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

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A goal of personalized medicine as applied to oncology is to identify compounds that exploit a defined molecular defect in a cancerous cell. A compound called procaspase-activating compound 1 (**PAC-1**) was reported that enhances the activity of procaspase-3 in vitro and induces apoptotic death in cancer cells in culture and in mouse xenograft models. Experimental evidence indicates that **PAC-1** activates procaspase-3 in vitro through chelation of inhibitory zinc ions. Described herein is the synthesis and biological activity of a family of **PAC-1** derivatives where key functional groups have been systematically altered. Analysis of these compounds reveals a strong correlation between the in vitro procaspase-3 activating effect and their ability to induce death in cancer cells in culture. Importantly, we also show that a fluorescently labeled version of **PAC-1** co-localizes with sites of caspase-3 activity in cancer cells. The data presented herein further bolster the hypothesis that **PAC-1** induces apoptosis in cancer cells through the direct activation of procaspase-3, has implications for the design and discovery of next-generation procaspase-3 activating compounds, and sheds light on the anti-apoptotic role of cellular zinc.

Introduction

Cancer recently overtook heart disease as the number one killer in the United States for individuals under 85 years of age.[1] Despite intensive research efforts toward targeted anticancer therapies, most modern chemotherapeutic regimens still rely on general cytotoxins that indiscriminately induce death in rapidly dividing cell types.[2] As resistance to apoptosis is a hallmark of cancer,[3−5] an intriguing approach to anticancer drug discovery is the reactivation of apoptotic cascades in cancer cells.[6] Indeed, burgeoning interest in apoptotic proteins as anticancer targets has led to the discovery of several promising classes of small molecules,[6−9] including those that bind Bcl-2,[10] MDM2,[11,12] and XIAP,[13,14] and those that suppress the expression of survivin.[15,16]

The caspase enzymes are cysteine proteases that both initiate and execute the apoptotic program.[17,18] The caspases are produced as low activity zymogens (proenzymes) that can be activated by various mechanisms.[19,20] For example, procaspase-9 is converted to caspase-9 through the formation of a multimeric protein complex called the apoptosome,[21] while procaspase-8 is converted to caspase-8 through a proximity model.[22,23] Active caspase-9 and caspase-8 can in turn directly proteolyze procaspase-3 and procaspase-7, forming active caspase-3 and caspase-7.[24,25] Caspase-3 is the key executioner caspase in the apoptotic cascade, and once activated, this enzyme cleaves scores of cellular substrates, committing the cell to apoptotic death.[26,27] On the basis of mouse knockout studies and other work, procaspase-7/caspase-7 may have a less important function relative to procaspase-3/caspase-3 in the demolition phase of apoptosis, although caspase-7 likely has some distinct cellular substrates.[28,29] We reported

the identification of a small molecule, called **PAC-1**[a] (compound **1** in Figure 1), which enhances the activity of procaspase-3 in vitro and induces apoptotic death in cancer cell lines and cells from primary tumor isolates.[30] **PAC-1** also showed efficacy in mouse models of lung and renal cancer and thus has promise as an anticancer agent.[30] **PAC-1** is the first small molecule discovered that induces the in vitro autoactivation of a proenzyme protease and as such could serve as a prototype for other protease activating compounds.

PAC-1 derivatives lacking the phenolic hydroxyl do not activate procaspase-3 in vitro and do not induce the death of cancer cells in culture.[30] This information, combined with the known tendency of *ortho*-hydroxy *N*-acyl hydrazones to bind metal ions,[31] led us to suspect metal ion binding as part of the mechanism of action of **PAC-1**. As caspase-3[32] and procaspase-3[33] are known to be inhibited by zinc, we focused on zinc and recently determined that **PAC-1** binds to zinc in a 1:1 stoichiometry with a dissociation constant of 52 nM.[33] In addition, we discovered that **PAC-1** enhances the activity of procaspase-3, caspase-3, procaspase-7, and caspase-7 in vitro through the sequestration of inhibitory zinc ions.[33] Described herein is the use of derivative synthesis and biochemical analysis to determine the exact **PAC-1** functional groups responsible for caspase activation, zinc chelation, and cell death. Importantly, the results of these experiments show a strong correlation between the ability of a compound to bind zinc, activate caspase-3 in vitro, and induce death in cancer cells in culture. On the basis of this structure−activity relationship, a novel fluorescent derivative of **PAC-1** was designed

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and synthesized. Subsequent confocal microscopy experiments indicate that fluorescently labeled **PAC-1** colocalizes with sites of caspase-3 activity in the cell. Overall, the results described herein support the hypothesis that **PAC-1** induces death in cancer cells through direct activation of procaspase-3 via the sequestration of inhibitory zinc ions, implicate zinc as an endogenous antiapoptotic agent, and suggest a shared mode of action for certain zinc-chelating anticancer compounds.

Results

Design and Synthesis of PAC-1 Derivatives. In an effort to fully define the structural requirements of PAC-1 for
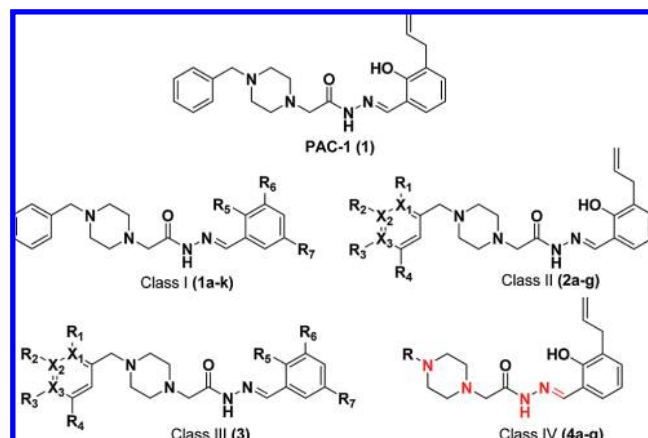

Figure 1. **PAC-1** and four classes of **PAC-1** derivatives. Class I compounds (**1a**−**k**) have modifications on the phenolic ring, Class II compounds (**2a**−**g**) have modification on the benzyl ring, Class III compounds (**3**) have modifications on both the phenolic and benzyl rings, and Class IV compounds (**4a**−**g**) have modifications to the key heteroatoms and bonds indicated in red.

procaspase-3/caspase-3 activation and cell death induction, we designed a series of derivatives with systematically altered functional groups that would allow a detailed structure−activity relationship (SAR) for **PAC-1** to be constructed. This SAR would in turn allow the design of a fluorescent version of **PAC-1** that could be used to determine the subcellular localization of this compound and its ability to co-localize with sites of caspase-3 activity in the cell. Thus, the twin goals of the SAR study were the determination of functional groups important for the biological activity of **PAC-1** and the discovery of regions on the **PAC-1** scaffold that could be altered with minimal loss in activity.

