Published OnlineFirst June 4, 2008; DOI: 10.1158/1535-7163.MCT-07-2308

Spotlight on Molecular Profiling

In vitro differential sensitivity of melanomas to phenothiazines is based on the presence of codon 600 BRAF mutation

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Abstract

The panel of 60 human cancer cell lines (the NCI-60) assembled by the National Cancer Institute for anticancer drug discovery is a widely used resource. We previously sequenced 24 cancer genes in those cell lines. Eleven of the genes were found to be mutated in three or more of the lines. Using a pharmacogenomic approach, we analyzed the relationship between drug activity and mutations in those 11 genes (*APC, RB1, KRAS, NRAS, BRAF, PIK3CA, PTEN, STK11, MADH4, TP53*, and *CDKN2A*). That analysis identified an association between mutation in BRAF and the antiproliferative potential of phenothiazine compounds. Phenothiazines have been used as antipsychotics and as adjunct antiemetics during cancer chemotherapy and more recently have been reported to have anticancer properties. However, to date, the anticancer mechanism of action of phenothiazines has not been elucidated. To follow up on the initial pharmacologic observations in the NCI-60 screen, we did pharmacologic experiments on 11 of the NCI-60 cell lines and, prospectively, on an additional 24 lines. The studies provide evidence that BRAF mutation (codon 600) in melanoma as opposed to RAS mutation is predictive of an increase in sensitivity to phenothiazines as determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt assay (Wilcoxon $P = 0.007$). That pattern of increased sensitivity to phenothiazines based on the presence of codon 600 BRAF mutation may be unique to melanomas, as we do not observe it in a panel of colorectal cancers. The findings reported here have potential implications for the use of phenothiazines in the treatment of V600E BRAF mutant melanoma. [Mol Cancer Ther 2008;7(6):1337–46]

Introduction

The NCI-60 panel is composed of 60 human cancer cell lines representing nine tissues of origin. The National Cancer Institute Developmental Therapeutics Program assembled the NCI-60 cell lines for an *in vitro* screen, and more than 100,000 compounds have been tested for anticancer activity in them since 1990 (1–5). As an adjunct to that extensive pharmacologic characterizations of the cells, they have been profiled more comprehensively by high-throughput studies at the DNA, RNA, protein, and functional levels than any other set of cells in existence (6–18). The resulting activity profiles have been proven information-rich in a variety of types of analyses and predictive algorithms (1, 19–26). Measures of the concentration required for 50% inhibition of growth (GI_{50}) of more than 42,000 compounds tested in the NCI-60 have been provided in a public database.

Recent studies have indicated that mutations in cancer genes may influence sensitivity to therapeutics targeted against the mutated gene product. Examples include the *BCR-ABL* fusion gene in chronic myeloid leukemia, which predicts sensitivity to imatinib (27), and kinase domain mutations of *EGFR* in lung cancer, which predict sensitivity to gefitinib (28, 29) and erlotinib (30). Moreover, there is evidence that mutations in cancer genes can indirectly affect the response to chemotherapy. For example, resistance to trastuzumab, a monoclonal antibody against overexpressed or amplified ERBB2 protein, is associated with the presence of mutations of *PTEN*, a downstream signaling effector of ERBB2 (31).

Received 2/1/08; revised 4/15/08; accepted 4/18/08.

Grant support: Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research and Wellcome Trust.

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doi:10.1158/1535-7163.MCT-07-2308

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The Cancer Gene Census designates 363 genes as ''cancer genes.''[6] More than 60 of them are causally implicated in cancer through the acquisition of small somatic intragenic mutations (32). In a previous study of the NCI-60, we characterized mutations in 24 of the 60 genes (13). In the present study, we identified an association between mutation in BRAF (codon 600) in melanoma and the antiproliferative activity of phenothiazine compounds. In a prospective manner, using an independent set of melanoma and colorectal lines, we then validated the prediction that BRAF mutation (codon 600), as opposed to RAS mutation, in melanoma is predictive of increased sensitivity to pharmacologic inhibition by phenothiazines.

Materials and Methods

Selection of Mutation Types for Statistical Analyses

In our mutation analysis of cancer genes in the NCI-60, we developed a pragmatic classification scheme for variants found in cancer cell lines based on information from previous mutational screens of both cancers and normal tissues (13). The classification has four strata and has been described previously (13). For the purposes of this study, we considered only the mutation types designated as likely oncogenic mutation and tentative oncongenic variant. Likely oncogenic mutation is defined as sequence changes that have been shown previously to be somatic mutations in human cancer and/or those consistent with the position and type of mutations for a given gene. This also included homozygous deletions of tumor suppressor genes. Tentative oncongenic variant is defined as sequence changes although located similarly to known cancer mutations are different from those reported previously or are present as heterozygous variants in tumor suppressor genes other than missense sequence changes in *TP53* (13).

Statistical Analyses of the Relationship between Cancer Gene Mutations and Drug Activity

In the first instance, we interrogated the relationship between mutations in 11 cancer genes and activity of 7,794 of the 42,000 compounds for which NCI-60 screening data have been made publicly available. The 7,794 compounds were selected according to the following criteria: (*a*) the compounds had to have been tested at least twice in the NCI-60 screen; (*b*) the compounds had to have GI_{50} values within the range of the dose-response curve for at least 50% of the cell lines; and (*c*) the SD of -log GI_{50} values of the NCI-60 cell lines had to be $\geq$1 log unit. Selecting based on those criteria provided us with a more robust set of data for analysis than if we had used the full dataset.

