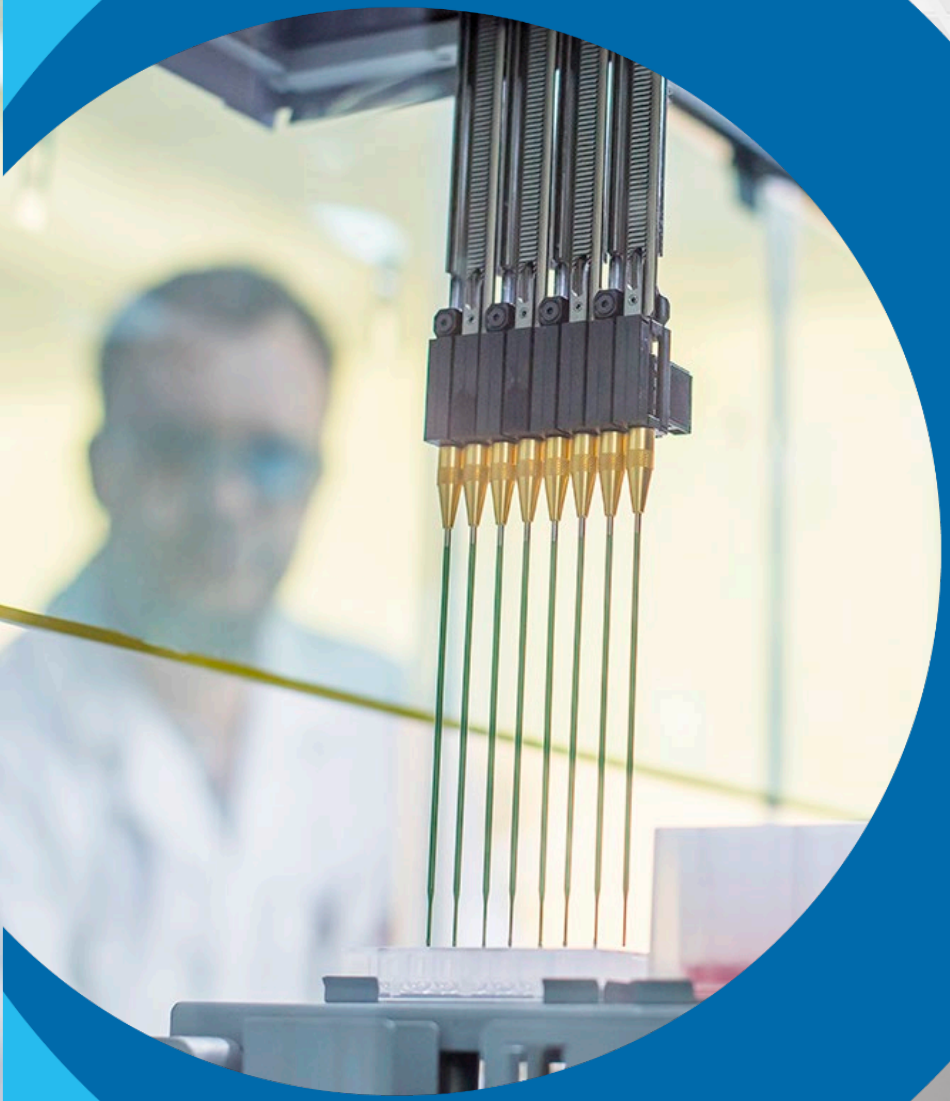


SYGNATURE DISCOVERY

Smarter IDO inhibitor profiling using biophysical, biochemical and cellular target engagement approaches

Dr Scott Pollack

Enabling Success

www.sygnaturediscovery.com

Sygnature Group Overview

- **Sygnature Discovery** was founded in 2004 in BioCity, Nottingham
- Provides high quality integrated or single discipline drug discovery support to Pharma, Biotech and NFP organisations
- Strong track record in drug discovery
  - 14 compounds delivered into the clinic (Phases I and II) since 2011
  - 13 other compounds into pre-clinical development
- 240 staff
  - 80% of scientists have PhDs
  - Considerable pharmaceutical industry R&D experience
- Private equity-backed company since September 2017
  - Senior management team are co-investors
  - Financially stable
  - Investment to fund expansion of capabilities & capacity

- **RenaSci** was founded in BioCity, Nottingham in 2001 and acquired by Sygnature in 2018
- Highly experienced team provide an integrated blend of consultancy and pre-clinical experimental services to Pharma, Biotech and NFP organisations
- Provide *in vivo* testing in drug abuse and dependence, CNS, obesity, NASH, and diabetes and its complications
- Collaborate with clients at all stages of the drug discovery and development process - from target identification to post registration
- Have facilitated more than 30 NCEs into clinical development and 10 drugs to the market

- **Peak Proteins** is an affiliate company and strategic partner for protein production and crystallography
  - Highly experienced team (Ex-AZ) with excellent track record in X-ray structure determination and protein biochemistry
  - Protein expression in a range of systems to 20 litre scale
  - Based in BioHub (Alderley Park)

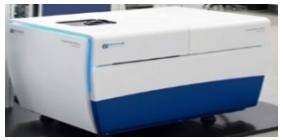
Sygnature Drug Discovery Scope

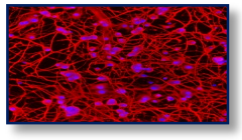
Target Validation	Hit Identification	Hit to Lead	Lead Optimisation	Translational Biology
Reproducing key literature experiments to confirm isolated reports	Fragment-based screening: SPR, MTS, NMR, X-ray Crystallography; Sygnature Fragment Library	Hit Validation and ADMET profiling	Early DMPK profiling *in-vitro* and *in-vivo*	Biomarker validation Gene Expression, Phosphoproteins, Cytokines, Neurotransmitters, Brain Microdialysis
Demonstration of target engagements and mechanism of action	HTS: Radiometric, Biochemical and Biophysical reads	High Throughput Chemistry Structure-based drug discovery	Efficient "Design-Make-Test-Evaluate" cycles	Robust *in vivo* pharmacological models
	MTS: Biochemical, Biophysical or Phenotypic/High content imaging	Fast Follower – Knowledge based approaches	Innovative chemistry to enable synthesis of complex target molecules	
	Virtual Screening: Commercial and Sygnature HTC enabled virtual libraries	Ligand based – scaffold hopping (Brood, Sygnature scaffolds)	*In-vivo* efficacy, PK/PD and dose in man predictions	

