

Chapter 2

Emerging Methods in Chemoproteomics with Relevance to Drug Discovery

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Abstract

A powerful interplay exists between the recognition of gene families, sensitive techniques in proteomics, and the interrogation of protein function using chemical probes. The most prominent methods, such as affinity capture, activity-based protein profiling and photoaffinity labeling, are extensively reviewed in the literature. Here we briefly review additional methods developed in the past 15 years. These include “stability proteomics” methods such as proteomically analyzed cellular thermal shift assays and the use of chemical oxidation as a probe of structure, the use of multiple bead-linked kinase inhibitors to analyze inhibitor specificities, and advances in the use of proteolysis-targeting chimeras for selective protein elimination.

Key words Chemoproteomics, CETSA, SPROX, DARTS, LiP-SRM, Proteomics, Kinobeads, PROTAC

1 Introduction

Proteomics has developed spectacularly in its short lifetime at a rate of growth and change rapid enough to unnerve the unwary. In its early days, two-dimensional gels were used to resolve hundreds of proteins into discrete spots in a way that highlighted meaningful variations [1], and digestion followed by Edman sequencing or peptide mass-mapping [2] was used to identify proteins of interest. This approach had the strong attribute that attention went directly to any proteins that changed under test, but its coverage of the proteome was generally low.

Rapid advances in mass spectrometry and the power of the SEQUEST algorithm soon converted the field to methods based on digestion and mass spectrometry, a teenage period that roughly covered the first decade of the twenty-first century [3]. As proteomics reaches its mid-twenties, label-based ratiometric methods or label-free alternatives that increase the depth and scope of comparative analyses form a basis for improving the quantitative rigor of global or broadband proteomic analyses. Meanwhile, specialized methods for targeted analysis have been established for cases in

which quantitation is required, as in the urgent but challenging hunt for protein biomarkers of human disease [4].

Interfacing proteomics with drug discovery has also been challenging. As some foresaw [5], seeking the origins of disease in altered protein expression patterns has not been an especially rewarding endeavor, if the criterion for reward is immediate actionability. Instead, it is applications based on the proteomic algorithm but directed to answering questions about the molecular action of drug-like molecules that are coming to the fore in the pharmaceutical industry.

2 Chemoproteomics: Principal Methods

The literature already offers many authoritative reviews of major methods in chemical biology. Rather than adding to the surfeit, we devote this article to a few specialized methods that have either proved their success or are currently raising hopes of new insights. The common thread running through these techniques is the involvement of a drug or potential drug molecule in the experiment, which is the origin of the term “chemoproteomics.” Often, but not always, it denotes an experimental approach composed of two parts, a first one in which affinity for a chemical probe is used to select proteins from a complex mixture, and a second in which the essential methods of proteomics (digestion, mass spectrometry and database searching) are used to identify those proteins. Already, however, this definition is being outdated by expansive new ideas.

Classical methods of this kind have already had real impact. Affinity capture of drug targets using small molecules immobilized on beads or captured after target binding via a biotin “handle” is an example. Handa and colleagues [6] used a refined version of this method employing methacrylate-coated nanobeads to uncloak the mechanism of action of the teratogenic drug thalidomide, rehabilitated now as a therapy for blood cancers. Their result has been incorporated directly into an exciting strategy for targeted protein degradation, which we discuss. Another example is photoaffinity labeling, which increasingly is used to identify specific ligand-binding sites on proteins rather than just to identify targeted proteins [7]. A third is the burgeoning strategy known as activity-based protein profiling [8, 9], which unifies previously disconnected chemical probes that react covalently with protein targets. Although these reagents function by a variety of mechanisms, some assisted by substrate-recognition elements of enzyme active sites and others targeting enzymes purely by complementary reactivity, they are now collectively designated as activity-based probes of enzyme targets. Their defining characteristic is an ability to label covalently a class of enzymes or other proteins that have an important feature in common, such as an exceptionally reactive thiol group or the common capacity to bind ATP.

Instead of providing still more coverage of these well-reported topics, we devote this section to a few technologies that may be new to readers who specialize in fields other than chemical biology or proteomics. These methods are not all new, but we share them in the spirit of colleagues sharing recent reading, as they are gaining momentum and the potential power of the methods reviewed deserves to be appreciated.

