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Rapid Screen for Tyrosine Kinase Inhibitor Resistance Mutations and Substrate Specificity

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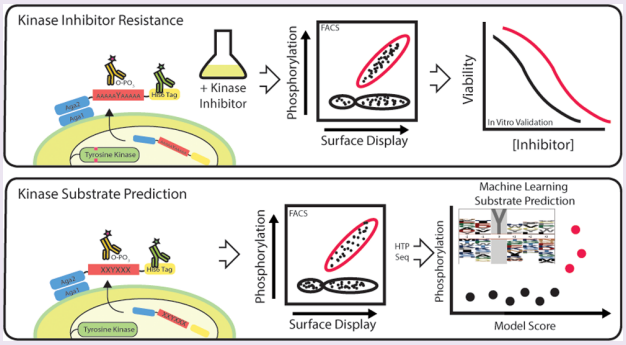
Supporting Information

ABSTRACT: We present a rapid and high-throughput yeast and flow cytometry based method for predicting kinase inhibitor resistance mutations and determining kinase peptide substrate specificity. Despite the widespread success of targeted kinase inhibitors as cancer therapeutics, resistance mutations arising within the kinase domain of an oncogenic target present a major impediment to sustained treatment efficacy. Our method, which is based on the previously reported YESS system, recapitulated all validated BCR-ABL1 mutations leading to clinical resistance to the second-generation inhibitor dasatinib, in addition to identifying numerous other mutations which have been previously observed in patients, but not yet validated as drivers of resistance. Further, we were able to demonstrate that the newer inhibitor ponatinib is effective against the majority of known single resistance mutations, but ineffective at inhibiting many compound mutants. These results are consistent with preliminary clinical and in vitro reports, indicating that mutations providing resistance to ponatinib are significantly less common; therefore, predicting ponatinib will be less susceptible to clinical resistance relative to dasatinib. Using the same yeast-based method, but with random substrate libraries, we were able to identify consensus peptide substrate preferences for the SRC and LYN kinases. ABL1 lacked an obvious consensus sequence, so a machine learning algorithm utilizing amino acid covariances was developed which accurately predicts ABL1 kinase peptide substrates.



The 90 tyrosine kinases of the human proteome play a central role in essential cellular processes, including regulation of proliferation, differentiation, and cell death. Protein tyrosine kinases catalyze the transfer of the gamma phosphate of adenosine triphosphate (ATP) to the hydroxyl-containing side chain of tyrosine, propagating cellular signaling by modulating activity of downstream enzymes or providing binding sites for phosphotyrosine-specific adaptor domains. Misregulation of tyrosine kinase activity is a hallmark of many human cancers, including myeloproliferative disorders and solid tumors of the lungs, breasts, and colon. Aberrant activity of tyrosine kinases may occur through activating mutations, including fusion proteins derived from chromosomal translocations, point mutations, or overexpression.[1−3]

The success of imatinib, approved by the United States Food and Drug Administration (FDA) in 2001, demonstrated the potential of targeted small molecule inhibition of oncogenic tyrosine kinase domains. Imatinib was first approved as an inhibitor of BCR-ABL1, a fusion protein resulting from a chromosomal translocation that is present in nearly all cases of chronic myelogenous leukemia (CML). The BCR-ABL1 oncogene exhibits constitutive activity and altered subcellular localization, leading to increased proliferation and genomic instability. In clinical trials, up to 76% of patients treated with imatinib achieved a complete cytogenetic response, compared to 14% of patients treated with the standard of care at the time, interferon-alpha plus cytarabine.[4] Due to a small population of BCR-ABL-positive cells that persist during treatment, imatinib therapy must be continued indefinitely. Since the approval of imatinib in 2001, more than two dozen tyrosine kinase inhibitors have been approved for therapeutic use. These target an array of kinases and cancer types, including EGFR and ALK inhibitors for nonsmall cell lung cancer, HER2 inhibitors for breast cancer subtypes, and FLT3 inhibitors in acute myeloid leukemia.[5]

