Determining the nanoBRET IC50 values of 30 legacy ACVR1/ALK2 inhibitors

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Background:

A large number of ACVR1/ALK2 inhibitors were previously synthesised by Paul Brenner's team (Target Discovery Center, University of Oxford) for the purpose of treating Fibrodysplasia Ossificans Progressiva (FOP). Although these compounds were not designed with blood-brain-barrier permeability in mind, they can serve as good bench-marks for my cellular assays. Therefore, 30 of these legacy compounds were chosen to be tested before other new bespoke Diffused Intrinsic Pontine Glioma (DIPG) compounds from Ontario Institute for Cancer Research (OICR) and Charles River Laboratories (CRL).

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

2) 2ul of 1000X compound stock was diluted to 10X in OptiMEM without phenol red fresh before experiment.

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 and M4K compounds
1) 1ul of 20X Tracer-6908 (for final concentrations of 65nM) 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.

96-well 10	1	2	3	4	5	6	7	8	9	10	11	12		
	0.1nM	0.5nM	1nM	5nM	10nM	50nM	100nM	250nM	500nM	1000nM	2500nM	5000nM	Compound	Location on plate
A													M4K1062	Plate 1-A
B													M4K1209	Plate 1-B
C													M4K1210	Plate 1-C
D													M4K1133	Plate 1-D
E													M4K1170	Plate 1-E
F													M4K1159	Plate 1-F
G													M4K1158	Plate 1-G
H													M4K1206	Plate 1-H
A													M4K1145	Plate 2-A
B													M4K1187	Plate 2-B
C													M4K1134	Plate 2-C
D													M4K1196	Plate 2-D
E													M4K1188	Plate 2-E
F													M4K1200	Plate 2-F
G													M4K1126	Plate 2-G
H													M4K1136	Plate 2-H
A													M4K1137	Plate 3-A
B													M4K1138	Plate 3-B
C													M4K1139	Plate 3-C
D													M4K1140	Plate 3-D
E													M4K1141	Plate 3-E
F													M4K1148	Plate 3-F
G													M4K1149	Plate 3-G
H													M4K1160	Plate 3-H
A													M4K1163	Plate 4-A
B													M4K1212	Plate 4-B
C													M4K1216	Plate 4-C
D													M4K1055	Plate 4-D
E													M4K1046	Plate 4-E
F													M4K1058	Plate 4-F
G														
H														

384-plate 1

	0.1		0.5		1		5		10		50		100		250		500		1000		2500		5000		nM	
A																									M4K1062	Plate 1-A
B																										
C																									M4K1209	Plate 1-B
D																										
E																									M4K1210	Plate 1-C
F																										
G																									M4K1133	Plate 1-D
H																										
I																									M4K1170	Plate 1-E
J																										
K																									M4K1159	Plate 1-F
L																										
M																									M4K1158	Plate 1-G
N																										
O																										
P	No Tracer		No Tracer		No Tracer		0nM		0nM		0nM															

384-plate 2

	0.1		0.5		1		5		10		50		100		250		500		1000		2500		5000		nM	
A																									M4K1206	Plate 1-H
B																										
C																									M4K1145	Plate 2-A
D																										
E																									M4K1187	Plate 2-B
F																										
G																									M4K1134	Plate 2-C
H																										
I																									M4K1196	Plate 2-D
J																										
K																									M4K1188	Plate 2-E
L																										
M																									M4K1200	Plate 2-F
N																										
O																										
P	No Tracer		No Tracer		No Tracer		0nM		0nM		0nM															

384-plate 3

	0.1		0.5		1		5		10		50		100		250		500		1000		2500		5000		nM	
A																									M4K1126	Plate 2-G
B																										
C																									M4K1136	Plate 2-H
D																										
E																									M4K1137	Plate 3-A
F																										
G																									M4K1138	Plate 3-B
H																										
I																									M4K1139	Plate 3-C
J																										
K																									M4K1140	Plate 3-D
L																										
M																									M4K1141	Plate 3-E
N																										
O																										
P	No Tracer		No Tracer		No Tracer		0nM		0nM		0nM															

