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A quantitative analysis of kinase inhibitor selectivity

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Kinase inhibitors are a new class of therapeutics with a propensity to inhibit multiple targets[1,2]. The biological consequences of multi-kinase activity are poorly defined, and an important step toward understanding the relationship between selectivity, efficacy and safety is the exploration of how inhibitors interact with the human kinome[2–4]. We present interaction maps for 38 kinase inhibitors across a panel of 317 kinases representing >50% of the predicted human protein kinome. The data constitute the most comprehensive study of kinase inhibitor selectivity to date and reveal a wide diversity of interaction patterns. To enable a global analysis of the results, we introduce the concept of a selectivity score as a general tool to quantify and differentiate the observed interaction patterns. We further investigate the impact of panel size and find that small assay panels do not provide a robust measure of selectivity.

A first step toward understanding how kinase inhibitor selectivity affects biological activity is to identify and quantify potential off-target kinase interactions[4,5]. We used a previously described *in vitro* competition binding assay[6] to evaluate 38 kinase inhibitors (**Supplementary Table 1** online) against a panel of 287 distinct human protein kinases (~55% of the predicted human protein kinome[7]), three lipid kinases and 27 disease-relevant mutant variants (**Supplementary Table 2** online). The compounds tested included 21 tyrosine kinase inhibitors, 15 serine-threonine kinase inhibitors, 1 lipid kinase inhibitor and staurosporine (classified according to the targets for which they were originally developed; **Supplementary Table 1**). Each compound was screened against the panel of 317 assays at a single concentration of 10 μM to identify candidate kinase targets, and for each interaction observed in this primary screen a quantitative dissociation constant (K_d) was determined. Among the 12,046 small molecule–kinase combinations queried, the primary screen revealed 3,175 binding interactions, including 2,993 with K_d < 10 μM, and 764 with K_d < 100 nM. For only 75 of the 3,175 (2.4%) interactions identified in the primary screen did the follow-up K_d measurement not confirm binding (positive predictive value = 0.976). To assess how many binding interactions may have been missed, we measured dissociation constants for sorafenib (Nexavar), VX-680/MK-0457, CHIR-258/TKI-258 and CHIR-265/RAF-265 against each kinase not identified as a binding target in the primary screen (233, 161, 163 and 258 kinases for sorafenib, VX-680/MK-0457, CHIR-258/TKI-258 and CHIR-265/RAF-265, respectively). The 10 μM primary screen was expected to detect interactions with K_d <~1 to 3 μM, and we therefore only considered as false negatives missed interactions with K_d < 1 μM. Three such interactions were identified (one for sorafenib, none for VX-680/MK-0457, two for CHIR-258/TKI-258 and none for CHIR-265/RAF-265; negative predictive value = 0.996; K_ds for these three interactions are included in **Supplementary Table 2**). The results presented here were obtained independently from those previously published for smaller kinase panels using the same technology[6,8], and the consistency between the data sets illustrates the robustness of the competition binding assay. Binding affinities for known targets of the compounds tested were in line with published results from conventional assays (**Supplementary Table 3** online). The new data represent the most comprehensive view of kinase inhibitor selectivity available to date, and reveal a diversity of interaction patterns of small molecules with the human kinome (**Fig. 1**; K_ds are shown in **Supplementary Table 2**).

Small molecule–kinase interaction maps provide a useful graphic overview of how compounds interact with the kinome[6] (**Fig. 1**). Although this view is quantitative in the sense that it represents the binding affinities of compounds for individual kinases, it provides only a qualitative overall impression of selectivity. To develop a more quantitative description, we calculated a selectivity score (S) for each compound by dividing the number of kinases found to bind with a dissociation constant <3 μM by the total number of distinct kinases tested (excluding mutant variants), in this case 290 (**Fig. 2a**). The selectivity score is an unbiased measure that enables quantitative comparisons between compounds and the detailed differentiation and analysis of interaction patterns. Scores ranged from 0.010 (three kinases bound with K_d < 3 μM) for lapatinib (Tykerb), an EGFR/ERBB2 inhibitor[9], to 0.87 (253 kinases bound with K_d < 3 μM) for staurosporine, a well characterized promiscuous kinase inhibitor (**Supplementary Table 4** online contains a complete list of selectivity scores). With the exception of staurosporine, which was substantially more promiscuous than any of the other compounds tested, there was a continuum of selectivities (**Fig. 2a**).

