BRAF V600E Is a Determinant of Sensitivity to Proteasome Inhibitors

Davide Zecchin1,3, Valentina Boscaro2, Enzo Medico1,3, Ludovic Barault3, Miriam Martini1,3, Sabrina Arena3, Carlotta Cancelliere3, Alice Bartolini3, Emily H. Crowley3, Alberto Bardelli1,3,4, Margherita Gallicchio2, and Federica Di Nicolantonio1,3

Abstract

A critical step toward defining tailored therapy in patients with cancer is the identification of genetic interactions that may impair—or boost—the efficacy of selected therapeutic approaches. Cell models able to recapitulate combinations of genetic aberrations are important to find drug-genotype interactions poorly affected by the heterogeneous genetics of human tumors. In order to identify novel pharmacogenomic relationships, we employed an isogenic cell panel that reconstructs cancer genetic scenarios. We screened a library of 43 compounds in human hTERT-HME1 epithelial cells in which PTEN or RB1 were silenced in combination with the targeted knockin of cancer-associated mutations in EGFR, KRAS, BRAF, or PIK3CA oncogenes. Statistical analysis and clustering algorithms were applied to display similar drug response profiles and mutation-specific patterns of activity. From the screen, we discovered that proteasome inhibitors show selectivity toward BRAF V600E-mutant cells, irrespective of PTEN or RB1 expression. Preferential targeting of BRAF-mutant cells by proteasome inhibitors was corroborated in a second BRAF V600E isogenic model, as well as in a panel of colorectal cancer cell lines by the use of the proteasome inhibitor carfilzomib. Notably, carfilzomib also showed striking in vivo activity in a BRAF-mutant human colorectal cancer xenograft model. Vulnerability to proteasome inhibitors is dependent on persistent BRAF signaling, because BRAF V600E blockade by PLX4720 reversed sensitivity to carfilzomib in BRAF-mutant colorectal cancer cells. Our findings indicated that proteasome inhibition might represent a valuable targeting strategy in BRAF V600E-mutant colorectal tumors. Mol Cancer Ther; 12(12); 2950–61. ©2013 AACR.

Introduction

Over the past decade, research has demonstrated that the clinical benefit from targeted therapies is dependent upon our knowledge of the presence of specific genetic aberrations within the tumor (1–9). To maximize therapy efficacy, treatment must be tailored to the genetic milieu of a specific tumor, to deliver what is referred to as 'precision medicine'.

This approach has led to progress in the treatment of specific malignancies including breast cancers overexpressing or harboring amplified EGF receptor 2 (HER-2) that can be successfully treated with trastuzumab (5). In addition, lung cancers carrying specific mutations in the EGF receptor (EGFR) are particularly sensitive to the EGFR tyrosine kinase inhibitors gefitinib and erlotinib (1–4). In addition, recent examples include selective clinical activity of the BRAF inhibitors vemurafenib or dabrafenib in melanomas with BRAF V600E mutation (6, 7), or the efficacy of the ALK inhibitor crizotinib for the treatment of lung cancers carrying translocation of the anaplastic leukemia kinase (ALK; refs. 8, 9).

However, only approximately 50% of patients with BRAF-mutant melanoma, 30% of patients with HER2-amplified breast cancer, and 60% of patients with EGFR-mutant or ALK-translocated lung cancer respond to blockade of the corresponding targets (1–9). Simple binary relationships between genetic aberrations and drug response are complicated in these cases by the intricate genetic landscape of solid tumors (10). Indeed, in most instances, multiple tumor suppressor mutations and oncogene variants occur in the same solid tumor [http://cancergenome.nih.gov/], and it is thought that,
together, these molecular alterations contribute to patients’ response to specific anticancer treatment. It has been reported, for instance, that the sensitivity of the EGFR-mutant lung cancer cells to EGFR tyrosine kinase inhibitors is reduced by inactivation of PTEN (11, 12). The activation of the phosphoinositide 3-kinase (PI3K) pathway, defined by PTEN loss and/or PIK3CA mutation, was also associated with poor response to trastuzumab and shorter survival time in HER-2-positive metastatic breast cancer (13, 14). This indicates that the influence of tumor complex genetics on therapy response warrants further consideration.

Nevertheless, there is a paucity of functional studies that systematically evaluate the effect of complex genotypes in the modulation of drug responses. We believe that such experimental approaches are fundamental in order to identify novel drug-genotype interactions that are unaffected by the concomitant presence of other common genetic alterations. On the other hand, these studies may improve our ability to predict response to existing anticancer therapies based on the plethora of genetic aberrations present in a solid tumor.