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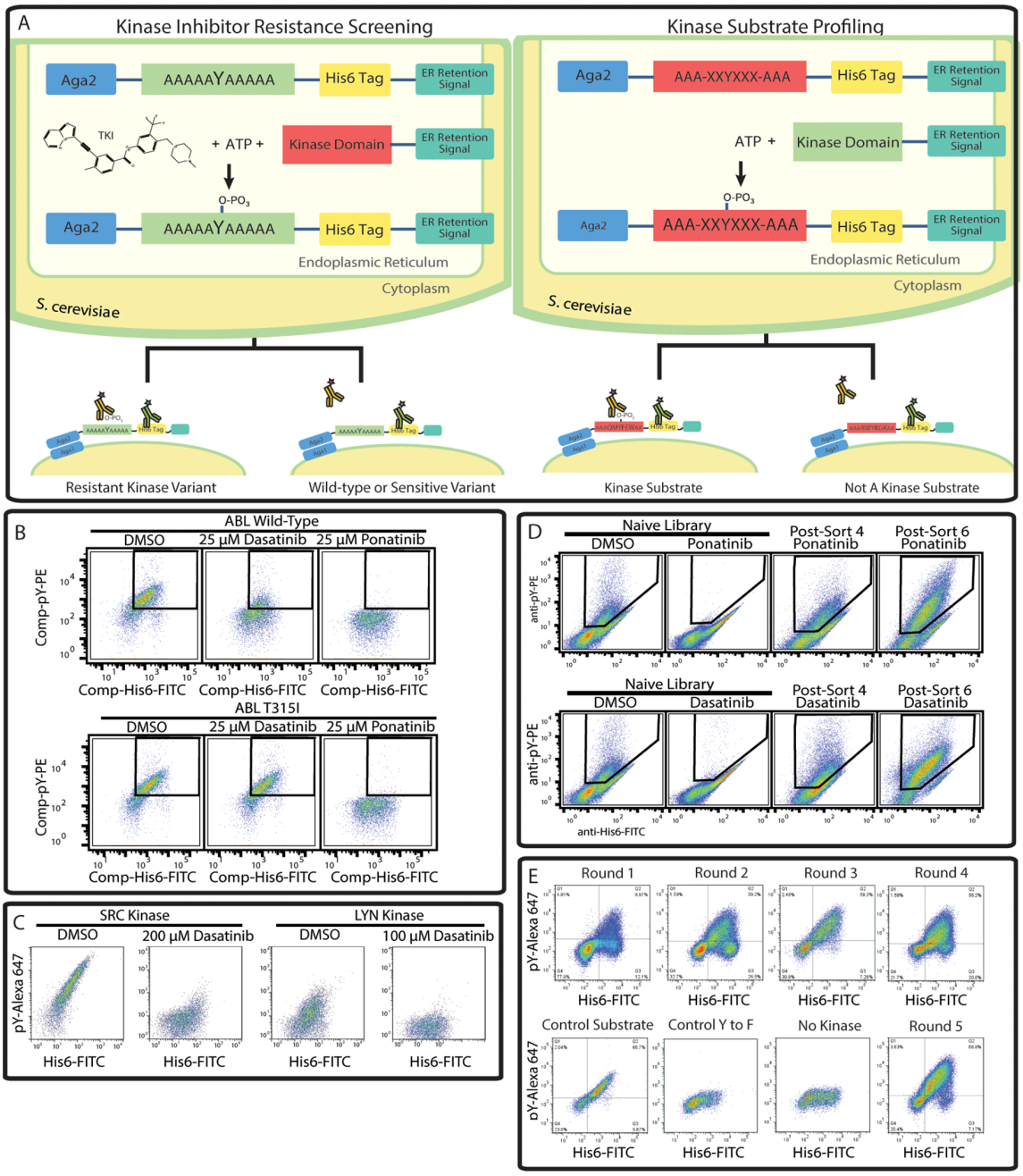

Figure 1. High-throughput screening for tyrosine kinase inhibitor (TKI) resistance mutations and substrate specificity. (A) Schematic of the assay system: The kinase domain and a kinase substrate peptide fused to Aga2 for display on the cell surface are both expressed with endoplasmic reticulum (ER) retention signals (FEHDEL) to enhance ER transit time, thus favoring phosphorylation of the Aga2-substrate. Cells expressing TKI-resistant kinase variants that display phosphorylated substrate are labeled with antiphosphotyrosine fluorescently labeled antibodies and can be isolated by FACS. (B) FACS of yeast cells expressing ABL1 or ABL1(T315I) mutation, which confers resistance to dasatinib but not ponatinib, cultured with vehicle (DMSO) or with TKI as shown. (C) Flow cytometry plots of cells expressing SRC or LYN in the presence or absence of dasatinib, which inhibits both SRC and LYN. (D) Enrichment of cells displaying phosphorylated substrate from a library of random ABL1 cultured in the presence of 25 μM TKI. Populations were sampled by long-read next-generation sequencing. (E) FACS enrichment of ABL1 kinase substrates from a combinatorial peptide library (XXYXXX). Cells with surface-displayed phosphorylated substrate are enriched over five rounds of sorting. Each round, including the unsorted cells, was sampled by short-read next generation sequencing.

Shortly after FDA approval, some patients who achieved an initial response to imatinib experienced subsequent disease progression due to point mutations within the kinase domain of BCR-ABL, which were confirmed by *in vitro* studies to confer resistance to imatinib.[6] Therefore, a suite of second-generation drugs was developed to treat imatinib-resistant patients, including bosutinib, dasatinib, and nilotinib.[7,8] Unfortunately, each of these drugs has since been shown to be susceptible to resistance mutations, both in patients and *in vitro*.[9] Most recently, the third-generation inhibitor ponatinib was introduced,[10] although due to the frequency of severe side effects, only for use in patients who have previously failed two

