High-Throughput Screening for Kinase Inhibitors

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Following G protein-coupled receptors (GPCRs), protein kinases have become the second most important class of targets for drug discovery over the last 20 years. While only four kinase inhibitors have reached the market to date (Fasudil for rho-dependent kinase, Rapamycin for TOR, Gleevec for BCR-Abl, and Iressa for EGFR), many more are already in clinical development. A historical overview of kinase inhibitors was recently published by Cohen.[1] After the previous successes, protein kinases are now regarded as attractive, well-drugable targets, and the analysis of the human genome has yielded 518 protein kinases.[2] We can thus expect screening for protein kinase inhibitors to become even more important in the future. In this review we will focus on the early steps of drug discovery programs producing new lead compounds. We will guide the reader through efficient state-of-the-art assay development and high-throughput screening of large chemical libraries for protein kinase inhibitors.

1. Development of High-Throughout Screening Compatible Protein Kinase Assays

1.1. Introduction

Most pharmaceutical companies use compound libraries that have now reached or passed the size of one million chemicals. So any biological assay developed for biomolecular screening faces the need for very high throughput. Searching for an inhibitor for a given kinase (and of course any other enzyme) nowadays means generating at least one million data points.

Under these circumstances, traditional techniques like gel-based assays, filter-binding assays, or enzyme-linked immunosorbent assays (ELISAs) have had to be replaced by assay technologies that circumvent the laborious and time-consuming washing and separation steps.

Well-established and widely used assay techniques matching the needs of screening are scintillation proximity (SP and flashplate), fluorescence resonance energy transfer (FRET), fluorescence polarization (FP), and Alphascreen assays. All these techniques are homogeneous mix-and-measure techniques that allow the addition of all reaction partners and detection reagents in one well of a microplate without the need for separation steps.

In screening large chemical libraries, cost pressure is an issue that drives the field towards miniaturization. The 96-well format is no longer able to meet the needs of industrial research. 384-well or 1536-well microplates have become standard, and to minimize reagent costs, assay volumes have been downscaled to 3–15 microliters.

Since even 5 μL assay volumes translate into a 5 L total assay volume and thousands of 384-well plates over an entire high-throughput screening (HTS) campaign, compound pooling comes into play as a further possibility to reduce costs. By pooling compounds the number of assays is greatly reduced and so are handling time and reagent costs. One has to consider the fact that compound interference, that is, influences of compounds on the detection system rather than the enzymatic activity to be tested, can cause serious problems, especially in pool screening. These problems have to be addressed by choosing suitable assay techniques and doing the right controls.

Decisions on HTS strategies are mostly general decisions made for the company, not for a certain project. So the researcher starting a new project to identify kinase inhibitors will not have to decide on pool screening but he or she will have to consider the probability of compound-interference problems and to find solutions, like early selectivity testing with the same assay technique to get rid of interfering compounds or retesting hits by using an alternative approach.

When a new kinase project is initiated, researchers have to decide on assay technology, substrates, the kinase (as a full-length protein or catalytic domain), and within certain limits the degree of miniaturization.

A choice has to be made between cellular or biochemical assays. Cell-based assays have the advantage that the target is in an intact cellular environment, probably correctly folded. For cellular assays stable cell lines are generated that provide a clear target-specific readout. Target classes like GPCRs and ion channels or screens for receptor agonists are candidates for cellular screening. A drawback of cell assays is the fact that many hits may be generated by inhibition of activities other than the addressed target ("off-target hits"). This will be the case if a long signal-transduction pathway is addressed and the readout results from the very last step, for example, reporter gene activity. In such a case, one is unwillingly screening several targets at once, since every step could be inhibited by library compounds. In the end, detailed mechanistic analysis has to follow the screening.

In addition to off-target hits, problems may arise by toxicity of compounds that reduce the readout of the assay without inhibition of the relevant target. Due to this fact, biochemical assays can be performed with higher compound concentra-

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tions than cellular assays, thereby raising chances of finding inhibitors in novel chemical classes. This is especially important when working on competitive fields that are already heavily patented.[3]

Unfortunately, both problems are more serious with pool screening, although pool screening is especially attractive for cellular assays, which are generally much more time consuming and more difficult to run with high throughput than biochemical asays.

For these reasons, biochemical assays form the gold standard in HTS laboratories, at least for soluble enzymes, since they allow for efficient logistics and rapid screening of large libraries, can be easily miniaturized, and do not suffer from off-target effects and toxicity issues.

We regularly use biochemical assays to screen for new kinase inhibitors and also focus on biochemical assays for this review. For a recent review on cellular kinase assays, see ref. [4].

1.2. Assay development

1.2.1. Choice of kinase protein: If the kinase to be screened is not commercially available, the investigator has to decide on expression and purification strategies. The development of cloning systems like Gateway[5] allows parallel expression in several hosts, like *Escherichia coli*, insect cells, and mammalian cells. This parallel approach maximizes the chance of getting active protein on time, but protein expression and purification still frequently remains a bottleneck for biochemical screening.

For purification purposes, addition of affinity tags like histidine tags or flag tags or expression of gutathion-S-tansferase (GST) fusion proteins is routine.

