An Efficient, Multifaceted Approach to Indoleamine 2, 3-Dioxygenase (IDO1) Hit Profiling

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Introduction

In small molecule drug discovery for intracellular enzyme inhibitors, it is essential to have an informative hit profiling strategy in place that ideally measures target engagement *in situ*. Using inhibitors of the immuno-oncology target indoleamine 2,3-dioxygenase 1 (IDO1) as an illustrative example, we have utilised biophysical, biochemical and cellular approaches to demonstrate a robust paradigm for hit profiling.

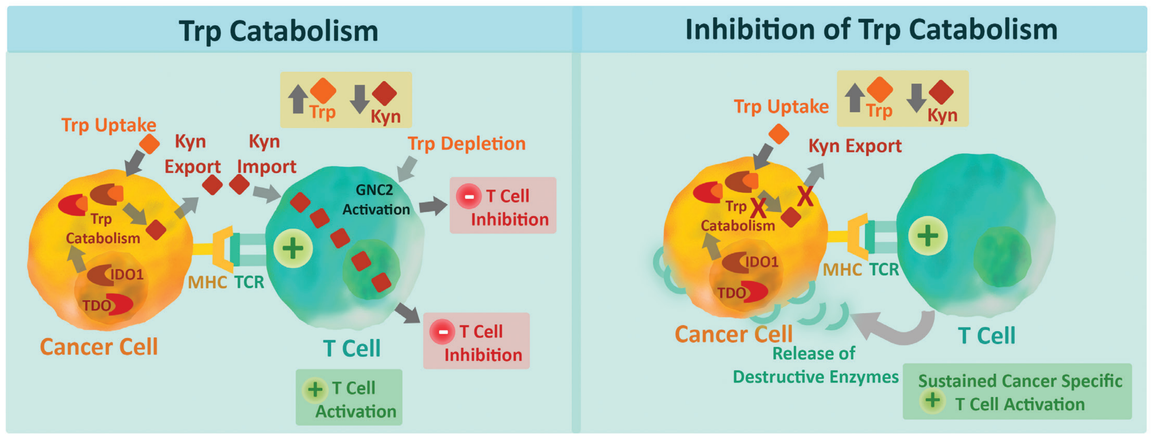
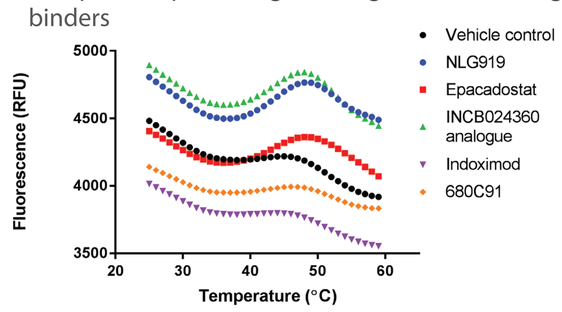


Figure 1: The role of IDO1 and tryptophan 2,3-dioxygenase (TDO) in immune evasion and the potential of IDO1 inhibitors in immuno-oncology (figure taken from BPS Bioscience website with permission).

Fluorescent Thermal Shift Assay (FTSA)

- The environmentally-sensitive fluorescent dye SYPRO orange quantifies the thermal stability of purified protein in the presence and absence of compounds
- Compounds producing a change in the melting temperature (ΔT_m) of the protein are identified as binders



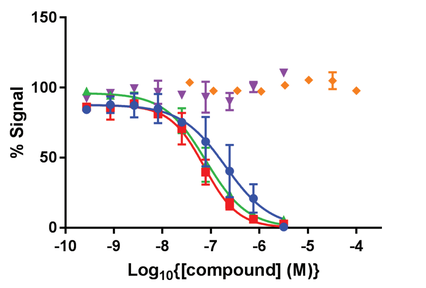
	ΔT_m (°C)
NLG919	1.1 ± 0.2
Epacadostat	1.2 ± 0.1
INCB024360 analogue	0.8 ± 0.4
Indoximod	-0.9 ± 0.1
680C91	0.3 ± 0.2

Figure 4: Effect of IDO1 and TDO inhibitors on the thermal stability of IDO1. Representative melting temperature shifts for 5 μM IDO1 in the presence of vehicle control (0.1 % (v/v) DMSO, black circle), 10 μM NLG919 (blue circle), 10 μM Epacadostat (red square), 10 μM INCB024360 analogue (green triangle), 3 μM Indoximod (pink triangle) and 100 μM 680C91 (orange diamond). Data were analysed by non-linear fitting to a Boltzmann sigmoidal curve where T_m occurs at the midpoint of the unfolding transition. ΔT_m are the mean of two independent experiments (N=2, n=4), errors denote standard deviation of the mean.

- Potent inhibitors result in a positive thermal shift, indicating they are IDO1 binders

Enzyme Assay

A commercially available product detection-based system (BPS Bioscience, 72021) was used to screen known IDO1-specific inhibitors; NLG919 (NewLink), Epacadostat (Incyte), INCB024360 analogue and Indoximod (NewLink) and the TDO-specific inhibitor 680C91 (Wellcome Research Laboratories), to validate this assay format for comparisons with orthogonal approaches.