We compiled 50% inhibition data (-$\log_{10}$ GI_{50}) for the 7,794 compounds from the Developmental Therapeutics Program Web site[7] and imported them as a matrix into the R statistical programming environment.[8] Another matrix, the mutation status of 11 cancer genes (*APC, RB1, KRAS, NRAS, BRAF, PIK3CA, PTEN, STK11, MADH4, TP53*, and *CDKN2A*) with salient mutations in the NCI-60, was also imported into R. We have identified previously by sequence analysis that those genes contained likely oncogenic mutations in three or more cell lines of the NCI-60. We also included cell lines with tentative oncogenic variants in any of those 11 genes (13). First, we used a Wilcoxon rank-sum test to interrogate the relationship between the mutations and the activity values of the 7,794 compounds. Second, we employed a pathway approach to the analysis to enrich for compounds whose activity appeared to be correlated with mutations of genes in the same pathway. That was done by combining cell lines with mutations of genes involved in the same pathway. For example, to identify active compounds that may act on the WNT pathway, cell lines mutant for either *APC* or *CTNNB1* were placed in the same group. We assessed the contributions to differential sensitivity of the following mutation combinations: *APC* and/or *CTNNB1, RAS* and/or *BRAF, RB1* and/or *CDKN2A, RAS* and/or *PIK3CA, PIK3CA* and/or *PTEN*, and *RAS* and/or *PIK3CA* and/or *PTEN*. A false discovery rate (FDR) of 0.25 was applied to the nominal Wilcoxon *P* values to correct for testing multiple hypotheses (15). Compounds with the most significant *P* values (adjusted $P < 0.05$) following correction for multiple testing were selected for follow-up analysis.

Cell Lines

We used a total of 35 cell lines in pharmacologic experiments to follow up on the NCI-60 screen data. Eleven of the 35 were from the NCI-60 panel: SKMEL-28, HT-29, UACC-257, M14, MALME-3M, SKMEL-2, SW620, DU145, A498, MDA-MB-231, and T47D. Twenty-four additional lines were provided by the Cancer Genome Project for experimental use: SKMEL-1, SKMEL-3, SKMEL-24, HT144, WM115, IGR-1, SKMEL-30, MEL-JUSO, IPC-298, HMVII, HMCB, MeWo, CHL-1, LS-411N, RKO, COLO-741, NCI-H508, LoVo, SW948, LS174T, LS123, NCI-H716, NCI-H630, and HT55.

Cell Culture

All cells were routinely maintained in RPMI 1640 (BioWhittaker) supplemented with 10% fetal bovine serum and 2 mmol/L L-glutamine (Invitrogen). All cultures were grown in an atmosphere of 5% CO_2 and 90% relative humidity at 37°C.

Compounds

Phenothiazine compounds, NSC 46061 [phenothiazine,10-[3-(4-methyl-1-piperazinyl)propyl]-2-(trifluoromethyl)-,disuccinate] and NSC 17474 [phenothiazine,10-[3-(4-methyl-1-piperazinyl)propyl]-2-(trifluoromethyl)-, dihydrochloride], were obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program. A stock 100 mmol/L solution of each compound was prepared in 99% DMSO (Sigma), and aliquots were stored at -80°C.

Proliferation Assay

Phenothiazine antiproliferative activity was determined by measuring formazan production from 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS; Promega), with drug

[6] http://www.sanger.ac.uk/genetics/CGP/Census
[7] http://dtp.nci.nih.gov/docs/cancer/cancer_data.html
[8] http://www.r-project.org

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concentrations tested in triplicate in each experiment. Serial dilutions of the phenothiazine stock solutions were prepared in medium at room temperature. Cell lines were seeded at a density of 5,000 to 7,000 per well. At 48 h after seeding, attached cell types were washed by aspiration of the supernatant and 150 μL drug-containing medium was added. Suspension cell types (SKMEL-1) were not aspirated after seeding and 150 μL drug-containing medium was directly added to the wells. Another 48 h later, the drug solution was aspirated from attached cell types, and 120 μL MTS-containing medium was added according to the protocol of the manufacturer (Promega). For suspension cell types, drug solution was not aspirated and 20 μL MTS was added directly to the wells. The plates were incubated at 37°C and read at 490 nm between 1 and 4 h.

Data Analysis

Using GraphPad Prism 4.02 (GraphPad Software), we log-transformed the drug concentrations and did nonlinear regression on the A_{490} data using the sigmoidal dose-response model with variable slope. Mean of the 50% effective concentration (EC_{50}), SE, and 95% confidence intervals were determined from the logistic fits. Data are presented as the mean EC_{50} of triplicate experiments.

Results

Gene-Compound Associations

In total, we interrogated the relationships between mutations in 11 individual genes and 6 gene combinations and the activity patterns of 7,794 drugs tested in the NCI-60. The data are presented as the distribution of nominal Wilcoxon *P* values (Fig. 1).

After we corrected the nominal *P* values for testing of multiple hypotheses with FDR, we found that 4 of the 17 gene mutation categories were significantly associated with any of the compounds. The two strongest single gene-compound associations were found for *CDKN2A* and *BRAF*. No significant associations were detected for individual genes *APC, RB1, KRAS, NRAS, TP53, PIK3CA, PTEN, STK11*, or *MADH4*.

For two of the gene combination pathways, RB1/CDKN2A and RAS/BRAF, there were significant gene-compound associations. Mutations of *APC* and *CTNNB1* in combination did not yield compounds that were statistically significant associations. Similarly, combinations consisting of *PIK3CA* and *PTEN, RAS* and/or *PIK3CA*, or *RAS* and/or *PIK3CA* and/or *PTEN* did not yield statistically significant associations with compounds.

