Bioorganic & Medicinal Chemistry 20 (2012) 1979–1989

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

# Unbiased binding assays for discovering small-molecule probes and drugs

Melissa M. Kemp, Michel Weïwer, Angela N. Koehler *

*Broad Institute of Harvard and MIT, Cambridge, MA 02142, USA*

ARTICLE INFO

*Article history:*
Available online 24 December 2011

*Keywords:*
Binding assays
Small-molecule probes
Thermodenaturation
Surface plasmon resonance
Small-molecule microarray
Mass spectrometry
DNA-encoded libraries

ABSTRACT

2011 marks the 10-year anniversary of milestone manuscripts describing drafts of the human genome sequence. Over the past decade, a number of new proteins have been linked to disease-many of which fall into classes that have been historically considered challenging from the perspective of drug discovery. Several of these newly associated proteins lack structural information or strong annotation with regard to function, making development of conventional in vitro functional assays difficult. A recent resurgence in the popularity of simple small molecule binding assays has led to new approaches that do not require knowledge of protein structure or function in advance. Here we briefly review selected methods for executing binding assays that have been used successfully to discover small-molecule probes or drug candidates.



## 1. Introduction

Over the past decade, the human genome sequence and subsequent genomic analyses have provided insight into the genes, proteins, and cellular pathways underlying disease. Systematic and comprehensive studies have led to the identification of over 2,850 genes associated with rare Mendelian diseases, over 1,100 loci associated with common diseases, and more than 150 new targets that are somatically mutated in cancers.[1] These genes encode many proteins that have been studied intensively by researchers in all sectors, including various enzymes, G-protein coupled receptors (GPCRs), and nuclear receptors that have been modulated successfully with small molecules.[2,3] The list also includes proteins that have been historically considered challenging targets for modulation by small molecules such as transcription factors,[4] extracellular growth factors,[5] and proteins that function primarily through protein–protein interactions or that lack known enzymatic function.[6] The list contains many proteins for which there is little information about structure or biological function. Small molecules should prove to be especially useful probes of function for such understudied proteins with relevance to disease and may help clarify their potential roles as therapeutic targets.

How does one design or discover small-molecule probes[7] for proteins in the absence of knowledge about structure or function? This question has led to a resurgence in the development of high-throughput and unbiased binding assays aimed at identifying small molecules that directly interact with a given protein target of interest.[8] Confirmed small-molecule binders to the target can serve as probes of a protein's function(s) in broad functional or cell-based phenotypic studies, an approach often referred to as reverse chemical genetics.[9,10] Unbiased and direct binding assays have already proven useful in identifying probes of challenging targets that function primarily through protein–protein or protein–DNA interactions.[5,11,12] Binding assays also provide the opportunity to discover structurally or mechanistically novel modulators of classically druggable targets. For example, binding assays involving enzymes may yield allosteric inhibitors of enzyme function as well as small molecules that bind distal to the active site and disrupt an orthogonal function such as a protein–protein interaction. Binding assays are also critical in fragment-based drug discovery approaches where small fragments with weak affinities are identified using numerous binding methods and coupled to NMR or X-ray crystallography to design more potent binders for various protein classes.[13] Finally, direct binding assays have facilitated structural biology efforts by identifying small molecules that can serve as aids in protein crystallization.[14,15]

This review will highlight several novel approaches to unbiased binding assays developed over the past decade that have already proven successful in small-molecule probe discovery and early-stage drug discovery. The review will emphasize non-competitive binding assay platforms that do not require prior knowledge of structure or function for a given protein target, including thermodenaturation-based methods, surface plasmon resonance (SPR), small-molecule microarrays (SMMs), affinity-selection-mass spectrometry (AS-MS), and selection methods involving DNA-encoded libraries (DEL). Where applicable, the reader is referred to more comprehensive reviews about a given platform.

* Corresponding author.
*E-mail address:* koehler@broadinstitute.org (A.N. Koehler).

0968-0896/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2011.11.071

## 2. Thermodenaturation assays

Binding of a ligand to a protein induces a thermodynamic change in the protein, often stabilizing the conformation relative to the free protein.[16–19] This phenomenon has been utilized to characterize binding of small molecules to proteins by monitoring thermal shifts in the denaturation profile of proteins in the presence of compound. This approach is independent of protein function and provides a simple method to study the binding of ligand to the protein by monitoring the thermodynamic changes of the receptor. Traditionally, the thermodynamic properties of proteins have been monitored using differential scanning calorimetry (DSC), where the thermal denaturation of proteins are monitored by the change in heat capacity.[20] However, like isothermal calorimetry (ITC), this method is relatively low-throughput and requires considerable amounts of protein and compound for each experiment. More recently, thermodenaturation methods involving differential scanning fluorimetry (DSF) and differential static light scattering (DSLS) have been developed into high-throughput platforms for identifying protein–small molecule interactions.[15] Thermodenaturation methods have also been used to complement other high-throughput screening (HTS) methods aimed at identifying modulators of biomolecular interactions such as AlphaScreen® and fluorescence polarization (FP).[21,22]

DSF involves incubation of protein and ligand with an environmentally sensitive fluorescent dye that is quenched in aqueous environments. As the protein unfolds with increasing temperature, the dye binds to the hydrophobic regions of the protein resulting in an increase in fluorescent signal.[20] The increase in fluorescence is monitored at increasing temperatures and the midpoint of the transition between the native state and the denatured state is referred to as the Tm. A change in Tm, or ΔTm, of protein alone and protein–ligand complex is a reflection of binding and is proportional to the affinity to which the ligand binds to the protein (Fig. 1).[23] Historically, the thermodynamics of proteins were measured in a stepwise manner by heating one sample at a time and monitoring the fluorescent change for each temperature until the highest desired temperature is reached.[24] Utilizing 384-well plates and a customized heating instrument with a fluorescent detection camera, 3-Dimensional Pharmaceuticals (now Johnson & Johnson) developed a high-throughput DSF platform called ThermoFluor®. This method has been used to rapidly screen proteins of interest for their ability to bind to various ligands.[20] Academic labs have also taken advantage of the technique's simplicity and adaptability for various targets; especially in the structural biology field, screening for stabilizing ligands to aid in protein crystallization.[15] Another thermodenaturation assay used routinely by structural biologists is differential static light scattering (DSLS). This technique monitors the difference in scattering of light of aggregating proteins upon increasing temperatures.[25,26] The readout is similar to DSF, with the inflection point of the sigmoidal curve referred to as Tagg. For a more detailed discussion of these methods the reader is referred to a review by Wasney and coworkers.[14]

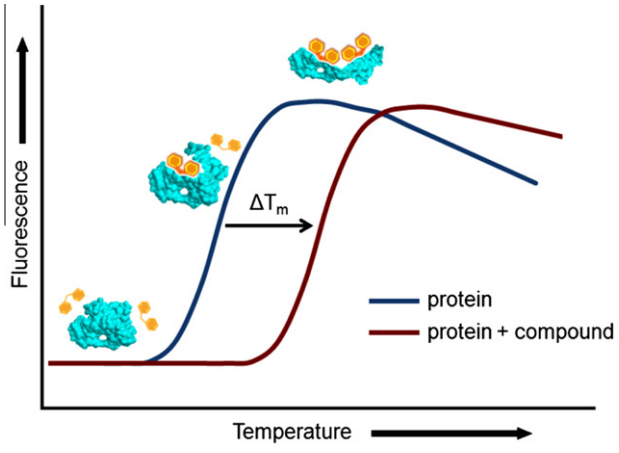



**Figure 1.** Representative shift in Tm values of differential scanning fluorimetry (DSF) curves upon the addition of compound.

As with any screening method, there are advantages and disadvantages to DSF. The method is highly versatile and requires minimal effort for assay development against a wide swath of targets. The reagent requirements are minimal compared with other methods, such as ITC and DSC. The method is compatible with a variety of standard real-time PCR detection instruments to systematically raise the temperature while detecting the fluorescence levels. DSF is semi-quantitative, offering an estimation of the binding affinity between ligand and protein by assuming a fixed enthalpy value.[20,27] With some protein families this method can work well in estimating affinities. Filippakopoulos et al. had successfully used DSF to determine affinity between a small molecule and bromodomain, and saw a linear correlation with ITC values.[28] However, accurate measurements of affinity constants ($K_D$) require that the enthalpy of the interaction be determined. Since DSF is not a true calorimetry, the approach cannot accurately determine the enthalpy of the interaction to obtain the $K_D$.[27,29] DSF and DSLS are typically used simply as primary 'yes/no' binding assays that are coupled to a secondary assay involving a complementary method, such as ITC or DSC, to determine affinity values. Other drawbacks can come from the physical properties of the target or small molecules being measured. Some autofluorescent compounds or compounds that interact with the hydrophobic dyes can produce artifacts in the assay. Small temperature changes arising from weak interactions are difficult to measure and can produce large errors in calculated binding constants.[27] Compounds that bind to multiple sites on the same protein have an additive ΔTm effect, potentially producing an artificially high affinity value.[20] It may be difficult to observe baseline thermal transitions for proteins that contain unstructured regions, such as many transcription factors, diminishing the utility of this approach for intrinsically disordered proteins.

Despite these drawbacks, thermodenaturation assays are increasingly popular for HTS. The generality of the approach has appealed to the structural genomics community where DSF has been used to evaluate and identify stabilizing ligands for proteins to aid in crystallization.[14,15] A recent report by the Structural Genomics Consortium noted that roughly 10% of all structures solved by the team were made possible only by the presence of identified ligands.[15] On the probe development side, DSF has been used for targets where conventional assay development has proven challenging, including pathogenic proteins of unknown function and proteins that function primarily through protein–protein interactions. CFE97, a *Streptococcus pneumoniae* protein of unknown function, was screened against a collection of 3,000 known bioactive compounds. Pyridoxal phosphate and pyridoxamine phosphate, along with nucleotides and nucleotide sugars, were found to bind to CFE97. Based on sequence alignments and the newly identified ligands, the authors hypothesized that the protein functions as an aminotransferase.[30] This approach might be applied to assign functions to other proteins.

