Determining whether bespoke nanoBRET tracers work with ALK5

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Special thanks:
David Drewry – Helped with designing the nanoBRET tracers
M4K pharma OICR chemist team – Synthesised the M4K1046-linker derivatives
Carrow Wells (UNC) – Conjugated the M4K1046 to nanoBRET fluorophore

Background:
I have always wanted to establish nanoBRET target engagement assay for ALK5. Relative to dual luciferase promoter assay and immunofluorescent staining, nanoBRET is many times faster. With a robust ALK5 nanoBRET assay, I will be able to rapidly screen for cellular off-target activity.

Since none of the commercial nanoBRET tracers worked with ALK5, we attempted to generate our own tracers. We chose to create these bespoke tracers based on M4K1046 because it has cellular IC50 of ~50nM for ALK5. David Drewry has helped to design linkers that will attach fluorophores (nanoBRET energy acceptor) to the solvent-facing end of M4K1046. Based on known structures, additional bulk in this region should not hinder the binding of the compounds to ALK5. Two versions of M4K1046 (with different linker length) were synthesised by the M4K pharma chemist team in OICR. They were subsequently sent to Carrow Wells for conjugation to nanoBRET fluorophore.

Experimental design:
I wanted to determine the binding of these tracers to ALK5-nanoluciferase fusion. Therefore, I incubated increasing concentrations of tracer with HEK293 cells expressing ALK5-nanoluciferase fusion protein. If the tracers can bind nicely to the ATP pocket of ALK5, incubation with increasing concentrations of tracer will result in increasing BRET signal (wavelength = 610 nm). The resulting magnitude of BRET and EC50 estimated from the curve are good indicators to whether these tracers can be used for nanoBRET assay with ALK5.

To identify any background signal, I have included a replicate of the above experiment with 10,000nM of parent M4K1046. These unlabelled compound will saturate ALK5 ATP pockets and prevent the binding of nanoBRET tracers. All signal from this set of experiment is contributed by non-specific background.

To be eliminate doubts of any technical or reagent issues with the nanoBRET experiment itself, I have replicated both experiments above, substituting ALK5 with ALK2.

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 TGFBR1 or 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
Name of tracers
UNC-CIW-001-011
UNC-CIW-001-012

Reconstitution of tracers
Tracers from UNC were reconstituted in DMSO to 4mM and stored in -80 degree Celsius. Working stock of 400µM was prepared by further dilution in DMSO.

Serial dilution of tracers
1) 20X tracers were made according to the following dilution scheme.

1	2	3	4	5	6	7	8	9	10	11	12	Start with 4µl of 400µM stock
DMSO	4	4	4	4	4	4	4	4	4	4	4	Volume of DMSO to add
0	32	16	16	16	16	16	16	16	16	16	16	Volume of Tracer Dilution Buffer to add
0	0.003906	0.007813	0.015625	0.03125	0.0625	0.125	0.25	0.5	1	2	4	Final Conc (uM)

Dilution of parent M4K1046
10X M4K1046 (final conc = 10µM) was prepared by first diluting 2µl of 50mM in 8µl of DMSO and subsequently in 990µl of OptiMEM. For experiment without parent M4K1046, 10µl of DMSO was diluted in 990µl of OptiMEM.

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 OptiMEM without phenol red 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 in OptiMEM without phenol red and filtered through nylon cell strainer to remove any clumps.
4) The cell density was determined using haemocytometer and adjusted to 2 x 10^5 ells/mL in OptiMEM without phenol red.
5) 17ul of cell suspension was dispensed into each well using multi-channel pipette.

