Advancing Biological Understanding and Therapeutics Discovery with Small Molecule Probes

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Abstract

Small-molecule probes can illuminate biological processes and aid in the assessment of emerging therapeutic targets by perturbing biological systems in a manner distinct from other experimental approaches. Despite the tremendous promise of chemical tools for investigating biology and disease, small-molecule probes were unavailable for most targets and pathways as recently as a decade ago. In 2005, the U.S. National Institutes of Health launched the decade-long Molecular Libraries Program with the intent of innovating in and broadening access to small-molecule science. This Perspective describes how novel small-molecule probes identified through the program are enabling the exploration of biological pathways and therapeutic hypotheses not otherwise testable. These experiences illustrate how small-molecule probes can help bridge the chasm between biological research and the development of medicines, but also highlight the need to innovate the science of therapeutic discovery.

Introduction

Despite remarkable advances in chemistry and biology, small molecule drug discovery remains a slow, costly, and low-yielding activity. In fact, productivity in drug discovery has fallen in recent years. It is estimated that only about 1 in 10 drug candidates entering the clinic are eventually approved – despite increasing investment (Hay *et al.*, 2014). A prime hurdle is our inability *to predict* the physiologic consequences of modulating candidate drug targets in humans. Currently, lack of sufficient efficacy is the cause of clinical candidates failing in about 50% of Phase-II trials (Arrowsmith, 2011a) and 66% of Phase-III trials (Arrowsmith, 2011b). This high rate of attrition results from an inadequate understanding of how diseases are caused and maintained in human populations.

Expectations regarding the therapeutic relevance of a target are often based on inferences from experiments that alter nucleic acids encoding the putative target (e.g., deleting a DNA sequence; degrading or overexpressing an RNA sequence). Yet most small-molecule drugs alter the functions of proteins, and the correlation between perturbing nucleic acids and their encoded proteins is imperfect. Modulating the functions of candidate drug targets with small molecules would provide insights with far greater relevance to drug discovery. However, the current absence of effective small-molecule probes or 'tool compounds' for the vast majority of proteins limits a systematic application of this approach to target validation.

Advances in understanding human biology, particularly 'experiments of nature' revealed with increasing frequency by human genetics, are now shining a light on many complex diseases and pointing to targets with a much higher likelihood of providing a therapeutic benefit safely than those derived from model systems (Plenge *et al.*, 2013). And the increasing availability of patient-derived cells and advances in precise genome-editing methods are providing more effective model systems for investigating the functional impact of observed human genetic variation.

But these insights also create a demand for small-molecule probes. The therapeutic implications of these genetic alterations are intriguing, but not certain. Not only do they raise the 'gene vs. protein' issue above – they often reflect heritable modulations from birth so the effect of targeting an implicated protein by a therapeutic in an ongoing disease is uncertain. Also, the therapeutic strategies suggested by these studies frequently point to targets that are inaccessible to protein-based drugs and for which small-molecule modulators are currently nonexistent – targets that fall outside traditional drug classes and have been labeled 'undruggable' by some within the pharmaceutical and academic communities. There is an urgent need to innovate chemistry and chemical biology in ways that enable small-molecule modulation of the challenging targets at the heart of disease.

In 2005, in an effort to bridge the chasm between basic biological research and the medicines that should in principle derive from insights into disease, the U.S. National Institutes of Health (NIH) initiated the decade-long Molecular Libraries Program (MLP) (Austin *et al.*, 2004). At that time, small-molecule discovery techniques, including high-throughput screening (HTS) and medicinal chemistry expertise, existed predominantly within pharmaceutical companies. Following a three-year pilot phase, the MLP launched the

Molecular Libraries Probe Production Centers Network (MLPCN), which comprised Comprehensive Screening Centers, Specialized Screening Centers and Specialized Chemistry Centers (Fig. 1). These centers were among the earliest efforts to bring systematic small-molecule screening into academic settings.

The MLP was designed as an experiment in integrating big science and individual investigator-led science. Individual investigators submitted funding applications to the NIH for assay development or screening projects. For screening projects that were selected through peer review, MLP Centers conducted high-throughput screens using an MLP small-molecule library that grew over the course of the program to a final size of about 390,000 compounds. Novel compounds emerging from the academic synthetic chemistry community, for instance small, chiral compounds having novel skeletons derived from diversity-oriented synthesis (DOS) and other compounds derived from the NIH-funded Chemical Methodologies and Library Development (CMLD) program, made up about 5% of the compound library and yielded small-molecule probes, including some highlighted below, that would not have been discovered otherwise. Following on the screens, the MLP pursued chemical optimization of hits with the aim of identifying small-molecule probes, including ones targeting proteins viewed as challenging, with sufficient potency and selectivity to provide 'high-quality' tool compounds (Frye, 2010; Oprea *et al.*, 2009; Workman and Collins, 2010). These probes were envisioned as a first step towards the testing of novel biological and therapeutic hypotheses.

With the conclusion of the program, we can now look back at the progress achieved over the decade of the MLP and forward at how to benefit from the lessons learned. We summarize advances in discovering biologically active small molecules and illustrate how these advances are yielding a rich armamentarium of novel small-molecule probes and, perhaps most notably, experimental paths to therapeutics discovery. The series of 'vignettes' described here provide examples of methodological development and small-molecule probe discovery that is enabling the testing of specific therapeutic hypotheses. These examples highlight the importance of small-molecule probes as critical tools for accelerating translational research, but also emphasize the tremendous hurdles that remain to be cleared if we are to capitalize fully on the rapid pace of biological advances to improve human health. If the lack of productivity of therapeutics discovery is to be reversed, transformative innovation will be needed to push back barriers at every stage of small-molecule therapeutics discovery.

Bridging the chasm

The most visible output of the MLP is a set of 375 small-molecule probes for exploring biology. MLP probes cover a diverse spectrum of target classes, including well-investigated classes such as kinases and G protein-coupled receptors (GPCRs) and less frequently investigated classes such as GTPases, proteases and RNA-binding proteins (Fig. 2). This broad coverage of target space highlights the intentional focus of the MLP Network on relatively under-explored proteins. Many of these compounds were 'first-in-class' probes that provided initial evidence for the chemical tractability of a previously recalcitrant target

or that modulated a target by a distinct mechanism from previous small molecules (Supplementary Table 1).

