Monolith Protocol MO-P-038

IDO1 – NLG919 Analogue

IDO1 (Indoleamine 2,3-dioxygenase 1) is a monomeric heme-containing dioxygenase that catalyzes the oxidative cleavage of L-Tryptophan. IDO1 shows elevated expression in some human cancers and is thought to play a role in the tumor immuno-editing process which sets up peripheral tolerance to tumor antigens. It has therefore gained interest as a drug target in cancer therapy, but no inhibitor has advanced to a drug so far. The interaction described in this protocol has been used as a positive control to validate fragments from a screening approach to identify new leads for IDO1 inhibitors.

protein – small molecule interaction | fragment screening

A1. Target/Fluorescent Molecule

Indoleamine 2,3-dioxygenase 1
uniprot.org/uniprot/P14902

A2. Molecule Class/Organism

Dioxygenase
Homo sapiens (Human)

A3. Sequence/Formula

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MAHAMENSWT ISKEYHIDEE VGFALPNPQE NLPDFYNDWM FIAKHLPDLI ESGQLRERVE KLNMLSIDHL TDHKSQRLAR
LVLGCITMAY VWGKGHGDVR KVLPRNIAVP YCQLSKKLEL PPILVYADCV LANWKKKDPN KPLTYENMDV LFSFRDGDCS
KGFFLVSLLV EIAAASAIKV IPTVFKAMQM QERDTLLKAL LEIASCLEKA LQVFHQIHDH VNPKAFFSVL RIYLSGWKGN
PQLSDGLVYE GFWEDPKEFA GGSAGQSSVF QCFDVLLGIQ QTAGGGHAAQ FLQDMRRYMP PAHRNFLCSL ESNPSVREFV
LSKGDAGLRE AYDACVKALV SLRSYHLQIV TKYILIPASQ QPKENKTSED PSKLEAKGTG GTDLMNFLKT VRSTTEKSLL
KEG
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A4. Purification Strategy/Source

Enzyme cloned from human cDNA, expressed in E. coli
Giotto Biotech (Giotto Biotech S.r.l., Firenze)

A5. Stock Concentration/Stock Buffer

1 mg/mL | 20 µM
20 mM Tris-HCl, pH 8.0, 100 mM NaCl

A6. Molecular Weight/Extinction Coefficient

45.3 kDa
51,380 $M^{-1}cm^{-1}$ (ε_{280})

A7. Dilution Buffer

50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 2 mM DTT, 0.05% TWEEN® 20

A8. Labeling Strategy

Monolith Protein Labeling Kit RED – NHS (MO-L001, NanoTemper Technologies GmbH)
1* Labeling Buffer NHS | 1* A-Column | 1* Dye RED-NHS 2nd Generation (10 µg) | 1* B-Column

A9. Labeling Procedure

1. Add 50 µL of Labeling Buffer NHS containing 0.05% TWEEN® 20 to 50 µL of 20 µM IDO1 to obtain 100 µL of a 10 µM solution.
2. Use the A-Column to perform a buffer exchange into Labeling Buffer NHS.
   a. Invert A-Column to suspend slurry and twist off bottom (twist slightly in both directions).
   b. Loosen the cap of the column and place it in a 1.5 mL microcentrifuge collection tube.
   c. Centrifuge at **1500 × g** for **1 min** to remove excess liquid.
   d. Add 300 µL of Labeling Buffer NHS and centrifuge at **1500 × g** for **1 min** (3x).
   e. Place 100 µL of the 10 µM IDO1 solution in the center of the resin.
   f. Place the sample in a **new** microcentrifuge collection tube and centrifuge at **1500 × g** for **2 min**.

