Direct and indirect approaches to identify drug modes of action

Lindsay B. Tulloch[1], Stefanie K. Menzies[2], Ross P. Coron[3], Matthew D. Roberts[4], Gordon J. Florence[1]* & Terry K. Smith[1]*

[1] *EaStChem School of Chemistry and School of Biology, Biomedical Sciences Research Complex*, University of St Andrews, St Andrews, Fife KY16 9ST, UK.

[2] *Current address: Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, Missouri 63110 USA*

[3] *Current address: Department of Parasites and Insect Vectors, Institut Pasteur, 25 Rue du Docteur Roux, 75015, Paris, France*

[4] *Current address: Department of Nutritional Sciences, Faculty of Health and Medical Sciences, University of Surrey, Guildford GU2 7WG, UK*

* Corresponding authors
Email: tks1@st-andrews.ac.uk (TKS) / gjf1@st-andrews.ac.uk (GJF)

Keywords: Drug mode of action, Target ID, Genomics, Transcriptomics, Proteomics, Metabolomics, Affinity chromatography, Photo-affinity labeling.

Abbreviations

cDNA	complementary DNA
CETSA	cellular thermal shift assay
CRISPR	clustered regularly interspaced short palindromic repeats
DARTS	Drug affinity response target stability
dsRNA	double-stranded RNA
ESI	electrospray ionisation
HIV	human immunodeficiency virus
ICAT	isotope-coded affinity tagging
IEX	ion exchange
iTRAQ	isobaric tags for relative and absolute quantitation
LC	liquid chromatography
LC-MS	liquid chromatography – mass spectrometry
LC-MS/MS	liquid chromatography – tandem mass spectrometry
MALDI	matrix-assisted laser desorption ionisation
MOA	mode of action
mRNA	messenger RNA
MS	mass spectrometry
MS/MS	tandem mass spectrometry
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
RNAi	RNA interference
RT-qPCR	reverse transcription – quantitative polymerase chain reaction
SDS-PAGE	sodium dodecyl sulphate – polyacrylamide gel electrophoresis
SILAC	stable isotope labelling with amino acids in cell culture
sgRNA	single guide RNA
SRM	selected reaction monitoring
TICC	target identification by chromatographic co-elution

Abstract

Phenotypic assays are becoming increasingly more common among drug discovery practices, expanding drug target diversity as lead compounds identified through such screens are not limited to known targets. While increasing diversity is beneficial to the drug discovery process and the fight against disease, the unknown modes of action of new lead compounds can hamper drug discovery as, in most cases, the process of lead compound optimisation is made difficult due to the unknown nature of the target; blindly changing substituents can prove fruitless due to the inexhaustible number of potential combinations, and it is therefore desirable to rapidly identify the targets of lead compounds developed through phenotypic screening. In addition, leads identified through target-based screening often have off-target effects that contribute towards drug toxicity, and by identifying those secondary targets the drugs can be improved. However, the identification of a leads mode of action is far from trivial and now represents a major bottleneck in the drug discovery pipeline. This review looks at some of the recent developments in the identification of drug modes of action, focusing on phenotype-based methods using metabolomics, proteomics, transcriptomics and genomics to detect changes in phenotype in response to the presence of the drug, and affinity-based methods using modified/unmodified drug as bait to capture and identify targets.

Introduction

Paul Ehrlich is widely regarded as the founder of modern drug discovery through his conception of chemotherapy – the use of a single molecule "magic bullet" with pathogen-specific binding to selectively kill a pathogen but not the host (1,2). By screening a library of small synthetic compounds against parasite-infected rodents (the process of phenotypic screening, or phenotype-based drug discovery), he identified compounds that were active against the parasites, then improved compound potency and selectivity (the process of lead optimisation) by adding substituents, and in 1907 produced Salvarsan, the first synthetic drug created by structure-based drug discovery (3).

Phenotype-based drug discovery remained the principle method of identifying new lead compounds until advances in molecular biology and genomics in the 1980s (namely PCR (4,5), nucleotide sequencing (6,7) and recombinant protein biology) and technological development (high-throughput chemical synthesis and high-throughput screening) allowed the switch to target-based drug discovery (8). This process involves the screening of compound libraries against purified recombinant target protein, typically targeting proteins with key roles in disease pathogenesis or proteins specific to the disease of interest (several reviews detailing and comparing phenotype- and target-based drug discovery methodologies are available (9–12) so will not be discussed herein). While there have undoubtedly been a large number of successes from target-based methods (11,13), attrition rates are high as lead compounds identified and optimised in vitro often fail in subsequent cell-based trials due to a range of issues including poor cell uptake, preferential off-target binding; unexpected toxicity in host cells; or even non-essentiality of the target (8). Risk of encountering some of these shortcomings can be minimised by restricting targets to those known to be essential and druggable, eliminating known and likely toxins from chemical libraries, and by restricting chemical libraries to compounds that are "drug-like". As such, drug targets in target-based methods are limited to those *believed* to be good and which can be expressed, purified and assayed, and therefore exclude the majority of exploitable targets from development; there are an estimated 600-1500 exploitable human targets (14), yet several analyses indicate that <700 of them are targeted by the ~2000 unique therapeutically active drugs currently approved for use in Western medicine (13,15). In addition, it is becoming ever more apparent that many drugs have complex modes of action, with primary and secondary targets of differing affinities and it may not be possible to anticipate such secondary effects from target-based screening practises.

Technological developments in high throughput screening, availability of chemical libraries and the availability/culturability of infectious organisms and cell lines as models for numerous human diseases, coupled with the shortcomings of target-based drug discovery have led to a resurgence in phenotypic screening both within academia and the pharmaceutical industry (11). As phenotypic screening does not focus upon any particular target directly, it removes target-bias and potentially expands the target repertoire by allowing the identification of new lead compounds with unknown targets and those previously thought undruggable. Furthermore, it allows the testing of drug combinations (i.e., drug pairs which have different targets) in their biological context and is proving effective in the fight against viruses (for example, HIV, (16)) and cancers (17–19). However, a major drawback of phenotypic

screening is that much of the time the target(s) and mode of action (MOA) of the newly developed drug are unknown, making the drug difficult to further optimise for potency and selectivity, and as a consequence target identification through drug MOA studies represents a major bottleneck in the drug discovery pipeline. Investigations of drug modes of action are not limited to studies following lead compound identification by phenotypic screening; lead compounds identified through target-based screening seldom bind solely to the target screened against and it is prudent to identify additional interacting proteins (which may include desirable secondary targets, undesirable off-targets or highlight drug uptake receptors/systems) to help improve compound efficacy/selectivity. Furthermore, by identifying the undesirable off-targets, lead compounds that would normally be rejected can instead be modified to reduce their off-target affinities, thereby reducing the high attrition rate that currently plagues drug discovery. A number of methodologies have recently been developed to help identify targets of drugs and drug modes of action, including direct/affinity-based methods that show the physical interaction between drugs and their targets, and indirect/phenotypic-based methods that show drug-induced changes in phenotype from which targets and modes of action can be inferred. Herein we review recent advances in drug MOA methodologies that can be applied to identify protein targets of newly-identified (or pre-existing) drugs using examples from different areas of drug discovery research.

Indirect / phenotype-based methods

A drugs mode of action can often be determined by comparing the phenotype of drug-treated cells with untreated cells. In some cases, the number of potential drug targets leading to the given phenotype may be relatively small and a focused analysis on those potential targets is likely suitable. However, in most cases the number of potential targets leading to the phenotype, such as cell death, is potentially enormous and global analyses are required to determine the drug targets. Some approaches investigate the acute effects of drug treatment to determine what happens to cells as they progress towards the end phenotype, while others investigate how cells adapt to chronic drug treatment and develop resistance mechanisms. Physical changes that occur during a cell-cycle or in cell morphology can sometimes indicate a drugs mode of action, but often the morphological change is too subtle or non-descript, and one needs to look at phenotypic changes at the molecular level. Over the past decades, advancements in the “omics” fields – genomics, transcriptomics, proteomics and metabolomics – have led to the generation of cost-effective, high throughput methodologies to analyse and compare molecular systems at an unprecedented scale. While they have been used primarily to characterise and understand disease, they can also allow analysts to determine how drugs work at the molecular level.

Genomics

The post-genomic era has significantly advanced/enhanced the drug discovery process, and large-scale genomic studies can be incorporated into techniques for drug target identification. The creation of organisms resistant to a candidate drug can be an effective method to identify specific drug targets (Fig 1A). For example, one study identified leucyl-tRNA synthetase as the target of antifungal tavaborole by culturing *Saccharomyces cerevisiae* in the presence of the drug to generate spontaneous resistance mutants, whose DNA was digested into 4-10 kb fragments, inserted into a yeast vector, and screened for the ability to protect tavaborole-susceptible cells by tavaborole selection (20). Sequencing of vector inserts from the small number of surviving colonies revealed that all contained mutations in leucyl-tRNA synthetase, which prevented the drug from binding. Advances in nucleotide sequencing technology allowed a similar technique to be applied by Ioerger and colleagues, who identified targets of several antibacterials in *Mycobacterium tuberculosis,* except entire genomes of resistant mutants were sequenced and aligned to genome sequences of the parental strain to detect deletions or resistance-associated polymorphisms (21). In this study the authors detected mutations in direct drug targets, transcription factors regulating the targets, and a pro-drug activator, demonstrating that this technique can not only identify targets in an unbiased manner, but also highlight potential resistance mechanisms, which may be of added value as it can guide the development of combination therapies to prevent the emergence of resistance in the field.

