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Target Engagement in Lead Generation

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ABSTRACT

The pharmaceutical industry is currently facing multiple challenges, in particular the low number of new drug approvals in spite of the high level of R&D investment. In order to improve target selection and assess properly the clinical hypothesis, it is important to start building an integrated drug discovery approach during Lead Generation. This should include special emphasis on evaluating target engagement in the target tissue and linking preclinical to clinical readouts. In this review, we would like to illustrate several strategies and technologies for assessing target engagement and the value of its application to medicinal chemistry efforts.

Great attention has been dedicated in the past few years to analysis of the current challenges in the pharmaceutical industry. In spite of the sequencing of the human genome and the exponential rate of learning in the area of human health, no significant improvements in clinical success rate have been realized. Multiple authors have analyzed the underlying causes for this inefficiency trend. Many concluded that a lack of efficacy in Phase 2 clinical studies was the major reason for failure.[1–3] The inability of preclinical disease models to predict clinical outcomes and the frequent irreproducibility of literature findings further increases the difficulty of modern drug discovery.[4,5] To both internal and external observers, it is clear the pharmaceutical industry is in a state of paradigm shift. The industry is moving away from older strategies and business models for selecting targets and molecules for clinical investigation. Recent strategies focus more on developing a deeper understanding of mechanisms of action, pathway biology, and the relation of a biological target to human disease. To increase the probability of technical success, it is crucial to start investing during preclinical research in target validation, target selection, and development of integrated drug discovery strategies.[2,6]

Identifying potential clinical readouts or biomarkers that can be used pre-clinically should help connect discovery research (from hit identification to candidate selection) to the ultimate test of the clinical hypothesis in man. At minimum, being able to demonstrate sufficient clinical target engagement at the site of action would unequivocally establish the validity of a given target for a specific disease indication.[7]

This concept is supported by recent analyses conducted by major pharmaceutical companies. A retrospective analysis by Pfizer of 44 drug programs in Phase 2 identified 'lack of efficacy' as the most common cause of attrition in their discovery programs.[8] To improve drug discovery effectiveness, the authors suggested a model of 'three pillars' for evaluating potential investment in non-validated drug targets: (1) sufficient exposure of ligands at the site of action; (2) proof of target engagement; (3) expression of functional pharmacological activity. The authors' conclusion was that projects being able to demonstrate all 'three pillars of survival' should have the highest probability of translating in human clinical studies.

AstraZeneca has also recently published an exhaustive review of their small molecule pipeline from 2005 to 2010.[9] They identified five critical technical determinants of project success (coined the 'five R's'): the right target, the right patient, the right tissue, the right safety and the right commercial potential. In particular, the 'right tissue' is defined as the appropriate exposure of the candidate drug in the target organ leading to sufficient pharmacological activity. To assess the 'right tissue', it is necessary to evaluate pharmacokinetic properties and target engagement to develop an understanding of the PK/PD correlations relative to the target organ. Interestingly, it was pointed out by the authors that less than 10% of the projects reviewed had demonstrated a strong correlation between target occupancy and pharmacological activity.

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This review is composed of two halves. The first half presents background and discussion on how target engagement can be used in Lead Generation drug discovery along with a brief overview of various established methods for its measurement. In the second half, we explore specific examples from recent literature where target engagement is being interrogated within Lead Generation and highlight emerging technologies that can assess target engagement.

Target engagement in Lead Generation: Traditionally, drug discovery teams build a testing scheme progressing compounds from in vitro testing (to measure binding, affinity, and selectivity) to assessing ADMET properties and efficacy in preclinical animal models.[10–12] A target engagement assay (Fig. 1) linking compound performance in vitro to compound performance in vivo is critical to ensure the appropriate compound concentration reached the intended target. Ideally, an integrated discovery approach will link target engagement with relevant clinical endpoints. It is important to emphasize that there are different types of biomarkers: (1) diagnostic markers, to assess the presence or absence of disease; (2) disease activity markers, to assess severity of the disease; (3) drug effect biomarkers, markers of target engagement and PD effects; (4) drug kinetic biomarkers, to assess genetic variants on drug metabolizing enzymes and drug transporters. For the purpose of this review, we will refer to drug effect biomarkers.

An excellent example of the use of drug effect biomarkers is the development of sitagliptin, a DPP4 inhibitor for the treatment of diabetes. Preclinical studies demonstrated that 80% inhibition of the enzyme generated maximal lowering of blood glucose. Similar degrees of DPP4 inhibition in the first human studies were associated with reduced glucose levels. Those correlations significantly facilitated Phase 2 clinical studies, and even shortened clinical development time.[13] A second example where target engagement has been clearly linked to efficacy is anti-psychotic drugs targeting the dopamine D2 receptor. It is now well established that achieving ~60% receptor occupancy correlates to positive benefits in patients.[14,15]

Being able to assess the degree of target engagement, pharmacodynamics and duration of effect (time on target) relative to preclinical measures of efficacy (e.g., behavioral measures, biomarkers, etc.) are crucial for compound selection and further hypothesis generation (Fig. 2). Once the correlation is built between in vitro activity, target engagement and in vivo efficacy, a target engagement assay should supply a mechanism for rapid decision making. Such an approach has the potential to require less use of iterative preclinical animal models, which supports the responsible use of animals for research.[16,17]

There is a wide variety of methods to measure target engagement biomarkers. The use of a particular method is influenced by the ease of access to the relevant tissue and the nature of the downstream pharmacological effect.[18] For example, within the field of chemical biology, Cravatt and co-workers have highlighted the use of chemical probes to engage their intended targets in vivo to validate protein function.[19] Optimized functional chemical probes can measure occupancy inside the cell and facilitate unbiased selectivity determination in a more physiologically relevant environment.

Imaging techniques like positron emission tomography (PET) have received great attention since they can enable non-invasive target engagement assays compatible with human clinical studies.[20,21] Recent developments in liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) methods, have enabled the rapid assessment of chemical space for a suitable tracer in a preclinical setting.[22] It takes advantage of the same biology that PET measures by comparing levels of the tracer in a total binding region to that of a null (target deficient) region, distinguishing specific binding from background. Several examples from different companies applying this methodology to their medicinal chemistry efforts will be discussed later in this review.

