mTOR and HDAC Inhibitors Converge on the TXNIP/Thioredoxin Pathway to **Cause Catastrophic Oxidative Stress and** Regression of RAS-Driven Tumors 🖉 🔮

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ABSTRACT

Although agents that inhibit specific oncogenic kinases have been successful in a subset of cancers, there are currently few treatment options for malignancies that lack a targetable oncogenic driver. Nevertheless, during tumor evolution cancers engage a variety of protective pathways, which may provide alternative actionable dependencies. Here, we identify a promising combination therapy that kills NF1-mutant tumors by triggering catastrophic oxidative stress. Specifically, we show that mTOR and HDAC inhibitors kill aggressive nervous system malignancies and shrink tumors in vivo by converging on the TXNIP/thioredoxin antioxidant pathway, through cooperative effects on chromatin and transcription. Accordingly, TXNIP triggers cell death by inhibiting thioredoxin and activating apoptosis signal-regulating kinase 1 (ASK1). Moreover, this drug combination also kills NF1-mutant and KRAS-mutant non-small cell lung cancers. Together, these studies identify a promising therapeutic combination for several currently untreatable malignancies and reveal a protective nodal point of convergence between these important epigenetic and oncogenic enzymes.

SIGNIFICANCE: There are no effective therapies for NF1- or RAS-mutant cancers. We show that combined mTOR/HDAC inhibitors kill these RAS-driven tumors by causing catastrophic oxidative stress. This study identifies a promising therapeutic combination and demonstrates that selective enhancement of oxidative stress may be more broadly exploited for developing cancer therapies. Cancer Discov; 7(12); 1450-63. © 2017 AACR.

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INTRODUCTION

Genetic alterations in oncogenes and tumor suppressors play an important causal role in tumor development. Accordingly, therapeutic efforts have largely focused on identifying and inhibiting key oncogenic drivers and/or downstream pathways (1). However, in addition to accumulating mutations, cancers also activate a variety of protective pathways that are not sufficient to drive tumorigenesis, but are nonetheless characteristic of the tumorigenic state (2). As such, these cellular adaptations represent potential cancer-cell dependencies that could be exploited for therapeutic purposes (2). Strategies designed to inhibit these protective pathways may be particularly useful for treating tumors that are not driven by a readily targetable protein. The challenge has been to identify key nodal points within essential adaptive pathways, which may vary by tumor type and/or genotype.

Redox homeostasis is important for the survival of both normal and cancer cells (3). However, many tumors possess elevated levels of reactive oxygen species (ROS) and exhibit signs of chronic oxidative stress, which is caused by oncogenic insults, hypoxia, metabolic defects, and proteotoxic stress (4). At sublethal levels, increased ROS are thought to enhance tumor development by causing mutations and altering cell signaling (5). However, to prevent excessive oxidative damage, tumors frequently upregulate antioxidant pathways (5, 6). Accordingly, many tumor cells are hypersensitive to perturbations in ROS levels (4). In fact, excessive oxidative stress is thought to contribute to the cytotoxic effects of chemotherapies, and efforts to potentiate ROS production in these settings are under way (7, 8). Nevertheless, because traditional cytotoxic agents also affect normal tissues, a targeted approach that selectively triggers catastrophic oxidative stress in tumor cells would offer a greater therapeutic window.

The *NF1* tumor suppressor encodes a RAS GTPase-activating protein and is mutated in a familial cancer syndrome and in an expanding number of sporadic tumors (9–13). *NF1*-mutant malignancies are driven by excessive RAS signaling and, like *KRAS*-mutant tumors, are largely unresponsive to current therapies (14). *NF1*-deficient nervous system tumors, known as malignant peripheral nerve sheath tumors (MPNST), develop sporadically and in individuals with neurofibromatosis type I (NF1) and are lethal in approximately 70% of patients (15). Therefore, new effective treatments are urgently needed.

The mTOR pathway has been shown to be critical in *NF1*-mutant malignancies (10, 16). In addition to its well-documented role in cell growth, proliferation, and protein translation, mTOR also regulates the production of reduced glutathione (GSH), one of the three major cellular antioxidants: GSH, thioredoxin, and catalase (4). Specifically, mTOR regulates SREBP, a transcription factor that



controls the production of glucose-6-phosphate dehydrogenase (G6PD), a rate-limiting enzyme in NADPH production and consequently glutathione reduction (17). Accordingly, mTOR inhibitors have been shown to suppress SREBP, G6PD, and GSH levels in MPNSTs (18). Nevertheless, mTOR inhibitors exert only cytostatic effects in these tumors (16, 18, 19). We therefore sought to identify other targeted agents that might cooperate with mTOR inhibitors to enhance oxidative stress beyond threshold levels, thereby killing these mTOR-driven malignancies. Here, we show that HDAC and mTOR inhibitors together impinge on a second major antioxidant pathway, the thioredoxin pathway, and trigger catastrophic oxidative stress, cell death, and most importantly tumor regression in vivo. Importantly, this combination also kills NF1-mutant and KRAS-mutant nonsmall cell lung cancers (NSCLC). Together, these studies identify a promising new therapeutic combination for these RAS-driven tumors and reveal a cooperative mechanism of action that may be more generally exploited for the development of other therapies.

RESULTS

HDAC and mTOR Inhibitors Cooperate to Kill NF1-Mutant Nervous System Malignancies

Because mTOR inhibitors suppress a major antioxidant pathway in MPNSTs, we sought to identify and combine other agents that stimulate ROS accumulation, as a potential strategy to induce catastrophic oxidative stress in these tumors. To facilitate clinical translation, we considered FDAapproved drugs that are known to enhance oxidative stress. Interestingly, HDAC inhibitors have been shown to elevate ROS in some tumor types (20). Indeed vorinostat, a panhistone deacetylase (HDAC) inhibitor, triggered an increase in ROS levels in human MPNST cells (Fig. 1A).

We then evaluated the combined effects of the mTOR kinase inhibitor sapanisertib and vorinostat in a variety of human MPNST cell lines and nontransformed cells. Sapanisertib was used because mTOR kinase inhibitors more effectively inhibit 4E-BP1 phosphorylation in these cells in vitro as compared with rapamycin (19), similar to observations in other cell lines (21, 22), and because the 4E-BP1/eIF4E pathway has been shown to be particularly important for MPNSTs (23). As previously reported, sapanisertib slowed the proliferation of MPNSTs, but cells did not die (23); however, when combined, sapanisertib and vorinostat potently killed MPNSTs (Fig. 1B). Within 72 hours, 59% of the cells died in response to this drug combination, whereas cells exposed to each agent alone continued to proliferate, albeit at a slower rate (Fig. 1B). These effects were observed in multiple MPNST cell lines, and cell death was not dependent on the presence or absence of TP53 mutations (Fig. 1B and C and Supplementary Fig. S1A). Using the Gaddum's (non-)interaction score, which is the most appropriate method to evaluate synergy of a cytotoxic combination when at least one agent is cytostatic (24), these agents exerted synergistic effects on the reduction in cell number in MPNSTs (Supplementary Fig. S1B). In contrast, sapanisertib and vorinostat did not kill nontransformed cells, demonstrating that this combination is not generally toxic (Fig. 1D).

