Genotype-Selective Combination Therapies for Melanoma Identified by High-Throughput Drug Screening

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ABSTRACT
Resistance and partial responses to targeted monotherapy are major obstacles in cancer treatment. Systematic approaches to identify efficacious drug combinations for cancer are not well established, especially in the context of genotype. To address this, we have tested pairwise combinations of an array of small-molecule inhibitors on early-passage melanoma cultures using combinatorial drug screening. Results reveal several inhibitor combinations effective for melanomas with activating RAS or BRAF mutations, including mutant BRAF melanomas with intrinsic or acquired resistance to vemurafenib. Inhibition of both EGF receptor and AKT sensitized treatment-resistant BRAF mutant melanoma cultures to vemurafenib. Melanomas with RAS mutations were more resistant to combination therapies relative to BRAF mutants, but were sensitive to combinations of statins and cyclin-dependent kinase inhibitors in vitro and in vivo. These results show the use of combinatorial drug screening for discovering unique treatment regimens that overcome resistance phenotypes of mutant BRAF- and RAS-driven melanomas.

SIGNIFICANCE: We have used drug combinatorial screening to identify effective combinations for mutant BRAF melanomas, including those resistant to vemurafenib, and mutant RAS melanomas that are resistant to many therapies. Mechanisms governing the interactions of the drug combinations are proposed, and in vivo xenografts show the enhanced benefit and tolerability of a mutant RAS-selective combination, which is currently lacking in the clinic. Cancer Discov; 3(1); 52–67. ©2012 AACR.


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INTRODUCTION

Inhibition of oncogenic drivers by targeted agents improves patient response rates (1–3), but primary and secondary resistance to these drugs is common (4–6). In melanoma, about half of patients harbor activating mutations in the BRAF oncogene (7). Most of these patients respond to the mutant BRAF inhibitor vemurafenib (PLX4032); however, some patients have primary resistance to therapy (8, 9). Acquired resistance occurs over a period of months in nearly all patients who do respond (8, 9). A large number of resistance mechanisms have been identified (10–18), and combinatorial therapies with vemurafenib will be needed to prevent disease progression and improve survival of patients.

Another roadblock to progress in melanoma therapeutics is the lack of effective therapies for mutant RAS-driven melanomas, which are found in up to one fifth of patients with melanoma (7, 19). RAS mutations in general (NRAS, KRAS, and HRAS) occur in up to a third of all human cancers (20) and are associated with relatively poorer prognoses in many cancer types, including lung and melanoma (21, 22). Furthermore, activating mutations in NRAS are one mechanism for secondary (acquired) vemurafenib resistance in a subset of mutant BRAF melanomas (14). Thus, an urgent need has arisen to identify effective drug combinations that attack mutant RAS-driven tumors and prevent treatment resistance.

To date, no reports have described any systematic method for identifying the most effective drug combinations for either mutant RAS or BRAF melanomas. Therefore, we have used a combinatorial high-throughput drug screening (cHTS) approach to evaluate the selectivity of drugs, alone and in pairs, in the context of BRAF- or RAS-activating mutations, using early-passage melanoma cultures.

We identify several unique cytotoxic combinations with agents commonly used in the clinic that have pronounced genotype selectivity for RAS mutant or BRAF mutant melanomas, including those with primary and acquired resistance to vemurafenib. Furthermore, we confirm the effectiveness of select combinations in vitro and in vivo and provide mechanistic reasoning for their cooperation. The results show that combinatorial drug screening illuminates positive drug interactions in melanoma otherwise obscured with single-agent screening alone, and that these findings may guide the implementation of new combinatorial drug trials.

RESULTS

Evaluation of Melanoma Responses to Single Agents

To address the impact of genotype on response to a range of anticancer agents, we curated a panel of 150 small-molecule compounds, including traditional chemotherapies and targeted agents (Supplementary Table S1), and tested the concentration-response behaviors in early-passage patient-derived melanomas characterized for activating mutations in
BRAF, RAS, or those wild-type for both (hereafter, WT; Supplementary Table S2). Concentration–response curves generally exhibited sigmoidal or exponential behavior and many agents elicited incomplete growth inhibition (Supplementary Fig. S1A), suggesting that additional agents are needed for full growth inhibition. Concentrations required to inhibit 50% of cell growth (GI50) were variable among cell lines (Supplementary Table S3). Compounds arrayed in duplicate on different plates yielded similar concentration-dependent responses (Supplementary Fig. S1B), as did compounds in related pharmacologic classes (Supplementary Fig. S1C).

Unsupervised clustering of single-agent efficacies (maximal growth inhibition achieved) was conducted to identify agents that selectively inhibit growth of genotypic subsets. Drugs were partitioned into 3 clusters, including agents lacking effect (Cluster 1), agents with uniform high efficacy (Cluster 2), and agents with variable efficacies across cell lines (Cluster 3). Reclustering of drugs showing variable efficacies confirmed mutant BRAF selectivity of vemurafenib, PLX4720, and GDC-0879 as well as clustering of other agents belonging to the same specific classes, including ErbB, SRC/ABL, and mitogen-activated protein kinase (MEK) inhibitors (Fig. 1B). In agreement with previous studies, growth-stimulatory effects of vemurafenib were seen in some mutant RAS and WT melanomas, and incomplete growth inhibition was observed for all mutant BRAF lines at concentrations up to 10 μmol/L vemurafenib (Fig. 1C; refs. 23, 24). Approximately 25% of mutant BRAF cultures were innately resistant to vemurafenib, showing a GI50 beyond 3 μmol/L (Fig. 1D), as observed elsewhere (25). It has been suggested that PTEN deficiency may hinder vemurafenib-induced apoptosis (16), whereas co-occurring PTEN and RB1 deficiency may attenuate growth inhibition by vemurafenib (18). Although we did not evaluate the contribution of apoptosis to growth inhibition in the limited number of lines deficient in PTEN (YUGEN8, YUMUT, and YUKADI) or RB1 (YUSAC2), we observed growth inhibition resistance to vemurafenib in the setting of normal PTEN and RB1 expression (Fig. 1E; Supplementary Table S2), suggesting that loss of either or both is not essential for primary vemurafenib resistance. Indeed, others have observed that primary resistance to vemurafenib can be altered by metastatic mechanisms, including increased MET/hepatocyte growth factor receptor (HGFGR) signaling (26, 27).