Perhaps the most exciting outcome of the MLP was the impact of probe-discovery efforts in highlighting paths for therapeutics discovery. Here, we describe a number of vignettes that illustrate the breadth of the translational impact of small-molecule probes identified through MLP research. These vignettes highlight advances in small-molecule science, as well as insights gained through the use of small-molecule probes in diverse disease contexts. Many of these and other MLP projects have directly catalyzed efforts to translate emerging biomedical insights (Table 1).

Targeting poorly characterized enzymes

Small-molecule probes can aid efforts to annotate the function of poorly characterized enzymes, including those that human genetic studies have implicated in disease. However, it is difficult to establish a typical biochemical high-throughput screening assay to identify modulators of an enzyme if the substrate is not known. Activity-based protein profiling (ABPP) makes use of reactive small-molecule ligands to label covalently the active sites of members of large enzyme families, including those that are uncharacterized (Nomura *et al.*, 2010). In this approach, broadly reactive ABPP probes can be used in a competition experiment to identify small molecules that selectively reduce labeling of a desired enzyme target. Despite the potential, this substrate-free screening approach has not been widely used because the current competitive ABPP methods relies on SDS-PAGE or mass spectrometry assays and can only be used to evaluate, at most, a few hundred compounds.

The MLP undertook a project to develop a high-throughput fluorescence polarization technology for ABPP-based screening (fluopol-ABPP) (Bachovchin *et al.*, 2009). A small fluorophore in solution rotates rapidly and emits a low fluorescence polarization signal, whereas a fluorophore bound to a protein rotates more slowly and emits a higher signal. Based on this known property, an ABPP assay was developed in which fluorescent ABPP probe labeling of an enzyme target generates a signal that is then reduced when a small molecule 'hit' effectively competes for binding. This approach proved generally applicable to high-throughput screening in multi-well format. For instance, screening 20,000 small molecules in 384-well plates led to the identification of multiple small molecules that blocked serine fluorophosphonate (FP)-rhodamine labeling of the serine hydrolase retinoblastoma-binding protein-9 (RBBP9), a cancer-associated enzyme. Secondary chemoproteomic assays in mouse brain fractions and human cell lysates subsequently identified the natural product emetine (ML081; Fig. 3) as the first RBBP9 inhibitor that is selective for this serine hydrolase family member (Bachovchin *et al.*, 2009).

Small molecule inhibitors of protein phosphatase methylesterase-1 PME-1, a regulator of protein phosphatase 2A (PP2A) that has been linked to cancer and neurodegeneration, had not previously been identified, in part due to the challenges of designing an *in vitro* biochemical assay suitable for monitoring the carboxymethylation state of PP2A. A fluopol-ABPP screen yielded initial hits, from which four novel aza-β-lactams were shown in follow-up assays to selectively inhibit PME-1 in human cells. The most potent inhibitor, ML174 (ABL127; Fig. 3), inhibited PME-1 with a low nanomolar IC_{50} and had no activity

against more than 50 other serine hydrolases. In mice, ML174 selectively inactivated PME-1 and increased the levels of PP2A carboxymethylation in the brain (Bachovchin *et al.*, 2011). Strikingly, the aza-β-lactam hits are examples of innovations resulting from diversity synthesis performed by an academic chemistry laboratory – such compounds are distinct from conventional screening compounds available from vendors. The aza-β-lactam scaffold has subsequently proven to be a versatile chemotype for developing selective serine hydrolase inhibitors (Zuhl *et al.*, 2012).

The MLP has also developed a modified ABPP method that can be used to read out cellular and *in vivo* target engagement, an often-missing link in the preclinical development of drug leads (Simon *et al.*, 2013). Using fluopol-ABPP, the first selective inhibitors of the serine hydrolases lysophospholipase 1 (LYPLA1) and lysophospholipase 2 (LYPLA2) were identified and found to act reversibly. For reversible inhibitors that lack known biomarkers such as endogenous substrates or products, demonstrating target engagement is challenging. To overcome this limitation, new ABPP probes were designed that reacted with serine hydrolases over an observable time window. In human cells and in mice, these moderately reactive ABPP probes could be used to accurately measure the target engagement and selectivity of the LYPLA1 and LYPA2 inhibitors. For instance, dosing mice with the inhibitors blocked more than 90% of LYPLA1 and LYPA2 activity in lung, heart and kidney tissue, whereas the activity of the enzymes was only reduced by about 50% in the brain and no detectable enzyme inhibition was seen in the liver (Adibekian *et al.*, 2012).

More generally, fluopol-ABPP has proven useful for enzyme classes beyond serine hydrolases and has been used for screening glutathione S-transferases (Tsuboi *et al.*, 2011), arginine methyltransferases (Dillon *et al.*, 2012) and protein arginine deiminases (Knuckley *et al.*, 2010).

Toward novel mechanisms of action compounds through multiplexed bioactivity data

Identifying small-molecule probes that modulate challenging targets often requires accessing new chemical starting points. Previous efforts to address this gap have typically focused on increasing diversity from a chemical structure perspective, a metric known to predict imperfectly the identity of compounds with new bioactivities. The MLP worked to develop high-throughput and inexpensive methods that would enable multiplexed cellular bioactivity measurements to guide directly the building of compound collections whose members have novel mechanisms of action.

Gene-expression studies have demonstrated the impact of unbiased, high-dimensional profiling data in defining disease states and also in inferring compound mechanism of action (Hughes *et al.*, 2000; Lamb *et al.*, 2006; Weinstein *et al.*, 1997). High-content imaging could provide a complementary measurement for capturing complex disease phenotypes in unbiased, high-throughput formats. However, current high-content screening assays have either focused on a specific cellular process of interest or a limited set of cellular readouts, often based on labeled proteins or qPCR of target transcripts, or have been limited in throughput (Loo *et al.*, 2007; Weinstein *et al.*, 1997; Young *et al.*, 2008). The MLP therefore focused on a 'cell-painting assay' that enables a more unbiased approach for characterizing alterations induced by disease states or small-molecule perturbagens

(Gustafsdottir *et al.*, 2013). Using six dyes measured across five channels, 824 distinct cellular morphological features could be quantitatively imaged. In a pilot assay, small molecules known to have similar bioactivities were accurately clustered together based on similarities between image-based profiles. As cell morphology is impacted by many factors ranging from genetic and epigenetic to metabolic and environmental, we anticipate that the low cost, but high-dimensional imaging assay will facilitate identification of unexpected cellular alterations associated with small-molecule perturbations and disease.

