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PHASE I STUDIES

Phase 1 clinical trial of the novel proteasome inhibitor marizomib with the histone deacetylase inhibitor vorinostat in patients with melanoma, pancreatic and lung cancer based on in vitro assessments of the combination

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Summary *Purpose* Combining proteasome and histone deacetylase (HDAC) inhibition has been seen to provide synergistic anti-tumor activity, with complementary effects on a number of signaling pathways. The novel bi-cyclic structure of marizomib with its unique proteasome inhibition, toxicology and efficacy profiles, suggested utility in combining it with an HDAC inhibitor such as vorinostat. Thus, in this study *in vitro* studies assessed the potential utility of combining marizomib and vorinostat, followed by a clinical trial with the objectives of assessing the recommended phase 2 dose (RP2D), pharmacokinetics (PK), pharmacodynamics (PD), safety and preliminary anti-tumor activity of the combination in patients. *Experimental Design* Combinations of marizomib and vorinostat were assessed *in vitro*. Subsequently, in a Phase 1 clinical trial patients with melanoma, pancreatic carcinoma or Non-small Cell Lung Cancer (NSCLC) were given escalating doses of weekly marizomib in combination with vorinostat 300 mg daily for 16 days in 28 day cycles. In addition to standard safety studies, proteasome inhibition and pharmacokinetics were assayed. *Results* Marked synergy of marizomib and vorinostat was seen in tumor cell lines derived from patients with NSCLC, melanoma and pancreatic carcinoma. In the clinical trial, 22 patients were enrolled. Increased toxicity was not seen with the combination. Co-administration did not appear to affect the PK or PD of either drug in comparison to historical data. Although no responses were demonstrated using RECIST criteria, 61% of evaluable patients demonstrated stable disease with 39% having decreases in tumor measurements. *Conclusions* Treatment of multiple tumor cell lines with marizomib and vorinostat resulted in a highly synergistic antitumor activity. The combination of full dose marizomib with vorinostat is tolerable in patients with safety findings consistent with either drug alone.

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Introduction

The ubiquitin-proteasome pathway plays a critical role in cell physiology by degrading ubiquitin tagged proteins. This includes those involved in signal transduction relevant to tumor cell growth and survival such as the NF-κB inhibitor IκB. This function is also important to tumor cell survival as it prevents accumulation of toxic levels of excess proteins in these highly metabolically active cells. The 26S proteasome contains a proteolytic core (20S proteasome) and 1–2 19S regulatory subunit caps, and has three major proteolytic activities: chymotrypsin-like (CT-L, β5), trypsin-like (T-L, β2) and caspase-like (C-L, β1). The proteasome was validated as an oncology drug target by the United States Food and Drug Administration (FDA) approval of the proteasome inhibitor bortezomib for the treatment of patients with multiple myeloma in 2003 and mantle cell lymphoma in 2006. However, drug induced or constitutive resistance to bortezomib and significant off-target toxicities such as peripheral neuropathy, thrombocytopenia and inhibition of multiple serine proteases, provide reasons to develop second generation proteasome inhibitors with broader activity and improved therapeutic ratio.

Marizomib (NPI-0052), a highly potent proteasome inhibitor, was discovered during the screening of extracts from *Salinispora tropica,* a newly discovered marine actinomycete. Initial studies demonstrated that marizomib inhibited the CT-L activity of human and rabbit 20S proteasomes (IC_{50} ~4 nM). Marizomib is also a potent inhibitor of the T-L activity of 20S proteasomes, and additionally inhibits the C-L activity at similar or lower potency compared to T-L. Marizomib has been shown to inhibit NF-κB activation and synthesis of cytokines such as TNF-a, IL-1 and IL-6 and adhesion molecules and growth factors such as ICAM-1 and VEGF [1, 2]. Marizomib, which is not based on a peptide backbone, has a markedly different structure and thus different properties, from other proteasome inhibitors in clinical use. As marizomib displays a longer duration and different pattern of proteasome inhibition compared to bortezomib, the toxicity and efficacy profile appears different than that of bortezomib.

Marizomib has been evaluated in a number of clinical trials as a single agent [3–5]. Pharmacodynamic data demonstrated that marizomib is a potent inhibitor of all three proteasome activities and that inhibition is dose, cycle and schedule dependent. Pharmacokinetic data indicate that the half-life is short with rapid clearance and a large volume of distribution. Exposure increases linearly with dose without indications of accumulation or induction of metabolism. Marizomib administered on a day 1, 8, and 15 schedule is generally tolerated up to an MTD of 0.6-0.7 mg/m^2 depending on the study. Marizomib does not appear to induce the common bortezomib associated toxicities of peripheral neuropathy, neutropenia and thrombocytopenia in spite of reaching levels of proteasome inhibition that equal or exceed those reported with bortezomib. Tumor assessments have indicated anti-tumor activity by marizomib, inclusive of patients with myeloma previously treated with bortezomib.

The histone deacetylase (HDAC) family of enzymes catalyzes the deacetylation of lysine residues within the amino-terminal tails of histones. The acetylation of histones and other proteins is generally associated with increased transcriptional activity whereas deacetylation results in transcriptional repression. Non-histone proteins such as the transcription factor NF-kB are also regulated by acetylation, which is also downregulated by proteasome inhibition. HDAC inhibition can induce growth arrest and apoptosis in cancer cells, in part through up regulation of growth inhibitory and pro-apoptotic genes such as p21. Similar effects can be seen with proteasome inhibition, and both classes of agents have complimentary effects on the epithelial-mesenchymal transition in tumor cells [6]. Importantly, HDAC inhibitors have also been shown to disrupt the aggresome, a key secondary cellular protein disposal unit after the proteasome. Finally, HDAC inhibition can downregulate proteasome subunit expression, providing additional rationale for the combination of HDAC and proteasome inhibitors. HDAC inhibitors have shown single-agent activity in patients, as validated by the FDA approval of vorinostat and romidepsin for the treatment of cutaneous T-cell lymphoma (CTCL) in 2006 and 2009 respectively [7].

Given the complementary effects on signal transduction and protein disposal pathways, it was reasonable to expect synergy between proteasome and HDAC inhibitors. This has in fact been reported in *in vitro* and *in vivo* studies. In particular, combinations of marizomib and HDAC inhibitors such as vorinostat, tubacin and SNDX-275 can induce a synergistic antitumor effect in human myeloma, leukemia and solid tumor models, including pancreatic carcinoma and melanoma [8, 9]. The combination of marizomib and vorinostat in particular has been assessed in animal models, where complementary efficacy was seen, without evidence of increased toxicity. In particular, when marizomib and vorinostat were assessed together in rats on the same dosing schedule of the present clinical trial, the combination was well tolerated and did not produce any new toxicity as compared to either compound alone [10]. These findings led to further *in vitro* assessment in lung cancer and melanoma models and the initiation of the current Phase 1 study in patients with advanced melanoma, NSCLC and

pancreatic carcinoma without other treatment options. In this study the dose and schedule of vorinostat was modified to overlap with marizomib administration and decrease dose density to maximize synergy while minimizing vorinostat related toxicity.

