Calphostin C Triggers Calcium-dependent Apoptosis in Human Acute Lymphoblastic Leukemia Cells

De-Min Zhu, Rama-Krishna Narla, Wei-Hua Fang, Nian-Cherng Chia, and Fatih M. Uckun

Departments of Immunology [D-M. Z., W.-H. F.], Experimental Oncology [R-K. N., N-C. C., F. M. U.], and Drug Discovery Program [R-K. N., F. M. U.], Parker Hughes Cancer Center, and the Children’s Cancer Group ALL Biology Reference Laboratory [R-K. N., F. M. U.], Hughes Institute, Roseville, Minnesota 55113

ABSTRACT

Recent studies have demonstrated that the naturally occurring perylenequinone antibiotic calphostin C is a potent inhibitor of protein kinase C and can induce apoptosis in some tumor cell lines by an as yet unknown mechanism. Here we demonstrate that calphostin C induces dose-dependent apoptosis in DT40 chicken lymphoma B-cells, and targeted disruption of lyn, syk, btk, PLCγ2, or IP3R genes does not prevent or attenuate its cytotoxicity. In our study, calphostin C also induced rapid apoptosis in human acute lymphoblastic leukemia (ALL) cell lines ALL-1 (BCR-ABL+ pre-pre-B ALL), RS4;11 (MLL-AF4+ pre-B ALL), NALM-6 (pre-B ALL), DAUDI (Burkitt’s/b-cell ALL), MOLT-3 (T-ALL), and JURKAT (T-ALL), whereas other potent PKC inhibitors did not. In biochemical studies, calphostin C was discovered to induce rapid calcium mobilization from intracellular stores of ALL cell lines, and its cytotoxicity against ALL cell lines was well correlated with the magnitude of this calcium signal. Calphostin C-induced apoptosis was markedly suppressed by BAPTA/AM, a cell-permeable Ca2+ chelator as well as NiCl2, an inhibitor of Ca2+/Mg2+-dependent endonucleases. Inhibition of the Ca2+/calmodulin-dependent phosphatase calcineurin with trifluoperazine dimadeate, a calmodulin antagonist, or cyclosporin A (a specific inhibitor of calcineurin) also reduced the magnitude of calphostin C-induced apoptosis in ALL cell lines. Calphostin C was capable of inducing calcium mobilization and apoptosis in freshly obtained primary leukemia cells from children with ALL. Taken together, our results provide unprecedented evidence that calphostin C triggers a Ca2+-dependent apoptotic signal in human ALL cells.

INTRODUCTION

Recurrence of leukemia continues to be a major obstacle to a successful outcome of multiagent chemotherapy or radiochemotherapy (in the context of bone marrow transplantation) in the treatment of ALL patients who have relapsed despite intensive multiagent chemotherapy (1-6). Consequently, the identification and development of new potent anti-ALL drugs have become focal points for translational leukemia research.

Calphostin C (C44H38O4, UCN-1028), is a naturally occurring perylenequinone antibiotic from the fungus Cladosporium cladosporioides FERM BP-1285, which was originally isolated from a block fence in Osaka, Japan. It has been shown to have a pleiotropic biological activity profile (6-14). A number of studies have demonstrated that this natural product can inhibit PKC (7, 8, 15-17), and cause, by an as yet undefined mechanism, apoptotic cell death (18-22).

The purpose of the present study was to examine the ability of calphostin C to kill human ALL cells. Here, we show that calphostin C, but not other PKC inhibitors, induce rapid apoptosis in ALL cells. Notably, both RS4;11, a highly radiation-resistant MLL-AF4 fusion transcript positive t(4;11) ALL cell line, and ALL-1, a multidrug-resistant BCR-ABL fusion transcript-positive t(9;22) ALL cell line, were exquisitely sensitive to calphostin C. In biochemical studies, calphostin C was discovered to induce rapid calcium mobilization from intracellular stores of ALL cells, and its cytotoxicity against ALL cells was correlated with the magnitude of this calcium signal. Calphostin C-induced apoptosis was markedly suppressed by BAPTA, a cell-permeable Ca2+ chelator as well as NiCl2, an inhibitor of Ca2+/Mg2+-dependent endonucleases. Inhibition of the Ca2+/calmodulin-dependent phosphatase calcineurin with trifluoperazine dimadeate, a calmodulin antagonist, or cyclosporin A, a specific inhibitor of calcineurin, also reduced the magnitude of calphostin C-induced apoptosis in leukemia cells. Taken together, our results provide unprecedented evidence that calphostin C triggers a Ca2+-dependent apoptotic signal in human ALL cells.

