Determining the dual luciferase ALK5 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). 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.

Protocol:

Transfect CAGA/RLTK in HEK293 (Monday)

10,000 cells are needed per well of the 96-well plate
30 compounds = 15 96-well plates = 6 384-well plates

Transfection mix composition (scale up according to need)

For each 200,000 cells in 1ml, 400ng CAGA and 100ng RLTK in 50ul OptiMEM without phenol red, 1.5ul FugeneHD, 20mins incubation

Trypsinise HEK293 cells and resuspend in medium without antibiotic, count and adjust concentration to 200,000 cells/ml
30 compounds requires 30*36 wells+ 96 control wells + 15% extra
= 1080+96+176 wells
= 1352 wells
= 13,500,000 cells
= 67.6ml of cells for transfection
= 70ml transfection cell suspension volume
1) Prepare transfection mixture. Mix 28ug CAGA + 7ug RLTK in 3500ul OptiMEM in a tube.
2) Add 105ul FugeneHD and mix by flipping tube and incubate for 20 minutes
3) Seed into 7 T75 flasks (10ml per flask)
Transfection control:
1) Transfect 200,000 cells in 2ml with 1000ng pEGFP-C1 in 100ul OptiMEM
2) Add 3ul FugeneHD, mix well and incubate 20mins
3) Mix with HEK293 cell suspension in tube and seed into 2 wells of 6-well plate

Re-seeding transfected HEK293 cells (Tuesday)

1) Trypsinise HEK293 cells and resuspend in complete medium
2) Filter through sterile nylon cell strainer to remove cell clumps, count and adjust concentration to 100,000 cells/ml
3) Seed 100ul into each well of 7 rows of each 96-well plate (10,000 cells per well)

Aliquoting serial dilutions (Monday)

Since off-target activities for M4K compounds are generally low, for ALK5 DLA, we will use double the concentrations used for ALK2 nanoBRET

Transfer 2ul of each compound into new 96-well PCR plates, seal with 3M plastic cover and keep in -20 freezer

96-well PCR plate aliquot layout for ligand and compound treatment

	Jong Fu's	Stock conc (mM)	
M4K1062	1	50	Plate 1
M4K1209	2	25	
M4K1210	3	50	
M4K1133	4	50	
M4K1170	5	50	
M4K1159	6	25	
M4K1158	7	50	
M4K1206	8	50	
M4K1145	9	50	Plate 2
M4K1187	10	50	
M4K1134	11	50	
M4K1196	12	50	
M4K1188	13	50	
M4K1200	14	50	
M4K1126	15	50	
M4K1136	16	50	
M4K1137	17	50	Plate 3
M4K1138	18	50	
M4K1139	19	50	
M4K1140	20	50	
M4K1141	21	50	
M4K1148	22	50	
M4K1149	23	50	
M4K1160	24	50	
M4K1163	25	50	Plate 4
M4K1212	26	25	
M4K1216	27	25	
M4K1055	28	50	
M4K1046	29	50	
M4K1058	30	50	

Perform treatment with TGFb and compounds (Wednesday)

Final volume of each well is 100ul (with 10ng/ml TGFb and 1X compound)

1352 wells in total

80ml (for TGFb dilution) +200ml (for compound dilution) of 1%serum medium needed

Ligand preparation

Thaw TGFb parent stock from -80 on ice

Prepare 75ml of 20ng/ml TGFb (2X conc)

Using white reservoir, transfer 165ul to 96-well flat-bottom plate

Compound dilution preparation

Prepare 2X conc compounds in 1%Serum=> dilute the 2ul aliquots of 500X into 498ul of 1%FCS medium (2X conc) 2ml 96-well block

Transfer 165ul to the 96-well flat-bottom plate with 165ul of 165ul of 20ng/ml TGFb

Mix well (Caution taken to not mix in between wells)

Preparation of compound series

0.2nM	1nM	2nM	10nM	20nM	100nM	200nM	500nM	1000nM	2000nM	5000nM	10000nM	Final Con
0.1uM	0.5uM	1uM	5uM	10uM	50uM	100uM	250uM	500uM	1000uM	2500uM	5000uM	500X Stoc

Addition of mixture to cells

Aspirate medium in 96-well plates with cells using multi-channel aspirator (Important!! keep interval short to avoid drying up of the cells)