384-plate 4

	0.1		0.5		1		5		10		50		100		250		500		1000		2500		5000			
A																									M4K1148	Plate 3-F
B																										
C																									M4K1149	Plate 3-G
D																										
E																									M4K1160	Plate 3-H
F																										
G																									M4K1163	Plate 4-A
H																										
I																									M4K1212	Plate 4-B
J																										
K																									M4K1216	Plate 4-C
L																										
M																									M4K1055	Plate 4-D
N																										
O																										
P	No Tracer		No Tracer		No Tracer		0nM		0nM		0nM															

384-plate 5

	0.1		0.5		1		5		10		50		100		250		500		1000		2500		5000			
A																									M4K1046	Plate 4-E
B																										
C																									M4K1058	Plate 4-F
D																										
E																										
F	No Tracer		No Tracer		No Tracer		0nM		0nM		0nM															
G																										
H																										
I																										
J																										
K																										
L																										
M																										
N																										
O																										
P																										

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:

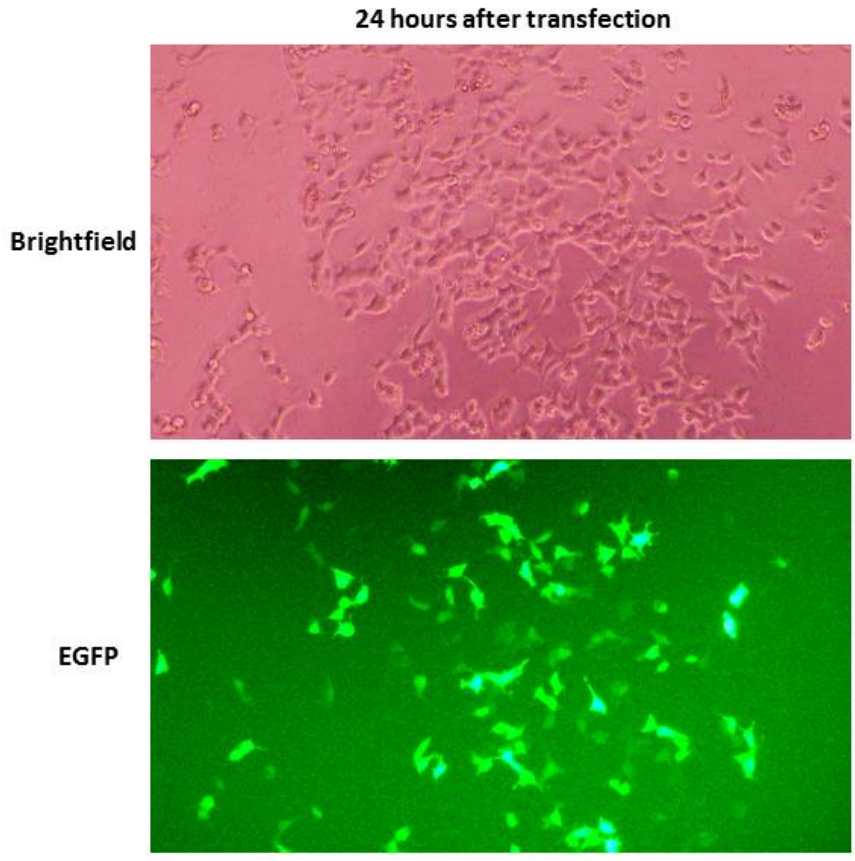


Figure 1. EGFP signal in transfected cells. HEK293 were transfected efficiently. Cells can be harvested for nanoBRET assay.

