Abstract

The development of vemurafenib resistance limits the long-term efficacy of this drug for treatment of metastatic melanomas with the V600E BRAF mutation. Inhibition of downstream MAPK signaling with vemurafenib induces apoptotic cell death mediated by caspase-3, suggesting that addition of a procaspase-3 activator could enhance anticancer effects. Here, we show that the combination of PAC-1, a procaspase-activating compound, and vemurafenib is highly synergistic in enhancing caspase-3 activity and apoptotic cell death in melanoma cell lines harboring the V600E BRAF mutation. In vivo, the combination displays a favorable safety profile in mice and exerts significant antitumor effects. We further demonstrate that addition of PAC-1 to the clinically useful combination of vemurafenib and a MEK inhibitor, trametinib, starkly enhances the caspase-3 activity and proapoptotic effect of the combination. Moreover, addition of low concentration PAC-1 also delays the regrowth of cells following treatment with vemurafenib. Finally, PAC-1 remains potent against vemurafenib-resistant A375VR cells in cell culture and synergizes with vemurafenib to exert antitumor effects on A375VR cell growth in vivo. Collectively, our data suggest that inhibition of MAPK signaling combined with concurrent procaspase-3 activation is an effective strategy to enhance the antitumor activity of vemurafenib and mitigate the development of resistance.

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The Combination of Vemurafenib and Procaspase-3 Activation Is Synergistic in Mutant BRAF Melanomas

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Introduction

Melanoma is the most common cutaneous malignancy and upon metastasis is considered the deadliest form of skin cancer (1). The discovery that approximately 50% of melanomas harbor the V600E mutation in the BRAF protein (2) spurred the development of V600E BRAF inhibitors (3, 4) and the subsequent approval of vemurafenib in 2011. V600E BRAF inhibitors like vemurafenib (and dabrafenib, approved in 2013) lead to impressive reduction in tumor burden within weeks of therapy, and extension of progression-free survival by 3 to 4 months (5, 6). Despite their initial antimalanoma activity, resistance to V600E BRAF inhibitors rapidly emerges. In the majority of resistant tumors, reactivation of the MAPK signaling pathway is observed (7), motivating the addition of MEK1/2 inhibitors (e.g., trametinib) to the treatment regimen for metastatic melanoma. Upfront combination therapy with MEK1/2 and V600E BRAF inhibitors is effective in delaying the median time to resistance by 3.7 to 4.1 months in patients who have not received prior V600E BRAF inhibition treatment (8, 9), but the addition of MEK1/2 inhibitor to patients who have already failed prior V600E BRAF inhibitor therapy only results in a marginal improvement in antitumor efficacy (10). Given the current clinical limitations of existing therapies, novel and rationally designed combination studies with other kinase inhibitors are being explored (11, 12). Despite all efforts to date, the development of resistance to targeted V600E BRAF therapies emerges in virtually 100% of patients treated; acquired drug resistance to this class of agents remains a significant obstacle to dramatically enhance survival benefits for metastatic melanoma patients.

In contrast to many studies that have focused on the combination of vemurafenib with inhibitors of diverse and druggable kinases, combination therapy of vemurafenib with agents that activate the apoptotic pathway has not been extensively explored. In part, this lack of exploration might be attributed to the fact that melanoma cells possess multiple defects in their apoptotic signaling pathways (13–15), rendering them resistant to many proapoptotic stimuli. We hypothesized that a suitable proapoptotic agent that induces apoptosis downstream of these apoptotic defects would be highly synergistic with V600E BRAF inhibitors. Given that the aberrations in the apoptotic signaling cascades in melanoma cells are upstream of the activation of procaspase-3, drugs that directly activate procaspase-3 are intriguing candidates for this combination therapy. In addition, because melanomas have elevated expression of procaspase-3 (16, 17), a procaspase-3 activator should be potent and selective for such cells. Furthermore, it is known that V600E BRAF inhibitors induce apoptotic cell death mediated by caspase-3; (3) thus, the combination of vemurafenib with a direct procaspase-3 activator could lead to dramatically enhanced caspase-3 activity and cancer cell death.

Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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relative to the effect of either single agent. PAC-1 (Fig. 1A) is a small molecule that directly activates cellular procaspase-3 via chelation of labile inhibitory zinc (18–22). Due to the over-expression of procaspase-3 in cancers of diverse origins (16, 17, 23–32), PAC-1 and its derivatives selectively induce apoptosis in cancer cells while sparing noncancerous cells (21, 25, 33, 34). PAC-1 exerts single-agent activity in multiple murine models of cancer (21, 34–36), including a xenograft model of melanoma (21). Importantly, in addition to favorable preclinical activity in murine tumor models, human cancer patients have been taking PAC-1 as part of a phase 1 clinical trial since March 2015 (NCT02355535).