While methods such as gene-expression profiling and the new image-based profiling assay contain rich information describing biological responses to small molecules, few approaches are available to use these data to understand structure–activity relationships (SAR). To enable more effective multi-parameter optimization of probes and therapeutic leads, the MLP developed a computational method to generate SAR information automatically from high-dimensional small-molecule profiling data (Wawer *et al.*, 2014a). \Gene expression profiling data and cell-painting data were collected for about 20,000 DOS molecules and just over 2000 bioactive compounds. Association-rule mining was then used to define 'rules' of varying strengths that connected chemical attributes (e.g., contiguous substructures within compounds, shared synthetic histories) to biological effect patterns in the gene expression and imaging data. In one application of the methodology, identification of a DOS-based molecule that co-clustered with structurally unrelated microtubule destabilizers provided an opportunity for 'scaffold hopping' based on the signatures derived from this high-content information.

Rooted in these methodological advances, the question became whether multiplexed cellular small-molecule activity data could be used to create a screening library with maximal *bioactivity* (performance) diversity. There is general consensus that a small-molecule library that perturbs as many biological targets and induces as many distinct cellular phenotypes as possible would be most effective for cell-based screening. However, the data needed to create *a priori* such a screening collection were not previously available. As a consequence, assembling diverse screening collections has thus far focused primarily on the computed diversity of chemical structures. In one notable recent paper, a retrospective analysis of high-throughput screening data was used to assemble a library on the basis of biological activity (Petrone *et al.*, 2012); however, this method, which is based on analysis of historical data, is not extensible to new candidate library members. The MLP measured gene-expression and image-based profiles for over 12,000 bioactive compounds and over 17,000 diversity-oriented synthesis (DOS)-based molecules for which HTS results from up to 178 cell-based screens were available (Wawer *et al.*, 2014b). We asked if the profiling data could select for compounds with diverse bioactivities, as judged by comparing patterns of activity in cells or against target proteins. Compounds selected based on varied activity in the imaging and gene expression assay profiles significantly outperformed compounds selected randomly or based on chemical diversity when their performance diversity in historical HTS assays was compared. In addition, we discovered thousands of compounds, many of which formed scaffold-based clusters, that induced cell morphology and gene expression patterns not seen within the 12,000 bioactives, likely hinting at novel mechanisms of action. These methods and others that yield multiplexed data reporting

complementary measures of compound activity offer a powerful route for prioritizing the synthesis and testing of small molecules more likely to modulate novel therapeutic targets.

New antiinfectives through chemical diversity

Many neglected infectious diseases are endemic to developing nations and the socioeconomic impact of these diseases is devastating. The impact of drug resistance and the lack of effective drugs are well exemplified by malaria and Chagas disease, respectively. In the case of malaria, there are several drugs available for treatment, but rapid emergence of resistant parasites poses a serious problem (Fidock, 2013). The currently available treatment options for Chagas disease are very limited and are efficacious only during the acute phase of the disease, not the chronic phase. Moreover, the severe adverse effects of the current anti-Chagas drugs lead to poor patient compliance (Bern, 2011). The development of safe new drugs for treating malaria and Chagas and other infectious diseases is urgently needed.

Identifying antimalarials and anti-trypanosomal agents with novel chemical structures and mechanisms of action may facilitate the development of therapies that are effective against parasites resistant to current drugs. DOS pathways have proven highly effective at rapidly generating novel, stereochemically rich compounds, including ones containing 8- to 14-membered rings (Gerard *et al.*, 2011; Lowe *et al.*, 2012; Marcaurelle *et al.*, 2010) and the MLP screening library contained more than 5,000 DOS compounds in this vein. These compounds, in conjunction with certain MLP probe-discovery projects that used a larger collection of 100,000 DOS compounds, have enabled the identification of small-molecule probes that act against these infectious agents through new mechanisms.

A cell-based malaria viability screen using an 8,000 DOS compounds led to the identification of a highly active hit with four stereogenic elements. Of the 15 other possible stereoisomers of the molecule that were present in the screening collection, only one had any detectable activity. Subsequent optimization, guided by these stereochemical insights, led to the identification of ML238 (Fig. 3), which is potent against both the chloroquine-resistant Dd2 strain ($IC_{50} = 0.4$ nM) and the wild-type 3d7 strain ($IC_{50} = 0.6$ nM) of *Plasmodium falciparum* (Heidebrecht *et al.*, 2012; Weiwer *et al.*, 2010). In addition, ML238 has good water solubility and does not lyse red blood cells ($EC_{50} > 40,000$ nM), which is important because it suggests a lack of broad cytotoxicity. To determine the compound's mechanism of action, MLP generated ML238-resistant parasites and conducted whole-genome sequencing. These studies revealed that ML238 acts at the quinone reductase site of the bc_1 complex (Q_i) (Lukens *et al.*, 2014), which is on the opposite side of the cell membrane from the quinol oxidase site targeted by the antimalarial drug atovaquone. ML238 and atovaquone lacked cross-resistance and were synergistic in combination, highlighting the quinone reductase site as a antimalarial therapeutic target that may overcome atovaquone resistance (Lukens *et al.*, 2014).

Similarly, a cell-based screen for molecules with selective lethality against *Trypanosoma cruzi*, the etiological agent of Chagas disease, identified ML341 (Fig. 3), a DOS-derived smallmolecule probe (Carmody *et al.*, 2010; Dandapani *et al.*, 2014). ML341 is potent against several strains of *T. cruzi* including the clinical cardiotropic isolate CA-1 strain, clone 72 (CA-I/72) ($IC_{50} = 1$ nM). Next, ML341 was evaluated for *in vitro* cidal activity

against a CA-I/72 strain in bovine skeletal muscle (BESM) cells. Emergence of the trypomastigote form of *T. cruzi* was monitored daily in infected BESM cells treated with ML341 or benznidazole, the current first line drug. In this stringent assay, ML341 was cidal at 40 nM, whereas benznidazole was cidal only at 6.6 uM.

In addition to this potency, ML341 was stable to mouse liver microsomes, which will facilitate the use of this probe in *in vivo* studies. Out of the 8 possible stereoisomers of ML341, only 2 stereoisomers were stable to mouse liver microsomes, a finding that helped guide further medicinal chemistry optimization. As seen in these two examples, having a comprehensive set of stereoisomers in the screening collection was crucial for evaluating rapidly stereochemistry-based SAR for potency and ancillary pharmacology.

