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Dual Small-Molecule Targeting of Procaspase-3 Dramatically Enhances Zymogen Activation and Anticancer Activity

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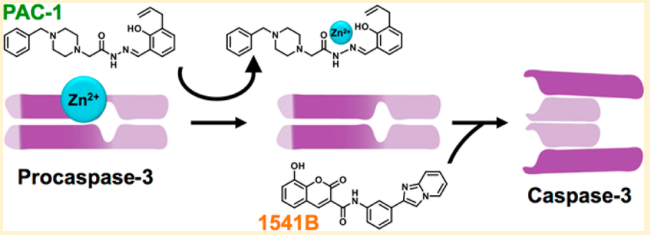
Supporting Information

ABSTRACT: Combination anticancer therapy typically consists of drugs that target different biochemical pathways or those that act on different targets in the same pathway. Here we demonstrate a new concept in combination therapy, that of enzyme activation with two compounds that hit the same biological target, but through different mechanisms. Combinations of procaspase-3 activators PAC-1 and 1541B show considerable synergy in activating procaspase-3 in vitro, stimulate rapid and dramatic maturation of procaspase-3 in multiple cancer cell lines, and powerfully induce caspase-dependent apoptotic death to a degree well exceeding the additive effect. In addition, the combination of PAC-1 and 1541B effectively reduces tumor burden in a murine lymphoma model at dosages for which the compounds alone have minimal or no effect. These data suggest the potential of PAC-1/1541B combinations for the treatment of cancer and, more broadly, demonstrate that differentially acting enzyme activators can potently synergize to give a significantly heightened biological effect.



INTRODUCTION

Combination therapy has become standard for treatment of cancer patients.[1,2] The goal of these drug cocktail regimens is to achieve additive or synergistic effects among chemotherapeutics, thereby maximizing summation dose-intensity with resultant enhanced anticancer activities and increased patient survival.[3−5] Combinations have been identified and developed both through unbiased approaches and by rational design,[5−8] and compounds that act on a single biochemical pathway are particularly strong candidates for synergy or potentiation. For example, inhibitors of poly(ADP-ribose)polymerase-1 (PARP-1), an enzyme that facilitates DNA damage repair, potently synergize with DNA damaging agents as demonstrated in cell culture and animal models.[9−11] Herein we describe an approach for potentiation not based on compounds acting on two targets within a single pathway, but rather with two compounds acting differentially to activate the same enzyme.

During apoptosis, the zymogen procaspase-3 is activated via proteolysis to caspase-3, and this active caspase-3 then cleaves scores of cellular substrates, executing the apoptotic program.[12] As procaspase-3 protein levels are elevated in various tumor histologies,[13−22] drug-mediated direct activation of procaspase-3 has gained significant interest as a selective anticancer strategy. Furthermore, caspase-3 has been shown to play critical roles in cardiomyocyte hypertrophy, cellular differentiation, and remodeling.[23−25] Thus development of a strategy to magnify the timing and level of caspase-3 activation in a specific and direct manner could greatly aid the study of active caspase-3 in these nonapoptotic processes. To date, two major classes of compounds have been disclosed that enhance the activity and automaturation of procaspase-3 in vitro and induce apoptosis in cancer cells in culture. Procaspase-activating compound-1 (PAC-1, Figure 1A) enhances the activity of procaspase-3 in vitro via the chelation of inhibitory zinc ions,[26,27] induces apoptosis in cancer cells in culture,[27−29] and has shown efficacy in multiple murine tumor models.[27] More recently, the compound 1541B (Figure 1A) was discovered to promote the automaturation of procaspase-3 to caspase-3 in vitro and to induce apoptotic death of cancer cells in culture.[30] Compound 1541B appears to activate procaspase-3 via a binding-induced shift in the on−off state equilibrium,[30] or through formation of nanofibrils.[31,32] PAC-1 and 1541B exert their activating effect on procaspase-3 by distinct biochemical mechanisms, suggesting the potential for synergistic effects in cell culture and in vivo.

In vitro, procaspase-3 has enzymatic activity that is dramatically lower than caspase-3, with estimates ranging from reductions of ∼200 to 10^7 fold.[32,33] Low micromolar levels of zinc inhibit the activity of procaspase-3 and caspase-3

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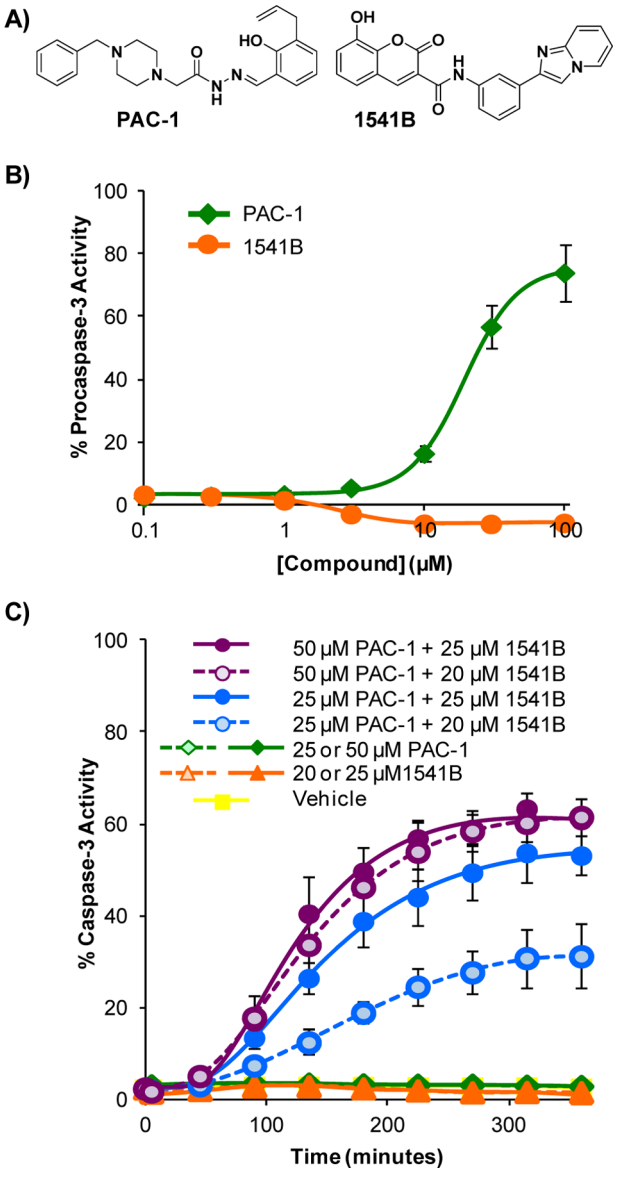

