Personalized Preclinical Trials in BRAF Inhibitor-Resistant Patient-Derived Xenograft Models Identify Second-Line Combination Therapies

Clemens Krepler1, Min Xiao1, Katrin Sproesser1, Patricia A. Brafford1, Batool Shannan1, Marilda Beqiri1, Qin Liu1, Wei Xu2, Bradley Garman2, Katherine L. Nathanson2, Xiaowei Xu2, Giorgos C. Karakousis2, Gordon B. Mills3, Yiling Lu3, Tamer A. Ahmed4, Poulikos I. Poulikakos4, Giordano Caponigro5, Markus Boehm5, Malte Peters5, Lynn M. Schuchter2, Ashani T. Weeraratna1, and Meenhard Herlyn1

Abstract

Purpose: To test second-line personalized medicine combination therapies, based on genomic and proteomic data, in patient-derived xenograft (PDX) models.

Experimental Design: We established 12 PDXs from BRAF inhibitor–progressed melanoma patients. Following expansion, PDXs were analyzed using targeted sequencing and reverse-phase protein arrays. By using multi-arm preclinical trial designs, we identified efficacious precision medicine approaches.

Results: We identified alterations previously described as drivers of resistance: NRAS mutations in 3 PDXs, MAP2K1 (MEK1) mutations in 2, BRAF amplification in 4, and aberrant PTEN in 7. At the protein level, re-activation of phospho-MAPK predominated, with parallel activation of PI3K in a subset. Second-line efficacy of the pan-PI3K inhibitor BKM120 with either BRAF (encorafenib)/MEK (binimetinib) inhibitor combination or the ERK inhibitor VX-11e was confirmed in vivo. Amplification of MET was observed in 3 PDX models, a higher frequency than expected and a possible novel mechanism of resistance. Importantly, MET amplification alone did not predict sensitivity to the MET inhibitor capmatinib. In contrast, capmatinib as single agent resulted in significant but transient tumor regression in a PDX with resistance to BRAF/MEK combination therapy and high pMET. The triple combination capmatinib/encorafenib/binimetinib resulted in complete and sustained tumor regression in all animals.

Conclusions: Genomic and proteomic data integration identifies dual-core pathway inhibition as well as MET as combinatorial targets. These studies provide evidence for biomarker development to appropriately select personalized therapies of patients and avoid treatment failures. Clin Cancer Res; 22(7); 1592–602. ©2015 AACR. See related commentary by Hartsough and Aplin, p. 1550

Introduction

The treatment of advanced melanoma has been significantly improved in recent years, enabled by BRAF and MEK inhibitors as new standard therapies in melanomas, with BRAF-V600E/K mutations (1, 2) and immune checkpoint inhibitors showing remarkably durable responses in a subset of patients (3–5). Although a majority of patients treated with BRAF or BRAF/MEK inhibitors experience a robust initial response, the excitement about the therapeutic success is dampened by the relapse of most patients. This is due to the development of acquired (secondary) resistance mediated by multiple mechanisms (6–10). Therefore, rational second-line combination therapies are urgently needed, and we expect that these therapies require individualization to the spectrum of resistance mechanism of each patient (11). There is a lack of translational models to study precision medicine approaches to resistance mechanisms found in patients, although a range of preclinical mouse melanoma models, including patient-derived xenografts (PDX), are in use (12). PDXs have been successfully established for solid tumors including melanoma by implanting fresh tumor material from patients directly into immunodeficient mice (13). Success rates vary significantly between tumor types, yet melanoma is highly suited to this experimental approach possibly due to the fact that even a few melanoma cells are sufficient to establish a tumor in NOD.Cg-PkdcsclidIl2rgtm1Wjl/Szl (14) mice. Tumor grafts generated in this way, and used as “avatars”, can predict therapeutic responses in cancer patients (15). Melanoma PDXs recapitulate the tumor architecture and genotype of the patient tumor (16), and metastatic behavior of these PDXs correlates with clinical outcome in patients (17). In this study, we developed PDXs from a cohort of patients who became resistant to and progressed on BRAF inhibitors. Using genomic and proteomic analysis, we were able to identify targets and test combinations of compounds in clinical development. However, we had an added advantage in that we were able to test multiple combinations in parallel due to an in vivo expansion strategy. These “preclinical trials” allowed us to
Translational Relevance

Basket trials to assign patients into treatment arms according to targetable alterations are an important development in personalized cancer medicine. However, selecting the appropriate combinations for each patient can be challenging. Especially, in patients with acquired resistance to kinase inhibitors, intratreatment heterogeneity and concurrent mutations within the same lesion occur frequently. In this study, we used PDxS from targeted therapy–progressed patients to test personalized combinations based not only on genomic data, but validated by proteomic analysis. By using a multi-arm preclinical trial design, we were able to identify efficacious precision medicine approaches and highlight the importance of assessing pathway activation status at the protein level.

define effective double and triple combination therapies, leading to complete tumor regression in all tumors of one PDx model treated. This translational approach towards improving personalized medicine in melanoma highlights the potential use of MET inhibitor combination therapy in a defined subset of melanoma patients.