Sphingosine-1-phosphate receptor modulators

Characterizing the mechanisms of action behind the anti-inflammatory properties of FTY720, a derivative of the fungal metabolite-derived serine palmityoltransferase inhibitor myriocin (Chen *et al.*, 1999), led to the discovery that FTY720 modulates sphingosine-1-phosphate (S1P) receptors to regulate lymphocyte tracking (Mandala *et al.*, 2002). This finding then paved the way for the subsequent clinical development of FTY720 (fingolimod), a sphingosine analog that modulates four of the five human S1P receptors, for the treatment of multiple sclerosis (Rosen *et al.*, 2008). S1P receptors are expressed predominantly in immune, neural, endothelial and smooth muscles cells, and are believed to play roles that include regulating immune cell trafficking, endothelial barrier integrity and blood pressure, suggesting the potential to modulate specific receptor subtypes to treat a variety of diseases (Rosen *et al.*, 2013).

To better validate individual S1P receptors as therapeutic targets, the MLP set out to identify small-molecule probes that selectively agonized or antagonized each receptor. A selective S1P1 antagonist (ML056, Fig. 3) with *in vivo* activity was developed through rational synthesis and used to explore the impact of perturbing this receptor on lymphocyte movement and function, providing evidence that the therapeutic effects of FTY720 are primarily mediated through this receptor (Sanna *et al.*, 2006). The concomitant reduction in lymphocytes by the antagonist, resulting in endothelial leakage and severe pulmonary edema, showed that agonist therapy was the only tolerable choice for autoimmune disease (Rosen *et al.*, 2013).

The poor water solubility of ML056 imparted by a hydrophobic strong zwitterion could be overcome both *in vitro* and *in vivo* by formulation as the reversible carbonate adduct. This derivatization was essential for the maintenance of complete receptor occupancy of stable S1PR1, which enabled the first successful crystal structure of a lipid-sensing GPCR (Hanson *et al.*, 2012). When combined with biochemical studies, the S1P1 structure also provided insights into a new agonist binding mode used by compounds that include the second-generation S1P1 agonist ML007 (Fig. 3). This series, identified in an allosteric agonist primary screen, was determined in a secondary assay to be subject to non-competitive inhibition by ML056, an orthosteric agonist, occupying the same site as the receptor's endogenous ligand. These studies defined a binding pocket distinct from S1P that affords

greater receptor selectivity and potentially a better pharmacokinetic profile and therapeutic index (Gonzalez-Cabrera *et al.*, 2008).

Agonizing the S1P1 receptor to modulate immune cell trafficking may provide a therapeutic approach for diseases beyond multiple sclerosis. Clinical outcomes following influenza infection are determined by properties instrinsic to the virus strain and also differences in the host immune response. In particular, high cytokine levels and immune cell recruitment correlate with a poor outcome. In mice infected with a 2009 H1N1 pandemic influenza viral isolate, CYM-5442, an optimized derivative of ML007 with *in vivo* activity, inhibited the secretion of cytokines, reduced immune cell infiltration into the lung, and improved survival – while having no impact on viral titers (Teijaro *et al.*, 2011; Walsh *et al.*, 2011).

The MLP has also identified agonists of the S1P2 (ML031; Fig. 3) (Satsu *et al.*, 2013), S1P3 (ML249; Fig. 3) (Guerrero *et al.*, 2010a; Jo *et al.*, 2012; Urbano *et al.*, 2013) and S1P4 (ML248; Fig. 3) (Guerrero *et al.*, 2010b; Guerrero *et al.*, 2012) receptors, as well as an S1P4 receptor antagonist (ML131; Fig. 3) (Guerrero *et al.*, 2011; Oldstone *et al.*, 2010; Urbano *et al.*, 2011a; Urbano *et al.*, 2011b), providing multiple first-in-class small-molecule probes for further eludidating the functions of this class of GPCRs. ML249, an allosteric agonist for the S1P receptor has been used to define orthosteric, allosteric and biopic space within the S1P3 receptor binding pocket (Jo et al. 2012).

Antagonists of PPARγ phosphorylation

The thiazolidinedione (TZD) class of antidiabetic drugs comprises PPARγ agonists that increase expression of the insulin-sensitizing hormone adiponectin and decrease expression of insulin resistance-inducing hormones – in part contributing to their potent insulin-sensitizing efficacy (Yu *et al.*, 2002). Although drugs in this class are marketed to treat type-2 diabetes, their clinical utility has been limited by serious side effects seen in a minority of patients, including fluid retention (Staels, 2005), congestive heart failure (Nesto *et al.*, 2003), and decreased bone density (Aubert *et al.*, 2010).

The antidiabetic efficacy of these drugs was long thought to result from receptor agonism. Strikingly though, as additional PPARγ modulators were developed, drug efficacy correlated more tightly with ligand binding affinity than with agonist activity, prompting a closer examination of the mechanism through which PPARγ ligands improve insulin sensitization. PPARγ ligands that were potent insulin sensitizers were found to be highly effective at reducing obesity-induced phosphorylation of PPARγ at S273 in mice, despite varying levels of receptor agonism. Also, the therapeutic efficacy of the TZD rosiglitazone correlated with how effectively the drug reduced PPARγ phosphorylation at S273 in individual patients. Expression profiling in adipocytes revealed that phosphorylation of PPARγ at S273 leads to repression of a subset of target genes associated with obesity and insulin sensitivity (Choi *et al.*, 2010). Recent studies have further shown that docking of the transcriptional repressor Thrap3 to phospho-S273 on PPARγ regulates the expression of diabetes-associated genes (Choi *et al.*, 2014). Collectively these results suggest that blocking phosphorylation of PPARγ at S273, by normalizing expression of insulin sensitizing genes, is the dominant therapeutic mechanism of this drug class. However, it was not clear if small molecules could

be identified that sufficiently separated the two activities of TZDs and, if found, what the efficacy and safety profile of such compounds would be.

Starting from known potent partial agonists of PPARγ, the MLP synthesized and tested analogs to look for molecules that bind the receptor tightly and inhibit phosphorylation of S273, but are devoid of receptor agonist activity. Identifying small molecules that block a post-translational modification of a receptor is a challenging objective that was aided by a toolbox of methods developed by the MLP for characterizing nuclear receptor structure-function (Marciano *et al.*, 2014a). These efforts led to the non-agonist (antagonist) probe ML244 (SR1664; Fig. 3) (Choi *et al.*, 2011; Kamenecka *et al.*, 2010). Hydrogen-deuterium exchange (HDX), which has proven particularly effective for characterizing ligand-induced conformational dynamics of nuclear receptors (Marciano *et al.*, 2014b), revealed that ML244 directly binds PPARγ, does not stabilize the Af2 surface of the receptor that is known to drive classical agonism, yet perturbs the surface of the ligand binding domain to induce a conformation that is not susceptible to phosphorylation of S273. Importantly, because ML244 binds PPARγ rather than directly inhibiting Cdk5, a kinase that mediates S273 phosphorylation of PPARγ, or any other kinase, the probe does not interfere with phosphorylation of other substrates in the proteome.