MATERIALS AND METHODS

Cell Lines. The following human ALL cell lines were used: (a) NALM-6, pre-B ALL; (b) RS4;11, t(4;11) pro-B ALL; (c) ALL-1, t(9;22) pre-pre-B ALL; (d) JURKAT, T-ALL; (e) MOLT-3, T-ALL; and (f) DAUDI, Burkitt’s lymphoma.

1 The abbreviations used are: ALL, acute lymphoblastic leukemia; PKC, protein kinase C; IP3R, inositol 1,4,5-trisphosphate receptor; TFPD, trifluoperazine dimadeate; CA, cyclosporin A; TdT, terminal deoxynucleotidyl transferase; TUNEL, TdT-mediated dUTP nick end labeling; CCG, Children’s Cancer Group; NCI, National Cancer Institute; LPC, leukemic progenitor cell; BCR, B-cell antigen receptor; PLCγ2, phospholipase C γ 2; BTK, Bruton’s tyrosine kinase.

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1 Supported in part by a Special Grant from Parker Hughes Trust.
2 To whom requests for reprints should be addressed, at Hughes Institute, 2665 Long Lake Road, Suite 330, Roseville, MN 55113. Phone: (612) 697-9228; Fax: (612) 697-1042.
Calphostin C Triggers Apoptosis in Human ALL Cells

Fig. 1 Calphostin C-induced apoptosis in DT40 chicken lymphoma B-cells. The chicken lymphoma B-cells were incubated with calphostin C at the indicated concentrations for 24 h, and the percentage of apoptotic nuclei was determined by TUNEL assays as described in the “Materials and Methods” section. A, concentration-dependence of calphostin C-induced apoptosis in chicken cells; SEs, ranged from 5 to 20% of the mean values (data not shown). B, apoptosis induced by 4 µM calphostin C. In C–F, chicken cells were incubated with 4 µM of calphostin C for 24 h, processed for the in situ apoptosis assay, and analyzed with a laser scanning confocal microscope. The green (yellow) fluorescence represents apoptotic nuclei, and the red fluorescence represents nuclei stained with propidium iodide.

phoma/leukemia. The ALL cells were cultured in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Summit Biotech, Ft. Collins, CO), 100 units/ml penicillin + 100 µg/ml streptomycin (Life Technologies). The DT40 chicken lymphoma B-cell lines including the wild type and those deficient for LYN, SYK, BTK, PLCγ2, or IP3R were cultured in RPMI 1640 (Life Technologies) supplemented with 5% heat-inactivated calf bovine serum (Summit Biotech), 1% chicken serum (Sigma, St. Louis, MO), and 100 units/ml penicillin + 100 µg/ml streptomycin (Life Technologies) (23–27). The cell lines were maintained in tissue culture at 37°C in a humidified 5% CO2 atmosphere.

Primary Leukemic Cells from Children with ALL. Mononuclear cell fractions that were composed primarily (≥90%) of leukemic cells were isolated by centrifugation on
Fig. 2 Calphostin C cytotoxicity against human ALL cells. In A, cells were incubated with calphostin C at different concentrations for 24 h, and the percent cell death was determined by viability assays, as described in the “Materials and Methods” section. Each data point represents the dead cells (mean ± SE) of three independent experiments. B, comparison of calphostin C with other PKC inhibitors. Cells were incubated with the specific inhibitor (each at 0.5 μM) for 24 h and analyzed with cell viability assay. Each data point represents the dead cells (mean ± SE) of three independent experiments.