Incubation with Tracers and Parent M4K1046

1) 1ul of 20X Tracer was added to each well with cells using multi-channel pipette.
2) Microplates were mixed for 60 seconds at 200rpm.
3) 2ul of 10X M4K1046 was added into each well. For wells without M4K1046, dilution of DMSO in OptiMEM was added.
4) Microplates were mixed again for 60 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
384-well aperture spoon installed

384-well polypropylene plate 1 (UNC-CIW-001-011 ALK5-Tracer-1)

Conc	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	
A	0		0.003906		0.007813		0.015625		0.03125		0.0625		0.125		0.25		0.5		1		2		4		TGFBR1-Luc
B	0		0.003906		0.007813		0.015625		0.03125		0.0625		0.125		0.25		0.5		1		2		4		
C	0		0.003906		0.007813		0.015625		0.03125		0.0625		0.125		0.25		0.5		1		2		4		
D		0		0.003906		0.007813		0.015625		0.03125		0.0625		0.125		0.25		0.5		1		2		4	TGFBR1-Luc
E		0		0.003906		0.007813		0.015625		0.03125		0.0625		0.125		0.25		0.5		1		2		4	+10uM M4K1046
F		0		0.003906		0.007813		0.015625		0.03125		0.0625		0.125		0.25		0.5		1		2		4	
G	0		0.003906		0.007813		0.015625		0.03125		0.0625		0.125		0.25		0.5		1		2		4		ACVR1-Luc
H	0		0.003906		0.007813		0.015625		0.03125		0.0625		0.125		0.25		0.5		1		2		4		
I	0		0.003906		0.007813		0.015625		0.03125		0.0625		0.125		0.25		0.5		1		2		4		
J		0		0.003906		0.007813		0.015625		0.03125		0.0625		0.125		0.25		0.5		1		2		4	ACVR1-Luc
K		0		0.003906		0.007813		0.015625		0.03125		0.0625		0.125		0.25		0.5		1		2		4	+10uM M4K1046
L		0		0.003906		0.007813		0.015625		0.03125		0.0625		0.125		0.25		0.5		1		2		4	
M																									
O																									
P																									
Q																									

384-well polypropylene plate 2 (UNC-CIW-001-012 ALK5-Tracer-2)

Conc	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	
A	0		0.003906		0.007813		0.015625		0.03125		0.0625		0.125		0.25		0.5		1		2		4		TGFBR1-Luc
B	0		0.003906		0.007813		0.015625		0.03125		0.0625		0.125		0.25		0.5		1		2		4		
C	0		0.003906		0.007813		0.015625		0.03125		0.0625		0.125		0.25		0.5		1		2		4		
D		0		0.003906		0.007813		0.015625		0.03125		0.0625		0.125		0.25		0.5		1		2		4	TGFBR1-Luc
E		0		0.003906		0.007813		0.015625		0.03125		0.0625		0.125		0.25		0.5		1		2		4	+10uM M4K1046
F		0		0.003906		0.007813		0.015625		0.03125		0.0625		0.125		0.25		0.5		1		2		4	
G	0		0.003906		0.007813		0.015625		0.03125		0.0625		0.125		0.25		0.5		1		2		4		ACVR1-Luc
H	0		0.003906		0.007813		0.015625		0.03125		0.0625		0.125		0.25		0.5		1		2		4		
I	0		0.003906		0.007813		0.015625		0.03125		0.0625		0.125		0.25		0.5		1		2		4		
J		0		0.003906		0.007813		0.015625		0.03125		0.0625		0.125		0.25		0.5		1		2		4	ACVR1-Luc
K		0		0.003906		0.007813		0.015625		0.03125		0.0625		0.125		0.25		0.5		1		2		4	+10uM M4K1046
L		0		0.003906		0.007813		0.015625		0.03125		0.0625		0.125		0.25		0.5		1		2		4	
M																									
O																									
P																									
Q																									

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:

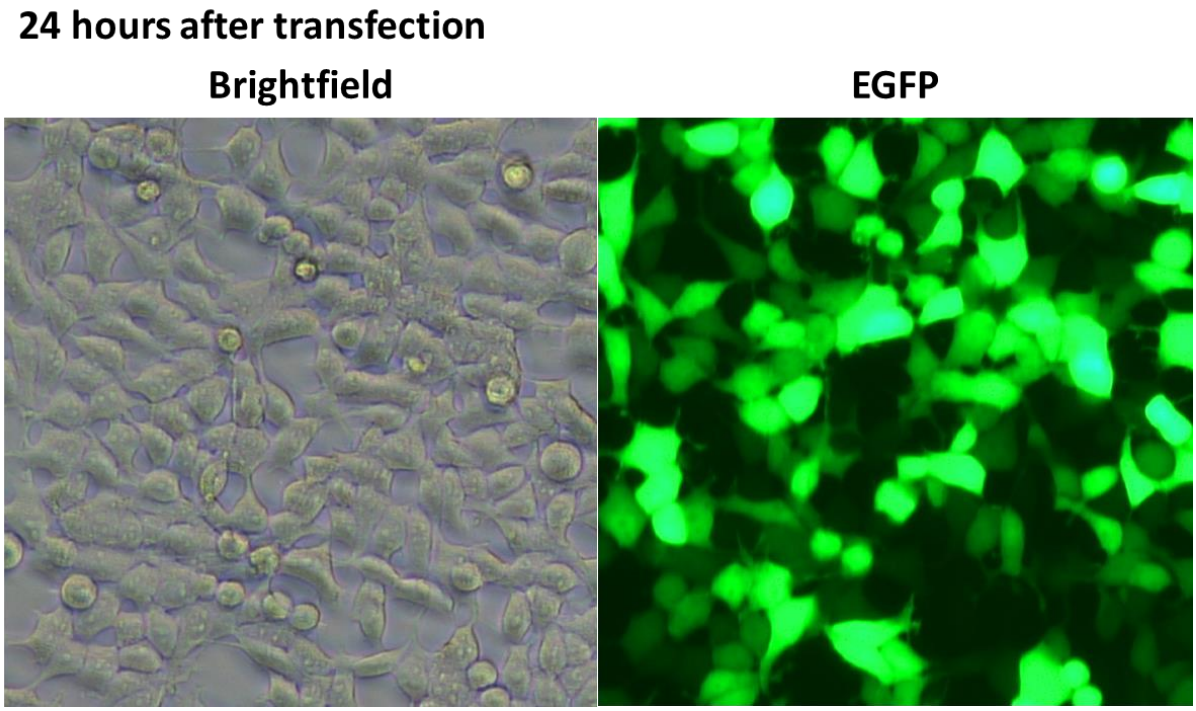

Figure 1. EGFP signal in transfected cells. HEK293 were transfected efficiently. Cells can be harvested for nanoBRET assay.