Methods

Patient samples and generation of PDxS

Biopsies from patients with a BRAF-V600E mutation who had progressed by RECIST on either vemurafenib or dabrafenib were included in this study. Tissue collection was approved by The Wistar Institute Institutional Review Board. Sterile tumor samples were placed in transport media (DMEM, Fungizone 0.1%, and 2 mL gentamicin 0.2%) on wet ice and processed within 24 hours under sterile conditions. Tumor tissue was finely minced using the cross blade technique, digested in collagenase IV for 20 minutes at 37°C with repeated trituration, followed by 2-minute incubation in trypsin. The tumor slurry was implanted with Matrigel (Corning Life Sciences) subcutaneously in NSG mice. When tumors reached a volume of 1,000 mm³ (determined by weekly caliper measurements using the formula W x W x 1/2), animals were sacrificed and tumors harvested. Tumor grafts were digested as above and either reimplanted within 24 hours or banked. All animal experiments were approved by The Wistar Institute Institutional Animal Care and Use Committee.

Targeted next-generation sequencing

PDx tumors were massively parallel DNA sequenced by Foundation Medicine (http://foundationone.com) for 315 cancer genes exons and 28 cancer gene introns for base pair change, insertions, deletions, copy number changes, and select fusions by next-generation sequencing (18). Copy number changes in genes known to be recurrently amplified in cancer were called as high-level (CN>8) and focal (CN>5) amplifications, nonfocal low-level (CN<8) amplifications, and homozygous deletions of genes known to be recurrently deleted in cancer.

Reverse-phase protein arrays

Frozen tumor tissue was ground in a mixer mill (Retsch) and lysed with 200-μL ice-cold lysis buffer [1% Triton X-100, 50 mmol/L HEPES pH 7.4, 150 mmol/L NaCl, 1.5 mmol/L MgCl₂, 1 mmol/L EGTA, 100 mmol/L NaF, 10 mmol/L Na pyrophosphate, 1 mmol/L Na₂VO₄, 10% glycerol, freshly added protease, and phosphatase cocktail tablets (Roche Applied Science Cat. #05056489001 and #0490637001)]. After two freeze-thaw cycles, samples were centrifuged at 13,000 rpm for 15 minutes at 4°C, and supernatants were collected. Protein concentration was determined by Bio-Rad protein assay (#500-0006). About 40-μL cell lysate (protein adjusted to 1–1.5 μg/μL) was mixed with 4× SDS sample buffer (40% glycerol, 8% SDS, 0.25 mol/L Tris_HCL pH 6.8, β-mercaptoethanol at 1/10 of volume without bromophenol added before use). The samples were then heated for 10 minutes at 100°C in a heat block and submitted for reverse-phase protein array (RPPA) processing. RPPA was performed by the MD Anderson Center RPPA core facility (Houston, TX) as previously described (19) and data reported as normalized log2. Several RPPA datasets were successfully merged using replicates-based normalization (RBN; ref. 20). Unsupervised hierarchical clustering was performed on RBN log2 median-centered protein values using Cluster 3.0 software (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm#crt). Results were visualized using TreeView software (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm#crt).

Compounds

PLX4720 200 ppm chemical additive diet was irradiated and heat-sealed (Research Diets) and fed to mice once tumors were established. PLX4720 was provided by Plexxikon. Encorafenib, binimetinib, VX-11e, capmatinib, and BKM120 were provided by Novartis. For in vivo oral gavage, compounds were suspended in 0.5% carboxymethylcellulose sodium (MC), 0.5% Tween 80 (encorafenib), 1% MC, 0.5% Tween 80 (binimetinib), 5% ethanol, 20% propylene glycol, 7.4% Tween 80 (VX-11e); 0.5% MC, 0.1% Tween 80 (capmatinib); 1% MC, 1% Tween 80 (BKM120) in water and dosed using feeding tubes (Instech Laboratories, Inc).

In vivo experiments

Human melanoma RPDX tumors were expanded in vivo using NSG mice prior to the therapy experiments. Pooled tumor chunks banked from early mouse passages were implanted into 50 NSG mice (1:10 expansion; refs. 3–5). These tumors were harvested when reaching the maximum volume allowed on the protocol (1,000 mm³), digested, and banked as live cells. The larger part of this stock was retained as a master bank, and the other part was implanted at a 1:5 ratio into NSG mice to use in the therapy experiments. The expansion phase was under continuous drug pressure with PLX4720 200 ppm chemical additive diet at approximately clinical plasma levels. The plasma levels of PLX4720 (103.7 μg/mL ± 3.2 after 7 days) were similar to steady-state levels in patients treated with vemurafenib 960 mg twice a day (130.6 μg/mL ± 71.78; ref. 21). When tumors have reached 200 mm³ per caliper measurement, animals were randomized into treatment groups followed by a 3-day washout phase. Tumor size was assessed twice weekly per caliper measurement. Mice were sacrificed after two weeks of treatment or when necessary for animal welfare. Dosing was prolonged when tumor control was achieved as indicated. Tumor tissue was conserved in formalin (for FFPE) and snap-frozen in
liquid N2 for protein extraction. Treatment groups were sacrificed 4 hours after last dose.