Materials and methods

In vitro assessment of proteasome inhibition and cytotoxicity

The human melanoma cell lines obtained from the American Type Culture Collection (Manassas VA) were divided into three groups (1) low metastatic including SB-2, DM4 and TXM13; (2) intermediate metastatic including Mel526, Me1624, Me1888, Me1938 and MeWo and highly metastatic including WM2664, WM293, WM793, WM35, A375SM, A375 and C8161. All melanoma cell lines were maintained in MEM supplemented with 10% FBS, MEM vitamin solution, L-glutamine, non-essential amino acids, sodium pyruvate and penicillin-streptomycin solutions in a 37°C incubator under an atmosphere of 5% CO_2 in the air.

Seven NSCLC cell lines including NCI-H157, -H226, -H322c, -H441, -H1341, HCC4006 and A549 and one SCLC cell line NCI-H196 were assessed. The cell lines were obtained through the Tissue Culture Core facility of the Univ. of Colorado Cancer Center (Aurora, CO). Cells were propagated in RPMI 1640 (InVitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 2.5% glutamine, 50,000 units penicillin and 80 μM streptomycin in a humidified 5% CO_2 atmosphere. Cell line identities were verified by genotyping using the DNA Sequencing & Analysis Core facility of the Univ. of Colorado Cancer Center (Aurora, CO).

Marizomib (Lot # 04166R05) was dissolved in dimethyl sulfoxide (DMSO) at 15 mg/ml, and used at concentrations ranging from 5 to 500 nM. Vorinostat (SAHA, Zolinza), obtained from Aton Pharma (Lot # AP-390-1, Tarrytown, NY), was dissolved in DMSO at 10 mM and used at 0.5 to 8 uM. Final DMSO concentrations in medium did not exceed 0.08%. Treatment times were as indicated in the text.

Growth inhibition of cell lines was determined by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay using CellTiter 96 Aqueous One reagent (Promega, Inc., Madison, WI). Briefly, cells were seeded in 96-well microtitre plates at 3000 cells per well and allowed to adhere overnight. Agents or vehicle were added to appropriate concentrations and incubation continued for an additional 72–96 h. The MTS reagent was then added and incubation continued for 1–2 h. Absorbance was measured at 490 nm in a 96-well plate reader (BioTek, Inc.). The combination index (CI) was calculated using CALCUSYN; synergy, additivity and antagonism were suggested by CI values <1.0, = 1.0 or> 1.0, respectively [11].

Apoptosis was measured by propidium iodide (PI) staining and fluorescence-activated cell sorting (FACS) analysis. After incubation with vorinostat and/or marizomib, cells were harvested by trypsinzation, pelleted by centrification and resuspended in phosphate buffered saline (PBS) containing 50ug/ml PI, 0.1% Triton X-100 and 0.1% sodium citrate. Samples were stored at 4°C and gently vortexed before FACS analysis using the FL-3 channel. The percent sub-G0/G1 population of each sample was determined.

The Proteasome Glo chymotrypsin-like (CT-L) activity assay was performed according to the manufacturer's instructions (Promega, Inc.). Briefly, 10,000 cells were plated in triplicate wells in a white 96-well microtitre plate with clear bottoms. Cells were allowed to attach for 4 h followed by the addition of NPI-0052 or DMSO. After 2 h, media were replaced with 25 ul fresh media, followed by 25 ul of the Proteasome Glo reagent. After 10 min incubation, luminescence was measured in a BioTek 96-well plate reader.

Patient selection

Patients with histologically-confirmed NSCLC, metastatic pancreatic adenocarcinoma or melanoma (lymphoma and multiple myeloma were allowed in a later amendment) for which a standard, approved therapy was not available and ≥18 years old were eligible. Patients were required to have Karnofsky Performance Status (KPS)≥70% and adequate hematopoietic, electrolyte, hepatic, renal, and cardiac laboratory results. Patients were excluded if they had received chemotherapy, biologic agents, immunotherapy, major surgery or other investigational agents within 28 days or if they had received radioimmunotherapies or intrathecal therapy in the prior 12 weeks. Additionally, patients were excluded if they had known brain metastases, significant cardiac disease, prior treatment with other HDACi or proteasome inhibitors, previous hypersensitivity reaction to vorinostat, propylene glycol or ethanol, had an active secondary malignancy, significant proteinuria, urinary tract infection, active urine sediment, kidney disease or were pregnant or breast-feeding. Patients who required systemic therapy; parenteral antibiotics or were HIV positive or had active Hepatitis A, B, or C infections, or any altered mental status or psychiatric condition were also excluded from the study.

The study was conducted in accordance with the Declaration of Helsinki and local laws and regulations. Ethics Committee approval and informed consent were obtained prior to participation.

Study design and drug treatment

Marizomib was administered via intravenous (IV) infusion over 1–10 min depending on infusate volume on days 1, 8 and 15 of 28 day cycles. In addition, patients took 300 mg vorinostat by mouth once daily on days 1–16 of each cycle. A modified accelerated dose titration design was used to escalate the dose of marizomib from a starting dose of 0.15 mg/m^2 [12]. Escalations were initially made in cohorts of at least 1 evaluable patient at approximately 100% increments, until grade 2 or higher of marizomib related adverse event was observed in cycle 1 or a dose level of 0.45 mg/m^2 was attained. Thereafter escalations were made in cohorts of at least 3 evaluable patients at≤50% increments. Dose-limiting toxicity (DLT) was defined as a drug related cycle 1 event including a grade 4 hematologic toxicity lasting>7 days, a clinically significant>grade 3 non-hematologic toxicity (excluding anorexia, fatigue), > grade 3 nausea, vomiting or diarrhea not controlled with optimal supportive care and/or prophylaxis), and any treatment delay>14 days secondary to recovery from drug related adverse events. If one patient in a cohort experienced a DLT, then the cohort was increased to at least six evaluable patients. If no further patients experienced a DLT, then the next dose level could be evaluated. If≥2 patients in a cohort experienced DLT, then the previous dose level was considered the maximum tolerated dose (MTD). If this had occurred at the first dose level, the dose of marizomib was to be decreased to 0.1 mg/m^2 and the dose of vorinostat to 200 mg per day in the next cohort, and escalation could be reinitiated from that point as described above. An intermediate dose level could be tested to more precisely define the MTD. DLT, dose modification and MTD could be assigned to one drug if the relevant event is attributed to that drug alone, with continued assessment of alternate dosing for the other drug. A recommended phase 2 dose (RP2D) could be selected below the MTD and/or escalation could be terminated at an RP2D below an MTD depending on safety, pharmacokinetics (PK), pharmacodynamic (PD) and response data, otherwise the MTD would constitute the RP2D. Once the MTD of marizomib had been reached the dose of marizomib could be reduced to 75% of the MTD and the dose of vorinostat escalated to 400 mg per day in the following cohort. If this did not exceed an MTD, the dose of marizomib could be re-escalated to the MTD in the next cohort. Any dose level at or below an MTD could be selected as the RP2D based on safety, pharmacokinetic, pharmacodynamic and response data. Once the RP2D was defined, up to an additional 20 patients could be enrolled at the RP2D dose level.