Fig. 3 Calphostin C-induced apoptosis in ALL cells. In A, NALM-6 cells (○) and MOLT-3 cells (●) were incubated with calphostin C at indicated concentrations for 24 h, and the percentage of apoptotic nuclei was determined by TUNEL assays as described in the “Materials and Methods” section. Each data point represents the mean (±SE) of three independent experiments. A (inset), DNA fragment of MOLT-3 and NALM-6 cells after 24-h incubation with indicated concentrations of calphostin C. In B, NALM-6 cells were incubated with 0.5 μM of calphostin C for 24 h, processed for the in situ apoptosis assay, and analyzed with laser scanning confocal microscope. When compared with controls (B.1), several of the cells incubated with calphostin C (B.2) showed apoptotic nuclei (yellow fluorescence). Red fluorescence represents all nuclei stained with propidium iodide.
Ficoll-Hypaque gradients from pretreatment bone marrow aspirate samples of children entered on CCG front-line treatment protocols CCG-1952 for standard-risk ALL and CCG-1961 for high-risk ALL or the CCG salvage protocol CCG-1941 for children with ALL in first bone marrow relapse. Diagnosis of ALL was based on morphological, biochemical, and immunological features of the leukemic cells, including lymphoblast morphology on Wright-Giemsa-stained bone marrow smears, positive nuclear staining for TdT, negative staining for myeloperoxidase, and cell-surface expression of two or more lymphoid differentiation antigens, as described below. Patients were classified based on their age and presenting WBC as standard risk or high risk according to NCI risk classification criteria (28). Each protocol was approved by the NCI and the Institutional Review Boards of the participating CCG-affiliated institutions. Informed consent was obtained from parents, patients, or both, as deemed appropriate, according to Department of Health and Human Services guidelines. All of the patient bone marrow samples were used following the guidelines of the Hughes Institute Committee on the Use of Human Subjects in Research for secondary use of pathological or surgical tissue. Immunophenotyping was performed centrally in the CCG ALL Biology Research.

Fig. 4 Concentration dependence of calphostin C-induced intracellular Ca\(^{2+}\) elevation in NALM-6 cells. The cells were loaded with a calcium indicator (Fluo-3; 1 μM for 30 min) and processed for calcium imaging. Concentration dependence of calphostin C triggered Ca\(^{2+}\) signals in NALM-6 cells. Each curve represents the normalized fluorescence intensity (F) averaged from 10 to 20 single cells. Arrow, cells were exposed to calphostin C. The concentrations of calphostin C were: line a, 0.1 μM; line b, 0.2 μM; line c, 0.5 μM; and line d, 0.5 μM. For line d, Ca\(^{2+}\) (1.8 mM) in the cell suspension was first chelated by 5 mM EGTA/NaOH (pH 7.4), and then calphostin C was added.

Fig. 5 Fluorescent images of NALM-6 cells treated by calphostin C. Number in upper left corner of each frame, time (min) when the image was taken after the addition of calphostin C (0.5 μM) at zero time. Color bar, scale of the fluorescence intensity. The burst of the fluorescence indicates the elevation of cytosolic Ca\(^{2+}\)
Fig. 6 Calcium dependence of calphostin C-induced apoptosis in leukemia cells. A, correlation between the amplitude of calphostin C-induced intracellular Ca\textsuperscript{2+} elevation and apoptosis in leukemia cells. The cell lines used were DAUDI (□), NALM-6 (●), RS4;11 (○), and ALL-1 (■). The concentrations of calphostin C were 0.1, 0.2, and 0.5 μM. Each data point, percentage of the apoptotic nuclei according to the maximum normalized fluorescence intensity observed (horizontal axis) at a given concentration of calphostin C. The fluorescence intensity was the average from 10–20 single cells, and the percentage (mean ± SE) of the apoptotic nuclei was obtained from the TUNEL assay of three independent experiments. B, The inhibitory effects of Ca\textsuperscript{2+} chelator and the inhibitors of Ca\textsuperscript{2+}-related enzymes on calphostin C-induced apoptosis in NALM-6 cells. The dark columns show the percent of death induced by the inhibitory reagents alone, and the open columns show the percent of death with the inhibitory reagents plus calphostin C. For the experiments with BAPTA, the cells were first incubated with 5 μM BAPTA/AM for 1 h in growth medium in the incubator, and then the cells were washed two times with fresh medium to remove excess BAPTA/AM from the medium and resuspended in medium containing 0.5 μM calphostin C. TFPD at 5 μM, CsA at 1 μM, and NiCl\textsubscript{2} at 0.5 mM were added to cell suspensions 1 h before the addition of 0.5 μM calphostin C. Each data point, mean value (± SE) from three or more independent experiments.