Combined HDAC and mTORC1 Inhibitors Trigger Potent Tumor Regression in a Genetically Engineered Mouse Tumor Model

Before dissecting the molecular mechanism of action, we first investigated whether this combination was effective in vivo. Many putative therapies have been reported to slow the growth of tumors in animal models; however, targeted agents that are effective in the clinic, such as BRAF inhibitors in melanoma and EGFR inhibitors in lung cancer, cause frank tumor regression in preclinical studies (25, 26). We therefore utilized a previously characterized genetically engineered mouse model to determine whether this combination could shrink tumors in vivo (Fig. 1E and ref. 27). Similar to human tumors, MPNSTs that develop in this model harbor null mutations in Nf1 and Trp53 and are histologically indistinguishable from human malignancies (27, 28). Tumors develop with an average latency of 5 months, and once detected grow rapidly in 10 days, mimicking the aggressive nature of human MPNSTs. Once palpable tumors were detected, animals were randomized and treated with HDAC and/or mTOR inhibitors. In mice and humans, rapalogs are able to suppress 4E-BP1 phosphorylation in many tissues (19, 29), perhaps due to the long half-life of the drug and/ or sequestration in immunophilin-rich red blood cells in vivo (30). Because rapamycin (sirolimus) is FDA approved for other indications, we selected this agent for initial in vivo studies (19). Rapamycin, which exclusively suppresses mTORC1, effectively inhibited its activity in vivo, as demonstrated by the loss of the hyperphosphorylated form of 4E-BP1 (Fig. 1F) and previously shown using both 4E-BP1 and phosphorylated S6 as pharmacodynamic biomarkers (19). The HDAC inhibitor vorinostat also effectively inhibited histone deacetylases in vivo, demonstrated by a sustained increase in acetylated H3K27 (Fig. 1F). Consistent with in vitro observations, vorinostat and rapamycin as single agents did not cause tumor regression; however, together they caused potent tumor shrinkage, on average by 38% and up to 76% with no signs of toxicity (Fig. 1G and Supplementary Fig. S2A). Notably, the dose of vorinostat used for this study (50 mg/kg) is predicted to be slightly less than the dose recently found to be tolerated when combined with rapamycin in humans (243 mg vs. 300 mg), underscoring the translational potential of this finding (31).

Multiple mTOR and HDAC Inhibitors Recapitulate the Therapeutic Response

To confirm that the observed therapeutic effects were due to on-target suppression of mTOR and HDAC, we sought to evaluate additional agents. In order to select an appropriate HDAC inhibitor, we first performed *in vitro* studies. Vorinostat inhibits class I, II, and IV HDAC complexes; however, more selective and/or potent agents have been developed (32). We therefore evaluated the effects of several structurally distinct HDAC inhibitors: panobinostat, entinostat, nexturastat A, and romidepsin. Panobinostat is also a broad HDAC inhibitor but is more potent than vorinostat (33). As such, panobinostat potently killed MPNSTs when combined with an mTOR inhibitor, and did so at much lower concentrations than vorinostat (20 nmol/L vs. 2 µmol/L; Fig. 1H vs. 1B). Romidepsin, a structurally unrelated compound that





Figure 1. Combined HDAC and mTOR inhibitors kill NF1-mutant malignancies in vitro and in vivo. A, S462 cells treated with either vehicle or vorinostat (2 µmol/L) for 24 hours were stained with dichlorofluorescin diacetate (DCFDA), a dye that measures ROS. Graph indicates relative mean DCFDA (arbitrary units, three independent experiments; *, P = 0.006, paired two-tailed Student t test). B, 90-8TL cells were treated with vehicle, sapanisertib (sap; 100 nmol/L), vorinostat (vor; 2 µmol/L), or sapanisertib + vorinostat for 3 days. The left y-axis indicates log2 of fold change in cell number at 3 days relative to day 0. The right y-axis indicates percent change in cell number at 3 days on a log₂ scale. Error bars ± SD from technical triplicates. At right, immunoblot shows levels of phosphorylated S6 (pS6) and histone H3 acetylation at lysine 9 (AcH3K9) after 24 hours of indicated treatment. S6 and Actin serve as controls. *, P < 0.000001. C, S462 cells were treated with vehicle, sapanisertib (200 nmol/L), vorinostat (2 µmol/L), or sapanisertib and vorinostat and evaluated as described in B. *, P = 0.009599; **, P = 0.000002. D, Nontransformed IMR-90 (lung fibroblast) cells treated and evaluated as described in C. E, Schematic of in vivo experimental design. F, In vivo pharmacodynamic analysis showing acetylated histone H3 at lysine 27 (AcH3K27) and 4E-BP1 in tissue from animals treated with vorinostat and rapamycin for the indicated amount of time. Lines indicate hyperphosphorylated and hypophosphorylated species of 4E-BP1. Tubulin serves as a control. G, Waterfall plot depicting change in tumor volume after 10 days of treatment with single and combined agents as indicated. Each of the four experimental groups of mice were treated as part of the same experiment. The data shown for the vehicle and rapamycin treatments are reproduced from Fig. 2A and C of ref. 19. The left y-axis depicts log₂ fold change in tumor volume after 10 days. The log₂ values on the left axis have been converted to the actual percent increase or decrease in tumor volume to best appreciate relative changes, shown on the right y-axis. Each bar represents an individual tumor. **H**, 90-8TL cells were treated with vehicle, sapanisertib (100 nmol/L), panobinostat (pan; 20 nmol/L), or sapanisertib + panobinostat and evaluated as described in **B**. *, *P* = 0.000021; **, *P* = 0.000001. **I**, 90-8TL cells were treated with romidepsin (rom; 1 nmol/L), sapanisertib (100 nmol/L), or romidepsin + sapanisertib and evaluated as described in **B**. *, P < 0.000001. **J**, Waterfall plot depicting log₂ fold change in tumor volume after 10 days of treatment with vehicle or panobinostat + sapanisertib. The log₂ values on the left axis have been converted to the actual percent increase or decrease in tumor volume to best appreciate relative changes, shown on the right u-axis. Red asterisk denotes tumor that was undetectable by palpation at 10 days. Tumor size was determined by measuring residual flat lesion/tissue after dissection.