In another example, Grasberger and colleagues described the use of HTS-DSF to identify a novel class of 1,4-benzodiazepine-2,5-diones (BDPs) that act as antagonists to the HDM2-p53 interaction. p53, a tumor suppressor protein involved in apoptosis and cell cycle arrest, has been implicated in many different cancers.[31,32] HDM2 interacts with p53, and is a negative regulator of its tumor suppressor functions. Crystal structures reveal an interaction in the

N-terminal transactivation domain of p53, promoting translocation to the cytoplasm, ubiquitination by the HDM2 E3 ligase domain, and subsequent proteolysis.[33] The crystal structures also revealed three key amino acids that are important for the interaction and a possible binding region for small-molecule disruptors.[34]

The ThermoFluor® assay was used to screen ~340,000 compounds against a truncated form of HDM2 that included the p53 binding domain. The 1,216 candidates from the primary assay included 116 BDPs with ΔTm values ranging from 1–4.9 °C, or at least three times the standard deviation at a standard concentration.[34,35] Affinity constants were estimated by varying the concentrations and comparing the difference in Tm. The values obtained were further validated using control peptides known to bind to the p53-binding domain of HDM2.[34,36,37] Assay positives were subjected to a secondary FP assay aimed at determining which binders were capable of displacing a fluorescein-labeled p53 peptide from HDM2.[35] The most potent BDPs served as starting points for structure–activity-relationship (SAR) studies and medicinal chemistry.[35,38] Stereochemistry was a determinant of activity as the lead compound **1** had a $K_D$ of 80 nM compared to the enantiomer **2** with a $K_D$ of 4.8 μM (Fig. 2). A crystal structure with HDM2 revealed that **1** binds in the same binding pocket as p53 and the orientation appears to mimic the α-helix of p53 peptide.[34] In JAR choriocarcinoma cells that overexpress p53 along with HDM2, the levels of p53 target genes, p21 and the HDM2 gene itself, increased upon treatment with **1**. Significant antiproliferative effects were observed only for the active stereoisomer. This study highlights the use of DSF to discover a small molecule that blocks a protein–protein interaction with cellular consequences.

## 3. Surface plasmon resonance

Surface plasmon resonance (SPR) technology is a commonly used real-time method for characterizing various biomolecular interactions, including interactions between proteins and small molecules.[39] The fundamental principle behind the SPR technique relies on a phenomenon that occurs when light is reflected off of thin metal films, such as gold and silver. The polarized light wave induces oscillation of plasmons between the metal and glass surface, which are sensitive to disturbances on the metal layer.[40] The activity at the metal surface is detected by the change in the refractive index of the medium near the surface. The reflected light at a specific angle of incidence is referred to as the SPR angle and the changes in this angle are plotted over time producing a sensorgram (Fig. 3). The changed SPR angle has a linear relationship with the mass absorbed onto the surface and can be used to measure biomolecular interactions in a label-free environment.[41] A more detailed discussion of the principles behind SPR biosensors is provided by Faegerstam et al.[42] A typical SPR experiment involves a biomolecule being immobilized to the surface of a sensor chip coated with a thin layer of gold and a dextran matrix. Another interacting molecule is passed over the chip and the binding interaction is monitored by the change in the refractive index at the surface over time. The resulting sensorgram can be interpreted in three phases—the association of the complex, equilibrium state, and dissociation of the two molecules. The analyte is injected at several concentrations to generate several full kinetic profiles containing all three phases. The data obtained is fit to a curve generated by an appropriate binding model, permitting the determination of a kinetic binding mechanism.[43] The on-rate ($k_{on}$) and off-rate ($k_{off}$) can be deduced and are used to calculate the $K_{D,}$ or dissociation constant, describing the affinity of the interaction.

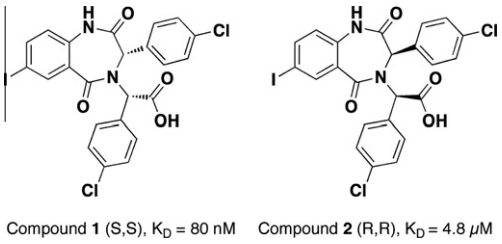



**Figure 2.** Structure of an HDM2 binder (compound **1**) and its enantiomer (compound **2**).

SPR is an attractive method for screening challenging protein targets because minimal information about protein function is required. SPR is growing in popularity and is frequently used in place of ITC, historically regarded as the gold standard to determine affinity constants. A key advantage of SPR is the minimal protein requirement; microgram quantities are often enough for immobilization to the surface of the sensor chip to get a reasonable signal for a small-molecule assay. SPR can also be run at higher throughput than ITC, and multiple ligands can be immobilized simultaneously to compare binding of an analyte. The system tolerates small amounts of organic co-solvents typically used in screening. The method also allows detection of wide ranges of molecular weights, kinetic constants ($k_{on} = 10^3$–$10^8$ $M^{-1}$ $S^{-1}$, $k_{off} = 10^{-6}$–1 $s^{-1}$), and binding affinities ($K_D$ = 1 mM–1 pM).[44]

As with all methods, there are disadvantages to the SPR approach.[14,45] SPR requires immobilization of at least one of the binding partners. Immobilization of protein to the surface of a SPR chip, either through affinity-based capture or direct coupling chemistry, can mask critical interaction sites or limit the conformational changes that can occur upon binding. Significant effort is often required to develop an appropriate surface capture approach for any given protein.[43] An additional drawback is the cost associated with this technique, since a dedicated instrument and specific sensor chips are needed to run an assay. A major limitation is the throughput of the method as compared with other methods described here. The leading distributor of SPR machines, Biacore (GE Healthcare Life Sciences), has released instruments with higher sensitivity and higher throughput, allowing for characterization of more interactions simultaneously. The T200 instrument is capable of automated and unattended analysis of 96-well and 384-well plates for up to 48 h, enabling 100–400 interactions per day, depending on the assay.[44,46] The A100 instrument can monitor up to 3,800 interactions per day using five detection spots. Advances in the use of SPR imaging may allow detection of the changes in SPR signal for thousands of interactions in an arrayed format on a single chip.[47,48] Others have employed the arrays for screening, immobilizing thousands of fragments on a chip and using SPR imaging to detect binding of the protein target to each of the compounds concomitantly.[49,50]

Despite these limitations, SPR was recently used to successfully screen for a small-molecule binder to a challenging intrinsically disordered protein. Erkizan et al. developed an SPR-based approach to discover and characterize binders of EWS-FLI1, demonstrating how SPR is useful for both primary screening and secondary mechanistic studies.[11] EWS-FLI1 is an oncogenic fusion protein resulting from the most common chromosomal translocation in Ewing sarcoma family tumors (ESFTs). The translocation event fuses *EWS* gene to an *ets* (erythroblastosis virus E26 transforming sequence gene) transcription factor, such as the *FLI1* gene or *ERG* and *ETV1*.[51,52] Previous studies have suggested that elimination of the EWS-FLI1 oncogenic fusion prolongs survival in ESFTs xenograft mouse models.[53] The interaction between EWS-FLI1 and RNA Helicase A (RHA) has shown to be crucial for oncogenesis.[11,54] The region of RHA that interacts with EWS-FLI1 is unique, and does not occupy other important binding sites in the protein, offering a potential target for anticancer treatment.

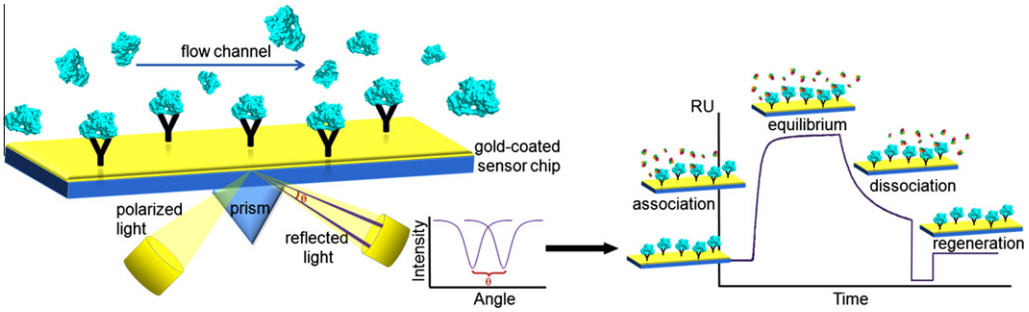



**Figure 3.** Schematic representation of a surface plasmon resonance (SPR) experiment and the resulting typical sensorgram obtained.

Toretsky and colleagues demonstrated that disruption of the protein–protein interaction with a peptide inhibits the growth of EWS-FLI1-positive ESFT cells.[11] Next, the group screened 3,000 small molecules from the National Cancer Institute Drug Target Program for direct binding to purified EWS-FLI1 using SPR. The compounds were evaluated in a ranking assay where a ratio between the observed change in RU and the theoretical maximal change in RU was used to prioritize candidates. One of the leads that came out of the screen, with a 0.9 ratio, was verified and subjected to chemical optimization in an effort to improve activity (Fig. 4). A more potent analog, YK-4-279, inhibits the protein–protein interaction of EWS-FLI1 with GST-RHA$_{647\text{-}1075}$. SPR was used to measure a steady-state affinity constant of 9.48 μM between EWS-FLI1 and YK-4-279. SPR was also used to execute a displacement assay with a peptide, E9R, which was shown to disrupt the binding between EWS-FLI1 and RHA. YK-4-279 (10 μM) reduced binding of E9R at various concentrations to EWS-FLI1. These results were validated using FP assays where E9R was displaced from EWS-FLI1 using 30 μM YK-4-279. The compound had a dose-dependent effect on EWS-FLI1-mediated transcription in a reporter assay, induced apoptosis in ESFT cells, and reduced the growth of ESFT orthotopic xenografts.

More recently, the same team used SPR to determine that YK-4-279 binds to other ETS transcription factors that share a highly homologous DNA-binding domain.[12,55] The translocation of the *TMPRSS2* gene to *ETV1* or *ERG* leads to their aberrant overexpression in prostate cancers.[56] Inhibition of these ETS transcription factors using either short interfering (si)RNA or short hairpin (sh)RNA led to inhibition of motility and invasion.[57] Rahim et al. calculated steady-state affinities ($K_D$) of 11.7 μM and 17.4 μM for ERG and ETV1, respectively. The authors evaluated the compound in a number of cell-based experiments and found that YK-4-279 inhibits the expression of ERG and ETV1 target genes involved in metastasis and breakdown of the extracellular matrix.[12] The compound also inhibits invasion and motility of ERG- and ETV-mediated metastatic cells. The mechanism of interaction was further investigated using SPR to determine that YK-4-279 does not directly block the interaction of ERG or ETV1 with ETS binding sites on DNA. The result was confirmed using chromatin immunoprecipitation of a specific ETS transcription factor promoter region in the presence of YK-4-279. The authors hypothesized that the mechanism of inhibition for ERG and ETV1 in prostate cancer is different from the mechanism of EWS-FLI1 inhibition in Ewing's sarcoma. Further investigation is needed to determine whether YK-4-279 inhibits specific protein–protein interactions involving ERG and ETV1 that are relevant to prostate cancer. The studies described here demonstrate how SPR can serve as a useful tool in both primary screens and detailed mechanistic studies for challenging targets such as intrinsically disordered transcription factors.