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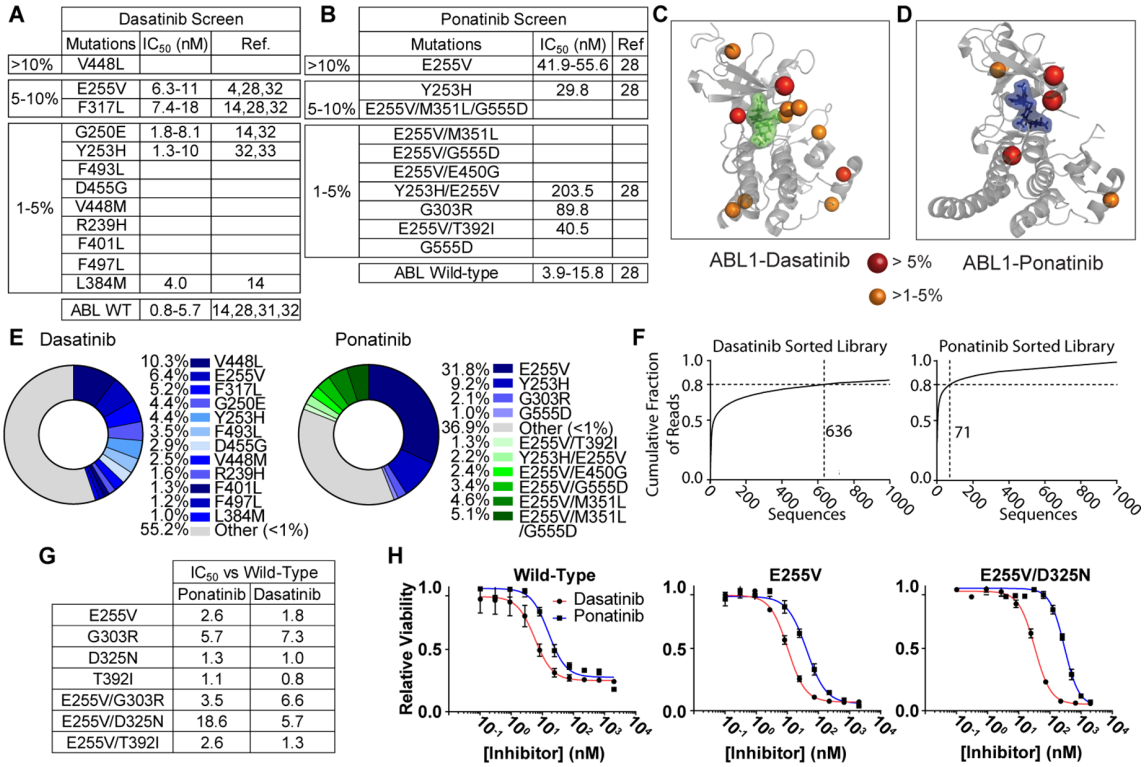

Figure 2. Tyrosine kinase inhibitor (TKI) resistance mutational landscapes. (A) High frequency ABL1 kinase mutant alleles enriched from cells grown in the presence of 25 μM dasatinib and sorted for four rounds. Data from 1.8×10^4 high-quality reads from amplicons isolated following round 4 (B). As in (A) for cells grown with 25 μM ponatinib after six rounds of sorting. Data from 9.9×10^3 high-quality reads from amplicons following round 6. (C) Mutations present in greater than 1% of mutant sequence reads are mapped onto ABL1 in complex with dasatinib (PDB ID: 2GQG). (D) As in (C), highly represented mutant sequence reads are shown on ABL1 in complex with ponatinib (PDB ID: 3OXZ). (E) Distribution of high-frequency alleles. Mutant alleles observed at a frequency > 1% are shown as slices, with single mutations in blue and compound mutations in green. (F) TKI-resistant ABL1 sequence diversity. The number of sequences accounting for 80% of mutant reads is indicated. (G, H) Effect of inhibitors on the growth of Ba/F3 cells expressing select BCR-ABL1 variants selected from yeast-based screening. Data are reported as ratio of IC_{50} for cells expressing BCR-ABL1 mutants over wild-type (see also Figure S5).

BCR-ABL inhibitors, or are known to harbor the potent T315I kinase domain mutation.

Current methods for surveying BCR-ABL inhibitor-resistant mutations rely on mammalian cell culture, either from CML-derived cells expressing BCR-ABL1[11,12] or by retroviral integration of the BCR-ABL1 oncogene into the murine Ba/F3 cell line.[13−17] Endogenous screens rely on increased mutational rate of CML cell lines, from both BCR-ABL expression and other genomic defects, to generate population diversity.[11,12] In contrast, retroviral methods generate diversity through mutagenesis[13,14] prior to transfection or *in situ* chemical mutagenesis of BCR-ABL1-expressing cells.[16,17]

