Parallel Synthesis and Biological Evaluation of 837 Analogues of Procaspase-Activating Compound 1 (PAC-1)

Danny C. Hsu,[†] Howard S. Roth,[†] Diana C. West,[†] Rachel C. Botham,[†] Chris J. Novotny,[‡] Steven C. Schmid,[†] and Paul J. Hergenrother*,[†,‡]

[†]Department of Chemistry and [‡]Department of Biochemistry, Roger Adams Laboratory, University of Illinois, Urbana, Illinois 61801, United States

Ⓢ *Supporting Information*

ABSTRACT: Procaspase-Activating Compound 1 (**PAC-1**) is an *ortho*-hydroxy *N*-acyl hydrazone that enhances the enzymatic activity of procaspase-3 in vitro and induces apoptosis in cancer cells. An analogue of **PAC-1**, called **S-PAC-1**, was evaluated in a veterinary clinical trial in pet dogs with lymphoma and found to have considerable potential as an anticancer agent. With the goal of identifying more potent compounds in this promising class of experimental therapeutics, a combinatorial library based on **PAC-1** was created, and the compounds were evaluated for their ability to induce death of cancer cells in culture. For library construction, 31 hydrazides were condensed in parallel with 27 aldehydes to create 837 **PAC-1** analogues, with an average purity of 91%. The compounds were evaluated for their ability to induce apoptosis in cancer cells, and through this work, six compounds were discovered to be substantially more potent than **PAC-1** and **S-PAC-1**. These six hits were further evaluated for their ability to relieve zinc-mediated inhibition of procaspase-3 in vitro. In general, the newly identified hit compounds are two- to four-fold more potent than **PAC-1** and **S-PAC-1** in cell culture, and thus have promise as experimental therapeutics for treatment of the many cancers that have elevated expression levels of procaspase-3.

KEYWORDS: *cancer, apoptosis, procaspase-3, zinc, anticancer compounds*
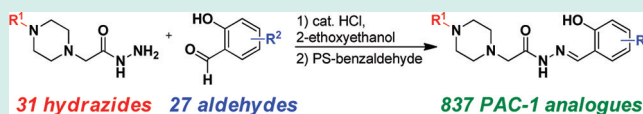
INTRODUCTION

Apoptosis is a process used by higher organisms to maintain homeostasis by removing cells that are in excess, damaged, or potentially dangerous. Critical to apoptosis is the activation of caspase enzymes, a class of cysteine proteases that cleave cellular substrates after recognition sequences with C-terminal aspartate residues.[1] There are two canonical apoptotic pathways, differing in that the apoptosis-initiating stimulus is intracellular (intrinsic pathway) or extracellular (extrinsic pathway).[2] These pathways converge at the cleavage of procaspase-3 to form the active caspase-3, the key "executioner" caspase that catalyzes the hydrolysis of hundreds of protein substrates,[3] leading to cell death.

One of the hallmarks of cancer is the ability of cancer cells to evade apoptosis, allowing for unchecked proliferation.[4] As such, reactivation of apoptosis in cells with defective apoptotic pathways is a promising anticancer strategy. Compounds such as p53-MDM2 disruptors (Nutlins),[5] Bcl-2 inhibitors (ABT-737),[6] and inhibitors of XIAP (SM-164),[7] act directly on proteins in the apoptotic cascade, inducing apoptosis and leading to death of cancer cells.

Complementary to the strategies described above, the direct activation of procaspase-3 with a small molecule has potential for the personalized treatment of cancer.[8] Procaspase-3 levels are elevated in certain cancers, including lymphomas,[9] leukemias,[10] melanomas,[11] pancreatic cancers,[12] liver cancers,[13] lung cancers,[14] breast cancers,[15] and colon cancers.[8,16] Because of the elevated levels of procaspase-3 in cancer cells, the

requirement of caspase-3 activation for apoptosis, and the relative downstream location of procaspase-3 in the apoptotic cascade, induction of apoptosis by the direct activation of procaspase-3 is being actively explored as a personalized anticancer strategy.[8,17]

In 2006, the discovery of Procaspase-Activating Compound 1 (**PAC-1**, Figure 1) was reported. **PAC-1** enhances the
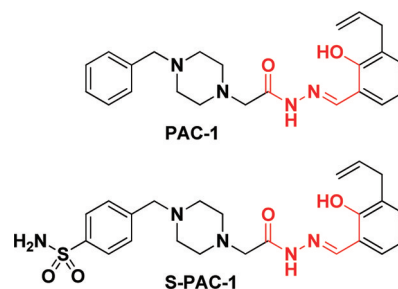

Figure 1. **PAC-1** and **S-PAC-1**, with the *ortho*-hydroxy *N*-acyl hydrazone motif highlighted in red.