We found that other drugs eliciting selectively higher growth inhibition in mutant BRAF melanomas included the SRC/ABL inhibitor bosutinib, the fibroblast growth factor receptor (FGFR) inhibitor dovitinib and the EGF receptor (EGFR) inhibitor gefitinib (Supplementary Fig. S1D). No drugs were selective exclusively for the WT melanomas, likely owing to the greater genetic heterogeneity of this group. MEK inhibitors U0126 and CIP-1374 were selective as single agents for both the mutant BRAF and RAS groups (Supplementary Fig. S1D), but were more effective for mutant BRAF lines. Lines with primary resistance to vemurafenib were also less sensitive to MEK blockade (Fig. 1B), in agreement with other reports (28). Interestingly, the 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoA reductase) inhibitor simvastatin, which indirectly interferes with posttranslational processing of RAS proteins (29), was the only drug to show a trend toward a higher degree of growth inhibition in the mutant RAS group (Fig. 1F). Collectively, these data reveal a limited number of single agents with genotype-selective efficacy (Supplementary Table S4). Moreover, unless used at high concentrations (10 μmol/L or greater), these single agents inhibited cell growth incompletely, which likely reflects that these agents are incompletely cytotoxic at lower concentrations.

Combinatorial Drug Screening

The limited responses to single agents prompted us to determine whether more defined genotype-selective patterns and higher efficacies could be observed using cHTS. Forty representative agents with high or variable efficacies (Fig. 1A, Clusters 2 and 3) were chosen for cHTS (Supplementary Table S5). As a group, these agents inhibit many of the signaling pathways important in cancer (Supplementary Fig. S2A). As melanoma lines varied in their sensitivities to each agent, we chose 3 consensus concentrations based on corrected median values for the GI50, GI25, and GI10 effect levels across all melanoma lines (Supplementary Fig. S2B), together giving 9 pairwise combinations per drug pair. Nineteen melanoma lines, including 8 mutant BRAF, 6 mutant RAS (5 NRAS mutants and 1 HRAS mutant), and 5 WT lines, were screened against more than 7,000 pairwise combinations in parallel with single-agent controls.

Relationships between genotype and drug combination efficacy were first assessed by unsupervised clustering of the most effective combination of the 9 tested for each drug pair per cell line (Fig. 2A). Two clusters are apparent, with one dominated by mutant BRAF melanomas that are sensitive to a substantial number of combinations, and the other dominated by mutant RAS and WT melanomas, which were generally less sensitive to the combinations. To ascertain genotype-selective combinations, we filtered data for drug pairings that yielded an average 15% or greater growth inhibition exclusively in a genotypic group. Mutant BRAF-selective drug pairs greatly outnumbered drug pairs selective for mutant RAS or WT groups (1,021; 75; and 22 combinations, respectively). Agents most frequently paired in combinations selective for the mutant BRAF group (“sensitizers”) included the FGFR inhibitor PD173074, the pan-BCL2 family inhibitor obatoclax, vemurafenib, bosutinib, the c-MET inhibitor Pha665752, the ErbB inhibitor lapatinib, and the AKT inhibitor MK-2206 (Fig. 2B). All of these agents were selective for BRAF mutant cultures at multiple combination concentrations (Supplementary Table S6), reinforcing their cooperative effect. Vemurafenib was not the most frequent sensitizer, possibly because of the lower concentrations used in combination screening (Supplementary Table S5) as well as the presence of 2 innately vemurafenib-resistant lines used in the screens (YUKSI and 501Mel, Fig. 1D). At the concentrations tested in cHTS, none of these sensitizers were exclusively selective for mutant BRAF melanomas in single-agent screening.

Strikingly, 52% of mutant RAS-selective drug combinations involved simvastatin (Fig. 2C), the only agent with a lower median GI50 in the mutant RAS group in single-agent screening (Fig. 1F). Despite the low number of mutant RAS-selective combinations, 25 other drugs paired with simvastatin showed greater effectiveness in the mutant RAS group, whereas only 4 drugs in combination with simvastatin were more effective in mutant BRAF lines, and were all general...
sensitizers of this group (Supplementary Table S7). Sixty-two of 64 combinations that elicited more than 50% inhibition in the mutant RAS group included simvastatin (Supplementary Table S8). Agents that combined with simvastatin at different concentrations selective for the mutant RAS group included the X-linked inhibitor of apoptosis protein (XIAP) inhibitor embelin, the novel allosteric MEK inhibitor CIP-1374, and the X-linked inhibitor of apoptosis protein (XIAP) inhibitor CIP-1374. None of these agents showed greater potency exclusively in the mutant RAS group with single-agent screening (Fig. 1B; Supplementary Table S4).

Finally, no drug combination partners were selective for the WT group of melanomas. Some combinations were effective (≥GI50) for all genotypic groups, although many included compounds that have not been used in the clinic, or were tested at higher concentrations of clinically tested agents shown to be genotype selective in combinations. Supplementary Table S8 collectively lists the genotype-selective and -nonselective combinations.