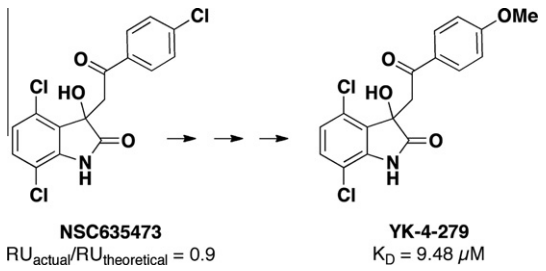



**Figure 4.** Structures of the original hit compound that bound to EWS-FLI1 and its analog with improved affinity for the protein.

## 4. Small-molecule microarrays

Small-molecule microarrays (SMMs) have proven to be a general, robust, and scalable platform for discovering protein–small molecule interactions that modulate protein function. They are easily fabricated, allowing screens of up to 12,000 compounds per slide with many different proteins of interest. Taking a cue from researchers working in the field of gene expression profiling and DNA microarrays, Schreiber and coworkers prepared microarrays containing covalently immobilized small molecules that can be probed for binding to a protein target of interest.[58] Since the initial report, a number of laboratories have described novel approaches to manufacturing SMMs, including covalent and noncovalent approaches, as well as novel approaches to detect binding.[59,60] Many of these laboratories recently contributed detailed protocols related to SMM fabrication, screening, and data analysis as part of a methods volume, edited by Uttamchandani and Yao, that is dedicated to SMMs.[59]

Typical SMM fabrication involves robotically depositing nanoliter volumes of compound stock solutions in an arrayed fashion on a substrate, such as a chemically reactive glass microscope slide. Microarray features are typically 50–200 μm in diameter and SMMs often contain more than 10,000 different small-molecule features per array.[61] SMMs containing diverse types of molecules have been reported and include commercial compound collections, known bioactives, FDA-approved drugs, purified natural products, natural product extracts, products of diversity-oriented syntheses,[62,63] carbohydrates, peptidomimetics, and fragment collections.[64–70] SMMs are incubated with a protein of interest and interactions are detected using either a fluorescence-based or SPR-based readout.[61,67] Fluorescent readouts typically involve incubating SMMs with protein followed by a fluorescently labeled antibody against the target or an epitope tag (Fig. 5). SMMs can be screened using pure proteins or proteins residing in cellular lysates. Detection of binding in lysate screens has involved fluorescent and expressible tags such as GFP,[65,68] labeled antibodies against overexpressed, epitope-tagged proteins,[65] and labeled antibodies against endogenously expressed proteins.[70] Screens

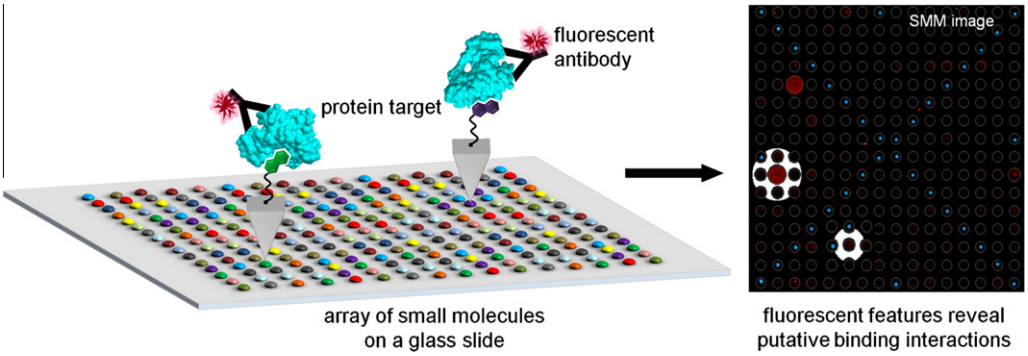



**Figure 5.** Schematic of a binding assay using a small-molecule microarray (SMM). SMMs are incubated with the protein of interest and binding is typically detected using a fluorescently labeled antibody against the protein or an epitope tag.

involving proteins in lysates may enable probe discovery for targets that reside in protein complexes and are challenging to work with in a purified form. The small molecule on the SMM may directly interact with the target of interest or an associated protein and secondary assays involving complementary methods such as SPR, DSF, ITC, or affinity-based pull-downs may be required to determine which protein complex member is the true receptor for the small molecule. SMMs have also been used for secondary assays involving peptoid ligands arising from a high-throughput, magnetic bead-based binding screen of a large combinatorial library.[64]

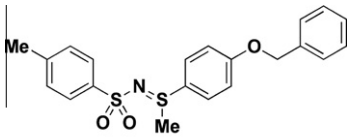
Advantages of the microarray assay format include miniaturization, throughput, accessible instrumentation, and generality with regard to screening protocols. The need to immobilize molecules on the surface may present a key disadvantage to this format as productive binding modes may be masked depending on the strategy used to fabricate the SMM, leading to false negatives. Multiple groups have published non-selective and unbiased immobilization approaches in an effort to increase both the types of compounds that may be immobilized as well as the number of orientations a given compound may have in a printed feature.[65,66] For example, fluorous chemistry was used to develop carbohydrate-based SMMs and has been employed to control the orientation of small-molecules to the surface of the slides, allowing for homogeneous display on surfaces.[70–72] Another limitation with SMMs is detecting binders that have weak affinities. 3D microarrays attempt to circumvent this issue with increased loading capacity per spot to improve sensitivity.[73,74] Like thermodenaturation-based assays, SMM assays are typically run as 'yes/no' primary assays followed by quantitative secondary binding assays such as SPR or ITC.[65,68] Despite these limitations, several ligands of varying affinities have been discovered using this approach as reviewed previously.[59,60,75–77] The approach has proven useful for discovering probes of many types of targets, including enzymes such as kinases,[78,79] proteases,[80,81] or deacetylases[70,82] and challenging targets such as transcriptional regulators,[83–85] extracellular growth factors,[5] or amyloid proteins.[86]

A recent example of a probe discovery effort using SMMs involves pirin, an Fe(II)-containing nuclear protein with little annotation regarding function.[68] Overexpression of human pirin has been observed in various tumor cell lines although the exact role of the protein in transformed or untransformed cells is undefined.[87] The protein has been shown to interact with the nuclear factor I/CCAAT box transcription factor (NFI/CTF)[88] and Bcl3, a proto-oncogene in chronic B-cell lymphocytic leukemia that interacts with the p50 and p52 NF-κB subunits and affects cell survival and proliferation.[89,90] In an effort to discover a direct small-molecule probe of pirin or pirin-associated proteins, Osada and coworkers screened SMMs containing more than 20,000 small molecules against mammalian cell lysates that expressed DSRed-fused pirin.[68] The screen was focused around a library of natural products and the molecules were immobilized on arrays using a photoaffinity strategy.[91] The screen yielded a small molecule named triphenyl compound A (TPh A) that bound to purified pirin with an affinity of 0.6 μM as measured by a secondary ITC assay (Fig. 6). A crystal structure of pirin complexed with TPh A revealed that the compound binds in a cavity with the metal binding site. TPh A is also capable of inhibiting the pirin-Bcl3 protein–protein interaction in vitro and in cells.

With a direct and functional small-molecule probe in hand, the authors proceeded to use the compound as a tool to understand the role of pirin by performing several phenotypic assays in the presence of compound. Interestingly, the compound inhibited melanoma cell migration, but not proliferation and downregulates *SNAI2* expression at 10 μM. This study provides a proof-of-concept for a primary binding screen involving SMMs probed with cell lysates that yielded a validated small-molecule probe for a protein of unknown function. The compound has already increased our knowledge about the role of pirin in cells.

## 5. Affinity selection-mass spectrometry

Affinity selection coupled to mass spectrometry (AS–MS) platforms allow the identification of protein–ligand interactions without the need for labels. During the affinity selection phase, the protein of interest is incubated with a mixture of small molecules. Small molecules that do not bind the protein target of interest are then washed away before the protein–ligand complex is analyzed by MS for ligand identification, either directly or indirectly, following a decomplexation step. A variety of AS–MS platforms for ligand discovery have been developed over the past decade and have been the subject of several excellent reviews.[92–94] These methods can be categorized in two groups, where the protein target is either immobilized on a solid support or free in solution (Fig. 7). While methods requiring immobilization of the protein target, such as frontal affinity chromatography–mass spectrometry (FAC–MS),[95–97] have proven useful in drug discovery, solution-based methods provide the advantage of probing the entire protein target in a biologically more relevant state. However, an extra step for the




**TPh A**
$K_D$ = 0.6 μM

**Figure 6.** Small-molecule antagonist of pirin discovered using SMMs.

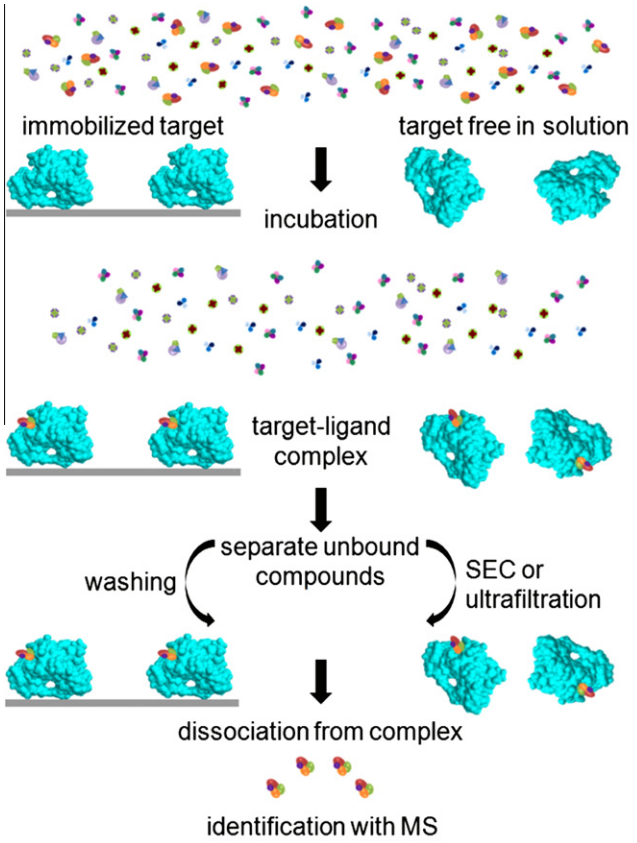



**Figure 7.** Approaches to affinity selection-mass spectrometry (AS–MS).

separation of unbound small molecules is necessary before MS analysis. This is typically achieved using size exclusion chromatography (SEC),[94,98] or by ultrafiltration.[99] Once freed from the unbound small-molecule pool, the protein–ligand complex is separated by denaturation of the protein using high temperature, organic solvents and/or by reverse-phase chromatography. MS then allows identifying the candidate binders.