Immunohistochemical analysis
Tumor tissue was fixed in 10% formalin, dehydrated, and embedded in paraffin. The immunohistochemical staining procedure followed the manufacturer’s protocol (Vector RTU Vectastain Kit. Universal Elite ABC kit #PK-7200). Primary antibody (Ki67; Vector #VP-RM04, 1:500; cleaved caspase-3; Cell Signaling Technology #9664s, 1:300) was added to each section and incubated overnight at 4°C in a humidity chamber. The color visualization was Vector Impact DAB kit (SK-4105), followed by counterstaining with hematoxylin.

Western blot analysis
Protein extraction was performed as described for RPPA. Fifteen microgram of protein extracts was subjected to electrophoresis on 10% SDS-PAGE gels and transferred on nitrocellulose membranes in the Bio-Rad Trans-Blot Turbo Transfer System. The membranes were blocked with Odyssey Blocking Buffer (#927-40000, 1:1 diluted in TBS; LI-COR) for 1 hour at room temperature and incubated at 4°C overnight with the following primary antibodies: pMet #3129, pAKT (s473) #4060s, pAKT (Thr308) #13038P, pERK #4370s, pMEK #9121S, MEK #2352, RSK #8408, pRSK #9344 (all Cell Signaling Technology), and β-Actin (Sigma #A5441). All primary antibodies were diluted 1:1,000 in 5% BSA TBS-0.1% Tween 20 buffer except β-actin, which was diluted 1:10,000. After washing and incubating with secondary antibodies (Thermo Scientific #P135571, Thermo Scientific #P135518, diluted 1:10,000 in 1:4 Odyssey Blocking Buffer), the bands were visualized by the LI-COR Odyssey Infrared Imaging System.

Statistical analysis
Progression-free survival (PFS) and overall survival (OS) of the patient were calculated using the Kaplan–Meier method. For in vivo experiments, statistical significance was determined using the trends of mean tumor volume over time. Treatment groups were compared using linear mixed models, and a likelihood ratio testing nested model was used to examine whether trends were overall significantly different among groups. P < 0.05 was considered to be significant.

Results
Establishment of PDXs from BRAF inhibitor progressed patient samples
We collected 12 tumor samples from 10 melanoma patients postprogression on a BRAF inhibitor (Fig 1A). In one patient, tissue from the same lesion was collected twice at different time points, and in another patient, a bowel and a brain metastasis sample was collected. The distribution between male and female was 6:4, and the median age at biopsy was 64.5 years. All patients, except one with an unknown primary, had cutaneous primaries, and all had distant metastatic disease, from which the PDX models were established, with 7 biopsies from subcutaneous metastases, one each from the parotid gland and bowel and 3 from brain metastases (Supplementary Table S1). Two patients had surgical complete responses after excision of their progressing lesions, 5 had partial responses, and 3 patients had stable disease as best response to BRAF inhibitor therapy. The median PFS of all patients in this set was 39 weeks, with a wide SD of 7.3 weeks (Fig. 1B). Median OS was 97.57 weeks with a SD of 45.64 weeks.

Whereas the majority of samples were from patients progressed on vemurafenib, 2 samples were from patients who had progressed on dabrafenib, a drug with similar clinical efficacy (22). All samples were successfully established as tumor grafts with a median latency until palpable of 5.75 weeks (Fig. 1C). The median growth rate was 120.3 mm3/weeks to sacrifice, measured from palpability to last follow-up (Fig. 1D). We did not observe any significant growth delay between untreated and chronically PLX4720–treated tumor grafts (Supplementary Fig. S1). The histologic examination of the original patient tumor and the tumor grafts grown in mice showed similarities with respect to morphologic and histopathologic criteria. Furthermore, PDX serially transplanted up to 5 passages in mice still resembled the initial lesion, even when these were grown under continuous drug pressure (Fig. 1B and Supplementary Fig. S2).

Identification of targetable resistance mechanisms
To characterize the resistance mechanisms in these models and assess how well they would recapitulate the known biology of resistance in patients, targeted next-generation sequencing was performed on all PDX expanded under BRAF inhibition with a median exon coverage of 713 using the FoundationOne panel (Foundation Medicine, Cambridge, MA). A median of 11.5 somatic short variants of known, likely, and unknown significance was identified, with one PDX containing 111 variants in the 343 exons and introns assessed, and complete results are provided in (Supplementary Fig. S3). The BRAF V600E variant was confirmed in all samples. Importantly, at least 2 and up to 9 known deleterious concomitant alterations (mutations, amplifications, and deletions) were found in each of the 12 PDX samples (Fig. 2A), including genes in the MAPK and PI3K pathways, the receptor tyrosine kinase (RTK) family, transcription regulators, and DNA repair genes. The most common alteration was loss of CDKN2A in 9 of 12 samples (23). Many genetic aberrations found through this approach were previously associated with resistance to BRAF inhibitors. For instance, 3 of 12 PDX had activating NRAS mutations (24), 2 of 12 had activating MAP2K1 mutations (Q56P and K57E; refs. 25, 6), 4 of 12 had BRAF amplification (8), and 7 of 12 samples had deletion or mutation of PTEN (8). Moreover, in several cases, multiple candidate resistance mechanisms co-occurred (e.g., PI3KCA and NRAS). Finally, some potentially actionable alterations detected were not previously described in the context of BRAF inhibitor resistance, such as MET amplification in 3 PDX models (WM3965s-2 with a calculated copy number of 16, WM3983 with a copy number of 9, and WM4007-1 with a copy number of 93). Matched samples were collected from several patients: WM4007 is a pretreatment lesion to WM3901 and does not have amplified BRAF; WM3936-1 and -2 are both from the same relapsed lesion at different time points and after aggressive growth under therapy, but are both remarkably similar; finally, WM407-1 and -2 are from therapy-resistant bowel and brain metastases, respectively, and although the 2 lesions have distinct mutation profiles, pERK, pAKT, and other protein levels were concordant in both PDX.