Four classes of **PAC-1** derivatives were envisioned (Figure 1): alterations on the phenolic ring (Class I, **1a**−**k**), on the benzyl ring (Class II, **2a**−**g**), simultaneous modification of both the benzyl and phenolic rings (Class III, **3**), and alterations to the **PAC-1** core (Class IV, **4a**−**g**, with modifications of the indicated atoms and bonds made in red). The majority of the Class I−III compounds were synthesized as shown in Scheme 1, via condensation of the appropriate hydrazides (**5**−**12**) and aldehydes (**13**−**23**). As shown in Scheme 1, yields for this condensation were between 72 and 95%, the exception being **1g**, which spontaneously forms a disulfide dimer, **1i**, resulting in a diminished yield (Scheme 2). Through such condensation reactions, we constructed 11 Class I derivatives (**1a**−**k**), 7 Class II derivatives (**2a**−**g**), and 1 Class III derivative (**3**). The hydrazide building blocks in Scheme 1 were synthesized through the reaction of various benzyl chlorides with piperazine, alkylation of the resulting products with ethyl chloroacetate, followed by reaction of the resulting ester with hydrazine (see Supporting Information for details). The aldehyde building blocks were either purchased or synthesized from simple starting materials (see Supporting Information for details).

Three Class IV derivatives could also be synthesized through analogous condensation reactions. Thus, compounds lacking

Scheme 1. Condensation of Hydrazides and Aldehydes to Create **PAC-1** Derivatives of Classes I−III

Hydrazide	Aldehyde	Product	Yield
5. $R_1, R_2, R_3, R_4 = H, X_1, X_2, X_3 = C$	**13.** $R_5 = OH, R_6 = Allyl, R_7 = H$	**1**	95%
5. $R_1, R_2, R_3, R_4 = H, X_1, X_2, X_3 = C$	**14.** $R_5 = OH, R_6, R_7 = H$	**1a**	72%
5. $R_1, R_2, R_3, R_4 = H, X_1, X_2, X_3 = C$	**15.** $R_5 = MeS, R_6, R_7 = H$	**1b**	88%
5. $R_1, R_2, R_3, R_4 = H, X_1, X_2, X_3 = C$	**16.** $R_5 = NH_2, R_6, R_7 = H$	**1c**	78%
5. $R_1, R_2, R_3, R_4 = H, X_1, X_2, X_3 = C$	**17.** $R_5 = COOH, R_6, R_7 = H$	**1d**	79%
5. $R_1, R_2, R_3, R_4 = H, X_1, X_2, X_3 = C$	**18.** $R_5 = CO_2Me, R_6, R_7 = H$	**1e**	72%
5. $R_1, R_2, R_3, R_4 = H, X_1, X_2, X_3 = C$	**19.** $R_5 = Cl, R_6, R_7 = H$	**1f**	94%
5. $R_1, R_2, R_3, R_4 = H, X_1, X_2, X_3 = C$	**20.** $R_5 = SH, R_6, R_7 = H$	**1g**	18%
5. $R_1, R_2, R_3, R_4 = H, X_1, X_2, X_3 = C$	**21.** $R_5, R_6, R_7 = H$	**1h**	75%
5. $R_1, R_2, R_3, R_4 = H, X_1, X_2, X_3 = C$	**22.** $R_5 = OH, R_6 = Allyl, R_7 = Allyl$	**1j**	93%
5. $R_1, R_2, R_3, R_4 = H, X_1, X_2, X_3 = C$	**23.** $R_5 = OMe, R_6 = Allyl, R_7 = H$	**1k**	85%
6. $R_1, R_2, R_3, R_4 = H, X_1 = N, X_2, X_3 = C$	**13.** $R_5 = OH, R_6 = Allyl, R_7 = H$	**2a**	83%
7. $R_1, R_2, R_3, R_4 = H, X_1 = C, X_2 = N, X_3 = C$	**13.** $R_5 = OH, R_6 = Allyl, R_7 = H$	**2b**	88%
8. $R_1, R_2, R_3, R_4 = H, X_1, X_2 = C, X_3 = N$	**13.** $R_5 = OH, R_6 = Allyl, R_7 = H$	**2c**	90%
9. $R_1, R_2 = H, R_3 = F, R_4 = H, X_1, X_2, X_3 = C$	**13.** $R_5 = OH, R_6 = Allyl, R_7 = H$	**2d**	--[a]
10. $R_1 = OMe, R_2, R_3 = H, R_4 = OMe, X_1, X_2, X_3 = C$	**13.** $R_5 = OH, R_6 = Allyl, R_7 = H$	**2e**	80%
11. $R_1, R_2 = H, R_3 = OMe, R_4 = H, X_1, X_2, X_3 = C$	**13.** $R_5 = OH, R_6 = Allyl, R_7 = H$	**2f**	81%
12. $R_1 = H, R_2 = NO_2, R_3, R_4 = H, X_1, X_2, X_3 = C$	**13.** $R_5 = OH, R_6 = Allyl, R_7 = H$	**2g**	74%
10. $R_1 = OMe, R_2, R_3 = H, R_4 = OMe, X_1, X_2, X_3 = C$	**14.** $R_5 = OH, R_6, R_7 = H$	**3**	82%

[a] See Supporting Information for the optimized synthetic route of **2d**.

