Synergistic melanoma cell death mediated by inhibition of both MCL1 and BCL2 in high-risk tumors driven by NF1/PTEN loss

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INTRODUCTION
Cutaneous melanoma accounts for the vast majority of skin cancer-related deaths. More than 100,000 newly diagnosed cases of melanoma are projected in the United States for 2020 together with ~6800 melanoma-related deaths [1]. The Cancer Genome Atlas (TCGA) classified cutaneous melanomas into four molecular subtypes: BRAF-mutant (47.5%), RAS-mutant (29%), NF1-mutant (9%), and triple-wild type (14.5%) [2]. The NF1-mutant category refers to cases lacking either BRAF or RAS mutations, whereas NF1 mutations can also arise as a mechanism of resistance to RAF/MEK-targeted therapies in BRAF-mutated melanoma [3, 4]. Thus, NF1 mutations have been reported in 13–17% of cutaneous melanomas overall [2, 5, 6].

The NF1 gene encodes neurofibromin, a 2818-amino-acid protein whose GTPase-activating protein-related domain negatively regulates Ras signaling by catalyzing the hydrolysis of Ras-GTP into Ras-GDP. Thus, one consequence of NF1-loss is the aberrant activation of Ras signaling [7]. In primary melanoma patient biopsies, NF1 mutations were not correlated with hot-spot BRAF mutations, a finding consistent with a redundant role for these two types of mutations in activating Ras-MAPK signaling.

Recent efforts to develop improved targeted therapies for melanoma have mainly focused on the BRAF-mutant subtype, leaving a paucity of treatment options for patients with NF1-mutant melanomas. It is unlikely that the FDA-approved BRAF-mutant-specific inhibitors will be beneficial against BRAF-wild-type, NF1-mutant melanomas. Moreover, analysis of multiple clinical trials indicate that the NF1-mutant subtype has the worst outcome among all metastatic melanomas [8]. Clearly, a better understanding of the molecular pathogenesis of NF1-mutant melanomas is needed to improve the design and hence the outcome of treatments for this subtype of melanoma.

A major impediment to the development of targeted therapies for patients with NF1-mutant melanomas has been the lack of suitable animal models. For example, both the BRAF-mutant and RAS-mutant subtypes of melanoma have been successfully modeled in mice [9] and zebrafish [10] by combining the melanoma-associated mutations in these genes with mutation or loss of p53 or Cdkn2a, which are both typically inactivated in human melanoma [2, 11]. However, NF1-loss was not sufficient to induce melanoma tumorigenesis in mice [3, 12] or zebrafish [13], either alone or in combination with p53 loss. As the NF1-mutant melanomas often harbor a high mutation load [14, 15], we reasoned that genetic or epigenetic alterations affecting genes other than p53 and Cdkn2a are likely required in combination with NF1-loss to initiate melanoma transformation in vivo. Because a significant subset of human NF1-mutant melanomas harbor genetic alterations leading to activation of the PI3K-AKT-mTOR

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pathway [2, 5], we hypothesized that targeting this pathway through inactivation of ptena/ptenb would drive melanomagenesis in nf1/p53-mutant zebrafish.

**RESULTS**

Loss-of-function mutations of nf1 and pten cooperate to drive melanomagenesis in p53-deficient zebrafish

We previously reported the development of nf1a−/−;nf1b−/− zebrafish lines with the loss of three of the four functional alleles of nf1 [13]. These animals develop spontaneous malignant peripheral nerve sheath tumors (MPNSTs) with low penetrance, but not melanomas, beginning at the age of 1.5 years, indicating that nf1-loss alone is not sufficient to drive melanomagenesis (Supplementary Fig. S1). When we bred the nf1a−/−;nf1b−/− line into a p53−/− background, the compound mutant fish developed MPNSTs or high-grade gliomas [13]. Although rare spontaneous melanomas were also detected, they had a very low penetrance (<2%) over the course of 40 weeks (Supplementary Fig. S1). Because human NF1-mutant melanomas often harbor gain-of-function alterations in the PI3K signaling pathway, including mutational inactivation of PTEN or overexpression of AKT3 [2, 5], we introduced pten loss-of-function mutations into nf1a−/−;nf1b−/−;p53−/−/M214K/M214K zebrafish by crossing with a previously established ptena−/−;ptenb−/− line [16, 17]. We then isolated nf1a−/−;nf1b−/−;ptena−/−;ptenb−/−;p53−/−/M214K fish and monitored the offspring for spontaneous tumor development every 2 weeks starting at 5 weeks of age. Very aggressive melanotic tumors began to appear in these fish at 7 weeks of age, with a penetrance of 80% by 20 weeks (Fig. 1a–e). Histopathologic study of the melanotic tumors revealed a dense, cellular neoplasm in which a subset of the neoplastic cells produced pigment, with an overall histology pathognomonic of malignant melanoma (Fig. 1a) [13]. These animals develop spontaneous malignant peripheral nerve sheath tumors (MPNSTs) with low penetrance, but not melanomas, beginning at the age of 1.5 years, indicating that nf1-loss alone is not sufficient to drive melanomagenesis (Supplementary Fig. S1). When we bred the nf1a−/−;nf1b−/− line into a p53−/− background, the compound mutant fish developed MPNSTs or high-grade gliomas [13].