Figure 1. PAC-1 synergizes with 1541B to enhance procaspase-3/caspase-3 activity in vitro. (A) Structures of PAC-1 and 1541B. (B) Procaspase-3 (100 nM) was incubated with PAC-1 or 1541B in zinc-supplemented caspase activity buffer. Enzymatic activity was assessed with Ac-DEVD-AFC. Data is normalized to procaspase-3 (100 nM) activity in zinc-free caspase activity buffer as 100% activity. Error bars represent SEM of three replicates. (C) Procaspase-3 (350 nM) in zinc-supplemented caspase activity buffer was incubated 1541B, PAC-1, or 1541B + PAC-1, and enzymatic activity was assessed with Ac-DEVD-pNA. Data is normalized to 100% activity = 350 nM caspase-3 in zinc-free caspase activity buffer. Error bars represent the SEM of three replicates.

in vitro.[26,34−37] Zinc colocalizes with procaspase-3/caspase-3 and inhibits its enzymatic activity in the cell.[38,39] PAC-1, a moderate affinity zinc chelator that has been shown to chelate the labile zinc pool in cells,[40] allows procaspase-3 to once again process substrates, including itself.[26] Described herein is the combination use of the two small molecule activators of procaspase-3, PAC-1 and 1541B. These compounds act synergistically to enhance procaspase-3/caspase-3 activity in vitro, induce rapid procaspase-3 processing and caspase-3 activity in cell culture, potently and rapidly cause apoptotic death in a variety of cancer cell lines, and have efficacy in a murine tumor model.

RESULTS

PAC-1 + 1541B Activate Procaspase-3 in vitro. As zinc colocalizes with cellular procaspase-3/caspase-3,[38,39] it was of interest to determine if 1541B could activate recombinantly expressed procaspase-3 (Supporting Figure S1A) in the presence of zinc; the activating effect of 1541B on procaspase-3 in vitro had previously only been evaluated under zinc-free conditions.[30] As shown in Figure 1B, compound 1541B does not enhance intrinsic procaspase-3 enzymatic activity in zinc-containing buffers, whereas PAC-1 relieves zinc-mediated inhibition and induces the anticipated activating effect.[41] This effect from PAC-1 is reliant upon its zinc binding ability, as an analogue that does not bind zinc (PAC-1a[26]) is not active in this experiment. In addition, an inactive derivative of 1541B (compound 1541D[30]) has no effect in this experiment (structures and data in Supporting Figure S1B−D). This orthogonal activity of PAC-1 and 1541B on procaspase-3 (PAC-1 activating zinc-bound procaspase-3, 1541B activating procaspase-3 with zinc removed) suggests synergistic potential, and indeed, the combination of PAC-1 and 1541B, when incubated with procaspase-3 in vitro, leads to dramatic caspase-3 activity far exceeding the effect of either compound alone (Figure 1C). Consistent with the established structure−activity relationships,[28,30] PAC-1a and 1541D as single agents and in combination were unable to facilitate procaspase-3 activation in vitro (Supporting Figure S1D).

PAC-1 + 1541B Activate Procaspase-3 in Cancer Cell Lines in Culture. To examine PAC-1 synergy with 1541B for activation of procaspase-3 in cancer cells in culture, a panel of cancer cell lines were treated with combinations of PAC-1 and 1541B and the caspase-3/-7 activity of the cell lysates was monitored with the fluorogenic caspase substrate Ac-DEVD-AFC. As seen in Figure 2A, cotreatment of U-937 (human lymphoma), BT-549 (human breast cancer), and A549 (human lung cancer) cells with PAC-1 and 1541B results in markedly more dramatic and rapid increases in DEVDase activity than treatment with either PAC-1 or 1541B as single agents. The combination treatment stimulates caspase activity that rivals or surpasses that induced by staurosporine (STS, 1 μM), one of the most rapid inducers of cellular DEVDase activity known, and is dose dependent (Supporting Figure S2).

To determine if the elevation of DEVDase activity was the result of enhanced cleavage of procaspase-3 to caspase-3 facilitated by compound cotreatment, cells were treated with PAC-1 and 1541B combinations and assessed by Western blotting. As shown in Figure 2B, dramatic activation of procaspase-3 to caspase-3 was observed in U-937, BT-549, and A549 cells upon treatment with the PAC-1/1541B combinations, whereas low/no procaspase-3 activation was observed with 1541B or PAC-1 alone at the times and concentrations evaluated. Analogous results in HL-60 (human leukemia), Hs578T (human breast cancer), and EL4 (murine lymphoma) cells are shown in Supporting Figure S3.