Scheme 2. Condensation of Hydrazide **5** and Aldehyde **20** Provides **1g** and Its Dimer, **1i**
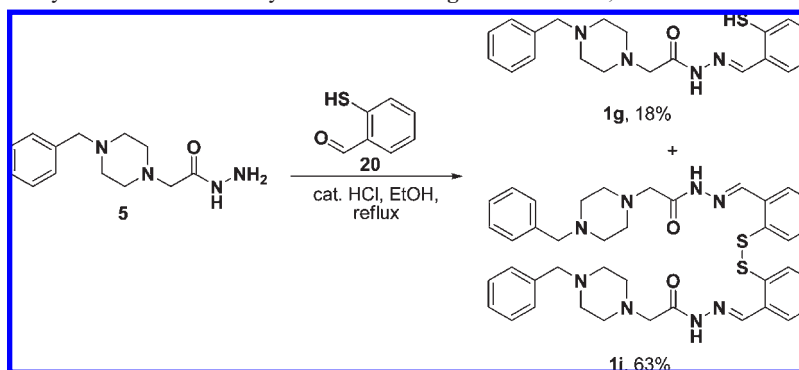
Scheme 3. Syntheses of Class IV Derivatives
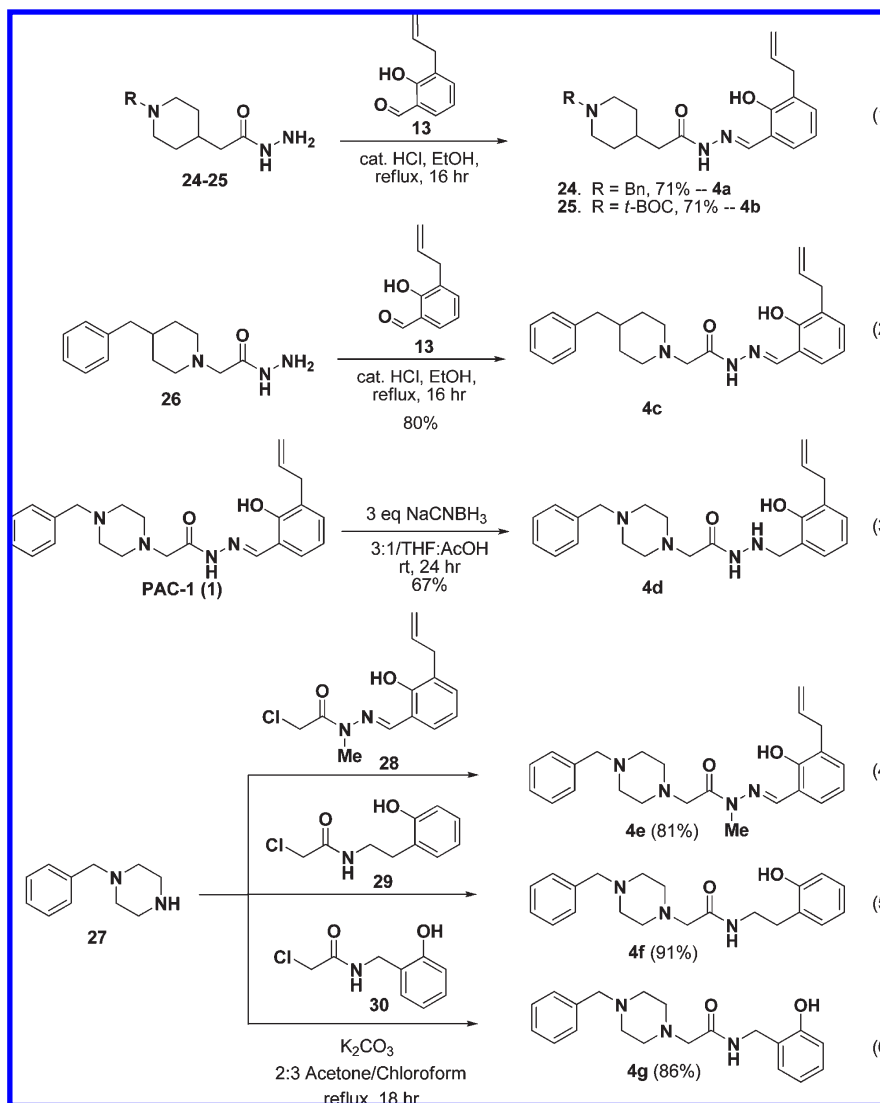
one of two piperazine nitrogens (**4a**−**c**) were synthesized from the corresponding hydrazides and aldehyde as shown in Scheme 3, eqs 1 and 2. However, a number of the derivatives in Class IV could not be synthesized via analogous condensation reactions and required alternative synthetic routes. Thus, compound **4d** was synthesized by reduction of the hydrazone in **PAC-1** using NaCNBH$_3$ (Scheme 3, eq 3). Compounds **4e**−**g** were synthesized through the reaction

of **27** with the appropriate chloride (**28**−**30**) (Scheme 3, eqs 4−6).

Evaluation of PAC-1 Derivatives. With 26 derivatives of **PAC-1** in hand, all compounds were evaluated for their ability to induce death in cancer cells, to relieve zinc-mediated inhibition of caspase-3 in vitro, and to bind zinc. Assessment of all compounds in these three assays provides a comprehensive data set used to formulate a structure−activity

relationship for **PAC-1**; brief descriptions of these assays are provided below.

The induction of cell death was determined using the U-937 (human lymphoma) cell line. U-937 cells grow in culture as a suspension, and previous studies with **PAC-1** have been conducted with these cells;[30,33] thus, they provide a convenient benchmark for the assessment of **PAC-1** analogues. For these experiments, cells were exposed to a range of compound concentrations for 72 h, cell death was quantitated via a sulforhodamine B assay,[34] and IC_{50} values were calculated from logistical dose−response curves.

We had previously shown that zinc inhibits both active caspase-3 and procaspase-3, and that **PAC-1** is able to enhance the activity of both of these enzymes.[33] Because of the low catalytic activity of procaspase-3 (∼200-fold less active than caspase-3),[33,35] we used the activation of caspase-3 as the method to evaluate and compare **PAC-1** and its derivatives. Therefore, all 27 compounds (**PAC-1** and its 26 derivatives) were tested at multiple concentrations with caspase-3 and zinc to determine their ability to relieve zinc-mediated inhibition of caspase-3 in vitro. The percent activation of caspase-3 at a compound concentration of 10 μM, the concentration at which most compounds showed maximal activity, is reported in Table 1. For these enzyme activation assays, all buffers were first treated with Chelex resin to remove divalent metal cations.

