Activated Calphostin C Cytotoxicity Is Independent of p53 Status and in Vivo Metastatic Potential

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ABSTRACT

The development of novel therapeutic agents to modulate programmed cell death independent of genetic background or malignant potential is a primary goal of modern cancer therapy. In this report, the light activation- and concentration-dependent cytotoxicity of calphostin C, a photosensitizable perylenequinone, is carefully evaluated using a series of nine well-characterized human and rodent prostate cancer cell lines representing the spectrum of disease progression (e.g., variations in metastatic ability, ploidy, and tumor suppressor gene status). Treatment of these cancer cell lines with nanomolar concentrations of calphostin C in combination with increasing amounts of light exposure established a relationship between light and dose dependence of calphostin C cytotoxicity.

The induction of apoptosis is rapid, as evidenced by the fact that immediately after treatment, cells exposed to calphostin C with light activation exhibit both morphological and biochemical changes consistent with apoptosis (cellular and nuclear shrinkage and chromatin condensation). For example, 78% of cells treated with 100 nM calphostin C in combination with 2 h of light activation underwent apoptosis within 24 h of treatment. DNA ladder formation could be detected within 12 h of treatment. In the absence of light activation, treatment with calphostin C at all concentrations tested had no acute or durable cytotoxic effects in any of the cell lines. Our findings demonstrate that calphostin C cytotoxicity is strictly light dependent. Furthermore, its efficacy is independent of the genetic background, p53 status, or in vivo malignant potential of a cell, making it a suitable candidate for the treatment of heterogeneous tumor cell populations.

INTRODUCTION

Normal tissue homeostasis is maintained by a balance between cell proliferation and cell death (1). Although recent studies suggest that all nucleated mammalian cells possess the functional biochemical pathways necessary for induction of apoptosis (2), cancer cells may evolve defects in the cellular pathways that initiate the cell death program (3). It is proposed that increasing the apoptotic fraction of tumor cells may increase the therapeutic index of chemotherapeutic agents and ionizing radiation (3). Based on these hypotheses, there has been a considerable effort during the past several years to develop compounds that activate the apoptotic pathways of heterogeneous tumor cell populations. The efficacy of many of these compounds, which include DNA-damaging agents and inhibitors of DNA repair processes, appears to be modulated by factors such as tumor suppressor gene status, growth rate, and malignant potential of the tumor cells (4, 5). Because at any given time a tumor is likely to be composed of cells with varying malignant potentials (6), a compound that could be specifically activated to induce apoptosis in tumor cells independent of these characteristics would be of therapeutic value.

Cal C is a member of the class of 4,9-dihydroxy-3,10-perylenequinones and has been used extensively as a specific inhibitor of the regulatory subunit of PKC (7). Like other PKC inhibitors, such as chelerythrine chloride, cal C has been shown to induce apoptosis [e.g., in HL60 and glioma cell lines (8, 9)]. For example, internucleosomal DNA fragmentation is induced after a 6-h exposure to 100 nM cal C (10). This work did not control for the light dependency of cal C activation, which is potentially the most interesting aspect of the drug. In fact, work by Bruns et al. (9) demonstrated that at nanomolar concentrations, cal C inhibits PKC activity in a light-dependent manner in in vitro biochemical assays. However, these studies did not evaluate the drug activity in proliferating cells. Research that did observe the inhibition of a nonneoplastic astrocyte cell line proliferation due to light activated cal C failed to show the induction of apoptosis (11). Based on its physical and chemical properties, it has been proposed that cal C may have applications as a photosensitizing (12) and anticancer agent (13). In studies of its photochemical activation, Diwu and Lown (12) demonstrated that under oxygenated conditions, photoactivation results in the formation of reactive oxygen species including $^{1}O_2$. The abbreviations used are: cal C, calphostin C; PKC, protein kinase C; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; CCD, charge coupled device.
O$_2$•-, .OH, and H$_2$O$_2$. Although cal C has been proposed as a new agent for photodynamic therapy and treatment of malignancies (14), to our knowledge, the studies reported previously did not carefully examine its light- and concentration-dependent cytotoxicity. To determine its potential utility as a photodynamic agent, these parameters, along with its ability to kill heterogeneous tumor populations and its efficacy relative to other well-characterized apoptosis-inducing agents, such as thapsigargin (15), need to be evaluated in established preclinical models of malignancy.

