The multiple orthogonal tools approach to define molecular causation in the validation of druggable targets

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Many genetic (gene deletion, interruption or mutation), epigenetic (such as antisense or small interfering RNA) and immunological methods are being applied in 'high-throughput target validation' studies of the novel potential targets arising from whole genome sequencing. Such applications often focus on 'loss of function' approaches. However, target validation is most reliable when multiple orthogonal approaches are used. Initiating a target-based discovery project based on correlative evidence is faster than awaiting causative evidence. Indeed, the multiple tools needed to generate firm proof usually include methods and reagents only generated after starting a discovery project with little evidence beyond correlations. Robust and rigorous tests of whether a drug candidate is efficacious *in vivo* because of its effects on a specific molecular particular target are best made by simultaneously applying multiple orthogonal tools. Examples of the orthogonal tools approach will be discussed.

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▼ Target validation, one of the most important tasks facing drug discovery scientists today, can be defined in many ways. The most scientifically rigorous definition for medicinal chemists is establishing proof that a molecular target has a crucial role in causation or symptoms of a human disease, and can be targeted by small molecules to clinically intervene in the disease or its symptoms [1]. This definition also encompasses proof of druggability. The most appropriate definition of target validation varies depending upon context. Here, we shall advocate the use of the multiple orthogonal tools approach to prove whether or not a putative target for a pharmacological agent is truly a necessary link in the chain of causation for that agent's effects. Such proof cannot be generated with single tool approaches.

The classical single tool approach is the use of a small molecule identified in a molecular screen to test a therapeutic concept in an animal disease model [2]. Another example is the use of an antisense [3] or small interfering RNA (siRNA) reagent [4], dosed into cells, tissues or animals, to test whether a specific molecular target is involved in a disease. The use of antibodies in the same way has been hampered by the difficulty of getting the reagents into cells (but see Ref. [5]). One of the most popular single tools is the genetic 'knockout', which could be a gene interruption or a partial or complete gene deletion, in a mouse oocyte followed by assessing the physiology of the resulting embryo or animal [6].

In the 'multiple orthogonal tools' approach for target validation, several tools are combined so that the target and the candidate therapeutic agent can be varied independently of each other (orthogonally). This approach is exemplified by drug resistance by target amplification, where overexpression of a putative target protein yields cells that are resistant to the effects of an otherwise cytostatic or cytotoxic small molecule. Increased potency of the small molecule in cells induced to express lowered amounts of the target is another example of a multiple orthogonal tools approach. Yet another example would be loss of efficacy or reduced potency for a small molecule drug candidate in cells (or whole animals) that are transgenic for a mutated form of the putative target, where the mutant protein has a much reduced affinity for the small molecule. Finally, another example would be a reduced therapeutic efficacy for a drug candidate that is an antisense RNA agent, in cells or tissues or animals where the target has been altered with (otherwise functionally silent) nucleotide sequence changes. Employed appropriately,

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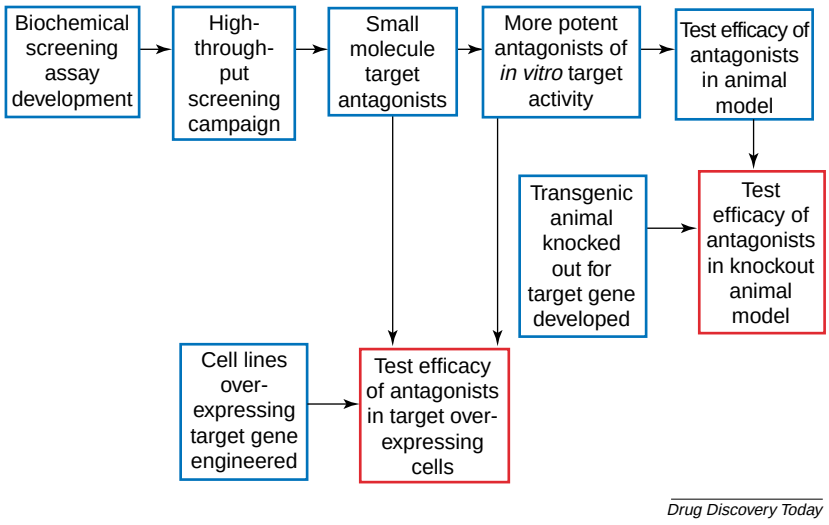

Figure 1. Combining target validation tools orthogonally (indicated by the tests in the red boxes) allows the rigorous assessment of whether a specific molecular target is causally involved in the efficacy of a drug candidate.