In patients where Grade 3 thrombocytopenia or decrease in platelets by≥100×10^9/L and to less than the lower limit of normal (LLN)was seen the dose of vorinostat was to be reduced by 100 mg / day without a treatment delay. If Grade 4 vorinostat-related hematological adverse events were seen, the dose of vorinostat was to be reduced by 100 mg / day upon re-treatment. If Grade 4 thrombocytopenia was seen, vorinostat dosing was to be held for 1 week and until the platelet count was≥75×10^9/L, and the dose then reduced by 100 mg / day upon re-treatment (this was not considered a DLT). If the level was not maintained at< Grade 2 on the new regimen the dosing regimen for that patient could be reduced from Days 1–16 to Days 1–3, 7–9, and 14–16. If the level was maintained at>LLN, re-escalation to the original dose could occur with the approval of the medical monitor. If the two events of Grade 4 thrombocytopenia or vorinostat related DLT were seen at any dose level, the vorinostat dosing regimen for subsequent patients was to be reduced from Days 1–16 to Days 1–3, 7–9, and 14–16.

Intrapatient dose escalation to the next highest dose tested could occur for individual patients (to occur at the start of the next cycle), provided at least 3 patients in the higher dose level cohort had completed at least one cycle with≤1/6 (e.g. 0/3) evaluable patients experiencing a DLT, and the patient considered for intrapatient dose escalation has not experienced prior drug related Grade 3 or greater adverse events.

Patients who were not evaluable (not having received 3 infusions of marizomib and 15 doses of vorinostat within cycle 1) were replaced. Treatment was continued in individual patients until progressive disease, toxicity of DLT level or requiring a 14 day treatment delay in spite of a dose reduction, or withdrawal from study by patient or investigator decision.

Study drug

Marizomib {marizomib: 6-oxa-2-azabicyclo[3.2.0]heptane-3,7-dione, 4-(2-chloroethyl)-1-[(S)-[(1S)-2-cyclohexen-1-yl] hydroxymethyl]-5-methyl-,(1R,4R,5S)} is a white crystalline solid. A liquid formulation of marizomib was initially used. It was supplied as a clear and slightly viscous solution (9 mL per vial) of marizomib (2.2 mg per vial) in 97.8% propylene glycol and 2.2% ethanol which is combined with 13 mL of citrate buffer / ethanol resulting in a marizomib concentration of 0.1 mg/mL (2.2 mg in 22 mL/vial). The resulting solution was mixed and kept refrigerated at 2-8°C prior to dispensation and administered via IV within 5 h of dilution. The starting dose of 0.15 mg/m^2 was based on prior clinical trials and repeated dose toxicology studies in rats and primates where the dose levels of 0.15 mg/m^2 and 0.12 mg/m^2 respectively resulted in no-observed-adverse-effects-levels (NOAELs).

A lyophile formulation of marizomib was subsequently introduced into clinical trials in 2009 to replace the liquid formulation. It was supplied as a white to light pink cake or

powder of 2 mg marizomib and 60 mg sucrose per vial. The parenteral dosing solution of marizomib is produced by reconstituting marizomib lyophile with 10 mL of diluent [55% propylene glycol, 5% ethanol, and 40% citrate buffer (10 mM, pH 5)], with a final concentration of marizomib equal to 0.2 mg/mL. The resulting reconstituted solution was kept at 2-8°C prior to dispensation and administered via IV within 8 h of reconstitution.

Vorinostat was supplied in 100 mg white, opaque, hard gelatin capsules. Each 100 mg vorinostat capsule contains 100 mg vorinostat and these inactive ingredients: microcrystalline cellulose, sodium croscarmellose and magnesium stearate. The capsule shell excipients are titanium dioxide, gelatin and sodium lauryl sulfate. The starting dose was 300 mg per day administered orally with food on Day 1–16 in the 28 day cycle.

Study assessments

Safety assessments performed at baseline and prior to each cycle included complete physical exam, KPS assessment, vital signs, safety labs (CBC, platelets, chemistry, coags, pregnancy at baseline), urinalysis, creatinine clearance and urine protein. During all cycles on days 8, 15 and 22, CBC, platelets and chemistry were obtained. Coagulation studies were obtained prior to each injection and on Day 22 during cycle 1. Further studies performed during cycle 1 include chemistry on days 2 and 4 and CBC and platelets on Day 2. ECGs were performed at baseline and on day 8 of each cycle. Vital signs and toxicity evaluations were completed on marizomib injection days and at each clinic visit. All safety assessments (except ECGs) were repeated at the End of Study visit. Tumor measurement and assessment by RECIST [13] using computed tomography or other methods as appropriate for each lesion, and/or tumor markers were obtained at baseline and at minimum during the rest period of cycle 1, 2 and every other cycle (approximately Day 15) thereafter or as clinically indicated and at End of Study.

Adverse events reported were described using MedDRA coding and graded by the National Cancer Institute Common Terminology Criteria for Adverse Events version 3.0 (NCI-CTCAEv3.0). Response was assessed per the Response Evaluation Criteria in Solid Tumors (RECIST) for patients with solid tumors.

Pharmacokinetics

For marizomib blood pharmacokinetic sampling was performed pre-dose, every 4 min during infusion (if a 10 min infusion), end of infusion, and 2, 5, 10, 15, 30 min after infusion during Cycle 1 on days 1 and 15. When quantifiable levels were observed at the last sampling time point, additional time points (45, 60, 90, and/or 120 min) were drawn. For vorinostat blood (2 mL each) was drawn into plain vacuum blood collection tubes prior to marizomib infusion on Cycle 1 Day 1; and prior to and 1, 2, 3, 4, and 6 h after vorinostat ingestion on Cycle 1 and Day 15.

Pharmacodynamics

Pharmacodynamic samples were obtained at baseline, immediately prior to, 1, 4 h after marizomib injection on Cycle 1 Day 1, 2 h prior to vorinostat administration, immediately prior to and 1 h following marizomib injection on Cycle 1 Day 15 and 1 h post marizomib injection on Cycle 2 Day 15. Blood samples were collected in sodium heparin-containing tubes (5 ml). Samples (1 ml aliquots) were centrifuged (2000 g, 10 min at RT) and the packed whole blood (PWB) pellet washed once with 5 times the pellet volume of ice-cold 1X Debucco's phosphate buffered saline (DPBS) with Ca^{+2}/Mg^{+2}. PWB pellets were lysed with 5 mM EDTA (pH 8.0) for 1 h on ice, after which cell lysates were cleared by centrifugation (19200 g, 10 min at 4°C) and glycerol was added at a final concentration of 10% (v/v). 20S proteasome activity was determined as described previously (Stein et al., 1996; Lightcap et al., 2000). The percentage proteasome inhibition was determined relative to the average proteasome activity observed in the baseline and Cycle 1 Day 1 pre- marizomib treatment sample.

