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REVIEW

A guide to picking the most selective kinase inhibitor tool compounds for pharmacological validation of drug targets

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To establish the druggability of a target, genetic validation needs to be supplemented with pharmacological validation. Pharmacological studies, especially in the kinase field, are hampered by the fact that many reference inhibitors are not fully selective for one target. Fortunately, the initial trickle of selective inhibitors released in the public domain has steadily swelled into a stream. However, rationally picking the most selective tool compound out of the increasing amounts of available inhibitors has become progressively difficult due to the lack of accurate quantitative descriptors of drug selectivity. A recently published approach, termed 'selectivity entropy', is an improved way of expressing selectivity as a single-value parameter and enables rank ordering of inhibitors. We provide a guide to select the best tool compounds for pharmacological validation experiments of candidate drug targets using selectivity entropy. In addition, we recommend which inhibitors to use for studying the biology of the 20 most investigated kinases that are clinically relevant: Abl (ABL1), AKT1, ALK, Aurora A/B, CDKs, MET, CSF1R (FMS), EGFR, FLT3, ERBB2 (HER2), IKBKB (IKK2), JAK2/3, JNK1/2/3 (MAPK8/9/10), MEK1/2, PLK1, PI3Ks, p38α (MAPK14), BRAF, SRC and VEGFR2 (KDR).

Abbreviations
PSID, Pubchem Substance Identification no.

The importance of selective kinase tool compounds

In 2002, Sir Philip Cohen predicted that protein kinases would become 'the drug targets of the 21st century' (Cohen, 2002). So far, kinases have lived up to this expectation. In the past 10 years, 15 small molecule kinase inhibitors and five anti-kinase antibodies have been approved by the U.S. Food and Drug Administration (Table 1). These successes (and the projects that failed along the way) have yielded a wealth of reference compounds in the public domain that are useful for investigating the role of specific protein kinases in cellular processes (for reviews, see Shokat and Velleca, 2002; Carlson and White, 2011; Hodgson and Schröder, 2011).

Kinase inhibitors are powerful tools for pharmacological validation because their effects give direct information on the effect of therapeutic targeting of the protein. However, many of them inhibit multiple kinases, in part because they target the highly conserved ATP-binding pocket. There are many instances where inhibition of an off-target kinase contributes to, or even is solely responsible for, the observed biological effects. A recent example comes from work implicating the kinase p38α in Wnt/β-catenin signalling (Verkaar *et al.*,

Table 1

Kinase inhibitors approved for therapeutic use

	Compound	Trade name	Company	Target	Cancer	Year of approval
1	Imatinib	Gleevec/Glivec (EU)	Novartis	BCR-ABL	CML	2001
				KIT	GIST	2008
2	Gefitinib	Iressa	Astra-Zeneca	EGFR	NSCLC	2003
3	Sorafinib	Nexavar	Bayer/Onyx	Multiple (VEGFR,	Renal cell carcinoma	2005
				PDGFR, RAF)	Liver cancer	2007
4	Erlotinib	Tarceva	Genentech/Roche	EGFR	NSCLC	2005
					Pancreatic cancer	2005
5	Dasatinib	Sprycel	BMS	Multiple (BCR-ABL, SRC)	CML	2006
6	Sunitinib	Sutent	Pfizer	Multiple (PDGFR, VEGFR, KIT)	Renal cell carcinoma	2006
					Imatinib-resistant GIST	2006
7	Temsirolimus	Torisel	Wyeth	mTOR[1]	Renal cell carcinoma	2007
8	Nilotinib	Tasigna	Novartis	BCR-ABL	Imatinib-resistant CML	2007
9	Lapatinib	Tykerb/Tyverb (EU)	GSK	EGFR, ERBB2	HER2+ breast	2007
					triple+ breast	2010
10	Everolimus	Zortress/Certican (EU)/Afinitor	Novartis	mTOR[1]	Advanced kidney cancer	2009
11	Pazopanib	Votrient	GSK	Multiple (PDGFR, VEGFR, KIT)	Renal cell carcinoma	2009
12	Vandetanib	Caprelsa	Astra-Zeneca	RET	Medullary thyroid cancer	2011
13	Ruxolitinib	Jakafi	Incyte	JAK1/2	Myelofibrosis	2011
14	Vemurafenib	Zelboraf	Roche	BRAF (V600E mutant)	Metastatic melanoma	2011
15	Crizotinib	Xalkori	Pfizer	ALK	NSCLC	2011

[1]These compounds inhibit the TORC1 cofactor of mTOR and are therefore not kinase inhibitors in a strict sense.

2011). p38α is a stress-activated serine/threonine kinase that mediates production of inflammatory cytokines. Multiple p38α inhibitors have been clinically evaluated for diseases of the immune system. Several researchers noted that administration of such p38α drugs to cell lines inhibits signalling through the Wnt/β-catenin pathway (Bikkavilli *et al.*, 2008; Cervenka *et al.*, 2010), an evolutionarily conserved signalling cascade critical for embryonic development and adult stem cell maintenance (Logan and Nusse, 2004; MacDonald *et al.*, 2009). However, recently released cross-screening data revealed that several widely used tool compounds for p38α (for instance, SB203580) also inhibit casein kinase Iδ and CKIε (Bain *et al.*, 2007; Verkaar *et al.*, 2011). Both kinases are well known to be activators of Wnt/β-catenin signalling (Peters *et al.*, 1999; Sakanaka *et al.*, 1999). Importantly, this cross-reactivity cannot be explained by sequence similarity, as p38α and CKIs are quite distant in the phylogenetic tree. Their pharmacological similarity could only be demonstrated by profiling inhibitors in biochemical assays. Another instance where compound promiscuity confounds scientific analysis is when the same compound is used as a tool inhibitor for more than one kinase. The spectrum selective inhibitor dasatinib was used as a 'typical' SRC inhibitor by Gnoni *et al.*, (2011), while An *et al.* (2010) used dasatinib as a 'typical' Abl inhibitor. Thus, it is important to thoroughly understand the selectivity of pharmacological tools in the kinase field, and to make sure that targets are validated with the most selective inhibitors (Davies *et al.*, 2000; Knight and Shokat, 2005; Bain *et al.*, 2007).

Whereas in the early days of kinase research, inhibitors were often named 'selective' on the basis of anecdotal evidence, the recent wealth of selectivity profiling data has greatly advanced the rational understanding of inhibitor promiscuity. In selectivity profiling, the activity/affinity of kinase inhibitors on a multitude of non-target kinases is tested in parallel. Here we give an overview of sources of profiling data, and illustrate how to interpret those data through new methods for quantifying selectivity. With this method, we have pinpointed the most selective inhibitors for the 20 most intensely investigated protein kinases. This review serves as a guide to picking the most selective tool compounds, thereby minimizing the chance that cross-reactivities will compromise target validation.

Technologies and study approaches in cross-screening

The most-used method to study kinase inhibitor selectivity is profiling in multiple parallel biochemical assays. Biochemical assays are preferred because the readout can be coupled with

Table 2

Providers of kinase panel profiling services

Provider	Location of profiling lab	Technology	Kinase panel size (wild-type kinases)	Website
Carna Biosciences	Kobe, Japan	Substrate mobility shift assay	305 (*272*)	http://www.carnabio.com
DiscoverX (Ambit)	San Diego, CA, USA	Phage display competitive binding	451 (*394*)	http://www.kinomescan.com
Millipore	Dundee, UK	Radioactive filter binding	*299* (*261*)	http://www.millipore.com
Life Technologies	Madison, WI, USA and Paisley, UK	Z′-Lyte™ and Adapta® binding assay	317 (*293*)	http://www.invitrogen.com
ProQinase	Freiburg, Germany	Radioactive filter binding	340 (*298*)	http://www.proqinase.com
Reaction Biology	Malvern, PA, USA	Radioactive filter binding	385 (*348*)	http://www.reactionbiology.com
MRC Centre for Kinase Profiling	Dundee, UK	Radioactive filter binding	126 (*122*)	http://www.kinase-screen.mrc.ac.uk

Kinase panel sizes as reported on November 2011. The order of suppliers is alphabetical. The authors have had compounds profiled with most of the listed labs, and obtained robust data from all of them. See provider's websites for more information on technologies and protocols used.

very high confidence to a particular target. Specialist profiling labs have emerged that offer selectivity profiling (Table 2). Typically, these labs run 100–400 kinase assays in parallel, using different assay formats. The largest panel even comprises 394 out of the 514 genes predicted to encode kinases in the human genome (Manning *et al.*, 2002; Table 2).

The classic, 'gold standard' format is the radioactive filter binding assay, which combines sensitivity with a generic readout of direct kinase function (i.e. transfer of a radioactive phosphate group), and is used by more than half of the commercial kinase profiling labs (Table 2). Non-radioactive alternatives include microfluidic detection of the mobility shifts of phosphorylated substrates (often referred to as the 'Caliper' format), or detecting substrate phosphorylation by its protease-protective effect on fluorescent energy transfer in a probe peptide (Z′-lyte™). Another often-employed format is a competitive binding assay in which kinases expressed on bacteriophages are prevented from binding to an immobilized probe ligand by a competing inhibitor of interest. The amount of bound kinase-phage is quantified by amplification of the phage DNA with the PCR (often called the 'Ambit' format, after the company that developed the technology, Fabian *et al.*, 2005).

