Potentiation of a Topoisomerase I Inhibitor, Karenitecin, by the Histone Deacetylase Inhibitor Valproic Acid in Melanoma: Translational and Phase I/II Clinical Trial

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Abstract

Purpose: The novel topoisomerase I inhibitor karenitecin (KTN) shows activity against melanoma. We examined whether histone deacetylase inhibition could potentiate the DNA strand cleavage, cytotoxicity as well as the clinical toxicity, and efficacy of KTN in melanoma.

Experimental Design: Apoptosis, COMET, and xenograft experiments were carried out as described previously. A phase I/II trial of valproic acid (VPA) and KTN was conducted in patients with stage IV melanoma, with any number of prior therapies, Eastern Cooperative Oncology Group performance status 0-2, and adequate organ function.

Results: VPA pretreatment potentiated KTN-induced apoptosis in multiple melanoma cell lines and in mouse A375 xenografts. VPA increased KTN-induced DNA strand breaks. In the phase I/II trial, 39 patients were entered, with 37 evaluable for toxicity and 33 evaluable for response. Somnolence was the dose-limiting toxicity. The maximum tolerated dose for VPA was 75 mg/kg/d; at maximum tolerated dose, serum VPA was ~200 μg/mL (1.28 mmol/L). At the dose expansion cohort, 47% (7 of 15) of patients had stable disease; median overall survival and time to progression were 32.8 and 10.2 weeks, respectively. Histone hyperacetylation was observed in peripheral blood mononuclear cells at maximum tolerated dose.

Conclusion: VPA potentiates KTN-induced DNA strand breaks and cytotoxicity. VPA can be combined at 75 mg/kg/d for 5 days with full-dose KTN without overlapping toxicities. In metastatic poor prognosis melanoma, this combination is associated with disease stabilization in 47% of patients. Further testing of this combination appears warranted.

Despite recent advances in our understanding of melanoma, this is a disease without effective therapy (1). We recently reported on a phase II trial of a novel topoisomerase (topo) I inhibitor, karenitecin (KTN), in metastatic melanoma (2). Given the synergy between histone deacetylase (HDAC) inhibitors and other classes of antineoplastics (especially topo II inhibitors; ref. 3), we examined if HDAC inhibition could potentiate the cytotoxicity and strand breakage caused by topo I inhibitors and if this combination could be tolerated and result in greater antitumor efficacy clinically.

HDAC inhibitors (HDACi) have emerged as an important class of agents for cancer therapy (4–6). These agents bind reversibly to the active site of HDAC (7), inhibiting it and causing histone hyperacetylation, chromatin decondensation, growth arrest, and differentiation (8–10). HDACi appear to be active as single agents in cutaneous T-cell lymphoma but not in common solid tumor malignancies (5). HDACi can potentiate the cytotoxicity of topo II inhibitors (3, 11, 12). The synergy appears to result from increased accessibility of DNA to topo inhibitors (3). The synergy appears to be sequence specific (13). Indeed, a recent phase I trial of the HDACi valproic acid (VPA) and epirubicin showed unexpected clinical efficacy in malignancies such as melanoma where previous trials have shown a lack of efficacy of anthracyclines (14). HDACi can inhibit different isotypes of these enzymes, so vorinostat may be more selective to HDAC 1, whereas VPA may show selectivity for HDAC 2 (15).

Materials and Methods

Chemicals and cell lines. VPA was purchased from Sigma-Aldrich. KTN was supplied by BioNumerik. All cell lines were purchased from the American Type Culture Collection and maintained in DMEM (Fisher Scientific) with 10% heat-inactivated fetal bovine serum

Translational Relevance

The preclinical data and phase I/II clinical trial described in this article explore the combination of a histone deacetylase inhibitor (HDACi) with a topoisomerase (topo) I inhibitor in melanoma. We show that HDACi pretreatment potentiates topo I inhibitor cytotoxicity in melanoma. Combined HDACi and topo I inhibitor treatment leads to increased DNA breakage compared with topo I treatment alone. The HDACi valproic acid could be combined at an active dose with a full dose of the topo I inhibitor, karenitecin. Histone hyperacetylation was observed in peripheral blood mononuclear cells. The combination was well tolerated. This study shows that a HDACi/topo I combination, using the agents described, maintains dose intensity and results in HDAC inhibition. This combination could have application not only for chemotherapy refractory cancers such as melanoma but perhaps also for malignancies where topo I inhibitors are more active such as colon cancer or ovarian cancer.

(Sigma-Aldrich), 2 mmol/L glutamine, and 50 units/mL penicillin and 50 μg/mL streptomycin (Fisher Scientific). Cells were incubated in a humidified atmosphere with 5% CO_2 at 37°C.

Apoptosis assays. Apoptosis assays were carried out as described previously (16). Briefly, log-phase cells were pretreated with 2 mmol/L VPA (A375 cells) or 5 mmol/L VPA (A2058, SK-Mel-5, and SK-Mel-28 cells) for 48 h followed by a 2 h treatment with KTN. Apoptosis was determined 24 h later. Cell nuclei were stained with 0.5 μg/mL bisbenzimide (Hoechst 33258). Apoptosis was scored by the presence of nuclear chromatin condensation and DNA fragmentation and evaluated by fluorescence microscopy. Two hundred cells were counted per experiment in at least six different fields (apoptotic nuclei/all nuclei $\times$ 100). For the sequence experiments, log-phase cells were pretreated, post-treated, or treated concurrently with 2 mmol/L VPA for 48 h and two doses of KTN for 2 h. Apoptosis was determined 24 h after KTN exposure for the pretreatment group or at the end of the VPA treatment for the post-treatment and concurrent VPA treatment groups.