This report examines the relationship between light and dose dependence of cal C cytotoxicity using a series of prostate cancer cell lines that have been well characterized both in vitro and in vivo. This panel of related cell lines was chosen because it represents the spectrum of disease progression. These cell lines have also been used extensively by numerous investigators to study the efficacy of chemotherapeutic agents in vivo. Given the heterogeneity of human disease, we chose this panel to represent variations in androgen responsiveness, tumorigenicity, and metastatic ability. In addition, cancer cell lines were chosen due to characteristics (e.g., p53 status and ploidy), which have been observed to alter the efficacy of chemotherapeutic agents (16–18). Our studies demonstrate that the cytotoxicity of cal C, at all concentrations tested, is exquisitely light dependent. Cells treated with cal C in the presence of visible light exhibited cellular and nuclear shrinkage, chromatin condensation, and DNA laddering consistent with the induction of apoptosis. The activation-dependent induction of apoptosis is independent of genetic background, p53 status, and in vivo malignant potential of the cell lines examined.

MATERIALS AND METHODS

Materials and Cell Lines. Stock solutions (1 mM) of cal C (Sigma Co.) or thapsigargin (Sigma Co.) were prepared in DMSO (Sigma Co.). The well-characterized human prostate cancer cell lines (DU 145, DuPro-1, TSU-Prl, LNCaP, and PC-3) and Dunning rat prostate cancer cell lines (AT3.1, AT6.1, AT2.1, and MAT LyLu) were used to evaluate the in vitro effect of cal C (Sigma Co.). The origins and characteristics of these cell lines have been described previously (16–25). The DU 145 and TSU-Prl cell lines have mutant p53 alleles (16, 18). PC-3 has a heterozygous deletion of the p53 allele (18). In addition, the DU 145 cell line expresses a truncated form of the Rb protein (17). The androgen-dependent LNCaP cell line expresses an intact p53 allele with a silent mutation in exon 5, codon 152 (18). The AT2.1 cell line is tumorigenic in vivo, whereas the AT3.1, AT6.1, and MAT LyLu cell lines are both tumorigenic and metastatic in both immunodeficient and syngeneic rodents (19–21). Except for AT6.1, which is pseudodiploid (21), all of the rest of the Dunning lines are aneuploid (19).

Cell Culture. Dunning prostate carcinoma cells were maintained in standard RPMI 1640 containing 8% FCS, 200 nM dexamethasone, 100 units/ml penicillin, and 100 units/ml streptomycin. The human cell lines were grown in standard RPMI 1640 containing 12% FCS, 100 units/ml penicillin, 100 units/ml streptomycin, and 100 nM dexamethasone. Tissue culture media and dexamethasone were purchased from Mediatech Corp. and Sigma Co., respectively. All other media additives were purchased from Life Technologies, Inc.

Cal C Treatment. Cells were harvested by trypsinization and resuspended in fresh media and 5 × 10$^5$ Dunning, or 1 × 10$^5$ human prostate cancer cells were plated per well on 96-well plates and incubated in 5% CO$_2$ at 37°C for 24 h. Frozen aliquots of stock solution were thawed immediately prior to addition to tissue culture media; unused portions were discarded. Repeated freeze/thaw cycles result in the rapid inactivation of cal C. All manipulations of the compound were performed in the dark using a Kodak GBX-2 safe light. The cells were pretreated with 10–1000 nM cal C (Sigma Co.) for 30 min in the absence of light. For protocols involving light exposure, standard fluorescent light (GE cool white light with an emission spectrum covering the range of 300–700 nm) was used as the activation source (26). The lids were removed from the tissue culture plates, and the cells were exposed to visible light at a distance of 780 cm below the light source in a laminar flow tissue culture hood (Baker Co.). After light exposure, the lids were replaced, and the plates were covered with aluminum foil to exclude any further light and then incubated under standard conditions. Controls consisted of cells treated with vehicle alone and exposed to visible light and cells treated with cal C without exposure to visible light. RPMI 1640 with DMSO served as the vehicle control.

