Sapphyrins induce apoptosis in hematopoietic tumor–derived cell lines and show in vivo antitumor activity

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Abstract
Sapphyrins are pentapyrrolic, metal-free, expanded porphyrins. In the present study, the activity of sapphyrins as anticancer agents in hematopoietic-derived tumor cells was explored. It was found that a dihydroxylated water-soluble sapphyrin derivative (PCI-2000) is a potent inducer of apoptosis in a wide variety of tumor cell lines including lymphoma (Ramos, DHL-4, and HF-1), leukemia (Jurkat and HL-60), and myeloma (8226/S, 1-310, C2E3, and 1-414). PCI-2000 triggers an apoptotic pathway in these tumor cells as shown by release of cytochrome c from mitochondria; activation of caspases 9, 8, and 3; cleavage of the caspase substrate poly(ADP-ribose) polymerase; and Annexin V binding. Apoptosis can be partially inhibited by overexpression of the antiapoptotic protein Bcl-2 or treatment with benzyloxy carbonyl-valine-alanine-aspartic acid-fluoromethylketone, a cell-permeable caspase inhibitor. Both PCI-2000 and PCI-2010, a tetrahydroxy bis-carbamate derivative of PCI-2000, resulted in increased levels of phosphorylated p38 mitogen-activated protein kinase. Inhibition of p38 mitogen-activated protein kinase phosphorylation resulted in a synergistic increase of PCI-2000 cytotoxicity. PCI-2010 showed less toxicity in mice than PCI-2000 and was active in slowing the growth of Ramos and HL-60 tumor xenografts in nude mice. These results provide preclinical rationale for the further study of sapphyrins for potential use in the treatment of hematopoietic-derived tumors.


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from PCI-2000 through the use of an active carbonate method (see Fig. 1 for chemical structures). Briefly, PCI-2000 (1 mmol) was treated with disuccinimidyl carbonate (5 mmol; Sigma-Aldrich, St. Louis, MO) in the presence of diisopropylethylamine (10 mmol) in anhydrous methylene chloride for 1 hour. The active carbonate intermediate was then reacted with diethanolamine (10 mmol) for 4 hours. Both reaction steps were carried out at room temperature. After the reaction was complete, the product was purified by flash chromatography on neutral alumina followed by column chromatography with tC18 Sep-Pak (Waters, Milford, MA). The purity of the final product was >94% as measured by reversed-phase high-performance liquid chromatography. The identity was ascertained by proton and carbon-13 nuclear magnetic resonance spectra, as well as by electrospray ionization mass spectrometry. PCI-2000 and PCI-2010 were formulated in 100% DMSO for the in vitro studies. For animal studies, PCI-2000 was formulated by dissolving in 5% mannitol followed by sterile ethanol followed by sterile filtration, whereas PCI-2010 was formulated by dissolving in 5% mannitol followed by sterile filtration.

**Cell Lines and Growth Conditions**

All cell lines were grown in RPMI 1640 with 10% fetal bovine serum in a 5% CO2/air incubator at 37°C. Cells at a density of 100,000 cells/mL were treated with sapphyrins for 24 or 96 hours and then assessed for apoptosis. Final DMSO concentrations in cell cultures were generally ≤0.02% and control experiments showed that DMSO concentrations used in this study had no effect on cell viability or growth. Cell numbers were determined using a model Z2 counter (Beckman-Coulter, Miami, FL). Addition of sapphyrins to tissue culture cells was done under subdued lighting and sapphyrin-treated cells were cultured in a dark incubator to minimize any possibility of cytotoxicity stemming from light exposure. Control experiments showed no enhancement of cell death in PCI-2000- or PCI-2010-treated cultures resulting from intentional exposure to bright fluorescent lighting for 1 minute.

**Apoptosis Assays**

Annexin V-FITC binding and propidium iodide exclusion were assayed with a FACSCalibur instrument (Becton Dickinson, San Jose, CA) using reagents from Biorad (Camarillo, CA) per protocol of the manufacturer. Caspase-3 activity was assayed using the EnzChek Caspase-3 Assay Kit #2 (Molecular Probes, Eugene, OR). Cells were harvested, rinsed in cold PBS, and analyzed according to the protocol of the manufacturer. Cell extracts were incubated in a reaction mixture containing Z-DEVD-R110 (0.5 mmol/L) at room temperature for 30 minutes, and fluorescence levels were determined at an excitation of 485 nm and emission of 510 nm using a fluorescence plate reader. For each cell line, measured fluorescence levels were normalized to fluorescence levels of nontreated cell lysates.