Melanomas arising in the nf1/p53−/− background were highly invasive into underlying musculature (Fig. 1b–d), and developed much earlier than melanomas in either the Tg(mitf:NRASQ61K);p53−/−/M214K or Tg(mitf:NRASQ61K);p53−/−/M214K zebrafish [18–21]. MPNSTs and glioblastomas appear in the nf1/p53 background after 30 weeks of age, while melanomas develop starting at 5 weeks of age in the nf1/p53 background and grow so rapidly that fish usually need to be sacrificed for humane reasons before 30 weeks of age, so melanomas are the only tumor-type observed in the nf1/p53 background. Importantly, these spontaneous melanomas arose exclusively in fish that were homozygous null for both nf1b and ptenb, heterozygous for nf1a and ptena mutant alleles, and either heterozygous or homozygous for p53−/−/M214K (Figs. 1 and S2). DNA PCR from melanoma tumors and adjacent normal tissue showed that the wild-type allele of nf1a and ptena is retained by the tumor cells (Supplementary Fig. S3). The nf1a−/−;nf1b−/−;ptena−/−;ptenb−/−;p53−/−/M214K tumors (designated “nf1/pent-mutant melanomas”) developed at random sites across the surface of the fish (Fig. 1a). The pigmented melanoma cells were highly invasive, infiltrating skeletal muscle adjacent to every tumor examined for histology (Fig. 1b–d). Hence, retention of only one allele of both nf1 and pten in a p53−/− background drives the development of highly invasive malignant melanoma in our zebrafish model.

The nf1/pent-mutant melanomas lack braf/nras hot-spot mutations

Since 80% of human cutaneous melanomas harbor activating hot-spot mutations in either BRAF or NRAS (e.g., BRAF600E, NRASQ61, or NRASQ61) [2], we examined the nf1/pent-mutant zebrafish melanomas for spontaneous mutations at equivalent sites in the zebrafish orthologues (Supplementary Fig. S4). Sequencing of eight tumors revealed only wild-type alleles of these two genes in each tumor (Supplementary Fig. S5). Hence, similar to the NF1-mutant class of human cutaneous melanomas [2], the loss of nf1 is sufficient to provide RAS pathway activation, and zebrafish melanomas in this background do not contain braf/nras hot-spot mutations.

nf1/pten-mutant melanomas exhibit aberrant activation of the RAS and PI3K pathways and are highly proliferative

Since NF1 and PTEN are well-established negative regulators of RAS and PI3K signaling [7, 22], respectively, we postulated that the nf1/pten-mutant melanomas would exhibit activation of effector pathways downstream of RAS and PI3K. Indeed, we detected high levels of phosphorylated ERK (pERK), phosphorylated AKT (pAKT), and phosphorylated S6 ribosomal protein (pS6, an mTOR downstream effector) by immunohistochemistry (IHC) in the nf1/pten-mutant melanomas (Fig. 1f), indicating hyperactivation of both RAS and PI3K pathways. Because these pathways drive proliferation, we next analyzed the proliferative capacity of nf1/pten-mutant melanomas, observing high levels of expression of proliferating cell nuclear antigen (PCNA) in 45% of tumor-cell nuclei but not the adjacent normal tissue (Fig. 1f), indicating a high tumor proliferative rate. Apoptotic cells were not observed in these melanomas, as indicated by the lack of detectable cleaved caspase-3 (Fig. 1f). Hence, combined activation of the RAS and PI3K pathways, a high proliferative rate, and the lack of apoptosis likely account for the rapid onset and high growth rate of nf1/pten-mutant melanomas.

nf1/pten-mutant melanomas can be serially transplanted into immunodeficient recipients