Genomic screens are limited to cells that spontaneously develop resistance, and to circumvent this, the Horn laboratory has applied genome-wide RNA interference (RNAi) libraries to identify proteins targeted by drugs or associated with resistance in *Trypanosoma brucei* (22–25), the parasite responsible for African sleeping sickness (Fig 1B). By transforming parasites with tetracycline-

inducible expression plasmids containing fragments of sheared *T. brucei* genomic DNA, they generated a library of cells, each expressing a random dsRNA to silence the expression of an individual gene, so that the entire genome could be knocked down in a mixed population of cells. The addition of sub-lethal drug concentrations then allowed parasites expressing irrelevant RNAi sequences to survive, while those expressing RNAi to proteins targeted by the drug were sensitised and killed, and those expressing RNAi to proteins involved in drug uptake thrived as less drug was taken up. Sequencing of RNAi species remaining using high throughput sequencing technology indicated which category each silenced gene fell into, implicating several proteins in drug uptake and drug activity. A complementary approach was used by Begolo et al (26), who developed an overexpression library in *T. brucei* by inserting genome fragments into ectopic expression vectors, providing cells with resistance to difluoromethylornithine and DDD85646 when overexpressing their

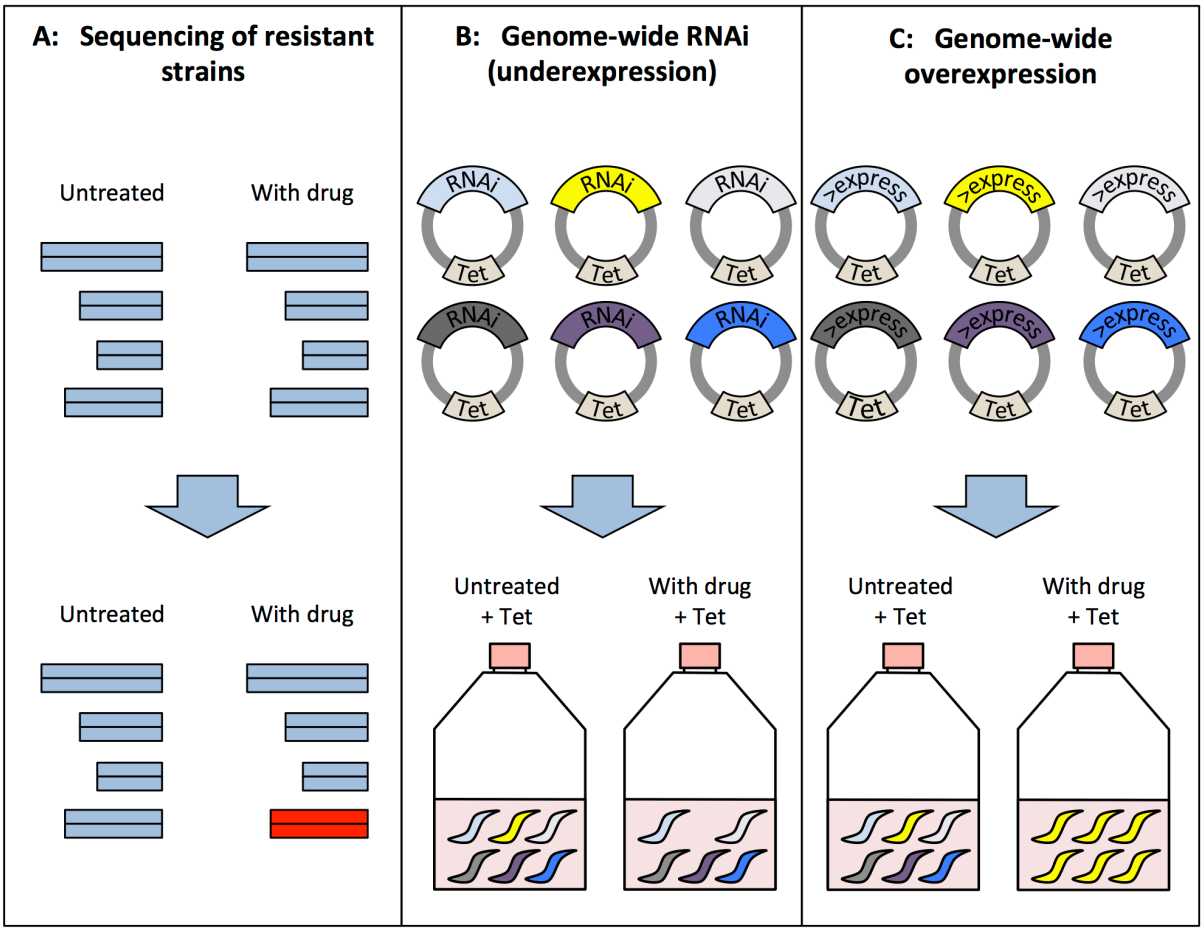

Fig 1. Genomics methods for target identification.

A) Sequencing of resistant strains. *Cells can adapt over time to overcome selective pressure from drugs (symbolised by the mutation of one or more genes, i.e. from blue to red). Genome-wide deep sequencing of resistant strains can reveal which genes have mutated to highlight potential targets of the drug, as well as indicate proteins that may be involved in drug uptake, trafficking and/or metabolism.*

B) Genome-wide RNAi (underexpression). *A genome-wide RNAi library can be generated by digesting an entire genome and inserting small DNA fragments with < 1 gene into RNAi expression plasmids. Transfection of the library into cells results in knock-down expression of a single gene in each cell. Cells expressing the RNAi for a given drug target (in this example, yellow) will be sensitised to the drug and killed at normally sub-lethal drug concentrations, removing that RNAi from the library. Sequencing the RNAi of surviving clones can reveal the drugs target by its absence from the drug-treated culture.*

C) Genome-wide overexpression. *Similar to RNAi, except gene products are expressed to overexpress one protein or protein fragment per cell. Cells overexpressing a given drug target will be desensitised to the drug and will survive normally lethal drug concentrations. Sequencing the overexpressed gene of surviving clones will reveal the drugs target and genes associated with resistance.*

targets, ornithine decarboxylase and N-myristoyltransferase respectively (Fig 1C), and together, these genome-wide knockdown/in techniques replicate the effects of classical reverse genetics in a high-throughput manner.

Not all organisms are amenable to RNAi, and the recent discovery of the CRISPR/Cas9 system, and its subsequent use in a wide range of organisms to study loss-of-function (reviewed in (27)), may replace the use of RNAi screens in future drug target identification efforts. Cas9 is a bacterial DNA repair endonuclease which, when expressed in eukaryotic cells, introduces double strand breaks in DNA matching the sequence of single guide RNA (sgRNA) templates. Endogenous DNA double-strand break repair pathways then repair the damage using a DNA template, and it is therefore possible to knockout individual genes in Cas9-transfected cells using DNA templates with frameshift deletions. Shalem and co-workers generated a library of A375 human melanoma cells in which >18,000 genes were singly knocked out, and they identified several genes involved in resistance to the BRAF protein kinase inhibitor vemurafenib following vemurafenib-selection (28). Another study has engineered Cas9 to act as a sgRNA-mediated DNA-binding protein to upregulate specific gene expression (29), and it is likely that this system will be valuable for drug target identification in eukaryotic overexpression libraries in the future.

Transcriptomics

While genomic approaches typically elude to chronic effects (genomic adaptions) of drug treatment, transcriptomics (several methodologies of which are reviewed in (30)) can be used to determine both chronic and acute effects, as the transcriptome contains genomic sequence data, but at levels that dynamically reflect the needs of the cell, and which change in response to drug treatment. Burczynski and co-workers analysed the effects of 100 drugs and toxic compounds on this latter aspect of transcriptomics by quantifying transcript levels in drug/compound-treated HepG2 cells using DNA microarrays (31). Following drug/compound treatment, they reverse-transcribed mRNA transcripts to Cy3/Cy5-fluorescently labelled complementary DNA (cDNA), which was subsequently hybridised to prefabricated chips containing arrays of DNA sequences covering 250 human genes, and quantified relative transcript abundance by fluorescence imaging (Fig 2B). They reported that compounds with similar modes of action had similar gene expression signatures, for example, DNA-damaging drugs such as cisplatin induced expression of DNA repair proteins, and microarrays have subsequently been used extensively for drug mode of action and toxicology investigations. Despite their widespread use, a major limitation of microarrays is that target sequences must be known and prefabricated on the chips, so unknown sequences cannot be detected. This may appear trivial as the number of organisms that have been genome-sequenced increases and the number of unknown sequences correspondingly decreases, but it means that mutations that occur in response to drug treatment may be missed. In addition, the analogue nature of the signal (emitted fluorescent light) makes it difficult to confidently detect and quantify low abundance species.

The development of next-generation sequencing that has facilitated genome-wide sequencing has also transformed transcriptomics, as direct sequencing of cDNA transcripts (RNA-seq) allows their simultaneous identification and binary quantitation (Fig 2C). Mortazavi and co-workers mapped and quantified the transcriptomes of three distinct mouse tissues by sequencing and scoring >40 million cDNA fragments per tissue and aligning each to a reference genome to determine the gene from which they were transcribed (32). In total they detected expression of >11000 genes to high certainty, ~5000 of which were expressed in a tissue-specific manner and included genes known to be specifically expressed in each of them. In addition, they detected splice variants for ~3500 genes, and RNA-seq should therefore be ideal for the analysis of the acute effects of drug treatments in cells. To assess the chronic effects of drug treatment, Wacker et al deep sequenced cDNA transcripts from human HTC-116 cell clones made resistant to BI2536, a Polo-like kinase 1 inhibitor, and detected two point mutations in the BI2536-binding site of Polo-like kinase 1 (33). Although their approach was similar the genome sequencing of drug resistant cells by Ioerger (21), non-coding introns, which are removed during mRNA splicing, are not sequenced during RNA-seq, making it more cost-effective than genome-wide sequencing.

