Preclinical Activity of the Rational Combination of Selumetinib (AZD6244) in Combination with Vorinostat in KRAS Mutant Colorectal Cancer Models

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Translational relevance

Colorectal cancer (CRC) is the third leading cause of cancer worldwide. Although current therapeutic regimens incorporate active chemotherapy and biological agents, patients eventually relapse without options for effective salvage therapy. Recent analysis of numerous clinical trials evaluating response rates of advanced CRC to epidermal growth factor receptor (EGFR) inhibitors demonstrated a correlation between activating mutations of the Kristen rat sarcoma viral oncogene homolog (KRAS) gene and resistance to these agents, thus identifying a large subset of patients in urgent need of alternative therapeutic approaches. In the present study, we utilized gene set enrichment analyses to support the rational combination of the MEK 1/2 inhibitor, selumetinib (AZD6244), and the histone deacetylase (HDAC) inhibitor, vorinostat (SAHA), against preclinical models of KRAS mutant CRC. Our data demonstrates that this combination exhibits potent, synergistic anti-tumor effects in, both in vitro and in vivo. These findings strongly support the clinical development of this rational combination in CRC patients with KRAS mutated tumors.

Abstract

Purpose: Despite the availability of several active combination regimens for advanced colorectal cancer (CRC), the 5-year survival rate remains poor at < 10%, supporting the development of novel therapeutic approaches. In this study, we focused on the preclinical assessment of a rationally-based combination against KRAS mutated CRC by testing the
combination of the MEK inhibitor, selumetinib, and vorinostat, a histone deacetylase (HDAC) inhibitor.

**Experimental Design:** Transcriptional profiling and gene set enrichment analysis (baseline and post-treatment) of CRC cell lines provided the rationale for the combination. The activity of selumetinib and vorinostat against the KRAS-mutant SW620 and SW480 CRC cell lines was studied in vitro and in vivo. The effects of this combination on tumor phenotype were assessed using monolayer and 3D cultures, flow cytometry, apoptosis, and cell migration. In vivo, tumor growth inhibition, 18F-fluoro-deoxy-glucose positron emission tomography (FDG-PET) and proton nuclear magnetic resonance (1H-NMR) were performed to evaluate the growth inhibitory and metabolic responses, respectively, in CRC xenografts.

**Results:** In vitro, treatment with selumetinib and vorinostat resulted in a synergistic inhibition of proliferation and spheroid formation in both CRC cell lines. This inhibition was associated with an increase in apoptosis, cell-cycle arrest in G1, and reduced cellular migration and VEGF-A secretion. In vivo, the combination resulted in additive tumor growth inhibition. The metabolic response to selumetinib and vorinostat consisted of significant inhibition of membrane phospholipids; no significant changes in glucose uptake or metabolism were observed in any of the treatment groups.

**Conclusion:** These data indicate that the rationally-based combination of the MEK inhibitor, selumetinib, with the HDAC inhibitor, vorinostat, results in synergistic anti-proliferative activity against KRAS mutant CRC cell lines in vitro. In vivo, the combination demonstrated additive effects that were associated with metabolic changes.
in phospholipid turnover, but not on FDG-PET, indicating that the former is a more sensitive endpoint of the combination effects.
Introduction

Selumetinib (AZD6244; ARRY-142886) is a small molecule, orally available, non-competitive inhibitor of mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2 (MEK1/2) (1). The RAS/RAF/MEK/ERK kinase cascade occupies a central role in mediating signal transduction from extracellular growth factors, cytokines and proto-oncogenes. Alterations in the control of RAS/MAPK pathway, resulting in its constitutive activation, have a well-established role in oncogenesis and tumor growth, mostly related to uncontrolled cell proliferation and suppression of apoptosis (2). The hyperactivation of this cascade is mediated through various mechanisms, including tyrosine kinase receptor over expression or mutations, or to KRAS or BRAF activating mutations. KRAS mutations are common in many different tumors, particularly in pancreatic (90%) and colon cancers (50%) (3, 4). Although BRAF mutations are more common in melanoma (63%), papillary thyroid cancer (45%), and low grade ovarian cancers (50%), the BRAF V600E mutation has also been found in colorectal cancers that also exhibit defective DNA mismatch repair (5, 6). The RAS/MAPK cascade is one of the major downstream signaling networks linking the epidermal growth factor receptor (EGFR), insulin-like growth factor-1 receptor (IGF-1R), and vascular endothelial growth factor receptor-2 (VEGFR-2) pathways to nuclear proteins. The safety profile and tolerability of selumetinib has been evaluated in a two-part, multi-center, ascending dose, Phase I clinical study (7). This trial demonstrated the tolerability of selumetinib, with the most common treatment-related toxicities being rash, diarrhea, nausea and fatigue. Several phase II trials are ongoing to evaluate the activity of selumetinib, as single agent or in combination with chemotherapy in NSCLC, melanoma and colorectal cancer (8-10).
Histone deacetylation by HDACs is a post-translational modification of lysine residues in nucleosomal histone proteins that affects chromatin structure and, thereby, gene regulation (11). Recently, HDAC activity has been shown to be up-regulated in cancer cells and it has been theorized that this results in repression of tumor suppressor gene products such as p53, making HDACs an attractive drug target (12, 13). In cell culture models, HDAC inhibitors (HDACi) have been shown to decrease proliferation, and induce apoptosis or autophagy-related death of several cell lines (14-16). Due to their relative specificity towards cancer cells, HDACi represent a new class of cancer treatment agents that are generally well tolerated. One such compound, vorinostat, (suberoylanilide hydroxamic acid, SAHA), has shown encouraging activity in early studies against several cancers including B-cell lymphoma (17), colon cancer (18, 19), non-small cell lung carcinoma (NSCLC) (20), and head and neck cancer and is currently approved for the treatment of cutaneous T-cell lymphoma (21). Several phase II studies have been conducted for breast (22), colon and lung (23, 24), head and neck (25), and ovarian cancer patients (26), however no consistent anti-tumor activity of vorinostat as single agent has been observed (11). The prevailing view is that further investigations to evaluate the safety and activity of vorinostat as a combination partner are needed to better evaluate the potential of this agent in cancer treatment (27). Several reports in the literature have demonstrated synergistic interaction between MEK and HDAC inhibitors. Yu et al (28, 29), demonstrated that combining HDAC with MEK inhibitors resulted in increased apoptosis, through the induction of oxidative damage and ROS generation (well known response markers for HDAC), as well as enhanced lethality in leukemia cells expressing the ABL/BCR mutation and resistance to imatinib.
Based on these data, and our own transcriptional profiling and gene set enrichment analysis, we hypothesized that targeting the MEK pathway would inhibit signal transduction pathways involved in CRC tumor cell proliferation, survival and angiogenesis, and that this could be potentiated by combination with vorinostat (30). Furthermore, we focused these preclinical studies on CRC tumors expressing KRAS mutations, since they are associated with resistance to other signal transduction inhibitors, and represent a subtype of CRC that is in dire need of new therapeutic strategies. Finally, we aimed to establish sensitive in vivo end-points for MEK and HDAC inhibitors in a murine CRC xenograft model.
Materials and Methods.