PAC-1 + 1541B Induce Procaspase-3 Activation before Cytochrome c Release from the Mitochondria. Apoptosis through the intrinsic pathway proceeds via an ordered series of events, with Bcl-2 family proteins acting on the mitochondria, stimulating cytochrome c release, formation of the apoptosome, caspase-9 activation, and subsequent cleavage of procaspase-3 to caspase-3.[42−44] Caspase-3 then

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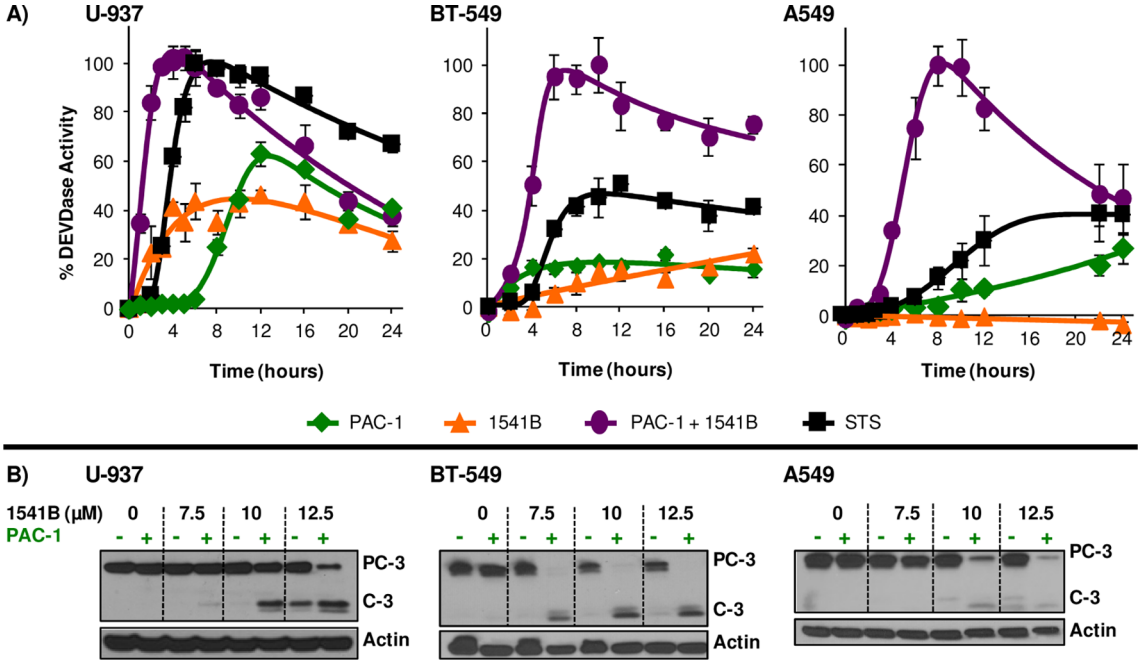

Figure 2. PAC-1/1541B combinations induce rapid and dramatic procaspase-3 activation and maturation in cancer cell lines in culture. (A) Cancer cell lines were treated with PAC-1 (30 μM), 1541B (15 μM), or the combination, cells were lysed at various time points, and caspase-3/-7 activity of the lysates was evaluated with the fluorogenic Ac-DEVD-AFC substrate. Error bars represent the SEM of three replicates. STS = staurosporine. (B) Western blot of various cancer cell lines after treatment with combinations of PAC-1 (30 μM) and 1541B. Treatment durations of 3, 4, and 8 h for U-937, BT-549, and A549 cells, respectively, were chosen on the basis of the timing of maximal caspase activity, as observed in cell lysate. Western blots are representative of at least two separate experiments.

cleaves scores of substrates, executing the apoptotic program.[12] To examine these events in cells treated with PAC-1 + 1541B, BT-549 cells were assessed for the timing of procaspase-3 activation relative to cytochrome c release from the mitochondria by Western blot of fractionated cell lysate. As shown in Figure 3A, analysis of cells treated with PAC-1 + 1541B shows the appearance of cleaved caspase-3 bands in the cytosolic fraction before cytochrome c levels diminish in the mitochondria. At these compound concentrations and relatively short treatment times, 1541B as a single agent is considerably less effective at inducing procaspase-3 activation (Figure 3B), and PAC-1 is not effective. Importantly, as shown in Figure 3C, staurosporine induces apoptosis through the canonical intrinsic pathway, with clear release of cytochrome c from the mitochondria prior to activation of procaspase-3.

BT-549 cells were assessed by phase contrast microscopy and monitored for the phenotypic changes associated with caspase activation and apoptosis (Supporting Figure S4). Consistent with the Western blot and caspase activity data, only the combination of PAC-1 and 1541B demonstrated substantial morphological changes indicative of apoptosis, such as blebbing.

PAC-1 + 1541B Synergize to Potently Induce Death of Cancer Cell Lines in Culture. The combination of PAC-1 and 1541B was evaluated for the capacity to induce apoptotic death in a variety of cancer cell lines in culture. These evaluations were performed at short incubation times, reflective of the timing of caspase activation observed in Figure 2 and where neither compound exerts a significant effect as a single agent. PAC-1 significantly synergizes with 1541B for potent proapoptotic activity in U-937, BT-549, and A549 cells (Figure 4A), and in HL-60, Hs578T, U-87, and EL4 cells (Supporting Figure S5; see Supporting Figure S6 for representative Annexin V-FITC/propidium iodide histograms); the dashed horizontal lines in each graph mark levels of cell death that would be observed from a strictly additive effect of PAC-1 and 1541B. While the non-blood-brain barrier permeable analogue S-PAC-1[29,40] possesses a comparable capacity for synergy as PAC-1 (Supporting Figure S7), synergy was not observed when either PAC-1a or 1541D were used in combination with 1541B or PAC-1 (Supporting Figures S8 and S9).

