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Poly-pharmacology of existing drugs: How to crack the code?

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ABSTRACT

Drug development in oncology is highly challenging, with less than 5% success rate in clinical trials. This alarming figure points out the need to study in more details the multiple biological effects of drugs in specific contexts. Indeed, the comprehensive assessment of drug poly-pharmacology can provide insights into their therapeutic and adverse effects, to optimize their utilization and maximize the success rate of clinical trials. Recent technological advances have made possible in-depth investigation of drug poly-pharmacology. This review first highlights high-throughput methodologies that have been used to unveil new mechanisms of action of existing drugs. Then, we discuss how emerging chemo-proteomics strategies allow effectively dissecting the poly-pharmacology of drugs in an unsupervised manner.

1. Introduction

Despite being a game changer when it was first introduced in the 1960's, chemotherapy is not sufficient to cure all cancer patients. The last decade has seen the emergence of multi-OMICS technologies allowing major advances in our understanding of cancer biology and in the discovery of molecular dependencies that can be therapeutically exploited. This has led to the development of targeted therapies against those newly identified vulnerabilities, which has formed the basis of personalized cancer treatments [1–3]. However, if these approaches look promising, they are yet to benefit the majority of cancer patients [4–8].

The difficulties in the clinic are evidenced by a success rate in clinical trials of less than 5% in oncology [9]. In fact, anticancer drug development still faces multiple challenges that lead to long process cycles for new therapeutics and ever-increasing costs, threatening healthcare systems around the world [10,11]. The high attrition rate of anticancer drugs can be explained by multiple factors. First, pre-clinical models used in research can overestimate the therapeutic potential of drug candidates, thus failing to accurately predict their efficacy in the clinic. Another reason lies in the absence of studies to explore all the biological effects of drugs and completely unravel their molecular mechanisms of action (MoA) [12,13]. Indeed, despite being highly efficient at developing on-target drugs, pharmaceutical companies rarely investigate the non-canonical interactors of their drug candidates using unsupervised approaches. It is now admitted that most existing drugs, if not all, act on

several targets [14,15], beyond the one they were originally developed for. This fact has even been demonstrated for targeted therapies [16]. It is currently estimated that approved drugs have at least 11.5 known molecular targets on average [14,15]. Although this number is likely underestimated since large-scale studies characterizing the full interactome of drugs in human cells are scarce. Thus, unveiling the poly-pharmacology of drugs can help better understand their efficacy as well as anticipate their toxicities, thus facilitating their transfer and optimal use in the clinic [17].

Moreover, this high degree of poly-pharmacology could open therapeutic avenues for drug repurposing, which consists in using existing drugs for new medical indications. This opportunity is perfectly illustrated by the repurposing of thalidomide as an anticancer agent [18,19]. Indeed in the 90's, major progress was made in deciphering its complex MoA. Thanks to the discovery of its anti-inflammatory and anti-angiogenic properties, it has been tested as an anticancer agent and was approved by the FDA for the treatment of multiple myeloma in 2006. Following this approval, analogues of thalidomide have been developed and in 2022, lenalidomide became the second most profitable anticancer drug. This exemplifies how deciphering the mechanisms of action of existing drugs can have a major impact in oncology and lead to both medical and financial benefits. Drug repurposing offers various advantages over developing an entirely novel drug for a given indication. Because repurposed drugs have already proven their safety in preclinical models and in humans, the risk of failure is lower. Moreover, most of the preclinical testing and safety studies have already been

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completed leading to a reduced time for drug development. The costs for bringing a repurposed drug to market are thus much lower since they are estimated to be around US\$300 million, compared with ~\$2–3 billion for a single new molecule approval [20]. However, despite the potential of this approach, only few fortuitous success stories have emerged in recent years such as the repurposing of thalidomide in multiple myeloma [18] as mentioned above and that of β -blockers for the treatment of infantile hemangiomas [21]. This could be explained by the lack of comprehensive assessment of the MoA of existing drugs in their new indication for rationalizing their therapeutic and adverse effects and maximizing the chances of clinical trial success. In addition, by characterizing the MoA of existing drugs in-depth in their new indications, new biological effects may emerge, thus unveiling novel targetable vulnerabilities that could be further therapeutically exploited.

The disulfiram story perfectly illustrates how deconvoluting the MoA of an already-approved drug can impact its clinical use and success. Disulfiram has been employed as a treatment for alcohol dependence for more than 70 years. In the mid 2000s, a large-scale high-throughput screening (HTS) highlighted its anti-tumor potential [22]. The MoA behind remained elusive until Skrott *et al.* [23] unveiled that disulfiram targets NPL4, an adaptor of p97 segregase in 2017, more than 100 years after its initial synthesis. Moreover, by highlighting the key role of copper metabolism in its anti-tumor properties [24], they developed a method to detect copper metabolite accumulation in cells and tissues to use as a predictive biomarker.

This success story shows that deciphering the MoA of a drug is critical for an optimized use of the molecule in the clinic but still remains a complex challenge. In this review article, we discuss the technological breakthrough that took place in the last decades and have progressively allowed characterizing the poly-pharmacology of drugs.