Single-cell gel electrophoresis (COMET) assay. The alkaline COMET assay was done as described by the manufacturer (Trevigen) and as described previously to show single- and double-strand DNA breaks (3, 17–19). A375 melanoma cells were treated with 2 mmol/L VPA followed by 1.5 h incubation with 0, 10, 50, or 500 nmol/L KTN. Cells were harvested by trypsinization and washed with cold PBS. Cells (5×10^3) were resuspended in 1% low-melting agarose (Bio-Rad) and layered on frosted glass slides. The slides were lysed (2.5 mol/L NaCl, 100 mmol/L EDTA, 10 mmol/L Tris-HCl, 1% sodium sarcosinate, 10% DMSO, 1% Triton X-100 containing 25 g/mL proteinase K) for 0.5 h at 4°C. The lysis buffer was removed, and the slides were washed four times with alkaline wash buffer [300 mmol/L NaOH, 1 mmol/L EDTA (pH 12.2)]. COMET tails were induced by electrophoresis for 20 min at 25 V in alkaline wash buffer. Slides were reequilibrated in TBE buffer [90 mmol/L Tris-HCl, 90 mmol/L boric acid, 2 mmol/L EDTA (pH 8.0)] for 1 h and stained with SYBR Green (Molecular Probes). Fifty images per slide were acquired (Vysis) and analyzed using ImageQuant
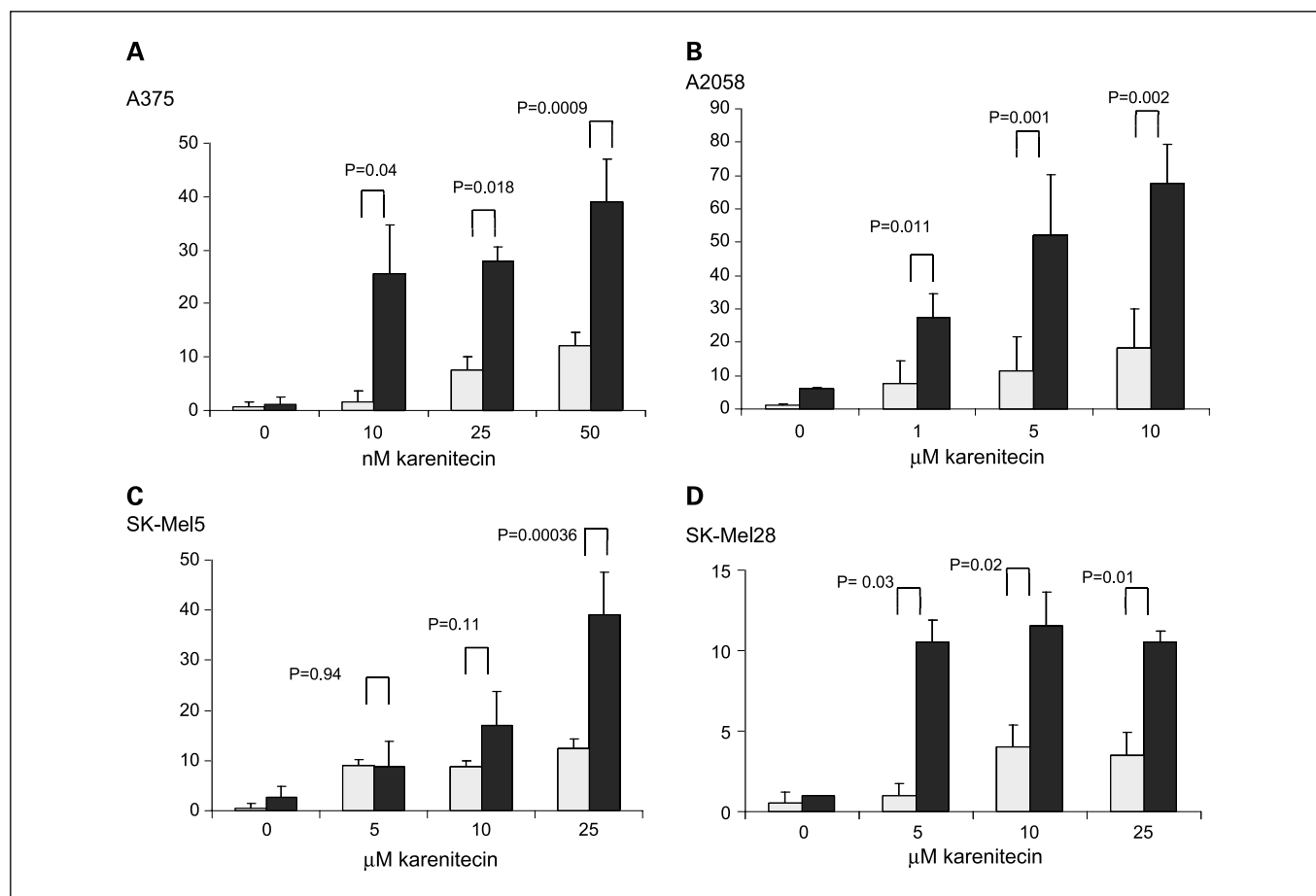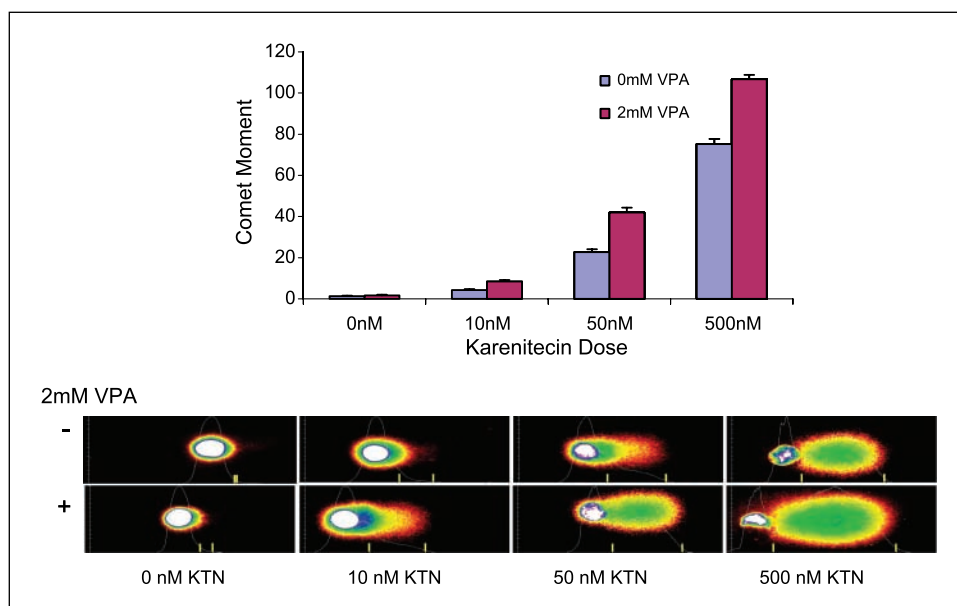

Fig. 1. Combination of VPA and KTN in melanoma. A375, A2058, SK-Mel-5, or SK-Mel-28 cells were pretreated for 48 h with VPA (0, 2, or 5 mmol/L) and KTN for 2 h and harvested, and % apoptosis was determined. *Bars,* SE. Results of two-tailed *t* tests comparing VPA-treated and untreated cells.