Herein we present a fast and efficient yeast-based flow-cytometry assay to screen for TKI-resistant mutations and identify kinase substrate sequence preferences. Briefly, a human kinase domain is coexpressed with a tyrosine-containing substrate peptide fused to the Aga2 protein for surface display.[18,19] Kinase and substrate are transiently colocalized in the endoplasmic reticulum (ER), where the phosphorylation reaction can take place prior to Aga2-substrate peptide export and eventual display on the yeast surface. The extent of substrate phosphorylation of the Aga2-peptide fusion is then detected by staining with fluorophore-labeled phosphotyrosine-specific antibodies. In media containing TKIs, cells with reduced kinase activity can be detected and enriched by fluorescence-activated cell sorting (FACS). As proof-of-principle, a library of ABL1 kinase mutants was screened in the presence of either ponatinib or dasatinib. We report on the facile isolation of TKI-resistant ABL1 mutations and show that our methodology; (i) recapitulates previously described dasatinib-resistant mutations isolated from patients; (ii) reveals novel mutations conferring resistance to ponatinib.[20] Significantly, our results showed that more than half of the most common ponatinib-resistant variants were compound mutants, whereas the majority of dasatinib-resistant variants contained a single mutation. This same yeast screening technology was also used in a complementary mode to analyze the substrate specificity of the kinase domains of human SRC, LYN, and ABL1. In this mode, high-throughput screening of combinatorial peptide substrate libraries, deep sequencing, and machine learning were combined to identify peptide substrate preferences for the SRC and LYN kinases as well as produce a predictive model for ABL1 specificity.

RESULTS AND DISCUSSION

Development of the Yeast Display System. In our yeast-based system, a kinase domain and the Aga2-substrate peptide fusion gene constructs are coexpressed with C-terminal ER-retention signals under the Gal1/10 promoter. The Aga2 fusion protein contains hexa-histidine and FLAG epitope tags flanking a minimal tyrosine kinase substrate sequence, AAAAAYAAAAA.[21] Coexpression of the kinase and transiting

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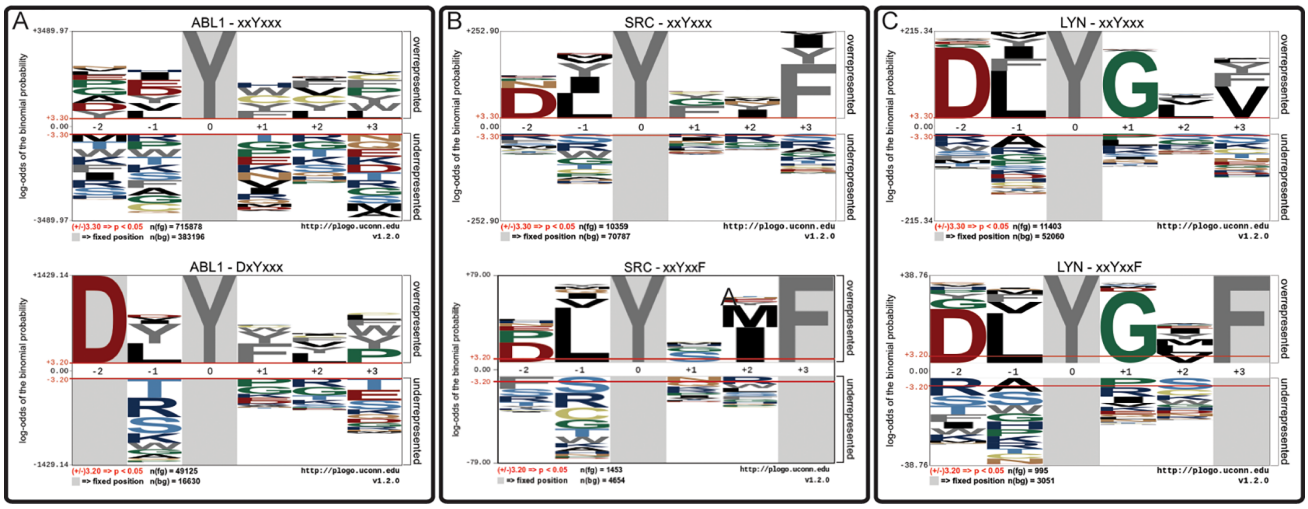

Figure 3. Sequence specificity of ABL1, SRC, and LYN kinase domains. Combinatorial libraries of the form XXYXXX, where X is encoded by the degenerate NNS codon. Results are plotted in the pLogo visualization tool (plogo.uconn.edu),[23] which calculates over- and under-representation of amino acids compared to a background set. Additionally, enriched amino acids may be "fixed" to examine covariance between substrate residues (displayed in gray) (A) ABL1 does not exhibit stringent specificity at the single amino acid level. Covariance between residues in the ABL1 substrate data set was further used to computationally predict substrate phosphorylation (Figure 4). (B, C) SRC and LYN kinase domains have broadly similar substrate specificity, but sampling bottlenecks prevented a similar analysis to the ABL1 data.

substrate in the ER results in phosphorylation of the substrate tyrosine, utilizing endogenous ATP. Ultimately, the substrate is transported to the yeast surface[18] and probed with phosphotyrosine and epitope tag antibodies for flow cytometry (Figure 1A). Cells expressing active ABL1 and substrate exhibit high antiphosphotyrosine (pY) and when analyzed by FACS. Culturing in TKI-supplemented media results in decreased phosphorylation, allowing for enrichment of inhibitor-resistant mutants (Figures 1B and S1). In addition to ABL1, the kinase domains of the proto-oncogenes SRC and LYN are also active in YESS and inhibited by dasatinib, indicating the generalizability of the method (Figure 1C). Cells expressing inhibitor-resistant kinase mutants or active substrates are enriched by FACS (Figure 1D and E).

