Kinase Pathway Dependence in Primary Human Leukemias Determined by Rapid Inhibitor Screening

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Running Title: Drug Screen to Identify Leukemia Pathway Dependence

The authors have no Conflicts of Interest to declare.
ABSTRACT

Kinases are dysregulated in most cancer but the frequency of specific kinase mutations is low, indicating a complex etiology in kinase dysregulation. Here we report a strategy to rapidly identify functionally important kinase targets, irrespective of the etiology of kinase pathway dysregulation, ultimately enabling a correlation of patient genetic profiles to clinically effective kinase inhibitors. Our methodology assessed the sensitivity of primary leukemia patient samples to a panel of 66 small-molecule kinase inhibitors over 3 days. Screening of 151 leukemia patient samples revealed a wide diversity of drug sensitivities, with 70% of the clinical specimens exhibiting hypersensitivity to one or more drugs. From this data set, we developed an algorithm to predict kinase pathway dependence based on analysis of inhibitor sensitivity patterns. Applying this algorithm correctly identified pathway dependence in proof-of-principle specimens with known oncogenes, including a rare FLT3 mutation outside regions covered by standard molecular diagnostic tests. Interrogation of all 151 patient specimens with this algorithm identified a diversity of gene targets and signaling pathways that could aid prioritization of deep sequencing data sets, permitting a cumulative analysis to understand kinase pathway dependence within leukemia subsets. In a proof-of-principle case, we showed that in vitro drug sensitivity could predict both a clinical response and the development of drug resistance. Taken together, our results suggested that drug target scores derived from a comprehensive kinase inhibitor panel could predict pathway dependence in cancer cells while simultaneously identifying potential therapeutic options.

Keywords: AML, CMML, ALL, CLL, MPN, CML, molecular diagnosis, personalized medicine
INTRODUCTION

Gene-targeted cancer therapies have achieved remarkable clinical outcomes in recent years(1-4). In particular, cell-permeable small molecules that exhibit inhibitory activity against tyrosine kinases have generated great interest. Tyrosine kinases represent a gene family widely implicated in cancer pathogenesis(5, 6), and dysregulation of specific tyrosine kinases has been observed in most hematologic malignancies, including chronic myeloid leukemia (CML)(7), chronic myelomonocytic leukemia (CMML)(8-11), other myeloproliferative neoplasms (MPN)(12-15), acute myeloid leukemia (AML)(9, 16-20), acute lymphoblastic leukemia (ALL)(21-26), and chronic lymphocytic leukemia (CLL)(27-30). Although a minority of patients with hematologic malignancies are successfully treated with kinase inhibitors, most patients remain ineligible for this form of targeted therapy due to lack of knowledge of the specific kinase pathways involved.

Many strategies exist to better understand kinase dysregulation in cancer including the recent development of deep sequencing techniques, which are accelerating our understanding of cancer genetics. Thus far, however, many studies of malignancies with predicted kinase pathway dependence have not found frequent mutations in kinase genes(31-34). These findings suggest that kinase pathway dependence in malignant cells often occurs due to complex genetic mechanisms. Hence, although deep sequencing represents an immensely powerful technique, it may not independently allow for prediction of kinase targets and kinase inhibitor therapies. Instead, understanding of the best kinase inhibitor therapies for patients will likely require the combination of deep sequencing with complementary studies that can define kinase targets regardless of mutational status. These functionally important kinase pathways can then be correlated with genetic profiles that have been revealed by deep sequencing.
To better define the utility of kinase inhibitor therapies in hematologic malignancies, we have developed a small-molecule kinase inhibitor panel designed to identify kinase pathway dependence in primary leukemia samples. To analyze kinase pathway dependence based on this functional data, we have developed an accompanying bioinformatics approach to predict the gene targets underlying inhibitor sensitivity profiles. This algorithm takes advantage of our knowledge of the gene products that are targeted by each drug, as well as the fact that these gene target profiles are partially overlapping. Using the overlap of effective drugs and eliminating gene targets of ineffective drugs, we are able to predict critical gene targets and signaling pathways for individual patient samples. These gene target predictions represent a manner by which functional data from drug screening could be integrated with genomics data such as deep sequencing to aid in prioritization of sequence variants and, thus, accelerate our understanding of molecular etiologies of cancer as well as application of individualized therapeutic approaches for patients.

MATERIALS AND METHODS

Kinase Inhibitors

Kinase inhibitors were purchased from or were generously provided by the sources outlined in Supplementary Table 7.

