Determining the nanoBRET AL2K IC50 values of 24 new ACVR1/ALK2 inhibitors

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Background:

In an effort to develop clinical compounds for Diffused Intrinsic Pontine Glioma (DIPG) treatment, new analogues of ACVR1/ALK2 inhibitors are continuously synthesised by Ontario Institute for Cancer Research (OICR) and Charles River Laboratories (CRL). I will provide prompt feedback of the cellular assay results to guide their design of new compounds.

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

Compound reconstitution in DMSO

Compound powder in glass vials were reconstituted in DMSO to 25mM. The ones that were not completely soluble were further diluted to 10mM with more DMSO.

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

Day 3

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.

Working compound dilution

1) 2ul of 1000X compound stock was diluted to 10X in OptiMEM without phenol red fresh before experiment.

96-well 10X compounds														
	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		Jong F
A													Plate 5-A	
B													Plate 5-B	
C													Plate 5-C	
D													Plate 5-D	
E													Plate 5-E	
F													Plate 5-F	
G													Plate 5-G	
H													Plate 5-H	
A													Plate 6-A	
B													Plate 6-B	
C													Plate 6-C	
D													Plate 6-D	
E													Plate 6-E	
F													Plate 6-F	
G													Plate 6-G	
H													Plate 6-H	
A													Plate 7-A	
B													Plate 7-B	
C													Plate 7-C	
D													Plate 7-D	
E													Plate 7-E	
F													Plate 7-F	
G													Plate 7-G	
H													Plate 7-H	
A													Plate 4-B	

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 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 60 seconds at 200rpm.
3) 2ul of 10X M4K compound serial dilution was added into each well.
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-plate 1

	0.1		0.5		1		5		10		50		100		250	
A																
B																
C																
D																
E																
F																
G																
H																
I																
J																
K																
L																
M																
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	
A																
B																
C																
D																
E																
F																
G																
H																
I																
J																
K																
L																
M																
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	
A																
B																
C																
D																
E																
F																
G																
H																
I																
J																
K																
L																
M																
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	
A																
B																
C																
D																
E																
F																
G																
H																
I																
J																
K																
L																
M																
N																
O																
P	No Tracer		No Tracer		No Tracer		0nM		0nM		0nM					

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:

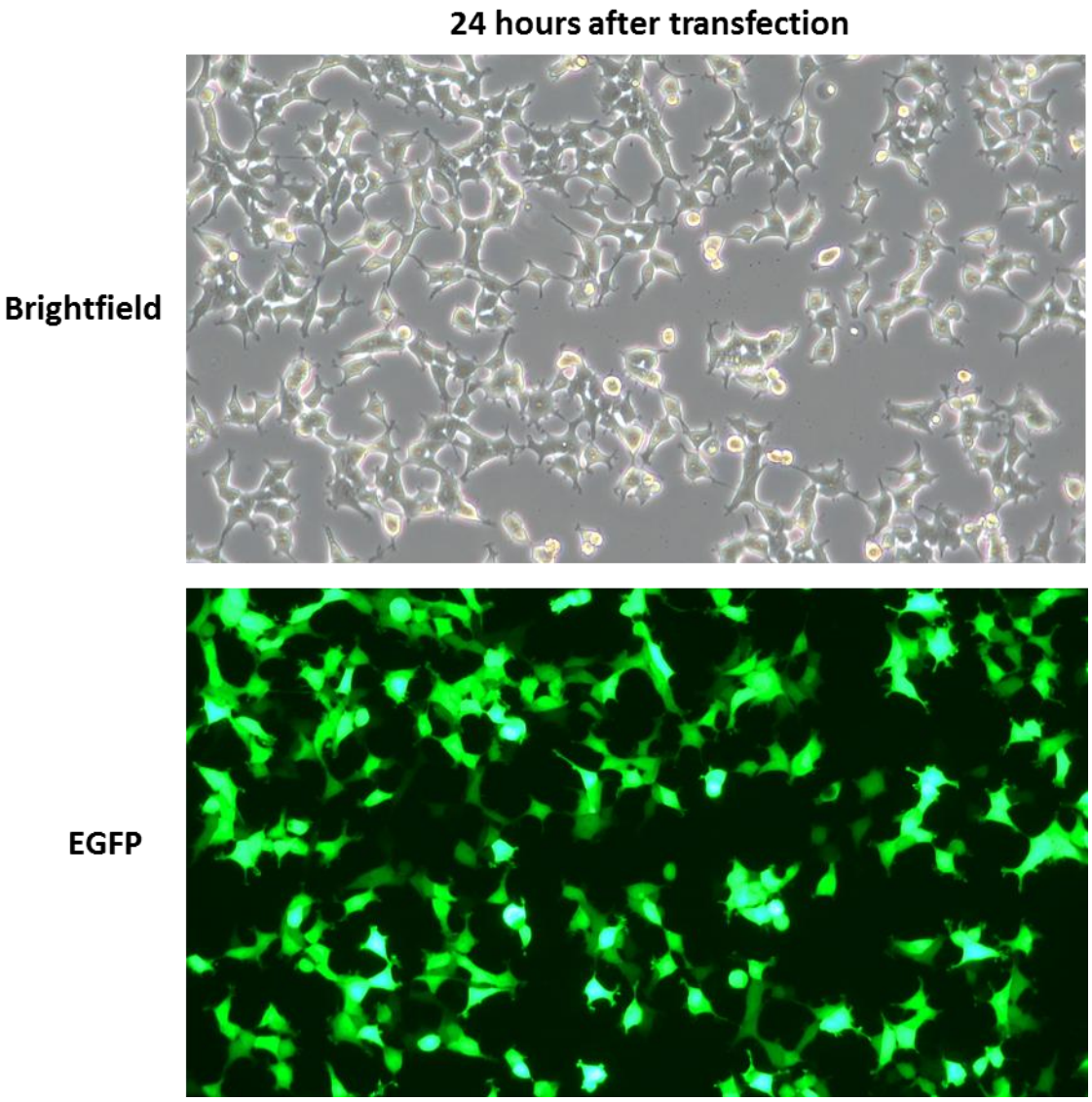

Figure 1. EGFP signal in transfected cells. HEK293 were transfected efficiently. Cells can be harvested for nanoBRET assay.

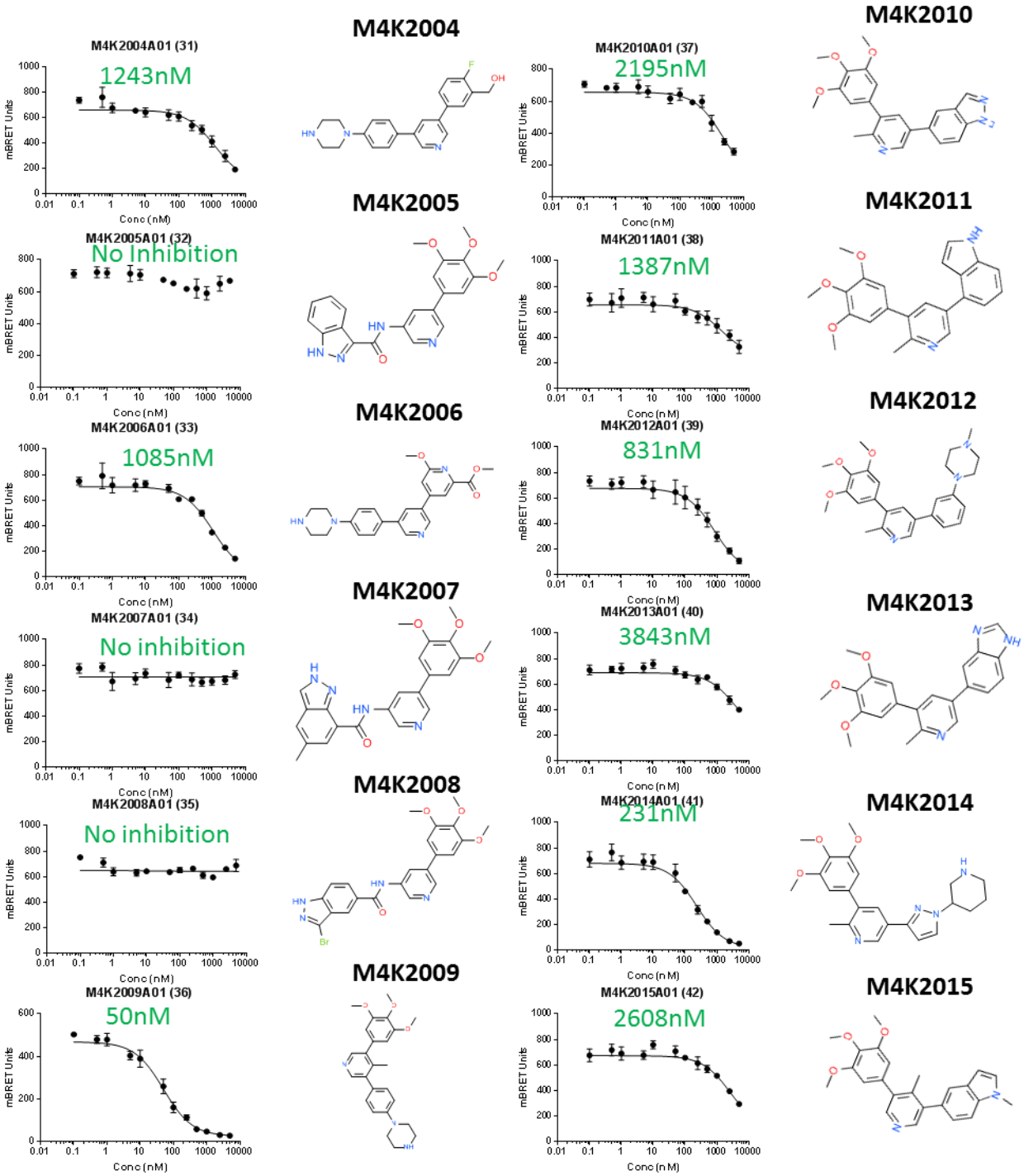

Figure 2. ALK2 NanoBRET IC50 curves and chemical structures of the first dozen of new ACVR1/ALK2 inhibitors. IC50 values estimated by GraphPad Prism are shown in green.