**Synergistic and Cytotoxic Drug Interactions in Mutant BRAF and RAS Melanomas**

Drug combinations were analyzed for additive, synergistic, or antagonistic interactions. Single-agent effects assessed in cHTS were plotted in relation to theoretical combinatorial effects using the Bliss independence model (30) and to their measured effects for each of the 9 concentration combinations per drug pair, depicted globally as drug “interaction signatures” (Fig. 3A). The majority of mutant BRAF melanomas shared a pattern of extensive synergies, whereas combinations in mutant RAS lines were more often antagonistic (i.e., less effective than predicted additivity; Fig. 3B). To identify genotype-selective synergistic drug pairs, we conducted unsupervised clustering of effect data from each of 36094 drug–drug combinations. The majority of mutant BRAF melanomas showed greater potency exclusively in the mutant RAS group with single-agent screening (Fig. 1B; Supplementary Table S4).

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clustering of average synergy values obtained from the 9 concentration combinations for each drug pair. Drugs that frequently appeared in combinations showing significantly higher synergies included lapatinib, vemurafenib, bosutinib, nilotinib, and PD173074 (Fig. 3C), and were usually observed in BRAF mutant melanomas. In contrast, these combinations were often antagonistic in mutant RAS melanomas (Fig. 3D).

Lapatinib was the most frequent synergizing partner showing specificity for mutant BRAF lines (Fig. 3E).

A significant number of patients with BRAF-mutated melanoma do not respond to vemurafenib (9). YUKSI and 501Mel cell lines harbor activating BRAF mutations but show primary resistance to vemurafenib (Fig. 1D). These lines also showed higher relative resistance against many drugs paired with vemurafenib, even at the highest concentrations tested (Fig. 3F). Nevertheless, both resistant lines were more sensitive to the same set of combinations that were effective for vemurafenib-sensitive melanomas, including vemurafenib paired with bosutinib, lapatinib, obatoclax, PD173074, or Pha665752 (Fig. 3F, highlighted area). Combinations that overlapped in our queries for highest average synergy and average efficacy specific to mutant BRAF lines often included lapatinib and MK-2206 (summarized in Table 1). This finding suggests a shared mechanistic susceptibility in BRAF mutants to inhibition of the targets of these agents, regardless of vemurafenib sensitivity. MK-2206 and lapatinib were not found to be selective for mutant BRAF melanomas in single-agent screens, indicating that the genotype selectivity of these agents in combination, as based on BRAF or RAS mutation status, was not predictable by single-drug screening alone. Combinations most selective for RAS-mutated melanomas were mainly additive and included simvastatin with the pan-CDK inhibitor flavopiridol or the HSP90 inhibitor 17-DMAG.

We next confirmed cytotoxic and synergistic properties of select drug pairs that overlapped in our analyses for highest average synergy and efficacy (Table 1). For mutant BRAF-specific combinations, we chose the AKT inhibitor MK-2206 paired with lapatinib or bosutinib (Fig. 3G; Supplementary Fig. S3A and S3D). For mutant RAS-specific combinations, we chose simvastatin with flavopiridol or with 17-DMAG (Fig. 3H; Supplementary Fig. S3B and S3E). Finally, vorinostat with flavopiridol was selected as a genotype-independent combination (Supplementary Fig. S3C). Isobologram analyses (31).
Figure 3. Efficacious drug combinations for RAS mutant and vemurafenib-resistant BRAF mutant melanomas. A, drug interaction signatures for each cell line representing all combinatorial data compiled in a 40 by 40 drug matrix. Insert at bottom: magnified view of the 9 concentration combinations tested for each drug pair (rows) for each melanoma line (columns). Inset at bottom: magnified view of the 9 concentration combinations for a representative drug pair within its drug interaction matrix, to indicate scale. Yellow bar is the Bliss independent sum of growth inhibition for single agents; red indicates synergy, and green, antagonism (see also Supplementary Fig. S3A–S3C). B, compilation of the total number of drug pair synergies, additivities, and antagonisms. P values calculated by χ² test. P < 0.05 for BRAF versus RAS for number of synergies and RAS versus BRAF or WT for number of antagonisms. C, unsupervised hierarchical clustering of average synergy values obtained from the 9 concentration combinations tested for each drug pair (rows) for each melanoma line (columns), and D, average antagonisms of the 9 combinations. Only drug pairs showing significant differences are shown. E, frequencies of drugs appearing in mutant BRAF-selective combinations yielding significantly higher relative average synergies. Lapatinib showed the largest number of synergies specific to mutant BRAF melanomas. F, percent growth inhibition of mutant BRAF lines treated with vemurafenib combined with other agents at maximal concentrations tested (see Supplementary Table S5). YUKSI (red), the line most intrinsically resistant to vemurafenib, was also less sensitive to these combinations. The second-most vemurafenib-resistant line 501Mel (black) used in cHTS was also less sensitive to many combinations. The most vemurafenib-sensitive line YULAC (green) was most sensitive to combinations with vemurafenib in almost all cases. Yellow highlight marks vemurafenib combinations that showed increased growth inhibition in the vemurafenib-resistant lines. G, drug combinations excluding vemurafenib, which showed highest selectivity toward mutant BRAF melanomas, were with lapatinib and the AKT inhibitor MK-2206. YUKSI and 501Mel were also sensitive to these combina- tions. Bars indicate means. Indicated P values calculated by Kruskal–Wallis ANOVA. Post hoc Fisher’s test showed P < 0.05 for the mutant BRAF group versus the other groups. H, drug combinations with the highest efficacy and selectivity toward RAS mutant melanomas included simvastatin with flavopiridol.
Table 1. Summary of drug pairs overlapping for highest efficacy and synergy

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<tr>
<th>Drug 1</th>
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<td>0.67</td>
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NOTE: Drug combinations overlapping for highest average synergy and effectiveness (GI80) with respect to a specific genotypic group (at least 15% higher growth inhibition than in the other 2 groups) or those without genotype selectivity. Average efficacy (percent as decimal) and average synergy values (percent as decimal over predicted Bliss additivity) of the 9 concentration combinations are shown for each drug pair. Drug pairs chosen for confirmation of cytotoxicity are bold-faced.

using a broader range of concentrations than those in cHTS confirmed the overall synergistic nature of these drug interactions, as determined by combination index calculations (Fig. 4A; Supplementary Table S9). Laptininib or bosutinib in combination with MK-2206 was highly synergistic on the representative mutant BRAF line YUMAC. Simvastatin with flavopiridol or 17-DMAG was also additive on the mutant NRAS line YUGASP at high concentrations, but synergistic at concentrations well below the range used in cHTS.

