Determining the IC50 of M4K1062 with ACVR1/ALK2-NanoLuc and different concentrations of Tracer-6908

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Background:

The EC50 of Tracer-6908 with ACVR1-c-nanoLuc and ideal conditions for the target engagement assay have been determined in the previous post. However, it is still necessary to verify that the IC50 values determined in the assay are closed enough approximation to the actual IC50 values of the compounds. If the IC50 values are strongly influenced by the concentration of Tracer-6908 used, multiple assays with progressively lower Tracer-6908 will need to be performed. The actual IC50 of the compounds can then be estimated by plotting the Tracer-6908 concentrations against the corresponding IC50 values and regressing to no Tracer-6908. To address this, multiple nanoBRET target engagement assays were setup with increasing concentrations of Tracer-6908 with the same serial dilution of M4K1962 (starting from the EC50 of 65nM).

Protocol:

Recipes:

Cell Culture Medium
90% DMEM (Life Technologies 11995)
10% FBS (HyClone SH30070.03)

Assay Medium
Opti-MEM WITHOUT PHENOL RED 100% (Life Technologies 11058)

NanoBRET Tracer Dilution Buffer
12.5mM HEPES, pH7.5 (Sigma H0887)
31.25% PEG-400 (Sigma 91893)

FuGENE® HD (Promega E2311)
Transfection Carrier DNA (Promega E4881)
Extracellular NanoLuc Inhibitor (30mM in DMSO) (CAS part # CS181047 or CS181048)
NanoBRET Nano-Glo® Substrate (CAS part # CS181045 or CS181046)

Day 1

HEK293 transfection

1) Medium was removed from cell flask via aspiration, cells were washed once with PBS and trypsinized for 5 minutes at 37°C to dissociate cells from the flask.
2) Trypsin was neutralised using growth medium and cells were pelleted via centrifugation at 500 x g for 3 minutes.
3) Medium was aspirated and cells were resuspended into a single cell suspension using cell culture medium (without antibiotic).
4) The cell density was adjusted to 2 x 10^5 cells/mL in cell culture medium.
5) Lipid:DNA complexes were prepared as following:
    a. 100uL solution of DNA in Opti-MEM without serum for every 400,000 cells.
        i. Plasmids combination:
        100ng ACVR1-c-nanoLuc + 900ng transfection carrier DNA
        100ng pEGFP-C1 + 900ng transfection carrier DNA
        ii. 100uL of Opti-MEM without phenol red
    b. Thoroughly mixed.

c. 3uL of FuGENE® HD was added into each 100uL of DNA mixture to form lipid:DNA complex. Caution was taken when adding FuGENE® HD so that it was pipetted directly into the liquid in the tube and did not touch the plastic side of the tube.

d. Mixture was inverted 5-10 times for thorough mixing.

e. Mixture was incubated at room temperature for 20 minutes to allow complexes to form.

6) 1 part (100uL) of lipid:DNA complex was added to 20 parts (2mL) of HEK293 cells in suspension at 2 x 10^5 cells/mL (400,000 cells total). Tube was mixed gently by 5 inversions.

7) Cells + lipid:DNA complex was dispensed into 6-well plate (200,000 cells per well). Transfection rate was checked the next day based on EGFP signal.

Day 2

M4K compound serial dilution

1) 1000X compound stock was prepared by serial dilution of the parent stock in DMSO.

Dilution guide									**parent stock=25mM=25,000uM**			
Well	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
	L	K	J	I	H	G	F	E	D	C	B	A
Final conc for NanoBRET	0.1nM	0.5nM	1nM	5nM	10nM	50nM	100nM	250nM	500nM	1000nM	2500nM	5000nM
1000x stock for NanoBRET	0.1uM	0.5uM	1uM	5uM	10uM	50uM	100uM	250uM	500uM	1000uM	2500uM	5000uM=1/5 parent
	4J+36	4I+36	4H+36	4G+36	4 F+36ul	4D+36	4C+36	4B+36	4A+36	8A+32	16A+16	12+48ul
Final volume (µl)	36	36	36	36	36	36	36	40	36	36	28	32

Tracer preparation

1) Dilution of 100X Tracer-6908 was prepared in DMSO.

2) 100X Tracer-6908 was diluted in NanoBRET Tracer Dilution Buffer to obtain 20X Tracer-6908.

Cell preparation

1) Medium was removed from cell flask via aspiration, cells were washed once with PBS and trypsinized for 5 minutes at 37°C to dissociate cells from the culture plate.