Fig. 2. DNA damage induced by topo I inhibitor with or without prior HDACi. Log-phase A375 cells were treated with or without 2 mmol/L VPA followed by a 1.5 h incubation with 0, 10, 50, or 500 nmol/L KTN. DNA damage was detected by alkaline COMET assay (sample assay at the *bottom*). Results from 50 images were plotted for each condition (*top*). Results of two-tailed *t* tests comparing VPA-treated and untreated cells.

software (Molecular Dynamics). The COMET moment was calculated using the following equation: COMET moment = 0 - n [(intensity of DNA at distance X) × (distance)] / intensity of total DNA. Experiments were repeated at least three times.

Xenograft studies. Female athymic nude mice were inoculated with 3×10^6 A375 cells suspended in Matrigel (1:1) subdermally on the left or right flank. Mice (six per cohort) were treated intraperitoneally with 0 or 500 mg/kg/d VPA given twice daily for five doses starting on day 1 followed by 0.8 mg/kg/d KTN given twice daily for four doses. Tumor volumes and weights were assessed four times weekly and calculated according to the following formula: $V = 0.52 \times a \times b^2$, where a is largest diameter and b is smallest diameter.

Trial design. This phase I/II trial was approved by the Scientific Review Committee and Institutional Review Board at the Moffitt Cancer Center and the University of South Florida, respectively. Patients received VPA on days 1 to 5 and KTN on days 3 to 8 of a 21-day cycle. The study was registered at the National Cancer Institute cancer trials Web site.[4] The trial was opened on March 2005 and completed accrual on April 2007.

Patients. Eligible patients had histologically documented, American Joint Committee on Cancer stage IV melanoma, Eastern Cooperative Oncology Group performance status of 0-2, and adequate bone marrow and renal and hepatic function [WBC >3.0×10^9/L, absolute neutrophil count >1.5×10^9/L, hemoglobin >9.0 g/dL, platelets >100×10^9/L, creatinine levels <1.5 times the upper limit of normal, bilirubin and serum glutamic oxaloacetic transaminase (aspartate transaminase) <1.5 times the upper limit of normal]. Patients with uncontrolled brain metastases or a history of seizure, women who were pregnant or nursing, and patients on medications known to interact with VPA were excluded.

Study treatment. VPA (Depakote; Abbott Labs) was given orally in two divided doses for 5 days. KTN was infused over 1 h daily for 5 days beginning on day 3 of each 3-week cycle. VPA (mg/kg/d) and KTN (mg/m²/d) were escalated as follows: by standard dose escalation procedure with 3 patients per cohort and expansion to 6 patients on occurrence of dose-limiting toxicities (DLT): 30/0.8 (cohort 1), 45/0.8 (cohort 2), 60/0.8 (cohort 3), 60/1 (cohort 4), 90/1 (cohort 5), and 75/1 (cohort 6). DLT were defined as first-cycle grade ≥3 (Common Terminology Criteria for Adverse Events version 3.0) nonhematologic toxicities, grade 3 thrombocytopenia >7 days, any grade 4 hematologic

toxicity (with the exception of asymptomatic grade 4 neutropenia or leucopenia lasting <8 days), or any toxicity requiring a dose delay of ≥2 weeks. Patients who were not evaluable for toxicity in the first cycle were replaced. To determine a possible interaction between KTN and VPA, patients in cohorts 1 to 4 did not receive VPA during cycle 2 of the trial.

Table 1. Demographic and patient characteristics

Characteristics	n (%)
Patients entered	39 (100)
Dose escalation	20 (51)
Maximum tolerated dose expansion	19 (49)
Assessable for toxicity	37 (95)
Assessable for response	33 (87)
Age (y), median (range)	61 (29-89)
Eastern Cooperative Oncology Group performance status	
0	9 (23)
1	24 (62)
2	6 (15)
Gender	
Male	29 (74)
Female	10 (26)
Race	
White	32 (82)
Other	7 (18)
Primary site	
Skin	31 (80)
Mucosal	2 (5)
Ocular	6 (15)
Prior chemotherapy regimens	
None	4 (10)
1	17 (44)
2	11 (28)
3	4 (10)
Disease stage	
IVA	9 (23)
IVB	5 (13)
IVC	24 (61)
Central nervous system metastasis	
Yes	6 (15)
No	33 (85)

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[4] cancer.gov (registration no. NCT00358319).

Table 2. Toxicity profile and DLT

Overall and DLT in the first cycle of the dose escalation cohort

Cohort (VPA/KTN)	Patient	Age	Prior regimen	DLT type/grade	All grades			
					Gastrointestinal			
					Nausea	Vomit	Diarrhea	Constipation
1 (30/0.8)	1	62	2	—	1	0	1	0
	2	77	1	—	0	0	0	0
	3	32	3	—	1	0	0	2
	4	72	1	—	0	0	0	0
2 (45/0.8)	5	37	3	—	0	0	0	0
	6	61	1	—	0	0	0	0
	7	59	1	—	0	0	0	0
3 (60/0.8)	8	49	0	—	1	0	0	1
	9	57	1	—	0	0	1	1
	10	66	3	—	0	0	0	1
4 (60/1.0)	11	55	2	—	1	1	0	1
	12	59	3	—	0	0	0	2
	13	63	1	—	0	0	0	2
	14	56	1	—	0	0	0	0
	15	33	1	—	0	0	0	1
	16	51	1	—	0	0	0	0
5 (90/1.0)	17	63	2	—	2	0	0	1
	18	52	0	Neuro/3	0	0	1	0
	19	89	0	Neuro/3	1	0	1	0