We found that the compounds significantly associated with *CDKN2A* mutation became slightly more statistically

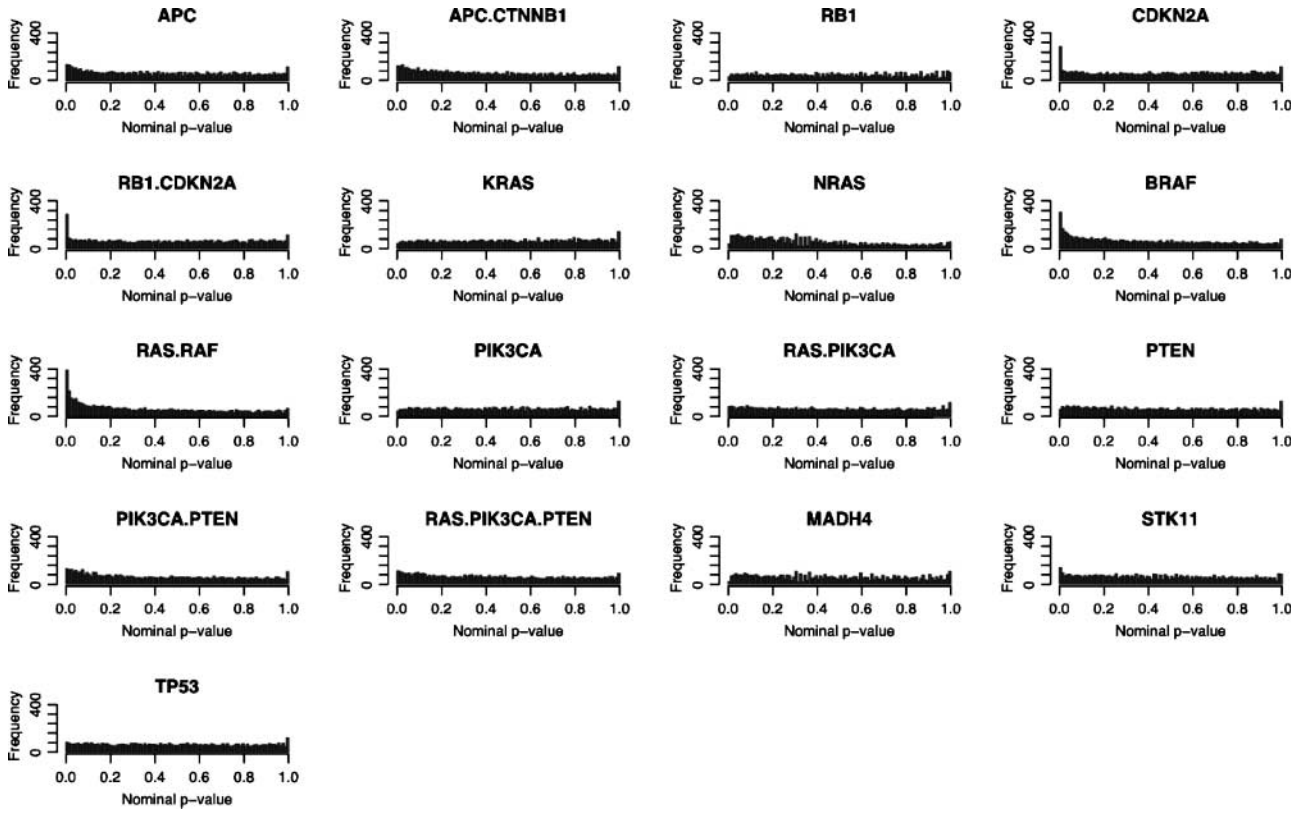

Figure 1. Histograms showing the relation between mutations of 11 individual gene mutation categories and 6 gene mutation combination categories and patterns of growth inhibition for 7,794 compounds tested in the NCI-60 cell line screen. The variable calculated for each drug has the form of a Wilcoxon rank-sum *P* value. $P < 0.05$, a compound that tends to be active in either wild-type or mutant cells for the gene or combination of genes.

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significant with the addition of cell lines with a *RB1* mutation (Supplementary Table S1).[9] Interestingly, the most significant association with mutation of *CDKN2A, RB1*, or the two in combination was camptothecin acetate (NSC 95382), an anticancer agent whose mechanism of action is inhibition of DNA topoisomerase I (Supplementary Table S1 and S2).[9] Cell lines mutant for *CDKN2A* were more sensitive to camptothecin acetate than were wild-type cells.

BRAF yielded the most statistically significant associations between mutation and compound activity (Table 1). In contrast, *KRAS* and *NRAS* did not yield any associations that met the stringent FDR criterion for statistical significance. One of the compounds, NSC 46061, identified as most significantly associated with mutation in RAS-BRAF pathway analysis was more significantly associated with *BRAF* mutation alone (Supplementary Table S3).[9]

Analysis of BRAF Mutation and Activity of 7,794 Drugs in the NCI-60

Table 1 shows the top 50 compounds (of the 7,794 analyzed) that were most statistically significantly related in their activity to BRAF mutation status. The most prominent drug group represented was the phenothiazine class, exemplified by NSC 676879, NSC 46061, NSC 17474, NSC 676963, NSC 677395, and NSC 674092. The second most prominent was the class consisting of MEK inhibitors, exemplified by NSC 706829 and NSC 354462. The third class consisted of the naphthazarins exemplified by NSC 661416 and NSC 661941.

The analysis showed ~6-fold difference in median (-log GI_{50}) values between BRAF mutant and BRAF wild-type cell lines for phenothiazine compounds (Supplementary Fig. S1).[9] There was a 15-fold difference in the median (-log GI_{50}) values between BRAF mutant and wild-type cell lines for the MEK inhibitor compounds (Supplementary Fig. S2).[9] That finding was recently confirmed using a different MEK inhibitor, CI-1040, by *in vitro* cell viability assays (15).