enzymatic activity of procaspase-3 in vitro, induces apoptotic cell death in cancer cells, and shows efficacy in multiple murine

tumor models.[8] Structure−activity relationship (SAR) studies revealed that the activity of **PAC-1** in vitro and in cell culture is dependent on the presence of the *ortho*-hydroxy *N*-acyl hydrazone moiety (highlighted in Figure 1),[18] a structural motif known to participate in metal chelation.[19] Indeed, zinc is a powerful inhibitor of procaspase-3 enzymatic activity,[20] and the mechanism by which **PAC-1** activates procaspase-3 in vitro is through chelation of inhibitory zinc from procaspase-3, which allows procaspase-3 to process itself to the active form.[18,20] This same basic mechanism appears to be operational in cell culture as well: approximately 10% of cellular zinc is not bound tightly but exists as the "labile zinc pool".[21] As zinc from the labile pool has been shown to colocalize with procaspase-3,[21] it appears that **PAC-1** chelation of this labile zinc inside the cells enhances procaspase-3 activity, leading to apoptosis.

PAC-1 can be safely administered to mice and research dogs at doses that give serum concentrations of ∼10 μM for 48 h.[22] A sulfonamide-containing derivative of **PAC-1**, called **S-PAC-1** (Figure 1), can be safely administered at doses that provide very high serum concentrations in mice (∼3.5 mM).[23] Encouragingly, a veterinary clinical trial of **S-PAC-1** (administered as a 24- or 72-h continuous IV infusion) in pet dogs with spontaneously occurring lymphoma revealed this compound to be safe in all veterinary patients and effective at reducing or stabilizing tumor growth in 4 out of 6 patients.[23] This result provides proof-of-concept for the notion that procaspase-3 activation via small molecule chelation of labile zinc can be a safe and effective anticancer strategy. In the continued search for more potent derivatives of **PAC-1**, we report herein the parallel synthesis of a combinatorial library of 837 **PAC-1** analogues, the evaluation of these compounds for their ability to induce death of cancer cells in culture, and further characterization of six analogues of **PAC-1** with enhanced potency.

■ RESULTS AND DISCUSSION

Design and Synthesis of Combinatorial Library Based on PAC-1. A library of **PAC-1** analogues was designed with the goal of identifying compounds capable of eliciting potent death of cancer cells in culture. As the maximal cytotoxicity of **S-PAC-1** is not reached until at least 24 h,[23] and both **PAC-1** and **S-PAC-1** exhibit short half-lives of 1−2 h in vivo,[22,23] a secondary goal of this study was to identify **PAC-1** analogues that could induce apoptosis more rapidly. Reported synthetic routes to **PAC-1** and **S-PAC-1**, as well as other **PAC-1** analogues, utilize the condensation of a hydrazide and an aldehyde as the final step in the synthetic scheme.[8,18,23,24] This modular nature of the **PAC-1** synthesis allows for a diverse array of functional groups to be conveniently incorporated into the **PAC-1** scaffold without altering the core *ortho*-hydroxy *N*-acyl hydrazone motif essential for procaspase-3 activation and induction of apoptosis.[18]

As shown in Figure 2, 31 hydrazides (**1**{*1−31*}) and 27 aldehydes (**2**{*1−27*}) were selected for building the library of 837 **PAC-1** analogues. The hydrazides were constructed from commercially available benzyl halide starting materials. The syntheses of hydrazides **1**{*1−6*} have been reported previously.[8,18,23] Hydrazides **1**{*7−27*} were synthesized according to Scheme 1. Substituted benzyl halides **4**{*7−27*} first reacted with piperazine to form substituted benzylpiperazines **5**{*7−27*}. A second alkylation of the piperazine ring with ethyl chloroacetate gave disubstituted piperazines **6**{*7−27*}, and the

esters were then converted to hydrazides **1**{*7−27*} by reaction with hydrazine. The synthetic routes toward hydrazides **1**{*28−31*} are detailed in Scheme 2. Synthesis of hydrazide **1**{*28*} began by the alkylation of piperazine with 4-vinylbenzyl chloride (**7**) to form monosubstituted piperazine **8** (Scheme 2, eq 1). A second alkylation with ethyl chloroacetate formed ester **9**, and reaction with hydrazine formed the hydrazide and reduced the olefin, giving hydrazide **1**{*28*}. The reduction of olefins with hydrazine typically involves the addition of an oxidizing agent,[25] but the presence of atmospheric oxygen was sufficient to achieve this transformation. Synthesis of hydrazide **1**{*29*} (Scheme 2, eq 2) began with the reaction of ethyl 2-(piperazin-1-yl)acetate (**10**), synthesized as previously reported,[8] with benzyl bromide **4**{*29*} to form intermediate **6**{*29*}. Reaction of **6**{*29*} with hydrazine then formed hydrazide **1**{*29*}. Hydrazide **1**{*30*} (Scheme 2, eq 3) was synthesized beginning with the reaction of 1-phenylpiperazine (**5**{*30*}) with ethyl chloroacetate to give disubstituted piperazine **6**{*30*}, and reaction with hydrazine formed hydrazide **1**{*30*}. Hydrazide **1**{*31*}, was synthesized by first protecting 4-methylbenzophenone (**11**) as the ethylene acetal (**12**), as shown in Scheme 2, eq 4. This compound was brominated under radical conditions to give benzyl bromide **13**. Reaction with monosubstituted piperazine **10** gave intermediate **14**, and reaction with hydrazine gave hydrazide **15**. Deprotection of the acetal with aqueous acid gave hydrazide **1**{*31*}.