Key breakthrough advances in imaging technology have allowed for an increase in imaging resolution resulting in a significant number of applications to early drug discovery. In vivo bioluminescent imaging (BLI) is a sensitive tool based on detection of light emission from cells or tissues. Reporter gene technology enables the analysis of specific cellular and biological processes in a living animal through imaging methods. Combining animal engineering with molecular imaging techniques, it is possible to conduct dynamic studies of specific molecular processes in living animals. BLI-based models founded on the same reporter assays used in high-throughput screening (HTS), could offer a bridge between in vitro biological assays and preclinical animal model efficacy studies. In comparison with animal models, mechanistic BLI reporter models require less resources. They also have a higher throughput and generate quantitative data for compound ranking in three formats: primary cells, tissues and whole animals.[23] This approach could dramatically impact cycle times during Lead Generation efforts. An interesting recent example is the generation of a bioluminescence transgenic mouse model for detecting ligand activation of GPCRs by Polites and co-workers at Sanofi.[24]

Another example of molecular imaging is biodistribution studies confirming that a compound reaches the target tissue. This also allows assessment of accumulation in non-target sites. Investigations in whole-animal imaging using micro-PET have garnered increased interest. This is due to the technique's ability to assess biodistribution not only for CNS targets but also for oncology targets where up-regulation of pumps excluding drugs from tumors are a significant issue.[25–27]

Target engagement studies are most valuable when there is a robust hypothesis regarding the extent of target engagement needed for a pharmacological effect. In those cases, data relating

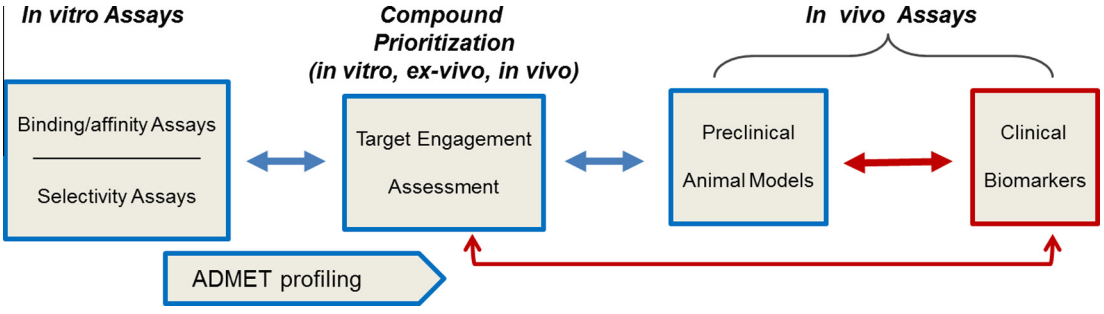


Figure 1. Flowscheme representation: building correlations across the drug discovery paradigm. Blue boxes represent preclinical assays and red boxes clinical readouts. There should be a connection between preclinical and clinical readouts. Clinical results should also inform future discovery projects. Arrows represent critical data correlations along the drug discovery paradigm.

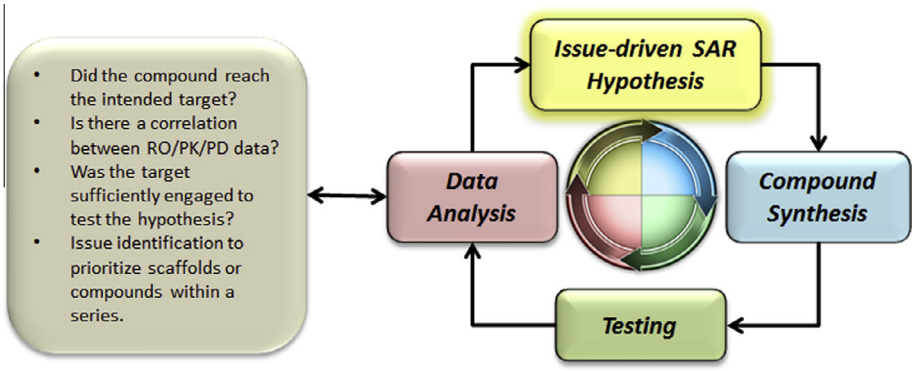


Figure 2. Iterative drug discovery process.

plasma concentration to target occupancy might guide dose selection directly. All preclinical drug discovery projects should have a hypothesis regarding the level and duration of target occupancy that is anticipated will deliver a desired in vivo outcome. Establishing and testing target engagement-pharmacology hypotheses are crucial for a project to ultimately select the optimal candidate molecules.

During Lead Optimization (LO) when additional toxicology data is available, a target engagement assay can be a very powerful tool to compare compounds. For example, Figure 3 shows the target engagement versus plasma compound concentration for two hypothetical compounds A and B which are ligands for target X. In this instance, it has been hypothesized that achieving a minimum of 60% target engagement is required to deliver the desired pharmacological effects (shown as a solid horizontal line on the graph). However, each molecule also has adverse effects driven by off-target activity which occur at unique plasma concentrations (shown as dashed vertical lines). Thus, each compound has a different margin of safety (MOS, see shaded rectangles).

Examples of target engagement in Lead Generation: Increasingly, we are seeing more researchers publishing on, and emphasizing the importance of the application of target engagement assays to their medicinal chemistry efforts. In the second part of this review, we will highlight a few selected examples of these integrated drug discovery approaches published recently. These examples span the known range of techniques, strategic approaches, stages of the drug development lifecycle, target classes, and disease opportunities. By design, each highlighted biological target has yet to be either validated (commercial launch) or invalidated (definitive clinical experiment) as a human drug target. These are truly ongoing efforts. In some cases, significant gaps in knowledge exist within the published literature. Our hope is that in presenting a broad overview of how others are approaching the fundamental questions of target engagement, inspiration will be gained to apply those learnings. Representative examples cover the following topics: target engagement in the CNS and lung; target engagement in the periphery; target engagement determination using chemical methods; target engagement in cells.

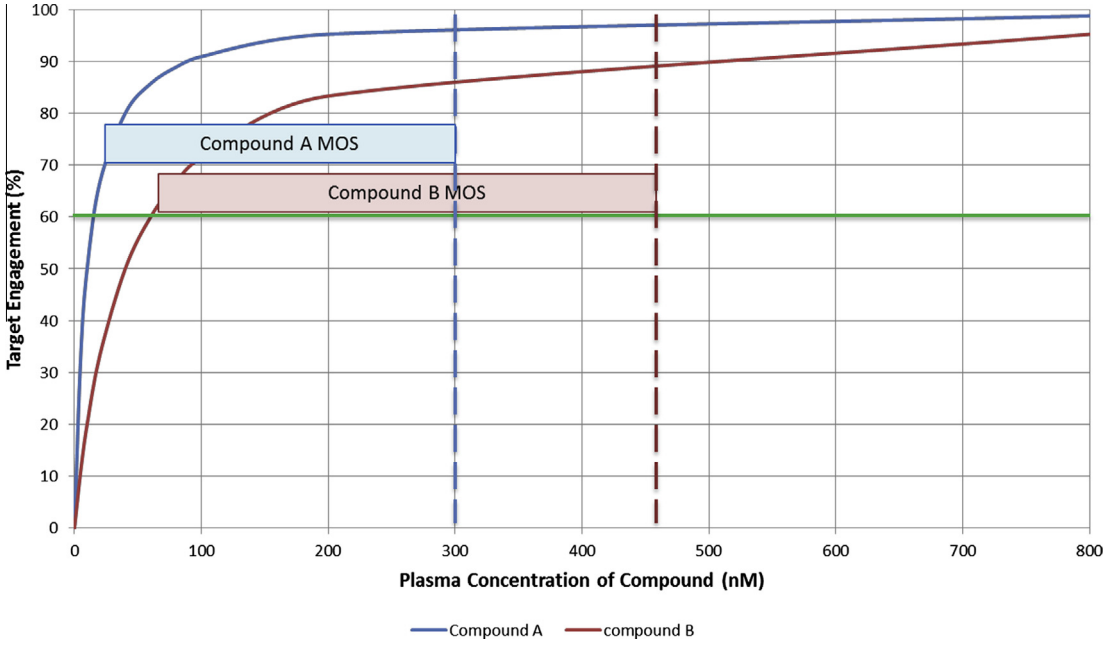


Figure 3. Hypothetical example illustrating how target engagement could help to differentiate compound performance. Graph shows target engagement versus plasma compound concentration for two structurally-different compounds, A and B, active for the same target (X). Green line represents the hypothesized minimum target engagement needed to achieve efficacy. Dashed lines represent plasma concentrations beyond which off-target toxicology is observed for each compound. Shaded areas represent margins of safety (MOS) for each compound.

