Pharmacokinetic Features and Metabolism of Calphostin C, A Naturally Occurring Perylenequinone with Antileukemic Activity

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Purpose. To examine the pharmacokinetic features and metabolism of calphostin C, a naturally occurring perylenequinone with potent antileukemic activity.

Methods. HPLC-based quantitative detection methods were used to measure calphostin C levels in lysates of leukemic cells and in plasma of mice treated with calphostin C. The plasma concentration-time data were analyzed using the WinNonlin program. In vitro esterases and a microsome P450 preparation in conjunction with a LC-MS(API-EI) system were used to study the metabolism of calphostin C.

Results. An intracellular exposure level ($AUC_{0\text{-}6h}$) of 257 μM•h was achieved after in vitro treatment of NALM-6 cells with calphostin C at a 5 μM final concentration in culture medium. After intraperitoneal (i.p.) injection of a 40 mg/kg nontoxic bolus dose of calphostin C, the estimated C_{max} was 2.9 μM, which is higher than the effective in vitro concentration of calphostin C against leukemic cells. Drug absorption after i.p. administration was rapid with an absorption half-life of 24.2 min and the estimated t_{max} was 63.0 min. Calphostin C was cleared with an elimination half-life of 91.3 min. An inactive and smaller metabolite (calphostin B) was detected in plasma of calphostin C-treated mice with a t_{max} of 41.3 min. Esterase (but not P450) treatment of calphostin C in vitro yielded an inactive metabolite (calphostin B) of the same size and elution profile.

Conclusions. Target plasma calphostin C concentrations of potent antileukemic activity can be reached in mice at nontoxic dose levels. This pilot pharmacokinetic study of calphostin C combined with the availability of the described quantitative HPLC method for its detection in cells and plasma provide the basis for future preclinical evaluation of calphostin C and its potential as an anti-leukemic drug.

KEY WORDS: pharmacokinetics; Calphostin C; HPLC; perylenequinone.

INTRODUCTION

Recurrence of leukemia continues to be a major obstacle to a successful outcome of multiagent chemotherapy in the treatment of acute lymphoblastic leukemia (ALL) patients especially those who have relapsed despite intensive multiagent chemotherapy (1). Consequently, the identification and development of new potent anti-ALL drugs have become the primary focus of translational leukemia research.

Calphostin C (Fig. 1) is a naturally occurring perylenequinone antibiotic from fungus *Cladosporium cladosporoides* FERM BP-1258, which was originally isolated from a block fence in Osaka, Japan (2,3). A number of studies have demonstrated that this natural product can inhibit protein kinase C (PKC) and induce apoptotic cell death (2–4). We have demonstrated that calphostin C can induce dose-dependent apoptosis in human ALL cell lines (5). In biochemical studies, we found that calphostin C induced 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 (5). Calphostin C was also capable of inducing calcium mobilization and apoptosis in freshly obtained primary leukemic cells from children with ALL regardless of their immunophenotype or NCI risk classification and it was equally potent against leukemic cells from children in bone marrow relapse (5). The antileukemic activity of calphostin C is due to its ability to induce calcium mobilization combined with its PKC inhibitory function (6).

Further development of calphostin C will require detailed pharmacodynamic studies in preclinical animal models. A quantitative HPLC-based analytical method for detecting calphostin C in plasma has recently been established in our laboratory (7). We now report the toxicity, pharmacokinetic features, and metabolism of calphostin C. Our findings provide unprecedented evidence that target plasma calphostin C concentrations exhibiting antileukemic activity can be reached in mice at nontoxic dose levels. This pilot pharmacokinetic study of calphostin C combined with the availability of the described quantitative HPLC method for its detection in cells and plasma provide the basis for future preclinical evaluation of calphostin C and its potential as an anti-leukemic drug.

MATERIALS AND METHODS

Chemicals and Drugs

Calphostin C (M.W. 790.6, purity 99%) was purchased from BIOMOL Research Labs, Inc. (Plymouth Meeting, PA). Cell culture medium (RPMI 1640) and antibiotic (10,000 U/ml penicillin G sodium; 10,000 μg/ml streptomycin sulfate; 25 μg/ml amphotericin B) were purchased from GIBCO BRL (Gaithersbury, MD). Fetal bovine serum was purchased from Summit (Fort Collins, CO) and was heat-inactivated at 56°C for 30 minutes prior to use. All other reagents (HPLC grade) were obtained from Fisher Chemicals (Fair Lawn, NJ).

Cell Lines and Cultures

NALM-6, a human B cell precursor leukemia cell line (German Collection of Microorganisms and Cell Cultures, Germany) was used in these studies. NALM-6 cells were propagated in RPMI 1640 media, supplemented with heat-inactivated FBS (10% v/v) and penicillin/streptomycin/amphotericin B (1%). All cell lines were maintained in liquid culture at 37°C in a humidified 95% air/5% CO_2 atomsphere.