The SAR of **PAC-1** derived from the synthesis and evaluation of ∼30 compounds demonstrated the necessity of the *ortho*-hydroxyl group,[18] so 27 salicylaldehyde building blocks were selected for library construction. Aldehydes **2**{*1−23*} were obtained from commercial sources, and the syntheses of aldehydes **2**{*24−26*} have been reported previously.[18,23,26] Aldehyde **2**{*27*} was synthesized via copper-catalyzed cyclo-addition of aldehyde **2**{*26*} with benzyl azide, as shown in Scheme 3.

Using a Büchi Syncore parallel synthesizer, each hydrazide was condensed with each aldehyde, with over 80 reactions performed simultaneously. Each aldehyde (5−15 mg) was allowed to react with excess hydrazide (1.7 equiv), and mass spectrometry was used to monitor the disappearance of the aldehyde from the reaction mixture. When the aldehyde had reacted completely, polystyrene-bound benzaldehyde was added as a scavenger resin to react with and remove the excess hydrazide. When mass spectrometry showed no hydrazide remaining, the beads were filtered, and the solutions were dried under high vacuum. Each of the 837 compounds was assessed by HPLC/MS. The purity of each compound is listed in Supporting Information Table S1, and the observed molecular ions are listed in Supporting Information Table S3. The library members had an average purity of 91%.

Evaluation of the PAC-1 Combinatorial Library. With 837 **PAC-1** analogues in hand, compounds were evaluated for their ability to induce apoptosis in cell culture. U-937 human lymphoma cells were exposed to the compounds for 24 h at a concentration of 20 μM. Both **PAC-1** and **S-PAC-1** display moderate potency (∼50% cell death) versus this cell line under these conditions. Apoptotic cell death was assessed by flow cytometry, using Annexin V-FITC/propidium iodide staining. Through this screening process, six compounds were identified and confirmed to induce >80% cell death under these conditions.

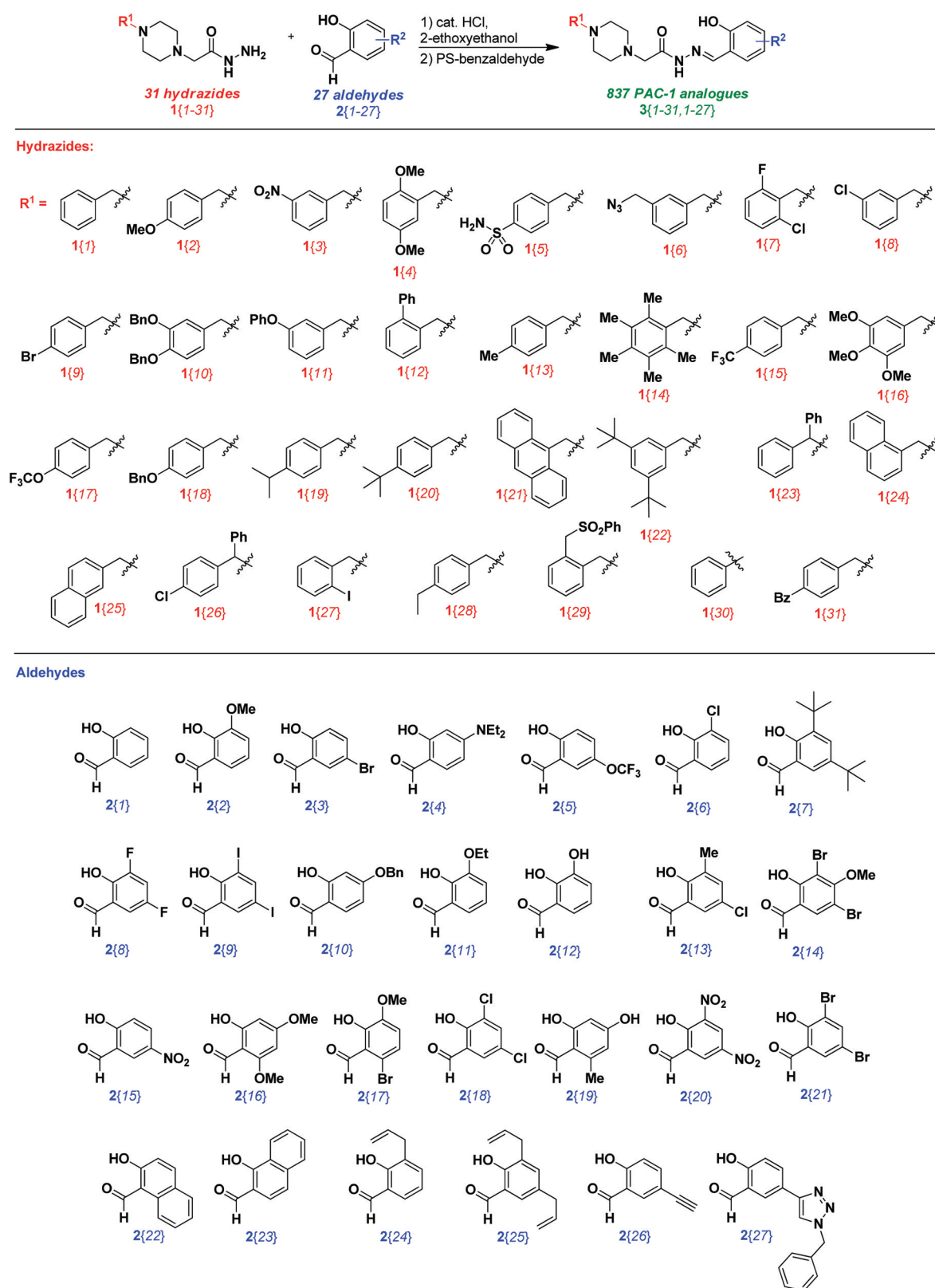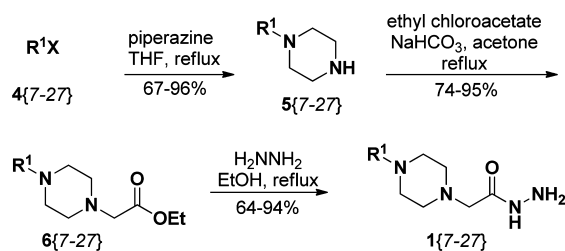**Figure 2.** Hydrazides and aldehydes used to construct 837-membered combinatorial library of **PAC-1** analogues.