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Interestingly, selectivity by this measure varied more than 50-fold among the seven small molecule kinase inhibitors tested here that are currently approved for use in humans (**Supplementary Table 1** online), from S = 0.010 for lapatinib to S = 0.57 for sunitinib (Sutent), with a fairly even distribution over this range. When only high-affinity interactions were taken into account and selectivity scores were calculated for binding interactions with K_d < 100 nM (S(100 nM)), it became apparent that most of the compounds bound only a relatively small number of kinases with high affinity (**Fig. 2b**). Among the exceptions were sunitinib and dasatinib (Sprycel), two of the marketed drugs, which both bound >15% of kinases tested with K_d < 100 nM (S(100 nM) = 0.18 and 0.16, respectively), whereas S(100 nM) values for gefitinib (Iressa;

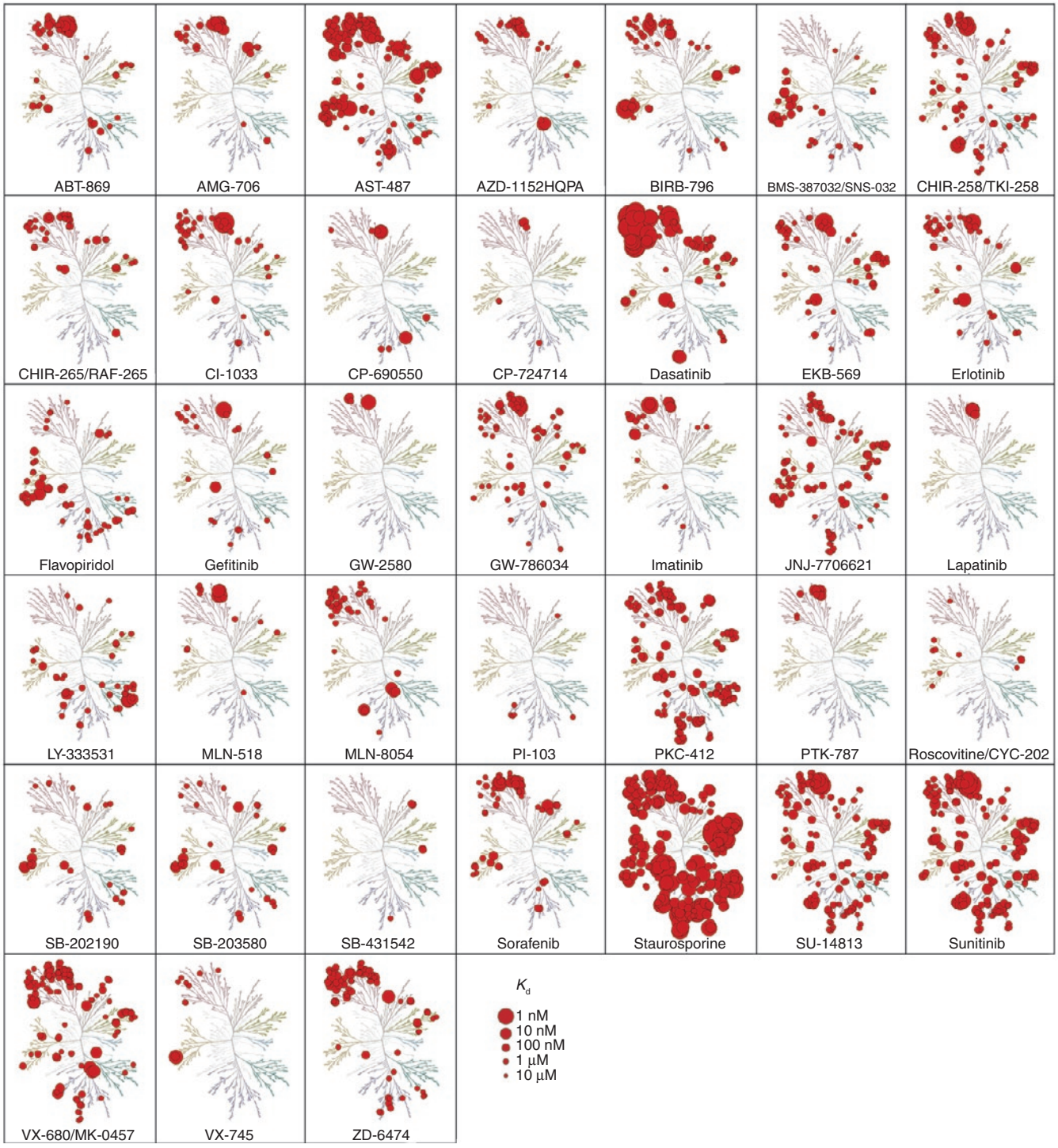

Figure 1 Small molecule–kinase interaction maps for 38 kinase inhibitors. Kinases found to bind are marked with red circles, where larger circles indicate higher-affinity binding. Interactions with K_d < 3 μM are shown. Complete results can be found in **Supplementary Table 2** online. The data set is also available through an interactive website (http://www.ambitbio.com/technology/publications). The kinase dendrogram was adapted and is reproduced with permission from *Science* (http://www.sciencemag.org/) and Cell Signaling Technology, Inc. (http://www.cellsignal.com/).