'chemical genetics' can be viewed as a multiple orthogonal tools approach to identify targets [7,8].

The speed demanded in the pharmaceutical industry for development of new drug candidates does not allow targets to be validated to an absolute level of certainty before a project begins. Pragmatically, the risk of starting a discovery project on a less-than-fully validated target must be balanced against the opportunity cost of delaying a project on a new target that might yield a blockbuster product. Indeed, the FDA and other regulatory authorities do not require that a drug's target be known, and many successful drugs act via unknown mechanisms. But in target-based discovery, effective lead optimization requires accurate knowledge of a relevant target. Moreover, as a target-driven discovery project proceeds, it is probable that the tools needed to verify rigorously that the lead compound's actions *in vivo* depend upon a specific molecular target will accumulate. Concerns about both clinical efficacy and safety, as well as the economic pressure to kill candidates quickly that are unlikely to succeed in the clinic, make it a good idea to do rigorous orthogonal tests as early as possible. Although the only validation that ultimately matters is human efficacy and safety, rigorously testing therapeutic concepts before clinical trials helps minimize costly failures.

Correlation versus causation

Proving a causal relationship between a molecular target and a disease is best performed not with a single tool or with a serial application of single tools, but by combining orthogonal tools (Figure 1). Orthogonal tools for high-throughput target validation, such as chemical and genetic or chemical and epigenetic approaches, are methods that can be applied to the target independently of each other, one affecting the target's activity chemically and the other affecting the amount of target present. Varying the level of a target *in vivo* independently of the concentration of an antagonist that has been identified *in vitro* allows a determination of whether the *in vivo* action of the antagonist arises from its effect on the putative target. Combining chemical and genetic or epigenetic approaches [2,9] (see Box 1) enables causal relationships to be tested rigorously, rather than providing merely correlative support for a target's disease relevance and druggability by applying single tools in serial fashion. Showing that a compound binds to a particular receptor *in vitro* and then showing that the same compound has an effect *in vivo* is a serial approach, and does not prove that the compound's *in vivo* effect is caused by its interaction with that particular receptor.

Target invalidation is as important as validation, because it allows early project termination and averts or minimizes the waste of resources on irrelevant or non-druggable targets [10–12]. Because the most rigorous proof requires that the fewest assumptions be made, multiple orthogonal methods are needed to minimize those assumptions. Several applications of multiple orthogonal tools in diverse therapeutic areas are discussed below to exemplify this multi-pronged strategy.

Why do we need the multiple orthogonal tools approach?

Despite the utility of gene knockouts and antisense methods, simply removing a protein completely can be uninformative or even misleading. Despite efforts to model drug efficacy with knockouts, many knockouts of the putative targets of drug candidates in the product pipelines of top pharmaceutical companies lead to fetal lethality, making the phenotypes difficult to interpret [13]. Ablating gene products by chromosomal knockouts or by antisense or siRNA methods can have pleitropic effects. Some proteins might function in multiple ways during development (e.g. transforming growth factor β [14] and uteroglobin [15]), or even in the fully developed adult organism (e.g. dipeptide peptidase IV [16], cytochrome C [17], phosphoglucose isomerase [18] and other moonlighting proteins [19]). Complete removal of such a multifunctional protein will probably have multiple

physiological consequences. Furthermore, 'no observable phenotype' in a knockout does not necessarily mean that the target has no important role in the adult organism. Functional flexibility in redundant systems can disguise the normal role of a target gene product. In such cases, the knockout animal does not predict physiology in the adult, because of the ability of the organism to compensate for the knockout during development. For instance, the first mice developed with a targeted disruption of the gene for the cAMP response element-binding (CREB) protein, a key regulator in the transcriptional control of many genes, yielded apparently healthy mice with no immediately obvious impairment [20]. This surprising result, from a disruption of what turned out to be CREB-α, spurred additional studies that revealed functional redundancy in the CREB family of transcription factors [21]. An indication of a key functional role for CREB-α in brain function was the effect of its deletion upon responsiveness to the small molecule morphine [22]. This indicates the value of the multi-pronged approach.