In mice fed a high-fat diet, ML244 reduced Cdk5-mediated phosphorylation of S273 on PPARγ, normalized serum glucose levels, and improved insulin sensitivity. In the leptin-deficient *ob/ob* mouse model of severe obesity, ML244 reduced S273 PPARγ phosphorylation and improved glucose tolerance as effectively as rosiglitazone. However unlike rosiglitazone, ML244 did not cause weight gain or fluid retention in these mice or reduce bone-cell mineralization in culture. Ongoing efforts are now focusing on increasing the drug-like properties of the ML244 scaffold and testing the impact of the resulting compounds following once-a-day oral administration in obese mice.

Allosteric modulators of muscarinic receptors

About 30% of currently marketed drugs modulate GPCRs. The vast majority of GPCR-targeted therapeutics act at an orthosteric site, These direct-acting ligands can have a number of potential drawbacks, including inducing toxicity and receptor desensitization or internalization. In addition, identifying selective orthosteric ligands has often been difficult (Conn *et al.*, 2009a). Muscarinic acetylcholine receptors (mAChRs) have attracted significant interest as therapeutic targets in multiple neurological disorders. In Phase-II and Phase-III clinical trials, pan-orthosteric agonists of the five human mAchRs led to cognitive improvements and had anti-psychotic effects in Alzheimer's disease (AD) and schizophrenia. However, adverse events, which were believed to result from activation of the M2 mAChR and M3 mAChR in the periphery, prevented further clinical development of the compounds. Despite significant effort, selectively targeting the acetylcholine binding site of the mAchRs did not prove possible (Conn *et al.*, 2009b) due to the high evolutionary conservation across subtypes for the endogenous acetylcholine ligand.

In contrast, allosteric sites are less evolutionarily conserved across receptor subtypes and have afforded exciting opportunities to achieve true subtype selectivity, as well as diverse pharmacology by stabilizing unique, activated conformations of the receptors. Moreover,

allosteric sites are generally lipophilic pockets, which could targeted by unique small-molecule chemotypes that are structurally distinct from endogenous ligands (Conn *et al.*, 2009b; Wenthur *et al.*, 2014). With this in mind, the MLP undertook screening efforts to identify selective positive allosteric modulators (PAMs) of mAChRs that could be used to dissect the individual contributions of these receptors to the therapeutic efficacy of pan-mAChR agonists. These efforts were aided by employing functional assays that enabled the identification of PAMs and negative allosteric modulators (NAMs) in a single kinetic calcium assay. While the HTS afforded hits, these compounds did not have the physiochemical properties and CNS penetration needed for in vivo target validation experiments. Achieving high-quality small-molecule probes required significant medicinal chemistry optimization of the initial screening hits, leading to the identification of an M1-selective PAM (ML169; Fig. 3) (Bridges *et al.*, 2010; Reid *et al.*, 2011; Tarr *et al.*, 2012), an M4-selective PAM (ML253; Fig. 3) (Le *et al.*, 2013; Niswender *et al.*, 2010) and an M5-selective PAM (ML326; Fig. 3) (Gentry *et al.*, 2010; Gentry *et al.*, 2013). All of these probes had submicromolar EC_{50}s, at least ten-fold selectivity over the other receptor subtypes and, importantly, were brain penetrant.

In a mouse model of AD, the M1-selective probe ML169 potentiated chemically-induced excitation in the striatum, which is believed to mediate anti-psychotic effects, and favored the production of soluble, rather than amyloid, APP (Tarr *et al.*, 2012). In a mouse model of schizophrenia, M4-selective ML253 reversed amphetamine-induced hyperlocomotion, recapitulating the effects of known anti-psychotic agents (Le *et al.*, 2013). Further experiments are now focusing on using these probes to explore the biology and potential therapeutic relevance of these receptors, including investigating the role of M5, about which little is currently known.

Small-molecule activators of a metabolic enzyme

Tumor growth is associated with metabolic adaptations that facilitate cell-autonomous nutrient uptake, an increased rate of aerobic glycolysis, and a shift towards more anabolic metabolism to support cancer cell growth and proliferation. Emerging mechanistic insights into how metabolic pathways are regulated in cancer are pointing towards potential new therapeutic targets; however, which targets represent true cancer dependencies versus nodes where metabolic plasticity will allow cancer cells to adapt and to escape metabolism-targeting therapeutics remains an open question. Small-molecule probes are urgently needed to study how metabolism is altered to support tumor growth and to identify cancer metabolism targets that could be exploited therapeutically.

Cancer cells in general adopt a less differentiated cell state and, analogous to less differentiated non-cancerous cells (e.g., adult stem cells), tend to rely on aerobic glycolysis. Pyruvate kinase, which catalyzes the final step in glycolysis, is derived from two gene products that each possesses two alternate splice isoforms. Cancer cells often express the M2 isozyme of pyruvate kinase (PKM2), which promotes aerobic glycolysis. In contrast, the M1 isozyme (PKM1) promotes glucose oxidation and is expressed in tissues with high ATP requirements such as muscle, heart and brain. The level of regulation of these isozymes is highly divergent. PKM1 has high constitutive activity, whereas PKM2 can adopt an active

or inactive conformation in response to signaling inputs. PKM1 expression increases oxidative glucose metabolism and can suppress both cell proliferation and tumor growth (Christofk *et al.*, 2008; Israelsen *et al.*, 2013; Lunt *et al.*, 2015). However, the extent to which ectopic PKM1 expression recapitulates smallmolecule activation of PKM2 was unknown, leaving uncertain whether small-molecule modulation of PKM2 activity would lead to changes in tumor growth or viability.