The differences in technologies used are potentially exacerbated by differences in construct sequences and expression systems that are used. Some laboratories express kinases in *Escherichia coli*, others in insect cells, which results in differences in the kinase phosphorylation status. Additionally, assay conditions may vary across labs, such as buffers and incubation temperatures, concentrations of ATP and, importantly, the nature of the peptide or protein substrate. Additionally, when compounds are slow-binding allosteric inhibitors, incubation times before the readout are critical because a read out has to take place in binding equilibrium.

For all these reasons, the IC_{50}s of reference inhibitors, as published on the websites of profiling labs, show the expected variation for IC_{50}s measured in different labs. However, encouragingly the available data indicate that most labs find similar selectivities for similar compounds. Results from 20 compounds in enzyme activity assays and ligand binding assays were found to be comparable (Fabian *et al.*, 2005). This was later confirmed in a massive effort where the potencies of ~10 000 inhibitors on 40 different targets were compared (Posy *et al.*, 2011). In a study where 16 compounds were profiled in either activity or binding assays, a single-value selectivity metric (see below) produced comparable values and similar selectivity rank ordering (Uitdehaag and Zaman, 2011).

Foregoing differences in technology and conditions used, profiling studies fall in one of the three following categories:

1. A dose-response binding experiment that gives a K_d for each target. This result is determined in the absence of ATP (Fabian *et al.*, 2005).
2. A dose-response activity assay that determines an IC_{50} for each target kinase. This result is dependent on the ATP concentration in the assay. If assays are performed at an ATP concentration equal to the kinase's $K_{M\text{-}ATP}$, then for a competitive inhibitor the Cheng–Prusoff relation states that $IC_{50} = 2K_i$ (Knight and Shokat, 2005). This K_i is the ATP-independent inhibition constant, and can be compared with the K_d.
3. A measurement at a single concentration of inhibitor. This results in a %-inhibition effect (or a %-remaining activity). Because fewer data points are needed, this experiment is easier to perform. However, %-inhibition data are more prone to variation than dose-response data (the difference between 20% and 80% inhibition can originate from a mere fivefold difference in IC_{50}). They are also less informative: an inhibitor of two kinases with IC_{50}s of 0.1 nM and 100 nM, a 1000-fold difference, would inhibit both at a similar ~100% in a 10 μM fixed concentration screen.

In our experience, the most efficient and cost-effective strategy is to determine the selectivity of a compound in two tiers: First, the compound is tested at a single concentration (in duplicate) to determine the target kinases. Subsequently, IC_{50}s are determined for all targets that are inhibited more than, for example, 70%. For IC_{50} determination, a 10-point dose-response curve is preferred, although a 5-point dose-response curve can already yield reliable data. If a compound is relatively selective, the follow-up is only a small study that makes the entire profiling study equivalent to a full-scale IC_{50}-based profiling.

Overview of published profiling studies

Selectivity profiles are increasingly found in publications where new inhibitors are presented (see below for examples), and this practice can only be encouraged. In addition, many very interesting studies exist in which entire inhibitor sets are selectivity profiled, allowing direct comparison of the selectivities of existing inhibitors. The first of these studies (Davies *et al.*, 2000) showed the selectivity profiles (single concentration data) of many common kinase reference compounds at the time. This was later extended with more reference inhibitors (again single concentration data, Bain *et al.*, 2007). Both studies gave clear guidelines on which inhibitors to use when investigating the biological actions of certain kinases.

Another milestone was the study by Fabian *et al.* who studied the selectivity of 20 kinase inhibitors that have been investigated in clinical trials in a dose-response binding assay on 119 kinases (Fabian *et al.*, 2005). This study demonstrated the promiscuity of some kinase drugs and drug candidates. It was followed by a larger dose-response studies of 38 clinically advanced kinase inhibitors on 317 kinases (Karaman *et al.*, 2008) and 72 inhibitors on 442 kinases (Davis *et al.*, 2011), which included the proposal of new tools for the quantification of selectivity. Both studies, published by Ambit, remain exquisite sources for the selectivity of known inhibitors.

Other profiling studies include the cross-reactivity of 156 commercially available protein kinase inhibitors on 60 human Ser/Thr kinases, using a single-concentration thermal shift assay (Fedorov *et al.*, 2007). Though thermal shifts are not necessarily IC_{50}s (Fedorov *et al.*, 2007), and the kinase panel is a particular subset of the kinome, this study provides selectivity data on many readily available and frequently used inhibitors. Even more such compounds were recently tested at single concentration in an activity assay (178 inhibitors on 300 kinases, Anastassiadis *et al.*, 2011). Both of these datasets provide an essential reference for interpreting old and new literature. Recently, even larger studies were released, such as the single point activity data of 577 inhibitors on 203 kinases, combined with IC_{50} profiles of 18 reference inhibitors (Bamborough *et al.*, 2008), and the massive single concentration profiling study of 21851 inhibitors on 317–402 kinases (Posy *et al.*, 2011). These studies generated interesting statistics on the pharmacological similarity of kinases. However, only few chemical structures were released. Nevertheless, they showed convincingly that, aided by X-ray structures, selectivity can be achieved on most kinase targets, starting from many different scaffolds (Johnson, 2009; Posy *et al.*, 2011).

A great contribution to the field was the recent large-scale publication of the binding K_ds of 3800 compounds against 172 kinases (Metz *et al.*, 2011). The data and structures of 1500 compounds were uploaded to the Pubchem database (http://pubchem.ncbi.nlm.nih.gov). The value of these data for tool compound discovery is hard to overstate. For virtually any of the kinases studied, new inhibitors were identified that are more selective than the known reference inhibitors, although the stability and cellular activity of these new tool compounds still needs to be established.

A new trend is the use of cross-screening in drug discovery (Goldstein *et al.*, 2008), as demonstrated by the recent profiling studies of 936 fragments on 30 kinases (Bamborough *et al.*, 2011), and 118 new compounds against 353 kinases (Miduturu *et al.*, 2011). The latter resulted in new tool compounds for previously untargeted kinases. Kinase cross-screening is an eminent method in drug discovery, because it generates potency and selectivity data in one study. Furthermore, and in contrast to high-throughput screening, it enables the picking of multi-targeted lead structures (Vieth *et al.*, 2004; Hopkins, 2008).

Finally, it must be stressed that kinase profiling for assessing selectivity is limited in two ways. First, it uses a fixed number of assays, while there are many more biological targets in a cell. To address this, various labs have developed proteomics-based methods to capture protein targets directly from cell lysates (Bantscheff *et al.*, 2009; Rix and Superti-Furga, 2009; Patricelli *et al.*, 2011). In addition, other profiling panels have been set up, for example, of GPCRs and targets implicated in drug safety (Hamon *et al.*, 2009; Heilker *et al.*, 2009). These approaches have shown that kinase inhibitors, as any drugs, can also bind to non-kinase targets and this should be kept in mind when interpreting kinase profiles.

Second, though kinase profiling provides a good view of an inhibitor's activity on individual targets, the situation inside a living cell is more complex. Efflux pump activity, ATP levels, viscosity, protein concentration, scaffolding, target location, etc., will all affect an inhibitor's ability to bind to a target, and these factors can change with a cell's metabolic and differentiated status. Therefore, biochemical selectivity must be seen as a firm base from which to explore cellular selectivity and cellular signalling as a whole.

Displaying and quantifying selectivity profiling data

The result from any selectivity profiling is a table that contains 200–400 IC_{50}s for each compound. The next step is then to represent those data in a meaningful way. Graphic methods to display selectivity data include heat maps (Fedorov *et al.*, 2007; Bamborough *et al.*, 2008), sorted activity on radar plots (Mihara *et al.*, 2008; Verkaar *et al.*, 2011) and dotting the phylogenetic tree of all human kinases (Manning *et al.*, 2002) with circles, where the diameter of each circle represents compound activity (Fabian *et al.*, 2005; Karaman *et al.*, 2008, Figure 1). Graphic representation has the advantage that the information on individual kinases is retained. However, many selectivity questions cannot be answered by qualitative diagrams, for example: 'what is the most selective inhibitor?' Such quantitative questions are very relevant when choosing tool compounds.

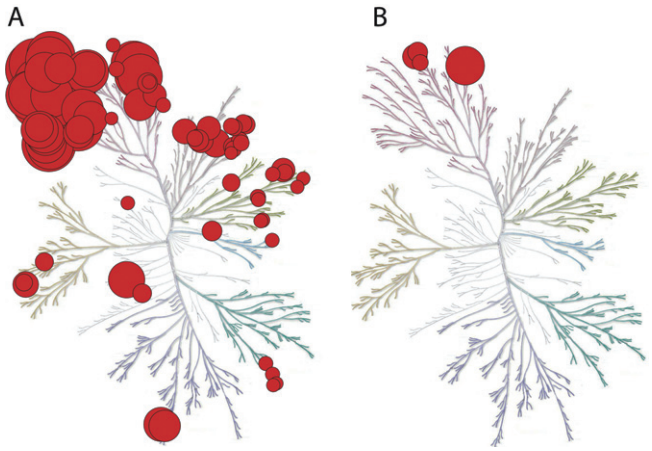


Figure 1

Graphical representation of inhibitor selectivity, based on the phylogenetic tree of human kinases (Manning *et al.*, 2002). (A) Dasatinib is an example of a promiscuous inhibitor. (B) GW2580 is an example of a selective inhibitor. The larger the diameter of the red circles, the more potent the binding affinity. The kinome tree was first introduced by Manning *et al.* (2002). Reproduced with permission from the paper by Karaman *et al.* (2008). The kinome tree was reproduced courtesy of Cell Signalling Technology, Inc. (http://www.cellsignal.com).