Tailored hit Identification and characterisation

- Development of a robust screening cascade, including orthogonal assays to ensure ‘true’ hits are identified
- Assay development via careful evaluation of potential parameters that may affect assay performance and data integrity
- Wealth of experience across a range of target classes and assay formats
- In depth mechanistic characterization via classical enzymological methods and biophysical characterisation

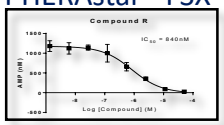
Multiple high-throughput Assay Readouts

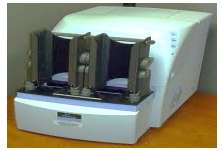




ImageXpress® Micro - Confocal

PHERAstar® FSX





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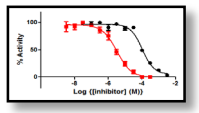
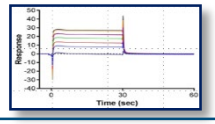
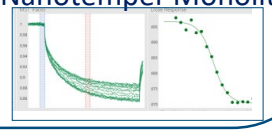


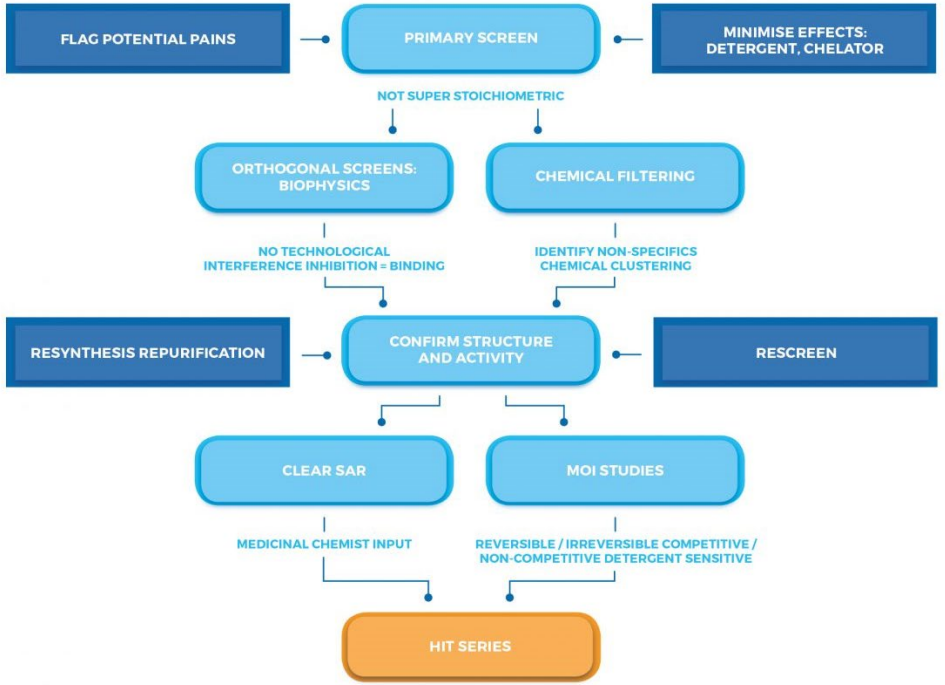
Biacore™ 8K



Nanotemper Monolith

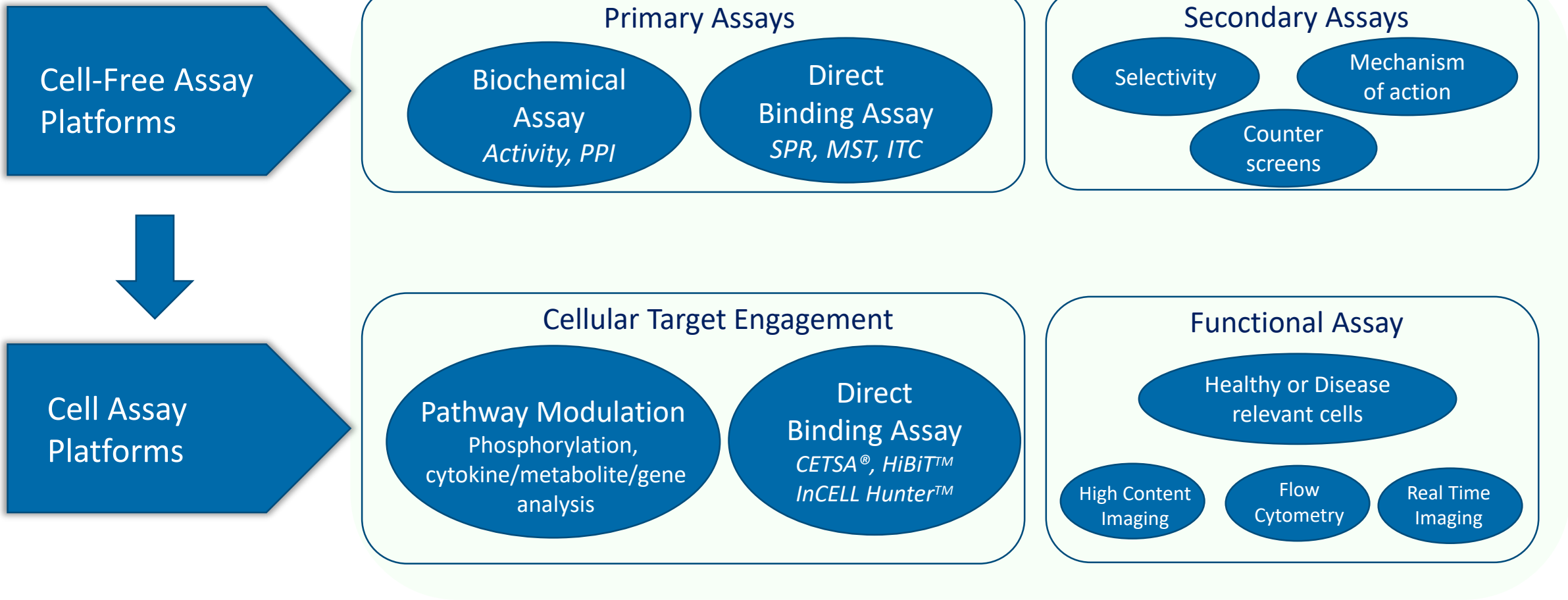


Tailored and flexible screening cascade



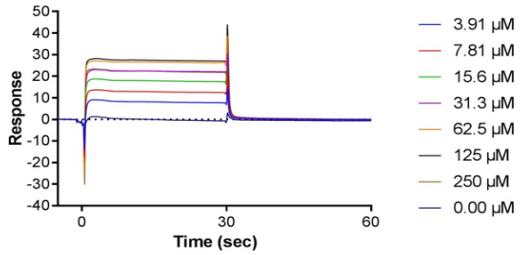
Intelligent screening cascade design

Inclusion and order of these steps depends on target and remit of screen

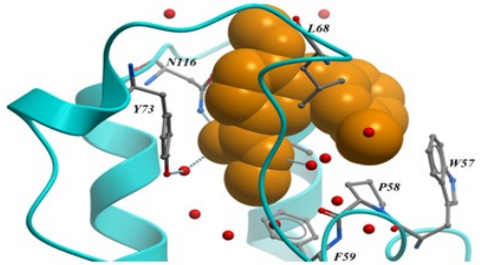


Target Engagement Supported by Multiple Biophysical Approaches

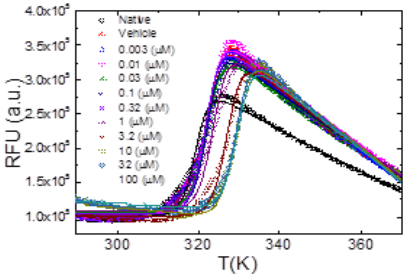
Sygnature has expertise in probing target engagement using a wide variety of different complementary biophysical assay techniques