Add 100ul of TGFb/compound mixture (add slowly and steady from the side to avoid detaching cells)

No stimulation and no compound (0nM) controls

Add 100ul of plain 1% Serum to each no stimulation control wells

Add 100ul of 1% Serum with 10ng/ml TGFb to each 0nM control wells

96-well layout for ligand and compound treatment

0.2nM	1nM	2nM	10nM	20nM	100nM	200nM	500nM	1000nM	2000nM	5000nM	10000nM		
												M4K1062	1
												M4K1209	2
No stim	No stim	No stim							0nM	0nM	0nM		

0.2nM	1nM	2nM	10nM	20nM	100nM	200nM	500nM	1000nM	2000nM	5000nM	10000nM		
												M4K1210	3
												M4K1133	4

0.2nM	1nM	2nM	10nM	20nM	100nM	200nM	500nM	1000nM	2000nM	5000nM	10000nM		
												M4K1170	5
												M4K1159	6

0.2nM	1nM	2nM	10nM	20nM	100nM	200nM	500nM	1000nM	2000nM	5000nM	10000nM		
												M4K1158	7
												M4K1206	8
No stim	No stim	No stim							0nM	0nM	0nM		

0.2nM	1nM	2nM	10nM	20nM	100nM	200nM	500nM	1000nM	2000nM	5000nM	10000nM		
												M4K1145	9
												M4K1187	10

0.2nM	1nM	2nM	10nM	20nM	100nM	200nM	500nM	1000nM	2000nM	5000nM	10000nM		
												M4K1134	11
												M4K1196	12
No stim	No stim	No stim							0nM	0nM	0nM		

0.2nM	1nM	2nM	10nM	20nM	100nM	200nM	500nM	1000nM	2000nM	5000nM	10000nM		
												M4K1188	13
												M4K1200	14

0.2nM	1nM	2nM	10nM	20nM	100nM	200nM	500nM	1000nM	2000nM	5000nM	10000nM		
												M4K1126	15
												M4K1136	16
No stim	No stim	No stim							0nM	0nM	0nM		

0.2nM	1nM	2nM	10nM	20nM	100nM	200nM	500nM	1000nM	2000nM	5000nM	10000nM		
												M4K1137	17
												M4K1138	18

0.2nM	1nM	2nM	10nM	20nM	100nM	200nM	500nM	1000nM	2000nM	5000nM	10000nM		
												M4K1139	19
												M4K1140	20
No stim	No stim	No stim							0nM	0nM	0nM		

0.2nM	1nM	2nM	10nM	20nM	100nM	200nM	500nM	1000nM	2000nM	5000nM	10000nM		
												M4K1141	21
												M4K1148	22

0.2nM	1nM	2nM	10nM	20nM	100nM	200nM	500nM	1000nM	2000nM	5000nM	10000nM		
												M4K1149	23
												M4K1160	24
No stim	No stim	No stim							0nM	0nM	0nM		

0.2nM	1nM	2nM	10nM	20nM	100nM	200nM	500nM	1000nM	2000nM	5000nM	10000nM		
												M4K1163	25
												M4K1212	26

0.2nM	1nM	2nM	10nM	20nM	100nM	200nM	500nM	1000nM	2000nM	5000nM	10000nM		
												M4K1216	27
												M4K1055	28
No stim	No stim	No stim							0nM	0nM	0nM		

0.2nM	1nM	2nM	10nM	20nM	100nM	200nM	500nM	1000nM	2000nM	5000nM	10000nM		
												M4K1046	29
												M4K1058	30

DLA measurement (Thursday)

Prepare 80ml 1X PLB by dilutiong 16ml of 5X PLB in 64ml of autoclaved water

Aspirate medium completely using multi-channel aspirator

(Important!! keep interval short to avoid drying up of the cells)

Add 50ul of 1X PLD per well, shake plate at RT for 20 minutes

(at least, slightly longer is alright)

Freeze plate in -80 if not measuring DLA immediately, can be stored long term

For DLA measurement, aliquot 10ul of lysed cells into 384-well plate

Use 2s DLA program in Pherastar FSX

(Important!! Lower gain to 2000, else signal will be over threshold)

Measure luminescent signal after injecting 25ul of LARII and again after injecting 25ul of Stop&Glo in each well