Although we prefer to screen with full-length proteins, the parallel expression of catalytic domains is a valid strategy to minimize risks in early project phases.

For lead optimization, protein crystallization and structure determination programs are started at the beginning and benefit from parallel expression strategy as well.

1.2.2. Choice of substrate: According to Copeland[6] natural substrates should be used for screening to ensure that the measured affinities of different inhibitors match the affinities that they will show in vivo. The first reason given was that, with small peptides as substrates instead of physiological protein substrates, one might miss inhibitors that block exosites instead of catalytic sites.

Exosites are substrate-binding sites outside the catalytically active site and are considered to work as a selectivity filter.

By definition, they function by protein–protein interactions, a type of interaction that is generally considered not to be drugable with small molecules.

In the field of small-molecule drug discovery, Lipinski and co-workers' "rule of five"[7] is a widely accepted guideline for the selection of compounds for HTS libraries based on their molecular properties, and compounds with molecular weights above 500 Da are hardly included in HTS libraries any more because of their often unfavorable physicochemical properties.

On the other hand, the examples given for exosite-binding inhibitors were peptides of 15[8] or even 85 residues.[9]

We think that most HTS libraries will not contain many inhibitors that will bind with high affinity to exosites, thus by using small peptide substrates instead of full-length protein substrates one will gain huge practical advantages without missing relevant hits.

Nevertheless, one should use substrates as natural as possible in order to ensure that the kinase, even with bound peptide substrate, is in the physiological conformation and that the properties of the catalytic site and its vicinity are similar to in vivo conditions. If the natural substrate is not available, one should try to use substrates as similar as possible. Based on the assumption that the natural substrate will be the one that is phosphorylated most efficiently, we routinely search for the best substrates in a library of 4500 peptides by testing activity.

Only if this approach fails to give substrates that are easy to handle do we use generic substrates such as poly-Glu–Tyr or poly-Glu–Ala–Tyr for tyrosine kinases or generic substrates such as maltose binding protein (MBP), casein, or histones for S/T kinases.

1.2.3. Choice of assay technology: Several technologies that allow high-throughput screening are available. The widely used methods have in common the use of homogeneous formats that do not involve any solid phases or washing steps. All steps from mixing reactants over incubation to addition of de-

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tection reagents and finally measuring a signal take place in one well of a microtiter plate. Traditional techniques like filter assays or ELISAs are no longer used in HTS campaigns. The development of homogeneous mix-and-measure techniques was a necessary precondition for the current level of miniaturization and throughput in screening laboratories.

The common readout in screening is optical, whether the signal is generated by scintillation, fluorescence, chemiluminiscence, or electrochemiluminiscence.

Assay techniques most frequently used for protein kinases are:

1. scintillation proximity assays (SPAs)
2. homogeneous time-resolved fluorescence (HTRF) assays
3. fluorescence polarization (FP) assays
4. Alphascreen assays

These and other techniques used in high-throughput screening are proximity based. A signal is generated if two components of a detection system are brought into close proximity. In the case of protein kinases the two components are recruited by the substrate peptide on one hand and the phosphate group on the other hand. HTRF assays, for example, use europium-labeled antiphospho-antibodies binding to the phosphorylated substrate peptide as the energy donor and streptavidin-tagged fluorophores that bind to the biotinylated peptide as the energy acceptor. The generation of signal strictly depends on the phosphorylation status of the peptide.

Several commonly used and well-established techniques and some new approaches are summarized in Table 1.

Assay techniques as simple and robust as possible are preferred to ensure high quality in screening. For this reason, the following general aspects should be considered in addition to the throughput and miniaturization issues.

Some assay technologies are inherently more sensitive to compound interference; these are fluorescence intensity, bioluminescence, and Alphascreen assays. Other technologies such as fluorescence lifetime, FRET, and time-resolved FRET (HTRF) assays are much more robust against compounds interfering with the detection system.[10]

Assay techniques that rely on coupled enzymatic reactions have the disadvantage that any compound inhibiting the secondary enzyme (for example, luciferase) instead of the target per se will be detected as a hit and this will require a higher number of confirmatory retests. Such a screening strategy requires secondary testing of all hits by using another technology or a selectivity test by using the same technology just to get rid of artifacts. Another problem with coupled enzymatic assay formats is the fact that optimization of reaction conditions may be a serious problem (for example, if the pH optima of the target and tool enzyme are different).

Another assay format with inherent problems is that of signal-decrease assays. Signal-decrease assays may be problematic because a relatively high turnover is needed to generate a statistically robust result, while a short linear range of the reaction would require only low substrate turnover. In a kinase reaction, detection of 0.1 μM phosphopeptide over a zero background is superior to detection of a decrease in adenosine triphosphate (ATP) from 1 μM to 0.9 μM!

In summary, we prefer homogeneous mix-and-measure assays that should work as signal-increase assays, avoid coupled enzyme assay formats, and have low sensitivity towards compound interference. Our favorite assay techniques are SPAs and HTRF assays. Among these we consider the HTRF assay to be superior due to its resistance against compound interference and sensitivity which allows further miniaturization and reagent cost savings. HTRF assays need up to 100-fold less enzyme than SPAs in the same format, thereby saving lots of work or money for protein production.[11]

1.2.4. Optimization of kinase assays for HTS: Since the aim of HTS is the identification of small molecules that bind and inhibit the screened target (here a protein kinase), optimization translates into maximizing sensitivity towards inhibitors while maintaining good statistical quality and keeping reagent costs low.