HPLC Determination of Cellular Calphostin C Levels

For determination of calphostin C levels in cell lysates, 100 μl of acetonitrile was added to a 50 μl cell lysate sample

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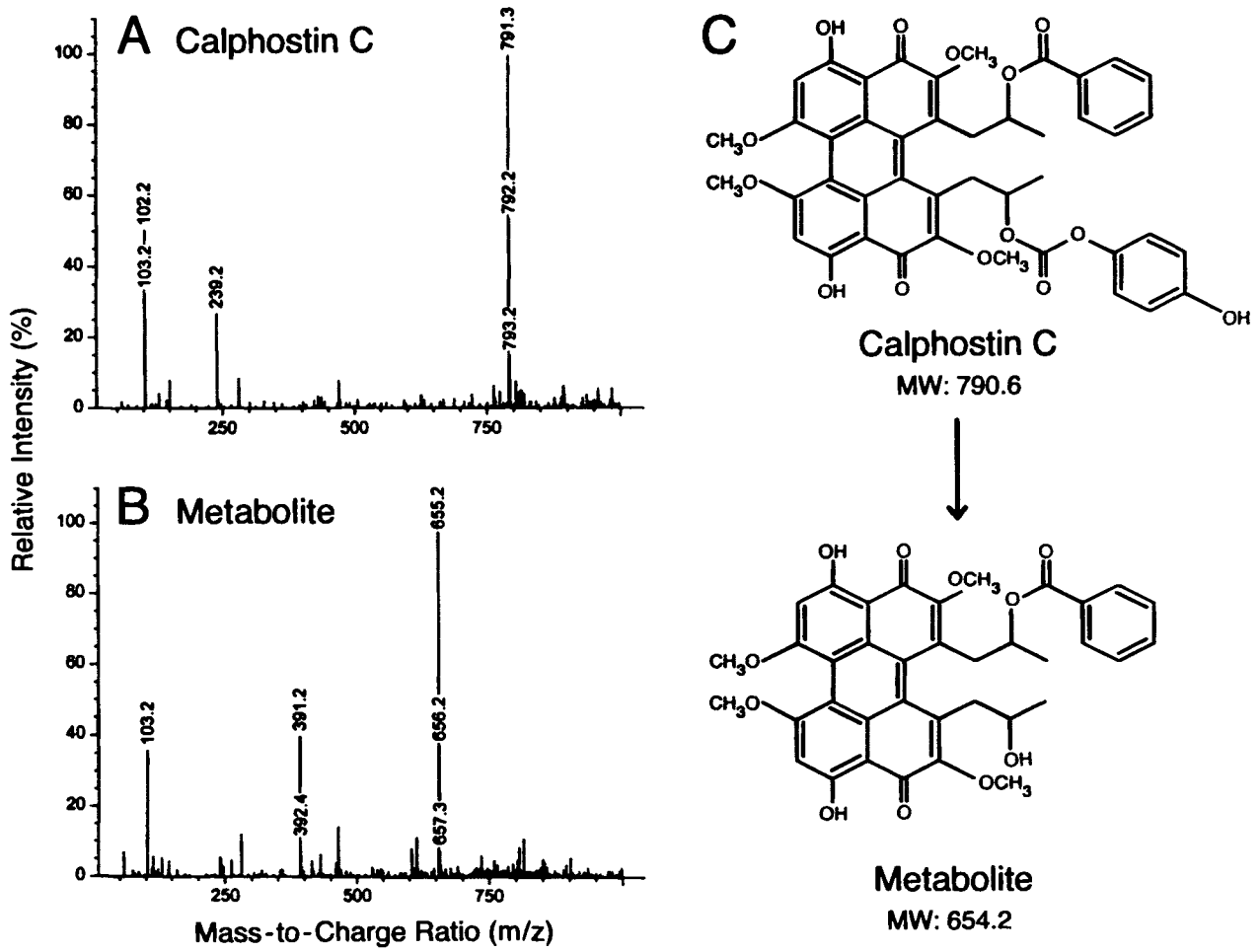

Fig. 1. API-EI mass spectra of parent calphostin C (A) and a putative metabolite (B). Proposed metabolism pathway of calphostin C (C).

containing calphostin C, and the mixture was vortexed and centrifuged. A 100 μl aliquot of the supernatant was injected for HPLC analysis using a recently reported HPLC conditions (7).

Calphostin C Stability in NALM-6 Leukemic Cell Lysates and in Plasma

Cell lysates and plasma samples were spiked with calphostin C to yield a final content of 40 pmol in cell lysate samples and a final concentration of 40 μM in plasma samples. Each of the samples were then divided into two aliquots, one of which was frozen at -20°C and the other placed in 37°C. At predetermined times, 50 μl aliquots of cell lysate and plasma samples were extracted as described above. The absolute peak area was used to examine the stability of calphostin C.