Drugs. Selumetinib (AZD6244) and Vorinostat (SAHA) were generously provided by AstraZeneca Pharmaceuticals LP and Merk Sharp and Dohme Corporation, respectively and National Cancer Institute, NIH. Selumetinib (AZD6244) was prepared as a 10 mM stock solution in DMSO for the in vitro experiments. For the in vivo studies, selumetinib was prepared in a solution of 0.5% methyl cellulose/0.1% Tween 80 in water. Vorinostat (SAHA) was prepared as a 10 mM stock solution in DMSO for the in vitro studies and as a solution of 45% PEG400 in water for the in vivo studies.

Culture of cell lines and assessment of cytotoxicity to selumetinib, vorinostat, or the combination. The following human colon cancer cell lines were obtained from American Type Culture Collection (Manassas, VA): HCT116 (KRAS mut), HCT15 (KRAS mut), HCT8 (KRAS mut), HT29 (KRAS wt), SW480 (KRAS mut), SW620 (KRAS mut), SW1417 (KRAS wt), LoVo (KRAS mut), LS513(KRAS mut), LS180 (KRAS mut), LS174T (KRAS mut), LS1034 (KRAS mut), Colo205 (KRAS wt), and the RKO (KRAS wt). Cells were grown in RPMI medium supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids, 1% penicillin/streptomycin and maintained at 37°C in an incubator under an atmosphere containing 5% CO₂. The cells were routinely screened for the presence of mycoplasma (MycoAlert, Cambrex Bio Science, Baltimore, MD). Cytotoxic effects were determined using the sulforhodamine B (SRB) method as previously described (19). Briefly, cells in logarithmic growth phase were transferred to 96 well flat bottom plates with lids. One hundred microliter of cell suspensions containing viable HCT116 (1,500 cells/well), HCT15 (2,000 cells/well), HCT8 (1,500 cells/well), GEO (2,000 cells/well), SW837(1,500/well), HT29 (3,000 cells/well), SW480
(2,500 cells/well), SW620 (3,000 cells/well), SW1417 (4,000 cells/well), LoVo (5,000 cells/well), LS513 (5,000 cells/well), GEO (2,000 cells/well), SW837 (1,500 cells/well), LS180 (3,000 cells/well), LS174T (3,500 cells/well), LS1034 (6,000 cells/well), Colo205 (8,000 cells/well), and RKO (1,500 cells/well) cells were plated into each well and incubated overnight prior to exposure to selumetinib or vorinostat. Initially, all of the cell lines were exposed for 24, 48 and 72 hrs to increasing concentrations of selumetinib (0-5 μmol/L) and vorinostat (0-10 μmol/L). Post drug treatment, media was removed and cells were fixed with cold 10% trichloroacetic acid (TCA) for 30 min at 4°C. Cells were then washed with water and stained with 0.4% SRB (Fisher Sci., Pittsburgh, PA) for 30 min at room temperature, washed again with 1% acetic acid, followed by stain solubilization with 10mM TRIS at room temperature on shaker for 15 min. The plates were then read on a plate reader (Biotek Synergy 2, Winooski, VT) set at an absorbance wavelength of 565 nm, and cell proliferation curves were derived from the raw absorbance (OD) data. The cells were classified as sensitive to the drug if their IC₅₀ was lower than 1μM or resistant if their IC₅₀ was higher than 1μM as previously described, (31).