To assess the transplantation potential of our melanoma model, we isolated nf1/pten-mutant melanoma cells and transplanted them intraperitoneally into the optically clear immunodeficient rag2−/−(caspfer2) zebrafish [23] (designated “rag2−/−”). Robust engraftment was observed at the site of injection. All recipient fish demonstrated rapidly growing melanotic tumor masses within 2 weeks (Fig. 1g). Because of the invasive properties of the primary nf1/pten-mutant melanomas (Fig. 1), we also tested the feasibility of their intramuscular transplantation into rag2−/− zebrafish, where the tumor cells not only expanded within muscle, but also invaded neighboring tissues such as the ventral fin (Supplementary Fig. S6). By contrast, non-transformed melanocytes (derived from normal pigmented melanocytes within the skin stripes) from nf1a−/−;nf1b−/−;ptena−/−;ptenb−/−;p53−/−/M214K zebrafish failed to engraft in rag2−/− zebrafish. Furthermore, although the melanomas arising from the Tg(mitf:BRADF600E);p53−/−/M214K zebrafish [24] can be serially transplanted into rag2−/− zebrafish, their post-transplantation growth rates were much slower, highlighting the extraordinarily high growth rate in vivo of the nf1/pten-mutant melanomas.

MEK and PI3K inhibitors lack efficacy against nf1/pten-mutant melanomas in vivo

Human NF1-mutant melanomas have the worst outcome among all metastatic melanomas [8], and PTEN-mutant melanomas are known to be resistant to T-cell mediated immunotherapy such as the immune checkpoint inhibitor [25]. Thus, there is a clear need for effective small-molecule inhibitors to overcome the aggressive growth properties of NF1/PTEN-mutant melanoma. Because targeting the RAS-MEK-ERK and PI3K-PTEN-AKT-mTOR signaling pathways might logically affect the growth of nf1/pten-mutant melanomas, we first transplanted these melanoma cells into 3-week-old rag2−/− zebrafish and treated the recipients with MEK (trametinib or cobimetinib) or pan-PI3K (buparlisib or apitolisib) inhibitors. The nf1/pten-mutant melanoma cells grew rapidly in DMSO-treated recipients and progressed from an inoculum of 500 cells to readily detectable pigmented tumors at 4–8 days post transplantation. Therefore, MEK and PI3K inhibitors are not effective against nf1/pten-mutant melanomas in vivo.
transplantation (Fig. 2a). Single-agent treatment with either MEK or PI3K inhibitors from days 2–8 post transplantation at each of their maximum tolerated dosages (MTDs; Supplementary Fig. S7a) did not affect the growth of tumors (Figs. 2b and S7b). Even when tumor-bearing recipient fish were treated with a combination of trametinib and buparlisib at their MTDs (Supplementary Fig. S7a), tumor growth was only transiently inhibited during treatment, followed by rapid regrowth after drug removal, resulting in the lack of improvement in overall survival (Fig. 2d).

Inhibition of mTOR suppresses the growth of nf1/pten-mutant melanomas in vivo

To broaden the coverage of candidate pathway inhibitors, we next tested a panel of antitumor drugs targeting the RAS-MEK-ERK and receptor tyrosine kinase-PI3K-AKT-mTOR pathways in our nf1/pten-mutant melanoma model by assessing tumor-cell growth and overall survival of recipient rag2−/− fish after 6 days of treatment (Figs. 2, S8 and S9). Among the 14 tested drugs, each at their MTD, only the rapamycin family of mTOR inhibitors (rapalog) showed selective activity against nf1/pten-mutant melanoma in vivo as single agents. Interestingly, four different mTOR kinase inhibitors did not show activity against nf1/pten-mutant melanomas at their MTD (Supplementary Fig. S9). During the 6-day treatment course, sirolimus (rapamycin) clearly suppressed the appearance of detectable tumors, and its inhibition of tumor growth persisted for 1 to 2 weeks post treatment, in marked contrast to the rapid tumor regrowth in fish treated with MEK and PI3K inhibitors (Figs. 2c, e, 3 and S10). We also treated nf1/pten-mutant melanomas with everolimus and temsirolimus, two FDA-approved analogs of sirolimus. The three rapalogs showed similar abilities to durably inhibit melanoma cell growth (Fig. 2c, e), which uniformly translated to improved overall survival, indicating that rapalogs may provide a useful treatment option for these melanomas in vivo.

Primary nf1/pten-mutant tumors are invariably melanotic, but after serial transplantation, the tumor cells often become amelanotic [26]. In order to track the melanoma cells using EGFP instead of melanin, we bred the sox10:EGFP fluorescent zebrafish into our nf1/pten−/− background, which was able to uniformly translate to improved overall survival, indicating that rapalogs may provide a useful treatment option for these melanomas in vivo.