Discovery of Inhibitor-Resistant Mutations. To screen for mutations that confer TKI resistance, a 3.2×10^7 member error-prone PCR ABL1 kinase library (error rate of 0.05%) was screened for activity in the presence of 25 μM dasatinib or ponatinib (Figure 1D). Previously, a titration with increasing concentrations of the inhibitors determined that 25 μM was required to observe strong inhibition with the yeast system. This relatively high concentration is presumably required due to diminished diffusion into the yeast ER (Figure S2). Libraries cultured with 25 μM inhibitor were sorted for high anti-pY/FITC ratios (Figure 1D). Greater than 10^7 cells were sorted in the first round. In subsequent rounds, previous sorting bottlenecks were exceeded by 10-fold to preserve diversity.

The fraction of pY-positive cells in dasatinib-treated cultures increased from 1.8% in the unsorted pool to 87.7% after six rounds of sorting. Phosphotyrosine-positive cells in ponatinib-treated cultures increased from 0.31% in the unsorted pool to 57.9% of cells after four rounds of sorting. Populations were sampled by PacBio sequencing, yielding approximately 10^4 sequences per population. Reads were aligned to the wild-type ABL1 kinase domain and filtered to remove those with insertions or deletions within 5 nucleotides of substitutions. The two sorted pools were highly polarized compared to the unsorted pool, with the ponatinib-treated pool more polarized and enriched for compound mutations (Figures 2E, S3, and S4). In the dasatinib-treated population, the top 636 variants accounted for 80% of the mutant sequences, while in the ponatinib-treated population the top 71 variants comprised nearly 80% of the mutant sequences (Figure 2F). In the ponatinib-sorted library, 46% of all mutants were compound mutants, representing a 4-fold increase over the unsorted pool (Figure S4). In addition, 6 of the 10 most frequent ponatinib-resistant variants contained two or more mutations, including the previously described Y253H/E255V amino acid substitutions isolated from a resistant patient, whereas none of the top 10 dasatinib-selected variants were compound mutants (Figure 2E).

The library was prepared with a low error rate (0.05%) so that greater than 90% of the library contained one or zero mutations,and 9.4% contained double mutations (Figure S4). Combined with the large library size (10^7), the ABL1 library was predicted to contain essentially complete coverage of single nucleotide substitutions and a significant fraction of possible double mutants.

We further characterized seven kinase domain variants for resistance to dasatinib and ponatinib in the murine Ba/F3 pro-B cell line (Figures 2G and S5). Three ponatinib-selected variants with compound mutations and the corresponding four variants with single mutations alone were assayed to investigate the relative importance of the compound mutations for ponatinib resistance. All three of the variants with double mutations (E255V/G303R, E255V/D325N, E255V/T392I) assayed had a significantly higher IC_{50} value for ponatinib than wild-type, validating our FACS data and selection strategy. Note that nanomolar concentrations of the inhibitors were used in these IC_{50} value determinations, consistent with known IC_{50} values derived from mammalian cell measurements. In our experiments, the E255V mutation alone conferred a 2.6-fold increase in IC_{50} for ponatinib, while the D325N mutation alone was not significantly different from wild-type. However, when combined, the E255V/D325N compound mutation combination resulted in an IC_{50} value 18-fold higher than wild-

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type, indicating that the effect of these two mutations was more than additive. Interestingly, the IC_{50} values for variants with the E255V/T392I and E255V/G303R compound mutations were not significantly different than the E225 V and G303R mutations alone, respectively (Figures 2G and S5). Furthermore, Western blots for phospho-CrkII Y221[22] showed that both E255V and E255V/D325N retained activity in the presence of 40 nM ponatinib, while wild-type BCR-ABL1 is inhibited (Figure S6).

Kinase Peptide Substrate Profiling. The yeast-based methodology was used in a complementary mode by screening a library of substrate peptide sequences for phosphorylation by wild-type kinase. Using this apparoach we were able to profile substrate sequence preferences for the SRC, LYN, and ABL1 kinases. Substrate libraries were constructed using degenerate codon primers surrounding a fixed tyrosine residue (XXYXXX), estimated to contain approximately 5×10^7 transformants per library. In the first round of sorting, both SRC and LYN phosphorylated substrates in just 0.4% and 0.1% of induced cells, respectively. In constract, ABL1 phosphorylated substrates in approximately 9% of induced cells in the initial round of sorting (Figure 1E). At the start of the fifth round of sorting, the percentage of phosphotyrosine-positive cells were 68%, 39%, and 70% for the SRC, LYN, and ABL1 libraries, respectively. For each population, plasmids were extracted from 10-times the population diversity. After quality filtering and barcode splitting, each ABL1 kinase sorting round contained between 9.3×10^5 and 3×10^6 sequence reads, corresponding to 3.5×10^5–8.4×10^5 unique substrate DNA sequences. SRC and LYN sequencing results contained between 3.4×10^5 and 2.0×10^6 sequences per round, with the exception of the second round of LYN substrate sorting, which contained only 1.7×10^4 DNA sequences. Amino acid enrichment relative to the presorted library are displayed in Figure 3.

