Vemurafenib Synergizes with Nutlin-3 to Deplete Survivin and Suppresses Melanoma Viability and Tumor Growth

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Abstract

Purpose: For patients with advanced melanoma, primary and secondary resistance to selective BRAF inhibition remains one of the most critically compelling challenges. One rationale argues that novel biologically informed strategies are needed to maximally cripple melanoma cells up front before compensatory mechanisms emerge. As p53 is uncommonly mutated in melanoma, restoration of its function represents an attractive adjunct to selective BRAF inhibition.

Experimental Design: Thirty-seven BRAF(V600E)-mutated melanoma lines were subjected to synergy studies in vitro using a combination of vemurafenib and nutlin-3 (Nt-3). In addition, cellular responses and in vivo efficacy were also determined. We also analyzed changes in the levels of canonical apoptotic/survival factors in response to vemurafenib.

Results: Dual targeting of BRAF(V600E) and Hdm2 with vemurafenib and Nt-3, respectively, synergistically induced apoptosis and suppressed melanoma viability in vitro and tumor growth in vivo. Suppression of p53 in melanoma cells abrogated Nt-3’s effects fully and vemurafenib’s effects partially. A survey of canonical survival factors revealed that both vemurafenib and Nt-3 independently attenuated levels of the antiapoptotic protein, survivin. Genetic depletion of survivin reproduces the cytotoxic effects of the combination strategy.

Conclusion: These results show preclinical feasibility for overcoming primary vemurafenib resistance by restoring p53 function. Moreover, it identifies survivin as one downstream mediator of the observed synergism and a potential secondary target. Clin Cancer Res; 19(16); 4383–91. © 2013 AACR.

Introduction

The central role of BRAF(V600E)-mediated oncogenesis in melanoma has been firmly established in both benchside (1) and bedside settings (2, 3). However, few patients exhibit complete responses, whereas many show disease progression by approximately 6 months, with less than 10% remaining progression-free beyond 12 months. Recently, combined BRAF and mitogen-activated protein/extracellular signal–regulated kinase (MEK) inhibition have improved this profile somewhat, but still less than 10% have complete responses and median progression-free survival is still less than 12 months (4, 5).

Despite the clear success of vemurafenib in recent trials, BRAF(WT) and a minor subset of BRAF(V600E) tumors are clearly unresponsive to selective BRAF inhibitors. MEK inhibition has also been established as an effective approach for BRAF(V600E)-mutant melanomas (5) and, unlike BRAF inhibitors, may serve as a backbone for targeted therapy regimens in certain BRAF(WT) populations. Maximizing the use of this new generation of anti-mitogen–activated protein kinase (MAPK) agents requires a two-prong strategy, which includes overcoming innate insensitivity (i.e., primary resistance) to the drug and decoding and disabling the secondary apparatus assembled by the cell to acquire resistance (i.e., secondary resistance). Numerous mechanisms of acquired resistance have been described including the emergence of activating NRAS mutations (6), drug-resistant splice variants of BRAF (V600E; ref. 7), MEK mutations (8), amplification of BRAF (9), and increased expression of COT/TPL2 (10). In addition, activation of the phosphoinositide 3-kinase pathway through acquired PTEN loss (11) or increased signaling through platelet-derived growth factor receptor or insulin-like growth factor receptors have been implicated in acquired resistance (12). Finally, production of stromal hepatocyte growth factor has recently been suggested as a mediator of primary resistance and monoclonal antibodies and small-molecule c-Met (the receptor for hepatocyte growth factor) inhibitors are in clinical development.
Translational Relevance
Over the course of a decade, the identification of activating BRAF mutations in melanomas culminated in a series of positive trials followed by the approval of the first selective BRAF inhibitor, vemurafenib. Despite these ostensible successes, not all BRAF(V600E) tumors fully respond to mitogen-activated protein kinase inhibitors and most patients on vemurafenib eventually succumb to relapse. As p53 is uncommonly mutated in melanoma, we set out to establish the preclinical feasibility of p53 restoration by combining vemurafenib with nutlin-3 (Nt-3), an Hdm2 antagonist. In this report, we show that the addition of Nt-3 to vemurafenib significantly induced apoptosis, reduced viability, and suppressed melanoma tumor growth in animals. Furthermore, we found that both vemurafenib and Nt-3 decreased levels of survivin, a known antiapoptotic protein in melanoma; genetic depletion of survivin reproduced the inhibitory drug effects. These results provide promising evidence that p53 rescue may represent one possible strategy to overcome primary vemurafenib resistance.

