

Chemical proteomics and its application to drug discovery

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The completion of the human genome sequencing project has provided a flood of new information that is likely to change the way scientists approach the study of complex biological systems. A major challenge lies in translating this information into new and better ways to treat human disease. The multidisciplinary science of chemical proteomics can be used to distill this flood of new information. This approach makes use of synthetic small molecules that can be used to covalently modify a set of related enzymes and subsequently allow their purification and/or identification as valid drug targets. Furthermore, such methods enable rapid biochemical analysis and small-molecule screening of targets thereby accelerating the often difficult process of target validation and drug discovery.

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Abbreviations

ABP	activity-based probe
AFPB	affinity-based probe
5'-FSBA	5'-fluorosulfonyl benzoyl adenosine
ICAT	isotope-coded affinity tag
SDS-PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis

Introduction

The completion of the human genome has created much excitement from the impact that this wealth of information is likely to have on the process of drug discovery and development [1–3]. It has been postulated that scientists could use genome information to identify and validate a host of new drug targets and tailor specific drugs based on an individual's detailed genetic makeup [4]. Although this new field of genomics holds much promise, it is clear that analysis of DNA or RNA content alone is not sufficient to understand cell biology and disease. Furthermore, the estimated 30 000–40 000 protein-encoding genes in the human genome could result in 10–100 times this number of unique proteins through post-transcriptional and post-translational processing and modification.

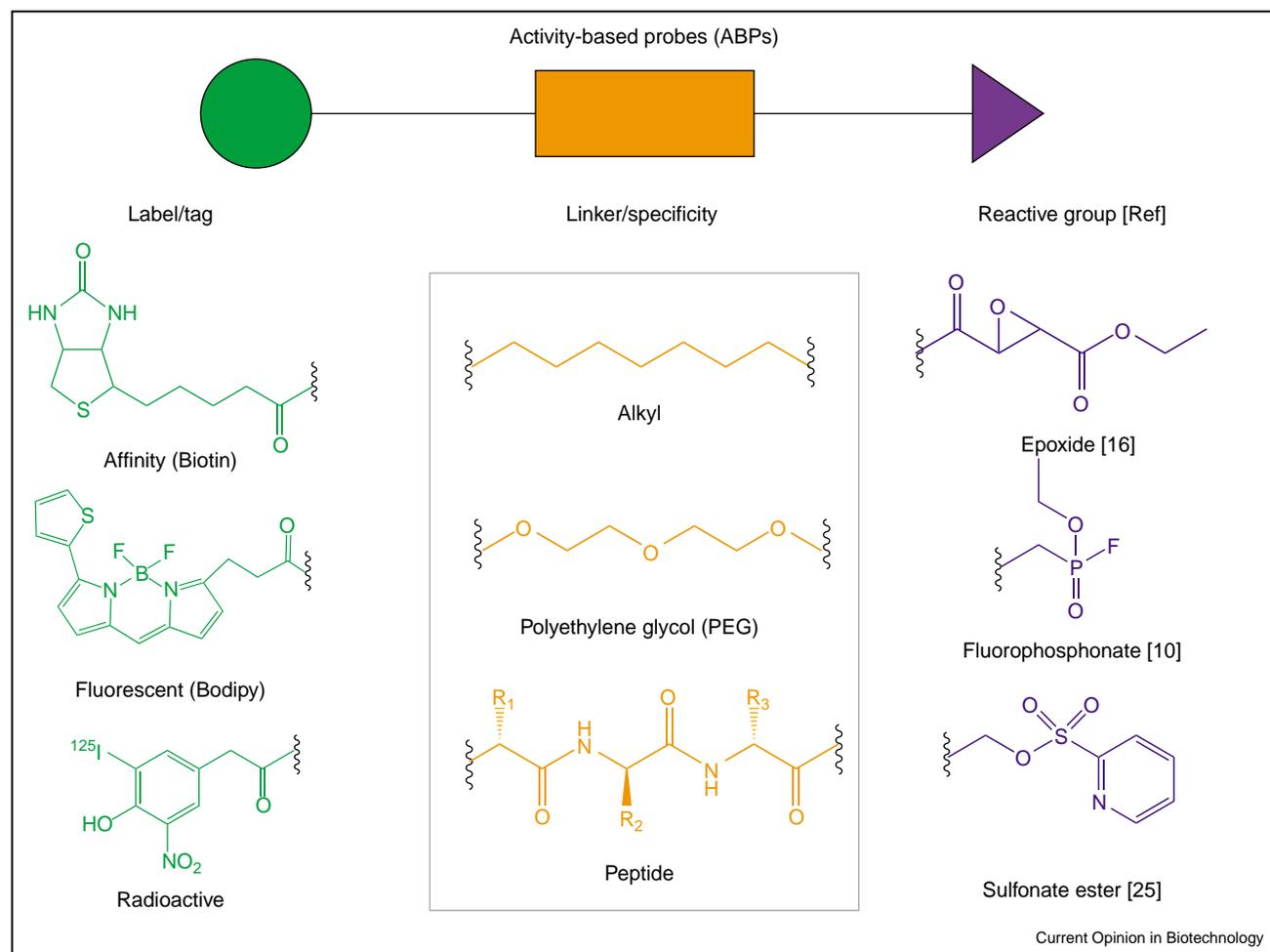
For this reason, the discipline of protein biochemistry has become an increasingly important component in efforts to reap maximum reward from the information provided by genomics. The term 'proteomics' has been coined to define the study of protein biochemistry on a genome wide scale.