The affinity of each compound for zinc was determined through the use of an EGTA titration according to the method of Zhu and co-workers.[36] For these experiments, **PAC-1** or derivatives were added in a Hepes buffer containing a large excess of EGTA, followed by incremental addition of zinc, and the fluorescence of the small molecule−zinc complex was read (excitation at 410 nm; emission at 475 nm). The resulting increase of fluorescence intensity was plotted against the calculated free zinc concentration in accord with similar protocols.[36] This plot constitutes a formation curve from which binding constants were calculated.

The results of these cell death, caspase-3 activation, and zinc binding experiments are shown in Table 1 (see Supporting Information Figures S1−S3 for individual data sets). As shown, there is a strong correlation between the ability of compounds to activate caspase-3 in vitro, bind zinc, and induce death in cancer cells in culture. Universally, compounds that do not activate caspase-3 in vitro do not bind zinc or induce significant death of the cancer cells in culture. For example, removal or alkylation of the *ortho*-hydroxyl (compounds **1h** and **1k**) results in compounds that do not bind zinc, activate caspase-3, or kill cells to any large degree. Likewise, replacement of this −OH with a thioether (**1b**), amine (**1c**), carboxylic acid (**1d**), methyl ester (**1e**), chloride (**1f**), or sulfhydryl (**1g**) results in compounds that are basically inactive in all three assays. However, if the *ortho*-hydroxyl is maintained, a variety of substitution patterns are tolerated on the phenolic ring. For example, (**1j**) contains allyl groups at two positions on this ring and is a potent caspase-3 activator, zinc binder, and cell death inducer, while the compound lacking any allyl groups (**1a**) is also reasonably potent in these assays.

The derivatives in which the nature of the benzyl ring was altered (Class II derivatives, **2a−g**) were also assessed. As shown in Table 1, these compounds universally retained the ability to induce death in U-937 cells, to activate caspase-3 in vitro, and bind zinc. The one Class III derivative (**3**) was also active in all three assays.

Compounds in Class IV were constructed to systematically probe the importance of the *ortho*-hydroxy *N*-acylhydrazone motif and the piperazine nitrogens on biological activity. Specifically, compounds were created in which the piperazine nitrogens were replaced with CH (compounds **4a−c**), that have the C−N double bond reduced (**4d**), that have an *N*-methylation (**4e**), and that have the hydrazone linkage replaced with an amide (**4f−g**). Assays reveal that all compounds with alterations to the *N*-acylhydrazone motif were inactive. No zinc binding could be detected for compounds **4d−g**; these four compounds also have no/low capacity for caspase-3 activation in vitro, and they do not strongly induce death in U-937 cells in culture (Table 1). In contrast, compound **4c**, in which one of the piperazine nitrogens is changed to a CH, showed cell death induction, caspase-3 activation, and zinc binding activity almost identical to those of **PAC-1**.

Clear SAR trends are thus apparent from the data in Table 1: (1) **PAC-1** derivatives that are unable to bind zinc do not activate caspase-3 in vitro, and do not appreciably induce death in U-937 cells in culture. This information suggests that the zinc binding capacity of **PAC-1** is important for its cell death-inducing properties. (2) The *ortho*-hydroxy *N*-acylhydrazone motif is critical for zinc binding. (3) Virtually all compounds that bind zinc activate caspase-3 in vitro and induce death in U-937 cells in culture. Only compounds **4a** and **4b**, which show no in vitro caspase-3 activation but are still cytotoxic to U-937 cells, are exceptions to this trend. Interestingly, we find that these two compounds (at a concentration of 10 μM) are reasonably potent inhibitors of caspase-3 enzymatic activity; thus, any activating effect of the compound may be masked by this inhibition (see Supporting Information, Figure S4). This inhibition effect is consistent with previous data showing that **PAC-1** will also inhibit procaspase-3/caspase-3 at high compound concentrations.[33]

Design, Synthesis, and Biochemical Characterization of a Fluorescent Version of PAC-1. On the basis of the information gleaned from the SAR study, above, a fluorescent version of **PAC-1** was designed that was predicted to retain the ability to bind zinc, activate caspase-3, and induce cell death. Specifically, as the SAR indicates that modifications on the benzyl ring are well-tolerated, a modified benzyl ring was used to conjugate **PAC-1** with a fluorophore.

To ensure orthogonal fluorescent signals in our proposed colocalization experiments, we choose a fluorophore that we could excite at 350 nm to conjugate to **PAC-1**, and of the possible dyes in this category, Alexa Fluor 350 was selected. To synthesize the fluorophore-conjugated **PAC-1**, Alexa Fluor 350 (activated as its *N*-hydroxysuccinimide (NHS) ester), **31**, was first treated with amine **32** to create alkynyl-Alexa Fluor 350 (compound **33**, Scheme 4). The Cu(I)-catalyzed variant of the Huisgen [3 + 2] cycloaddition reaction[37] was then used to link azido-PAC-1 (compound **34**, whose synthesis is described in the Supporting Information) with **33** to form **2h** (Alexa Fluor 350-conjugated PAC-1 or **AF350-PAC-1**, Scheme 4). Compound **2h** was characterized by HRMS and ^{1}H NMR (see Supporting Information). Once in hand, **2h** was evaluated for its ability to induce death in U-937 cells in culture, activate caspase-3 in vitro, and bind zinc. Compound **2h** has activity similar to that of **PAC-1** in these assays (IC_{50} versus U-937 cells = 14.8 ± 3.2 μM; caspase-3 activation at 10 μM = 6.3 ± 1.6% and at 50 μM = 20 ± 3%; K_d for zinc = 61 ± 9 nM; full details in Supporting Information, Figure S5)