35ml of LARII and Stop&Glo needed

384-well layout for DLA Pherastar FSX measurement

Aliquot each triplicate in criss-crossed manner to minimise bleed-through of signal from neighbouring wells, since aperture spoon cannot be used

384-w 1	1	2	3	4	5	6	7	8	9	10	11	12			
A	0.2	1	2	10	20	100	200	500	1000	2000	5000	10000	96-w 1	M4K1062	1
B															
C															
D														M4K1209	2
E															
F															
G													96-w 2	M4K1210	3
H															
I															
J														M4K1133	4
K															
L															
M													96-w 3	M4K1170	5
N															
O															
P	No stim	No stim	No stim							0nM	0nM	0nM			

384-w 2	1	2	3	4	5	6	7	8	9	10	11	12			
A														M4K1159	6
B															
C															
D													96-w 4	M4K1158	7
E															
F															
G														M4K1206	8
H															
I															
J													96-w 5	M4K1145	9
K															
L															
M														M4K1187	10
N															
O															
P	No stim	No stim	No stim							0nM	0nM	0nM			

384-w 3	1	2	3	4	5	6	7	8	9	10	11	12			
A													96-w 6	M4K1134	11
B															
C															
D														M4K1196	12
E															
F															
G													96-w 7	M4K1188	13
H															
I															
J														M4K1200	14
K															
L															
M													96-w 8	M4K1126	15
N															
O															
P	No stim	No stim	No stim							0nM	0nM	0nM			

384-w 2	1	2	3	4	5	6	7	8	9	10	11	12			
A														M4K1136	16
B															
C															
D													96-w 9	M4K1137	17
E															
F															
G														M4K1138	18
H															
I															
J													96-w 10	M4K1139	19
K															
L															
M														M4K1140	20
N															
O															
P	No stim	No stim	No stim							0nM	0nM	0nM			

384-w 3	1	2	3	4	5	6	7	8	9	10	11	12			
A													96-w 11	M4K1141	21
B															
C															
D														M4K1148	22
E															
F															
G													96-w 12	M4K1149	23
H															
I															
J														M4K1160	24
K															
L															
M													96-w 13	M4K1163	25
N															
O															
P	No stim	No stim	No stim							0nM	0nM	0nM			

384-w 2	1	2	3	4	5	6	7	8	9	10	11	12			
A														M4K1212	26
B															
C															
D													96-w 14	M4K1216	27
E															
F															
G														M4K1055	28
H															
I															
J													96-w 15	M4K1046	29
K															
L															
M														M4K1058	30
N															
O															
P	No stim	No stim	No stim							0nM	0nM	0nM			

Results:

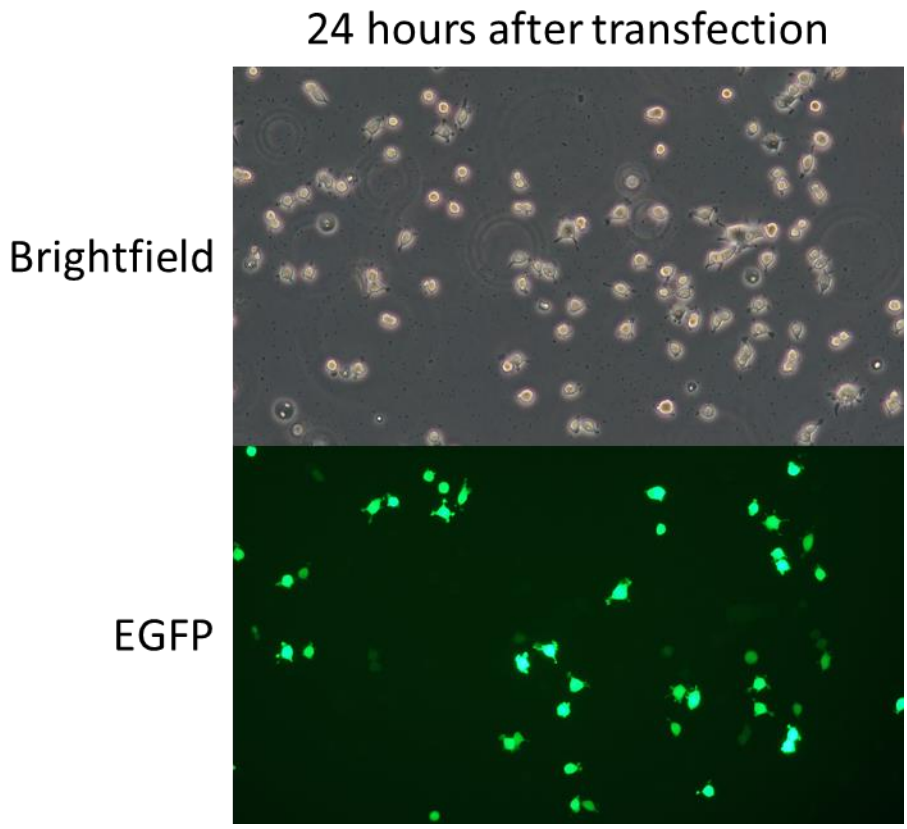

Figure 1. EGFP signal in transfected cells. HEK293 were transfected efficiently. Cells can be harvested and re-seeded into 96-well plates for ALK5 dual luciferase assay.