The broad field of proteomics has as its primary goal the understanding of the structure, function, expression, cellular localization, interacting partners, and regulation of every protein produced from a complete genome [5,6]. At the heart of this field are many new technologies and techniques that are being developed to facilitate this daunting task. This review focuses on the multidisciplinary field of chemical proteomics, which makes use of synthetic organic chemistry, cell biology, biochemistry, and mass spectrometry. A central component of this field is the design of specific protein-modifying reagents that can be used for functional studies of distinct enzyme families within a complex proteome. These chemical probes are designed to covalently modify a target enzyme in such a way that it can be subsequently identified and/or purified [7]. Through synthetic organic chemistry, these probes can be designed to react with mechanistically or functionally distinct families or subfamilies of enzymes. In the cases where enzymes utilize a nucleophilic attack mechanism, probes can be designed to modify specific active-site residues in a manner that requires enzymatic activity of the target. Such probes have been termed activity-based probes (ABPs) to reflect their need for an active enzyme to facilitate covalent modification. Other chemical probes have been designed that target non-catalytic residues on proteins and enzymes. These affinity-based probes (AFBPs) require highly selective, tight binding to targets to be useful probes for distinct protein/enzyme families. Still other chemical probes have been designed to modify proteins *en masse* for quantitative studies of complete proteomes. Regardless of their mechanism of action, chemical probes are finding increasing use in the field of proteomics and have great potential to aid in the process of target identification, target validation, and drug discovery.

Anatomy of a chemical probe

In their most basic form, chemical probes consist of three distinct functional elements (Figure 1): a reactive group for covalent attachment to the enzyme; a linker region that can modulate reactivity and specificity of the reactive group; and a tag for identification and purification of modified enzymes. We outline the chemical structures of each of these reactive groups and highlight specific examples of how they are used in chemical probes.

Figure 1



Structure of a chemical probe. A chemical probe has three basic components: a reactive group for covalent attachment to the enzyme of interest; a linker region to provide spacing and specificity; and a tag to allow for identification and/or purification. Specific examples of each are shown with references where relevant. See text for details.