To determine the potential long term toxicity of cal C treatment in the absence of visible light, a standard growth curve of AT6.1 cells was generated. Cells were plated at a density of 70 cells/cm$^2$, allowed to attach overnight, pretreated with 100 nM cal C (Sigma Co.) or vehicle alone, wrapped in foil to exclude light, and incubated under standard tissue culture conditions. To determine the dark toxicity (IC$_{50}$ in the absence of light) of cal C, increasing concentrations (up to 1000 nM) of the compound were added to exponentially growing AT3.1 cells and incubated for 24 h in the absence of light, and viability was measured by MTT assay.

Cell Morphology and Nuclear Visualization. Cell morphology was also examined immediately after cal C-treated cells were exposed to visible light. Dunning rat and human prostate cancer cells were plated at a concentration of 50 and 100 cells/cm$^2$, respectively. The cells were treated as described in the preceding section and examined immediately under light microscopy.

Exponentially growing Dunning prostate cancer cells and human prostate cancer cells were treated with 100 nM cal C (Sigma Co.) and exposed to visible light (with covers removed) for varying lengths of time. After light exposure, covers were replaced, and the dishes were wrapped in aluminum foil to exclude any further light exposure. Cells were then incubated under standard tissue culture conditions for the indicated time periods. Floating and attached cells were combined and collected by centrifugation at 750 × g and resuspended in ~100 μl cell culture media. The cell suspension was then mixed with 100 μl of 1 μg/ml 4,6-diamidino-2-phenylindole (Sigma) prepared in PBS containing 1% Triton X-100. Antifade p-phenylenediaz...
Evaluation of the activation-dependent and concentration-dependent cytotoxicity of cal C. The activation and concentration dependence of cal C cytotoxicity was examined in all eight prostate cancer cell lines. Representative data from MAT LyLu (A) and TSU-Prl (B) cell lines are shown. Cells were seeded on 96-well plates and treated with 0 nm (D), 25 nm (E), or 100 nm (F) cal C in combination with 0, 1, 3, or 6 h of activation as described in "Materials and Methods." The viability of the cells after treatment was determined by MTT assay. Each data point represents six separate determinations, and each experiment was repeated two to four times. Bars, SE.

**RESULTS**

Initial experiments were designed to evaluate the light activation requirement(s) of cal C and to determine its concentration dependence for in vitro cytotoxicity. Cell viability was measured by MTT assay (28). Cell lines were treated with fixed concentrations of cal C (i.e., 0, 25, and 100 nm) in combination with increasing exposures to light (i.e., 0, 1, 3, and 6 h). The results from studies of two of the nine cell lines are shown in Fig. 1. The rat cell line MAT LyLu (Fig. 1A) is euploid and highly malignant in vivo, and the human cell line TSU-Prl (Fig. 1B), which was derived from a lymph node metastasis, has a mutated p53 gene. Cells were pretreated in the dark with the indicated concentration of cal C, exposed to light for the time indicated, and incubated in the absence of light under normal cell culture conditions for an additional 18 h. Thus, the control cell lines (i.e., 0 h of light exposure, □) were exposed to cal C for ≤24 h. In the absence of activation (0 h light exposure), MAT LyLu and TSU-Prl cells treated with 0, 25, or 100 nm cal C showed no decrease in viability (Fig. 1). In contrast, cells treated with activated cal C demonstrated a decrease in cell viability with increasing light exposure and cal C concentration (Fig. 1). For example, 45% of the MAT LyLu cells were viable after treatment with 25 nm of cal C and 3 h of activation (Fig. 1A, ■), whereas ~20% of the cells were viable after treatment with 100 nm cal C and the same amount of activation (Fig. 1A, □). In comparison, TSU-Prl cells were less sensitive to 3 h of
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light exposure in combination with 25 nM cal C (Fig. 1B, □). However, treatment with 100 nM cal C under this condition resulted in a decrease in viability similar to that observed in the MAT LyLu cell line (Fig. 1B, □). The remaining seven cell lines examined demonstrated similar results (data not shown). Because treatment of cells for 24 h with cal C in the absence of light had no detectable toxicity, an additional time course experiment was conducted. The growth of AT6.1 rat prostatic cancer cells in the presence 100 nM cal C or vehicle alone without light activation was monitored over a 6-day period (Fig. 2). As is shown by the growth curve, in the absence of light, 100 nM cal C (-----) caused no measurable inhibition of growth, demonstrating that cal C toxicity is strictly light activation dependent.