Here, we report the synergistic activity of PAC-1 + vemurafenib and PAC-1 + vemurafenib + trametinib in enhancement of caspase-3 activity and apoptotic cell death in V600EBRAF melanoma. As a result of increased apoptotic cell death, the PAC-1 + vemurafenib combination induces significant reduction in tumor volume in a murine xenograft model of V600EBRAF melanoma, beyond the antitumor effects of the individual agents. In addition, this enhancement of apoptotic death in vemurafenib-sensitive melanoma by the addition of PAC-1 significantly delays the regrowth of cells after exposure to vemurafenib. Finally, PAC-1 remains effective in vemurafenib-resistant A375VR cells in culture and synergizes with vemurafenib to retard tumor growth of these cells in vivo, suggesting utility of this combination in melanomas that have progressed beyond BRAF-inhibitor treatment, for which few options for treatment are currently available.

Materials and Methods

Cell culture and reagents

A375 (CRL-1619) and CHL-1 (CRL-9446) were purchased from the ATCC on November 5, 2014, and November 18, 2014, respectively. A375SM was provided by Professor Isaiah Fidler (MD Anderson, Texas) on October 30, 2014. All cell lines except B16-F10, H460, and HCT 116 were cultured in DMEM supplemented with 10% FBS (Gemini). B16-F10, H460, and HCT 116 were cultured in RPMI with 10% FBS. Vemurafenib, trametinib, and Annexin V–FITC (10040-02) were purchased from LC Laboratories, Med-ChemExpress, and SouthernBiotech, respectively. The following antibodies were purchased from Cell Signalling Technology: anti–PARP-1 (9542), anti–caspase-3 (9662), anti–β-actin (4967), anti-phospho-ERK1/2 (Thr202/Tyr204) (4370), anti-ERK1/2 (4695), and anti-rabbit IgG HRP linked (7074). Anti–cleaved-PARP-1 (ab32561) antibody was purchased from Epitomics. PAC-1 and PAC-1a were synthesized as previously reported (34).

Cell line authentication

All human cell lines (A375, A375SM, CHL-1, H460, HCT 116, MIA PaCa-2, SK-MEL-5, and UACC-62) have been authenticated using the PowerPlex16HS Assay (Promega): 15 Autosomal Loci, X/Y at the University of Arizona Genetics Core. The results of the test have been performed for the A375 cell line using the Mycoplasma detect PCR at the University of Illinois Veterinary Diagnostic Lab on May 13, 2015. Results can also be found in the Supplementary Information. Mycoplasma testing has been performed for the A375 cell line using the Mycoplasma detection assay by the sulforhodamine B (SRB) assay.

Cellular proliferation assays

A total of 1,000 to 2,000 cells were seeded per well in a 96-well plate and allowed to adhere before DMSO solutions of PAC-1 or vemurafenib were added to each well. Proliferation was assessed by Annexin V–FITC/PI staining after treatment with vemurafenib (10 μmol/L) and PAC-1 (12 μmol/L) for 24 hours, whereas this combination has negligible effect on cell lines with wild-type BRAF. Dashed horizontal lines represent the level of cell death expected from a mere additive effect of the two agents. Values are reported as mean ± SEM of at least three independent experiments. P values shown for two-way interaction to determine if the combination for induction of apoptosis is different from an additive effect (dashed horizontal lines) of individual agents are statistically significant (*, P < 0.05; **, P < 0.01; and ***, P < 0.001).

Annexin V/PI flow cytometry analysis

A total of 70,000 cells were seeded in 12-well plates and allowed to adhere before addition of compounds. Cells were treated with compounds for 24 hours at 37 °C, after which they were harvested and resuspended in 450 μL of cold buffer (10 mmol/L HEPES,
140 mmol/L NaCl, 2.5 mmol/L CaCl₂, pH 7.4) premixed with Annexin V–FITC and propidium iodide (PI: 0.55 μg/mL) dyes. Samples were analyzed on a BD Biosciences LSR II flow cytometer, and data analysis was performed using FCS Express V3.2.

**Caspase-3/7 activity assay**

A total of 5,000 to 8,000 cells were plated in 96-well plates and allowed to adhere. Cells were treated with 1 μmol/L of staurosporine for 24 hours or with 13 μmol/L of raptinal (37) for 3 hours as positive control, DMSO as negative control, and indicated concentrations of PAC-1 and vemurafenib for 0, 2, 4, 7, 10, 12, 16, 20, or 24 hours. Plates were then assessed for caspase-3/7 activity via addition of bifunctional lysis and activity buffer (200 mmol/L HEPES, 400 mmol/L NaCl, 40 mmol/L EDTA, 0.4 mmol/L EDTA, 1% Triton-X, pH 7.4) with 20 μmol/L of Ac-DEVD-AFC (Cayman Chemicals) as the fluorogenic substrate (Δλ₄₅₀ = 400 nm; Δλ₅₀₅ = 505 nm). Plates were preincubated at 37°C at 30 minutes in the Synergy multi-reader mode (BioTek) and then read for 30 minutes at 3-minute intervals. The slopes for each well were calculated. Activity is expressed as normalized to minimal and maximal activity observed within the assay.