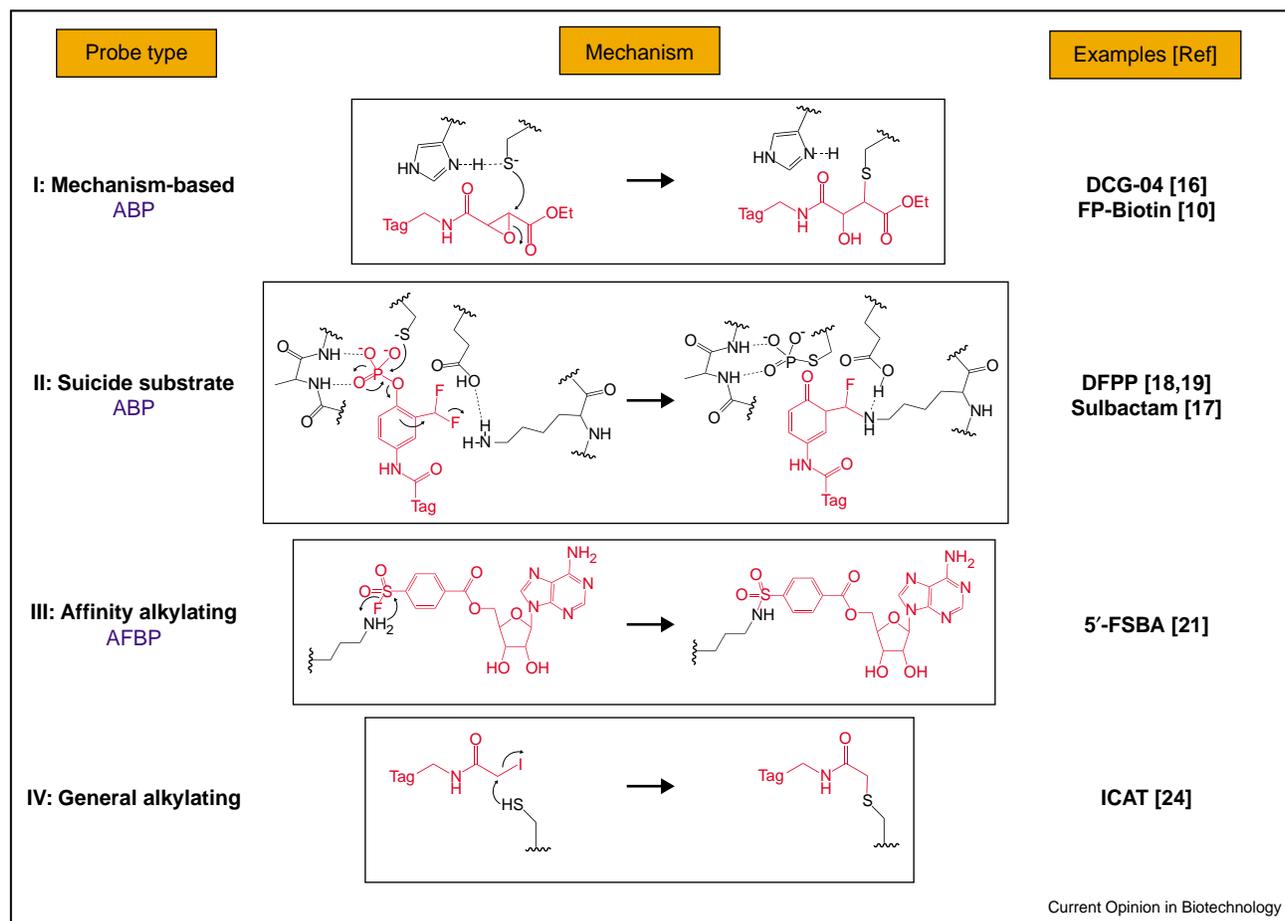
Structure of the reactive group

Perhaps the greatest challenge in the design of a chemical probe is selection of a reactive group that provides the necessary covalent modification of a target protein. The difficulty lies in the duality of this functional group as it must be both reactive towards a specific residue on a protein and inert towards other reactive species within the cell or cell extract. In general, the reactive groups of most of the successfully designed chemical probes have been based on the chemistries of covalent, mechanism-based inhibitors of various enzyme families. Such inhibitors rely on mechanistic differences of individual enzyme classes as a means for selective targeting. For example, serine and cysteine proteases utilize a catalytic amino acid nucleophile in the active site, yet each has a different nucleophilic residue and a distinct catalytic mechanism allowing for the design of chemistries that react with one class and not the other. In fact, protease

inhibitors provide a rich source of reactive groups that have been designed based on subtle differences in reaction mechanisms for the major protease families. Not surprisingly, many of the best examples of chemical probes have been designed to target proteolytic enzymes [8–12] (for review see [7]).