One rationale argues that novel biologically informed strategies are needed to maximally cripple melanoma cells up front before compensatory mechanisms emerge. Relatively few reports have posited tractable strategies for overcoming de novo resistance to either MEK or BRAF inhibition. To this end, we recently reported that restoration of wild-type p53 with Nt-3 (Nt-3, an Hdm2 antagonist) synergistically enhanced melanoma apoptosis when deployed with MEK inhibitors in vitro (13). We now report that Nt-3 can synergize with vemurafenib to suppress viability in vitro and tumor growth in vivo. Moreover, we also show that one possible mediator of this synergism is survivin, a known survival factor in melanoma. Melanoma cells treated with either vemurafenib or Nt-3 led to dramatic reductions in survivin levels, whereas depletion of survivin by genetic or pharmacologic means reproduced the growth suppressive effects of vemurafenib. These results offer one possible approach to overcome primary resistance to vemurafenib and implicate survivin as one molecule that may contribute to the observed synergism between MAPK inhibition and Hdm2 antagonism.

Materials and Methods

Reagents
Reagents were obtained from the following sources: Nt-3 (Cayman Chemicals), lentiviral-based short hairpin RNA (shRNAs; Sigma-Aldrich, Addgene), PLX4720/PLX4032 (vemurafenib; Selleck Chemicals), anti-pERK, anti-extracellular signal-regulated kinase (ERK), anti-cyclin D1 (cycD1), anti-survivin, and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies (Cell Signaling Technology), anti-p53 and anti-p21 (Santa Cruz Biotechnology), caspase inhibitor I (EMD), and Caspase-Glo 3/7 assay kit (Promega).

Cell culture
Melanoma cell lines have been previously published (14–16) and were validated against public datasets (http://www.sanger.ac.uk/genetics/CGP/cosmic/) by sequence and/or comparative genomic hybridization information when available. Lines were obtained from the American Type Culture Collection (ATCC). Lines were cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% FBS in humidified atmosphere of 5% CO₂/95% air at 37°C to confluence. Medium was replaced every 2 to 3 days. The A375(sh-GFP) and A375(sh-p53) lines have been previously described (13). The A375(VEH-R) line was generated using escalating doses of vemurafenib (survival curves for the two lines are shown in Supplementary Fig. S2; vemurafenib GI₅₀ for sensitive = 42 nmol/L and resistant = 9.7 μmol/L).

Drug treatment and cell viability assay
Cell viability was determined by AlamarBlue (Molecular Probes) fluorescence assay, PrestoBlue (Molecular Probes) fluorescence assay, and CellTiter-Glo (Promega) luminescence assay. Approximately 12 hours before drug treatment, cells were seeded at a density of 3,000 cells (100 μL) per well in a 96-well plate. The plates were incubated with drugs for 48 hours. Ten microliters of AlamarBlue or PrestoBlue was added to each well and incubated for 3 hours and 10 minutes, respectively, at 37°C. AlamarBlue and PrestoBlue are fluorescent substrates reduced by mitochondrial enzyme activity in viable cells. Alternatively, 30 μL of CellTiter-Glo was added to each well and incubated, protected from light on an orbital shaker, for 10 minutes. CellTiter-Glo contains luciferase, which catalyzes the oxygenation of luciferin (creating light) according to the amount of ATP present. Fluorescence or luminescence intensity was determined using a Molecular Devices plate reader with an excitation filter centered on 540 nm and an emission filter centered on 590 nm or with an integration time of 500 milliseconds and measuring total light emitted, respectively.