The knockout of a receptor gene can yield animals with changes in other signaling systems that compensate or even over-compensate for the loss of function [23–29]. For example, several of the differing subunits of the nicotinic acetylcholine receptor appear to be redundant when examined by gene knockouts [23]. In another example, a genetic knockout of the serotonin receptor subtype 5HT1B yielded animals that are more sensitive to the effects of cocaine, even though a small molecule antagonist specific to the 5HT1B receptor blocks many of the pharmacological effects of cocaine in wild-type animals [24]. The effects of the knockout have been interpreted to arise from an over-sensitization, because of one receptor subtype's loss, of other receptors affected by cocaine [25]. A third example involves the β2 adrenergic receptor (AR-β2), which is the primary conduit for communication between the sympathetic nervous system and the cells of the immune system [26]. Mice that had homozygous deletions of the AR-β2 gene developed normally with fully functional immune systems, except for an inability to respond to norepinephrine [27]. The compensation that allowed normal development of the AR-β2 deletion mice appeared to involve non-adrenergic receptor systems [27]. The power of combining small molecule agonists and antagonists with gene knockouts, knockins and antisense studies is evident in the applications of this orthogonal approach to G-protein coupled receptors (GPCRs), which have been extensively reviewed [28,29].

The opportunity cost of relying on single methods for target validation

Despite uncertainties in interpreting the phenotypic effects of gene knockouts, such as those described above, it is tempting to invoke Occam's razor and interpret lack of evidence ('negative' constructs) as evidence for lack of physiological significance [30]. This approach has its costs. Some valuable targets will be missed in high-throughput technologies if a single tool approach is used to validate a drug discovery portfolio. For example, CREB (discussed above)

Box 1. Definitions of orthogonal tools for validation tests of potential drug targets

In general, the genetic and epigenetic tools can be used to alter the intracellular level or nature of the putative target, and the chemical and other protein-directed tools can be used to alter the function of the target, either by inhibiting or stimulating target activity. The genetic and epigenetic tools are generally orthogonal to the chemical and other protein-directed tools.

Genetic or epigenetic

Knockout = homozygous (–/–) target gene disruption or deletion; null mutations with loss of function might also be used; heterozygous (+/–) deletion leaves partial target expression

Gene replacement (also known as knockin or transgenic) = replacement of target gene in an animal with, for example, the homologous human gene

Overexpression = engineered increase in level of target protein; can be altered by transcriptional regulation or gene amplification

Dominant-negative allele = gene expressing a mutant target protein which blocks the activity of other (wild-type) proteins with which it interacts

RASSL = receptor designed to be activated solely by a synthetic ligand

ASKA = analog-sensitive kinase allele (gene encoding a protein kinase engineered to interact with a synthetically modified substrate or a specific inhibitor)

Ortholog = an alternate protein with same activity as putative target protein; might be selected for its intrinsic resistance to an inhibitor

Antisense = nucleic acid designed to hybridize to mRNA to induce its degradation by RNase H

siRNA = small interfering RNA; short double-stranded RNA designed to specifically block the expression of a gene containing homologous sequences

Chemical (or other protein-directed agent)

Small molecule = synthetic or natural product; could be an inhibitor, agonist, antagonist, or reverse agonist

Anti-target antibody = monoclonal or less frequently polyclonal antibody or antibody fragment selected for high affinity target binding and/or inhibition

Aptamer = synthetic nucleic acid selected for high affinity target binding and/or inhibition

Nutritional rescue = providing a metabolite downstream of an inhibited biosynthetic enzyme.