In this report, we employed a previously characterized panel of isogenic human cell lines that recreate possible molecular scenarios observed in human cancer (15). Using a homologous recombination, we introduced the activating mutations EGFR delE746-A750, PIK3CA H1047R, PIK3CA E545K, KRAS G13D, and BRAF V600E into the genome of the nontransformed human cell line hTERT-HME-1 (abbreviated as HME-1), which already harbors the C176F on TP53. This TP53 mutation was previously reported to impair the TP53 checkpoint response to genotoxic stress in HME-1 cells (15). PTEN or RB1 tumor suppressor genes have been systematically silenced in these isogenic cell lines generating a combinatorial model referred to as the “matrix” (See Supplementary Fig. S1).

Using the HME-1 matrix, we investigated the role of single or multiple cancer genetic alterations in modulating the response to antineoplastic drugs. This approach uncovered a novel pharmacogenetic interaction between proteasome inhibitors and the BRAF V600E allele.

The BRAF V600E mutation occurs in 5% to 8% of advanced colorectal cancer samples. Patients with metastatic colorectal cancer with BRAF-mutant tumors have a poor prognosis and do not respond to BRAF inhibitors in monotherapy (16, 17). Accordingly, the development of therapeutic strategies for metastatic BRAF mutated colorectal cancer represent an urgent and unmet clinical need. We, therefore, elected to evaluate the activity of proteasome inhibitors in BRAF-mutant colorectal cancer models. Finally, we investigated the ability of selective BRAF targeted agents to modulate response to the proteasome inhibitor carfilzomib in BRAF-mutant colorectal cancer cells.

### Materials and Methods

#### Cells and cell culture reagents

The HME-1 cell line was obtained from the American Type Culture Collection (ATCC; LGC Standards S.r.l., Milan, Italy) in October 2005. The CACO2, NCI-H716, HuTu80, COLO201, SW1417, and LS411N cell lines were purchased from the ATCC in June 2010; COLO320 and HCA7 were obtained from the European Collection of Cell Cultures (ECACC) in September 2009 (distributed by Sigma-Aldrich Srl, Milan, Italy). CaR1 and OUMS523 cell lines were purchased from the Japanese Collection of Research Bioresources (JCRB) (Tokyo, Japan) in January 2011. The HDC135 cell line was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) repository (Braunschweig, Germany) in November 2010. The NCI-H630, KM20, and SNU-C5 cell lines were purchased from the Korean Cell Bank (Seoul, Korea) in February 2011. VACO432 and RKO cells were obtained from Horizon Discovery (Cambridge, United Kingdom) in March 2011. The LIM1215, LIM2405, and LIM2537 cell lines (18, 19) were provided by Prof. R. Whitehead, Vanderbilt University, Nashville, with permission from the Ludwig Institute for Cancer Research, Melbourne branch, Australia in August 2011. The DiFi and OXCO1 cell lines were a kind gift from Dr J. Baselga in November 2004 (Oncology Department of Vall d’Hebron University Hospital, Barcelona, Spain) and Dr V. Cerundolo in March 2010 (Weatherall Institute of Molecular Medicine, University of Oxford, Oxford, United Kingdom), respectively. The genetic identity of the cell lines used in this study was confirmed by STR profiling (Cell ID, Promega) no longer than 6 months before drug-profiling experiments. All cells were cultured as previously described (15) or according to the manufacturers’ instructions. All cell culture media were supplemented with 10% FBS or 5% for HME-1 (Sigma-Aldrich), 50 U/mL penicillin, and 50 mg/mL streptomycin. Geneticin (G418) was purchased from Gibco and puromycin from Sigma-Aldrich.

#### Construction of isogenic models

The generation of the HME-1 matrix has been previously reported (15). All experimental procedures for BRAF V600E targeting vector construction, adeno-associated virus (AAV) production, cell infection, and screening for recombinants have been described previously (20, 21).