Calphostin C Pharmacokinetics in NALM-6 Leukemia Cells

NALM-6 cells in log phase were allowed to proliferate overnight to achieve a 1×10^6 cells/ml cell density. In each experiment, calphostin C was added to the cell cultures to achieve a final concentration of 5 μM, and cells were placed in a humidified 5% CO_2 incubator. At predetermined time points (10, 30 min and 1, 2, 4, and 6 h), cells were transferred to 16 × 125 mm Pyrex tubes and pelleted. The cell pellets were washed twice with fresh culture medium. Then the cells were lysed in 100 μl deionized water and saved at −20°C until processing. The quantity of calphostin C in the cell lysate samples was determined by the HPLC method described above.

Calphostin C Pharmacokinetics in Mice

Female CD-1 mice (~25 g) purchased from Charles River Laboratories (Wilmington, MA) were housed in a controlled environment (12-h light/12-h dark photoperiod, 22 ± 1 °C, 60 ± 10% relative humidity), which is fully accredited by the USDA (United States Department of Agriculture). Mice were allowed free access to pelleted food and tap water throughout the experiments. Animal studies were approved by the Hughes Institute Animal Care and Use Committee and all animal care procedures conformed to the Principles of Laboratory Animal Care (NIH publication #85-23, revised 1985). Four mice were injected intraperitoneally with a 40 mg/kg bolus dose of calphostin C. Blood samples (~0.2 ml) were collected from the ocular plexus via retro-orbital venipuncture prior to and at 5, 15, 45 min, and 2 and 6 h (for 2 mice) and 10, 30 min and 1, 4 and 9 h (for other 2 mice) after drug administration. No anesthetizing agent was used throughout the animal experiments. These blood samples were heparinized and centrifuged

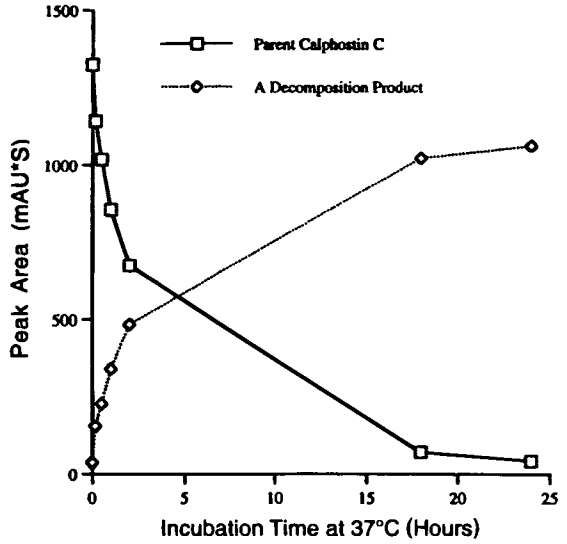

Fig. 2. Incubation of calphostin C with mouse plasma at 37°C.

at 7,000 × g for 10 min in a microcentrifuge to obtain plasma. The plasma samples were immediately extracted and analyzed by an established HPLC assay (7).

HPLC and LC-MS Analysis of Calphostin C Metabolism

We set up two systems to examine the possible mechanism for production of a metabolite of calphostin C. In order to evaluate the role of esterases on metabolism of calphostin C, calphostin C (30 μM) was incubated in a final volume of 200 μl with 20 U porcine liver esterase (Sigma) in 50 mM Tris-HCl buffer for 3 h at 37°C. The reaction was stopped by addition of 400 μl methanol. Incubations without the drug and incubation of drug with buffer without esterases served as controls. In order to evaluate the role of the cytochrome P450 system, calphostin C (30 μM) was incubated in a final volume of 200 μl with 400 μg of liver microsomes prepared from CD-1 mice in the presence or absence of 1 mM NADPH for 1 h at 37°C. The reaction was stopped by addition of 400 μl methanol. Calphostin C samples incubated in Tris-HCl buffer with NADPH without microsomes served as control.

The modified HPLC conditions with a mobile phase of acetonitrile/0.1% trifluoacetic acid (80/20, v/v) and flow rate of 0.6 ml/min were used in LC-MS analysis of parent calphostin C and its putative metabolite. Mass spectrum analysis was carried out using atmospheric pressure ionization-electrospray (API-ES) and a high-energy-dynode (HED) electron multiplier (Hewlett Packard, Palo Alto, CA). The conditions for mass spectrum analysis were set at fragmentor of 70 volts, drying gas flow of 10 l/min, nebulizer pressure of 25 psig and drying gas temperature of 350°C.