2. High-throughput screening

The classical process to develop a new compound begins with the identification of a molecular target dysregulated in cancer cells thus showing a high therapeutic potential. Then, a completely new molecule can be developed or an existing drug that has a biological activity against this target can be found. To do so, in both cases, scientists have widely taken advantage of HTS of large chemical libraries. Thanks to technological advances made in the last decades, this kind of approaches has reduced the cost while increasing the efficiency of drug development. It is estimated that the number of data points generated by large screening programs was about 200,000 at the beginning of the 1990s, and rose to around 50 million in the early 2000s [25].

2.1. High-throughput screening of compound libraries: target-centric approaches

Historically, HTS has been used as a “target-centric” approach, which means that they are set up to identify specific modulators of a given target. Usually, it involves the generation of small compound libraries, which are screened *in vitro* at high concentrations against the target of interest. Then, medicinal chemistry programs are developed to enhance the potency, selectivity, target engagement, efficacy as well as absorption, distribution and safety of the selected molecules [26]. The HTS step can sometimes involve a library of already existing drugs. This kind of pharmacological HTS against a specific target can then unveil a completely novel target for a given drug.

2.1.1. *In vitro* screening on purified proteins

In vitro HTS implies the screening of large-scale molecule libraries against a purified protein of interest (Fig. 1A). It enables the identification of compounds that can bind or inhibit the activity of the target. One of the most successful studies relying on this kind of approach was undertaken by Hosseini *et al.*, who unveiled a new target of camptothecin and its analog SN-38, the active metabolite of irinotecan, two

widely used chemotherapeutic drugs. By screening a library of 1280 FDA-approved drugs in AlphaScreen, they found that camptothecin and SN-38 inhibit the binding of FUBP1 to its DNA target sequence FUSE [27]. This discovery adds a further possible MoA for these drugs, which, until now, had mostly been linked to the inhibition of topoisomerase I [28,29].

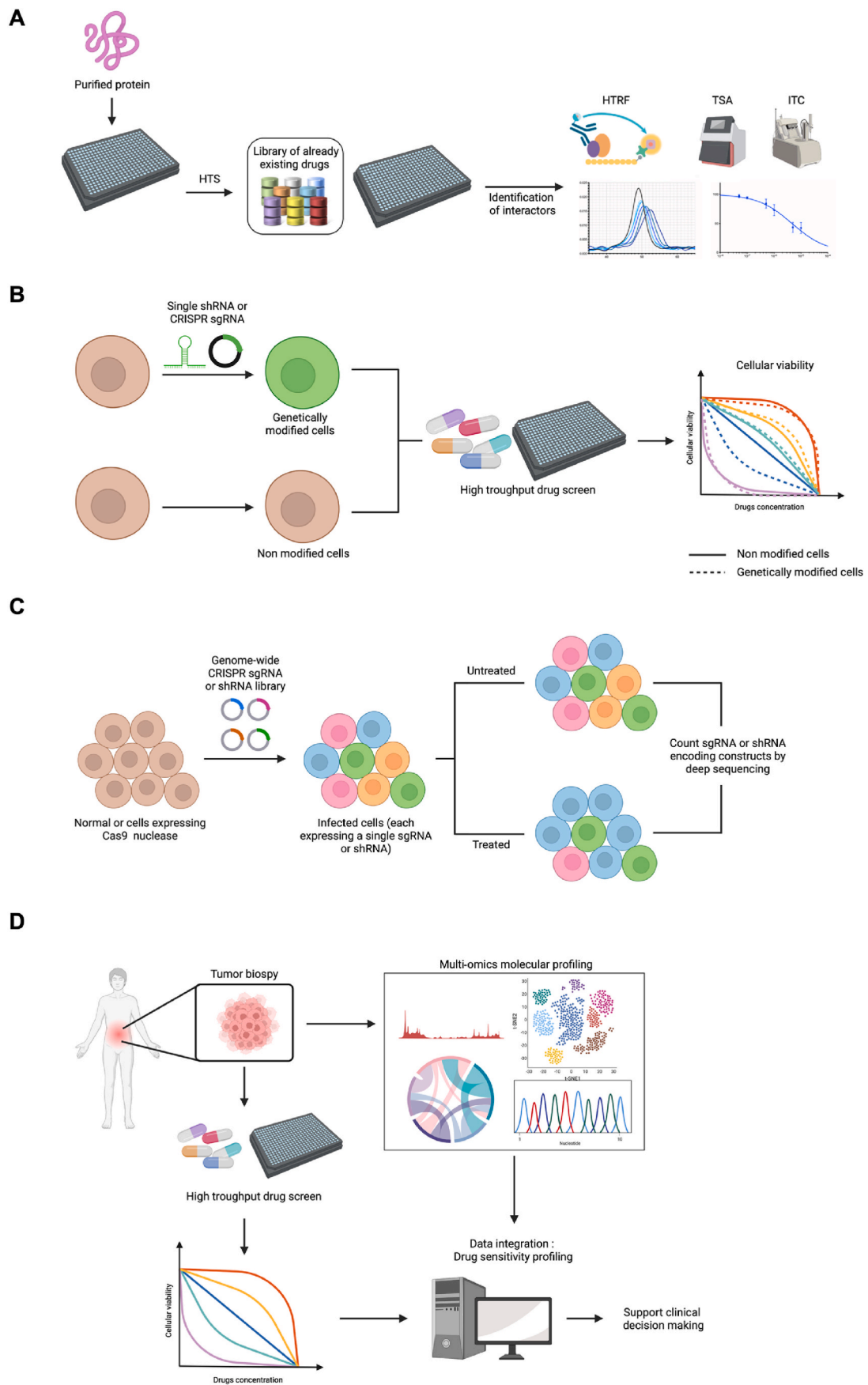
However, the main limitation of this approach is that it relies on purified and recombinant proteins. Beyond the technical difficulty that this implies, the *in vitro* model is not complex enough and cannot guarantee that the interaction found *in vitro* actually occurs *in cellulo*. The emergence of functional genomics coupled with pharmacological HTS has partly answered this limitation as it has allowed to identify new molecules targeting a given protein *in cellulo*.