BRAF short-splicing variants have been reported in BRAF inhibitor–progressed patient samples at a frequency of 13% to 32%
However, all of our PDX models were determined to be negative for BRAF splice variants by protein and RNA analysis (Supplementary Fig. S4). To complement genomic profiling with an assessment of pathway activation status, RPPAs were run for all PDX. To differentiate between genomic/epigenomic changes versus signaling feedback loops due to continued BRAF inhibition, an analysis of differential protein signaling between all untreated PDX by unsupervised hierarchical clustering was performed (Supplementary Fig. S2A). Principal component analysis (PCA) was performed on three groups identified in the clustering but failed to distinguish among the groups due to the lack of similarly expressed proteins (Supplementary Fig. S2B). Furthermore, attempting to identify signaling feedback loops, we analyzed protein fold changes between treated and untreated tumors using unsupervised hierarchical clustering (Supplementary Fig. S2C). Again, PCA did not succeed in defining commonly changed pathway. Instead, it highlighted the heterogeneity of resistance mechanisms within our relatively small tumor subset (Supplementary Fig. S2D). However, MAPK pathway reactivation was identified as a putative mechanism of resistance in the majority of PDX (Fig. 2B). Fold change in pAKT levels between BRAF inhibitor treated versus untreated PDX tumors indicated the PI3K pathway as a possible compensatory mechanism in 5 PDX models (Fig. 2C). Although we did not see a negative correlation between pERK and pAKT, the increase of pAKT while on drug indicates that continued pathway inhibition in the resistant setting might lead to upregulation of PI3K signaling through cross-talk between these two pathways (27).

Rational dual MAPK and PI3K pathway inhibition inhibits tumor growth in vivo

To test the hypothesis of dual-core pathway inhibition based on genomic and proteomic data, we selected a MAPK and PI3K hyperactivated model for a multi-arm PDX in vivo study. The patient whose tumor tissue was used in this study had received dabrafenib in a clinical trial with an excellent clinical response but developed a new subcutaneous thigh lesion after 9 months of therapy, which was then biopsied (WM3936-1). The patient was transitioned to commercial vemurafenib, but aggressive growth of that same lesion was observed under therapy so that this progressing thigh lesion was surgically excised after 3 months on vemurafenib (WM3936-2). We found that both PDX had similar...
mutation profiles and had acquired NRAS and PIK3CA mutations. Available next-generation sequencing data of a pretherapy lesion biopsy indicated NRAS wild-type and PIK3CA wild-type status at least to the depth of sequencing performed. WM3936-1 and -2 were both derived from the same patient lesion progressing on dabrafenib and subsequently vemurafenib, and both harbored NRAS Q61K heterozygous, PTEN C105Y homozygous, and PIK3CA H1047Y heterozygous mutations as potential resistance mechanisms that would be expected to lead to re-activation of the MAPK and compensatory activation of the PI3K pathway, as confirmed in the RPPA data. Neither the PIK3CA or NRAS mutations were detected in a pretherapy patient lesion; PTEN status could not be assessed. On the basis of genomic and RPPA (Fig. 2A and C) data, we designed a rational second-line combination therapy to target all candidate resistance mechanisms centered on the pan-PI3K inhibitor BKM120 (28) in combination with either a BRAF/MEK inhibitor combination using encorafenib and binimetinib currently in clinical trials (NCT01543698 and ASCO 2015 abstract 9007) or the ERK inhibitor VX-11e (29).

For this 6-arm in vivo study, the WM3936-2 PDX model was expanded until tumor grafts could be implanted simultaneously into a cohort of 60 NSG mice. Once tumors were established, animals were dosed for 2 weeks. As expected, the tumors were resistant to BRAF/MEK inhibitor combination therapy as well as to PI3K inhibition alone. The ERK inhibitor inhibited tumor growth as a single agent compared with control (P < 0.0001) but targeting both MAPK and PI3K signaling using either of the 2 strategies: triple combination encorafenib/binimetinib/BKM120 (P < 0.0001) or double combination VX-11e/BKM120 (P < 0.0001) resulted in significantly improved tumor growth control (Fig. 3A). The difference between triple and double therapy was not significant. This result confirmed the utility of rationally designed MAPK/PI3K inhibitor combination therapy based on genomic and proteomic data. Tumor tissue harvested at the end of study was assessed for pathway inhibition; BKM120 as a single agent did not decrease pAKT signaling, and this difficulty in assessing PI3K inhibition at the level of AKT has been previously described. The combination of encorafenib/binimetinib resulted in a modest inhibition of pRSK as a downstream target of the MAPK pathway and pS6, downstream of MAPK and PI3K pathways. Similarly, ERK inhibition by VX-11e resulted in robust inhibition of pRSK reflected in tumor growth inhibition. However, only the encorafenib/binimetinib/BKM120 triple combination led to a complete inhibition of both pRSK and pS6 (Fig. 3B).

