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Proteomic tools to study drug function

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Abstract

With the ever growing repertoire of drugs being developed, new unbiased methods are urgently needed that allow fast screening of protein targets and off-targets. Ideally, these methods are capable of studying target engagement in a cellular context and provide a link between drug and cellular phenotype. Mass spectrometry based strategies provide an excellent way to study drug-target interactions as well as drug effects in a cellular context with excellent sensitivity and depth. In order to perform unbiased drug target screening several methods have been developed over the last years. In this review, we discuss affinity pull-down approaches to study direct drug-target interaction, methods which use alterations in protein stability as a measure for drug binding and the biological relevance of PTM enrichments to study the effect of inhibitors on cellular signalling.

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Introduction

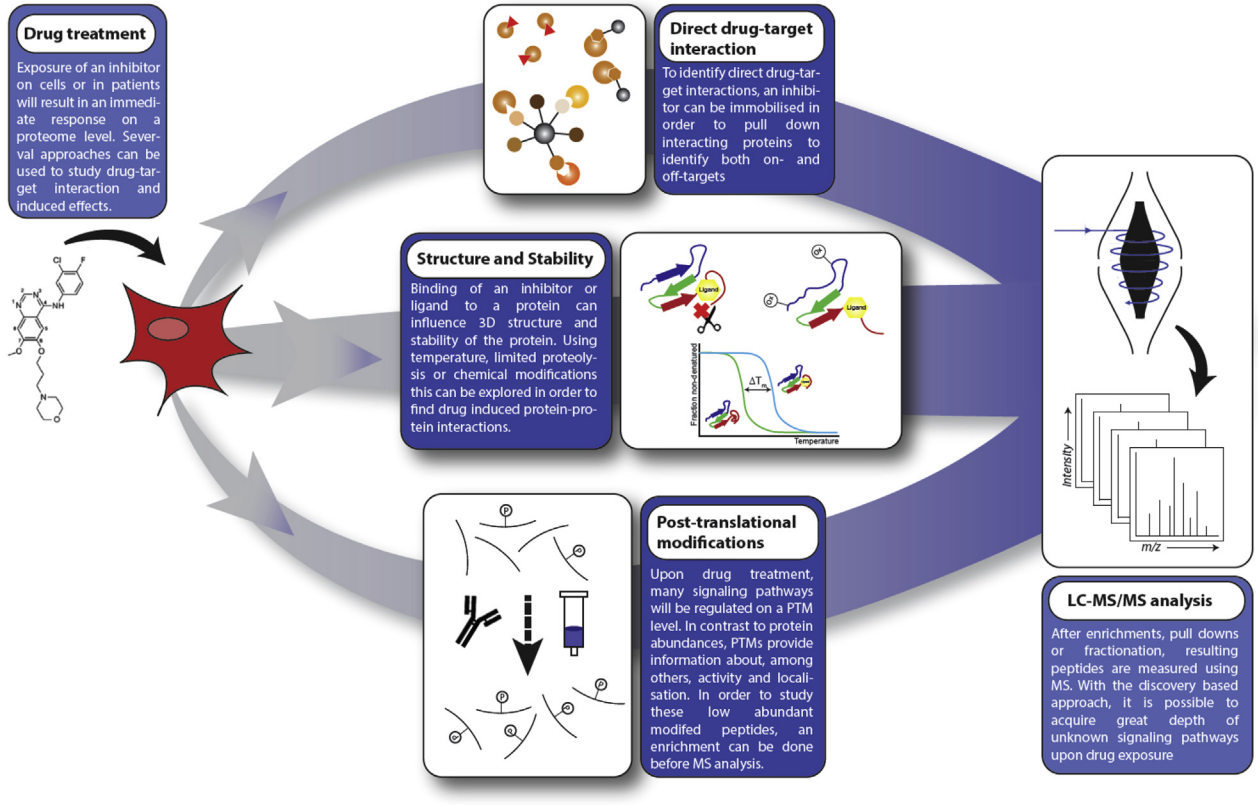
An increasing number of small molecule inhibitors are being approved by the FDA, calling for methods enabling the dissemination of drug function at a molecular level. Especially drug target engagement studied in a cellular context is extremely important as it will provide a link between drug and cellular phenotype and could explain possible side-effects or resistance mechanisms. Genetic approaches are well suited to identify novel drug targets and genes which play a role in drug function and development of resistance [1]. However, genetic approaches only reveal drug-target interactions indirectly. A change in drug sensitivity upon mutation or overexpression of a specific gene merely shows a possible influence of the gene, but does not prove direct drug-target interaction [2]. The same limitations hold true for transcriptomic data. Even though the extent of gene transcription can be determined, the correlation between mRNA levels and protein abundance is still a subject of debate [3]. More importantly, both genomics and transcriptomics lack the ability to determine the role of often short lived and reversible events like post-translational modifications (PTMs), protein localisation and protein−protein interactions. On the contrary, this information is retained within proteomics data, providing essential information on the regulation of cellular signalling during drug treatment which might explain off-targets and side effects and provide clues to the development of drug resistance which is not caused by mutations. Shotgun proteomics focusses on proteins in a discovery-based manner, resulting in an unbiased representation of the phenotype and molecular landscape [4]. Recent advances made in the proteomics field, such as the development of the Orbitrap mass analyser and Fusion Tribrid instruments, have made it possible to measure and quantify the proteome with high accuracy and great depth. By using optimised sample preparation, fractionation and enrichment strategies preceding LC-MS/MS analysis, different aspects of the proteome can be explored.