Fig. 7 Confocal images of apoptotic nuclei—effect of NiCl\textsubscript{2}. A, control NALM-6 cells. In B, cells were treated with 0.5 μM calphostin C for 24 h. In C, cells were treated with 0.5 mM NiCl\textsubscript{2} for 24 h. In D, cells were treated with 0.5 mM NiCl\textsubscript{2} 1 h before the addition of 0.5 μM calphostin C for 24 h. Green (yellow) fluorescence represents the apoptotic nuclei. Red fluorescence represents the ALL nuclei stained with propidium iodide.
Reference Laboratory by indirect immunofluorescence and flow cytometry using monoclonal antibodies reactive with the following differentiation antigens: CD2, CD3, CD5, CD7, CD10, CD19, CD24, and CD34 as described previously (1,3).

Table 1  Inhibitory effects of NiCl2 on calphostin C-induced apoptosis in ALL cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Control</th>
<th>Calphostin C</th>
<th>NiCl2 (0.5 μM)</th>
<th>NiCl2 (0.5 mM)</th>
<th>calphostin C (0.5 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NALM-6</td>
<td>1.8 ± 0.4</td>
<td>49.9 ± 2.1</td>
<td>16.3 ± 1.3</td>
<td>20.5 ± 1.3</td>
<td>3.2 ± 0.9</td>
</tr>
<tr>
<td>MOLT-3</td>
<td>1.7 ± 0.6</td>
<td>44.6 ± 1.4</td>
<td>15.6 ± 0.9</td>
<td>33.2 ± 2.8</td>
<td>2.5 ± 0.7</td>
</tr>
<tr>
<td>RS4;11</td>
<td>0.4 ± 0.2</td>
<td>41.0 ± 1.6</td>
<td>12.1 ± 0.7</td>
<td>10.1 ± 1.1</td>
<td>3.5 ± 0.8</td>
</tr>
<tr>
<td>ALL-1</td>
<td>3.3 ± 0.6</td>
<td>42.7 ± 1.7</td>
<td>10.6 ± 1.3</td>
<td>9.6 ± 1.1</td>
<td>2.1 ± 0.5</td>
</tr>
</tbody>
</table>

The percent (mean ± SE) of apoptotic nuclei of the cells obtained from TUNEL assay of three independent experiments.

Table 2  Cytotoxic activity of calphostin C against freshly isolated primary leukemia cells from children with ALL