Most reactive groups take the form of suicide/mechanism-based inhibitors or affinity labeling reagents. These have been used for decades as drugs (e.g. aspirin), as tools to identify the active-site residues in enzymes, and as tools to understand the mechanism and function of enzyme catalysis *in vivo* [13,14]. There are far too many examples of mechanism-based inhibitors or affinity labeling reagents to cover within the scope of this review, so we use specific examples to highlight the general types of reagents that have potential applications in probe design.

Figure 2



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Mechanism of action of the four primary classes of chemical probes. Examples of the four general types of reactive groups that can be incorporated into chemical probes with the mechanism of action of an example reactive group for each shown in the middle column. The right-hand column contains specific examples of chemical probes/inhibitors that belong to each class with the relevant references listed.

There are four general classes of reactive groups that have been used to design chemical probes (Figure 2). The first two classes (type I and type II) are ABPs based on true mechanism-based or suicide inhibitors and require active target enzymes to react with the probe. In the case of the mechanism-based probes (type I), the key nucleophile is the catalytic residue of the enzyme normally involved in attack of a substrate. This type of probe can therefore be made selective based on knowledge of catalytic mechanism and is often tailored to the reactivity of the specific nucleophilic atom used by the enzyme class (i.e. a sulfur used by cysteine proteases). The second class of ABPs (type II) contain a 'masked electrophile' that is uncovered after the probe functions as substrate for the target enzyme. The unmasked electrophile is able to react with nearby, non-catalytic, nucleophilic residues in the active site. Affinity alkylating probes (AFBPs, type III) contain affinity-based labeling groups that require only a strong nucleophile or electrophile in the vicinity of the active-site pocket and do not require the enzyme to be fully

active. Probes carrying this class of reactive group must rely on the selectivity of the probe scaffold to direct modification to specific enzyme/protein classes. The final class of reactive groups used for probe design (type IV) contains non-specific alkylating groups that react with targets based only on the intrinsic reactivity of a specific amino acid residue such as cysteine. This class of probes has recently been shown to be valuable for bulk proteomic analysis using mass spectrometry methods [15].

Examples of type I reactive groups in chemical probes include peptide acyloxymethyl ketones and epoxides that have been shown to efficiently and selectively label cysteine proteases of the caspase and papain families, respectively [11,16] (Figure 2). These reactive groups make use of an electrophilic carbon that is susceptible to attack by the active-site nucleophile of the enzyme. In the case of an epoxide probe the result is the formation of a covalent bond between the active-site thiol residue and the electrophilic carbon in the epoxide ring (Figure 2). In

theory, any reactive group that mimics a substrate and has an electropositive carbon could be used to covalently modify the active-site residue of an enzyme that uses a nucleophilic attack mechanism (e.g. through cysteine or serine residues). Furthermore, many other enzyme classes (e.g. phosphatases, DNA repair enzymes, esterases) utilize a nucleophilic attack mechanism at some point during the process of catalysis. To date, type I reactive groups are the most commonly used in ABPs and this is likely to continue to be the case as new probes are developed for other enzyme classes.

Two examples of type II mechanism-based inhibitors are sulbactam, a β -lactamase inhibitor used in combination with other antibiotics to combat bacterial antibiotic resistance [17], and DFPP, a probe that has been used to alkylate protein phosphatases [18,19]. The proposed mechanism of action against phosphatases for DFPP involves initial attack by the active-site nucleophile on the phosphotyrosine mimetic resulting in dephosphorylation of the probe. This leads to production of a reactive quinone methide that can react with a nucleophilic side-chain, lysine or cysteine for example, found in the active site, resulting in irreversible alkylation of the enzyme. One drawback to this type of reactive group is that it can lead to diffusion of unmasked electrophiles from the active-site pocket and alkylation of other sites on the target enzyme or other proteins that carry nucleophilic residues on their surface. This in fact is the mechanism of action of a similar probe, ortho-(difluoromethyl)aryl- β -D-glucoside, that can alkylate β -galactosidase without inhibiting its enzymatic activity [20]. This problem can potentially be overcome by adding specificity elements to the probe (see below) to increase its affinity for the active site of the enzyme and keep the unmasked electrophile bound long enough for specific alkylation of the desired enzyme to occur.