**Release of Cytochrome c from Mitochondria**

The distribution of cytochrome c in the cytoplasm and pellet containing mitochondria was determined using Western blotting after Dounce homogenization and differential centrifugation of sapphyrin-treated and control cells. Mouse anti–cytochrome c antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used to detect cytochrome c, and mouse monoclonal antibody to cytochrome c oxidase II, a protein found in the inner mitochondrial membrane, was used to track the mitochondrial fraction (Cell Signaling Technologies, Inc., Beverly, MA).

**Western Blotting**

Cells were lysed in triple-detergent lysis buffer [50 mmol/L Tris-Cl (pH 8.0), 150 mmol/L NaCl, 0.1% SDS, 0.5% deoxycholic acid, 1.0% NP40, supplemented with 100 mmol/L phenylmethylsulfonyl fluoride and Complete Protease Inhibitor Cocktail (Roche Molecular Biochemicals, Indianapolis, IN)] on ice for 10 minutes. After centrifugation at 10,000 × g for 10 minutes, the supernatants were analyzed for protein concentration, with equal amounts of protein being resolved on SDS-polyacrylamide gels (Bio-Rad, Hercules, CA). The gels were transferred to a polyvinylidene difluoride membrane using a Semi-dry Transfer Cell (Bio-Rad). Antibodies to caspases and poly(ADP-ribose) polymerase were used, which specifically recognized the full-length and cleaved forms of their respective antigens (Cell Signaling Technologies). Antibodies to p38 MAPK and phosphorylated p38 MAPK (both antibodies recognize all four isoforms) were obtained from Cell Signaling Technology. All membranes were blotted with antibodies for loading controls. Protein bands were imaged and quantified in the linear range and normalized to control proteins using the Odyssey Infrared Imaging System (LI-COR, Lincoln, NE).

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**Figure 1.** Structure of sapphyrins PCI-2000 and PCI-2010. The sapphyrins were synthesized as described in Materials and Methods.
**Inhibition of Sapphyrin-Induced Apoptosis by Bcl-2 or Caspase Inhibition**

HL-60 neo and HL-60 Bcl-2 cells were incubated with PCI-2000 and apoptosis was assessed by Annexin V FITC and propidium iodide. Caspases were inhibited by treating cells with 100 \( \mu \text{mol/L} \) benzoylloxycarbonyl-valine-alanine-aspartic acid-fluoromethylketone (z-VAD-fmk) for 15 minutes before addition of PCI-2000 to Ramos or HL-60 cells.

**Results**

**PCI-2000 Localizes in the Cytoplasm of Ramos Cells**

PCI-2000 is a fluorescent compound that can be detected by fluorescence microscopy with a Texas red filter set. To determine the subcellular localization of PCI-2000, Ramos cells were treated with 1 \( \mu \text{mol/L} \) of PCI-2000 for 4 hours, centrifuged onto glass slides, and visualized using a Nikon Eclipse fluorescence microscope equipped with Nomarski optics. PCI-2000 was distributed in the cytoplasm but not the nucleus of cells (Fig. 2).

**PCI-2000 Induces Apoptosis in Leukemia-, Lymphoma-, and Myeloma-Derived Tumor Cell Lines**

We treated T-lineage leukemia (Jurkat), B-lineage tumors (Ramos, HF-1, and DHL-4), and myeloma cell lines (1-310, 1-414, and C2E3) with PCI-2000 (1 \( \mu \text{mol/L} \)) or vehicle (DMSO). Within 24 hours, cultures treated with PCI-2000 showed a large proportion of cells with morphologic features of apoptosis whereas those treated with DMSO did not (data not shown). Consistent with induction of apoptosis, treatment with PCI-2000 (1 \( \mu \text{mol/L} \)) for 24 hours resulted in 60% to 90% Annexin V-positive cells, activation of caspases, and cleavage of poly(ADP-ribose) polymerase in all cell lines tested (Fig. 3). Although 0.5 \( \mu \text{mol/L} \) of PCI-2000 did not induce apoptosis within the first 24 hours of treatment, >90% of the cells underwent apoptotic death within 96 hours (data not shown).