**In vitro resistance assay**

A total of 800 A375 or UACC-62 cells were plated in 96-well plates and allowed to attach overnight. The next day, vemurafenib (5 or 10 μmol/L) or PAC-1 (1 μM) were treated in six technical replicates for 5, 10, or 20 days. Fresh media and compounds were added every 2-3 days for the duration of the study. At the end of 5, 10 or 20 days, the wells were fixed with 10% cold trichloroacetic acid for 1 h at 4°C. The wells were then washed, allowed to dry and stained with 0.5% SRB dye for 30 minutes at room temperature. The wells were then washed with 0.1% acetic acid and allowed to dry. At this point, images of the plates were taken with CellDoc XR (BioRad). Finally, 200 μL of 10 mmol/L Tris base (pH > 10.4) was added into well, and the absorbance at 510 nm was read using SpectraMax Plus (Molecular Devices). The absorbance at 510 nm is plotted against the days after treatment as an indication of cell proliferation over the time course of the experiment.

**Immunoblotting**

Cells and tumor tissues were lysed using RIPA buffer containing phosphatase and protease inhibitor cocktail (Calbiochem). The protein concentration of each sample was determined by the BCA assay (Pierce). Cell lysates containing 20 μg of protein were loaded into each lane of 4% to 20% gradient gels (BioRad) for SDS-PAGE. Proteins were transferred onto PDVF membrane for Western blot analysis.

**PCR and sequencing**

A375 and A375VR cells were lysed and RNA extracted using the RNeasy Kit (Qiagen). Note that 900 ng of RNA was used for reverse transcription reaction using the iScript cDNA synthesis Kit (BioRad). qPCR reactions were run on the 7900HT fast real-time PCR system (Applied Biosystems). Regular PCR reactions were run using the MyFi Mix PCR kit (Bioline) for 35 cycles and ran on a 1% agarose gel. Target amplicons were gel extracted and sequenced at the University of Illinois at Urbana-Champaign (UIUC) core sequencing facility. Primers used can be found in the Supplementary Information.

**A375 and A375VR xenograft model**

All animal studies were performed in accordance with University of Illinois at Urbana-Champaign (UIUC) Institutional Animal Care and Use Committee guidelines (protocol no. 14292). 0.1 mL of A375 or A375VR in 1:1 DEMEM/matrigel (Corning) was injected into the right flank of 6 to 7 (A375) or 5 (A375VR) weeks old female athymic nude mice (Charles River). In both the models, the mice were randomized into four groups: control, 100 mg/kg PAC-1, 10 mg/kg vemurafenib, and the combination of 100 mg/kg PAC-1 and 10 mg/kg vemurafenib. Initial tumor volume measurements were taken, and dosing was initiated for a period of 15 days. Vemurafenib was formulated as 5% DMSO in 1% methyl cellulose and given twice daily by oral gavage (p.o.). PAC-1 was formulated in 200 mg/mL hydroxypropyl-β-cyclodextrin at pH 5.5 and given by i.p. injection. Tumor length and width measurements were taken three times a week, and volume was calculated as 0.52 × L × W². At the end of the study, the mice were euthanized and tumors were excised. The tumors were weighed and used for Western blot and immunohistochemistry (IHC).

**IHC of A375 tumors and quantification of Ki-67 index**

IHC was performed on 4-μm-thick formalin-fixed paraffin-embedded A375 tumors after hematoxylin and eosin staining confirmed the presence of a neoplastic cell population along with adequate tissue integrity. Antibody against Ki-67 (Biocare Medical; #CRM325) was used for IHC, and staining was visualized using the IntelliPATH FLX DAB chromogen Kit (Biocare Medical; #IPK 5010 G80). Human tonsil was used as the positive control tissue. Polymer negative control serum (mouse and rabbit; Biocare Medical; #NC499) was substituted for the primary antibody as a negative control. For quantification of Ki-67 index, 2,000 neoplastic cells were counted, and the percentage of positive cells was calculated. In tumors too small to quantify 2,000 cells, the maximal number of neoplastic cells was counted. All slides were reviewed by a single veterinary pathologist (K.L. Wycislo).