Collection of Patient Samples and Cell Culture

All clinical samples were obtained with informed consent with approval by the Institutional Review Boards of Stanford University, Oregon Health & Science University, the Children’s Oncology Group, and Erasmus Medical Center/Sophia Children’s Hospital. Blood or bone
marrow from patients was separated on a Ficoll gradient and mononuclear cells were treated with ACK lysis buffer. The only exceptions to this procedure were cases of atypical CML or chronic neutrophilic leukemia, where samples were only processed with ACK lysis buffer to preserve the neoplastic granulocytes that would otherwise be lost on the Ficoll gradient. Cells from myeloid leukemia samples were cultured in R10 (RPMI-1640 medium supplemented with 10% FBS (Atlanta Biologicals, Lawrenceville, GA), L-glutamine, penicillin/streptomycin (Invitrogen, Carlsbad, CA), and fungizone (Invitrogen)) supplemented with $10^{-4}$ M 2-mercaptoethanol (Sigma). Cells from lymphoid leukemia samples were cultured in R20 (RPMI-1640 medium supplemented with 20% FBS (Atlanta Biologicals, Lawrenceville, GA), L-glutamine, penicillin/streptomycin (Invitrogen, Carlsbad, CA), and fungizone (Invitrogen)) supplemented with $10^{-4}$ M 2-mercaptoethanol (Sigma) insulin-transferrin-sodium selenite (Invitrogen).

**Kinase Inhibitor Screen**

Kinase inhibitors were stored at 10-100 mM in DMSO (stock concentration was 1000 times the final concentration of the highest tested dose). Drugs were used at final concentrations shown in Supplementary Table 4. For creation of replicate plates of the library, each drug concentration was diluted to twice the final concentration and 50 μl were plated into 96-well plates using a Hydra 96-channel automated pipettor (Matrix Technologies, Hudson, NH). Plates were sealed with adhesive lids (Bio-Rad; microplate seal B), wrapped in aluminum foil, and stored at -20°C until use. Upon receipt of a patient sample, plates were thawed at 37°C, 5% CO₂ for 1 hour and centrifuged at 800 x g prior to removal of adhesive lids. Subsequently, patient samples were suspended into culture media at a concentration of 1,000,000 cells per ml, such that addition of
50 μl to each well would deliver 50,000 cells to that well (this also dilutes the drugs to their final, desired concentration). Cells were incubated for 3 days at 37°C, 5% CO₂ and subjected to a CellTiter 96 AQueous One solution cell proliferation assay (Promega, Madison, WI). Each plate contained 7 wells without any drug. The average absorbance value of these 7 wells was used for data normalization and the kill curve of each drug gradient was assessed relative to this average no-drug point.

Quantification of Patient Response and Effective Drug Targets

An algorithm was designed and implemented using Excel and Visual Basic to provide automated IC₅₀ calculation and therapeutic target identification. IC₅₀ values were calculated using second-degree polynomial regression curves fit through five data points (average of no drug wells and four serial dilution points). All curves were manually inspected and a small number of IC₅₀s were corrected in two circumstances: (1) The curve fit intersected the IC₅₀ at two distinct points – the lower concentration intersect was used in these instances; (2) the polynomial curve fit yielded an artificial IC₅₀ not reflected in the data points (generally due to increasing cell viability over the course of the drug titration). For a given sample, drug IC₅₀ values were considered effective if less than or equal to 5-fold below the median IC₅₀ for all samples tested. Where an IC₅₀ was not achieved for a given drug, an IC₅₀ value equal to the highest drug concentration used was arbitrarily assigned. After effective and ineffective drugs were determined for each sample, a drug target score was assigned by the program for each potential therapeutic target.

The drug target score is based on the IC₅₀ measured effectiveness of panel drugs against a given therapeutic target for a given patient. Each drug is associated with a “tiered” ranking of target genes for which the drug has been shown to biochemically associate(35, 37-43). Weights
are used to give a stronger quantitative emphasis to targets with a higher ranking (Figures 4A and 4C). Scores are determined empirically for a given sample by assigning positive weighted scores to targets of effective drugs and negative weighted scores to targets of ineffective drugs. Drug effectiveness threshold and tiered ranking weights were determined empirically using patient samples and cell lines with known kinase signaling abnormalities. The algorithm generates a cumulative drug target score for each target according to the following equations:

\[
\text{Effective Drug Target Score} = \sum_{i=1}^{n} \text{Weight}_{\text{Tier}}(i)
\]

Where \( \text{Weight}_{\text{Tier}}(i) \) is a given drug target’s tier ranking for drug \( i \) and \( n \) is the number of effective drugs.