Stable infections
Lentiviral transduction was used to alter the levels of survivin. shRNA against survivin was cloned in either pLKO.1-puro vector (Sigma-Aldrich) or pMKO.1-puro vector (Addgene), whereas CDNA for survivin was cloned into the CDS16B2 vector (Systems Bioscience). Lentiviral supernatant was produced by transient transfection of HEK293T cells (ATCC) using lipofectamine (Invitrogen Life Technologies, Inc.), according to the manufacturer’s instructions. The viral-containing supernatants were harvested 48 hours after transfection and filtered through a 0.45 μm filter unit. To transduce melanoma cells with lentivirus, logarithmically growing melanoma cells were seeded at a density of 2 × 10⁵ cells per well in 6-well plates. A total of 0.5 mL of lentivirus suspension and 8 μg/mL of polybrene were added...
Volumes were calculated using the following ellipsoid formula:

\[ V = \frac{4}{3} \pi \frac{l^2}{2} \times \frac{d^2}{2} \]

in which \( V \) represents the volume, \( l \) represents the small diameter, and \( d \) represents the large diameter. PLX4720 and Nt-3. Tumor size was measured every day with vehicle dimethyl sulfoxide (DMSO), PLX4720, Nt-3 (100 mg/kg), or combination of both day with vehicle dimethyl sulfoxide (DMSO), PLX4720, Nt-3 (100 mg/kg), or combination of both day with vehicle dimethyl sulfoxide (DMSO).

Animal studies

Athymic nude mice, ages 6 to 8 weeks and weighing approximately 15 to 20 g, were purchased from Charles River Laboratories. All animal experiments were carried out in accordance with protocols approved by the Institutional Animal Care and Use Committees. Four million A375 cells in 0.1 mL of PBS were injected subcutaneously into the right lateral flank. Once the tumor reached around 5 mm in diameter, the mice were injected intraperitoneally every day with vehicle dimethyl sulfoxide (DMSO), PLX4720 (50 mg/kg), Nt-3 (100 mg/kg), or combination of both PLX4720 and Nt-3. Tumor size was measured every day with a Mitutoyo 500-196-20 Digital Caliper for 2 weeks. Tumor volumes were calculated using the following ellipsoid formula:

\[ V = \frac{4}{3} \pi \left( \frac{L \times (S')}{2} \right)^2 \]

in which \( L \) represents the large diameter of the tumor and \( S' \) represents the small diameter.

Immunoblotting analysis

Equal amounts of protein (5–30 μg) were loaded onto 10% or 4%–20% SDS polyacrylamide mini-gels (Bio-Rad), transferred to polyvinylidene difluoride membranes. After being blocked in 5% milk in TBS-Tween for 1 hour, blots were incubated with primary antibodies overnight, followed by horseradish peroxidase-conjugated secondary antibody (1:5,000) for 45 minutes. Antigen–antibody complexes were detected by enhanced chemiluminescence.

Caspase-3/7 activity assay

Melanoma cells were grown in 96-well plates and treated with inhibitors for 48 hours. The Caspase-Glo 3/7 reagent was then added to each well and incubated for 30 minutes. Caspase-3/7 activity was detected using Caspase-Glo 3/7 Assay System (Promega #G8091) and conducted as per manufacturer’s protocol.

Cell-cycle analysis

Cell-cycle stage was determined using flow cytometry. Briefly, treated melanoma cells were fixed with 70% ethanol at −20°C for 1 hour. The cells were resuspended in 1 mL of PBS containing propidium iodide (PI; 5 μg/mL) and RNase A (0.1 mg/mL) and were incubated at 37°C for 30 minutes. The cells (10° cells/analysis) were examined by flow cytometry (Coulter), and the cell-cycle distribution was determined by DNA content. Cells distributed in sub-G1 were defined as apoptotic according to the criteria described by others (17).

Apoptosis was also determined by Annexin V–fluorescein isothiocyanate (FITC). Briefly, the quantification of apoptosis was determined by staining cells with Alexa Fluor 480 AnnexinV (Invitrogen Eugene) and PI. Survivin knockdown assay was determined by Annexin V–fluorescein isothiocyanate (FITC). Briefly, treated melanoma cells were fixed with 70% ethanol at −20°C for 1 hour. The cells were resuspended in 1 mL of Annexin V–binding buffer, stained with 5 μL of Annexin V for 15 minutes, and 1 μL of PI (added just before analysis) at room temperature in the dark. The fluorescent signal of the cells was measured with a flow cytometer (Beckman Coulter).