**Results**

The combination of PAC-1 and vemurafenib enhances apoptosis in cells with the V600E BRAF mutation

In a panel of nine cell lines of diverse origins and BRAF-mutational status, vemurafenib is potent (IC₅₀ values between 200 and 550 mmol/L) only in cell lines harboring the V600E/BRAF mutation, consistent with previously reported values (Fig. 1B; ref. 3). Evaluation of PAC-1 in the same panel of cell lines shows that PAC-1 retains similar activity in all cell lines (IC₅₀ values between 1 and 4 mmol/L), regardless of BRAF mutational status (Fig. 1B). The ability of the combination of PAC-1+vemurafenib to induce apoptotic cell death was then assessed in these cell lines. Under conditions (24-hour incubation with compounds) where neither vemurafenib nor PAC-1 induced significant apoptotic death (≤10%) as single agents, the PAC-1+vemurafenib combination induces significant apoptosis (20%–45%) in cell lines with the V600E/BRAF mutation (Fig. 1C). A similar trend was also observed when a lower concentration of vemurafenib (0.5 μmol/L) was evaluated in combination with PAC-1 in V600E/BRAF cell lines (Supplementary Fig. S1). However, the PAC-1+vemurafenib combination does not induce synergistic apoptosis in cell lines with WT/BRAF (Fig. 1C).
PAC-1 and vemurafenib synergize to enhance caspase-3 activity and apoptosis in A375, SK-MEL-5, and UACC-62 cells

In order to more broadly explore the observed synergy, apoptotic death was assessed in three human V600E BRAF melanoma cell lines treated with a matrix of concentrations of PAC-1 and vemurafenib that induce minimal apoptosis as single agents. In these experiments, large increases in the populations of apoptotic cells (beyond the additive effect of single agents alone) were observed in A375 (Fig. 2A), SK-MEL-5 (Supplementary Fig. S2A), and UACC-62 (Supplementary Fig. S2B). To quantify the synergy of this drug combination, combination indices (CI) were calculated. A drug combination that is synergistic will have a CI value less than 1, whereas a value of 1 reflects an additive effect (38). Ninety-three percent of the calculated CI values are less than 1 (A375 in Fig. 2B, SK-MEL-5 in Supplementary Fig. S2B, and UACC-62 in Supplementary Fig. S3B), indicating synergism for the combination across all three cell lines tested.

To assess if the increase in apoptosis was a result of increased activation of executioner procaspases, caspase-3/7 enzymatic activity was evaluated in A375 cells (after lysis) using a fluorogenic substrate. In A375 cells treated with vemurafenib or PAC-1 alone (at the same concentrations used in Fig. 1C), negligible increases in caspase-3 activity were observed at these time points and concentrations (Fig. 2C). However, when A375 cells were treated with PAC-1 and vemurafenib, a significant increase in caspase-3 activity was observed as early as 7 hours after treatment (Fig. 2C). In Western blot analyses, neither of the single agents had an effect on PARP-1 cleavage at these time points and concentrations; however, the combination resulted in significant cleaved PARP-1 (Fig. 2D), a result of the increased caspase-3/7 activity in cells treated with the PAC-1-vemurafenib combination. After treatment with the combination for 24 hours, near-complete cleavage of PARP-1 was observed in A375 cells (Fig. 2D). Similar results for the caspase-3/7 activity assay and cleavage of PARP-1 were also observed in SK-MEL-5 (Supplementary Fig. S2C and S2D) and UACC-62 cells (Supplementary Fig. S3C and S3D).

The PAC-1 derivative PAC-1a (Fig. 1A) lacks the zinc chelating motif and thus does not activate procaspase-3 or induce apoptosis (18, 34). Use of PAC-1a in combination with vemurafenib did not result in a significant increase in the proportion of apoptotic cells (beyond the additive effect of single agents alone) were observed in A375 (Fig. 2B, SK-MEL-5 in Supplementary Fig. S2B, and UACC-62 in Supplementary Fig. S3B), indicating synergism for the combination across all three cell lines tested.
of cells undergoing apoptosis in A375, SK-MEL-5, or UACC-62 cells [Supplementary Fig. S4A–S4C]. This result is also consistent with the absence of increased PARP-1 cleavage in cells treated with the PAC-1a and vemurafenib combination (Supplementary Fig. S4D), indicating that the cells did not undergo apoptotic death.