Target engagement in the CNS and lung: The CNS and lungs[28] are target compartments which can be challenging for drug molecules to access. The blood brain barrier (BBB) limits exposure of molecules from the plasma to brain/spinal cord because the cell junctions of the membrane are much tighter than peripheral membranes.[29–34] Further, the presence of multiple efflux transporters within the BBB cells creates additional barriers to compound delivery.[35,36] Additionally, the critical life sustaining functions of the CNS and the brain's encapsulation within the skull, effectively limit the ability to sample brain tissues directly to measure biomarkers without euthanizing the lab animal or harming human patients. CSF can be easily tapped in patients for biomarker assessment. However, for brain focused targets this can still be an indirect measure of target engagement depending on the biology in question. Thus, discovery efforts in this arena have sought methods to assess target engagement through imaging or other indirect methods.

In the case of the lungs, the lung architecture presents unique challenges for direct delivery of drugs due to its highly branched structure.[37] Efficient delivery of drug to the lung for disease treatment depends on multiple factors such as particle size, density, shape, velocity, charge, etc. Further the deeper the target is within the lung, the more difficult it can be to obtain efficient drug delivery.[38] Thus, when a drug is inhaled, determining how much drug is delivered to the target is important.[39] We highlight in this review a preclinical Itk program that illustrates this challenge.

Phosphodiesterase enzymes: The phosphodiesterases are a family of enzymes that hydrolyze cyclic phosphate nucleotides (cAMP and cGMP). The enzymes make up 11 different families, each containing multiple isoforms, which are differentially expressed throughout the tissues of mammals. Because cAMP and cGMP are important biological signaling molecules and tissue distribution for some PDEs is specific, PDE inhibitors have been identified as potential drug targets. To date, multiple PDE5 inhibitors have been successfully developed for human use in treating erectile dysfunction.

In attempting to validate PDEs as drug targets, several challenges have been encountered. The first is the transient nature of the substrates and the fact that the product nucleosides are also produced by other non-PDE related pathways. Thus, substrate measurement rather than product measurement must be used to interrogate the enzyme reaction in vivo. The second is target location. Several of the highest interest PDEs (PDE2, PDE9, and PDE10) are found in the CNS. The third challenge is the high level of homology between the PDE families and their respective isoforms. The approaches that have been taken to demonstrate pre-clinical and clinical target engagement for the PDEs have varied but generally fall into three distinct camps: (1) estimations using compound tissue concentration relative to potency as a surrogate; (2) use of tracer molecules; (3) direct measure of substrate in the target compartment.

PDE10A: PDE10A has been a target of intense interest within the pharmaceutical industry, initially for the treatment of schizophrenia and more recently for Huntington's disease. In 2009, Pfizer announced the first clinical compound, **PF-02545920** (also known as MP-10, Fig. 4).[40] Since then, several additional efforts have been disclosed with up to four compounds in registered clinical trials.[41] Pfizer selected **PF-02545920** for development based on efficacy and its ability to demonstrate a PD effect (5 fold increase in cGMP) in the striatum (the major brain area of PDE10 expression) at a low dose. Thus, they connected tissue PD effects with in vitro potency. In 2012, Pfizer disclosed that **PF-02545920** failed to reach its primary efficacy end-point in a trial with schizophrenia patients and has since begun to explore the opportunities of developing **PF-02545920** as a treatment for Huntington's Disease.[42]

In this example, preclinical activity did not translate to clinical outcomes. The availability of PDE10 tracers would be valuable to allow clinical assessment of target engagement to ensure that the clinical hypothesis had been adequately tested. Notably, Pfizer has initiated its own clinical trial to establish receptor occupancy of **PF-02545920** in humans using PET.[41] Several other PDE10 tracers have recently been described in the literature. Among these, **IMA-107** (Fig. 4) from Imanova Ltd has been advanced into humans and initial human data has been disclosed.[43] Janssen pharmaceuticals has also reported a number of potential PET and radioligand tracers.[44–47]

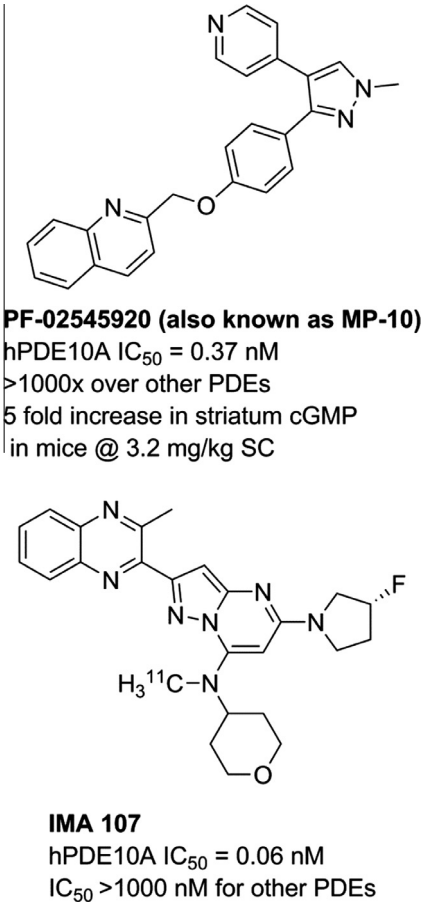
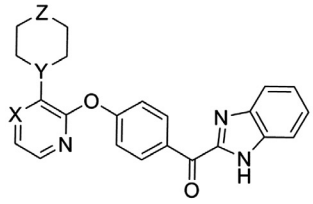


Figure 4. The first PDE10A clinical candidate PF-02545920 and recently developed radioligand IMA 107.

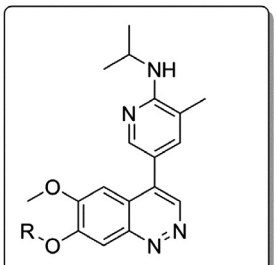
In 2014, Amgen disclosed their own clinical PDE10A candidate **1e** (Fig. 5).[48] Their approach to identification of a PDE10A inhibitor relied on a combination of elements including target occupancy (TO), efficacy, and preclinical species PK performance. To establish TO, the Amgen scientists used tracer **2** (Fig. 5) developed using a LC–MS/MS approach.[49] The advantage to the LC–MS/MS approach was that the team could evaluate multiple potential tracers in vivo prior to performing the costly radiolabeling. In evaluating compounds **1a** and **1d** from their SAR, the Amgen team noted that within scaffold **1**, very small changes had significant impact on the compounds' ability to engage the target.[48] That prompted them to prepare four other close analogs which were assessed with regard to their ability to engage the target, elicit efficacy in rat PCP models, and cross-species pharmacokinetic performance. Notably, compounds **1b**, **1e**, and **1f** had strong target engagement and low minimum efficacious doses (MED) in the rat PCP model. Ultimately, compound **1e**, had a more attractive cross-species PK performance leading to its being selected for advancement into the clinic.