The SpeedScreen platform was originally developed at Novartis as an approach to discover inhibitors of orphan genomic targets and protein targets that have proven intractable using conventional assays.[94] The automated 96-well assay format involves incubating the protein target, typically at a concentration of 2 μM, with pools containing 400 compounds. Each compound is screened at ~10 μM in aqueous media with 2–5% DMSO as a co-solvent. After the incubation phase, separation of the target protein from the small-molecule pool is performed using the SpeedScreen 'sandwich', consisting of an application plate, a size exclusion plate, and a collection plate. After a brief centrifugation step, the protein target is recovered in the collection plate. The protein–ligand complex is then subjected to reverse-phase HPLC at elevated temperature in order to separate the protein from the small-molecule binder. Data analysis is performed using software that compares the mass of the binder with the original pool of small molecules. A cycle time of 4 min/injection leads to the analysis of 144,000 potential binders/day (assuming 400 small molecules/well).

The automated ligand identification system (ALIS), originally developed at NeoGenesis, is a highly efficient solution-based AS–MS technique that has been used for small-molecule drug discovery.[98] The protein of interest is first incubated with a mixture of mass encoded compound library members (typically 2,500). After equilibrium is reached, SEC is used to separate unbound small molecules from the protein of interest. A UV detection system (230 nm) is used to monitor the protein and an automated valve system directs the protein peak toward a reverse-phase chromatography column where the protein–ligand complex is dissociated. Elution of eventual ligands into an ESI–MS system allows identification of putative small-molecule binders of the protein target.

The ALIS approach has been used to discover inhibitors for a wide range of targets. For example, ALIS has been used to discover novel ligands of GPCRs such as the muscarinic $M_2$ acetylcholine receptor.[100] New ligands of this GPCR were identified and a mechanistic study was performed using ALIS.

The use of known orthosteric site antagonists in competition experiments demonstrated that NGD-3350 is an orthosteric antagonist of $M_2$ and NGD-3366 is an allosteric ligand of $M_2$ (Fig. 8). This study highlights the strength of the ALIS platform both for discovering new ligands but also to better understand their mode of action. The ALIS platform has also been used to look for inhibitors of numerous enzymes, including an *Escherichia coli* dihydrofolate reductase,[101] the β-secretase enzyme BACE-1[102] implicated in Alzheimer's disease, the lipid phosphatase SHIP2,[103] a potential target in the treatment of type 2 diabetes, and the kinesin spindle protein ATPase (KSP).[104] A non-ATP competitive inhibitor of the MK2 kinase was recently discovered using ALIS.[105] The binding mode was confirmed using saturation transfer difference NMR and medicinal chemistry efforts led to the development of a low nanomolar inhibitor of MK2. This compound only inhibited one other kinase out of a panel of 150 and clearly shows the advantage of developing non-ATP competitive inhibitors of kinases. Another advantage of ALIS is the possibility to accurately determine protein–ligand binding affinity ($K_D$), allowing ranking of ligands and thus facilitating triage of a list of hit compounds.[106–108]

Abbott's ultrafiltration-based ufAS–MS method is also an efficient approach to discovering new protein–ligand interactions.[109] In 2004, Qian et al. described using FP and AS-MS as complementary methods to screen for inhibitors of Bcl-xL.[110] Two new classes of micromolar Bcl-xL inhibitors were identified by both methods and their binding was confirmed using NMR. In 2006, Comess et al. also reported the identification of ATP-competitive inhibitors of the DNA damage checkpoint kinase Chk1 using AS-MS.[111] A triazolopyridazine compound was found to be active in both the kinase assay and in the Chk1-dependent H1299 cell line.

More recently, Comess and colleagues at Abbott reported on the discovery of non-ATP competitive inhibitors of the mitogen activated protein kinases (MAPK), Jnk-1 and p38.[99] In the AS-MS screen, the kinase was incubated with large mixtures of compounds (500,000 small molecules in total) and non-binders were removed from the desired protein–ligand complex using ultrafiltration. After a chemical extraction step, MS analysis was performed to identify candidate binders. Of 68 candidate binders to Jnk-1, 41 were confirmed using NMR. The majority of these compounds bound to the ATP-site with two compounds showing binding to an allosteric site (Compound **3**, Fig. 8). Although the allosteric compounds were found to be inactive in a Jnk-1 kinase assay, they inhibited phosphorylation of Jnk-1 by MKK7, thus preventing activation of Jnk-1 and inhibiting the MAPK activation cascade. X-ray crystallography studies revealed that the compounds bind to the MAP insert region of Jnk-1. This example shows how one can identify a kinase inhibitor without necessarily inhibiting its kinase activity, but rather by inhibiting its activation, something that may not be measured using a conventional biochemical assay. The p38 kinase used for AS–MS screen was partially phosphorylated, thus partially activated, in order to look for potential binders of all possible forms of p38. After validation of the target using known inhibitors, 520,000 compounds were screened and 145 candidate binders were identified, containing a large number of known ATP-competitive p38 chemotypes. A selected set of 68 compounds was characterized for their binding mode using

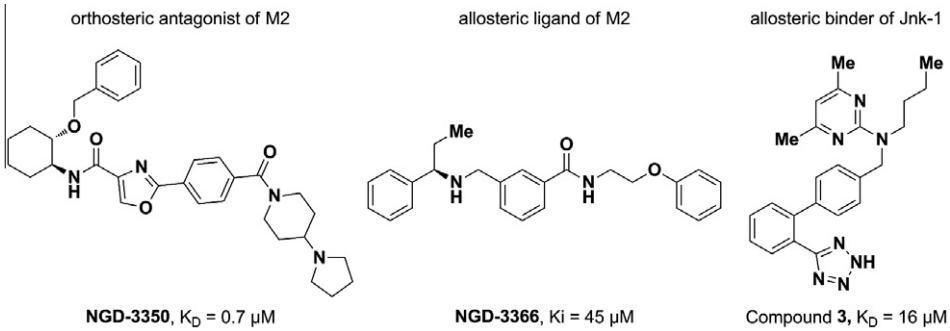



**Figure 8.** Representative small molecules discovered using AS–MS approaches.

two-dimensional $^{13}C$–1H NMR. While the majority of confirmed ligands bound at the ATP-site, 2 compounds bound p38 outside of the ATP-site. Unlike the Jnk-1 allosteric inhibitor, these compounds inhibit p38 kinase activity with low micromolar $IC_{50}$. They also inhibit phosphorylation of p38 by MKK6 and phosphorylation of MK2 by p38 with similar potency to the p38 alone. Selectivity profiling also showed that the molecules were inactive against highly related isoforms of p38α: the p38β, p38γ and p38δ kinases, and inhibited only 6 other kinases on a panel of 129. These examples illustrate the high impact role of AS–MS in identifying ligands with novel modes of action in an unbiased format, and provide excellent starting points for further medicinal chemistry efforts.

## 6. DNA-encoded libraries

Brenner and Lerner introduced the concept of DNA-encoded libraries (DELs) in 1992[112] and the idea matured into a powerful strategy for drug discovery over the past decade. Building DELs relies on the use of DNA either as a barcode to record the synthetic history of each member of the library (DNA-recorded synthesis) or as a template to drive chemical reactions (DNA-templated synthesis) (Fig. 9).[113–116] After synthesis, the library can be interrogated with a protein of interest using several rounds of affinity selection. Small-molecule binders are then easily identified using their DNA tag through amplification and sequencing.

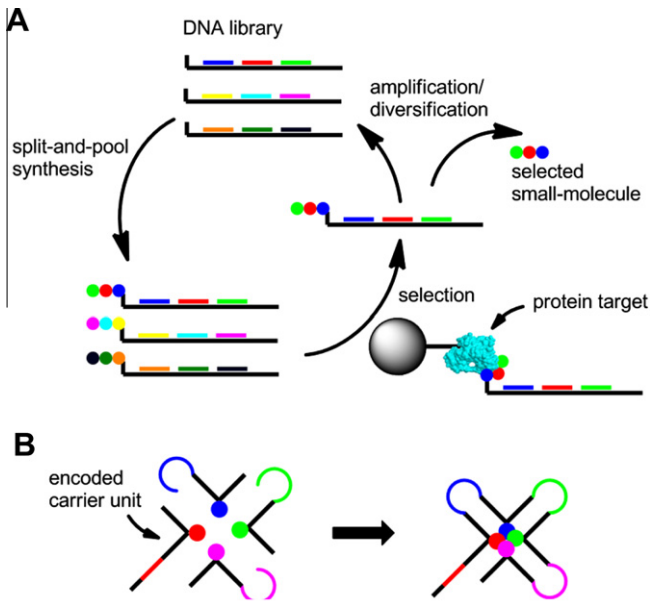



**Figure 9.** DNA-encoded libraries (DELs) built using DNA as a barcode to record synthetic history (**A**) or as a template to drive synthetic reactions (Vipergen Yoctoreactor, **B**).

Liu and coworkers provided proof-of-concept for DEL selections in 2003 using known small molecules that bind glutathione *S*-transferase, carbonic anhydrase and streptavidin, among others.[117] The group pioneered DNA-templated synthesis, first demonstrating feasibility of multi-step DNA-driven library synthesis,[118,119] and explored a wide range of aqueous-compatible organic reactions in the context of DNA-templated synthesis.[120–122] Using this strategy, Liu and colleagues recently reported the synthesis of a 13,000-member library of macrocycles of four different ring sizes based mainly on amide-bond coupling reactions and Wittig olefinations.[123] They later reported on affinity selections against the library using a panel of 36 different protein targets, which led to the identification of a highly selective submicromolar inhibitor of Src kinase (*trans*-A10-B1-C5-D6, Fig. 10).[124] Based on the DNA-templated approach, Ensemble Therapeutics has developed a library of around three million macrocycles for use in selections to identify inhibitors of protein–protein interactions in the fields of oncology, diabetes, inflammation and pain.[125] The work of Vipergen ApS is also of great interest in the field of DNA-templated synthesis.[126] The Vipergen YoctoReactor® technology platform allows encoded reactants to come into close proximity and drive the reactions to completion. Its compatibility with high temperature and organic solvents also extend the range of possible chemistries and thus library sizes.

