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Emerging Concepts

How Physiologic Targets Can Be Distinguished from Drug-Binding Proteins

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ABSTRACT

In clinical trials, some drugs owe their effectiveness to off-target activity. This and other observations raise a possibility that many studies identifying targets of drugs are incomplete. If off-target proteins are pharmacologically important, it will be worthwhile to identify them early in the development process to gain a better understanding of the molecular basis of drug action. Herein, we outline a multidisciplinary strategy for systematic identification of physiologic targets of drugs in cells. A drug-binding protein whose genetic disruption yields very similar molecular effects as treatment of cells with the drug may be defined as a physiologic target of the drug. For a drug developed with a rational approach, it is desirable to verify experimentally that a protein used for hit optimization in vitro remains the sole polypeptide recognized by the drug in a cell.

SIGNIFICANCE STATEMENT

A body of evidence indicates that inactivation of many drug-binding proteins may not cause the pharmacological effects triggered by the drugs. A multidisciplinary cell-based approach can be of great value in identifying the physiologic targets of drugs, including those developed with target-based strategies.

Introduction

Phenotypic (i.e., whole-cell) screening (Low et al., 2009; Swinney, 2013; Sykes and Avery, 2013; Chatelain and Ioset, 2018) has made important contributions to the discovery of hits and drugs for infectious diseases (Jacobs et al., 2011; Mesu et al., 2018). As investigators turn their attention to possible mechanisms of action for the drugs, it is instructive to inform those studies with reports that therapeutic effects of several drugs in clinical trial are due to their off-target activity (Lin et al., 2019). Similarly, some drugs that were optimized in vitro against purified proteins have other targets in cells (Lackey, 2006; Dai et al., 2008; Karaman et al., 2008; Dolloff et al., 2011; Dunne et al., 2011; Hafner et al., 2019). A possible explanation for these observations is that studies used to identify drug targets were incomplete. Rational drug development does not guarantee exclusive recognition of the in vitro target of a medicinal chemistry campaign in cells. Is there a way forward amid this uncertainty?

Many paths, all producing a list of candidates that need experimental validation, can be used to identify drug targets [reviewed in Schenone et al. (2013), Haanstra and Bakker (2015), Zhu et al. (2015), Manchado et al. (2016), and Muller and Hemphill (2016)]. In the case of single-cell eukaryote pathogens (e.g., *Trypanosoma*, *Leishmania*, and *Plasmodium* spp.), three general strategies, alone or in combination, are employed: 1) knockdown of the candidate gene to show that it is essential for cell viability; 2) overexpression of the gene, leading to reduced susceptibility of the cell to the drug (or selection of resistant lines); and 3) detection of complexes between drugs and proteins. In all these strategies, it is not routine practice for the molecular effect resulting from a target gene's loss (or overexpression) to be compared directly to the molecular effects of drug treatment on cells. In the absence of this information, it may be difficult to conclude that identified genes are physiologic targets of the drugs in question.

Identification of Physiologic Targets of Drugs

A physiologic target of a drug (PTD) may be defined as a gene whose knockdown or overexpression yields very similar/identical molecular effects as treatment of cells with the drug. Cell death is not a good surrogate for molecular effects in this regard, since many pathways lead to that endpoint. Many drug-binding proteins may not be the physiologic targets. For example, plasma proteins bind to drugs (Berezhkovskiy, 2008; Cho et al., 2010; Svennebring, 2016) but are not considered physiologic targets of the small molecules.

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CEC, cytocidal effective concentration; ITZ, itraconazole; OSBP, oxysterol-binding protein; PTD, physiologic target of a drug; TbGSK3β, trypanosome glycogen synthase kinase-3β; Tf, transferrin.

Physiologic changes in organisms originate in biochemical and molecular stimuli in cells. Thus, data from 1) molecular genetics, 2) molecular cell biology/biochemistry, and 3) perturbation of cells are relevant for identification of physiologic targets of a drug. A central role of molecular cell biology in this effort is difficult to ignore. Although some drug-binding proteins are described as targets because knockdown of their gene products compromises cell viability, this conclusion could use further support from experiments that directly link the events being monitored at a molecular level. Drug binding to a protein needs to be directly linked to disruption of a molecular function of the target, since knockdown of a target by itself can compromise cell viability in absence of drug. For these reasons, it is important to functionally separate drug-binding proteins from physiologic targets of drugs.