2.1.2. *In cellulo* screening in genetically-modified model

Genetically-modified models coupled with screening programs have first used RNA interference technology and have quickly gained popularity with the CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats) revolution to modulate the expression of a given gene before screening large-scale libraries of compounds. This method is based on revealing cellular sensitivity and/or resistance to a drug caused by single gene modulation (Fig. 1B). This approach allows the discovery of many new pharmacological modulators of proteins of interest in oncology, as well as many other fields of medical research. As an example, in order to identify effective fatty acid uptake inhibitors in tumors, Chu *et al.* [30], generated a cell line sensitive to those inhibitors by knocking out acetyl-CoA carboxylases ACC1 and ACC2. They screened 1,560 FDA-approved drugs on this double KO cell line and identified nortriptyline, a tricyclic antidepressant, as an inhibitor of fatty acid uptake. This result could open therapeutic avenues for both cancer and metabolic diseases. Nevertheless, studies demonstrating that nortriptyline-mediated fatty acid uptake inhibition could also contribute to its antidepressant efficacy are still lacking.

The story of the development of imipridone ONC201 greatly highlights the benefits and limitations of these methodologies. ONC201 is the first therapeutic agent to show a small benefit in the incurable Diffuse Intrinsic Pontine Glioma (DIPG) in more than 60 years of failed clinical trials and therefore arouse the interest of the whole scientific community [31]. The MoA behind its anti-cancer activity remains elusive. ONC201 has been first described as a dopamine receptor (DRD2) antagonist. However, it has been demonstrated that DRD2 KO cells are still sensitive to ONC201 cytotoxicity [32], suggesting that other targets may be involved in its anticancer activity. Elsewhere, by performing a screen of the National Cancer Institute Diversity Set II against the apoptosis-inducing protein TRAIL, Allen *et al.* identified ONC201 as a potent p53-independent TRAIL inducer [33,34]. Furthermore, Ishizawa *et al.* [35] conducted a drug screening using a library of 747 molecules and showed that ONC201 and its homolog ONC212 are also potent agonists of the mitochondrial protease ClpP, leading to ClpP-mediated mitochondrial proteolysis and selective cancer cell death. The agonist activity of ONC201 on ClpP adds a new layer in the deconvolution of its MoA and could serve as a biomarker for treatment response. More recently, Venneti *et al.* [31] showed using H3K27M-mutant DIPG patient samples that ONC201 treatment induce a downregulation of TCA cycle metabolism, increase glutamine metabolism and partially restore H3K27me3 levels. These studies highlight the need to combine multiple approaches to be able to properly describe the MoA of a drug. They also suggest that drug poly-pharmacology may play a critical role in their anti-tumor efficacy.

Overall, although “target-centric” methods are powerful strategies to identify modulators of a given protein, eventually revealing novel drug targets, they are not suited to fully unravel all the interactors of a given drug.



(caption on next page)

Fig. 1. Using high-throughput screens to identify new targets of a drug. (A) *In vitro* high-throughput screening using purified protein. A large-scale library of already existing drug is screened against a purified protein of interest. The potential interaction between the protein and the drugs is then evaluated by biophysical test such as Homogeneous Time Resolved Fluorescence (HTRF), Isothermal Titration Calorimetry (ITC), or Thermal Shift Assay (TSA). (B) *In cellulo* high-throughput screening. Functional genomic (RNA interference technology or CRISPR/Cas9) is used to modulate the expression of a single gene before screening a large-scale library of compounds. Then, drug response is compared between modified and non-modified cells to reveal cellular sensitivity and/or resistance to drugs caused by the single gene modification. (C) Genome wide shRNA or CRISPR/Cas9 high-throughput screening. Infected cells, each expressing a single sgRNA or shRNA using a genome wide shRNA or CRISPR/Cas9 library, are treated with a compound of interest or vehicle. By deep sequencing, genes that are important for cellular sensitivity and/or resistance to the drug tested are then identified. (D) Chemo-genomics & multi-OMICs profiling to guide clinical decision making. Tumor samples from patient are used to: i) perform a high-throughput drug screening to reveal chemosensitivity, and ii) realize a multi-OMICs molecular profiling. Data are then integrated to generate a drug sensitivity molecular profile to guide clinicians. Created with [BioRender.com](https://www.biorender.com).