**PCI-2000 Induces Release of Cytochrome c from Mitochondria**

Many chemotherapy drugs affect the mitochondrial pathway of apoptosis by releasing cytochrome c, which then activates a caspase cascade through the formation of a complex with apoptotic protease activating factor 1 and caspase-9, thereby resulting in autocleavage of caspase-9 (11). To determine if mitochondria are involved in sapphyrin-induced apoptosis, Ramos cells were treated with PCI-2000 (1 \( \mu \text{mol/L} \) for 16 hours) and fractionated into soluble and membrane (including mitochondria) fractions. In carrier vehicle-treated cells (DMSO), most of the cytochrome c was found in the membrane fraction. In cells treated with PCI-2000, more cytochrome c was found in the soluble fraction, leading to the conclusion that it had been released from the mitochondria (Fig. 4).

**Activation of Caspases 9, 3, and 8 in PCI-2000-Treated Cells**

There are two primary pathways for caspase activation—one triggered by receptor activation on the cell surface and the other by mitochondrial damage (11, 12).
Release of cytochrome c suggested activation of the mitochondrial pathway. To show caspase activation, we treated Ramos cells with increasing amounts of PCI-2000 for 8 hours and also did a time course with a fixed dose of PCI-2000. We found that cells treated with 2.5 μmol/L of PCI-2000 for 8 hours or with 5 μmol/L of PCI-2000 for 4 hours had increased caspase activity (Fig. 5A). To determine which caspases were activated, cells were harvested and protein subjected to Western blotting to monitor cleavage (activation) of caspases 9, 3, and 8 and the substrate poly(ADP-ribose) polymerase. Consistent with activation of the mitochondrial pathway, caspase-9 was activated early followed by caspase-3 (Fig. 5B). Caspase-3 (a downstream target of caspase-9) was cleaved as was the caspase-3 substrate poly(ADP-ribose) polymerase (Fig. 5B). Caspase-8 cleavage occurred after cleavage of caspases 9 and 3, consistent with a potential feedback loop from caspase-3 resulting in caspase-8 cleavage (Fig. 5B; ref. 13).

Overexpression of Bcl-2 or Inhibition of Caspase Activity Blocks PCI-2000–Induced Apoptosis

One function of Bcl-2, an oncogene with antiapoptotic properties, is to block the activity of Bcl-2 homology domain 3-only members to release cytochrome c from mitochondria (14). We hypothesized that Bcl-2, through inhibition of cytochrome c release, could potentially inhibit sapphyrin-induced apoptosis. To test this hypothesis, wild-type HL-60 cells and HL-60 cells overexpressing Bcl-2 were treated with 1 μmol/L PCI-2000 (15). Overexpression of Bcl-2 resulted in fewer Annexin V–positive cells and less caspase-3 activity compared with the control cell line (Fig. 6 A and B). Although Bcl-2 inhibited apoptosis in PCI-2000–treated cells, Bcl-2 did not circumvent the growth inhibition caused by PCI-2000 (Fig. 6C). These results show that HL-60 cells resistant to sapphyrin-induced apoptosis through Bcl-2 overexpression are still growth inhibited.

Caspase activation leads to the morphologic and biochemical changes associated with apoptosis (16). To
determine if caspase activation played a direct role in sapphyrin-induced apoptosis, we treated Ramos cells with 1 μmol/L PCI-2000 or with PCI-2000 and 100 μmol/L z-VAD-fmk (a cell membrane-permeable, irreversible, caspase inhibitor). Most cells treated with PCI-2000 were apoptotic as shown by Annexin V binding whereas most cells treated with the combination of PCI-2000 and z-VAD-fmk were Annexin V negative (Fig. 6D). These results show that PCI-2000 triggers the caspase pathway resulting in caspase-dependent apoptosis.