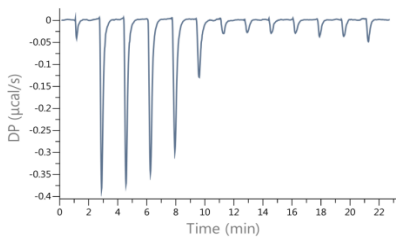
Surface Plasmon Resonance



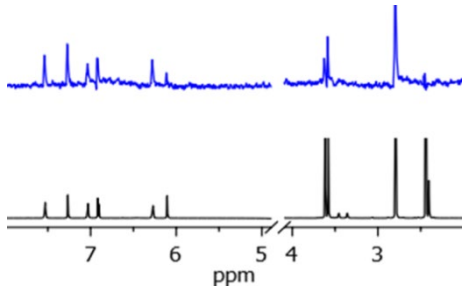
Crystallography



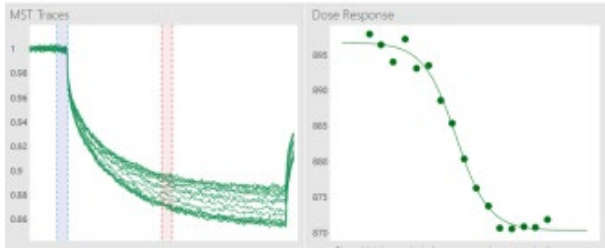
Fluorescent Thermal Shift Assays



Isothermal Titration Calorimetry



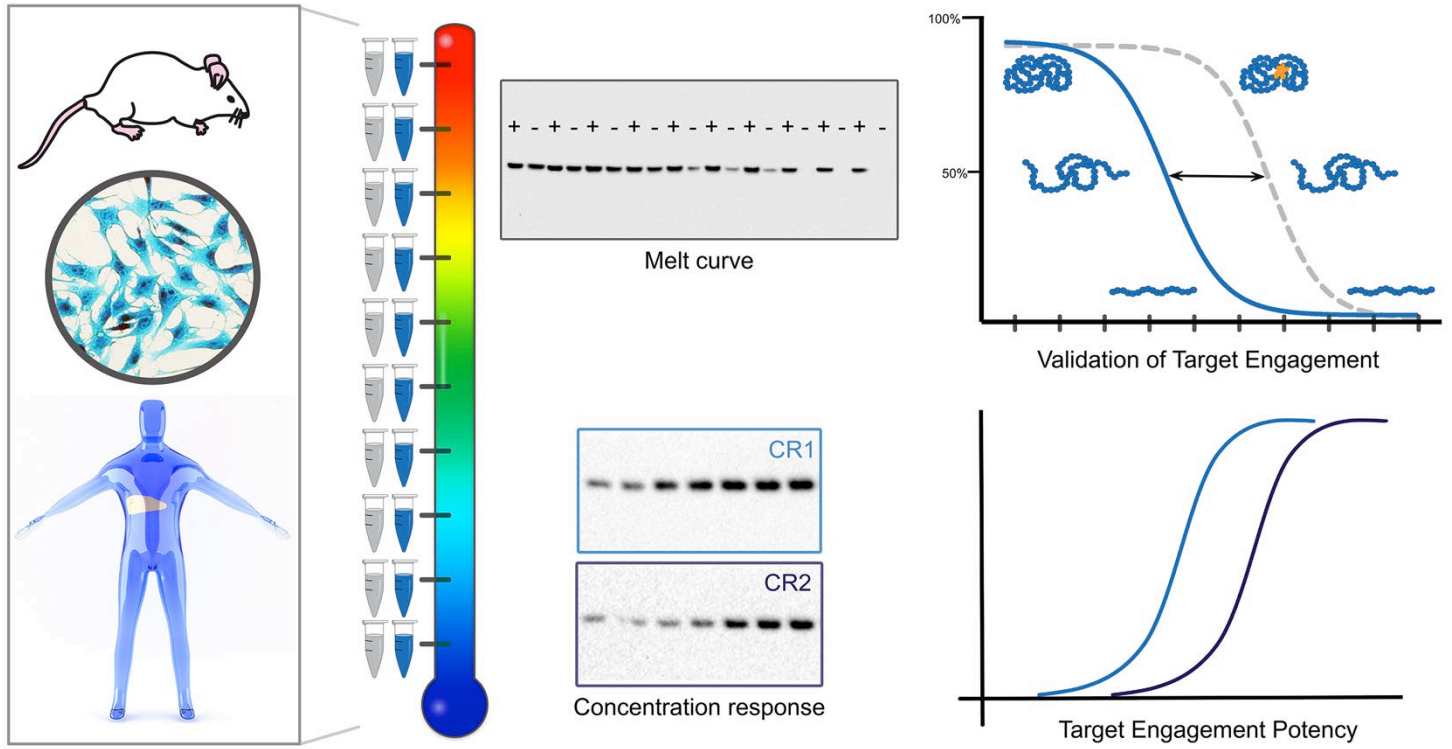
NMR



Microscale thermophoresis

Target Engagement Supported by strategic collaboration with Pelago Biosciences

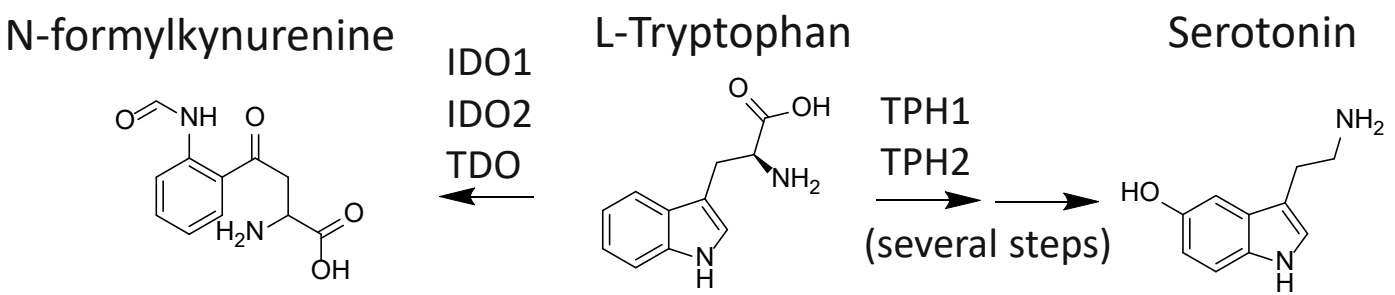
CETSA® (Cellular thermal shift assay): A powerful approach to assess cellular target engagement

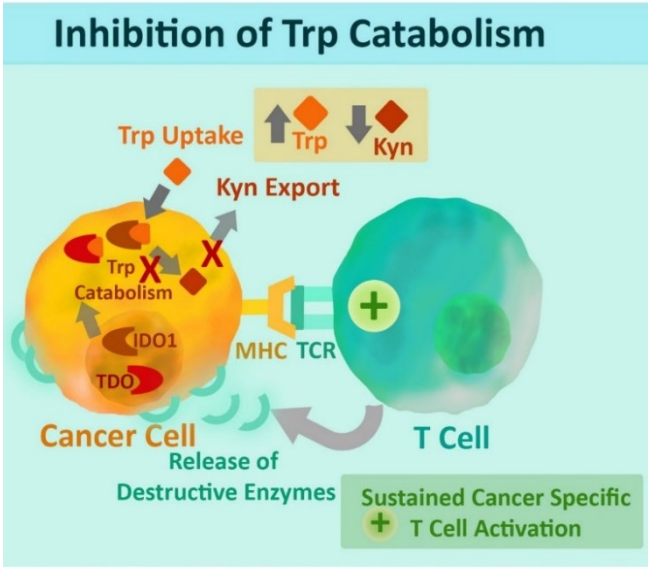


Science
AAAS

Monitoring Drug Target Engagement in Cells and Tissues Using the Cellular Thermal Shift Assay
Daniel Martinez Molina *et al.*
Science **341**, 84 (2013);
DOI: 10.1126/science.1233606

Indoleamine 2,3-dioxygenase 1 (IDO1) in the Tryptophan Catabolism Pathway





- IDO1 and Tryptophan 2,3-dioxygenase (TDO) play key roles in tryptophan catabolism
- IDO1 expression in some cancers leads to Trp depletion and kynurenine accumulation
- This combination leads to immunosuppression
- By enhancing T cell activation IDO1 inhibitors are a potential immuno-oncology target
- **IDO is a good test case for evaluating assays relevant to a robust screening cascade including biochemical and cellular target engagement**

Figures adapted with permission from BPS Bioscience

IDO1 as an illustrative target for a smart drug discovery hit finding approach

- Small molecule drug discovery requires an efficient hit profiling strategy
- Important to assess several approaches including cellular target engagement as part of that strategy