3 Stability Proteomics: Cellular Thermal Shift Assay and SPROX

Typical proteomics studies correlate stress on a system (e.g., the presence of a disease state or bioactive compound) with shifts in the expression levels or modifications of specific proteins. Changes in protein abundance or levels of post-translational modification are inferred from the intensity ratios of peptide ions. This type of approach has been used with increasing success for two decades to study protein networks and lay a foundation for systems biology. Despite their power, these techniques are less suited to detecting indirect effects such as changes in protein interactions that leave protein expression levels unchanged. Some recently developed proteomic techniques combine the power of LC-MS with biophysical or biochemical manipulation to monitor the ligand-induced thermodynamic stabilization of proteins, and we refer to these techniques collectively as “Stability Proteomics”.

All stability proteomics techniques use or are compatible with bottom-up (i.e., digest-based) mass spectrometric measurements as a readout for the thermodynamic stability of proteins. Experiments often compare results obtained in the absence or presence of a compound with the intent of identifying proteins that become thermodynamically stabilized or destabilized by the compound of interest. An advantage of these methods is that no chemical derivatization or immobilization of the ligand molecule or target proteins is required. In addition, they can all detect on- and off-target protein–drug interactions as well as direct and indirect binding events. Perhaps the most anticipated potential of stability proteomics techniques is the possibility to identify previously unknown off-target (or even unknown on-target) protein–drug interactions.

Methods of this kind detect changes in the thermodynamic stability of proteins using a variety of biophysical and biochemical mechanisms. The cellular thermal shift assay (CETSA) (Fig. 1) was originally developed using a readout based on western blots [10] but has been recently adapted to LC-MS [11]. CETSA relies on the loss of protein solubility in the thermally denatured state to generate thermal denaturation curves. LC-MS approaches generate these melting curves from reporter ion intensities which approximate the abundance of soluble proteins through their peptide surrogates. The experimental protocol for CETSA is attractively simple, but analysis of the data appears challenging.

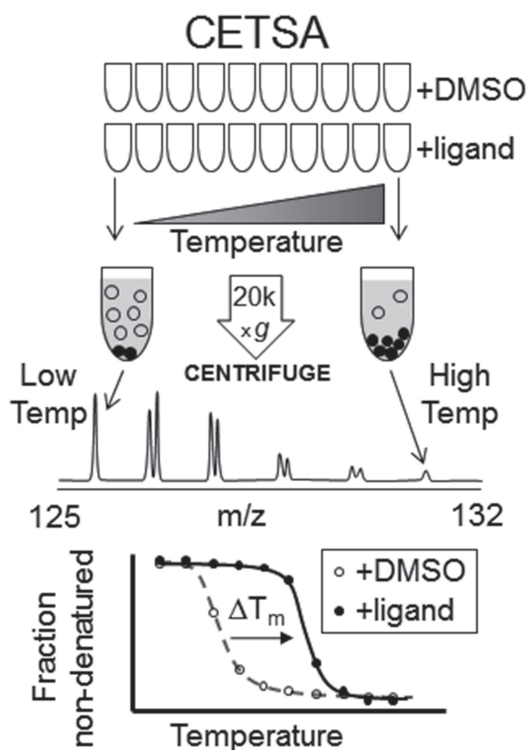


Fig. 1 The CETSA (cellular thermal shift assay) protocol uses ten aliquots of a control (DMSO-treated) sample and ten aliquots of sample treated with ligand. The aliquots can originate from a lysate or intact cell sample. Each aliquot in a set is exposed to one of a series of temperatures and denatured/aggregated proteins are then removed by centrifugation. The fraction of non-denatured protein remaining in each aliquot is measured by TMT (Tandem Mass Tag™) reporter ion intensities from peptides in its tryptic digest, and the reporter ion intensities are used to construct thermal melting curves. Ligand-induced shifts in the T_m values for these curves indicate ligand-induced effects on the thermodynamic stabilities of proteins

An additional method, the “stability of proteins from rates of oxidation” (SPROX) technique (Fig. 2) follows the chemical denaturation of proteins by measuring the hydrogen peroxide-mediated oxidation of structurally protected methionines over a range of concentrations of chemical denaturant [12–14]. Unlike the other stability proteomics techniques, SPROX provides additional information regarding affinity (K_d), binding pocket location and domain-specific interactions, but is limited at the peptide level to globally protected methionine-containing peptides.

