Novel, Improved Cell-Based Assays to Enable Immunotherapy Drug Development for Checkpoint Receptors

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Abstract

Regulation of immune responses is tightly controlled through a balance of co-stimulatory and inhibitory checkpoint receptors, often exploited by many cancers. Therefore, therapeutics that block inhibitory receptors or activate immuno-stimulatory checkpoint receptors have proved to be powerful agents to restore anti-tumor immune responses. However, developing drugs targeting these checkpoint proteins has proved to be quite challenging, as cell-based assays used to screen for functional drugs are often difficult to create, involve the use of human primary cells, and have long, complicated protocols. Here, we present data for the new PathHunter® Checkpoint assays that target clinically relevant co-inhibitory and co-stimulatory checkpoint receptors and measure receptor activation and signaling, using the industry-validated Enzyme Fragment Complementation (EFC) technology. These assays facilitate the development of relevant therapeutics, enabling rapid and sensitive screening of biologics and small molecules. Furthermore, the robustness and reproducibility of these assays lend themselves well for use in characterization, relative potency, and QC lot release testing of immunotherapy drugs. These mechanism of action-based, cell-based assays do not require human primary cells, and provide a highly sensitive response, with an easy-to- use protocol that delivers results in a day. In this poster, we present data for bioassays targeting PD-1, and the emerging IO target: SIRPα/CD47 signaling axis.

SHP Recruitment Assay for PD-1 Checkpoint Target Concept

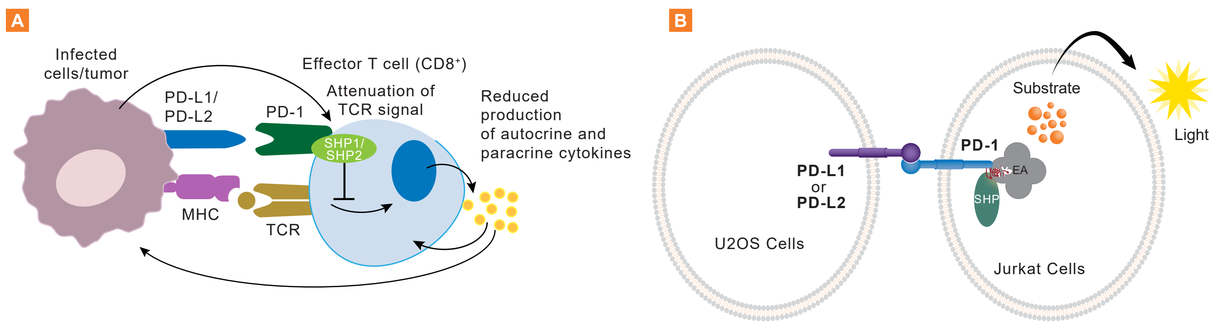
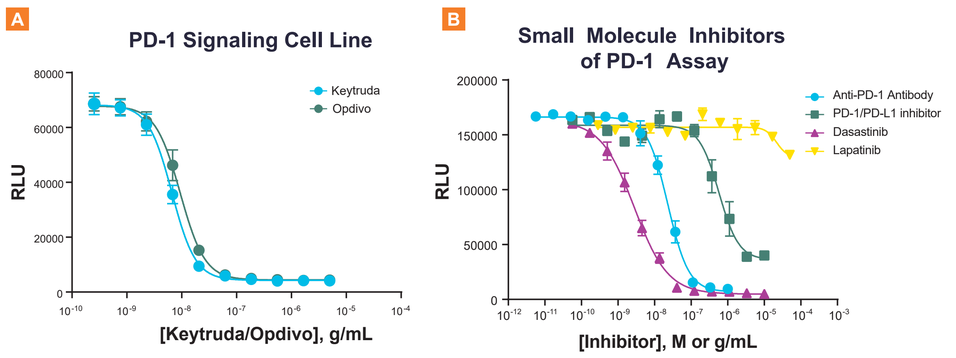