1.2.4.1. Buffers and additives: Enzymatic activity is optimized in order to save protein and to ensure sensitivity against inhibitors. Optimized conditions allow a reduction of enzyme concentration, thus leading to improved resolution among high-affinity hits (see below). Furthermore, protein stability is a big issue in HTS laboratories. Depending on the throughput and degree of automation, enzymes as well as reagents have to be stable for hours at room temperature. Thus, the stability of enzymes in different buffers should always be tested, in addition to mere activity. Since library compounds are dissolved in dimethyl sulfoxide (DMSO), the enzyme stability against DMSO has to be checked and, if necessary, the screening conditions should be modified accordingly.

A huge number of factors can influence enzymatic reactions and should be tested (Table 2). In many cases, however, preliminary knowledge from the literature or target-class-specific experiences, as well as technical requirements, limit the number of factors that are tested on a routine basis.

At Schering, we start assay optimization by screening for pH value and salt optima, requirements for bivalent cations (like Mn^{2+} and Mg^{2+}) and many additives that may influence stability, and for the activity of the enzyme.

To improve efficiency, we handle this first step of assay development in a standardized manner, starting with a screen for factors and interactions that have significant effects. For this purpose, design of experiments (DOE) software (Design Expert (StatEase)) is used together with automated pipetting (Biomek, AAO software, Beckman Coulter).[24] This allows efficient randomized screening of many variables and their interactions. In a first step, data for dependence on sodium, magnesium, and manganese as well as the influence of glycerol and detergents are generated with a fractional factorial design of high resolution that identifies all effects and two-factor interactions with confidence. Information from this experiment is used to build conditions for selecting the best buffer compound and pH value optimum. With pH value and buffer substances at optimum conditions, step 1 is repeated to ensure that the results are still valid under the changed conditions.

Table 1. *Assay techniques for high-throughput screening of kinases.*

Technique	Principle	Pros and cons
scintillation proximity assay (SPA)[11]	Emission of beta particles in close proximity to scintillants leads to emission of light. ATP^{33} is used as the substrate, biotinylated peptides are bound to streptavidin-coated scintillant beads, and only phosphorylated peptides generate a signal due to the limited pathlength of P^{33}.	+ no restrictions in choice of substrate peptide + low costs – safety considerations – ATP concentration is limited (background increases with P^{33}) – at least 10% ATP turnover is required for a good signal-to-background ratio – limited potential for miniaturization due to statistical nature of radioactive decay
homogeneous time-resolved fluorescence (HTRF)[11] assay	Biotinylated substrate peptides are bound to streptavidin-labeled with allophycocyanin or other fluorophores. Phosphorylation is monitored by generation of a FRET signal of europium-labeled antiphospho-antibodies that bind to the phosphorylated peptide and come into proximity with the FRET acceptor.	+ nonradioactive, ratiometric, and time-resolved measurement + low enzyme need + high miniaturization potential + bead free + low costs – availability of specific antibodies or use of generic substrates for which antibodies are available
fluorescence polarization (FP)[12,13] assay	Depolarization of polarized light is dependent on molecule size, with small molecules depolarizing light faster. Fluorophore-labeled tracer peptides are used to give a signal after binding to antiphospho-antibody during the detection step. The signal generated by tracer binding is decreased by phosphopeptides generated in the reaction step.	+ nonradioactive + low enzyme need + high miniaturization potential + bead free – signal-decrease assay – tracer and antibody needed – peptide size limited
IMAP[14] assay	Special form of fluorescence-polarization assay. Fluorophore-labeled peptides have to be used as substrates and phosphorylation is monitored through mass increase by binding of detection beads coated with trivalent metal ions.	+ nonradioactive – peptide size limited to 5 kDa, so protein substrates cannot be used – 20–30% peptide turnover is required for a good signal
amplified luminescence proximity homogeneous assay (ALPHA)[15]	A donor bead that generates singlet oxygen upon illumination is brought into proximity with an acceptor bead that generates light by chemoluminescence depending on this singlet oxygen. Proximity is mediated by the biotin–streptavidin interaction and phosphoserine/phosphotyrosine–antibody interaction.	+ nonradioactive + low enzyme need – very sensitive to compound interference[10]
Caliper[16] assay	Electrophoretic separation of substrate and phosphopeptides in microfluidic devices. Reactions may be performed on-chip or off-chip	+ nonradioactive. + high accuracy due to measurement of substrate consumption and product formation + highly miniaturized on-chip format – 20–30% peptide turnover required for a good signal – limited throughput
electrochemiluminiscence (ECL, MSD)[17] assay	Signal is generated in an electrochemical reaction that depends on recruitment of ruthenium complexes to electrodes.	+ nonradioactive – availability of specific antibodies – relatively high costs
electrochemiluminiscence (ECL, ORIGEN)[18]	Signal is generated in an electrochemical reaction that depends on recruitment of ruthenium complexes to electrodes in a kinase assay. Biotinylated peptides are recruited to a magnetic electrode through streptavidin dynabeads and a signal is generated if ruthenylated antiphospho-antibody binds to the phosphorylated peptide.	+ nonradioactive + very low background and compound interference due to flow chamber approach + limited throughput due to long cycle times in the flow chamber approach – relative large assay volumes – availability of specific antibodies
IQ (Pierce)[19] assay	Phosphorylation of peptides allows binding of a proprietary quencher, thereby reducing fluorescence	+ nonradioactive – signal-decrease assay – compound interference