In Supplementary Fig. S11 [24], when transplanted into 3-week-old rag2−/− zebrafish and treated for 6 days with multiple different inhibitors, the EGFP-expressing amelanotic cells responded poorly to single-agent treatment with either trametinib or buparlisib, had only temporary responses to the trametinib–buparlisib combination, but showed more durable responses to sirolimus and temsirolimus (Supplementary Fig. S12). Thus, the amelanotic melanoma cells appear to respond in a similar fashion to the melanotic melanoma cells,
reinforcing the dependence of both subtypes of melanoma on mTOR signaling for malignant cell growth in vivo.

**Cell growth in nf1/pten-mutant melanomas depends on mTOR signaling**

The RAS-MEK-MAPK and PI3K-AKT-mTOR pathways negatively regulate each other, such that a drug-induced blockade of one pathway results in increased activity of the other [27, 28]. To test whether these drugs act on the expected pathways in inhibitor-treated nf1/pten-mutant melanomas, we analyzed treated tumors by IHC, observing that treatment with the MEK inhibitor trametinib led to a reduction in pERK levels (Fig.4a, b), as expected; while levels of pAKT and pS6 are increased (Fig. 4a, c, d), reflecting the loss of RAS-MEK-MAPK-mediated cross-inhibition of PI3K-AKT-mTOR signaling [27]. Similarly, treatment with the PI3K inhibitor buparlisib led to a reduction in pAKT and pS6 levels, with loss of RAS-MEK-MAPK-mediated cross-inhibition, resulting in increased pERK levels (Fig. 4a–d). This concomitant upregulation of an alternative pathway explains why neither buparlisib nor trametinib alone inhibited tumor-cell proliferation (Fig. 4a, e). The trametinib–buparlisib combination readily inhibited both the RAS and PI3K pathways, leading to a significant, though modest, decrease in tumor-cell proliferation (Fig. 4). Thus, these two pathways appear to function redundantly in driving the proliferation of nf1/pten-mutant melanomas. Interestingly, 2 days of sirolimus treatment resulted in undetectable levels of pS6 staining (Fig. 4a, d), reflecting mTOR inhibition with transient increase and then sustained loss of pERK levels (Figs. 4a, b and 5) and suppression of proliferation (Fig. 4). Thus, the sustained compensatory upregulation of the ERK pathway induced by buparlisib was not evident when mTOR-mediated phosphorylation was specifically inhibited by sirolimus.

To assess the durability of pathway suppression by inhibitor treatment, we treated nf1/pten-mutant-melanoma recipients with the inhibitors for 6 days, then analyzed the tumors after 4 days in the absence of the drugs. Sirolimus led to sustained reductions in pERK, pAKT, pS6, and PCNA levels at 4 days post treatment (Fig. 5a–c), as part of a cytoprotective autophagy stress response (Fig. 6a). By contrast, the initial signaling and antiproliferative effects of the trametinib-buparlisib combination (Fig. 4) were short-lived, as 4 days after drug removal, the pERK, pAKT, pS6, and PCNA levels were returning to normal (Fig. 5). Similar to sirolimus, temsirolimus also induced durable inhibition of pS6 and sustained suppression of pERK, pAKT, and tumor proliferation (Supplementary Fig. S13). Thus, in contrast to combined inhibition of PI3K and MEK, mTOR inhibition alone leads to the sustained suppression of RAS and PI3K pathways and tumor-cell growth in transplanted melanomas.

**Co-inhibition of BCL2 and MCL1 synergizes with sirolimus to cause apoptotic cell death nf1/pten-mutant melanomas in vivo**

It is important to emphasize that while either sirolimus or temsirolimus can induce prolonged proliferative arrest based on the absence of PCNA staining, we did not detect cleaved caspase 3 in treated tumor cells (Figs. 4a, 5a, and S13), indicating that...
neither agent is cytotoxic as a single agent. To query further the proliferative arrest induced by these rapalogs, we studied the autophagy marker LC3 by IHC. This analysis revealed autophagy not only of the tumor cells by LC3 staining, but also striking levels of autophagy in the brain and liver of the sirolimus-treated animals (Fig. 6a). The absence of a cytotoxic effect and initiation of autophagy as a cell survival mechanism would likely limit the therapeutic potential of rapalogs in \( nf1/pten \)-mutant melanomas. Thus, we analyzed a panel of antitumor drugs to identify those with the potential to synergize with sirolimus by inducing