#### Drug proliferation assay

Parental and mutated cells were seeded in 100 μL complete growth medium at a density of $3 \times 10^4$ cells/well in 96-well plastic culture plates. After serial dilutions, 100 μL of drugs in serum-free medium were added to cells with a multichannel pipette. Vehicle- and medium-only–containing wells were added as controls. Plates were incubated at 37°C in 5% CO2 for 96 hours, after which cell viability was assessed by ATP content using the CellTiter-Glo Luminescent Assay (Promega). To account for clonal variability, two independent isogenic knockin (KD) clones infected with scramble short hairpin RNA (shRNA), or with shRNAs targeting PTEN or RB1 were tested. All luminescence measurements (indicated as relative light units (RLU)) were recorded by the Victor X4 Multilabel Plate Reader (PerkinElmer). In Supplementary
Table S1, we have reported a list of tested compounds, their chemical formula, their molecular weight (MW), the solvent used for suspension, the concentration of stock solutions, the concentrations tested in the experiments, and the storage conditions used for the stock. Each compound was preliminarily tested on HME-1 parental cells infected with scramble shRNA to determine the concentration referred to as the highest no-observed effect level (NOEL), the IC50, and the IC90 values, as previously reported (20). The three concentrations of each compound tested on the entire isogenic cell panel were selected on the basis of these premises.

**Proteasome activity assay**

Proteasome activity was assayed using Proteasome-Glo Chymotrypsin-Like Cell-Based Assay (Promega). Cells were seeded 16 hours prior to drug treatment. Proteasome activity was measured after 2-hour incubation with proteasome inhibitors, according to the manufacturer’s instructions.

**SDS-PAGE and Western blot analysis**

Cell lysates were prepared in boiled Laemmli buffer (2.5% SDS, 125 mmol/L Tris–HCl, pH 6.8). Lysates were sonicated, cleared by centrifugation at 14,000 rpm for 10 minutes at room temperature, and the supernatant containing soluble protein was removed. The protein concentration of the supernatant was determined by micro-BCA protein assay (Pierce). An equal amount (25 μg) of whole-cell lysate per lane was boiled in lithium dodecyl sulfate (LDS) buffer and reducing agent, according to the manufacturers’ instructions, and separated by SDS-PAGE on 10% precasted polyacrylamide mini-gels (Invitrogen). The separated lysates were then transferred to a nitrocellulose membrane. The blot was then incubated with blocking buffer [Tris-buffered saline (TBS)–10% BSA] for 1 hour at room temperature and incubated overnight with the primary antibody (diluted according to the manufacturer’s instructions in TBS–5% BSA) at 4°C. The blot was then washed 3 times for 10 minutes in washing buffer (TBS containing 0.2% Tween 20), incubated with secondary antibody horseradish peroxidase (HRP)-conjugate (Sigma; diluted 1:10,000–1:2,000) and washed a further 3 times. ECL solution (Enhanced Chemiluminescence System, Amersham) was then added to the filter, and the chemiluminescent signal was acquired by the LAS4000 Image reader (Fujifilm). Antibodies used for immunoblotting were: anti-P21, anti-PARP, anti-PTEN, and anti-RB1 (Cell Signaling Technology); anti-EGFR (clone 13G8, Enzo Life Sciences); anti-Ubiquitin (Santa Cruz Biotechnology); and anti–actin (Sigma-Aldrich).

**Pharmacology data analysis (“Pharmarray”)**

Cell viability at each drug concentration was initially normalized to vehicle-treated cells for each cell line, and triplicate observations within the same experiment were averaged. We then calculated, within each experiment, the drug response as follows: we considered the difference between the Log2 viabilities of the parental cell line and the Log2 viability of a given mutant/genotype. All drug concentrations were tested on each cell line at least 3 times. Drug responses associated to a given mutant and obtained in individual experiments were considered as distinct entities in the subsequent clustering analysis. Similarly, responses to different concentrations of each compound were analyzed as distinct elements in the clustering experiments. All data were clustered and visualized using the publicly available GEDAS software (ref. 22; http://sourceforge.net/projects/gedas).

An array of data was generated in which the red color indicates higher sensitivity (i.e., lower Log2 viability respect to the parental cell line) of a given mutant to a specific drug concentration whereas the green color indicates lower sensitivity (i.e., higher Log2 viability respect to the parental cell line).

The genotypes of the cell lines tested in individual experiments were displayed on the horizontal axis, and we performed an unsupervised, average linkage hierarchical clustering by an uncentered Pearson correlation coefficient. Different drug concentrations were listed in the vertical axis and clustered by the C-means Fuzzy algorithm using an average cosine correlation coefficient. The different clustering metrics were chosen based on the results of the clustering optimization tool included in GEDAS.