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inhibits class I HDAC complexes, but not class II or IV, was also effective in this context (Fig. 1I). However, more selective HDAC inhibitors such as nexturastat A and entinostat, which suppress HDAC6 and only a subset of Class I HDACs, respectively (34, 35), did not kill cells when combined with mTOR inhibitors (Supplementary Fig. S3A and S3B), suggesting that the broad inhibition of Class I HDAC complexes is required for cell death when combined with mTOR inhibitors.

We therefore replaced vorinostat with panobinostat, and rapamycin with the mTOR kinase inhibitor sapanisertib for in vivo analysis. These agents also promoted dramatic tumor regression in vivo, ranging from 45% to undetectable in one instance, further supporting the conclusion that mTOR and HDAC inhibitors represent a promising therapeutic combination for these malignancies (Fig. 1J and Supplementary Fig. S2B). The observation that rapamycin is effective in vivo mechanistically demonstrates that mTORC1 suppression is sufficient for mediating these effects; however, it remains to be formally established whether rapalogs or mTOR kinase inhibitors will more effectively inhibit mTORC1 at tolerable doses in humans. Nevertheless, given the substantially higher potency of panobinostat, we believe that the superior efficacy of panobinostat/sapanisertib observed here was largely due to the activity of panobinostat. This conclusion is supported by in vitro observations showing that panobinostat induces more cell death than vorinostat when combined with a constant dose of sapanisertib, and does so at 1% of the concentration of vorinostat (Fig. 1B versus 1H).

Oxidative Stress Precedes and Is Required for Cell Death Triggered by HDAC and mTOR Inhibitors

Our initial hypothesis was that HDAC and mTOR inhibitors might function by triggering catastrophic oxidative stress in these malignancies. To determine whether enhanced oxidative stress was preceding and possibly contributing to the cytotoxic effects of HDAC/mTOR inhibition, expression profiles of MPNSTs were examined 24 hours after treatment, prior to robust cell death observed at 72 hours. Notably, multiple gene sets associated with oxidative stress and ER stress, a tightly linked stress response triggered by ROS-induced protein misfolding, were among the gene sets that were differentially expressed in the HDAC/mTOR inhibitor-treated cells (Fig. 2A; Supplementary Table S1). Signatures related to senescence, aging, and hypoxia, additional processes linked to oxidative stress, were also among the recurrent, differentially expressed signatures.

We then investigated whether mTOR inhibitors and HDAC inhibitors were cooperatively enhancing ROS levels in MPNSTs. Although HDAC inhibition alone increased ROS, mTOR and HDAC inhibitors together triggered a significantly greater, sustained increase, raising levels by 120% in MPNST cell lines [Fig. 2B (90-8TL, *P* = 0.001) and Supplementary Fig. S4; S462, P = 0.007]. By contrast, in normal cells, in which these agents were unable to trigger cell death (Fig. 1D), ROS induction was substantially lower (Fig. 2B, P = 0.000009). Most importantly, when MPNSTs were cotreated with n-acetyl cysteine (NAC), a broad-spectrum ROS scavenger, the cooperative effect of sapanisertib and vorinostat on cell death was abolished, demonstrating that the increase in ROS is required for the observed cytotoxicity (Fig. 2C; P = 0.009). It should be noted that NAC had no effect on target inhibition (Fig. 2D), and it also did not disrupt the individual effects of mTOR and HDAC inhibitors on cell proliferation (Fig. 2C), suggesting that oxidative stress does not mediate the cytostatic effects of these agents individually, but is required for the unique, cooperative response that triggers cell death. Finally, we looked for signs of severe oxidative and ER stress in vivo. Indeed, electron microscopic analysis of tumors exposed to combined HDAC and mTOR inhibitors in vivo revealed severe mitochondrial damage and massive swelling of the endoplasmic reticulum after only 7 hours of treatment (Fig. 2E). Together, these observations suggest that severe oxidative stress precedes cell death and is required for the observed cytotoxicity of this combination in these malignancies.

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Figure 2. The therapeutic effects of HDAC and mTOR inhibitors are mediated by the suppression of class I HDACs and require oxidative stress A, 90-8TL cells were treated with vehicle (veh), sapanisertib (sap; 100 nmol/L), vorinostat (vor; 2 µmol/L), or combined sapanisertib + vorinostat for 24 hours, and a microarray analysis was performed. Gene sets related to oxidative or proteotoxic stress significantly altered in the combination treated cells relative to the other treatment groups (vehicle and monotherapies) are shown. The p-values for LS permutation, KS permutation, and Efron-Tibshirani GSA test for each gene set are shown, with P < 0.005 highlighted in red. A complete list of the gene sets that were recurrently differentially expressed is shown in Supplementary Table S1. **B**, Graph depicts relative mean fluorescence intensity of 90-8TL (black) and IMR-90 (gray) cells stained with dichlorofluorescin diacetate (DCFDA), a dye that measures ROS, and treated as indicated for 48 hours (90-8TL cells treated as in A, IMR-90 cells treated as in Fig. 1D). Error bars indicate SD from three technical triplicates. *, P = 0.002889; **, P = 0.000009. **C**, S462 cells were treated with vehicle, sapinsertib (200 nmol/L), vorinostat (2 µmol/L), or sapinsertib + vorinostat with (black) or without (white) 5 mmol/L N-acetyl cysteine (NAC). The left y-axis indicates log₂ fold change in cell number after 3 days. The log₂ values on the left axis have been converted to the actual percent increase or decrease in cell number after 72 hours to best appreciate relative changes, shown on the right y-axis. Error bars, SD of technical triplicates. *, P = 0.009. D, S462 cells were treated as in C; immunoblot depicts phosphorylated S6 (pS6) and acetylated histone H3 at lysine 9 (AcH3K9) after 24 hours of indicated treatments. Total S6 and vinculin serve as controls. E, Transmission electron microscopy (TEM) image of tumor cells after 7 hours of treatment with vorinostat and rapamycin. Red circles indicate representative endoplasmic reticulum (ER), and blue arrows indicate representative mitochondria (M). Scale bars are in white. F, S462 cells were treated with vehicle, buthionine sulfoximine (BSO; 200 µmol/L), vorinostat (2 µmol/L), or BSO + vorinostat. The left y-axis depicts log₂ fold change in cell number after 3 days. The log₂ values on the left axis have been converted to the actual percent increase or decrease in cell number after 72 hours to best appreciate relative changes, shown on the right y-axis. Error bars indicate SD of technical triplicates. *, P = 0.000003; **, P < 0.000001. **G**, Waterfall plot depicting log₂ fold change in tumor volume after 10 days of treatment with BSO (black) or \dot{B} SO + panobinostat. The log₂ values on the left axis have been converted to the actual percent increase or decrease in tumor volume to best appreciate relative changes, shown on the right y-axis. Vehicle and sapinsertib + panobinostat data (gray) are reprinted from Fig. 1J for clarity. As in Fig. 1J, red asterisk denotes tumor that was undetectable by palpation at 10 days. Tumor size was determined by measuring residual flat lesion/tissue after dissection. H, S462 cells overexpressing either LACZ (white) or G6PD (black) were treated with vehicle or sapinsertib (200 nmol/L) + vorinostat (2 µmol/L). The left y-axis indicates log₂ of fold change in cell number after three days. The log₂ values on the left axis have been converted to the actual percent increase or decrease in cell number after 72 hours to best appreciate relative changes, shown on the right y-axis. At right, immunoblot depicts G6PD after 16 hours of treatment with sapanisertib + vorinostat. Actin serves as a control. *, P = 0.000875.