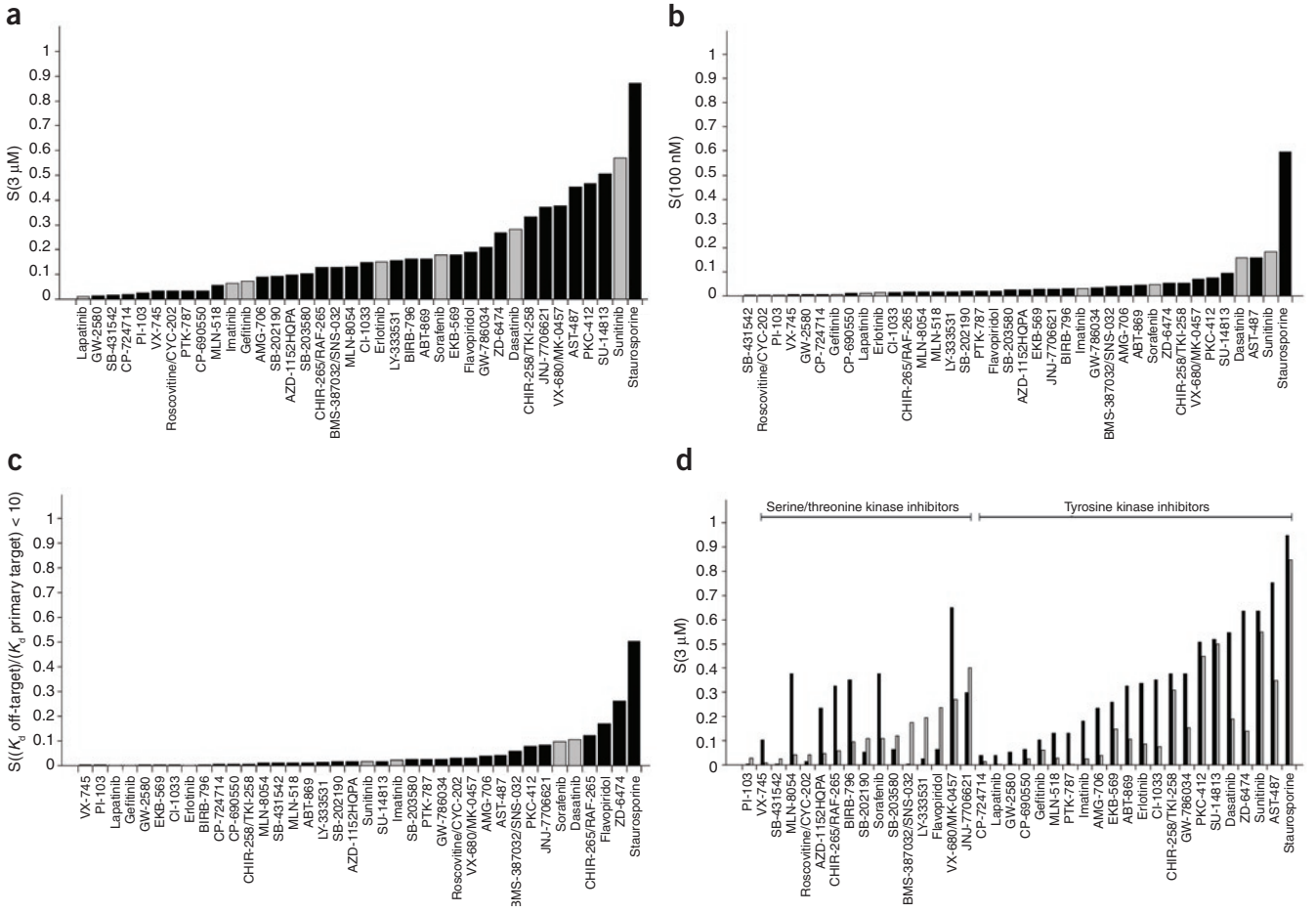

Figure 2 Selectivity scores as a quantitative measure of specificity. (**a**) Selectivity scores calculated for binding interactions with K_d < 3 μM. S(3 μM) = (number of binding interactions with K_d < 3 μM/number of kinases tested (290); the 27 mutant variants were excluded from this analysis). Compounds approved for use in humans (as of August, 2007) are highlighted (gray bars). (**b**) Selectivity scores calculated for binding interactions with K_d < 100 nM. S(100 nM) = (number of binding interactions with K_d < 100 nM/number of kinases tested (290)). Compounds approved for use in humans (as of August, 2007) are highlighted (gray bars). (**c**) Selectivity relative to primary targets. The ratio of binding affinities for off-targets relative to the primary target for each compound was calculated (primary targets are shown in **Supplementary Table 1**; for compounds with multiple primary targets the first listed was used; for LY-333531 the primary target was not represented in our panel, and a literature IC_{50} value was used to calculate K_d ratios[27]). The number of off-targets for which the (K_d off-target/K_d primary target) ratio is less than 10 was divided by the total number of kinases tested (excluding primary targets and the 27 mutant variants) to arrive at S((K_d off-target/K_d primary target) < 10). For each compound, additional primary targets listed in **Supplementary Table 1** were not considered off-targets in this calculation. (**d**) Comparison of selectivity scores (S(3 μM)) for tyrosine kinases (black bars) and serine-threonine kinases (gray bars). There are 77 tyrosine kinases and 210 serine-threonine kinases (including atypical protein kinases) in the panel. The lipid kinases PIK3CA, PIP5K1A and PIP5K2B were excluded from this analysis. A complete list of selectivity scores is provided in **Supplementary Table 4** online.

0.007), lapatinib (0.010), erlotinib (Tarceva; 0.014), imatinib (Gleevec; 0.031) and sorafenib (0.048) were all <0.05, and at least threefold lower than those for sunitinib and dasatinib.