Quantitative questions can be solved by the emerging science of deriving a single selectivity value from profiling tables. Conceptually, such a value can be compared with an IC_{50} or logP to indicate potency or lipophilicity, but instead indicating the general selectivity of a compound. Selectivity values can be used to rank compounds, greatly facilitating the identification of suitable tool compounds.

The first proposed methods to quantify selectivity were threshold methods such as the promiscuity score (Fedorov *et al.*, 2007) and the selectivity score (Karaman *et al.*, 2008). The selectivity score divides the number of kinases hit below a certain threshold (usually 3 μM) by the number of kinases tested. The method has the advantage that it is simple to calculate, but has the drawback that it is arbitrary. For instance, the score is equally high if an off-target kinase is inhibited at 1 μM or is inhibited at 1 nM, whereas the first IC_{50} is highly preferred in a selective inhibitor. In this way, the use of a cut-off can lead to the erroneous conclusion that more potent kinase inhibitors are less selective (Posy *et al.*, 2011).

A method that avoids thresholds is the Gini score, which quantifies the curvature of a graph of sorted %-inhibition values (Graczyk, 2007). The higher the curvature, the more selective the inhibitor. The score is named after Corrado Gini, who used it to quantify income distribution disparities. The Gini score, thus, is a rare example of a quantitative method that has crossed over from the social sciences to the natural sciences. The Gini score has no physical–chemical meaning, and works with %-inhibition data.

A method that does have physical meaning, and which uses K_d and IC_{50} data, is the partition coefficient (Cheng *et al.*, 2010). The partition coefficient determines the theoretical distribution of inhibitor molecules in a hypothetical kinase mixture, and calculates the fraction of inhibitor molecules bound to an assigned reference kinase. A similar partition coefficient is used to quantify selectivity in analytical chemistry (Peters *et al.*, 2009). The assignment of a reference kinase makes the partition coefficient a biased approach. Depending on the project it is used for, an inhibitor can have different selectivity scores. The method also assigns similar values to an inhibitor that potently inhibits one off-target kinase and an inhibitor that less potently inhibits a plethora of off-target kinases (Cheng *et al.*, 2010; Uitdehaag and Zaman, 2011).

The selectivity entropy

To remedy the drawbacks of the said methods, we recently proposed a selectivity entropy value. In many fields, such as bio-informatics and physical chemistry, entropy values are used to quantify the specificity of an effect by the broadness of the frequency distribution of all possible effects (Godden *et al.*, 2000). The selectivity entropy uses this background to quantify the theoretical binding distribution of inhibitor molecules over all kinases in the selectivity panel (Uitdehaag and Zaman, 2011). It can also be regarded as a weighted summation of all possible partition coefficients in the kinase panel (Uitdehaag and Zaman, 2011).

High selectivity entropy indicates a promiscuous compound, whereas low selectivity entropy specifies selective compounds. The pan-kinase inhibitor staurosporine has an entropy score of 2.9, whereas compounds that only hit one kinase in the entire profile have an entropy score of 0 (Table 3). The selectivity entropy does not require assignment of a reference kinase, uses K_d and IC_{50} values, and has thermodynamic meaning. Selectivity entropy can be easily calculated in Excel (for instructions, see Uitdehaag and Zaman, 2011). In addition, we have built a website (accessible via http://www.entropy.99k.org) where a table of IC_{50} values can automatically be converted into a set of selectivity entropies.

Recently, all methods for selectivity quantification were compared (Uitdehaag and Zaman, 2011). In general, the more advanced methods (Gini score, partition coefficient, selectivity entropy) give comparable rankings of compounds. However, in a test where 16 compounds were profiled in two different labs, the entropy method gave most consistent values, indicating that the entropy score is preferred when comparing selectivity profiles from separate labs.

Statistics of the selectivity entropy

The universality of selectivity entropy is demonstrated by large profiling datasets all showing similar average entropies and entropy distributions (Figure 2). The binding data of Karaman *et al.* (2008) show an average entropy of 1.8 and a median of 1.9. (2.0 and 2.1, respectively, in the follow-up work by Davis *et al.*, 2011). A large activity-based dataset has values of 1.9 and 2.0 respectively (Uitdehaag, 2010, Figure 2). The data from Metz *et al.* (2011) show average and median entropies of 2.2 (Figure 2). The similarity of all these values confirms that the average selectivity entropy in a panel of about 200 kinases is 2.0 (Uitdehaag and Zaman, 2011). Compounds with an entropy score lower than 2.0 are therefore 'more selective than average'. However, compounds with entropies below 1.0 are frequently found in cross-screens

Table 3

Recommended tool compounds for important kinase targets

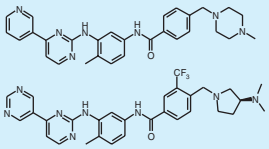
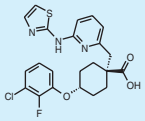
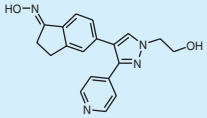
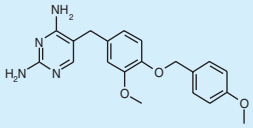
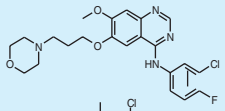
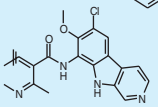
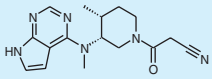
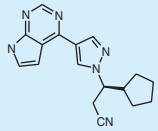
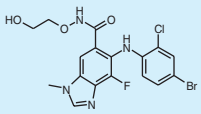
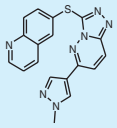
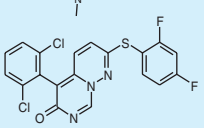
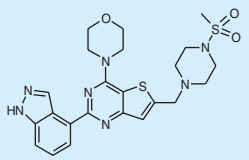
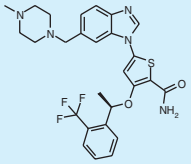
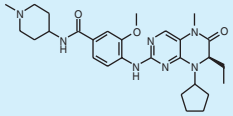
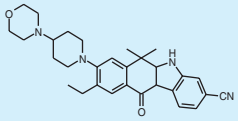
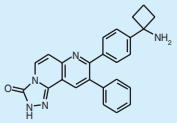
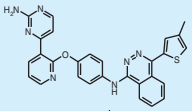
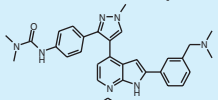
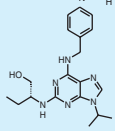
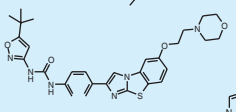
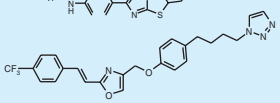
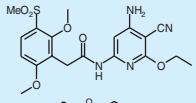
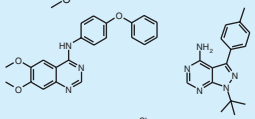
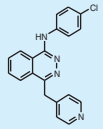
Target	Structure	Tool compound	Target Ki or IC50 (nM)[1]	Selectivity entropy (panel size)	Cellular activity (nM)	References	Vendor
Inhibitors meeting criteria							
Abl		Imatinib/ Bafetinib	12/9	1.0 (290)/nd	200–1500/ 10–1000	Fabian *et al.*, 2005; Deguchi *et al.*, 2008; Karaman *et al.*, 2008 Rix *et al.*, 2010	3
Aurora A		MK5108	0.064/14/12[2]	0.4 (233)	160	Shimomura *et al.*, 2010	4
BRAF		GDC0879	0.19	0.2 (383)	63	Hansen *et al.*, 2008; Davis *et al.*, 2011	3
CSF1R		GW2580	1.6	0.3 (290)	330	Conway *et al.*, 2005; Karaman *et al.*, 2008	3
EGFR		Gefitinib	1	0.4 (290)	5–20	Jiang *et al.*, 2005; Karaman *et al.*, 2008	3
IKBKB		MLN120B	19	0.7 (383)	2500	Hideshima *et al.*, 2006; Davis *et al.*, 2011	4
JAK1/2/3		CP690550	5/2.2[2]	0.9 (383)	21	Changelian *et al.*, 2008; Davis *et al.*, 2011	3
JAK2		Ruxolitinib	0.036	0.4 (383)	127	Quintás-Cardama *et al.*, 2010; Davis *et al.*, 2011	3
MEK1/2		AZD6244	14	0.02 (222)	10	Yeh *et al.*, 2007; Uitdehaag and Zaman, 2011	3
MET		SGX523	2.7	0.01 (213)	12	Buchanan *et al.*, 2009;	3
p38α		VX745	2.8	0.3 (290)	30	Karaman *et al.*, 2008; Verkaar *et al.*, 2011	3
PI3KCA		GDC0941	1.1	1.0 (383)	100–1000	Edgar *et al.*, 2010; Davis *et al.*, 2011	3