And

\[
\text{Ineffective Drug Target Score} = \sum_{j=1}^{m} \text{Weight}_{\text{Tier}}(j)
\]

Where \( \text{Weight}_{\text{Tier}}(j) \) is a given drug target’s tier ranking for drug \( j \) and \( m \) is the number of ineffective drugs.

\( n+m = 66 \).
Hierarchical clustering was performed using GenePattern software (Broad Institute, Cambridge, MA). Sample clustering and two-way clustering by row (drug) and column (patient sample) were performed using Pearson correlation distance shown in Figure 6 and supplemental Figures 2 and 3.

RESULTS

Development of a kinase inhibitor panel for analysis of primary leukemia specimens

The ubiquitous role of tyrosine kinases in regulating critical cellular processes leading to malignancy suggests that a large percentage of leukemia (and other malignancy) patient samples would exhibit sensitivity to inhibition of one or more kinase pathways. To test this hypothesis, we compiled a library of 66 small-molecule kinase inhibitors with collective activity against two-thirds of the tyrosine kinome (Supplementary Table 1). Since many non-tyrosine kinases are also critical regulators of cellular growth/survival, we also included drugs with activity against select families of non-tyrosine kinases including PI3K/AKT, PKC, PKA, IκK, RAF/MEK/ERK, JNK, p38, AMPK, aurora kinases, and cyclin-dependent kinases (Supplementary Table 1). Each inhibitor was plated at four graded concentrations that bracket the predicted on-target IC\textsubscript{50} value. Primary patient samples were incubated with this panel of drugs for three days at which point a tetrazolium-based cell viability assay (MTS) was performed for assessment of cell viability. All values were normalized to cells incubated in the absence of drug (Supplementary Figure 1).
Analysis of 151 leukemia patient samples with small-molecule kinase inhibitor panel

Over a two-year period, we accrued and tested 151 fresh, primary leukemia patient samples against this panel of kinase inhibitors. The cohort was comprised of 34 AML, 42 ALL, 31 MPN, and 44 CLL patients. Detailed clinical and demographic information about this patient cohort can be found in Supplementary Table 2. Assessment of kinase inhibitor hypersensitivity profiles of these 151 leukemia patient samples revealed a wide diversity of responses to kinase inhibitors, even when patients were grouped according to diagnostic subsets and kinase inhibitors grouped according to predicted gene target spectra (Figure 1). Despite this heterogeneity of responses, certain trends emerged, such as more frequent sensitivity to PI3K/AKT inhibitors in lymphoid samples. In addition, select cases could be identified with universal sensitivity to whole families of kinase inhibitors. For example, AML case 07335 exhibited universal sensitivity to all ERBB-family inhibitors on the panel, suggesting involvement of an ERBB family member in maintenance of the viability of malignant cells from that patient (Figure 1). Overall 70% of patients exhibited hypersensitivity to one or more kinase inhibitors (IC\textsubscript{50} Data for each drug for each patient specimen listed in Supplementary Table 3; Raw data points used to generate these IC\textsubscript{50}s listed in Supplementary Table 4). Nine of the drugs on our panel are currently approved by the FDA (imatinib, nilotinib, dasatinib, sunitinib, erlotinib, gefitinib, lapatinib, sorafenib, and pazopanib), and approximately 40% of samples exhibit hypersensitivity to one or more of these nine drugs. Hypersensitivity to a drug was determined by comparison of the response of each individual sample with the response of all other samples (patient IC\textsubscript{50} compared with whole cohort median IC\textsubscript{50}). In this way, we could define outlier samples that were truly hypersensitive to a given drug versus responses at higher
concentrations that might occur due to off-target toxicity of the compound. Rank ordering of patient IC\textsubscript{50}s for each drug helps illustrate this point (Supplementary Figure 2). To better distinguish inhibitor sensitivity profiles that were similar from patient to patient, we applied one-way Pearson correlation for hierarchical clustering of the data (Supplementary Figure 3). Notably, although drug responses are clearly not uniform among diagnostic subsets, there were large groups of patients diagnosed with the same type of leukemia (notably ALL (shaded red) and CLL (shaded yellow); Supplementary Figure 3) with similar responses to these kinase inhibitors. However, the segregation of sample responses by cell type was far from complete with a number of isolated lymphoid samples clustering amidst myeloid cases and vice versa. In addition, two-way hierarchical clustering revealed drugs with similar activity profiles across patients (Supplementary Figure 4). Many of these drugs clustered in groups that would be predicted based on known target profiles of the compounds such as BIRB-796 and VX-745 (both p38 inhibitors), flavopiridol and BMS-387032 (both CDK inhibitors), and EKB-569 and CI-1033 (both ERBB-family inhibitors).