The biologically most relevant measure of selectivity may be how many and which targets are inhibited with potency similar to or greater than an intended, primary target. To quantify the frequency of off-target interactions with affinities comparable to those for primary targets, we calculated for each compound the fold-difference between the affinity for each off-target and a primary target, K_d(off-target)/K_d(primary target) (primary targets are shown in **Supplementary Table 1**; for compounds with multiple primary targets, only the first listed was used in this calculation), and determined a selectivity score by counting only off-target interactions with K_d(off-target)/K_d(primary target) < 10 (**Fig. 2c** and **Supplementary Table 4**). Most of the compounds tested did not bind many off-targets with affinities similar to that for primary targets, and by this measure were quite selective. Among the exceptions were sorafenib and dasatinib, which both bound ~10% of the kinases tested with affinities within tenfold of that for the primary target considered in the analysis. These compounds therefore have the potential to interact with a large number of kinases at concentrations required for potent inhibition of their primary targets. The highest affinity off-targets often were kinases in the same group or family as the primary targets[7] (**Supplementary Fig. 1**). For example, the primary target for PTK-787 is VEGFR2, and the highest affinity off-targets were closely related receptor tyrosine kinases. Similarly, all but two of the off-targets with affinities within tenfold of the affinity of a primary target for the ABL1/SRC inhibitor dasatinib were tyrosine kinases. There were a number of compounds, however, with high-affinity off-targets that were not closely related to their primary targets. The receptor tyrosine kinase inhibitor ZD-6474, the cyclin-dependent kinase inhibitor flavopiridol, the aurora kinase inhibitor VX-680/MK-0457 and the class III receptor tyrosine kinase inhibitor AST-487, for example, each bound

