# COMMENTARY

# Medicinal chemistry and the effect of assay design

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**ABSTRACT**

Medical chemists use a wide variety of biochemical and cellular assays to direct compound optimization. Structure-Activity Relationship (SAR) campaign decisions are influenced by the information gathered from these tests. As a result, it is crucial for medicinal chemists to comprehend both the advantages and disadvantages of each assay used to evaluate produced analogs. We argue that early collaboration between assay biologists, informaticians, and medicinal chemists is essential for the successful execution of a medicinal chemistry campaign, their combined skill sets are essential for not only creating reliable assays but also for implementing a successful screening cascade in which numerous orthogonal and counter assays are chosen to confirm the activity and target(s) of the synthesized compounds. We examine various instances of drug and chemical probe discovery from joint National Center for Advancing Translational Sciences/ National Institutes of Health projects and scientific literature where the evaluation of substances in secondary or orthogonal assays resulted in the discovery of unexpected activities, compelling a reevaluation of the original assay design that was used to discover the biological activity of the compound. The objective of this Perspective is to steer toward the creation of physiologically appropriate assays that may accurately capture the genuine bioactivity of compounds being generated in a medicinal chemistry campaign using these retrospective case studies.

## INTRODUCTION

The National Center for Advancing Translational Sciences (NCATS) of the National Institutes of Health's Division of Preclinical Innovation (DPI) conducts research with a focus on medicinal chemistry (NIH). Medicinal chemists working with assay biologists and informaticians on a variety of projects inside the DPI. Most research initiatives start as partnerships with outside researchers who are very knowledgeable on a particular biological target or pathway or the pathology of a disease. In such collaborations, small molecule probes that can be employed in proof-of-concept studies are frequently found using High-Throughput Screening (HTS), which is followed by medicinal chemistry optimization of the hits. The ability to cross boundaries, particularly in the direction of an understanding of assay biology, is another crucial trait of a translational scientist. While domain expertise, particularly in synthetic organic chemistry, is a requirement for medicinal chemists to conduct translational science, we encourage chemists to take this into consideration as well. This perspective includes a few high-yield case studies from the NCATS DPI and the literature where drugs unexpected actions were revealed through orthogonal assay evaluation. These illustrations emphasize how crucial it is for medicinal chemists to demonstrate the bioactivity of their compounds through assay design and the construction of a strong screening tree. Recombinant protein and fluorogenic substate-based cell-free enzymatic assays are still a hallmark of HTS and medicinal chemistry-based optimization. In order to find small-molecule modulators of lysosomal hydrolases linked to uncommon diseases as Gaucher, Pompei, Wolman, and Fabry disease, NCATS has modified and downsized a wide range of such tests. Glucocerebrosidase (GCase) deficiency leads to an excessive buildup of glucocerebrosides or glycosphingolipids in lysosomes, which is the hallmark of Gaucher Disease (GD), a recessive monogenic lysosomal disorder. The most frequent missense mutation in type 1 GD patients, N370S, results in protein misfolding and degradation via the ER-associated protein pathway. Iminosugars,

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including the GCase inhibitor isofagomine, are substrate mimics that can act as pharmacologic chaperones. They are thought to bind to the protein, aiding in the folding process and boosting GCase trafficking to the lysosome. In order for the mutant enzyme to digest the stored lipids and lessen the illness phenotype while still being catalytically less active than Wild-Type (WT) GCase, the inhibitor must be replaced by natural glycosphingolipids once in the lysosomes. Numerous kinase inhibitors have received clinical approval for the treatment of cancer since the discovery of the particular Bcr-Abl inhibitor imatinib, and hundreds more are currently undergoing clinical studies. HTS and Structure-Activity Relationship (SAR) optimization studies that frequently search for inhibition of recombinant kinases in cell-free formats are the origin of many kinase inhibitors. The example from NCATS below illustrates how kinase engagement in cells may be drastically different from the kinase profile activity of an inhibitor with cell-free kinases. The dystrophin protein, which binds intracellular actin to the extracellular matrix in myofibrils, is completely lost in Duchenne Muscular Dystrophy (DMD), a rare X-linked progressive muscular degenerative disease that affects 1 in 5000 boys. In one partnership, NCATS looked for substances that could potentially compensate for the loss of dystrophin by upregulating 71 integrin, another structural transmembrane protein in myofibers. To evaluate target engagement with the alleged target CDK2 and other kinases in myotubes, a commercial platform technology (ActiveX Biosciences, San Diego , CA) was used. With an acylated biotinylated group at the third phosphate position, the KiNativ probe is a biotinylated Adenosine Triphosphate (ATP)-mimetic that binds irreversibly to conserved lysine residues at the outer pocket of most kinases. Myotubes from DMD patients were lysed, then the probe was incubated with them. Kinases that incorporated the biotinylated substrate were then enriched by pull down using streptavidin immobilization (streptavidin is a protein with a very high affinity for biotin with a K d of 10-15 M; it can be immobilized on solid support and used to purify/pull molecules attached to biotin). When myotubes were pretreated with compound before lysis, a concentration-dependent reduction of the peptides associated with the kinase that is binding to the compound was noticed. Subsequent sample proteolysis and Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) were used to identify signature peptide fragments from about 300 kinases. The authors hypothesized that SU9516 inhibits the stress-sensing SPAK/OSR1 kinase in DMD myocytes as the mechanism by which it downregulates p65-NF-B activation downstream, reversing the DMD disease phenotype. At 100 n M, there was approximately 45% binding to CDK2, in addition to 81% and 79% binding to OSR1 and STLK3, respectively. In addition, kinase profiling for SU9516 using the HotSpot (Reaction Biology, Malvern, PA) test platform against 300 recombinant kinases had already been documented. OSR1 and STLK3, kinases that were active at 100 n M in myotubes in the cellular assay, were barely inhibited by 500 n M SU9516.