Figure 1. A. Many inhibitory checkpoint receptors (e.g. PD-1, TIGIT) harbor immunoreceptor tail tyrosine (ITT)-like and ITIM motifs in their cytoplasmic tails (motifs that recruit SH2 domain proteins to phosphorylated tyrosine residues). These SH2 domain proteins, such as the SHP phosphatases are critical in mediating the downstream pathway responses for the receptor. B. Full-length PD-1 receptor was engineered with a small β-gal fragment (PK in red) fused to its C-terminus, and the SH2-domain of SHP-1 was engineered with the complementing β-gal fragment (EA). These constructs were stably expressed in Jurkat cells, while untagged full length PD-L1 or PD-L2 were stably expressed in U2OS cells (ligand-presenting cells). Ligand engagement, through co-culture with ligand-presenting cells, results in phosphorylation of PD-1-PK fusion protein, leading to the recruitment of SHP-1-EA which forces complementation of the EFC components to create an active β-gal enzyme. This active enzyme hydrolyzes substrate to create chemiluminescence as a measure of receptor activity. (Figure 1. adapted from Okazaki *et al.*, 2013. *Nature Immunology* 14, 1212-1218.)

PD-1 Assay is Suitable for Detection of Biologic and Small Molecule Inhibitors

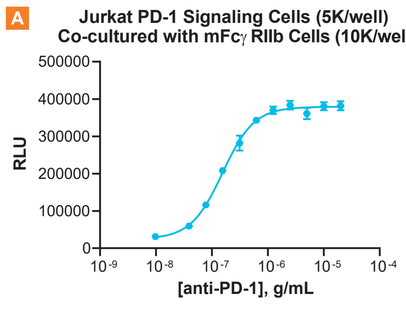


	Keytruda	Opdivo
S/B ratio	15.9	15.4

	Anti-PD-1 Antibody	PD1/PD-L1 inhibitor	Dasastinib	Lapatinib
Hill Slope	-1.515	-1.601	-0.9441	-
IC_{50}	23.09 ng/mL	575.7 nM	2.79 nM	-

Figure 2. A. Jurkat PD-1 cells were treated with serial dilutions of Keytruda® (blue) or Opdivo® (green) for 1 hour prior to stimulation with U2OS PD-L2 cells for 2 hours at room temperature. Expected rank order of the two therapeutic antibodies is observed in the assay with low ng/mL sensitivity for the two marketed drugs. Keytruda and Opdivo are registered trademarks of Merck and BMS, respectively. B. The PathHunter PD-1 assay, when treated with small molecules known to inhibit Src family kinases, has exhibited reduced activity (Dasatinib). Additionally, a low molecular weight inhibitor of PD-L1 interaction with PD-1 (PD-1/PD-L1 inhibitor) also reduces the assay signal in a dose-dependent manner, although not as potently as the anti-PD-1 antibody. These data demonstrate that the PathHunter PD-1 Signaling Assay can be used to identify novel small molecule inhibitors of PD-1, including kinase inhibitors and disruptors of PD-1 interaction with its ligands, PD-L1 and PD-L2.

Analysis of Anti-PD-1 Agonist Activity in the PD-1 Signaling Assay



B

Experiment No.	S/B	EC_{50}, ng/mL
Exp 1, Plate 1	17.9	236
Exp 1, Plate 2	19.5	247
Exp 2, Plate 1	19.9	173
Exp 2, Plate 2	19.2	161
Exp 3	15.9	155
Mean	18.5	194.4
% RSD	8.80%	22.50%

Figure 3. A. Representative dose curve demonstrating the agonist activity of a commercial anti-PD-1 antibody (which is based on a murine IgG1) in the Jurkat PD-1 Signaling Cells facilitated by co-culture with cells expressing mFcγR2b. U2OS mFcγR2b cells were seeded into the assay plate, opsonized with the anti-PD-1 antibody for 1 hour at 37°C, then incubated with Jurkat PD-1 Signaling cells for 2 hours at room temperature. B. Reproducibility of the Jurkat PD-1 agonist assay mode (in co-culture with U2OS mFcγR2b cells) with commercial anti-PD-1 antibody. Low variability in the assay window (S/B) as well as EC50 is observed for the control antibody. The assay is suitable for rank ordering human antibodies (using U2OS human FcγRIIb clustering cells) and for use as a relative potency assay for agonist antibodies.