For the ABL1 sequencing results, we applied a machine learning algorithm utilizing amino acid covariances to predict substrate sequences beyond the depth of the sequencing data. From both the presorted and sorted data, frequencies of each amino acid and each combination of two and three amino acids were calculated. For any given sequence of the form XXYXXX, a model score was calculated by computing the product of the conditional probabilities for each three amino acid combination, the conditional probabilities of each two amino acid combination, and the prior probabilities of each single amino acid. The log-likelihood score is the ratio of this calculation from the sorted and unsorted rounds. When calculated from the round three sorted data, log-likelihood scores for all possible peptides (3.2×10^6) ranged from −80 to +55. To validate that these scores predict phosphorylation by ABL1, 32 peptides were cloned individually into the YESS system and the extent of substrate phosphorylation was measured by FACS (Figure 4). It should be noted that 24 of these 32 peptides did not occur in any of the sequencing data and were therefore inferred to be substrates by amino acid covariance. To validate these results *in vitro*, phosphorylation of 12 synthetic peptides ABL1 was measured by LC-MS. Each of the seven peptides which were phosphorylated in the YESS system were also phosphorylated in the in vitro reaction, and each of the five peptides which were not phosphorylated in YESS were not phosphorylated *in vitro*.

The yeast-based approach presented here is unique in that it is capable of both the rapid and effective screening of tyrosine

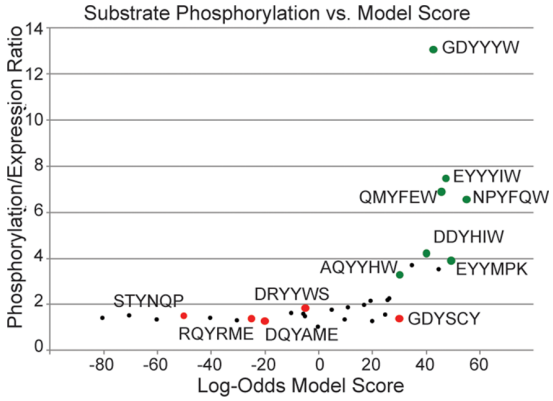

Figure 4. Model score accurately predicts substrate phosphorylation by ABL1 kinase domain. Thirty two peptides, ranging from the highest to lowest model score of all possible 5-mer peptides, assayed in yeast. Phosphorylation was observed for all peptides with a model score greater than 30. Furthermore, 24 of these 32 peptides were not present in the sequencing data set. Twelve peptides were further analyzed by a binary in vitro phosphorylation assay. Peptides which were phosphorylated in vitro are indicated by green dots, and those where no phosphopeptide was detected are indicated by red dots.

kinase inhibitor resistance as well as determining peptide substrate specificity in a comprehensive fashion. First, we investigated how well the new method can identify relative susceptibilities to resistance mutations elicited by different tyrosine kinase inhibitors. We sought to develop a facile method to identify, early in the drug development cycle, those drug candidates with the best potential for long-term efficacy. In contrast to mammalian cell-based techniques,[6,13−17,24] our yeast-based method can screen all possible single mutations and a significant portion of double (compound) mutations in under 2 weeks. Additionally, by directly assaying the phosphorylation of a synthetic substrate, our screen is more broadly applicable than mammalian screens, each of which requires a unique cell line with an intrinsic, selectable phenotype of kinase activity. Importantly, our yeast kinase libraries also offer the distinct advantage of being amenable to aliquoting and long-term storage, removing the need to produce a mutant library each time a new screen is undertaken. The large data sets generated by our approach therefore uniquely allow accurate comparisons between multiple inhibitors screened in parallel against an identical pool of kinase mutations. Finally, beyond the extensive work with ABL1, our yeast-based inhibitor assay has been applied successfully to SRC and LYN kinases, strongly supporting the general applicability of the method (Figure 1C).

The mutations recovered from our yeast-based screen reported here using dasatinib and ponatinib can be compared to mutations in inhibitor-resistant patients treated for CML.[25] In the case of dasatinib, our data recapitulated all eight of the previously described single mutations that lead to disease relapse in the clinic and have been verified as significantly resistant in vitro. Of these eight clinical mutations, four were observed in our top five and seven were observed among our top 20 most sequenced dasatinib resistance-conferring mutations.[20] In addition, many mutations seen from deep-sequencing of patients are also observed in our data, though the role of these mutations in disease relapse has not yet been reported. Note that none of the most common dasatinib variants were compound mutations. Although less clinical data

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has been reported so far, preliminary indications are that ponatinib retains efficacy against an array of known imatinib-resistant single mutants, but is still susceptible to compound mutations such as Y253H/E255V in CML patients[20,26−28] (Figure 2B). Our ponatinib screening data is consistent with these initial clinical findings in that 6 of our top 10 most common ponatinib-resistant variants contained two or more mutations, including Y253H/E255V. Note that our yeast-based screen utilized only soluble kinase domains, so we were not able to examine any clinical mutations outside of these regions of the kinases.