Table 3

Continued

Target	Structure	Tool compound	Target Ki or IC50 (nM)	Selectivity entropy (panel size)	Cellular activity (nM)	References	Vendor
PLK1		GSK461364	0.094	0.1 (383)	50	Davis *et al.*, 2011; Schöffski 2009	3
PLK1/2/3		BI2536	0.2/0.8/4[2]	0.9 (383)	3–30	Steegmaier *et al.*, 2007; Davis *et al.*, 2011	3
Other useful inhibitors							
ALK		CH5424802	1.9	2.4 (402)	33	Sakamoto *et al.*, 2011	3
Akt		MK2206	5/12/652[2]	nd	40–1000	Lindsley *et al.*, 2005; Hirai *et al.*, 2010; Tan *et al.*, 2011	3
Aurora A/B/C		AMG900	5/4/1[2]	1.6 (353)	2.1	Payton *et al.*, 2010	3
Aurora B/C		GSK1070916	0.38/1.5/490[2]	1.3 (328)	7	Adams *et al.*, 2010	3
CDK2/5		Roscovitine	700/160[2]	2.0 (290)	5000	Meijer *et al.*, 1997; Karaman *et al.*, 2008	3
FLT3		AC220	0.63	1.7 (383)	0.56	Zarrinkar *et al.*, 2009; Davis *et al.*, 2011	3
ERBB2		TAK165	6	nd	5	Nagasawa *et al.*, 2006;	
JNK1/2		6s	38/150	0.7	1300	Szczepankiewicz *et al.*, 2006	4
SRC		Srcl1/PP1	180/53	nd	nd/500	Bain *et al.*, 2007	3
VEGFR		PTK787	62	1.5 (290)	5–30	Karaman *et al.*, 2008; Wood *et al.*, 2000	3

Top section: inhibitors meeting criteria outlined in Figure 4. Bottom section: useful inhibitors missing some criteria.
Vendor 3 is available at one or more of the following vendors: Selleck Chemicals, LC Labs, MedKoo Biosciences, ActiveBiochem, Axon MedChem or Merck-Chemicals. In general, vendors can be found most easily by searching on compound name and 'mg' in a web browser.
Vendor 4 is not commercially available at time of print.
[1]Value as reported in the reference, from activity or binding assay, not corrected for ATP concentration.
[2]Activity on the respective isoforms as mentioned in the left column.

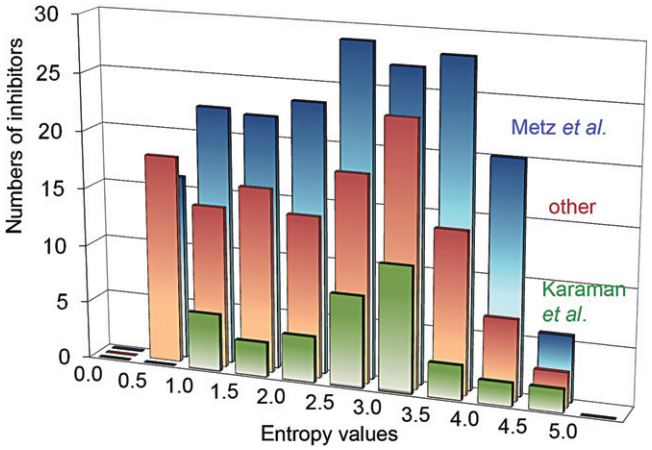


Figure 2

Distribution of the selectivity entropies of inhibitors in profiling datasets. The bars indicate the numbers of inhibitors in the indicated datasets with entropies between values at the *x*-axis. The distribution indicated 'other' originates from an activity-based dataset of 127 cpds profiled on 222 kinases (Uitdehaag, 2010). For the Metz *et al.* (2011), dataset, numbers on the *y*-axis need to be multiplied by 20. All three independent datasets have a similar entropy distribution.

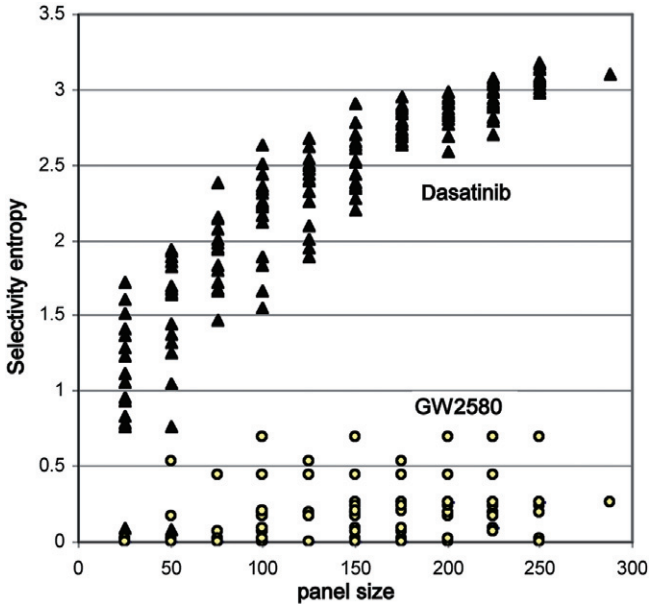


Figure 3

Dependency of the selectivity entropy on panel size. The Karaman *et al.* (2008) dataset was divided into 20 random subpanels with 11 different sizes. The selectivity entropies for the promiscuous dasatinib and the selective GW2580 were calculated for each panel. For dasatinib, a reasonable entropy guess can be made from a 200-kinase panel. For GW-2580, which inhibits only two kinases, variation is in practice independent of panel size. For every panel size, SDs were calculated for GW2580 and dasatinib. For a panel size of 200, the SD for dasatinib is 0.10, and the SD for GW2580 is 0.19. If all SDs from all panel sizes are averaged, the final average SD is 0.2.

(Figure 2). Such highly selective tool compounds are much more useful for validating kinase targets than those 'more selective than average'.

Another crucial value is the SD on a single selectivity entropy determination for a particular compound. This is a measure of the statistical relevance of entropy differences. Errors in entropy values can arise from assay differences, different panel sizes and non-overlapping panel composition. In an experiment where 16 compounds were profiled in two different labs, all three error sources played a role resulting in an SD of 0.3 (Uitdehaag and Zaman, 2011).

When looking at the individual sources of variation, panel size is important because the entropy value tends to increase when more kinases are tested (Figure 3) (Brandt *et al.*, 2009). When values originating from widely different panel sizes are compared, ideally a logarithmic panel size correction needs to be applied (Uitdehaag and Zaman, 2011, Figure 3). In reporting entropy values, panel sizes should therefore be given (Table 3). Secondly, variation due to panel composition can be assessed by recalculating entropies from random sample panels (Figure 3). For the archetypal promiscuous inhibitor dasatinib (Figure 1), determining an entropy score in different panels of 200 kinases results in an SD of 0.10 (Figure 3). For the selective inhibitor GW2580 (Figure 1), this is 0.19 (Figure 3). Finally, variation due to assay reproducibility was modelled by multiplying assay data (Karaman *et al.*, 2008) with a random factor between 0.1 and 10, and recalculating entropies. Repeating this 50 times with different random factors, results in an SD of 0.20 for dasatinib, and 0.32 for GW2580. Assay reproducibility thus seems the major source of error in entropy determination, and for the selectivity entropy of any compound, an SD of about 0.3 should be taken into account.

General approach for selecting tool compounds

The advent of single-value selectivity scores allows more rational picking of selective tool compounds (Figure 4). To start such a process, the selectivity entropy scores of publicly available inhibitors need to be calculated and rank ordered. In general, the most selective of these should be chosen. If the selectivity entropies are derived from kinase panels with largely differing sizes, the inhibitors that are profiled over many kinases (>100) are preferred. The best tool inhibitors have an entropy smaller than one, but if no such exquisitely selective compounds exist, multiple compounds can be selected for use in parallel (Figure 4). However, if no compounds with an entropy score smaller than two are available, cross-reactivity becomes an unacceptable risk even when multiple inhibitors are used, and one should rather start developing a new tool compound than planning any biological experiment with one of the available inhibitors.

The parallel use of multiple compounds is only meaningful if these compounds are structurally dissimilar and do not exhibit comparable cross-reactivity profiles. The latter could potentially be excluded by calculating correlation coefficients of both inhibitor kinase profiles, but visual inspection of the cross-screening data is often more practical.

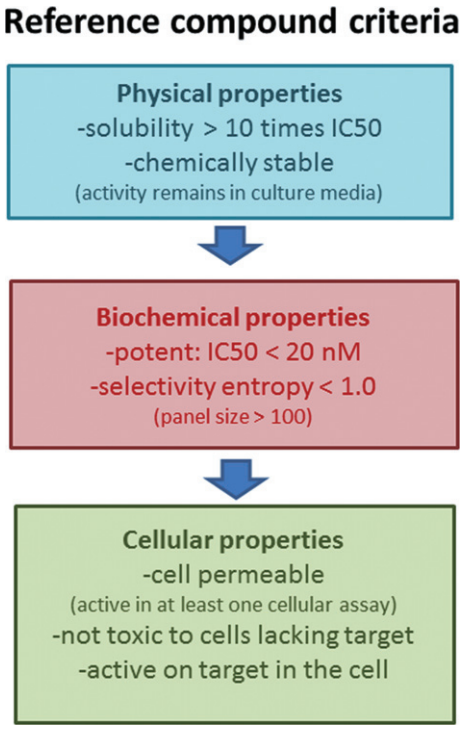


Figure 4

Flowchart for the selection of a good kinase tool inhibitor. Physical, biochemical and cellular properties should be assessed before using an inhibitor as reference. These criteria were used for the inhibitor selection of Table 3. Most *in vivo* or clinically tested inhibitors will have suitable solubility, stability and activity characteristics, leaving only the selectivity to be assessed.