The examples cited for PDE10 illustrate a variety of approaches to exploring a novel target. It is not yet known if PDE10 inhibitors will be successful for human medicinal use. With the recent availability of clinically useful tracers, well established pre-clinical target engagement, and the initiation of clinical trials to determine



1

Compd	X	Y	Z	PDE10A IC50 (nM)	Occ at 10 mg/kg	Occ ED_{50} (nM_{total})	PCP MED (mg/kg)
a	N	N	O	4.5	21%	ND	ND
b	N	C	O	0.8	91%	117	1.0
c	C	C	O	0.1	69%	ND	ND
d	N	N	NAc	5.1	57%	ND	ND
e	N	C	NAc	0.1	86%	711	0.3
f	C	C	NAc	0.1	86%	965	0.3



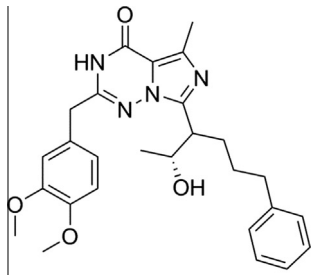
2
R = CH_3 or CT_3
hPDE10A IC_{50} = 1.9 nM
>700x over other PDEs
Good permeability
Low PGP efflux
PK LC/MS characteristics:
AUC Ratio (striatum/thalmus) = 9.4
% injected dose/g of striatum = 1.2

Figure 5. Amgen PDE10A clinical candidate **1** and tracer **2**.

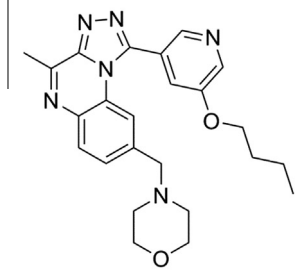
receptor occupancy using PET it should be possible to determine if PDE10 is a valid drug target for human disease in the near future.[41]

PDE2A: A recent report on the design of selective PDE2A inhibitors from Janssen Pharmaceuticals uses multiple techniques of establishing in vivo target engagement in the pursuit of pre-clinical target validation.[50] PDE2A has been proposed as a potential target for CNS disorders affecting memory, learning, and cognition. The initial pre-clinical in vivo target validation data for PDE2 was generated with inhibitor **Bay 60-7550** (Fig. 6). However, some reports have also suggested that **Bay 60-7550** has poor uptake in the brain.[50] Thus, Janssen set out to find a brain penetrant and selective PDE2A inhibitor.[50] Beginning with a non-selective PDE2/10 inhibitor, they optimized potency and selectivity using X-ray structural data culminating in inhibitor **3**. Compound **3** displayed exceptional selectivity against other PDE enzymes. It also displayed rapid uptake into the brain and maintained reasonable brain concentrations for up to 2 h after a 10 mg/kg SC dose. Unbound brain levels at 1 h were determined to be 67 nM (115 ng/g). The authors used tritium labeled **3** to establish ex-vivo target engagement in rat brain slices. Co-administration of the selective PDE10 inhibitor **MP-10** (Fig. 4) showed that 1 h post a 10 mg/kg SC dose, PDE2 occupancy was ~80%. Thus, with multiple lines of evidence predicting compound **3** strongly engages PDE2 in vivo, the authors measured cGMP levels in rat striatum and hippocampus 1 h post dose and found that in both regions the substrate levels were 300% the levels of the vehicle controls. In the disclosure, no experiments attempting to link target engagement and PD to relevant pre-clinical efficacy were described.

Pfizer has recently reported on its efforts to develop a PET ligand for PDE2.[51] The authors used a properties focused approach to design an optimal PET ligand for PDE2. Their approach involved constructing a database of 62 PET ligands that successfully reached the clinic and 15 others that failed in late-stage clinical development as a negative control. Their analysis led them to conclude that an optimal range of parameters for PET ligand design for novel targets could be identified (see Table 1). The authors then set out to identify a suitable starting point for PET ligand design using these parameters. This approach identified compound **4** as a suitable lead molecule (Fig. 7).



Bay 60-7550
hPDE2 IC_{50} = 0.8 nM
rPDE10 IC_{50} = 832 nM



3
hPDE2A IC_{50} = 10 nM
rPDE10A IC_{50} = 4183 nM
hPDE11A IC_{50} = 2138 nM
hPDE1A, hPDE3B,
hPDE4D,
hPDE5A, hPDE6AB,
hPDE7A, hPDE9A IC_{50}
>10000 nM

Figure 6. BAY60-7550 and Janssen pharmaceuticals PDE2A inhibitors.

The Pfizer team then designed and synthesized seven potential PET ligands (**5**–**11**) by varying the phenyl substitution and/or

Table 1
Pfizer's preferred design/selection parameters for novel CNS PET ligands

Pharmacology	Non-specific binding	Brain permeability	Desirable physiochemical properties[a]
$Brain_{max}/K_d$ >10 >30–100 × selectivity	F_{u_brain} >0.05 F_{u_plasma} >0.05	RRCK P_{app} AB >5 × 10^{-6} cm/s MDR BA/AB ⩽ 2.5	$cLogP \leqslant 3$; $cLogD \leqslant 2$ MW ⩽ 360 amu 40 < PSA ⩽ 90 Hydrogen-bond donors ⩽ 0.5 $pK_a \leqslant 8$

[a] *Note*: the authors used a previously published multi-parameter method to weight physiochemical properties into a composite score.[51] Values for what the authors indicate are the more desirable ranges of specific properties are shown in the table rather than the composite scores.

incorporation of a fluoroazetidine. All compounds showed good potency against PDE2. Compound **5** showed good in vivo brain penetration and was selected for radiolabeled synthesis. Evaluation of [^{18}F]-**5** in non-human primates demonstrated the compound had rapid and high uptake in the striatum (high PDE2 expression) and low uptake in the cerebellum (low PDE2 expression). Further, the binding of [^{18}F]-**5** could be blocked with a non-labeled and specific PDE2 inhibitor.

PDE2A research is less advanced than that for PDE10. However, the development of selective inhibitors as well as PET ligands should facilitate the progression of the science to determine the validity of the target.