Cell Death Induction and Relief of Zinc-Mediated Inhibition of Procaspase-3 by Hit Compounds. After resynthesis of the hits (**3**{2,7}, **3**{4,7}, **3**{18,7}, **3**{20,24}, **3**{25,7}, and **3**{28,7}), analytically pure samples of the compounds were evaluated in further biological assays. These structures and the biological results are shown in Table 1. The compounds were evaluated, at a range of concentrations, for their ability to induce cell death in U-937 cells. All six of these hits were found to be two- to four-fold more potent in cell culture than **PAC-1** and **S-PAC-1** in a 72-h treatment.

Scheme 1. Synthesis of Hydrazides 1{7−27}

Scheme 3. Synthesis of Aldehyde 2{27}
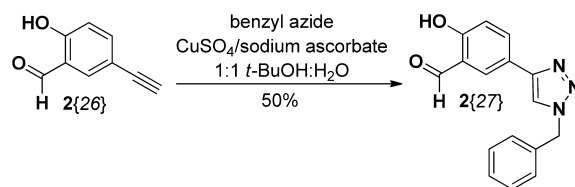
iodide positive). The novel analogues were found to be more potent than **PAC-1** under these 24 h conditions.

The six confirmed hits were then evaluated in vitro for their ability to relieve zinc-mediated inhibition of procaspase-3 (Table 1 and Figure 4). In this experiment, procaspase-3 was incubated with $ZnSO_4$, conditions under which procaspase-3 has no enzymatic activity.[18,20] All compounds were able to enhance procaspase-3 enzymatic activity under these conditions (as assessed by the cleavage of the colorimetric caspase-3 substrate Ac-DEVD-pNA, synthesized as previously reported[27]), and five of the six hit compounds showed greater activity than **PAC-1** in

In a second experiment, flow cytometry analysis with Annexin V-FITC/propidium iodide staining was performed on U-937 cells that were exposed to the compounds at a single concentration (7.5 μM) for 24 h (Table 1 and Figure 3). As demonstrated by the histograms in Figure 3, within 24 h the majority of the compound treated cells were undergoing apoptosis (cells in the lower right quadrant of the histogram − Annexin V positive, propidium iodide negative), or were in a late apoptotic/necrotic stage (upper right quadrant − Annexin V positive, propidium

Scheme 2. Synthesis of Hydrazides 1{28−31}

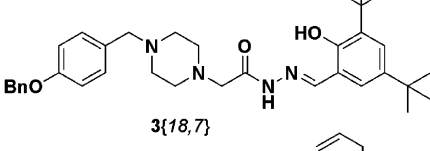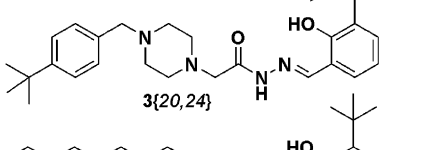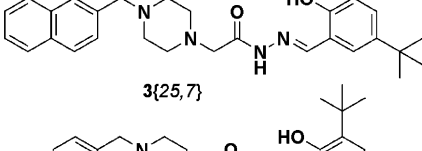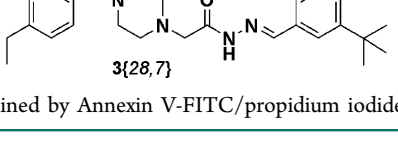**Table 1. Six Library Compounds Induce Potent Cell Death of U-937 Cells (Human Lymphoma) in both 24- h[a] and 72 h[b] Experiments and Enhance Enzymatic Activity of Procaspase-3 in Vitro**