**Sapphyrin Treatment Results in Increased Phosphorylation of p38 MAPK**

During the course of this study, PCI-2010, a tetrahydroxy bis-carbamate derivative of PCI-2000, was synthesized and evaluated for cytotoxicity. PCI-2010 was found to be as efficacious as PCI-2000 in inducing apoptosis in Ramos cells as shown by Annexin V and caspase activity assays (Fig. 7A and B). PCI-2010 treatment also resulted in caspase cleavage and poly(ADP-ribose) polymerase cleavage (data not shown).

p38 MAPKs are protein kinases that are activated (phosphorylated) by a wide variety of stress-inducing stimuli including chemotherapy, DNA damage, and oxidative stress (17, 18). To determine if sapphyrins activated p38 MAPK, we treated Ramos cells with increasing doses of PCI-2000 and PCI-2010 for 8 hours.
and did analysis for phosphorylated p38 and total p38 MAPK. Both PCI-2000 and PCI-2010 resulted in increased levels of phosphorylated p38 MAPK (Fig. 7C). Activation of the p38 MAPK pathway through phosphorylation may be either proapoptotic or antiapoptotic depending on cell types and conditions (18). To determine the relevance of p38 phosphorylation to sapphyrin-induced cytotoxicity, we treated Ramos cells with PCI-2000 and an inhibitor of p38 phosphorylation, SB 203580. We found that the inhibitor alone was not cytotoxic but stimulated the cytotoxicity of PCI-2000 (Fig. 7D). For example, PCI-2000 at 1 μmol/L resulted in 26.9% Annexin V–positive cells but combined treatment with PCI-2000 and the p38 phosphorylation inhibitor resulted in 60.5% Annexin V–positive cells in both Ramos and HL-60 cells.

PCI-2010 Is Active in Xenograft Tumor Models

Single-dose toxicity studies showed that PCI-2010 had a higher lethal dose in mice than did PCI-2000 (Table 1). Because PCI-2000 and PCI-2010 have approximately equivalent in vitro apoptosis-inducing activity, PCI-2010 was selected for further in vivo study to determine efficacy in two different animal models. The first was a minimal disease model, wherein Ramos xenograft–bearing animals were treated with PCI-2010 (20 μmol/kg daily for two doses) or vehicle control (5% mannitol) before development of palpable tumors. In this model, animals treated with PCI-2010 showed a substantial delay in tumor growth compared with the vehicle-treated animals (Fig. 8A). Comparison of mean tumor volumes in controls and PCI-2010–treated animals using a Student’s t test (assuming equal variances) revealed a significant difference ($P < 0.05$) beginning on day 5 through study completion. As a further demonstration of efficacy, a second, palpable

<table>
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<th>Number of deaths within 14 days</th>
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disease model was used. In this case, animals with palpable HL-60 tumors (average, 5 mm diameter) were injected with vehicle control or PCI-2010 (10 μmol/kg every 4 days for five doses). Treatment with PCI-2010 was found to delay the growth of tumors compared with control animals (Fig. 8B). Tumors in control animals were significantly different from those in PCI-2010–treated animals (P < 0.05) beginning on treatment day 17 through study completion.

Discussion

Porphyrrins and expanded porphyrins such as texaphyrins and sapphyrins have potential medical utility, including cancer imaging and therapy (1, 19, 20). For instance, the porphyrin mixture, porfimer sodium, is a drug used for the treatment of esophageal and endobronchial lung cancer via photodynamic therapy (19). Sapphyrins are expanded metal-free porphyrins with interesting chemical properties. However, only limited information is available regarding their biological activity. Like porphyrins, sapphyrins are known to localize to tumor tissues through mechanisms that are not understood (8). Sapphyrins have been investigated in the context of photodynamic therapy (5, 6). One sapphyrin has been previously reported to exhibit cytotoxic activity in the absence of photoactivating light (9). In that study, which was focused primarily on the biological activity of a pentapyrrollic expanded porphyrin, it was found that the sapphyrin was able to induce apoptosis in tumor cells. To explore the mechanism of sapphyrin-induced apoptosis, the phosphorylation of p38 MAPK was studied. Figure 7 shows the results of this analysis. A phospho-antibody specific for p38 MAPK was used to detect the phosphorylated form of the protein. The results indicate that sapphyrin treatment leads to an increase in phosphorylated p38 MAPK, suggesting that this is a key signaling event in sapphyrin-induced apoptosis.
porphyrin termed pentaphyrin, only the inhibitory dose (ID$_{50}$) for Jurkat cells was presented and only for one particular sapphyrin. To the best of our knowledge, there has been no follow-up article published and no other report on the biological activity manifested by sapphyrins in the absence of light.