In this review, we will list and discuss different ways of fractionation and enrichment before LC-MS/MS analysis to study both drug function and perturbation of cellular signalling induced by small molecule inhibitors, with a focus on direct drug-target interactions, structural changes and PTMs (see Figure 1).

Direct drug-target interaction

The classical *in-vitro* workflow used to identify drug-target interactions is an affinity-based protein profiling (AfBPP) approach. This approach is based on a single specific compound immobilized on a solid support, which is incubated with a complex cell lysate. After elution, interacting proteins are detected with LC-MS/MS analysis (see Figure 2) [5]. These methods are suitable to screen for both on- and off-targets, to evaluate drug specificity (by performing a competition experiment in which increasing concentrations of free drug are titrated into the lysates or on cells), and to detect changes in binding affinity or interactome between different conditions [6]. Examples of the successful implementation of this compound centric approach are the target profiling of the chronic myeloid leukaemia drug Dasatinib, originally designed to target Bcr-Abl and Src [7] and Gefitinib, a first generation EGFR inhibitor [8] (More examples are listed in

Figure 1

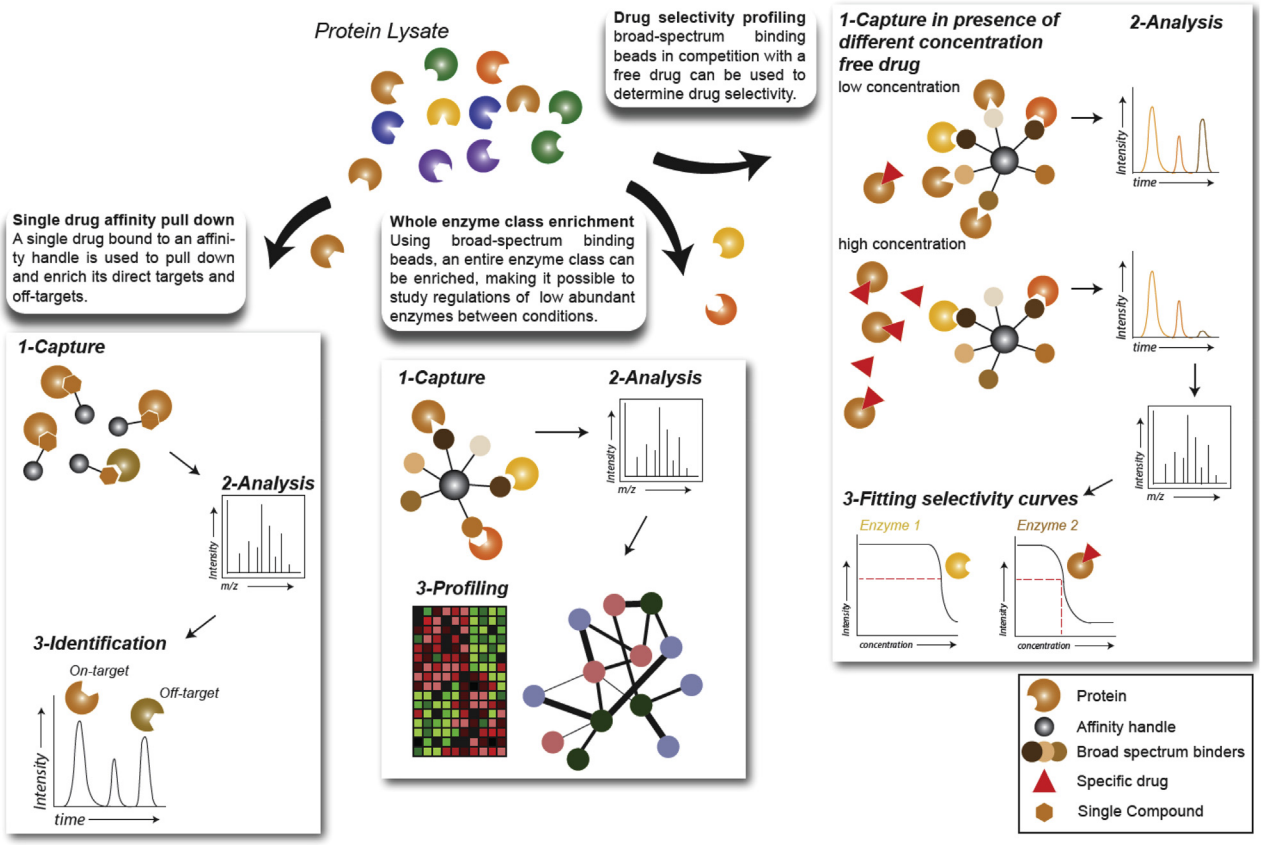


Overview of discussed topics. Mass spectrometry is a suitable technique to study drug function in complex systems. Moreover, several strategies of sample preparation preceding MS analysis can be used to focus on a particular part of the proteome. Here, we highlight methods to study drug-induced effects on the proteome, with the focus on pull downs to study direct drug-target interactions, methods to study structural changes induced by ligand binding and complex formation and enrichment strategies to study post-translational modifications.