Results

In vitro assessments

Growth inhibition data (MTS assays) with seven NSCLC cell lines (A549, H226, H441, H157, H322c, HCC4006 and H1341) and one SCLC cell line (NCI-H196) demonstrated that all cell lines showed marked sensitivity to marizomib, and there was an approximate 30-fold difference in IC_{50}s ranging from 10 nM to 300 nM . Six NSCLC cell lines (H157, A549, H322c, H358, H441 and H460) were used for marizomib-vorinostat combinations. These initial experiments used one level of HDACi; 2 uM vorinostat. The results of growth inhibition studies using marizomib alone compared with marizomib plus 2 uM vorinostat showed substantial shifts in IC_{50} values in some cases (i.e., from ~40 nM to ~15 nM in H157 cells). More detailed studies utilizing isobologram analyses were therefore completed for the one SCLC (H196) and 7 NSCLC cell lines. Concentrations for marizomib (nM) were 5, 10, 25, 50, 100, 200, 500 nM and 0.5, 1, 2, 4, 8 uM for vorinostat. The findings demonstrated CI indexes ranging from ~0.9 to ~0.15 suggesting strong synergy of the combination. Bar graph plots of CI values are provided for two cell lines H322c and H196. Marizomib and vorinostat were further evaluated on a panel of

human melanoma cells lines with low, intermediate and high metastatic potential. Similar to the findings with the NSCLC cell lines, treatment with vorinostat and marizomib combinations significantly enhanced the induction of apoptosis as determined by PI staining and FACS analysis. Interestingly, the greatest activity of the combination was evident in the panel of highly metastatic melanoma cell lines (Fig. 1.).

NCI-H322c and H196 were further evaluated for changes in proteasome CT-L activity and its relationship to cytotoxic sensitivity. As shown in Fig. 2, 10 nM marizomib inhibited proteasome CT-L activity by >90% in both cell lines. Thus the sensitivity profiles did not correlate with cytotoxic activity of marizomib.

Patient demographics

In total, 22 patients were enrolled in the clinical trial. Table 1. summarizes patient demographics and tumor histologies. Secondary to the practice patterns and protocol allocation of the investigators the majority of patients had

a. Sensitivity to lung cancer cell lines to marizomib alone assessed by growth inhibition on MTS assay.

Human Lung Tumor Type	Cell Line	IC_{50} ± SD (nM)
Large Cell Carcinoma	**H1341**	**10 ± 1**
Small Cell Carcinoma	**H196**	**20 ± 1**
Large Cell Carcinoma	**H157**	**30 ± 2**
Squamous Cell Carcinoma	**H226**	**30 ± 3**
Adenocarcinoma	**H441**	**50 ± 6**
Adenocarcinoma	**A549**	**160 ± 20**
Bronchioloalveolar Carcinoma	**H322C**	**280 ± 22**
Adenocarcinoma	**HCC4006**	**300 ± 30**

b. Sensitivity of lung cancer cell lines to marizomib and vorinostat assessed by growth inhibition on MTS assay (SAHA = vorinostat; NPI = marizomib).

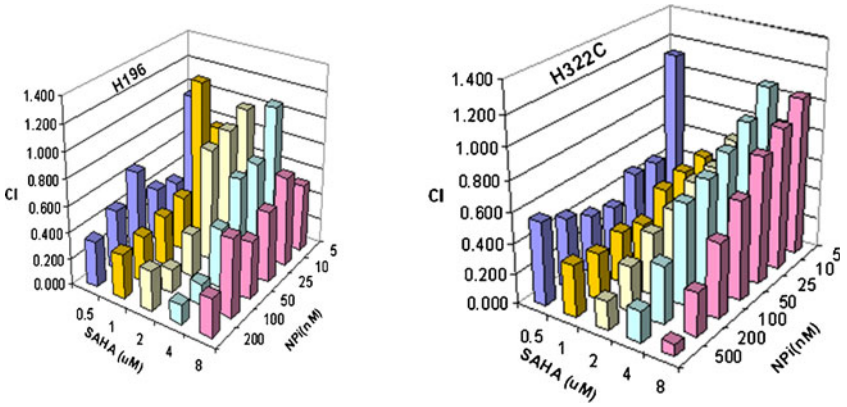

Fig. 1 Sensitivity of cell lines to marizomib and vorinostat. **a** Sensitivity to lung cancer cell lines to marizomib alone assessed by growth inhibition on MTS assay. **b** Sensitivity of lung cancer cell lines to marizomib and vorinostat assessed by growth inhibition on MTS assay (SAHA = vorinostat; NPI = marizomib). **c** Sensitivity of highly metastatic melanoma cell lines to marizomib and vorinostat assessed by percent sub-G0/G1 population on FACS (SAHA = vorinostat; NPI = marizomib). FACS analysis of highly metastatic melanoma cell lines after 48 h treatment

c. Sensitivity of highly metastatic melanoma cell lines to marizomib and vorinostat assessed by percent sub-G0/G1 population on FACS (SAHA = vorinostat; NPI = marizomib).

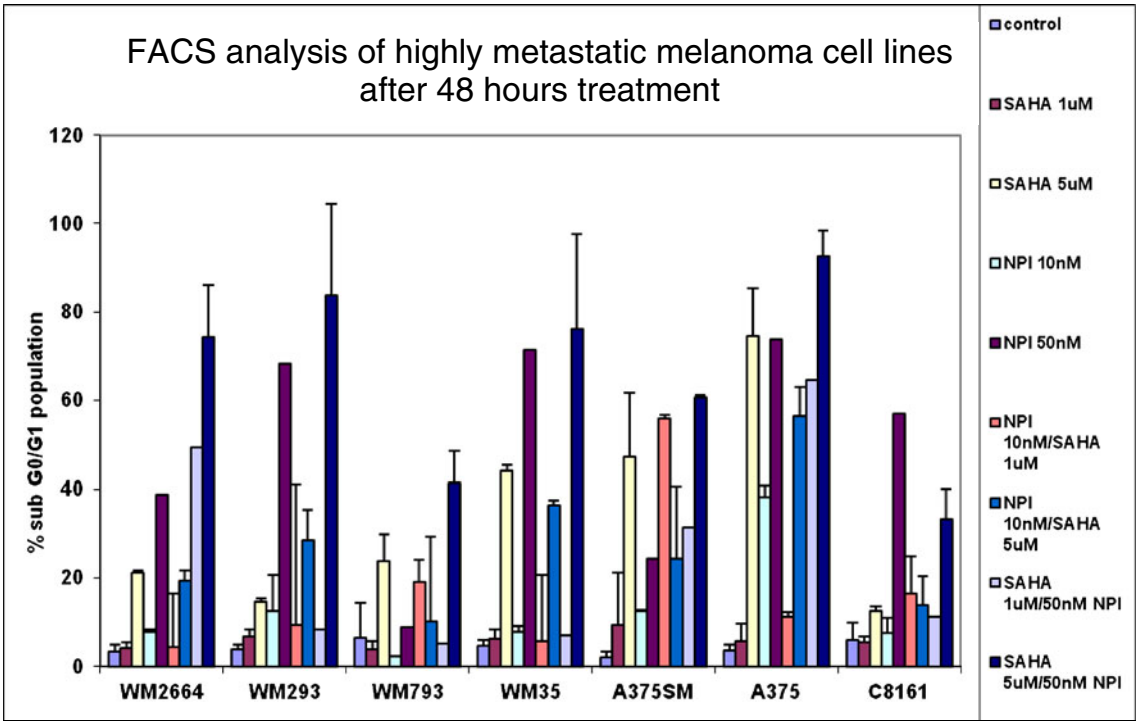

Fig. 1 (continued)

melanoma. Subsequently, the male:female ratio was approximately 2:1. Performance status was good in the majority of patients, with only 14% of patients having a Karnofsky Performance Status (KPS) <80%.