We sought to evaluate the antitumor properties of sapphyrins on their reported ability to concentrate in neoplastic tissues (8). In this study, we describe the cytotoxic (PCI-2000 and PCI-2010) and antitumor (PCI-2010) activities of sapphyrins that justify their further study as potential anticancer agents. We showed that sapphyrins induce apoptosis in numerous cell lines including those derived from lymphoma, leukemia, and multiple myeloma in the absence of sufficient light to induce photodynamic effects. The biochemical pathway through which sapphyrins induce apoptosis has not yet been fully elucidated. Similar to other stress-inducing stimuli, sapphyrin treatment results in the phosphorylation of p38 MAPK, which may then transduce either survival or death signals (17, 18). The combination of PCI-2000 with a specific inhibitor of p38 MAPK phosphorylation resulted in synergistic cytotoxicity, suggesting that p38 MAPK phosphorylation was protective in PCI-2000–treated Ramos cells. A similar situation has been described with bortezomib, a proteasome inhibitor that is used in the treatment of multiple myeloma. Bortezomib induces the phosphorylation of p38 MAPK, and inhibition of p38 MAPK phosphorylation enhances bortezomib-induced cytotoxicity (21). The synergistic combination of a sapphyrin and a p38 MAPK phosphorylation inhibitor may someday be clinically relevant.

Sapphyrins activate the mitochondrial pathway of apoptosis as shown by release of cytochrome c from mitochondria and cleavage of caspase-9. Interestingly, sapphyrins have been shown to interact directly with isolated mitochondria in an in vitro situation (22). Future studies will determine if sapphyrins localize to mitochondria in intact cells. Overexpression of Bcl-2, a protein that modulates mitochondrial-mediated apoptosis through binding of Bcl-2 homology domain 3 proteins, inhibits sapphyrin-induced apoptosis but not growth inhibition. Therefore, like many other cytotoxic agents, sapphyrin acts upstream of Bcl-2 to trigger an apoptotic pathway. Regardless of the mechanism(s) used to trigger mitochondrial release of cytochrome c, sapphyrins activate a caspase cascade that results in cell death in the absence of Bcl-2 overexpression. Sapphyrin-induced apoptosis is caspase dependent because an inhibitor of caspases (z-VAD-fmk) blocks apoptosis. The ability of sapphyrins to induce apoptosis is not limited to hematopoietic tumor–derived cell lines because they also induce apoptosis in solid tumor–derived cell lines.

In an effort to ascertain whether sapphyrins warrant further consideration as potential anticancer agents, their toxicity profiles in normal mice were determined and their efficacy in mouse tumor models was assessed. Single injections of escalating doses of PCI-2000 and PCI-2010, followed by observation for morbidity and mortality and histologic examination, revealed that PCI-2010 had a more acceptable toxicity profile than PCI-2000. There was no decrease in the peripheral WBC count, RBC count, or platelet count in sapphyrin-treated mice, suggesting that bone marrow suppression is not a prominent side affect of sapphyrins. We focused our tumor efficacy studies on PCI-2010 exclusively because it was found to be better tolerated in mice than PCI-2000. As detailed in Results, PCI-2010 was found to delay tumor growth in two different xenograft models, a Ramos xenograft minimal disease model and an HL-60 palpable tumor model. As yet, we have not done sufficient experiments to determine the optimal dosing regiments. Nonetheless, the experiments done to date serve to highlight the fact that sapphyrins such as PCI-2010 may have a role to play as potential anticancer agents. This class of compounds is particularly attractive.

3 D. Magda and L. Naumovski, unpublished observations.
4 Unpublished observations.
because it is readily amenable to chemical manipulation. Accordingly, efforts are currently under way to prepare other sapphyrin derivatives and to evaluate their toxicity and efficacy profiles.

References
Molecular Cancer Therapeutics

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