In our analysis, the strongest statistical correlation observed was that between BRAF mutation and activity of phenothiazines. Six of the 14 phenothiazines in the data set were in the top 50 of the 7,794 compounds in their statistical association with BRAF mutation. That observation strongly suggested that phenothiazines, as a class, are significantly associated with BRAF mutation (Table 1).

A display of the GI_{50} values of the top phenothiazine compounds shows that BRAF mutant cell lines were most sensitive to inhibition (Fig. 2; Supplementary Figs. S3 and S4).[9] We also observed that the melanoma cell line panel was the most sensitive to growth inhibition. However, there was a strong association between melanoma status and BRAF mutation status (8 of the 9 NCI-60 melanoma cell lines have V600E BRAF mutations). However, there were circumstantial suggestions that the association is with the mutation. First, the only NCI-60 melanoma cell line (SKMEL-2) that is wild-type for BRAF (a RAS mutant) was less sensitive to the phenothiazine compounds than were the rest of the melanoma cell lines in the panel (Fig. 2; Supplementary Figs. S3 and S4).[9] Second, the tendency toward increased sensitivity of BRAF mutant cell lines to phenothiazines was also observed in colorectal cancers. That is, the GI_{50} values of the two V600E BRAF mutant colorectal cancer cell lines, HT29 and COLO205, showed a similar trend; they were more sensitive to phenothiazines than were the RAS mutant colorectal cancer cell lines, SW620, HCC-2998, HCT-116, and HCT-15.

Confirmation of the Antiproliferative Activity of Phenothiazine Compounds in the NCI-60 Cell Lines

We selected 11 BRAF mutant and wild-type subsets of the NCI-60 cells and assessed the antiproliferative activity of piperazine phenothiazine compounds (NSC 46061 and NSC 17474) in those lines. Included were one colorectal and five melanoma V600E BRAF mutants, the one G464V BRAF mutant line (which also has a RAS mutation). The BRAF wild-type cell lines were selected to include the single melanoma line in the NCI-60 without a BRAF mutation and one RAS mutant colorectal cancer cell line. The other BRAF wild-type lines were a renal, a breast, and a prostate cancer cell line, each selected as representative of the tissue type.

Overall, the V600E BRAF mutant lines appeared to be somewhat more sensitive to phenothiazines than were the G464V BRAF mutant, RAS mutant, or RAS/BRAF wild-type lines. The EC_{50} values averaged ~2-fold higher in V600E BRAF wild-type cell lines, including the one NRAS (Q61R) mutant melanoma, SKMEL-2 (Supplementary Tables S4 and S5; Supplementary Figs. S5 and S6).[9] Excluding the EC_{50} value of the G464V BRAF mutant cell line, the differential response to NSC 46061 for V600E BRAF mutants and wild-type cell lines was statistically significant (Wilcoxon $P = 0.020$). The differential response to NSC 17474 for V600E BRAF mutant and wild-type cell lines was also statistically significant (Wilcoxon $P = 0.007$). The consistency of the antiproliferative effect of piperazine phenothiazines is shown by the use of identical compounds, NSC 46061 and NSC 17474, with different salt forms.

Validation of the Differential Sensitivity of V600E BRAF Mutant, RAS Mutant, and RAS/RAF Wild-type Melanoma to Phenothiazine NSC 17474

Following the confirmation of differential phenothiazine activity in the NCI-60 cell lines based on V600E BRAF mutation, we sought to replicate the finding. First, we examined a larger and independent set of melanoma cell lines with various BRAF and RAS mutations,[10] by performing MTS assays following 48 h of treatment with piperazine phenothiazine compound NSC 17474.

Based on the EC_{50} values of an independent set of 13 melanoma cell lines, we found that BRAF mutation at