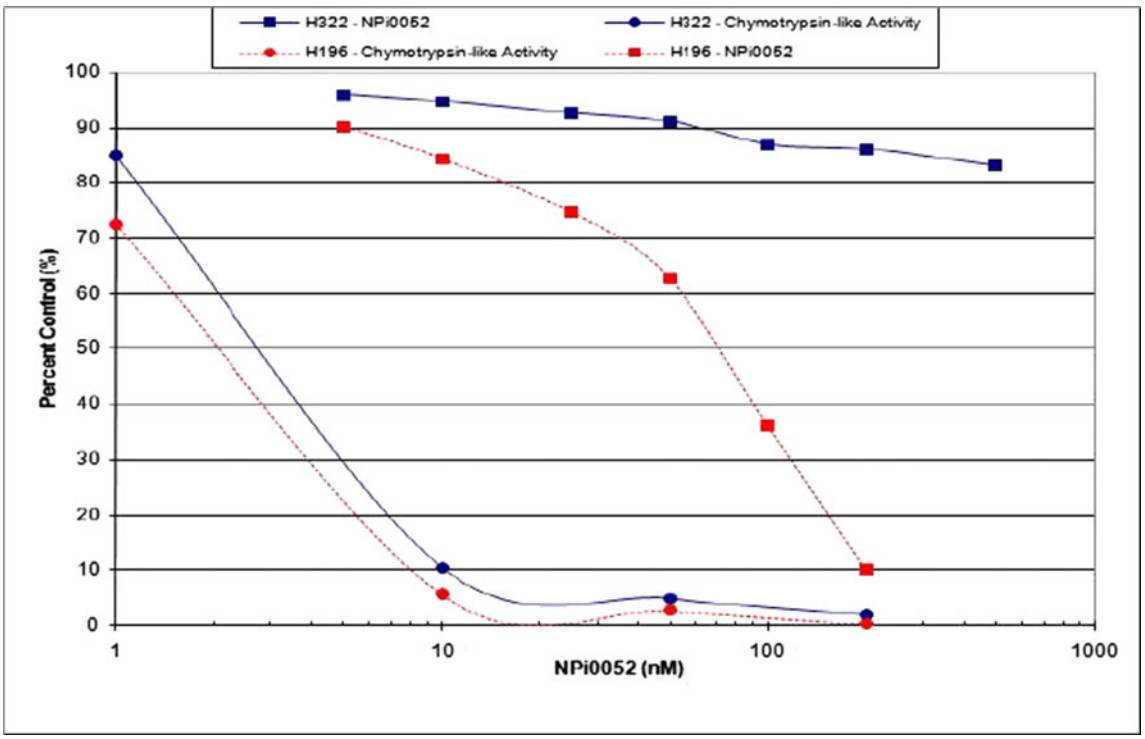

Fig. 2 Sensitivity profiles to marizomib in NSCLC and SCLC cell lines are not related to differences in inhibition of proteasome CT-L activity

Table 1 Demographics and patient characteristics

Characteristics	Patients
Median age (years)	60.5 (37–77)
Male/Female	14/8 (64%/36%)
KPS≥80	19 (86%)
KPS<80	3 (14%)
Patient Diagnoses	Patients (#)
Non-Small Cell Lung Cancer	1
Pancreatic Cancer	4
Melanoma	17
Lymphoma	0
Multiple Myeloma	0
Prior Treatment	Patients (%)
Surgery (%)	18/22 (82%)
Radiation (%)	11/22 (50%)
Chemotherapy (%)	22/22 (100%)
Chemoradiation (%)	1/22 (5%)
Number of Regimens (median)	4

Treatment delivered

The dose of vorinostat was maintained at 300 mg / day throughout the study. For the marizomib, dose escalation: 9 patients were treated in Cohort 1 (0.15 mg/m^2) as 1 patient experienced a DLT of thrombocytopenia related to vorinostat requiring a cohort of ≥6 patients, but no patients experienced a Grade ≥2 marizomib related adverse event in cycle 1 or a DLT allowing a ≤100% escalation and ≥1 patient in the next cohort; 1 patient in Cohort 2 (0.3 mg/m^2) as no patients experienced a Grade ≥2 marizomib related adverse event in cycle 1 or a DLT allowing a ≤100% escalation and ≥1 patient in the next cohort; 5 patients in Cohort 3 (0.6 mg/m^2) as a patient experienced a Grade ≥2 marizomib and vorinostat related adverse event of nausea and vomiting in cycle 1 and a dose level of 0.45 mg/m^2 had been attained with no patients experiencing a DLT, allowing a ≤50% escalation and ≥3 patients in the next cohort; and 7 patients in Cohort 4 (0.7 mg/m^2) as no patients experienced a DLT, but the MTD of marizomib as a single agent had been attained (Fig. 3.). The median number of cycles received was 2 and mean time on treatment was 1.4 months. Dose escalation was continued up to the single agent full dose of marizomib 0.7 mg/m^2 without meeting the study defined MTD definition and thus escalation was stopped. The first 20 patients were treated with the liquid formulation of marizomib, and the last 2 patients were treated with the lyophile formulation. Adverse events related to vorinostat (fatigue, nausea, diarrhea and anorexia) were also significant at the 300 mg dose level without apparent sensitivity to the marizomib dose level, thus it was elected not to escalate the vorinostat dose. Therefore, being less than an MTD, this dose level was selected as the RP2D.

Safety

Table 2 lists all adverse events reported for all patients. Nausea, vomiting, constipation, diarrhea and fatigue were the most common adverse events reported. Fatigue, nausea, diarrhea, vomiting, and dysgeusia / anorexia were the most