Toxicity in any cycle in the dose expansion cohort (VPA 75 mg/kg/d)

Patient	Age	Prior regimens	Worst grade toxicity considered probable or likely related to treatment				
			Constitutional	Gastrointestinal			
			Fatigue	Nausea	Vomit	Diarrhea	Constipation
20	57	1	1	1	0	1	0
21	74	2	1	1	0	0	1
22	50	2	0	0	0	1	0
23	65	1	0	0	0	0	0
24	29	1	0	1	0	1	1
25	69	2	1	2	1	0	1
26	67	2	1	0	0	0	0
27	67	2	0	1	0	0	0
28	38	4	0	1	1	0	0
29	71	3	0	0	0	0	0
30	62	3	0	0	0	0	0
31	71	3	0	1	1	0	0
32	69	0	1	1	1	0	0
33	62	0	1	2	1	0	1
34	57	3	1	0	0	0	0
35	42	2	1	1	0	0	1
36	49	2	0	1	2	0	1

(*Continued on the following page*)

Treatment assessment. Baseline evaluations included history, physical examination, and complete blood count with differential, metabolic, hepatic, and renal function assessments. Because VPA-related pancreatitis has been reported, amylase and lipase levels were also checked. These tests were repeated weekly during the first two cycles and then once every 3 weeks in all succeeding cycles. A 12-lead ECG was done on day 1 of VPA treatment to rule out preexisting QT-prolongation. Somnolence and confusion were assessed according to Common Terminology Criteria for Adverse Events version 3 before and after VPA treatment via mini-mental status tests. Disease restaging was done every two cycles (6 weeks).

Pharmacokinetic studies. Samples for evaluation of VPA pharmacokinetics were collected before and 4 h after the morning dose immediately before KTN infusion on day 3 of cycle 1. Blood samples (5 mL) were collected in heparinized tubes, processed within 30 min after collection, and stored at -20°C. Total and free fraction drug concentrations for therapeutic monitoring of VPA were determined by Quest Diagnostics (Nichols Institute). Fluorescence polarization assaying was done by an Abbott TDxFLx instrument for free levels and by an Abbott AxSym for total levels. Free fractioned plasma for free VPA testing was obtained by using Amicon Centrifree filtration devices (Millipore). Calibration was linear from 1 to 150 μg/mL for total VPA and from 2 to 25 μg/mL for free VPA. Dilutions were made for all samples outside of these ranges as these have been validated previously. Inter- and intra-assay variability was <5% for both tests. Unknown sample concentrations were extrapolated from the regression analysis of the standard curves.

Table 2. Toxicity profile and DLT (Cont'd)

| All grades | | | | | |
| Neurological | | | Hematological | | |
Somnolence	Confusion	Ataxia	Absolute neutrophil count	Hemoglobin	Platelet
1	0	0	2	1	0
0	0	0	0	0	0
0	0	0	1	2	0
0	0	0	0	0	0
0	0	0	3	3	1
1	0	0	0	0	0
0	0	0	0	2	0
1	0	0	3	0	0
1	0	0	2	2	0
1	0	0	0	2	0
1	0	0	0	1	0
2	2	0	3	2	0
2	0	1	0	2	1
0	0	0	0	0	0
1	0	0	0	3	0
0	0	0	0	0	1
2	0	0	3	2	0
3	3	2	0	0	0
1	3	1	0	1	0

| Worst grade toxicity considered probable or likely related to treatment | | | | | | |
| Neurological | | | Hematological | | | |
Somnolence	Confusion	Ataxia	Absolute neutrophil count	Neutropenia	Hemoglobin	Platelet
1	0	0	0	0	1	0
0	1	1	4	0	2	3
1	0	0	3	0	2	1
2	0	0	4	0	4	3
1	0	0	2	0	4	1
1	0	0	3	0	2	1
2	0	0	0	0	0	1
2	1	0	3	0	1	0
2	0	0	3	0	4	3
2	1	1	4	2	0	3
2	0	0	0	1	1	0
2	1	1	4	2	1	3
2	1	0	1	0	0	0
2	0	0	2	0	1	0
1	0	0	2	0	1	0
2	1	0	3	0	2	2
2	1	0	0	0	2	1

Abbreviations: DLT, dose limiting toxicity; KTN, karenitecin; VPA, valproic acid.

KTN concentrations were determined in plasma by high-performance liquid chromatography using a modification of the previously published method by Smith et al. (20). Heparinized blood samples (7 mL) were obtained at 0, 1, 2, 4, 6, 24, 25, 48, 49, 72, 73, 96, 97, 100, 102, and 120 h after infusion. Samples were prepared using solid-phase extraction in a 96-well plate format. Varian solid-phase extraction C18 well plates were used with the Varian Captivac 96-well solid-phase extraction apparatus. Before extraction, all standards, controls, and unknowns were diluted 1:1 with 5% hydrochloric acid followed by the addition of the internal standard, topotecan. All solid-phase extraction steps were carried out with 200 µL volumes, except for elution, which was 120 µL. Wells were conditioned with methanol and water followed by the loading of the plasma sample. Wells were washed with water followed by 60% methanol in water. Elution was achieved with a mobile phase of 0.01 mol/L ammonium acetate and acetonitrile. The eluted solid-phase extraction samples were injected to an Agilent Technologies 1100 high-performance liquid chromatography system using the aforementioned mobile phase. Separation was carried out on a C18 column at a flow rate of 1.0 mL/min with a gradient profile starting at 60% acetonitrile, holding for 3 min, then increasing to 90% acetonitrile across 4 min, and then returning to 60% acetonitrile over 1 min with a total run time of 15 min. Fluorescence detection was monitored at an excitation wavelength of 370 nm and emission of 430 nm. The method is linear from 0.5 to 50 ng/mL. Inter- and intra-assay variability was <6% and 12.3%, respectively, and the assay had a relative mean error of <10%. Unknown sample concentrations were

extrapolated from the regression analysis of the standard curves. This methodology was tested and validated under current International Conference on Harmonization/Food and Drug Administration criteria according to the Guidance for Industry on Bioanalytical Method (21).