MET amplification alone is not sufficient to predict effective therapy

We then analyzed the activation status of RTKs present on the RPPA (Fig. 3C), as these have been reported as potential resistance
Figure 3.
Dual pathway inhibition controls tumor growth. A, tumor growth curves of WM3936-2 PDX. Animals were treated with vehicle control, encorafenib 20 mg/kg every day + binimetinib 3 mg/kg every day (Enc + Bin), BKM120 30 mg/kg every day, the triple combination encorafenib + binimetinib + BKM120, VX-11e 50 mg/kg twice a day (VX), or VX-11e + BKM120. Dosing was started with established tumors; all compounds were administered orally, n = 10/group; error bars are SEM; * indicates P < 0.0001. B, immunoblot of tumors harvested at the end of study, 4 hours post last dose. The membrane was probed with indicated antibodies. β-Actin was included to ensure equal loading. C, levels of RTK proteins assessed by RPPA, red higher than median, green lower than median, unsupervised hierarchical clustering; data is mean of 3 biologic replicates. D, relative tumor growth (final volume/days to maximum volume) of WM3983 PDX relative to vehicle control. In two separate experiments, animals were treated with (i) vehicle control, encorafenib 20 mg/kg every day (Enc + Bin) 3 mg/kg every day and (ii) vehicle control, capmatinib 25 mg/kg every day (Cap). In both experiments, dosing was started with established tumors. All compounds were administered orally for 14 days, n = 10/group; error bars are SEM. E, immunoblot of tumor grafts harvested after 3 days of dosing 4 hours post last dose. The membrane was probed with indicated antibodies. β-Actin was included to ensure equal loading. + denotes the WM3965 tumor graft tissue included as a positive control with elevated levels of MET and pMET. F, immunohistochemical staining for MET of melanoma lesion of the patient. The pre-BRAF inhibitor biopsy shows a strong membrane stain for MET, but only in a subpopulation. The post-progression biopsy is negative for MET; the positive cells are macrophages (this lesion was used to establish the PDXs).
mechanisms in melanoma (30). Clusters of PDX with upregulation in the EGFR/HER2/HER3 family of RTKs, c-Kit upregulation, as well as MET were identified. We selected the proto-oncogene MET (31, 32) as another promising target for second-line therapies. The 25% (3/12) incidence of MET amplifications in this set of BRAF inhibitor–progressed PDX was significantly higher than in the melanoma Cancer Genome Atlas (TCGA) at 8/129 BRAF hotspot mutated, 0/17 BRAF non-hotspot, and 3/149 BRAF wild-type yielding a total of 11/299 or 3.7%. Thus, MET amplification and BRAF hotspot mutation had a significant tendency towards cooccurrence (P = 0.035). In the TCGA database, out of 9 MET-amplified melanomas with available RPPA data, only 2 showed more than 2-fold increase in pMET. Indeed, in one of the three MET-amplified PDXs, an isolated progression of a scalp lesion, pMET was not increased compared with the median. This was possibly due to being part of a much broader amplicon including EGFR and possibly the entire chromosomal arm 7, and despite being amplified 9-fold. In a study of 1,115 patients, MET amplifications were detected in 2.5% of solid tumors (melanoma 2/61) and these patients presented with more metastatic sites then non-MET–amplified tumors (26). This led us to hypothesize that MET amplifications represent either preexisting or acquired mechanisms of resistance and would predict response to the MET antagonist capmatinib (33). Indeed, MET pY1235 (pMET) levels significantly higher than the median were confirmed in 2 of 3 PDX models with MET amplification (Fig. 3C). High levels of pMET were seen in WM3965 and WM4071-P DX tumors, with the latter derived from a bowel metastasis in a brain metastasis from the same patient and also collected post progression (WM4071-1) did not have amplified MET or increased pMET, although this could relate to difference in sequencing depth and an ability to make amplification calls.

To test whether genomic data alone would be sufficient to design a rational second-line therapy in a MET-amplified setting, we expanded the WM3983 PDX in vivo and confirmed it to be completely resistant to encorafenib (even exhibiting increased proliferation as compared with vehicle control) and encorafenib/binimetinib combination therapy (Fig. 3D). Importantly, while WM3983 demonstrated amplified MET, it did not have pMET signaling (Fig. 3C). Thus, the MET inhibitor capmatinib had no antitumor effect in this model in vivo (Fig. 3D), concordant with undetectable levels of pMET in control tumors harvested at the end of study (Fig. 3E). The lack of pERK and pMEK inhibition in encorafenib/binimetinib–treated versus untreated animals (Fig. 3E) supported a MAPK reactivation mechanism of resistance in line with the NRAS-mutant; BRAF-amplified genotype of this tumor. To rule out the possibility that the PDX had lost its pMET phenotype due to changes in the murine environment, we analyzed patient samples from before and after BRAF inhibitor progression. Although MET-positive subpopulations of tumor cells were found in the pretherapy lesion, these had disappeared in the progression biopsy used to generate the PDX (Fig. 3F).