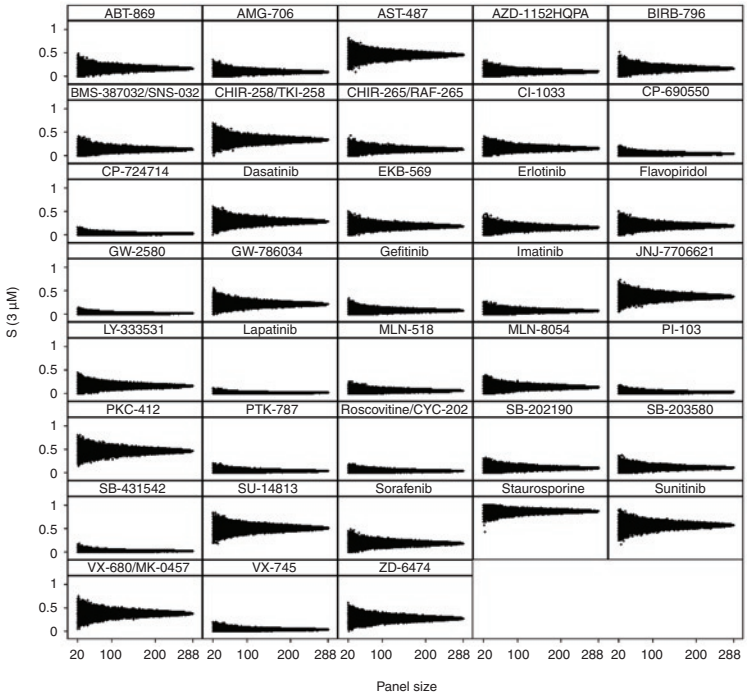

Figure 3 The effect of panel size on apparent selectivity. Subpanels ranging in size from 20 to 288 kinases were randomly chosen from the full panel of 290 distinct kinases. Selectivity scores (S(3 μM)) were calculated for 500 individual panels of each size, as described in the text. The total number of scores plotted for each compound is 134,500 (500 panels × 269 panel sizes).

kinases outside of their primary target's group[7] with similar affinity as the primary targets.

From the kinase interaction maps (**Fig. 1**) it is apparent that some compounds bound with comparable frequency to multiple groups of kinases, whereas others strongly discriminated between groups. To begin to quantify these differences, we calculated separate selectivity scores for tyrosine kinases (S_{TK}) and serine-threonine kinases (S_{STK}) for each of the 38 compounds (**Fig. 2d** and **Supplementary Table 4**). The results highlight, for example, the distinctly different activity patterns of sunitinib and dasatinib. Both compounds were developed as tyrosine kinase inhibitors[10,11] and bound a substantial fraction of kinases tested (S(3 μM) = 0.57 for sunitinib and 0.28 for dasatinib). Yet sunitinib did not have a strong preference for tyrosine or serine-threonine kinases (S_{TK}(3 μM) = 0.64, S_{STK}(3 μM) = 0.55), whereas dasatinib favored tyrosine kinases and was quite selective against serine threonine kinases (S_{TK}(3 μM) = 0.55, S_{STK}(3 μM) = 0.19). This difference was even more pronounced when only high-affinity interactions were considered (S(100 nM) = 0.18 for sunitinib and 0.16 for dasatinib; S_{TK}(100 nM) = 0.21 and S_{STK}(100 nM) = 0.17 for sunitinib; S_{TK}(100 nM) = 0.47 and S_{STK}(100 nM) = 0.048 for dasatinib). In general, whereas compounds originally described as tyrosine kinase inhibitors bound tyrosine kinases more frequently than serine-threonine kinases, many of the compounds originally described as serine-threonine kinase inhibitors bound tyrosine kinases more frequently (**Fig. 2d**). Examples include the aurora kinase inhibitors VX-680/MK-0457, MLN-8054 and AZD-1152HQPA, which had a substantially higher S_{TK}(3 μM) (0.65, 0.38 and 0.23, respectively) than S_{STK}(3 μM) (0.27, 0.043 and 0.048, respectively). Sorafenib, initially described as an inhibitor of the RAF serine/threonine kinase[12], also preferred tyrosine kinases (S_{STK}(3 μM) = 0.11, S_{TK}(3 μM) = 0.38; S_{STK}(100 nM) = 0.0095, S_{TK}(100 nM) = 0.16), and at least some of the antitumor effects of this compound are likely due to inhibition of tyrosine kinases[13–15].