**Transcriptional profiling of the CRC cell lines.** The baseline gene expression of all of the human CRC cell lines was assessed using the Affymetrix U133 Plus 2.0 microarrays. Additionally, post-treatment profiling was performed on HCT8 (selumetinib-R/vorinostat-S), HT29 (selumetinib-S/vorinostat-R), SW480 (R/R), and SW620 (S/S) cells, treated with vorinostat and selumetinib as single agents and in combination. The cells were plated in T75 flasks for 24 hrs, and then treated with vorinostat or selumetinib for 72 hrs. All cell lines were treated at doses equivalent to the IC₅₀ of the most sensitive cell line. After treatment, RNA was extracted with a RNeasy Plus Mini Kit (Qiagen Inc,
Valencia, CA) and analyzed by the Gene Array Core at the University of Colorado Cancer Center. Absolute intensity signals from the microarray gene expression profiles were extracted using Affymetrix Power Tools (APT) and probe sets representing the same gene were collapsed based on maximum values. Next, the gene expression levels were converted to a rank-based matrix and standardized (mean = 0, standard deviation = 1) for each microarray. Using this pre-processing method, the same cell lines from different data sets were clustered based on their gene expression profiles. Data analyses were performed on this rank-based matrix.

**Gene set enrichment analysis.** Gene set analysis was performed using the GSEA software Version 2.0.1 obtained from the Broad Institute (http://www.broad.mit.edu/gsea). Gene set permutations were performed 1000 times for each analysis. We used the nominal p-value and Normalized Enrichment Score (NES) to sort the pathways enriched in each phenotype. We used the 313 pathways defined by BioCarta database as the gene set in this study (www.biocarta.com). One hundred and eighty eight gene sets passed the gene set size filter criteria (min = 10, max = 500).

**In vitro evaluation of the combination effects of selumetinib and vorinostat.** Synergy was determined for the KRAS-mut cell lines SW837, LS1034, HCT116, GEO, SW480 and SW620. These cell lines were selected because all of them contain KRAS mutations but exhibit divergent sensitivity to the single agents. To evaluate synergy cells were exposed for 72 hrs to vorinostat at 0.375, 0.75, 1.5 μM and selumetinib at 0.125, 0.250, 0.5 and 1 μM (SW480), or 0.06, 0.125, 0.250 and 0.5 μM (SW620), in all possible combinations. The results of the combined treatment were analyzed according to the isobolographic method of Chou and Talalay (32), using the CalcuSyn software program.
(Biosoft, Cambridge, UK). The resulting Combination Index (CI) was used as a quantitative measure of the degree of interaction between the two drugs. A CI equal to 1 denotes additivity, CI greater than 1 antagonism, and CI values less than 1 indicate synergism.

The effect of selumetinib and vorinostat was further evaluated in the SW480 cell line which is KRAS mut, resistant to selumetinib as well as vorinostat (R/R), and in the SW620 cell line, which is KRAS mut but sensitive to both drugs (S/S) using a 3D culture system. Briefly, 100μl of matrigel (BD Biosciences, Bedford, MA) was plated in 8-well chamber slides (BD Biosciences, Bedford, MA) to form a base layer. Cells were then seeded at the density of 2500 cells/well in 2% matrigel-supplemented media to form the upper layer. After an initial incubation to allow for tumor spheroid formation, vehicle or drugs were added to media and changed every three days for a total of 15 days. The SW480 (R/R) were exposed to vorinostat (0.750 μM) or selumetinib (0.125 μM) or the combination, whereas SW620 (S/S) cells were treated with selumetinib (0.06 μM), vorinostat (0.750 μM), or the combination. After 15 days of treatment, the plates were photographed at 20X magnification on an inverted microscope. Spheroids with a diameter higher than 1 micron were counted. All of the **in vitro** experiments were conducted in triplicate.

**Flow cytometric analysis of cell cycle distribution.** For cell cycle analysis, cells were seeded in 6-well plates (2.5 x 10⁵ cells/well) for 24 hours. The cells were then treated with vorinostat at 0.750 μM, or selumetinib at doses of 0.125 μmol/L for SW480 (R/R) and 0.06 μmol/L for SW620 (S/S), or with the respective combination for 24 hours. Culture media was removed and the cells were washed in PBS, harvested, centrifuged for
5 min at 1500 rpm, and resuspended in Krishan’s stain. After incubation at 4°C for 24 hours, the samples were analyzed by flow cytometry at the University of Colorado Cancer Center Flow Cytometry Core Facility. Experiments were conducted in triplicate.

**Apoptosis assay.** SW480 and SW620 cells were seeded in 96-well, white walled plates and allowed to plate for 24 hours. SW480 (R/R) cells were treated with vorinostat (0.750 μmol/L) or selumetinib (0.125 μmol/L) or the combination for 24, 48 and 72 hours, after which apoptosis was determined by the measurement of caspase 3/7 activity using a luminometric Caspase-Glo 3/7 assay (Promega, Madison, WI) according to the manufacturer’s protocol and read using a 96-well plate reader. Cellular apoptosis was expressed as the fold-increase over untreated control cells. Similarly, SW620 (S/S) cells were treated with selumetinib (0.06 μmol/L) or vorinostat (0.750 μmol/L) or the combination for 24, 48 and 72 hours and apoptosis was measured as described above. Experiments were conducted in triplicate.

**Immunoblotting of downstream effector proteins.** SW480 and SW620 cells were seeded into 6-well plates (2.5 x 10^5 cells/well) for 24 hrs prior to treatment and exposed to each drug alone or in combination for an additional 24 hrs. After treatment, cells were harvested in RIPA buffer containing protease inhibitors, EDTA, NaF, and sodium orthovanadate. The total protein in samples was determined using the BioRad DC Protein Assay (BioRad, Hercules, CA). Fifty micrograms of total protein was loaded onto a 4-20% gradient gel, electrophoresed and then transferred to nitrocellulose using the I-Blot (Invitrogen). Membranes were blocked for 1 hour in blocking buffer [0.1% casein solution in 0.2 phosphate buffered saline (PBS)], prior to overnight incubation at 4°C with one of the following primary antibodies: phospho-ERK1/2, cleaved PARP,
acetylated histone H3 or β-actin (Cell Signaling Technology). Following primary antibody incubation, membranes were then washed 4 times (10 minutes each) in TBS-Tween (0.1%) and then incubated with the appropriate secondary anti-rabbit or antimouse IgG1 horseradish peroxidase (HRP)-linked antibody at 1:15,000 (Jackson ImmunoResearch) for 1 hour at room temperature. After four additional washes, blots were developed using the Odyssey Infrared Imaging System (LI-COR Biosciences). Immunoblot experiments were done in triplicate for each antibody.