Integrating genomic and proteomic data for the design of second-line combination therapies

On the basis of these findings, we hypothesized that integration of genomic and protein data may provide greater information content than either alone and selected the MET amplified and high pMET signaling PDX model WM3965 for a multi-arm combination study centered on capmatinib (Fig. 4A). The patient whose tumor was used to generate the PDX had received vemurafenib in a neoadjuvant setting, but after only 3 months, developed early progressive disease (PD) in the right parotid gland, which was surgically excised and used to generate the PDX. The 6-arm design included a vehicle control group showing rapid tumor growth, encorafenib single agent (accelerated tumor growth, P = 0.020) and encorafenib/binimetinib combination arms (no antitumor effect) confirming the aggressive and MAPK pathway inhibitor resistant phenotype of this PDX model. Remarkably, the tumors in the other 3 dosing groups (capmatinib single agent, capmatinib/encorafenib, and capmatinib/encorafenib/binimetinib) all rapidly regressed after as short as 3 days of dosing. This trend continued until more than 2 weeks of daily dosing, at which point a separation of the growth curves became apparent, whereas the tumor grafts on capmatinib single agent and capmatinib/encorafenib developed therapy resistance, albeit with variable tumor growth kinetics, the capmatinib/encorafenib/binimetinib (triple combination) treated tumors showed complete tumor regression in 10 of 10 animals after 21 days of dosing with no evidence of therapy resistance (Fig. 4A).

High levels of pMET protein were confirmed in vehicle treated tumors by Western blot, and high pERK and pAKT levels indicated active signaling through both MAPK and PI3K pathways, respectively (Fig. 4B). Furthermore, the highly proliferative phenotype could be demonstrated by strong Ki67 staining (Supplementary Fig. S6A). In contrast, animals dosed for 3 days with capmatinib showed complete abrogation of pMET (Fig. 4B). Importantly, capmatinib single agent did not lead to meaningful decreases in pAKT or pERK signaling, whereas the triple combination resulted in almost complete inhibition of pAKT and pERK. This observation correlated with increased apoptosis as measured by cleaved caspase-3 staining at this early time point (Supplementary Fig. S6B).

Tumor tissue of vehicle-treated, capmatinib ± encorafenib–responding and progressing animals was submitted for RPPA analyses. Intriguingly, three distinct clusters could be observed: one containing all early responders and the other two randomly distributed untreated and progressed tumors (Fig. 4C). The capmatinib–responding tumor cluster was predominately defined by pMET, pEGFR, and pHER2 downregulation in association with downregulation of their downstream effectors pMAPK, pBR, cyclin D1, pAKT, p4E-BP1, IGFBP2, and FOXM1. Interestingly, glycogen synthase (GYS) phosphorylation was inversely upregulated, indicating a decrease in glycogen production ability in these tumors. The observation that the control and progression samples were interspersed is consistent with a loss of MET inhibitory effect and reversal of the signaling profile back to the untreated state. Still, two distinct populations were apparent in this PDX model as defined by protein expression profile, confirming the heterogeneity found in PDX tumors. We then performed PCA comparing the 2 clusters and found evidence for differences in metabolism, PI3K signaling, and RTKs (Supplementary Fig. S7), although these did not correspond with time to progression. As resistance occurred at roughly the same time and relatively quickly after initial regression, this may suggest a common (adaptive) resistance mechanism.

Finally, patient biopsies from the therapy-naive primary lesion (Fig. 4D) and a postprogression metastasis, used to generate the PDXs (Fig. 4E), both stained highly positive for MET, indicating that the amplification of MET might have been
preexisting in the primary melanoma and thus acted as an intrinsic mechanism of resistance leading to early relapse with only 3 months PFS.

Discussion

Patient-derived xenografts provide sustainable models for personalized therapy. The key advantage of these models is their surprising biologic and genetic stability when implanted into mice, as reflected in our current study. This allowed for a comprehensive analysis of drivers of resistance to targeted therapy and the design of effective second-line combination therapies tailored to each model. Although patient-specific real-time "co-clinical" trials are feasible, obstacles such as timing and regulatory issues may hinder progress. Instead, the development of biomarkers using this approach might offer a higher benefit ratio. A single patient-based approach is further complicated by the fact that PDX models currently do not allow for a comprehensive assessment of immunotherapies, which are rapidly becoming first-line therapy for melanoma patients. Although efforts are ongoing to move this technology into "humanized" mice, which have a reconstituted human immune system, this will prove both challenging and extremely costly. Our studies are therefore restricted to direct targeting of signaling pathways in melanoma cells and