The approach known as “drug affinity-responsive target stability” (DARTS) [15, 16] and a similar energetics-based method for target identification [17] (Fig. 3) rely on the classical observation that ligand binding often stabilizes proteins against proteolytic degradation. Peptide peak intensities in proteomic analysis are used as the readout for these techniques. DARTS has the

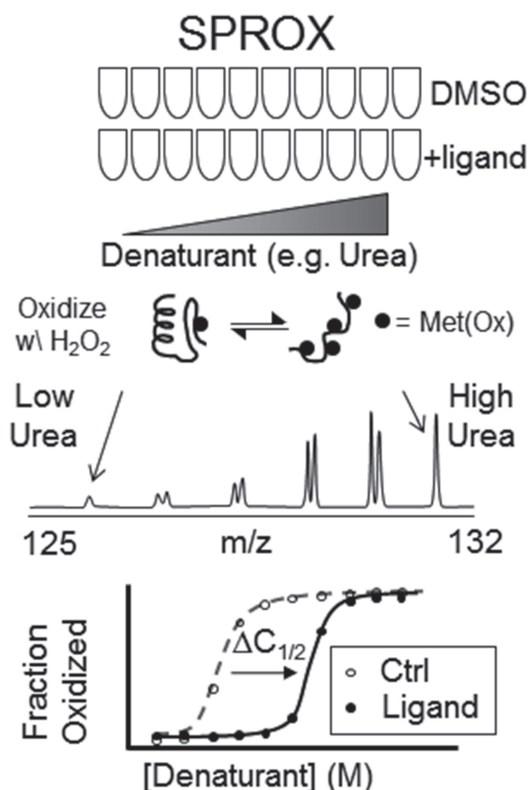


Fig. 2 As with CETSA, the SPROX protocol starts with aliquots of a protein mixture treated with either DMSO (as a control) or ligand, but in this case chemical denaturant is used to shift the folding equilibria of the proteins. Hydrogen peroxide is then added to the aliquots so that solvent-exposed methionine side chains are labeled with oxygen. The extent of methionine oxidation is used to report on the folding equilibrium at each denaturant concentration, typically measured by reporter ions. The data are used to generate denaturation curves, or SPROX curves, and the transition midpoints ($C_{1/2}$) from the SPROX curves for each protein/peptide in the presence and absence of ligand are used to determine changes to proteins' thermodynamic stability in solution at the time of labeling

advantage of being label-free, but the experiment requires prior optimization of conditions.

Limited proteolysis (LiP) coupled with single reaction monitoring (SRM) is similar to DARTS (Fig. 3), but follows the partial digestion step with an additional alternative proteolytic digestion step to increase the number of peptides amenable to bottom up LC-MS and takes advantage of SRM for improved quantitative accuracy. However, LiP-SRM requires additional optimization steps and the quantitative advantage provided by SRM requires prior knowledge of the targets. As such, Lip-SRM may prove to be particularly useful as a follow-up technique for the other stability proteomics techniques that validates potential hits with greater sensitivity.

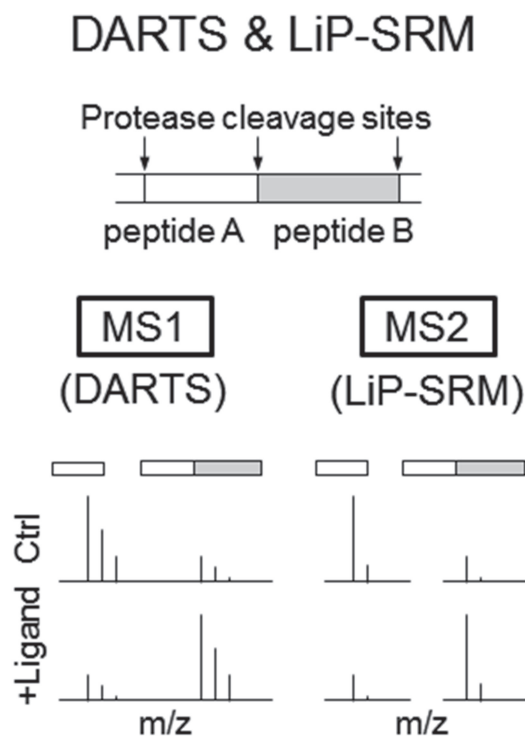


Fig. 3 DARTS (drug affinity-responsive target stability) and LiP-SRM (limited proteolysis coupled with single reaction monitoring) both rely on a protein's susceptibility to protease-catalyzed cleavage. The premise of these techniques is that specific regions of proteins are less susceptible to proteolytic cleavage when they are stabilized by a ligand. Protease efficiency is measured at the peptide level in DARTS and at the peptide fragment level in LiP-SRM. All of the proteomic stability techniques are capable of detecting direct on-target and off-target interactions with ligands (stabilization) as well as indirect effects such as the disruption of a protein–protein interaction (destabilization)

The remainder of this section will focus on CETSA and SPROX, which are the only stability proteomics methods that can generate denaturation curves. Shifts in these curves induced by compound binding can be instructive.