Concepts from the field of receptor signaling have relevance for attempts to discover physiologic targets of drugs. Not all proteins that bind a ligand can transduce signals and thus qualify for classification as a receptor. Similarly, not all drug-binding proteins are physiologic targets whose inhibition leads to the same molecular defects observed when the drug is added to cells. How can we distinguish between drug-binding proteins and physiologic targets of the drugs?

Three stages may be described for this challenging task. First, drug-binding proteins can be identified using one of many strategies, including affinity chromatography and photoaffinity labeling (Campos et al., 1996; Wang et al., 2011, 2012; Tulloch et al., 2017; Das, 2019; Muskens et al., 2019; Xiao and Li, 2020). Second, molecular effects of a drug can be documented in molecular studies after perturbing cells with drug concentrations that are not toxic ((e.g., concentration of drug which on short-term exposure (less than 6 h) reduces proliferation by 25%) [CEC_{25} (Bachovchin et al., 2019)]. Third, genes of drug-binding proteins can be knocked down, and the molecular effects in cells can be compared with those obtained from drug perturbation studies. The expectation is that when a physiologic target is knocked down, the molecular defects observed are very similar to those obtained when the drug is added to cells. In the case of polypharmacology (Anighoro et al., 2014; Proschak et al., 2019), more than one gene may need to be knocked down to recapitulate all the molecular defects obtained by adding the drug to cells. It is also conceivable that a physiologic target does not directly bind the drug, because it interacts with a drug-binding protein.

Unbiased Strategies for Discovery of Drug-binding Proteins in Cells

Drugs are optimized to promote health by modulating aspects of human physiology. Since the smallest unit of human life is a cell (found in many differentiated states and organized into tissues and organs), it stands to reason that drugs exert their primary effect on cells. From this view point, the effects of drugs on cells that are associated with specific diseases deserves to be highlighted in molecular pharmacology studies.

Studies to identify physiologic targets of drugs (PTDs) begin with discovering cellular macromolecules that bind the small molecule, even for drugs designed with rational approaches (Yang and Huang, 2006; Chintakrindi et al., 2012; Milligan et al., 2013; Cozza, 2017; Wang et al., 2018). Although a host of methods are used to find drug-binding proteins, usually termed targets (Bunnage et al., 2015), confidence in data obtained using different techniques is not equivalent, so it is important to interpret the information cautiously.

Photoaffinity labeling of proteins using drugs functionalized with reactive moieties (i.e., warheads) represents a powerful strategy for identifying drug-binding proteins when combined with proteome-wide analysis enabled by mass spectrometry (Fischer et al., 2010, 2012; Hamouda et al., 2014; Kawamura et al., 2014; Phizackerley et al., 2017; Thomas et al., 2017; Hill and Robertson, 2018). On-target labeling of proteins using this approach can be detected by adding excess untagged drug to outcompete modification of the specific protein by probe (Parker et al., 2017). When more than one protein is labeled in this technique, approaches that rely on physical protein-drug interactions come in handy to verify true targets (see next paragraph).

Biochemical/chemical approaches, judiciously used, offer a direct path to finding drug-binding proteins because the assays (e.g., photoaffinity labeling) track physical interactions between drug and protein targets. Column affinity chromatography, in which drugs are coupled to matrices, has been used to identify drug-binding proteins (Mercer et al., 2011; Shi et al., 2012; Katiyar et al., 2013; Schenone et al., 2013; Jones et al., 2015; Rylova et al., 2015; Thomas et al., 2017). Here, it is important to remember that proteins eluted from the column, even after rigid washing protocols (Mensa-Wilmot et al., 1995; Amarasinghe and Jin, 2015; Mishra, 2020), might not bind the drug that is coupled to the chromatography matrix; some eluted proteins may be present on the column as a result of interaction with other proteins that bind directly to the drug. For this reason, a second set of studies that measure direct interactions between drugs and proteins [e.g., surface plasmon resonance (Nikolovska-Coleska, 2015; Douzi, 2017), microscale thermophoresis (Wienken et al., 2010; Khavrutskii et al., 2013; Hellinen et al., 2020), thermal shift assays (Martinez Molina et al., 2013; Alshareef et al., 2016; Lucet and Murphy, 2017; Dziekan et al., 2019), activity-based proteomic profiling (Cravatt et al., 2008; Golkowski et al., 2017), or drug inhibition of purified enzyme activity (Labar et al., 2007; Guyett et al., 2016; Warrilow et al., 2016; Simčič et al., 2014)] are essential for confirming drug-binding properties of proteins identified with affinity chromatography techniques.