Table 1). Even though this approach is straightforward, introduction of a chemical modification, which is needed to immobilize the compound to a support, might influence the bioactivity of the small molecule. In addition, compounds that cannot be chemically immobilised cannot be analysed using a compound centric approach. An elegant solution to this problem is exemplified by approaches like the kinobead [9], mixed inhibitor bead (MIB) [10] or HDAC probes [11] in which a mixture of broad spectrum inhibitors is immobilized on beads. Using these beads, an entire enzyme class can be enriched from a complex cell lysate. Similar to a compound centric approach, these broad-spectrum binding probes can then be used in a competition experiment with a free drug. However, in contrast to a compound-centric approach, these probes are suitable for high-throughput screening, since it is no longer necessary to produce a compound-matrix structure for each individual compound of interest because the same probe can be used for each experiment [••12]. In addition, these broad binding probes can be used as global enrichment tools for enzyme classes of interest. Several enzyme classes, like kinases or HDACs, are often low abundant, despite their essential role in modulating cellular signalling, and therefore difficult to study using shotgun proteomics. By enriching a subset of enzymes, rather than profiling the proteome, changes in protein abundances and alterations of PTMs which are induced by drug treatment can now be studied in more depth. Some studies using broad-spectrum kinase enrichment have claimed a correlation between affinity and activity [10], however this has recently been opposed [•13].