The CETSA technique (Fig. 1) essentially performs a thermal shift assay (TSA) on every protein in a given test mixture. As with TSA of a pure protein, in which the protein is carried through its denaturation equilibrium over a series of increasing temperatures, the CETSA technique gauges the relative amount of each protein that remains folded over a range of temperatures. Unlike most TSA techniques for single pure proteins, CETSA estimates denaturation based on solubility rather than fluorescence. The CETSA derived “ T_m ” value may not be accurate for proteins that unfold irreversibly, but this does not prevent the technique from detecting ligand-induced shifts in CETSA curves. The fact that CETSA can

screen for ligand interactions from (potentially) every peptide/protein detected in an LC-MS experiment makes this new technique appear very appealing. In addition, it is reported to be able to be performed on live cells as well as on cell lysates.

The process by which denaturation curves are generated involves dividing a protein mixture or cell population into equal aliquots. Each aliquot is heated to a different temperature that will contribute a point to the thermal melting curve for each protein detected. The temperature-specific samples are lysed, if necessary, and centrifuged to remove proteins which are no longer soluble as a result of denaturation. After digestion, isobaric tagging and pooling, the sample is analyzed by LC-MS and thermal denaturation curves are generated for each detected peptide from the isobaric tag reporter ions. These peptide-specific data must then be pooled to give data at the protein level.

An additional sample containing a compound of interest is prepared likewise and the CETSA denaturation curves are compared between conditions to identify proteins that were stabilized or destabilized by the compound. In addition, CETSA can also generate isothermal dose-response (ITDR) profiles that resemble dose-response curves.

The SPROX technique provides results fundamentally similar to chemical denaturation curves from CD or fluorescence-based studies on pure proteins [14, 18]. SPROX methodology calls for diluting a protein mixture into a series of chemical denaturants that shift the unfolding equilibria of proteins in each denaturant-containing mixture (Fig. 2). Solvent-accessible Met side chains are then labeled (oxidized) with hydrogen peroxide. The oxidant is quenched, and the protein samples are digested, labeled with isobaric tags and pooled. SPROX uses the denaturant-dependent oxidation of Met side chains to generate denaturation curves and measure thermodynamic properties of proteins. Reporter ion intensities from oxidized and unmodified methionine-containing peptides are used to generate this data and the folding free energy (ΔG), m -value, and K_d can be derived by fitting this data to SPROX equations. However, these ΔG values may not be accurate for cases where oxidation affects protein equilibria. Although the K_d values may still be accurate when ΔG estimation is compromised, the accuracy of K_d values is affected when oxidation interferes with compound binding. The number of proteins identified from an LC-MS experiment that can be useful in a SPROX analysis is also limited to those proteins for which digestion yields Met-containing peptides. In theory the SPROX approach could generate dose-response data similar to PLIMSTEX (protein-ligand interaction by mass spectrometry) [19], but this has yet to be demonstrated.

As with all proteomic studies, novel hits from stability proteomics techniques require validation using orthogonal techniques such as western blotting with immunodetection. Nonetheless, the

various emerging methods of stability proteomics present researchers with the exciting potential to discover novel on- and off-targets of their compounds. Having knowledge of all targets for a given drug candidate could enable medicinal chemists to design around off-target interactions that could be potentially detrimental *in vivo* and in the clinic. On a more cautionary note, the widespread feasibility and applicability of these techniques remains to be seen, as the few novel target identifications secured by these methods to date all come from the labs in which the techniques were developed. Although the SUPREX technique (stability of unpurified proteins from rates of H/D exchange), on which SPROX was based, has seen broader use among the scientific community [20, 21], time will tell if these new stability proteomics techniques will be adopted into the tool kits of external laboratories.