**Logical prediction of oncogenic signaling pathways using inhibitor sensitivity profiles**

Clinical and research interest in the application of kinase inhibitors has led to a concerted effort to develop techniques that characterize the targets to which each compound can effectively bind(35-38). Since a majority of the kinase inhibitors on our panel have been characterized in this manner, we realized that this information could be used to predict the critical gene targets and signaling pathways that underlie the observed kinase inhibitor sensitivity patterns. Development of this bioinformatics approach relies on the fact that all kinase inhibitors on our panel bind multiple targets and the target spectra for these drugs are partially overlapping. Thus, if a sample exhibits sensitivity to two different drugs, the gene targets that are commonly
inhibited by both drugs are implicated as most likely to explain this sensitivity pattern (unless there are two different pathways operational in that sample). A second step can further narrow the candidate gene list by elimination of gene targets of drugs to which a sample is not sensitive.

The results from AML patient 08024 illustrate our target identification strategy. Malignant cells from this patient were hypersensitive to three drugs: AST-487, sunitinib, and SU-14813. As noted above, drug hypersensitivity is defined by comparison of the IC₅₀ values for this individual sample with the median IC₅₀ values that these drugs achieved across the entire cohort. Cells from patient 08024 exhibited IC₅₀ values that were at least five-fold lower than the cohort median IC₅₀ values for each of these three drugs (Figure 2A). Analysis of the known gene targets of these three compounds revealed four genes, KIT, PDGFRβ, CSF1R, and FLT3, that are common targets between all three drugs (Figure 2B). Since this sample was also unaffected by the two drugs, dasatinib and nilotinib (no difference between patient 08024 IC₅₀ and median IC₅₀; Figure 2A), the gene targets of these two drugs can be eliminated from consideration as genes that could mechanistically explain cell viability in this patient sample. The gene targets that are effectively inhibited by dasatinib and/or nilotinib are shaded blue while the genes not targeted by either dasatinib or nilotinib are shaded red (Figure 2B). This reveals that the only gene target in common between AST-487, sunitinib, and SU-14813 that is also not a gene target of dasatinib or nilotinib is FLT3. Further analysis of AML patient 08024 revealed presence of the FLT3-ITD and no wild type FLT3 alleles (Figure 2C).

Development of a customized algorithm to automate oncogenic pathway prediction
Application of the logic illustrated in Figure 2B can be performed manually for a small number of kinase inhibitors, however, expansion of this process to evaluate data from the entire panel of 66 drugs requires computational support. We have developed an algorithm to carry out the same logical steps outlined in Figure 2, for all 66 kinase inhibitors in 4 steps (Sweave document with instructions for generating application to perform this algorithm found in Supplemental Material). First, the gene target information for each drug was curated from published sources(35, 37-43) into a database (Figure 3A and Supplementary Table 1). Second, the K_D or IC_{50} values (depending on the format of assay used to define drug targets) for each drug were subdivided into five tiers on a Log_{10} scale. The first tier is defined as the gene with the lowest K_D or IC_{50} value as well as all other genes with K_D or IC_{50} values less than or equal to 10-fold that lowest value. Genes with K_D or IC_{50} values ranging from 10-fold to 100-fold the lowest value are considered tier 2 targets. Each subsequent Log_{10} increase in K_D or IC_{50} comprises a new tier of gene targets (Figure 3A; lower portion). Next, the IC_{50} results from an individual patient sample are subdivided into drugs to which the sample was hypersensitive (defined by a patient-specific IC_{50} that is five-fold less than the cohort median IC_{50}) as well as the drugs to which the sample is not sensitive (Figure 3B). We next devised a scoring system that assigns points to each gene based on whether inhibitors defined to block that gene target were effective (positive points awarded to the gene’s score) or ineffective (negative points deducted from the gene’s score). The addition or subtraction of points is performed in a graded manner with tier 1 genes having the most points added or subtracted from their score and the score for genes from each subsequent tier being modified to lesser degrees (Figure 3C). Finally, the cumulative scores for each gene are tabulated and ranked such that the highest scoring genes for any given patient are those genes predicted to be most probable in explaining the observed drug response.
and, therefore, the most probable in playing a pathogenic role for that patient’s malignancy (Figure 3D).