Further assessment (vs U-937 cells) of a broad range of concentrations for both compounds clearly shows the dramatic synergy between these two agents. Shown in Figure 4B is the cell death induced by the compound matrix, with synergy apparent from simple comparisons of the percent cell death induced by the single agents versus the combinations; this is true at both short (6 h, Figure 4B) and long (24 h, Supporting Figure S10) time points. Synergism is frequently quantified in such experiments though calculation of Combination Indices (CI). Combinations describing synergistic interactions possess CI values <1, while antagonistic interactions have CI value >1; additive effects occur when the CI value equals 1.[45−47] Thus, the lower the CI value, the stronger the synergy. When U-937 cells were treated with a range of PAC-1 and 1541B compound concentrations in combination, synergistic interactions were observed broadly, with several combinations considered strongly synergistic (Figure 4B and C and Supporting Figure S10).

The proapoptotic effect of the PAC-1/1541B combination was markedly reduced with the pan-caspase inhibitor Q-VD-OPh, consistent with the involvement of caspases in the mode of cell death (Figure 5A). To further investigate the connection between activation of procaspase-3 and the cell death induced by the drug combination, MCF-7 cells were used, a cell line that does not express procaspase-3,[48] together with a matched

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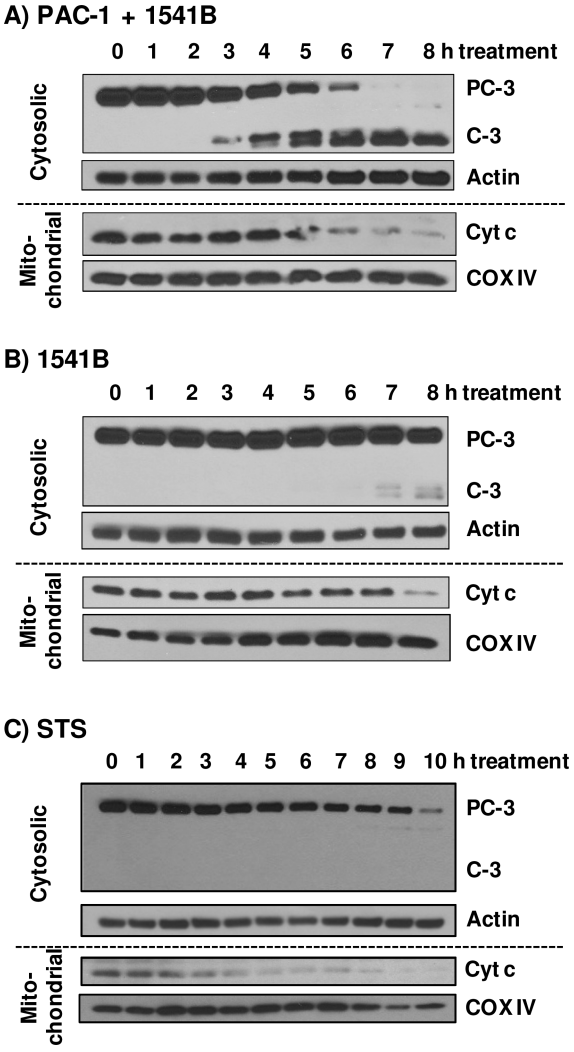

Figure 3. Rapid procaspase-3 activation is observed in cells treated with PAC-1 + 1541B, occurring prior to release of cytochrome c from the mitochondria. (A) BT-549 breast cancer cells were treated with PAC-1 (30 μM) and 1541B (10 μM) over 8 h and assessed in the cytosol for procaspase-3 activation, and in the mitochondria for cytochrome c levels. (B) BT-549 cells treated with 1541B (10 μM) and assessed for 8 h. (C) BT-549 cells treated with STS (1 μM) and assessed for 10 h.

MCF-7 cell line where procaspase-3 is expressed via a plasmid (Figure 5B). The combination of PAC-1/1541B has minimal effect on MCF-7 cells transfected with control plasmid (MCF-7 VRL), but dramatically induces apoptosis in procaspase-3 expressing MCF-7 cells (MCF-7 C-3) (Figure 5C), an effect correlated with procaspase-3 activation (Figure 5D).

PAC-1 + 1541B Have Efficacy in a Murine Tumor Model. To explore the therapeutic utility of the dual procaspase-3 activation strategy, the combination of PAC-1 and 1541B was assessed in vivo. HPβCD was used as vehicle, and compounds were administered via IP injection. Prior to beginning efficacy studies, the tolerability and toxicity of 1541B as a single agent and in combination with PAC-1 was determined (Supporting Tables S1, S2, and S3). On the basis of these results, a maximum combination dosage of 125 mg/kg of PAC-1 followed by 17.5 mg/kg of 1541B was chosen for the efficacy model. The hematologic and nonhematologic toxicity of this treatment of PAC-1 and 1541B was determined following 3 consecutive daily IP administrations of HPβCD, PAC-1, 1541B, or combination (PAC-1 + 1541B). No clinically significant evidence for myelosuppression, renal injury, or hepatic toxicity was identified in any of the treatment cohorts (Supporting Table S3). For mice receiving HPβCD, PAC-1, or 1541B alone, all collected target organs were devoid of histologic evidence for inflammation and necrosis. In mice receiving combination IP PAC-1 and 1541B, pathologic changes were noted in the lung parenchyma of 2 out of 3 mice. These mice had eosinophilic and histiocytic perivascular and peribronchiolar infiltrates, which were mild to moderate in severity. The clinical significance of this finding is unclear, as mice in these groups were asymptomatic and did not demonstrate weight loss compared to controls.