Oncogenic and Epigenetic Drugs Trigger Lethal Oxidative Stress

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Differentially expressed gene sets	LS Permutation	KS Permutation	Efron- Tibshirani's GSA
OXIDATIVE STRESS			
Kyng_Response_to_H2O2	0.00039	0.00039	0.07
Chuang_Oxidative_Stress_Response_UP	0.00066	0.02977	<0.005
Chuang_Oxidative_Stress_Response_DN	0.00361	0.11379	0.57
Weiget_Oxidative_Stress_by_TBH_and_H2O2	0.00933	0.00047	0.36
Mootha_ROS	0.09478	0.25818	<0.005
Gargalovic_Response_to_Oxidized_Phospholipids_Turquoise_DN	0.0004	0.01311	0.62
Gargalovic_Response_to_Oxidized_Phospholipids_Turquoise_UP	0.00062	0.00005	0.085
PROTEOTOXIC STRESS			
Reactome_Unfolded_Protein_Response	0.00353	0.0029	0.545
Reactome_Activation_of_Chaperones_by_IRE1_alpha	0.01542	0.0042	0.32
Reactome_Activation_of_Chaperone_Genes_by_ATF6_alpha	0.01644	0.00462	0.06
Reactome_Activation_of_Chaperones_by_ATF6_alpha	0.0311	0.00488	0.075















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Figure 3. HDAC and mTOR inhibitors function by converging on the thioredoxin interacting protein (TXNIP) and activating ASK1. **A**, Microarray analysis of 90-8TL cells after 24 hours of treatment with vehicle (veh), sapanisertib (sap; 100 nmol/L), vorinostat (2 vor; µmol/L), or sapanisertib + vorinostat. Heat map depicts the uniquely upregulated genes (dark blue) or downregulated genes (light blue) from cells treated with sapanisertib + vorinostat, as compared with all other treatment groups, with a *P* < 0.001 and a fold change of 1.5 or more. *TXNIP* is highlighted in red, as a gene of particular interest within this signature. **B**, Immunoblot depicts protein levels of TXNIP, phosphorylated S6 (pS6), and acetylated H3K9 (AcH3K9) after 16 hours of treatment with sapanisertib (200 nmol/L), vorinostat (2 µmol/L), or sapanisertib + vorinostat in S462 cells. Actin serves as a control. Below, numbers indicate relative TXNIP protein levels quantified and normalized to actin levels, with vehicle levels set to 1. **C**, 90-8TL cells were treated with sapanisertib (100 nmol/L), vorinostat (2 µmol/L), or sapanisertib + vorinostat and analyzed as in **B**. **E**, S462 cells were infected with lentiCRISPRv2-expressing guides against *LACZ* or *TXNIP* as indicated. Immunoblot depicts TXNIP, pS6, and AcH3K9 protein levels fater 16 hours of treatment as in **B** and **D**. Actin and S6 serve as controls. **F**, As in **E**, S462 cells were infected with lentiCRISPRv2-expressing guides against *LACZ* or *TXNIP* as indicated and treated with sapanisertib (200 nmol/L). The left *y*-axis indicates the log₂ of fold change in cell number after 72 hours to best appreciate relative changes, shown on the right *y*-axis. Error bars, SD of technical triplicates. *, *P* = 0.000007; **, *P* < 0.000001. (*continued on following page*)

mTOR Inhibitors Contribute to the Therapeutic Response by Suppressing G6PD and GSH

Previous studies have shown that mTOR inhibitors affect oxidative stress, in part, by suppressing the expression of G6PD and consequently inhibiting the production of reduced GSH (18), one of three major cellular antioxidant systems. To investigate whether sapanisertib was indeed functioning through the G6PD/GSH pathway, sapansertib was replaced by buthionine sulfoximine (BSO), which inhibits glutathione production through a different mechanism (36). Importantly, BSO phenocopied sapanesertib: specifically, BSO exerted modest cytostatic effects as a single agent, but potently killed MPNSTs when combined with HDAC inhibitors in vitro (Fig. 2F). Moreover, when administered to tumor-bearing animals, BSO alone did not promote tumor regression, but did so when combined with panobinostat, albeit slightly less well than sapanesertib and panobinostat as might be expected (Fig. 2G). Conversely, G6PD overexpression prevented cell death triggered by mTOR and HDAC inhibitors (Fig. 2H). Thus, both gain-of-function and loss-of function experiments, corroborated by in vivo observations,

further support the conclusion that cell death is mediated by excessive oxidative stress and that mTOR inhibitors function, in part, by suppressing G6PD and GSH.