**(Effects of selumetinib and vorinostat on ligand-induced cell migration.** The effects of selumetinib and vorinostat on SW620 and SW480 cell migration were measured using the modified Boyden chamber assay. Assays were performed using uncoated 8.0 micron transwell inserts placed in 24 well plates (Becton Dickinson, Franklin Lakes, NJ). Cells were resuspended in 250 μl of standard growth media (RPMI supplemented with 10% FBS, 1% non-essential amino acids, 1% penicillin/streptomycin) and seeded at a density of 10 x 10⁴ cells/well onto the inserts (upper chamber of the well) and the lower chambers of each well were filled with 600 μl of standard growth media. After 24 hours, the media in the upper chambers was replaced with media containing 0.1% FBS in presence or absence of selumetinib or vorinostat or the combinations (selumetinib at the doses of 0.125μmol/L; vorinostat at the dose of 0.750 μmol/L). The media in the bottom of the 24 well plate was replaced with fresh media containing 10% FBS with or without the drugs (selumetinib 0.125μmol/L or vorinostat, at the dose of 0.750 μmol/L). After 48 hours, the filters were fixed with 4% formaldehyde for 15 min, washed three times with D-PBS, and migrating cells were stained with DAPI for 30 min, washed with D-PBS 3x15 min and photographed at 20X magnification on an inverted microscope. Five fields
per well were counted manually and averaged. Selumetinib, vorinostat and the combination effects on epithelial growth factor (EGF)- or insulin-like growth factor (IGF-I)-induced migration was explored using SW480 (R/R) cells because the SW620 cells were not sufficiently migratory. Briefly, 10 x 10⁴ cells/well were resuspended in 250μl of standard growth media and plated in the upper chamber. The lower chamber of the wells was filled with 600 μl of standard growth media. After 24 hr, the media in the upper and lower chambers of the plate was replaced with 0.1% FBS media and incubated overnight in the presence or absence of treatment. Cell migration was then stimulated by replacing media in the lower chamber with media containing either 10% FBS or EGF (100 ng/ml), IGF (200 ng/ml) in absence or presence of treatment (selumetinib at the dose of 0.125μmol/L; vorinostat at the dose of 0.750 μmol/L). Cell migration was evaluated after 48 hours from growth factor stimulation, as described above. After 48 hours the filters were fixed with 4% formaldehyde for 15 min and washed three times with D-PBS, and migrating cells were stained with DAPI for 30 min, washed with D-PBS 3x15 min and photographed at 20X magnification on an inverted microscope. Five fields per well were counted manually and averaged.

**Assessment of the selumetinib and vorinostat combination in vivo.** Female athymic nude mice, 4-6 weeks old, were purchased from Harlan Laboratories (Harlan Laboratories online). Animals were housed in polycarbonate cages and maintained on a 12-hour light/dark cycle in the University of Colorado Center for Comparative Medicine, a facility accredited by the American Association for Accreditation of Laboratory Animal Care. Animals were housed 3-5 per cage and food and water were provided *ad libitum*. All studies were conducted in accordance with the NIH Guidelines for the Care and Use
of Laboratory Animals. SW620 (S/S) and SW480 (R/R) cell lines were harvested in exponential growth phase and resuspended in a 1:1 mixture of media:matrigel. One million SW620 and five million of SW480 per injection were injected subcutaneously in both flanks of each mouse. When tumors reached an average of 150-300 mm$^3$ of volume, mice were randomized into four groups (n=8 tumor per group). Animals received either vehicle, 25 mg/kg selumetinib, 75 mg/kg vorinostat, or a combination of 25mg/kg selumetinib and 75mg/kg vorinostat. Selumetinib was administered by oral gavage twice a day (BID), 7 days per week. Vorinostat was administered by intraperitoneal injection (IP) daily, 5 days per week. For combination treatment, animals received selumetinib followed immediately by vorinostat injection. Mice were monitored daily for signs of toxicity and were weighed twice weekly. Tumor size was evaluated twice per week by caliper measurements using the following formula: tumor volume = [length × width$^2$] / 0.52. Tumor volume and body weight data was collected using the Study Director Software package (Studylog Systems, South San Francisco, CA). Animals were treated for 28 days, and then euthanized by isoflurane anesthesia. These studies were carried out under an approved University of Colorado Animal Care (IACUC) protocol.