Table 1. Evaluation of PAC-1 and Its Derivatives

Compound	U937 (IC$_{50}$, μM)	Caspase-3 (% Activation at 10 μM)	Zinc Binding (K_d, nM)
PAC-1 (1)	4.8 ± 1.0	45.8 ± 4.8	52 ± 2
1a	15.3 ± 2.0	30 ± 2	77 ± 2
1b	61 ± 22	0	> 1 × 10^4
1c	>100	0	> 1 × 10^4
1d	>100	0	> 1 × 10^4
1e	>100	0	> 1 × 10^4
1f	57 ± 5	0	> 1 × 10^4
1g	>100	0	> 1 × 10^4
1h	>100	0	> 1 × 10^4
1i	>100	0	> 1 × 10^4
1j	1.8 ± 0.4	59 ± 5	43 ± 9
1k	32 ± 12	0	> 1 × 10^4
2a	9.5 ± 1.8	48 ± 3	68 ± 10
2b	14 ± 2	20.6 ± 1.6	62 ± 9
2c	21 ± 6	26.3 ± 0.1	80 ± 14
2d	2.0 ± 0.2	24.2 ± 1.7	48 ± 3
2e	1.0 ± 0.1	31 ± 3.6	39 ± 3
2f	2.7 ± 0.8	24 ± 3.4	47 ± 5
2g	4.6 ± 1.0	19.5 ± 1.8	45 ± 13
3	12 ± 2	30.7 ± 1.2	65 ± 4
4a	2.8 ± 1.1	0^a	44 ± 2
4b	6.5 ± 3.6	0^a	48 ± 7
4c	2.7 ± 1.1	36 ± 3	49 ± 2
4d	59 ± 14	17.1 ± 1.2	> 1 × 10^4
4e	22 ± 3	0	> 1 × 10^4
4f	>100	4.1 ± 1.0	> 1 × 10^4
4g	>100	4.4 ± 0.9	> 1 × 10^4

^{a} These compounds exhibit a significant inhibitory effect at 10 μM, masking any activation (see text). All errors represent the standard error of the mean, $n = 3$.

Once it was determined that **2h** retained activity similar to that of **PAC-1** in these three assays, confocal microscopy experiments were conducted. The goals of these experiments were (1) to determine the subcellular localization of **2h**; (2) to determine if **2h** binds zinc in the cell; (3) to determine if **2h** can activate procaspase-3/-7 to caspase-3/-7 inside the cell; and
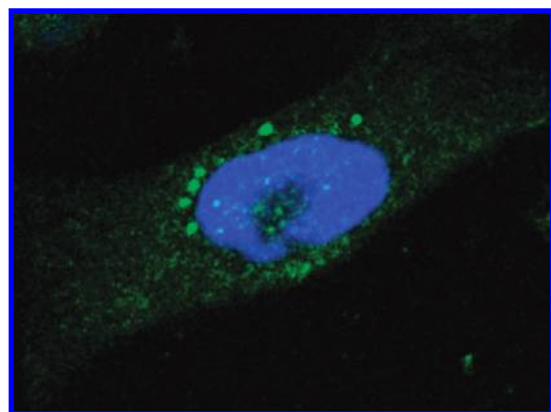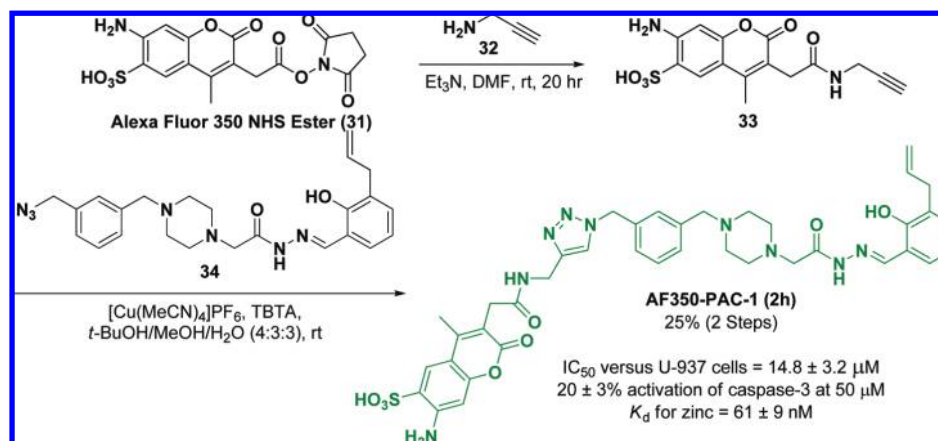
Scheme 4. Synthesis of **2h**, a Fluorescent Version of **PAC-1**

Figure 2. Compound **2h** (pseudocolored green) localizes to the cytoplasm in SK-MEL-5 cells and gives punctate staining similar to that of other fluorescent zinc chelators (see text). DNA was stained with SYBR green and pseudocolored blue.

(4) to determine if **2h** colocalizes with sites of caspase-3/-7 activity inside the cell.

Subcellular Localization of 2h in Fixed Cells. Toward the first and second goals, live cell imaging was used with **2h** and SK-MEL-5 (human melanoma) cells; these adherent cells are a convenient cell line for microscopy studies. SK-MEL-5 cells were treated with $25 \, \mu M$ **2h** for 1 h, washed with media lacking phenol red, and further incubated for 2 h. Cells were then imaged using a Leica multiphoton confocal microscope. Cells treated with **2h** show clear fluorescence in the cytoplasm, with little staining observed in the nucleus (Figure 2); the DNA in this experiment was stained with the SYBR green nucleic acid dye and pseudocolored blue in Figure 2. The image in Figure 2 is representative of wider field images (see Supporting Information, Figure S6) that show a similar staining pattern in ~50% of cells. The other ~50% of cells are either dead or lack staining all together. Furthermore, a punctate cytoplasmic staining pattern is observed for **2h** (Figure 2). This punctate staining is similar to the localization pattern observed by other fluorescent zinc chelators[36,38-41] and is hypothesized to be a visualization of the zincosome.[42-45] The punctate staining pattern indicates that **2h** readily crosses the cell membrane in a short period of time (< 1 h) and suggests that **2h** chelates zinc in the cell.