The pharmacokinetic parameters of each compound were then determined (Supporting Table S4). Following a single IP injection at a dose of 125 mg/kg, PAC-1 in HPβCD was rapidly absorbed and achieved a maximal plasma concentration of 32720.0 ng/mL within 20 min postinjection. PAC-1 was distributed quickly throughout the body with a mean distribution half-life of 20 min and a mean terminal elimination half-life of 4.2 h. Likewise, 1541B in HPβCD was absorbed and reached a maximal plasma concentration of 2699.9 ng/mL within 20 min of intraperitoneal administration. The distribution half-life of 1541B was 20 min and the terminal elimination half-life was 5.4 h.

The EL4 syngeneic murine lymphoma model was chosen to explore the therapeutic utility of PAC-1 and 1541B combinations, as PAC-1 and 1541B synergize to induce dramatic activation of procaspase-3 and cell death versus this cell line (Supporting Figures S3C and S5D), and it is a challenging treatment model due to its rapid doubling time (less than 24 h[49]) and uniformly high tumor formation rate (~100%) when inoculating greater than 1×10^6 cells subcutaneously in C57BL/6 mice.[50] PAC-1 was chosen over S-PAC-1 for the combination studies as it has greater activity versus the EL4 cell line in culture.[29] C57BL/6 mice implanted with EL4 cells were treated with PAC-1 alone (125 mg/kg), 1541B alone (17.5 mg/kg), or sequential treatments of PAC-1 + 1541B (125 mg/kg and 17.5 mg/kg, respectively) once-a-day for three days, formulated in HPβCD. Once tumors in vehicle-treated mice had reached their maximum allowed size of ~1500 mm^3 (8 days), all mice were sacrificed, and tumors were excised and weighed. As shown in Figure 5E, 1541B treatment had no effect, and PAC-1 had a small but statistically significant effect on tumor growth in this model. However, the combination of PAC-1 and 1541B dramatically retarded tumor growth.

■ DISCUSSION

While there is clear benefit to anticancer strategies utilizing combinations of drugs that act on different targets, the work described herein demonstrates that dramatic synergy can be observed with compounds that act through orthogonal mechanisms on the same biological target. This multitargeting approach may have particular advantages when activation of an enzyme is sought. PAC-1 chelates the labile inhibitory zinc from procaspase-3/caspase-3, thus priming the enzyme for robust and efficient activation by 1541B. In cell culture at concentrations where neither compound significantly induces death at 6–12 h, dramatic enhancement of cell death (over the additive effect) is observed with the PAC-1/1541B combination. This cell death is tied to the ability of the PAC-1/1541B combination to induce a rapid conversion of procaspase-3 to

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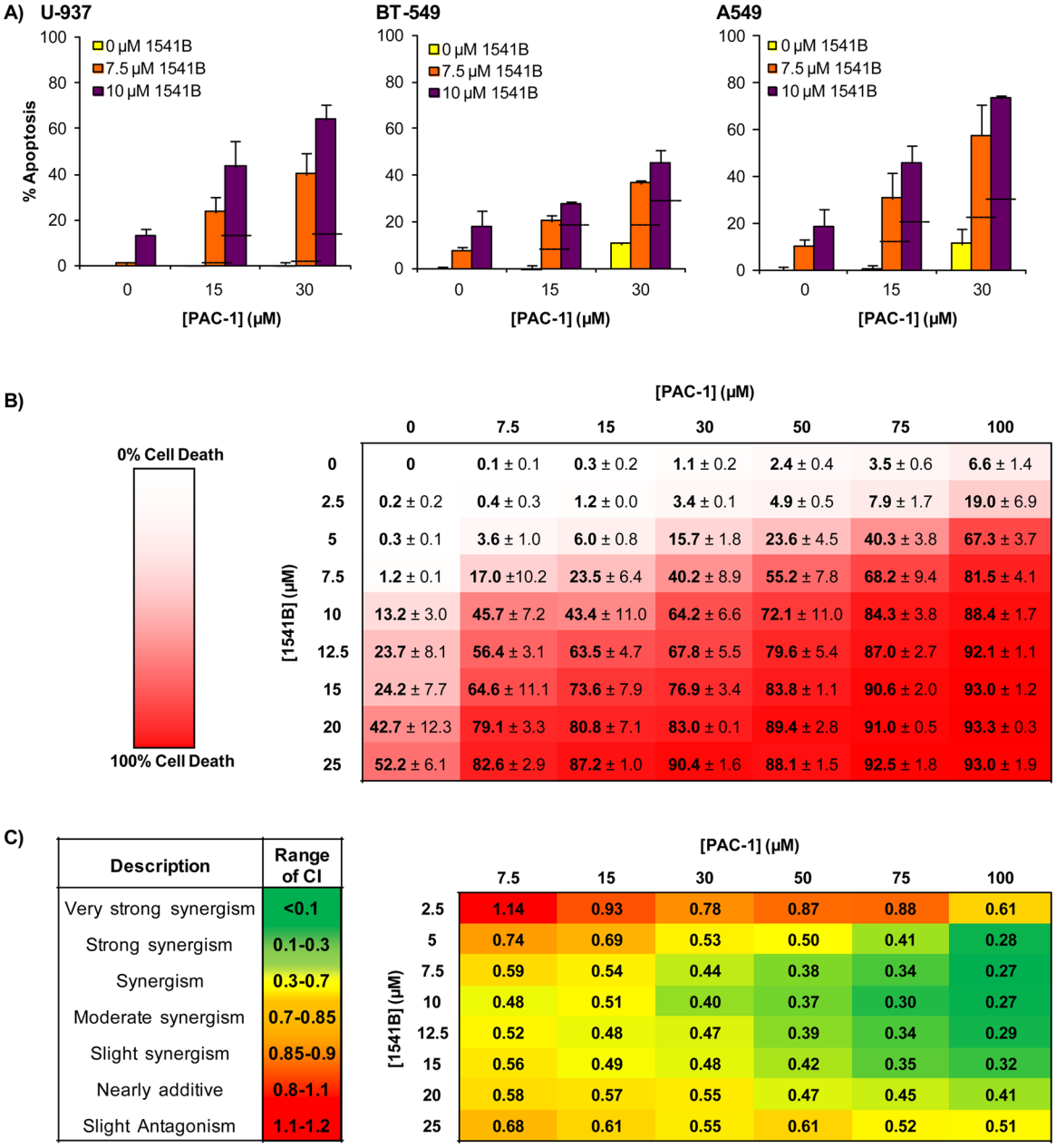