HDAC and mTOR Inhibitors Cooperate by Converging on the Thioredoxin Interacting Protein and Activating Apoptosis Signal-Regulating Kinase 1

To deconstruct the molecular mechanism by which these agents cooperatively induce catastrophic oxidative stress and cell death, we examined the transcriptional profiles of cells exposed to combined mTOR and HDAC inhibitors and identified genes that were differentially expressed in response to the combination relative to other treatment groups. Notably, one of the most significantly upregulated genes was thioredoxin interacting protein (*TXNIP*; *P* = 0.0000807). *TXNIP* mRNA expression was modestly elevated in response to sapanisertib and vorinostat alone, but its expression was substantially enhanced by the combination (Fig. 3A). Interestingly, the TXNIP protein binds and inhibits thioredoxin (TRX), a second major cellular antioxidant (37). Moreover,

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Figure 3. (Continued) **G**, S462 cells with indicated CRISPR guides were treated with vehicle or sapinsertib (200 nmol/L) + panobinostat (20 nmol/L), and analyzed as in **E**. *, *P* = 0.000004; **, *P* = 0.000001. **H**, 90-8TL cells were treated with vehicle, sapinsertib (100 nmol/L), auranofin (aur; 750 nmol/L) or auranofin + sapinsertib. The left *y*-axis indicates the log₂ of fold change in cell number after 3 days. The log₂ values on the left axis have been converted to the actual percent increase or decrease in cell number after 72 hours to best appreciate relative changes, shown on the right *y*-axis. Error bars, SD of technical triplicates. *, *P* = 0.000004; **, *P* < 0.000001. **I**, Immunoblot depicting phosphorylated P38 (pP38), TXNIP, AcH3K9, and p56 levels in S462 cells after 16 hours of treatment with sapanisertib (200 nmol/L) and panobinostat (pan; 20 nmol/L). P38 serves as a control. **J**, Immunoblot depicting pP38, TXNIP, AcH3K9, and p56 levels in 90-8TLs after 16 hours of treatment with sapanisertib (100 nmol/L) and vorinostat (2 µm0l/L). P38 serves as a control. **J**, and vorinostat (2 µm0l/L). P38 serves as a control. **K**, 90-8TL (left) and S462 (right) cells were transfected with pooled siRNAs targeting *MAP3K5* (ASK1) or nontargeting (CTRL) and treated with vehicle, sapanisertib (100 nmol/L 90-8TL, 200 nmol/L S462), vorinostat (2 µm0l/L), or sapanisertib + vorinostat. The left *y*-axis indicates the log₂ of fold change in cell number after 3 days. The log₂ values on the right *y*-axis. Error bars, SD of technical triplicates. *, *P* = 0.003935; **, *P* < 0.000001. **L**, 90-8TL cells were transfected to the actual percent increase or decrease in cell number after 72 hours to best appreciate relative changes, shown on the right *y*-axis. Error bars, SD of technical triplicates. *, *P* = 0.003935; **, *P* < 0.000001. **L**, 90-8TL cells were transfected with pooled siRNAs targeting (CTRL), and treated with vehicle, sapanisertib (100 nmol/L), vorinostat (2 µmol/L), or sapanisertib + vorinosta

the TRX/TXNIP system plays a major role in regulation of redox homeostasis, and TXNIP has been shown to mediate cell death caused by oxidative stress in some settings (38, 39). The cooperative effects of mTOR and HDAC inhibitors on TXNIP protein levels in MPNST cell lines (S462 and 90-8TLs) was confirmed and quantified by Western analysis, which demonstrated a 10- to 90-fold increase in protein expression (Fig. 3B–D). TXNIP expression was induced by both vorinostat/sapanisertib (Fig. 3B and C) as well as panobinostat/ sapanisertib (Fig. 3D). Notably, the potential involvement of TRX/TXNIP in this therapeutic setting raised the intriguing possibility that the mTOR/HDAC inhibitor combination might be disengaging two of the three major oxidant pathways in MPNSTs: the glutathione and thioredoxin pathways.

To determine whether TXNIP was required for the therapeutic effects of combined HDAC/mTOR inhibition, the *TXNIP* gene was genetically ablated using the CRISPR/Cas9 system. Two CRISPR guide sequences were utilized which both suppressed TXNIP protein induction in drug-treated cells (Fig. 3E). Notably, both sg*TXNIP-1* and sg*TXNIP-2* suppressed cell death induced by HDAC and mTOR inhibitors (Fig. 3F and G). Effective *TXNIP* suppression prevented cell death triggered by either vorinostat or panobinostatbased combinations and did so in both S462 (Fig. 3F and G) and 90-8TLs (Supplementary Fig. S5A and S5B), demonstrating that TXNIP upregulation is essential for the therapeutic effects of this combination.

To complement these loss-of-function studies we evaluated the effects of auranofin, which inhibits thioredoxin through a different mechanism, specifically by suppressing thioredoxin reductase (40). If mTOR and HDAC inhibitors kill cells by concomitantly suppressing GSH and thioredoxin, then auranofin should be sufficient to functionally replace HDAC inhibitors in this setting. Importantly, auranofin potently cooperated with sapanisertib and triggered MPNST cell death (Fig. 3H). Together with the genetic *TXNIP* ablation studies, these findings confirm the importance of the TXNIP/ thioredoxin pathway in this therapeutic response.

In addition to inhibiting the antioxidant function of thioredoxin directly, TXNIP specifically induces oxidative

stress-induced cell death by triggering the dissociation of apoptosis signal-regulating kinase 1 (ASK1) from thioredoxin, resulting in its activation (39, 41). Importantly, we found that combined mTOR/HDAC inhibitors activated ASK1, as demonstrated by the increased phosphorylation of its downstream target, p38, in both MPNST cell lines in response to either vorinostat- or panobinostat-based combinations (Fig. 3I and J). However, to confirm a functional role for ASK1 in cell death, ASK1 expression was ablated using pooled siRNAs targeting the gene that encodes ASK1, MAP3K5, in S462 and 90-8TL cells. Similar to the effects of TXNIP ablation, both ASK1-deficient MPNST cell lines were protected from cell death (Fig. 3K and Supplementary Fig. S5C, 90-8TL *P* < 0.0000001, S462 *P* = 0.004). These findings demonstrate that ASK1 activation is essential for cell death triggered by combined HDAC and mTOR inhibitors.

Finally, although these observations suggest that ASK1 mediates the cytotoxic effects of TXNIP, TXNIP has also been shown to inhibit the expression and membrane localization of the glucose transporter GLUT1 (42). To investigate a potential role for GLUT1 suppression in this response, we ablated the expression of the GLUT1 gene *SLC2A1* and determined whether its loss might cooperate with either agent alone or enhance the cytotoxic effects of the combination. GLUT1 suppression did not promote cell death on its own, did not trigger cell death when combined with either agent individually, and if anything slightly ameliorated the cytotoxicity of the combination (Fig. 3L and Supplementary Fig. S5D). Taken together, these observations suggest that cell death in this setting is primarily mediated through TXNIP's effects on ASK1 activation, rather than GLUT1 suppression.