2.2. High-throughput screening: compound-centric approaches

Compound-centric approaches offer powerful and unbiased tools to unravel the MoA of bioactive compounds and their pharmacological targets. These are also based on the idea of identifying sensitivity and resistance [36] to a biological compound caused by gene modulations but this time with genome-wide coverage (Fig. 1C). It first began with genome-wide RNAi strategy, but efforts have been compromised by the lack of on-target activity and significant observed off-target effects [37–39]. Recently, the CRISPR/Cas9 technology appeared as a new strategy for genome-wide chemo-genomic profiling.

The effectiveness of this approach was first demonstrated by Zhang and colleagues almost 10 years ago in a melanoma model [40] where they were searching for genes whose loss induced resistance to vemurafenib, a mutated B-Raf inhibitor. Using a CRISPR/Cas9 screen they validated *NF1* and *MED12* as mediators of RAF activity, but also found *NF2*, *CUL3*, *TADA2B*, and *TADA1* as novel hits, which tweaked the comprehension of vemurafenib activity, and moreover, demonstrated the promise of this type of screening programs.

Later on, many studies confirmed the potential of deciphering the MoA of drugs using CRISPR-Cas9 technology. Lin *et al.* [41] suggested that the anti-cancer activity of drugs is frequently mediated by interactions with non-canonical targets. For instance, ricolinostat is known to be a selective HDAC6 inhibitor [42]. In this study, the authors showed that sensitivity to ricolinostat is partly independent from HDAC6 expression, suggesting that other targets could be involved. Their results also demonstrate that the putative PBK (PDZ binding kinase) inhibitor OTS964 has been mischaracterized and acts in fact by blocking CDK11 activity. Until then, no CDK11-specific inhibitor had been described [43]. The discovery of OTS964 as the first CDK11 inhibitor could therefore open new therapeutic avenues by targeting a previously undruggable mitotic CDK. Overall, their findings provide evidence that the poly-pharmacology of anti-cancer drugs is a common property involved in their anti-cancer effects.

At first, it was thought that genome-wide CRISPR/Cas9 profiling would completely supplant RNAi screening. However, emerging evidence demonstrate the complementarity of using both approaches [44]. Due to their specificities – shRNA (short hairpin RNA) inducing only partial reduction in gene expression vs CRISPR/Cas9 inducing complete knock-out – the combined use of shRNA and CRISPR screening enables to discriminate essential and non-essential genes involved in the MoA of drugs that may be difficult to detect using either screening approach alone. Deans *et al.* thus performed parallel genome-wide shRNA and CRISPR-Cas9 screens to identify the molecular target of GSK983 [44], an initial broad spectrum antiviral agent. The overlap between both techniques was not perfect. Indeed, genes required for essential biological processes were found as top hit in the shRNA screen but were not among the top hits in the CRISPR-Cas9 screen. Conversely other genes were found as top hits only in the CRISPR-Cas9 screen, probably because the knockdown of these genes by shRNA was not sufficient to induce a significant phenotype. By integrating the data of both techniques, they found DHODH (dihydroorotate dehydrogenase) as the target of GSK983, explaining its MoA against the dengue virus and highlighting the complementarity of using both screening method. This result makes GSK983 a potential anticancer agent since DHODH is a targetable

vulnerability in many cancers [45]. Moreover, to improve the robustness of genome-wide screening results, computational methods have emerged, allowing design optimization of sequence libraries and taking into account the limitations of CRISPR and shRNA screening technologies [46,47]. More recently, Wang *et al.* established an integrated framework using CRISPR, shRNA and molecular cell profiles. Their results demonstrated an increase in the accuracy of identified cancer dependencies, which could facilitate drug target discovery [48].

HTS profiling has been a helpful tool in the field as it allows the identification of targets and pathways mediating hypersensitivity and resistance relevant to the compound's MoA. However, these approaches remain prone to artefacts and therefore require extensive validation experiments. Furthermore, they mostly reveal modulators of the efficacy of the compound and their direct targets may be missed.