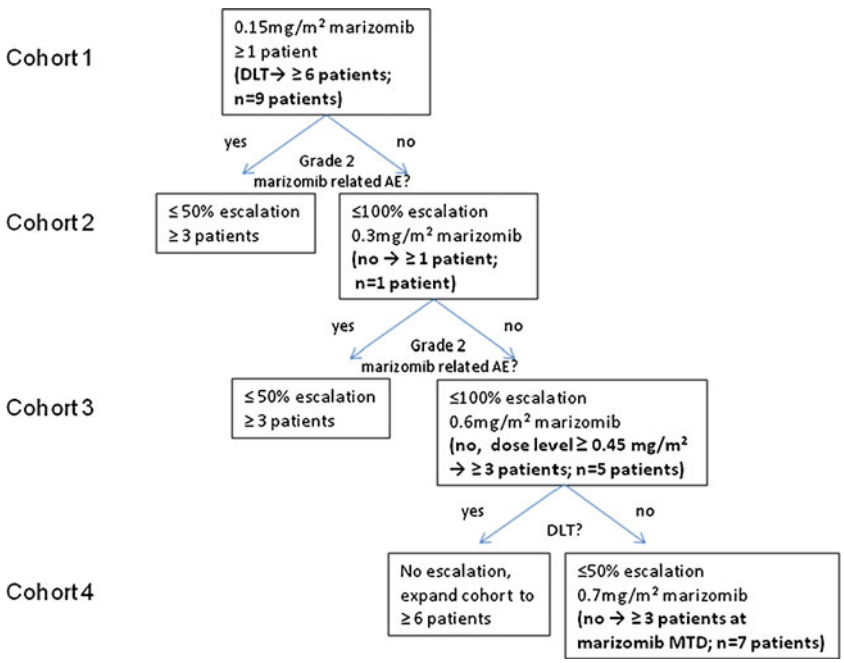

Fig. 3 Dose escalation schema

Table 2 All adverse events reported

	0.15 mg/m^2		0.3 mg/m^2		0.6 mg/m^2		0.7 mg/m^2	
	n=9		*n*=1		*n*=5		*n*=7	
	Grade 1-2	Grade 3-4	Grade 1-2	Grade 3-4	Grade 1-2	Grade 3-4	Grade 1-2	Grade 3-4
Nausea	8				5		5	
Fatigue	9		1		2		4	1
Vomiting	4				4		3	
Diarrhoea	3				2		5	
Anorexia	3				1		4	
Dyspnoea	2				3		2	
Headache	2				2		3	
Infusion site pain	5						2	
Dysgeusia	1		1		1		2	
Back pain	2	1			1			
Blood creatinine increased	1				2		1	
Decreased appetite	3				1			
Oedema peripheral	2				2			
Abdominal pain	1						2	
Blood lactate dehydrogenase increased	1				1		1	
Blood urea increased					2		1	
Constipation	2						1	
Deep vein thrombosis		3						
Haemoglobin decreased					2		1	
Lower respiratory tract infection	1				1	1		
Anaemia	1	1						
Asthenia					1		1	
Blood albumin decreased					2			
Blood glucose increased	1							1
Confusional state		1					1	
Dehydration	1				1			
Dysuria	2							
Haematocrit decreased					2			
Hallucination, auditory					1		1	
Hepatomegaly	2							
Hot flush	1				1			
Infusion site reaction	2							
Insomnia					1		1	
Joint swelling	1				1			
Lethargy					2			
Liver palpable subcostal	1						1	
Muscle spasms	1						1	
Pain					1	1		
Pain in extremity	1				1			
Urinary incontinence	1				1			
Arthralgia					1			
Ascites	1							
Balance disorder							1	
Blood bilirubin decreased	1							
Blood calcium decreased					1			

Table 2 (continued)

	0.15mg/m^2		0.3mg/m^2		0.6mg/m^2		0.7mg/m^2	
	n=9		*n*=1		*n*=5		*n*=7	
	Grade 1-2	Grade 3-4	Grade 1-2	Grade 3-4	Grade 1-2	Grade 3-4	Grade 1-2	Grade 3-4
Blood magnesium decreased					1			
Blood potassium decreased					1			
Blood triglycerides increased					1			
Blood urine present					1			
Breath sounds abnormal					1			
Catheter site related reaction							1	
Clubbing					1			
Coordination abnormal							1	
Cough					1			
Dizziness	1							
Dry mouth	1							
Dyspepsia							1	
Dysphagia	1							
Dysphasia		1						
Eye pain					1			
Faecal incontinence	1							
Fall							1	
Flatulence	1							
Fluid intake reduced	1							
Flushing							1	
Gait disturbance							1	
Gastroenteritis	1							
Gastroesophageal reflux disease	1							
Gingival bleeding	1							
Glucose urine present	1							
Haemoptysis	1							
Hallucination					1			
Herpes zoster	1							
Hiccups	1							
Hydropneumothorax	1							
Hyperglycaemia		1						
Hypertension	1							
Hyperuricaemia					1			
Hypoaesthesia					1			
Hypomagnesaemia	1							
Infusion site swelling							1	
Injection site bruising	1							
Irritability	1							
Lice infestation	1							
Limb crushing injury	1							
Limb discomfort	1							
Lip dry	1							
Mobility decreased	1							
Mucosal inflammation	1							
Nodule					1			

Table 2 (continued)

	0.15mg/m^2		0.3mg/m^2		0.6mg/m^2		0.7mg/m^2	
	n=9		*n*=1		*n*=5		*n*=7	
	Grade 1-2	Grade 3-4	Grade 1-2	Grade 3-4	Grade 1-2	Grade 3-4	Grade 1-2	Grade 3-4
Oral candidiasis	1							
Palpitations					1			
Petechiae	1							
Pitting oedema					1			
Pleural effusion	1							
Protein total decreased					1			
Pruritus	1							
Pyrexia					1			
Red blood cell count decreased					1			
Respiratory tract congestion	1							
Salivary hypersecretion	1							
Shock					1			
Syncope	1							
Thrombocytopenia		1						
Tremor	1							
Upper respiratory tract infection	1							
Viral infection					1			
Visual disturbance							1	

common adverse events considered potentially related to marizomib, with fatigue, nausea, vomiting, diarrhea and anorexia being the most common adverse events considered potentially related to vorinostat. Serious adverse events reported included deep venous thrombosis, thrombocytopenia, fever, back pain, lower respiratory tract infection, nausea/vomiting and accidental fall. Of serious adverse events, only one (Grade 2 nausea and vomiting) was considered at least possibly related to marizomib and vorinostat, and one (Grade 4 thrombocytopenia) to vorinostat alone; both are consistent with the known safety profiles of the agents. It is not clear whether the remaining adverse events represent any difference from what is expected as a baseline incidence in this patient population. No patient was seen to have a mean QTcF of ≥500 msec or increased ≥60 msec from baseline, and only 4 patients were seen to have single timepoints with ≥30 msec increases compared to baseline. Significant trends in changes of hematologic, chemistry or coagulation parameters were not identified. Only one DLT (thrombocytopenia) was reported at a dose of 0.15 mg/m^2 marizomib and 300 mg vorinostat leading to expansion of this cohort. This was considered related to vorinostat and not related to marizomib, given the known effect of vorinostat on platelets and paucity of thrombocytopenia seen in marizomib studies. No further DLTs were reported through the highest dose tested 0.7 mg/m^2 marizomib and 300 mg vorinostat. Of note, toxicities reported to be associated with vorinostat and marizomib in the literature were seen at equal to lower than expected rates in this study, and unexpected toxicities were not noted.

Tumor response

No confirmed responses were reported, however, a notable stable disease rate of 61% [11 patients (all with melanoma) / 18 evaluable patients after 2 cycles] was observed, with 39% (7/18) of evaluable patients having decreases in tumor measurements (up to 25%). Four of these patients maintained stable disease for≥4 months. The best response in tumor measurements of each patient is diagramed in Fig. 4.