It is very important that selected compounds have proven activity and stability in a cell, disqualifying unproven screening hits as pharmacological tools. As a guideline, Figure 4 lists properties that are essential for any tool compound for use *in vitro*. For *in vivo* applications, compounds additionally need to be stable enough to monitor the biological effect after application via the desired route and lack acute toxicities. As informal guideline, compounds that have been clinically evaluated and meet the selectivity criteria will almost certainly qualify as good tool compounds.

As not all tool compounds are equally selective, we recommend that in all cases where kinase tool compounds are used in biological studies, selectivity values are mentioned, with reference to the profiles in the literature. However, study of the individual profiles remains very important, particularly if biological validation focuses on particular off-target kinases, for instance isoforms. Only with access to all information, a pharmacological validation with kinase inhibitors can be properly assessed.

Tool compounds for clinically relevant kinases

To illustrate our guidelines for picking selective tool compound from cross-screening data, we have selected the most suitable tool inhibitors for several frequently investigated kinase drug targets, using the criteria of Figure 4 (Table 3). Targets were selected on the basis of the number of articles in Pubmed and the progression of compounds hitting these targets in the clinic. Throughout the text, the word spectrum-selective is used to indicate an inhibitor that predominantly inhibits members of a particular kinase subfamily. The suffix pan- (as in pan-Aurora) is used to indicate inhibitory activity on all isoforms of that target. Hugo Gene Nomenclature Committee-approved names for kinases are listed in the section titles (in brackets where a kinase has a dominant trivial name). For entropy calculations, all activities on non-human and mutant kinases were discarded, leading to reduced panel sizes compared with the literature (e.g. 290 kinases were included from Karaman *et al.*, 2008 and 383 kinases from Davis *et al.*, 2011).

Abl (ABL1)

Chronic myeloid leukaemia (CML) is caused by a chimeric *BCR-ABL* gene that is a driver of malignant transformation (Hantschel and Superti-Furga, 2004). The clinical success of the Abl inhibitor imatinib in treating CML heralded the emergence of kinase inhibitors as a drug target class (Druker *et al.*, 2001). The initial euphoria brought about by the efficacy of imatinib was followed, however, by the emergence of resistant tumours (Hantschel and Superti-Furga, 2004). Resistance is caused by point mutations in Abl that render imatinib incapable of binding. Since then, second-generation Abl inhibitors that target mutant Abl and display improved potency were generated (Manley *et al.*, 2010). Broadening the spectrum of Abl mutants being hit and losing selectivity may have gone hand in hand, because these follow-up molecules are generally less selective than imatinib. For instance, the entropy scores for nilotinib (1.7), DCC2036 (2.9), dasatinib (3.2) and PD173955 (3.3) are considerably higher than that of imatinib (0.8) (Karaman *et al.*, 2008; Manley *et al.*, 2010; Chan *et al.*, 2011; Davis *et al.*, 2011). For other Abl inhibitors, only limited selectivity information has been published, though it can be inferred that they are not exquisitely selective. For example, the dual Src/Abl inhibitor saracatinib inhibited 11 kinases with IC_{50}s below 100 nM out of a panel of 23 kinases tested (Green *et al.*, 2009), and bosutinib inhibited 63 kinases for more than 80% at 1 μM out of a panel of 272 kinases tested (Remsing-Rix *et al.*, 2009; Rix and Superti-Furga, 2009). Probably, the most selective out of these second-generation Abl inhibitors is INNO406/bafetinib, which inhibited 23 kinases for more than 80% at 1 μM out of 272 kinases tested (Rix *et al.*, 2010). We advise the use of imatinib in parallel with bafetinib to pharmacologically investigate the role of Abl or Bcr-Abl in physiologic processes.

ALK

Alk is a tyrosine kinase of which translocations drive anaplastic lymphoma, and a significant percentage of non-small cell lung cancers (Lovely *et al.*, 2011). A clinical refocus of the dual MET/ALK inhibitor PF02341066/crizotinib has led to marked patient responses, opening the ALK field as an area of intense research. In one publication (Lovely *et al.*, 2011), %-inhibition data are given on 96 kinases at 1 μM crizotinib, indicating activity (>80% inhibition) against in at least 20

kinases, including Abl, Axl, CSF1R, MET and ALK (Zou *et al.*, 2007). Small profiles were released for NVP-TAE684 (Galkin *et al.*, 2006), and more extensive profiles for X376 (Lovely *et al.*, 2011) and AP261113 (Katayama *et al.*, 2011). The spectrum selectivity of crizotinib and NVP-TAE684 was revealed recently by Davis *et al.* (2011), where they showed entropies of 3.0 and 3.7 respectively. Another thoroughly profiled and selective ALK reference compound in the public domain is CH5424802. In a 402 kinase profile at two concentrations, it inhibits ALK, LTK and GAK at single-digit nanomolar concentration, and 27 others at 10–1000 nM IC_{50} (Sakamoto *et al.*, 2011). On the basis of these data, the selectivity entropy of CH5424802 is 2.4. Two compounds that only have been characterized biochemically (Metz *et al.*, 2011), PSID (Pubchem substance ID) 103904390 and 103904391, are more selective than CH5424802. Both only hit ALK in a 172 kinase profile, and have a selectivity entropy of 0.01. Awaiting further characterization of PSID 103904390 and 103904391, CH5424802 is the most suitable tool compound for ALK.

Akt (AKT1)

Akt (or PKB) was initially identified as the gene driving oncogenic transformation through the retrovirus Akt-8 (Bellacosa *et al.*, 1991). Consequently, Akt was found to be up-regulated in a plethora of cancers and to be instrumental for tumour growth (Mitsiades *et al.*, 2004; Cheng *et al.*, 2005). The allosteric Akt inhibitor MK2206 has been suggested to be a highly selective inhibitor (Lindsley *et al.*, 2005), but no cross-screening data have been disclosed. The only reference to its selectivity over other kinases than Akt isoforms is the reiterated statement that MK2206 is selective over PKA, PKC and SGK (Lindsley *et al.*, 2005; Hartnett *et al.*, 2008; Wu *et al.*, 2008; Zhao *et al.*, 2008). Also for perifosine, which is currently under clinical evaluation for the treatment of neuroblastoma, no selectivity data are available in the public domain. The Akt inhibitors for which selectivity data have been published are not uniquely selective for Akt. For instance, GSK690693 has a calculated selectivity score of 2.7, based on a panel of 94 kinases (Rhodes *et al.*, 2008), and A674563 has a selectivity entropy of 2.0, calculated from a profiling effort encompassing 383 kinases (Davis *et al.*, 2011). Other Akt inhibitors include CCT128930, which inhibited seven kinases >80% at 10 μM over 47 kinases tested (Yap *et al.*, 2011), and AT7867, which inhibited 5 out of 19 kinases tested with IC_{50}s below 100 nM (Grimshaw *et al.*, 2010).

Due to its allosteric binding mode, MK2206 is likely to be very selective for Akt in comparison with other Akt inhibitors. Nevertheless, the data demonstrating its selectivity should be published (Table 3).

Aurora kinases A (AURKA) and B (AURKB)

Because of the importance of Aurora kinases in mitosis, these serine/threonine kinases were among the first kinases pursued for oncology indications (Katayama and Sen, 2010). However, no Aurora inhibitors have reached the market so far. Of the three Aurora isoforms (A, B and C), both Aurora A and Aurora B have been identified as targets, and as a result, pan-Aurora inhibitors as well as those with specificity towards Aurora A or Aurora B have been developed (Yan *et al.*, 2011).

Many Aurora inhibitor profiles have been published. A single-concentration profile of SU6668 (Bain *et al.*, 2007), a 35-kinase profile of danusertib/PHA739358 (Carpinelli *et al.*, 2007) and a full IC_{50} profile of VX680/MK0457/tozasertib (entropy 3.1, Karaman *et al.*, 2008) have been published. These studies revealed all three inhibitors as promiscuous. Of ZM447439, which is mentioned to be 'a more specific inhibitor', unfortunately only a 16-kinase profile has been published (Ditchfield *et al.*, 2003).

Full profiling showed better selectivity for the Aurora B/C selective AZD1152HQPA (entropy 1.9) and the Aurora A selective MLN8054 (entropy 1.9) (Karaman *et al.*, 2008; Davis *et al.*, 2011). Furthermore, recent efforts identified even more selective inhibitors, such as the pan-Aurora inhibitors SNS314 (entropy 1.4 in a 219 kinase panel, Arbitrario *et al.*, 2010) and AMG900 (entropy 1.6 in a 353 kinase profile, Payton *et al.*, 2010). AMG900 is about equally active on all Auroras. GSK1070916 inhibits Aurora B and Aurora C about 1000-fold more potent than Aurora A and has an entropy of 1.3 in a 328 kinase panel (Adams *et al.*, 2010). The Aurora A inhibitor MK5108 is selective over B and C and has an entropy of 0.44 in a 233 kinase panel (Shimomura *et al.*, 2010). All these inhibitors have single-digit nanomolar biochemical and cellular potencies (Table 3). This makes the Aurora kinase field well equipped with good tool compounds.