[9] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

[10] http://www.sanger.ac.uk/CGP/COSMIC

Published OnlineFirst June 4, 2008; DOI: 10.1158/1535-7163.MCT-07-2308

Table 1. Top 50 compounds associated with BRAF mutation

NSC ID	Chemical name	Nominal *P*	Adjusted *P*	Mean fold difference
676879	Phenothiazine, 2-azido-10-[4-(4-methyl-1-piperazinyl)butyl]-difumarate	2.30E-07	1.80E-03	5.6
46061	Phenothiazine, 10-[3-(4-methyl-1-piperazinyl)propyl] -2-(trifluoromethyl)-, disuccinate	5.70E-07	2.20E-03	5.5
658874	No name	4.80E-06	1.20E-02	6
644902	Benzo[*b*]naphtha[2,3-d]furna-6,11-diome, 4-chloro-3-hydroxy	3.30E-05	2.10E-02	5.7
708550	No name	3.10E-05	2.10E-02	5.5
661193	Propanamide, 2-[4-[[4-chlorophenyl]carbonyl]-2-chlorophenoxy]-2-methyl-*N*-[2-(dimethylamino)ethyl]	1.20E-05	2.10E-02	5.5
678932	1H-Benzimidazole-4-carboxamide, *N*-[2-(dimethylamino)ethyl]-2-(4-pyridinyl)-, hydrochloride	2.80E-05	2.10E-02	5.7
658443	2-Chloro-3-amino-5,8-dihydroxy-1,4-naphthoquinone	1.90E-05	2.10E-02	5.8
717507	No name	1.40E-05	2.10E-02	4.7
715767	No name	1.90E-05	2.10E-02	5.2
689620	No name	2.20E-05	2.10E-02	5.6
664565	No name	2.80E-05	2.10E-02	4.6
626482	1,5,10-Trihydroxy-7-methoxy-3-methyl-1H-naphtho[2,3-c]pyran-6,9-dione	5.80E-05	2.30E-02	6.3
686324	1-Methyl-3-(4-[2-dimethylaminoethoxy]phenyl)-2-phenylindolizine	6.00E-05	2.30E-02	5.6
17474	Phenothiazine, 10-[3-(4-methyl-1-piperazinyl)propyl]-2-(trifluoromethyl)-dihydrochloride	4.90E-05	2.30E-02	5.6
708551	No name	5.30E-05	2.30E-02	5.2
303612	Mequitazine	6.30E-05	2.30E-02	5.6
718579	No name	5.80E-05	2.30E-02	5.6
676963	3-Azido-10-[4-(4-(4-benzoylphenyl)methyl)-1-piperazinyl) butyl]phenothiazine, bismaleate salt	4.30E-05	2.30E-02	5.6
706829	1,6-Bis[4-(4-aminophenoxy)phenyl]diamantine	5.00E-05	2.30E-02	6.1
715580	No name	5.60E-05	2.30E-02	4.8
661941	2-(3-Chloropropyloxy) naphthazarin	7.20E-05	2.50E-02	6.5
661416	2-(2-(2-Methoxyethoxy)ethoxy)naphthazarin	7.50E-05	2.50E-02	6.3
354462	Hypothemycin	9.50E-05	3.00E-02	6.6
635366	No name	1.10E-04	3.30E-02	5.6
707847	No name	1.00E-04	3.30E-02	4.6
676931	1-Amino-2-hydroxy-3-naphthoic acid hydrochloride	1.10E-04	3.30E-02	4.7
708073	No name	1.20E-04	3.50E-02	4.7
677395	2-Azido-10-[(4-dimethylamino)butyl]phenothiazine, oxalate salt	1.40E-04	3.70E-02	5.7
656204	Discorhabdin G	1.50E-04	3.70E-02	5.6
721393	No name	1.50E+04	3.70E-02	5.6
699452	No name	1.50E-04	3.70E-02	5.6
627991	Benzo[*g*]pteridine-2,4-dione,8-chloro-10-(4-chlorophenyl)-3-methyl-	1.60E-05	3.80E-02	5.6
79563	No name	1.60E-05	3.80E-02	4.6
617131	No name	1.70E-05	3.80E-02	5.7
674092	Quinoline-4-carboxamide,*N*,*N*′-[(1,4-piperizinediyl)bis (3,1-propanediyl)]bis(2-phenyl-,dihydrochloride	1.70E-05	3.80E-02	5.4
682223	2H-Pyrano[3,2-g]quinoline-5,10-dione,4-hydroxy-2,2,6-trimethyl-	1.90E-04	4.00E-02	5.7
708864	No name	1.90E-04	4.00E-02	4.7
717827	No name	2.00E-04	4.10E-02	4.3
669995	No name	2.10E-04	4.20E-02	5.4
658450	2-Acetamido-6-methyl-8-hydroxy-1,4-naphthaquinone	2.60E-04	4.80E-02	5.6
649750	No name	2.50E-04	4.80E-02	4.7
90829	No name	2.90E-04	5.10E-02	4.8
681603	No name	2.90E-04	5.10E-02	4.7
713546	No name	2.80E-04	5.10E-02	5.5
707452	No name	3.00E-04	5.10E-02	4.7
708075	No name	3.10E-04	5.20E-02	5.6
13028	No name	3.30E-04	5.40E-02	5.6
689078	No name	3.50E-04	5.70E-02	5.5
656211	No name	4.00E-04	6.10E-02	5.6

NOTE: NSC ID, chemical name, Wilcoxon nominal *P* values, and FDR-adjusted *P* values of the top 50 compounds associated with BRAF mutation.

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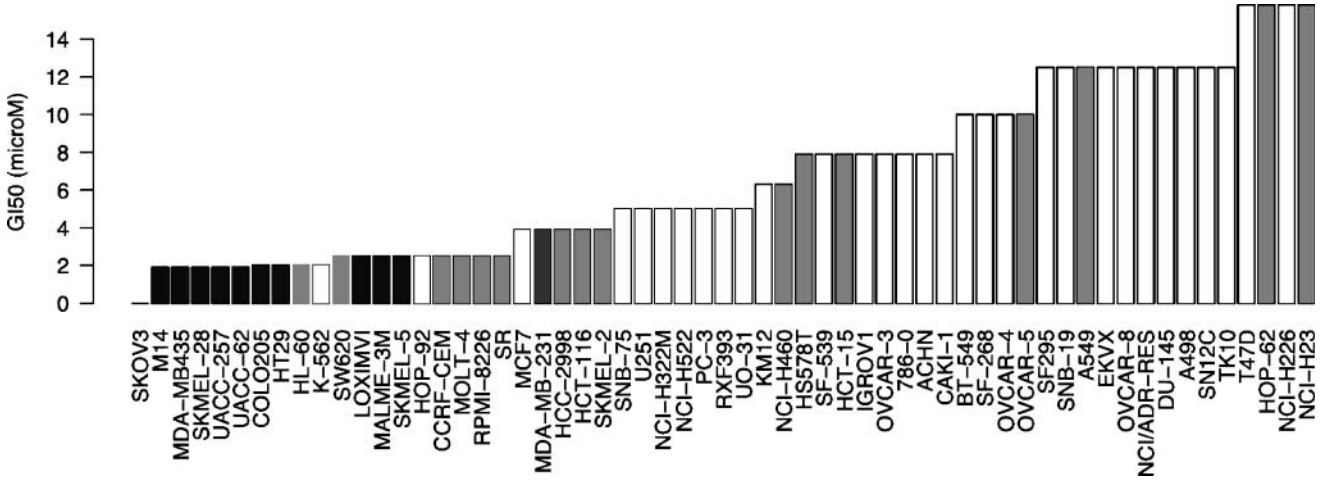