Figure 4. PAC-1 and 1541B synergize to induce rapid cell death in cancer cell lines. (A) Cancer cell lines were treated with the indicated concentrations of PAC-1 (0, 15, and 30 μM) and 1541B (0, 7.5, 10 μM) for six hours, and apoptotic death was assessed using flow cytometry with Annexin V-FITC/propidium iodide staining. Error bars represent the SEM of three replicates. The dotted horizontal lines represent the level of cell death expected from a mere additive effect of PAC-1 and 1541B for each drug combination. (B) Percent cell death observed after 6 h treatment of U-937 cells with PAC-1 + 1541B combinations in matrix format. Values are heat mapped with white equal to 0% cell death and red equal to 100% cell death. Error represents the SEM of three replicates. (C) Combination Index values calculated for each combination with Combosyn software (<1 indicates synergistic interaction with values <0.3 indicating strong synergism). Values are heat mapped with lowest values in green and highest values in red.

caspase-3, as shown by the Western blots and the caspase-3 enzymatic activity in cell lysates. PAC-1 is safe in mice and dogs,[51] and a derivative of PAC-1, S-PAC-1, showed promising activity in a phase I clinical trial of pet dogs with lymphoma,[29] thus the observed synergy with 1541B could have clinical significance.

PAC-1 has been utilized as a tool for studying direct procaspase-3 activation in various systems,[23,52] and data presented herein suggest that the PAC-1/1541B combination will also be valuable as a tool for rapidly activating procaspase-3. As interest in activating enzymes with small molecules is increasing rapidly and has considerable medicinal potential,[53−57] our data suggest that targeting strategies using two small molecules with different activation mechanisms could be a general approach for dramatic enhancement of the intended biological effect.

METHODS

In Vitro Activation of Procaspase-3. Procaspase-3 and caspase-3 were expressed as described previously and purified with Qiagen nickel-NTA resin.[58] Increasing concentrations of PAC-1, 1541B, PAC-1a and 1541D were assessed for their capacity to enhance activity of

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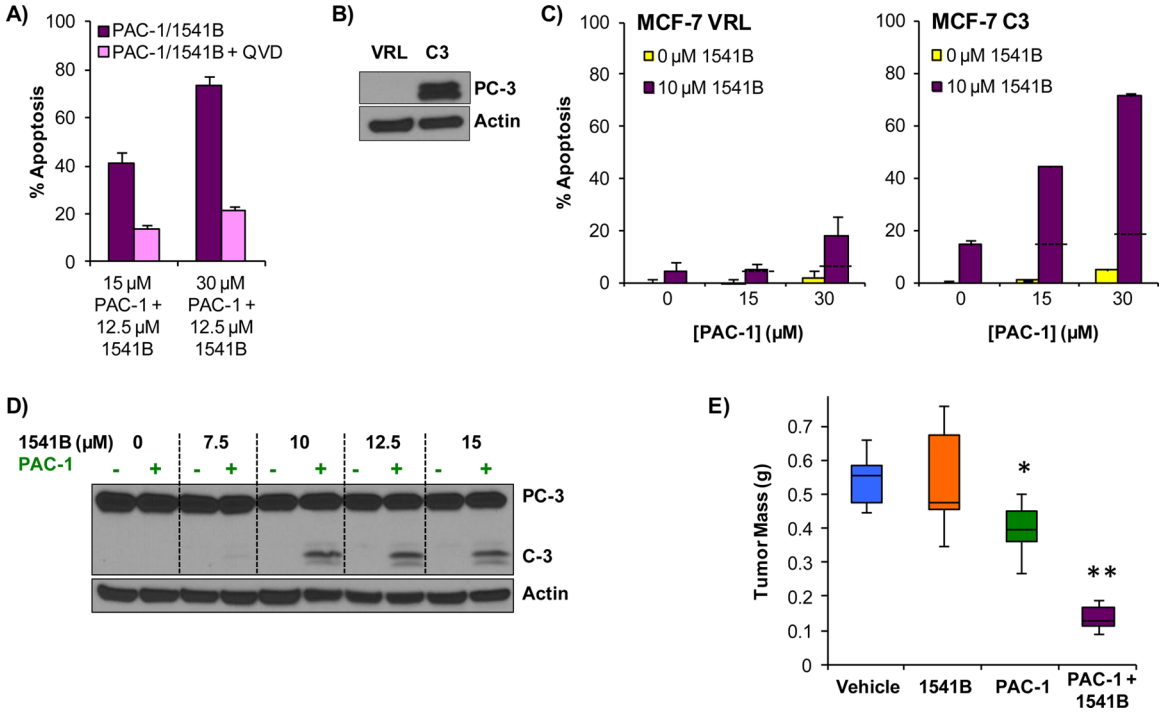