Animal studies

Athymic nude mice, ages 6 to 8 weeks and weighing approximately 15 to 20 g, were purchased from Charles River Laboratories. All animal experiments were carried out in accordance with protocols approved by the Institutional Animal Care and Use Committees. Four million A375 cells in 0.1 mL of PBS were injected subcutaneously into the right lateral flank. Once the tumor reached around 5 mm in diameter, the mice were injected intraperitoneally every day with vehicle dimethyl sulfoxide (DMSO), PLX4720 (50 mg/kg), Nt-3 (100 mg/kg), or combination of both PLX4720 and Nt-3. Tumor size was measured every day with a Mitutoyo 500-196-20 Digital Caliper for 2 weeks. Tumor volumes were calculated using the following ellipsoid formula:

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Apoptosis genes in response to vemurafenib

For details, see Supplementary Data.

Statistical analysis

Differences between the fractional survivals were statistically assessed with the Student t test for normally distributed samples and the Mann–Whitney test for nonparametric comparisons. All analyses were conducted using SigmaStat.

Results

Vemurafenib and Nt-3 act in concert to suppress melanoma growth and tumorigenesis

To determine whether Nt-3 can potentiate vemurafenib effects, we subjected 27 BRAF(V600E) melanoma lines to a 3 × 4 dose–dose combination analysis (0.2 μmol/L, 2.0 μmol/L, 20 μmol/L, Nt-3 × 1 μmol/L, 3 μmol/L, 10 μmol/L, 30 μmol/L vemurafenib). Figure 1A shows the individual and combined survival curves and the isobologram for a representative cell line (NAE), which showed significant synergism. Figure 1B shows the combination indices (CI) for all of the lines as an integrated heatmap in a diagram. Forty-four percent and 77% of the lines showed at least one dose point with “very strong” (i.e., CI < 0.1) or “strong” synergy (i.e., CI < 0.3), respectively. The level of synergy seemed to increase with higher concentrations of Nt-3 though there were no distinct thresholds of effect.

We next explored the physiologic basis for the observed synergism. In melanoma cell lines A375 and WM239 (Fig. 2A), exposure to vemurafenib alone led to the expected decreases in pERK and cycD1 with little effect on p53 and p21. Separate treatment of the same cell lines with Nt-3 alone led to increases in p53 and p21 without significant changes in cycD1 levels. Concurrent exposure to both drugs (vemurafenib + Nt-3) led to simultaneous and largely independent effects in these lines suggesting that dual drug application does not create functional interference. As Mdm4 has recently been implicated in melanoma biology (18), we examined the comparative effects of Nt-3 and vemurafenib on both Hdm2 and Mdm4. As shown in Supplementary Fig. S2, Nt-3 induced Hdm2 but not Mdm4, whereas vemurafenib had no effect on either protein; the basal levels of Mdm4 seem to be slightly higher than Hdm2.

To further characterize the cellular response, we treated both lines with vemurafenib and Nt-3 and observed an increased apoptotic response when both agents were used (Fig. 2B). In a three-dimensional culture system (Supplementary Fig. S3), there was a notable reduction in viable A375 cells with vemurafenib alone (number of green cells) but little increase in dead cells (red cells). The addition of
Nt-3 both alone and in conjunction with vemurafenib led to a greater number of dead cells. Finally, we treated animals with DMSO, PLX4720 (50 mg/kg), Nt-3 (100 mg/kg), or a combination of PLX4720 + Nt-3 (Fig. 2C). Both PLX4720 and Nt-3 led to an intermediate level of growth suppression, whereas the combination showed strong additive effects (PLX4720 vs. PLX4720 + Nt-3, \( P < 0.01 \)). Taken together, these results provide evidence that the combination of vemurafenib and Nt-3 can effectively potentiate apoptosis and enhance suppression of both viability \textit{in vitro} and tumor growth \textit{in vivo}.

