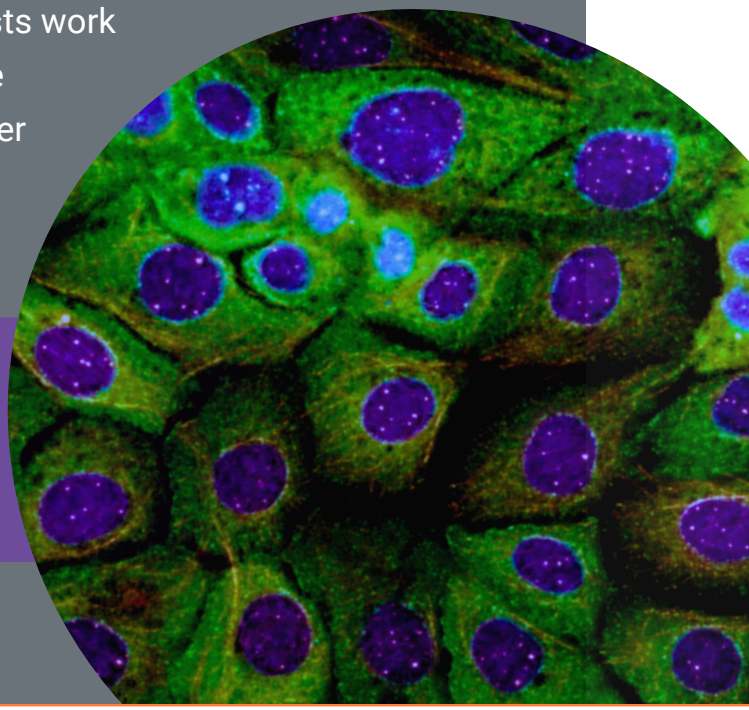
Paraza

Cellular On-Target Activity

Following initial biochemical characterization, cellular assays demonstrating on-target activity are essential to confirm that compounds bind to their intended target within the complex cellular environment, where permeability, metabolism, and intracellular localization affect efficacy. Understanding compound pharmacodynamics in cellular and in-vivo contexts are critical steps to support informed decision-making during lead optimization and preclinical development.

At Paraza, we have strong expertise in addressing cellular assay challenges for emerging targets. Our expert scientists work closely with client partners to design and customize target engagement and pharmacodynamic biomarker assays to drive confident decision-making and accelerate drug discovery.



- Cellular target engagement assays
- Functional assays
- Genetic exploration of on-target activity
- In-vivo pharmacodynamics

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Direct Cellular Target Engagement Assays

Cell-based target engagement assays enable quantitative assessment of compound–target interactions under physiologically relevant conditions. Probe-displacement assays can be implemented using a variety of readouts, including radioligand competition, NanoBRET, TR-FRET, and alphaLISA.

Probe displacement assay

Developing cellular target engagement assays for less-well characterized enzyme classes remains challenging, requiring robust readouts that reflect true target engagement and enable compound ranking using high-throughput screening. In this example, our medicinal chemistry team designed a BODIPY590-conjugated probe for a novel target, derived from an initial lead. It was used to develop a BRET-based probe displacement assay in 384-well format with excellent performance, enabling assessment of compound cellular activity to guide SAR.