BRAF

The BRAF and RAF1 (CRAF) isoforms play an essential role in cell proliferation. The BRAF mutant V600E is a driving mutation in the majority of melanomas and some other cancers (Flaherty *et al.*, 2010). Many pharmaceutical companies have developed BRAF inhibitors, culminating in astounding clinical success (Solit and Sawyers, 2010). RAF-selective tool compounds could help to contribute to our understanding of wild-type RAF signalling. A large IC_{50}-based profile of the RAF inhibitor sorafenib revealed that it has below average selectivity (entropy 2.2, Karaman *et al.*, 2008). For GW5074 and ZM336372, single concentration testing indicates substantial off-target activities (Bain *et al.*, 2007). For SB590885 and SB-699393, only partial profiles have been published (Takle *et al.*, 2008). In another publication (Hansen *et al.*, 2008), a profile on 212 kinases is mentioned, unfortunately without disclosing the underlying data. More selective is PLX4720 that only substantially inhibits BRAF, RAF1 and Brk in a 65-kinase profile (Tsai *et al.*, 2008), but in a 383 kinase profile surprisingly shows more potent activity on MEK5 than on BRAF (Davis *et al.*, 2011). The best BRAF reference inhibitor is therefore GDC-0879, which only inhibits BRAF and RAF1 in panels of 140 kinases (Hoeflich *et al.*, 2009) and383 kinases (entropy 0.2, Davis *et al.*, 2011). (Table 3).

CDKs

Cyclin-dependent kinases (CDKs) are a highly conserved subfamily of 13 kinases, involved in regulating the cell cycle and transcription. CDKs are highly pursued potential cancer drug targets (Malumbres and Barbacid, 2009). Early drug discovery efforts resulted in the identification of several 'classic' CDK inhibitors, such as roscovitine/seliciclib for which the profiling in a small panel was already published in 1997 (Meijer *et al.*, 1997). Subsequently, more extensive profiling (Fabian *et al.*, 2005; Bain *et al.*, 2007; Karaman *et al.*, 2008; Davis *et al.*, 2011) confirmed that roscovitine/seliciclib is

averagely selective (entropy 2.0). Less selective are flavopiridol/alvocidib (entropy 2.5) and SNS032/BMS387032 (entropy 2.4), and the dual Aurora/CDK inhibitor JNJ7706621 (Emanuel *et al.*, 2005), which is highly promiscuous (entropy 3.7).

Unfortunately, more recently developed compounds were only profiled in smaller panels, such as PD0332991 (Fry *et al.*, 2004), PHA793887 (Brasca *et al.*, 2010), P276-000 (Joshi *et al.*, 2007), AZD5438 (Byth *et al.*, 2009) and BS181 (Ali *et al.*, 2009). Where newer compounds have been profiled more extensively, they have not shown high selectivity, such as R547 (entropy 2.2), AT7519 (entropy 2.4), EXEL2880 (entropy 3.2) (Squires *et al.*, 2009; Davis *et al.*, 2011). Nevertheless, the data suggest that it is possible to design more selective inhibitors. For instance, PHA793887 is a pan-CDK inhibitor that in a panel of 44 off-target kinases only hits GSK3β (Brasca *et al.*, 2010). Selectivity within the CDK family is also attainable: for instance, the CDK7 selective BS181 has 40–2000 times specificity over other family members and only hits two other kinases in a 69-kinase panel (Ali *et al.*, 2009). For proper comparison, the selectivity of these compounds needs to be quantified in larger panels. Until then, roscovitine remains one of the best CDK tool compounds (Table 3).

CSF1R

CSF1R (or Fms) is a tyrosine kinase that plays an important role in macrophage development and differentiation. CSF1R inhibitors target macrophages in inflammation and oncology (Hamilton, 2008). Many well-known tyrosine kinase inhibitors, such as imatinib, sorafenib and dasatinib, have nanomolar CSF1R activity, but profiling shows these are all spectrum-selective inhibitors (Karaman *et al.*, 2008). An exception is GW2580 (Conway *et al.*, 2005), which only hits CSF1R and Trk kinases in a panel of 290 kinases, and has a selectivity entropy of 0.3 (Karaman *et al.*, 2008; Uitdehaag and Zaman, 2011). In a recent cross-comparison of CSF1R inhibitors in a panel of different assays, we confirmed that GW2580 is a very selective inhibitor (Uitdehaag *et al.*, 2011).

EGFR and ERBB2

EGFR (Her1) and ERBB2 (Her2) are closely related receptor tyrosine kinases that are aberrantly expressed and/or activated in a plethora of cancers, most notably breast and lung cancer (Zhang *et al.*, 2007). EGFR and ERBB2-targeting therapeutics include the antibodies cetuximab, panitumumab (both EGFR-specific) and trastuzumab (ERBB2), and the small molecule kinase inhibitors erlotinib, gefitinib, vandetanib and lapatinib (Zhang *et al.*, 2007).

Fabian *et al.* (2005) and Karaman *et al.* (2008) showed that gefitinib and erlotinib are both very selective inhibitors of EGFR, with entropy scores of 0.4 and 0.9 respectively (Table 3). They do not possess cross-reactivity towards ERBB2 or other ERBB family members, and only significantly inhibit GAK (Karaman *et al.*, 2008). Several other compounds, such as CI1033/canertinib (entropy score of 0.2), BIBW2992/afatinib (0.4), GW-2016 (0.6), lapatinib (0.7) and EKB569/pelitinib (1.4) have excellent selectivity but do not distinguish between EGFR and ERBB2 and are therefore not suitable as tool compounds (Fabian *et al.*, 2005; Karaman *et al.*, 2008; Davis *et al.*, 2011). The marketed VEGFR/EGFR inhibitor vandetanib/ZD6474 is a promiscuous EGFR inhibitor, having an entropy score of 2.6, derived from a 119-kinase profile (Fabian *et al.*, 2005).

Compounds that inhibit ERBB2 but not EGFR have been described (Barbacci *et al.*, 2003; Lippa *et al.*, 2007; Moasser, 2007). TAK165/mubritinib is the most selective over EGFR (Nagasawa *et al.*, 2006). However, extensive selectivity data on other kinases is lacking for this and other ERBB2-specific inhibitors. The only presumed ERBB2-specific compound that has undergone rigorous selectivity testing is CP724714 (entropy score of 1.1), which was initially described to display 600-fold selectivity for ERBB2 over EGFR (Jani *et al.*, 2007), but in binding assays appears equipotent on both kinases (Karaman *et al.*, (2008). An enticing ERBB2-selective alternative is a compound from the Metz database with Pubchem Substance ID (PSID) 103905568). This inhibits ERBB2 with an IC_{50} of 1.6 nM and only cross-reacts with ERBB4 (IC_{50}: 25 nM), yielding a selectivity entropy of 0.4 in a panel of 172 kinases.

FLT3

A translocation of the *FLT3* gene (FLT3-ITD) is the driver mutation in certain types of leukaemia (Zarrinkar *et al.*, 2009). Despite the fact that many tyrosine kinase inhibitors have FLT3 activity (Karaman *et al.*, 2008), very few are selective for FLT3. When profiles of clinically used FLT3 inhibitors were compared (Zarrinkar *et al.*, 2009), it appeared that MLN518/tandutinib and AC220/quizartinib are the most selective, with entropies of 1.6 and 1.8 respectively. A second profiling study showed both entropies to be 1.7 (Davis *et al.*, 2011), which is consistent. Both compounds hit CSF1R, KIT, the PDGFRs and other receptor tyrosine kinases. AC220 is preferred as an FLT3 tool compound, because of its higher cellular potency compared with MLN518. In addition, it would be worthwhile to characterize the cellular activity of the 0.8 nM FLT3 inhibitor with PSID 103904858 (Metz *et al.*, 2011), which hits only KIT, CSF1R, KDR and FLT1/4, and has a selectivity entropy of 1.0.

IKBKB

IKBKB (inhibitor of NF-κB kinase β, also known as IKK2 or IKKβ) is a crucial mediator of activation of NFκB signalling, which is centrally important in inflammation and cancer (Karin and Greten, 2005). For this reason, many labs have sought to develop IKBKB inhibitors, with or without specificity for its oligomeric partner IKK1. Profiles have been determined of first-generation IKBKB inhibitors such as TPCA-1 (Bamborough *et al.*, 2008), PS1145 (Wen *et al.*, 2006), as well as Calbiochem IKK inhibitor VII and Calbiochem inhibitor 401483 (Fedorov *et al.*, 2007). In all cases, these studies revealed great promiscuity.