Figure 5. PAC-1 and 1541B induce rapid caspase-dependent cell death in cancer cell lines. (A) The pan-caspase inhibitor Q-VD-OPh (25 μM) protects against PAC-1/1541B-mediated cell death in U-937 cells. Error bars represent the SEM of three replicates. (B) Western blot analysis of MCF-7 cells transformed with empty plasmid (MCF-7 VRL) and MCF-7 cells transformed with a plasmid containing the gene for procaspase-3 (MCF-7 C-3). (C) MCF-7 VRL and MCF-7 C-3 cells were treated with the indicated concentrations of PAC-1 and 1541B and apoptotic death was assessed using flow cytometry with Annexin V-FITC/propidium iodide staining. Error bars represent the SEM of three replicates. The dotted horizontal lines represent the level of cell death expected from a mere additive effect of PAC-1 and 1541B for each drug combination. (D) Western blot of MCF-7 C-3 cells treated for 6 h with combinations of PAC-1 (30 μM) and 1541B. (E) The combination of PAC-1 and 1541B has an antitumor effect in vivo. EL4 cells were injected subcutaneously into C57BL/6 mice (10 million cells per mouse), the animals were split into four groups and treated once-a-day for three days via IP injection with vehicle (2-hydroxypropyl-β-cyclodextrin, HPβCD), 1541B (17.5 mg/kg in HPβCD), PAC-1 (125 mg/kg in HPβCD), or 1541B + PAC-1 (17.5 and 125 mg/kg, respectively, in HPβCD). After eight days the mice were sacrificed and the tumors excised and weighed. n = 6–7 mice per group. P-values are relative to vehicle control; *, $P < 0.005$; **, $P < 5 \times 10^{-6}$.

100 nM procaspase-3 in an inhibitory zinc-based system (2 μM $ZnSO_4$, 50 mM HEPES, 50 mM KCl, 1.5 mM TCEP, 0.1% CHAPS, pH 7.6). Compounds were added to zinc-inhibited PC-3, incubated for 30 min, and activity was assessed by cleavage of Ac-DEVD-AFC (50 μM) and was normalized to 100 nM procaspase-3 in zinc-free caspase activity buffer (50 mM HEPES, 50 mM KCl, 1.5 mM TCEP, 0.1% CHAPS, pH 7.6).

1541B (20 and 25 μM) and PAC-1 (25 and 50 μM) were evaluated separately and in combination for their capacity to activate 350 nM procaspase-3 over time in a mildly inhibitory zinc-based system (750 nM $ZnSO_4$, 50 mM HEPES, 50 mM KCl, 5 mM TCEP, pH 7.6, 0.1% TritonX-100). At designated time points aliquots were assessed by cleavage of Ac-DEVD-pNA (200 μM). 350 nM caspase-3 in a zinc-free caspase activity buffer (50 mM HEPES, 50 mM KCl, 5 mM TCEP, pH 7.6, 0.1% TritonX-100) was used for 100% caspase-3 activity.

Caspase Activation in Cell Lysate. U-937 (50 000 cells per well), BT-549 (10 000 cells per well) and A549 (10 000 cells per well) were plated in 96-well plates, allowed to adhere overnight (BT-549 and A549), and incubated with 1 μM Staurosporine (STS), 1541B (7.5, 10, 12.5, or 15 μM), PAC-1 (30 μM), 1541B (7.5, 10, 12.5, or 15 μM) and PAC-1 (30 μM) or DMSO (final concentration 1%) in phenol-red free RPMI media. Plates were assessed for executioner caspase activity via addition of a 4× bifunctional lysis activity buffer (200 mM HEPES, 400 mM NaCl, 40 mM DTT, 0.4 mM EDTA, 1% TritonX-100, 20 μM Ac-DEVD-AFC). Fluorescence was measured after a one-hour incubation. Activity is expressed as normalized to minimal and maximal activity observed within the assay.

Western Blot Assessment of Procaspase-3 Cleavage to Caspase-3. For Western analysis one million suspension (U-937, HL-60 and EL4) or adherent cells at 75% confluency in 6-well plates were treated with increasing concentrations from 7.5 to 12.5 μM 1541B in the presence or absence of 30 μM PAC-1 for a duration reflective of near maximal caspase activity as seen in the cell lysate experiment. At the conclusion of treatment, the medium and Trypsin-aided detached cells were pelleted, lysed on ice in RIPA buffer (50 mM Tris base, 150 mM NaCl, 1% TritonX-100, 0.5% Na-deoxycholate, 0.1% SDS, pH7.4, with a 1:100 dilution of Protease Inhibitor Cocktail Set III), and clarified, and protein content was normalized by BCA Protein Assay reagent (Pierce). Samples were denatured, separated by SDS-PAGE (4–20%), and transferred to a membrane for Western blot analysis of pro- and active caspase-3 (Cell Signaling 9662). Blots were stripped and reprobed for β-Actin (Cell Signaling 4970) as a loading control.

Western Blot Assessment of Procaspase-3 Activation and Cytochrome c Release Timing. BT-549 cells at ~75% confluency (allowed to adhere for >24 h) in 6-well plates were treated with 10 μM 1541B and 30 μM PAC-1, 10 μM 1541B or 1 μM STS for up to 10 h. At each time point, medium and Trypsin-aided detached cells were pelleted, washed in PBS and suspended in cold digitonin permeabilization buffer (75 mM NaCl, 1 mM sodium phosphate monobasic, 8 mM sodium phosphate dibasic, 250 mM sucrose, 200 μg/mL of digitonin, protease cocktail inhibitor, pH 7.5) and placed on ice for 5 min. Permeabilized cells were centrifuged (14 000 rcf, 5 min), and the supernatant (cytosolic fraction) was saved. The pellet (mitochondrial fraction) was washed with digitonin permeabilization buffer, lysed in RIPA buffer, and clarified. Protein content for all samples was normalized (Pierce BCA Protein Assay). Samples were denatured, separated by SDS-PAGE and transferred to a membrane for Western blot analysis probed for the presence of pro- and active caspase-3 (Cell Signaling 9662), and cytochrome c (Cell Signaling 4272). Western blots were stripped and reprobed for β-Actin (Cell

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Signaling 4970) and COX IV (Cell Signaling 5247) as loading controls.