Cell Cytotoxicity Assays

The viability of NALM-6 cells treated with calphostin C or its metabolite was evaluated using the mixture of calcien/AM and ethidium homodimer (Molecular Probes Inc., Eugene, OR) coupled with laser scanning confocal microscopy (MRC

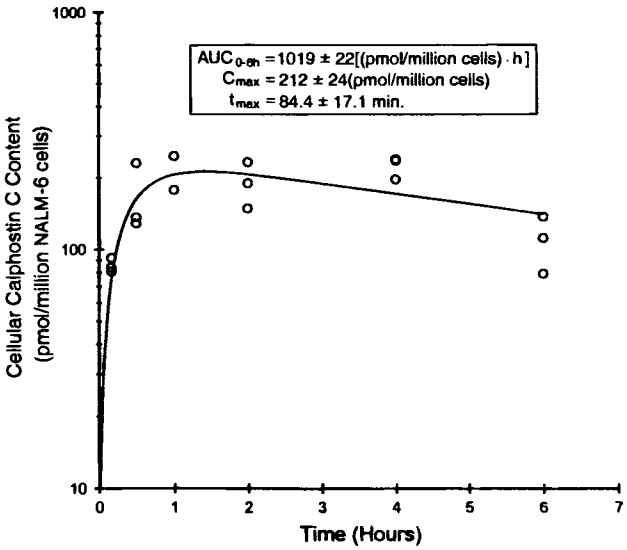

Fig. 3. Cellular calphostin C concentration-time profiles in NALM-6 cells following incubation with 5 μM of calphostin C in culture medium.

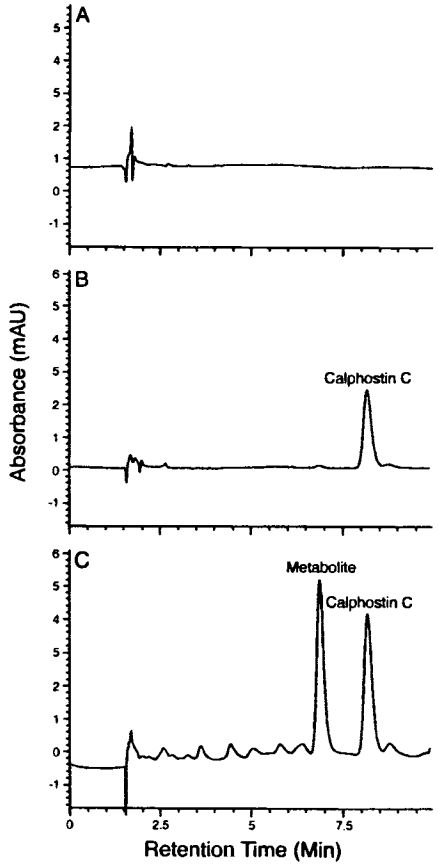

Fig. 4. Representative chromatograms of (A) blank plasma; (B) blank plasma spiked with calphostin C; and (C) a plasma samples 15 min following i.p. administration of 40 mg/kg calphostin C (the concentration of calphostin C was 1.2 μM).

1024, BioRad, Inc., Richmond, CA), as described previously (5). In brief, the cells were incubated with 2 μM calcien and 4 μM ethidium homodimer in PBS for 30 min, and then transferred onto slides. Fluorescent images of the cells were taken by laser scanning confocal microscope. The excitation wavelength is 488 nm for calcien, and 514 nm for ethidium homodimer. The percentage of viable cells exhibiting a homogeneous green fluorescence from calcien staining and non-viable cells exhibiting red fluorescence from the DNA-bound ethidium homodimer were determined in order to evaluate calphostin C and its metabolite for cytotoxicity against leukemic cells.

Apoptosis Assays

The merocyanine (MC) 540 binding (as an early marker of apoptosis) and propidium iodide (PI) permeability (as a marker of advanced stage apoptosis) were measured simultaneously in NALM-6 cells 24 h after exposure to calphostin C or its metabolite by quantitative multiparameter flow cytometry, as described previously (8). Phosphatidylserine, which is normally confined to the intracellular compartment, is exposed on the surface of apoptotic cells because of a loss of plasma membrane phospholipid asymmetry. The randomized packing of membrane phospholipids allows such cells to exhibit increased binding of the lipophilic dye MC540 and to be detected by flow cytometry. Loose packing of membrane phospholipid head groups and cell shrinkage precede DNA fragmentation in apoptotic cells, thereby providing MC540 binding as an early marker for apoptosis. Plasma membrane permeability to PI develops at a later stage of apoptosis. MC540 binding and PI permeability were simultaneously measured. Whole cells were analyzed with a FACStar Plus flow cytometer. All analyses were done at 488 nm excitation from an argon laser.