Survivin is mutually downregulated by both vemurafenib and Nt-3

To identify possible survival factors which could be coregulated by both vemurafenib and Nt-3, we examined the levels of 58 known apoptotic factors (Supplementary Table S1) in response to 3\( \mu \)mol/L vemurafenib; these data are part of a more complete microarray analysis that will be published as part of another communication. Among the 33 survival-related genes with available expression data (Fig. 3A), \textit{BIRC5} exhibited the greatest change in levels in response to vemurafenib (–2.99 log2 or 7.9-fold decrease with vemurafenib). \textit{BIRC5} encodes survivin, which is a member of the inhibitor of apoptosis family, a known negative target of p53 and a well-established melanoma survival marker (19). With these results, we hypothesized that survivin is coregulated by vemurafenib and Nt-3 and thus one mechanistic nexus between the MAPK and p53 pathways.

As p53 is known to downregulate survivin (20), we next showed that Nt-3 dramatically reduced survivin levels in several melanoma lines (Supplementary Fig. S4). As shown in Fig. 3B, A375 and WM239 cells which were exposed to either vemurafenib or Nt-3 or a combination of both all underwent depletion of survivin levels in response to these treatments, as predicted by the microarray results. Next, if the apoptotic and growth inhibitory effects of vemurafenib and Nt-3 are mediated by a decrease in survivin levels, one would then expect that depletion of survivin in melanoma cells should reproduce some of the cytotoxic effects of these compounds. Indeed, suppression of survivin levels in A375 and WM239 lines led to a significant induction of apoptosis as evidenced by increases in both the sub-G1 (Fig. 3C) and FITC-Annexin (Fig. 3D) subpopulations in both lines. Survivin overexpression also attenuated some of the apoptotic response to vemurafenib (Supplementary Fig. S4).

We next determined whether these two drugs perturbed independent pathways of survivin control. First, we used
an isogenically matched A375 cell line in which p53 has been stably suppressed, i.e., A375(sh-p53) line (13). The induction of p53 by Nt-3 was completely abrogated in the A375(sh-p53) cells, whereas the inhibition of pERK by vemurafenib remained intact (Fig. 4A). Compared with the control A375 line [i.e., A375(sh-GFP)], Nt-3 had no effect on survivin whereas vemurafenib still decreased survivin levels albeit with some residuum. Conversely, we also set out to prove that Nt-3 can fully suppress survivin in A375 cells that have been rendered resistant to vemurafenib [A375(VEM-R); Supplementary Fig. S1, dose response]. Relative to the parental A375 line, the A375(VEM-R) line exhibited no significant changes in either pERK or survivin levels in response to vemurafenib. In contrast, Nt-3 successfully induced p53 in the A375(VEM-R) line while effectively downregulating survivin without any appreciable impact on pERK signaling.

To assess whether these independent signal controls correlate with cell viability, the effects of Nt-3 and vemurafenib on the matched A375 lines were also examined. As expected, there was complete abrogation of Nt-3’s inhibitory effects upon p53 depletion. Interestingly, in the A375 (sh-p53) line, there was also a partial but consistent rescue from vemurafenib inhibition when compared with
the A375(sh-GFP) line. However, in genetically diverse melanoma lines, p53 mutation status did not seem to predict vemurafenib sensitivity (Supplementary Fig. S6). In cell lines rendered resistant to vemurafenib [i.e., A375 (VEM-R)], there was little effect on response to Nt-3 though response to vemurafenib was completely attenuated. Taken together, these findings show that vemurafenib and Nt-3 jointly downregulate survivin, and possibly other mediators, though full suppression of this molecule (Fig. 4A) and viability (Fig. 4B) by vemurafenib may require basal p53 function.

Discussion

In these current studies, we provide evidence that (i) p53 rescue through Hdm2 antagonism potentiates the suppressive effects of vemurafenib and enhances both melanoma cytotoxicity in vitro and melanoma growth in vivo, (ii) both vemurafenib and Nt-3 downregulate survivin, and (iii) direct attenuation of survivin by genetic means reproduces the inhibitory effects of vemurafenib and Nt-3 on melanoma cells. Through this analysis, it seems that survivin represents one possible mediator of the observed synergism and as such, a potential therapeutic target that may be exploited to overcome primary vemurafenib resistance.