Once differences between expression levels in particular genes have been ascertained, they can be verified using reverse transcriptase coupled with quantitative PCR (RT-qPCR, Fig 2D), which involves the amplification of specific transcript cDNAs with primers specific to the gene of interest and then

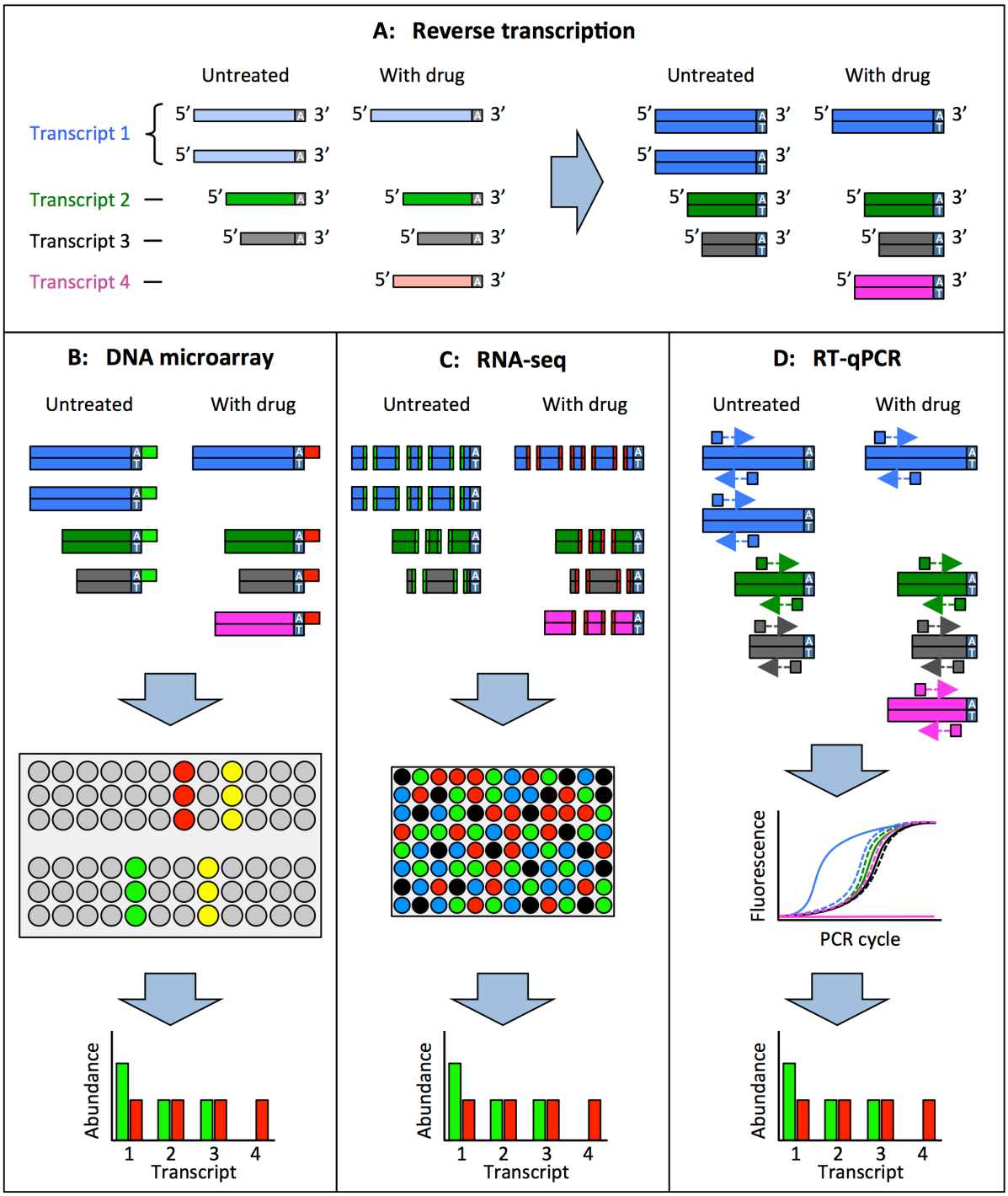

Fig 2. Transcriptomics methods for target identification.

A) Reverse transcription. *To quantify the number of mRNA transcripts mRNA is first reverse transcribed to cDNA, which is more stable and amenable to downstream applications. In this example, the drug causes a decrease in expression of transcript 1 and an increase in transcript 4 expression.*

B) Dual-colour DNA microarray. *cDNA is labelled with a sample-specific fluorescent probe and then hybridised to an array of prefabricated gene sequences. Relative fluorescence shows which sequences are present in each sample and their relative abundances. A disadvantage is that sequences that are not on the chip cannot be detected.*

C) RNA-seq. *cDNA is restriction-digested and tagged with sample-specific octameric sequences (shown as green/red). All fragments are simultaneously sequenced using next-generation sequencing to provide accurate quantitation and information on splice variation and generation of single nucleotide polymorphisms.*

D) Reverse transcription quantitative-PCR (RT-qPCR). *Primers specific to the transcripts of interest are used to amplify those cDNA. The use of fluorescent probes in TaqMan assays quantifies the relative number of amplicons to accurately show the relative abundance of each transcript in the sample. Impractical for transcriptome-wide analyses, but ideal for following up hits.*

quantifying the amount of cDNA amplified (34). Different methods have been reported for transcript cDNA quantitation, but the gold standard is the TaqMan real-time assay (35), which requires the addition of a short transcript-specific probe labelled with a fluorophore at one end and quencher at the other. The quencher absorbs the energy emitted by the fluorophores when in close proximity in the intact probe (through Förster resonance energy transfer), either in free solution or bound to the target cDNA, but as the Taq polymerase elongates the unlabelled primer, it degrades the labelled probe bound to the target allowing the released fluorophore to emit light. The fluorescence is measured after every PCR cycle and the increase in fluorescence is proportional to the amount of amplicon synthesised, resulting in an accurate quantitation of target transcript.

Proteomics

As mRNA transcripts are translated into proteins, the effects that drugs have on a cells transcriptome may be reflected in the cells proteome, and can be determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and peptide mass fingerprinting (36–38). In shotgun mass fingerprinting, which is the most widely-used form of proteomics, proteins from a complex mixture (such as an entire protein extract from cells) are enzymatically digested and peptides are separated and analysed by data-dependent acquisition, whereby parent ions (peptides) are sampled and the most abundant species are fragmented to yield fragmentation 'fingerprints' from which parent ions can e sequenced (Fig 3A). Sequences are then assigned to proteins when compared against a reference database by the MASCOT software (39), and relative protein abundance determined from the number of reads obtained. While genome-wide and transcriptome sequencing analyses allow the identification of new (mutated) sequences in response to drug adaption by aligning the sequences against genomic templates, no such application currently exists for proteomic analyses; peptides containing single or multiple mutations that are not in the reference database will not be assigned to a protein, so peptide mass fingerprinting cannot readily identify drug targets from the generation of mutant proteins. However, peptide mass fingerprinting is ideal for detecting changes in protein expression levels or in their post-translational modifications. It is possible to analyse drug-treated and untreated samples separately, however, their direct quantitative comparison by shotgun proteomics is difficult due to the somewhat inconsistent nature of data-dependent acquisition, and it is often preferable to differentially label proteins, during or after drug treatment, from each sample and subsequently analyse them simultaneously to reduce sampling variation – reviewed by Steen and Pandey (40). For example, Oda and colleagues used stable isotope labelling with amino acids in cell culture (SILAC (41)) to label proteins from one sample by growing cells in medium supplemented with heavy (^{15}N-labelled) amino acids, while cells from a second sample were grown in unlabelled medium. Proteins incorporating the heavy amino acid were characteristically heavier than those from cells in unlabelled medium, differentiating the proteins from the different samples and allowing their relative quantitation. Similar techniques have been used to label proteins from primary cells, which often cannot be cultured for sufficient time to allow for SILAC. For example, isotope-coded affinity tagging (ICAT, (42)) involves chemically adding differential tags to cysteine residues immediately prior to LC-MS/MS so that it can be determined from which sample each Cys-containing peptide came from. Alternatively, isobaric tags for relative and absolute quantitation (iTRAQ, (43)) labels primary amines with differential tags, allowing every peptide to be allocated to a sample, and was the method utilised by Yang et al to determine the mode of action of a bactericidal houttuyfonate by dissecting the global protein alterations in *Streptococcus pneumoniae* in response to the compound (44). Recently SWATH-MS (45) has been developed as a data-independent alternative to shotgun peptide mass fingerprinting and overcomes some of the limitations of data-dependent acquisition. Rather than sampling parent ions and fragmenting the most abundant species, SWATH-MS fragments every parent ion over a given m/z range to more accurately and reproducibly count ions, allowing the label-free comparison of different samples from separate analyses.

A targeted approach often follows the 'global' analysis, and proteins of interest can be enriched to detect low-abundance species. For example, Pandy et al identified several targets of the epidermal growth factor receptor, which phosphorylates target tyrosine residues following receptor activation, by enriching tyrosine-phosphorylated proteins with an anti-phosphotyrosine antibody and analysing captured proteins by MS/MS (46). Using a similar technique, Pan et al determined the effects of U0126 and SB202190, two mitogen-activated protein kinase inhibitors, on the phosphoproteome of cancer cells by capturing phosphoproteins through titanium oxide affinity chromatography coupled with MS/MS (47).