5-HT$_{1A}$ receptor: Recent pharmacology research has shown that infusion of the 5-HT$_{1A}$ receptor agonist 8-OH-DPAT in rats decreased the volume threshold for bladder voiding.[52–55] Conversely, a 5-HT$_{1A}$ antagonist (WAY-100635) has been shown to reverse these effects.[53] These reports led Eisai pharmaceuticals to explore the effects of their 5-HT$_{1A}$ receptor antagonist **E2110** (Fig. 8) as a potential therapy for overactive bladder (OAB).[56] To verify the previous findings, the Eisai team set out to use micro-PET technology to establish the level of central receptor occupancy (RO) required for achieving efficacy in a rat model of OAB. Such a correlation would hopefully provide assistance to the design of clinical experiments with drug candidate **E2110** in humans. To that end, [^{11}C] WAY-100635 was used to establish the dose dependence of **E2110** receptor occupancy in rat brains relative to plasma concentration. The authors then used this data to establish a model of plasma compound concentration versus RO. In separate pharmacology experiments using two different models (surgical and chemical) of OAB in rats, the authors established the minimum efficacious dose of **E2110**. Based on the plasma levels, the authors used their PK-RO model to project the minimum RO required to achieve efficacy (defined as increased intervals between micturition) in both models which was approximately 60%.

In this example, the authors hypothesized that central occupancy of 5-HT$_{1A}$ quantified by in vivo micro-PET should be a useful surrogate biomarker to measure anti-OAB effects. Although micturition is supposedly regulated by several neurotransmitter pathways within the central and peripheral nervous system, their approach clearly outlines a hypothesis for how to determine effective doses in preclinical models which might be clinically transferrable. Future clinical studies will ultimately determine the validity of this approach for OAB treatment.

Itk: IL-2 inducible kinase (Itk) plays an important role in antigen receptor signaling in T cells. Thus, it has been proposed as a potential drug target for anti-inflammatory diseases. GlaxoSmithKline has reported on a pre-clinical research effort to identify Itk inhibitors as potential therapies for asthma.[57] Itk as an asthma target presents an interesting drug discovery challenge because compounds must be highly potent in cells, selective, and delivered to the lung. There are >500 kinases and there is a high structural similarity within the ATP binding site. In addition, cells contain a high ratio of endogenous ATP to Itk's $K_{m,app(ATP)}$ (endogenous ATP concentrations are ~1–2 mM while $K_{m,app(ATP)}$ for Itk is ~5 μM).[58] Therefore, the GSK team chose to pursue covalent irreversible inhibitors in hopes that they could achieve high cell potency and selectivity within a molecular framework that could be delivered to the target in the lung. From their research, compound **12** emerged as a highly soluble, permeable and potent Itk inhibitor (Fig. 9). Notably, compound **12** showed good selectivity over other kinases. Selectivity is driven by its kinetic profile showing a rapid initial non-covalent binding event followed by a covalent modification of the enzyme Cys-442. The authors found that Itk had a slow turn-over rate after inactivation by compound **12** which allowed prolonged cellular inhibition of the kinase post-dose.

The authors note that the use of animal efficacy models for asthma may not be predictive of human outcomes. Therefore, to examine the PD activity of their compounds, the authors developed a rat model in which the compound was aerosolized and delivered to the lungs via inhalation (2.3 mg/kg dose was delivered over 20 min). Animals were then sacrificed, lungs perfused, and a cell suspension was prepared. To determine target engagement the amount of the compound delivered to the target was assessed. Notably, the lung tissue concentration was found to be ~6 μM post perfusion, but most of the compound was not cell associated. Upon preparation of a cell suspension the concentration was found to be much lower (8 nM). The cells were then stimulated with anti-CD3 for 18 h and T cell activation was determined by flow cytometry. In the event, **12** showed almost complete inhibition of T cell activation. Based on the low level of compound the authors found to be delivered to the cells (vide supra), this robust effect was consistent with the slow turnover of Itk as measured in their in vitro experiments.

Target engagement in the periphery: In drug discovery focused on the periphery as opposed to the CNS, it can be significantly more common for compound plasma levels to be equivalent or nearly equivalent to those in the target tissue. Therefore, plasma compound levels or biomarkers can be powerful tools for assessment of target engagement. Still, some areas of peripheral drug discovery can derive significant benefit from alternative methods for establishing target engagement. In this section, we highlight recent literature on a class of enzymes located not just in the periphery, but within the cell nucleus.

Histone deacetylases (HDACs) are a family of enzymes which remove acetyl groups from lysines of histones.[59] Inhibitors of HDACs have been approved for human use in the treatment of cancers but have also been proposed as drug targets for CNS diseases as well. The HDAC family contains a large number of isoforms and has a significant substrate scope. Different inhibitor biodistribution and enzyme selectivity profiles would be hypothesized to carry different efficacy and safety benefits/risks. Unfortunately, technologies to assess HDAC inhibitor biodistribution and action have not been available until recently.

Wang et al. have recently described HDAC PET ligand **13** (Fig. 10).[60] Ligand **13** can be used to explore both CNS and periph-

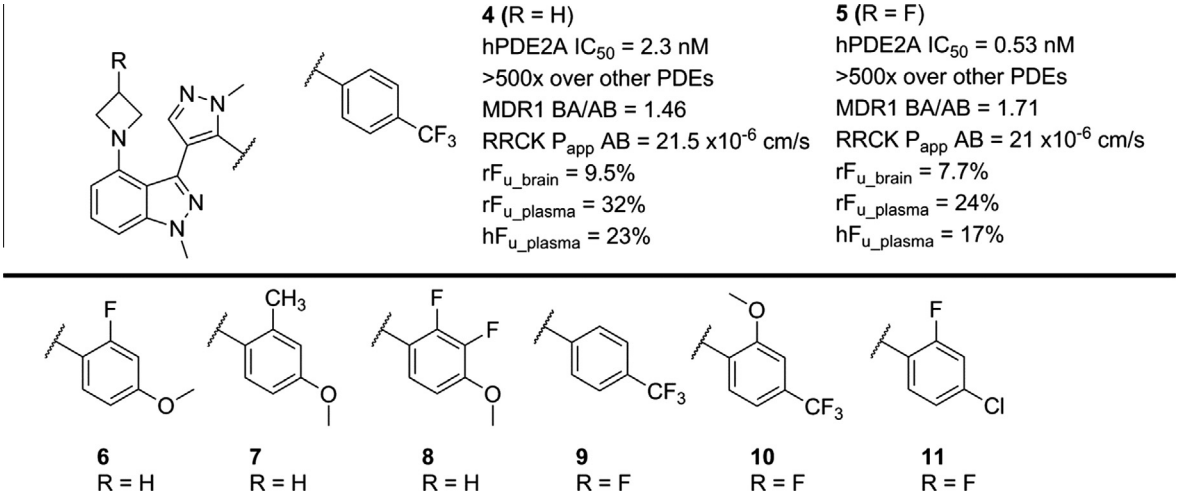


Figure 7. Pfizer PDE2A inhibitors and PET ligands.

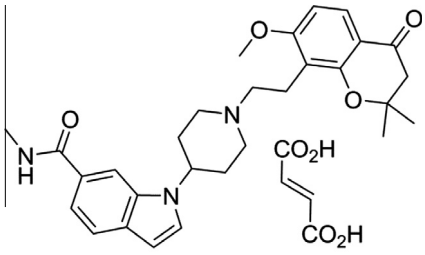


Figure 8. 5-HT_{1A} receptor antagonist E2110.