2.3. Multi-OMICs profiling

The past decade has seen the emergence of multi-OMICs technologies allowing the molecular profiling of cancers. This deep characterization has been used to identify patterns and markers of drug sensitivities (Fig. 1D). In early 2010's the Broad Institute performed several sequencing of multiple cell lines for gene expression, chromosomal copy number variations coupled with pharmacological profiling [49,50]. Cross-data analysis resulted in the discovery of several candidates that can be used as drug sensitivity biomarkers. As an example, Barretina *et al.* [49] found that DNA-damage responsive protein SLFN11 expression can predict sensitivity to topoisomerase inhibitors, and that the expression of transcription factor AHR correlates with MEK inhibitor efficacy in NRAS-mutant lines. Since then, several studies arose using extensive multi-omics profiling coupled with pharmacological screening on more and more cell lines and patient tumor samples [51,52]. More recently this kind of approaches has even been transferred to the clinic, with the use of whole genome, transcriptome and methylome sequencing to profile the molecular alterations of each patient tumor and guide clinicians towards a personalized treatment [4–8]. Unfortunately, success in the clinic so far remains minimal. There are several putative explanations to that. First, the OMICs datasets used are still limited and should be extended with other molecular and functional data (e.g., proteomics, metabolomics, drug sensitivity profiling, single-cell sequencing...). As an example, Mayoh *et al.* recently showed that integrating high-throughput drug screening results with molecular profiling could improve drug recommendation accuracy [53]. Second, the link between a targeted therapy and its target expression/activity is often not sufficiently characterized. This is perfectly illustrated by the lack of correlation between HDAC6 expression and response to ricolinostat as mentioned earlier [41] but also between BRAF V600E mutation and response to vemurafenib [54], or between CDK4/6 expression and response to its inhibitors [8]. Lastly, it is also well-recognized that combination therapies should be used upfront to maximize treatment efficacy. In line with this, the National Cancer Institute has recently launched the NCI-ComboMATCH program to improve genomic-driven combination therapy in cancer [55].

Collectively, the rise of high-throughput screening, multi-OMICs and functional genomics technologies was thought to revolutionize patient care by identifying new therapeutic targets and developing rationally-

designed drug combinations. However, to date, the progress made in the clinic remains marginal. On top of the explanations described above, we could add that knowledge on the poly-pharmacology of drugs is still partially lacking to better support drug treatment recommendations.

3. Chemo-proteomics

As previously described, recent advances in OMICs technologies have benefited drug discovery. Proteins being the main targets of almost all pharmacological agents, the recent technical developments in Mass Spectrometry (MS) have enabled the emergence of chemo-proteomics as a valuable strategy for drug development [56,57].

Chemo-proteomics allows the detection of direct and indirect interactions of a pharmacological compound with proteins [58]. These approaches classically rely on two different methodologies. The first one is based on the isolation of drug targets by chemical probes derived from the compound under investigation, often referred as compound-centric chemo-proteomics (CCCP), while the second one uses the impact of the compound binding on protein structure and their biophysical properties such as their thermal stability or resistance to proteolysis.

3.1. Compound-centric chemo-proteomics

The identification of drug interactors using CCCP approaches is based on affinity enrichment. This specificity allows the enrichment of low-abundance targets. The objective is the isolation, identification and quantification of proteins that interact with the molecule of interest. Most of the time this is achieved by adding a chemical group to the compound to enable its immobilization and the pull down of its interactors [59–61]. Then, quantitative MS is used to quantify the isolated interactors (Fig. 2A). Hammam *et al.* [62] elegantly demonstrated the potential of CCCP to uncover the MoA of existing drugs. They aimed at unraveling how the tyrosine kinase inhibitor, masitinib, sensitizes cancer cells to the antimetabolite drug gemcitabine [63]. They used an NH₂-modified version of masitinib allowing its cross-linking to beads to capture its interactors and quantify them by LC-MS/MS. As a result, they identified the deoxycytidine kinase (dCK) as a new target of masitinib that mediates its impact on gemcitabine sensitivity.

Another example of successful use of CCCP was brought by Graves and colleagues. They confirmed, in an unbiased approach, the activity of ONC201 and its analogues ONC206 and ONC212 against the human mitochondrial caseinolytic protease P ClpP [64]. They performed an affinity proteomics approach to isolate the interactors of the ONC compounds by coupling them to agarose beads and incubating them with HeLa cell lysates to confirm ClpP as a specific target. Moreover, they set up a competition assay, demonstrating that the analog compounds ONC206 and ONC212 bound ClpP with ~10-fold higher affinity compared to the parental compound ONC201. This study explains, for the first time, the difference in efficacy between ONC201 and its analogues and pinpoints ClpP agonism as the critical MoA of these drugs.

Classical affinity purification MS methods are performed on cell lysates, but the emergence of click chemistry has allowed the use of CCCP *in cellulo*. Click chemistry is a class of biocompatible chemical reactions that enable the combination of a specific molecule with a chosen substrate [65]. Its application in biology has been widely studied [66–68], but Rodriguez and colleagues [69] were the first to use click chemistry to study the interactome of pharmacological agents in cells. To unveil the interactome of enoxacin, a small antibiotic that can inhibit cancer cell growth, they developed a Click-qMS method that uses a click reaction to couple streptavidin beads with enoxacin, allowing the pull down of its interactors from treated cells. They found PIWIL3 as a target of enoxacin, which explains its specificity to cancer cells since PIWIL3 is over-expressed in cancers [70,71] but limited to testis in healthy tissues [72]. Click chemistry furthermore allowed them to determine enoxacin cellular localization by confocal fluorescence microscopy by coupling it with an Alexa Fluor 594. Since then, click chemistry-based proteomics

has been increasingly used to uncover non-canonical drug targets [73, 74].