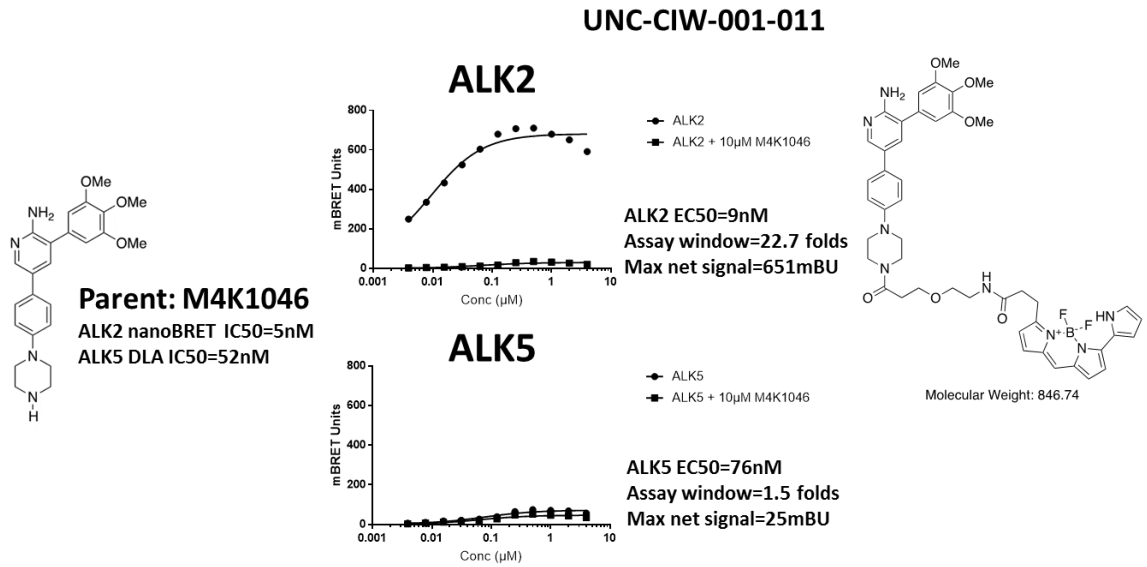

Figure 2. EC50 curves of the first bespoke ALK5 tracer (UNC-CIW-001-011) with ALK2-nanoluc and ALK5-nanoluc. The chemical structures of parent M4K1046 and the bespoke tracer are shown on the side of the graphs. This tracer cannot bind to ALK5.

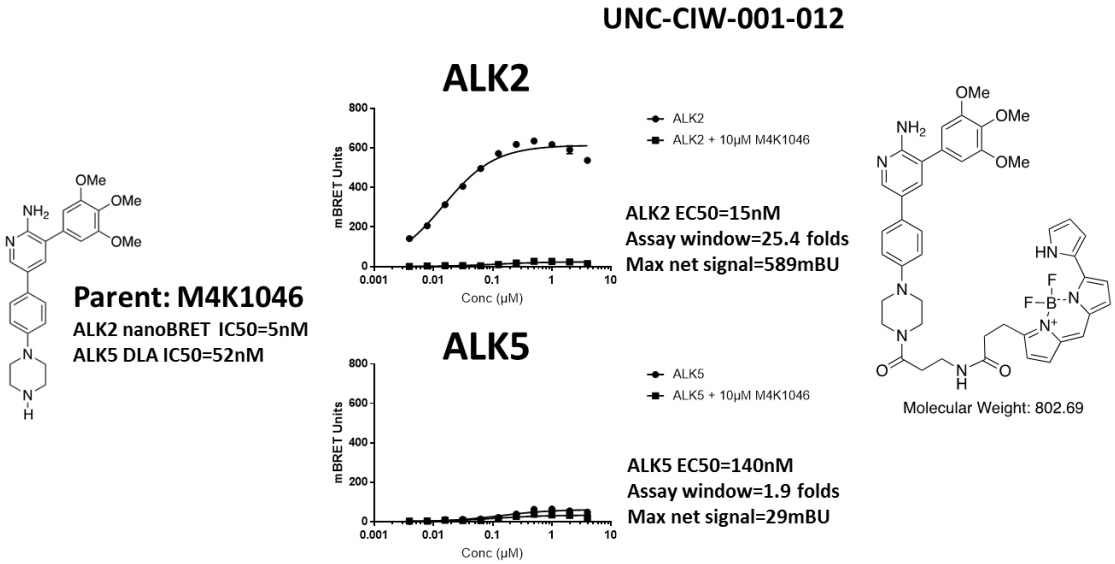

Figure 3. EC50 curves of the second bespoke ALK5 tracer (UNC-CIW-001-012) with ALK2-nanoluc and ALK5-nanoluc. The chemical structures of parent M4K1046 and the bespoke tracer are shown on the side of the graphs. This tracer cannot bind to ALK5.

Conclusion:

Both bespoke tracers cannot be used for ALK5 nanoBRET.

Both of them did not bind to ALK5. The nanoBREt experiment itself was a success since both tracers worked well with ALK2 (over 20 folds assay windows). The background noise was low for both tracers.

Information gained:

ALK5 seems to intrinsically not tolerate bulky additions on the solvent end of M4K1046. This might be additional avenue for improving ALK2 vs ALK5 selectivity.