Type III AFBPs differ from ABPs (type I and II) in that they do not require an active enzyme for modification. In most cases they are substrate analogs that contain a reactive center that is susceptible to attack by an electrophile or nucleophile in the active site of the enzyme or that can be activated through the subsequent addition of chemicals or UV light. An example of an affinity-based labeling reagent is the nucleotide analog 5'-fluorosulfonylbenzoyl adenosine (5'-FSBA) that has been used extensively to identify the active site of nucleotide-binding enzymes [21] (Figure 2). This ATP mimetic binds to the active site of an enzyme bringing the reactive fluorosulfonyl group into close proximity to active-site nucleophiles such as lysine and cysteine. This results in alkylation of the enzyme and loss of activity. 5'-FSBA has been shown to be a potent inhibitor of protein kinases through alkylation of the invariant active-site lysine [22], as well as an inhibitor of many other nucleotide-binding proteins [21]. Similarly, GTP analogs that can be acti-

vated by UV light or chemical reduction can be used as affinity labels for GTP-binding proteins [23]. The potential exists for the use of affinity labeling reagents to modify a wide variety of enzymes that bind small molecule substrates (such as ATP) or cofactors.

The final class of reactive groups (type IV) makes use of a general alkylating reagent. The iodoacetamide group of the isotope-coded affinity tag (ICAT) [24] is designed to react with free sulfhydryl groups on cysteine residues (Figure 2). The result is the covalent attachment of the probe to all free sulfhydryls on a protein or in a protein mixture. This reactive group has been shown to be very useful in the quantitation of relative bulk protein levels in two different proteomes and is reviewed elsewhere [15].

Structure of the linker region

The linker region of a chemical probe connects the reactive group to the tag used for identification and/or purification (Figure 1). The linker region can serve multiple purposes. Its primary function is to provide enough space between the reactive group and the tag to prevent steric hindrance that could block access of the reactive group or accessibility of the tag for the purpose of purification. This is often accomplished using a long-chain alkyl or polyethylene glycol (PEG) spacer. The alkyl linker can be particularly useful to modulate hydrophobicity and allow entry into live cells or tissues, whereas the PEG linker can confer more solubility to hydrophobic probes in aqueous solutions.

The linker can also incorporate specificity elements used to target the probe to a desired enzyme or family of enzymes. To date, these specificity elements normally take the form of a peptide or peptide-like structure, particularly for the ABPs used that target proteases (see below) where two to four amino acids are incorporated chemically into the probe to provide specific binding to protease active sites. Yet other examples of a chemical probes exist in which the main specificity elements are contained within the structure of the reactive group [25*].

The use of very large peptides or proteins to provide a high degree of target specificity to a chemical probe also holds much promise [26,27**]. Recently, techniques have been developed that can be used to modify a recombinantly expressed ubiquitin with an electrophilic reactive group. The resulting protein probe can then be used to covalently modify proteases that process ubiquitin *in vitro*. This method is particularly important because it has been difficult to chemically synthesize specificity elements for de-ubiquinating enzymes due to the need for a very long (>70 amino acids) stretch of amino acids. This technique of making ABPs from recombinant proteins could be very useful in the design of probes for other enzymes or protein-binding domains that require substantial protein recognition elements for specificity.

Structure of the tag

The purpose of the tag on a chemical probe is to allow quick and simple identification and purification of probe-modified proteins. In general, the tag is the primary element that distinguishes an ABP or AFBP from a stand-alone mechanism-based inhibitor. The most commonly used tags are biotin, fluorescent, and radioactive tags (Figure 1). Because the simplest and most cost-effective method to separate proteins involves the use of protein gels, tags used in probe design must be compatible with SDS-PAGE methods. Biotin facilitates detection by simple western blot approaches using a reporter avidin molecule in place of the standard secondary antibody. Fluorescent and radioactive tags can be visualized by direct scanning of gels with a fluorescent scanner/phosphorimager such as the Typhoon scanner from Amersham Biosciences [28]. Fluorescent and radioactive tags have several advantages over biotin. They are typically faster to use because they involve minimal time and handling. They also are more sensitive and have a greater dynamic range than streptavidin-biotin detection methods, especially when using the recently developed fluorescent tags such as the Alexa Fluors from Molecular Probes [29]. Finally, they have the added advantage of allowing multiplexing of samples based on non-overlapping excitation/emission spectra of the tags. This allows one to use probes with different colored fluorescent tags in different experiments and readily obtain all results on a single gel [30[•],31[•]].