One of the major questions for characterizing kinase inhibitors is how many kinases should compounds be screened against to obtain an accurate measure of selectivity. To directly and quantitatively address this question, we selected subpanels ranging in size from 20 to 288 assays from among the 290 distinct kinases in our full panel (excluding mutant variants). For each panel size we randomly selected five hundred different, individual panels and calculated selectivity scores (S(3 μM)) for each compound against each panel (**Fig. 3**). The data show that as the number of kinases sampled decreased, the variability in the selectivity scores for a given panel size increased (also reflected in higher coefficients of variation; see **Supplementary Fig. 2**). Results were similar when, instead of choosing kinases entirely at random, the proportionate representation of kinases from each group[7] was maintained relative to the full panel (data not shown). The exact composition of a panel therefore considerably affects apparent selectivity, particularly for small assay panels. These findings are consistent with the observation that the targets of small-molecule inhibitors are not uniformly distributed in kinase space, and that the distribution of targets across kinase groups and families varies for each compound (**Fig. 1**). Data from a single, small panel are thus unlikely to yield a robust measure of selectivity or to properly place compounds in rank order by overall selectivity. Furthermore, identification of specific, individual off-targets can lead to unanticipated clinical applications of existing compounds. For example, the discovery that imatinib inhibits KIT in addition to ABL1 and the PDGF receptors first suggested the use of imatinib for the treatment of gastrointestinal stromal tumors[16–18], and the discovery that the aurora kinase inhibitor VX-680/MK-0457 has activity against the drug-resistant ABL1(T315I) mutant kinase has led to the successful use of this compound in chronic myeloid leukemia patients with the ABL1(T315I) mutation[8,19]. In both of these cases the discovery was made only after the compound had already entered clinical trials for other indications. Novel, potentially clinically important targets are most likely to be found when screening large numbers of kinases and important information, therefore, remains concealed when using limited assay panels to assess the interaction of compounds with the human kinome.

The data presented here provide the most comprehensive description of selectivity for small-molecule kinase inhibitors available to date. As such they are an important resource for guiding studies to understand the biological activity of the inhibitors tested, to explore how chemical structure affects interaction patterns across the kinome, and to model small-molecule/kinase interactions computationally[20]. To obtain such a broad assessment of selectivity, we used an efficient *in vitro* competition binding assay[6]. The binding assays are active-site directed[6] and not designed to identify allosteric interactions, yet binding of at least some non-ATP competitive compounds, such as the MEK inhibitor CI-1040 (ref. 21), can be detected and appropriate binding affinities measured (data not shown). As with any cell-free *in vitro* binding or enzymatic assay, it is important to recognize that the activities of compounds in cells will not exactly mirror those observed here, and there are many potential causes for differences[4]. Our intent here has been to explore the

intrinsic ability of compounds to bind to kinase active sites. To understand the potency and impact of the interactions identified in a biologically relevant context will require follow-up in cellular assays.

It has been recognized for some time that selectivity is a critical issue for small-molecule kinase inhibitors[22–25], and that the relationship between selectivity, kinome interaction patterns and biological activity needs to be explored and more clearly defined. However, the study of selectivity has been qualitative and not systematic. Compound selectivity has often been assessed by screening against small numbers of kinases and without objective, standardized criteria[26–28]. It has therefore been difficult to compare results for different compounds and from multiple studies. The increasing availability of large assay panels[29–32] and of novel inhibitors for a growing number of kinases that have a range of selectivities provides an opportunity for a more systematic approach. To fully exploit the data generated will require novel analytical tools to organize and objectively analyze large-scale, small molecule–kinase interaction data sets[31,33]. We therefore introduce here the concept of a selectivity score, which may be used to quantify and explore selectivity, and which provides a general and simple means for communicating results from kinase inhibitor profiling experiments. Selectivity scores may be determined with data from any assay technology and from any size study and will facilitate comparisons not only between compounds, but also across independent studies and assay platforms. Selectivity scores may be calculated using a variety of criteria, as illustrated here. The scores quantify not only the overall selectivity of compounds, but may also be used to address more specific questions to explore kinase interaction patterns in detail.