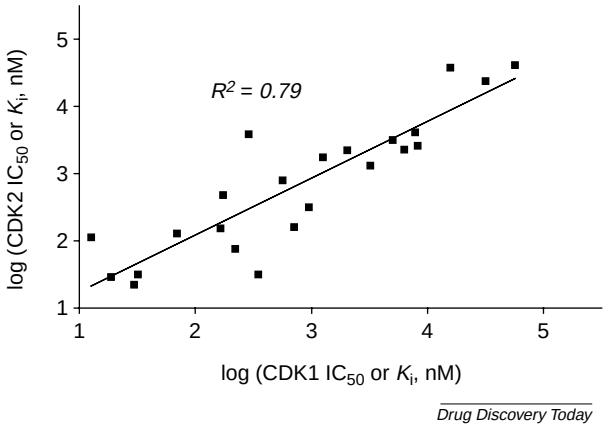

Figure 2. Correlation between the inhibitory potencies against CDK1 and CDK2 for small molecule inhibitors from ten different structural classes (IC_{50} and K_i values are from Ref. [49]). The line was generated by least squares fit of the points shown to the equation for a straight line.

appeared to be non-essential in the initial analysis of CREB-targeted mice. Numerous other examples exist in studies of GPCR knockouts [28,29]. For instance, mice that were constructed with a knockout of the M_5 muscarinic receptor gene showed no differences from their +/+ littermates developmentally, nor in many behavioral and pharmacological tests [31]. However, the ability of cerebral arteries to respond to acetylcholine, a powerful vasodilator, was completely lost in the M_5 muscarinic receptor knockout mice [31]. Non-cerebral arteries were unaffected by this knockout. The specific detailed studies required to elucidate this phenotype were prompted by a careful analysis of the tissue distributions of M_5 receptor mRNA. A decade earlier, it had been shown by *in situ* hybridization histochemistry with isotype-specific oligonucleotide probes [32] that the only cerebral arteries that expressed the M_5 muscarinic receptor mRNA were those of dopamine-containing neurons of the substantia nigra pars compacta. However, the physiological meaning of this receptor subtype in that region of the brain was not certain until the genetic tool (knockout mouse) was combined with the small molecule (acetylcholine) [31]. The use of acetylcholine with the knockout demonstrated a clear physiological role for the M_5 muscarinic receptor, despite the lack of an agent that was selective against the M_5 muscarinic receptor.

Another example involves cyclophilin A, a protein that is essential for the action of one of the most important immunosuppressant drugs, cyclosporin [33]. Homozygous disruption of both alleles of the gene encoding cyclophilin A in mouse embryonic stem cells did not affect the ability of the cells to grow normally and to differentiate into hematopoetic precursor cells [34]. Yet, the drug that requires cyclophilin A for its action has revolutionized solid organ transplantation, and is one of the most valuable drugs in the medicine chest. In many other cases, the physiological effect of a gene knockout appears subtle or non-existent until the correct small molecule is applied to the correct tissue [29].

Pros and cons of small molecules

The advantage of a small molecule is that it can intervene with a single site on a multifunctional protein, leaving other functions unmodified. However, small molecules cannot be assumed to be selective, and indeed it is the difficult business of the medicinal chemist to make them so [35]. Just because a small molecule is potent *in vitro* and shows an effect in an animal, this does not mean that the *in vitro* activity is the root cause of the *in vivo* effect. Correlation is not causation. Again, cyclosporin provides an instructive example [36]. In 1989, it was discovered that the high affinity binding protein for cyclosporin, cyclophilin, is identical to an abundant protein previously characterized as a peptidylprolyl isomerase (PPI) [37,38]. PPIs catalyze the *cis-trans* isomerization of peptidylprolyl bonds, one of the slowest steps in the folding of some protein domains [39]. Cyclosporin was shown to potently inhibit the PPI activity of cyclophilin [37,38]. Proposals were quickly advanced that protein folding might be a crucial and limiting step in T lymphocyte maturation [37,39–41]. However, within several years this hypothesis was eliminated [12]. One clue was that the tissue concentrations of cyclophilin are a thousand-fold higher than the concentrations of cyclosporin required to block T cell proliferation [42]. Moreover, the inhibitory potencies of synthetic cyclosporin analogs for blocking T cell proliferation did not correlate with their potencies against the PPI activity of cyclophilin [42,43], suggesting that the latter was not the physiologically relevant event. Instead, it was shown that the inhibition of a protein phosphatase (calcineurin) by the cyclosporin–cyclophilin complex is the limiting event responsible for the immunosuppressive activity of cyclosporin [44]. As a result, synthetic efforts to generate a cyclosporin analog with optimal inhibition of the PPI activity of cyclophilin were replaced by efforts to synthesize an analog that would, when complexed to cyclophilin, potently inhibit the phosphatase activity of calcineurin (see, for example, Refs [45,46]).