Laptininib and MK-2206 individually induced approximately 20% cell death each (normalized to vehicle control) by 3 days in the BRAF mutant line YUMAC (Fig. 4B and C). The combination of these 2 drugs at the same concentrations increased cell death by 50% (87% actual, 36% Bliss model prediction), consistent with their synergy seen in cHTS and isobologram analyses. These agents alone or in combination lacked appreciable cytotoxicity in the mutant NRAS line YUGASP. Conversely, simvastatin paired with flavopiridol was more cytotoxic in YUGASP (80% actual, 63% predicted), compared with the BRAF mutant line YUMAC (36% actual, 35% predicted). The combination of simvastatin with 17-DMAG, or bosutinib with MK-2206, similarly resulted in genotype-selective cytotoxicity in the mutant RAS and BRAF lines, respectively (Fig. 4C; Supplementary Fig. S3F). The genotype-unbiased combination of vorinostat and flavopiridol was also cytotoxic to both RAS and BRAF lines at high concentrations.
Figure 4. Confirmation of synergy and cytotoxicity of genotype-selective drug pairs. A, formal assessment of synergy of lapatinib or bosutinib with MK-2206, and simvastatin with flavopiridol or 17-DMAG on representative BRAF mutant and RAS mutant melanomas, by Chou–Talalay isobologram analysis. Data are normalized, with connecting line at X and Y = 1 corresponding to the line of additivity. Datapoints falling below line are synergistic, along or near the line are additive, and above the line are antagonistic (see key). Data represent averages for 3 separate experiments. Combination indices and growth inhibition values can be found in Supplementary Table S9. B, flow cytometry of Annexin-V and propidium iodide viability markers after lapatinib (2.5 μmol/L) and MK-2206 (5.0 μmol/L) treatment alone or in combination, or simvastatin (2.5 μmol/L) and flavopiridol (0.1 μmol/L) alone or in combination with representative lines YUMAC (BRAF mutant) or YUGASP (NRAS mutant). Numbers in the lower right quadrant correspond to early apoptotic cells, whereas numbers in the top right and left quadrants correspond to late apoptotic and necrotic cells, respectively. Flow cytometry for vehicle-only controls and other combinations are shown in Supplementary Fig. S3F. C, percent viability for combinations tested by flow cytometry, as in B. N = 3 for all experiments. Error bars, mean ± SD. P values calculated by Student t test; *, P < 0.05. D, evaluation of drug-class effects. Representative concentration-response curves of single agents or dual-agent combinations showing enhanced growth inhibition in mutant BRAF cells, using the EGFR inhibitor gefitinib with MK-2206 (top row) or atorvastatin with flavopiridol in mutant RAS cells (bottom row) in relation to the predicted Bliss independence model. See also Supplementary Fig. S4. For statin combinations with flavopiridol, top and bottom x-axes represent concentrations used for each drug with those same concentrations used in combinations. Same-concentration combinations were used for gefitinib and MK-2206.
Most target classes were represented by only one compound in eHTS, so we verified that the genotype-selective effects produced by these combinations were related to their target classes. The EGFR inhibitors gefitinib or afatinib/BIBW2992 paired with the AKT inhibitors MK-2206 or GSK692094 produced similar combination responses to lapatinib and MK-2206, and were more effective in mutant BRAF lines, including the line most intrinsically resistant to vemurafenib (YUKSI; Fig. 4D; Supplementary Fig. S4A). Likewise, the combination of statins, including lovastatin and atorvastatin, combined with the pan-CDK inhibitors flavopiridol and AT7519 produced similar responses that were somewhat effective in mutant BRAF lines, but more effective in NRAS mutant and HRAS mutant lines (Fig. 4D; Supplementary Fig. S4B).

Vemurafenib-Resistant Mutant BRAF Cells Regain Sensitization to Vemurafenib upon Concomitant Inhibition of EGFR and AKT

We further evaluated the efficacy of the lapatinib/MK-2206 combination with flow cytometry, clonogenic assays, and soft agar assays on mutant BRAF melanomas sensitive or resistant to vemurafenib, using repeated administration of lower concentrations of these agents to minimize off-target effects (see Methods). This treatment strategy was minimally effective for patient-derived lines with the greatest primary resistance to vemurafenib, including YUKSI and YUKOLI, as indicated by maintained cell viability and colony numbers with reduced colony size (Fig. 5A and B), but relatively more effective for the vemurafenib-sensitive lines, including YULAC (Supplementary Fig. S5A). Vemurafenib-resistant cell lines selected from YULAC and YUCOT lines (Supplementary Fig. S5B), hereafter, YULAC-R and YUCOT-R, respectively, were also less sensitive to this regimen (Fig. 5B; Supplementary Fig. S5C). At 500 nmol/L and above, lapatinib and MK-2206 effectively suppressed their targets p-EGFR and p-AKT, respectively (Fig. 5C), despite moderate impact on viability and clonogenicity of these agents singly and in combination.