TXNIP Expression Is Induced through Cooperative Effects on Chromatin and Transcription

In other settings, HDAC inhibitors have been shown to enhance TXNIP expression through direct effects on histone H4 acetylation near the TXNIP transcription start site (43), consistent with the modest increase in expression we observe in response to HDAC inhibitors alone (Fig. 3A-D). Accordingly, we found that treatment with vorinostat increased acetylation of histone H4 near the transcriptional start site of TXNIP in MPNSTs (Fig. 4A). However, given the cooperative effects of mTOR and HDAC inhibitors, we sought to determine how mTOR inhibitors were contributing to TXNIP upregulation. Interestingly, mTOR has been shown to negatively regulate MondoA, a basic helix-loop-helix leucine zipper transcription factor that functions in an obligate heterodimer with MLX. The MondoA-MLX interaction is suppressed by mTOR activation (44) and is one of two transcriptional complexes known to regulate TXNIP expression (43, 45). To determine whether MondoA-MLX was regulating TXNIP transcription in this therapeutic context, MondoA expression was ablated using pooled siRNAs that target the gene that encodes MondoA, MLXIP (Fig. 4B). MondoA ablation potently inhibited TXNIP mRNA (Fig. 4C) and protein expression (Fig. 4D), indicating that the MondoA-MLX complex is the primary transcriptional regulator of TXNIP in this therapeutic setting. Together, these findings explain the observed cooperativity between HDAC and mTOR inhibitors, which respectively open chromatin at the TXNIP promoter

and induce *TXNIP* transcription through activation of the MondoA–MLX complex.

Combined HDAC and mTOR Inhibitors Kill NF1- and KRAS-Mutant Lung Cancers

Finally, to determine whether this combination might be effective in other RAS pathway-driven tumors we evaluated these agents in NSCLC lines that harbored either NF1 or KRAS mutations, as NF1 and KRAS mutations occur in 11% and 33% of human NSCLC, respectively (46). Notably, the HDAC/mTOR inhibitor combination killed both NF1deficient NSCLC lines and 2 out of 3 KRAS-mutant lines (Fig. 4E). Moreover, similar to findings in the autochthonous MPNST model, combined HDAC/mTOR inhibitors triggered robust tumor regression in vivo in a xenograft model of KRAS-mutant NSCLC (Fig. 4F and G). These observations suggest that utility of this combination may extend beyond nervous system malignancies and represents a promising therapeutic approach for at least a subset of NF1- and KRASmutant lung cancers for which there are currently no effective treatments.

DISCUSSION

The ability to identify and inhibit specific oncogenic drivers in cancer has changed the standard of care for many diseases (1). Nevertheless, relatively few tumor types harbor single, targetable driving alterations, and even those that do often acquire resistance to such therapies. These observations suggest that additional therapeutic strategies are needed. One approach may be to concomitantly target key oncogenic pathways along with other cancer-specific vulnerabilities. However, the challenge has been both to identify critical dependencies in a given tumor type and to selectively target these vulnerabilities while sparing normal tissue.

Chronic oxidative stress has been proposed to represent a potential cancer-specific vulnerability (2, 4). Indeed, the excessive production of ROS in already-sensitized tumor cells is thought to contribute to the efficacy of some chemotherapies (7, 8). However, given the general toxicity of these agents, a more targeted approach, designed to selectively induce oxidative stress in tumors, is needed. Here, we identify a promising drug combination that functions by triggering irresolvable, catastrophic oxidative stress in NF1-mutant malignancies in vitro and in vivo (Fig. 5). Specifically, we show that mTOR and HDAC inhibitors suppress thioredoxin, a major antioxidant, by potently inducing the expression of its direct inhibitor, TXNIP, through cooperative effects on chromatin and transcription. Importantly, cell death is preceded and mediated by excessive oxidative stress as well as ASK1 activation, an apoptotic kinase normally suppressed by thioredoxin. Notably, mTOR inhibitors have previously been shown to suppress another antioxidant, GSH; however, mTOR inhibitors are not sufficient to induce irresolvable oxidative stress or cell death. Here, we show that the HDAC/mTOR inhibitor combination is effective because it inhibits a second major antioxidant pathway in these cancers. As such, two of the three major antioxidant pathways are suppressed by this combination. Importantly, although the HDAC/mTOR inhibitor combination kills NF1-mutant nervous system malignancies as well as





Figure 4. TXNIP expression is induced through cooperative effects on chromatin and transcription. **A**, 90-8TL cells were treated with vehicle (veh) or vorinostat (vor) for 6 hours. Graph shows percent input of *TXNIP* (left) and *GAPDH* (right) relative to respective vehicle treatments, after chromatin immunoprecipitation of acetylated histone H4. IgG chromatin immunoprecipitation is shown as a control. *, *P* < 0.0001. **B**, 90-8TL cells were transfected with pooled siRNAs targeting *MLXIP* (MondoA) or nontargeting (CTRL), and treated with vehicle, sapanisertib (sap; 100 nmol/L), vorinostat (2 µmol/L), or sapanisertib + vorinostat. Graph depicts quantitative PCR of *MLXIP* transcript levels in indicated treatment condition, 72 hours after transfection with indicated siRNA, and 24 hours after indicated treatment. Data points indicate relative mRNA expression, ± SD of three replicates. **C**, Cells were transfected and treated as in **B**; graph depicts quantitative PCR of *TXNIP* transcript levels in indicated treatment condition, 72 hours after transfection with indicated siRNA, and 24 hours after treatment as in **B**. Data points indicate relative mRNA expression, ± SD of three replicates. **D**, Immunoblot depicts TXNIP, phosphorylated S6 (pS6), and acetylated lysine 9 of histone H3 (AcH3K9) in 90-8TL cells 72 hours after transfection with indicated siRNA and 24 hours after treatment as in **B**. Unculin serves as a control. **E**, Indicated human NSCLC cells were treated with vehicle, sapanisertib (200 nmol/L), vorinostat (2 µmol/L), or sapanisertib + vorinostat. Graphs depict for expresented he percent change in cell number, relative to day 0 ± SD, on a log₂ scale. *NF1* and *KRAS* mutation status is noted. **F**, Diagram of *in vivo* experimental design. **G**, Waterfall plot depicting change in tumor volume after 10 days of treatment with single and combined agents as indicated. *KRAS*-mutant xenografts (H1573 cells) were used for this analysis. Percent change in tumor volume after 10 days is graphed on a log₂ sca

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Figure 5. Model depicting the mechanism by which HDAC and mTOR inhibitors promote catastrophic oxidative stress and kill tumor cells. HDAC and mTOR inhibitors trigger irresolvable oxidative stress and cell death by converging on two of the three major antioxidant pathways and activating ASK1. Specifically, as single agents mTOR inhibitors suppress the glutathione pathway, through effects on GGPD (17, 18). However, when combined with HDAC inhibitors, these agents together induce TXNIP expression through cooperative effects on chromatin and the MondoA–MLX transcriptional complex. Importantly, TXNIP inhibits a second major antioxidant pathway in tumors (thioredoxin) and triggers ASK1 activation, and together these events cause catastrophic oxidative stress, cell death, and tumor regression. This model is supported by both gain-of-function and loss-of-function studies. TXNIP ablation, GGPD overexpression can be phenocopied by BSO.