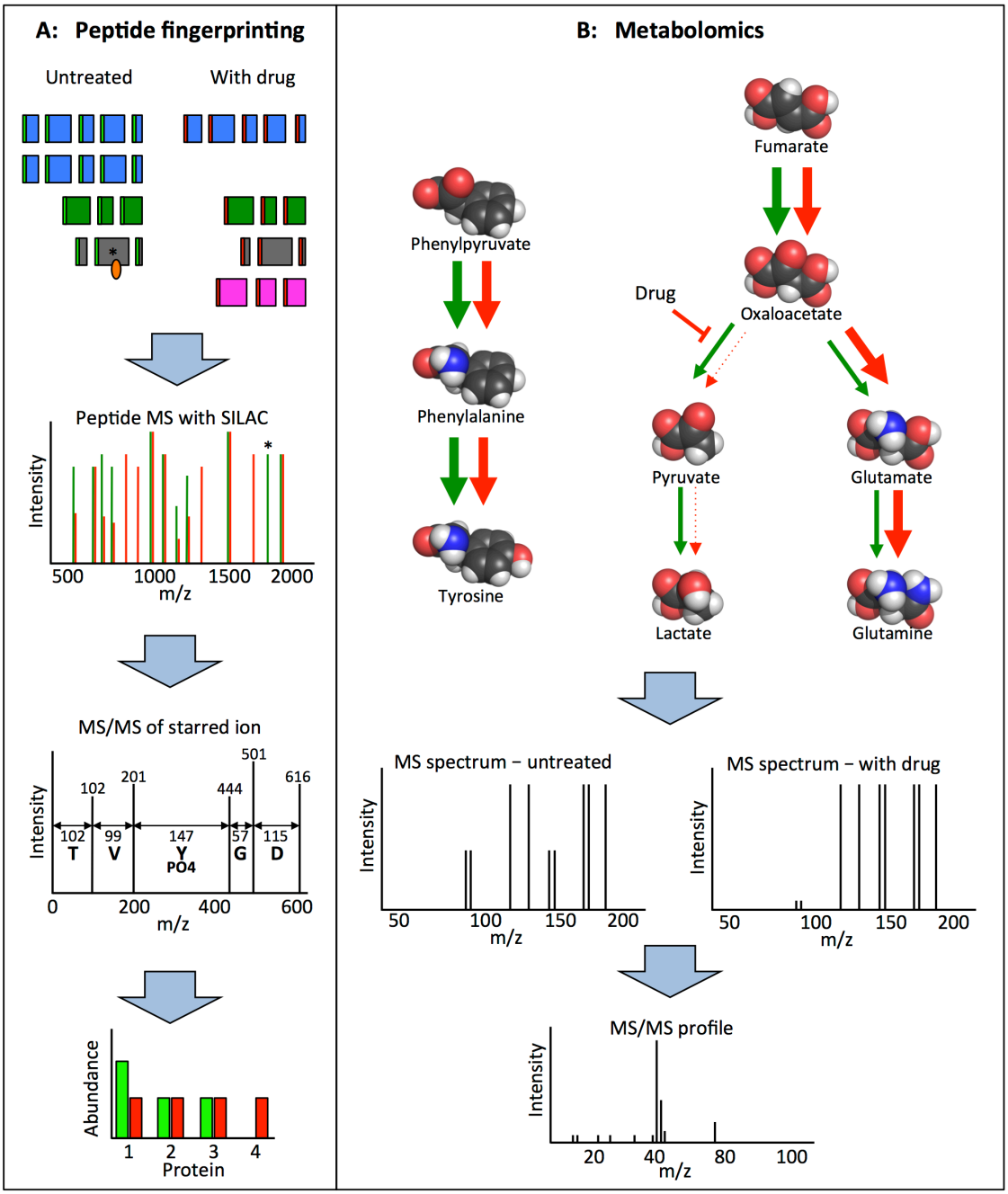

Fig 3. Mass spectrometry methods for target identification.

A) Proteomics and peptide mass fingerprinting using SILAC. *Continuing the example from Fig 3, the drug causes a decrease in expression of protein 1 and an increase in protein 4 expression. In addition, it inhibits the phosphorylation (orange oval) of protein 3. Proteins from untreated cells are unlabelled (green), while proteins from drug-treated cell grown in ^{15}N-labelled medium are heavy (red). Proteins are digested and peptides analysed by LC-Ms/MS. The first round of MS detects the mass and abundance of each parent ion (peptide). As light and heavy peptides differ slightly in mass, their relative abundance from each sample can be determined. Subsequent fragmentation of a selected parent ion at a peptide bond yields a unique peptide mass fingerprint from which the sequence and posttranslational modifications can be determined, allowing peptides to be assigned to proteins for quantitation.*

B) Metabolomics. *Schematic representation of two metabolic pathways showing metabolic flux. The size of the arrows represents the rate of flux in untreated (green arrows) and drug-treated (red arrows) cells. In this example the drug inhibits the conversion of oxalic acid to pyruvate, decreasing the amount of pyruvate and lactate produced, and excess oxalic acid is instead metabolised through an alternative route to glutamic acid and glutamine. Relative metabolite concentrations can be determined by MS, and a comparison between untreated and drug-treated can reveal which metabolites change. Subsequent fragmentation of a selected parent ion reveals a metabolites fingerprint from which the relative quantities of isomers can be determined.*

Changes in protein abundance or posttranslational modification need to be verified, and western blotting provides a simple means to do this. Western blotting can readily be applied to show differences in protein levels or posttranslational modification using primary antibodies raised against the target, while dual colour scanning of blots using secondary antibodies conjugated to near far-red fluorophores (700 nm and 800 nm) allows test and control samples to be analysed simultaneously.

Metabolomics

Drugs that inhibit metabolic enzymes may cause accumulation of their substrates and depletion of their products (Fig 3B), so it is possible to determine a drugs target by detecting metabolomic changes in response to drug treatment. It is often desirable first to compare the 'global' metabolomes of drug-treated and untreated cells in an untargeted, unbiased manner. Mass spectrometry (MS) methods, such as electrospray ionisation (ESI, (48)) or matrix-assisted laser desorption ionisation (MALDI, (49)), are ideally suited to the comparative untargeted analysis of entire metabolomes from whole cell extracts as, depending on the device and sensitivity used, MS can differentiate molecules differing by a single electron and it is therefore theoretically possible to determine the precise chemical makeup of virtually every mass peak detected, assign each to a metabolite from a mass library, and quantify the relative abundance of each chemical entity based on peak intensity. However, the inability to distinguish different metabolites with the same chemical formulae (isomers) makes MS alone unpractical for comparative metabolomics. Tandem mass spectrometry (MS/MS) can resolve this by fragmenting sample ions of interest (precursor or parent ions) into product or daughter ions, with each molecule yielding a unique fragment fingerprint which can be used to identify the isomers present and their relative abundance. To assist in metabolite identification, the METLIN metabolomics database (50,51) and Human Metabolome Database, HMDB (52), provide comprehensive libraries, with excellent online tools for querying MS/MS data. Alternatively, liquid chromatography (LC) can be used to separate samples into compound classes prior to MS, and LC-MS has been used to identify the effects of miltefosine on lipid metabolism in *Leishmania* (53) and for mechanistic investigations of several antiprotozoal drugs (54). While the application of LC offers great selectivity for the separation of biomolecules/drugs, it should be highlighted that an MS compatible solvent system is required and can reduce separation efficacy. Additionally, a higher sample concentration is required to mitigate chromatographic losses, resulting in an overall compromise in sensitivity compared to MS alone. Further detail on the applications of LC-MS in drug development can be found in reviews by Lee (55) and Lu (56), while considerations for untargeted metabolomics generally are detailed extensively by Dunn (57) and Patti (58).

Once an untargeted, global approach has identified a metabolic area that appears affected by treatment with a drug of unknown mode of action, a targeted approach can be used to focus the investigation and gain sensitivity and/or certainty that the changes detected are in the metabolites presumed (reviewed in (59)). One method is to reduce sample complexity by extracting the relevant metabolite species for analysis, such as a lipid extract for lipidomics (returning to the miltefosine mode of action as example (53)), rather than analysing the whole cell extract. An alternative approach is to use selected reaction monitoring (SRM) methods, whereby the mass spectrometer is set to only select and record specific precursor/product ion combinations from the unfractionated complex mixture, greatly increasing detection sensitivity as the majority of a sample can then be rejected and ignored. The use of stable isotope-labelled (commonly deuterium, ^{15}N or ^{13}C) metabolites can also be highly informative in focused analyses, as they allow the analyst to follow metabolic flux from a given metabolite (60,61) and verify changes detected in global analyses.