Pharmacokinetic Analyses

Data fitting and pharmacokinetic parameter calculations were carried out using the pharmacokinetic software, WinNonlin program, Standard version 2.0 (Pharsight Inc., Palo Alto CA). Cellular calphostin C pharmacokinetics was modeled with the assumption that the cellular drug uptake followed first order kinetics, where the area under the concentration-time curve

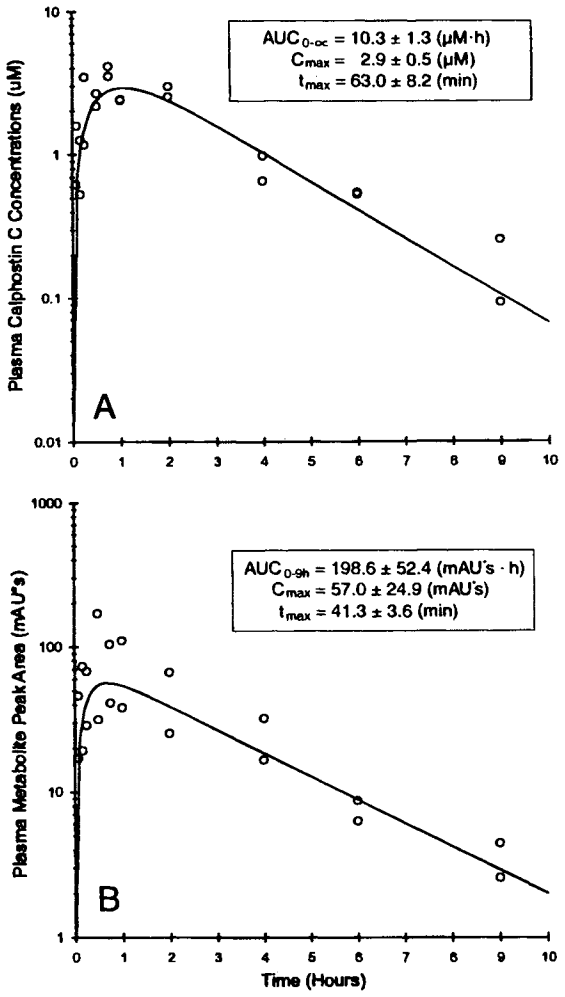

Fig. 5. (A) Plasma calphostin C concentration-time profile in mice following the intraperitoneal administration of 40 mg/kg bolus dose of calphostin C (N = 4). (B) Plasma calphostin C metabolite peak area-time profile in mice following intraperitoneal administration of calphostin C at bolus dose of 40 mg/kg (N = 4).

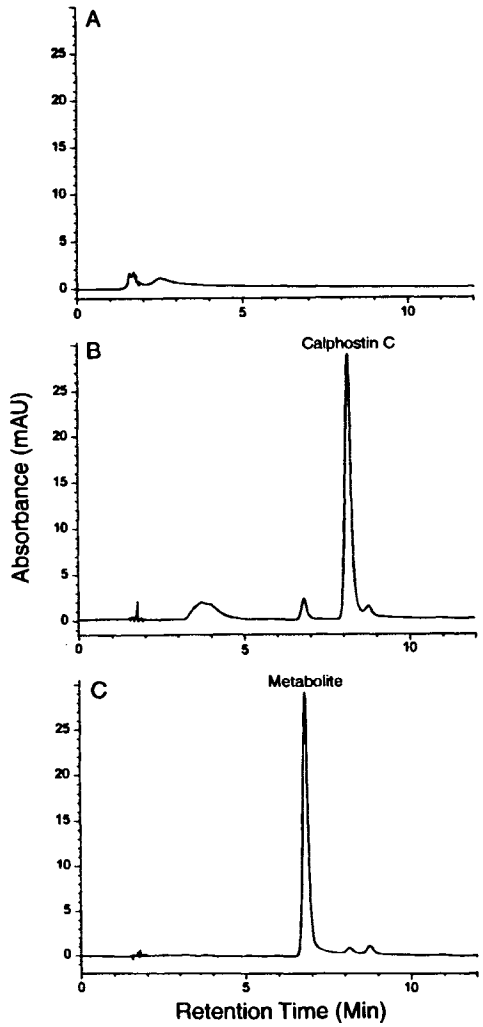

Fig. 6. Effect of esterases on metabolism of calphostin C. (A) esterase alone without calphostin C; (B) calphostin C without esterase; (C) calphostin C was completely converted to its metabolite which eluted at a retention time of 6.9 min in the presence of esterases.