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**Fig. 3** Sirolimus, but not trametinib or buparlisib, prevents rapid relapse of \( nf1/pten \)-mutant melanoma following treatment. Three-week-old \( rag2^{-/} \) zebrafish transplanted with pigmented \( nf1/pten \)-mutant melanoma cells were treated for 6 days with DMSO, 80 nM trametinib, 2 \( \mu M \) buparlisib, the combination of 80 nM trametinib and 2 \( \mu M \) buparlisib, or 20 \( \mu M \) sirolimus. **a, c, e, g, and i** Representative zebrafish at the end of the 6-day drug treatment. **b, d, f, h, and j** Representative zebrafish at 4 days following the end of drug treatment. **k** Quantification of melanotic \( nf1/pten \)-mutant tumor-cell area at the end of the 6-day course of drug treatment (left), and 4 days later (right). ns \( p > 0.05 \), * \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \) by two-tailed, unpaired \( t \)-test. Scale bar = 1 mm.
apoptosis, thus converting “cytostatic autophagy” to “cytotoxic autophagy” [29]. This evaluation included MEK inhibitors trametinib and cobimetinib, the PI3K inhibitors buparlisib and apitolisib, the pan-RAF inhibitor sorafenib, the PARP inhibitor olaparib, the autophagy inhibitor chloroquine, and inhibitors of the BCL2 family of pro-survival proteins including sabutoclax, obatoclax, venetoclax, and S63845 (MTD determination see Supplementary Fig. S14).

As shown in Supplementary Fig. S15, none of the drugs delayed tumor progression when given alone to 3-week-old fish-bearing nf1/pten-mutant melanomas, and only sirolimus in combination with venetoclax showed overall survival benefit compared to...
sirolimus alone. In particular, the autophagy inhibitor chloroquine markedly delayed tumor progression when combined with sirolimus, presumably by blocking the ability of the autophagosomes to fuse with lysosomes, thus preventing both tumor and normal cells from accessing the nutrients sequestered in the autophagosome [30–32]. However, its use with sirolimus caused massive post-treatment death of the recipient fish as early as 4 days after drug administration, presumably due to autophagy of normal tissues such as liver (Figs. 6 and S15). Thus, we sought to identify drugs that would modify the autophagy response not directly as in the case of chloroquine but selectively by promoting apoptosis.

Fig. 5 | Sirolimus induces a durable cytostatic effect in nf1/pten-mutant melanomas. a Representative tissue sections from a transplanted nf1/pten-mutant melanoma tumor at 4 days after a 6-day drug treatment with DMSO (CTR), 80 nM trametinib, 2 μM buparlisib, the combination of 80 nM trametinib and 2 μM buparlisib, or 20 μM sirolimus. Sections were immunostained using antibodies to detect pERK, pAKT, pS6, PCNA, and CC3. pERK-, pAKT- and pS6-positive tumor areas, as well as PCNA-positive nuclei, are quantified in (b–e). “T + B” refers to trametinib plus buparlisib. ns p > 0.05, *p < 0.05, **p < 0.01, ***p < 0.0001 by Mann–Whitney test. Scale bar = 20 μm.

|                | CTR      | Trametinib | Buparlisib | T+B      | Sirolimus |
|----------------|----------|------------|------------|----------|-----------|
| pERK           |          |            |            |          |           |
| pAKT           |          |            |            |          |           |
| pS6            |          |            |            |          |           |
| PCNA           |          |            |            |          |           |
| CC3            |          |            |            |          |           |

Table: 4 days after the 6-day treatment
Pro-survival members of the BCL2 family of proteins are required for the survival of cells undergoing autophagy [33], with tumor cells typically showing greater dependence on these pro-survival effects because of their higher-than-normal expression of BH3-only initiators of apoptosis, leading to an increased propensity to undergo apoptosis through a mechanism called “apoptotic priming” [34]. Thus, since pro-survival BCL2 family proteins are essential in the high-stress environment induced by sirolimus, ...
their inhibition would be expected to induce tumor cells to undergo apoptosis before normal cells [35–38]. Therefore, inhibitors of pro-survival BCL2 family proteins should have a therapeutic index based on synergy with the effects of sirolimus in targeted therapy for “primed” NF1/PTEN-mutant tumor cells, while sparing normal tissues.

To test this hypothesis, we focused on two inhibitors, venetoclax (inhibiting BCL2) [39] and S63845 (inhibiting MCL1) [40]. Interestingly, although venetoclax alone had no effect on tumor growth at a dose of 7.5 μM, its combination with 10 μM sirolimus significantly delayed tumor progression (Fig. 6b, c). Similarly, S63845 alone did not affect tumor growth at a dose of 5 μM, but in combination with 10 μM sirolimus, it augmented the growth suppressive effects of sirolimus (Fig. 6d, e).