Harbury and coworkers used a split and pool strategy coupled to solid-phase chemistry and employed non-encoded chemical building blocks, thus simplifying the generation of large libraries.[127–129] Using this strategy, they prepared a 100 million-member library of 8-mer peptoids based on amide bond coupling and nucleophilic substitutions. The library construct is made of a 340-mer ssDNA possessing 8 coding regions (one for each building block of the 8-mer peptoid final product), each having 10 possible sequences (coding for 10 potential building blocks at each position of the peptoid) which allows for the encoding of a $10^8$-member library.[130] This library has been successfully subjected to affinity selection for the N-terminal SH3 domain of the proto-oncogene Crk and led to the identification of a low micromolar binder ($K_D$ = 16 μM).

In Harbury's approach, the DNA barcode is preinstalled and also serves as a handle for the split and pool strategy. An alternative approach consists in introducing the DNA barcode during the stepwise synthesis of the library. Neri and colleagues have reported several DELs[131–134] and recently described the preparation of a 100 million-member library based on amide bond coupling reactions and Diels–Alder cycloadditions.[135] This library was interrogated against carbonic anhydrase IX and led to the identification of a new bis-sulfonamide inhibitor with a submicromolar $IC_{50}$ that also showed activity in hypoxic tumors in vivo.

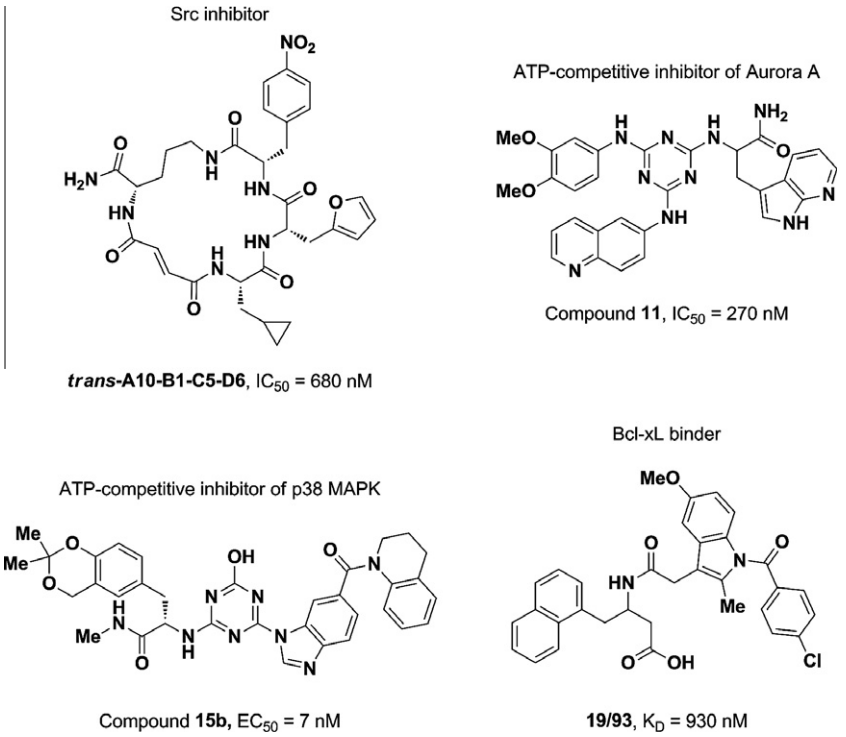



**Figure 10.** Representative small molecules discovered using selection strategies involving DNA-encoded libraries (DELs).

Morgan and coworkers at Praecis Pharmaceuticals (now part of GlaxoSmithKline) recently published an 800 million-member library, the largest ever reported to date.[136] Uniquely, the GSK group used dsDNA to record the stepwise synthesis (Fig. 11) in an effort to protect the heterocyclic bases within the helix and limit chemical alterations of the DNA during the library synthesis. A short DNA duplex 'headpiece' is first functionalized with a spacer (Fmoc-15-amino-4,7,10,13-tetraoxapentadecanoic acid). After Fmoc removal, the amino group serves as the starting point for the small-molecule library synthesis. The non-linked DNA side of the headpiece contains a 2-base 3' overhang, which is used as the substrate for further ligation of the coding tags. The coding tags used to record the synthetic history are 7-base dsDNA sequences containing a unique 3' overhang to ensure that each set of tags can only ligate to the preceding cycle's tag.

After enzymatic tagging, the free amino group of the spacer is acylated with a library of 192 Fmoc-protected amino acids. Removal of the Fmoc protecting group is followed by a second enzymatic tagging, installation of the activated triazine core and SNAr using 32 amino acids or hydroxy acids. A third DNA tag is then installed and followed by a second SNAr reaction using 340 amines. Finally, a fourth DNA tag is introduced and amide bond coupling reactions are performed using 384 amines. After purification and primer ligation, an 802,160,640-member DEL was obtained. As a proof of concept, VX-680, a known Aurora A kinase inhibitor was functionalized with a similar DNA barcode, included into a smaller

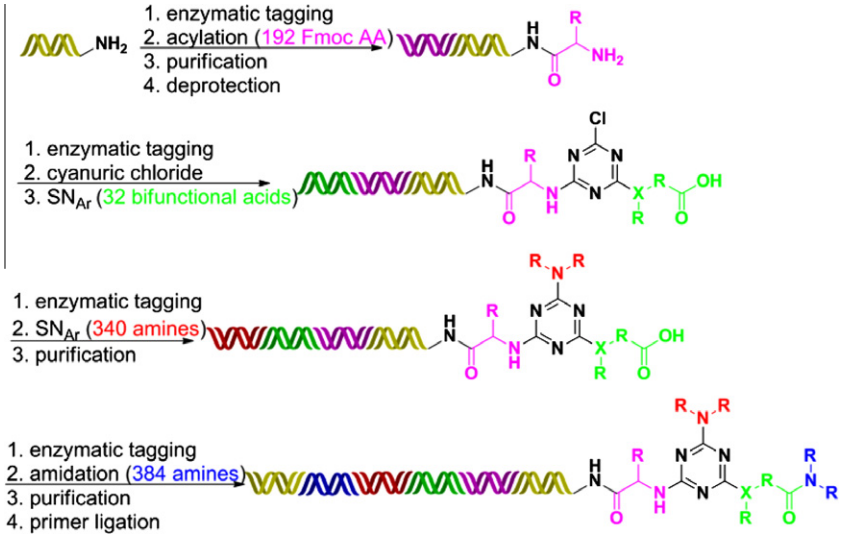



**Figure 11.** Stepwise DNA-encoded library (DEL) synthesis leading to an 800-million member library.[136]

7 million-member library, and affinity selection was performed against Aurora A. After 3 rounds of selection, a 100,000-fold enrichment of the VX-680 DNA tag was observed, validating the method. Affinity selection performed on Aurora A and p38 MAPK, led to the discovery of low nanomolar ATP-competitive inhibitors (Compound **11**, Fig. 10). The first scaffold that was found to bind to Aurora A contained a 6-aminoquinoline as the cycle 2 synthon and the second scaffold contained a 7-azatryptophan as the cycle 1 synthon. In order to elucidate the binding mode of these novel binders, the ATP-competitive inhibitor VX-680 was added to the buffer and the affinity selection was repeated. Disappearance of the previously selected scaffolds suggested that they interact with the ATP-binding site. The smaller 7 million-member library, in which 3-amino-4-methyl-N-methoxybenzamide (AMMB) was included in cycle 2 as a known pharmacophore fragment for p38 MAPK, was also screened against that kinase. As expected, the hits were strongly enriched in structures containing the AMMB synthon. The p38 MAPK was then screened against the 800 million-member library and yielded a family containing a benzimidazole-5-carboxylate at the privileged cycle 2 synthon. Resynthesis of a representative compound afforded a strong p38 MAPK inhibitor. The cycle 3 amine synthon was found to have very low influence on the affinity for p38 MAPK. Several analogs lacking this synthon were low nanomolar inhibitors of p38 MAPK (Compound **15b**, Fig. 10). This example illustrates the advantage of the built-in SAR of these massive libraries.

While most published DEL examples involved druggable targets, recent work in the Neri group demonstrates that small DELs consisting of roughly 4000 compounds can afford potent binders of challenging proteins such as the anti-apoptotic protein Bcl-xL.[137] The identified hit compound was able to compete with a Bak-BH3 peptide, a proapoptotic antagonist of Bcl-xL (19/93, Fig. 10). Although it's not clear how useful this approach may be for selected DNA-binding proteins, it is likely that more examples of small-molecule probes of challenging targets will be enabled by DEL-based approaches as this method gains popularity. The extraordinary development of DNA sequencing technologies should also enable further expansion of DEL technology utility in drug discovery.

## 7. Concluding remarks

As outlined in this review, unbiased binding assays using a variety of technology platforms enable probe discovery for both conventional and challenging targets. Importantly, binding-based approaches provide a path forward to discover and develop probes for proteins of unknown function that are associated with disease. Each of the technologies presented in this review may be more or less appropriate for a given target depending on the availability and stability of the protein or the tendency to engage in multiprotein complexes. Many of the assay formats described here involve a trade-off between quantitative information and throughput in the primary assay. For example, SPR provides high-value kinetic information about binding but the throughput of the assay is relatively low in comparison to some of the other methods. Conversely, the SMM format has a higher throughput but typically is not used as a quantitative assay. Emerging technologies that combine the throughput of the microarray format with a kinetic readout may improve prioritization of assay positives from the primary screen.[138,139] Efforts are also underway to increase the throughput of other 'gold-standard' calorimetric methods so that they may prove useful in primary assays.[29,140] Although advanced knowledge of protein structure is required, NMR-based screening methods have also witnessed recent increases in throughput and have proven very useful in direct or competitive assays for fragment-based screens.[141,142] Finally, coupling improvements in sensitivity of biophysical methods with advances in microfluidics may also reduce sample requirements for both the target and small molecules.[143–145] All of these emerging technologies are likely to make general binding assays more attractive as an initial step in probe and drug discovery.