(AUC) was estimated by the linear trapezoidal method to the last point. For modeling calphostin C pharmacokinetics in mice, an appropriate pharmacokinetic model was chosen to fit the plasma calphostin C concentration-time curves based on the lowest weighted squared residuals, lowest Schwartz Criterion (SC), lowest Akaike's information criterion (AIC) value, lowest standard errors of the fitted parameters, and dispersion of the residuals. The area under the plasma concentration time curve (AUC) was calculated by the linear trapezoidal rule between first (0 h) and last sampling time plus C/k, where C is the concentration of last sampling and k is the elimination rate constant. All pharmacokinetic parameters are presented as estimates from pooled data (±SEM).

RESULTS AND DISCUSSION

Accuracy and Sensitivity of the Analytic HPLC Method for Detection of Calphostin C in Cell Lysates

Under the described chromatographic conditions (7), the retention time for calphostin C was 8.2 minutes. At the retention time of calphostin C, no interfering peaks were detected in the blank cell lysate controls. Under the above described extraction procedures, the average extraction recovery of calphostin C from cell lysates was 90.9 ± 1.6%. The calibration curve for calphostin C showed a linear relationship between the quantity of calphostin C in the cell lysates (2.5–500 pmol) and the absolute peak areas. The calibration curve could be described by the regression equation: $Y = 0.0366X + 0.0411$ ($r > 0.999$), in which Y is the agent recovered in pmol and X is absolute peak area. The test for the linearity (Instat Program, V.3.0, GraphPad Software, San Diego, CA) did not detect any significant departure from linearity. The lowest detection limit of calphostin C in a cell lysate samples was 0.02 μM (i.e., 1 pmols in a 50 μl sample) at a signal to noise ratio of ~3.

The overall accuracy of this method was 101.0 ± 4.7% with a coefficient of variation less than 7%. One-way analysis of variance (ANOVA) (Instat program V. 3.0) showed no significant differences between the spiked versus experimentally measured calphostin C levels. Thus, the procedures described above were satisfactory with respect to both accuracy and precision.