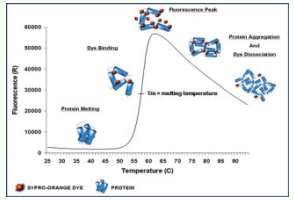
Cell-Free Assay Platforms

Primary Assays

Biochemical Assay
IDO activity assay

IDO
TRP → KYN
⊤
INH

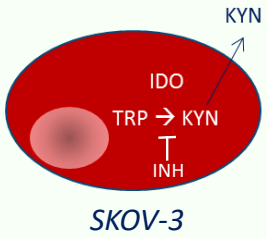
Direct Binding Assay
Thermal Shift



Cell Assay Platforms

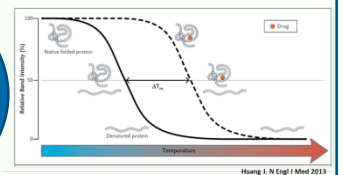
Cellular Target Engagement

Product detection
Cellular Kynurenine secretion

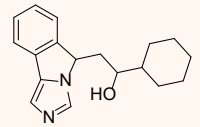


SKOV-3

CETSA
Direct measure of target binding

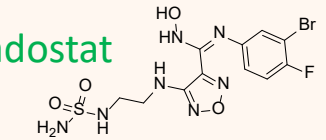


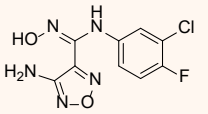
Tool Compounds



NLG919

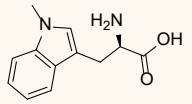
Epacadostat

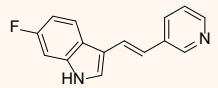




INCB024360 analogue

Indoximod

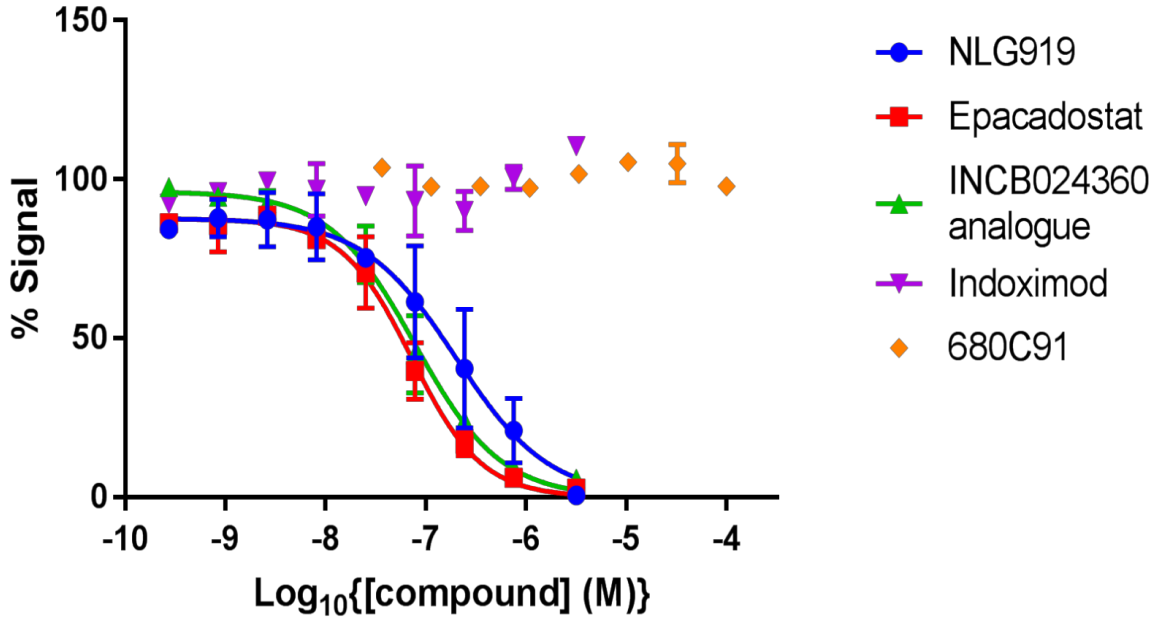




680C91 (TDO)

IDO1 enzyme activity assay

- Biochemical assay for enzyme activity
- Commercially-available product detection based system (BPS Bioscience)



Compound	Enzyme activity assay IC_{50} (nM)
NLG919	200
Epacadostat (INCB024360)	72
INCB024360 analogue	78
Indoximod	> 3000
680C91	> 100,000

- Inhibitors showed expected ranking of potency and selectivity

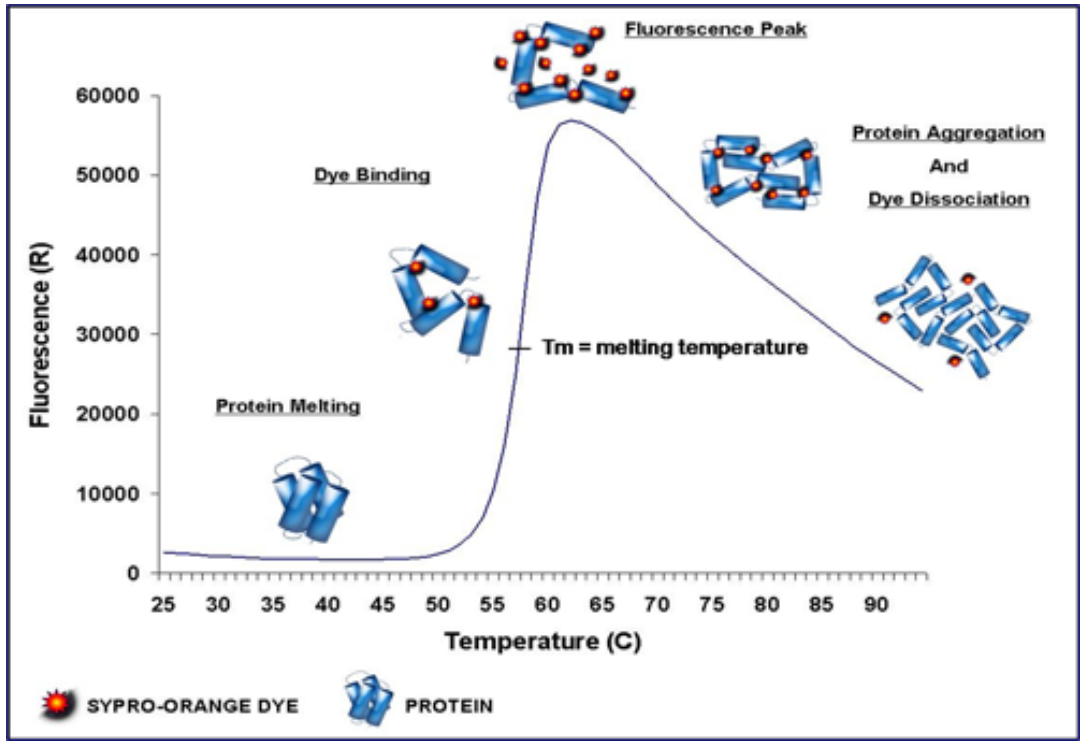
Activity determined by measuring the absorbance of reaction product, N-formyl kynurenine, at λ = 322 nm
Mean of 2 independent experiments (N=2, n=4)

Biophysical assay for target engagement

- Detection of direct interaction of IDO1 with inhibitors by fluorescence thermal shift (FTSA)

Fluorescence thermal shift assays (FTSA)