A more recent example of how reliance on small molecules alone can be deceiving is provided by inhibitors of the serine/threonine kinase, cyclin-dependent kinase 2 (cdk2). This kinase appears to cooperate with several homologous enzymes in controlling the advancement of cells through the cell cycle and mitosis [47]. Many groups in both industrial and academic laboratories have developed

potent inhibitors of this enzyme, with the goal of treating cancer and other proliferative diseases such as restenosis [48–50]. The potencies of the compounds in blocking the kinase activity of cdk2 *in vitro* correlate well with their potencies in blocking cell proliferation [49]. However, it was recently shown [51], using a dominant-negative form of the enzyme as well as antisense and siRNA methods in several cancer cell types, that cdk2 is not essential for cancer cell proliferation. It is probable that the cellular activity seen with small molecule inhibitors of cdk2 is actually because of their effects upon a different enzyme. Perhaps the actual target is cdk1, because the potencies of cdk1 and cdk2 inhibition are highly correlated, as illustrated in Figure 2 [49]. Proving that cdk1 is the actual target for these inhibitors would require the use of cells engineered specifically to overexpress activated cdk1, or to express an activated form of a mutant cdk1 that is resistant to the inhibitors.

Combining multiple orthogonal tools

For a target-based drug discovery project to be effective, the target must be physiologically relevant to the disease's cause or symptoms. Most discovery projects begin with only supportive correlations that fall short of proof. Proving such relevance requires the simultaneous application of several tools orthogonally (Figure 1). A single tool, or the serial application of single tools cannot provide such proof, although they can provide evidence needed to begin a discovery project on a promising new target. This might lead to more correlative evidence and sufficient tools to perform an orthogonal test. Such orthogonal tests become more vital as discovery projects proceed to later stages.

An application of multiple orthogonal tools is the creation of drug-resistant bacteria by amplification or point mutation of a causal target. When a small molecule inhibits a molecular target *in vitro* and also has antimicrobial activity, the correlation of these two 'single tool' observations suggests that the antimicrobial activity arises because of the *in vivo* blockade of that target. However, this correlation is not proof, because there might be other targets for the small molecule that are more important. Combining these orthogonal tools, by increasing or decreasing the *in vivo* target concentration and measuring the resulting effect on the *in vivo* potency of the small molecule, allows one to eliminate the possibility that the target being assayed *in vitro* is not physiologically relevant. When amplification or mutation of the proposed molecular target yields drug resistance, this proves a causal relationship. Cell rescue instigated by introducing an alternate pathway, or by feeding an intermediate or essential metabolite downstream of an anabolic blockade, are other methods for proving the physiological relevance of a target for a small molecule inhibitor. Classic examples of such evidence exist for the enzyme dihydrofolate reductase (DHFR), the target for the antifolate trimethoprim. Trimethoprim is a widely prescribed antibacterial drug which potently inhibits many bacterial DHFRs [52]. DHFR is responsible for replenishing a fully reduced folate cofactor required for the synthesis of an essential DNA precursor (thymidine 5′-monophosphate, or thymidylate). The requirement for thymidylate can be met by feeding thymine or thymidine, which can be converted to thymidylate by bacteria, rendering the bacteria resistant to trimethoprim in the presence of thymidine or thymine (nutritional rescue) [53]. Alternately, either the mutation of the chromosomal DHFR to a trimethoprim-resistant form [54] or the introduction of a plasmid-encoded trimethoprim-resistant ortholog [55] are sufficient to render bacteria resistant to the drug, proving that DHFR is the *in vivo* target for trimethoprim.