Compound	72-hour IC$_{50}$ (μM)	% Cytotoxicity (24 hours at 7.5 μM)	Procaspase-3 (% Activity at 25 μM)
PAC-1	3.8 ± 0.4	21	42 ± 1.8
S-PAC-1	4.4 ± 0.7	23	4 ± 0.6
3{2,7}	1.8 ± 0.2	90	53 ± 4.1
3{4,7}	1.6 ± 0.2	53	64 ± 2.5
3{18,7}	1.4 ± 0.2	97	36 ± 1.6
3{20,24}	0.9 ± 0.03	83	82 ± 2.4
3{25,7}	1.0 ± 0.04	50	69 ± 5.3
3{28,7}	2.0 ± 0.2	70	60 ± 2.4

[a]Cell death determined by Annexin V-FITC/propidium iodide staining. [b]Biomass quantified using the sulforhodamine B assay.

this assay. These data indicate that the compounds enhance the activity of procaspase-3 in vitro through chelation of inhibitory zinc, and suggest that in the cell the compounds chelate zinc from the labile pool, allowing procaspase-3 to be processed to active caspase-3, leading to apoptotic cell death.

The direct modulation of apoptotic proteins is an attractive anticancer strategy, and many such compounds are advancing through clinical trials. **PAC-1** and its derivative **S-PAC-1**, which chelate labile cellular zinc and induce apoptosis in cancer cells, have shown promise in various preclinical antitumor models. However, derivatives that induce cell death more rapidly and more potently could be even more attractive as experimental therapeutics. Using parallel synthesis and guided by the known SAR, we constructed 837 **PAC-1** analogues and evaluated them for their cell death inducing properties. The six compounds shown in Table 1 emerged from this effort; these compounds are two- to four-fold more potent than **PAC-1** at induction of cancer cell death in both 24-h and 72-h assays.

Given the general hydrophobicity of the hit compounds relative to **PAC-1**, it is possible that this enhanced potency and enhanced rate of cell death is driven by enhanced cell permeability. These qualities are likely to be advantageous as the compounds are moved forward in vivo. In addition, it is possible that other members of this library will emerge as viable in vivo candidates as alternate properties (such as propensity to cross the blood-brain barrier, improved metabolic stability, improved solubility/ formulation for in vivo studies, etc.) are examined. Thus, this library of 837 compounds will be a rich source from which to develop next-generation procaspase-3 activating compounds.

■ EXPERIMENTAL PROCEDURES

General Procedure for the Synthesis of PAC-1 Analogues. To a 16 × 150 mm test tube were added hydrazide (1.7 equiv.), aldehyde (1.0 equiv.), 2-ethoxyethanol (1 mL), and 1.2 M HCl (10 mol %). The tubes were shaken on a Büchi Syncore parallel synthesizer at 110 °C until all aldehyde
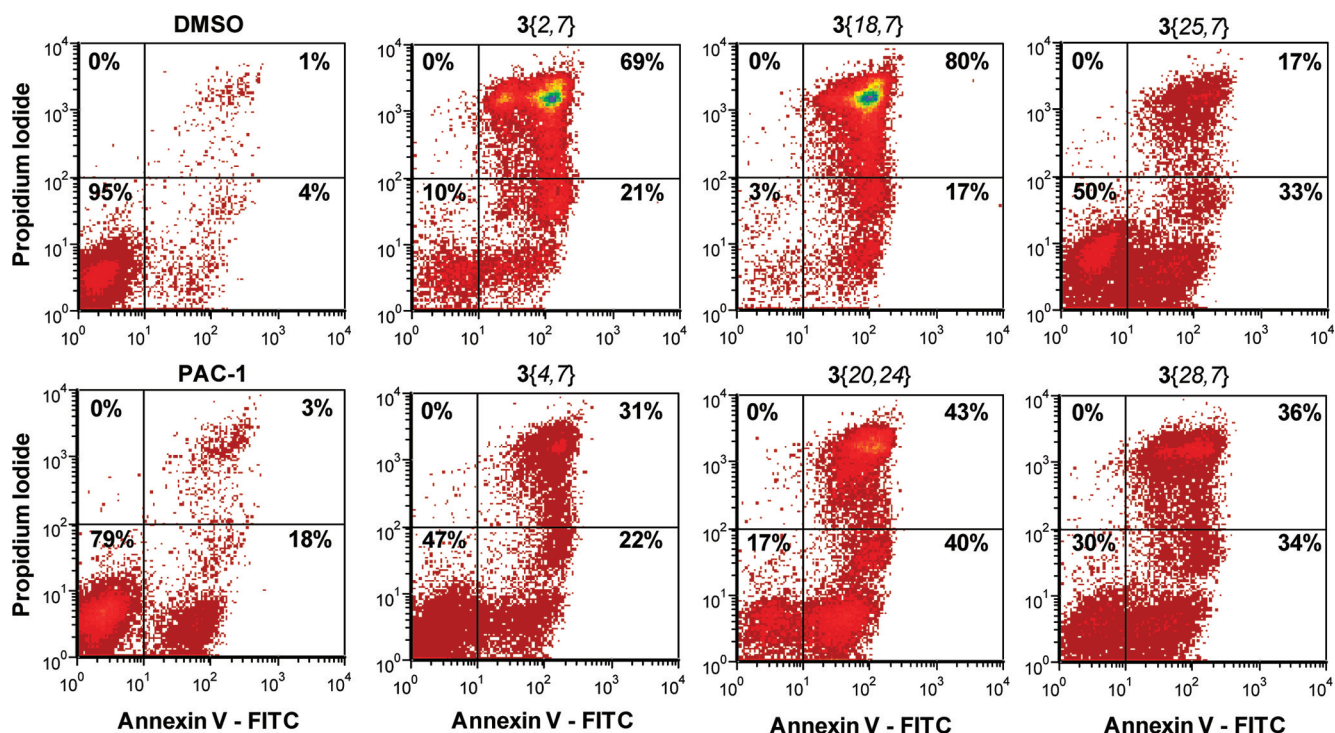
Figure 3. Annexin V-FITC/propidium iodide staining of U-937 cells treated with 7.5 μM of each compound for 24 h.