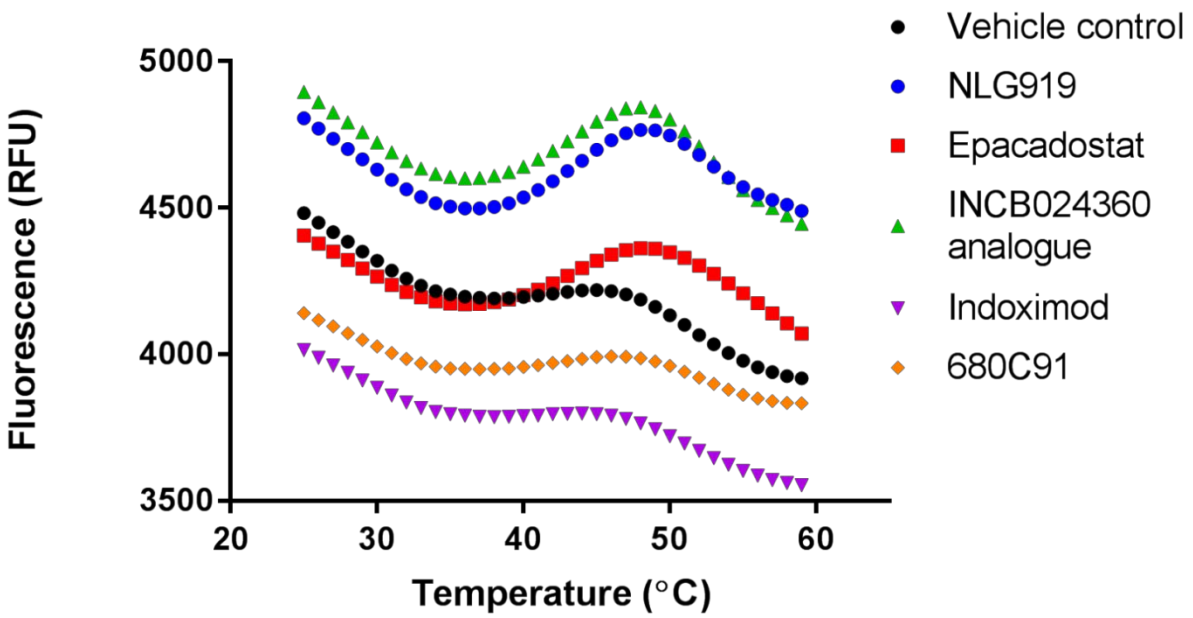
- When a solution of protein is exposed to a temperature gradient the protein will melt at a specific temperature
- This can be measured with an environmentally sensitive dye
- Other agents in solution which interact with the protein will change the temperature required to melt the protein (T_m)
- By understanding the effects upon melting temperature, we can determine ligand binding mechanism and affinity



Ref.: http://www.bio.anl.gov/molecular_and_systems_biology/Sensor/sensor_images/assay_theory_figure.png

Biophysical assay – Thermal shift (FTSA)

- FTSA is a straightforward method that quantifies T_M shifts (ΔT_M) from which affinities can be calculated*



Mean of 2 independent experiments (each n=4) ± SD

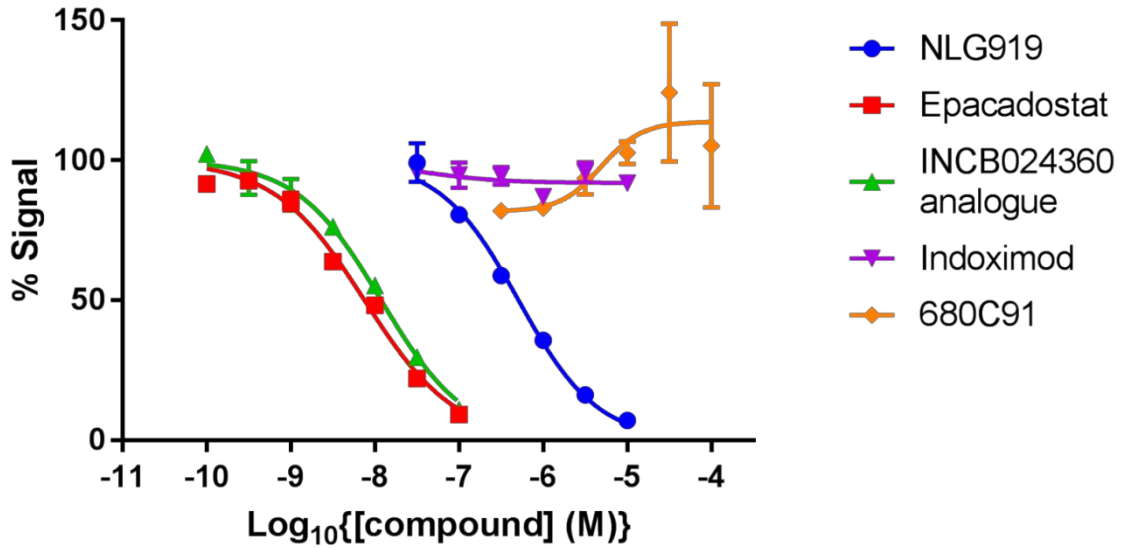
Compound	ΔT_m (°C)	Active?
NLG919	1.1 ± 0.2	+
Epacadostat (INCB024360)	1.2 ± 0.1	+
INCB024360 analogue	0.8 ± 0.4	+
Indoximod	-0.9 ± 0.1	-
680C91	0.3 ± 0.2	-

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

*Redhead, M. *et al.*, 2017, Biochemistry 56:6187-6199

Cellular IDO1 activity assay

- Cellular IDO1 activity measured in SKOV-3 ovarian carcinoma cells
- Express IDO1 constitutively but negligible expression of IDO2 and TDO*
- Commercially-available product detection based system (BPS Bioscience)



Activity was determined by measuring the absorption of kynurenine at λ = 480 nm
Mean of 2 independent experiments (each n=4)

*Litzenburger, U. M. *et al.* 2014, Oncotarget 5:1038-1051

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

- Rank order of compounds matches that observed in the enzyme assay
- Epacadostat and its analogue more potent in cellular assay
  - Could be due to differences in MOI, cellular access, IDO1 protein, substrate conc., assay time frame and conditions