**Application of oncogene prediction algorithm to four proof-of-principle examples**

To test this algorithm, we chose three specimens from leukemia patients with known, dysregulated tyrosine kinase pathways and one specimen from a patient without a known kinase mutation. The first example, AML patient 08024, was described in Figure 3 whereby the FLT3-ITD gene target was predicted based on analysis of the response pattern of the cells to 5 small-molecule kinase inhibitors. To determine whether our algorithm would also successfully identify FLT3 as a high probability gene target when applying data from all 66 drugs, we performed the algorithm on kinase inhibitor panel screening outcomes from this patient sample. This exercise revealed that FLT3 was the second highest scoring gene on the target list with a score of 89 points (Figure 4A). The highest scoring gene (EGFR) scored 90 points due to near complete hypersensitivity of this specimen to ERBB-family inhibitors. Analysis of target profiles of these ERBB-family inhibitors reveals that they do not exhibit off-target effects against FLT3(37), indicating that there may be crosstalk between the FLT3-ITD oncogene and ERBB-family members. We also applied this screen to cells from a CML patient in blast crisis. In this case, the algorithm correctly identified ABL1 as the top-scoring gene (Figure 4B). Finally, we applied this technique to cells from an MPN patient positive for the oncogene, MPL-W515L. In this case, three of the top five genes are JAK-family kinases, which represent therapeutic targets downstream of MPL (MPL is not a kinase, but signals through JAK kinases)(44, 45) (Figure 4C). Thus, application of this algorithm to three proof-of-principle examples shows that the
approach correctly identifies known oncogenic signaling pathways in each case. In addition, an AML patient (08102) with no known mutations in tyrosine kinases exhibited hypersensitivity to 12 of the kinase inhibitors on our panel (Figure 4D). Among the genes predicted by our algorithm to be likely involved in pathogenesis of this specimen was FLT3, which is commonly mutated in AML (Figure 4E). However, as noted above, this patient did not exhibit any of the common FLT3 abnormalities. This prompted us to perform more extensive sequencing of FLT3, and we identified a point mutation in the extracellular region of FLT3 (S451F) (Figure 4F), this rare mutation has been previously shown to exhibit transformative capacity(46).

**Algorithmic prediction of oncogenic signaling pathways in 151 patient samples**

To better understand the gene targets that might underlie drug sensitivity patterns in our entire cohort of leukemia patient samples, we applied our target prediction algorithm to all 151 specimens that were interrogated by the kinase inhibitor panel. The data reveal a heterogeneous list of probable gene targets that arise in a highly patient-specific manner (Supplementary Table 5). One clear conclusion from these results is the need for a personalized approach to application of gene-targeted therapies. However, the data can also be utilized to identify genes that may be more frequently implicated in any one diagnostic subset of malignancy. As such, we tabulated the average gene scores for each patient within the broad diagnostic categories of ALL, CLL, AML, CML, CMML, and other MPN. Gene scores were normalized for comparison between patients by dividing all scores of a given profile by the maximum score for that profile. Negative scores were removed resulting in a normalized profile score between 0 and 1. Next, we computed average normalized scores for each gene across diagnostic groups. Finally, the gene scores for each diagnostic group were again normalized to the highest scoring gene within each group so that each diagnostic category would be represented on the same scale. These values
were then expressed on a heat map to visually represent the genes that are predicted to be more frequently involved in the pathogenesis of each of these broad diagnostic subsets of hematologic malignancies (Figure 5; input data found in Supplementary Table 6). As anticipated, ABL1 is the highest cumulative scoring gene among CML patients tested, serving as a proof-of-principle for this strategy. Interestingly, CaM kinases appear to score highly in lymphoid but not myeloid malignancies. One possible link between that CaM kinases and lymphoid malignancies may be the activation of CBP. Recent studies have identified mutations in CBP (and loss of activity) as a strong correlation with relapse in ALL(47). It has been previously established that phosphorylation of CBP by CaM kinase IV is important for its activity(48). Therefore, inhibition of CaM kinases may provide further insight to the functional importance of CBP in lymphoid viability. PI3K and AKT isoforms score highly in lymphoid malignancies as well as myeloproliferative neoplasms, but are infrequently represented in AML patients. BTK scores highly in CLL patients, consistent with the notion that B-cell receptor signaling, which depends partly on BTK, is critical for viability of cells from many CLL patients(28, 30). Cyclin-dependent kinases are predicted to be involved more frequently in MPN than in AML or lymphoid malignancies. Ephrin receptors score strongly across all diagnostic groups. In addition, p38 appears frequently involved in all malignancy subsets, and especially so in AML and CMML. There are a wide diversity of genes and signaling pathways that are predicted to play a role in the pathogenesis of each type of hematologic malignancy. However, our drug target scoring algorithm indicates particular pathways are more frequently represented in some diagnostic subsets than in others. Follow-up investigation will be required to validate the complete genetic etiology of these observations.