Recently, more specific inhibitors were published. For S1627, only a 12-kinase profile was published (Tegeder *et al.*, 2004), but it was revealed that S0100230, from the same chemical class, only hits IKBKB in a 200 kinase profile (Ritzeler, 2011). Another related compound, SAR113945, is currently in clinical trials. PHA408 shows excellent specificity (350-fold) over IKK1 and only hits Pim-1 in a 30-kinase panel (Mbalaviele *et al.*, 2009). Isoquinoline inhibitors, such as compound 21 in Christopher *et al.* (2009), only hit ROCK1 in

a 59-kinase panel and have 25-fold selectivity over IKK1. Selectivity data for IMD1041, IMD0254 and BMS345541 are not available, unfortunately. A better characterized inhibitor is BI5700, a 9 nM IKBKB inhibitor that essentially only weakly hits off-target IKK1 and FLT3 in a 59-kinase profile, and has a cellular IC_{50} of 290 nM for IκBα phosphorylation (Huber *et al.*, 2010). However, the best IKBKB inhibitor is MLN120B, which is related to PS1145 and was recently profiled (Davis *et al.*, 2011), revealing 50-fold selectivity over IKK1 and a very low selectivity entropy of 0.7 (Table 3).

JAK2 and JAK3

Mutations in JAK2 have been implicated in polycythemia vera, whereas JAK3-deficient humans are severely immunodeficient, identifying these JAKs as targets in oncology and immunology respectively. The best known JAK inhibitor is tasocitinib/CP690550 (Changelian *et al.*, 2008), which binds to JAK1, 2 and3 and TYK2 in a panel of 383 kinases (Davis *et al.*, 2011), resulting in a selectivity entropy of 0.9. Tasocitinib is therefore an excellent pan-JAK reference inhibitor.

Selectivity for one specific JAK isoform seems most easily achieved for JAK2. The JAK2 specific inhibitor R723 was profiled in a 200-kinase panel, hitting 13 kinases (including JAK3) at 100 nM, and only JAK2 at 20 nM (Shide *et al.*, 2011). SB1518 is 50-fold selective for JAK2 over JAK3, but also inhibits TYK2 and FLT3 in a 58-kinase panel (Hart *et al.*, 2011). AZ-690 is even more selective for JAK2. When the compound was profiled at three concentrations on 83 kinases, it inhibited 11 significantly (>50%) at 100 nM, but none of the JAKs except JAK2 (Gozgit *et al.*, 2008). The related AZD-1480 has comparable selectivity (Hedvat *et al.*, 2009). The recommended JAK2 specific inhibitor, however, is INCB018424/ruxolitinib, a 36 pM (!) inhibitor of JAK2, which in a 383-kinase panel only significantly inhibits Tyk2 as off-target (entropy 0.4, Davis *et al.*, 2011).

For JAK1, the dual JAK1/JAK2 reference inhibitor CYT387 has no JAK3 activity in a 141-kinase panel, profiled at two concentrations, although it potently inhibits six other kinases (Pardanani *et al.*, 2009, estimated entropy 2.8). A fully selective JAK3 inhibitor has not yet been described.

JNK1/2/3 (MAPK8/910)

The JNK kinase family (JNK1, 2 and 3) consists of three independent genes that are activated upon a large range of cellular stressors, including cytokines, mitogens and osmotic stress (Manning and Davis, 2003). JNK2, and to a lesser extent JNK1, have been implicated in chronic inflammatory diseases, such as rheumatoid arthritis and asthma, and evidence supports a role for JNK3 in neurodegenerative disorders (Manning and Davis, 2003). The first clinically evaluated JNK inhibitor is SP600125 (Bennett *et al.*, 2001). SP600125 has been used in over 800 articles to implicate JNK in cellular processes (Bogoyevitch and Arthur, 2008), despite the fact that profiling efforts suggested that the compound is not selective (Fabian *et al.*, 2005; Bain *et al.*, 2007). Indeed, the selectivity entropy score of SP600125 is 2.5, ranking it as a below-average selective inhibitor (Fabian *et al.*, 2005). Although several inhibitors for JNK have since been described, most of these have only been termed selective without disclosure of the actual data (Scapin *et al.*, 2003; Swahn *et al.*, 2006; Ma *et al.*, 2007). For instance, Kamenecka and colleagues describe a JNK inhibitor (compound 9l) which anecdotally inhibited 11 out of 400 kinases when tested at a concentration of 3 μM (Kamenecka *et al.*, 2010). The most selective JNK inhibitor for which selectivity data have been published is compound 6s (Szczepankiewicz *et al.*, 2006), which only inhibited JNK1, -2, -3 and ERK2 out of a panel of 74 kinases, with a selectivity entropy score of 0.7. Whereas all the said inhibitors exhibit only limited selectivity over JNK isoforms, a research compound (compound 5) developed by GSK (Angell *et al.*, 2007) only inhibited JNK-3 >80% in a panel of 214 kinases which included JNK-1 and -2 (Bamborough *et al.*, 2008). However, its cellular activity has not been demonstrated yet (Angell *et al.*, 2007). In conclusion, we advise the use of compound 6s for general JNK inhibition.

MEK1/2 (MAP2K1/MAP2K2)

MEK1 and MEK2 are functionally overlapping MAP kinase kinases that act downstream of RAF. Many MEK1/2 inhibitors are currently undergoing clinical testing (Trujillo, 2011). Early on, allosteric and very selective, so-called type III inhibitors were discovered (Dudley *et al.*, 1995; Ohren *et al.*, 2004). All reported MEK inhibitors are dual MEK1/2 inhibitors, including UO126, PD184352, AZD6244, PD0325901, CH498765, TAK733, XL518, RDEA119 and GSK1120212, and all belong to the same chemical class. The single concentration profiles of UO126, PD184352 and PD0325901 revealed that they are very selective (Bain *et al.*, 2007). More recently, the 222 kinase IC_{50} profiles of AZD6244 and PD0325901 confirmed their exquisite selectivity, with respective entropies of 0.02 and 0.55 in a panel of 222 kinases (Uitdehaag and Zaman, 2011). Of these, AZD6244/selumetinib is the most selective, essentially inhibiting no other kinases but MEK1/2 in the entire profile and exhibiting potent cellular and *in vivo* activity (Yeh *et al.*, 2007).

MET

MET (or HGFR) is a tyrosine kinase of which activating mutations cause hereditary papillary renal carcinoma, and which has been implicated in many other malignancies (Munshi *et al.*, 2010). Well-known MET inhibitors are SU11274, PHA665752 and MGCD265. However, broad kinome profiles of these inhibitors have not been published. SU11274 was profiled in a Ser/Thr kinase panel, where it inhibits at least seven kinases, most potently LOK (Fedorov *et al.*, 2007). PF02341066/crizotinib, the dual ALK/MET inhibitor, is also not selective (see above). Recently, two highly selective inhibitors were published. ARQ197 is a non-ATP competitive inhibitor with a K_i of 355 nM, that at 10 μM only inhibits four other kinases out of a panel of 230 (Munshi *et al.*, 2010). Even more potent and selective is SGX523, an ATP-competitive inhibitor (K_i = 2.7 nM) that at 1 μM only inhibits MET from a panel of 213 kinases (entropy 0.0, Buchanan *et al.*, 2009), which was confirmed in a panel of 383 kinases (entropy 0.0, Davis *et al.*, 2011). SGX523 is therefore the preferred MET reference compound.

p38α (MAPK14)

p38α is a highly pursued target for inflammatory diseases, such as rheumatoid arthritis, Crohn's disease, psoriasis and

chronic obstructive pulmonary disease (Genovese, 2009; Lindstrom and Robinson, 2010). Clinical development of the earliest compounds was stopped due to liver, brain or skin toxicity (Genovese, 2009; Lindstrom and Robinson, 2010). Subsequently developed p38α inhibitors are increasingly selective. For instance, the entropy score of the clinically tested p38α inhibitor VX745 is 0.3, ranking it among the most selective tool inhibitors (Karaman *et al.*, 2008; Uitdehaag and Zaman, 2011). Other highly selective p38α inhibitors include SCIO469 and ORG48762-0, both of which appear to have virtually no cross-reactivity towards kinases other than p38α and p38β, although they were only characterized on a limited set of kinases (Mihara *et al.*, 2008; Verkaar *et al.*, 2011). Of note, these kinase inhibitors are more specific than the most frequently used p38α tool inhibitors: SB203580 (entropy 2.3) and BIRB796 (entropy 1.2), the latter, for instance, being a potent inhibitor of JNK (Bain *et al.*, 2007; Karaman *et al.*, 2008). The *in vitro* potency of VX745 and SCIO469 is comparable with that of SB203580 (Verkaar *et al.*, 2011).

PI3K family (PI3KCA/B/C/D/G and mTOR)

The PI3K family consists of 15 kinases that have pleiotropic roles in cellular signalling, such as cell growth, survival and differentiation (Katso *et al.*, 2001). Of these kinases, mTOR and p110α, -β, -γ and –δ have been pursued for several indications. For instance, mutations in p110α are common in solid tumours (Samuels *et al.*, 2004) and p110β is a target for the treatment of thrombosis (Jackson *et al.*, 2005). Drug development for other PI3K isoforms (p110γ and δ) centres around inflammatory and auto-immune diseases (Rommel *et al.*, 2007).