Selection of genes that confer resistance, or reduced susceptibility, to drugs offers an indirect approach for discovery of potential drug-binding proteins. In isolated cases, reduced cell susceptibility to a drug against a metabolic enzyme occurs by mutations in the enzyme itself (Wilson et al., 1992; Dicker et al., 1993; Yun et al., 2008). However, the myriad of pathways employed by cells to counter susceptibility to drugs, including efflux and rewiring of metabolic pathways, is astounding (Aouida et al., 2004; Barrett et al., 2011; Chitanga et al., 2011; Seyhan et al., 2012; Claus et al., 2014; Dermawan et al., 2014; Gatti et al., 2015; Chang et al., 2019; Zhang et al., 2019b; Haider et al., 2020; Yobi et al., 2020). For these reasons, it is prudent to delay conclusions that genes identified by sequencing of genomes from drug-resistant cells are targets of the drugs until confirmatory data from other studies are obtained.

When crystal structures or appropriate homology models are available, computational docking studies can have a role in verifying drug targets (Abdolmaleki et al., 2017; Zhang et al., 2019a; Sharma et al., 2020). Understandably, the most convincing data from such predictions are supported by biochemical/biophysical studies using a library of small molecules to test predictions from docking studies. Unfortunately, validation of data from docking studies is rarely performed.

Targets Are Unknown for a Large Number of Drugs

For decades, metformin has been used as an antidiabetic drug, and it now appears to be on track for repurposing to treat prostrate and breast cancer (Dowling et al., 2007; Wang et al., 2014). In cells, modes of action of metformin include inhibition of 1) mitochondrial glycerophosphate dehydrogenase (Madiraju et al., 2014), 2) thiamine transport by OCT1 (Chen et al., 2014), and 3) protein synthesis initiation (Dowling et al., 2007). It is highly unlikely that all these actions of metformin are mediated by a single protein target. Thus, although no evidence for direct binding of metformin to a specific protein is available, the range of its modes of cellular action point to the drug having multiple targets.

Aspirin is an analgesic with anti-inflammatory properties; it can inhibit cyclooxygenase-2, 5-lipoxygenase (Kaur et al., 2012), and IKK-β kinase (Yin et al., 1998), and it prevents movement of activating transcription factor 6 from the Golgi to the cell nucleus (Mugge and Silva, 2017). Such diverse cellular effects hint at a multiplicity of targets for the drug. Indeed, aspirin acetylates over 120 proteins (Wang et al., 2015), suggesting a unifying theme for pleiotropic actions for the drug: extensive post-translational acetylation of proteins. Further work is needed to identify physiologic targets of aspirin in each of the cell types in which the drug disrupts specific biologic pathways.

Lapatinib (Tykerb) is an Erb1/2 (EGFR/Her2) kinase inhibitor used for treatment of breast cancer (Lackey, 2006). Lapatinib binds three other human protein kinases (Karaman et al., 2008), inhibits drug efflux by ABCG1 and ABCB2 transporters (Kuang et al., 2010; Chun et al., 2015), and activates JNK/c-Jun signaling through unknown effectors (Dolloff et al., 2011). In the single-cell eukaryote *Trypanosoma brucei*, which lacks EGFR/Her2, lapatinib forms complexes with three protein kinases (Katiyar et al., 2013; Behera et al., 2014), disrupts assembly of the paraflagellar rod, and inhibits endocytosis of transferrin (Guyett et al., 2017). Human cell resistance to lapatinib treatment is associated with PAK 2 (Chang et al., 2018), NF-$\kappa\beta$ signaling (Wetterskog et al., 2013), and ABC transporters (Dai et al., 2008; Kuang et al., 2010; Sodani et al., 2012). Recent acceptance of kinase-independent prosurvival functions of EGFR (Refaat et al., 2015; Tan et al., 2015; Cossu-Rocca et al., 2015 Thomas and Weihua, 2019) suggests a need to determine physiologic targets of lapatinib in human and trypanosome cells to understand the drug's mechanisms of action.

Molecular Outcomes of Target Engagement by Drugs Can Be Validated In Vivo

We will use two studies to illustrate feasibility of identifying physiologic targets of drugs, although it was not the intention of the authors of selected publications to address issues raised in this publication.