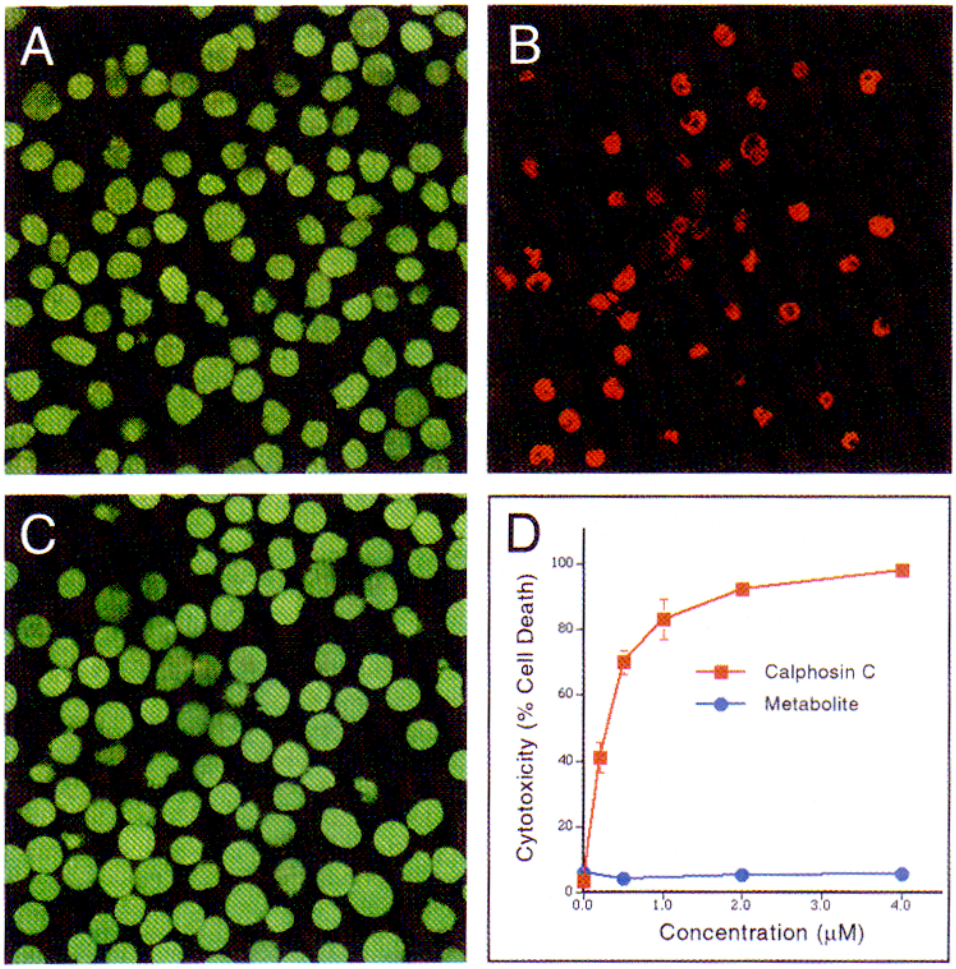

Fig. 7. Cytotoxicity of calphostin C and its metabolite against NALM-6 ells. The cytotoxicity (or the % death of the cells treated by the reagents) was measured by Cell Cytotoxicity Assay as described in Materials and Methods. The fluorescent confocal images represent the control cells (A), cells treated by 2 μM calphostin C (B), and by 4 μM metabolite (C), respectively. The homogeneous green fluorescence indicates the survival of the cells cells, and the red fluorescence indicates the death of the cells. Cytotoxicity was presented as the percentage of cell death of BALM-6 cells (mean ± SEM, N = 3) (D).

Stability of Calphostin C in Cell Lysates and in Plasma

Our results demonstrated that calphostin C is very stable in cell lysates for at least 5 days if stored at −20°C and for one day at 37°C. After longer incubations, calphostin C showed slow decomposition, reaching 7.8% at 5 days of 37°C incubation. Similar stability results were obtained when calphostin C was incubated in culture medium, with a 15.0% decomposition after 5 days of incubation at 37°C.

We next examined the stability of calphostin C in plasma. If stored at −20°C, calphostin C was stable for one day in plasma. However, when incubated in plasma at 37°C, calphostin C decomposed rapidly. The decomposition rates of calphostin C after 10 min, 30 min, 1h, 2h, 18 h, and 24 h of incubation in plasma at 37°C were 13.6%, 23.1%, 35.5%, 49.2%, 94.7% and 96.8%, respectively. Correspondingly, the peak area of a decomposed compound increased over time (Fig. 2).

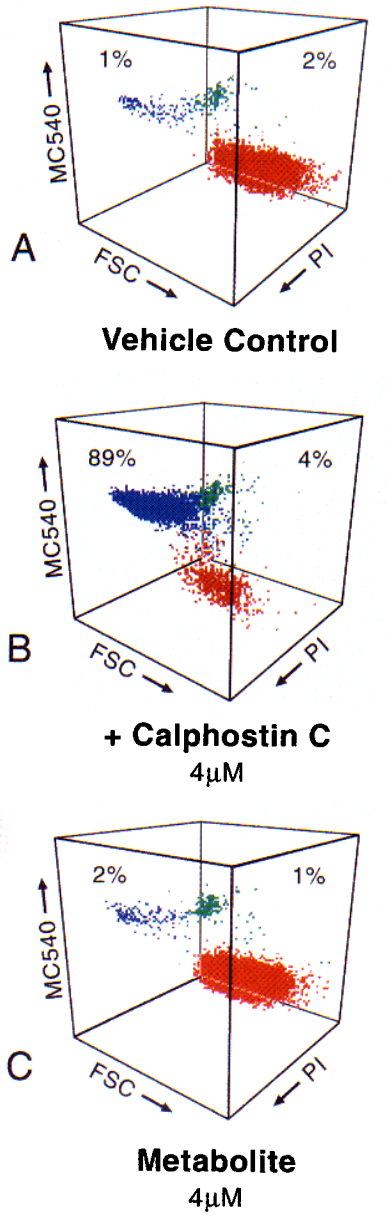

Fig. 8. Calphostin C-induced apoptosis in NALM-6 cells. Fluorescence-activated cell sorting (FACES) correlated three-parameter displays of NALM-6 cells stained with MC540 and PI 24 h after treatment with 4 μM calphostin C and its metabolite as described in Materials and Methods. The percentages indicate the fraction of cells at an early stage of apoptosis, as measured by single MC540 (in green), and the fraction of cells at an advanced stage of apoptosis, as measured by dual stain of MC540 / PI (in blue). The non-apoptotic cells were indicated by red color. The decrease in FSC of apoptotic cells indicates the size shrinkage of those cells.

Cellular Pharmacokinetics of Calphostin C in NALM-6 Cells

The cellular concentration-time curve of calphostin C following treatment of NALM-6 cells with 5 μM calphostin C fit a first order pharmacokinetic model (Fig. 3). The calculated cellular pharmacokinetic parameters are shown in the inset of Fig. 3. Calphostin C exhibited a rapid uptake with a first order kinetics (t_{max} = 84.4 min). The predicted C_{max} (maximum cellular content) and AUC_{0-6h} of calphostin C in NALM-6 leukemic cells achieved by the 5 μM extracellular concentration were 212 pmol/1 × 10^6 cells (0.212 fmols/cell or ~50 μM for an average cell volume of 4.2 × 10^{-12} L) and 1080 pmol/1 × 10^6 cells*h (~257 μM·h), respectively.