More rigorously testing the potential of this therapeutic approach and better understanding the role of pyruvate kinase activity in cancer biology required small-molecule activators of PKM2, yet activating protein function is considered a tall order for small molecules. To address this gap, the MLP conducted a screen to identify molecules that increased PKM2 activity in a biochemical assay. Three different chemotypes were identified and confirmed to be bona fide activators of PKM2. Subsequent optimization of these hits resulted in the identification of ML203 (DASA-58; Fig. 3) and ML265 (TEPP-46; Fig. 3), the first two reported PKM2 activators (Boxer *et al.*, 2010). Further characterization demonstrated that the probes increase activity by stabilizing formation of the PKM2 tetramer, the more active form of the enzyme. This tetramer stabilization is further enhanced in the presence of fructose-1,6-bisphosphate (FBP), an endogenous activator of PKM2. Structural studies revealed that the synthetic small-molecule *activators* occupy a binding site at a monomer-monomer interface distinct from the allosteric site bound by FBP (Anastasiou *et al.*, 2012). Thus, these MLP probes enhance enzyme activity by stabilizing a protein-protein interaction – a mechanism of action generally viewed as challenging to accomplish (Thiel *et al.*, 2012).

Next, the probes were used to explore the cancer contexts in which activating PKM2 might provide a therapeutic benefit. In cancer cell lines grown in culture, ML203 and ML265 reduced cellular proliferation and had an impact on metabolite levels distinct from the changes observed with ectopic PKM2 expression (Anastasiou *et al.*, 2012). A change in serine metabolism was among the most dramatic changes observed, and PKM2 activation can synergize with serine deprivation to slow the growth of cancer cells (Kung *et al.*, 2012). Pyruvate kinase activation can also impair the growth of xenograft tumors with oral dosing of ML265 causing increased tumor latency and reduced tumor size (Anastasiou *et al.*, 2012). Although these results provide initial evidence that activating PKM2 is tumor suppressive, the effect was relatively modest and the molecules had the highest efficacy in slowing tumor initiation.

A critical next step will be determining if there are established tumor contexts that are particularly dependent on low PK activity or if there are combination treatments that confer particular sensitivity to PKM2 activation, for instance modulators of serine metabolism. Beyond its role in tumors, regulated PK activity is also found in non-cancerous tissues, including the liver and red blood cells. Thus, activation of PKM2 or another PK isoform using small molecules could be beneficial in both cancer and non-cancer disease states. To this end, PK activators are currently being explored as therapeutics for pyruvate kinase deficiency, an inborn error of metabolism that specifically affects the red blood cell isoform of PK (Zanella *et al.*, 2007).

Stabilizing a folded lysosomal enzyme while retaining its activity

Gaucher's disease is a rare, autosomal disorder that results from a deficiency in the lysosomal enzyme glucocerebrosidase (GCase). Currently approved treatments for the disease include enzyme-replacement therapy, which is effective for some patients who have the most common form of the disease; however it is also expensive (Ortolano *et al.*, 2014). When enzyme-replacement therapy is not an option, patients may be treated with a small molecule inhibitor of glucosylceramide synthetase, decreasing production of glucocerebrosides, the substrate of GCase that accumulates in disease. This therapy is associated with a number of side effects including weight loss, tremors and peripheral nerve damage (Larsen *et al.*, 2012). Neither of these treatments is effective against neuropathic forms of the disease (Suzuki, 2013).

Many GCase mutants found in Gaucher's patients are enzymatically active, but are improperly folded by endoplasmic reticulum (ER) chaperone proteins, causing ER accumulation and degradation before the protein reaches the lysosome (Orvisky *et al.*, 2002). As a result, current drug-discovery efforts are focusing on identifying chemical chaperones that bind mutant GCase to increase the fraction of properly folded enzyme and promote greater trafficking to the lysosome. Chemical chaperones identified to date are transition-state mimetics that stabilize the enzyme, but also inhibit enzymatic activity (Suzuki, 2013). The most advanced chemical GCase chaperone, isofagomine, was recently tested in Phase-II clinical trials. Isofagomine increased GCase levels in the white blood cells of all patients treated, demonstrating its capacity to increase trafficking. However, the treatment only led to a clinical improvement in one of 18 patients who completed the study, probably due to isofagomine's inhibition of GCase activity (Panicker *et al.*, 2012).

Chemical chaperones that stabilize the enzyme without inhibiting activity may provide a therapeutic approach with improved efficacy. To identify such compounds, the MLP developed a high throughput dose response assay that used a spleen homogenate solution from a patient with Gaucher's disease, thus creating an assay using mutant enzyme in a physiological environment. This assay enabled the identification of a pyrazolopyrimidine-based molecule that was optimized to the small-molecule probe ML198 (Fig. 3) (Goldin *et al.*, 2012; Rogers *et al.*, 2010). In three patient-derived dermal fibroblast lines with distinct genotypes, ML198 increased the activity and lysosomal localization of mutant GCase. Moreover, in macrophages from Gaucher's patients, ML198 reduced aberrant lipid accumulation and normalized phagocytic and efferocytosis function. The MLP probe also displayed good pharmacokinetics and brain penetration in mice (Patnaik *et al.*, 2012).

Innovating small-molecule discovery

Human biology is providing new insights into therapeutic targets. For example, human genetics is revealing risk and protective variants of genes across a range of diseases. Biochemical investigations of variant proteins can provide a blueprint or recipe to follow that defines the activity that a drug should confer on the more common version of the protein. However, the activities suggested by these 'experiments of nature' (Plenge *et al.*, 2013) are challenging and generally unfamiliar to historical drug discovery. For example, rather than pointing to the need for inhibiting a specific protein kinase, they might highlight

the need for stabilizing a specific protein such as APP or ApoE to reduce levels of a post-translational modification (Jonsson *et al.*, 2012; Mahley and Huang, 2012). Drugs don't yet exist that have these mechanisms of action. To realize the promise of precision medicine there is a great need to innovate chemistry, chemical biology and other areas of science and engineering related to therapeutics discovery if we are to uncover the kinds of mechanism of action compounds suggested by human biology.

The MLP has been an early pioneer in efforts to develop small-molecule probes with novel mechanisms of action. One of the lessons learned was the power of close collaborations of academic biologists and clinicians with chemists and chemical biologists for developing effective and informative probes that facilitate studies of emerging biology. Another insight gained was the critical importance of devoting sufficient resources to chemical optimization. Hits straight from screens rarely have the potency and selectivity necessary to be effective tool compounds – and if used with insufficient caution can lead to non-robust conclusions (Begley and Ellis, 2012). Some percentage of hits identified in screens are likely promiscuous compounds unlikely to ever yield tool compounds (Baell and Walters, 2014). Other hits are good starting points, but require a substantial investment in "hit-to-probe" chemistry. When this investment is made, the resulting compounds can provide unique avenues for exploring biology. For instance, the MLP has discovered many small-molecule probes that modulate targets via mechanisms that are difficult or impossible to explore by genetic perturbations, such as altering the rate of protein modifications (noted above), disrupting protein–protein interactions, increasing protein stability and inhibiting protein dimerization (Supplementary Table 1).