<table>
<thead>
<tr>
<th>UPN number</th>
<th>NCI risk group</th>
<th>Immuneotype</th>
<th>C1</th>
<th>C2</th>
<th>0.5 μM</th>
<th>2.0 μM</th>
<th>4.0 μM</th>
<th>C1</th>
<th>C2</th>
<th>0.5 μM</th>
<th>2.0 μM</th>
<th>4.0 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Standard</td>
<td>B</td>
<td>11</td>
<td>9</td>
<td>61</td>
<td>100</td>
<td>100</td>
<td>852 (836, 840)</td>
<td>710 (580, 840)</td>
<td>0 (0, 0)</td>
<td>99.0</td>
<td>0 (0.0, 99.0)</td>
</tr>
<tr>
<td>2</td>
<td>High</td>
<td>B</td>
<td>3</td>
<td>2</td>
<td>12</td>
<td>100</td>
<td>100</td>
<td>2812 (2792, 2832)</td>
<td>2438 (2424, 2452)</td>
<td>860 (800, 920)</td>
<td>65</td>
<td>0 (0.0, 99.9)</td>
</tr>
<tr>
<td>3</td>
<td>High</td>
<td>T</td>
<td>4</td>
<td>6</td>
<td>12</td>
<td>88</td>
<td>100</td>
<td>2316 (2236, 2396)</td>
<td>1878 (1866, 1888)</td>
<td>946 (916, 976)</td>
<td>50</td>
<td>0 (0.0, 99.9)</td>
</tr>
<tr>
<td>4</td>
<td>Standard</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>1314 (1180, 1448)</td>
<td>1238 (1052, 1424)</td>
<td>4 (4.4)</td>
<td>99.9</td>
<td>0 (0.0, 99.9)</td>
</tr>
<tr>
<td>5</td>
<td>High</td>
<td>T</td>
<td>12</td>
<td>5</td>
<td>88</td>
<td>97</td>
<td>100</td>
<td>1016 (896, 1136)</td>
<td>928 (880, 976)</td>
<td>0 (0.0, 99.9)</td>
<td>99.9</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Standard</td>
<td>B</td>
<td>11</td>
<td>13</td>
<td>66</td>
<td>95</td>
<td>98 (%)</td>
<td>922 (792, 1052)</td>
<td>874 (864, 884)</td>
<td>626 (576, 676)</td>
<td>28</td>
<td>0 (0.0, 99.9)</td>
</tr>
<tr>
<td>7</td>
<td>First relapse</td>
<td>B</td>
<td>11</td>
<td>1</td>
<td>60</td>
<td>100</td>
<td>100</td>
<td>1814 (1780, 1848)</td>
<td>1298 (1164, 1432)</td>
<td>6 (6.8)</td>
<td>99.9</td>
<td>0 (0.0, 99.9)</td>
</tr>
<tr>
<td>8</td>
<td>First relapse</td>
<td>B</td>
<td>11</td>
<td>1</td>
<td>60</td>
<td>100</td>
<td>100</td>
<td>1080 (972, 1188)</td>
<td>772 (748, 796)</td>
<td>37 (30.4, 44)</td>
<td>95</td>
<td>0 (0.0, 99.9)</td>
</tr>
</tbody>
</table>

UPN, unique patient number; C1, untreated controls; C2, vehicle (0.1% DMSO)-treated controls; ND, not determined.

a For blast colonies, the numbers in parentheses are the actual colony numbers in duplicate Petri dishes. The percent inhibition of LPC is shown in brackets.
Lymphoma B Cells. Treatment with calphostin C caused...apoptosis by targeted gene disruption (Fig. 1), providing genetic evidence that calphostin C does not use the BCR-linked apoptotic pathway. We have previously shown that radiation-induced apoptosis in DT40 cells is mediated by BTK, and BTK-deficient DT40 cells do not undergo apoptosis after exposure to γ-rays or hydrogen peroxide (23). In contrast, calphostin C-induced apoptosis was not prevented or attenuated in BTK-deficient DT40 cells (Fig. 1). The potent apoptotic activity of calphostin C against wild-type and mutant DT40 lymphoma B-cell clones prompted the hypothesis that this perylenequinone could elicit significant cytotoxicity against human ALL cells.