had reacted (monitoring by ESI-MS). The reaction mixture was cooled to room temperature, and polystyrene-benzaldehyde (3.5 equiv.) was added. The reaction mixture was shaken at 25−80 °C until no hydrazide remained (monitoring by ESI-MS). The reaction mixture was cooled to room temperature, and the resin was filtered and washed with 2-ethoxyethanol. The filtrate was dried under high vacuum to afford the **PAC-1** analogue.
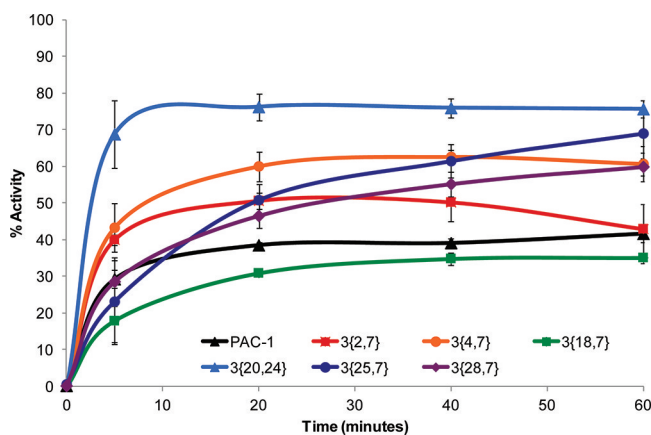

Figure 4. Relief of zinc-mediated inhibition of 1 μM procaspase-3 by **PAC-1** and hit compounds, as measured by the processing of the Ac-DEVD-pNA substrate. Compounds were tested at a concentration of 25 μM with 3.5 μM ZnSO$_4$ and normalized to a DMSO-treated control (0%) and a zinc-free control (100%), and substrate cleavage was monitored at 405 nm.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Detailed experimental procedures for the synthesis of hydrazides **1**{*7−31*}, aldehyde **2**{*27*}, and **PAC-1** analogues **3**{*2,7*}, **3**{*4,7*}, **3**{*18,7*}, **3**{*20,24*}, **3**{*25,7*}, and **3**{*28,7*}; purity

and mass spectral data for **3**{*1−31,1−27*}; characterization data and copies of ^{1}H NMR, ^{13}C NMR, and ^{19}F-NMR of all new compounds; and full biological protocols. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author
*E-mail: hergenro@uiuc.edu.
Author Contributions
D.C.H. and H.S.R. contributed equally to this work. D.C.H., H.S.R., D.C.W., R.C.B., and P.J.H. designed the research plan. D.C.H., H.S.R., C.J.N., and S.C.S. synthesized the library building blocks. D.C.H., H.S.R., and D.C.W. synthesized the combinatorial library. D.C.H., D.C.W., and R.C.B. screened the library for cytotoxicity. R.C.B. performed secondary biological assays. H.S.R. and P.J.H. prepared the manuscript and Supporting Information.

■ REFERENCES

(1) Hengartner, M. O. The biochemistry of apoptosis. *Nature* **2000**, *407*, 770−776.

(2) Elmore, S. Apoptosis: A review of programmed cell death. *Toxicol. Pathol.* **2007**, *35*, 495−516.