Visualization of PAC-1-Induced Activation of Procaspase-3 in Live Cells. Because **PAC-1** is known to relieve zinc-mediated inhibition of both procaspase-3/7 and active
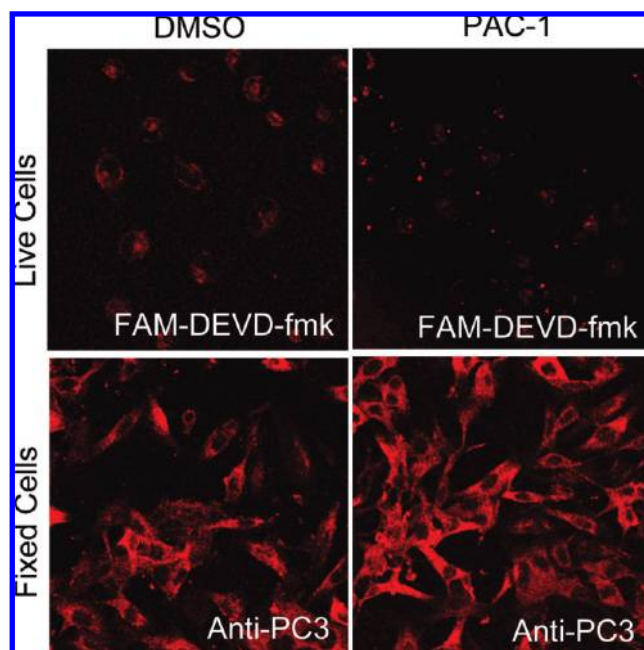

Figure 3. (Top) Activity of caspase-3 in vehicle-treated and **PAC-1** treated SK-MEL-5 cells. Cells were treated with either DMSO or **PAC-1** ($25 \, \mu M$) and then treated with FAM-DEVD-fmk ($25 \, \mu M$), a caspase-3/-7 inhibitor that will covalently append a fluorophore to enzymes that possess caspase-3/-7 activity. (Bottom) Visualization of the entire procaspase-3/caspase-3 population of SK-MEL-5 cells using an antibody to caspase-3.

caspase-3/7 in vitro,[33] we sought to investigate this phenomenon in cell culture. To determine the effect of **PAC-1** treatment on the activity of procaspase-3/-7 and caspase-3/-7 in the cell, **PAC-1** (not **2h**) was utilized in conjunction with a fluorescently labeled, irreversible peptidic inhibitor of caspase-3 and -7, known as FAM-DEVD-fmk. FAM-DEVD-fmk is a cell permeable carboxyfluorescein conjugated peptide that irreversibly alkylates the active site cysteine in active caspase-3 and -7 via its fluoromethyl ketone (fmk) warhead;[46-48] thus, staining with this reagent provides a means to visualize caspase-3/-7 activity in the cell. In the experiment, SK-MEL-5 cells were treated with **PAC-1** ($25 \, \mu M$) and FAM-DEVD-fmk ($25 \, \mu M$). After incubation with these compounds for 1 h, the cells were washed with media lacking phenol red and further incubated for 2 h. Cells were then imaged using a Leica multiphoton confocal
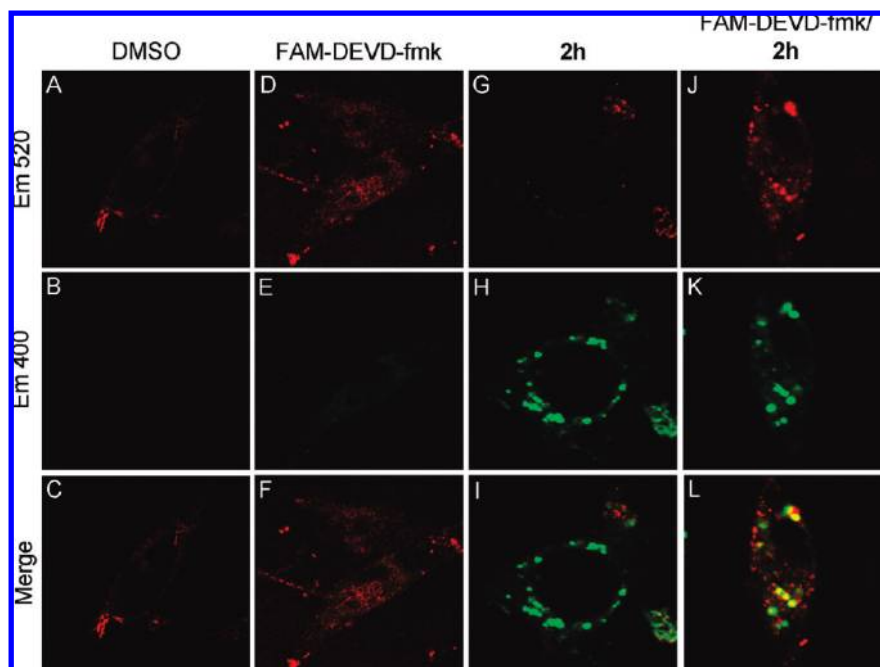
Figure 4. Colocalization of **2h** with sites of caspase-3/-7 enzymatic activity. SK-MEL-5 cells treated with DMSO show background levels of staining in both the red and green channels (A−C). Cells treated only with FAM-DEVD-fmk do not show any staining above background levels (D−F). Cells treated only with **2h** show punctate staining in the cytoplasm with no increase in the emission at 520 nm (G−I). Cells treated concurrently with **2h** and FAM-DEVD-fmk show spots of intense caspase-3/-7 activity (pseudocolored red) and punctate staining of **2h** (pseudocolored green), which exhibit good colocalization in the merged image (J−L).

microscope. In these live cell images, the active caspase-3/-7 population within cells can be observed with minimal interference from the inactive procaspase-3/-7. As shown in Figure 3 (top), in vehicle treated cells only minimal staining is apparent; however, upon treatment with **PAC-1**, the cells exhibit spots of intense fluorescence, indicative of caspase-3 activity. This result indicates that the procaspase-3 in these areas has been activated to caspase-3.

To determine how these localized areas of caspase-3 activity compared to the total population of procaspase-3, we utilized a rabbit anticaspase-3 antibody that recognizes both active caspase-3 and procaspase-3. SK-MEL-5 cells were treated with either DMSO or 25 μM **PAC-1** for 1 h. The cells were then washed with media lacking phenol red and allowed to incubate further for 2 h. At this point, the cells were fixed and stained with the anticaspase-3 primary antibody, followed by an antirabbit Alexa Fluor 647 secondary antibody, and mounted on slides. All cells were imaged on a Leica multiphoton confocal microscope. In the immunofluorescent images, procaspase-3/caspase-3 localization is confined to the cytoplasm (Figure 3, bottom) as is consistent with results in the literature.[49] Thus, upon short treatments with **PAC-1**, there is no obvious change in the amounts or localization of procaspase-3/caspase-3. Analysis of the images in Figure 3 suggests that a subpopulation of the procaspase-3 is being activated to caspase-3 in the **PAC-1** treated cells.