Matrix combination screening

Combination therapies are gaining momentum as an efficient means of tackling disease. Indications that can rapidly adapt to selective pressure, such as HIV and cancer, can quickly overcome monotherapies, but by combining drugs that have different targets or mechanisms of action, the number of resistance mutations required to gain selective advantage are increased (16). Drug combinations can also be used to reduce toxic side effects by allowing lower concentrations of toxic drugs to be used where they work synergistically. High throughput matrix combination screening is now being used to identify suitable drug combinations for cancer (17–19) and malaria (62), whereby cell viability assays are carried out with drug pairs in perpendicular serial dilution checkerboards using 384-well or 1536-well tissue culture plates. Such checkerboards can show whether drug pairs synergise (for example by targeting different targets in parallel pathways), antagonise (by hitting

different targets in the same pathway) or work additively (by targeting the same or unrelated targets). By screening a drug of unknown action with drugs of known action the analyst can build an interaction network for the drug of unknown action to indicate how the drug works. Drugs with the same modes of action typically have similar checkerboard profiles when tested pairwise with other drugs, so by comparing the checkerboard profiles of the unknown drug with those of known action it may be possible to determine its target. High-throughput matrix combination screening is very much in its infancy and is likely to become a powerful tool in drug target identification, particularly as drug discovery shifts towards combination therapies generally to protect new discoveries from the emergence of resistance.

Direct / affinity-based methods

Affinity-based methods are able to demonstrate direct physical interaction between a drug and its target but usually require prior chemical modification of the drug. For example, the binding of radiolabelled or fluorescent drug analogues to their targets in native protein gels or microscopy can show the size and/or cellular localisation of their targets, while drug immobilised to a matrix can be used to capture targets (described in more detail below) for their identification by peptide fingerprinting. However, the direct interaction between an unmodified drug and its target can also be detected by changing the stability or rigidity of the target through their physical interaction, and applications detecting these changes are also discussed.

Direct interactions with modified drug

Ideally a drugs target can be identified by peptide mass fingerprinting (36,37) following its direct and selective capture by the drug immobilised to a matrix using affinity chromatography (Fig 4A). The principle of affinity chromatography was first demonstrated by Lerman in 1953 when a tyrosinase was partially purified from a crude mushroom extract through its binding to cellulose-immobilised p-azophenol (a substrate-like inhibitor) and subsequent elution by mass action of competitive inhibitor in solution (63). Since this pioneering work, affinity chromatography has been used extensively for target identification purposes. A limitation of affinity chromatography is that target(s) seldom enrich alone as many proteins non-specifically bind to the matrix, so to address this Ong and colleagues combined affinity capture with SILAC to distinguish targets of several kinase inhibitors (64), including the broad-spectrum kinase inhibitor K252a, from non-specific binders. In one example they used one K252a-bound matrix to capture ^{13}C-labelled target proteins and another to capture unlabelled target proteins pre-incubated with competing soluble K252a, and analysed both eluates simultaneously by LC-MS/MS. They identified 48 potential targets (proteins absent from the control eluate), 46 of which were known kinases or kinase-associated proteins from a background of >500 non-specific contaminants (proteins present in the control eluate), demonstrating the sensitivity and selectivity of the procedure. While the principle of affinity capture is simple, the process is not always trivial as the coupling of the inhibitor to the matrix may impede target-binding (either by the covalent attachment of a required binding atom, such as an H-bonding amide, directly to the matrix, or by steric hindrance from the matrix if the attached drug cannot reach into the targets binding cavity); the drug-target interaction may not be strong enough to retain the target while washing away non-targets; the target may not be sufficiently soluble (or solubilised) for it to wash on and off the matrix. Many of these factors are reviewed by Rix and colleagues (65) so will not be discussed here.

Inhibitors with only weak interactions with their targets (K_i >50 μM) may not be amenable to affinity chromatography directly, but photoaffinity labelling (reviewed by Smith and Collins (66)) can resolve this issue by covalently attaching a photoreactive analogue of the drug to its target (Fig 4B). Suitable photoreactive substituents include azides, benzophenones and diazirines, which decompose under UV-irradiation to highly reactive nitrenes, diradicals or carbenes respectively, which then rapidly covalently bind to neighbouring molecules. We recently developed bi-functional photoaffinity analogues of trypanocidal bis-tetrahydropyran 1,4-triazole compounds to identify their targets in *T. brucei* by attaching diazirine and alkyne moieties to our lead compound (67). After employing a pulse-chase methodology to traffic the bi-functional compound to its target in live cells, we photo-activated the diazirine to covalently conjugate the compound to its target in its biological context. Subsequently we “clicked” on fluorescent Cy5.5-azide by copper-catalysed azide-alkyne cycloaddition (67,68) to microscopically visualise the cellular localisation of its target, and “clicked” on biotin-azide for target isolation by streptavidin affinity pull-down and target identification (68), utilising the ultra-high affinity of streptavidin for biotin (K_d <1 pM (69)). The versatility of the alkyne moiety coupled with its small size

makes it an ideal chemical handle for such photoaffinity labelling techniques. In addition, the alkyne, which is typically absent from biological tissue, can be used as a Raman label to visualise drug uptake in live cells by spontaneous Raman microscopy, which detects laser-induced vibrations of specific molecular functional groups (reviewed by Tipping and co-workers (70)).

Direct interaction with unmodified drug

The modification of a drug to show direct interaction with its targets is not always ideal as it may involve lengthy structure-activity-relationship analyses and time/skill synthesising analogues. Several "label-free" methods have been employed to overcome these limitations and identify targets from complex protein mixtures using unmodified drugs. Target identification by chromatographic co-elution (TICC) is one such method and involves detecting a change in the chromatographic retention time of a drug when it binds its target, and identifying the target by LC-MS/MS. As proof of principle, Chan and colleagues demonstrated the methotrexate-dihydrofolate reductase interaction by TICC using non-denaturing ion exchange chromatography (IEX) coupled with LC-MS in SRM mode to scan for the presence of methotrexate in IEX fractions (71). They first performed IEX-LC-MS on methotrexate alone to determine the methotrexate retention time and suitable daughter ions to scan for, and then analysed methotrexate with purified dihydrofolate reductase and observed a shift in the retention time of methotrexate to that of dihydrofolate reductase, indicating that methotrexate was bound to the protein. Finally, they added methotrexate and dihydrofolate reductase to an *E. coli* cell lysate to represent a complex protein mixture. Again, the retention time of methotrexate was different from unbound drug, and peptide fingerprinting of the relevant fraction confirmed that dihydrofolate reductase was present. Chan and colleagues also demonstrated the drug-target interaction for several other drugs using the same procedure, demonstrating the general utility of this method for identifying drug targets.

A drug-protein interaction can often change the thermostability of the protein by, for example, holding the protein in a more rigid conformation, or exerting pressure to destabilise the protein. Lomenick et al developed a method named drug affinity responsive target stability (DARTS, Fig 4C)) that relies on the reduced susceptibility of proteins to proteolysis when a protein is bound by a drug, due to an increased stability of target protein upon drug binding (72). They subjected purified immunophilin FKBP12 to proteolysis by the protease subtilisin in the presence/absence of several drugs including FKBP12 inhibitors rapamycin and FK506, and demonstrated that FKBP12 was only protected from proteolysis when its own inhibitors were present. To test the method at a proteome-wide scale, they subjected human Jurkat cell lysates to proteolysis in the presence/absence of the EF-1α inhibitor didemnin, and SDS-PAGE revealed that a protein of the same mass as EF-1α was protected by the inhibitor. Protein sequencing by LC-MS/MS confirmed that the band was EF-1α, demonstrating that DARTS can be used to identify targets of drugs in a label-free manner. Advantages to this technique are that the targets of weak inhibitors are able to be identified, and whole cell lysates, as opposed to purified protein, can be utilized (72). Furthermore, this technique can be used in any organism, and is not restricted to those in which genetic knockdown or overexpression libraries can be easily created. The authors describe limitations to the technique, such as the influence of binding affinity, protein abundance, and the susceptibility of the protein to endogenous proteolysis. Further refinement of DARTS is currently aimed at identifying conditions under which lower abundance proteins can be identified and the optimal mass spectrometry conditions for peptide fragment identification (72).

In a similar vein to DARTS, proteins interacting with ligand are less sensitive to heat-induced unfolding. Thermal shift (also known as differential scanning fluorimetry) is a rapid and inexpensive technique which has been used to study this thermal stabilisation in a wide range of proteins under varying conditions or upon the binding of an interacting ligand. In this method, the temperature at which a protein unfolds is measured by an increase in fluorescence caused by the binding of an environmentally sensitive dye (73,74) (e.g. SYPRO Orange) to exposed hydrophobic regions (Fig 4D). The increase in fluorescence can be monitored in a real-time PCR instrument (75) and is plotted as a function of temperature, generating a sigmoidal curve that can be described by a two-state transition. The inflection point of the transition curve is then calculated using the Boltzmann equation. The binding of low molecular weight ligands can increase the thermal stability of a protein (76–79) and the binding affinity measured from the shift in the unfolding temperature (or melting point) termed ΔT_m.