4 Kinobeads

The central importance of protein kinases in biology places them among the most attractive drug targets, but the extent of their mutual similarity initially made specific inhibition of particular kinases appear to be a difficult task [22]. Persistence has transformed the picture and kinase inhibitors now appear in the clinic in the form of a variety of life-saving and life-enhancing medicines. In some cases, they are less specific than was originally intended, and this apparent shortcoming has turned out to be a strength [23]. Therefore, the ability to define in quantitative terms the specificity of protein kinase inhibitors is a topic of great importance.

Kinase selectivity screens using panels of enzymes [24] and a notably successful ATP-based covalent probe [25] have provided two routes to acquiring the needed information, but one of the most streamlined and elegant approaches is the use of kinobeads [26] (Fig. 4). This approach exploits the ability of multiple kinase inhibitors linked to agarose beads to capture a high fraction of the protein kinases present in a cell or tissue lysate. Inhibitors present in soluble form can compete against the bead-linked compounds, allowing a direct means of gauging the relative affinities of different enzymes and inhibitors.

The kinobead approach has some attractive features. Coupling kinase inhibitors that belong to several different classes to agarose beads has allowed the capture and MS-based identification of as many as several hundred kinases per assay, while the use of isobaric chemical tags (initially iTRAQ) [27] allows for ratiometric quantitation of their binding or displacement by competitors. Varying the concentrations of soluble inhibitor during the capture step allows derivation of an IC₅₀ for any detected kinase.

To introduce the concept and its utility, Bantscheff and coworkers investigated the inhibitory activities of several drugs, imatinib

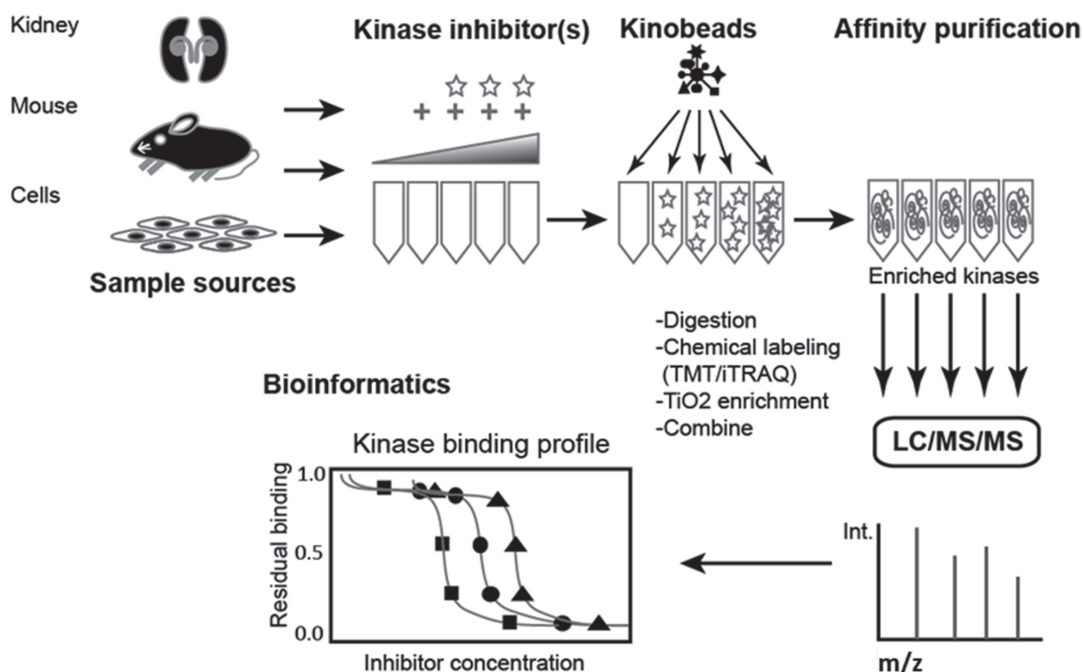


Fig. 4 Kinobeads workflow. Kinase-containing samples are first incubated with increasing concentrations of a kinase inhibitor. Equal amounts of kinobeads (polymeric beads carrying multiple immobilized kinase inhibitors) are then added to each sample, leading to capture of protein kinase molecules while inhibitor-bound kinase molecules remain in solution. The beads are washed, the proteins bound to them are digested, and the multiple digests are first chemically labeled with isobaric tagging reagents such as TMT or iTRAQ, and then combined for ratiometric quantitation of the kinases bound to the beads and the effect on their binding of the soluble competitor. Label-free quantitation (LFQ) might be used as an alternative, in which case no mixing would occur. Kinase binding as a function of inhibitor concentration is extracted from the result, and the respective affinities of the soluble inhibitor for different kinases can be calculated