Induction of Apoptosis. The induction of apoptosis was measured by Annexin V-FITC/Propidium iodide staining and flow cytometry. Either 1 000 000 suspension (U-937, HL-60, EL4) cells or adherent cells at ∼75% confluency in 6-well plates were treated with combinations of PAC-1 and 1541B (final concentration of DMSO <1%). The entire contents of each well was then transferred to flow cytometry tubes, pelleted, and suspended in 450 μL of Annexin V-FITC binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, 1% BSA, pH 7.4), premixed with Annexin V-FITC and Propidium iodide dyes. Staining was assessed by flow cytometry (10 000 events per sample). The Annexin V-FITC (−)/Propidium iodide (−) population was confirmed to be greater than 90% viable and normalized as the live control. Percent apoptosis was determined as 100% minus the viable quadrant. The role of caspases in the induction of apoptosis was assessed by cotreatment with 25 μM Q-VD-OPh (CalBioChem).

1541B and PAC-1 + 1541B MTD. All experimental procedures were reviewed and approved by the University of Illinois Institutional Animal Care and Use Committee. 6−8 week old C57BL/6 mice were used in all experiments (Charles River). The maximum tolerated dose (MTD) of 1541B was determined. Beginning at 5 mg/kg, (formulated at 0.5 mg/mL in HPβCD, pH 5.5) mice (groups of three) were treated for 5 consecutive days and monitored for signs of toxicity. MTD was the highest dosage for which all mice survived and acceptable (<20%) weight loss was observed. The MTDs of PAC-1 (200 mg/kg) and 1541B (20 mg/kg) were used as the base unit for determining the combined MTD. Beginning with 1/10 of each MTD (20 mg/kg PAC-1 + 2 mg/kg 1541B), mice were treated with PAC-1, followed by 1541B one hour later. Groups of 3 mice were treated with 1/10, 1/6, 1/4, 1/3, 1/2 and 3/4 of each single agent's MTD. Given the tolerability of 1/2 and the toxicity of 3/4, an additional treatment of 125 mg/kg PAC-1 + 17.5 mg/kg 1541B was evaluated and determined to be the combined MTD.

Toxicity Assessment. Ten week old, C57BL/6 female mice (n = 3/cohort) were administered three consecutive daily intraperitoneal injections of hydroxypropyl-β-cyclodextrin (vehicle), PAC-1 (125 mg/kg), 1541B (17.5 mg/kg), or PAC-1 + 1541B (125 mg/kg +17.5 mg/kg, respectively), and then humanely sacrificed 24 h later. Heparinized whole blood was collected for assessment of total white blood cells, neutrophils, lymphocytes, hematocrit, platelets, creatinine, blood urea nitrogen, albumin, alanine aminotransferase, alkaline phosphatase, and total bilirubin. Mice were necropsied, and heart, lung, kidney, liver, spleen, gastrointestinal tract and brain were collected for histopathology. Tissue samples were fixed overnight in 10% neutral buffered formalin, processed, and paraffin embedded for histopathology using routine methods. Tissue blocks were sectioned into 3 μm tissue sections and stained with hematoxylin and eosin. All slides were systematically evaluated by a single board certified veterinary pathologist (LBB) for evidence of acute or chronic inflammation and toxicity. All lesions were characterized, recorded, and scored for severity (minimal = 1, mild = 2, moderate = 3, and severe = 4).

Pharmacokinetics. Ten week old, C57BL/6 female mice were administered a single intraperitoneal dose of PAC-1 at 125 mg/kg or 1541B at 17.5 mg/kg, and sacrificed in cohorts of 3 at predetermined time points (0, 10, 20, 30, 40, 60, 120, 240, 360, 720, and 1440 min). Blood was collected and centrifuged, and plasma separated for quantification of PAC-1 or 1541B by HPLC methods (UIUC Metabolomics Center, Urbana, IL). Pharmacokinetic analyses were performed, using a nonlinear regression program (Winnonlin, version 5.1, Pharsight Corporation, Cary, NC).

EL4 Syngeneic Tumor Model. Ten million EL4 cells were prepared in HBSS and injected subcutaneously on the right flank of sedated (ketamine/xylazine) mice (day 0). By three hours post-injection the injection bleb was no longer evident, and soft tumors appeared the following morning. Mice were randomized into four treatment groups: vehicle, 1541B alone, PAC-1 alone, and 1541B + PAC-1. Mice were treated on days 1, 2, and 3. Compounds were formulated in HPβCD (1541B at 0.5 mg/mL at pH 5.5 and PAC-1 at 12.5 mg/mL at pH 5.5). All mice received two treatments, one hour apart: vehicle (HPβCD + HPβCD), 1541B (HPβCD + 1541B), PAC-1 (PAC-1 + HPβCD) and 1541B + PAC-1 (PAC-1 + 1541B). After 8 days the largest tumors had achieved maximal size; mice were sacrificed, and tumors were excised and weighed.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supporting figures and tables, as described in the text (Figures S1−S10 and Tables S1−S4). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): The University of Illinois has filed patents on PAC-1.

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