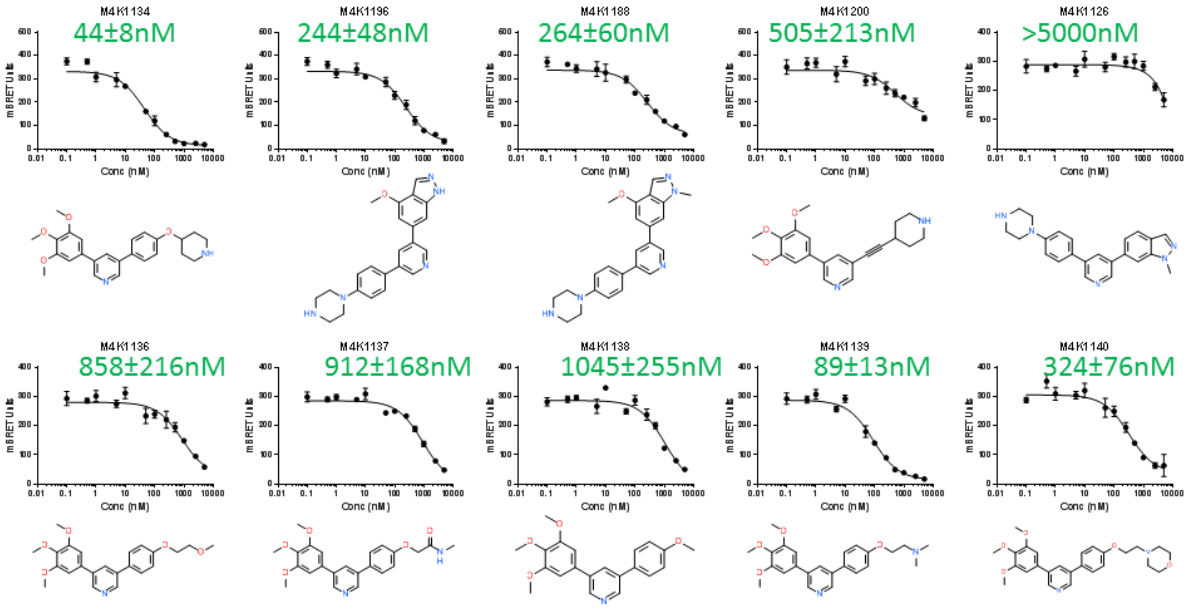
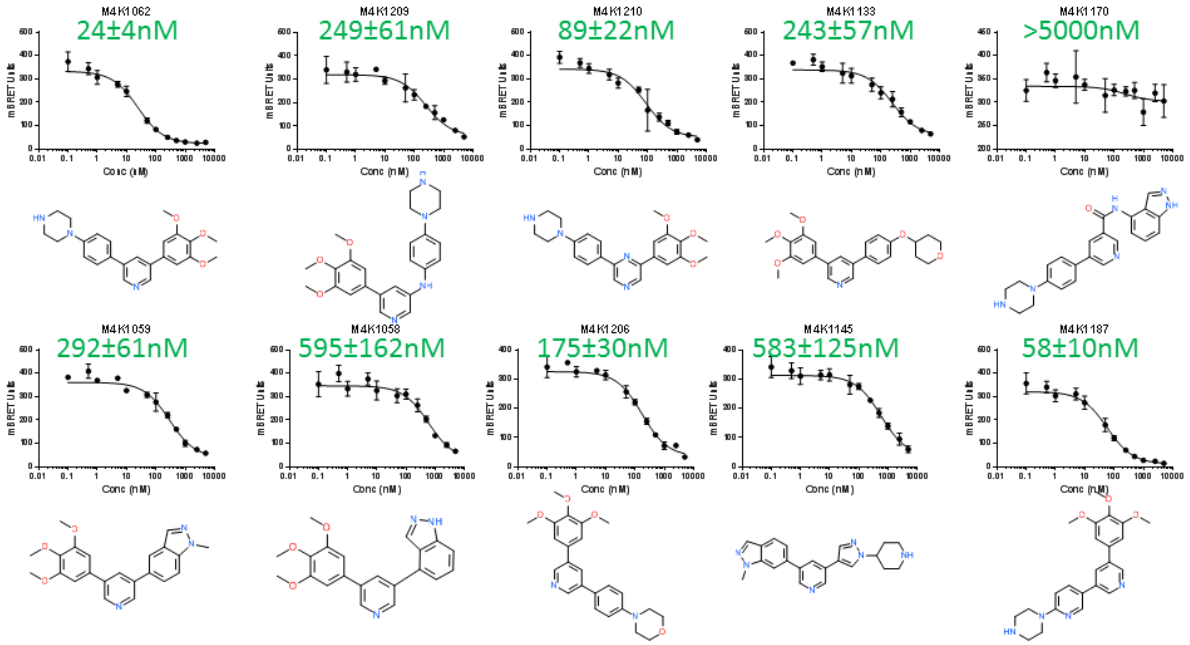


Figure 2. Chemical structures and NanoBRET IC50 curves of the first 10 legacy ACVR1/ALK2 inhibitors. IC50 values estimated by GraphPad Prism are shown in green.