Table 1. (Continued)

Technique	Principle	Pros and cons
Kinase-Glo (Promega)[20]	ATP consumption in a kinase reaction is monitored through luciferase activity.	+ nonradioactive – signal-decrease assay – off-target hits due to the coupled enzyme format, so need for additional selectivity testing
DiscoveRx (Hithunter, ED-NSIP)[21]	Relies on β-Gal complementation. Complementation is either dependent on displacement of the β-Gal fragment labeled tracer from antiphosphoserine or -threonine antibody by products of the kinase reaction (Hithunter) or on displacement of β-Gal fragment labeled staurosporine from the kinase by the test compound (ED-NSIP).	+ nonradioactive + ED-NSIP: no substrate needed – Hithunter: need for antiphospho-antibodies that bind reaction product and detection tool-peptide (or custom tool-peptide synthesis may be required) – off-target hits due to the coupled enzyme format, so need for additional selectivity testing – ED-NSIP: high enzyme need
PKLight (Cambrex)[22] assay	ATP consumption by the kinase is measured. Remaining ATP is detected by bioluminescence.	+ nonradioactive – signal-decrease assay – off-target hits due to the coupled enzyme format, so need for additional selectivity testing
Z′-Lite (Invitrogen)[23] assay	Phosphorylation of a double-labeled peptide inhibits cleavage by a protease that otherwise separates two partner dyes of a FRET system.	+ nonradioactive, ratiometric readout – peptide needs two labels and a protease cleavage site in the vicinity – off-target hits due to the coupled enzyme format, so need for additional selectivity testing

The third and last step of this routine for kinases uses response-surface design to determine optimal concentrations for the most important factors identified in steps 1 and 2. This approach ensures that general reaction optima are found.

An advantage of this procedure is that we can handle frozen replicas of master plates for steps 1 and 2, on which only enzyme, substrates, and detection reagents have to be added, thus saving lot of time.

The influence of unstable reagents (antioxidants, protease inhibitors, etc.) is then checked individually.

Factors that also have to be optimized individually are the choice and concentration of detection reagents, which vary with substrate concentrations on one hand and with the assay technique on the other hand (for example, the amount of radioactivity and concentration of SPA beads for scintillation proximity assays). Since these parameters have to fit to the concentration range of reaction products that have to be detected, optimization of these detection reagents is a late step that is done after determination of kinetic parameters like the Michaelis constant (K_m) value and linear range of the reaction (see below).

An important factor to consider is the tolerance of the enzyme towards DMSO. HTS library compounds are dissolved in DMSO and most HTS assays are run with final DMSO concentrations of 0.5–1.5 %. If a given kinase does not tolerate these conditions, one has to adapt the screening routine to the requirements, for example, by dilution of compounds and thus screening at lower compound concentration. This is only possible for assays of excellent quality since sensitivity is decreased and has to be compensated by lower hit criteria.

During these early steps of assay optimization, we always not only maximize the absolute activity but also focus on signal-to-background ratios, data scatter, and the robustness of the assay, parameters that have a big impact on the statistical performance (see below).

1.2.4.2. Substrate concentrations and reaction kinetics: Kinase activity, as all enzymatic activities, is best monitored by continuous measurements of substrate depletion and product formation. In a typical reaction, the velocity will decrease with decreasing substrate concentration.

Due to the requirement for high throughput, however, single-time point assays are performed in most screening campaigns. It is necessary that a single-time point measurement reflects the true velocity of the reaction and this is only the case for the initial velocity. Since reaction velocity decreases over time, effects of inhibitors are most prominent in the initial phase of the reaction.

Testing the length of the initial linear phase is part of assay development, along with balancing the substrates in a bimolecular reaction to avoid early slowdown due to low concentration of one of the substrates.

The scientist optimizing an assay for HTS purposes has also to think about inhibition types and the assay has to reflect the inhibition type that is looked for. Drug-like small molecules will bind with high affinity only to sites that evolved to bind small substrates or allosteric regulators. It is not probable that small molecules will block protein–protein interactions such as the binding of a protein substrate to the kinase and experience shows that no such inhibitors have been found yet. Thus, ATP-

Table 2. Buffer components routinely tested in assay development.

buffer compounds	β-morpholinoethanesulfonic acid (MES), 3-(*N*-morpholine)propanesulfonic acid (MOPS), 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), tris(hydroxymethyl)aminomethane (Tris)
pH value	5–9
monovalent cations	sodium, potassium
bivalent cations	magnesium, manganese
carrier proteins	bovine serum albumin (BSA), casein, ovalbumin
detergents	NP-40, Tween-20, CHAPS, Triton-X-100
reducing agents	1,4-dithiothreitol (DTT), reduced glutathione (GSH)
protease inhibitors	ethylenediaminetetraacetate (EDTA), phenylmethylsulfonyl fluoride (PMSF), aprotinin, leupeptin, commercial preparations ("complete", Roche)
osmotic regulators	glycerol, sucrose, poly(ethylene glycol) (PEG)
blocking reagents	poly(ethylene imine) (PEI), milk powder

competitive inhibitors and noncompetitive inhibitors that bind to distant allosteric sites are the main focus of drug discovery programs.