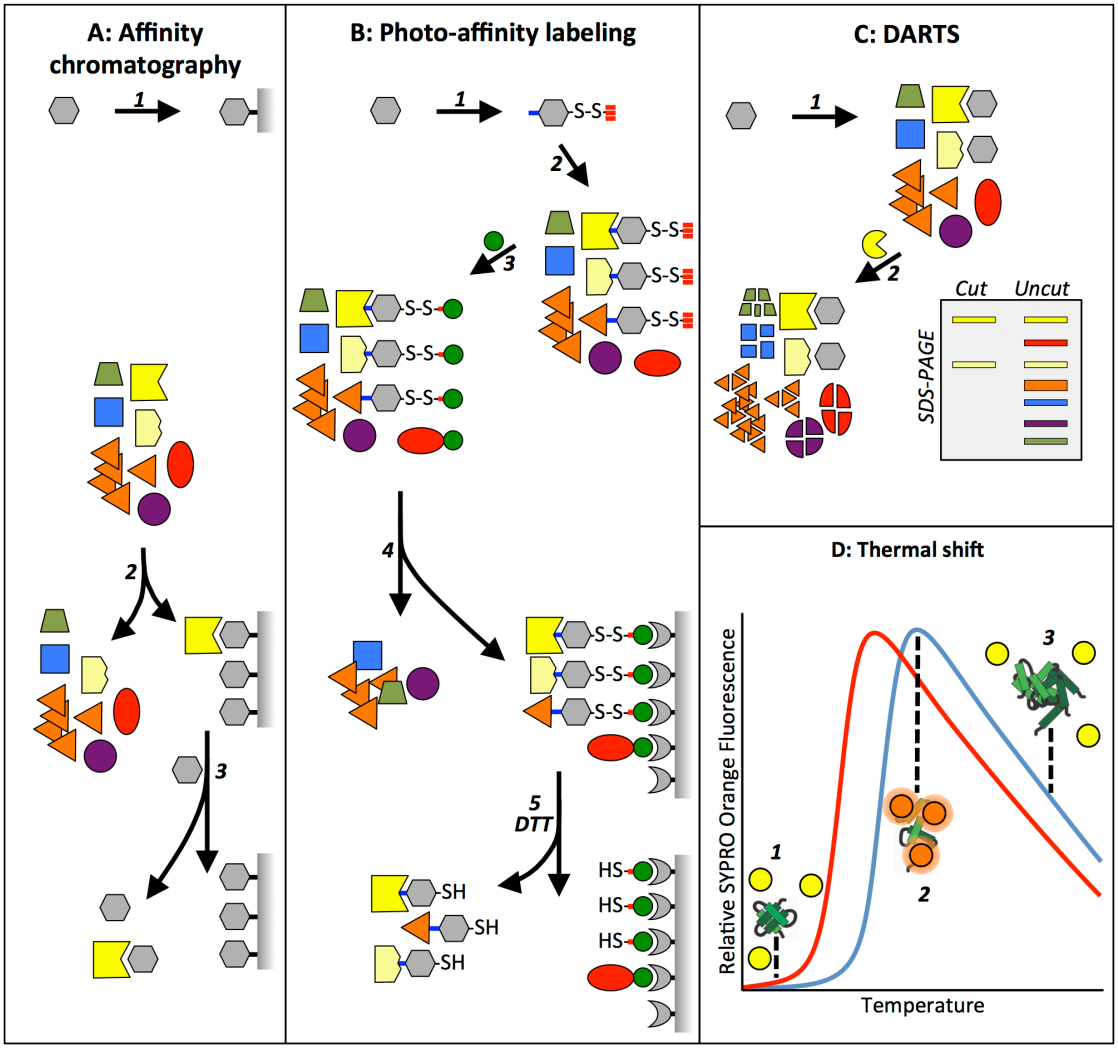

Fig 4. Affinity-based methods for target isolation for subsequent identification.

A. Affinity chromatography: *1) The drug (grey hexagon) is first modified by immobilising it to a matrix. 2) The high affinity target (yellow shape) is captured by passing the lysate over the immobilised drug and non-bound proteins are washed away. Note that the low-affinity target (light yellow shape) is not captured by this method. 3) The high-affinity target is eluted by the addition of soluble drug.*

B. Photo-affinity labeling: *1) The drug (grey hexagon) is functionalised to a photo-affinity probe with photoreactive moiety (blue line) and alkyne handle (red lines) attached via a disulfide bond. 2) The photo-affinity probe is trafficked to its target(s) in vivo and then covalently cross-linked to them following UV-activation of the photoreactive moiety. Both high- and low-affinity targets (dark and light yellow shapes respectively) are labeled specifically with this technique, while highly abundant proteins (orange triangles) may be non-specifically labeled due to UV-activation of probe not bound to target. 3) Proteins are extracted and an azide-affinity tag (e.g., biotin-azide, green circle) is "clicked" on. Some non-specific binding of the tag to non-target proteins (red oval) may occur. 4) Biotin affinity tagged proteins are captured on streptavidin agarose (grey cups) and non-tagged proteins are washed away. 5) Photo-affinity labeled proteins can be eluted through cleavage of the disulfide bond with reducing agent (e.g., DTT). High- and low-affinity targets will be enriched, along with highly abundant contaminants labeled non-specifically, while proteins bound non-specifically to the affinity tag will remain attached.*

C. DARTS: *1) The unmodified drug (grey hexagon) is added to a cell lysate. 2) The presence of the drug stabilises the protein, making it less susceptible to proteolysis by proteases (yellow pacman). When cut and uncut samples are run in a gel the targets should remain visible following SDS-PAGE and subsequent staining. The bands can then be cut out and target protein extracted from the gel for subsequent identification by MS.*

D. Thermal shift: *1) The protein in its native conformation binds no SYPRO orange (yellow circles) as hydrophobic regions of the protein are buried. 2) As the temperature increases, the protein unfolds due to thermal instability and SYPRO orange binds to hydrophobic regions as they become exposed, causing the dye to emit fluorescence at 610 nm (orange circles). 3) Following a peak in intensity, a gradual decline in fluorescence is observed as protein precipitates and aggregates, displacing bound SYPRO orange (yellow circles). The binding of a drug to its purified target can stabilize the target's secondary structure and make it less susceptible to heat denaturation (red versus blue lines).*

Thermal shift can be utilised for the screening of small-molecule and ligand libraries in the drug discovery process. Furthermore, the assay has also been utilised to screen buffer conditions in efforts to maximise protein stability during purification, storage, biochemical characterization, and crystallography trials (80,81). Unlike many other biochemical / biophysical techniques, thermal shift does not require a prior knowledge of the targets' function. Furthermore, the technique is amenable to high-throughput screening due to its multiwell format, low protein concentration requirement, and rapid protocol (82).

A number of thermal shift assay variations have been described in recent years. In addition to SYPRO Orange, alternative dyes or probes may be utilised, allowing for the screening of proteins in the presence of surfactants (DCVJ (83)) or analysis of membrane proteins (CPM (84)), BFC (85)), etc. In Fast Parallel Proteolysis (FASTpp), the thermal melting of protein domains can be determined in cell lysates through the use of a thermostable protease (86). Similarly, the Cellular Thermal Shift Assay (CETSA) allows for thermal shift measurements in the context of cell lysates, whole cells, or tissues; preserving posttranslational modifications, expression levels, and the local environment of the endogenous protein (87). Irrespective of the specific technique or dye utilised, thermal shift may not be amenable for a small number of intrinsically disordered proteins. Furthermore, the assay provides initial evidence of ligand interactions, potentially requiring alternative, biochemical validation. Caution must also be taken during data analysis and compound ranking as the ΔT_m value of a protein in the presence of a ligand may not be a true reflection of their relative affinities.

Perspective

The identification of drug modes of action is becoming an essential component of the drug discovery process. The resurgence of phenotypic screening, yielding lead compounds with unknown modes of action creates a bottleneck, in that further lead optimisation is often difficult while their targets remain unknown. In addition, there is growing realisation that lead compounds identified through target-based screening do not solely interact with the target screened against, but also likely interact with several unknown targets, which potentially include desirable secondary targets, undesirable off-targets and components of drug uptake and efflux systems. These unknown targets can often result in lead compound rejection later in the drug discovery process after a significant investment of time and money has been spent optimising a lead compound against a purified target. However, by identifying the additional targets (and particularly off-targets), lead compounds can be further optimised to prevent their interaction and rejection from further development, thereby lowering the high attrition rate in drug discovery.

There are now a growing number of tools that greatly aid the identification of drug modes of action. Several methods can be employed to isolate targets through their direct interaction with drugs for their subsequent identification. Traditionally, a drug would be modified by coupling it to a matrix and targets identified through affinity chromatography, however, the technique is limited by the strength of thedrug-target interaction and weak interactions may be missed as weakly-bound proteins may wash too easily off the matrix. To circumvent this limitation, a drug can be modified to include a bi-functional tag comprising photo-affinity and molecular handle moieties to covalently cross-link the drug to its targets, allowing weak interactions to be detected. In addition, the versatility of the molecular handle allows the modified drug to be used in a variety of different applications. While both affinity chromatography and photo-affinity labelling can be ideal for target isolation and identification, they both require the chemical modification of the drug, and considerable time and experimental experience may be required to chemically synthesise and test suitable drug analogues that retain biological activity. For projects that lack this ability, or for an alternative approach, methods that instead show direct interaction between targets and unmodified drugs can be employed. TICC uses MS in SRM mode to identify chromatographic protein fractions containing unmodified drug, but requires a specialised mass spectrometrist, and as with affinity chromatography, weak interactions may be missed. However, DARTS and thermal shift, which take advantage of the changes in protein thermostability in response to drug-binding, require little specialist skill and can therefore be applied in any laboratory.