CETSA® MELT AND SHIFT CURVE IN INTACT CELLS

- Assay also undertaken with compound and heat treatment of lysed cells

Viability determined

Cell suspension + cmpd. or vehicle

Detach intact whole cells
Mix equal volumes 2X compound/DMSO and 2X cells

↓ Incubation: 60 min 37 °C

Viability determined

Heat Treatment

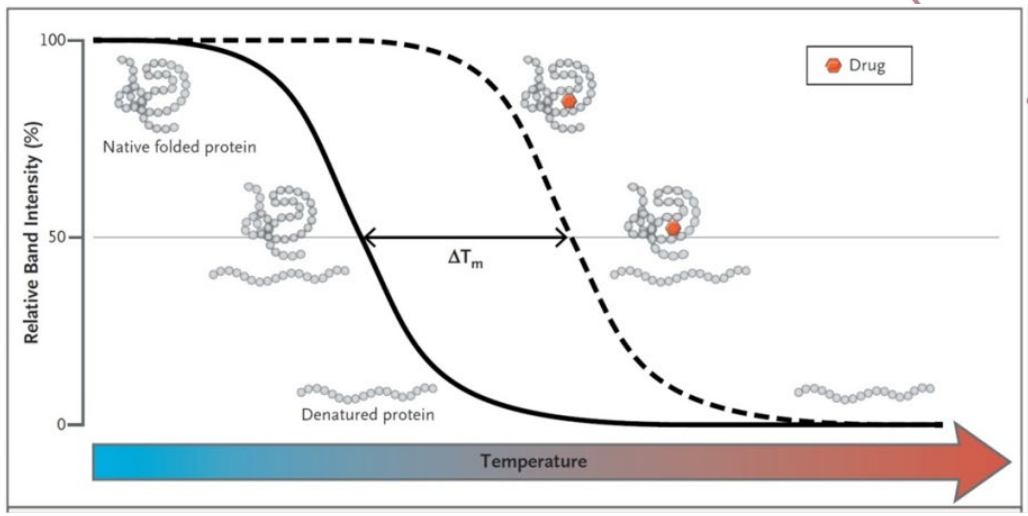
Aliquot cell suspension/cmpd mix
Each aliquot heated to a single temperature of a temperature gradient

↓

Cell Lysis — 3X freeze thawing

↓ Separation: 20,000 x g 20 min

Detection of soluble proteins by Western Blot

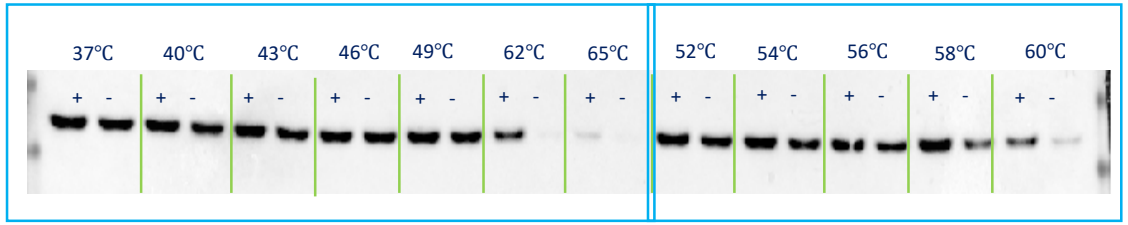


Huang J. N Engl J Med 2013

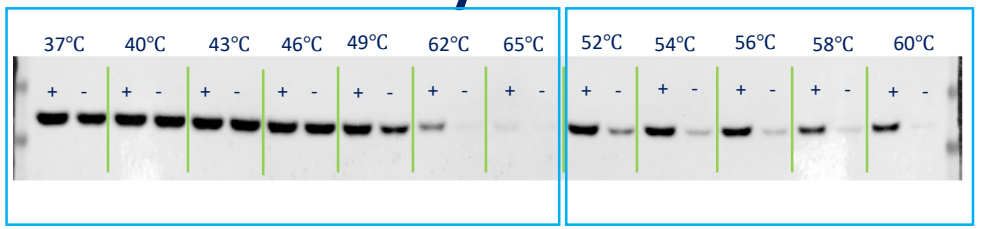
Cellular thermal shift assays (CETSA®)

- Detection of target engagement of IDO1 with inhibitors in intact cells and cell lysates