**Tumor metabolic response of SW480 xenografts by $^{18}$FDG-PET and $^1$H-NMR.** $^{18}$F-fluoro-2-deoxy-D-glucose positron emission tomography (FDG)-PET was performed on SW620 (S/S) xenografts to measure the effect of selumetinib, vorinostat, or the combination on tumor metabolic activity. When tumors reached an average of 150-200 mm$^3$, mice were randomized into four groups (n = 8 tumors per group). Treatment groups were the same as the first *in vivo* study and animals were treated for a total of 21 days as described in the previous section. FDG-PET was performed the day before the start of
treatment (baseline; BL) and on days 4 (D4) and 21 (D21) of treatment. Animals were fasted for 4-8 hours prior to FDG injection and glucose blood levels were monitored. Approximately 250 μCi of FDG, obtained through the University of Colorado Hospital (PetNet Solutions, Knoxville, TN), was administered by tail vein injection (IV) to conscious animals. Animals were maintained in cages on a heated water pad for one hour to allow for FDG uptake in tumors. Under isoflurane anesthesia (2.5%), animals were placed on a warm pad (m2m Imaging, Cleveland, OH) and a 10-minute emission scan was acquired with a Siemens Inveon micro-PET scanner (Siemens Medical, Knoxville, TN). Image analysis was performed with AsiProVM (Concorde Microsystems, Knoxville, TN) software. Regions of interest (ROIs) were drawn with the trace command around the tumors on axial slices and the total activity of all tumor slices was summed. Total activity was divided by the time-corrected dose delivered (time corrected dose = dose injected x exp (-0.006317*t), where t is the time between the injection and scan time and is presented as the percentage of the respective tumor’s baseline scan.

By the end of the study (day 22) CRC xenografts were collected, snap frozen in liquid nitrogen and extracted for an expanded metabolic profile by 1H-NMR. The exact extraction and NMR acquisition protocols were extensively reported previously by our group (36, 37). All quantitative data sets (presented as μmol of metabolite per gram tissue) were included in custom-build metabolic fluxes analyzers and data interfaces and presented as metabolic heat maps (38).

**Statistical Analysis.** The statistical analyses of the *in vitro* and *in vivo* data were carried out using Prism version 4.02 program (GraphPad Software, Inc). A one-way ANOVA test was performed to evaluate statistically significant changes between the study groups.
P values <0.05 were considered statistically significant. Bar represents the mean of three independent experiments performed in triplicate.
Results

**Gene array and pathway analyses.** To identify genes and pathways that are correlated with single-agent vorinostat sensitivity, we compared the baseline gene expression across the CRC cell line panel using GSEA. HDAC pathway was among the top enriched pathways in the sensitive lines as compared to the resistant lines. We next sought to identify which genes and pathways were enriched in the resistant cell lines after vorinostat treatment. Comparing the baseline and post-treatment expression profiles of the two resistant cell lines (HT29 and SW480) revealed that MAPK signaling pathway is among the top enriched pathways after vorinostat treatment (Figure 1 A). The core genes of the MAPK signaling pathway are illustrated in Figure 1 B and C. This suggests that treating these vorinostat resistant cell lines with selumetinib may synergize the combination drug effects. Indeed, when we compared the core genes in the MAPK signaling pathway of the two vorinostat-resistant cell lines after selumetinib and the combination, the gene expression patterns were altered (Figure 1 B).

**Effects of the Selumetinib and Vorinostat combination on proliferation of KRAS-mutant CRC cell lines in vitro.** To seek for a new active treatment for KRAS-mut CRC patient we studied the combination of selumetinib and vorinostat on GEO, HCT116, SW837, SW620 and LS1034. In an initial study, conduct on a panel of colorectal cancer cell lines to evaluate the activity of selumetinib and vorinostat as single agent, we observed that the SW837 and the SW480 were resistant to both selumetinib and vorinostat (R/R), the GEO were resistant to selumetinib and sensitive/intermediate for vorinostat (R/I-R), the LS1034 were sensitive to selumetinib and resistant to vorinostat (S/R), and the SW620 were sensitive to both drugs (S/S) as showed in Supplemental Data.
Figure S1. Then, cells were treated concurrently for 72 hours at clinically relevant doses, according to phase I trial results (7, 39, 40). Our results, as calculated by the method of Chou and Talalay (32) showed that selumetinib and vorinostat treatment resulted in clear synergy for the all cell lines analyzed (Supplemental Data Figure S2), with a strongest effect observed for the SW620 (S/S) cell line (Figure 2 A, B) with CI values between 0.354-0.736, and for the SW480 (R/R) cell line (Figure 2 C, D) with CI values of 0.07-0.777. Based on these results, we chose to use for the subsequent experiments the SW620 and the SW480, treated at the following doses of selumetinib and vorinostat for subsequent experiments: SW620 (S/S): 0.06 μM selumetinib and 0.750 μM vorinostat; SW480 (R/R): 0.125 μM selumetinib and 0.750 μM vorinostat.

To assess the efficacy of selumetinib, vorinostat and the combination in a more physiological model, we next performed a 3 dimensional (3D) culture analysis with tumor spheroid formation in matrigel as the endpoint. As depicted in Figure 3 A-D, the SW620 S/S and SW480 R/R cells generated spheroids when growth in matrigel which were inhibited by both single agents and more strikingly, by the combination. Interestingly, the SW480 R/R cells displayed a sensitive phenotype to selumetinib and vorinostat in 3D culture but a resistant phenotype in monolayer growth, suggesting that these agents require more intact tumor architecture to exert their full effects.

Effects of selumetinib and vorinostat on cell cycle distribution, apoptosis, and downstream signaling pathways. To further elucidate the mechanism(s) responsible for the observed synergistic effects on CRC cell proliferation, we investigated the impact of selumetinib in combination with vorinostat on cell cycle distribution, apoptosis, and intracellular signaling pathways. By flow cytometry, cell cycle analysis following
treatment of SW620 S/S cells with single-agent selumetinib or vorinostat resulted in a significant increase in the percentage of cells in G1, which was further increased in the combination and associated with a reduction of cells in S phase (Figure 4A). By contrast, in the SW480, R/R cells, G1 arrest was induced by treatment with selumetinib which was maintained in the combination (Figure 4B). Next, we evaluated the ability of selumetinib, vorinostat, or the combination to induce apoptosis in SW620 and SW480 cells after 24 hours of exposure to these agents using a caspase 3/7 activity assay. While apoptosis was modestly increased by the single-agents, the combination showed a significant induction of apoptosis in both cell lines, as depicted in Figure 4 C, D.