Pharmacokinetics

Pharmacokinetic data indicated the half life of marizomib was <15 min and volumes of distribution were Vz=73.9± 44.7 L and Vss=80.7±53.6 L, which did not seem to be dose dependent. This is consistent with historical data as the Cmax, AUCtotal, half-life, clearance and volume of distribution data are not statistically different from those values observed with single agent marizomib studies, and are generally within 5-10% of the single agent PK values (Table 3). The pharmacokinetic parameters of vorinostat were likewise not statistically different from, and were generally within 15% of, the published data (T1/2 of 1.69±

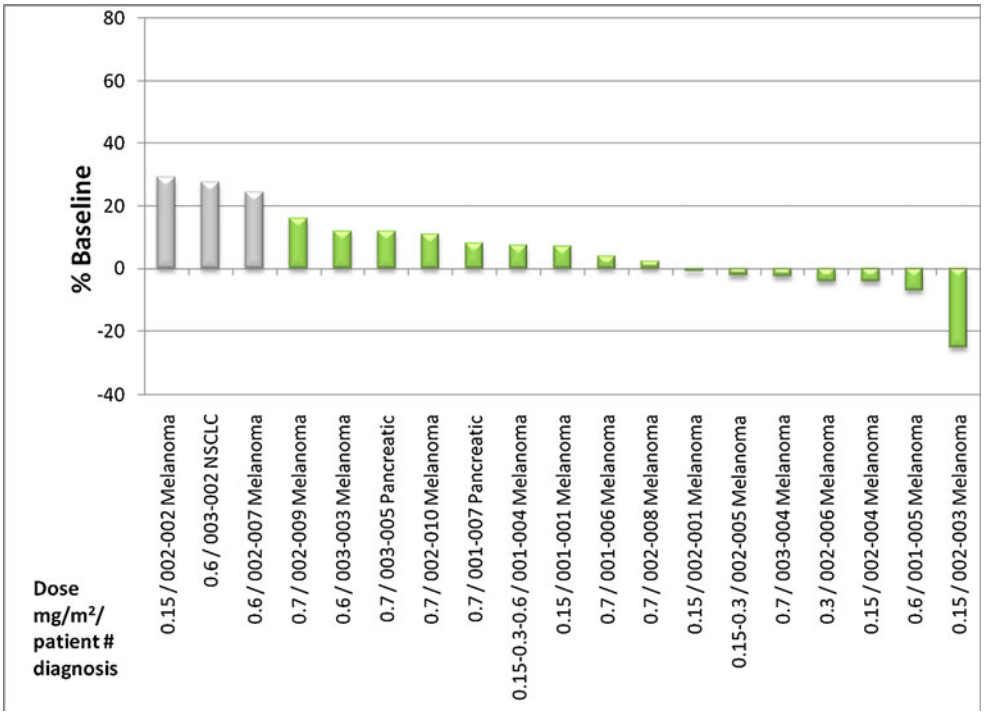

Fig. 4 Best response in tumor measurements on study

0.86 h and Vz/F of 1040.1±458.3 L) (Table 3). These results do not suggest a pharmacokinetic interaction between marizomib and vorinostat.

Pharmacodynamics

Assessment of proteasome activity in blood samples collected from patients before and after marizomib treatment at 0.15 and 0.3 mg/m^2 and vorinostat at 300 mg/day demonstrated a dose dependent inhibition of the packed whole blood CT-L activity. Inhibition up to 61%±14% and 78% respectively was observed at Cycle 2 Day 15. To evaluate the effect of vorinostat on marizomib induced proteasome inhibition, during Cycle 1 vorinostat was given 2 h after marizomib injection on Day 1 and 2 h before marizomib injection on Day 15. Comparison of proteasome inhibition in Cycle 1 Day 1 samples collected 1 h post 0.15 mg/m2 marizomib (before vorinostat administration) and 4 h post marizomib injection, (2 h after vorinostat administration) showed similar inhibition (23%±13% and 24%±10%resp.). In addition proteasome inhibition in Cycle 1 Day 15 samples collected 2 h before marizomib injection (just prior to vorinostat administration) and just before marizomib injection (2 h after vorinostat administration) was also similar 31%±16% and 30%±12% respectively. A comparable trend was observed in samples collected when marizomib was administered at 0.3 mg/m^2 (Fig. 5.). These results illustrate that the addition of vorinostat to marizomib does not appear to affect the marizomib induced inhibition of CT-L proteasome activity in packed whole blood preparations.

Discussion

In vitro studies confirmed synergistic effects of marizomib and vorinostat in multiple tumor cell types, leading to the

Table 3 Pharmacokinetics

STUDY	Cmax ng/mL	AUCtotal ng/mL*min	Half-Life Min	Clearance L/Min	Vss L
Marizomib Pharmacokinetic Parameters					
Current	30.2±15.0	310.2±119.3	14.1±3.9	5.55±3.22	75.11±39.68
All Single Agent Studies	31.3±19.8	300.4±143.4	7.61±3.5	6.19±5.43	77.89±18.09
STUDY	Cmax ng/mL	AUCtotal ng/mL*h	Half-Life Hr	Clearance L/Hr	Vz L
Vorinostat Pharmacokinetic Parameters					
Current	250.1±97.0	744.2±282.4	1.69±0.86	484.4±256.8	1040.1±458.3
Literature	297±111	802±364	2.25±0.6	444±244	1016±476

Comparison of pharmacokinetic data with data from single agent studies (marizomib at 0.7 mg/m^2, 10 min infusion weekly; vorinostat at 300 mg orally daily)

Mean±SD

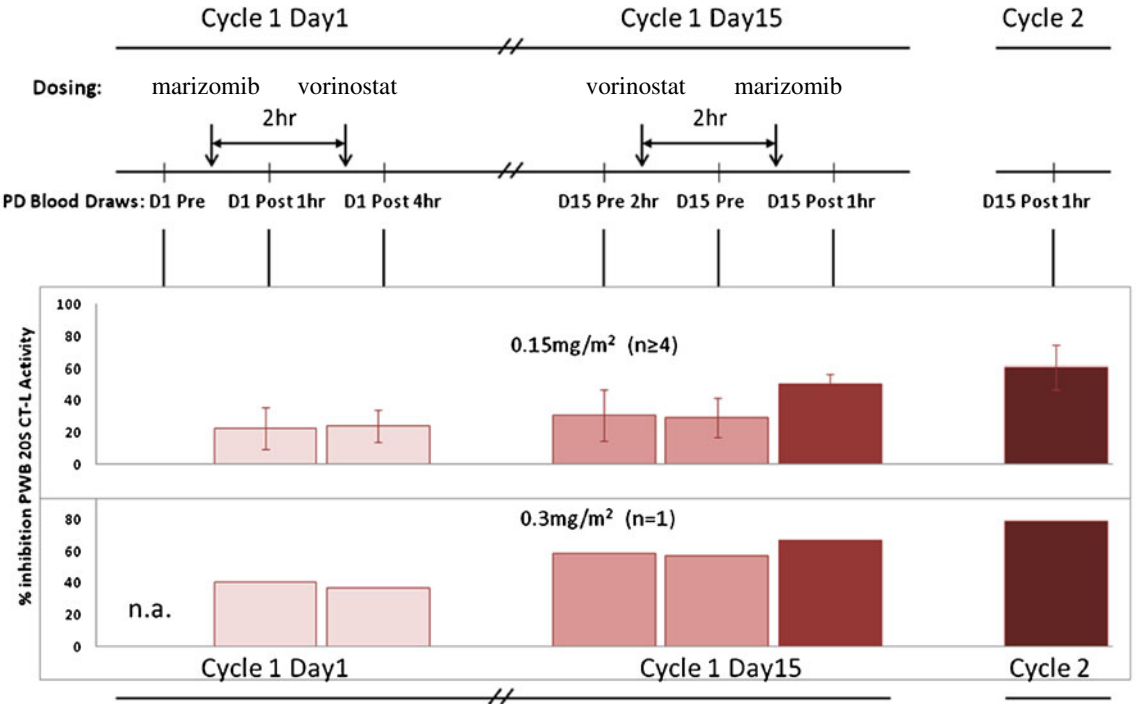

Fig. 5 Proteasome Inhibition. Comparison of proteasome inhibition by marizomib before and after vorinostat administration

initiation of the clinical trial. In this first clinical trial to evaluate a non-peptide based proteasome inhibitor in combination with an HDAC inhibitor, the full dose of marizomib was attained in the dose escalation without demonstration of unexpected or unacceptable toxicity. The RP2D was 0.7 mg/m^2 marizomib on days 1, 8 and 15 of 28-day cycles with 300 mg/day of vorinostat on days 1–16. Dose escalation of vorinostat was not attempted beyond the 300 mg per day starting dose, which is the low end of the range recommended in the prescribing information, as toxicity was notable, but still tolerable at this level. The safety profile of the combination did not appear different than what would be expected with the drugs administered alone. Pharmacokinetic and pharmacodynamic assessments likewise did not demonstrate any adverse interactions between the drugs. Indications of antitumor activity were seen in patients with melanoma with some decreases in tumor measurements and stable disease.