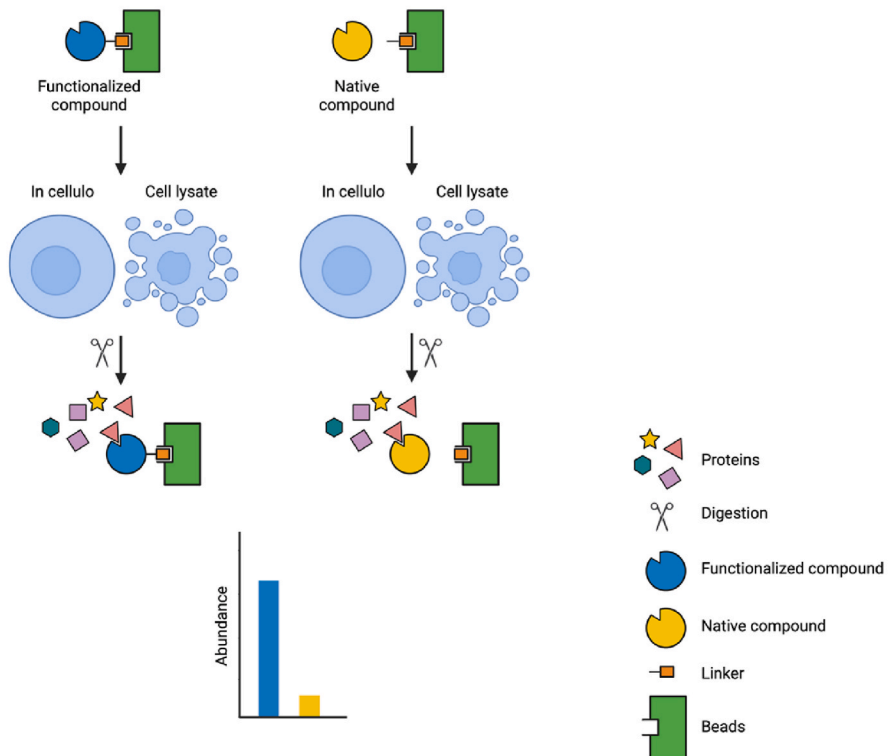
Although powerful, CCCP methods require functionalized molecules, which entails two main limitations: i) they can be difficult to synthesize, ii) the modification of the parental compound can induce a bias, and iii) for click applications, Cu²⁺ is toxic *in vivo*. Recently, other proteomic strategies that rely on ligand-induced effects on the biophysical properties of target proteins have emerged. Indeed, the interaction of a compound with a given protein can impact its thermal stability, resistance to proteolysis or surface exposure of amino acid side chains [75–81]. Quantitative MS allows the detection of these changes and can be applied to proteome-wide scale.

3.2. Ligand-induced effects on biophysical properties of target proteins

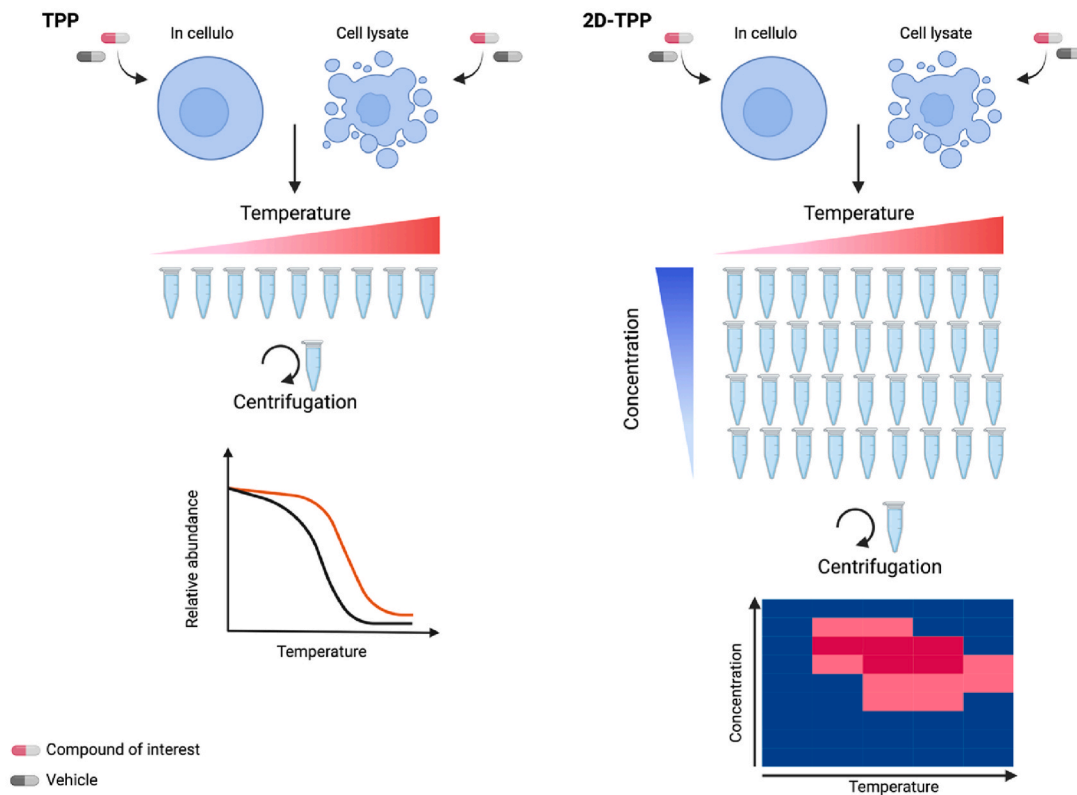
The binding of a compound to a protein can impact its biophysical properties. This can then be exploited to reveal the interactome of pharmacological compounds. For example, drug binding can locally alter the accessibility of proteases, which is taken advantage of in two different methodologies. The first is called “Drug affinity-responsive target stability” (DARTS) and detects the reduction in protease susceptibility of the target protein upon direct binding of a bioactive compound. The second method is called limited proteolysis-coupled mass spectrometry (LIP-MS), which first uses a non-specific protease and then trypsin digestion to generate proteolytic peptides that act as reporters of protein folding in the presence of a compound [77,78,81,82]. Although highly effective in theory, these methods have not been extensively used to date to characterize the MoA of anticancer drugs as one of the main limitations of these methods is that they can only be applied to cell lysates.