**Inhibition of ERK1/2 phosphorylation and activation of procaspase-3 are required to enhance apoptotic cell death**

Consistent with the data in Fig. 1C, no enhancement in caspase-3 activity or PARP-1 cleavage was observed in two V600E BRAF cell lines when treated with the combination of PAC-1 + vemurafenib (Supplementary Fig. S5A—S5C). The lack of PAC-1 + vemurafenib synergy in cell lines harboring V600E BRAF suggests that inhibition of ERK1/2 and activation of procaspase-3 are both required to induce the dramatic enhancement of apoptotic cell death. Indeed, after 24 hours of treatment with vemurafenib, inhibition of ERK1/2 phosphorylation was not observed in V600E BRAF cell lines even at high concentrations (30 μmol/L) of vemurafenib (Supplementary Fig. S5B and S5C). This observation is consistent with previous reports where vemurafenib does not inhibit ERK1/2 phosphorylation in V600E BRAF cells, but paradoxically activates it (3). To further investigate this, A375 (harboring V600E BRAF) cells were treated with PAC-1, vemurafenib, or the combination and probed for the presence of cleaved PARP-1 and ERK1/2 phosphorylation. After 24 hours, phospho-ERK1/2 bands were not observed in cells treated with vemurafenib (at 0.5 and 1.0 μmol/L) and the combination (Fig. 2E). However, significant increases in the amount of cleaved PARP-1 were only observed in cells treated with both PAC-1 and vemurafenib (Fig. 2E). Similar results were also observed in SK-MEL-5 (Supplementary Fig. S2E) and UACC-62 cells (Supplementary Fig. S3E). At low concentrations of vemurafenib (0.1 and 0.25 μmol/L), where incomplete inhibition of ERK1/2 phosphorylation was observed, a slight increase in PARP-1 cleavage over that of single-agent effects was also observed (Fig. 2E). This result suggests that even with incomplete inhibition of ERK1/2 phosphorylation, procaspase-3 activation, which is downstream of ERK1/2 signaling, can be enhanced with the addition of PAC-1 to vemurafenib treatments. Taken together, the data show that procaspase-3 activation via PAC-1 dramatically enhances the proapoptotic effect of vemurafenib in cell lines with V600E BRAF mutation.

**Addition of PAC-1 to vemurafenib and trametinib enhances caspase-3 activity and apoptosis**

Addition of a MEK1/2 inhibitor, such as trametinib, is widely used in the clinic to enhance the efficacy of vemurafenib in V600E BRAF melanomas (8, 9). To explore the effect of PAC-1 with this combination, cells were treated with vemurafenib + trametinib, in the presence or absence of PAC-1, and apoptosis was assessed. In both A375 and UACC-62 cell lines, vemurafenib + trametinib cotreatment led to mere additive increases in the population of apoptotic cells (Fig. 3A). In contrast, the addition of PAC-1 led to a large increase in the population of apoptotic cells, beyond the additive effect of single agents alone (Fig. 3A). Vemurafenib + trametinib cotreatment did not lead to PARP-1 cleavage, whereas addition of PAC-1 led to near quantitative cleavage of PARP-1 (Fig. 3B). To explore if the increased apoptotic cell death in the presence of PAC-1 is a result of enhanced enzymatic activity of executioner caspases, the caspase-3/7 activity of A375 and UACC-62 cells treated with vemurafenib + trametinib, plus or minus PAC-1, was assessed. Again, a dramatic increase in caspase-3/7 activity was observed when PAC-1 was included, an effect that was absent without addition of PAC-1 (Fig. 3C).

The combination of vemurafenib and PAC-1 significantly reduces tumor burden in an A375 xenograft model

To determine the antitumor effect of the PAC-1 + vemurafenib combination in vivo, an A375 xenograft model (39) was used. In this model, nude mice were inoculated subcutaneously with A375 cells, and after allowing the tumors to grow, mice were randomized based upon tumor volume into four groups [F = 0.03 < Fcritical (3.01)] and dosed with PAC-1, vemurafenib, or the combination for 15 days. Treatment with PAC-1 alone led to minimal reduction in tumor mass and volume compared with untreated control mice (Fig. 4A and B). Mice dosed with vemurafenib alone experienced a moderate reduction (53%; P = 0.04) in tumor volume and mass compared with control (Fig. 4A and B), with 3 of 8 mice having comparable tumor mass as the control mice (Fig. 4B). In contrast, mice treated with the combination of PAC-1 and vemurafenib had significantly smaller tumor burden compared with control mice (Fig. 4A and B; Supplementary Fig. S6). In these mice, a 78% reduction in tumor volume was observed (Fig. 4A; P = 0.0008 vs. control), with 6 of 8 mice having tumors less than 0.2 g in mass (Fig. 4B), suggesting that addition of PAC-1 enhances the anti-tumor effects of vemurafenib in vivo and reduces the variability in response to treatment.