Cellular Colocalization of 2h with Sites of Caspase-3 Activity. To determine if the spots of caspase-3/7 activity observed in the image in Figure 3 are due to direct induction of procaspase-3/-7 activation by **PAC-1**, we sought to determine if **2h** will colocalize with caspase-3/-7 activity inside the cell. SK-MEL-5 cells were treated with DMSO or **2h** and/or FAM-DEVD-fmk for 1 h. Cells were then washed in media lacking phenol red and incubated for an additional 2 h before

confocal imaging. Visualization at 400 nm (pseudo colored green) and 520 nm (pseudo colored red) reports on the cellular localization of **2h** and FAM-DEVD-fmk, respectively, and representative images are shown from the red channel, green channel, and a merge of the two (top, middle, and bottom, respectively in Figure 4). Cells treated with vehicle alone showed minimal staining in the green and red channels (Figure 4A,B,C). In cells that are stained with the FAM-DEVD-fmk caspase-3/-7 inhibitor, there is very little additional staining observed over background (Figure 4D, E,F). Cells that were treated with **2h** showed punctate cytoplasmic staining in the green channel with no bleedthrough to the red channel, consistent with data in Figure 3 (Figure 4G,H,I). However, when cells are treated with both **2h** and FAM-DEVD-fmk, staining corresponding to active caspase-3/-7 is observed in a staining pattern similar to the **2h** staining (Figure 4J,K,L). In fact, the majority of this staining overlaps in the merged image (Figure 4L); this overlap was quantified as 52% using Image J software over 12 cells (see Supporting Information, Figure S7).[50] Combined, the results suggests that there is low/no basal cellular caspase-3/-7 activity in these cells, but that procaspase-3/-7 becomes rapidly activated in the presence of **2h**. In addition, the sites of intense cellular caspase-3/-7 enzymatic activity overlap considerably with the subcellular localization of **AFC350-PAC-1**, suggesting that **AFC350-PAC-1** is responsible for procaspase-3/-7 activation at these sites.

Induction of Apoptosis by PAC-1 Derivatives. To assess if **PAC-1** derivatives induce apoptotic death, the effect of two of the more potent compounds (**2d** and **4c**) was evaluated versus U-937 cells. For these experiments, each compound (at 50 μM) was incubated with U-937 cells for 12 h, at which point cells were costained with propidium iodide (PI) and FITC-labeled Annexin V. Cells that are negative for PI staining but positive for Annexin V are considered to be in
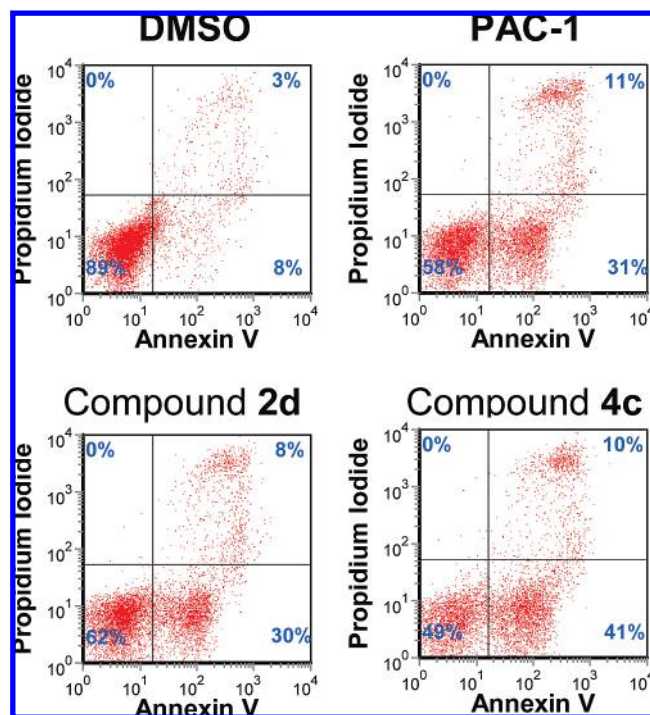
Figure 5. Assessment of apoptotic induction by **PAC-1** derivatives. U-937 cells were treated with **PAC-1**, **2d**, and **4c** (50 μM each) or vehicle control for 12 h, then stained with propidium iodide (PI) and FITC-labeled Annexin V. All three compounds induce apoptosis as assessed by the Annexin V positive/PI negative populations. The results shown are representative of three replicate experiments with each compound.

the early stages of apoptosis.[51] Such dual staining experiments were previously used to show that **PAC-1** induces apoptotic death in cancer cells in culture.[30] The result of this PI/Annexin V experiment with the **PAC-1** derivatives clearly show that each compound induces apoptotic death (Figure 5).

Discussion

PAC-1 is a novel experimental therapeutic that has shown efficacy in several mouse models of cancer.[30] It was recently discovered that **PAC-1** enhances the activity of procaspase-3, procaspase-7, caspase-3, and caspase-7 in vitro through the chelation of inhibitory zinc ions.[33] To probe the relevance of this in vitro mechanism to the anticancer activity of **PAC-1** in cell culture, we determined a full structure–activity relationship for **PAC-1** with respect to its ability to induce death in cancer cells in culture, its ability to activate caspase-3 in vitro, and its ability to chelate zinc. We then used this information to construct a fluorescent derivative of **PAC-1** that could be used to track subcellular localization. The information obtained through these experiments sheds light not only on the mechanism by which **PAC-1** induces death in cancer cells but also on the role of cellular zinc as an endogenous antiapoptotic agent.