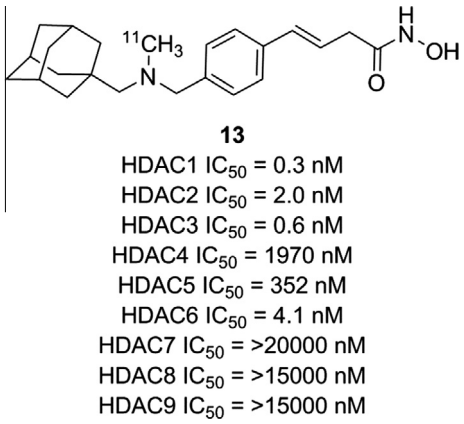


Figure 10. HDAC PET ligand.

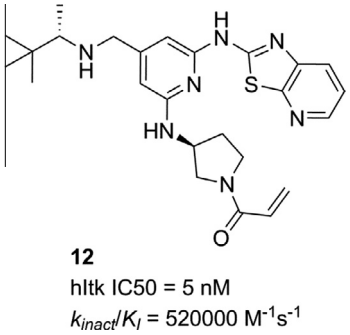


Figure 9. Itk inhibitor.

eral distribution of HDAC inhibitors. Ligand **13** has good potency for HDACs 1, 2, 3, and 6. Notably, ligand **13** had strong uptake in heart, kidney pancreas, and spleen. This uptake could be blocked with the broad spectrum HDAC inhibitor SAHA.

Not only is biodistribution in the area of HDAC inhibitors important but having an understanding of how HDAC inhibitors affect enzyme action in living systems has also been identified as a critical need to advance these epigenetic targets as therapies for human disease. To that end, Munteanu et al. recently reported a MALDI MS (Matrix-assisted laser desorption/ionization mass spec) method that allows the determination of histone acetylation levels in both cells in vitro and in vivo.[61] Cells treated with inhibitor could be analyzed whether they were suspended or fixed. The authors were also able to establish EC_{50}s for compounds using this technology. This cellular approach could then be extended to use in mice. Using HDAC inhibitor LBH-589 the authors were able to quantify, in a time dependent fashion, the level of acetylation on Histone H4. Further, imaging of tumor cross sections could be accomplished allowing the use of this technology for histological assessment of compound action.

These two recent advances set the stage for additional understanding of HDACs as targets for human disease. Ligand **13** demonstrates the ability to develop imaging tools for the assessment of biodistribution and target engagement of new HDAC inhibitors. Coupled with the MALDI technology for assessing histone acetylation levels, with these tools one can begin to attempt to link compound target engagement and in vivo effects.

Target engagement determination using chemical methods: In some scenarios, facile measurement of biomarkers or the use of imaging agents to determine target engagement in vivo are impractical for technical, safety, or cost reasons. In such cases, alternative strategies to assess ligand–target interaction are needed. In some of these instances, organic synthesis and

medicinal chemistry can be used to develop target specific agents to assess ligand-target protein interaction. Such technologies are often used in chemoproteomics.[62,63] When properly developed, these technologies have the ability to be used all the way from pre-clinical target validation work to human clinical trials. In this section, we highlight two recent examples from the field of kinases which demonstrate how these approaches can be used across the drug discovery spectrum for both peripheral and CNS based targets.

LRRK2: Leucine-rich repeat kinase-2 (LRRK2) mutations that increase enzyme activity have been linked to Parkinson's disease.[64–66] Thus, LRRK2 inhibitors represent potential therapeutic agents for Parkinson's disease. LRRK2 is expressed in the CNS and in the periphery. While numerous substrates have been proposed, to date, the disease relevant substrate(s) for LRRK2 has not been confirmed.[67–72] The absence of a clear disease relevant substrate and the fact that LRRK2 is found both peripherally and in the CNS increases the challenge of pre-clinical target validation and drug discovery efforts. Novartis recently disclosed their efforts to find brain penetrant, potent, and selective LRRK2 inhibitors (Fig. 11).[73] In order to establish target engagement, the authors wanted to identify a scaffold which could be cross-linked to a solid support and used to develop a LRRK2 pull-down assay. The team began their SAR using the non-selective kinase inhibitor Sunitinib (Fig. 11). The authors generated a number of analogs. From this set, compound **16** (Fig. 11) was found to be a highly selective LRRK2 inhibitor. It also contained the requisite attachment site (a primary amine) to link to a solid support. With a pull-down assay tool compound in hand, they set out to find a compound to use in in vivo studies. Fusion of the pyrrole and transposition of the pendant amide ultimately led to an SAR that delivered two compounds, **14** and **15**, which were selective and had PK properties supporting brain penetration in mice. Using their pull-down assay, the authors also showed that compound **14** dose dependently engaged LRRK2 in mouse brain after oral dosing. Hopefully, future research will help to establish if there is a link between target engagement and PD effects for this target.

Btk: Bruton's tyrosine kinase (Btk) is expressed in B cells. Because Btk activation is a critical element of B cell response to antigens, Btk has been identified as a potential therapeutic target for autoimmune and B-cell malignant diseases.[74] Notably, cysteine 481 in Btk is not conserved in many other kinases which opens the possibility of using irreversible inhibition as a means of achieving selective inhibition within the broader gene family. To that end, Celgene developed clinical compound **CC-292** as a covalent inhibitor of Btk which reacts with C481, inactivating the enzyme (Fig. 12).[74]

In order to assess the target engagement of Btk by **CC-292**, the researchers developed biotinylated inhibitor **CNX-500** (Fig. 12).[74] As outlined in Figure 13, **CNX-500** could be used to assess target engagement in both mice and human subjects. After dosing of **CC-292**, B cells are isolated from plasma and lysed. Treatment of the lysate with **CNX-500** inactivates any Btk not already covalently bound to **CC-292**. The **CNX-500** protein adduct is then captured using streptavidin beads and quantified using ELISA to establish the level of target engagement of the treatment group relative to vehicle controls. The use of **CNX-500** allowed the authors to establish timelines for Btk protein turnover, **CC-292** PK–PD relationships, and **CC-292** target engagement efficacy relationships in a collagen induced arthritis model in mice. They also used **CNX-500** to establish the PK–PD relationship of **CC-292** in healthy human volunteers.

Recent efforts to establish target engagement in cells: The establishment of target engagement at the cellular level is extremely challenging because there are few established technologies. While cellular assays can be developed for many targets, the measured signal in these assays often is an indirect outcome of drug–target interaction. In some cases, direct measures of function are possible (i.e., substrate depletion, product formation, etc.) but sometimes the measured signals are more distant, creating potential uncertainty regarding the link between the observed outcome and the anticipated target–drug interaction.