To quantitate the relative cytotoxicity of cal C, the IC_{50} for the drug in combination with 3 h of activation was determined. Representative data from the MAT LyLu and TSU-Pr1 cell lines are shown in Fig. 3. As determined from these experiments (summarized in Table 1), the IC_{50}s for this treatment condition ranged from 7–60 nM and appear to be independent of the p53 status, ploidy, or malignant potential of the cells. The concentration dependence of the cell kill in all eight of the cell lines was similar to that of MAT LyLu and TSU-Pr1 (Fig. 3) in that ≤20% cell viability was observed in cells treated with ≥100 nM activated cal C (data not shown). In contrast, cells treated with up to 1000 nM cal C in the absence of activation showed >90% viability. Because of the solubility properties of cal C and the toxicity threshold of DMSO in tissue culture, we have not been able to achieve a sufficient concentration of cal C to cause detectable cytotoxicity in the absence of light.

In our studies, treatment of cells with activated cal C appeared to induce a rapid loss of cell viability. To test this observation, cells were pretreated with 100 nM cal C, exposed to light for 3 h, and immediately examined by light microscopy. Representative data from the TSU-Pr1 (A) and MAT LyLu (B) cell lines are shown; bars, SE. ---, cells treated with cal C in the absence of light; ----, cells treated with cal C in combination with light activation. The IC_{50}s derived for the remaining cell lines are summarized in Table 1.

**Table 1** Determination of the IC_{50}s of the nine prostate cancer cell lines after treatment with cal C and 3 h of light activation as examined by MTT

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC_{50} (nM)</th>
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<tbody>
<tr>
<td>AT6.1</td>
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<td>AT3.1</td>
<td>20</td>
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<tr>
<td>AT2.1</td>
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<tr>
<td>MAT LyLu</td>
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<tr>
<td>TSU-Pr1</td>
<td>50</td>
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<tr>
<td>DuPro-1</td>
<td>60</td>
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<tr>
<td>PC-3</td>
<td>50</td>
</tr>
<tr>
<td>LNCaP</td>
<td>7</td>
</tr>
<tr>
<td>DU145</td>
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To characterize further the light-dependent induction of apoptosis by cal C, cells were treated and prepared as described in "Materials and Methods" and examined by fluorescence microscopy. Representative data from a AT3.1 cells treated with 100 nM cal C, in combination with 2 h of light activation, are shown in Fig. 5A. AT3.1 cells treated with 100 nM cal C in the absence of light activation (Fig. 5B) had a normal nuclear and cytoplasmic appearance, similar to that of cells treated with vehicle (DMSO) in the presence of light (Fig. 5A). As a quantitative measure of the induction of apoptosis, the percentage of cells showing nuclear shrinkage after cal C treatment was determined. In cells treated with 100 nM cal C in the presence of 2 h of light activation, 78% of cells were scored as apoptotic after a 24-h incubation (Fig. 5C), 98% after 48 h (Fig. 5D), and 99% after 72 h (Fig. 5E) compared with only 6% of cells treated with unactivated cal C after 72 h (Fig. 5B). Cells treated with 500 nM thapsigargin, an agent known to induce apoptosis, mirrored our findings with cal C.
Fig. 4 Microscopic examination of cells after treatment with activated cal C. Cells were pretreated with 100 nm cal C and then exposed to 0 or 3 h of visible light. Cell morphology was examined by light microscopy (×100) immediately after the time indicated. A, TSU-Prl treated with 100 nm cal C and 0 h of light exposure. B, TSU-Prl treated with 100 nm cal C and 3 h of light exposure. C, AT6.1 treated with 100 nm cal C and 0 h of light exposure. D, AT6.1 treated with 100 nm cal C and 3 h of light exposure.