It is known that each member of the pro-survival BCL2 family proteins, including BCL2 and MCL1, can bind and sequester BH3-only proteins independently and thereby prevent these BH3-only proteins from inducing apoptosis by activating BAX and BAK [39]. We previously discovered in vivo synergistic anti-leukemia activity of venetoclax and S63845, as each drug causes marked compensatory upregulation of MCL1 and BCL2 protein levels when used as single agent in zebrafish [41]. Hence, we reasoned that co-inhibition of BCL2 and MCL1 in nf1/pten-mutant melanoma cells might produce an even greater synergistic antitumor effect than observed with either inhibitor given individually with sirolimus. Indeed, when we combined 7.5 μM venetoclax and 2.5 μM S63845 with 10 μM sirolimus, we observed greatly enhanced growth suppression of nf1/pten-mutant melanoma cells (Fig. 6f, g). To determine the basis for this boosted effect, we analyzed the contributions of these three agents to tumor-cell proliferation and apoptosis. 7.5 μM venetoclax and 2.5 μM S63845 had no effect on proliferation or apoptosis, while 10 μM sirolimus significantly inhibited proliferation but failed to induce apoptosis (Fig. 6h, i). In combination, however, the three drugs effectively inhibited proliferation, and dramatically increased levels of apoptosis (Fig. 6h, i). Importantly, the fish tolerated this drug combination without noticeable toxicity. Thus, our results indicate that tumor cells sensitized by sirolimus become more dependent than normal cells on BCL2 and MCL1 for sustained survival, thus increasing their susceptibility to apoptosis in the absence of these key pro-survival proteins.

Co-inhibition of BCL2 and MCL1 synergizes with sirolimus to induce apoptosis in human NF1/PTEN-deficient melanoma cells

To validate the efficacy of our three-drug combination, we turned to studies using human NF1/PTEN-deficient melanoma cells. For this purpose, we first evaluated the expression level of neurofibromin and PTEN in a panel of human melanoma cell lines and identified one cell line, WM-3246, that lacked detectable expression of either neurofibromin or PTEN (Fig. 7a). Then, using WM-3246 cells, we tested the effects of sirolimus, venetoclax and S63845 on the viability of NF1/PTEN-deficient melanoma cells. As a single agent, sirolimus induced only modest levels of cytostatic growth suppression at concentrations >50 nM (Fig. 7b–d). Venetoclax did not produce effects on WM-3246 cell growth at concentrations up to 250 nM, whereas S63845 suppressed cell growth in a dose-dependent manner at doses >5 nM (Fig. 7b, c). The greatest impact on cell growth was evident when sirolimus was tested in combination with venetoclax and S63845 (Fig. 7c, d); synergy was obtained by isobologram analysis over a range of drug concentrations (Fig. 7e), indicating that these cells depend on both BCL2 and MCL1, as well as on mTOR signaling, for cell growth and survival. Western blot analysis showed compensatory upregulation of MCL1 in cells treated with venetoclax (Fig. 7f), confirming the molecular basis for the synergy between S63845 and venetoclax in sirolimus-treated WM-3246 cells. Furthermore, cleaved caspase 3 in WM-3246 cells treated with the three-drug combination but not with sirolimus alone (Fig. 7g), validating the induction of apoptosis by co-inhibition of BCL2 and MCL1 in sirolimus-sensitized NF1/PTEN-deficient human melanoma cells.

We also tested the three-drug combination identified in our NF1/PTEN-mutant melanoma model in BRAF-mutant melanomas with PTEN mutations, because BRAF activation by mutation is more prevalent than biallelic inactivating mutations of NF1. Although each of these drugs demonstrated little or no activity as single agents, the three-drug combination showed significant activity against the BRAF-mutant melanoma cells harboring PTEN-mutation (Supplementary Fig. S16). Furthermore, the venetoclax-S63845 combination potentiated melanoma cell killing caused by the BRAFV600E inhibitor darenfrib in BRAF-mutant melanoma cells (Supplementary Fig. S16), suggesting that co-inhibition of BCL2 and MCL1 as a strategy to enhance the induction of apoptosis has broad utility as a means to potentiate the activity of targeted therapies in disseminated human melanomas.

