How Physiological Targets Can Be Distinguished from Drug-binding Proteins
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Abstract

Some drugs in clinical trial owe their effectiveness to “off-target” activity. This and other observations raise a possibility that many studies to identify targets of drugs are incomplete. If “off-target” proteins are pharmacologically important it will be worthwhile to identify them early in the development process, for a better understanding of the molecular basis of drug action. Herein, we outline a multidisciplinary strategy for systematic identification of physiological 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 physiological 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 physiological targets of drugs, including those developed with target-based strategies.

Running title: Drug-Binding Proteins May Not Be Physiological Targets

Non-standard abbreviations: CEC, cytocidal effective concentration; PTD, physiological target of drug.
Introduction

Phentotypic (i.e., whole cell) screening (Chatelain and Ioset, 2018; Low et al., 2009; Swinney, 2013; Sykes and Avery, 2013) has made important contributions to 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 (Dai et al., 2008; Dollof et al., 2011; Dunne et al., 2011; Hafner et al., 2019; Karaman et al., 2008; Lackey, 2006; Nathan G. Dollof, 2011). 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, amidst this uncertainty?

Many paths, all producing a list of candidates that need experimental validation, can be used to identify drug targets (reviewed in (Haanstra and Bakker, 2015; Manchado et al., 2016; Muller and Hemphill, 2016; Schenone et al., 2013; Zhu et al., 2015)). In the case of single cell eukaryote pathogens (e.g., Trypanosoma, Leishmania, Plasmodium spp.), three general strategies, alone or in combination, are employed; (i) knockdown of the candidate gene to show that it is essential for cell viability; (ii) over-expression of the gene leading to reduced susceptibility of the cell to the drug (or selection of resistant lines); and (iii) 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 over-expression) 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 physiological targets of drugs in question.

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Identification of Physiological Targets of Drugs

A “physiological target of a drug” (PTD) may be defined as a gene whose knockdown or over-expression 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 physiological targets. For example, plasma proteins bind to drugs (Berezhkovskiy, 2008; Cho et al., 2010; Svennebring, 2016) but are not considered physiological targets of the small molecules.

Physiological changes in organisms originate in biochemical and molecular stimuli in cells. Thus, data from; (a) molecular genetics, (b) molecular cell biology/biochemistry, and (c) perturbation of cells, are relevant for identification of physiological 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, the 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 physiological targets of drugs.

Concepts from the field of receptor signaling have relevance for attempts to discover physiological targets of drugs. Not all proteins that bind a ligand can transduce signals, qualifying for classification as receptors. Similarly, not all drug-binding proteins are physiological 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 physiological 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; Das, 2019; Muskens et al., 2019; Tulloch et al., 2017; Wang et al., 2012; Wang et
al., 2011; 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 (CEC$_{25}$ concentration, for example (Bachovchin et al., 2019)). Third, genes of drug-binding proteins can be knocked down, and the molecular effects in cells compared to those obtained from drug perturbation studies. The expectation is that when a physiological 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 physiological 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 physiological targets of drugs (PTDs) begin with discovering cellular macromolecules that bind the small molecule, even for drugs designed with rational approaches (Chintakrindi et al., 2012; Cozza, 2017; Milligan et al., 2013; Wang et al., 2018; Yang and Huang, 2006). While 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 are 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