Beyond just recapitulating clinical data, our yeast-based screening data collectively predict that ponatinib is generally less susceptible to resistance compared to dasatinib. In particular, the isolated ponatinib-resistant population was both more highly polarized and more enriched for compound mutations, indicating that single mutations are generally less likely to confer resistance to ponatinib compared with dasatinib. For example, in our unsorted library treated with 25 μM ponatinib, just 0.31% of cells expressed resistant mutants, indicating that these mutations were relatively rare compared to the dasatinib-treated sample (Figure 1D). Similarly, following sorting, the ponatinib-treated library was more highly polarized than the dasatinib-treated library (Figure 2F). Finally, the proportion of compound mutations in the ponatinib-screened library overall (>46%) and in the most common sequence reads (Figure 2A) was significantly higher than the dasatinib screen (35%, Figure 2B). Validation of these results in the murine Ba/F3 cell line showed that each of three compound mutations were at least as resistant as the E255V mutation, and the E255V/D325N compound mutation is 7-fold more resistant than either single mutation alone (Figure 2G).

Note that most clinical studies to date have involved patients treated sequentially with imatinib and second-line TKIs, including dasatinib and ponatinib, following the appearance of imatinib resistance.[26,29] For that reason, many clinical isolates obtained from patients treated with ponatonib or dasatinib also contain the T315I mutation that is known to arise during first-line treatment. Because we only investigated ponatinib alone, it is not surprising that T315I mutations were not found to dominate in our studies. Nevertheless, it is interesting that the single inhibitor approach reported here did identify several compound mutations in the case of ponatinib treatment. Perhaps due to this difference in inhibitor exposure in the current experiment versus clinical practice, we also identified many dasatinib-resistant single mutations and some ponatinib-resistant compound mutations not yet reported based on clinical data.

We next investigated the second application of our new yeast-based method involving the analysis of the active-site peptide sequence preferences of tyrosine kinases. To accomplish this we used our yeast system in a different mode, retaining the sequence of the wild-type tyrosine kinase but screening against a large random library of substrates. Using this approach, consensus sequence preferences were obtained for the SRC and LYN kinases, but the ABL1 kinase yielded preferences that were far less obvious. Applying machine learning to millions of ABL1 peptide substrates allowed us to predict accurately whether a peptide will be phosphorylated, regardless of it being present in the sequencing data. Although high-throughput screening has been utilized for kinase substrate screening, prior to the present study it had yet to be combined with next-generation sequencing and machine learning, which in this study allows us to predict accurately whether a peptide may be phosphorylated by ABL1 kinase *in vitro*.

The results reported here have established the utility of our new yeast-based method for two aspects of kinase biology: peptide substrate specificity and inhibitor resistance. First, the system was able to reproduce and therefore likely predict clinical findings of TKI resistance, providing a facile and inexpensive screen for predicting potency to known and novel resistance mutations early in the development cycle of next-generation clinical TKI candidates. Furthermore, by combining our high-throughput screening with next generation sequencing of kinase substrates we are able to predict accurately kinase substrate peptides, an advance which could add greatly to the understanding of tyrosine kinase biology.

This report should be considered as just the beginning of what can be accomplished with YESS in the tyrosine kinase arena. We are currently investigating other FDA-approved TKIs. Ideally, we will be able to create a comprehensive database of resistance mutations for all of the approvided TKIs that will be useful for clinicians attempting to choose the best alternative TKI once a given clinical resistance mutation has been identified in their patient. Such a database would also aid those developing next generation TKIs, providing a rapid method to screen numerous candidate molecules for resistance early in the drug development cycle. Finally, we also hope to learn more about the substrate specificities of tyrosine kinase active sites to aid in the basic understanding of these important enzymes.

At a higher level, as the list of pharmaceuticals such as TKIs used to treat disease continues to grow, it is important that we shift attention toward finding new drug candidates with improved long-term efficacy including the ability to evade common resistance mechanisms.[30,31] Assays such as the one presented here will be able to greatly assist in these efforts.

METHODS

Vector Construction. Amino acids 237−630 of human ABL1 isoform 1b were cloned into the pESD vector under the GAL10/GAL1 bidirectional promoter in place of TEV protease.[18,19] TEV protease substrate was replaced with a minimal kinase substrate (AAAAAYAAAAA).[21] Yeast receptor adhesion subunit Aga2, ER retention signal, and hexahistidine and FLAG epitope tags were retained from the pESD vector.[18,19]

Yeast-Based Inhibitor Resistance Assay. ABL1 wild-type and T315I mutant expression was induced by growth in SG-UT[32] medium containing 125 uM dasatinib, ponatinib, or DMSO for 40 h at 20 °C. Cells were washed three times with TBST+BSA (TBS + 0.5% BSA + 0.05% Tween20), then stained with anti-His6-FITC (Thermo Fisher, MA1-81891) and antiphosphotyrosine-PE (BioLegend) at 4 °C for 30 min, followed by three washes with TBST+BSA. FACS analysis was performed with the FACS Aria IIu or FACSCalibur (BD Biosciences).