Analysis of downstream signaling pathways by immunoblotting demonstrated that in both cell lines selumetinib reduced ERK phosphorylation and vorinostat induced histone H3 acetylation, as expected (Figure S3). Interestingly, H3 acetylation was markedly increased in the combination treatment (Figure S3). Increased PARP cleavage was observed in both cell lines after treatment with vorinostat or selumetinib, however, levels of cleaved PARP were markedly increased in SW620 S/S line after the combination treatment, and to a lesser extent in the resistant cell line, SW480 (R/R) consistent with an increase in apoptosis as observed using the caspase 3/7 activity assay (Figure S3; Figure 4, C, D).

Effects of selumetinib, vorinostat, and the combination on ligand-induced migration of SW480 cells. As cancer cell migration is mediated by growth factor pathways such as the RAS/RAF/MEK/ERK pathway (41-47), we evaluated the effects of selumetinib and vorinostat alone and in combination on cell migration using a modified Boyden chamber assay. As depicted in Supplementary Data Figure S4, the combination of selumetinib and
vorinostat significantly reduced both FBS- and IGF-stimulated migration compared to EGF, which exhibited little induction of migration, regardless of treatment.

**Antitumor activity of selumetinib in combination with vorinostat in SW620 S/S and SW480 R/R xenograft models.** To further evaluate the efficacy of selumetinib in combination with vorinostat as a potential treatment option for CRC we tested the tumor growth inhibitory potential of these drugs in a murine xenograft model. Tumor growth curves for these studies are depicted in Supplementary Data **Figure S5** for the SW480 R/R xenograft and in **Figure 5** A for the SW620 S/S xenograft. In the SW620 S/S model, vorinostat alone demonstrated little anti-tumor growth effects while selumetinib and the combination were similar, demonstrating a trend towards significant tumor growth inhibition compared to control (*p<0.05). Treatment of the SW480 R/R xenograft resulted in more modest growth inhibitory effects, which also appeared to be dominated by the single-agent effects of selumetinib.

**Assessment of SW620 S/S xenograft tumor metabolic response by $^1$H-NMR and $^{18}$FDG-PET.** An expanded metabolic profile, calculated from $^1$H-NMR extracts of treated and untreated tumors, showed that no changes in lactate, the end product of glycolysis, were observed in the treatment groups (**Figure 5** B). In fact, no significant metabolic changes were detected for the HDAC inhibitor, vorinostat. The most striking changes observed with MEK inhibition by selumetinib were related to highly decreased levels of membrane phospholipids (such as phosphatidylcholine, its precursors phosphocholine and phosphatidylinositol). Accumulation of nucleosides and adenosines were also seen in the selumetinib treated group. The combination group mostly reflected the metabolic effects of single-agent selumetinib, with broader decreases in membrane
phospholipids. To assess the effects of selumetinib in combination with vorinostat on glucose uptake, $^{18}$FDG-PET was performed on mice bearing SW620 tumors at baseline, before starting treatment, after four days of treatment to evaluate early drug effects and after 21 days of treatment with either vehicle, selumetinib, vorinostat, or the combination. No significant decreases in glucose uptake were observed in either study group when compared to untreated controls after three days of treatment (data not shown), while a trend of decrease in glucose uptake was observed day 21 in the selumetinib and combo group compared with vorinostat (Figure 5 C, D *p<0.05).

Discussion

Recent efforts in cancer therapeutics have focused on the development of targeted therapies directed towards specific molecules related to cancer cell biology in order to achieve a more specific and effective treatment, and at the same time, avoid some of the systemic toxicities associated with chemotherapy (51, 52). To optimize this therapeutic approach, patient selection for clinical trials has become of critical importance (53-56). For example, recent studies have shown a correlation between CRC tumors with activating KRAS mutations and resistance to EGFR-targeted therapies. Therefore, there is an emerging need for alternative therapeutic approaches for this patient population (5, 56, 57). In the present study, we utilized in vitro and in vivo models of CRC to evaluate the efficacy of selumetinib in combination with vorinostat as a potential therapeutic strategy for the treatment of CRC patients expressing mutant KRAS. Our data demonstrated that this combination exerts potent anti-tumor effects on in vitro models of CRC including synergistic inhibition of cell proliferation, G1 cell cycle arrest, induction of apoptosis, and spheroid formation in 3D culture. Interestingly, this combination
inhibited CRC cell migration, which suggests that this treatment not only inhibits tumor growth, but may play a role in the metastatic process as well. *In vivo* studies using KRAS mutant CRC xenograft models in nude mice confirmed the anti-tumor effects of the combination of selumetinib and vorinostat, although synergy was not observed, likely due to the single-agent effects of selumetinib.

In our initial studies, we evaluated selumetinib and vorinostat as single agents against a panel of CRC cell lines that were selected based on differential KRAS, BRAF, and p53 mutational status. Several reports have suggested a relationship between KRAS and BRAF mutation status and response to MEK inhibitors (1). Other investigators demonstrated that the blockage of the MAPK pathway with a MEK inhibitor induced cell death only in cells that were KRAS mutant but p53 wild type (58). Although we failed to demonstrate any significant correlations between cell line sensitivity to selumetinib or vorinostat and KRAS, BRAF, or p53 status (data not shown), a recent study by Dry, et. al. showed that a gene signature of functional MEK activation was consistently elevated in cell lines sensitive to selumetinib, while BRAF/KRAS mutation or ERK activation varied across cell lines, indicating that expression profiling may be a better predictor of drug response than individual biomarkers or gene mutations for this class of agents (59).