A similar method to study low abundant enzymes and therefore drug function is the use of an activity-based pull down strategy (ABPs). Here a warhead binds a class of enzymes based on their specific enzymatic activity. The warhead is bound to a fluorescent or radioactive probe, which can be visualised on a denaturing gel [14]. With the introduction of MS, ABPs tags were redesigned, in which the reactive group is combined with an affinity tag like biotin. This allows the interactors to be purified and subsequently analysed from complex samples, an approach which is called activity-based protein profiling (ABPP) (see Figure 2) [15].

Figure 2



Overview of discussed pull down approaches to study direct drug-target interactions. An inhibitor immobilised on an affinity handle can be used to purify direct interactors from a complex lysate, which are subsequently analysed with MS. This approach can be used to identify both on- and off-targets. An alternative approach is the use of a broad-spectrum binding probe, which can be used to study an entire enzyme class, or in a competition assay combined with a free drug to determine drug selectivity.

Like broad-spectrum binding probes, as described, ABPPs can be used as a global enrichment strategy to study regulation of low abundant, but crucial, enzymes, or in a competition experiment with a free drug. Theoretically, an ABPP probe can be made for every enzyme class, for example methyltransferases [16], proteases [17] hydrolases [18] and kinases [9,10,19,20] (summarised in Table 1). Out of all enzyme classes, kinases are of special interest as they have been implicated in many human diseases [21], therefore several different approaches have been developed to study this enzyme class. Despite enriching the same proteins, these methods all use slightly different methodologies, making them partially complementary [22]. In either method, a consecutive PTM enrichment makes it possible to study modifications determining the regulation of the enzyme with high confidence [23].

Structural changes as evaluation of drug-target interaction

Besides affinity based chemical proteomic approaches, other techniques can be used to measure drug target engagement. Drug-target interactions will cause changes in structure and stability of the target protein, thereby changing its behaviour, which can be detected using limited proteolysis, chemical modifications or thermal denaturation (see Figure 3).

Limited proteolysis: DARTS and LiP-MS

In the Drug Affinity Responsive Target Stability assay (DARTS), a low concentration of broad specificity protease (e.g. thermolysin or Pronase) is added to cell lysate, which causes partial proteolysis at exposed regions of the proteins [24]. When a protein binds to a ligand or compound, this protein might be stabilized and protected from proteolysis, giving rise to a distinct proteolytic pattern of peptides. By comparing the intensity of proteins on a denaturing gel or the intensity of peptides by MS, proteins which are stabilized by the compound can be identified [25,•26].

An improvement of this technique is to combine the partial proteolysis with a tryptic digestion step and subsequently measure these peptides using MS [27].

Table 1

Literature overview of pull down approaches. Table of literature describing pull down approaches. Listed are affinity matrix: single or multiple (i.e. single inhibitor/compound or library immobilised on stationary phase), enzyme class targeted by the probe, a description of the design of the affinity or activity compound used for pull down and predicted targets, if listed in the respective paper.