NF1- and *KRAS*-mutant lung cancers, it does not kill normal cells and it is not toxic to mice *in vivo*.

Although the majority of this study has focused on MPNSTs, the therapeutic effect of these agents in KRAS- and NF1-mutant lung cancer is striking and warrants further investigation. MPNSTs that arise in individuals with NF1 are inherently more homogeneous as compared with lung cancer, in that they are initiated by NF1 mutations and progress due to a specific set of additional genetic alterations (47-49). In contrast, lung cancers are much more genetically and biologically heterogeneous (50, 51). Although 4 of 5 of the RAS pathway-driven lung cancers evaluated in this study were sensitive to these agents, further study is needed to establish whether NF1 and KRAS mutations are predictive biomarkers, and/or if other predictive biomarkers exist. Sensitivity to this combination may ultimately be dictated by the specific antioxidant pathways that are upregulated in a given tumor, and whether or not mTOR and HDACs serve as critical buffering pathways. Nevertheless, given that there are currently no effective therapies for both NF1- and RAS-mutant lung cancers, these findings reveal a potential therapeutic strategy that can be further investigated.

Currently, there are no effective treatments for any *NF1*- or *KRAS*-mutant cancers. Moreover, although promising agents designed to target a subset of mutant KRAS proteins are being developed (52, 53), even if they are successful, durable regressions are likely to require a drug combination rather than a single agent. These studies demonstrate that enhanced oxidative stress represents a tractable vulnerability in these

RAS-driven tumors, a finding that can be used to inspire clinical trials now and in the context of future combinations. Fortunately, HDAC inhibitors and rapalogs are currently being evaluated in the clinic for other indications, based on an unrelated mechanistic rationale (clinicaltrials.gov). Therefore, tolerable doses of relevant drug combinations have been established (31, 54) and doses of additional combinations will become available in the near future (NCT00918333 and NCT01341834). Nevertheless, efficacy in solid tumors may require the most potent and/or specific agents. Our data support the evaluation of combinations using either potent pan-HDAC (e.g., panobinostat) or Class I-specific HDAC inhibitors and suggest that selective HDAC 1/3 or HDAC 6 inhibitors will not be effective in these tumors. Regardless, these studies have identified a promising new therapeutic combination for these currently untreatable tumors and demonstrate that approaches to selectively enhance oxidative stress in cancer cells may be more broadly exploited for the development of effective combination therapies.

METHODS

Cell Lines and Reagents

SNF96.2 (2009), S462 (2003), H1435 (2014), H1838 (2014), H1573, and IMR-90 (2011) were purchased from the ATCC in the year indicated. H23 and H1792 were generously provided by Dr. Pasi Janne (Dana-Farber Cancer Institute, Boston, MA) in 2008. 90-8TL was generously provided by Dr. Eric Legius (KU Leuven, Belgium) in 2002. 88-14 was generously provided by Dr. Jonathan Fletcher

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(Dana-Farber Cancer Institute, Boston, MA) in 2012. T265 was generously provided by Dr. Eduard Serra (Institut de Medicina Predictiva i Personalitzada del Càncer, Barcelona, Spain) in 2016. No further authentication of these cell lines was performed. 90-8TL and S462 were tested for Mycoplasma in March 2013 (negative). The other cell lines in this study have not been tested for Mycoplasma. Cells were used for the experiments in this article within 15 to 20 passages from thawing, with the exception of IMR-90 cells, which were used within 10 passages. H23, H1435, S462, 88-14, T265, SNF96.2, and 90-8TL cells were cultured in DMEM supplemented with FBS (10%) and L-glutamine. H1792, H1838, and H1573 cells were cultured in RPMI supplemented with FBS (10%) and L-glutamine. IMR-90 cells were cultured in MEM supplemented with FBS (10%) and L-glutamine. Cells were grown in normoxic conditions, and all experiments were performed in normoxic conditions. Antibodies were obtained from the following sources: Cell Signaling Technologies: pS6^{S235/236} (2211), S6 (2217), TXNIP (14715), vinculin (4650), pP38^{T180/Y182} (9211), P38 (9212), ASK1 (8662), GLUT1 (12939); Sigma Aldrich: Actin (A2066), Tubulin (T5168); EMD Millipore: Acetylated H3K9 (06-942), Bethyl: G6PD (A300-404A). Sapanisertib, Nexturastat A, romidepsin, panobinostat (in vitro), entinostat, and vorinostat were purchased from Selleck Chemicals. Rapamycin and panobinostat (in vivo) were purchased from LC Labs. Auranofin was purchased from Santa Cruz Biotechnology. BSO was purchased from Sigma Aldrich. Carboxy-H2DCFDA was purchased from Life Technologies (#C400).

RNAi

Nontargeting, HDAC1, HDAC2, HDAC3, HDAC6, MLXIP, MAP3K5, and SLC2A1 siRNA pools were purchased from GE Healthcare/ Dharmacon (D-001810-10, L-003493-00, L-003495-02, L-003496-00, L-003499-00, L-008976-00, L-003584-00, and L-007509-02, respectively). siRNAs were transfected overnight using RNAiMax lipofectamine from Invitrogen.

CRISPR

CRSIPR guides targeting *LACZ* (GCTGGAGTGCGATCTTC CTG), and TXNIP (sg*TXNIP*-1: GGGACATGCGCATCATGGCG; sg*TXNIP*-2: CAGAAGTTGTCATCAGTCAG) were generated and cloned into lentiCRISPRv2 by Dr. Gerald Marsischky at the Genome Engineering Production Group (GEPG) at Harvard Medical School.

Chromatin Immunoprecipitation (ChIP)

90-8TL cells were treated with vehicle or vorinostat for 6 hours and then cross-linked for 10 minutes with an 11% formaldehyde solution, and cell lysis was performed according to the Agilent mammalian ChIP-on-Chip protocol. Lysates were sonicated on ice for 45 minutes, 20 seconds on, 40 seconds off, in a Misonix Cup Horn Sonicator at 4°C. Chromatin was immunoprecipitated overnight at 4°C with acetylated histone H4 antibody (Active Motif) or rabbit IgG (Millipore), which had first been conjugated to protein G magnetic beads (Life Technologies 10004D).