(Gleevec), dasatinib (Sprycel), and bosutinib (Bosulif) [26]. Lysates deriving from K562 cells, which express BCR-ABL fusion protein, were first incubated (separately) with an increasing concentration of each kinase inhibitor (100 pM–10 μ M), after which the lysates were exposed to kinobeads. Kinases captured by the beads despite the competitive presence of the soluble inhibitor were digested, labeled with iTRAQ reagent, and detected by mass spectrometry with reporter ion readouts providing a gauge of the extent of binding at each concentration of the competitor. This allowed IC₅₀ values for all three drugs to be derived.

An additional benefit of the work was its potential to identify nonkinase drug targets. In the introductory paper [26], potent inhibition of the oxidoreductase NQO2 by imatinib was indicated by the kinobeads binding protocol and directly confirmed in an enzyme assay.

Several improvements to the workflow and new applications for the method have now been reported [28, 29]. The original

format held the possibility that endogenous ATP or related compounds might compete with soluble kinase inhibitors for occupancy of ATP binding sites on kinases during the first step, potentially causing IC₅₀ values to be inconsistent between experiments. To correct for this, lysates were first depleted for cofactors by gel filtration before addition of inhibitors. As expected, the concentrations of endogenous nucleotide factors can greatly affect the observed affinities of inhibitors for target kinases [29].

Although the kinobeads method was devised to profile kinase inhibition, it has been adapted to measuring differences in the expression of kinases between two cell lines. To do this, kinobeads were used to capture kinases from different cell lines without any resort to competition from soluble inhibitors. The relative expressions of kinases were derived by taking the summation of their corresponding three largest peptide intensities [29, 30].

To broaden the scope of the kinobead method, Médard et al. synthesized a new generation of kinobeads capable of capturing a wider selection of kinase families [28]. The workflow was streamlined by performing competition assays in 96-well plates and label-free quantitation in the powerful MaxQuant program [31] was used instead of iTRAQ. The improved method allowed 216 protein kinases to be captured and, therefore, potentially to be the targets of competitive binding studies.

Finally, comparison of the kinobeads method with covalent capture of kinases using acylphosphate ATP analogs indicated that the two methods are complementary [32] and can be used in tandem when maximum coverage of the kinase complement of a sample is required.

5 Targeted Protein Degradation

As we noted above, affinity-based protein capture allied to sensitive protein identification was the method that resolved the molecular mechanism of thalidomide [6]. The drug and its relatives bind to cereblon, the substrate recognition module of a certain E3 ubiquitin protein ligase complex (there are more than 600 [33]), and can modulate its specificity for certain protein substrates. For example, exquisite SILAC-based proteomic analysis was used to demonstrate the extraordinarily specific effect of lenalidomide in bringing about the ubiquitination and degradation of casein kinase 1 α as well as two transcription factors, IKZF1 and IKZF3 [34]. The latter effects provide the clinical efficacy of lenalidomide in multiple myeloma.

Increased understanding of the mode of action of these compounds has led to their incorporation into the existing strategy of using a double-headed drug to bring a protein targeted for elimination into close proximity to an E3 ubiquitin ligase. One end of the agent should have affinity for the targeted protein, and has been a drug-like small molecule from the outset. The element to

be recognized by the E3 ubiquitin ligase was originally a peptide [35], but difficulty with cell-permeability caused this to give way to an E3-targeting small molecule [36, 37]. Impressive preliminary demonstrations of the use of a thalidomide-related E3-targeting group have appeared [38, 39], and interest in this strategy is sure to continue to grow despite concerns that the method requires agents that embody two drug-like moieties and consequently will tend to have molecular weights beyond the preferred range for good pharmacokinetic properties.

6 Conclusion

The power and scope of rapidly emerging methods in chemical biology is one of many developments resulting from the genomic revolution that occurred mainly in the 1990s. This resulted in massive enhancement of our understanding that related proteins are derived from families of related genes. Together with the emergence of methods for protein recognition based on the essential algorithm of proteomics, the stage has been set for adventurous exploration of the potential of chemical probes—agents that address proteins with respect to their functions—to elucidate details of the subtle differences that may exist between related family members. As proteins will presumably continue to account for the great majority of drug targets, this additional capability to monitor their functional aspects will be an important complement to mainstream drug discovery in the coming decades.

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