A) **Intact cells**



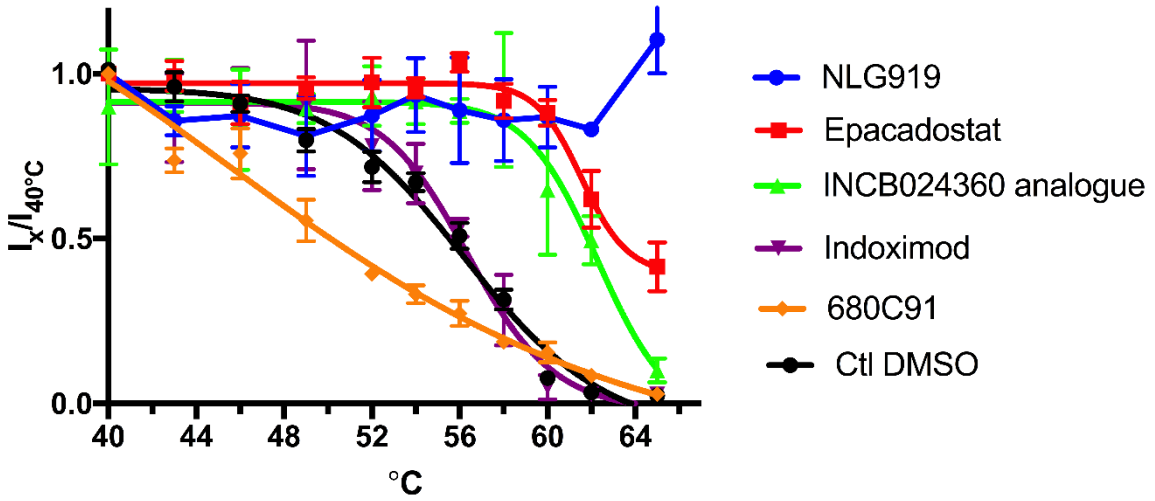
B) **Cell lysates**



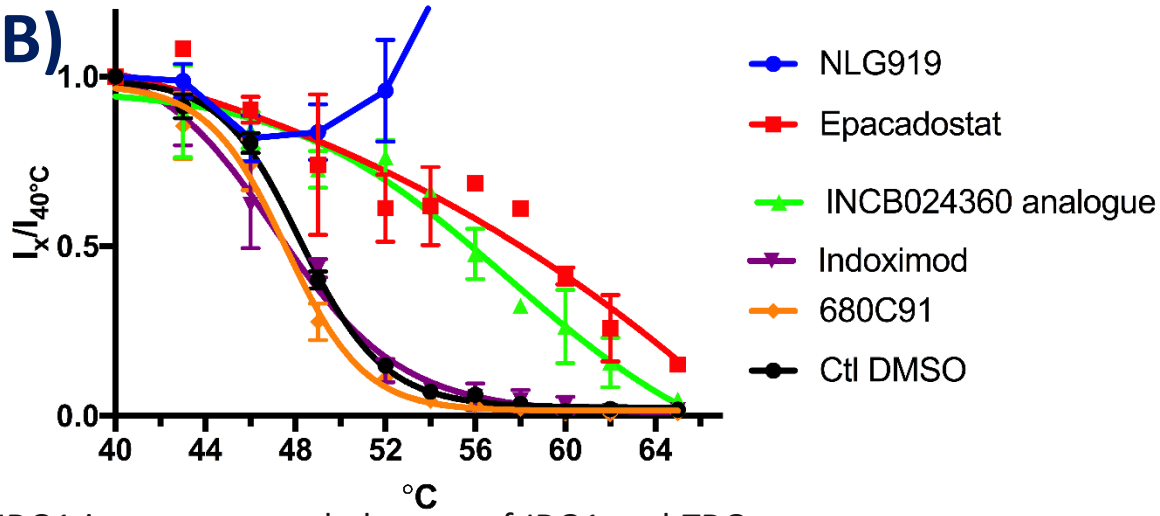
Representative Western blots to determine quantity of IDO1 in soluble fraction of intact SKOV-3 cells (A) and SKOV-3 lysates (B) in the presence and absence of INCB024360 analogue

- Similar compound trends observed in whole cells and lysates

A)



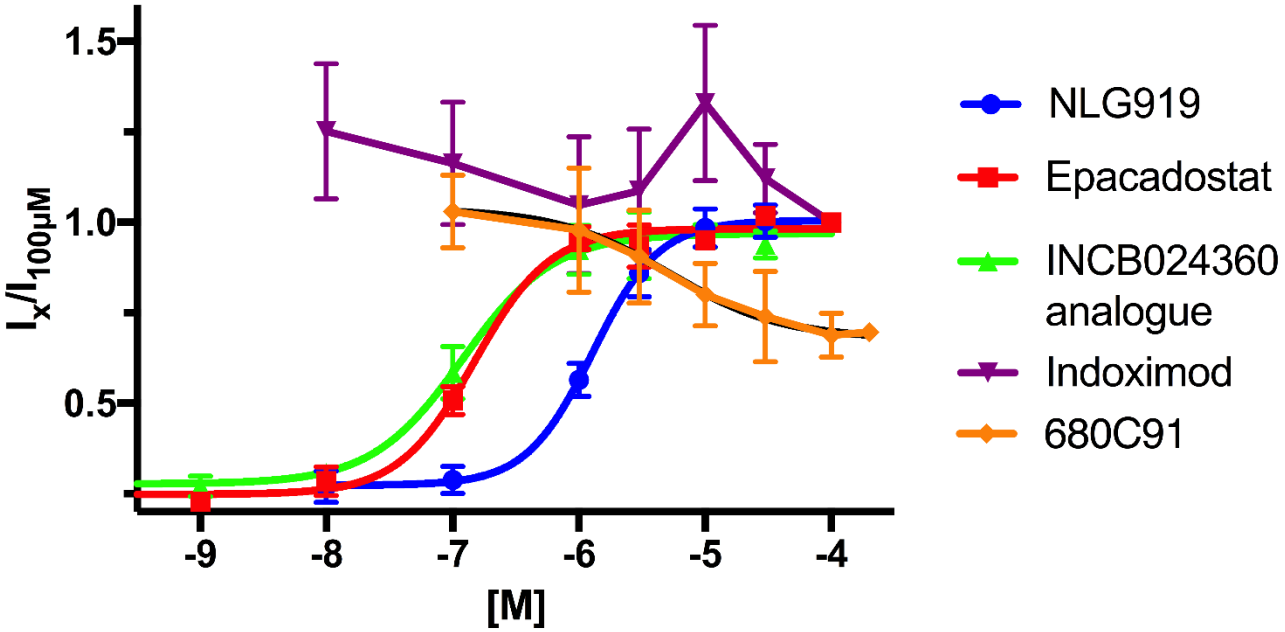
B)



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

Cellular thermal shift assays (CETSA®)

- Focusing on optimal temperature for analysis (58 °C)



IDO1 in intact SK-OV-3 cells, concentration-response at 58 °C (N=3, n=9), (52 °C for 680C91 N=2, n=6)
*Destabilisation observed with 680C91

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

- Rank order of compounds matches that observed in the enzyme activity assay

Overall results - summary

- Rank order of compound potencies generally consistent among all 4 assays
- Cellular target engagement via CETSA in good agreement with enzyme assay
- Thermal shift agrees qualitatively with other assays but may not be robust for this target
- Specific differences in potency between biochemical and cellular activity assay
  - Could be due to differences in MOI, cellular access, IDO1 protein, substrate conc., assay time frame and conditions