Several methodologies exist to determine drug modes of action indirectly, by attributing changes in cell phenotype to drug activity. Advances in the "omics" fields, primarily developed as of cost-effective, high throughput methodologies for the detailed characterisation and understanding of disease, now allow global analyses of entire genomes, transcriptomes, proteomes and metabolomes

such that drug-treated cells can be compared with untreated cells at the molecular scale. For example, genomics and transcriptomics can be used to detect genetic mutations that arise from selection through chronic drug treatment. The genome-wide or transcriptome-wide sequencing of resistance mutants can reveal mutations in primary and secondary targets, as well as in drug trafficking systems, providing valuable insight not only into the drugs mode of uptake and action, but also as to how cells adapt to drug pressure to overcome the effects of the drug. This in turn can direct researchers towards suitable drugs to pair with the newly identified drug to develop drug combinations that make resistance less likely in the field, which not only protects the newly-developed drug from premature obsolescence, but improves clinical outcomes, which is the main focus of drug discovery. Several options are available for the generation of genetic mutants, including the spontaneous formation of resistance mutants, or their forced creation using genome-wide RNAi, genome-wide overexpression, or CRISPR/Cas9 systems, where they are available and amenable to the disease of interest. Transcriptomics, proteomics and metabolomics can be used to detect changes in cell signalling and metabolism in response to acute drug treatment, whereby transcriptomics and proteomics may be most suitable for drugs that affect signalling, while metabolomics may be most suitable for drugs that affect metabolism. There is a large degree of overlap between the “omics” methodologies and it may be worthwhile applying more than one strategy to gain confidence in the findings and also to develop a greater understanding of how the drug of interest works. Ultimately there is no ‘one-size-fits-all’ strategy and the optimal methodologies for drug mode of action study that are adopted by any given project may be dictated by the tools available to the laboratory, coupled with the methods applicable for their disease of interest and chemical properties of the drug.

References

1. Kaufmann SHE. Paul Ehrlich: founder of chemotherapy. Vol. 7, Nature reviews. Drug discovery. England; 2008. p. 373.
2. Bosch F, Rosich L. The contributions of Paul Ehrlich to pharmacology: a tribute on the occasion of the centenary of his Nobel Prize. Pharmacology. 2008;82(3):171–9.
3. Steverding D. The development of drugs for treatment of sleeping sickness: a historical review. Parasit Vectors. 2010 Mar;3(1):15.
4. Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, et al. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science. 1985 Dec;230(4732):1350–4.
5. Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, et al. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science. 1988 Jan;239(4839):487–91.
6. Maxam AM, Gilbert W. A new method for sequencing DNA. Proc Natl Acad Sci U S A. 1977 Feb;74(2):560–4.
7. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci U S A. 1977 Dec;74(12):5463–7.
8. Lindsay MA. Target discovery. Nat Rev Drug Discov. 2003 Oct;2(10):831–8.
9. Swinney DC. Phenotypic vs. target-based drug discovery for first-in-class medicines. Clin Pharmacol Ther. 2013 Apr;93(4):299–301.
10. Zheng W, Thorne N, McKew JC. Phenotypic screens as a renewed approach for drug discovery. Drug Discov Today. 2013 Nov;18(21–22):1067–73.
11. Moffat JG, Rudolph J, Bailey D. Phenotypic screening in cancer drug discovery - past, present and future. Nat Rev Drug Discov. 2014 Aug;13(8):588–602.
12. Moffat JG, Vincent F, Lee JA, Eder J, Prunotto M. Opportunities and challenges in phenotypic drug discovery: an industry perspective. Nat Rev Drug Discov. 2017 Aug;16(8):531–43.
13. Rask-Andersen M, Almen MS, Schioth HB. Trends in the exploitation of novel drug targets. Nat Rev Drug Discov. 2011 Aug;10(8):579–90.
14. Hopkins AL, Groom CR. The druggable genome. Nat Rev Drug Discov. 2002 Sep;1(9):727–30.
15. Santos R, Ursu O, Gaulton A, Bento AP, Donadi RS, Bologa CG, et al. A comprehensive map of molecular drug targets. Nat Rev Drug Discov. 2017 Jan;16(1):19–34.
16. Bock C, Lengauer T. Managing drug resistance in cancer: lessons from HIV therapy. Vol. 12, Nature reviews. Cancer. England; 2012. p. 494–501.
17. Held MA, Langdon CG, Platt JT, Graham-Steed T, Liu Z, Chakraborty A, et al. Genotype-selective combination therapies for melanoma identified by high-throughput drug screening. Cancer Discov. 2013 Jan;3(1):52–67.
18. Mathews Griner LA, Guha R, Shinn P, Young RM, Keller JM, Liu D, et al. High-throughput combinatorial screening identifies drugs that cooperate with ibrutinib to kill activated B-cell-like diffuse large B-cell lymphoma cells. Proc Natl Acad Sci U S A. 2014 Feb;111(6):2349–54.
19. Schaffer M, Chaturvedi S, Davis C, Aquino R, Stepanchick E, Versele M, et al. Identification of potential ibrutinib combinations in hematological malignancies using a combination high-throughput screen. Leuk Lymphoma. 2017 Jul;1–10.

20. Rock FL, Mao W, Yaremchuk A, Tukalo M, Crepin T, Zhou H, et al. An antifungal agent inhibits an aminoacyl-tRNA synthetase by trapping tRNA in the editing site. Science. 2007 Jun;316(5832):1759–61.
21. Ioerger TR, O'Malley T, Liao R, Guinn KM, Hickey MJ, Mohaideen N, et al. Identification of new drug targets and resistance mechanisms in Mycobacterium tuberculosis. PLoS One. 2013;8(9):e75245.
22. Baker N, Alsford S, Horn D. Genome-wide RNAi screens in African trypanosomes identify the nifurtimox activator NTR and the eflornithine transporter AAT6. Mol Biochem Parasitol. 2011 Mar;176(1):55–7.
23. Alsford S, Eckert S, Baker N, Glover L, Sanchez-Flores A, Leung KF, et al. High-throughput decoding of antitrypanosomal drug efficacy and resistance. Nature. 2012 Jan;482(7384):232–6.
24. Horn D. High-throughput decoding of drug targets and drug resistance mechanisms in African trypanosomes. Parasitology. 2014 Jan;141(1):77–82.
25. Glover L, Alsford S, Baker N, Turner DJ, Sanchez-Flores A, Hutchinson S, et al. Genome-scale RNAi screens for high-throughput phenotyping in bloodstream-form African trypanosomes. Nat Protoc. 2015 Jan;10(1):106–33.
26. Begolo D, Erben E, Clayton C. Drug target identification using a trypanosome overexpression library. Antimicrob Agents Chemother. 2014 Oct;58(10):6260–4.
27. Shalem O, Sanjana NE, Zhang F. High-throughput functional genomics using CRISPR-Cas9. Nat Rev Genet. 2015 May;16(5):299–311.
28. Shalem O, Sanjana NE, Hartenian E, Shi X, Scott DA, Mikkelson T, et al. Genome-scale CRISPR-Cas9 knockout screening in human cells. Science. 2014 Jan;343(6166):84–7.
29. Konermann S, Brigham MD, Trevino AE, Joung J, Abudayyeh OO, Barcena C, et al. Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. Nature. 2015 Jan;517(7536):583–8.
30. Lowe R, Shirley N, Bleackley M, Dolan S, Shafee T. Transcriptomics technologies. PLoS Comput Biol. 2017 May;13(5):e1005457.
31. Burczynski ME, McMillian M, Ciervo J, Li L, Parker JB, Dunn RT 2nd, et al. Toxicogenomics-based discrimination of toxic mechanism in HepG2 human hepatoma cells. Toxicol Sci. 2000 Dec;58(2):399–415.
32. Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B. Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nat Methods. 2008 Jul;5(7):621–8.
33. Wacker SA, Houghtaling BR, Elemento O, Kapoor TM. Using transcriptome sequencing to identify mechanisms of drug action and resistance. Nat Chem Biol. 2012 Feb;8(3):235–7.
34. Becker-Andre M, Hahlbrock K. Absolute mRNA quantification using the polymerase chain reaction (PCR). A novel approach by a PCR aided transcript titration assay (PATTY). Nucleic Acids Res. 1989 Nov;17(22):9437–46.
35. Heid CA, Stevens J, Livak KJ, Williams PM. Real time quantitative PCR. Genome Res. 1996 Oct;6(10):986–94.
36. Henzel WJ, Billeci TM, Stults JT, Wong SC, Grimley C, Watanabe C. Identifying proteins from two-dimensional gels by molecular mass searching of peptide fragments in protein sequence databases. Proc Natl Acad Sci U S A. 1993 Jun;90(11):5011–5.
37. Wolters DA, Washburn MP, Yates JR 3rd. An automated multidimensional protein identification technology for shotgun proteomics. Anal Chem. 2001 Dec;73(23):5683–90.
38. Yates JR 3rd. The revolution and evolution of shotgun proteomics for large-scale proteome analysis. J Am Chem Soc. 2013 Feb;135(5):1629–40.
39. Perkins DN, Pappin DJ, Creasy DM, Cottrell JS. Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis. 1999 Dec;20(18):3551–67.
40. Steen H, Pandey A. Proteomics goes quantitative: measuring protein abundance. Trends Biotechnol. 2002 Sep;20(9):361–4.
41. Oda Y, Huang K, Cross FR, Cowburn D, Chait BT. Accurate quantitation of protein expression and site-specific phosphorylation. Proc Natl Acad Sci U S A. 1999 Jun;96(12):6591–6.
42. Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, Aebersold R. Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. Nat Biotechnol. 1999 Oct;17(10):994–9.
43. Ross PL, Huang YN, Marchese JN, Williamson B, Parker K, Hattan S, et al. Multiplexed protein quantitation in Saccharomyces cerevisiae using amine-reactive isobaric tagging reagents. Mol Cell Proteomics. 2004 Dec;3(12):1154–69.
44. Yang X-Y, Shi T, Du G, Liu W, Yin X-F, Sun X, et al. iTRAQ-Based Proteomics Revealed the Bactericidal Mechanism of Sodium New Houttuyfonate against Streptococcus pneumoniae. J Agric Food Chem. 2016 Aug;64(32):6375–82.
45. Gillet LC, Navarro P, Tate S, Rost H, Selevsek N, Reiter L, et al. Targeted data extraction of the MS/MS spectra generated by data-independent acquisition: a new concept for consistent and accurate proteome analysis. Mol Cell Proteomics. 2012 Jun;11(6):O111.016717.
46. Pandey A, Podtelejnikov A V, Blagoev B, Bustelo XR, Mann M, Lodish HF. Analysis of receptor signaling pathways by mass spectrometry: identification of vav-2 as a substrate of the epidermal and platelet-derived growth factor receptors. Proc Natl Acad Sci U S A. 2000 Jan;97(1):179–84.
47. Pan C, Olsen J V, Daub H, Mann M. Global effects of kinase inhibitors on signaling networks revealed by quantitative phosphoproteomics. Mol Cell Proteomics. 2009 Dec;8(12):2796–808.
48. Fenn JB, Mann M, Meng CK, Wong SF, Whitehouse CM. Electrospray ionization for mass spectrometry of large biomolecules. Science. 1989 Oct;246(4926):64–71.
49. Tanaka K, Waki H, Ido Y, Akita S, Yoshida Y, Yoshida T, et al. Protein and polymer analyses up to m/z