We next assessed the interaction of vemurafenib with lapatinib or MK-2206. Dual combination of vemurafenib with either of these agents moderately increased efficacy relative to single-agent treatments in primary-resistant lines YUKSI and YUKOLI (Fig. 5D and E) and in acquired-resistant lines YULAC-R and YUCOT-R (Fig. 5E; Supplementary Fig. S5D and S5E). Vemurafenib and MK-2206 together were more effective in acquired-resistant lines; however, the triple combination of lapatinib, MK-2206, and vemurafenib greatly reduced p-ERK, and moderately reduced p-AKT inhibition with MK-2206, as all dual and triple combinations resulted in nearly complete loss of mutant NRAS activity and abolished colony growth in both primary and secondary resistance settings. Importantly, the dual and triple combinations did not induce substantial cytotoxicity in the mutant NRAS line YUGASP and only moderately reduced colony formation (Supplementary Fig. S5F).

Receptor tyrosine kinase (RTK) activation often contributes to vemurafenib resistance in mutant BRAF melanomas (13, 14, 26, 27). In primary vemurafenib-resistant YUKSI and YUKOLI BRAF mutant melanoma cells, inhibition of AKT with MK-2206 single-agent treatment effectively suppressed p-AKT but increased levels of p-EGFR and p-ERK by 24 hours (Fig. 5C and F; Supplementary Fig. S6A and S6B). Conversely, vemurafenib or lapatinib alone did not change p-AKT levels in these lines. In the triple combinations, the activity of MAPK and phosphoinositide 3-kinase (PI3K)/mTOR complex (mTORC) pathways was effectively suppressed, as suggested by a strong reduction in p-ERK, p-AKT, and p-P70S6K (Fig. 5F), although some rescue of p-ERK signal was observed. This rescue was likely due to cross-pathway activation of ERK upon p-AKT inhibition with MK-2206, as all dual and triple combinations with MK-2206 partially restored p-EGFR and p-ERK.

The impact of these agents on MAPK and PI3K signaling in acquired vemurafenib-resistant lines (YULAC-R and YUCOT-R) was similar to that in primary vemurafenib-resistant lines. Vemurafenib or MK-2206 alone depleted or partially restored p-ERK levels, respectively (Fig. 5G; Supplementary Fig. S6C). In contrast, single-agent lapatinib treatment in acquired vemurafenib-resistant lines resulted in initial suppression, but subsequent elevation of p-ERK and p-AKT levels relative to baseline (Fig. 5G), whereas it had little impact on p-AKT and p-ERK in primary vemurafenib-resistant lines. Nevertheless, the 3 combined agents effectively suppressed both p-AKT and p-ERK levels in the vemurafenib-sensitive (Supplementary Fig. S6D and S6E) and vemurafenib-resistant lines tested.

Finally, we assessed the single-, dual-, and triple-agent treatments in preventing the long-term emergence of resistant colonies (Fig. 5H). As expected, the parental line YULAC readily formed colonies with lapatinib or MK-2206 alone or combined (data not shown). Dual-agent combinations with vemurafenib performed more effectively; however, a lower number of resistant clones did emerge. Only the vemurafenib/lapatinib/MK-2206 triple combination was found to impede the emergence of resistant colonies completely.

Mechanistic and In Vivo Evaluation of the Mutant NRAS-Selective Simvastatin/Flavopiridol Combination

Statins interfere with isoprenyl RAS modifications required for plasma membrane localization and activity (32–34). Indeed, membrane localization of NRAS was nearly eliminated with simvastatin treatment (Fig. 6A), confirming an association between loss of membrane-anchored NRAS and induction of cytotoxicity. Still, a large number of other proteins, including cancer-relevant RAC and RHO proteins, are also isoprenylated and may be concomitantly affected by statins. We therefore carried out RNA interference (RNAi)-mediated knockdown experiments to compare the effects of NRAS reduction with statin-induced NRAS inhibition. NRAS siRNA treatments resulted in nearly complete loss of mutant NRAS protein, greatly reduced p-ERK, and moderately reduced p-AKT in mutant NRAS YUGASP cells (Fig. 6B). The combination of NRAS siRNA and flavopiridol treatment fully suppressed p-ERK levels and substantially increased BIM and PARP cleavage by 96 hours. This finding was associated with increased cytotoxicity relative to either treatment alone, as measured by flow cytometry (Fig. 6C). As with NRAS siRNA, repeated administration of lower concentration simvastatin reduced activity of the MAPK and PI3K pathways. This effect was enhanced in the presence of flavopiridol in NRAS mutant and HRAS mutant lines (Fig. 6D; Supplementary Fig. S7A and S7B), along with a significant reduction in viability and clonogenicity (Fig. 6E; Supplementary Fig. S7C and S7D). In contrast, cytotoxicity and clonogenic inhibition following...
Figure 5. Combined targeting of EGFR and AKT re-establishes vulnerability to vemurafenib. A, representative 2D clonogenic (i), soft agar (ii), and flow cytometry (iii) assays on the YUKSI primary vemurafenib-resistant mutant BRAF line, which also shows marked resistance to lapatinib and MK-2206 used alone or combined. D, DMSO vehicle; L, lapatinib (1.5 μmol/L); M, MK-2206 (1.5 μmol/L). B, reproduced experiments as described in A for the primary-resistant lines YUKSI and YUKOLI, as well as acquired resistant line YULAC-R. Normalized clonogenic fraction refers to 2D clonogenic and soft agar assays, whereas normalized survival fraction refers to flow cytometry analyses. Lapatinib and MK-2206 both used at 1.5 μmol/L. C, immunoblotting to assess target engagement of EGFR and AKT by lapatinib and MK-2206, alone or combined. Increase of p-EGFR can be seen upon AKT inhibition by MK-2206. D, 2D clonogenic (i), soft agar (ii), and flow cytometry (iii) assays on the YUKSI line showing enhanced susceptibility to vemurafenib upon treatment with lapatinib and MK-2206 combination at 1.5 μmol/L each. V, vemurafenib (10 μmol/L); other designations same as in A. E, reproduced experiments as in D for the vemurafenib-resistant lines YUKSI, YUKOLI, and YULAC-R. F, Molecular consequences of vemurafenib, lapatinib, and MK-2206 as single agents or as dual- or triple-agent combinations for downstream activity indicators of MAPK and PI3K pathways, including p-ERK and p-AKT. Primary vemurafenib-resistant YUKSI cells were treated with drugs for 24 hours before protein extraction. Concentrations of drugs in μmol/L are shown in parentheses after drug letter designations. G, p-ERK and p-AKT activity in the in vitro–selected vemurafenib-resistant YULAC-R line after 1- or 24-hour drug treatments. High levels of cleaved PARP could be detected clearly by 24 hours in the triple-agent combination in association with complete loss of p-ERK and p-AKT. F, full-length PARP; C, cleaved PARP. H, long-term 2D clonogenic assay on the parental YULAC line that was sensitive to vemurafenib. Cells were plated at 2 × 10³ cells per well and treated every other day with fresh drug for 2 weeks, followed by 4 weeks of recovery. Only the triple-agent combination completely prevented the emergence of colonies.
Simvastatin sensitizes mutant NRAS cells to flavopiridol in vitro and in vivo. A, immunofluorescence on YUGASP mutant NRAS cells showing depletion of membrane-associated NRAS after simvastatin treatment at 5 μmol/L for 24 hours, or 1 μmol/L for 72 hours, with fresh drug added daily. B, NRAS knockdown by siRNA nearly depletes p-ERK protein and attenuates active AKT. Addition of flavopiridol at 0.1 μmol/L during the final 48 hours of siRNA treatment completely abrogated p-ERK and resulted in increased levels of proapoptotic BIM and cleaved PARP relative to siRNA or flavopiridol treatment alone. EL, L, and S mark the 3 BIM isoforms; F, full-length PARP; C, cleaved PARP. C, flow cytometry for viability confirmation in YUGASP mutant NRAS cells after NRAS knockdown with or without flavopiridol (FLAV) treatment at 0.1 μmol/L. SCR, scrambled siRNA control. (continued on following page)