Since ATP-binding sites are conserved between kinases, many inhibitors will block more than one kinase. For this reason, a selectivity profiling of HTS hits is extremely important (see below). To compare the affinities of a given inhibitor towards several different kinases, reaction conditions have to be comparable in terms of ATP concentrations. Performing all assays at $[ATP] = K_m(ATP)$ ensures that the IC_{50} values obtained will be a good measure of the inhibitors' potential to displace ATP from the catalytic site.

Another reason for the common practice of fixing ATP concentrations at the K_m value is the fact that it ensures that all types of inhibitors can be found. Sensitivity towards competitive inhibitors decreases with substrate concentrations, especially above the K_m value of the substrate. Since most inhibitors will be competitive because ATP-binding sites are the sites where small molecules can bind with high affinity, it is very important not to work far above the K_m(ATP) values. (This would saturate the enzyme with ATP, thereby reducing the chance of finding inhibitors that have a similar affinity to bind the ATP-binding site.)

On the other hand, it is argued that one needs sufficiently high substrate concentrations to be able to detect uncompetitive inhibitors (inhibitors that bind to the enzyme–substrate complex). Since the third type of inhibition, noncompetitive inhibition is independent of substrate concentration, the conclusion is that working exactly at the K_m value will ensure maximum sensitivity towards all types of inhibition.[6]

Although the aim is to perform assays at $[S] = K_m$ for both substrates, there may be reasons to break this rule, like assay quality in terms of signal-to-background ratio, solubility, limits of the detection system, problems in balancing a bisubstrate reaction, and even costs. If it is not possible to work at $[S] = K_m$, the best way to solve the problem is to work below the K_m value since this will not affect sensitivity to noncompetitive inhibitors and will even increase the sensitivity towards competitive inhibitors. Only the sensitivity towards uncompetitive inhibitions will be lowered. However, we consider the probability of finding uncompetitive kinase inhibitors in HTS libraries to be very low anyway.

If, for example, the peptide has to be used below its K_m value one could theoretically miss an inhibitor that is uncompetitive in relation to the peptide. But where in the enzyme would this inhibitor bind to exert an uncompetitive inhibition? If a peptide-uncompetitive inhibitor binds in the ATP site it will be detected as an ATP-competitor and won't be missed in screening! Only if an inhibitor binds together with the peptide into the peptide-binding site without obstructing the ATP-binding site will it be missed in screening below the K_m(peptide) value. It seems very improbable that HTS libraries should contain compounds small enough to work as such a pure uncompetitive inhibitor.

1.2.4.3. Quality control: Before the start of high-throughput screening, the assay used has to be validated. Enzyme and reagents have to be stable. The assay has to be within a linear range of time (initial phase) and show a linear dependence on enzyme concentration. Whenever available, a reference inhibitor should be used and its IC_{50} value has to be reproducible.

The main quality parameter in HTS laboratories nowadays is the z' factor, as described by Equation (1), where σ is the standard deviation and μ is the mean of the standard (s) or the negative (c) control (100% inhibition by a reference inhibitor).[25]

$$z' = 1 - \frac{3\sigma_s + 3\sigma_c}{|\mu_s - \mu_c|} \quad (1)$$

z' factors above 0.5 indicate a large separation band between the values for the positive and negative controls (100% activity and 0% activity). The z' factor has the advantage of expressing the noise in relation to the signal window and, thus, gives a more complete estimation of assay quality than signal-to-background or signal-to-noise ratios alone would do.

Before starting automation and HTS, the assay should have been optimized to $z' > 0.5$ and this quality level has to be maintained during screening.

2. High-Throughput Screening

Effective HTS has three main success factors:

- good biological assays,
- a high-quality HTS compound library,
- the ability to test the library in the assay in a timely and cost-effective manner.

The first topic has been already discussed and the latter one will be discussed in Section 2.2.

2.1. Compound libraries

As the outcome of a screening campaign cannot per se be better than the input, a good compound library is crucial for the success of HTS.

At the beginning of HTS in the early 1990s, many companies greatly expanded their historical compound collections by first-generation combinatorial chemistry (combichem) and extensive compound purchases. First-generation combichem often produced compounds of high molecular weight and unfavorable physicochemical properties, for example, low solubility. Early commercial compound collections, often purely quality controlled, were of very variable quality. In addition to these inherent problems, quite often compound storage and retrieval conditions were not optimal for maintaining the integrity of the compounds. Most companies have reacted to this and have significantly improved their compound collection by substantial investments into automated compound stores, library clean-up, new compound purchases, and improved automated synthesis. Full LC/MS quality control of purchased and combichem compounds is meanwhile standard. In addition, improved knowledge about desirable properties and structural features has led to several "smart" filters which are applied routinely before compound purchase or in the design of new combichem libraries. Examples are Lipinski and co-workers' "rule of five"[7] or "lead-likeness" criteria[26,27]) and filters for unwanted structural elements,[3] "promiscuous inhibitors",[28] or "frequent hitters".[29]

As a result, diverse high-quality libraries containing in the order of 0.5–1 million compounds are now standard, even for medium-size pharmaceutical companies like Schering.