   The collected flow-through should yield around 100 µL of ~7.5 µM IDO1 (70 – 80% recovery).
3. Add 30 µL of DMSO to 10 µg RED-NHS dye to obtain a ~470 µM solution. Mix the dye thoroughly by vortexing and make sure that all dye is dissolved.
4. Mix 8.5 µL of the 470 µM dye solution with 91.5 µL of Labeling Buffer NHS to obtain 100 µL of a 39.8 µM dye solution (5x protein concentration).
5. Mix IDO1 and dye in a 1:1 volume ratio (200 µL final volume, 5.8% final DMSO concentration).
6. Incubate for 30 minutes at room temperature in the dark.
7. In the meantime, remove the top cap of the B-Column and pour off the storage solution. Remove the bottom cap and place with adapter in a 15 mL tube.
8. Fill the column with dilution buffer and allow it to enter the packed resin bed completely by gravity flow. Discard the flow through collected. Repeat this step 3 more times.
9. Add 200 µL of the labeling reaction from step 5 to the center of the column and let sample enter the bed completely.
10. Add 300 µL of dilution buffer after the sample has entered and discard the flow through.
11. Add 600 µL of dilution buffer and collect the eluate discarding the first 3 drops (~2 µM).

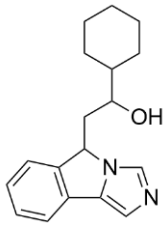
A10. Labeling Efficiency

Measurement of protein concentration and degree of labeling (DOL) using a UV-VIS-NIR JASCO V570 Spectrophotometer of JASCO Inc. (Easton, Maryland, USA):
nanotempertech.com/dol-calculator

Absorbance A_{280}	0.01193	Protein concentration	2.033 µM
Absorbance A_{650}	0.01193	Degree-of-labeling (DOL)	1.04

B1. Ligand/Non-Fluorescent Binding Partner

NLG919 Analogue



B2. Molecule Class/Organism

Small molecule fragment / IDO1 inhibitor

B3. Sequence/Formula

$C_{18}H_{22}N_2O$

B4. Purification Strategy/Source

Selleckchem.com
S7111

B5. Stock Concentration/Stock Buffer

14.1 mg/mL | 50 mM
DMSO

B6. Molecular Weight/Extinction Coefficient

282.4 Da

B7. Serial Dilution Preparation

1. Add 8 µL of DMSO to 2 µL of the NLG919 Analogue stock to obtain 10 µL of a 10 mM solution.
2. Mix 2 µL of the 10 mM NLG919 Analogue solution with 48 µL of dilution buffer to obtain 50 µL of a 400 µM NLG919 Analogue solution.
3. Mix 8 µL of DMSO with 192 µL of dilution buffer to obtain 200 µL of a 4% DMSO solution.
4. Prepare a PCR-rack with 16 PCR tubes. Transfer 20 µL of the 400 µM NLG919 Analogue solution into tube **1**. Then, transfer 10 µL of the 4% DMSO solution into tubes **2** to **16**.
5. Prepare a 1:1 serial dilution by transferring 10 µL from tube to tube. Mix carefully by pipetting up and down. Remember to discard 10 µL from tube **16** to get an equal volume of 10 µL for all samples.
6. Mix 9 µL of labeled IDO1 with 191 µL of dilution buffer to obtain 200 µL of ~90 nM IDO1.
7. Add 10 µL of ~90 nM labeled IDO1 to each tube from **16** to **1** and mix by pipetting.
8. Incubate for 10 minutes at room temperature in the dark before loading capillaries.

C. Applied Quality Checks

Validation of structural integrity of IDO1 using circular dichroism[1]:

Element	Sample	t = 0'	t = 30'	t = 60'	t = 90'	t = 120'
α-helix	rhIDO1	28.6%	28.6%	27.2%	27.1%	26.7%
	NT647-rhIDO1	24.5%	24.3%	24.7%	24.0%	24.1%
Antiparallel β-sheet	rhIDO1	12.7%	12%	13.7%	14.1%	14.5%
	NT647-rhIDO1	15.3%	15.4%	15.0%	15.7%	15.8%
Parallel β-sheet	rhIDO1	8.9%	8.9%	9.0%	9.0%	9.0%
	NT647-rhIDO1	9.4%	9.4%	9.3%	9.4%	9.3%
β-turn	rhIDO1	17.5%	17.5%	17.5%	17.5%	17.6%
	NT647-rhIDO1	17.1%	17.2%	17.2%	17.1%	17.2%
Random coil	rhIDO1	32.3%	32.2%	32.6%	32.3%	32.2%
	NT647-rhIDO1	33.7%	33.7%	33.8%	33.8%	33.6%
Total	rhIDO1	100%	100%	100%	100%	100%
	NT647-rhIDO1	100%	100%	100%	100%	100%