Pharmacokinetic Features of Calphostin C in Mice

Calphostin C, when administered as a single bolus injection intravenously or intraperitoneally (i.p), was nontoxic to mice (5 mice/group) at any of the 5 different dose levels ranging from 0.05 mg/kg to 50 mg/kg. During the 30 day observation following the administration of calphostin C, no significant toxic signs (e.g., weight loss, decrease in activity, diarrhea, ataxia etc) or fatalities were observed in any of the 25 mice treated with this drug. No toxic lesions were detected histopathologically in any of the organs (brain, large intestine, small intestine, stomach, liver, kidney, lung, heart, ovary, pancreas, skeletal muscle, skin, spinal cord, uterus, urinary bladder) of the calphostin C-treated mice (data not shown). After i.p. injection of a 40 mg/kg nontoxic bolus dose of calphostin C, the estimated C_{max} was 2.9 μM, which is higher than the effective in vitro concentration of calphostin C against leukemic cells. The representative HPLC chromatograms of plasma samples from mice injected intraperitoneally with a 40 mg/kg bolus dose calphostin C are shown in Fig. 4 and the change in plasma concentration of calphostin C over time is best described as a one compartment, first order pharmacokinetic model (Fig. 5A). Some of the pharmacokinetic parameters of calphostin C following i.p. administration are presented in inset of Fig. 5A. The results showed that calphostin C is rapidly absorbed with an absorption half-life of 24.2 min and the time to reach the maximum plasma calphostin C concentration (t_{max}) was 63.0 min. Calphostin C was eliminated with an elimination half-life of 91.3 min. The predicted maximum plasma calphostin C concentration was 2.9 μM following i.p. administration of 40 mg/kg calphostin C, which is higher than the concentration required for killing >99% leukemic cells in vitro (5,6). Intriguingly, we observed that a possible metabolite of calphostin C (retention time at 6.9 min) emerges in plasma very early with a t_{max} of 41.3 min, which is earlier than the time to reach the

maximum parent calphostin C concentrations in plasma (Fig. 4C & Fig. 5B). The AUC of this metabolite was 198.6 mAU*s·h, which is 74.7% of the AUC of the parent compound calphostin C (265.8 mAU*s·h), assuming both calphostin C and its metabolite have the same extinction coefficient.

Metabolism of Calphostin C

When treated with porcine liver esterase in vitro (N = 2), all calphostin C was converted to its putative metabolite eluting at the retention time of 6.9 min within 3 h (Fig. 6C). Therefore, esterases may be responsible for observed metabolism of calphostin C *in vivo*. Since we used a relatively high concentration of calphostin C in these studies, the actual rate of esterase-mediated degradation may be faster. No metabolite formation was induced when calphostin C was incubated with mouse liver microsomes in the presence of NADPH. Thus the cytochrome P450 system is unlikely to be involved in the calphostin C metabolism.

LC-MS results shown in Fig. 1A and 1B demonstrated that the putative metabolite has a m/z of 655 (M + H), which is consistent with the molecular weight of 654 deduced (3), while parent calphostin C has a m/z of 791 (M + H). These findings prompt the hypothesis that the ester bond of calphostin C is the likely target for esterase-mediated metabolism in vivo (Fig. 1C). Therefore, this metabolite was tentatively determined as calphostin B (3).

In order to test the biological activity of this metabolite, calphostin C was hydrolyzed in 0.2N NaOH for 2 h at room temperature. The solution was then supplemented with 0.2 N HCl to adjust the pH to 7.0. The hydrolysis was complete within 2 h, as determined by HPLC and LC-MS. This hydrolysate possesses the same retention time, similar UV spectra (spectrum match factor of >955) and same MS spectrum as the metabolite from plasma and esterase hydrolysis of calphostin C. NALM-6 pre-B leukemia cells were incubated with the metabolite for 24 h at 37°C. Cell viability and apoptosis analysis did not show any toxicity of this putative metabolite to NALM-6 cells (Fig. 7C, 7D and 8). However, calphostin C induced apoptosis, resulting in 100% leukemic cell death (Fig. 7B). Thus, the identified metabolite (calphostin B), which was previously reported to be a weak inhibitor of protein kinase C (3), appears to be inactive, and esterases may be critical for the in vivo metabolism of calphostin C.

In summary, we have employed a highly sensitive and accurate analytical HPLC method for quantitative detection of the potent anti-leukemic agent calphostin C in plasma and leukemic cells lysates. This first pharmacokinetic analysis of calphostin C and the availability of the described HPLC method will provide the basis for future pharmacodynamic and pharmacokinetic studies of calphostin C in preclinical animal models. Furthermore, our findings prompt the hypothesis that esterase-resistant calphostin C analogues may have superior antileukemic activity, which will be tested in future structure-activity relationship analyses of calphostin C analogues in SCID mouse models of human ALL.

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