Error-Prone Library Construction. ABL1 kinase domain was amplified with an error-prone variant of KOD polymerase to generate a pool of random mutants. Vector was prepared by digestion of pESD-ABL1 plasmid with *Sal*I-HF, XhoI, and NcoI-HF. Electrocompetent EBY100 were prepared as described previously.[18,32] In each of three 2 mm electroporation cuvettes (Thermo Fisher Scientific), 350 μL of electrocompetent EBY100 cells was combined with 10 μg of ABL1 error-prone PCR product and 3 μg of digested vector to a maximum volume of 400 μL. Library size was estimated by dilution series of transformed cells plated on SD-UT agar.

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Library Screening by FACS. Library cells were induced by growth in 10 mL of SG-UT with 25 μM inhibitor (Selleckchem) or an equivalent volume of DMSO. Wild-type ABL1 with inhibitor or DMSO used to determine the location of the sorting gates. Phosphotyrosine+/His6+ cells were collected and re-sorted, then transferred to SD-UT medium for growth at 30 °C until dense, 1−2 days. Subsequent rounds were performed identically until phosphotyrosine+/His6+ cells accounted for 60−90% of the population.

Substrate Library Construction. Primers encoding the XXYXXX peptide, where X encoded by the NNS degenerate codon, were used to amplify the C-terminal half of the Aga2-substrate fusion gene. Overlap extension PCR with the N-terminal half of the Aga2-substrate fusion created an amplicon encoding the entire gene, which was cotransformed with *Eco*RI and PacI-digested parental vector into the EBY100 yeast display strain. Transformation efficiency was assayed as described above. Kinase and substrate expression were induced by 48 h growth at 20 °C in SG-UT medium.

High-Throughput Sequencing of Inhibitor Resistance Libraries. Plasmids were recovered from saturated overnight cultures using the ZymoPrep II kit (Zymo Research). DNA from unsorted, dasatinib-sorted, and ponatinib-sorted libraries was barcoded by PCR. After barcoding PCR, concentrations were quantified by Qbit (Thermo Fisher). Pooled samples were sequenced with the PacBio RSII at the Arizona Genomics Institute at the University of Arizona.

High-Throughput Sequencing of Substrate Libraries. Plasmids from each round were isolated from 10-fold the estimated population diversity using the ZymoPrep II kit (Zymo Research). Substrate genes were amplified using primers containing 5-mer barcodes, followed by purification by agarose gel extraction and pooling for sequencing. Full MiSeq 2 × 250 bp (ABL1) or 1 × 300 bp (SRC and LYN) runs were performed by the University of Texas Genomic Sequencing and Analysis Facility (GSAF). Sequence reads were filtered for quality reads (qphred > 25) with the fastx toolkit (http://hannonlab.cshl.edu/fastx_toolkit/).

Substrate Sequence Analysis. Sequences were aligned to wild-type ABL1 kinase domain using an implementation of NCBI BLAST on the Texas Advanced Computing Core. Mutations in aligned sequences were then translated and compiled into a database. Sequences were counted and assembled into Python dictionaries, which were then used to calculated frequencies of amino acids and conditional frequencies of all possible two and three amino acid combinations.

Ba/F3 Validation of Resistance Mutants. Mutants were generated on a pDONR Bcr-Abl p210 template using QuickChange site-directed mutagenesis (Agilent) and transferred to the pfMIG retroviral expression vector using Gateway cloning (Thermo Fisher Scientific). The murine pro B-cell line Ba/F3 (DSMZ ACC-300) was retrovirally transduced with the human Bcr-Abl p210 wildtype and mutant cDNAs as previously described.[33] The transduced cells were FACS-sorted for GFP (coexpressed with Bcr-Abl) and Bcr-Abl expression levels were checked by immunoblotting.

Transduced BaF3 cells were grown in RPMI medium (Lonza) supplemented with 10% FBS, 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin. Cells thawed from freezer stocks were passaged twice in media additionally supplemented with 10 ng mL^{-1} IL-3 (Peprotech), followed by two passages in the absence of IL-3. Then 5 ×10^4 cells were seeded in 100 μL in each well of a 96-well plate. Next, 50 μL of inhibitor in RPMI + FBS + Pen/Strep was added to each well. Cell viability was assayed after 24 h using CellTiter Glo (Promega) according to the manufacturer's instructions, except that the reagent was diluted 1:5 in PBS. Luminescence was detected using a plate reader (Tecan). Titration curves were fitted using a four-parameter dose−response curve using GraphPad Prism. Immunoblotting was performed with Phospho-CrkII Tyr211 and GAPDH antibodies (Cell Signaling Technologies) according to manufacturer's instructions after 2 h incubation with inhibitor. Membranes were incubated with anti-Rabbit IgG (Biolegend) for 1 h at RT, then visualized using the G:BOX system (Syngene)

In Vitro Validation of ABL1 Substrates. A concentration of 1 μM peptide, where the indicated sequence is flanked by three alanines (GenScript), was incubated with 100 nM GST-ABL1 (ProQinase) overnight at 25 °C in 50 mM Tris HCl pH 8.0, 150 mM NaCl, and 500 μM ATP. Reactions were quenched at 98 °C for 2 min. LC-MS was performed on an Agilent 6130 instrument.[30,31]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.9b00283.

Flow cytometry control experiments, sequence analysis, and *in vitro* validation of resistance mutations (PDF)

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Notes

The authors declare no competing financial interest.

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