The MLP has not been alone in recognizing the critical experimental niche filled by small-molecule probes (Edwards *et al.*, 2015; Edwards *et al.*, 2011; Frye, 2010; Workman and Collins, 2010). Numerous academic and industrial efforts, including public–private partnerships such as the one led by the Structural Genomics Consortium, now focus on identifying and publishing potent and selective small molecules. As a result, the use of small-molecule probes has become a more established part of the biologist's toolkit.

Once high-quality small-molecule probes are developed, one of the biggest current hurdles to translation is that preclinical models do not accurately predict efficacy and toxicity in humans. Academically initiated human experiments, for example, involving Phase-0 (the FDA exploratory IND path) or Phase-I trials – if performed with safe compounds – could be used to look for target engagement and the modulation of biomarkers likely to be predictive of efficacy. Testing disease hypotheses emerging from human biology with on-mechanism small molecules in humans, although insufficient for drug approval, would almost certainly increase the probability of successful drug development and eventual approval.

In addition to enabling advances in biological understanding, small-molecule probes are more directly impacting therapeutic discovery. For instance, in multiple cases, biotechnology or pharmaceutical companies have licensed the intellectual property around MLP probes (Table 1). This investment speaks to the potential for academic small molecule probe discovery to impact drug discovery efforts. However, there remains an alarming

disconnect between the pace at which potential therapeutic targets are uncovered and our ability to develop small molecules with the desired activity.

In contrast to a decade ago, the infrastructure and expertise for small molecule discovery is firmly rooted in academic settings. Innovative small molecule science is now embedded in academia alongside deep and rapidly advancing biomedical knowledge (Reed *et al.*, 2012). Advancing small-molecule discovery in this environment, particularly when methods, data and small-molecule probes are made openly available, may provide an effective bridge towards more rapidly identifying small molecules that modulate the challenging mechanisms illuminated by human biology to accelerate the testing of emerging therapeutic hypotheses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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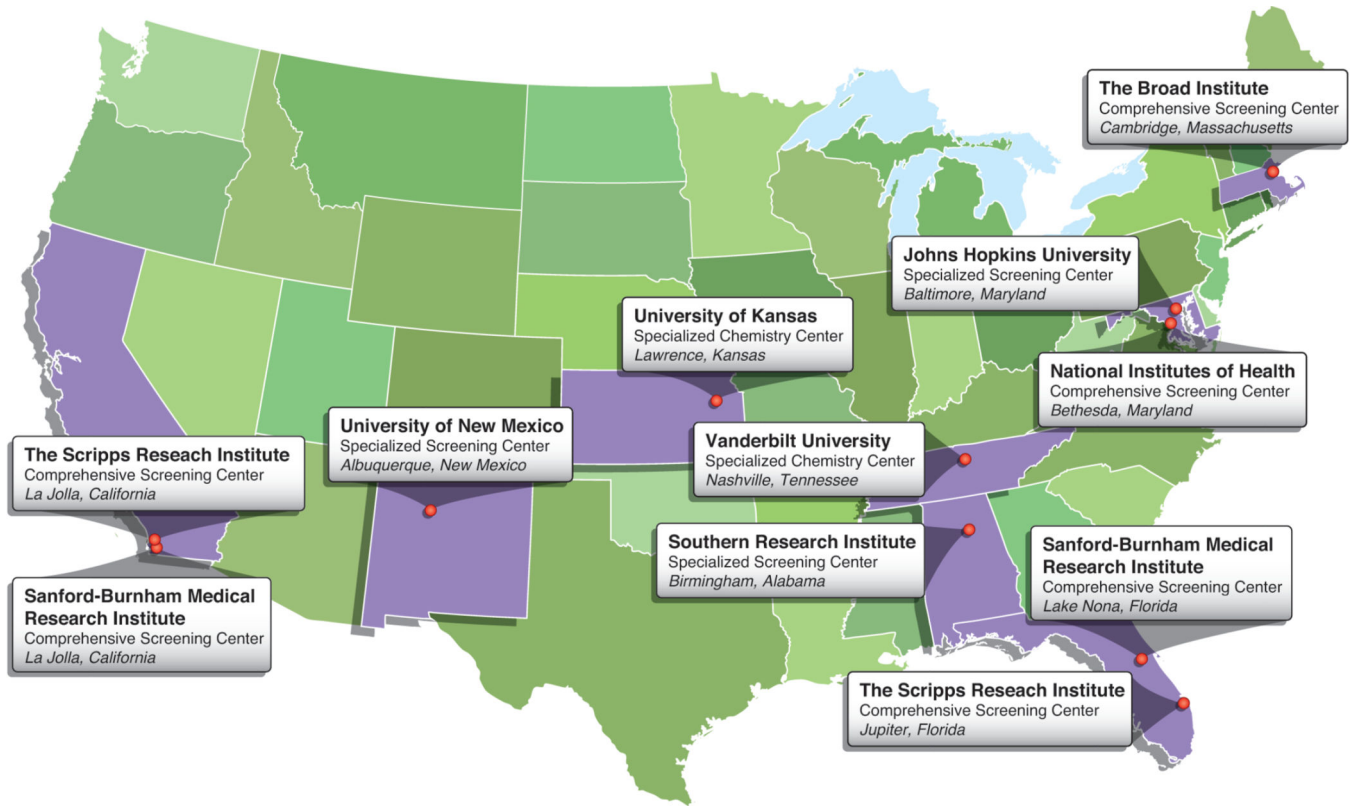
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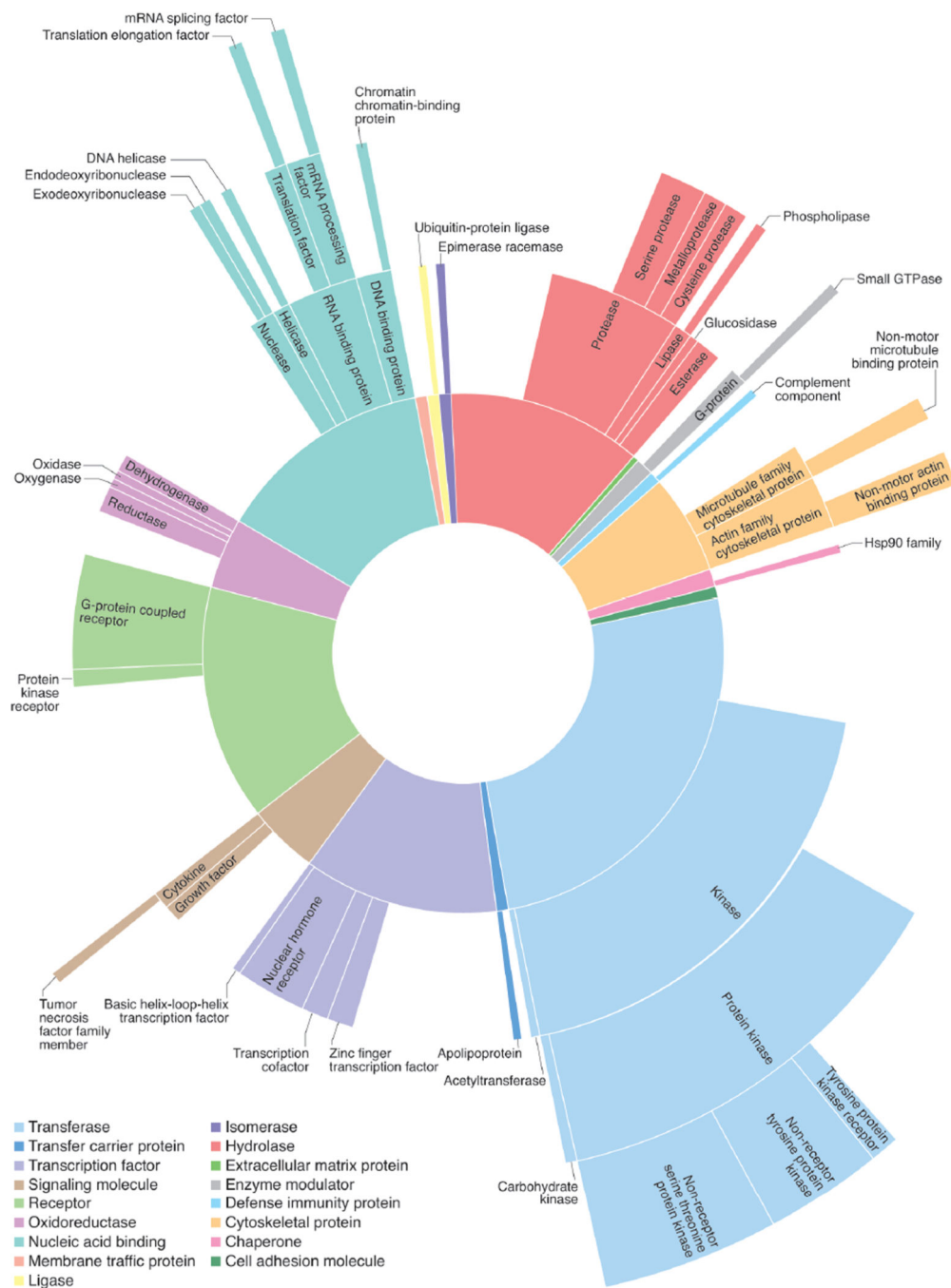**Fig. 1. Centers comprising the MLPCN Network**