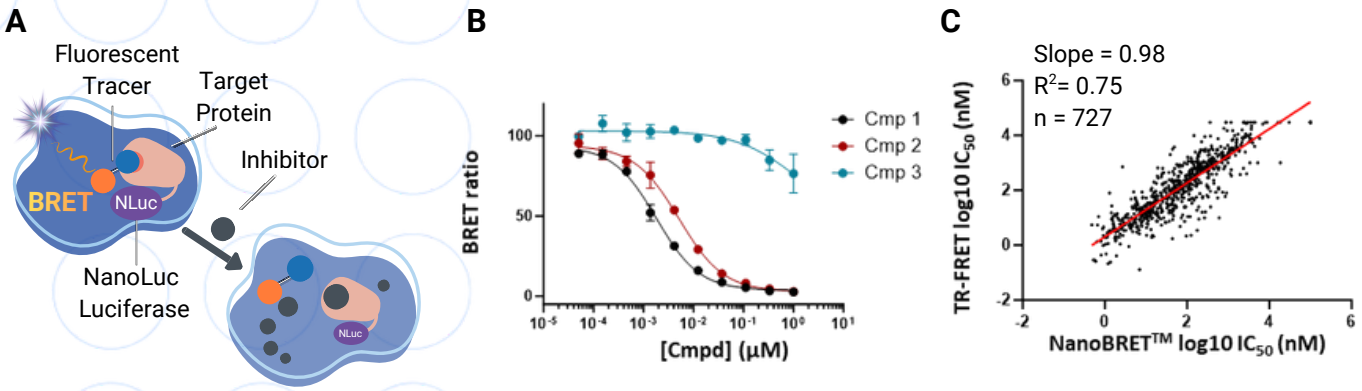

Fig 1. Probe displacement assay developed for a novel target within a less characterized enzyme class.
A) Schematic of NanoBRET probe displacement assay. B) Example of dose response assay in 384-well format. Compounds of varying potencies are shown. C) Correlation between NanoBRET and a biochemical TR-FRET probe displacement assay is shown.

Kinase Autophosphorylation by AlphaLISA

Monitoring autophosphorylation is a common approach to evaluate target engagement in the kinase inhibitor space. AlphaLISA™ kinase assays use antibodies for total and phosphorylated substrate detection to generate a fluorescent/luminescent signal. Like TR-FRET, AlphaLISA technology is sensitive, robust and high-throughput. Both assays are available as pre-validated kits for many targets, and when there is no commercial kit available, we are experts in custom-development of assays.

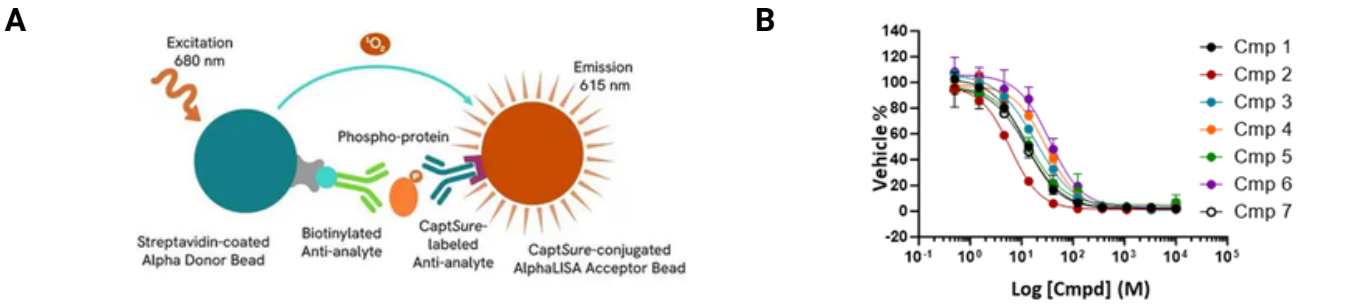

Fig 2. Evaluation of kinase inhibition by AlphaLISA™ assay.
A) Schematic of AlphaLISA™ assay (image source: www.revvity.com). B) A fully automated dose-response AlphaLISA™ assay was optimized to screen compounds targeting the kinase of interest in cells. Examples of tested compounds are shown.

Functional Readouts of On-Target Activity

Direct measurement of compound-target binding may not be feasible for some targets or therapeutic strategies. To circumvent the lack of direct measurement, functional engagement can be assessed by monitoring target-dependent molecular changes.

RT-qPCR Assessment of Splicing Modulators

Small molecule or RNA-based splicing modulators are an emerging drug class that influences pre-mRNA splicing. They promote exon skipping or inclusion in order to restore normal patterns of expression, or to generate non-functional transcripts that lead to gene silencing. At the moment, there is no high-throughput assay that can directly measure drug-ribonucleoprotein (or drug-RNA) interactions. We quantify splicing modulation using RT-qPCR, providing a sensitive and accurate readout that confirms on-target effects and supports compound ranking in a cellular context.