Regardless of the benefits of fluorescent and radioactive tags, biotin remains the most commonly used tag because of its ability to provide both a gel-based method of detection and a method for purification of labeled enzymes on streptavidin-agarose beads. Purification and identification of probe-bound proteins is the key step for the application of chemical probes to proteomics (for a review of methods for protein identification in proteomics see [32[•],33]). The biotin-streptavidin bond is one of the strongest known non-covalent interactions with an association constant of 10^{15} M^{-1} [34] allowing for quantitative binding of low abundance biotinylated enzymes. However, this tight binding interaction comes at a price, as it can be difficult to elute biotinylated proteins from streptavidin resins without using harsh conditions that result in the elution of non-specifically bound and endogenously biotinylated proteins. For this reason, a major focus in probe design in the future will be the inclusion of cleavable linkers between the reactive group and the biotin tag that allow mild and selective elution of the probe-bound proteins from the solid support. A photocleavable linker has been reported for use with ICAT probes [35[•]] and it is anticipated that other types of cleavable linkers will also be developed.

Applications of chemical proteomics

Chemical probes can be used to study all aspects of proteomics from protein expression and identification

to cellular localization and regulation. Here we discuss the applications of chemical probes primarily in the context of drug discovery and development, but these methods also have many important applications in basic research.

Applications to target identification and validation

One of the primary challenges facing pharmaceutical companies is allocating their drug discovery resources towards therapeutically relevant protein targets. The process of target selection is complex and choosing a poor target can cost time and money, especially in competitive markets. It is estimated that each new drug candidate that enters the clinic costs 250 full-time employee years and \$70 million [36]. Part of this cost stems from the high attrition rate of targets during the early stage of drug discovery, with an estimated sixty new targets required to generate three new marketed drugs every year [4]. Chemical probes provide a method to focus initial new target identification efforts towards proteins that are more easily validated and most likely to be effective drug targets, thereby creating a higher potential for success. Drug targets that come from chemical proteomic screens are pre-selected as susceptible to inhibition by small-molecule drugs by virtue of the method of their identification. Chemical probes can also be used in validation experiments in animal or cellular models both at early stages of disease model selection and during initial target validation experiments. Additionally, reactive groups and specificity elements of the chemical probe can serve as a starting point for small-molecule inhibitor design.

Chemical probes have already been used to identify cysteine proteases involved in processes such as apoptosis [12], cataract formation [37], and malarial infection [38^{••}]. Other probes have been used to profile enzymes involved in clinically relevant conditions such as cancer progression and cancer cell invasiveness [25[•],39[•]]. In all of these cases, complex disease states and proteomes were distilled into a few biochemically tractable enzymes that could then be studied in more detail.

Chemical probes have the potential to rapidly increase the number of new drug targets. Recent studies have estimated that of the 483 known drug targets, only 122 have been targeted by orally available small-molecule inhibitors that are marketed to treat human disease [40,41]. Almost half of these are enzymes that have the potential to be targeted with chemical probes. These studies also estimate that there are ten times as many possible drug targets remaining to be discovered. Chemical proteomics has the potential to uncover these targets in a rapid, systematic and comprehensive manner through design, synthesis, and application of relevant probes.

Among published chemical probes, those designed to target the cysteine protease family may have limited

potential for new target identification given the small size of this enzyme family in humans. By contrast, chemical probes designed to target serine hydrolases [42] hold a greater potential given that approximately 1% of the human genome is predicted to encode members of this enzyme family [2,3]. Furthermore, a generally reactive probe containing a sulfonate ester electrophile was found to be useful for identifying abundant enzymes that are not sequence related but might share common features in substrate binding or catalytic mechanism [25^{*}]. It will be interesting to see how profiling of these mechanistically related enzymes can be used to identify drug targets and increase our biological understanding of disease.