Examination of procaspase-3 levels in the tumor samples by Western blot showed an appreciable and consistent reduction in the amount of procaspase-3 only in tumor samples derived from mice that received the combination treatment, versus variable responses for the other dosing groups (Fig. 4C and D). Using immunohistochemical staining, a significant reduction in the percentage of Ki-67–expressing cells in tumors treated with PAC-1 + vemurafenib was observed (Fig. 4E), indicating that the PAC-1 + vemurafenib combination was capable of not only amplifying procaspase-3 activation, but also attenuating cell proliferation. Finally, in mice treated with PAC-1 + vemurafenib, no hematologic toxicities were observed (Supplementary Table S1), indicating a favorable safety profile for the combination.

Taken together, the in vivo data are consistent with the cell culture results showing that the synergy of PAC-1 + vemurafenib leads to increase in caspase-3 activity and induction of apoptotic cell death, as well as reduction in cell proliferation.

Long-term treatment with PAC-1 prevents cell regrowth, and addition of PAC-1 to vemurafenib delays the onset of cell regrowth.

The Fmax of vemurafenib (the percent cell death induced by high concentrations of compound; ref. 40) in A375 cells is 96.8% ± 0.3% after 5 days (Fig. 5A), indicating that approximately 3% of A375 cells are insensitive to vemurafenib. Under the same conditions, PAC-1 has an Fmax of 99.4% ± 0.7% (Fig. 5A), suggesting that PAC-1 kills A375 cells quantitatively, with very few insensitive cells. We therefore hypothesized that long-term treatment with vemurafenib would lead to regrowth of cancer cells, whereas treatment with PAC-1 should prevent regrowth. To investigate this hypothesis, A375 and SK-MEL-5 cells were plated at low densities and treated continuously with PAC-1 (4 μmol/L) or vemurafenib (10 μmol/L) for up to 30 days. In A375 and SK-MEL-5 cells treated
with vemurafenib, regrowth of cells was observed in as early as 20 days (Fig. 5B). However, in wells treated with PAC-1, no regrowth was observed even after 30 days (Fig. 5B). Thus, consistent with the higher E_{max} value, PAC-1 is able to quantitatively kill cells thereby preventing regrowth.

To investigate if addition of low concentrations of PAC-1 could combine with vemurafenib to prevent cancer cell regrowth, A375 and UACC-62 cells were plated at low densities in 96-well plates and treated continuously with PAC-1 (1 μmol/L), vemurafenib (10 μmol/L), or the combination for up to 20 days. After 5 days, treatment with PAC-1, vemurafenib, or the combination each resulted in significant reduction in cell number compared with the control (A375: Fig. 5C and D; UACC-62: Supplementary Fig. S7A and S7B). On day 10, there is no observable difference between the PAC-1–treated wells and the control. In wells treated with 5 or 10 μmol/L vemurafenib, cell death was 89.4% ± 1.4% and 93.2% ± 1.1%, respectively. However, in wells where A375 cells were treated with 1 μmol/L PAC-1 and 5 or 10 μmol/L vemurafenib, increased cell death was observed, 96.1% ± 1.0% and 97.9% ± 0.7% respectively. Consequent to achieving more complete cell death, a smaller proportion of cells remain in wells treated with both PAC-1 and vemurafenib. After 20 days of treatment, significant regrowth of colonies was observed in vemurafenib-only–treated wells but not in wells receiving the cotreatment (A375: Fig.
This result indicates that the more complete cell death induced by cotreating cells with PAC-1 and vemurafenib is effective in delaying the regrowth of A375 and UACC-62.

**PAC-1 synergizes with vemurafenib in vemurafenib-resistant melanoma in vivo**

To assess if PAC-1 remains active in a cell line that has acquired resistance to vemurafenib, a vemurafenib-resistant A375VR cell line was generated by growing A375 parental cell line in sequentially higher concentrations of vemurafenib (0.5 to 1.0 μmol/L) for 2 months. To determine the mechanism of resistance of A375VR, genes for MEK1/2, NRAS, and AKT were sequenced, but no commonly reported mutations that would confer resistance were found (41). Similarly, splice variant of the V600E BRAF mRNA was also not observed (42). Through qPCR, A375VR cells have approximately 3-fold higher levels of MDRI mRNA compared with A375. However, compared with up to 1,000-fold higher levels of MDRI mRNA in ovarian cells resistant to doxorubicin or cisplatin (43), the level of MDRI mRNA overexpression is