Mechanism of Action of Anti-CD47 Therapeutics: Blocking the CD47/SIRPα Axis

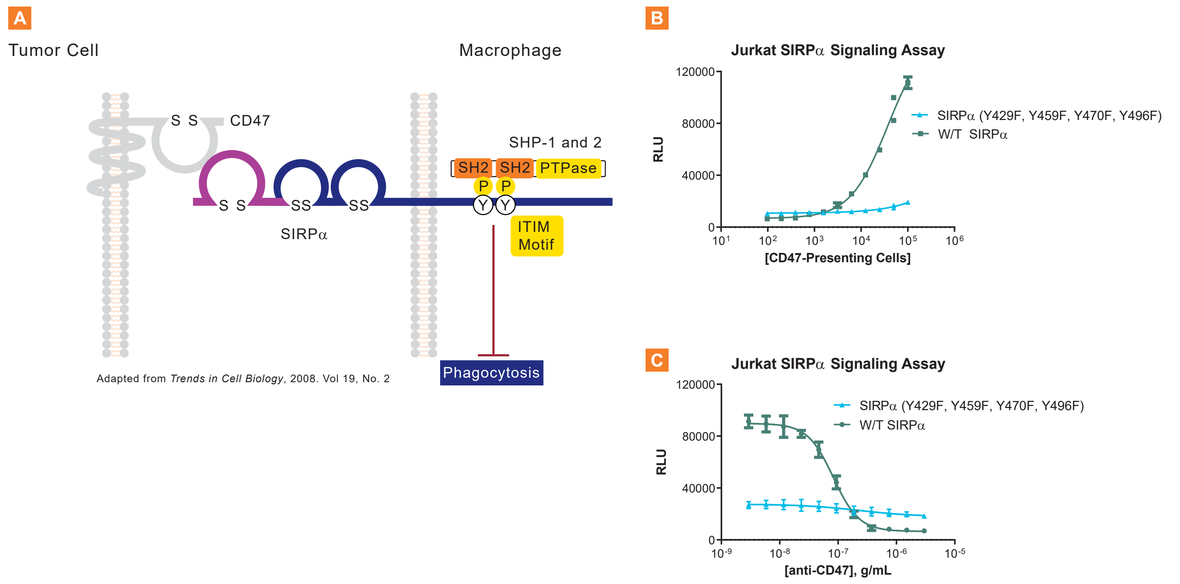
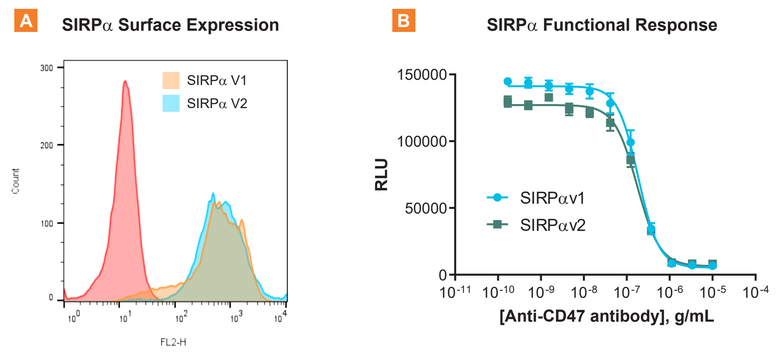