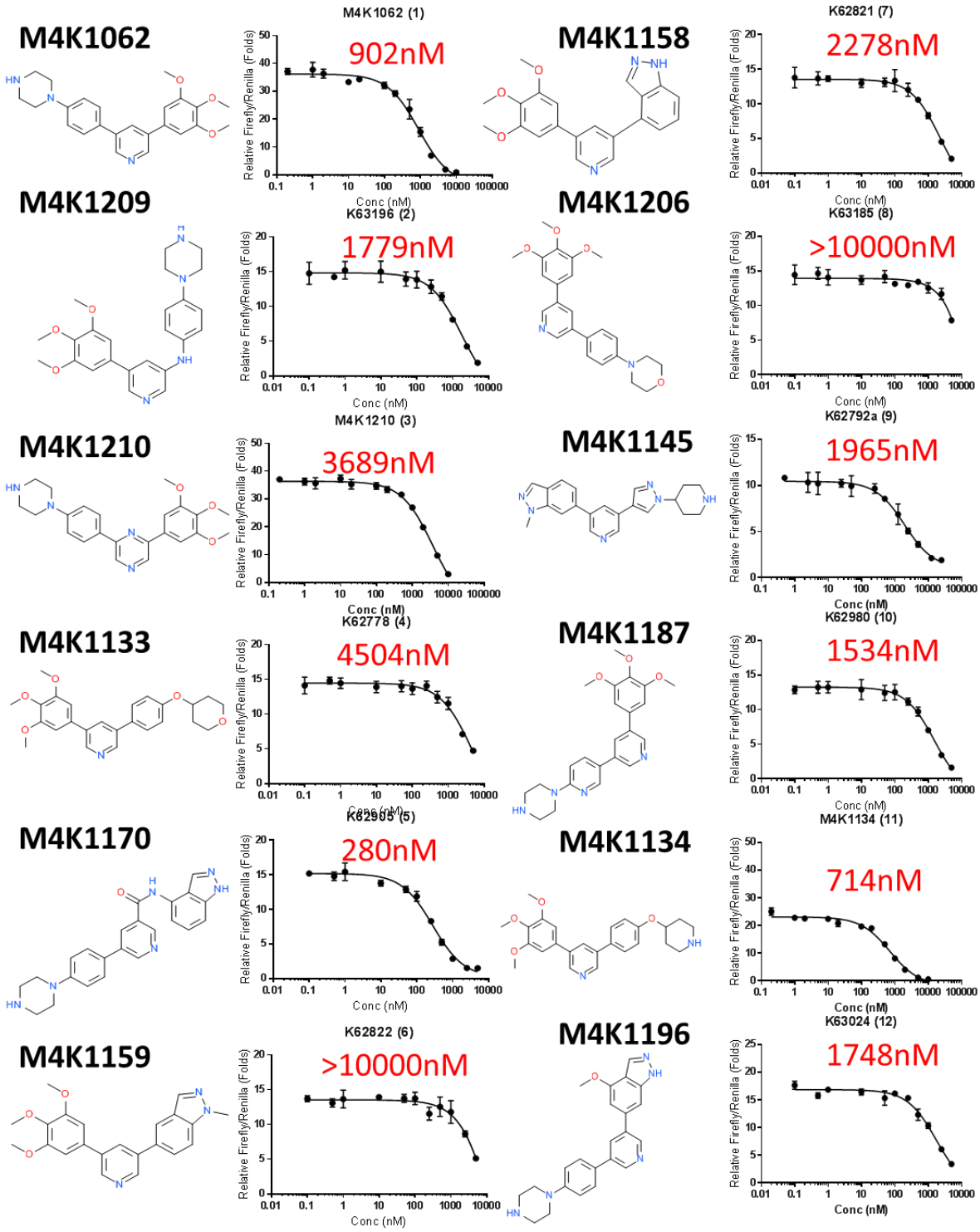

Figure 2. Chemical structures and DLA IC50 curves of the first 12 legacy ACVR1/ALK2 inhibitors. IC50 values estimated by GraphPad Prism are shown in red.

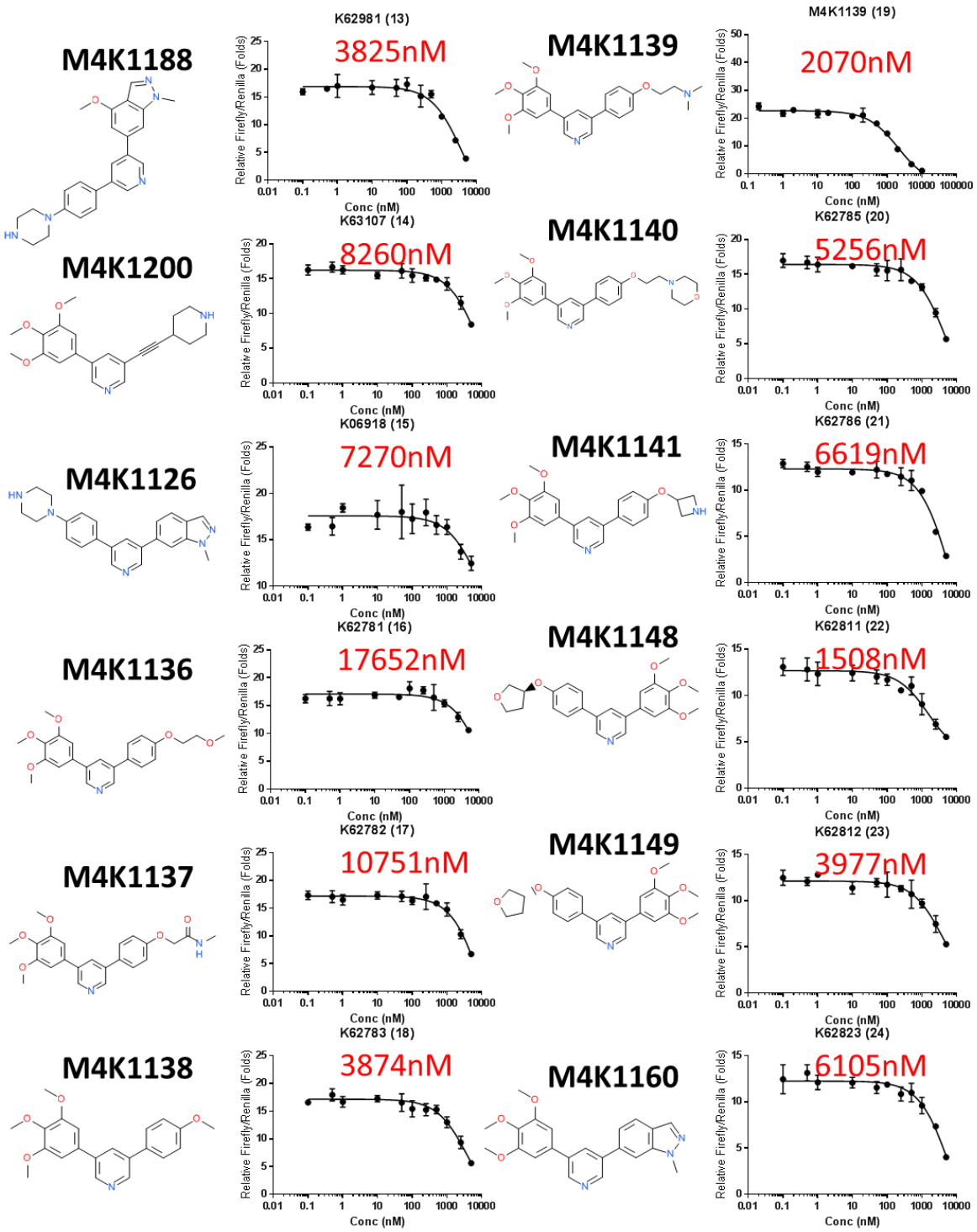

Figure 3. Chemical structures and DLA IC50 curves of the next 12 legacy ACVR1/ALK2 inhibitors. IC50 values estimated by GraphPad Prism are shown in red.