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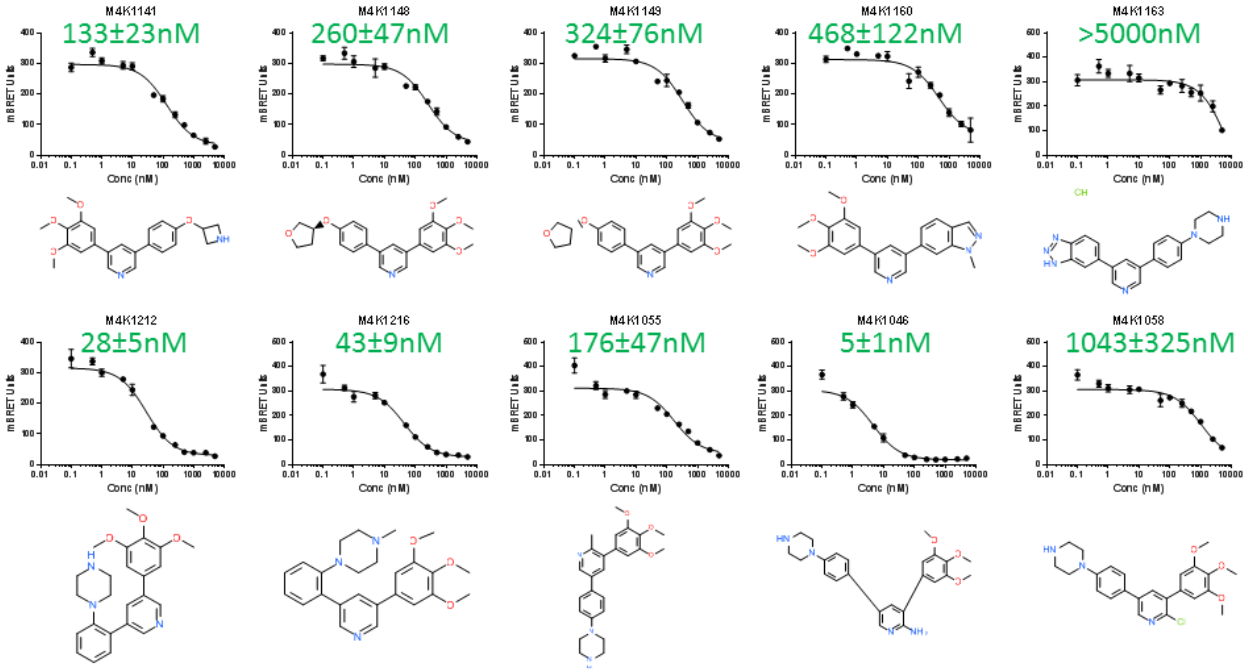

Figure 4. Chemical structures and NanoBRET IC50 curves of the last 10 legacy ACVR1/ALK2 inhibitors. IC50 values estimated by GraphPad Prism are shown in green.

Conclusion:

Some of the legacy ACVR1/ALK2 inhibitors are very potent (M4K1062, M4K1210, M4K 1187, M4K1134, M4K1139, M4K1212, M4K1216, M4K1046). However, their off-target activity towards TGFBR1/ALK5 still needed to be determined. Inhibition of TGFBR1/ALK5 leads to cardiac toxicity. Since I have not yet been able to establish a robust nanoBRET target engagement assay for TGFBR1/ALK5, I will have to resort to dual luciferase promoter assay (orthologous assay) for the time being.