2) Trypsin was neutralised using assay medium and cells were pelleted via centrifugation at 300 x g for 5 minutes.

3) Medium was aspirated and cells were resuspended into a single cell suspension using complete assay medium.

4) The cell density was adjusted to 2 x 10^5 ells/mL in assay medium.

5) Cell suspension was dispensed into each well using multi-channel pipette at the following volume: 17ul or 38ul for 384-well microplate

Incubation with Tracer-6908

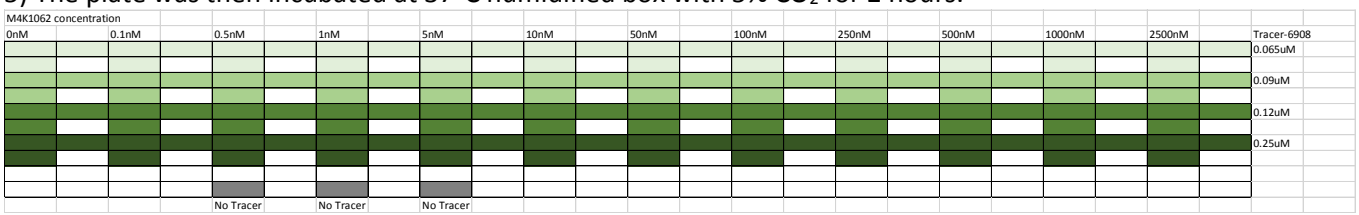
1) 1ul of 20X Tracer-6908 (for final concentrations of 65nM, 90nM, 120nM or 250nM) was added to each well with cells using multi-channel pipette.

2) Microplates were mixed for 30 seconds at 200rpm.

3) 2ul of 10X M4K compound serial dilution was added into each well.

4) Microplates were mixed again for 30 seconds at 200rpm.

5) The plate was then incubated at 37°C humidified box with 5% CO_2 for 2 hours.



NanoBRET measurement

1) The plate was removed from incubator and allowed to cool down to room temperature for 15 minutes.

2) Less than 20 minutes before NanoBRET measurement, NanoLuc substrate solution was prepared by diluting Nano-Glo substrate (1:166) and Extracellular NanoLuc Inhibitor (1:500) in assay medium.
3) 10ul of NanoLuc substrate solution was added to each well.
4) BRET signal was measured using Pherastar FSX with the following settings:
Top optic
610/460 luminescent optical module simultaneous dual emission (LUM 610-LP 460-80)
3600/1879 gain
0.1s setting time
1s to normalize the results

NanoBRET calculation
1) NanoBRET signal in mBRET units was calculated using the following formula:

$$\left(\frac{Sample\ 610nm}{Sample\ 460nm} - \frac{No\ tracer\ 610nm}{No\ tracer\ 460nm}\right) \times 1000$$

Results:

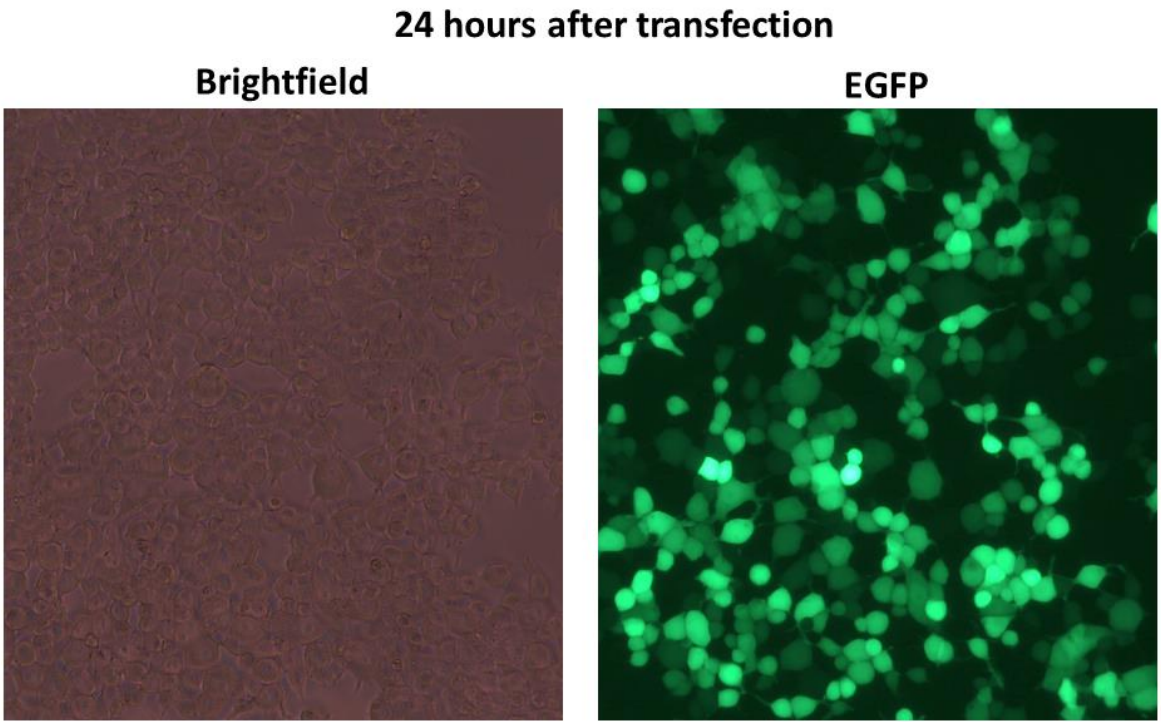


Figure 1. EGFP signal in transfected cells. HEK293 were transfected efficiently. Cells can be harvested for nanoBRET assay.

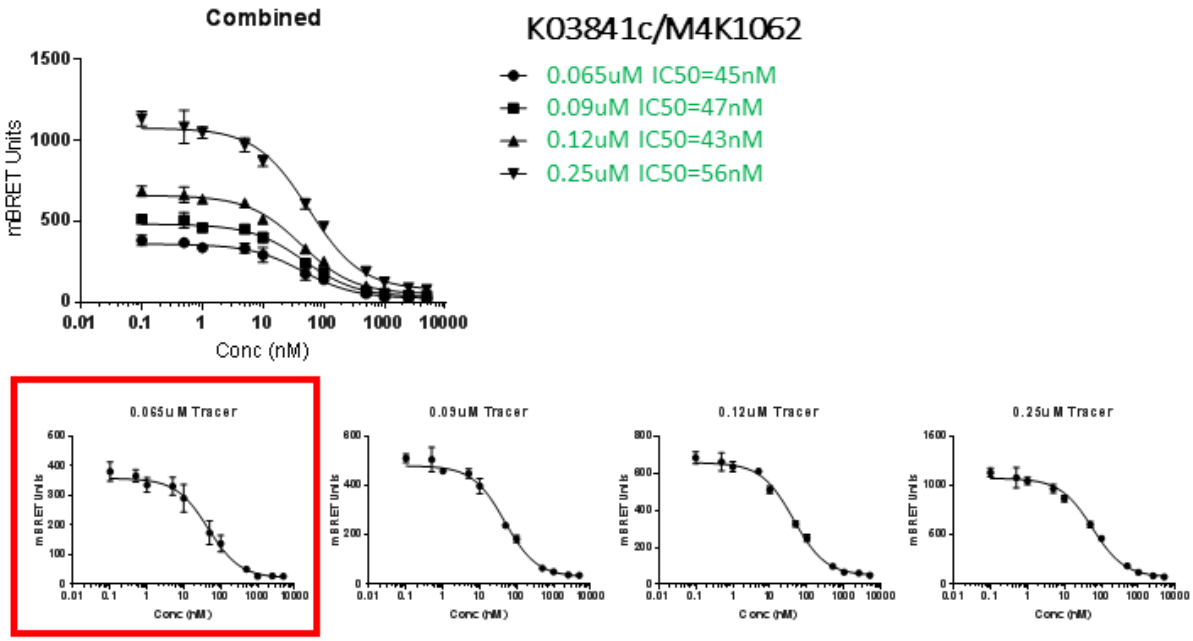


Figure 2. NanoBRET IC50 of compound M4K1062 using increasing concentrations of Tracer-6908 with ACVR1/ALK2-c-nanoLuc transiently expressed in HEK293 cells. Final chosen Tracer-6908 concentration is bracketed in red rectangle.

Conclusion:

In cells transfected with ACVR1-c-nanoLuc, the IC50 values of the same compound are almost identical at lower concentrations of Tracer-6908. Therefore, the IC50 values determined using these concentrations of Tracer-6908 should be good approximation of the actual compound IC50. 65nM Tracer-6908 is chosen for future nanoBRET ACVR1/ALK2 assays because it will be most cost efficient. Even though the maximum signal at this tracer concentration is lower, the standard deviation among experimental replicates remained small, indicating that the quality of the data is not compromised.