## References and notes


1. Lander, E. S. *Nature (London U.K.)* **2011**, *470*, 187–197.
2. Edwards, A. M.; Isserlin, R.; Bader, G. D.; Frye, S. V.; Willson, T. M.; Yu, F. H. *Nature (London U.K.)* **2011**, *470*, 163.
3. Schreiber, S. L. *Proc. Natl. Acad. Sci. U. S. A.* **2011**, *108*, 6699.
4. Koehler, A. N. *Curr. Opin. Chem. Biol.* **2010**, *14*, 331.
5. Stanton, B. Z.; Peng, L. F.; Maloof, N.; Nakai, K.; Wang, X.; Duffner, J. L.; Taveras, K. M.; Hyman, J. M.; Lee, S. W.; Koehler, A. N.; Chen, J. K.; Fox, J. L.; Mandinova, A.; Schreiber, S. L. *Nat. Chem. Biol.* **2009**, *5*, 154.
6. Berg, T. *Curr. Opin. Drug Discovery Dev.* **2008**, *11*, 666.
7. Frye, S. V. *Nat. Chem. Biol.* **2010**, 6.
8. Kodadek, T. *Nat. Chem. Biol.* **2010**, *6*, 162.
9. Schreiber, S. L. *Bioorg. Med. Chem.* **1998**, *6*, 1127.
10. Stockwell, B. R. *Nat. Rev. Genet.* **2000**, *1*, 116.
11. Erkizan, H. V.; Kong, Y.; Merchant, M.; Schlottmann, S.; Barber-Rotenberg, J. S.; Yuan, L.; Abaan, O. D.; Chou, T.-H.; Dakshanamurthy, S.; Brown, M. L.; Uren, A.; Toretsky, J. A. *Nat. Med. (N. Y., NY, U. S.)* **2009**, *15*, 750.
12. Rahim, S.; Beauchamp, E. M.; Kong, Y.; Brown, M. L.; Toretsky, J. A.; Uren, A. *PLoS One* **2011**, *6*, e19343.
13. Coyne, A. G.; Scott, D. E.; Abell, C. *Curr. Opin. Chem. Biol.* **2010**, *14*, 299.
14. Vedadi, M.; Arrowsmith, C. H.; Allali-Hassani, A.; Senisterra, G.; Wasney, G. A. *J. Struct. Biol.* **2010**, *172*, 107.
15. Vedadi, M.; Niesen, F. H.; Allali-Hassani, A.; Fedorov, O. Y.; Finerty, P. J., Jr.; Wasney, G. A.; Yeung, R.; Arrowsmith, C.; Ball, L. J.; Berglund, H.; Hui, R.; Marsden, B. D.; Nordlund, P.; Sundstrom, M.; Weigelt, J.; Edwards, A. M. *Proc. Natl. Acad. Sci. U. S. A.* **2006**, *103*, 15835.
16. Brandts, J. F.; Lin, L. N. *Biochemistry* **1990**, *29*, 6927.
17. Pace, C. N.; McGrath, T. *J. Biol. Chem.* **1980**, *255*, 3862.
18. Schellman, J. A. *Biopolymers* **1975**, *14*, 999.
19. Straume, M.; Freire, E. *Anal. Biochem.* **1992**, *203*, 259.
20. Pantoliano, M. W.; Petrella, E. C.; Kwasnoski, J. D.; Lobanov, V. S.; Myslik, J.; Graf, E.; Carver, T.; Asel, E.; Springer, B. A.; Lane, P.; Salemme, F. R. *J. Biomol. Screening* **2001**, *6*, 429.
21. Bobkova, E. V.; Weber, M. J.; Xu, Z.; Zhang, Y.-L.; Jung, J.; Blume-Jensen, P.; Northrup, A.; Kunapuli, P.; Andersen, J. N.; Kariv, I. *J. Biol. Chem.* **2010**, *285*, 18838.
22. Wan, K. F.; Wang, S.; Brown, C. J.; Yu, V. C.; Entzeroth, M.; Lane, D. P.; Lee, M. A. *Cell Cycle* **2009**, *8*, 3943.
23. Matulis, D.; Kranz, J. K.; Salemme, F. R.; Todd, M. J. *Biochemistry* **2005**, *44*, 5258.
24. Pantoliano, M. W.; Rhind, A. W.; Salemme, F. R.; Springer, B. A.; Bone, R. F.; Petrella, E. C. **1997**, 1997-US8154, 9742500.
25. Buchner, J.; Schmidt, M.; Fuchs, M.; Jaenicke, R.; Rudolph, R.; Schmid, F. X.; Kiefhaber, T. *Biochemistry* **1991**, *30*, 1586.
26. Senisterra, G. A.; Markin, E.; Yamazaki, K.; Hui, R.; Vedadi, M.; Awrey, D. E. *J. Biomol. Screening* **2006**, *11*, 940.
27. Zhu, Z.; Cuozzo, J. *J. Biomol. Screening* **2009**, *14*, 1157.
28. Filippakopoulos, P.; Qi, J.; Picaud, S.; Shen, Y.; Smith, W. B.; Fedorov, O.; Morse, E. M.; Keates, T.; Hickman, T. T.; Felletar, I.; Philpott, M.; Munro, S.; McKeown, M. R.; Wang, Y.; Christie, A. L.; West, N.; Cameron, M. J.; Schwartz, B.; Heightman, T. D.; La Thangue, N.; French, C.; Wiest, O.; Kung, A. L.; Knapp, S.; Bradner, J. E. *Nature (London, U.K.)* **2010**, 468.
29. Chaires, J. B. *Annu. Rev. Biophys.* **2008**, *37*, 135.
30. Carver, T. E.; Bordeau, B.; Cummings, M. D.; Petrella, E. C.; Pucci, M. J.; Zawadzke, L. E.; Dougherty, B. A.; Tredup, J. A.; Bryson, J. W.; Yanchunas, J., Jr.; Doyle, M. L.; Witmer, M. R.; Nelen, M. I.; DesJarlais, R. L.; Jaeger, E. P.; Devine, H.; Asel, E. D.; Springer, B. A.; Bone, R.; Salemme, F. R.; Todd, M. J. *J. Biol. Chem.* **2005**, *280*, 11704.
31. Levine, A. J. *Cell (Cambridge, Mass.)* **1997**, *88*, 323.
32. Soussi, T.; Dehouche, K.; Beroud, C. *Hum. Mutat.* **2000**, *15*, 105.
33. Iwakuma, T.; Lozano, G. *Mol. Cancer Res.* **2003**, *1*, 993.
34. Grasberger, B. L.; Lu, T.; Schubert, C.; Parks, D. J.; Carver, T. E.; Koblish, H. K.; Cummings, M. D.; LaFrance, L. V.; Milkiewicz, K. L.; Calvo, R. R.; Maguire, D.; Lattanze, J.; Franks, C. F.; Zhao, S.; Ramachandren, K.; Bylebyl, G. R.; Zhang, M.; Manthey, C. L.; Petrella, E. C.; Pantoliano, M. W.; Deckman, I. C.; Spurlino, J. C.; Maroney, A. C.; Tomczuk, B. E.; Molloy, C. J.; Bone, R. F. *J. Med. Chem.* **2005**, *48*, 909.
35. Parks, D. J.; LaFrance, L. V.; Calvo, R. R.; Milkiewicz, K. L.; Gupta, V.; Lattanze, J.; Ramachandren, K.; Carver, T. E.; Petrella, E. C.; Cummings, M. D.; Maguire, D.; Grasberger, B. L.; Lu, T. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 765.
36. Bottger, A.; Bottger, V.; Garcia-Echeverria, C.; Chene, P.; Hochkeppel, H. K.; Sampson, W.; Ang, K.; Howard, S. F.; Picksley, S. M.; Lane, D. P. *J. Mol. Biol.* **1997**, *269*, 744.
37. Bottger, V.; Bottger, A.; Howard, S. F.; Picksley, S. M.; Chene, P.; Garcia-Echeverria, C.; Hochkeppel, H. K.; Lane, D. P. *Oncogene* **1996**, *13*, 2141.