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proteome-wide analysis enabled by mass spectrometry (Fischer et al., 2010; Fischer et al., 2012; Hamouda et al., 2014; Hill and Robertson, 2018; Kawamura et al., 2014; Phizackerley et al., 2017; Thomas et al., 2017). 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., photo-affinity labeling) track physical interactions between drug and protein targets. Column affinity chromatography in which drugs are coupled to matrices have been used to identify drug-binding proteins (Jones et al., 2015; Katiyar et al., 2013; Mercer et al., 2011; Rylova et al., 2015; Schenone et al., 2013; Shi et al., 2012; Thomas et al., 2017). Here it is important to remember that proteins eluted from the column, even after rigid washing protocols (Amarasinghe and Jin, 2015; Mensa-Wilmot et al., 1995; Mishra, 2020), might not bind the drug that is coupled to the chromatography matrix; some eluted proteins may be present on the column due to interaction with other proteins that bound 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 (Douzi, 2017; Nikolovska-Coleska, 2015); microscale thermophoresis (Hellinen et al., 2020; Khavrutskii et al., 2013; Wienken et al., 2010); thermal shift assays (Alshareef et al., 2016; Dziekan et al., 2019; Lucet and Murphy, 2017; Martinez Molina et al., 2013); activity-based proteomic profiling (Cravatt et al., 2008; Golkowski et al., 2017) or drug inhibition of purified enzyme activity (Guyett et al., 2016; Labar et al., 2007; Simcic et al., 2014; Warrilow et al., 2016) is 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

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to a drug against a metabolic enzyme occurs by mutations in the enzyme itself (Dicker et al., 1993; Wilson et al., 1992; 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; Chang et al., 2019; Chitanga et al., 2011; Claus et al., 2014; Dermawan et al., 2014; Gatti et al., 2015; Haider et al., 2020; Seyhan et al., 2012; Yobi et al., 2020; Zhang et al., 2019b). 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; Sharma et al., 2020; Zhang et al., 2019a). 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 now appears to be on track for "repurposing" to treat prostate and breast cancer (Dowling et al., 2007; Wang et al., 2014). In cells, modes of action of metformin include inhibition of (a) mitochondrial glycerophosphate dehydrogenase (Madiraju et al., 2014), (b) thiamine transport by OCT1 (Chen et al., 2014), and (c) 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 (COX-2), 5-lipoxygenase (5-LOX) (Kaur et al., 2012), IKK-β kinase (Yin et al., 1998), and it prevents movement
of activating transcription factor 6 (ATF6) 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 physiological targets of aspirin in each of the cell types where the drug disrupts specific biological 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), it inhibits drug efflux by ABCG1 and ABCB2 transporters (Chun et al., 2015; Kuang et al., 2010), 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 (Behera et al., 2014; Katiyar et al., 2013), disrupts assembly of the paraflagellar rod, and inhibits endocytosis of transferrin (Guyett et al., 2017). Human cell resistance to lapatinib treatment is associated with PAK2 (Chang et al., 2018), NF-kappaβ 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 pro-survival functions of EGFR (Cossu-Rocca et al., 2016; Refaat et al., 2015; Tan et al., 2015; Thomas and Weihua, 2019) suggest a need to determine physiological targets of lapatinib in human and trypanosome cells in order 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 physiological 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 physiological target of the drug.

Studying the eukaryotic microorganism Trypanosoma 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 physiological 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 modes of action studies can be used to validate the outcome of drug engagement of a physiological target in a cell.

**The Value of Knowing Physiological Targets of a Drug**

There are multiple benefits of knowing physiological targets of drugs. First, the concept makes it easier to accept a possibility that “off-target” proteins may be biological effectors. By definition, drugs are foreign to cells, which contain over twenty-thousand proteins (Venter et al., 2001; Venter et al., 2015). One expects a drug to bind multiple proteins in a cell (Davis et al., 2011; Fabian et al., 2005; 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 physiological targets enables investigators to make a direct link between protein binding of drug and molecular changes elicited by target engagement (see last section). Re-evaluation of targets for drugs 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; Venter et al., 2015), so an analog of a hit that emerges from optimization as 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 physiological 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 physiological targets from drug-binding proteins could foresee issues that arise late in drug development or, more likely, in the post-marketing 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, due to additional costs involved the multi-disciplinary 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 physiological 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

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inhibition may cause selective toxicity, and lead to cost saving from attrition of some candidate drugs (Choi et al., 2014; Flynn et al., 2017; von Kleist et al., 2016; Zuhl et al., 2016).

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