Histone H4 and H3 acetylation. Peripheral blood mononuclear cells were isolated with Ficoll centrifugation (Ficoll-Paque; GE HealthCare). Mononuclear cells were lysed in SDS lysis buffer containing 2% SDS, 0.06 mol/L Tris (pH 6.8), and 10% glycerol. Protein (25 μg) was separated by gel electrophoresis, and acetylated H3 and H4 histones were visualized using anti-acetylated histone H3 and H4 (Upstate Biotechnology; polyclonal, 1:2,000) by Western blot analysis as described previously (13). GAPDH (Chemicon; monoclonal) was used as a loading control and also provided a standard for comparing signal from gel to gel. MCF-7 cells (25 μg) treated with or without 2 mmol/L VPA for 48 h were used to standardize the H3 and H4 signal.

Statistical analyses. Descriptive statistics were used to summarize the study patients. Toxicity was graded by Common Terminology Criteria for Adverse Events version 3.0, and tumor response was defined by the Response and Evaluation Criteria in Solid Tumors criteria (22). SPSS version 16 (SPSS) was used to plot Kaplan-Meier survival distributions.

Results

VPA and KTN combination in melanoma cell lines. Melanoma cell lines were treated with either KTN alone or both KTN and VPA. As shown in Fig. 1, an increase in KTN-induced apoptosis was seen in A375 (13-fold), SK-Mel-5 (3-fold),

A2075 (10-fold), and SK-Mel-28 (11-fold) when cells were pretreated with VPA compared with KTN alone. These results were essentially unchanged if vorinostat was used instead of VPA or topotecan was used instead of KTN (data not shown). In addition, we also performed clonogenic assays following KTN alone or VPA followed by KTN in the A375 cell lines. We found that the IC_{50} for KTN alone was 2.95 mmol/L, whereas the IC_{50} for combined VPA and KTN was 0.86 nmol/L KTN with 0.125 mmol/L VPA. We tested whether schedule was important with four conditions: KTN only, simultaneous VPA and KTN, VPA followed by KTN, or KTN followed by VPA. As shown in Supplementary Fig. S1, pre-exposure with VPA and simultaneous treatment with both agents were more effective than post-exposure with VPA. Next, we evaluated if this combination was effective in animal xenografts. Female athymic mice with A375 xenografts were treated with saline, VPA, KTN, or VPA followed by KTN (Supplementary Fig. S2). Reduced tumor volume was seen in mice pretreated with VPA followed by KTN compared with the other treatments. Because the mechanism of action of KTN involves binding to DNA and causes single-strand breaks, we tested whether pretreatment with the HDACi could potentiate DNA breakage (Fig. 2). In this experiment, pretreatment with either no VPA or 2 mmol/L VPA was followed by exposure to KTN in the alkaline COMET assay as reflected in the lengthened COMET moment (*bottom*) and by
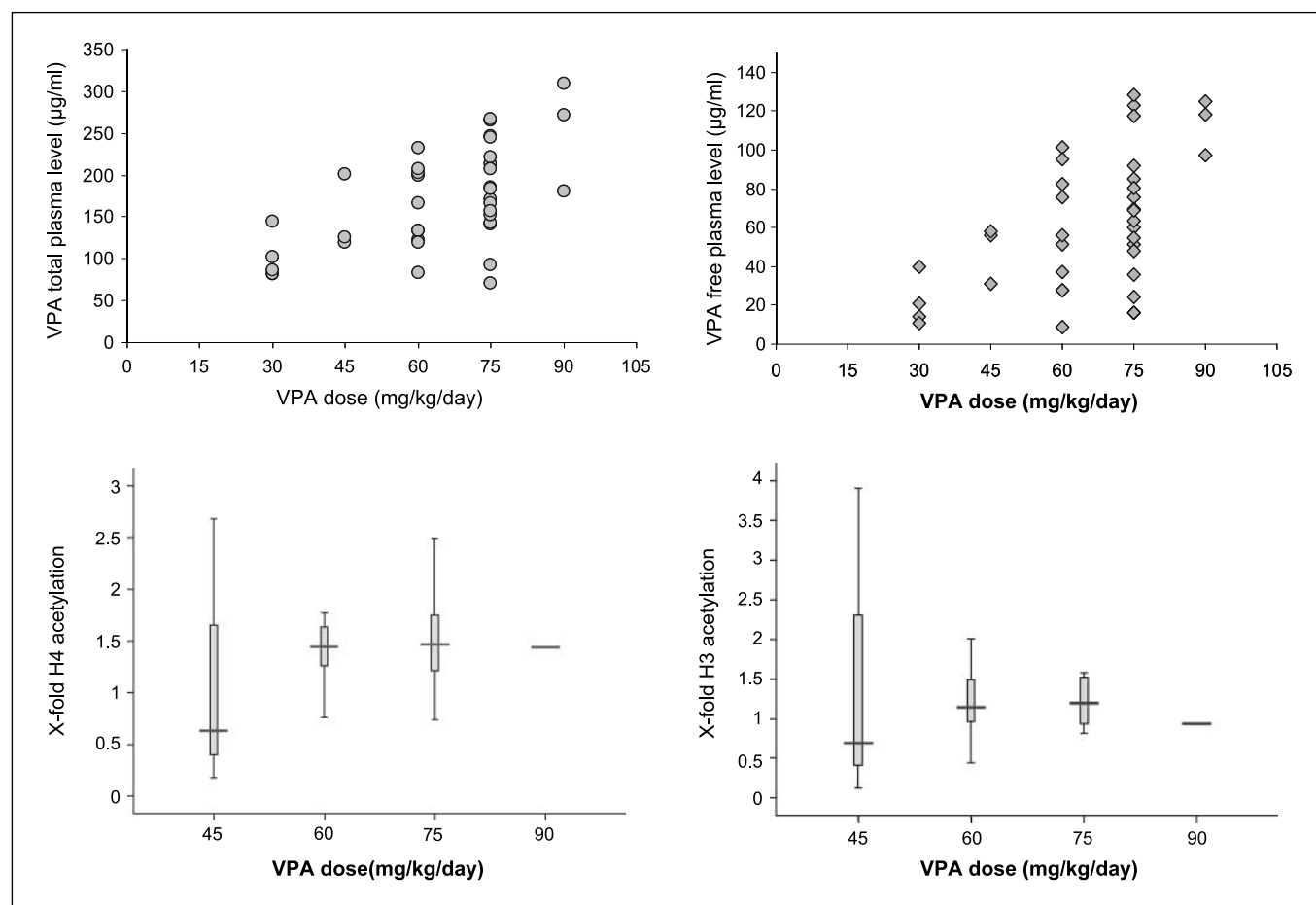