Figure 4.
The PAC-1-vemurafenib combination retards tumor growth in an A375 subcutaneous mouse xenograft model of melanoma. A, the effect of PAC-1, vemurafenib, and their combination in the A375 model. Mice bearing subcutaneous tumors were dosed for 15 days. Mice were dosed with PAC-1 once daily at 100 mg/kg (n = 6) via i.p. injection, vemurafenib twice daily at 10 mg/kg (n = 8) by p.o., or the PAC-1-vemurafenib combination (n = 8). The black line below the x-axis indicates the dosing period for the mice during the study. Tumor volumes are plotted as mean ± SEM. B, masses of the excised tumors. C, tumor lysates were analyzed by Western blot for changes in procaspase-3 levels. Actin was used as loading control. Band intensity was quantified using ImageJ. D, plot of procaspase-3 levels normalized to the actin loading controls. E, percentage of cells that are positive for Ki-67 following immunohistochemical staining of formalin-fixed tumor samples. A total of 2,000 cells were counted in each sample for each of the four treatment groups. P values shown are with respect to control mice (*, P < 0.05; **, P < 0.01; and ***, P < 0.001).
considered low, indicating that resistance is unlikely due to dramatic upregulation of MDR phenotype.

Vemurafenib kills the A375VR cell line with a 5-day IC50 value of 1.5 μmol/L, 12-fold less potent compared with the sensitivity of the parental A375 (Fig. 6A). Moreover, the vemurafenib Emax for A375VR is 79% ± 6.3%, which is 14% lower than the parental A375 cell line. Although treatment of parental A375 cells with vemurafenib (0.5 or 1 μmol/L) for 2 hours results in complete inhibition of ERK1/2 phosphorylation, this effect is not observed in A375VR, consistent with resistance of A375VR to vemurafenib and continued MAPK signaling (Fig. 6B). In contrast, PAC-1 retains activity against A375VR with an IC50 value of 2.4 μmol/L (vs. 1.2 μmol/L for the parental cell line; Fig. 6C) and a similar Emax. We hypothesized that despite the inability of vemurafenib to inhibit ERK1/2 phosphorylation and MAPK signaling in the resistant A375VR cell line, the combination might retain partial capacity to exert a synergistic effect based on the PARP-1 cleavage observed for PAC-1 + vemurafenib treatment, even under conditions of incomplete inhibition of ERK1/2 phosphorylation (Fig. 2E). To investigate if PAC-1 can resensitize A375VR cells to vemurafenib-induced apoptosis, A375VR cells were treated with PAC-1 in combination with low concentrations of vemurafenib. This combination treatment led to an increase in the proportion of cells undergoing apoptosis (Supplementary Fig. S8A and S8C), suggesting that the addition of PAC-1 can bypass the resistance mechanism of A375VR to vemurafenib. This effect was abolished when inactive variant PAC-1a was used (Supplementary Fig. S8C). The PAC-1 + vemurafenib combination was synergistic, inducing an

Figure 5.
Low concentrations of PAC-1 (1 μmol/L) significantly delay cell regrowth in combination with vemurafenib in long-term cell culture experiments. A, comparison of Emax values in A375 cells treated with vemurafenib and PAC-1. B, A375 and SK-MEL-5 cells treated with PAC-1 (4 μmol/L) or vemurafenib (10 μmol/L) for a duration of 30 days. C, A375 cells were treated with PAC-1 (1 μmol/L), vemurafenib (5 or 10 μmol/L), or the combination. After 5, 10, or 20 days, the wells were fixed with 10% trichloroacetic acid, stained with 0.5% sulforhodamine B (SRB) dye, and imaged with BioRad GelDoc RX. Day 20 images of control and PAC-1 samples are not shown because the cells were unviable due to overcrowding. D, quantification of C where the SRB dye is dissolved in 10 mmol/L Tris base at pH 10.4, and the absorbance read at 510 nm. Corrected absorbance at 510 nm was plotted against the days of continuous treatment by normalizing against absorbance on day 0 before the start of treatment. Values are reported as mean ± SEM of at least three experiments. T test performed between wells treated with vemurafenib only versus vemurafenib and PAC-1 (1 μmol/L). On day 10, only the wells treated with vemurafenib (10 μmol/L) and PAC-1 (1 μmol/L) are significantly different from vemurafenib (10 μmol/L) only (P = 0.049) treatment. On day 20, wells treated with vemurafenib (5 or 10 μmol/L) and PAC-1 (1 μmol/L) are significantly different from vemurafenib (5 or 10 μmol/L), as indicated (*, P < 0.05; **, P < 0.01; and ***, P < 0.001).
average of 7.5% higher population of apoptotic cells than predicted by the Bliss independence model (ref. 44; Supplementary Fig. S8A and S8B). Finally, to determine if PAC-1 can synergize with vemurafenib in vivo, A375VR cells were implanted subcutaneously in nude mice, and the mice were dosed daily for 15 days with vemurafenib (10 mg/kg), PAC-1 (100 mg/kg), or the combination. Treatment with vemurafenib or PAC-1 alone does not exert any antitumor affect in this in vivo model, whereas treatment with the PAC-1 þ vemurafenib combination led to significant reduction in tumor volume compared with the untreated control (Fig. 6D).