Consistent with this report, we observed constitutive over-expression of HDAC and MAPK pathways in the cell lines sensitive to vorinostat or selumetinib, respectively. Interestingly our data showed that cell lines resistant to vorinostat exhibited a constitutive over-expression of the MAPK pathway, and that the MAPK pathway was upregulated in response to treatment with vorinostat, providing a functional rationale for exploring the use of vorinostat in combination with selumetinib in our preclinical models of CRC.
To further evaluate the synergistic effects of this combination, we chose to focus on several KRAS mutant CRC cell lines. Our results showed that the combination of selumetinib and vorinostat resulted in a synergistic antiproliferative effects in all cell lines studied. Mechanistically, our results suggest that this antiproliferative effect of combination of selumetinib and vorinostat is mediated by an increase in apoptotic cell death as measured by caspase 3/7 activity, cleaved PARP and perhaps increased acetylated histone H3 levels. This is in accordance with previous reports demonstrating that MEK inhibition was synergistic with HDAC inhibitor through the induction of cell death via apoptosis (29). We also evaluated the effects of the combination on cell cycle distribution and apoptotic events. It has been previously reported that vorinostat increases the expression of cyclin-independent kinases such as p21<sup>CIP1/WAF1</sup> in bladder cancer, and p27<sup>KIP1</sup> in gliomas, and induces G2/M cell cycle arrest in CRC cell lines, while selumetinib induced G1 cell cycle arrest and induction of apoptosis in a CRC xenograft model (1, 19, 60). In our study, we observed a G1 cell cycle arrest in the SW620 S/S cell line after each single agent treatment that was significantly increased after exposure to the combination. In the SW480 R/R cell line, vorinostat showed no effects on cell cycle distribution, while single-agent selumetinib and combination treatment resulted in a G1 arrest, suggesting that the combination effects were mediated by selumetinib.

Because cell proliferation is only one phenotypic endpoint of malignant behavior, we used other <i>in vitro</i> models such as a modified Boyden chamber and three-dimensional culture (3D) and found that the combination of these agents resulted in significant decreases in cell motility and spheroid formation, respectively. As MEK is a downstream effector of the EGFR, VEGFR and IGF-1R signaling pathways, we hypothesized that
MEK activation could play a role in ligand-induced cell migration. For example, previous data has shown that treatment of NSCLC with HDAC inhibitors induces re-expression of E-cadherin mRNA, induction of the epithelial phenotype, and decreased migratory activity (61). By contrast, our findings indicated that stimulation of EGFR pathway did not affect the migration of SW480 cells, although both FBS and IGF-1 induced cell migration that was inhibited by both single agents and substantially by the combination. The insulin-like growth factor I receptor (IGF1R) and other components of the IGF system have been shown to be over-expressed in colon cancer and are associated with advanced stage of disease, metastasis, and reduced survival (62). These results suggest that the combination of vorinostat and selumetinib may act downstream of this pathway to block the phenotypic effects of IGF-induced CRC migration. In three-dimensional culture, cancer cells are able to form tumor spheroids that are thought to more closely recapitulate the cancer cell-extracellular matrix interactions that play a fundamental role in tumor growth and metastasis (63). The inhibitory effects of the combination were confirmed in the 3D model in both the sensitive (SW620) and resistant (SW480) cell lines. Of interest was the finding that the SW480 cells appeared to be sensitive to selumetinib in the 3D model, supporting the limitation of 2D assays in assessing more global impacts on cellular phenotype in vitro.

In our in vivo studies of both SW620 (S/S) and SW480 (R/R) cell line xenografts, combination treatment demonstrated significant tumor growth inhibition compared to untreated controls or vorinostat, but not to single-agent selumetinib. As would be expected from the 3D results, the SW480 xenograft was sensitive to selumetinib in vivo, which we hypothesize could be related to MEK-dependent effects on the tumor
microenvironment and tumor cell-matrix interactions. Although the single-agent activity of selumetinib at 25 mg/kg obfuscated the detection of synergy in vivo, our assessment of metabolic end-points led to some interesting findings. Surprisingly, no significant inhibition of glucose uptake (FDG-PET) or metabolism (lactate from \(^1\)H-NMR) was observed for either selumetinib or vorinostat, alone or in combination, compared to untreated controls. While HDAC inhibition did not appear to induce any metabolic changes, the MEK inhibitor selumetinib had a profound inhibitory effect on membrane phospholipid turnover (decrease in total phospholipids as well as phosphatidyl-choline and phosphatidyl-inositol). This, together with an accumulation of adenosine and nucleotides, is reflective of the anti-proliferative effects observed in this study for selumetinib and the combination. These data demonstrate that effects on glucose metabolism are not observed with all targeted agents and that preclinical metabolic studies should be conducted to more precisely determine the most appropriate readout for functional imaging. For example, these results suggest that in human clinical trials, incorporation of \(^{18}\)F-choline PET, rather than FDG-PET, might be the most relevant imaging endpoint for treatment response to MEK inhibitors.