A recent approach to assessing target engagement of drugs in cells has been reported by Molina et al.[75,76] The authors refer to

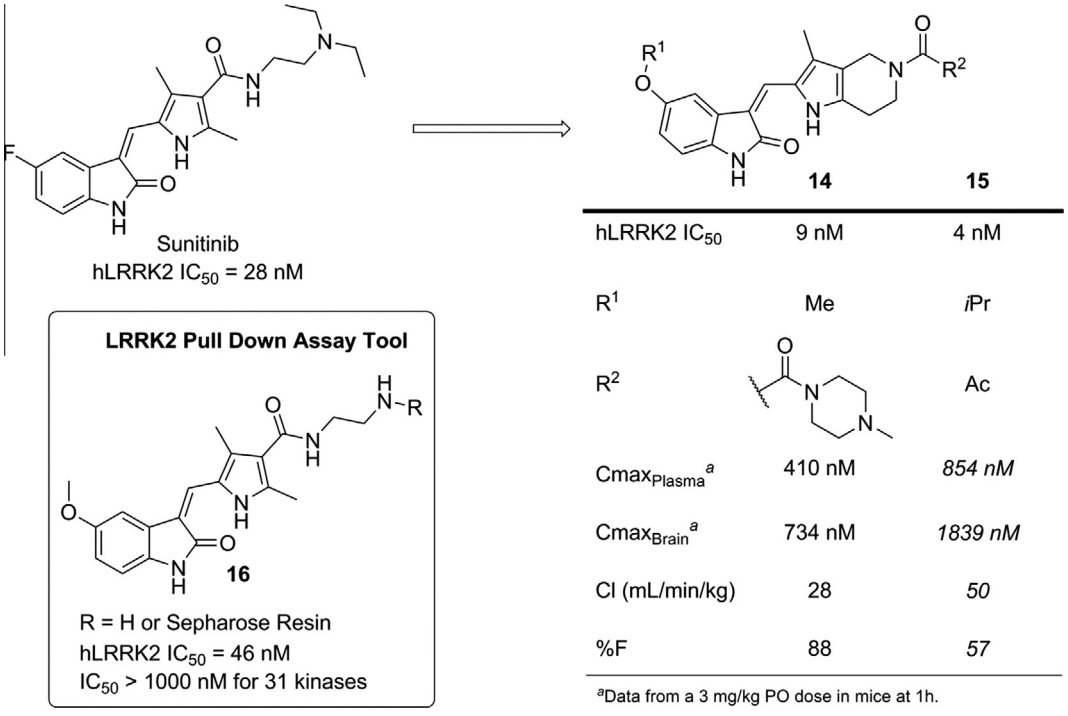


Figure 11. LRRK2 inhibitors.

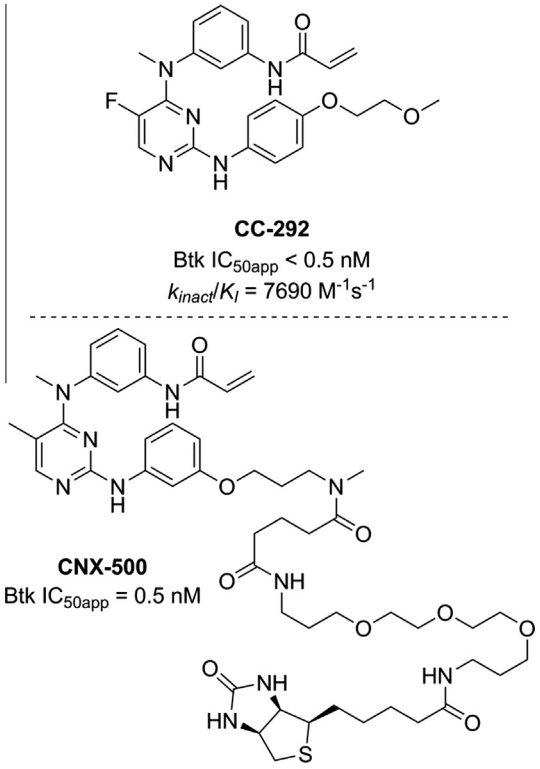


Figure 12. Btk inhibitor **CC-292** and tool compound **CNX-500**.

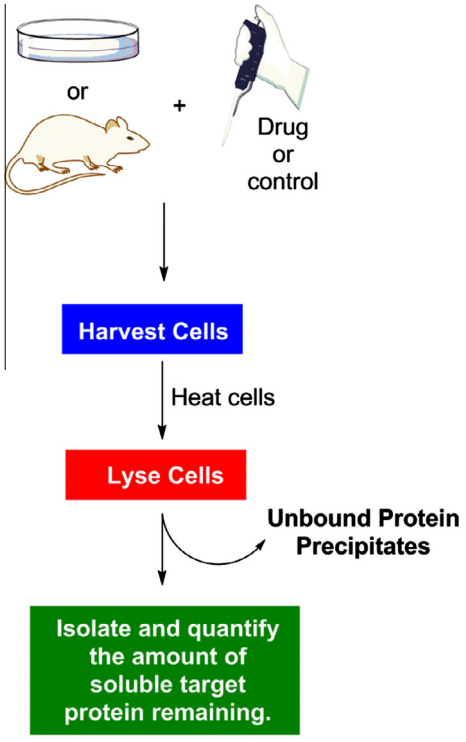


Figure 14. CETSA allows assessment of target engagement in cells.

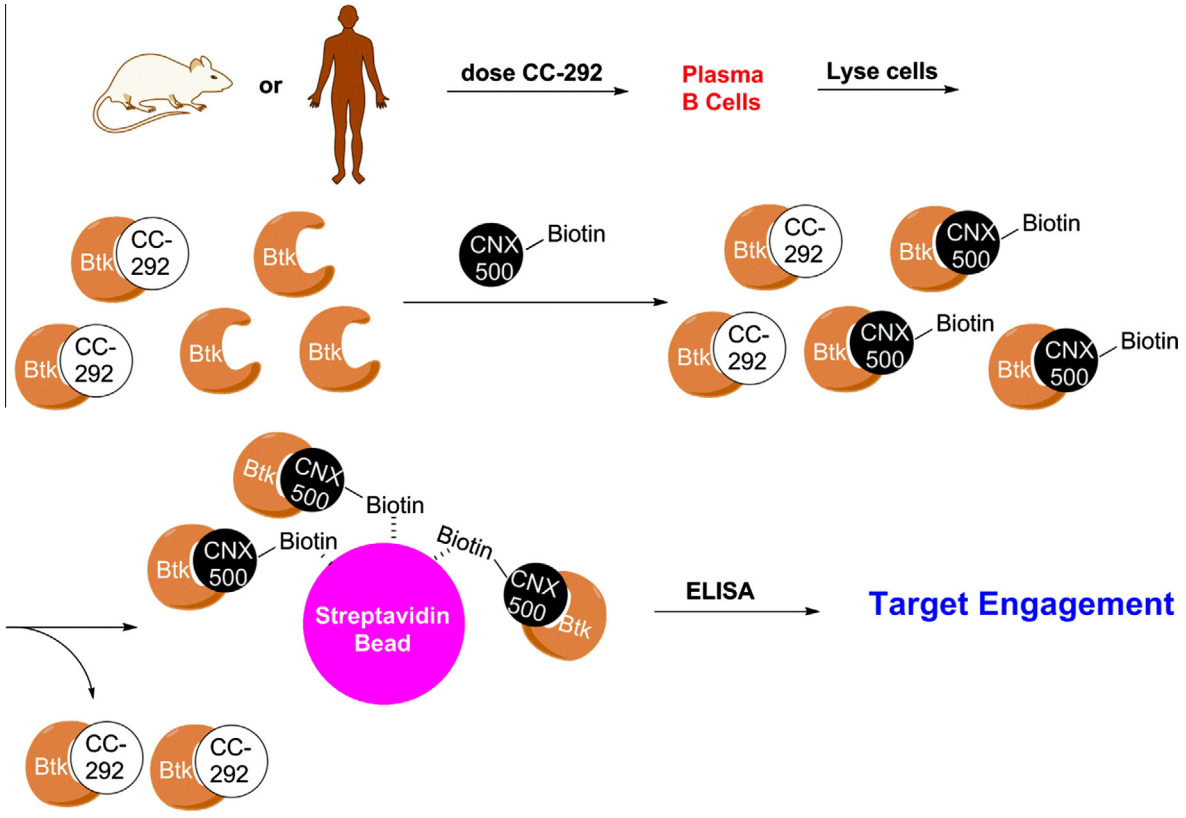


Figure 13. **CNX-500** is used to assess engagement of Btk by clinical candidate **CC-292**.