Numerous researchers have pursued DHFR inhibitors to treat the infectious parasites that cause the human protozoan diseases leishmaniasis, Chagas' disease and African trypanosomiasis [56]. However, these organisms can bypass the blockade of DHFR by expression of a novel pteridine reductase [57,58]. An alternate strategy would be to develop dual inhibitors of both DHFR and pteridine reductase [59]. A collection of quinazoline and 2,4-diaminopyrimidine inhibitors was identified that inhibited both of these *Leishmania* enzymes, and some also inhibited the growth of *Leishmania* [59]. However, a sub-set of the growth-inhibitory compounds must have another target in these parasites besides DHFR or pteridine reductase, because either overexpression or deletion of the reductase targets yielded recombinant strains of *Leishmania* with unchanged sensitivity to that sub-set of inhibitors, compared with the sensitivity of wild-type organisms [59].

Other studies with *Leishmania* by Ullman and colleagues support the idea that ornithine decarboxylase (ODC) is the target for α-difluoromethylornithine, an irreversible inhibitor of ODC [60,61]. ODC catalyzes the formation of putrescine (1,4-diaminobutane, a biosynthetic precursor to spermidine), and controls the rate of biosynthesis of polyamine biosynthesis in most organisms. Although clinically α-difluoromethylornithine is used as an anticancer drug, the drug is also cytotoxic for infectious protozoa such as *Leishmania* species [60]. Supplementing the protozoan diet with either spermidine or its precursor putrescine rescues *Leishmania* completely from either the cytotoxic effects of α-difluoromethylornithine treatment [60] or the lethality caused by deletion of the ODC gene [61].

Examples of the use of multiple orthogonal tools in a variety of therapeutic areas are given in Table 1. Many of

Table 1. Applications of simultaneous multiple orthogonal tools in target validation studies

	Proposed target	Small-molecule (drug or pharmacological tool)	Phenotype or pharmacological/ toxicological effect	Method to alter target – small molecule interaction	System	Results	Ref.
1	Bcr-Abl kinase and imatinib-resistant Bcr-Abl kinase	Imatinib	Drug sensitivity of cells expressing Bcr-Abl kinase	Anti-Bcr-Abl siRNA (transfected)	Human cell lines	The antitumor activity of imatinib is because of the drug's inhibition of Bcr-Abl kinase	[62]
2	*Toxoplasma gondii* cGMP-dependent protein kinase	Antiprotozoal small-molecule (trisubstituted pyrrole)	Sensitivity of parasites in infected mice to killing by the small molecule	Replacement of wild-type kinase gene with drug-resistant allele	Mice infected by wild-type or mutant parasites	The antiprotozoal action of the trisubstituted pyrrole small molecule in mice is because of inhibition of the parasite cGMP-dependent protein kinase	[63]
3	Xenobiotic receptor CAR-mediated transcriptional activation	Acetaminophen	Hepatotoxicity induced by CYP3A4-catalyzed metabolism of acetaminophen	CAR gene knockout	Whole mouse	Acetaminophen induces transcription of the gene encoding CYP3A4, the enzyme that converts it to a hepatotoxin, by activating hepatic transcription factor CAR	[64]
4	Bacterial β-ketoacyl-acyl carrier protein synthase I (fabB)	Thiolactomycin	Drug sensitivity for growth	Replacement of wild-type fabB gene with drug-resistant allele	Bacteria grown *In vitro*	The antibacterial activity of thiolactomycin is because of inhibition of the wild-type bacterial β-ketoacyl-acyl carrier protein synthase I	[65]
5	Farnesyl-transferase action on RhoB	L-744832	Tumor induction by tumor cell graft	RhoB gene knockout	Whole mouse with graft of engineered tumor cells	The antitumor activity of L-744832 requires RhoB to become a substrate for geranylgeranyltransferase, upon blockade by L-744832 of normal RhoB farnesylation	[66]
6	CD26 dipeptidyl-peptidase IV	Valine-pyrrolidide	Glucagon-like peptide 1 (GLP-1) stability and glucose-stimulated insulin secretion	CD26 gene knockout	Whole mouse	The stabilization of serum GLP-1 and increased glucose tolerance caused by valine-pyrrolidide is because of the inhibition of CD26	[67]