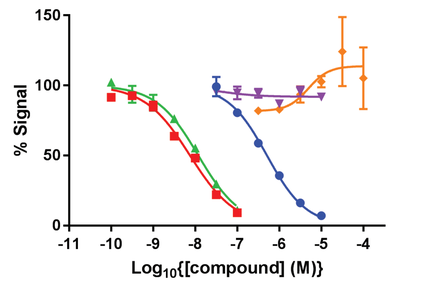


	Enzyme assay IC50 (nM)
NLG919	200
Epacadostat	72
INCB024360 analogue	78
Indoximod	> 3000
680C91	> 100000

Figure 2: Effect of IDO1 and TDO inhibitors on *in vitro* human IDO1 activity. IDO1 and inhibitors were added to L-tryptophan substrate and incubated for 3 hrs at room temperature. Activity was determined by measuring the absorbance of reaction product, N-formylkynurenine, at λ = 322 nm. Data were normalised to high and low controls and fitted with a 4PL equation. Data reflect the mean of two independent experiments (N=2, n=4) with error bars denoting standard deviation.

Cellular Assay

- IDO1 activity measured in SKOV-3 ovarian carcinoma cells using IDO1 cellular activity QuickDetect™ supplements (BPS Bioscience, 62000-1)
- IDO1 constitutively expressed in SKOV-3 cells, IDO2 and TDO expression negligible[1]



	Cellular assay IC_{50} (nM)
NLG919	510
Epacadostat	7.6
INCB024360 analogue	12
Indoximod	> 10,000
680C91	> 100,000

Figure 3: Effect of IDO1 and TDO inhibitors on IDO activity in SKOV-3 cells. Cells were treated with inhibitors and supplements for 24 hrs. Activity was determined by indirect measurement of kynurenine levels by analysing absorbance at λ = 480 nm. Data were normalised to high and low controls and fitted with a 4PL equation. Data reflect the mean of two independent experiments (N=2, n=4) with error bars denoting standard deviation.

- Rank order of compounds matches that observed in the enzyme assay
- Epacadostat and its analogue are more potent in the cellular assay

Cellular Thermal Shift Assay (CETSA®)

- CETSA® involves heating multiple aliquots of cell lysate or intact cells and quantifying the presence of target protein in the soluble fraction by western blotting
- Compound binding to the target alters the proteins thermal stability and is observed as a shift in T_m allowing cellular target engagement to be assessed
- Inhibitor potencies determined via isothermal concentration-responses

Figure 5 : Representative example of western blot to determine quantity of IDO1 in soluble fraction of (A) intact SKOV-3 cells and (B) SKOV-3 lysates in the presence and absence of INCB024360 analogue.

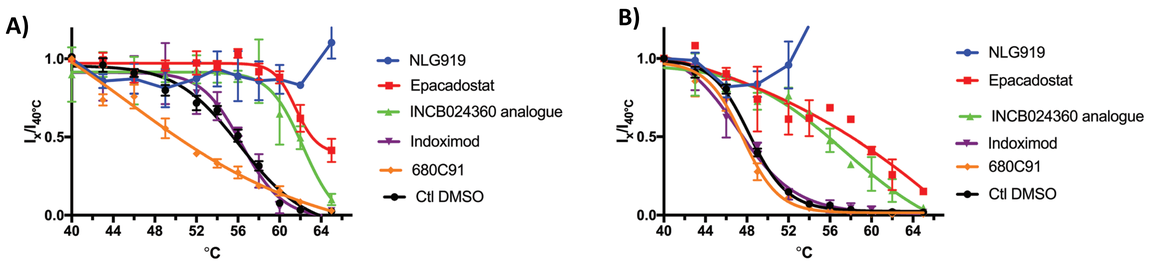


Figure 6 : CETSA® melt curves in (A) intact SKOV-3 cells and (B) SKOV-3 lysates for IDO1 in presence and absence of IDO1 and TDO specific inhibitors.

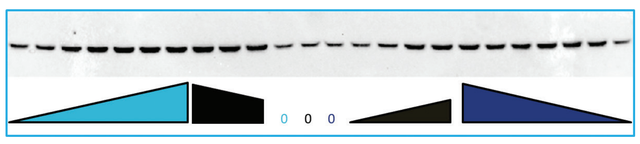
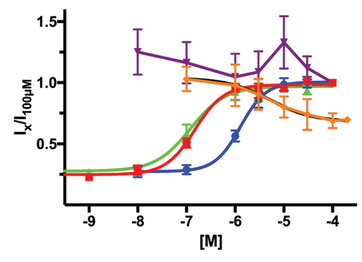


Figure 7 : Representative example of western blot used to determine potency of INCB204360 analogue in intact SKOV-3 cells. The western blot depicts one biological experiment (N) with three technical repeats (n) of increasing ligand concentration per membrane. 0 = DMSO control.



	CETSA intact EC_{50} (nM)
NLG919	1300
Epacadostat	100
INCB024360 analogue	100
Indoximod	> 100,000
680C91	5200

Figure 8 : Isothermal concentration-responses of IDO1 and TDO specific inhibitors in intact SKOV-3 cells. Concentration-responses performed at 58°C for IDO1 inhibitors and 52°C for the TDO inhibitor 680C91

- Rank order of compounds matches those observed in the enzyme and cellular assays

References

1) Litzenburger, U. M. et al. (2014). *Oncotarget*, **5** (4): 1038-1051. 2) Hou, D. Y. et al. (2007). *Cancer Res*. **67**(2): 792-80. 3) Yue, E. W. et al. (2009). *J. Med. Chem.*, **52** (23): 7364-7367. 4) Liu, X. et al. (2010). *Blood*, **115** (17): 3520-3530. 5) Chen, Y. et al. (2016). *Nat. commun*. **7**, 13443

Acknowledgements

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Summary

- Qualitative agreement between tailored set of techniques with rank order of compounds matching those observed in the literature[2,3,4,5]
- Correlation of target engagement and efficacy adds value to hit identification, however, to fully understand a compound's pharmacology a tailored screening cascade is required