38. Parks, D. J.; LaFrance, L. V.; Calvo, R. R.; Milkiewicz, K. L.; Jose Marugan, J.; Raboisson, P.; Schubert, C.; Koblish, H. K.; Zhao, S.; Franks, C. F.; Lattanze, J.; Carver, T. E.; Cummings, M. D.; Maguire, D.; Grasberger, B. L.; Maroney, A. C.; Lu, T. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 3310.
39. Rich, R. L.; Myszka, D. G. *J. Mol. Recognit.* **2010**, *23*, 1.
40. Homola, J. *Chem. Rev. (Washington, DC, U. S.)* **2008**, *108*, 462.
41. Mitchell, J. *Sensors* **2010**, *10*, 7323.
42. Faegerstam, L. G.; Frostell-Karlsson, A.; Karlsson, R.; Persson, B.; Roennberg, I. *J. Chromatogr.* **1992**, *597*, 397.
43. Myszka, D. G. *Curr. Opin. Biotechnol.* **1997**, *8*, 50.
44. Myszka, D. G.; Rich, R. L. *Pharm. Sci. Technol. Today* **2000**, *3*, 310.
45. Campbell, C. T.; Kim, G. *Biomaterials* **2007**, *28*, 2380.
46. Situ, C.; Buijs, J.; Mooney, M. H.; Elliot, C. T. *TrAC, Trends Anal. Chem.* **2010**, *29*, 1305.
47. Boozer, C.; Kim, G.; Cong, S.; Guan, H.; Londergan, T. *Curr. Opin. Biotechnol.* **2006**, *17*, 400.
48. Neumann, T.; Junker, H. D.; Schmidt, K.; Sekul, R. *Curr. Top. Med. Chem. (Sharjah, United Arab Emirates)* **2007**, *7*, 1630.
49. Dickopf, S.; Frank, M.; Junker, H.-D.; Maier, S.; Metz, G.; Ottleben, H.; Rau, H.; Schellhaas, N.; Schmidt, K.; Sekul, R.; Vanier, C.; Vetter, D.; Czech, J.; Lorenz, M.; Matter, H.; Schudok, M.; Schreuder, H.; Will, D. W.; Nestler, H. P. *Anal. Biochem.* **2004**, *335*, 50.
50. Vetter, D. *J. Cell. Biochem.* **2002**, 79.
51. Delattre, O.; Zucman, J.; Melot, T.; Garau, X. S.; Zucker, J.-M.; Lenoir, G. M.; Ambros, P. F.; Sheer, D.; Turc-Carel, C., et al *N. Engl. J. Med.* **1994**, *331*, 294.
52. Im, Y.-H.; Kim, H. T.; Lee, C.; Poulin, D.; Welford, S.; Sorensen, P. H. B.; Denny, C. T.; Kim, S.-J. *Cancer Res.* **2000**, *60*, 1536.
53. Hu-Lieskovan, S.; Heidel, J. D.; Bartlett, D. W.; Davis, M. E.; Triche, T. J. *Cancer Res.* **2005**, *65*, 8984.
54. Toretsky, J. A.; Erkizan, V.; Levenson, A.; Abaan, O. D.; Parvin, J. D.; Cripe, T. P.; Rice, A. M.; Lee, S. B.; Ueren, A. *Cancer Res.* **2006**, *66*, 5574.
55. Wei, G.-H.; Badis, G.; Berger, M. F.; Kivioja, T.; Palin, K.; Enge, M.; Bonke, M.; Jolma, A.; Varjosalo, M.; Gehrke, A. R.; Yan, J.; Talukder, S.; Turunen, M.; Taipale, M.; Stunnenberg, H. G.; Ukkonen, E.; Hughes, T. R.; Bulyk, M. L.; Taipale, J. *EMBO J.* **2010**, *29*, 2147.
56. Seth, A.; Watson, D. K. *Eur. J. Cancer* **2005**, *41*, 2462.
57. Tomlins, S. A.; Laxman, B.; Dhanasekaran, S. M.; Helgeson, B. E.; Cao, X.; Morris, D. S.; Menon, A.; Jing, X.; Cao, Q.; Han, B.; Yu, J.; Wang, L.; Montie, J. E.; Rubin, M. A.; Pienta, K. J.; Roulston, D.; Shah, R. B.; Varambally, S.; Mehra, R.; Chinnaiyan, A. M. *Nature (London, UK)* **2007**, *448*, 595.
58. MacBeath, G.; Koehler, A. N.; Schreiber, S. L. *J. Am. Chem. Soc.* **1999**, *121*, 7967.
59. Uttamchandani, M.; Yao, S. Q.; Editors, *Small Molecule Microarrays: Methods And Protocols. [In: Methods Mol. Biol. (N.Y., NY, U.S.), 2010; 669]*. Springer: New York, NY, 2010; p 228.
60. Vegas, A. J.; Fuller, J. H.; Koehler, A. N. *Chem. Soc. Rev.* **2008**, *37*, 1385.
61. Bradner, J. E.; McPherson, O. M.; Koehler, A. N. *Nat. Protoc.* **2006**, *1*, 2344.
62. Galloway, W. R. J. D.; Isidro-Llobet, A.; Spring, D. R. *Nat. Commun.* **2010**, 1.
63. Schreiber, S. L. *Science (Washington, D.C.)* **2000**, 287.
64. Astle, J. M.; Simpson, L. S.; Huang, Y.; Reddy, M. M.; Wilson, R.; Connell, S.; Wilson, J.; Kodadek, T. *Chem. Biol. (Cambridge, MA, U.S.)* **2010**, *17*, 38.
65. Bradner, J. E.; McPherson, O. M.; Mazitschek, R.; Barnes-Seeman, D.; Shen, J. P.; Dhaliwal, J.; Stevenson, K. E.; Duffner, J. L.; Park, S. B.; Neuberg, D. S.; Nghiem, P.; Schreiber, S. L.; Koehler, A. N. *Chem. Biol. (Cambridge, MA, U. S.)* **2006**, *13*, 493.
66. Kanoh, N.; Asami, A.; Kawatani, M.; Honda, K.; Kumashiro, S.; Takayama, H.; Simizu, S.; Amemiya, T.; Kondoh, Y.; Hatakeyama, S.; Tsuganezawa, K.; Utata, R.; Tanaka, A.; Yokoyama, S.; Tashiro, H.; Osada, H. *Chem.-Asian J.* **2006**, *1*, 789.
67. Kanoh, N.; Kyo, M.; Inamori, K.; Ando, A.; Asami, A.; Nakao, A.; Osada, H. *Anal. Chem.* **2006**, *78*, 2226.
68. Miyazaki, I.; Simizu, S.; Okumura, H.; Takagi, S.; Osada, H. *Nat. Chem. Biol.* **2010**, *6*, 667.
69. Schmitz, K.; Haggarty, S. J.; McPherson, O. M.; Clardy, J.; Koehler, A. N. *J. Am. Chem. Soc.* **2007**, *129*, 11346.
70. Vegas, A. J.; Bradner, J. E.; Tang, W.; McPherson, O. M.; Greenberg, E. F.; Koehler, A. N.; Schreiber, S. L. *Angew. Chem., Int. Ed.* **2007**, *46*, 7960.
71. Ko, K.-S.; Jaipuri, F. A.; Pohl, N. L. *J. Am. Chem. Soc.* **2005**, 127.
72. Pohl, N. L. *Angew. Chem., Int. Ed.* **2008**, 47.
73. Marsden, D. M.; Nicholson, R. L.; Ladlow, M.; Spring, D. R., *Chem. Commun. (Cambridge, U. K.)* **2009.**
74. Marsden, D. M.; Nicholson, R. L.; Skindersoe, M. E.; Galloway, W. R. J. D.; Sore, H. F.; Givskov, M.; Salmond, G. P. C.; Ladlow, M.; Welch, M.; Spring, D. R. *Org. Biomol. Chem.* **2010**, 8.
75. Duffner, J. L.; Clemons, P. A.; Koehler, A. N. *Curr. Opin. Chem. Biol.* **2007**, *11*, 74.
76. Nicholson, R. L.; Welch, M.; Ladlow, M.; Spring, D. R. *ACS Chem. Biol.* **2007**, *2*, 24.
77. Winssinger, N.; Pianowski, Z.; Debaene, F. *Top. Curr. Chem.* **2007**, *278*, 311.
78. Kwon, S. J.; Lee, M.-Y.; Ku, B.; Sherman, D. H.; Dordick, J. S. *ACS Chem. Biol.* **2007**, *2*, 419.
79. Miao, H.; Tallarico, J. A.; Hayakawa, H.; Muenger, K.; Duffner, J. L.; Koehler, A. N.; Schreiber, S. L.; Lewis, T. A. *J. Comb. Chem.* **2007**, *9*, 245.
80. Urbina, H. D.; Debaene, F.; Jost, B.; Bole-Feysot, C.; Mason, D. E.; Kuzmic, P.; Harris, J. L.; Winssinger, N. *ChemBioChem* **2006**, *7*, 1790.
81. Uttamchandani, M.; Lee, W. L.; Wang, J.; Yao, S. Q. *J. Am. Chem. Soc.* **2007**, *129*, 13110.
82. Kemp, M. M.; Wang, Q.; Fuller, J. H.; West, N.; Martinez, N. M.; Morse, E. M.; Weiwer, M.; Schreiber, S. L.; Bradner, J. E.; Koehler, A. N. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 4164.
83. Clemons, P. A.; Bodycombe, N. E.; Carrinski, H. A.; Wilson, J. A.; Shamji, A. F.; Wagner, B. K.; Koehler, A. N.; Schreiber, S. L. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107*, 18787.
84. Koehler, A. N.; Shamji, A. F.; Schreiber, S. L. *J. Am. Chem. Soc.* **2003**, *125*, 8420.
85. Kuruvilla, F. G.; Shamji, A. F.; Sternson, S. M.; Hergenrother, P. J.; Schreiber, S. L. *Nature (London, U. K.)* **2002**, *416*, 653.
86. Chen, J.; Armstrong, A. H.; Koehler, A. N.; Hecht, M. H. *J. Am. Chem. Soc.* **2010**, *132*, 17015.
87. Bergman, A. C.; Alaiya, A. A.; Wendler, W.; Binetruy, B.; Shoshan, M.; Sakaguchi, K.; Bergman, T.; Kronenwett, U.; Auer, G.; Appella, E.; Joernvall, H.; Linder, S. *Cell. Mol. Life Sci.* **1999**, *55*, 467.
88. Wendler, W. M. F.; Kremmer, E.; Foerster, R.; Winnacker, E.-L. *J. Biol. Chem.* **1997**, *272*, 8482.
89. Courtois, G.; Gilmore, T. D. *Oncogene* **2006**, *25*, 6831.
90. Dechend, R.; Hirano, F.; Lehmann, K.; Heissmeyer, V.; Ansieau, S.; Wulczyn, F. G.; Scheidereit, C.; Leutz, A. *Oncogene* **1999**, *18*, 3316.
91. Kanoh, N.; Kumashiro, S.; Simizu, S.; Kondoh, Y.; Hatakeyama, S.; Tashiro, H.; Osada, H. *Angew. Chem., Int. Ed.* **2003**, *42*, 5584.
92. Annis, D. A.; Nickbarg, E.; Yang, X.; Ziebell, M. R.; Whitehurst, C. E. *Curr. Opin. Chem. Biol.* **2007**, *11*, 518.
93. Jonker, N.; Kool, J.; Irth, H.; Niessen, W. M. A. *Anal. Bioanal. Chem.* **2011**, *399*, 2669.
94. Zehender, H.; Mayr, L. M. *Expert Opin. Drug Discovery* **2007**, *2*, 285.
95. Calleri, E.; Ceruti, S.; Cristalli, G.; Martini, C.; Temporini, C.; Parravicini, C.; Volpini, R.; Daniele, S.; Caccialanza, G.; Lecca, D.; Lambertucci, C.; Trincavelli, M. L.; Marucci, G.; Wainer, I. W.; Ranghino, G.; Fantucci, P.; Abbracchio, M. P.; Massolini, G. *J. Med. Chem.* **2010**, *53*, 3489.
96. Chan, N.; Lewis, D.; Kelly, M.; Ng, E. S. M.; Schriemer, D. C. *Methods Princ. Med. Chem.* **2007**, *36*, 217.
97. Ng, E. S. M.; Chan, N. W. C.; Lewis, D. F.; Hindsgaul, O.; Schriemer, D. C. *Nat. Protoc.* **2007**, *2*, 1907.
98. Annis, A.; Chuang, C. C.; Nazef, N. ALIS: An affinity selection-mass spectrometry system for the discovery and characterization of protein–ligand interactions. In *Methods and Principles in Medicinal Chemistry*; Mannhold, R., Kubinyi, H., Folkers, G., Eds.; Wiley-VCH, 2007; pp 121–184.
99. Comess, K. M.; Sun, C.; Abad-Zapatero, C.; Goedken, E. R.; Gum, R. J.; Borhani, D. W.; Argiriadi, M.; Groebe, D. R.; Jia, Y.; Clampit, J. E.; Haasch, D. L.; Smith, H. T.; Wang, S.; Song, D.; Coen, M. L.; Cloutier, T. E.; Tang, H.; Cheng, X.; Quinn, C.; Liu, B.; Xin, Z.; Liu, G.; Fry, E. H.; Stoll, V.; Ng, T. I.; Banach, D.; Marcotte, D.; Burns, D. J.; Calderwood, D. J.; Hajduk, P. J. *ACS Chem. Biol.* **2011**, *6*, 234.
100. Whitehurst, C. E.; Annis, D. A. *Comb. Chem. High Throughput Screening* **2008**, *11*, 427.
101. Annis, D. A.; Athanasopoulos, J.; Curran, P. J.; Felsch, J. S.; Kalghatgi, K.; Lee, W. H.; Nash, H. M.; Orminati, J.-P. A.; Rosner, K. E.; Shipps, G. W.; Thaddupathy, G. R. A.; Tyler, A. N.; Vilenchik, L.; Wagner, C. R.; Wintner, E. A. *Int. J. Mass Spectrom.* **2004**, *238*, 77.
102. Coburn, C. A.; Stachel, S. J.; Li, Y.-M.; Rush, D. M.; Steele, T. G.; Chen-Dodson, E.; Holloway, M. K.; Xu, M.; Huang, Q.; Lai, M.-T.; DiMuzio, J.; Crouthamel, M.-C.; Shi, X.-P.; Sardana, V.; Chen, Z.; Munshi, S.; Kuo, L.; Makara, G. M.; Annis, D. A.; Tadikonda, P. K.; Nash, H. M.; Vacca, J. P. *J. Med. Chem.* **2004**, *47*, 6117.
103. Annis, D. A.; Cheng, C. C.; Chuang, C.-C.; McCarter, J. D.; Nash, H. M.; Nazef, N.; Rowe, T.; Kurzeja, R. J. M.; Shipps, G. W., Jr. *Comb. Chem. High Throughput Screening* **2009**, *12*, 760.
104. Sheth, P. R.; Shipps, G. W., Jr.; Seghezzi, W.; Smith, C. K.; Chuang, C.-C.; Sanden, D.; Basso, A. D.; Vilenchik, L.; Gray, K.; Annis, D. A.; Nickbarg, E.; Ma, Y.; Lahue, B.; Herbst, R.; Le, H. V. *Biochemistry* **2010**, *49*, 8350.
105. Huang, X.; Shipps, G. W.; Cheng, C. C.; Spacciapoli, P.; Zhang, X.; McCoy, M. A.; Wyss, D. F.; Yang, X.; Achab, A.; Soucy, K.; Montavon, D. K.; Murphy, D. M.; Whitehurst, C. E. *ACS Med. Chem. Lett.* **2011**, *2*, 632.
106. Adam, G. C.; Meng, J.; Athanasopoulos, J.; Zhang, X.; Chapman, K. T. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 2404.
107. Annis, D. A.; Nazef, N.; Chuang, C.-C.; Scott, M. P.; Nash, H. M. *J. Am. Chem. Soc.* **2004**, *126*, 15495.
108. Annis, D. A.; Shipps, G. W., Jr.; Deng, Y.; Popovici-Mueller, J.; Siddiqui, M. A.; Curran, P. J.; Gowen, M.; Windsor, W. T. *Anal. Chem. (Washington, DC, U. S.)* **2007**, *79*, 4538.
109. Kawai, M.; BaMaung, N. Y.; Fidanze, S. D.; Erickson, S. A.; Tedrow, J. S.; Sanders, W. J.; Vasudevan, A.; Park, C.; Hutchins, C.; Comess, K. M.; Kalvin, D.; Wang, J.; Zhang, Q.; Lou, P.; Tucker-Garcia, L.; Bouska, J.; Bell, R. L.; Lesniewski, R.; Henkin, J.; Sheppard, G. S. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 3574.
110. Qian, J.; Voorbach, M. J.; Huth, J. R.; Coen, M. L.; Zhang, H.; Ng, S.-C.; Comess, K. M.; Petros, A. M.; Rosenberg, S. H.; Warrior, U.; Burns, D. J. *Anal. Biochem.* **2004**, *328*, 131.
111. Comess, K. M.; Trumbull, J. D.; Park, C.; Chen, Z.; Judge, R. A.; Voorbach, M. J.; Coen, M.; Gao, L.; Tang, H.; Kovar, P.; Cheng, X.; Schurdak, M. E.; Zhang, H.; Sowin, T.; Burns, D. J. *J. Biomol. Screening* **2006**, *11*, 755.
112. Brenner, S.; Lerner, R. A. *Proc. Natl. Acad. Sci. U. S. A.* **1992**, *89*, 5381.
113. Clark, M. A. *Curr. Opin. Chem. Biol.* **2010**, *14*, 396.
114. Kleiner, R. E.; Dumelin, C. E.; Liu, D. R., *Chemical Society Review* **2011.**
115. Melkko, S.; Dumelin, C. E.; Scheuermann, J.; Neri, D. *Drug Discovery Today* **2007**, *12*, 465.
116. Scheuermann, J.; Neri, D. *ChemBioChem* **2010**, *11*, 931.
117. Doyon, J. B.; Snyder, T. M.; Liu, D. R. *J. Am. Chem. Soc.* **2003**, *125*, 12372.
118. Gartner, Z. J.; Kanan, M. W.; Liu, D. R. *J. Am. Chem. Soc.* **2002**, *124*, 10304.