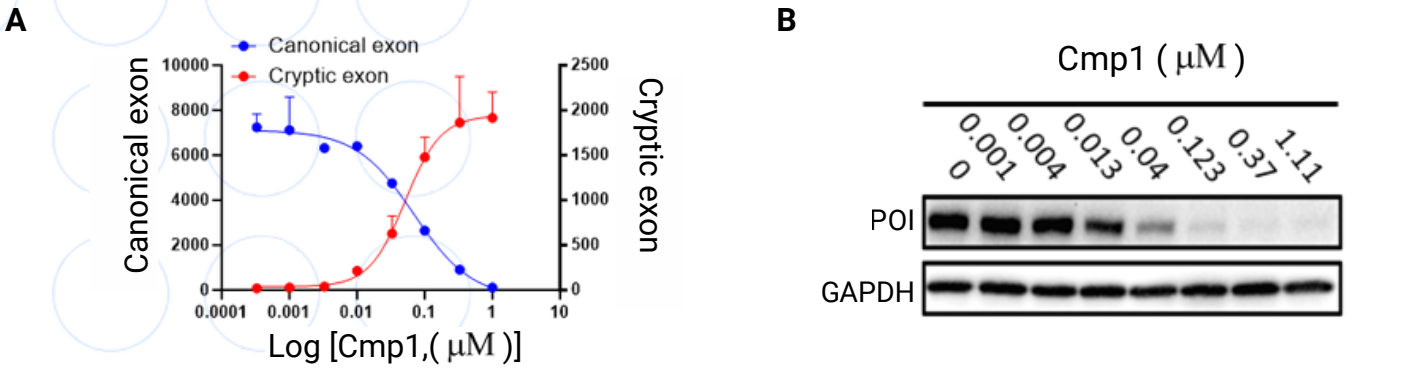

Fig 3. Splice modulator target engagement assay.
A) RT-qPCR assay in a 384-well plate format to quantify canonical transcript and alternative transcript levels following compound treatment. B) Western Blot analysis confirming the suppression of the protein of interest (POI) following treatment with Compound 1.

MoA Confirmation by Genetic Knockdown

For some novel targets suitable antibodies are not available. In such cases genetic perturbation offers an approach to confirm compound mechanism of action. In the example below, target gene knockdown greatly reduced the activity of specific inhibitors, providing support for the expected mechanism of action. Such results obtained in the complex cellular environment complement selectivity observed in biochemical experiments to build the case for specific on-target activity.

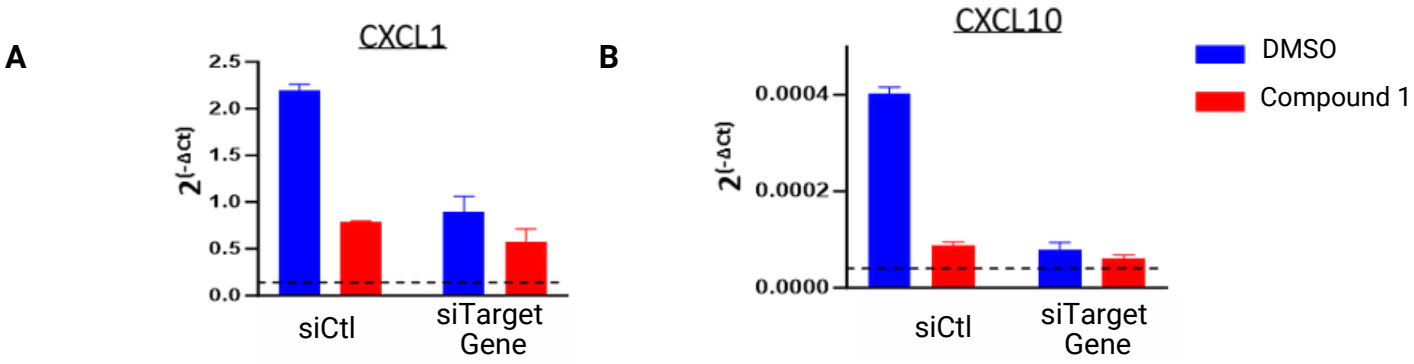

Fig 4.Silencing POI abolishes compound activity, providing indirect evidence of on-target MoA.
Knockdown of expression of POI impaired the ability of the Compound 1 to inhibit TNFalpha-induced CXCL1 (A) and CXCL10 (B) gene expression, as analyzed by RT-qPCR.

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Pharmacodynamic biomarkers in murine models

Assessing compound pharmacodynamics (PD) in animal models is critical for preclinical decision-making and translational confidence. At Paraza, in vivo analyses are conducted on plasma and tissue samples collected after compound administration. Multiple technologies can be employed to provide complementary evidence of compound activity.

Biomarker quantification using MesoScale Discovery (MSD)

The MesoScale Discovery (MSD) assay uses electrochemiluminescence to detect target proteins in complex biological samples. MSD technology enables sensitive, multiplexed biomarker measurements with low background and a wide dynamic range. Whether using a ready-made kit or through development of a custom assay, we have successfully established reliable pre-clinical quantification methods for PD biomarkers for multiple targets, in cells and tissues.