The induction of apoptosis was further confirmed by the detection of DNA ladders 12 h after the treatment of cells with activated 100 nm cal C in combination with 2 h of light activation. The extent of intranucleosomal laddering detected was similar to that detected in AT3.1 cells treated with 500 nM thapsigargin for 24 h. Neither vehicle control cells nor cells treated with 100 nm cal C in the absence of light showed any DNA fragmentation (Fig. 6). It is important to note that the detection of ladder formation represents an early event in the apoptotic pathway, thus the ladders detected by treating cells with activated cal C was strictly time dependent. That is, ladders could not be detected at later time points (24 or 48 h), a finding in agreement with the state of cells as observed by fluorescence microscopy.

DISCUSSION

Failure to control local tumor growth remains a leading cause of cancer deaths (29). In spite of improvements in surgical techniques, chemotherapeutic regimens, and radiation therapy approaches, ~142,000 of the 439,400 deaths from the most common types of cancer reported in the United States in 1990 could be directly attributed to metastatic lesions (29). Thus, improved methods of local tumor control are of critical importance. Therapeutic strategies for the treatment of malignancy are complicated by a variety of factors, including the heterogeneity of tumor cell populations, the ability of tumor cells to adapt and survive combination therapies, and the achievement of optimal therapeutic ratios. Recently, approaches based on the identification of cellular apoptosis as a therapeutic target have been the focus of considerable study (3). Although promising, even these strategies are complicated by the fact that certain genetic changes (i.e., p53 mutation; Ref. 4) and malignant properties (i.e., acquisition of metastatic ability; Ref. 5) can confer increased resistance to apoptosis-inducing agents. Taken together, these findings demonstrate a need for the development of new therapeutic modalities that can be specifically activated to kill heterogeneous tumor cell populations.

One approach to the design of such strategies has been the identification and development of photosensitizing agents. This approach is based on the observation that photosensitizers are cytotoxic when excited by light but are relatively innocuous in the absence of light activation (30). The attainment of high therapeutic ratios using such agents is dependent upon several factors including their photodynamic yields, their physical application, and their controlled activation. Although preliminary results have been promising in specific cancers, the use of photosensitizing agents, such as hematoporphyrin derivative, has shown many of the limitations of traditional chemotherapeutic strategies, i.e., host toxicity and suboptimal target specificity (31). These limitations motivated efforts to identify and characterize alternative photosensitizing agents including members of the perylenequinonoid family of pigments (30). One such compound, cal C, is an ideal candidate for such studies because it is activated specifically by the absorption of visible light (9, 12) and is also a potent inhibitor of PKC (7, 13, 32), a target of chemotherapeutic strategies for the induction of apoptosis (3). Interestingly, other PKC-targeted agents have been used in translational work alone, or in combination with known chemotherapeutic agents, to enhance tumor cell kill (33–35). We hypothesize that the unique photoactivatable properties of cal C may enable it to increase the local control of disease, compared with other compounds such as staurosporine, H7, chelerythrine chloride, and bryostatin.

In this report, nine well-characterized cancer cell lines were used to establish a relationship between light- and concentration-dependent cytotoxicity of cal C. For example, 24 h after
treatment, the cytotoxicity seen in TSU-Prl cells treated with 100 nM cal C in combination with 1 h of light activation was comparable with that seen in TSU-Prl cells treated with 25 nM cal C in combination with 6 h of light activation. Interestingly, the onset of the apoptotic cascade appears to follow immediately after treatment with activated cal C. This interdependency of time and concentration effects must be taken into consideration in the experimental design of studies addressing the properties of cal C and other photodynamic agents.

We chose a standard treatment protocol of 3 h of light exposure to determine the IC_{50} of activated cal C. Under these conditions, the IC_{50}s ranged from 7-60 nM in all of the cell lines tested. Unlike the rest of the cell lines tested, LNCaP had a 3-6 fold lower IC_{50} value. At this point, it is unclear whether the increased sensitivity of this cell line to activated cal C is due to the presence of wild-type p53. In the absence of activation, cells exposed to ≤1000 nM cal C for up to 24 h had no detectable decrease in viability. Because of the solubility properties of cal C and the limiting cell toxicity of DMSO, we were unable to achieve a concentration sufficient to determine an IC_{50} of cal C in the absence of light (i.e., the dark toxicity). Consequently, we are unable to accurately determine the photodynamic ratio (IC_{50, dark}/IC_{50, light}) of this compound and thus cannot offer an accurate comparison between cal C and other known photodynamic agents.