Fig. 3. Pharmacokinetic profiles of VPA and increases in histone acetylation. VPA total and free plasma levels are shown, with levels in μg/mL depicted on the *X axis* and doses on the *Y axis* (*top*). *Bottom*, fold histone H4 or H3 expression is plotted on the *Y axis* in peripheral blood mononuclear cells plotted against VPA dose on the *X axis*.
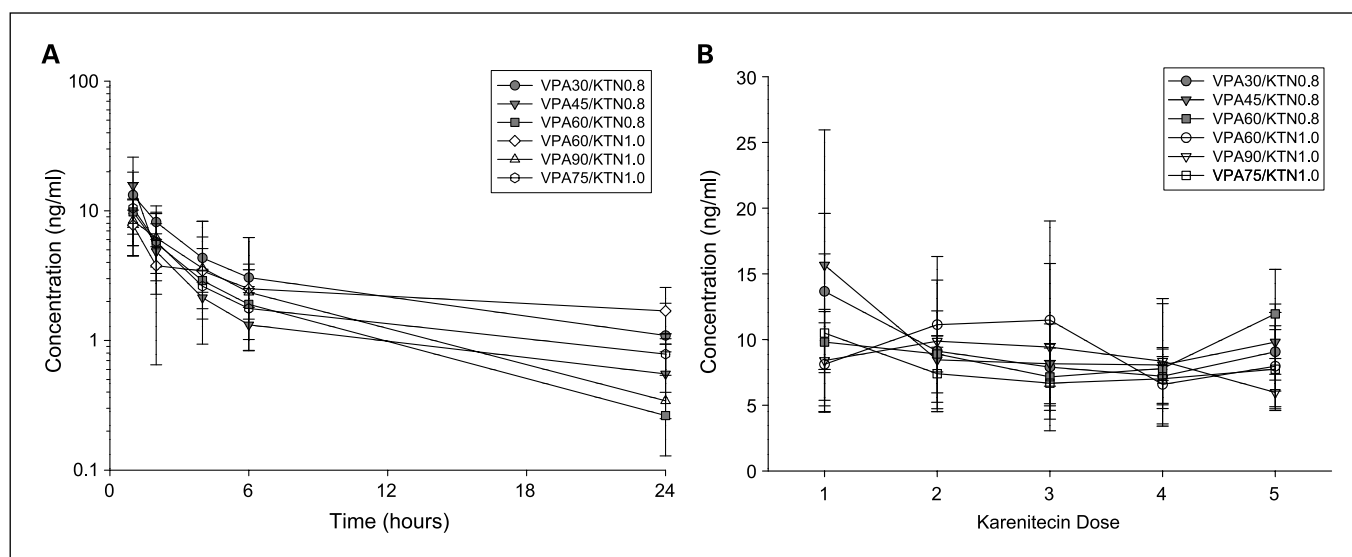
Fig. 4. Pharmacokinetic profile of KTN. Plasma concentration-time profiles for the fifth dose of KTN (mean ± SD; *A*) and C_{max} for the first five doses of KTN (mean ± SD; *B*). KTN = 0.8 mg/m² is plotted as *closed circles* (VPA 30 mg/kg/d), *triangles* (VPA 45 mg/kg/d), or *squares* (VPA 60 mg/kg/d). KTN = 1 mg/m² is plotted as *open diamonds* (VPA 60 mg/kg/d), *triangles* (VPA 90 mg/kg/d), or *circles* (VPA 75 mg/kg/d).

the histogram (*top*). Based on these results, we designed and conducted a phase I/II trial of the combination of VPA and KTN in melanoma.

Patients. Thirty-nine enrolled patients participated in this trial. Thirty-seven patients were evaluable for toxicity and 33 patients were evaluable for response. Patient demographics and tumor characteristics are summarized in Table 1. Most patients were previously treated with at least one chemotherapy regimen, and 28% of patients had failed two prior chemotherapy regimens. Sixty-one percent of patients had advanced stage IVC disease and 15% of patients had a prior history of central nervous system involvement.

Treatment administration and drug delivery. If no DLT was observed, a cohort was limited to 3 patients each. If a given patient received less than one complete cycle of treatment (for any reason, except drug-related toxicity), that patient was replaced. Toxicities experienced in the first cycle are described in Table 2.

DLT and other toxicities. We started with a VPA dose of 30 mg/kg/d for 5 days and escalated VPA to 60 mg/kg/d for 5 days, with KTN at 0.8 mg/m²/d. We then escalated KTN to 1.0 mg/m²/d for 5 days and continued escalating VPA to 90 mg/kg/d. We observed DLT at this VPA dose in 2 patients on cohort 5 (Table 2). Both patients experienced grade 3 neurologic toxicity (somnolence and confusion), which was transient. We deescalated to 60 mg/kg/d VPA and again observed no neurologic DLT. We then reescalated to 75 mg/kg/d VPA, and no DLT were seen at this level; this cohort was considered the maximum tolerated dose and was expanded to a total of 15 evaluable patients. Toxicities for the dose expansion cohort (Table 2) included grade 3 and 4 hematologic toxicities (4 absolute neutrophil count grade 4 and 5 absolute neutrophil count grade 3) and grade 1 and 2 neurologic toxicities (reversible somnolence: 11 with grade 2 and 5 with grade 1).