D1. MST System/Capillaries

Monolith NT.115 Red (NanoTemper Technologies GmbH)
Premium Capillaries Monolith NT.115 (MO-K025, NanoTemper Technologies GmbH)

D2. MST Software

MO.Affinity Analysis v2.1 (NanoTemper Technologies GmbH)

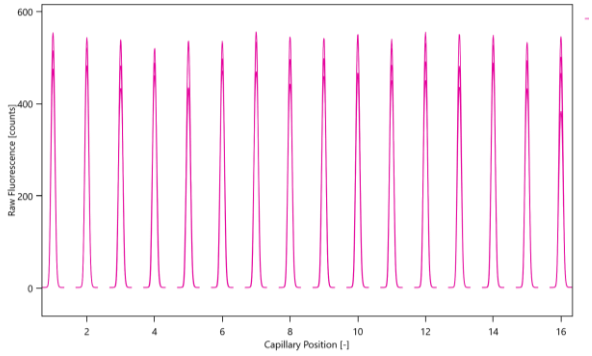
D3. MST Experiment (Assay Buffer/Concentrations/Temperature/MST Power/Excitation Power)

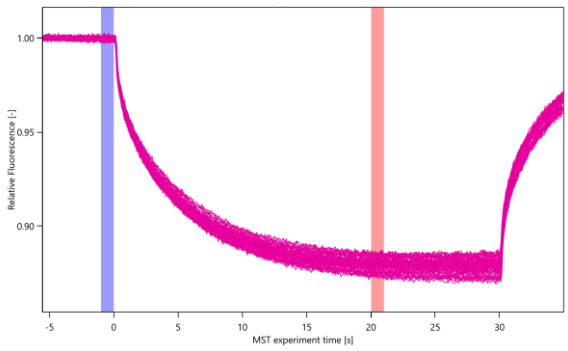
50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 2 mM DTT, 0.05% TWEEN® 20, 2% DMSO
45 nM IDO1 | 200 µM – 6.1 nM NLG919 Analogue | RT (~23°C) | medium MST power | 20% excitation power

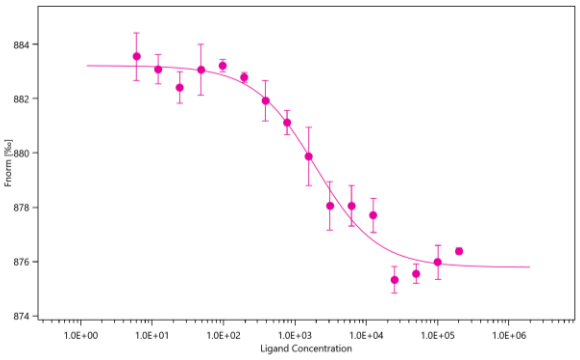
[1] The stability of NT647-rhIDO1 and unmodified rhIDO1 protein was checked using circular dichroism. The spectra of both proteins were recorded using a Jasco810 spectrophotometer with 1 mm path-length quartz cuvettes at room temperature (~22°C). The sensitivity was 100 millidegrees and the scanning speed was 20 nm/min for an accumulation of 2 scans. CD data was collected between 180 and 260 nm for both samples at a concentration of 0.1 mg/mL in phosphate buffer (PPB; 50 mM K_2HPO_4, pH 7.4). The deconvolution of the spectra was performed with CDNN 2.1 software.

D4. MST Results (Capillary Scan/Time Traces/Dose Response)

K_d = 3.3 ± 0.4 µM Coletti et al., Eur J Med Chem. 141, 169-177 (2017)







D5. Reference Results/Supporting Results

EC_{50} = 75 nM Cell-based activity assay

Mautino et al., Proceedings: AACR 104th Annual Meeting 2013; Apr 6-10, 2013

E. Contributors

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