Quantitative PCR

RNA was extracted from cells after indicated treatments using TRIzol (Invitrogen). Primers for *TXNIP* (gene expression) have been previously described (55) and were ordered from Invitrogen. Primers for *MLXIP* were (5'-3'): GCCAACAGGTGAGAATGAGA (forward) and TTCCTTTGAAGGATGTTCCC (reverse). Primers for *MAP3K5* were (5'-3'): AGACATCTGGTCTCTGGGC (forward) and AACATTCC CACCTTGAACAT (reverse). All samples were normalized to human *HPRT1*. Primers for *HPRT1* were (5'-3') GCCGGCTCCGTTATGG (forward) and AACCTGGTTCATCATCACTA (reverse). ChIP-PCR primers for *TXNIP* were (5'-3'): CAAGCATTCCTTATCACACAGATG (forward) and GTGATCAAAGGAGGGCAAGATA (reverse). ChIP- PCR primers for *GAPDH* have been previously published (43). Quantitative real-time PCR was performed using qScript SYBR green.

Cell Growth Studies

Approximately 175,000 cells per well were seeded into 6-well plates. For siRNA experiments, cells were seeded 12 to 16 hours after transfection. Twenty-four hours after plating, day 0 counts were taken using a hemocytometer. For inhibitor experiments, drug treatments were started at this time. Final cell counts were taken 72 hours after day 0 counts. Unless otherwise indicated, drug concentrations were as follows: vorinostat (2 µmol/L), romidepsin (1 nmol/L), panobinostat (20 nmol/L), sapanisertib (200 nmol/L for all cell lines except 90-8TLs, where sapanisertib was used at 100 nmol/L), auranofin (750 nmol/L), and BSO (200 µmol/L). The concentration of sapanisertib (200 nmol/L) was chosen based on previously published studies (23, 56, 57). Then, 100 nmol/L was used in 90-8TLs for historical reasons, but 200 nmol/L produces the same results. We confirmed that this concentration of sapanisertib effectively inhibited mTOR in all instances and induced a maximal cytostatic response as a single agent. The concentration of vorinostat was selected based on previous studies which typically use 1 to 5 μ mol/L (58). The 2 μ mol/L concentration was selected because this concentration effectively inhibited histone deacetylation and exerted minimal effects on cell viability on its own. Dose-response curves were initially performed to identify concentrations of panobinostat and romidepsin. Final concentrations were selected that effectively inhibited histone deacetylation and induced minimal or no cell death as single agents. For agents that did not have an effect (Nexturastat A and entinostat), a range of concentrations are shown to demonstrate that these are indeed inactive even at the highest concentrations.

In Vivo Drug Treatments

Animal procedures were approved by the Center for Animal and Comparative Medicine in Harvard Medical School in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act (Protocol #03379). C56/BL6 NPcis mice have been previously described (27). A power analysis was used to determine the number of mice per treatment group. Both male and female mice were used. Mice were treated daily with rapamycin via i.p. injection at 5 mg/kg, which was prepared as previously described (16). Vorinostat was administered at 50 mg/kg once daily via i.p. injection. Vorinostat was dissolved directly into (2-hydroxypropyl)-βcyclodextrin (Sigma Aldrich C0926). Panobinostat was administered via i.p. injection at 10 mg/kg once daily. Panobinostat was dissolved into DMSO, and then diluted into 10% (2-hydroxypropyl)-betacyclodextrin. Sapanisertib was prepared in a solution of 5% N-methyl-2-pyrrolidone and 15% polyvinylpyrrolidone, and administered via oral gavage once daily at 0.8 mg/kg. BSO was dissolved directly into the drinking water and administered at 20 mmol/L.

Tumor Volume Measurements

Treatment was initiated when tumors reached approximately 200 to 700 mm³. Tumor measurements were taken using a vernier caliper. Tumor volume was calculated using the standard formula: $L \times W^2 \times 0.52$.

Microarray and Analysis

RNA was isolated from 90-8TL cells 24 hours after treatment with indicated drugs. RNA was isolated using TRIzol, following the manufacturer's protocol, and RNA cleanup was performed using the Qiagen RNeasy kit (#74104). The Molecular Biology Core Facilities at Dana-Farber Cancer Institute hybridized RNA to the Affymetrix Human 1.0 STS array chip. To determine genes and gene sets differentially expressed amongst treatment groups a class comparison

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analysis was performed using BRB-Array tools developed by Dr. Richard Simon (National Cancer Institute, NIH, Rockville, MD) and the BRB ArrayTools Development team. Microarray data can be accessed from the Gene Expression Omnibus (GEO) database (accession number: GSE84205).

DCFDA Staining

Cells were treated as indicated. Cells were then stained with H2DCFDA at 10 $\mu mol/L$ and analyzed by flow cytometry.

Electron Microscopy

Tumor samples were collected 7 hours after a single treatment with 5 mg/kg rapamycin and 100 mg/kg vorinostat. Tissue was cut into 1 mmol/L cubes and fixed in 2% glutaraldehyde and 4% paraformaldehyde in 0.1 mol/L sodium cacodylate buffer, and then processed by the Electron Microscopy Facility at the University of Chicago as previously described (59). Images were collected and analyzed by Dr. Kay Macleod (University of Chicago).

Xenograft Model

For xenograft study, 2.5 million H1573 cells in 50% matrigel were injected into the flanks of female nude mice, and tumors were allowed to form. When tumors were between 130 mm³ and 350 mm³, animals were assigned to a treatment group. Tumors were measured every 2 to 3 days with a vernier caliper. Body condition and weight loss were monitored as signs of toxicity.

Statistical Analysis

Quantitative measurements are graphed as mean ± standard deviation (SD) of three technical replicates, unless otherwise indicated. To determine significance, an ANOVA analysis was performed, followed by either a two-tailed unpaired t test or Bonferonni's multiple comparison test when multiple groups were considered. For quantitative experiments, a P value of less than 0.05 was considered significant, and P values are shown when statistical tests were used. For microarray analysis (class comparison analysis of genes and gene sets) lower significance thresholds were used (unadjusted *P* < 0.001 and unadjusted *P* < 0.005, respectively) to narrow the focus to the genes and pathways most significantly impacted by the combination treatment. P values shown are adjusted for multiple hypothesis testing when applicable, unless otherwise noted. All data were graphed and analyzed using Prism 7, with the exception of the microarray data, which were analyzed using BRB-Array tools.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: C.F. Malone, M. Haigis, K. Cichowski Development of methodology: C.F. Malone, M. Haigis Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.F. Malone, C. Emerson, R. Ingraham, W. Barbosa, S. Guerra, H. Yoon, K.F. Macleod, O. Maertens Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C.F. Malone, C. Emerson, R. Ingraham, L.L. Liu, F. Michor, M. Haigis, K.F. Macleod, O. Maertens, K. Cichowski

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