7	Leptin receptor signaling	Leptin	Leptin-dependent changes in eating behavior and body fat accumulation	Protein phosphatase 1B gene knockout	Whole mouse	The signaling cascade that is activated by binding of leptin is regulated by protein phosphatase 1B [68]
8	Farnesoid X receptor (FXR)-mediated transcriptional activation	Guggulsterone	Hypolipidemic effect in animals with high dietary cholesterol	FXR gene knockout	Whole mouse	The lowering of blood lipids induced by guggulsterone is caused by activation of the transcription factor, FXR [69]
9	PPAR-α-mediated transcriptional activation	Clofibrate	Hepatic peroxisome proliferation and liver damage	PPAR-α gene knockout	Whole mouse	Clofibrate-induced mouse hepatotoxicity must occur via PPAR-α activation [70]
10	5-HT_7 receptor signaling	Serotonin; oleamide	Hypothermia	5-HT_7 receptor gene knockout	Whole mouse	The 5HT_7 receptor mediates serotonin-induced hypothermia; oleamide induces hypothermia through some other receptor (even though oleamide binds to the 5HT_7 receptor) [71]
11	Neurotrophin-induced neuron activation	Neurotrophin	Neuronal membrane depolarization	Antisense RNA expression	Human neuro-blastoma cell line	Brain-derived neurotrophic factor elicits neuron excitation by activation of sodium channel Nav1.9 [72]
12	TNF-α	Bacterial lipopoly-saccharide (LPS)	Sepsis induced by TNF-α expression, secondary to bacterial LPStreatment	Anti-TNF-α siRNA (intraperitoneal injection)	Whole mouse	The septic response in mice treated with LPS requires the induction of TNF-α [73]
13	Cannabinoid CB1 receptor	Rimonabant (small molecule CB1 receptor antagonist)	Indomethicin-induced intestinal ulcers, and LPS-induced TNF-α release	CB1 receptor gene knockout	Whole mouse	Rimonabant blocks LPS- induced TNF-α release by interaction with CB1 receptor, but must block indomethicin-induced ulceration through another mechanism [74]
14	Thromboxane A2 (TXA2) receptor	Arachidonic acid; small molecule agonist U-46619	Vascular effects of arachidonic acid	(TXA2) receptor gene knockout	Whole mouse	Arachidonate-induced shock and the effects of TXA2 receptor agonist U-46619 on blood pressure and platelet aggregation are mediated by TXA2 receptor [75]
15	P2Y12 ADP receptor	ADP; clopidogrel	ADP-induced platelet aggregation	P2Y12 ADP receptor gene knockout	Whole mouse and platelets	Major portion of ADP-induced platelet aggregation is because of the P2Y12 receptor; the antagonism of ADP by clopidogrel occurs exclusively through P2Y12 receptor [76]

the studies incorporate new twists on the classic drug resistance approach. For example, siRNA-induced target suppression [62] and an inhibitor-resistant variant [63] of protein kinases (entries 1 and 2) were used to show that the physiologically significant target for a small molecule inhibitor was the specific kinase in question. With the constitutive androstane receptor (CAR), the multiple orthogonal tools approach was employed (entry 3) to prove that CAR is required to induce the events that result in the hepatotoxicity of the widely used drug, acetaminophen [64]. In these cases, the nutritional rescue method is out of the question. However, the basic concepts are the same, and the multiple orthogonal tools approach is broadly applicable to many different types of proteins and across many therapeutic areas, including antibacterials (Table 1, entries 2 and 4), oncology (entries 1 and 5), metabolic diseases (entries 6–9), CNS (entries 10 and 11), inflammation (entries 12 and 13), and cardiovascular diseases (entries 14 and 15).