Figure 4.
Integrating genomic and protein signaling results in an effective triple therapy preclinical in vivo trial. A, tumor growth curves of WM3965 PDX (MET amplified, high pMET); dosing was started with well-established tumors expanded on BRAF inhibitor diet followed by a washout period before the start of dosing. Animals received either vehicle control, encorafenib (Enc) 20 mg/kg every day, binimetinib (Bin) 3 mg/kg every day, capmatinib (Cap) 25 mg/kg every day as single agents or combinations as indicated. All compounds were administered orally, n = 10/group; error bars are SEM; * indicates P < 0.05. B, immunoblot of tumors harvested after 3 days of dosing and 4 hours post last dose. The membrane was probed with indicated antibodies and β-Actin was included to ensure equal loading. C, RPPA analysis of tumors harvested either at the end of the efficacy experiments (A) or 3 days of dosing (B). Mice without palpable tumors were not included (all triple combo animals). Color coding on the lower x axis denotes the following groups: blue are control animals (vehicle), orange are the 6 animals treated with Cap or Enc/Cap for 3 days (early responders), and pink were treated with Cap or Enc/Cap in the efficacy cohorts and progressed after initial response (progression). Proteins with similar expression along all samples were excluded. The proteins that vary across the samples over a cutoff of 0.4 SDs are shown on the y axis. D, MET immunohistochemical staining (brown) of FFPE patient tissue. The pre-BRAF inhibitor sample is the safety margin around the primary lesion and shows residual melanoma nests in the dermal layer (black circle). E, lymph node metastasis of the same patient after progression (this biopsy was used to establish the PDX).
possibly the murine stroma. These PDX models with acquired resistance to targeted therapy provide an effective way to expand tissue for multiple methods (sequencing, RPPA, etc.) that do not face the same challenges as growing these cells on plastic (loss of architecture and single cell clonality), and as we show in this study, provide alternative therapeutic targets in relapsed patients. Overall, PDX can play a major role in exploring novel targets and combination therapies based on the increasingly detailed picture of genomic and proteomic aberrations in cancer cells.

In this study, we utilized tumor samples from patients who reflected the wide range of responses seen in the clinic (34). The relative uniformity in tumor grafting in vivo and invariable resistance to dosing with a BRAF inhibitor indicated that once resistance had occurred, all tumors were capable of tumor initiation in vivo irrespective of time to progression in patients. Next-generation sequencing of patient lesions using targeted platforms is transforming personalized cancer therapy by uncovering actionable genomic alterations in a majority of solid tumors such as lung cancer (35). We used the same approach to uncover possible mechanisms of intrinsic and acquired resistance. Many of these alterations such as NRAS (24) and MAP2K1 (25, 36) mutations as well as PTEN deletion and BRAF amplification (8) were previously described to confer resistance to BRAF inhibitors. MAPK pathway–hyperactivating alterations (BRAF or RTK amplification, NRAS and MAP2K1 mutations) were found in 11 of 12 samples (most often mutually exclusive), and RPPA analyses confirmed active MAPK signaling under drug pressure in the majority of PDX concordant with recently published results (6, 37). Interestingly, all that brain metastasis–derived PDX had aberrant PTEN, whereas only one of the extracerebral metastases had a PTEN deletion, which had been previously described as a poor prognostic marker (38). Taking into account the intrapatient heterogeneity and possible clonal selection in the generation of a PDX model, it is likely that not the whole spectrum of tumor cell population in a patient will be represented in a mouse avatar. Thus, mapping of patients for driver mutations and their possible convergence on the same biologic pathways (39) can be studied by generating multiple PDX from the same patient.

The identification of multiple concomitant alterations per PDX further emphasized the challenges of personalized therapy selection in the clinic and was consistent with published reports (27). To determine whether multiple genomic aberrations integrated into a limited number of pathways, we extended our analysis to signaling on the phospho protein level using the RPPA platform. RPPA relies on validated antibodies and well characterized targets, allowing us to confirm sustained signaling through the MAPK pathway in the majority of PDX and increased activation of the PI3K pathway in a smaller subset. We did not focus our study on the discovery of novel targets as studies of patient samples directly are much more suited to this approach. Rather, the strength of our PDX platform lies in translating findings to in vivo target validation.

Indeed, using either a BRAF/MEK inhibitor or ERK inhibitor combination strategy with the pan-PI3K inhibitor BKM120 proved effective in abrogating tumor growth in a MAPK and PI3K pathway–activated mouse avatar. This confirmed previous reports in cell lines with acquired NRAS mutations resistant to dabrafenib (40) and in those with increased RTK signaling leading to increased PI3K signaling (41). Therefore, we could establish that genomic profiling and assessment of associated signaling pathway activity is a viable strategy to design rational second-line combination therapies in vivo. Also, we can postulate that the corresponding patient likely would not have benefited from dabrafenib/trametinib combination therapy, but that the inhibition of both MAPK and PI3K pathways would have been critical in achieving a response. This combination efficacy may be the first clear example of co-occurrence of redundant mechanisms of resistance.