Clinical relevance of in vitro drug sensitivity/resistance
The clinical utility of this type of test is predicated on a meaningful correlation between in vitro and an in vivo response to kinase inhibitors. As a proof of concept, we tested this correlation in a patient with refractory AML. A 36 year-old patient with a white blood cell (WBC) count of 133,000 was diagnosed with AML with inversion of chromosome 2 and trisomy 8. The FLT3-ITD was noted to be weakly positive with an allelic ratio of 0.02. Following leukapheresis and a standard 7+3 induction therapy, he was found to have refractory AML and was re-induced with HAM chemotherapy. Although he achieved a remission and soon after underwent an unrelated donor transplant, he relapsed 60 days later. The donor was not available for donor leukocyte infusions and the patient was refractory to FLAG-IDA salvage therapy. Our inhibitor panel showed dramatic sensitivity to numerous kinase inhibitors, including several drugs that are already FDA-approved and have been used for treatment of AML (Figure 6A). Since this patient had no other standard therapeutic options, he elected treatment with one of the inhibitors predicted to be effective by the inhibitor panel assay. Daily treatment with this drug, Sorafenib, induced a rapid normalization of WBC counts with decreased blasts in both the peripheral blood and bone marrow that was maintained for over two months (Figure 6B). At the time of relapse, a repeat kinase inhibitor sensitivity panel showed the in vitro response to Sorafenib was approximately 3 logs less than the pre-treatment cells (Figure 6C). Interestingly, the analysis also showed that this relapsed AML remained highly sensitive to another FDA-approved kinase inhibitor, Sunitinib. (Figure 6D). Treatment with daily Sunitinib resulted in a significant initial response with rapid reduction in WBC counts and peripheral leukemic blasts for a period of 4 weeks.

DISCUSSION
Here we demonstrate that functional screening of primary cells from leukemia patients with a panel of kinase inhibitors can identify effective kinase inhibitors in 70% of patients in just three days, including 40% of patient samples that were hypersensitive to drugs already approved for clinical use. We also demonstrate an algorithm that utilizes the partially overlapping gene target spectra for each drug for prediction of critical gene targets that underlie kinase inhibitor sensitivity patterns. Finally, using this algorithm, we identify and rank probable gene targets in 151 patients with a variety of hematologic malignancies.

The predicted gene targets for these patient samples show a great deal of heterogeneity, even within diagnostic subsets. This finding highlights one of the strengths of this kinase inhibitor screen—gene targets and patient-specific therapeutic options are detected regardless of the frequency with which these targeted therapies would be applicable within the given disease subset. In addition, although our target prediction algorithm can suggest candidate pathways for follow-up validation, this technique is empirical in that it does not require specific knowledge of any genetic lesion or biomarker to uncover potential therapeutic options for patients.

It is likely that the algorithm shown here exhibits both false-positive and false-negative gene targets. Since the algorithm is driven by our knowledge of potential gene target spectra of kinase inhibitors that are present on the panel, there is certainly an opportunity for gene target bias due to pathways that are over- or under-represented on the panel. As drug development continues and the panel expands to include other drugs that offer more complete and even coverage of the kinome, this pitfall will be diminished. In addition, further profiling of these inhibitors to fully delineate target spectra will also improve the algorithm output since this output is largely driven, and limited, by our knowledge of these gene target spectra.
It is also possible that the setup of the assay could lead to false-negative results, since the technique relies on a short 3-day window for assessment of drug sensitivity. For this reason, interpretation of negative results from the drug assay (i.e. lack of sensitivity to a particular drug) must be taken with caution. Correlation of results from this screen with clinical cases in which patients are treated with drugs from the panel will help inform the full clinical relevance of both positive and negative assay results.

It will be critical to integrate this kinase inhibitor screen with other cutting edge techniques. We have already begun to apply this assay in conjunction with a related siRNA screen (49, 50), and initial results obtained within four days of receiving fresh primary samples indicate a high concordance of siRNA and kinase inhibitor sensitivities. In addition, application of genomics techniques such as gene expression microarray and deep sequencing in parallel with these functional screening tools will undoubtedly accelerate our understanding of the precise molecular events that underlie the observed gene sensitivity patterns. Matching of these patient genotypes with drug sensitivity patterns will ultimately enable patients to be treated on the basis of tumor genotypes, and this drug assay and gene target algorithm offer useful modalities for translation of tumor genotypes into therapeutically relevant clinical strategies. Hence, the kinase inhibitor screen illustrated here represents an advance towards individual patient-tailored cancer therapy. Malignant cells from cancer patients can be economically screened to identify effective targeted therapies within three days, thus matching the appropriate drugs with the appropriate patients on a timescale that affords opportunity for informed, mechanism-based intervention.
ACKNOWLEDGEMENTS