Figure 2. GI_{50} values for phenothiazine NSC676879 in the NCI-60 cell line panel. *Black,* V600E BRAF mutant lines; *light gray,* RAS mutant lines; *dark gray,* G464V BRAF mutant and RAS mutant line; *white,* BRAF and RAS wild-type lines. SKOV3 ovarian line was not tested.

codon 600 is predictive of an increase in sensitivity to inhibition by NSC 17474 when compared with all other genotypes studied (Fig. 3; Supplementary Table S6; Supplementary Fig. S7).[9] The results also show that the presence of RAS mutation and/or non-codon 600 BRAF mutation is associated with decreased sensitivity to inhibition by NSC 17474 when compared with codon 600 BRAF mutants (Wilcoxon $P = 0.007$). Similarly, absence of both RAS and BRAF mutation in melanoma cell lines was associated with decreased sensitivity to inhibition by NSC 17474 in comparison with V600E BRAF mutants.

Validation of Differential Sensitivity of V600E BRAF Mutant, RAS Mutant, and RAS/RAF Wild-type Colorectal Lines to Phenothiazine NSC 17474

After validating the predicted differential sensitivity between V600E BRAF mutant and RAS mutant melanomas to NSC 17474, we hypothesized that the phenomenon might extend to other tissue types. We have observed previously suggestions of a similar pattern of preferential sensitivity to phenothiazines in V600E BRAF mutant colorectal lines of the NCI-60, but there are only two such lines. Therefore, we acquired an independent set of 11 colorectal lines with various BRAF and RAS mutations.[10] As was done for the melanomas, we did MTS assays on that larger set of colorectal lines following 48 h of treatment with NSC 17474.

Based on the EC_{50} values we obtained for the independent set of 11 colorectal lines, the pattern of response to NSC 17474 is different from that observed in the melanoma cell lines (Fig. 4; Supplementary Table S7).[9] *BRAF* and *RAS* mutant colorectal lines had similar patterns of response to NSC 17474 (Wilcoxon $P = 1.0$). Although the *RAS/BRAF* wild-type colorectal lines were ~2-fold less sensitive on average than were the BRAF mutant and RAS mutant lines, there was considerable overlap between the groups (Supplementary Table S7; Supplementary Fig. S8).[9]

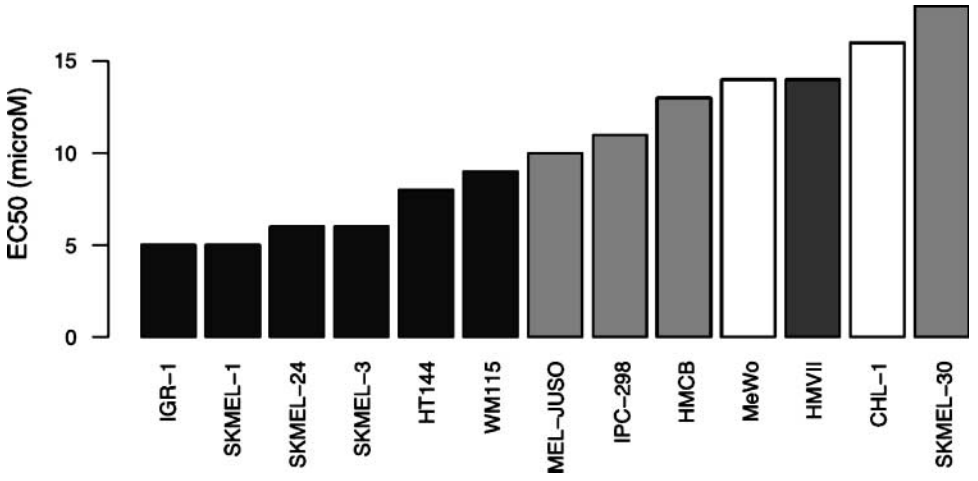

Figure 3. EC_{50} values for phenothiazine NSC 17474 in an independent set of melanoma cell lines. *Black,* codon 600 BRAF mutant lines; *light gray,* RAS mutant lines; *dark gray,* G469V BRAF mutant and RAS mutant line; *white,* BRAF and RAS wild-type lines.

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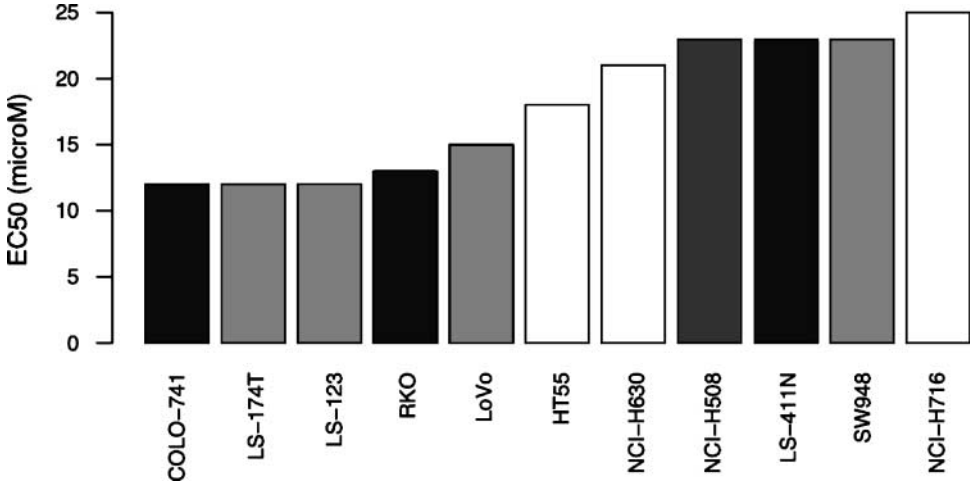

Figure 4. EC_{50} values for phenothiazine NSC 17474 in an independent set of colorectal cell lines. *Black,* V600E BRAF mutant lines; *light gray,* RAS mutant lines; *dark gray,* G596R BRAF mutant line; *white,* BRAF and RAS wild-type lines.