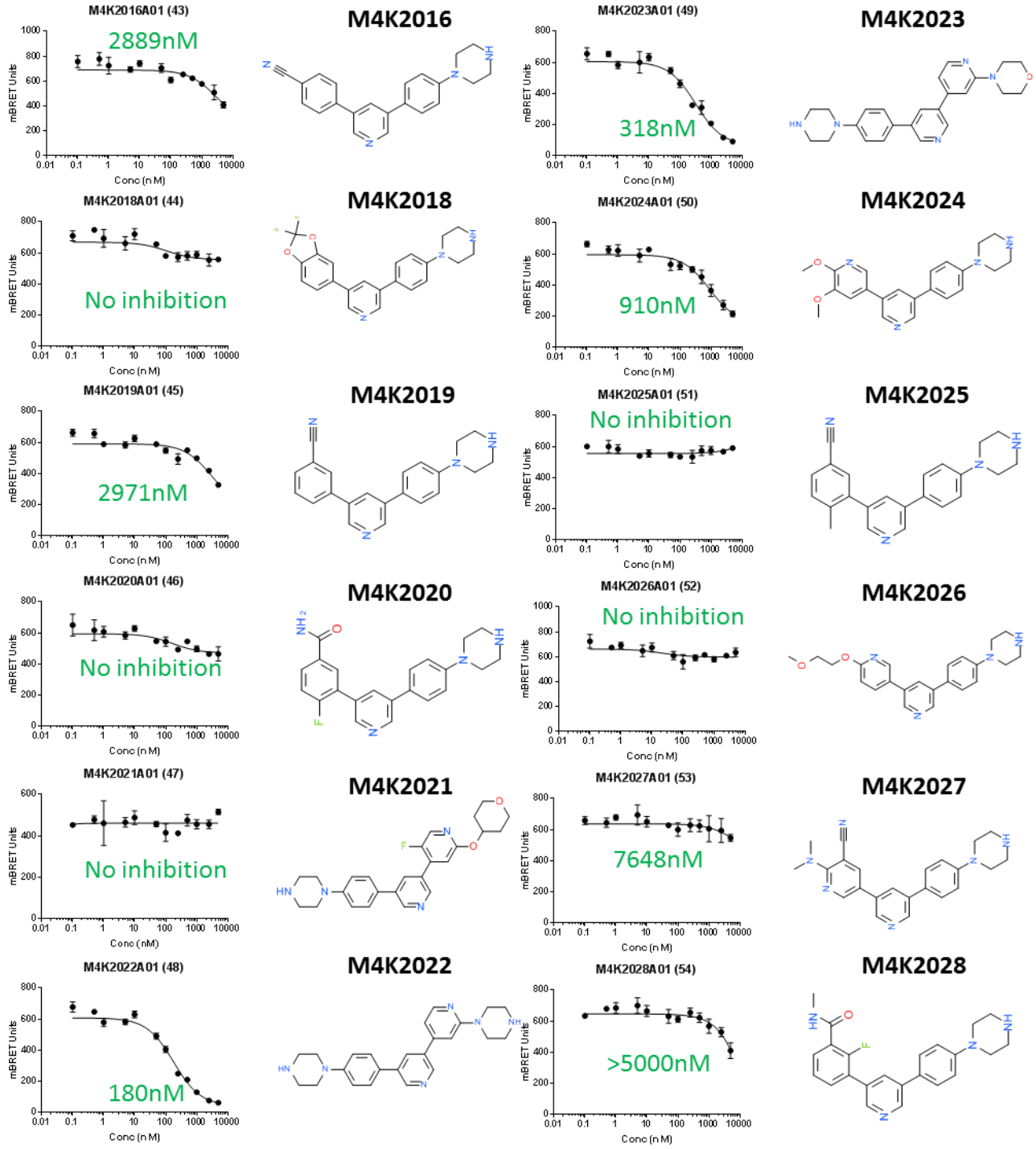

Figure 3. ALK2 NanoBRET IC50 curves and chemical structures of the second dozen of new ACVR1/ALK2 inhibitors. IC50 values estimated by GraphPad Prism are shown in green.

Conclusion:

Most of the modifications resulted in lower potency towards ALK2. Only M4K2009 has similar potency compared to parental compound. Since a robust nanoBRET tracer is not available for TGFBR1/ALK5, I will use dual luciferase promoter assay (orthologous assay) to determine the off-target activity of these compounds towards TGFBR1/ALK5.