2.2. Assay formats

In the past several years, the use of homogeneous assay techniques has enabled a great miniaturization.

Low-volume 384-well plates with assay volumes of 15–20 μL have become standard, even in medium-sized HTS labs. Several HTS labs have already moved to standard assay volumes of 3–5 μL, either in 1536-well or 384-well low-volume formats. The latter is still an attractive alternative for some companies, as the assay variability tends to be lower than in 1536-well and the move to 1536-well plates might require substantial capital investment for new dispensers and readers.[30]

The limiting factor for further miniaturization is not the screening itself but the format of the compound storage and logistics. The compounds are normally stored as solutions in neat DMSO in the 384-well format. The smallest volume of compound solution that can be accurately transferred in a fast and highly paralleled manner is 50 nL by using the Hummingbird (Genomic solutions) 384-well capillary pipettor. With a final DMSO concentration in the assay of 1–1.5%, 50 nL of compound solution correspond to 3–5 μL as the minimal assay volume that can be achieved without an additional step for compound dilution and transfer.

The choice of the concentration of the test compounds is a balance between the positive rate and the relevance of the concentration. Preferred concentrations are in the range of 3–30 μM,[3,31c] depending on the respective lead discovery team.

2.3. Different approaches to effective screening

2.3.1. Diverse versus focused library screening: Many companies have built up smaller focussed libraries dedicated for the screening of kinases, either in addition to or instead of a general screening library. The design of focused kinase inhibitor libraries—driven by the vast empirical knowledge on suitable scaffolds for kinase inhibitors and predictions based on the rapidly growing structural information—is described by Prien[32] in this issue. The hit rates of these libraries are, in many screens for kinase inhibitors, significantly higher than those of the normal HTS libraries. At Schering, it is not unusual that the number of hits from the focused kinase inhibitor library is nearly as high as the number of hits found in addition by screening of the nearly 100-fold larger full HTS library. However, the hit rates can be also very low if the ATP-binding site of the kinase specifically deviates from a "normal" kinase ATP-binding site. In addition, even if the number of hits is high, the compounds often belong to only a few clusters and exhibit less structural diversity than the hits from the full library. As the focused libraries are based on well-known scaffolds or structural features, one has to consider as well that the patent situation is often more problematic for these hits.

The usage of focused libraries differs from company to company. Some companies regard a focused kinase-oriented library as a tool to reduce the screening costs or to run more sophisticated/time-consuming assays when full library screening would take too long. The full diverse library is only screened if the focused library did not yield good hits. Particularly for smaller companies entering drug discovery, working only with focused libraries is a serious option in order to avoid the high costs associated with the set-up of a large diverse library and a full HTS infrastructure.

Other companies regard screening of the full diversity-oriented library and a kinase-oriented focused library as complimentary approaches which are normally carried out in parallel. At Schering, for example, every new kinase target is screened against a focused kinase-inhibitor library (in duplicate as single compounds) and the full diverse HTS library (in pools, as described in Section 2.3.2.). When it is taken into account that the preceding costs of a project (for target identification and validation, enzyme production, assay development) are mostly much higher than the costs of testing an additional 500 000 compounds, screening of the full library is a meaningful investment in order to fully exploit the potential lying in the target and in the compound collection. A more detailed overview about the pros and cons of both strategies—not only with respect to kinases—has been given by Valler and Green.[33]

2.3.2. Single-compound screening versus pool screening: Another way to reduce the number of test samples and thereby the costs associated with screening is to test mixtures of compounds ("pool screening") instead of single compounds. This strategy has been widely used, with typical pool sizes of 2–

10 compounds. Sometimes a part of the reduction in sample numbers is reinvested to screen the pools in duplicate, as testing the compounds only once is commonly regarded as the main disadvantage of single-compound screening. A special format is the orthogonal pooling procedure first described by Devlin et al.[34] In this approach, which is also used by Schering, every compound is tested in two completely different sets of compounds. This allows the identification of the active component of a pool without testing of all pool members, simply by electronic deconvolution of the assay data, as long as the hit rates are not too high.

Surprisingly, despite of the large impact of this strategy and the old discussion[35] about the pros and cons, there is very little literature data comparing the results of both strategies. The only case study published[34] is nearly ten years old and is meanwhile of very limited relevance as it does not reflect the current situation of miniaturized homogeneous assays. This lack might be explained by the high cost of a serious comparison or simply by the fact that the investments made into the logistics for pool screening or the screening capacity necessary for single-compound screening represent a significant hurdle to a change of strategy. For the same reason, a discussion of the pros and cons of pool screening cannot be limited to kinase assays but has to take into account also the other assays (for example, GPCR assays) typically run in HTS labs.