In addition to the increasing number of reported chemical probes, many mechanism-based inhibitors or affinity labeling reagents have already been identified that could be used to develop new classes of chemical probes. Of the major classes of enzymes that have been the focus of drug discovery efforts, the two that are most amenable to chemical proteomics applications are protein kinases and protein phosphatases. These enzymes are critical regulators of cell signaling and metabolism and have received much attention as possible drug targets [43,44].

The protein kinase family of enzymes is perhaps the most likely to benefit from a chemical proteomic strategy. This family is made up of over 500 members with diverse substrate requirements and cellular functions [2,3]. For this reason, it is unlikely that any single chemical probe will be developed that will target the entire kinase family. Rather, many subclass-specific reagents will need to be identified. Interestingly, no chemical probes for kinases, other than 5'-FSBA mentioned above, have appeared in the literature. However, covalent inhibitors of epidermal growth factor receptor (EGFR) kinases and phosphatidylinositol-3 (PI3) kinases have been identified [45,46]. It is not clear whether these reactive groups could in fact be used to generate specific chemical probes for these families of kinases.

Protein phosphatases are also emerging as desirable drug targets as they are involved in many cell-signaling events [47]. Phosphatases are particularly suitable for analysis with type I reactive groups on chemical probes because the reaction mechanism involves a nucleophilic thiolate group on a cysteine residue in the active site [48]. In addition to the di-fluoromethylphenols mentioned above ([18,19]; Figure 2), haloacetophenones have been used as tyrosine phosphate mimetics to covalently modify phosphatases [49]. One problem with these probes is the low specificity with which they bind phosphatases. With K_1 values in the range of 0.04 mM to >2 mM, they may not be specific enough to identify low abundance phosphatases in a complex protein mixture. Specificity elements in the linker region or perhaps even on the reactive group may be needed before these types of

probe can be useful to study protein phosphatases in a complex setting.

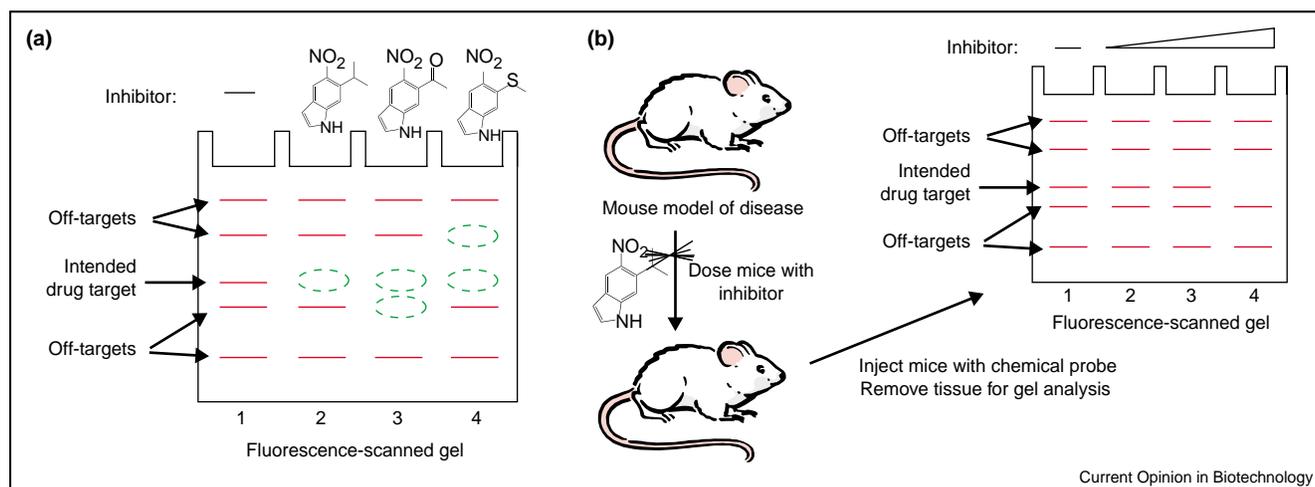
Applications to drug screening and drug efficacy studies

The process of new target identification often provides little information regarding the function of potential targets in a given disease state. One strategy for target validation is to quickly identify lead compounds that inhibit the target and use them to assess the functional role of the target in a disease model. However, identification of these lead compounds can be costly and time-consuming. Identifying small-molecule inhibitors of enzymatic drug targets usually involves designing a high-throughput assay that can be automated and used to screen large libraries of potential inhibitors. These standard enzymatic screens often require large quantities of recombinantly expressed target proteins that can be difficult to produce. In addition to the desired target, several closely related enzymes are often produced and screened to test for drug selectivity.