Discussion

Given that the aberrations in the apoptotic signaling cascades in melanoma cells are upstream of the activation of procaspase-3, small molecules that directly activate procaspase-3 can induce apoptosis by bypassing the defective apoptotic circuitry. Activation of procaspase-3 with PAC-1 has been shown previously to have single-agent efficacy against melanoma cells in culture (21, 33, 34), and now we show that PAC-1 þ vemurafenib, or PAC-1 þ vemurafenib þ trametinib, are powerfully synergistic in the induction of caspase-3 activity and apoptotic cell death in melanomas with V600E BRAF mutation. Besides melanomas, the V600E BRAF mutation has been reported in several other cancers, including Erdheim–Chester disease (ECD, 54%; ref. 45), Langerhans’-cell histiocytosis (LCH, 57%; ref. 45), non-small cell lung cancer (NSCLC, 1.5%; ref. 46) and hairy-cell leukemia (100%; ref. 47). In two recent phase II trials, efficacy of vemurafenib in several nonmelanoma cancers harboring the V600E BRAF mutation was reported, with promising results seen in patients with NSCLC, ECD, LCH, and refractory hairy-cell leukemia (48, 49). Given these clinical data and our current work showing potent synergy between PAC-1, vemurafenib, and trametinib in V600E BRAF melanomas, these PAC-1/drug combinations could have efficacy in other malignancies harboring the V600E BRAF mutation.

The Emax parameter is a useful metric to assess the ability of a compound to quantitatively kill cancer cells in culture (40); Emax values less than 100% imply heterogeneity in the ability of the drug to kill the cancer cell population. Here, we show that vemurafenib has an Emax of approximately 97% in V600E BRAF-mutant A375 cells, but the Emax value for PAC-1 approaches 100%. Because of this, no regrowth of A375 or SK-MEL-5 cells is observed in long-term experiments with PAC-1. However, extensive regrowth was observed in A375, UACC-62, and SK-MEL-5 cells treated only with vemurafenib for 20 days. With the addition of a low concentration of PAC-1 (1 μmol/L) to vemurafenib, little to no regrowth was observed in cells. These results suggest that addition of low concentrations of PAC-1 (1 μmol/L, a
PAC-1 concentration that is readily achieved in vivo; ref. 50) could be effective clinically in delaying resistance. The significant increase in caspase-3 activity, followed by massive induction of apoptosis early on during the combination treatment, likely kills off a large proportion of the cells that were initially insensitive to vemurafenib. Consequently, there is a significantly smaller residual population of cells that are unaffected by the treatment, crucial to delaying the regrowth of cells.

Currently, few options exist for patients who have developed vemurafenib-resistant melanomas. The MEK1/2 inhibitor, trametinib, though approved for melanomas with V600E BRAF mutation, exerts limited activity in combination with BRAF inhibitor in patients who have failed prior therapy (10). Our results show that PAC-1 still synergizes with vemurafenib to exert antitumor effects in vemurafenib-resistant tumors. Therefore, addition of PAC-1 might be a viable and alternative therapeutic option for patients whose melanomas have progressed after vemurafenib treatment. The PAC-1-vemurafenib combination is well tolerated, has a good safety profile, and exhibits significant antitumor effects in vivo. PAC-1 is currently in a phase I clinical trial (NCT02355535), and both vemurafenib and trametinib are approved first-line treatment for V600E BRAF melanoma. There is thus a clear path to translate the preclinical demonstration of synergy described in this work to clinical trials where this novel combination can be assessed in human patients with cancers harboring the V600E BRAF mutation.

Disclosure of Potential Conflicts of Interest
T.M. Fan has ownership interest (including patents) in, and is a consultant/advisory board member for, Vanquish Oncology. P.J. Hergenrother is Founder/CSO of, has ownership interest (including patents) in, has received a commercial research grant from, and is a consultant/advisory board member for, Vanquish Oncology. No potential conflicts of interest were disclosed by the other authors.

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Conception and design: J. Peh, P.J. Hergenrother Development of methodology: J. Peh, P.J. Hergenrother Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Peh, T.M. Fan Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Peh, T.M. Fan, P.J. Hergenrother Writing, review, and/or revision of the manuscript: J. Peh, T.M. Fan, K.L. Wycislo, H.S. Roth, P.J. Hergenrother Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K.L. Wycislo Study supervision: P.J. Hergenrother Other (overseas immunohistochemical staining procedures and performed all immunohistochemical analysis/quantification): K.L. Wycislo Other (synthesis of compounds): H.S. Roth

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Vemurafenib and PAC-1 Are Synergistic in Mutant BRAF Melanomas


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