In summary, we utilized gene array and gene set enrichment analyses to develop a rational basis for testing the combination of selumetinib and vorinostat as a novel therapeutic option for KRAS mutant CRC. Moreover, the preclinical findings described in this report, utilizing both in vitro and in vivo models of CRC, demonstrated that dual inhibition of MAPK signaling and HDAC activity results in inhibition of several biological processes associated with tumor growth and progression. Together, these data
suggest that clinical testing of selumetinib in combination with vorinostat should be explored as a potential therapy for KRAS mutant CRC.

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References


63. Rieber M, Rieber MS. Signalling responses linked to betulinic acid-induced apoptosis are antagonized by MEK inhibitor U0126 in adherent or 3D spheroid melanoma irrespective of p53 status. Int J Cancer 2006; 118:1135-43.

Figure legends

Figure 1. Pathway analysis of colorectal cancer cell lines at baseline and after treatment with vorinostat, selumetinib, or the combination. A. Top five pathways enriched in the vorinostat resistant cell lines (HT29 and SW480) after vorinostat treatment. B. Heatmap of the core genes in the HDAC and MAP Kinase signaling pathway at baseline, after treatment with vorinostat, selumetinib, and the combination. Pathway diagram derived from BioCarta where the core genes are circled. Red and green in the heatmap represent gene over-expression and under-expression, respectively. C. Pathway diagram derived from BioCarta where the core genes are colored in red.

Figure 2. Selumetinib in combination with vorinostat demonstrates synergistic inhibition against colorectal cancer cell lines. SW620 S/S (A, B) and SW480 R/R (C, D) were plated in 96 well plates for 24 hours and treated for 72 hours with varying doses of selumetinib and vorinostat. Proliferation was assessed by the SRB assay. Raw proliferation data were expressed as percent of viable cells and CI values were analyzed according to the Chou and Talalay method for drug interactions using the CalcuSyn software package.

Figure 3. Effects of selumetinib and vorinostat on CRC spheroid formation in 3D culture. CRC cell lines were plated in 8-well chamber slides on matrigel pads at a density of 2500 cell/well resuspended in 2% matrigel-supplemented RPMI plus 10% FBS. SW620 (S/S) and SW480 R/R cells were treated with indicated doses of selumetinib, vorinostat or the combination for 15 days. SW620 (A, B) and SW480 (C, D) spheroids were photographed and counted after 7 and 15 days of treatment. Spheroids with a diameter greater than 1 micron were counted. Data represent the mean of three
independent experiments. *p < 0.05 selumetinib, vorinostat or combination treatment versus control; **p < 0.05 combination versus control, and single agents. Bars represent the mean of three independent experiments performed in triplicate.

**Figure 4.** Selumetinib and vorinostat induce G1 cell cycle arrest and apoptosis in CRC cell lines. SW620 S/S (A, C) and SW480 R/R (B, D) CRC cell lines were treated with selected doses of selumetinib and vorinostat for 24 hours. For cell cycle analysis, cells were stained with Krishanaposs stain followed by flow cytometric analysis. Apoptosis was analyzed by fluorometric measurement of caspase -3 and -7 activity. *p < 0.05 selumetinib, vorinostat or combination treatment versus control; **p < 0.05 combination versus control, and single agents. Bars represent the mean of three independent experiments performed in triplicate.

**Figure 5:** Antiproliferative and metabolic responses of the SW620 S/S cell line xenograft *in vivo.* A. Curves represent tumor growth rate, expressed as “percent of day one”, when treated with either vehicle, selumetinib (25mg/kg), vorinostat (75mg/kg) or the combination; B. Metabolic heat-maps based on quantitative FDG-PET and 1H-NMR spectroscopic data sets in SW620 xenografts on day 22. The data represents mean ± SEM of 8 biopsies/group. The metabolites, their ratios and metabolic fluxes were grouped based on their biochemical relevance. For the control group, all intracellular metabolite levels are presented as μmol per cell wet weights; metabolite ratios and glucose uptake are unitless. Metabolic pathways which were undisturbed by treatment are presented as yellow blocks. A decrease in a metabolic endpoint is indicated by red, while an increase is depicted in green. Statistical significance for metabolite changes are based on
multivariate analysis of metabolic fluxes with *p<0.02. The interactive metabolic profile array database was custom-based (38).

C. Representative PET images and their quantification (D) (presented as the percent of normalized glucose uptake to the baseline) acquired on days 4 and 21 post-treatment. Decreases of $^{18}$FDG uptake in tumors can be measured as early as 4 days post-treatment, with no significant differences between either treatment group compared with control but with a trend of selumetinib compared with vorinostat measured at day 21.
Figure 2

A

SW620 (S/S)

SELUMETINIB

VORINOSTAT

B

Dose-effect curve

Effect

Dose

x COMBO + SELUMETINIB o VORINOSTAT

C

SW480 (R/R)

SELUMETINIB

VORINOSTAT

D

Dose-effect curve

Effect

Dose

x COMBO + SELUMETINIB o VORINOSTAT
Figure 3

SW620 (S/S)

A

![Graph showing number of colonies per field for SW620 (S/S) over 7 and 15 days with and without treatments.](image)

B

- CONTROL
- SELUMETINIB
- VORINOSTAT
- COMBO

SW480 (R/R)

C

![Graph showing number of colonies per field for SW480 (R/R) over 7 and 15 days with and without treatments.](image)

D

- CONTROL
- SELUMETINIB
- VORINOSTAT
- COMBO
Figure 4

A  SW620

B  SW480

C  SW620

D  SW480
Preclinical Activity of the Rational Combination of Selumetinib (AZD6244) in Combination with Vorinostat in KRAS Mutant Colorectal Cancer Models

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