Response. The best response seen was prolonged stable disease, up to 50 weeks. Stable disease was seen in 39% (13 of 33) of evaluable patients and in all cohorts, including

cohort 1 (Supplementary Fig. S3). The median time to progression and overall survival of the entire trial population were 8.9 weeks (confidence interval, 5.7-12.1) and 31.8 weeks (confidence interval, 19.8-43.8), respectively. In the dose expansion cohort, 47% (7 of 15) of evaluable patients had stable disease, and median time to progression was 10.3 weeks (confidence interval, 5.3-14.7); the median overall survival was 32.9 weeks (confidence interval, 9.3-54).

Pharmacokinetic results. We profiled VPA levels on day 3, just before the first KTN infusion. Total and free VPA levels increased linearly with VPA dose (Fig. 3, *top*). At maximum tolerated dose (75 mg/kg/d VPA), the median level of VPA was 184 μg/mL (1.27 mmol/L VPA). We also examined the pharmacokinetics of KTN with and without VPA in the first 10 patients. No significant differences were seen (data not shown). We examined KTN concentration-time profiles as shown in Fig. 4A and Supplementary Table S1 for the fifth KTN dose period in combination with VPA. The data for first dose period produced similar profiles (data not shown). The mean C_{max} for the first five doses of KTN in combination with VPA is represented in Fig. 4B. The mean AUC (0-24) for the fifth dose of KTN was 59.0 ± 22 μg h/L for cohorts receiving 1.0 mg/m². The terminal half-life for the fifth dose (*n* = 6) was calculated at 11.0 ± 1.9 h, and the clearance was determined to be 37.0 ± 17.3 L/h. The concentration-time profile and AUC of KTN showed limited variation within each dose level of KTN regardless of the dose of VPA, suggesting that the escalating doses of VPA had little effect on the pharmacokinetics of KTN (mean AUC of KTN as a single agent was 87.1 ± 52 and 73.8 ± 32 when combined with VPA).

Histone acetylation: pharmacodynamic studies. Peripheral blood mononuclear cells pre-VPA and day 3 post-VPA were isolated and acetyl histone H3 or H4 was determined. As shown in Fig. 3 (*bottom*), there appears to be increased histone H4 and H3 hyperacetylation with an apparent plateau at 60 mg/kg/d VPA. It should be noted that only 1 patient was sampled at 90 mg/kg/d and no patients were sampled at 30 mg/kg/d.

Discussion

Current therapy for metastatic melanoma remains highly unsatisfactory. Standard first-line dacarbazine chemotherapy in metastatic melanoma is associated with a median progression-free survival of only 6 to 8 weeks and median overall survival of only 5.6 to 7 months in phase III clinical trials (23–26). Combinations or variations of existing chemotherapy or biotherapy agents have not appreciably improved clinical outcomes (23, 24, 26–28). The introduction of novel non-cross-resistant drugs combinations is therefore vital to improve outcomes for the vast majority of patients who progress on front-line chemotherapy.

We have shown previously that KTN, a novel topo I inhibitor, has a moderate degree of activity in melanoma (2) with prolonged stable disease seen in 33% of patients. We asked if HDAC inhibition, which can cause chromosomal decondensation and increased sensitivity to topo II inhibitors (3, 13, 16, 29), may do the same with topo I inhibitors. We determined the effects of dose and sequence of treatment in a series of preclinical studies (Figs. 1 and 2; Supplementary Figs. S1 and S2). We found that if HDACi pretreatment was followed by topo I inhibitor, increased apoptosis was seen in multiple melanoma cell lines. This increased cytotoxicity was schedule specific and required HDACi pretreatment and was associated with increased DNA strand breaks, apoptosis, and reduced tumor growth in xenografts. It should be noted that the doses of KTN used in the preclinical experiments were sometimes substantially larger depending on the assay used than the actual KTN concentrations achieved in humans (Figs. 1, 2, and 4; Supplementary Figs. 1 and 2; Supplementary Table S1). Also, the apoptosis assays predominantly used in the preclinical studies (Fig. 1; Supplementary Fig. S1) reflect cell death and measure only one aspect of chemotherapy response and other types of assays (such as clonogenic assays or metabolic assays) can reflect other important aspects of response to chemotherapy. Based on these preclinical results, we conducted a phase I/II trial of the HDACi VPA plus KTN in melanoma.

This combination was well tolerated, and toxicities were largely as expected and nonoverlapping (Table 2). The maximum tolerated dose was 75 mg/kg/d VPA for 5 days with 1 mg/m^2/d KTN (Table 2). We subsequently expanded this cohort and accrued 19 patients at this level, of whom 15 were evaluable for both toxicity and response. Based on results from this trial, we would recommend VPA at this dose if a 5-day continuous administration is being considered in other oncology trials.

The best response seen in this trial was stable disease (similar to the previous KTN phase II trial in melanoma; Supplementary Fig. S3); however, this was seen in almost half of evaluable patients. The median overall survival was 32.9 weeks with a median progression-free survival of 10.3 weeks in the dose expansion cohort. These results, albeit in the context of a single-institution phase I/II trial, compare favorably with recent trial data in melanoma, considering that the vast majority of patients in this trial had previously progressed on chemotherapy and had M$_{1c}$ disease. In this trial, 7 of 15 (47%) patients had stable disease with median progression-free survival of 10.3 weeks versus 34% with stable disease with median progression-free survival of 7.9 weeks shown in patients on the previous single-agent KTN phase II study.

In conclusion, the addition of HDACi VPA appears to be a promising modality to increase sensitivity to topo I inhibition in preclinical models in melanoma and is tolerable *in vivo* with disease stabilization occurring in nearly half of treated patients at maximum tolerated dose. Clearly, these data need to be tested in randomized trials to determine whether there is indeed increased efficacy compared with current standard treatments. Although our preclinical and clinical trial data relate to this combination in melanoma, other diseases such as colon or ovarian cancer, where topo I inhibitors are better established, may be even more responsive to this combination.