A separate study was conducted to determine the potential long-term toxicity of cal C treatment in the absence of visible light. The results of this work showed that cells grown in the presence of 100 nM cal C without light activation over a 6-day period were not growth inhibited compared with control cells. This is of particular significance because this concentration of cal C in combination with 3 h of light activation results in 80% cell kill in all of the cell lines tested. These results clearly demonstrate that cal C cytotoxicity is exquisitely light activation dependent. Furthermore, the observation that activated cal C is equally effective against all of the cell lines tested suggests that its cytotoxicity is independent of the genetic background or malignant potential of the cell.

How do our findings of activated cal C cytotoxicity compare with other reports in the literature? Although other published reports state that treatment of cal C in the presence of visible light caused apoptotic cell death, it is not clear that these studies were...
controlled adequately for variations in light exposure. This compound is highly sensitive to light, and as such, we have found that seemingly small variations in its handling and manipulation ultimately lead to highly variable results in cytotoxicity assays. We were able to virtually eliminate this variability by meticulously controlling for light exposure as described by Bruns et al. (i.e., working under a safelight and a completely dark room, among others; Ref. 9). Our finding of the complex interdependence between cal C concentration, time of visible light exposure, and time course of apoptosis posttreatment is contrary to those of other investigators. For example, Weller et al. (8) “found only minor enhancement of cell killing during continuous light exposures.” Because of such fundamental differences, it is difficult to compare our IC50's with those reported in the literature. Similarly, in our experience, treatment of cells with 1000 nM cal C in combination with 1 h of light activation would result in complete loss of cellular viability and integrity, making gene expression or signal transduction studies seemingly impossible (36). Based on the photochemistry and photobiology of this compound and other perylenequinones, we would posit that these discrepancies are the result of inadvertent degradation of the compound, resulting in decreased photodynamic yields.

Is cal C worthy of further preclinical studies? Our present findings, as well as those published previously, demonstrate that the phototoxicity of cal C could be tightly regulated. Furthermore, activated cal C rapidly induces a cell death mechanism that is apparently conserved in a wide range of tumor cell lines with varying genetic defects. Studies reported by Weller et al. (8) suggest that this is not due to “random oxidative damage” because free radical scavengers did not block the phototoxicity of cal C in their studies. The observations of rapid onset of apoptotic morphologies and the apparent inability of cycloheximide to block this process suggest that new protein synthesis is not required for activated cal C-induced apoptosis (8). It is also unclear as to whether activated cal C is working through a PKC-dependent pathway (8). According to current literature, cal C works by inhibiting the conserved diglyceride/sphinganine binding site within the regulatory domain of PKC (7). In addition, sphingoid bases have been reported to act as endogenous PKC inhibitors (37-39). Previous studies have also demonstrated that inhibition of PKC with cal C enhances the lethal effects of exogenous ceramide and sphingosine while also increasing their endogenous production (39, 40). It is conceivable that cal C action is more complex than PKC inhibition alone. Thus, the biochemical mechanism of the “phototoxic switch” of activated cal C remains equivocal.

These promising in vitro results suggest that cal C may have potential utility for photodynamic control of local tumors. Because a significant percentage of all cancer deaths are the result of failure of local tumor control (29) and increasing local control can potentially enhance control of metastatic disease, cal C may be poised to fill an important clinical role. With this in mind, we conducted a pilot animal toxicity study using single i.p. injections 1, 2.5, and 20 mg/kg of unactivated cal C in Swiss nude mice. After administration of the compound, animals were followed for a 2-week period, during which no durable weight loss of overt toxicity was observed.6 In summary, the low toxicity of cal C in the absence of light activation, its high photodynamic yields, and the intriguing possibility that it works by a tightly controlled and conserved apoptotic mechanism demonstrate that cal C is worthy of further preclinical evaluation.

ACKNOWLEDGMENTS

We thank Dr. Raphael Espinosa for generous assistance in the preparation of CCD images and Grace Chae for library research contributions. We also thank Dr. M. Eileen Dolan, The University of Chicago Section of Hematology Oncology, for advice on the design and implementation of the pilot animal toxicity studies. We are also very grateful to Dr. Charles Brendler for enthusiastic support of our work.

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