Discussion

We have identified compounds for which sensitivity of response was statistically significantly associated with mutations of *CDKN2A* or *BRAF* or with combinations of genes representing pathways in which those genes reside (*RB1* and/or *CDKN2A* and *RAS* and/or *BRAF*). However, we did not identify compounds statistically significantly associated with the remaining 13 gene mutation categories (FDR-adjusted $P < 0.05$). In some cases (e.g., with mutations of *APC, RB1, NRAS, STK11*, and *MADH4*), perhaps we lack the statistical power to make adequate comparisons between mutant and wild-type response to drugs. There is, however, reasonable statistical power for *CDKN2A* and *TP53*, and we do find compounds statistically significantly associated with mutation of *CDKN2A.* However, we do not find any compounds statistically significantly associated with mutation of *TP53* by the stringent FDR condition chosen.

We hypothesized that grouping cell lines with mutation of genes in the same pathway might aid in identifying compounds acting on that particular pathway. However, applying that approach to the APC-CTNNB1, RAS-PIK3CA, PIK3CA-PTEN, and RAS-PIK3CA-PTEN pathways did not yield more statistically significant associations between drug activity and mutations in those gene combinations. We did, however, identify statistically significant associations between pathway mutations of RB1 and/or CDKN2A and mutations of RAS and/or BRAF that had been identified through analysis of CDKN2A and BRAF independently.

The lack of effectiveness of the pathway approach for RAS and BRAF deserves particular note. There is evidence that RAS can signal through BRAF. That might lead us to expect that drugs showing particular effectiveness in cell lines with BRAF mutations might show similar effectiveness in RAS mutant lines. That did not seem to be the case for RAS mutations either separately or combined with BRAF. The reasons are unclear. It may be that there are several compensatory outlets of mutant RAS signaling and that abrogation of the MEK-extracellular signal-regulated kinase-mitogen-activated protein kinase pathway therefore has little effect. It may also be that mutant RAS predominantly signals through CRAF and that the drugs that have an effect with V600E BRAF mutations do not influence pathways modulated by CRAF.

There are several reports that the loss of PTEN is associated with resistance to several targeted chemotherapeutic agents including trastuzumab in breast cancer (31), gefitinib, in breast (33), endometrial (34), and prostate cancer cell lines (35) and resistance to imatinib in acute lymphoblastic leukemia (36). However, we did not observe those associations with trastuzumab, gefitinib, and imatinib because those chemotherapeutics were not included in the larger 42,000 compounds screened in the NCI-60 and subsequently not in the subset of 7,794 compounds. Even if those chemotherapeutic agents had been screened for anticancer activity in the NCI-60, we would likely lack statistical power to detect those associations given the small number of lines representative of each tissue type and relatively few PTEN mutant lines in the NCI-60.

BRAF yielded the most statistically significant associations between mutations and drug activity. Phenothiazine compound NSC 676879 was the most significant compound associated with mutation of *BRAF*. The activity pattern of three phenothiazine compounds in the NCI-60 showed that BRAF mutant cell lines were more sensitive to growth inhibition than were the RAS mutant and BRAF wild-type cell lines. We therefore prioritized the statistical association between increased phenothiazine activity and presence of BRAF mutation for follow-up experimental studies.

Overall, there appears to be a statistical association between increased antiproliferative activity of phenothiazines and BRAF mutation in melanoma. In the NCI-60, the increased activity of phenothiazine compounds in BRAF mutant cell lines of the NCI-60 seemed to be based on the presence of V600E BRAF mutation and did not extend to the G464V BRAF mutation. However, we did not have enough cell lines of differing BRAF and RAS mutation

Published OnlineFirst June 4, 2008; DOI: 10.1158/1535-7163.MCT-07-2308

status in the NCI-60 panel to make that assertion with confidence. In a larger and independent set of melanoma cell lines, however, we showed that the increased phenothiazine activity in melanomas is most apparent with codon 600 BRAF mutant melanomas. We found that cells mutant in BRAF at codons other than 600 and also RAS mutant and RAS/RAF wild-type melanomas were less sensitive to piperazine phenothiazines than were the codon 600 BRAF mutants.

The kinase activities of different codon 600 BRAF mutants are similar to one another (37). Therefore, it seems reasonable to assume *a priori* that all codon 600 BRAF mutants would respond similarly to drug inhibition, as we indeed found for the phenothiazines. However, we had only two additional codon 600, non-V600E BRAF mutants and would need larger numbers of such mutants to confirm that observation. The caveat is that codon 600 BRAF mutants other than V600E are rare.