The most commonly used inhibitors to target PI3Ks are wortmannin, a fungal metabolite, and LY294002, a quercetin derivative. Both compounds selectively inhibit p110s and closely related kinases, such as mTOR and DNA-PK (Davies *et al.*, 2000; Vanhaesebroeck *et al.*, 2001; Raynaud *et al.*, 2007), but display virtually no cross-reactivity towards 400 non-lipid kinases tested at 10 μM (Anastassiadis *et al.*, 2011). The best-characterized pan-PI3K inhibitors are PI103 and GDC0941. Both compounds inhibit all p110 isoforms, mTOR1 and -2 with high potency (Knight *et al.*, 2006; Davis *et al.*, 2011). The selectivity entropy of PI103, as derived from the Karaman dataset (in which lipid kinases are relatively under-represented), is 0.05 (Karaman *et al.*, 2008; Uitdehaag and Zaman, 2011). The entropy of PI103 calculated from a recent profiling effort that incorporated a larger panel of PI3K family members was 1.5, in which GDC0941 had an entropy of 1.0 (Davis *et al.*, 2011). It should be noted that both compounds display virtually no cross-reactivity outside the PI3K family and are therefore excellent pan-PI3K-selective inhibitors (Davis *et al.*, 2011). PI103 and GDC0941 are preferred over other pan-PI3K inhibitors like PP242 and TG-100–115, which have entropy scores of 3.3 and 2.1 respectively (Davis *et al.*, 2011).

More recent drug optimization projects have yielded inhibitors that display isoform specificity. For instance, the p110γ-specific inhibitor AS-605240 displays about 10-fold selectivity over other PI3Ks (Camps *et al.*, 2005).

Several p110δ-specific inhibitors have been described. IC287114 is approximately 10- to 20-fold selective for p110δ over other PI3Ks and does not inhibit any kinase out of 110 kinases, when tested at 10 μM (Knight *et al.*, 2006; Jamieson *et al.*, 2011). The IC287114 derivatives PIK293 and PIK294 inhibit p110δ with somewhat improved potency (10 nM vs. 130 nM for PIK294 and IC287114, respectively), while retaining 10- to 20-fold selectivity over other PI3Ks (Knight *et al.*, 2006; Jamieson *et al.*, 2011). Unfortunately, no selectivity data for PIK293 and PIK294 towards other kinases have been disclosed (Knight *et al.*, 2006). A slightly more selective (at least 40-fold) p110δ inhibitor is CAL101, which has anecdotally been described to inhibit no kinase other than PI3K isoforms out of a panel of 402 kinases (Lannutti *et al.*, 2011). Although these data should be disclosed or verified, CAL101 is probably the best p110δ-selective inhibitor.

PIK75 was previously described to be a p110α/γ-specific inhibitor (Knight *et al.*, 2006), but a recent screening effort showed that apart from PI3K isoforms, PIK75 inhibited 68 out of 110 kinases at 10 μM (Jamieson *et al.*, 2011). A more selective p110α-selective inhibitor is A66, which is 40-fold selective over other PI3Ks and only inhibited CLK4 and PI4Kβ out of 318 kinases tested at 10 μM (Jamieson *et al.*, 2011).

Probing of p110β function is best performed with TGX221, which is 10-fold selective over other PI3Ks and inactive on 110 kinases when tested at 10 μM (Jackson *et al.*, 2005; Jamieson *et al.*, 2011).

Finally, the individual roles of mTORs can be dissected with rapamycin, a highly selective allosteric inhibitor of mTOR (Bain *et al.*, 2007).

PLK1

PLK1 (polo-like kinase 1) is centrally important in mitosis and therefore an interesting drug target in oncology. Initial clinical tests in a variety of cancers showed great promise, especially in combination therapy (Schöffski, 2009). To further study the role of PLK1 in a non-therapeutic setting, inhibitors such as ON01910, HMN214 and LFM-A13 are less suited because of their activities on other kinases, including cell cycle kinases such as CDKs (Steegmaier *et al.*, 2007; Uckun *et al.*, 2007; Schöffski, 2009). More selective inhibitors are BI2536, BI6727 and GSK461364. The profiling data of two compounds related to GSK461364 show off-target activity (within a 100-fold window) on NEK2 and PDGFR1β (but not on Plk2) in a 57 kinase profile (Emmitte *et al.*, 2009). For BI6727/volasertib, a 50-kinase profile was mentioned, but not published (Rudolph *et al.*, 2009). For BI2536, a 63 kinase profile was published, showing essentially no off-target activity, except on Plk2 and Plk3 (Steegmaier *et al.*, 2007). Recently, the first full profiling studies of BI2536 and GSK461364 were published, demonstrating their exquisite selectivity (entropies 0.9 and 0.1, respectively Davis *et al.*, 2011). Both compound have a potent nanomolar cellular activity (Steegmaier *et al.*, 2007; Schöffski, 2009). However, whereas BI2536 is a PLK1/2/3 inhibitor, GSK461364 is exclusively selective for PLK1, making it the most suitable tool compound.

SRC

SRC belongs to a closely related family of non-receptor tyrosine kinases, comprising nine members. SRC has been assigned critical roles in cellular survival and proliferation, and elevated SRC activity and/or expression has been

observed in a variety of cancers (Belsches-Jablonski *et al.*, 2005). Many SRC inhibitors have been reported, but most are not selective. For example, saracatinib, bosutinib and dasatinib all inhibit SRC, but also many other kinases (Karaman *et al.*, 2008; Green *et al.*, 2009; Remsing-Rix *et al.*, 2009). More selective SRC inhibitors are SU-6656 (Blake *et al.*, 2000), SRC-I1 (Tian *et al.*, 2001), PP1 and PP2 (Hanke *et al.*, 1996). These inhibitors have been profiled at a single concentration against 73 kinases by Bain and colleagues (Bain *et al.*, 2007). Their analysis revealed that SU-6656 inhibited nine kinases more than 80% at 1 μM, whereas the other compounds were slightly more selective (SRC-I1, PP1 and PP2 inhibited 5, 4 and 4 kinases at 1 μM respectively). Several other inhibitors have been suggested to be selective for SRC, such as A-419259 (Wilson *et al.*, 2002), AZM475271 (Yezhelyev *et al.*, 2004), AP23846 (Summy *et al.*, 2005), MNS (Wang *et al.*, 2007) and PH006 (Ma *et al.*, 2010), but these have not been profiled extensively. The biochemical analysis of Metz and colleagues revealed an interesting compound (PSID 103905316) that inhibits SRC (IC_{50} of 3.2 nM), and significantly cross-reacts with Fyn and EGFR only (IC_{50}s of 1 and 32 nM, respectively) and has an entropy score of 1.1.

As PP1 and PP2 are structurally similar compounds with overlapping cross-reactivities, we recommend using either of these inhibitors in parallel with SRC-I1 to probe SRC function, as previously suggested by Bain and colleagues (Bain *et al.*, 2007).

VEGFRs (FLT1/KDR/FLT4)

Members of the VEGFR family of receptor tyrosine kinases (VEGFR1/FLT1, VEGFR2/KDR/FLK1 and VEGFR3/FLT4) are important mediators of pro-angiogenic signals, and VEGFR signalling is imperative for tumour angiogenesis (Rini, 2007; Bhargava and Robinson, 2011). Three compounds that inhibit VEGFRs have reached the market in the last 5 years: sorafenib, sunitinib and pazopanib. Treatment with any of these compounds is associated with adverse events, and subsequent drug development activities have centred on improving selectivity (Bhargava and Robinson, 2011). Indeed, sunitinib and sorafenib are not selective VEGFR inhibitors, with entropy scores of 2.0 and 2.2 (Uitdehaag and Zaman, 2011). Likewise, pazopanib has an entropy score of 2.0 when tested over a panel of 61 kinases (Kumar *et al.*, 2009). Other VEGFR inhibitors that were profiled by Karaman *et al.* (2008) include ZD-6474/vandetanib (entropy score of 2.9), CHIR265 (2.5), AMG706 (2.4), GW786034 (2.1), ABT869 (1.9) and PTK787/vatalanib (1.5). Vatalanib inhibits VEGFR1-3 and significantly cross-reacts with several related kinases, such as KIT, CSF1R and PDGFRs. Other VEGFR inhibitors, such as KRN951/tivozanib (Nakamura *et al.*, 2006) and AZD2171/cediranib (Wedge *et al.*, 2005), are also not selective over these closely related kinases. A possible exception is brivanib, which is inactive on PDGFRs, but which has only been profiled on nine kinases (Bhide *et al.*, 2006). Vatalanib is therefore recommended as a pan-VEGFR tool compound.

Some VEGFR2-specific compounds have been described. Of these, ZM323881 is probably the most selective over VEGFR1 (Whittles *et al.*, 2002; Spencer *et al.*, 2009). However, its activity towards other kinases was not adequately covered by a selectivity panel, as this panel consisted of only five kinases (Whittles *et al.*, 2002).

Conclusion

Cross-reactivity is an inherent property of most kinase inhibitors. Nonetheless, it is still common practice to describe compounds as 'selective' based purely on anecdotal evidence. Fortunately, it is becoming increasingly customary to accompany the description of a new kinase inhibitor with kinase panel screening data. These data are supplemented with large-scale cross-screening initiatives, which expand our knowledge of the selectivity of established reference inhibitors. Such activities will not only fill our compound tool box, but will also provide information on which tools to use for pharmacological experiments.

We have provided a guideline for picking the most selective tool compound for a large set of clinically relevant and intensively pursued kinase drug targets. Tool compounds for many other kinases can be found in the compound literature and cross-screening studies. For the targets discussed here, the recommended inhibitors provide a snapshot of the publicly available inhibitors and their cross-reactivities known at this moment in time. Updates will follow in due course.

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Conflict of interest

The authors declare no conflicts of interest for this paper.

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