Cytotoxicity of Calphostin C against Human ALL Cell Lines. We first examined the antileukemic activity of calphostin C against established human ALL cell lines using standard viability assays. As shown in Fig. 2A, calphostin C killed both B-lineage ALL and T-lineage ALL cells in a dose-dependent fashion. Notably, both RS4;11, a highly radiation-resistant MLL-AF4 fusion transcript-positive t(4;11) ALL cell line, and ALL-1, a multidrug resistant BCR-ABL fusion transcript-positive t(9;22) ALL cell line, were exquisitely sensitive to calphostin C. Unlike calphostin C, other potent PKC inhibitors, including Ro31 8220 [IC50(PKC) = 1.9 μM; Refs. 39–41], Go 6983 [IC50(PKC) = 7 nM; Refs. 42 and 43], and Gd 6976 [IC50(PKC) = 7.9 nM; Refs. 44–46] exhibited minimal cytotoxicity against the ALL cells (Fig. 2B).

Calphostin C-induced Apoptosis in Human ALL Cells. We next set out to determine whether the cytotoxicity of calphostin C against human ALL cell lines was due to induction of apoptosis. To this end, we used TUNEL assays combined with confocal laser scanning microscopy as well as DNA fragmentation assays. As shown in Fig. 3, exposure of NALM-6 B-lineage ALL as well as MOLT-3 T-lineage ALL cells to calphostin C resulted in a dose-dependent apoptosis, as evidenced by the ladder-like fragmentation pattern of nuclear DNA and digoxigenin-11-UTP labeling of exposed 3'-hydroxyl end of fragmented nuclear DNA in the presence of TdT.

Calphostin C-induced Calcium Mobilization in Human ALL Cell Lines. Calcium mobilization from intracellular stores has been shown to play an important role in the regulation of apoptosis in lymphoid cells (38). Therefore, we hypothesized that calphostin C could trigger apoptosis in human ALL cells by altering the intracellular free calcium concentration. Exposure of the ALL cells to calphostin C resulted in a time- and dose-dependent transient elevation of the cytoplasmic calcium concentration. Higher concentrations of calphostin C resulted in both faster onset and greater magnitude of the calcium signal (Fig. 4). The "lag period" from the addition of calphostin C to the burst of Ca2+ was 50 s at 0.2 μM (line a), 35 s at 0.5 μM (line b), and 20 s at 1 μM (line c). At all of the calphostin C concentrations tested, cytoplasmic Ca2+ concentrations returned to baseline levels within approximately 10 min (Figs. 4 and 5). The calphostin C-induced Ca2+ signal was not dependent on extracellular Ca2+; the kinetics and magnitude of the signal...
were not altered when the extracellular Ca$^{2+}$ (1.8 mM) was chelated by 5 mM EGTA (line d, Fig. 4). Therefore, the observed Ca$^{2+}$ elevation in calphostin C-treated ALL cells results from a Ca$^{2+}$ release from intracellular Ca$^{2+}$ stores rather than a Ca$^{2+}$ influx through the plasma membrane channels.

**Cause-Effect Relationship between Calphostin C-induced Calcium Mobilization and Apoptosis in Human ALL Cell Lines.** The cytotoxicity of calphostin C against ALL cells was correlated with the magnitude of the induced Ca$^{2+}$ signal (Fig. 6A). Notably, the cell-permeable Ca$^{2+}$ chelator BAPTA/AM reduced calphostin C-induced apoptosis from 70% to 26%, implicating elevation of Ca$^{2+}$ levels as the triggering signal for apoptosis (Fig. 6B).

It has been reported that inhibition of calcineurin (i.e., protein phosphatase 2B), a Ca$^{2+}$/calmodulin-dependent serine/threonine phosphatase, protects cells against Ca$^{2+}$-triggered apoptosis (47-51). When NALM-6 cells were treated with 5 μM TFPD, a calmodulin antagonist, for 1 h before the addition of calphostin C (0.5 μM), calphostin C-induced apoptosis was significantly suppressed with only 40% (instead of 70%) of cells showing apoptotic nuclei (Fig. 6B). Similarly, when NALM-6 cells were pre-treated by 1 μM CsA, a specific inhibitor of calcineurin, the percentage of apoptotic cells was reduced to 30% (Fig. 6B). These studies demonstrated the importance of calcineurin in calphostin C-induced apoptosis and provided further support for the hypothesis that calphostin C-induced apoptosis is Ca$^{2+}$-dependent.

