accentuated finding was recorded in the WSU-$DLCL_2$ cell line. After 24 h of CA-4 exposure, cells entering G_2M increased from 25.3% in control to 74.7% in cells exposed to 5 nM CA-4 (Figure 4D). There was a reciprocal decrease in G_0/G_1 and S phases in all cell lines commensurate with the increase in G_2/M.

Discussion

CA-4 was found to display potent toxicity toward tumor vasculature,[11,19,20] and has activity against human solid tumors[10] and murine leukemia cell lines.[21] The present study was designed to investigate the growth inhibitory effects, cell cycle modulation and apoptosis induction in four malignant human B cell lines as assessed by a number of parameters including morphology, cell growth and cell cycle analysis.

CA-4 showed a concentration–dependent effect on all cell lines tested. Five different concentrations ranging from 0.5 to 5 nM were tested for 4 days. Significant growth inhibition at 3, 4 and 5 nM was observed in Reh, WSU-CLL and WSU-WM, while lower concentrations (1 and 2 nM) were active against WSU-$DLCL_2$. This cell line appears more sensitive to CA-4. WSU-$DLCL_2$ is a prototype of aggressive but curable NHL. CA-4's effects are cell line dependent, not differentiation-stage dependent, as the WSU-CLL cell line represents a stage of B cell differentiation that is between that of WSU-$DLCL_2$ and WSU-WM. Our studies show that the growth inhibition is mediated by cell cycle arrest in G_2/M, which is also more pronounced in WSU-$DLCL_2$ than in the other cell lines tested.

To understand the mechanism by which cells die after treatment with CA-4, we analyzed cells by flow cytometry after annexin-V staining and by light microscopy. Annexin-V staining showed only a small number of cells dying by apoptosis (Figure 3 and Table 2) which was confirmed by microscopic examination (Figure 2). We recorded higher levels of annexin-V

staining in WSU-DLCL$_2$ and WSU-WM cell lines after 24 h of CA-4 treatment. We did not, however, observe higher levels of apoptosis by morphology. We therefore believe that after 24 h of CA-4 treatment, cell membranes are allowing annexin-V to enter the cells and stain the PS on the inner surface of the membrane which would account for a false positivity in annexin-V staining in the WSU-DLCL$_2$ and WSU-WM cell lines. Microscopic observation also showed characteristics of mitotic catastrophe (Figure 2) as defined by the formation of giant, multinucleated cells. The phenomenon of mitotic catastrophe has been reported in cells after DNA damage by irradiation[22,23] or chemotherapy.[24]

The concept of arresting cells in mitosis and inducing cell death is clinically plausible. There are many standard chemotherapeutic drugs that are used in the clinic to treat NHL, which affect the cell cycle, like vincristine and etoposide. CA-4 has the characteristics of both an antimitotic and antiangiogenesis agent. Other tubulin binding agents like vinblastine, flavone acetic acid and vincristine were reported as having antivascular effects,[25–27] but those effects were evident only at doses approaching the maximum tolerated dose (MTD). In comparison, CA-4 has been reported to induce vascular shutdown within tumors at doses less than one-tenth of the MTD.[11,20] Therefore CA-4 is a more interesting drug than those currently used, having the same characteristics, in the clinic. Further studies in a pre-clinical, *in vivo* mouse model are being conducted in our laboratory to investigate the vascular shutdown in B cell lymphoma.

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