Drug binding can also lead to a change in the stability of the target protein. This property can be exploited in different approaches such as Thermal Proteome Profiling (TPP) and stability of proteins from rate of oxidation (SEPROX). SEPROX monitors the oxidation rate of methionine residues upon addition of H₂O₂ using LC-MS/MS during a chemical denaturation gradient. As with previous methods SEPROX can only be applied to cell lysates [80,83]. TPP, also known as Cellular Thermal Shift Assay (CETSA)-MS, is a biophysical test based on the principle that interaction of proteins with a ligand can impact their thermal stability [75,76]. Briefly, cell samples, previously incubated with either a drug or its vehicle, are heated to different temperatures to induce the denaturation of the proteome. Ultracentrifugation is used to separate the denatured proteins that precipitate in the pellet, while the native/unaffected ones remain in the soluble fraction. MS can then be used to identify and quantify these proteins (Fig. 2B). This *in cellulo* methodology allows the detection of direct protein interactors of a drug within a cell as well as secondary downstream effects such as post-transcriptional modifications (PTMs) [84,85]. TPP experiments can also be performed on cell lysates, and comparison with the *in cellulo* experiment allows the discrimination between primary and secondary targets [75]. Besides *in cellulo* and lysate-based experiments, TPP has also been applied to tissues and blood samples to measure the engagement of drugs with their canonical and non-canonical targets *in situ* [76,86]. Moreover, a newer version of TPP called two-dimensional thermal proteome profiling (2D-TPP) has recently emerged. This development integrates the conventional TPP experiment where a single dose of the drug is tested at different temperatures, with an isothermal ligand concentration-dependent experiment. Here, a range of concentrations of the drug is used, at a temperature close to the melting points of individual targets (Fig. 2B). Using 2D-TPP, Becher *et al.* [87] unveiled novel targets of the HDAC inhibitor, panobinostat, including phenylalanine hydroxylase, whose inhibition leads to increase in phenylalanine and decrease in tyrosine levels. This explains some of the hypothyroidism adverse effects of panobinostat observed in the clinic and could open repurposing opportunities for the treatment of tyrosinemia. The 2D-TPP protocol partly addresses a limitation of conventional TPP experiment,

A



B



(caption on next page)

Fig. 2. Chemo-proteomics approaches to unveil the interactome of drugs. (A) Compound-centric chemo-proteomics (CCCP). Using a specific linker, functionalized molecule is synthesized from a native compound of interest. Cells or cell lysate are treated with functionalized or native compound and proteins interacting are isolated thanks to the linker allowing the immobilization of the compound by binding with beads or by affinity matrix. Interacting proteins are identified and quantified by mass spectrometry. (B) Thermal Proteome Profiling approaches (TPP). Cells or cell lysates are treated with vehicle or the compound of interest at a single dose for TPP (left panel) or at several concentrations for 2D-TPP (right panel). Cells are then heated to a range of temperatures. Analysis is based on physical properties of each protein using its specific denaturated melting temperature. Given that a drug interacting with a protein impacts its thermostability changing its melting temperature, it allows to reveal the drug interactome. Practically, denaturated and native proteins are separated by ultracentrifugation since denaturated proteins drop in the pellet while native ones remain in the soluble fraction. Proteins in the soluble fraction are then identified and quantified by mass spectrometry. Created with BioRender.com.