Affinity matrix	Enzyme class	Affinity/Activity compound	Known/predicted targets	Reference
Single	Kinase	Dasatinib	c-Src; c-Abl	[7]
Single	Kinase	Gefitinib	EGFR	[8]
Single	Methyltransferase	3-Deazaneplanocin A (DzNeP)	*S*-adenosyl-L-homocysteine hydrolase (SAHH); EZH2	[61]
Single	Lipase	Orlistat	Fatty acid synthase (FAS)	[62]
Single	E3 ligase complex	Thalidomide		[63]
Single	PARP1/2 family	XAV939	Wnt pathway	[6]
Single	Kinase	MLN8237	Aurora A Kinase (AKA)	[64]
Single	Kinase	PF-6274484; PCI-32765; Inhibitor 5[a], 7[b]; Afatinib	EGFR; BTK	[65]
Single	Kinases (ATP probe)	Lysine in the ATP pocket		[20]
Single	Proteases	Active site cysteine or serine		[17]
Single	Glycoside hydrolases	2-deoxy-2-fluoroxylobioside		[18]
Single	Methyltransferases	*S*-adenosyl-L-homocysteine (SAH) core		[16]
Single	Serine hydrolases	TAMRA-fluorophosphonate (FP-TAMRA)		[66]
Multiple	Methyltransferases	12 small molecules containing tetrazole library (Gn; $n = 1-12$)	DNMT 1	[67]
Multiple	HDACs	SAHA, givinostat		[11]
Multiple	Kinases (CTx-0294885)	Multiple kinase inhibitors		[68]
Multiple	Kinases (kinobeads)	Several inhibitors; beads		[9,12,19]
Multiple	Kinases (MIBs)	Several inhibitors; multi-column		[10]
Multiple	Metalloproteases	Probe library of succinyl Hx scaffolds (HxBPyne probes)		[69]

[a] Inhibitor 5: (R,E)-1-(3-(4-Amino-3-(4-phenoxyphenyl)-1H-pyrazolo [3,4-d]pyrimidin-1-yl)piperidin-1-yl)but-2-en-1-one.

[b] Inhibitor 7: (E)−N-(4-((3-Chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl)-4-(dimethylamino)but-2-enamide.

This allows proteins to be measured on the proteome-wide level, instead of only detecting high abundant proteins using gel-based strategies. Others have used the same approach but focussed the MS analysis on the peptide level, called limited proteolysis mass spectrometry (LiP-MS) [28,•29]. By comparing the intensity of specific peptides in the limited proteolysis condition versus a trypsin-only control, the exact region in which a structural change occurs can be determined.

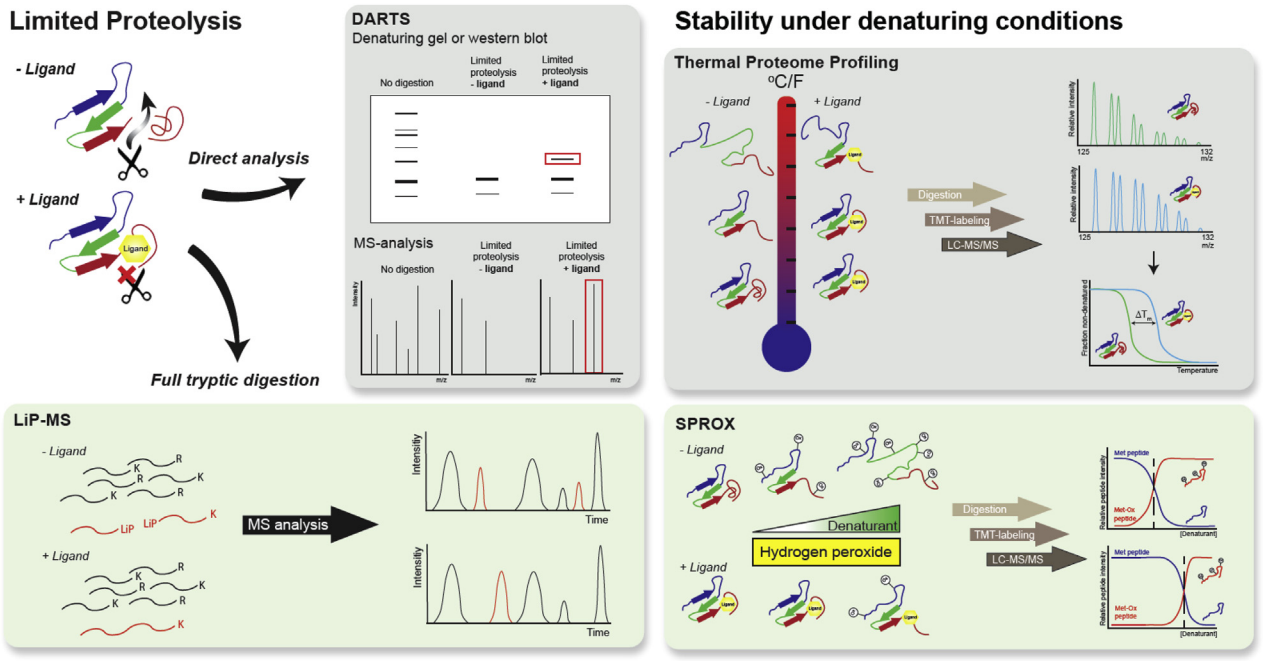
A limitation of DARTS or LiP-MS is the possible laborious optimization of the partially proteolytic conditions for every experiment. In addition, it might be difficult to detect the proteolytic protected peptides in the complex background of unaffected peptides, although this could be circumvented using fractionation. Furthermore, limited proteolysis is only applicable to soluble proteins due to the native extraction conditions.