Combination effects of PLX4720 and carfilzomib were assessed using the method established by Poch and colleagues (23). We elected to employ the methods of Poch and colleagues as they propose a corrective factor for dose–response curves having a slope different from 1, such as those shown by PLX4720 in most BRAF-mutant colorectal cancer cell lines. For this reason, the Poch method results in a more accurate estimation of combination effects when the agents show a relatively flat dose–response curve.

**Statistical analysis**

Unsupervised clustering analysis was paralleled by statistical evaluation of the genotype-specific differences of the drug responses performed by a t test. Specifically, the statistical test compared the responses of the different mutant cell lines to a given compound with the response of the wild-type (WT) scramble control cells. With this aim, a heteroscedastic two-tailed t test was employed for all mutants as well as for all compounds (see Supplementary Table S2 for the complete list of t tests). In the other experiments, statistically significant differences between groups were determined by using the heteroscedastic Student two-tailed t test. A P value less than 0.05 was considered statistically significant.

**Xenograft studies**

All animal procedures were approved by the Ethical Commission of the Institute for Cancer Research and Treatment and by the Italian Ministry of Health. RKO cells were injected subcutaneously into the right posterior
flanks of 7-week-old female CD-1 nude mice (six animals per group; Charles River, Calco, Italy). Tumor volumes were determined using \( [D \times (d)^2]/2 \), in which \( D \) represents the largest diameter of the tumor, and \( d \) represents the largest perpendicular volume to \( D \). When tumors reached a volume of approximately 200 to 250 mm\(^3\), mice were randomly assigned to treatment with vehicle or drug. For *in vivo* experiments, carfilzomib was formulated in an aqueous solution of 10% (w/v) sulfobutylether-\( \beta \)-cyclodextrin (Captisol, a free gift from CYDEX Pharmaceuticals Inc) and 10 mmol/L sodium citrate (pH 3.5). Carfilzomib solutions were diluted daily with vehicle before tail-vein injections. Carfilzomib was administered on days 1, 2, 8, 9, 15, and 16 in 28-day cycles at a dose of 4 mg/kg.

**Results**

**The isogenic "matrix" of genotypes recapitulates known interactions between drugs and multiple genetic alterations**

We initially assessed whether the HME-1 cellular matrix could recapitulate pharmacogenomic relationships previously established experimentally and clinically.

Previous research showed that EGFR kinase inhibitors are more effective in cells carrying EGFR mutations, but the concomitant loss of PTEN impairs this response (11, 12).

Therefore, we focused on HME-1 isogenic cell lines KI for the EGFR E746-A750 allele and on their isogenic counterpart lacking PTEN expression. We evaluated the genotype-dependent response of these models to EGFR tyrosine kinase inhibitors as a test case.

We observed that erlotinib, canertinib, and lapatinib, classified as inhibitors of the HER family receptor tyrosine kinase, as well as the dual EGFR-VEGFR inhibitor vandetanib affected the growth of HME-1 isogenic cell lines in which the *EGFR* E746-A750 allele was knocked in. Concomitant inactivation of PTEN partially rescued this phenotype (Fig. 1A and B).

In addition, we observed that the KI of BRAF V600E allele conferred resistance to EGFR inhibitors (Fig. 1A) confirming previous findings (24, 25).

These results indicate that HME-1 isogenic models harboring multiple genetic alterations can recapitulate complex drug–genotype relationships found in patients.

**Drug screening of isogenic cell lines carrying combinations of genetic alterations**

Next, we exploited the isogenic HME-1 matrix to seek novel pharmacogenetic interactions.

We assembled a library of 43 compounds (Supplementary Table S3), including: (i) molecules targeting tyrosine kinase receptors (RTK) or their effectors (e.g., anti-HERs, anti-MEK, anti-SRC, anti-AKT, anti-mTOR); (ii) compounds that do not target members of the RTK signaling pathways, but are employed as anticancer therapies (PARP, proteasome, HSP90 inhibitors, epigenetic modulators); (iii) drugs in clinical use aside from cancer therapy but that have been shown to have anti-proliferative and antineoplastic activity (indomethacin, statins). Most of the drugs included in the list are approved by the U.S. Food and Drug Administration (FDA)/European Medicines Agency (EMA) or are undergoing clinical trials in patients with cancer.