One example of the approach involves a peptidase that has potential value as a target for diabetes therapy (entry 6). CD26 is a multifunctional glycoprotein with dipeptidyl peptidase activity that cleaves and inactivates several peptide hormones [16] including glucogon-like peptide 1 (GLP-1). The intact form of GLP-1 enhances insulin secretion, and hence small molecule inhibitors of CD26 are being tested clinically by Merck and Novartis as potential drugs to treat diabetes [77]. Treatment of rodents or humans with CD26 inhibitors increases the stability of circulating GLP-1, enhancing insulin secretion and increasing glucose tolerance [78]. Knocking out the CD26 gene in mice has a similar effect, and the resulting CD26 knockout mice show no further enhancement of insulin secretion or glucose tolerance with the addition of the CD26 inhibitor valine-pyrrolidide [67]. Prior to the knockout study, the assignment of the actual target for CD26 inhibitors was uncertain because of the numerous enzymes exhibiting similar activity *in vivo* (see Ref. [67] and references cited therein). The effect of the small molecule CD26 inhibitor NVP-DPP728 is also abolished in aged rats that lack CD26 activity [79]. These studies prove that CD26 is the only enzyme whose blockade is necessary for the *in vivo* effects of NVP-DPP728 or valine-pyrrolidide. However, mice in which the GLP-1 gene has been knocked out are still responsive to valine-pyrrolidide [67], demonstrating that CD26 affects blood glucose regulation by cleaving more substrates than just GLP-1.

An example of the orthogonal tools approach in cardiovascular diseases involves the thromboxane A2 (TXA2) receptor (Table 1, entry 14). Homozygous deletion of the TXA2 receptor gene yields mice that have prolonged bleeding times, and that are no longer responsive to the synthetic TXA2 receptor agonist U-46619, which proves that the cardiovascular effects of U-46619 are mediated solely through the TXA2 receptor [75]. The vascular effects of U-46619 are competitively antagonized completely by ifetroban, which is in Phase II clinical trials for various cardiovascular indications [80]. This strongly suggests that at least some of ifetroban's efficacy is because of its interaction with the TXA2 receptor, even without a direct examination of the effects of ifetraban itself in a TXA2 receptor knockout, or with a similar genetic tool.

The studies described here and others listed in Table 1 show clearly that modern methods to ablate, alter, or increase the concentration of a target *in vivo*, in combination with an exogenous pharmacological agent, can be employed to definitively assess whether the target is a link in the chain of causation for that agent's effects.

Applying multiple orthogonal tools to prove direct interactions

The use of multiple orthogonal tools does not necessarily prove that the target in question interacts directly with the pharmacological agent. Definitive proof of causality that depends upon a direct interaction requires the use of allele-specific agonists or antagonists, as exemplified by Shokat's work with protein kinases [81], and by Conklin's work with GPCRs [82]. This technique requires that the target protein be mutated specifically to interact only with a cognate synthetic ligand. In Shokat's case, the kinase target is referred to as an ASKA (analog sensitive kinase allele). In Conklin's case, the engineered receptor is called a RASSL (receptor activated solely by synthetic ligand). The goals are similar in both cases: to identify the physiological function of the kinase or GPCR *in vivo*, without the convolutions arising from effects of non-selective ligands on multiple targets. A chimeric RASSL can also be used to define receptor function when there is no small molecule known to activate the receptor of interest. To do this, the chimera must contain binding determinants from another receptor which has an available small molecule activator. The approaches pioneered by Shokat, Conklin and others can be extended to other potential target proteins [83].

Perspective

Many existing drugs have been developed without knowledge of the actual target, or against targets validated with single tool approaches. However, the need to integrate information from multiple techniques for effective target-based drug discovery is increasing [1,2,9]. New discovery projects with novel targets will require that target validation continue and become more rigorous as projects mature.

The application of multiple techniques in an orthogonal manner allows the *in vivo* level or nature of a putative target to be manipulated independently of the concentration of the pharmacological agent. Combining multiple orthogonal tools can yield definitive deductions, and thereby validate or invalidate a potential drug target, as well as its druggability. Such deductive evidence yields far more powerful proof than the correlative evidence provided by the serial application of single tools, and will be used increasingly by progressive companies to set priorities among competing advanced projects.

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