treatment with these agents was not remarkable in mutant BRAF lines resistant to vemurafenib (Supplementary Fig. S7E and S7F). Collectively, these data show enhanced effects of statins combined with CDK inhibitors in NRAS mutant or HRAS mutant melanomas that may, in part, be mediated by reduction of NRAS activity.

Finally, we tested the impact of the simvastatin/flavopiridol combination in reducing tumor growth, using a preclinical xenograft model. YUGASP cells were injected subcutaneously into immunocompromised mice, and a dose-escalation study was conducted to ensure tolerability up to predefined maximum doses of the 2 drugs combined. No toxicity was observed with single agents or dual agents in the ranges of doses tested (data not shown). Simvastatin and flavopiridol modestly reduced tumor growth as single agents, with flavopiridol having a greater effect (Fig. 6F and G). The combination of simvastatin and flavopiridol significantly reduced tumor growth and resulted in initial tumor regression within the first week of treatment. The combination was well tolerated (Supplementary Fig. S7G), and histologic assessment at the 2-week mark indicated a trend toward increased cell death, as indicated by pyknotic cell index and reduced mitotic index in the combination treatment group relative to the single-agent and mock treatment arms (Supplementary Fig. S7H).

**DISCUSSION**

Resistance to therapies in cancer is a major clinical hurdle, creating a compelling need to discover more effective combinations of agents that are currently available. We present results from a drug combinatorial screen designed to probe positive drug interactions in mutation-defined subgroups of cancer. This screen has identified previously undescribed drug interaction patterns and several combinations with potential for high efficacy in melanoma (Table 1). Among these, statins with CDK inhibitors were validated as selective for NRAS and HRAS mutants, and a triple combination consisting of vemurafenib, EGFR, and AKT inhibitors was selective for BRAF mutant lines, including those with primary or in vitro–selected resistance to vemurafenib.
In patient-derived BRAF mutant melanomas with vemurafenib resistance, the EGFR inhibitors gefitinib, lapatinib, and afatinib were minimally cytotoxic when used alone, as were the AKT inhibitors MK-2206 and GSK692094, but more effective when combined at high concentrations than at lower concentrations. Yet, combinations of lapatinib and MK-2206 at reduced concentrations still effectively sensitized resistant cells to vemurafenib, giving credence to the idea that the major targets of these agents are critical mediators of vemurafenib resistance.

Cross-pathway feedback control between the MAPK and PI3K pathways is an important feature of many cancers (35, 36). In all vemurafenib-sensitive or -resistant BRAF mutant lines tested, we found an increase in ERK activity upon AKT inhibition with MK-2206. These effects were robust enough in primary vemurafenib-resistant lines to partially rescue p-ERK levels in spite of vemurafenib treatment. Thus, the consequences of MK-2206 treatment are likely 2-pronged: suppression of the survival role of the AKT pathway and alteration of feedback inhibition resulting in increased MAPK signaling, potentially through receptor kinases.

Melanomas with primary vemurafenib resistance were found to have higher baseline EGFR levels, similar to those in colorectal cancers insensitive to this agent (37, 38). In contrast to these studies, we found that combined EGFR and mutant BRAF inhibition was not effective for primary vemurafenib-resistant melanoma cells, or in preventing the emergence of resistant clones in vemurafenib-sensitive cells. Previous work in breast cancer showed upregulation of multiple RTKs, including EGFR, upon AKT inhibition with MK-2206 (36). Thus, it is plausible that primary-resistant melanomas will require AKT inhibition as a prerequisite for enhancing reliance upon MAPK signaling through RTKs and mutant BRAF. In addition, the partial reduction in p-ERK with EGFR inhibition seen in these cells may increase sensitivity to further MAPK inhibition by vemurafenib treatment. More generally, this idea suggests that combinations of specific RTK inhibitors with AKT inhibitors could likewise resensitize acquired vemurafenib-resistant lines to vemurafenib, and indeed, we found
that the triple-agent combination was superior in these cases. Moreover, this regimen also blocked the long-term development of resistant clones in parental vemurafenib-sensitive cells, suggesting a route for prevention of vemurafenib resistance, which otherwise develops over a period of months (8, 9).