Supported in part by The Leukemia and Lymphoma Society, the TJ Martell Foundation, and the Doris Duke Charitable Foundation. JWT is supported by grants from the National Cancer Institute (NCI; grant # 5K99CA151457-02) as well as grants from the William Lawrence and Blanche Hughes Foundation and the Oregon Clinical and Translational Research Institute (OCTRI) grant number UL1 RR024140 from the National Center for Research Resources (NRCC), a component of the NIH, and NIH Roadmap for Medical Research. BJD is an investigator of the Howard Hughes Medical Institute.
REFERENCES


FIGURE LEGENDS

Figure 1: Response of 151 leukemia patient samples to 66 small-molecule kinase inhibitors.

Mononuclear cells were obtained from 151 primary leukemia patient samples and IC₅₀ values for 66 small-molecule kinase inhibitors were tabulated as described in Supplementary Figure 1. The percent of median IC₅₀ for each patient for every drug is expressed on this heat map where darkest red indicates most sensitive (most sensitive patient sample to any drug was 0.07% of median) and white indicates completely insensitive (IC₅₀ for patient is at or above 100% of median). Patient samples are arranged by diagnosis (ALL – red; AML – green; CLL – yellow; MPN – blue). Kinase inhibitors are arranged based on family of primary gene targets for each drug. Numerical input for this heat map is found in Supplementary Table 3.

Figure 2: Logical prediction of gene targets based on drug sensitivity/resistance.

To predict operational gene targets in Patient 08024, response to 5 kinase inhibitors is considered. (A) Patient 08024 exhibits at least 5-fold lower IC₅₀ than median to AST-487, SU-14813, and sunitinib (effective drugs), while no difference is seen between median and Patient 08024 IC₅₀ for dasatinib and nilotinib (ineffective drugs). To predict gene targets that underlie this sensitivity pattern, the overlap of gene targets between the three effective drugs (AST-487, SU-14813, and sunitinib) is shown in (B) revealing that only KIT, PDGFR, CSF1R, and FLT3 are common gene targets between these drugs. To distinguish between these four gene targets, their responsiveness to the ineffective drugs (dasatinib and nilotinib) is considered. The gene targets in blue (KIT, PDGFR, CSF1R) are good targets of dasatinib and/or nilotinib, while the gene targets in red (FLT3) are not effectively inhibited by dasatinib or nilotinib. Elimination of
these dasatinib/nilotinib targets (in blue) leaves only FLT3 as a potential gene target for explanation of the functional response to these 5 drugs. Follow-up PCR analysis revealed this AML patient sample to exhibit FLT3-ITD with loss of the wild-type FLT3 allele (C).

Figure 3: Development of an algorithm for gene target prediction based on kinase inhibitor efficacy.

Automation of gene target prediction based on kinase inhibitor sensitivity patterns proceeds in several steps:

(A) The kinase targets of each kinase inhibitor are curated into a single database from published sources. The gene target with lowest $K_d/IC_{50}$ for a given inhibitor as well as all gene targets with $K_d/IC_{50}$ less than or equal to 10-fold that lowest value are considered tier 1 targets. Gene targets with $K_d/IC_{50}$ within each subsequent 10-fold increase in $K_d/IC_{50}$ are considered to be in tiers 2-5, respectively.

(B) Drug IC$_{50}$s for an individual patient sample are compared to median drug IC$_{50}$s to determine effective and ineffective drugs, as described in Figure 1.

(C) Gene targets of effective drugs are given positive points and gene targets of ineffective drugs receive negative points, both in a tiered fashion based on the gene target tiers defined in part (A).

(D) Total scores are tabulated for every gene target and ranked such that gene targets with the highest points are predicted to be most probable for operational involvement in preservation of
cell viability and, therefore, most likely to mechanistically underlie the observed drug sensitivity pattern.