which is false negatives. Indeed, drugs can induce very small or no thermal shifts upon binding to their targets. The magnitude of the changes in thermal stability depends on the concentration and affinity of compounds but also on the structure of ligands and target proteins. Thus, using one single concentration of a small ligand binding to large multidomain proteins can lead to false negative results [75,76,88–92]. Additional limitations of TPP should also be discussed. First, despite the improved resolution and accuracy of mass spectrometry data acquisition and analyses, current technologies do not cover the whole proteome, leading to non-identified proteins. Second, some proteins, mostly small and hydrophilic ones, are thermostable at temperatures that are incompatible with the structural integrity of cells meaning that they are not denaturated and therefore do not precipitate [88]. Third, membrane proteins can be challenging to isolate, even though recent protocols enable the detection of membrane proteins [89–91]. A fourth limitation of conventional TPP and 2D-TPP is that they produce numerous samples requiring extensive MS acquisition time (up to several weeks or even months) for a single experiment, plus time-consuming analyses that require advanced expertise. This is why multiplexing using isobaric labelling with tandem mass tags (TMTs) and data dependent acquisition (DDA) MS has traditionally been used. Pooling samples decrease mass spectrometry acquisition time and reduces technical variability. However, the main problems with this approach are cost, sample preparation time and analysis variability when using multiple batches to include biological replicates. New workflows have started to emerge to overcome this issue. Proteome integral solubility alteration (PISA) for instance relies on pooling all the samples from a set of temperatures in a single one, allowing to decrease acquisition time, the amount of data generated and increasing throughput [93–95]. However, pooling samples together from a wide temperature range can make it even harder to detect discreet thermal shifts, leading to an increase in false negative results. New MS acquisition methods that do not use TMT isobaric tagging and DDA MS acquisition have recently appeared. They notably rely on non-labelling approaches using data independent acquisition (DIA) MS. It has been demonstrated on a TPP workflow that this new strategy outperforms the conventional TMT-DDA method with a reduced costs and sample preparation steps [96].

In summary, CCCP approaches are powerful tools for specific enrichment of low-abundance targets, which may be difficult to see in global profiling. However, these approaches rely on functionalized small molecules which can be challenging to synthesize and these modifications can induce bias. Target profiling based on biophysical or biochemical principles, such as TPP and LIP-MS, do not require compound modification and for TPP can be used to identify intracellular targets in living cells. They are progressively becoming more attractive thanks to the increasing sensitivity and depth of coverage of the proteome by MS. However, they are still hampered by the large amount of time they require, false negatives, and incomplete coverage of the proteome (Table 1).

4. Take home message and discussion

There is an urgent need to improve the success rate of clinical trials in oncology. Currently, multi-omics and functional genomics techniques are widely used to establish the molecular and clinical features of cancers and thus unveil their vulnerabilities. The generated data are mostly

Table 1
Pros and cons of CCCP vs TPP approaches.

CCCP approaches		TPP approaches	
Advantages	Disadvantages	Advantages	Disadvantages
Compatible with <i>in vivo</i> , <i>in cellulo</i> or <i>ex cellulo</i> experiments (Click chemistry)	Requires functionalized small molecules that can induce a bias	Any parental compounds and combinatory treatment can be tested	Time consuming and high expertise needed
Enrichment of low-abundance proteins	Requires functionalized small molecules: some molecules are complex and can be difficult to synthesize	Compatible with <i>in vivo</i> , <i>in cellulo</i> or <i>ex cellulo</i> experiments	No functional applications
Functional applications: Click chemistry is compatible with imaging and cytometry: drug kinetic and cellular localization	CU ²⁺ is toxic <i>in vivo</i>	2D-TPP uses a range of concentration of the drug	False negatives & incomplete coverage of the proteome

used to rationally design drug combinations but they could also be exploited to launch specific programs that focus on developing engineered poly-pharmacology. As such, the Panacea program represents a good example aiming at developing multi-targeted drugs (<https://www.darpa.mil/program/panacea>). Elsewhere, unveiling the poly-pharmacology of already existing drugs will enable to exploit cancer dependencies in a more efficacious and less toxic way. This review article focused on the technological breakthroughs that enabled to develop new tools in the last decades allowing to decipher more precisely the poly-pharmacology of existing drugs. Overall, the potential of chemo-genomic, HTS, and chemo-proteomics to unveil new MoAs of drugs and/or refine the comprehension of their biological activity has been clearly established in recent landmark studies and reviewed herein. All the above-mentioned methods have advantages and limitations, and thus should be combined and integrated as illustrated by the ONC201 story in DIPG. Indeed, functional genomics screening approach together with a chemo-proteomic method were needed to ascertain ClpP as the main target of ONC201 in DIPG [64], emphasizing that combining several methodologies is essential to precisely dissect the activity of a drug. Finally, a good example of how knowledge on the poly-pharmacology of existing drugs should be used to design more effective and less toxic drug combinations to overcome drug resistance, is the study by S. Klaeger *et al.* that unveiled the target landscape of 243 clinical kinase inhibitors using a kinobeads approach [16]. The identified atlas of the poly-pharmacology of those kinase inhibitors has been then exploited to guide their use in a more effective and less toxic way by targeting several hyperactive kinases with low-dose drug combinations in pancreatic ductal adenocarcinoma [97]. Very recently, the kinobeads approach was further extended to reveal the target landscape of 1,000 kinase inhibitors [98].

In conclusion, defining the poly-pharmacology of already existing

drugs will help to better assess their biological effects, thus guiding their optimal use in the clinic, and could pave the way to the development of new “designed” poly-pharmacological drugs.

CRedit authorship contribution statement

Baptiste Mouysset: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation. **Marion Le Grand:** Writing – review & editing, Validation, Supervision, Methodology. **Luc Camoin:** Writing – review & editing, Methodology. **Eddy Pasquier:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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