Evaluation of 26 **PAC-1** derivatives suggests an intimate link between the metal chelating properties of the compounds and their ability to induce caspase-3 activation in vitro and to induce death in U-937 cells in culture. Significantly, any alteration to the *ortho*-hydroxy *N*-acylhydrazone motif results in compounds with no ability to bind zinc, low/no activity in caspase-3 activation, and cell death assays. This data is consistent with a large body of literature showing that *ortho*-hydroxy *N*-acylhydrazones are metal chelators.[31,52–58] Conversely, other sites on **PAC-1** could be altered without

losing activity in these three assays. Minimally, this data suggests that the metal chelating moiety of **PAC-1** is critical to the anticancer properties of this compound.

Procaspase-3 is known to reside in the cytoplasm[49,59] where it remains in an inactive form until it is cleaved by upstream apoptotic caspases.[18] Several studies have shown that procaspase-3 is capable of autoactivation in vitro,[19,33,35] although the relevance of this mode of activation in vivo is unclear. Our hypothesis for the mode of action of **PAC-1**-induced cell death is that it relieves the zinc-mediated inhibition of procaspase-3, allowing procaspase-3 to use its enzymatic activity to cleave another molecule of procaspase-3, resulting in active caspase-3.[33] Once this occurs, the process accelerates; any caspase-3 generated can then cleave procaspase-3 (this is known to occur in vitro[35]) in a feed-forward cycle. We conducted a series of experiments designed to investigate if **PAC-1** directly induces caspase-3 activity in the cell and if **PAC-1** does so through the direct activation of procaspase-3.

In an initial experiment, the population of active caspase-3 in **PAC-1** treated cells was examined relative to vehicle treated controls and relative to the population of cellular procaspase-3. As might be expected, we found that there is very little caspase-3 activity in actively growing SK-MEL-5 cells in culture (as assessed by the fluorescent caspase-3/-7 inhibitor). In contrast, the cytoplasm shows considerable procaspase-3 (as assessed with antibody staining). These results are shown in the left panels of Figure 3. Treatment of cells with **PAC-1** for 1 h induced strong localized spots of caspase-3/-7 activity (Figure 3, top right), indicating that **PAC-1** causes the rapid elevation of caspase-3/-7 enzymatic activity. By employing a fluorescently labeled version of **PAC-1** (compound **2h**) we were then able to determine that **2h** colocalizes to the spots of intense caspase-3/-7 activity (Figure 4). This colocalization data is perhaps the strongest evidence to date supporting the notion that **PAC-1** directly induces procaspase-3 activation inside the cancer cell.

Interestingly, our recent data show that **PAC-1** will activate both procaspase-3 *and* caspase-3; thus, another possibility for the **PAC-1** mode of action would be that **PAC-1** relieves the zinc-mediated inhibition of caspase-3. As the FAM-DEVD-fmk inhibitor only reports on *active* caspase-3, the experiments described in this article do not directly rule out this possibility. However, this scenario seems less likely given that cancer cell procaspase-3/caspase-3 ratios are typically strongly weighted in favor of procaspase-3,[60–62] although in some primary cancer cell types and cell lines, there does appear to be significant levels of caspase-3.[63–66]

The data in this article also suggest a prominent role for zinc as an endogenous antiapoptotic agent, and there is a lot of literature to support this notion.[42,67–69] It is estimated that as much as 10% of cellular zinc exists loosely bound to various cellular proteins or contained in the zincosomes. Perhaps most provocatively, zinc has been shown to colocalize with procaspase-3,[70,71] and we have shown that zinc inhibits procaspase-3 enzymatic activity in vitro.[33]

Thus, the weight of the available evidence suggests the following mechanism for the **PAC-1**-mediated induction of apoptosis in cancer cells: **PAC-1** rapidly enters the cell where, with a dissociation constant of ~50 nM,[33] it competes favorably for the loosely bound zinc pool. Enzymes whose activity was previously inhibited by zinc, such as procaspase-3, are now freed to carry out their catalytic function. Even with an enzymatic rate 200-fold less than that of caspase-3, procaspase-3 is active enough to catalyze the hydrolysis of other

molecules of procaspase-3, thus activating them to caspase-3. Active caspase-3 can cleave its > 100 cellular substrates to execute the apoptotic program, and it can also cleave more procaspase-3, further stimulating the feed-forward cycle.

This model for the cytotoxicity of **PAC-1** is supported by several lines of evidence: Rapid caspase-3/-7 activity is seen in cells treated with **PAC-1**, both in whole cell experiments where caspase-3 activity is measured with chromogenic substrates[30] and in microscopy experiments where caspase-3 activity can be assessed with a fluorescent inhibitor (Figure 3). In addition, caspase-3 activity is observed *before* mitochondrial membrane depolarization in **PAC-1** treated cells.[30] Also, exogenous zinc inhibits the apoptosis-inducing effect of **PAC-1**,[33] and zinc colocalizes with cellular procaspase-3.[70,71] Finally and perhaps most significantly, a fluorescent version of **PAC-1** colocalizes with sites of cellular caspase-3 activity (Figure 4).

Relative to other zinc chelators, **PAC-1** appears to have the proper combination of moderate affinity for zinc while still retaining the ability to rapidly penetrate cells, modest zinc affinity allowing for chelation of the loosely bound zinc pool, with sufficient hydrophilicity to promote cell permeability. It is unlikely that **PAC-1** is special in this regard; other cell-permeable, moderate-affinity zinc chelators are likely to induce death through a mechanism similar to that of **PAC-1**. It is also unlikely that **PAC-1** removes zinc only from procaspase-3. The full effect of chelating the loosely bound cellular zinc pools remains to be uncovered; indeed, the results of **PAC-1** may explain the mode of action of other known zinc-chelating anticancer agents.[72,73]

In summary, the SAR we have established herein for **PAC-1** paves the way for the development of even more potent versions of this compound. The design, synthesis, and evaluation of **2h** has allowed us to gather further data supporting the hypothesis that **PAC-1** directly activates cellular procaspase-3 through the chelation of inhibitory zinc ions. As **PAC-1** is the first small molecule reported to directly activate procaspase-3, the data reported herein is critical for the continued understanding and advancement of procaspase-3 activation as an anticancer strategy. The development and in vivo testing of more potent versions of **PAC-1** and further investigations into the antiapoptotic role of cellular zinc are underway in our laboratories, and these results will be presented in due course.

Supporting Information Available: Full experimental protocols and compound characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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