As already mentioned, the main advantage of pool screening is the saving in costs and time. The main disadvantage of pool screening is that active compounds might be masked by a counteracting activity of any other compound in the pool/mixture. A counteracting activity could either affect the assay reaction itself (for example, cytotoxic compounds in cell-based assays), interfere with the detection readout (for example, fluorescent compounds), or interfere with the compound itself (for example, by reacting with the compound[36] or nucleating its precipitation). Interference with the assay biology itself is especially problematic in cellular assays as cytotoxicity is a quite common phenomenon at the relatively high total concentrations of test compounds reached in the mixture screening. It might be tolerable in screens for antagonists where the cytotoxicity only leads to a higher number of "false positives". However, it is a severe problem in screens for agonists where an active compound would not be identified in the presence of a cytotoxic compound in the pool ("false negative"). For this reason, some HTS labs use pool screening only for biochemical assays and run cell-based assays in single-compound mode. As already discussed, compound interference with the detection readout strongly depends on the assay technique chosen. In consequence, the same is true for the relevance of the higher compound interference associated with pool screening. We have had rather good experiences with pool screening of HTRF assays or SPAs, even at fairly high compound concentrations (10 μM per compound, >100 μM total concentration of test compounds), whereas we had to completely omit pool screening of Alphascreen assays. FP assays are borderline, depending on the type and concentration of the fluorescent label used; the application of red-shifted dyes like *N*,*N*′-bis-carboxypentyl-5,5′-disulfonatoindiodicarbocyanine (Cy5TM) instead of the commonly used fluorescein is highly recommended.[13]

The pros and cons of single-compound screening have already been implicitly discussed as the cons and pros of pool screening. Nevertheless, some points should be explicitly mentioned. First, another important advantage of single-compound screening, besides the higher data quality, is the higher flexibility. Library changes, compound additions as well as removals, can take place faster. In addition, there are more possibilities for taking the benefit from the data beyond the respective individual projects, for example, by usage of activity fingerprints for hit prioritization in other projects.[35b]

On the other hand, a major limitation is that single-compound screening of large libraries is generally done only once due to the high costs. Compared to the duplicate testing often done in pool screening, this comprises a higher risk for false negatives.

In the last few years, the importance of the time and cost savings associated with pool screening has declined due to the significant improvement in our capabilities to screen large chemical libraries: The extended use of homogenous assay technologies and miniaturized higher density plate formats has strongly reduced the expenditure of time and money per data point. As this reduction has far exceeded the concurrent growth of the compound collections, the costs and the duration of a single screening campaign have strongly decreased and so, in consequence, have the savings associated with pool screening. In addition, the number of validated targets did not increase as greatly as expected a few years ago, thereby making a single target more valuable. This reduced the focus on costs and throughput and increased the willingness to pay the price associated with single-compound screening for the higher data quality in order to optimize the outcome of the high investments made into target validation and compound libraries.

2.3.3. Fully automated systems versus workstations: In principle there are two different philosophies in HTS, integrated full automation and unit automation ("workstation approach").

In the first approach, samples, reagents, and plates are supplied to an integrated system of robotic plate manipulators, liquid-handling instruments, and detectors. Scheduling software then controls the flow of plates and conducts the entire assay totally unattended.[37] In the best cases, human intervention is limited to feeding test samples, reagents, and plates and disposing of the waste.

In the other approach, automation is limited to individual workstations, independent systems that are highly specialized to perform a single function or task as efficiently as possible.[38] Typical examples are a plate reader with integrated stackers and barcode reader or a 384-well pipettor with plate stackers, tip washer, and refillable reservoir. The transfer of the plates (mostly in batches) between the different workstations is done by humans.

The discussion about the advantages and disadvantages of both strategies has already leasted for several years.[39]

A big advantage of the workstation approach is its flexibility. The workstations can be combined in whatever order and in quantities are optimal for a specific assay. The decoupling of the different unit operations allows an easy allocation of addi-

tional capacity to a bottleneck step and the sharing of unique devices between different screening projects. This facilitates a high throughput as well as an effective utilization of the equipment. In addition, having several equal workstations instead of a single fully integrated system reduces the impact of instrument breakdowns.

Automated systems have, once the assay is established, a higher throughput and require less personnel. For a standard 384-well homogeneous kinase assay with separately prepared 384-well compound plates and 3 addition steps (enzyme, substrate mix, and stop/detection reagent mix), the throughput is typically about 100 000 wells per day with an automated system compared to 25 000–40 000 wells per day and per person with workstations. On the other hand, workstations allow a faster assay set-up and require less capital expense. The overall efficiency of both approaches thus depends on the number of samples tested per screen and the number of screens run per year. Fully automated systems are more prominent in larger HTS labs which have to test many compounds against a high number of targets, whereas the use of workstations is more common in medium-sized HTS labs.

The largest difference between the two approaches might concern the people situation. Fully automated HTS systems are complex systems. The setup of the system and the establishing of suitable standard operating procedures (SOPs) for its usage and maintenance require experienced personnel and have long learning curves. Training is a very important factor and normally HTS labs have people fully dedicated to the operation of these systems. In consequence, development and screening of the assay is normally done by different people. However, once the SOPs are established and the automated assay is seriously validated before the screening campaign, a constant data quality and throughput can be expected that does not depend on the daily form of the lab technicians. This is a clear advantage over the workstation approach, where both quality and throughput can vary significantly according to the technicians' capabilities and interest in processing the assay plates as accurately, quickly, and efficiently as possible. In comparison to the operation of fully automated systems, efficient usage of workstations is easier and has only a short learning curve. In consequence, it does not require dedicated personnel and the assay can be developed and run by the same person. This eliminates a potentially troublesome interface and saves time. In addition, it increases the accountability, as a single person now has full responsibility for the screening results.