Compound	Enzyme IC_{50} (nM)	Cell activity IC_{50} (nM)	FTSA Active ?	CETSA EC_{50} (nM)
NLG919	200	510	+	1300
Epacadostat (INCB024360)	72	7.6	+	100
INCB024360 analogue	78	12	+	100
Indoximod	Inactive	Inactive	-	Inactive
680C91 (TDO inhibitor)	Inactive	Inactive	-	5200*

*Destabilisation with 680C91

Conclusions

- Small molecule drug discovery approaches require efficient hit identification strategy
- Important to assess cellular target engagement (e.g., CETSA) as part of that strategy
- IDO provided a useful example of applying this approach
- Qualitative agreement observed among tailored set of techniques (4 assays)
  - Rank order of potencies match literature values
- No single technique is sufficient to fully interpret the relationship between enzyme and cellular IDO pharmacology

→ This example with IDO illustrates Sygnature's tailored approach to rigorous hit identification cascade design and demonstrates that the ideal screening/profiling cascade should be suited to the target

Acknowledgements

Sygnature Discovery Team

Chris Tomlinson (see poster 103)
Rupert Satchell

Pelago Biosciences

Daniel Martinez
Michael Dabrowski
Jakob Karén

Kris Clark – Poster 22 on Immuno-oncology assay platforms
Jamie Patient – Poster 115 on strategies in drug discovery toxicology

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