Figure 4. A. Recruitment of SHP1 phosphatase to the C-terminal tail of SIRPα in response to ligand (CD47) engagement is thought to be mediated by phosphorylation of four tyrosine residues in the ITIM motif (Y429, Y459, Y470 and Y496). To demonstrate that signaling through SIRPα is mediated by phosphorylation of these residues, we generated tyrosine to phenylalanine mutations at each of these residues to create a functionally dead SIRPα receptor. B. Analysis of functional response of wild type (W/T) SIRPα and SIRPα (Y429F, Y459F, Y470F, Y496F) to CD47 ligand (agonist mode). C. Analysis of functional response of W/T SIRPα and SIRPα mutants (Y429F, Y459F, Y470F, Y496F) to anti-CD47 after stimulation with CD47 ligand (ligand neutralizing mode). Single mutations disrupted signaling to different degrees (data not shown), but mutation of 3 or more tyrosine residues completely abrogated CD47-mediated SHP recruitment in both agonist and antagonist mode.

Jurkat SIRPα Signaling Assay



C

Cell Line	S/B	IC_{50}, ng/mL
SIRPα V1	21.6	183.5
SIRPα V2	16.6	170.3

Figure 5. At least 10 SIRPα variants have been described in the literature, however, variants 1 and 2 (V1 and V2) are the most prevalent in the population. We have developed signaling assays for both V1 and V2. A. Comparison of surface expression of SIRPα in the Jurkat SIRPα V1 and V2 Signaling Cell Lines by flow cytometry. Comparable levels of surface expression are observed in the two cell lines. B. An antagonist antibody to human CD47 was used to compare the responses of Jurkat SIRPα V1 and V2 Signaling cell lines. A dilution series of anti-CD47 antibody was pre-incubated with Jurkat CD47-presenting ligand cells for 1 hour followed by co-culture with either Jurkat SIRPα V1 or V2 Signaling cells for 5 hours at 37°C. Very similar IC_{50} values were observed for both SIRPα variants, consistent with SPR data in the literature showing similar binding affinities for CD47 between the two variants. A modest difference in assay window (S/B) is observed, with S/B for SIRPα V1 slightly larger than for SIRPα V2.

SIRPα Bioassay Qualification with Commercial anti-CD47 Antibody: Preliminary Data

A

Analyst	Day	Estimated Relative Potency, %		Relative Bias, %
		Plate 1	Plate 2	
1	1	97.8	103.2	0.5
1	2	103.6	105.7	4.65
1	3	96.5	96.8	-3.35
2	1	97.8	97.3	-2.45
2	2	98.1	98.2	-1.85

Average RP: 99.45% Relative Bias: 0.55%
Repeatability: 3.8% Intermediate Precision: 3.34%

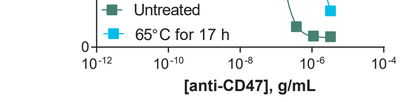


Figure 6. A. Preliminary assay qualification data for the Jurkat SIRPα signaling assay. Intermediate precision of the assay was evaluated by two analysts who compared a 100% anti-CD47 test sample to a 100% reference standard on 10 different plates over 3 days. Excellent repeatability (3.8%) and intermediate precision (3.34%) was obtained with this preliminary dataset. B. The Jurkat SIRPα signaling assay is stability-indicating. A 100% sample of anti-CD47 antibody was incubated at 65°C for 17 hours prior to testing a dose response curve of the heat treated antibody in the Jurkat SIRPα signaling assay relative to the 100% reference standard. A noticeable shift in potency was observed for the heat-treated sample.

Summary

- No primary cells – Get biologically-relevant responses without primary cells
- Easy-to-use protocol with fast results – Increase efficiency with an "add-and-read" protocol and get results in 5-8 hours
- Highly sensitive response – Better sensitivity than competitor assays allows screening of early stage and dilute development samples
- Multiple applications – Drive development of biologic and small molecule drugs
- Support broader drug program – Cell-based assay for functional screening, lead optimization, and bioanalytical QC lot release applications