An attractive and straightforward alternative is called Pulse Proteolysis [30,31]. In Pulse Proteolysis, the lysate is shortly incubated with a broad specificity protease in the presence or absence of a ligand and at different concentrations of denaturant. By only shortly incubating the lysate with protease, folded proteins will be unaffected while unfolded proteins will be digested. Subsequently, the lysate is analysed on a gel and by comparing the intensities of the bands at different denaturant concentrations, a conclusion can be made concerning the stability of the protein. Bands of interest can then be subjected to in-gel digestion and identified using mass spectrometry. This technique can also be used to measure the stability of membrane proteins [32].

Chemical modification: SPROX and SMTA

An alternative method of determining protein structure and stability is Stability of Proteins from Rate of Oxidation (SPROX) [33,34]. In SPROX, hydrogen peroxide induced oxidation of globally protected methionine residues at increasing concentrations of chemical denaturant is used as a measure for stability. The addition of chemical denaturant will unfold the protein, making previously protected methionine residues susceptible for oxidation. By MS-based quantification of the rate of oxidation at increasing concentration of denaturant, a conclusion can be made on the stability of the protein in either the presence or absence of drugs [35−37]. Subsequently, it is possible to measure binding affinity and domain-based stabilization. A limitation of this technique is the sole application to proteins containing protected methionine residues. In addition, SPROX needs a large amount of input material. Lastly, SPROX is not amendable to

Figure 3



Structural changes as evaluation of drug-target interactions. Overview of the different principles to evaluate protein-drug target engagement effects. In limited proteolysis a broad specificity protease is used to cleave exposed regions of the protein. This altered digestion pattern is detected using denaturing gel in DARTS, after which the protein can be identified using MS. In LiP-MS the limited proteolysis step is followed by total digestion using trypsin and detection using MS. In TPP, the ligand stabilizes the protein against thermal denaturation, which can be detected by a shift in melting curve. This method can be used to identify drug-ligand interactions on a protein level. Similarly, in SPROX the rate of unfolding due to a chemical denaturant is determined by oxidizing exposed methionine residues by hydrogen peroxide. This method is able to gain detailed information of the structural changes due to protein–protein interactions on a peptide level.

membrane proteins, where the chemical induced denaturation is limited due to the lipid bilayer. To increase the scope of SPROX to peptides which do not contain methionine residues, s-methyl thioacetimidate (SMTA) can been used, which causes amidination of buried lysine residues [38]. Since SPROX and SMTA protocols target different amino acids, they are complimentary, thereby improving the effectiveness of this technique [38].