Kuppeveld and colleagues studied itraconazole (ITZ) inhibition of enterovirus EV71 replication in human HeLa R19 cells (Strating et al., 2015). The authors found, using microscale thermophoresis, that ITZ bound human oxysterol-binding protein (OSBP) and OSBP-related protein 4 (Wienken et al., 2010). Genetic knockdown of OSBP enhanced susceptibility of virus replication to a reduced concentration of ITZ. In addition, OSW-1, an antagonist of OSBP (Burgett et al., 2011), exerted stronger inhibition of EV71 replication under these circumstances. Finally, overexpression of OSBP restored replication of EV71 in the presence of ITZ, confirming that inhibition of viral replication by ITZ depended on OSBP as a physiologic target of the drug.

Studying the eukaryotic microorganism *T. brucei*, Guyett and colleagues showed that the drug TWS119 (Ding et al., 2003) killed trypanosomes and prevented endocytosis of transferrin (Tf) as a mode of action. Employing a combination of genetic, chemical perturbation, and molecular cell biology tools, they validated trypanosome glycogen synthase kinase-3β (TbGSK3β) as a physiologic target of TWS119 (Guyett et al., 2016). Evidence presented was as follows. First, TWS119 inhibited Tf endocytosis in *T. brucei*. Second, TWS119 inhibited enzyme activity of purified TbGSK3β, demonstrating that TWS119 binds to the protein. Third, genetic knockdown of TbGSK3β reduced Tf endocytosis, whereas overexpression of TbGSK3β increased Tf uptake, firmly establishing a positive role of TbGSK3β in endocytosis of Tf. Finally, TWS119 blocked the increase in Tf endocytosis caused by overexpression of TbGSK3β, consistent with TWS119 inhibition of TbGSK3β activity in vivo.

The two studies summarized in this section illustrate how a judicious choice of molecular-mode-of-action studies can be used to validate the outcome of drug engagement of a physiologic target in a cell.

The Value of Knowing Physiologic Targets of a Drug

There are multiple benefits of knowing physiologic targets of drugs. First, the concept makes it easier to accept a possibility that off-target proteins may be biologic effectors. By definition, drugs are foreign to cells, which contain over 20,000 proteins (Venter et al., 2001; Venter et al., 2015). One expects a drug to bind multiple proteins in a cell (Fabian et al., 2005; Davis et al., 2011; Katiyar et al., 2013). None of those binding-proteins should be termed off-target, even if that protein is not our preferred one.

Second, a focus on physiologic targets enables investigators to make a direct link between protein binding of drug and molecular changes elicited by target engagement (see the last section). Re-evaluation of targets for drug optimization in vitro against purified proteins is appropriate given these considerations. One cannot assume that the target for medicinal chemistry optimization in vitro is the sole binding protein in a cell for the drug. Human cells contain tens of thousands of proteins (Venter et al., 2001, 2015), so an analog of a hit that emerges from optimization of the drug may have new cellular targets. Experiments to determine cellular specificity of new analogs will enable medicinal chemists to know whether

optimized hits remain on-target during drug development (Lepovitz et al., 2020).

Third, overexpression of a protein may reduce a cell's susceptibility to a drug. However, knockdown of the protein could produce a different molecular change than that obtained by perturbing cells with the drug. It is important to obtain both sets of data before conclusions about physiologic drug targets are made when a protein overexpression strategy is used for target identification.

For the pharmaceutical industry, these ideas may be viewed from several perspectives. Ability to distinguish true physiologic targets from drug-binding proteins could foresee issues that arise late in drug development or, more likely, in the postmarketing phase. For a new therapeutic agent, alternative targets that produce unanticipated pharmacological effects could be identified, and that information could inform repurposing of the drug for other indications. Nevertheless, because of additional costs involved, the multidisciplinary studies described here are unlikely to be applied to every hit in a medicinal chemistry project. However, it will be feasible to perform these studies for clinical candidates, since physiologic targets of drugs are likely to include off-target proteins whose inhibition might be the basis of drug action (Lin et al., 2019). Further, these studies are likely to reveal targets whose inhibition may cause selective toxicity and lead to cost saving from attrition of some candidate drugs (Choi et al., 2014; von Kleist et al., 2016; Zuhl et al., 2016; Flynn et al., 2017).

Authorship Contributions

Performed data analysis: Mensa-Wilmot.

Wrote or contributed to the writing of the manuscript: Mensa-Wilmot.

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