Each compound was tested on parental HME-1 cells and on their derivatives infected with a scramble (non-target) shRNA to verify whether and to what extent infection by lentivirus impacted drug response. No significant differences in response were detected (data not shown). Furthermore, no significant differences were also observed when we evaluated the effect of lentiviral infection on proliferation of the KI cells carrying oncogenic mutations in *KRAS, BRAF, PIK3CA*, or *EGFR* as compared with their WT counterpart (Supplementary Fig. S2).
The matrix was subsequently assayed for drug responses by a proliferation assay, using at least three drug concentrations and two clones for each different KI genotype. All drugs were tested at least 3 times on each cell line. Drug responses of mutant cells were normalized to the response of parental scramble HME-1 as described in the Materials and Methods section. Normalized data was then clustered and plotted on an array using the GEDAS software (22). This approach was previously developed and described for the analysis of differential drug activity in KI isogenic models and is defined as a “Pharmarray” (20). Cell lines and drugs were clustered on the basis of their response profile. The entire analyzed dataset is shown in Supplementary Fig. S3. Magnification of a drug cluster is shown in Fig. 2 as a relevant example. The Pharmarray analysis revealed that, in most cases, the genotypes sharing a KI mutation or a knocked down tumor suppressor gene were clustered together.

The presence of these clusters suggested that the genotype of HME-1 isogenic models strongly influenced the pattern of response of these cells to the compounds.

**BRAF-mutant isogenic HME-1 cells show increased sensitivity to proteasome inhibitors**

The cluster of drugs in Fig. 2 preferentially inhibited the **PIK3CA E545K, KRAS G13D**, and **BRAF V600E** mutated genotypes. Intriguingly, this cluster of compounds included three different concentrations of the proteasome inhibitor bortezomib. We focused further on the effect of this compound toward **BRAF-mutant** cells, as this drug–genotype interaction was the most novel in our panel and of potential translational relevance. Indeed, no influence of PTEN or RB1 knockdown on bortezomib activity was observed in the cluster.

The preferential targeting of HME-1 **BRAF** KI clones by proteasome inhibitors was confirmed using the nonboronic agent carfilzomib (Fig. 3A and B). These results point at proteasome per se as a key molecular determinant of the pharmacologic response.

In order to elucidate the relationship between **BRAF** mutated cell lines and proteasome inhibitors, we measured the amount of ubiquitinated proteins following bortezomib treatment. We found that **BRAF-mutant** cells accumulated more ubiquitinated protein with respect to the WT counterpart (Fig. 3C). This was also observed following carfilzomib treatment (Supplementary Fig. S4).

Treatment of HME-1 BRAF V600E with clinically relevant concentrations of bortezomib resulted in increased p21 levels and PARP cleavage (Fig. 3C). Proteasome inhibitors appear, therefore, to elicit a greater growth inhibition and apoptotic response in BRAF V600E KI cell lines likely due to an accumulation in ubiquitinated protein.

Increased accumulation of ubiquitinated protein in **BRAF** KI cell lines following treatment with proteasome inhibitors may be due to a higher basal proteasome activity in these cells. To investigate this hypothesis, we measured the proteasome activity in WT versus BRAF V600E cell lines under basal conditions and following proteasome inhibitor treatment. To this aim, we employed a cell-based proteasome activity assay, which determines the chymotrypsin-like activity associated with intact proteasomes toward a luminogenic peptide substrate. We showed that **BRAF** mutated HME-1 had higher basal chymotrypsin-like activity with respect to WT cells under basal conditions. Bortezomib treatment reduced activity to comparable levels in all isogenic cell lines (Fig. 3D). The greater fold inhibition of proteasome activity correlates with the increased rate of ubiquitinylated protein accumulation in **BRAF** V600E with respect to the WT.

**BRAF-mutant colorectal cancer cells display increased sensitivity to proteasome inhibitors**

We then elected to assess the interaction between **BRAF** inhibitors and response to proteasome inhibitors in colorectal cancer, a malignancy in which the **BRAF** mutation confers poor prognosis in the metastatic setting. To this end, we generated a **BRAF** V600E isogenic cell line using LIM1215 cells, which are WT for **KRAS, BRAF, and PIK3CA** (18, 26). Using a previously developed methodology (20), we infected LIM1215 cells with a recombinant adeno-associated viral vector carrying the **BRAF** V600E allele. After selection, we isolated two independent clones in which the mutation was introduced (KI) by homologous recombination in heterozygosity under the gene’s own promoter. Bortezomib and carfilzomib preferentially inhibited the growth of **BRAF** KI clones with respect to the parental counterpart (Fig. 4A and B). This confirmed that our findings in the breast cancer HME-1 matrix can also be applied to the colorectal cancer cell line LIM1215.