Temperature based: CETSA and TPP

When a protein is subjected to high temperatures, it will start to irreversibly unfold, revealing the hydrophobic core and precipitate. However, when a ligand or compound is bound, the protein stabilizes and the melting point of the complex increases. Consequently, an increased melting point of a protein is indicative of complex formation. In the Cellular Thermal Shift Assay (CETSA), aliquots of lysate or intact cells, which are treated with a drug or vehicle, are subjected to a range of temperature points after which the remaining soluble protein amount is determined using western blot [39,•40]. Drug-target interactions can be detected by a difference in protein intensity in vehicle versus drug conditions. The limitation of this method lies in the antibody based detection, as it requires prior knowledge of the target to be analysed as well as the availability of specific antibodies for the target.

In order to deal with these limitations and to allow full proteome probing, this technique has been combined with quantitative MS, called thermal proteome profiling (TPP) [41]. The use of TMT 10 plex for ten different temperature points makes it possible to measure a complete melting curve in a single LC-MS/MS run. A big advantage of temperature based profiling is that it can be used on both intact cells and lysates. When drug treatment is performed on intact cells, the cellular environment remains intact. Therefore, it might be possible to not only detect the direct target of the compound but also downstream processes due to (in) activation of the target protein.

However, this method has some limitations. Firstly, TPP does not achieve peptide level resolution, when compared to LiP-MS, therefore it is difficult to determine thermal sensitivity of a specific protein domain. Also, in the original protocol, only soluble proteins were analysed while insoluble proteins, such as membrane proteins, were removed before analysis. Later however, it was shown that by adding a mild detergent to the lysis buffer membrane proteins are solubilized, making them

amendable for analysis [42]. Furthermore, some proteins do not show thermal stabilization due to ligand binding, limiting the detection of target engagement effects of these proteins. Lastly, the huge amount of data generated by measuring the whole proteome at the different temperature points can make it difficult to extract valuable information.

Numerous variations on TPP exist. Savitski et al. [41] developed a TPP-concentration range assay, which can be used to measure the affinity between ligand and proteins. Becher et al. [••43] developed 2D-TPP, in which they combined 12 temperature points with 5 different drug concentrations. The 2D-TPP assay is more sensitive and can be used to directly measure affinities of stabilized proteins. Lastly, Leuenberger et al. [••44] combined the possibilities of TPP with LiP-MS, which they used to measure thermostability on a proteomic scale with domain-level resolution. Recently, Tan et al. [45] developed a variant of TPP which they called thermal proximity coaggregation, which can be used to monitor intracellular protein complex dynamics.

Post-translational modifications

Many small molecule inhibitors that are being developed target enzymes that catalyse post-translational modification (PTMs) of effector proteins. A multitude of possible PTMs affect protein function in terms of turnover, activity, localisation and complex formation, thereby changing a variety of signalling pathways in the cell [46]. Therefore, it is equally important to study direct compound-target interactions as well as the indirect effects of drugs on the PTM landscape upon drug exposure, during treatment and upon development of drug resistance. Peptides carrying PTMs have low stoichiometry compared to their unmodified counterpart, making enrichment before MS analysis a recommended step. PTM enrichments have been developed for several different PTMs, using various approaches [••47]. A large body of literature exists on enrichment techniques (a selection has been listed in Table 2), therefore this is not further addressed in this review.

The analysis of PTMs can be used to answer a variety of biological questions in the study to discover drug function. First of all, the discovery based MS approaches are able to determine both direct on-target [48,49], as well as off-target effects [50]. For example, differences in phenotype and additive effect upon treatment with two MEK inhibitors, targeting the same pathway, could be explained by resolving drug induced changes on a PTM level [51]. In addition to detecting immediate effects, changes on a PTM level induced by long-term treatment can explain drug resistance development. For example, the study of the phospho-proteome of a Lapatinib resistant breast cancer cell line led to the discovery of altered signalling pathways responsible for the acquired resistance, in addition to genetic mutations and changes in protein expression [52]. Lastly, since PTMs can determine the activity or function of a protein, they can be used as a biomarker. By integrating data of phosphoproteomes combined with information on responses, mutation and other metadata, a phospho-proteomic signature reveals hallmarks of disease related signalling [53]. Subsequently, a prediction can be made about the drug response in a specific patient, which can play a role in the determination of the type of treatment.