**DISCUSSION**

Loss-of-function mutations of the NF1 tumor suppressor in human melanoma cells were first identified by us and others in the early 1990s [42, 43]. The TCGA program subsequently undertook a multiplatform characterization of cutaneous melanoma samples at the DNA, RNA, and protein levels, in which NF1-mutant melanoma emerged as an important subtype within a genomic classification framework [2]. Although highly useful as a means to identify cooperative molecular aberrations that might serve as druggable targets or predictive biomarkers, this genomic approach did not suggest a therapeutic strategy for tumors linked to NF1 loss. Using a zebrafish experimental system that models human NF1-mutant melanomas, we show that activation of both the RAS and PI3K pathways in a background of pten loss is required to initiate melanomas in nf1-deficient animals. However, the RAS and PI3K pathways function redundantly in tumor maintenance, due to compensatory upregulation of either pathway when the other is inhibited (Figs. 4 and 5). Even simultaneous inhibition of both pathways only transiently inhibited the growth of nf1/pten-mutant melanomas, such that the overall survival of tumor-bearing fish was unaffected (Figs. 2, S7 and S10). This result contrasts with findings in basal-like breast cancer cell lines, in which the combination of MEK and PI3K inhibitors produced cytotoxic and tumor effects [44].

Given the superior of sirolimus in suppressing the growth of transplanted nf1/pten-mutant melanomas while inducing autophagy in normal tissues, we faced a major challenge: to identify drugs that could selectively cause apoptosis in sirolimus-sensitized melanoma cells. Such studies require an animal model that allows one to simultaneously assess both antitumor effects and toxicity to normal tissues, a criterion that was readily met by our zebrafish model. Indeed, while the antitumor response of nf1/pten-mutant melanomas to the combination of sirolimus and chloroquine initially appeared promising, the treated fish died due to toxicity to normal tissues (Supplementary Fig. S15), illustrating the importance of analyzing this drug combination in an in vivo model system. By contrast, the combination of sirolimus with inhibitors of the anti-apoptotic proteins BCL2 (venetoclax) and MCL1 (S63845) was both well tolerated by normal tissues and highly active in inducing apoptosis in tumor cells (Figs. 6, 7, and S15). This selectivity apparently results from the fact that the malignant cells are “primed” to undergo apoptosis, while normal cells do not harbor the same levels of upregulation of BH3-only death proteins and can survive and maintain mitochondrial integrity despite the simultaneous inhibition of two major pro-survival proteins.

Our results underscore the advantages of using a reliable in vivo preclinical model to analyze the effects of simultaneously
inhibiting multiple pathways with small-molecule drugs. Given its greater efficiency and lower costs compared to murine models, our zebrafish experimental system appears ideal for pursuing additional classes of pathway inhibitors in NF1/PTEN-mutant melanomas, as single agents and in combination, to define their clinical translational potential. Thus, the three-drug combination of sirolimus, venetoclax, and S63845 is well tolerated at effective dosages in vivo and shows activity against human as well as zebrafish NF1/PTEN-deficient melanoma cells, providing preclinical evidence justifying an early-stage clinical trial in patients with melanomas of this high-risk genomic subtype. Notably, the three-drug combination identified in our NF1/PTEN-mutant melanoma model also showed anti-melanoma activity in BRAF-mutant melanoma cells harboring PTEN-mutation (Supplementary Fig. S16). Furthermore, the venetoclax-S63845 combination potentiated melanoma cell killing caused by the BRAFV600E inhibitor dabrafenib in BRAF-mutant melanoma cells (Supplementary Fig. S16). Thus the potentiation of apoptosis induced by co-inhibition of BCL2 and MCL1 is a strategy with wide applicability to enhance the anti-melanoma activity by targeted therapies in malignant melanoma.

MATERIALS AND METHODS
Zebrafish
Zebrafish experiments and animal husbandry were performed in accordance with Dana-Farber Cancer Institute IACUC-approved protocol #02-107.
Melanoma tumor watch

The cell lines were tested for mycoplasma contamination using MycoAlert short tandem repeat analysis using the PowerPlex 1.2 system (Promega). The cell lines were excised from tumor-bearing mice, and offspring were monitored every week, starting at 3 weeks, once crossed, and scored as hyperpigmented cell masses indicative of melanoma tumors. Once a hyperpigmented cell mass was identified, the individual fish was separated and carefully monitored weekly for at least 3 weeks for tumor progression. Only fish with expanding hyperpigmented cell masses were scored as tumor fish and analyzed further by H&E staining and immunohistochemical assays. All fish were genotyped for nfta1/nftb1/ptena/ptenb/ps3 and nfta1/nftb1/ptena/ptenb/ps3Δ14KΔ4K mutant zebrafish were aneuthetized with 0.003% tricaine (Sigma-Aldrich, St. Louis, MO) and positioned on a 10-cm Petri dish coated with 1% agarose. Primary and serially passaged tumors derived from nfta1/nftb1/ptena/ptenb/ps3Δ14KΔ4K and nfta1/nftb1/ptena/ptenb/ps3Δ14KΔ4K/Tg(sox10:EGFP) zebrafish lines were excised from tumor-bearing fish and mechanically dissociated with a razor blade in 0.9× PBS + 5% FBS (Life Technologies, Carlsbad, CA) at room temperature. The collected cell suspension was filtered through a 40-μm cell strainer (Falcon, Corning, NY) and resuspended in 0.9× PBS + 5% FBS. For the intraperitoneal and intramuscular transplantation into 3- to 4-month-old adult zebrafish, a 26 s/2/2 Hamilton 80300 syringe (Hamilton, Reno, NV) was used [23]. For the intraperitoneal transplantation into 3-week-old juvenile zebrafish, cell suspensions were loaded into borosilicate glass capillary needles (1 mm o.d. × 0.78 mm i.d.; Harvard Apparatus, Holliston, MA), and the injections were performed with a Pneumatic PicoPump and a manipulator (WPI, Sarasota, FL) [45].