The effects of the vemurafenib/lapatinib/MK-2206 combination were minor on mutant NRAS cells, reaffirming its selectivity for lines with BRAF mutations. Generally, RAS mutant melanomas were resistant to most single-agent and dual-agent treatments in comparison with mutant BRAF melanomas and melanomas with WT BRAF and RAS, which is consistent with the slightly poorer clinical prognosis associated with NRAS mutation (22). Many drug combinations that were synergistic in mutant BRAF cells tested in cHTS, including RTK inhibitor combinations, were more often antagonistic in mutant RAS cells. This finding likely reflects the greater pleiotropy of RAS-dependent signaling, as RAS activates a variety of effector pathways, including MAPK, JAK-STAT, RAL-GDS, and PI3K signaling (20, 34).

Mutant RAS-selective combinations detected in cHTS often involved the HMG-CoA reductase simvastatin. Clinical studies have not supported use of statins as cancer monotherapies (39), and epidemiologic studies have not conclusively substantiated reduced cancer risk in individuals chronically treated with statins at the somewhat lower concentrations used for hypercholesterolemia control (39, 40). Still, it remains to be seen if statins are more functional for the specific prevention or treatment of RAS mutant cancers.

Here, we find that the impact of statins on NRAS mutant melanomas may be mediated through direct interference with the function of NRAS, as we confirmed that it is relocalized from the plasma membrane under these conditions, an important component of RAS signaling (41). Moreover, NRAS knockdown yielded biologic phenotypes similar to those with statin treatment, both alone and in combination with flavopiridol. Nonetheless, NRAS knockdown was more effective in suppression of p-ERK than was simvastatin; and it is possible that the myriad proteins reliant on isoprenoid modifications—including additional GTP-binding proteins important in cancer, such as RAC and RHO—and more global effects on lipid metabolism contribute to these differences.

Concentrations of statins used in combinations to elicit complete cytotoxicity were by themselves only partially cytotoxic. Moreover, tumor growth inhibition with simvastatin alone in preclinical xenografts was not remarkable. RNAi-mediated knockdown of oncogenic NRAS elicited only partial cytotoxicity, similar to results seen with KRAS mutant cancers and mutant NRAS melanomas with or without activating BRAF mutations (14, 42–44). However, cHTS identified multiple second agents combined with simvastatin that were more effective in mutant RAS melanomas. Thus, these data show that loss of mutant NRAS, whether induced by small-molecule agents or by synthetic oligonucleotides, is mainly a critical priming event for cytotoxicity induced by a second agent such as an HSP90, MEK, or CDK inhibitor.

The pan-CDK inhibitor flavopiridol alone was not selective for mutant RAS melanomas, so this drug in combination with statins presumably works to forestall residual cell-cycle activation through RAS-dependent pathways or through pathways acting in parallel (45). In support of this idea, synthetic lethality has been shown elsewhere with combinatorial knockdown of mutant KRAS and CDK4 in non–small-cell lung cancer mouse models (46). One phase II trial of flavopiridol as monotherapy in patients with stage IV melanoma resulted in stable disease in approximately half of patients, for up to half a year or more (47). Candidate oncogenic drivers were not evaluated, and so the efficacy of CDK inhibition in conjunction with oncogenic driver blockade, such as mutant RAS, remains unknown in humans. Our in vivo mouse studies show the tolerability and enhanced efficacy of flavopiridol in combination with simvastatin for mutant NRAS melanoma. This combination may also prove superior for preventing or treating vemurafenib-resistant BRAF V600* melanomas that acquire de novo mutations in RAS (14, 48).

Over the past 2 decades, the discovery of novel targeted agents that inhibit the oncogenic drivers of cancers has paved the way for more favorable patient outcomes and, importantly, more tolerable therapies. However, even the most precisely targeted therapies when used alone are limited in their ability to promote cytotoxicity in some cancer cells (30). Cancers associated with poor prognoses, such as advanced-stage melanomas, will require combination therapies to obstruct the outgrowth of resistant cells (49). Combinatorial drug screening has allowed for the discovery and experimental confirmation of a number of effective combination regimens that in the correct genotypic setting may prove more effective and tolerable in patients while avoiding selection for resistance through inadequate dosing.

METHODS
Melanoma Cell Cultures
Patient-derived melanoma lines, with the exception of 501Mell, were collected as previously described (24). Cell lines were derived directly from human melanoma metastases using a Yale Institutional Review Board–approved protocol with informed consent. Tumor lines were further confirmed by expression profiling and Sanger sequencing for BRAF and NRAS mutational status (Supplementary Table S2) and compared with the independent clinical genetic evaluation of the pathologic resection specimens in many cases. These studies served to validate that the short-term cultures expressed melanoma markers and were of the correct designated genotype. Cell lines were cultured in basal medium [OptiMEM (Invitrogen) supplemented with 10% FBS and 1% penicillin/streptomycin (P/S)] and maintained in a 37°C incubator maintained at 5% CO2. Acquired vemurafenib-resistant lines (YUCOT-R and YULAC-R) were generated by exposing parental lines to 3 μmol/L vemurafenib every 2 days for approximately 10 to 12 weeks.