Some general challenges in PTM enrichment have been described, of which some excellent reviews have been written [••47]. A major technical challenge is the large amount of starting material often necessary. Increased enrichment specificity and MS sensitivity are necessary to perform larger scale, high-throughput experiments and/or to perform analysis on clinical material [54]. In addition, some modifications, like cysteine oxidation, are highly unstable, so sample handling should be optimised to preserve these PTMs. In addition to specific enrichment, MS/MS settings can be optimized, fitted to

Table 2

Literature overview of PTM enrichment strategies. Many different approaches of PTM enrichment have been developed over recent years. The method of enrichment is largely determinded by the type of PTM, although different approaches are possible, allowing for different applications. A large body of literature exist on the topic, therefore a small sample of PTM enrichment approaches described in literature are listed here as a starting point of further exploration of the desired topic.

Post-translational modification	Method of enrichment	Reference
Phosphorylation (pTyr)	immunopurification	[70]
Ubiquitination	immunopurification	[71,72]
SUMOylation	IMAC and immunopurification	[73,74]
Lysine and arginine Methylation	immunopurification	[75,76]
Lysine acetylation	immunopurification	[77,78]
O-GlcNAcylation	immunopurification	[79]
Phosphorylation (pHis)	Immunopurification	[80]
Cysteine oxidation	Affinity purification	[81,82]
ADP-ribosylation	PARP/PARG inhibitors combined with affinity-purification	[83,60]
Glycosylation	Leptin magnetic bead array (LeMBA)	[84]
O-GlcNAcylation	biotin alkyne probe combined with click-chemistry	[85,86]
Glycosylation	Boronic acid purification	[87]
Ubiquitination	ThUBD	[88]
Phosphorylation (pSer, pThr)	Fe3+-IMAC	[89]
Phosphorylation (pSer, pThr, pTyr)	TiO4 chromatography; pY antibodies	[90]
Phosphorylation (pSer, pThr)	TiO4 chromatography	[91]

the PTM in question, to get the best quality data [•55]. Different fragmentation methods (HCD [56], ETD [57], EtHCD [58], or a combination of techniques [59,60]) are available for optimal fragmentation of (labile) PTMs. These methods allow both the high confidence sequence determination as well as reliable site localization of modified peptides.

Outlook

MS based methods are able to provide detailed knowledge which advances our understanding of unexplored cellular signalling pathways, creating opportunities to resolve drug function. MS-based technology can find novel drug targets, determine off-targets or show an unknown potential in an alternative application than a drug was designed for. Furthermore, proteome wide screening of biological systems can result in understanding of resistance development against an inhibitor or can reveal useful biomarkers. Despite its potential, clinical application of MS technology is still in an early phase. MS experiments are time consuming when analytical depth is required, moreover, a great part of the scientific community does not have access to MS facilities.

Over the last decades, exciting technical advances in the MS field have made it possible to reach further in the proteome than ever before. The current challenge, however, is the vast amount of data produced, risking the true meaning of it to be lost. Therefore, it is important to focus the data acquisition by combining different analytical tools with MS analysis in order to reduce complexity and extract the most useful information from separate parts of a proteome. For this purpose, existing protocols can be altered to make them compatible with MS. Although several examples already exist in literature, more development needs to be done for chemical strategies to be implemented into MS based research. Ultimately, the right combination of inventive methods coupled together with the extensive reach of MS analysis will bring the most unambiguous answers to biological challenges.

Conflict of interest

The authors declare no conflict of interest.

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- •• of outstanding interest

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