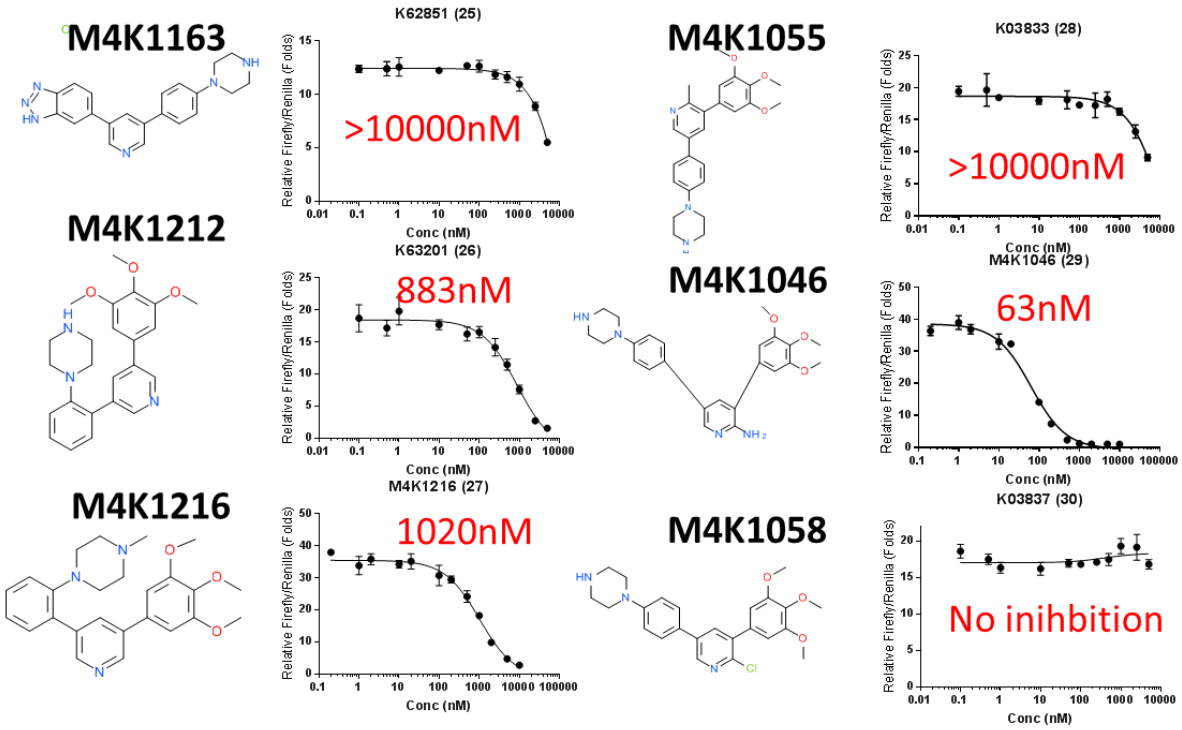

Figure 4. Chemical structures and DLA IC50 curves of the last 6 legacy ACVR1/ALK2 inhibitors. IC50 values estimated by GraphPad Prism are shown in red.

Conclusion:

The off-target ALK5 inhibition by most of the legacy ACVR1/ALK2 inhibitors are not high. Only a handful of legacy compounds had ALK5 IC50 <1000nM (M4K1062, M4K1134, M4K1212 and M4K1046). Among them M4K1046 has an ALK5 IC50 of 63nM. This shows that the legacy compounds designed for FOP treatment had been well designed to minimise the risk of cardiac toxicity. However, in all cases, potency towards ALK2 and ALK5 appeared to be coupled. Differentially increasing the potency towards ALK2 while minimising ALK5 potency is highly desirable for future M4K designs. More careful analysis of the structure-activity-relationship (SAR) of these legacy compounds is necessary to draw useful lessons for new M4K compound design.