Taking advantage of recent molecular profiling efforts in which the genomic landscape of a large panel of cell lines was characterized (27, 28), we sought to further validate this pharmacogenomic relationship using 12 colorectal cancer cell lines harboring **BRAF** V600E mutations. In addition, we selected eight colorectal cancer cell lines WT for **BRAF** and **KRAS** as negative controls. We independently verified by Sanger sequencing the mutational status of selected hotspots, including **BRAF** exon 15, **KRAS** exons 2–3–4, **NRAS** exons 2–3, and **PIK3CA** exons 9–20. We observed that colorectal cancer cell lines with **BRAF** V600E mutations were particularly responsive to carfilzomib, whereas WT cells were significantly less affected (P < 0.05; Fig. 4C). In addition, we showed that sensitivity to carfilzomib is independent of the PTEN or RB1 expression status in colorectal cancer cell lines (Fig. 4D).

These results confirmed that oncogenic **BRAF** is a novel determinant of sensitivity to proteasome inhibitors. However, the presence of few outlier nonresponder cell lines highlighted the potential influence of additional factors, beyond PTEN or RB1 in shaping drug response.

We have recently proposed that EGFR expression can be a determinant of resistance to **BRAF** or **MEK** inhibitors in **BRAF-mutant** colorectal cancer cells (29). We asked whether EGFR expression could also be related to the lack of activity of the proteasome inhibitor in some colorectal cancer models. However, we did not detect any
association between response to carfilzomib and EGFR expression in this panel of cancer cells, independently from BRAF status (Fig. 4D).

Next, we sought to investigate whether the addiction of BRAF-mutant cells to proteasome function was dependent upon the activity of the BRAF V600E-mutant

Figure 2. Drug profiling of isogenic HME-1 cells uncovers bortezomib as a preferential inhibitor of BRAF/KRAS/PIK3CA–mutant cells. Magnification of a drug cluster from the Pharmarray panel. The cell line genotype is shown on the vertical axis. Genotypes are defined according to the color code indicated in the legend. Cells carrying the indicated genetic alterations were clustered using a hierarchic unsupervised algorithm based on their response profile versus the whole library; drug names included in the cluster are listed on the horizontal axis at the top of the panel. For each compound, the lowest concentration used was annotated with the number 1, intermediate concentration with 2, and the highest with 3. The drug name is followed by the molecular target on which the compound is reported to act (in brackets). Red-colored boxes indicate drugs that, at the indicated concentrations, preferentially inhibited the growth of mutated cells, whereas green boxes show compounds to which mutated cells were more resistant compared with the WT counterpart. Black boxes indicate no significant difference in response between mutant and parental cells, whereas gray boxes indicate experiments not performed.
protein. To this aim, two BRAF-mutant colorectal cancer lines sensitive to proteasome inhibition (SNU-C5 and LS411N) were treated with carfilzomib and with the BRAF V600E inhibitor PLX4720, alone or in combination. Indeed, PLX4720 cotreatment impaired the response to the proteasome inhibitor and antagonism between these drugs was observed in both cell lines (Fig. 5). These findings suggest that the persistent activation of BRAF V600E signaling is required for the activity of proteasome inhibitors in BRAF-mutant colorectal cancer cell lines.

Finally, we tested the in vivo efficacy of the proteasome inhibitor carfilzomib as single agent on BRAF-mutant xenografts. To this aim, we used immunodeficient mice xenografted with human RKO cells. Nineteen days after cell injection, palpable tumors were present in all animals, and cohorts of mice were treated with vehicle or carfilzomib. Figure 6A shows that treatment of mice with...
carfilzomib generally elicited a potent growth inhibition of RKO colorectal cancer tumors. Moreover, proteasome inhibitor promoted severe shrinkage in most of the individual treated tumors (Fig. 6B). These encouraging results support the clinical testing of carfilzomib in BRAF-mutant metastatic patients with colorectal cancer.

Discussion

Establishing pharmacogenomic relationships between genetic aberrations and targeted therapies is an important goal for researchers and clinicians in the era of “precision medicine”. However, the presence of a single genetic lesion that is known to be a potential driver of malignant proliferation in a particular cancer does not always predict a priori response to treatment. Indeed, recent clinical evidence has indicated that combinations of genetic alterations within the same tumor can influence drug response (12, 14). Thus, the development of a model in which mutations can be systematically combined and tested for drug sensitivity or resistance is of increasing importance.