100 000 by laser ionization time-of-flight mass spectrometry. Rapid Commun Mass Spectrom [Internet]. 1988;2(8):151–3. Available from: http://dx.doi.org/10.1002/rcm.1290020802
50. Smith CA, O'Maille G, Want EJ, Qin C, Trauger SA, Brandon TR, et al. METLIN: a metabolite mass spectral database. Ther Drug Monit. 2005 Dec;27(6):747–51.
51. Tautenhahn R, Cho K, Uritboonthai W, Zhu Z, Patti GJ, Siuzdak G. An accelerated workflow for untargeted metabolomics using the METLIN database. Nat Biotechnol. 2012 Sep;30(9):826–8.
52. Wishart DS, Tzur D, Knox C, Eisner R, Guo AC, Young N, et al. HMDB: the Human Metabolome Database. Nucleic Acids Res. 2007 Jan;35(Database issue):D521-6.
53. Vincent IM, Weidt S, Rivas L, Burgess K, Smith TK, Ouellette M. Untargeted metabolomic analysis of miltefosine action in Leishmania infantum reveals changes to the internal lipid metabolism. Int J Parasitol Drugs drug Resist. 2014 Apr;4(1):20–7.
54. Creek DJ, Barrett MP. Determination of antiprotozoal drug mechanisms by metabolomics approaches. Parasitology. 2014 Jan;141(1):83–92.
55. Lee MS, Kerns EH. LC/MS applications in drug development. Mass Spectrom Rev. 1999;18(3–4):187–279.
56. Lu W, Bennett BD, Rabinowitz JD. Analytical strategies for LC-MS-based targeted metabolomics. J Chromatogr B, Anal Technol Biomed life Sci. 2008 Aug;871(2):236–42.
57. Dunn WB, Erban A, Weber RJM, Creek DJ, Brown M, Breitling R, et al. Mass appeal: metabolite identification in mass spectrometry-focused untargeted metabolomics. Metabolomics [Internet]. 2013 Mar;9(1):44–66. Available from: https://doi.org/10.1007/s11306-012-0434-4
58. Patti GJ, Yanes O, Siuzdak G. Innovation: Metabolomics: the apogee of the omics trilogy. Nat Rev Mol Cell Biol. 2012 Mar;13(4):263–9.
59. Dudley E, Yousef M, Wang Y, Griffiths WJ. Targeted metabolomics and mass spectrometry. Adv Protein Chem Struct Biol. 2010;80:45–83.
60. Srivastava A, Kowalski GM, Callahan DL, Meikle PJ, Creek DJ. Strategies for Extending Metabolomics Studies with Stable Isotope Labelling and Fluxomics. Metabolites. 2016 Oct;6(4).
61. Sauer U. Metabolic networks in motion: 13C-based flux analysis. Mol Syst Biol. 2006;2:62.
62. Mott BT, Eastman RT, Guha R, Sherlach KS, Siriwardana A, Shinn P, et al. High-throughput matrix screening identifies synergistic and antagonistic antimalarial drug combinations. Sci Rep. 2015 Sep;5:13891.
63. Lerman LS. A Biochemically Specific Method for Enzyme Isolation. Proc Natl Acad Sci U S A. 1953 Apr;39(4):232–6.
64. Ong S-E, Schenone M, Margolin AA, Li X, Do K, Doud MK, et al. Identifying the proteins to which small-molecule probes and drugs bind in cells. Proc Natl Acad Sci U S A. 2009 Mar;106(12):4617–22.
65. Rix U, Gridling M, Superti-Furga G. Compound immobilization and drug-affinity chromatography. Methods Mol Biol. 2012;803:25–38.
66. Smith E, Collins I. Photoaffinity labeling in target- and binding-site identification. Future Med Chem. 2015;7(2):159–83.
67. Fraser AL, Menzies SK, Gould ER, King EF, Tulloch LB, Zacharova MK, et al. Design and synthesis of broad spectrum trypanosomatid selective inhibitors. ACS Infect. Dis. 2018 In press.
68. Tulloch LB, Menzies SK, Fraser AL, Gould ER, King F, Zacharova MK, et al. Photo-affinity labelling and biochemical analyses identify the target of trypanocidal simplified natural product analogues. PLoS Negl Trop Dis. 2017;11:e0005886.
69. Green NM. Avidin. Adv Protein Chem. 1975;29:85–133.
70. Tipping WJ, Lee M, Serrels A, Brunton VG, Hulme AN. Stimulated Raman scattering microscopy: an emerging tool for drug discovery. Chem Soc Rev. 2016 Apr;45(8):2075–89.
71. Chan JNY, Vuckovic D, Sleno L, Olsen JB, Pogoutse O, Havugimana P, et al. Target identification by chromatographic co-elution: monitoring of drug-protein interactions without immobilization or chemical derivatization. Mol Cell Proteomics. 2012 Jul;11(7):M111.016642.
72. Lomenick B, Hao R, Jonai N, Chin RM, Aghajan M, Warburton S, et al. Target identification using drug affinity responsive target stability (DARTS). Proc Natl Acad Sci USA. 2009 Dec;106(51):21984–9.
73. Poklar N, Lah J, Salobir M, Macek P, Vesnaver G. pH and temperature-induced molten globule-like denatured states of equinatoxin II: a study by UV-melting, DSC, far- and near-UV CD spectroscopy, and ANS fluorescence. Biochemistry. 1997 Nov;36(47):14345–52.
74. Pantoliano MW, Petrella EC, Kwasnoski JD, Lobanov VS, Myslik J, Graf E, et al. High-density miniaturized thermal shift assays as a general strategy for drug discovery. J Biomol Screen. 2001 Dec;6(6):429–40.
75. Lo M-C, Aulabaugh A, Jin G, Cowling R, Bard J, Malamas M, et al. Evaluation of fluorescence-based thermal shift assays for hit identification in drug discovery. Anal Biochem. 2004 Sep;332(1):153–9.
76. Schellman JA. Macromolecular binding. Biopolymers [Internet]. 1975;14(5):999–1018. Available from: http://dx.doi.org/10.1002/bip.1975.360140509
77. Pace CN, McGrath T. Substrate stabilization of lysozyme to thermal and guanidine hydrochloride denaturation. J Biol Chem. 1980 May;255(9):3862–5.
78. Brandts JF, Lin LN. Study of strong to ultratight protein interactions using differential scanning calorimetry. Biochemistry. 1990 Jul;29(29):6927–40.
79. Straume M, Freire E. Two-dimensional differential scanning calorimetry: simultaneous resolution of intrinsic protein structural energetics and ligand binding interactions by global linkage analysis. Anal

Biochem. 1992 Jun;203(2):259–68.
80. Ericsson UB, Hallberg BM, Detitta GT, Dekker N, Nordlund P. Thermofluor-based high-throughput stability optimization of proteins for structural studies. Anal Biochem. 2006 Oct;357(2):289–98.
81. Vedadi M, Niesen FH, Allali-Hassani A, Fedorov OY, Finerty PJJ, Wasney GA, et al. Chemical screening methods to identify ligands that promote protein stability, protein crystallization, and structure determination. Proc Natl Acad Sci U S A. 2006 Oct;103(43):15835–40.
82. Niesen FH, Berglund H, Vedadi M. The use of differential scanning fluorimetry to detect ligand interactions that promote protein stability. Nat Protoc. 2007;2(9):2212–21.
83. Menzen T, Friess W. High-throughput melting-temperature analysis of a monoclonal antibody by differential scanning fluorimetry in the presence of surfactants. J Pharm Sci. 2013 Feb;102(2):415–28.
84. Alexandrov AI, Mileni M, Chien EYT, Hanson MA, Stevens RC. Microscale fluorescent thermal stability assay for membrane proteins. Structure. 2008 Mar;16(3):351–9.
85. Hofmann L, Gulati S, Sears A, Stewart PL, Palczewski K. An effective thiol-reactive probe for differential scanning fluorimetry with a standard real-time polymerase chain reaction device. Anal Biochem. 2016 Apr;499:63–5.
86. Minde DP, Maurice MM, Rudiger SGD. Determining biophysical protein stability in lysates by a fast proteolysis assay, FASTpp. PLoS One. 2012;7(10):e46147.
87. Jafari R, Almqvist H, Axelsson H, Ignatushchenko M, Lundback T, Nordlund P, et al. The cellular thermal shift assay for evaluating drug target interactions in cells. Nat Protoc. 2014 Sep;9(9):2100–22.