On the other hand, a MET–amplified BRAF and combined BRAF/MEK inhibitor–resistant model did not respond to MET inhibition in vivo, and we therefore concluded that amplification of MET is not sufficient to define it as a driver of resistance and that a second readout, such as pMET protein levels would be necessary. This was in line with a previously published study of a large cohort of over 1,000 patients where MET amplification did not correlate with response to a MET inhibitor and where MET protein levels were not assessed (42). Targeting MET has proved effective in MET–amplified gastric cancer using the inhibitor volitinib (43), and MET has been described as a novel target for adjuvant therapy for melanoma (44). In our study, the triple combination of MET, BRAF, and MEK inhibition was exceptionally effective in vivo, with profound MAPK pathway inhibition. This observation might be explained by HGF/MET–mediated RAS activation (45) leading to BRAF dimerization and thus resistance to vemurafenib (46). We therefore propose that increased MET protein phosphorylation with or without MET amplification should be assessed as a biomarker of response to MET inhibitor combination therapies. This will be of highest priority in MAPK pathway inhibitor–resistant patients as increases in MET RNA levels have been described at an increased frequency in this patient cohort (47). However, as targeted genomic sequencing is currently the gold standard of personalized therapies, a preselection of patients for protein signaling analysis currently not in widespread use for clinical treatment selection will be advantageous.

Although we did not extend our study to patient treatment after determining efficacious second-line therapies in the PDX models, a clinical trial with parallel assignment into 5 treatment arms based on sequencing data is currently ongoing in relapsed melanoma patients (ClinicalTrials.gov: NCT02159066). The results from the PDX preclinical studies clearly argue that genomic and proteomic approaches should be integrated to increase the success rates of personalized cancer therapies, as this approach allowed us to outline and confirm personalized medicine strategies. These models can be used to refine precision medicine approaches and to develop biomarkers of response for future clinical trials and avoid treatment failures for patients. Future studies using humanized mouse models with reconstituted T-cell function will be of major importance to integrate the findings described here into an immunotherapy landscape of melanoma.

Disclosure of Potential Conflicts of Interest

C.B. Mills reports receiving commercial research grants from Adelson Medical Research Foundation, AstraZeneca, Critical Outcome Technologies, Komen Research Foundation, and Nanostring; speakers bureau honoraria from AstraZeneca, ISIS Pharmaceuticals, Nuevolution, and Symphogen; holds ownership interest (including patents) in Catena Pharmaceuticals, Myriad Genetics, PIV Ventures, and Spindletop Ventures; and is a consultant/advisory board member for Adventist Health, AstraZeneca, Blend, Catena Pharmaceuticals, Critical Outcome Technologies, HanAL Bio Korea, ImmunoMET, Millennium Pharmaceuticals, Nuevolution, Precision Medicine, Provia Diagnostics, Signalchem Pharmaceuticals, and Symphogen. D.E. Removal reports receiving commercial research grants from ImmunoMET, Millennium Pharmaceuticals, and Nuevolution; speakers bureau honoraria from AstraZeneca and Symphogen; and is a consultant/advisory board member for Adaptive Biotechnologies, AstraZeneca, Blend, Catena Pharmaceuticals, Critical Outcome Technologies, HanAL Bio Korea, ImmunoMET, Millennium Pharmaceuticals, Nuevolution, Precision Medicine, Provia Diagnostics, Signalchem Pharmaceuticals, and Symphogen.
Lifesiences, and Symphogen. M. Peters holds ownership interest (including patents) in Novartis. No potential conflicts of interest were disclosed by the other authors.

Disclaimer
The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

Authors’ Contributions
Conception and design: C. Krepler, K. Sproesser, G.B. Mills, G.C. Caponigro, M. Boehm, M. Peters, M. Herlyn
Development of methodology: C. Krepler, K. Sproesser, X. Xu, T.A. Ahmed, M. Herlyn
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Krepler, M. Xiao, K. Sproesser, P.A. Brafford, B. Shannan, M. Boehm, M. Herlyn
Writing, review, and/or revision of the manuscript: C. Krepler, K. Sproesser, G.B. Mills, L.M. Schuchter, A.T. Weeraratna, M. Herlyn
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Sproesser, B. Shannan, M. Boehm, X. Xu, P.I. Poulikakos, M. Herlyn

References

Study supervision: C. Krepler, W. Xu, G. Caponigro, M. Boehm, M. Peters, M. Herlyn
Other (performed RPPA and provided the raw data): Y. Lu

Acknowledgments
The authors thank the Animal and Imaging Core Facilities at the Wistar Institute and the Tumor Tissue and Biospecimen Bank at the University of Pennsylvania Abramson Cancer Center. The authors also thank Gideon Boillag at Plexaxon for providing PLX4720.

Grant Support
Support for shared resources utilized in this study was provided by Cancer Center Support Grant (CCSG) P30CA010815 to the Wistar Institute. This work was also supported by NIH grants P01 CA114046, P01 CA052874, P30 CA010815, R01 CA047159, and a research grant by Novartis (to M. Herlyn). CCSG grant P30CA016672 to G.B. Mills, R01 CA174746-01 to A.T. Weeraratna, and the Dr. Miriam and Sheldon G. Adelson Medical Research Foundation.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received July 23, 2015; revised November 16, 2015; accepted December 3, 2015; published OnlineFirst December 16, 2015.
32. Boccaccio C, Comoglio PM. MET, a driver of invasive growth and cancer.
Clemens Krepler, Min Xiao, Katrin Sproesser, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-15-1762

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2015/12/16/1078-0432.CCR-15-1762.DC1

Cited articles
This article cites 47 articles, 11 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/22/7/1592.full#ref-list-1

Citing articles
This article has been cited by 3 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/22/7/1592.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.