Several previous studies have attempted to unveil cancer pharmacogenomic relationships. Isogenic cell models able to effectively recapitulate single genetic aberrations have been employed extensively to establish binary drug-genotype associations (20, 30, 31). Nevertheless, limited efforts have been dedicated to dissect the role of combinations of mutations in determining drug response. Among these studies, it is worth mentioning the use of isogenic cell lines to evaluate the influence of KRAS or TP53 status on the sensitivity to...
specific anticancer therapies in defined genetic backgrounds (32–35).

This report aimed to identify new pharmacogenomic relationships by screening 43 selected compounds on a panel of isogenic models harboring multiple cancer associated alterations. In comparison with previous studies, two major improvements have been implemented. First, we employed epithelial human cell models that closely recapitulated combinations of cancer mutations. Indeed, KI of nucleotide changes in oncogenes and knockdown of tumor suppressor genes were coupled to build a genetic ‘matrix’ in the human breast epithelial cell line HME-1 (15). The advantage of this matrix is that expression levels of mutant oncoproteins are similar to levels observed in human tumors, as they are controlled by endogenous genomic elements. Second, we screened a panel of 43 selected compounds, including a number of last generation and FDA-approved targeted therapies, to maximize the translational impact of the screening outcome.

The ‘Pharmarray’ approach that we previously developed (20) was then applied to analyze the drug screening profiles of the combinatorial HME-1 ‘matrix’ (15). By the use of the cell matrix, previous observations that EGFR KI cells show increased sensitivity to the EGFR kinase inhibitors gefitinib and erlotinib (20) were extended to novel HER-targeted agents such as vandetanib, canertinib, and lapatinib. Consistent with previous studies, this phenotype was partially rescued by the silencing of the PTEN tumor suppressor gene, confirming that our model system can recapitulate complex pharmacogenomic relationships found in lung tumors (12). We envision that the use of more dedicated methods of analysis of the Pharmarray data will unveil other similar interactions in which the drug response is impacted by PTEN or RB1 silencing in specific KI genotypes. Indeed, those relationships might become evident by systematically analyzing drug responses normalized versus different genotypes instead of WT cells.

However, in the present work, we aimed to show novel pharmacogenomic relationships that were not significantly affected by the concomitant inactivation of the tumor suppressor genes analyzed in the matrix. Pharmarray analysis showed that the BRAF-mutant cells were preferentially targeted by the proteasome inhibitor bortezomib independently from the silencing of PTEN or RB1. The genotype-specific activity of bortezomib was corroborated by the use of an irreversible proteasome inhibitor, carfilzomib, suggesting that this effect was due to target inhibition and not to peculiar pharmacologic properties of bortezomib. Interestingly, this pharmacogenomic relationship was also observed in KRAS G13D-mutant cells albeit less pronounced than in BRAF V600E cells. Our results, therefore, support claims that proteasome inhibitors, such as MG132 and bortezomib, display synthetic lethality with respect to KRAS mutations in cancer cells (36).

Selective targeting of BRAF-mutant cells by proteasome inhibitors was not affected by concomitant inactivation of PTEN or RB1 tumor suppressors. This is different from what was previously reported for BRAF/MEK inhibitors...
as concomitant mutational inactivation of PTEN or RB1 diminished response to those agents in melanomas harboring BRAF V600E (37). Therefore, targeting the proteasome may represent a valuable alternative to BRAF or MEK kinase inhibition for the treatment of BRAF-mutant tumors harboring PTEN or RB1 alterations.

BRAF inhibitors showed poor activity also in BRAF mutated metastatic colorectal cancer (17, 29), and novel treatment strategies are required to improve treatment options and survival in such patients.

Although bortezomib was the first proteasome inhibitor to show antitumor activity against a variety of hematologic malignancies, it demonstrated poor efficacy as a single agent in both phase 1 and 2 trials on a broad range of solid tumors, including colorectal cancer (38–40). Carfilzomib is the first new-generation FDA-approved proteasome inhibitor. Differently from bortezomib, carfilzomib is an irreversible inhibitor, characterized by a potent and persistent proteasome inhibition and greater selectivity for the chymotrypsin-like activity of the proteasome (41). It showed antitumor activity on xenografted cancer cell lines originating from solid tumors (42). A phase 1 study of carfilzomib has shown that it is well tolerated with consecutive day dosing (43), and a phase 1b/2 study on relapsed solid tumors is ongoing [http://www.clinicaltrials.gov].

