

Identification of false positives in a HTRF® screen for small molecule inhibitors of PD-1/PD-L1

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- 2 methods are described to assess the incidence of false positives in a HTRF PD-1/PD-L1 interaction screen.
- Comparing reads at the start and end of drug incubation can be used to identify false positives.
- Abnormal changes in 620nm raw data can be used as an indication of compound interference.

Introduction

The programmed cell death protein 1 (PD-1) receptor (CD279), expressed on the surface of activated T cells, and its ligand PD-L1 (CD274), expressed on the surface of dendritic cells or macrophages, are important targets in multiple cancers. The PD-1 and PD-L1 interaction functions as an immune checkpoint that helps maintain immune homeostasis and inhibits autoimmunity. In cancer, immune checkpoint mechanisms often suppress the nascent anti-tumour immune response. Blocking the PD-1 - PD-L1 interaction is promising for cancer immunotherapies.

Monoclonal antibodies (e. g. rituximab, nivolumab) have been approved for treatment of various cancers. However, to date there have been no approved small molecule PD-1/PD-L1 inhibitors. Small molecules have advantages over monoclonal antibodies, including simpler manufacturing, more stability and reduced likelihood of immunogenicity. A PD-1/PD-L1 assay was developed to screen small molecules from the BioAscent diversity library for inhibitors that are able to block the PD-1/PD-L1 interaction and serve as suitable starting points for drug development.

Assay Principle

Homogenous Time Resolved Fluorescence (HTRF) uses a long fluorescence lifetime FRET pair to assess molecular interactions. Donor and acceptor molecules are coupled to either protein, PD-L1 or PD-1, via tags or antibodies. If PD-1 and PD-L1 are in close proximity then excitation, at 337 nm, causes energy transfer between donor and acceptor molecules and results in a FRET signal (read at 620 nm and 665 nm). If the binding partners are not in close proximity (PD-1 and PD-L1 not bound together) then FRET does not occur.

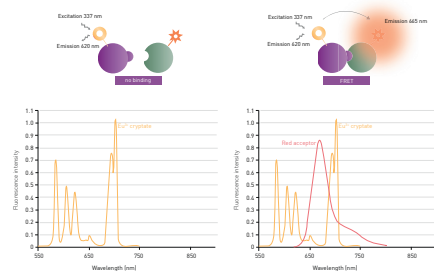


Fig. 1: CisBio HTRF assay principle

Materials & Methods

- White small volume 384 well plate (Greiner)
- Human 6His-PD-L1 (Abcam)
- Human IgG-PD-1 (R&D Systems)
- pAb Anti-human IgG-Tb (CisBio)
- mAb Anti 6His-d2 (CisBio)

Experimental Procedure

1. 80 nL of compound at 100x final concentration is added to white 384 well plates using an ECHO® acoustic dispenser (Labcyte) – 80 nL of reference to 0.1 mM. compound or DMSO is added to positive and negative control wells respectively.
2. 4 µL of PD-1 (at 2x final concentration) is added to all plates using a Preddator bulk reagent dispenser (Redd & Whyte)
3. After 30 min at room temp (in the dark) 4 µL of PD-L1/HTRF mixture (at 4x final concentration) is added to all plates using the Preddator.
4. The plates are then incubated for 30-60 min before reading on the PHERAstar®.

Instrument Settings

Optic Settings	Time resolved, endpoint Top optic	
	Optic module	HTRF 337 665 620
	Excitation source	Flash lamp
	Integration start	50 µs
	Integration time	200 µs
General settings		Number of flashes 200 with flash lamp
		Settling time 0.5 s

False positive - specific considerations for HTRF

HTRF false positive can be due to:

- Auto-fluorescence or quenching of fluorescence
- Compounds acting as fluorescence donors
- Tag/antibody binders
- Common/target specific false positive modalities, e. g. aggregators, denaturants, redox, metal contaminants.

Two methods were used to identify false positives prior to orthogonal testing:

1. HTRF/PD-1/PD-L1 complex was equilibrated for 30 min. Test compounds were then added and the effect was measured at time = 0 min and time = 60 min - compounds binding to the PD-1/PD-L1 interaction surface should show little activity at 0 min due to the time required to reach a new equilibrium.



2. Assessment of the raw emission wavelength (620 nm and 665 nm) for each compound - genuine positives should only affect the 665 nm data.

Results & Discussion

A selection of 5168 BioAscent Library compounds were screened at 20 μM for PD-1/PD-L1 inhibition with 112 compounds producing $> 20\%$ effect relative to controls, which were further screened for potency.

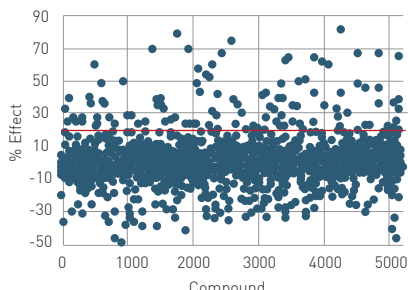


Fig. 2: Scatter plot of all single point screening data [Red line represents 20% selection cut off].

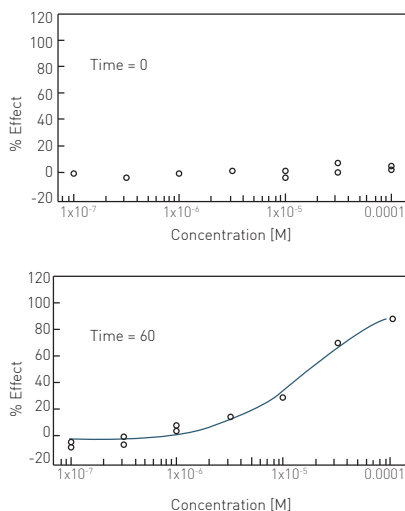


Fig. 3: Example data for genuine positive compound. Little/no activity shown against the pre-formed PD-1/PD-L1 complex at 0 min but developing activity after 60 min and not interfering with 620 nm wavelength. % effect was calculated from the HTRF ratio from minimum (0% = no inhibition) and maximum (100% = complete inhibitor binding) control wells.

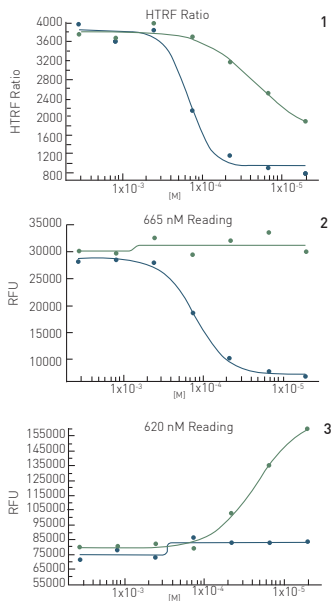


Fig. 4: Example data showing the assessment of analysed [ratio] and raw [620 nm and 665 nm] data. The ratio data [1] shows a dose response for the reference molecule (blue) and compound (green). An increase in the 620 nm reading [665 nm reading stays the same] indicates the compound is interfering with the HTRF technology and is classified as a false positive.

Conclusion

28 of the 112 hit compounds produced a dose response curve and were tested for false positive activity using the two methods as described. Thereof, 5 compounds did not show false positive behaviour and are most likely to genuinely interrupt the PD-1/PD-L1 interaction. The remaining compounds interfered with the HTRF technology and were excluded from further testing.

The 5 most promising compounds identified from this screen were further tested in orthogonal biophysical assays [Differential light scattering and Microscale Thermophoresis] and formed the backbone of a medicinal chemistry synthesis campaign for PD-1/PD-L1 inhibitors.

Acknowledgements

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