Fig. 2. Spectrum of targets modulated by MLP probes

Target classes modulated by MLP probes are visualized based on HTS data and user-defined thresholds. Arc lengths represent the number of distinct targets within a target class that are modulated by this collection of probes. This graph was generated using the BioAssay Research Database (BARD; bard.nih.gov), which was generated by the MLP to enable users to mine and visualize small-molecule activity data. The graph depicts the bioactivity of about 65% of the total number of MLP probes across 415 unique assays associated with 460 targets, which were categorized using the Panther database into 110 protein categories. The

breadth of target classes highlights the diverse biology and target space explored by the MLP.

Fig. 3. MLP probes highlighted in vignettes

Table 1

Translational trajectory for MLP probes.

Targets	MLP probes	Post-MLP trajectory
Serine hydrolases	ML081, ML174, ML211, ML225, ML226, ML256, ML257, ML294, ML295, ML296	Screening and assay platforms, serine hydrolase-directed small-molecule libraries and serine hydrolase inhibitors, resulting in part from MLP projects, have been licensed to Abide Therapeutics, which is pursuing the further identification, preclinical, and clinical development of serine hydrolase inhibitors for a variety of indications, in particular neurological, immunological and metabolic diseases.
P. falciparum, T. cruzi	ML238, ML341	The identification of the MLP malaria probe led to a Gates Foundation-funded Chemical Diversity Initiative that has explored the use of the DOS collections more broadly in infectious disease and has yielded promising compounds that target the etiological agents of malaria tuberculosis via novel mechanisms. In work supported by the Global Health Innovation Technology (GHIT) fund and in collaboration with Eisai, the MLP anti-trypanosomal probe is being optimized to enable *in vivo* proof of concept studies and a DOS-based antimalarial agent is being optimized towards IND-enabling studies.
S1P1 agonists	ML007	Receptos licensed intellectual property surrounding S1P1 agonists including the racemate of its clinical candidate RPC1063, which is currently in two Phase- III studies in relapsing multiple sclerosis and about to enter Phase-III testing in ulcerative colitis and Phase-II testing in Crohn's disease.
M4 mAChR	ML108, ML253	AstraZeneca has licensed intellectual property associated with the M4 PAMs and the company is pursuing preclinical development of the compounds as a potential treatment for the neuropsychiatric symptoms associated with AD and schizophrenia.
GCase	ML198	This probe and related analogs have been licensed to Lysosomal Therapeutics Inc., which is pursuing preclinical development of these non-inhibitory GCase chaperones.
Integrin $\alpha_{IIb}\beta_3$ receptor antagonist	ML165	Rockefeller Medical School and NCATS are pursuing an IND based on a derivative of ML165 indicated for pre-hospital therapy of patients with ST segment elevated myocardial infarction (STEMI).
P97 AAA ATPase inhibitor	ML240	Cleave BioSciences licensed intellectual property surrounding ML240. A derivative of this probe, CB-5083, is currently in two Phase-I studies, one in relapsed and refractory multiple myeloma and the other in solid tumors refractory to the standard-of-care.