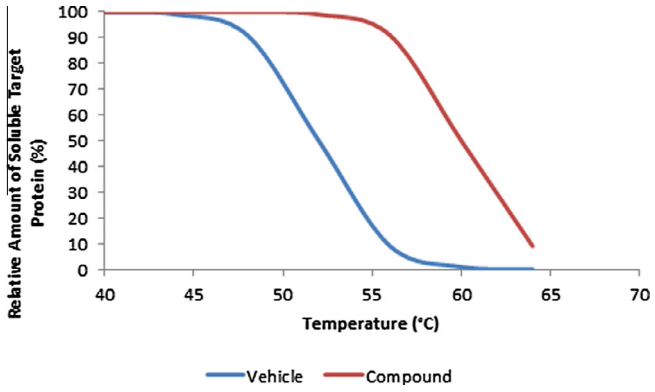


Figure 15. Hypothetical output of a CETSA experiment.

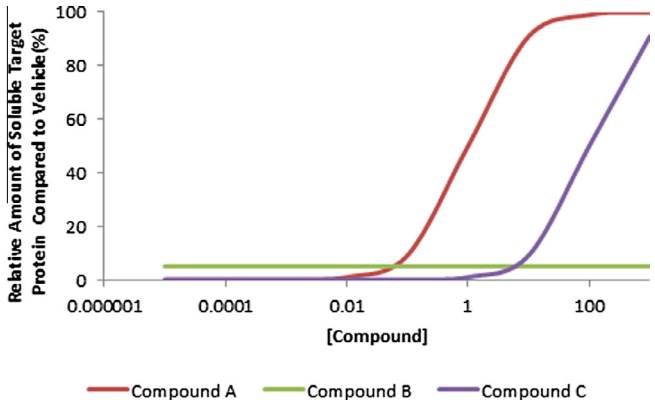


Figure 16. Hypothetical output of an $ITDRF_{CETSA}$ experiment. Compound A engages the target protein more effectively than compound C. Compound B does not appear to be able to engage the target protein.

their approach as CETSA which stands for cellular thermal shift assay. This technology leverages the same thermal stability assay used broadly in structural biology to assess compound binding to proteins except that, rather than using purified proteins, it uses cell lysates. In their approach, cells or animals can be dosed with compound and vehicle (Fig. 14). Cells/tissues are then harvested for analysis from each group. In CETSA, aliquots of the cells are then incubated at various temperatures for a given time. The cells are lysed and the amount of soluble target protein remaining is quantified and reported as its relative intensity. Because heating the cells causes native proteins to denature and precipitate, binding of a ligand shifts the thermal stability curve showing proof of compound and target protein interaction (Fig. 15).

This approach can be modified to explore the compound concentration effects of binding in an experiment that the authors refer to as an isothermal dose–response fingerprint CETSA ($ITDRF_{CETSA}$). Here the animals or cells are treated with compound in a dose response experiment. Cells from each dosing group are harvested and incubated at a set temperature prior to lysing and soluble protein quantification. Plotting the relative intensity of the soluble protein versus compound concentration allows comparison of target engagement effectiveness within a series of compounds. For example, in the hypothetical experiment shown in Figure 16, compound A engages the target protein more effectively than compound C. Compound B does not appear to be able to engage the target protein.

The authors demonstrated the potential value of the approach by comparing the PARP-1 inhibitors iniparib and olaparib.[75] Iniparib failed to meet phase 3 efficacy endpoints while olaparib was recently approved by the FDA.[77,78] Using CETSA and $ITDRF_{CETSA}$ the authors showed that, within their experimental protocol, iniparib did not engage PARP-1 while olaparib did shift the thermal stability. Based on these results, they suggest that iniparib acts through an alternative mechanism to PARP-1.

Drug discovery is currently experiencing a paradigm shift due to the multiple challenges facing the pharmaceutical industry. It is important to assess the target engagement of a potential drug within the target tissue to properly interpret PD effects and efficacy both pre-clinically and clinically. In this review, we have exemplified several cases across different indications that highlight the use of target engagement assays during Lead Generation.

To capitalize on the benefits of using target engagement techniques along the drug discovery process, it is important to prospectively build an integrated flowscheme (Fig. 17). In early Lead Generation, foundational work to enable target engagement assays is critical and should yield improved decision making. For example, initial investment in tracer identification in Lead Generation could allow target engagement assessment of multiple scaffolds or individual compounds. Moving toward Lead Optimization, quantification of target occupancy could provide better differentiation of toxicity profiles as well as guide dose selection. Ultimately, these data linking target engagement to PD effects and preclinical efficacy should enhance the design of the clinical experiment and provide a definitive target validation answer earlier.

In summary, the examples from recent literature described in this review demonstrate the value of target engagement techniques in medicinal chemistry. As the pharmaceutical industry and drug discovery science evolve, we anticipate that the trend to incorporate target engagement into early drug discovery will continue to increase. Hopefully, these efforts will allow the pharmaceutical industry to improve its overall efficiency.

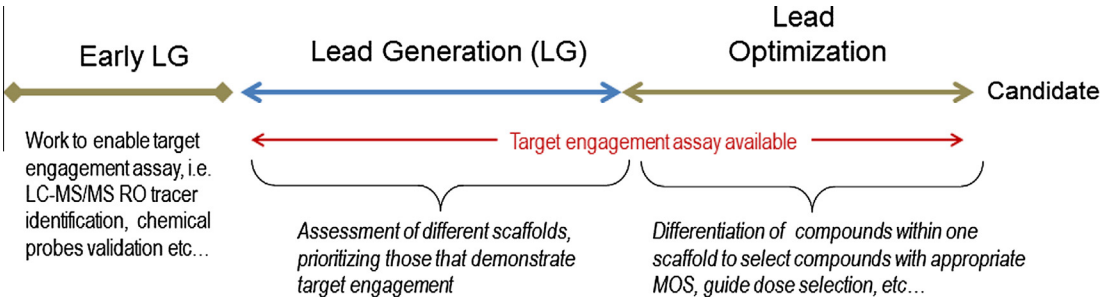


Figure 17. Proposed drug discovery paradigm incorporating target engagement assays.

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