119. Gartner, Z. J.; Tse, B. N.; Grubina, R.; Doyon, J. B.; Snyder, T. M.; Liu, D. R. *Science (Washington, DC, U. S.)* **2004**, *305*, 1601.
120. Chen, Y.; Kamlet, A. S.; Steinman, J. B.; Liu, D. R. *Nat. Chem.* **2011**, *3*, 146.
121. Gartner, Z. J.; Kanan, M. W.; Liu, D. R. *Angew. Chem., Int. Ed.* **2002**, *41*, 1796.
122. Rozenman, M. M.; Kanan, M. W.; Liu, D. R. *J. Am. Chem. Soc.* **2007**, *129*, 14933.
123. Tse, B. N.; Snyder, T. M.; Shen, Y.; Liu, D. R. *J. Am. Chem. Soc.* **2008**, *130*, 15611.
124. Kleiner, R. E.; Dumelin, C. E.; Tiu, G. C.; Sakurai, K.; Liu, D. R. *J. Am. Chem. Soc.* **2010**, *132*, 11779.
125. Ensemble Therapeutics, Ensemble Pipeline. 2010. http://www.ensembletx.com/pipeline/index.html.
126. Hansen, M. H.; Blakskjaer, P.; Petersen, L. K.; Hansen, T. H.; Hoejfeldt, J. W.; Gothelf, K. V.; Hansen, N. J. V. *J. Am. Chem. Soc.* **2009**, *131*, 1322.
127. Halpin, D. R.; Harbury, P. B. *PLoS Biol.* **2004**, *2*, 1022.
128. Halpin, D. R.; Harbury, P. B. *PLoS Biol.* **2004**, *2*, 1015.
129. Halpin, D. R.; Lee, J. A.; Wrenn, S. J.; Harbury, P. B. *PLoS Biol.* **2004**, *2*, 1031.
130. Wrenn, S. J.; Weisinger, R. M.; Halpin, D. R.; Harbury, P. B. *J. Am. Chem. Soc.* **2007**, *129*, 13137.
131. Buller, F.; Zhang, Y.; Scheuermann, J.; Schafer, J.; Buhlmann, P.; Neri, D. *Chem. Biol. (Cambridge, MA, U. S.)* **2009**, *16*, 1075.
132. Dumelin, C. E.; Truessel, S.; Buller, F.; Trachsel, E.; Bootz, F.; Zhang, Y.; Mannocci, L.; Beck, S. C.; Drumea-Mirancea, M.; Seeliger, M. W.; Baltes, C.; Mueggler, T.; Kranz, F.; Rudin, M.; Melkko, S.; Scheuermann, J.; Neri, D. *Angew. Chem., Int. Ed.* **2008**, *47*, 3196.
133. Mannocci, L.; Zhang, Y.; Scheuermann, J.; Leimbacher, M.; De Bellis, G.; Rizzi, E.; Dumelin, C.; Melkko, S.; Neri, D. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 17670.
134. Melkko, S.; Zhang, Y.; Dumelin, C. E.; Scheuermann, J.; Neri, D. *Angew. Chem., Int. Ed.* **2007**, *46*, 4671.
135. Buller, F.; Steiner, M.; Frey, K.; Mircsof, D.; Scheuermann, J.; Kalisch, M.; Buhlmann, P.; Supuran, C. T.; Neri, D. *ACS Chem. Biol.* **2011**, *6*, 336.
136. Clark, M. A.; Acharya, R. A.; Arico-Muendel, C. C.; Belyanskaya, S. L.; Benjamin, D. R.; Carlson, N. R.; Centrella, P. A.; Chiu, C. H.; Creaser, S. P.; Cuozzo, J. W.; Davie, C. P.; Ding, Y.; Franklin, G. J.; Franzen, K. D.; Gefter, M. L.; Hale, S. P.; Hansen, N. J. V.; Israel, D. I.; Jiang, J.; Kavarana, M. J.; Kelley, M. S.; Kollmann, C. S.; Li, F.; Lind, K.; Mataruse, S.; Medeiros, P. F.; Messer, J. A.; Myers, P.; O'Keefe, H.; Oliff, M. C.; Rise, C. E.; Satz, A. L.; Skinner, S. R.; Svendsen, J. L.; Tang, L.; van Vloten, K.; Wagner, R. W.; Yao, G.; Zhao, B.; Morgan, B. A. *Nat. Chem. Biol.* **2009**, *5*, 647.
137. Melkko, S.; Mannocci, L.; Dumelin, C. E.; Villa, A.; Sommavilla, R.; Zhang, Y.; Gruetter, M. G.; Keller, N.; Jermutus, L.; Jackson, R. H.; Scheuermann, J.; Neri, D. *ChemMedChem* **2010**, *5*, 584.
138. Fei, Y.-Y.; Landry, J. P.; Sun, Y.-S.; Zhu, X.-D.; Wang, X.-B.; Luo, J.-T.; Wu, C.-Y.; Lam, K. S., *J. Biomed. Opt.* **2010**, *15*, 016018/1.
139. Zhu, X.; Landry, J. P.; Sun, Y.-S.; Gregg, J. P.; Lam, K. S.; Guo, X. *Appl. Opt.* **2007**, *46*, 1890.
140. Torres, F. E.; Recht, M. I.; Coyle, J. E.; Bruce, R. H.; Williams, G. *Curr. Opin. Struct. Biol.* **2010**, *20*, 598.
141. Grant, T. D.; Luft, J. R.; Wolfley, J. R.; Tsuruta, H.; Martel, A.; Montelione, G. T.; Snell, E. H. *Biopolymers* **2011**, *95*, 517.
142. Kobayashi, M.; Retra, K.; Figaroa, F.; Hollander, J. G.; Ab, E.; Heetebrij, R. J.; Irth, H.; Siegal, G. *J. Biomol. Screening* **2010**, *15*, 978.
143. Hong, J.; Edel, J. B.; deMello, A. J. *Drug Discovery Today* **2009**, *14*, 134.
144. Okagbare, P. I.; Soper, S. A. *Electrophoresis* **2010**, *31*, 3074.
145. Trivedi, V.; Doshi, A.; Kurup, G. K.; Ereifej, E.; Vandevord, P. J.; Basu, A. S. *Lab. Chip.* **2010**, *10*, 2433.