(3) (a) Nicholson, D. W. Caspase structure, proteolytic substrates, and function during apoptotic cell death. *Cell Death Differ.* **1999**, *6*, 1028−1042. (b) Earnshaw, W. C.; Martins, L. M.; Kaufmann, S. H. Mammalian caspases: Structure, activation, substrates, and functions during apoptosis. *Annu. Rev. Biochem.* **1999**, *68*, 383−424. (c) Slee, E. A.;

Adrain, C.; Martin, S. J. Executioner caspase-3, -6, and -7 perform distinct, non-redundant roles during the demolition phase of apoptosis. *J. Biol. Chem.* **2001**, *276*, 7320−6. (d) Sakahira, H.; Enari, M.; Nagata, S. Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. *Nature* **1998**, *391*, 96−99.

(4) (a) Hanahan, D.; Weinberg, R. A. The hallmarks of cancer. *Cell* **2000**, *100*, 57−70. (b) Hanahan, D.; Weinberg, R. A. Hallmarks of cancer: the next generation. *Cell* **2011**, *144*, 646−74.

(5) Vassilev, L. T.; Vu, B. T.; Graves, B.; Carvajal, D.; Podlaski, F.; Filipovic, Z.; Kong, N.; Kammlott, U.; Lukacs, C.; Klein, C.; Fotouhi, N.; Liu, E. A. In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. *Science* **2004**, *303*, 844−8.

(6) Oltersdorf, T.; Elmore, S. W.; Shoemaker, A. R.; Armstrong, R. C.; Augeri, D. J.; Belli, B. A.; Bruncko, M.; Deckwerth, T. L.; Dinges, J.; Hajduk, P. J.; Joseph, M. K.; Kitada, S.; Korsmeyer, S. J.; Kunzer, A. R.; Letai, A.; Li, C.; Mitten, M. J.; Nettesheim, D. G.; Ng, S.; Nimmer, P. M.; O'Connor, J. M.; Oleksijew, A.; Petros, A. M.; Reed, J. C.; Shen, W.; Tahir, S. K.; Thompson, C. B.; Tomaselli, K. J.; Wang, B. L.; Wendt, M. D.; Zhang, H. C.; Fesik, S. W.; Rosenberg, S. H. An inhibitor of Bcl-2 family proteins induces regression of solid tumours. *Nature* **2005**, *435*, 677−681.

(7) Lu, J.; Bai, L.; Sun, H.; Nikolovska-Coleska, Z.; McEachern, D.; Qiu, S.; Miller, R. S.; Yi, H.; Shangary, S.; Sun, Y.; Meagher, J. L.; Stuckey, J. A.; Wang, S. SM-164: a novel, bivalent Smac mimetic that induces apoptosis and tumor regression by concurrent removal of the blockade of cIAP-1/2 and XIAP. *Cancer Res.* **2008**, *68*, 9384−93.

(8) Putt, K. S.; Chen, G. W.; Pearson, J. M.; Sandhorst, J. S.; Hoagland, M. S.; Kwon, J. T.; Hwang, S. K.; Jin, H.; Churchwell, M. I.; Cho, M. H.; Doerge, D. R.; Helferich, W. G.; Hergenrother, P. J. Small-molecule activation of procaspase-3 to caspase-3 as a personalized anticancer strategy. *Nat. Chem. Biol.* **2006**, *2*, 543−550.

(9) Soini, Y.; Paakko, P. Apoptosis and expression of caspases 3, 6 and 8 in malignant non-Hodgkin's lymphomas. *APMIS* **1999**, *107*, 1043−50.

(10) Estrov, Z.; Thall, P. F.; Talpaz, M.; Estey, E. H.; Kantarjian, H. M.; Andreeff, M.; Harris, D.; Van, Q.; Walterscheid, M.; Kornblau, S. M. Caspase 2 and caspase 3 protein levels as predictors of survival in acute myelogenous leukemia. *Blood* **1998**, *92*, 3090−7.

(11) Fink, D.; Schlagbauer-Wadl, H.; Selzer, E.; Lucas, T.; Wolff, K.; Pehamberger, H.; Eichler, H. G.; Jansen, B. Elevated procaspase levels in human melanoma. *Melanoma Res.* **2001**, *11*, 385−93.

(12) Virkajarvi, N.; Paakko, P.; Soini, Y. Apoptotic index and apoptosis influencing proteins bcl-2, mcl-1, bax and caspases 3, 6 and 8 in pancreatic carcinoma. *Histopathology* **1998**, *33*, 432−9.

(13) Persad, R.; Liu, C.; Wu, T. T.; Houlihan, P. S.; Hamilton, S. R.; Diehl, A. M.; Rashid, A. Overexpression of caspase-3 in hepatocellular carcinomas. *Mod. Pathol.* **2004**, *17*, 861−7.