Tumor-cell transplantation

The identity of cell lines used in this study was verified, and offspring were monitored every week, starting at 3 weeks, for tumor progression. Only fish with expanding hyperpigmented cell masses were scored as tumor fish and analyzed further by H&E staining and immunohistochemical assays. All fish were genotyped for nfta1/nftb1/ptena/ptenb/ps3 and nfta1/nftb1/ptena/ptenb/ps3Δ14KΔ4K mutant zebrafish were aneuthetized with 0.003% tricaine (Sigma-Aldrich, St. Louis, MO) and positioned on a 10-cm Petri dish coated with 1% agarose. Primary and serially passaged tumors derived from nfta1/nftb1/ptena/ptenb/ps3Δ14KΔ4K and nfta1/nftb1/ptena/ptenb/ps3Δ14KΔ4K/Tg(sox10:EGFP) zebrafish lines were excised from tumor-bearing fish and mechanically dissociated with a razor blade in 0.9× PBS + 5% FBS (Life Technologies, Carlsbad, CA) at room temperature. The collected cell suspension was filtered through a 40-μm cell strainer (Falcon, Corning, NY) and resuspended in 0.9× PBS + 5% FBS. For the intraperitoneal and intramuscular transplantation into 3- to 4-month-old adult zebrafish, a 26 s/2/2 Hamilton 80300 syringe (Hamilton, Reno, NV) was used [23]. For the intraperitoneal transplantation into 3-week-old juvenile zebrafish, cell suspensions were loaded into borosilicate glass capillary needles (1 mm o.d. × 0.78 mm i.d.; Harvard Apparatus, Holliston, MA), and the injections were performed with a Pneumatic PicoPump and a manipulator (WPI, Sarasota, FL) [45].

Cell culture

Melanoma cell lines Mewo, WM-3246, WM-3622, WM-3629, WM-3670 and WM-3918 were purchased from Rockland (Rockland Immunochemicals Inc, Limerick, PA), and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, l-glutamine, and penicillin/streptomycin. Melanoma cell lines COLO829 and C32 were purchased from ATCC (ATCC, Manassas, VA) and maintained according to the provided Culture Methods. HEK-293T cells were purchased from ATCC, and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, l-glutamine, and penicillin/streptomycin. Jurkat cells were maintained in RPMI-1640 medium supplemented with 10% FBS, l-glutamine, and penicillin/streptomycin. Jurkat cell lines were excised from tumor-bearing fish and mechanically dissociated with a razor blade in 0.9× PBS + 5% FBS (Life Technologies, Carlsbad, CA) at room temperature. The collected cell suspension was filtered through a 40-μm cell strainer (Falcon, Corning, NY) and resuspended in 0.9× PBS + 5% FBS. For the intraperitoneal and intramuscular transplantation into 3- to 4-month-old adult zebrafish, a 26 s/2/2 Hamilton 80300 syringe (Hamilton, Reno, NV) was used [23]. For the intraperitoneal transplantation into 3-week-old juvenile zebrafish, cell suspensions were loaded into borosilicate glass capillary needles (1 mm o.d. × 0.78 mm i.d.; Harvard Apparatus, Holliston, MA), and the injections were performed with a Pneumatic PicoPump and a manipulator (WPI, Sarasota, FL) [45].

Statistical analysis

Statistical analysis was performed with Prism 5 software (GraphPad). Kaplan-Meier methods and the log-rank test were applied to assess the rate of tumor growth in Figs. 1 and S1, and tumor progression in Figs. 2, 6, S7, S8, S9, S10 and S15. The quantitative data in Figs. 3, 4, S5 and S12 are reported as median values. A Mann–Whitney test with confidence intervals of 95% was used for the analyses in Figs. 3, 4, 5, 6 and S12.

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COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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