Single- and Dual-Agent Screening
Cells were deposited into 384-well microtiter plates at 750 cells per well using a multidrop dispenser (Thermo) in 20 μL basal medium. Drug stock plates for 1,000× single agents were created by serial 1:2 dilution, typically from 10 mmol/L (Supplementary Table S1), using an expandable multichannel pipette (MatrixTechCorp). A PlateMate Plus automated instrument (MatrixTechCorp) was used for pin transfer of 20 μL drug volume from drug stock plates into 384-well microtiter cell plates. A total of 0.1% and 20% DMSO was used as negative and positive controls, respectively. For dual-agent screens, 96–deep well plates were used for single-agent concentration stocks, and pairwise combinations of drugs were generated...
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using hit-picking automation (Freedom EVO, Tecan). Each of 780 unique drug pairs at 9 concentration combinations was generated spanning twenty-two 384-well microtiter stock plates. All experiments were carried out in triplicate at the Yale Center for Molecular Discovery (YCMD, New Haven, CT). Cells were exposed to drug for 72 hours, followed by growth inhibition measurement with the CellTiter-Glo ATP detection assay (Promega) based on amenability for HTS (50). Only experiments with high Z-factor quality indices (>0.5) were analyzed.

Statistical Analyses

Data were compiled into a relational database using PostgreSQL RDBMS (PostgreSQL.org). Statistics were computed with built-in functions of PostgreSQL or with R (R-project; http://www.r-project.org).

Flow Cytometry

Cells were plated at 1 x 10^5 cells per well in 6-well format and allowed to adhere overnight. Lifted cells were combined with adherent cells harvested by trypsinization. Repeated administration of agents was conducted every other day for 3 total treatments using fresh medium and drugs, and analyzed by flow cytometry the following day. Cells were stained using the BD Pharmingen Apoptosis Detection Kit II according to the manufacturer’s protocol (BD). Samples were analyzed with the BD LSRII flow cytometer, with recording of at least 10,000 events per sample. Cells were analyzed by doublet discrimination, and compensation was applied for each experiment. Each line was treated independently, and gates were fixed based on negative control signals. Plots were generated using FlowJo 7.6.5.

Immunofluorescence

Cells were plated on 8-chamber culture slides (BD Falcon) at 1,000 cells per well and allowed to adhere overnight before drug treatments. Cells were fixed using 4% paraformaldehyde at room temperature for 15 minutes, followed by quenching with 100 mmol/L glycine and permeabilization with 0.1% Triton X-100/PBS, and blocked with 5% bovine serum albumin (BSA)/phosphate-buffered saline with Tween 20 (PBST) for 30 minutes at 37°C. The anti-NRAS antibody (Santa Cruz Biotechnology; clone F155), which recognizes both mutant and bovine serum albumin (BSA)/phosphate-buffered saline with Tween 20 (PBS) with 2% P/S (2x RPMI complete). The top layer consisted of 750 μL 0.6% agarose combined with 750 μL 2x RPMI complete medium containing 1 x 10^5 cells per well in 6-well plate format. Colony spheres were fixed and stained with 0.01% crystal violet [50 mL MeOH, 10 mL glacial acetic acid, 4 mL 0.5% crystal violet, 36 mL D H2O] for 2 hours at room temperature, followed by destaining with water. The 2D clonogenic plates were scanned with a VersaDoc Model 3000 imager (Bio-Rad) and Quantity One software. Soft agar plates were photographed. Both 2D and soft agar colonies were enumerated using ImageJ software version 1.46r.

Immunoblotting

Immunoblots were conducted with the following primary antibodies, all at 1:1,000: NRAS (cat. no. sc-31), GAPDH (sc-25778), and EGFR (sc-03; Santa Cruz Biotechnology); phospho-AKT (Ser473; cat. no. 4060), AKT (cat. no. 9272), phospho-p42/44 MAPK (Thr202/Tyr204; cat. no. 9106), p42/44 MAPK (cat. no. 4695), phospho-p70S6K (Thr421/Ser424; cat. no. 9204), p70S6K (cat. no. 9203), EGFR (cat. no. 9202), phospho-EGFR (Tyr1068; cat. no. 3777), BIM (cat. no. 2819), PARP (cat. no. 9542), β-actin (cat. no. 4970), PTEN (cat. no. 9559), and Rb1 (cat. no. 9309; Cell Signaling Technology); and ImmunoPure donkey anti-rabbit (cat. no. 31458) and goat anti-mouse (cat. no. 31432) horseradish peroxidase (HRP)-conjugated secondary antibodies (Thermo).

RNA Interference

The NRAS siRNA ON-TARGET plus SMARTpool (Dharmacon) was used for in vitro knockdown experiments. Ten microliters of FuGENE HD transfection reagent (Promega) was added to 190 μL of basal media without P/S, mixed by inversion, and allowed to equilibrate for 10 minutes. In parallel, 10 μL siRNA was added to 190 μL of basal media without P/S at 500 nmol/L and combined 1:1 with the transfection reagent/basal media mixture, resuspended, and added to cells at 30% to 50% confluence in 6-well plate format in 1,600 μL media (final siRNA concentration, 50 nmol/L). Fresh siRNA/transfection reagent mixture was added again 24 hours later, as described above, and cells were collected for protein or flow cytometry after 96-hour total exposure to siRNA. Flavopiridol (0.1 μmol/L) was added 48 hours before protein or cell collection, where indicated.

Xenografts

A total of 5 x 10^6 cells were resuspended in basal medium and injected subcutaneously into both flanks of 5- to 6-week-old NCr nude mice (Taconic). Tumors were grown until palpable at 200 mm^3 before treatments. Simvastatin was slowly dissolved in prewarmed PBS and delivered daily by oral gavage at 10 mg/kg. Flavopiridol was dissolved in prewarmed 0.1% saline solution and delivered intraperitoneally every other day. Tumors were measured by digital caliper every other day, and volume was estimated using the equation Volume = (width)^2 x length/2.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Authors’ Note

A recent article describes another approach to combination screening in melanoma (Roller et al., Mol Cancer Ther 2012;11:2505–15).

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