It is known that mutations of the glycine-rich loop of BRAF (e.g., G464V and G469V) do not confer the same kinase activation on the BRAF protein as do codon 600 mutations. The G464V BRAF mutation is known to increase the kinase activity of BRAF modestly compared with mutations of codon 600 BRAF (38). Whereas the codon 600 BRAF mutation confers constitutive kinase activity on the BRAF protein, the glycine-rich loop BRAF mutations primarily disrupt the glycine-rich loop and kinase domain interaction, destabilizing the inactive BRAF conformation and stimulating BRAF activity (39). The kinase activity of the G469V mutant has not been tested. However, a different amino acid substitution at codon 469 (G469A) has been shown to confer higher basal kinase activity than does V600E mutation (38). Our drug response data show that the G469V BRAF mutant melanoma cell line was not as sensitive as the codon 600 BRAF mutants to inhibition by phenothiazines. Based on this observation, it would be interesting to test the hypothesis that small-molecule inhibitors of CRAF such as sorafenib may be particularly effective in melanomas that rely on CRAF signaling such as those with RAS mutation and/or glycine-rich loop BRAF mutation.

The two V600E BRAF mutant colorectal cancer cell lines in the NCI-60 seemed to show the same trend as did the V600E melanomas. That is, they were more sensitive to the phenothiazines than were the RAS mutant colorectal lines. However, in a larger and independent set of 11 colorectal cancer cell lines, we observed that the presence of V600E BRAF mutation was not associated with an increased sensitivity to phenothiazine. There was also no difference in the EC_{50} values among the V600E BRAF mutant, G596R BRAF mutant, and RAS mutant colorectal cancers. In fact, the EC_{50} values of the BRAF mutants, RAS mutants, and RAS/RAF wild-type colorectal lines overlapped.

That finding may mirror the different biological contexts of BRAF mutation in melanomas and colorectal cancers. The V600E BRAF mutation is the most common BRAF mutation in both types of cancer, but there are differences in the biology. BRAF mutation is an initiating event in melanoma. BRAF mutant and RAS mutant melanomas activate extracellular signal-regulated kinase differently. Mutant BRAF, especially V600E, directly activates MEK, whereas RAS mutant melanomas activate MEK indirectly through wild-type CRAF (37).

In colorectal tumor development, BRAF mutation occurs in the adenoma-to-carcinoma sequence, identical to the stage at which KRAS mutations occur (40). Therefore, it has been proposed that BRAF and KRAS mutations in colorectal cancers lead to similar phenotypic patterns (40).

Biologically, then, V600E BRAF and RAS mutations in melanomas are quite distinct from one another in their consequences, whereas in colorectal cancers V600E BRAF and RAS mutation seem to confer similar phenotypes. The difference we see between melanomas and colorectal cancers in their pharmacologic response to phenothiazines may reflect those biological distinctions.

Nevertheless, additional BRAF mutant colorectal lines would need to be screened to conclude a lack of association between V600E BRAF mutation and sensitivity to phenothiazines. Despite the relatively small numbers of melanoma cell lines we have tested, the results support a statistically significant association between the presence of V600E BRAF mutation and increased phenothiazine activity. Importantly, we have shown that the phenothiazine activity is not solely due to melanoma status but is due to the presence of the codon 600 BRAF mutation within a melanoma context. It would also be interesting to further investigate whether that differential effect of phenothiazines also occurs in other cancer types, for example, thyroid, with similar BRAF and RAS mutations.

To our knowledge, this is the first report of a differential in sensitivity of V600E BRAF mutant and RAS mutant melanoma cell lines to pharmacologic inhibition by phenothiazines. The differential response of melanomas may be evidence of the underlying antiproliferative mechanism of action of phenothiazine compounds. Several different mechanisms have been proposed to explain the antiproliferative activity of phenothiazines: inhibition of protein kinase C activity (41), inhibition of DNA-dependent protein kinase (42), reversal of multidrug resistance (43), calcium channel blockade, and antagonism of calmodulin (44). However, the underlying mechanism is not yet clear. There is evidence that phenothiazine compound, NSC 17474, has the ability to modulate the AKT pathway by inhibiting relocalization of transcription factor FOXO1a to the nucleus in PTEN-null cells (45). To explore further the mechanism of the increased sensitivity of codon 600 BRAF mutant melanoma to phenothiazines, it would be important to evaluate the downstream effectors of BRAF, such as MEK and extracellular signal-regulated kinase, and possibly AKT following phenothiazine treatment. In addition, it would be interesting to investigate whether the *in vitro* differential sensitivities translate in an *in vivo* system. Results of such studies may have potential implications for the use of phenothiazines or its derivatives for the treatment of melanoma.

Published OnlineFirst June 4, 2008; DOI: 10.1158/1535-7163.MCT-07-2308

In this study, we carried out a systematic statistical analysis of correlations between mutations in cancer genes and differential drug sensitivity in the NCI-60. We identified an association between the antiproliferative activity of phenothiazines and presence of BRAF mutation. Using pharmacologic assays, we subsequently confirmed and prospectively validated the association in a larger and independent set of cell lines. This is the first comprehensive analysis of mutations in cancer genes and drug activity carried out in the NCI-60 cell line set. In a broader sense, our work shows the complexity of exploring associations between drug activity and molecular genetic profiles in cancers. Nevertheless, the analysis presented here shows how that combination of studies can generate useful information on drugs and their potential uses.

Disclosure of Potential Conflicts of Interest

The other authors disclosed no potential conflicts of interest.

Acknowledgments

We thank Bill Reinhold for kindly providing the NCI-60 cell lines used in this study and Sarah Edkins and Claire Stevens for kindly providing the independent set of melanoma and colorectal lines used in the validation studies.

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Published OnlineFirst June 4, 2008; DOI: 10.1158/1535-7163.MCT-07-2308

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Mol Cancer Ther 2008;7:1337-1346. Published OnlineFirst June 4, 2008.