2.4. Evaluation of hits

Once an HTS campaign is completed, the potency of the identified hits is assessed by measuring of their IC_{50} values. As the next step, the hits are typically evaluated with regard to compound purity and structural tractability, as well as other chemical and physical filter criteria (for example, log *P* and aqueous solubility). Compounds surviving these initial filters are then subjected to more detailed evaluation concerning efficacy, selectivity, pharmacokinetic parameters, etc. The whole process, often named "hit-to-lead" or "lead generation", will not be discussed in detail as it would be a subject for a review on its own. (For recent reviews, see ref. [31].)

Instead, the focus will be directed towards two topics that are particularly important in the evaluation of kinase inhibitors, the selectivity and the reversibility and kinetics of inhibitor binding. Since nearly all kinase inhibitors address the ATP-binding site, a suitable selectivity profile is very important. It should be one of the main criteria for the selection between different hit clusters, especially if one aims for a nononcological indication. Although significant progress has been made in understanding the structural basis of selectivity between different kinases,[40] profiling against a broad panel of kinases still yields unexpected results.[41] Thus, the selectivity profiling should be done broadly and not be restricted to closely related kinases.

For selectivity-profiling assays the situation is somehow different than for HTS assays, as they have to balance two partially conflicting requirements.

One requirement is the highly sensitive detection of the inhibition of other kinases. The other is to get a good estimation of the relative potency against various kinases under cellular conditions or, in other words, the selectivity profile should reflect the in vivo conditions as much as possible to reduce the risk of project failure in later stages.

Whereas the best sensitivity is reached if the assays are run with ATP concentrations individually adjusted to the K_m values of the respective kinases, the best comparability with the cellular situation is given if all assays are run at the same millimolar cellular ATP concentration. Testing all the kinases at their respective K_m values or at a fixed ATP concentration below the respective K_m values would be misleading, as the resulting IC_{50} values would translate differently to cellular millimolar ATP levels and would not reflect the profile under physiological conditions. Determination of inhibition constant (K_i) values instead of IC_{50} values would not solve this problem and is also not practical for the high number of compounds that usually result from an HTS campaign.

A practical approach to overcome this problem is to run all the kinase assays at a fixed ATP concentration that is just above the K_m values of all the kinases. In the Kinase Profiler at Upstate (Dundee, UK)—currently the largest commercially accessible kinase selectivity panel—the K_m values for ATP are mostly in the range of 5–100 μM. When this is taken as a model, running all kinase selectivity assays at 100 μM ATP is a good compromise between the different demands.

A convenient way to assess the reversibility and kinetics for a high number of compounds is to measure apparent IC_{50} values after different preincubations of enzyme and test compound.[6] In brief, enzyme and test compounds are incubated at 10–20-fold higher concentrations then usual. After a reasonable equilibration time (30–60 min), the samples are diluted to their normal concentrations. The reaction is started by the addition of the substrates either directly (conditions A) or after a second incubation (conditions B). If inhibitor binding is rapidly reversible, the IC_{50} values after the compound preincubation (conditions A and B) will be comparable to the values in the normal assay. A significantly lower IC_{50} value with the compound preincubation (conditions A and B) indicates irreversible

(or very slowly reversible) inhibitors. Irreversible inhibitors are mostly considered not tractable as drug candidates and are discarded. A lower IC_{50} value directly after compound preincubation (conditions A) that rises back to its original value after the second incubation (conditions B) could indicate slowly reversible inhibitors. Slow-binding kinetics are often a sign for enzyme conformational changes prior to binding. As this implies the chance to enhance selectivity, these compounds might be worthwhile for further evalutation, even if they have lower potencies. The most prominent example for a slowly reversible kinase inhibitor is Gleevec.

3. Summary and Outlook

Over the last few years, significant improvements have been made in our capability to screen for low-molecular-weight kinase inhibitors. The development and performance of a biologically relevant, economically viable, one million sample screen is normal and can be confidently predicted. Although there will be continuous improvements—for example, ongoing miniaturization and standardization to a single, nonradioactive, highly sensitive assay technology—assay development and primary screening are no longer bottlenecks. The focus has shifted to later stages of the drug discovery process.

Based on the growing importance of selectivity profiling, HTS labs will become, more and more, data factories that support not only hit-identification but also the hit-to-lead and lead optimization processes. Independently of the project, all new kinase inhibitors will be tested dose dependently in all available kinase assays. This will not only speed up lead optimization but will also build up a comprehensive data basis for the rational prediction of compounds with improved potency and selectivity. The lever for real progress will be in the interplay between the effective data generation by the wet HTS and structural biology, good approaches in rational drug design and virtual screening turning this information into new structures, and the rapid supply of these new compounds by effective compound logistics and automated medicinal chemistry.

Keywords: assay development · drug discovery · high-throughput screening · inhibitors · kinases

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