In cell culture systems and in animal models, Nt-3 clearly potentiates the inhibitory effects of vemurafenib. These results build upon and greatly expand our earlier studies showing synergism between MEK inhibitors and Hdm2 antagonism (13). More specifically, the addition of Nt-3 seems to induce a more cytotoxic profile compared with vemurafenib or anti-MEK agents (13) alone. Although Nt-3 is the prototypic Hdm2 antagonist, there is now emerging evidence that broader approaches may be needed to fully restore p53 function especially in light of Mdm4’s recently described role in melanoma (18). In our experience, basal levels of Mdm4 were higher than those for Hdm2 although Nt-3 dramatically induced Hdm2 without significant impact on Mdm2. Although Nt-3 itself has poor pharmacologic properties in vivo, analogs and Hdm2...
antagonists with completely novel structures are currently in clinical development (NCT01605526, NCT01877382, and NCT01462175). Our results, along with those already reported, substantiate the Mdm family as potential therapeutic targets in melanoma.

We identified survivin as a negative target for both vemurafenib and Nt-3. Survivin ranks among one of the most tightly linked markers to melanoma aggression and serves dual physiologic roles as both an inhibitor of apoptosis and a critical component of the chromosomal passenger complex (19). Our results are consistent with prior studies which document suppression and induction of survivin by p53 (20) and MAPK stimulation (21), respectively. Although survivin levels can be independently attenuated by either vemurafenib or Nt-3, vemurafenib alone was not able to fully suppress survivin levels in the absence of p53, which is consistent with the partial rescue of cellular viability in A375 (sh-p53) cells upon vemurafenib exposure (Fig. 4). Thus, baseline p53 activity may be required for the full inhibitory effects of vemurafenib. Whether survivin levels or p53 status correlate with vemurafenib resistance clinically is the subject of ongoing studies. It is also not clear how vemurafenib modulates survivin levels as the BIRC5 promoter harbors binding sites for a large set of transcription factors including p53, c-myc, Egr-1, p300, and HNF-4a (http://genome.ucsc.edu), it is conceivable that transcriptional repression is one possible means of regulation although posttranscriptional mechanisms are also possible. From the treatment perspective, a prior single-agent phase II study of the survivin inhibitor, YM155, failed to show a significant survival benefit (22) though a more recent antisurvivin phase II vaccine trial did detect an overall survival advantage in patients with survivin-specific T-cell reactivity (median 19.6 vs. 8.6 months; P = 0.0077; ref. 23). Future
development of more specific antisurvivin agents may represent a powerful adjunct to anti-MAPK or immunologic approaches, if not as a standalone agent.

In more general terms, drug combinations such as vemurafenib and Nt-3 may achieve its net effects through drug-specific and shared molecular responses. For example, CCND1 (cyclD1) and CDKN1A (p21) represent genes that are selectively regulated by vemurafenib and Nt-3, respectively, whereas survivin exemplifies a gene which is regulated by both agents (Fig. 5). This suggests that the mode of synergism could be either collaborative (e.g., concurrent cyclD1 suppression and p21 induction) or convergent (e.g., survivin repression). As multipathway cocktails become developed, these molecular details will allow for a more precise mapping of synergistic and antagonistic interactions.

In summary, we document an effective pharmacologic partnership between selective BRAF inhibition and Hdm2 antagonism that enhances melanoma cytotoxicity and reduces tumorigenesis. We have identified survivin as a coregulated target of vemurafenib and Nt-3 and one, among possibly many, rich opportunities for harmonizing MAPK and p53 pathways in melanoma therapeutics.

Disclosure of Potential Conflicts of Interest

K.T. Flaherty is as a consultant/advisory board member of Roche and Genentech. H. Tsao has a commercial research grant from Piramal and has been a consultant/advisory board member of Genentech and Quest Diagnostics. No potential conflicts of interest were disclosed by the other authors.

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