Our earlier findings in ALL and AML and pancreatic carcinoma models indicated that marizomib and vorinostat combinations resulted in a highly synergistic anti-tumor activity *in vitro* [8, 14]. The *in vitro* studies performed here in melanoma and NSCLC cell lines further supported assessing this combination in patients with these types of tumors. It is proposed that proteasome inhibitors induce apoptosis in cells by preventing degradation of key proapoptotic and anti-growth proteins as well as promoting proteotoxic protein accumulation and protein aggregation which increases cellular stress. Treatment with proteasome inhibitors results in the accumulation of ubiquitin-conjugated proteins that are organized into structures called aggresomes. These structures are formed as a cytoprotective mechanism. In contrast to proteasome inhibitors, HDAC inhibitors have been shown to block aggresome formation. Blocking of aggresome formation by HDAC inhibitors can further increase cellular stress. Tumor cells, particularly tumor types such as myeloma, have an extremely high protein turnover and possess a well developed endoplasmic reticulum. Thus, the ability of proteasome and HDAC inhibitors to significantly enhance cellular stress may contribute to the marked synergy with these two drug combinations. Interestingly the differences in cytotoxic activity of marizomib in these various cells lines was not related to the inhibition profiles for the proteasome CT-L activity. Additional studies indicated similar enhanced activity of marizomib when combined with other HDAC inhibitors with different class specificities such as tubacin and SNDX275 (MS275) in multiple myeloma cells lines (Chauhan and Anderson unpublished observations). The findings suggested that changes in sensitivity were not related to changes in proteasome sensitivity to marizomib and support the use of combination therapy that targets other distinct or overlapping pathways. Resistance may be mediated by intrinsic genetic or epigenetic characteristics of individual tumors, as has been suggested in studies with bortezomib indicating the degree of NF-kB activation to be vital.

Combining marizomib with vorinostat in patients was seen to be feasible and tolerable, with the most common

adverse events being nausea, vomiting, constipation, diarrhea and fatigue. Thus, antidiarrheals, stool softeners and antiemetics, such as serotonin antagonists, could be used as needed and prophylactic anti-emetic use was recommended at doses of marizomib>0.3 mg/m^2. This is not much different than what has been reported with both drugs alone. Fatigue, nausea, vomiting, dizziness, headache and diarrhea have been commonly reported with marizomib alone [15]. For vorinostat the most common adverse reactions are diarrhea, fatigue, nausea, thrombocytopenia, anorexia and dysgeusia [7]. This is markedly different than the boronated peptide based proteasome inhibitor bortezomib, for which the most commonly reported adverse reactions include asthenic conditions, diarrhea, nausea, constipation, peripheral neuropathy, vomiting, pyrexia, thrombocytopenia, psychiatric disorders, anorexia and decreased appetite, neutropenia, neuralgia, leukopenia and anemia. Further, studies combining bortezomib with vorinostat have reported myelosuppression as a common grade 3 toxicity [16, 17]. Other similar toxicities included fatigue, nausea, diarrhea and QT prolongation. There was not evidence of a significant effect on QT interval in this study in spite of the known effect of vorinostat on QTc interval [18]. It, however, seems reasonable to monitor electrolytes and electrocardiogams with this combination as is recommended for vorinostat alone. Thus, the combination of marizomib with vorinostat in the study did not demonstrate adverse drug-drug interactions, or other significant new safety findings.

Pharmacokinetic data indicate a short half-life of marizomib and large volumes of distribution, which do not seem to be dose dependent, consistent with historical data. The pharmacokinetic parameters of vorinostat were likewise in agreement with published data. Co-administration of vorinostat with marizomib therefore does not appear to affect the kinetics of either drug. This is not unexpected as the proposed molecular targets and elimination pathways of the drugs are markedly different (glucuronidation for vorinostat vs. spontaneous hydrolysis in plasma for marizomib) [16].

It has been demonstrated that proteasome inhibitors such as bortezomib can also regulate the expression of class I HDACs and conversely, HDAC inhibitors can regulate the expression of proteasome 20S subunits [19, 20]. These recent findings of additional downstream targets for these drugs and the induction of cellular stress mechanisms may explain why myeloma and other tumor cell types are highly sensitive to the combination of proteasome and HDAC inhibitors. Pharmacodynamic data from the patient samples show that marizomib inhibits proteasome activity into the predicted minimum effective range at doses≥0.15 mg/m^2. Inhibition is dose and time dependent, and reached that obtained with therapeutic doses of bortezomib without producing the adverse event profile reported with bortezomib. At the doses and scheduling evaluated, inhibition of the proteasome by marizomib did not appear to be altered by vorinostat. This was consistent with the *in* vitro results. However, the effect of vorinostat on nucleated cells that can generate new proteasomes and generally have shorted half-lives versus the non-nucleated RBCs was not evaluated.

Patient efficacy assessments were encouraging, albeit not definitive. Stable disease, including prolonged stable disease, was reported in 61% of patients (all having melanoma; the 3 patients with pancreatic carcinoma and NSCLC having evaluable disease all had progressive disease as a best response), with 39% of patients having decreases in tumor measurements, and could be maintained with chronic dosing without the emergence of significant toxicity. Efficacy results were equivalent to better than those reported with the combination of bortezomib and vorinostat in solid tumors [21], although significant activity has been reported with this combination in myeloma [17, 18]. Both vorinostat and bortezomib have been assessed separately in patients with advanced NSCLC and melanoma without much difference in outcome [22–24]. Albeit promising data with marizomib in this histology from laboratory studies and another clinical trial lead to particular interest in testing the combination of marizomib and vorinostat in patients with melanoma, it was perhaps not the most obvious target for the initial evaluation of this combination given that the principal activity for both classes of drugs has been in hematologic malignancies. Further, although there have been some recent successes, melanoma has generally demonstrated therapeutic intractability to a majority of agents.

Laboratory studies indicate the combination of proteasome and HDAC inhibition offers significant therapeutic potential based on complimentary inhibition of protein disposal which is critical to tumor cell survival. This strategy is just beginning to be translated into clinical trials with some success. High response rates have been reported with the combination of bortezomib and vorinostat in a Phase 3 study in multiple myeloma [18]. Marizomib offers potential advantages in terms of proteasome inhibition profile and safety as illustrated by this study. It is hoped this can be carried through to further clinical trials in other malignancies where superior efficacy might be clearly demonstrated.

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