For the abovementioned reasons, we sought to confirm the preferential targeting of BRAF-mutant cells by carfilzomib in cancer models of colorectal cancer origin. Importantly, we demonstrated that carfilzomib preferentially inhibited the growth of mutant BRAF with respect to the WT colorectal cancer cell lines, corroborating the results obtained in the isogenic models. Carfilzomib exhibited a potent antineoplastic activity as single agent also in vivo on the RKO BRAF-mutant colorectal cancer cell line.

The encouraging results from preclinical trials and the findings reported in this work suggest that new-generation irreversible proteasome inhibitors may represent a valuable approach to target colorectal cancer cells harboring the BRAF V600E mutation.

It should be acknowledged that our screening showed exceptions to the selective targeting of BRAF-mutant colorectal cancer cells by carfilzomib. We believe that the characterization of those outliers is important to understand the basis of the genotype-specific inhibition by antiproteasome compounds. In addition, this approach may help in better defining the subset of patients that most likely would benefit from antiproteasome treatments because there are currently no predictive biomarkers for this class of agents.

We hypothesized that some of those exceptions may be explained by known mechanisms of resistance to BRAF inhibitors in BRAF-mutant colorectal cancer cells. It was shown that EGFR expression confers primary resistance to BRAF inhibition in BRAF V600E–mutant colorectal cancer (29). Nevertheless, EGFR expression did not correlate with sensitivity to proteasome inhibitors in our cell panel. Another hypothesis relies on the observation that BRAF-mutant colorectal cancer samples are frequently associated to microsatellite instable (MSI) phenotype (44). As many BRAF-mutant cell lines employed in the present study are MSI, including the LIM1215 KI BRAF isogenic models, we cannot rule out the possibility that microsatellite instability contribute together with BRAF V600E mutation to increase the sensitivity to proteasome inhibition.

To our knowledge, this is the first report to show that proteasome inhibition could act preferentially on cancer cells with oncogenic BRAF. Mechanistically, we speculate that BRAF-mutant cells may experience a nononcogenic addiction to the proteasome function because the protein degradation mediated by the ubiquitin–proteasome system is needed to counterbalance the proteotoxic stress induced by the mutant protein. Indeed, different oncogenes have been associated to proteotoxic stress responses (45) and phenomena of nononcogenic addiction to the proteasome activity have been shown also for KRAS-mutant cells (36, 46). The evidence that—following proteasome blockade—a higher accumulation rate of ubiquitinated proteins occurred in mutant cells with respect to the WT supports our model. Importantly, in support of our hypothesis, we also showed that the dependence from
proteasome function is dependent upon the persistence of BRAF V600E activity.

In conclusion, we have shown that isogenic models in the “matrix” described in this article can be exploited for synthetic lethality screenings to identify drugs that are selectively toxic for cancer cells carrying complex tumor genotypes. Indeed, this analysis led to the identification of the new, potentially relevant drug–genotype correlation between BRAF mutation and proteasome inhibition.

We acknowledge that combinations of multiple drugs are often needed to maximize the antitumor effect and to delay the onset of resistance (47). The next challenge of personalized medicine will be tailoring the right combinatorial therapy to the right complex tumor genotype. Indeed, approaches to perform high-throughput screenings of combinations of compounds have been published and showed remarkable results also in the analysis of BRAF-mutant melanoma cells (48, 49). Therefore, we envision that the screening of drug combinations on specific components of the matrix, such as genotypes harboring BRAF mutation, will represent a valuable strategy to unveil more effective therapy–genotype correlations.

Disclosure of Potential Conflicts of Interest
A. Bardelli has ownership interests, including patents, is a shareholder, and is a member of the Scientific Advisory Board at Horizon Discovery Ltd, United Kingdom, to which some of the cell lines described in this article have been licensed through the University of Turin. No potential conflicts of interest were disclosed by the other authors.

References
Proteasome Inhibitors Are Active on BRAF-Mutant Cells


Molecular Cancer Therapeutics

**BRAF V600E Is a Determinant of Sensitivity to Proteasome Inhibitors**

Davide Zecchin, Valentina Boscaro, Enzo Medico, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-13-0243

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2013/10/09/1535-7163.MCT-13-0243.DC1

Cited articles
This article cites 48 articles, 17 of which you can access for free at:
http://mct.aacrjournals.org/content/12/12/2950.full#ref-list-1

Citing articles
This article has been cited by 4 HighWire-hosted articles. Access the articles at:
http://mct.aacrjournals.org/content/12/12/2950.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, use this link
http://mct.aacrjournals.org/content/12/12/2950.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.