Chemical probes have recently been shown to have great potential to facilitate the process of lead drug identification (Figure 3a). DCG-04, a chemical probe directed towards the papain family of proteases, was used to identify preliminary selective inhibitors of individual cathepsin proteases [30^{**}]. A fluorescently tagged version of DCG-04 was used in rat liver extracts in competition with a small library of inhibitors to identify compounds that could selectively inhibit a desired target protease. This method does not require, *a priori*, knowledge of the target enzyme, and screens performed in complex mixtures provide information regarding the potency and selectivity of each compound with respect to other closely related family members. However, initial screening methods depend upon the use of gel-based separation techniques, allowing for only low-throughput applications. Further development will be required to make this approach amenable to high-throughput analysis. For example, the use of fluorescent probes with different excitation and emission profiles can be used in conjunction with a high-throughput capillary electrophoresis system, thereby allowing for unattended screening of thousands to tens of thousands of compounds per day.

Chemical probes could also be applied in drug efficacy studies (Figure 3b). This process involves the identification of the intended drug target in a complex proteome using a simple competition assay between the drug candidate and the chemical probe. The efficacy of the drug candidate can be tested against the native enzyme in a more physiologically relevant milieu than standard enzymological assays. Furthermore, the ability of a drug candidate to inhibit a specific target and not other related targets can be assessed within a relevant tissue sample. Such methods for evaluation of drug potency and selectivity

Figure 3



Applications of chemical probes to methods of drug discovery. **(a)** Use of a chemical probe in drug lead selection. Image of a fluorescence-scanned SDS-PAGE gel is shown depicting the reactivity of a fluorescently tagged chemical probe in a complex proteome (lane 1, red lines depict labeled enzymes). Hypothetical lead drug candidates are added in competition with the chemical probe (lanes 2–4). Enzyme family members whose labeling is inhibited by addition of a drug indicates active-site binding (dotted green circles). The drug lead in lane 2 shows the most specificity for the intended drug target. **(b)** Evaluation of drug efficacy in an animal model for disease using a chemical probe. In preclinical studies, an animal is treated with a lead drug candidate to assess efficacy. The animals can be injected with a fluorescently tagged chemical probe to evaluate the efficacy and selectivity of the drug towards the intended target *in vivo*. Lanes 2–4 of the fluorescence-scanned SDS-PAGE gel show that the drug candidate is indeed inhibiting the intended drug target (as measured by competition for labeling of the target by the fluorescent probe) in a dose-dependent manner and shows no activity towards related family members.

using chemical probes could be carried out in a variety of systems including purified proteins, cells lysates, live cells, and even live animals. Information gained from such an analysis could help focus drug development efforts to increase potency and selectivity of a drug candidate within the context of a cellular or animal disease model.

Conclusions

The sequencing of the human genome has had tremendous impact on science and medicine. To maximize the benefit from this rich resource of information, scientists must continue to advance the fields of genomics and proteomics. The possibility of defining the function of every gene or protein in an entire genome now seems possible, although it will require many years of work and will depend on continued technological innovation.

The field of chemical proteomics represents the crossroads of many disciplines that, when applied together, are well suited to advance our understanding of biology and drug development. Without a doubt, the most important tool of this field is the chemical probe that must be carefully designed to covalently attach to proteins of interest and allow purification and/or identification. The chemical probes that have been developed to date have been shown to be useful for the identification of new protease drug targets in a variety of different diseases. They have also been used to rapidly identify drug leads. These applications could have significant impact on the

speed with which quality drug candidates enter into the clinic.

The continued success of chemical proteomics depends on the design of new probes and probe classes that can specifically target diverse sets of enzyme families. As chemical probes become more widespread in their use, there will undoubtedly be other families of enzymes whose mechanism of action is particularly suited for investigation using chemical proteomic methods.

Acknowledgements

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