Disclosure of Potential Conflicts of Interest

Acknowledgments

References

1. Flaherty KT. Chemotherapy and targeted therapy combinations in advanced melanoma. Clin Cancer Res 2006;12:2366–70s.
2. Daud A, Valkov N, Centeno B, et al. Phase II trial of karenitecin in patients with malignant melanoma: clinical and translational study. Clin Cancer Res 2005;11:3009–16.
3. Marchion DC, Bicaku E, Daud AI, Richon V, Sullivan DM, Munster PN. Sequence-specific potentiation of topoisomerase II inhibitors by the histone deacetylase inhibitor suberoylanilide hydroxamic acid. J Cell Biochem 2004;92:223–37.
4. Rasheed WK, Johnstone RW, Prince HM. Histone deacetylase inhibitors in cancer therapy. Exp Opin Invest Drugs 2007;16:659–78.
5. Marchion D, Munster P. Development of histone deacetylase inhibitors for cancer treatment. Expert Rev Anticancer Ther 2007;7:583–98.
6. Dokmanovic M, Clarke C, Marks PA. Histone deacetylase inhibitors: overview and perspectives. Mol Cancer Res 2007;5:981–9.
7. Finnin MS, Donigian JR, Cohen A, et al. Structures of a histone deacetylase homologue bound to the TSA and SAHA inhibitors. Nature 1999;401:188–93.
8. Gurvich N, Tsygankova OM, Meinkoth JL, Klein PS. Histone deacetylase is a target of valproic acid-mediated cellular differentiation. Cancer Res 2004;64:1079–86.
9. Munster PN, Troso-Sandoval T, Rosen N, Rifkind R, Marks PA, Richon VM. The histone deacetylase inhibitor suberoylanilide hydroxamic acid induces differentiation of human breast cancer cells. Cancer Res 2001;61:8492–7.
10. Marks PA, Richon VM, Rifkind RA. Histone deacetylase inhibitors: inducers of differentiation or apoptosis of transformed cells. J Natl Cancer Inst 2000;92:1210–6.
11. Kim MS, Blake M, Baek JH, Kohlhagen G, Pommier Y, Carrier F. Inhibition of histone deacetylase increases cytotoxicity to anticancer drugs targeting DNA. Cancer Res 2003;63:7291–300.
12. Tsai SC, Valkov N, Yang WM, Gump J, Sullivan D, Seto E. Histone deacetylase interacts directly with DNA topoisomerase II. Nat Genet 2000;26:349–53.
13. Marchion DC, Bicaku E, Daud AI, Sullivan DM, Munster PN. *In vivo* synergy between topoisomerase II and histone deacetylase inhibitors: predictive correlates. Mol Cancer Ther 2005;4:1993–2000.
14. Munster P, Marchion D, Bicaku E, et al. Phase I trial of histone deacetylase inhibition by valproic acid followed by the topoisomerase II inhibitor epirubicin in advanced solid tumors: a clinical and translational study. J Clin Oncol 2007;25:1979–85.
15. Khan N, Jeffers M, Kumar S, et al. Determination of the class and isoform selectivity of small-molecule histone deacetylase inhibitors. Biochem J 2008;409:581–9.
16. Marchion DC, Bicaku E, Turner JG, Daud AI, Sullivan DM, Munster PN. Synergistic interaction between histone deacetylase and topoisomerase II inhibitors is mediated through topoisomerase IIβ. Clin Cancer Res 2005;11:8467–75.

17. Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res 1988;175:184–91.

18. Singh NP, Danner DB, Tice RR, McCoy MT, Collins GD, Schneider EL. Abundant alkali-sensitive sites in DNA of human and mouse sperm. Exp Cell Res 1989;184:461–70.

19. Collins AR. The comet assay for DNA damage and repair: principles, applications, and limitations. Mol Biotechnol 2004;26:249–61.

20. Smith JA, Hausheer F, Newman RA, Madden TL. Development of a high-performance liquid chromatographic method to determine the concentration of karenitecin, a novel highly lipophilic camptothecin derivative, in human plasma and urine. J Chromatogr B Biomed Sci Appl 2001;759:117–24.

21. Department of Health and Human Services, Food and Drug Administration. Guidance for Industry on Bioanalytical Method Validation. Federal Register 2001;66 28526 docket no. 98D-1195.

22. Therasse P, Arbuck SG, Eisenhauer EA, et al. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. New guidelines to evaluate the response to treatment in solid tumors. J Natl Cancer Inst 2000;92:205–16.

23. Bedikian AY, Millward M, Pehamberger H, et al. Bcl-2 antisense (oblimersen sodium) plus dacarbazine in patients with advanced melanoma: the Oblimersen Melanoma Study Group. J Clin Oncol 2006;24:4738–45.

24. Chapman PB, Einhorn LH, Meyers ML, et al. Phase III multicenter randomized trial of the Dartmouth regimen versus dacarbazine in patients with metastatic melanoma. J Clin Oncol 1999;17:2745–51.

25. Avril MF, Aamdal S, Grob JJ, et al. Fotemustine compared with dacarbazine in patients with disseminated malignant melanoma: a phase III study. J Clin Oncol 2004;22:1118–25.

26. Middleton MR, Grob JJ, Aaronson N, et al. Randomized phase III study of temozolomide versus dacarbazine in the treatment of patients with advanced metastatic malignant melanoma. J Clin Oncol 2000;18:158–66.

27. Atkins ME, Lee S, Flahert L, J S, Sondak V, Kirkwood JM. A prospective randomized phase III trial of concurrent biochemotherapy (BCT) with cisplatin, vinblastine, dacarbazine (CVD), IL-2 and interferon α-2b (IFN) versus CVD alone in patients with metastatic melanoma (E3695): an ECOG-coordinated Intergroup study. Chicago (IL): ASCO; 2003.

28. Rosenberg SA, Yang JC, Schwartzentruber DJ, et al. Prospective randomized trial of the treatment of patients with metastatic melanoma using chemotherapy with cisplatin, dacarbazine, and tamoxifen alone or in combination with interleukin-2 and interferon α-2b. J Clin Oncol 1999;17:968–75.

29. Marchion DC, Bicaku E, Daud AI, Sullivan DM, Munster. PN. Valproic acid alters chromatin structure by regulation of chromatin modulation proteins. Cancer Res 2005;65:3815–22.

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