(14) (a) Tormanen-Napankangas, U.; Soini, Y.; Kahlos, K.; Kinnula, V.; Paakko, P. Expression of caspases-3, −6 and −8 and their relation to apoptosis in non-small cell lung carcinoma. *Int. J. Cancer* **2001**, *93*, 192−8. (b) Krepela, E.; Prochazka, J.; Liul, X.; Fiala, P.; Kinkor, Z. Increased expression of Apaf-1 and procaspase-3 and the functionality of intrinsic apoptosis apparatus in non-small cell lung carcinoma. *Biol. Chem.* **2004**, *385*, 153−68.

(15) (a) O'Donovan, N.; Crown, J.; Stunell, H.; Hill, A. D.; McDermott, E.; O'Higgins, N.; Duffy, M. J. Caspase 3 in breast cancer. *Clin. Cancer Res.* **2003**, *9*, 738−42. (b) Zapata, J. M.; Krajewska, M.; Krajewski, S.; Huang, R. P.; Takayama, S.; Wang, H. G.; Adamson, E.; Reed, J. C. Expression of multiple apoptosis-regulatory genes in human breast cancer cell lines and primary tumors. *Breast Cancer Res. Treat.* **1998**, *47*, 129−40.

(16) Roy, S.; Bayly, C. I.; Gareau, Y.; Houtzager, V. M.; Kargman, S.; Keen, S. L.; Rowland, K.; Seiden, I. M.; Thornberry, N. A.; Nicholson, D. W. Maintenance of caspase-3 proenzyme dormancy by an intrinsic "safety catch" regulatory tripeptide. *Proc. Natl. Acad. Sci. U. S. A.* **2001**, *98*, 6132−7.

(17) Wolan, D. W.; Zorn, J. A.; Gray, D. C.; Wells, J. A. Small-Molecule Activators of a Proenzyme. *Science* **2009**, *326*, 853−858.

(18) Peterson, Q. P.; Hsu, D. C.; Goode, D. R.; Novotny, C. J.; Totten, R. K.; Hergenrother, P. J. Procaspase-3 activation as an anti-cancer strategy: Structure−activity relationship of procaspase-activating compound 1 (PAC-1) and its cellular co-localization with caspase-3. *J. Med. Chem.* **2009**, *52*, 5721−5731.

(19) Charkoudian, L. K.; Pham, D. M.; Franz, K. J. A pro-chelator triggered by hydrogen peroxide inhibits iron-promoted hydroxyl radical formation. *J. Am. Chem. Soc.* **2006**, *128*, 12424−5.

(20) Peterson, Q. P.; Goode, D. R.; West, D. C.; Ramsey, K. N.; Lee, J. J. Y.; Hergenrother, P. J. PAC-1 activates procaspase-3 in vitro through relief of zinc-mediated inhibition. *J. Mol. Biol.* **2009**, *388*, 144−158.

(21) Truong-Tran, A. Q.; Grosser, D.; Ruffin, R. E.; Murgia, C.; Zalewski, P. D. Apoptosis in the normal and inflamed airway epithelium: role of zinc in epithelial protection and procaspase-3 regulation. *Biochem. Pharmacol.* **2003**, *66*, 1459−1468.

(22) Lucas, P. W.; Schmit, J. M.; Peterson, Q. P.; West, D. C.; Hsu, D. C.; Novotny, C. J.; Dirikolu, L.; Churchwell, M. I.; Doerge, D. R.; Garrett, L. D.; Hergenrother, P. J.; Fan, T. M. Pharmacokinetics and derivation of an anticancer dosing regimen for PAC-1, a preferential small molecule activator of procaspase-3, in healthy dogs. *Invest. New Drugs* **2011**, *29*, 901−11.

(23) Peterson, Q. P.; Hsu, D. C.; Novotny, C. J.; West, D. C.; Kim, D.; Schmit, J. M.; Dirikolu, L.; Hergenrother, P. J.; Fan, T. M. Discovery and canine preclinical assessment of a nontoxic procaspase-3-activating compound. *Cancer Res.* **2010**, *70*, 7232−41.

(24) Hergenrother, P. J.; Putt, K. S.; Sandhorst, J. S.; Peterson, Q. P.; Fako, V. Compositions and methods including cell death inducers and procaspase activation. International Patent Application WO 2008/134474.

(25) Miller, C. E. Hydrogenation with diimide. *J. Chem. Educ.* **1965**, *42*, 254−&.

(26) Chang, K. H.; Huang, C. C.; Liu, Y. H.; Hu, Y. H.; Chou, P. T.; Lin, Y. C. Synthesis of photo-luminescent Zn(II) Schiff base complexes and its derivative containing Pd(II) moiety. *Dalton Trans.* **2004**, 1731−8.

(27) Peterson, Q. P.; Goode, D. R.; West, D. C.; Botham, R. C.; Hergenrother, P. J. Preparation of the caspase-3/7 substrate Ac-DEVD-pNA by solution-phase peptide synthesis. *Nat. Protoc.* **2010**, *5*, 294−302.