The ability to quantify selectivity will become increasingly important as larger numbers of compounds, and ultimately entire compound libraries, are profiled against large kinase panels[31]. The resulting library profiles will simultaneously provide information about compound selectivity and potency and will reveal general patterns of how chemical structure affects reactivity across the kinome, as well as the detailed interaction patterns within kinase subfamilies and with therapeutically relevant kinases. Finally, knowledge of the interaction patterns of kinase inhibitors in development may help to interpret the biological activity observed in the clinic or in preclinical models. The insights gained should substantially improve the efficiency of kinase inhibitor discovery.

METHODS

Compounds. Staurosporine, SB-203580 and LY-333531 were purchased from A.G. Scientific, Inc. GW-2580, JNJ-7706621, SB-202190 and Roscovitine/CYC-202 were purchased from Calbiochem. SB-431542 was purchased from Tocris Bioscience. Flavopiridol was purchased from Qventas, Inc. Sunitinib and imatinib were custom synthesized by Saichem, Inc. GW-786034, MLN-518, MLN-8054, VX-680/MK-0457, PTK-787, ZD-6474, lapatinib, erlotinib, and BIRB-796 were custom synthesized by CiVentiChem L.L.C. CHIR-258/TKI-258, AST-487, ABT-869, AZD1152HQPA, gefitinib, CI-1033, BMS-387032/SNS-032 and PI-103 were custom synthesized by Shangai SynCores Technologies, Inc. VX-745 and EKB-569 were custom synthesized by WuXi Pharmatech. AMG-706 and SU-14813 were custom synthesized by BioDuro, Inc. CP-690550 was custom synthesized by SynChem, Inc. Dasatinib was provided by Bristol-Myers Squibb. CHIR-265/RAF-265, CP-724714, PKC-412 and sorafenib were synthesized at Ambit Biosciences.

Competition binding assays. Competition binding assays were developed, validated and performed as described previously[6]. For most assays, kinases were produced as fusions to T7 phage[6]; for the remaining assays, kinases were expressed in HEK-293 cells and subsequently tagged with DNA for PCR detection (data not shown). In general, full-length constructs were used for small, single-domain kinases, and catalytic domain constructs for large multidomain kinases (**Supplementary Table 5** online). For the binding assays, streptavidin-coated magnetic beads were treated with biotinylated affinity ligands for 30 min at 25 °C to generate affinity resins. The liganded beads were blocked with excess biotin and washed with blocking buffer (SeaBlock (Pierce), 1% BSA, 0.05% Tween 20, 1 mM DTT) to remove unbound ligand and to reduce nonspecific binding. Binding reactions were assembled by combining kinase, liganded affinity beads and test compounds in 1× binding buffer (20% SeaBlock, 0.17× PBS, 0.05% Tween 20, 7 mM DTT). Test compounds were prepared as 100× stocks in DMSO and rapidly diluted into the aqueous environment. DMSO was added to control assays lacking a test compound. Primary screen interactions were performed in polypropylene 384-well plates in a final volume of 34 µl, whereas K_d determinations were performed in polystyrene 96-well plates in a final volume of 135 µl. The assay plates were incubated at 25 °C with shaking for 1 h, and the affinity beads were washed extensively with wash buffer (1× PBS, 0.05% Tween 20) to remove unbound protein. The beads were then resuspended in elution buffer (1× PBS, 0.05% Tween 20, 2 µM nonbiotinylated affinity ligand) and incubated at 25 °C with shaking for 30 min. The kinase concentration in the eluates was measured by quantitative PCR. Each kinase was tested individually against each compound. K_ds were determined using eleven serial threefold dilutions.

Note: Supplementary information is available on the Nature Biotechnology website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturebiotechnology/.

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