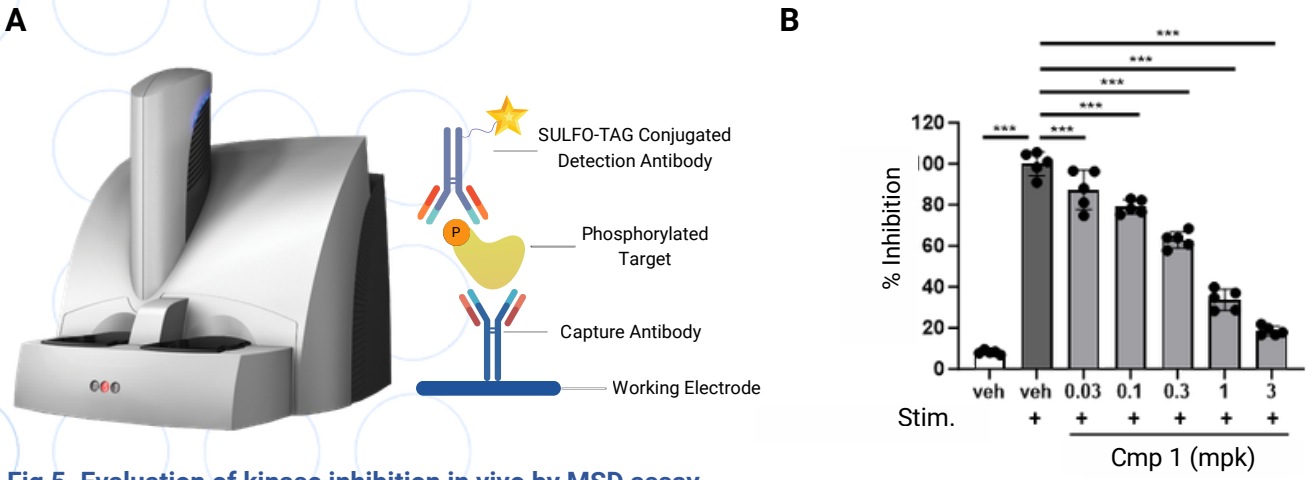

Fig 5. Evaluation of kinase inhibition in vivo by MSD assay.
The phosphorylation of the POI was investigated by MSD in mouse lung tissues A) MSD instrument and schematic representation of technology. B) Fifteen minutes after in vivo mouse stimulation, a strong phosphorylation of the POI was observed. This phosphorylation was inhibited in a dose-dependent manner after oral administration of Compound 1.

LC-MS/MS quantification of metabolites

Quantitative mass spectrometry enables direct identification of molecules based on the mass-to-charge ratios and fragmentation pattern, affording extreme flexibility for assessment of endogenous and exogenous chemicals, peptides and lipids in multiple sample types. For instance, by measuring levels of both an enzyme inhibitor and its PD biomarker, it is possible to achieve translational evaluation of pharmacokinetics and pharmacodynamics from the same tissue samples. MS-based readouts provide high sensitivity and specificity, making them well suited for preclinical and translational studies.

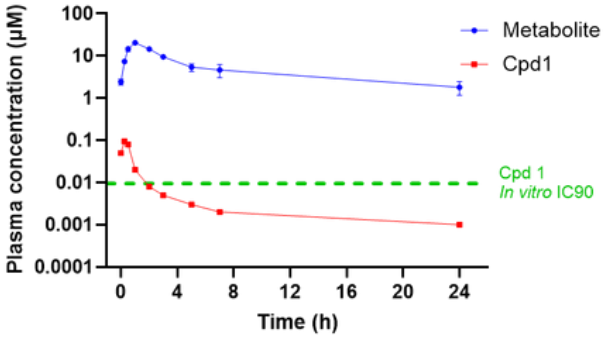

Fig 6. Evaluation of enzyme activity in plasma.
Pharmacokinetic (PK) profiles of inhibitor and POI substrate measured by mass spectrometry in rat plasma using Vanquish Flex UPLC coupled with TSQ Quantis triple quadrupole mass spectrometer (Thermo). Both analytes showed comparable profiles, with the expected slight delay in substrate accumulation relative to the injected Compound 1.

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