**Figure 4: Algorithm gene target scores for four leukemia patient samples with known oncogenic lesions.**

Proof-of-principle of performance of algorithm described in Figure 3. The algorithm gene target scores from three patient samples with known genetic etiology are considered. The first (A) is an AML patient with FLT3-ITD, and FLT3 ranks as the second-highest gene target. EGFR is the highest scoring gene in this example due to complete hypersensitivity of the specimen to ERBB family inhibitors. This may reveal a mechanistic link between FLT3 and ERBB family signaling, since the ERBB family inhibitors do not exhibit affinity for FLT3. The second sample (B) is derived from a CML patient in blast crisis with no kinase domain mutations in BCR-ABL. ABL1 is the top scoring gene for this sample. The third example (C) is an MPN patient with a MPL-W515L mutation. Although MPL is not a kinase (and is not scored by the algorithm), it signals primarily through JAK kinases. Notably, JAK1, JAK2, and TYK2 are three of the top five scoring genes for this example. A fourth patient with no known kinase mutations exhibited sensitivity to a variety of kinase inhibitor on our panel (D). The algorithm described in Figure 3 predicted this pattern to be driven by dependence on a variety of type III receptor tyrosine kinases, including FLT3, which is commonly mutated in AML (E). Since this patient did not exhibit any of the FLT3 mutations commonly observed in AML, we sequenced full-length FLT3 and identified a rare point mutation in the FLT3 extracellular domain (S451F), previously shown to exhibit transformative capacity. The sequence trace of this mutant FLT3 allele is shown in (F).
Figure 5: Cumulative algorithm gene target scores by diagnosis.

To gain insight into gene targets that are frequently and infrequently predicted in various diagnostic subsets of leukemia, we tabulated cumulative gene target scores for ALL, AML, CLL, CML, CMML, and other MPN. Gene target scores for all 151 patients were tabulated by the algorithm described in Figure 4 (individual patient target scores found in Supplementary Table 5). To compute cumulative scores, all negative values were eliminated and remaining positive values were normalized for each patient to that patient’s respective highest scoring gene, such that all gene scores were on the same scale from 0 to 1 for every patient. The mean of these normalized gene scores for each gene was then calculated for every patient within the above diagnostic leukemia subsets. These average gene target scores for each leukemia subset were once again normalized to the highest scoring respective gene from within each leukemia subset such that each subset was represented on the same 0 to 1 scale. These values are illustrated on a heat map where darkest blue indicates genes most frequently predicted to be operationally important within each respective leukemia subset and white indicating no evidence for functional importance. Notably, as a proof-of-principle, ABL1 is the highest cumulative scoring gene in CML. The numerical input for this heat map is found in Supplementary Table 6.

Figure 6: Correlation of in vitro sensitivity with clinical patient response.

(A) A specimen from a multiply relapsed AML patient was evaluated on the kinase inhibitor panel and exhibited hypersensitivity to a variety of kinase inhibitors, including two that are FDA-approved (Sorafenib and Sunitinib).
(B) This patient was treated on Sorafenib and subsequently treated on Sunitinib and responded well to both treatments with rapid reductions in white blood cell counts, peripheral blast percentage, and bone marrow blast percentage.

(C) In vitro response of this patient’s cells to Sorafenib mimicked the clinical response whereby pre-treatment cells were highly sensitive and cells taken after Sorafenib relapse exhibited an IC$_{50}$ greater than logs higher than the pre-treatment cells.

(D) In vitro response of this patient’s cells to Sunitinib were consistent with the clinical response whereby cells were highly sensitive to Sunitinib before Sorafenib treatment and at Sorafenib relapse, at which time the patient responded beneficially to treatment with Sunitinib.
**Figure 3**

### d) Gene Name vs. Cumulative Score

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Cumulative Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLT3:</td>
<td>52</td>
</tr>
<tr>
<td>KIT:</td>
<td>50</td>
</tr>
<tr>
<td>RET:</td>
<td>44</td>
</tr>
<tr>
<td>PDGFRB:</td>
<td>34</td>
</tr>
<tr>
<td>LCK:</td>
<td>33</td>
</tr>
</tbody>
</table>

**a) Diagram of Gene Tree:**
- Tier 1 Genes: +20.00  
- Tier 2 Genes: +10.00  
- Tier 3 Genes: +5.00  
- Tier 4 Genes: +2.50  
- Tier 5 Genes: +1.25

**b) Graphs of Drug Effectiveness:**
- **All Drugs**
- **Effective Drugs**
- **Ineffective Drugs**
Figure 4

(a) AML, FLT3-ITD+
(b) CML, BCR-ABL+
(c) MFN, MPL W515L+

Genotype Unknown
Algorithm Result (Top 30 Genes Ranked by Score)

(f) FLT3 WT
FLT3 S451F

FIGURE 4
Figure 6
Kinase Pathway Dependence in Primary Human Leukemias Determined by Rapid Inhibitor Screening

Jeffrey W Tyner, Wayne F Yang, Armand Bankhead, et al.

Cancer Res Published OnlineFirst October 18, 2012.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-12-1906

Supplementary Material
Access the most recent supplemental material at:
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