When the free Ca$^{2+}$ concentration is increased in the cytosol, Ca$^{2+}$ ions diffuse into the nucleus and activate Ca$^{2+}$/Mg$^{2+}$-dependent endonucleases, which are capable of causing apoptotic DNA fragmentation (38, 52, 53). The role of Ca$^{2+}$/Mg$^{2+}$-dependent endonucleases in calphostin C-induced apoptosis was examined using NiCl$_2$ (0.5 mM), an inhibitor of these endonucleases (52, 53). As illustrated in Fig. 6B and Fig. 7, a 1-h incubation with NiCl$_2$ before the addition of calphostin C inhibited calphostin C-induced apoptosis in NALM-6 cells. Similar results were obtained using MOLT-3, RS4;11, and ALL-1 cell lines (Table 1). Thus, NiCl$_2$-sensitive Ca$^{2+}$/Mg$^{2+}$-dependent endonucleases seem to play a key role in calphostin C-induced apoptosis of human ALL cells.

**Cytotoxic Activity of Calphostin C against Freshly Isolated Primary Leukemic Cells from Children with ALL.** We next examined the in vitro antileukemic activity of calphostin C against freshly isolated bone marrow blasts from eight children with ALL, including six patients with newly diagnosed ALL (UPN 1–6) and 2 children (UPN 7 and 8) with B-lineage ALL in bone marrow relapse. Of the six children with newly diagnosed ALL, three (UPN 1, 4, 6) had standard-risk B-lineage ALL, one (UPN 2) had high-risk B-lineage ALL, and two (UPN 3 and 5) had high-risk T-lineage ALL (Table 2). In 6 of these 8 cases, we had sufficient cells to evaluate the effects of calphostin C on intracellular calcium levels (Fig. 8). Exposure of freshly obtained leukemic cells from each of these six subjects to calphostin C resulted in a time- and dose-dependent transient elevation of the cytoplasmic calcium concentration. Higher concentrations of calphostin C resulted in both faster onset and greater magnitude of the calcium signal in these primary leukemic cells (Fig. 8), similar to the results obtained using established ALL cell lines. In all of the eight cases, calphostin C inhibited LPC-derived blast colony formation in a dose-dependent fashion and induction of apoptosis was confirmed in 6 of 6 cases analyzed by TUNEL assays as well (Table 2). Thus, calphostin C is capable of killing primary leukemic cells from children with newly diagnosed ALL regardless of their immunophenotype or NCI risk classification, and it is equally potent against leukemic cells from children in bone marrow relapse.

In summary, we evaluated the activity of the naturally occurring perylenequinone antibiotic calphostin C against human ALL cells and found that this agent induces dose-dependent and rapid apoptosis in human ALL cell lines as well as primary leukemic cells freshly obtained from children with ALL. In biochemical studies, calphostin C was discovered to induce rapid calcium mobilization from intracellular stores of ALL cell lines, and its cytotoxicity against ALL cell lines was well correlated with the magnitude of this calcium signal. Calphostin C-induced apoptosis was markedly suppressed by BAPTA/AM, a cell-permeable Ca$^{2+}$ chelator as well as NiCl$_2$, an inhibitor of Ca$^{2+}$/Mg$^{2+}$-dependent endonucleases. Inhibition of the Ca$^{2+}$/calmodulin-dependent phosphatase calcineurin with TFPD, a calmodulin antagonist, or CsA, a specific inhibitor of calcineurin, also reduced the magnitude of calphostin C-induced apoptosis in the ALL cell lines. Calphostin C was capable of inducing calcium mobilization and apoptosis in freshly obtained primary leukemic cells from children with ALL. Taken together, our results provide unprecedented evidence that calphostin C triggers a Ca$^{2+}$-dependent apoptotic signal in human ALL cells. However, our results do not formally exclude the possibility that calphostin C may also activate calcium-independent apoptotic pathways.

**REFERENCES**


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Clinical Cancer Research

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D M Zhu, R K Narla, W H Fang, et al.


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