Evaluating the effect of 8 M4K compounds on the viability of DIPG patient-derived cells (SU-DIPG-VI, HSJD-DIPG-007 and SU-DIPG-IV)

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Background:

Viability assay using Celltiter Glo kit has been chosen as the main method for evaluating the efficacy of ALK2 inhibitors on DIPG patient-derived cells due to its robustness. As a pilot experiment, 8 ALK2 inhibitors with differing potencies were chosen and evaluated in 3 DIPG patient-derived cell lines (one with wild-type ALK2 and two with mutant ALK2).

Experimental design:

DIPG cells will be seeded into wells coated with laminin at densities optimised in previous experiments. After allowing the cells to adhere overnight, they will be treated with serial dilutions of various M4K compounds. After 7 days of compound treatment, the area occupied by the cells, the intensity of propidium iodide signal and Celltiter Glo luminescence signal will be determined.

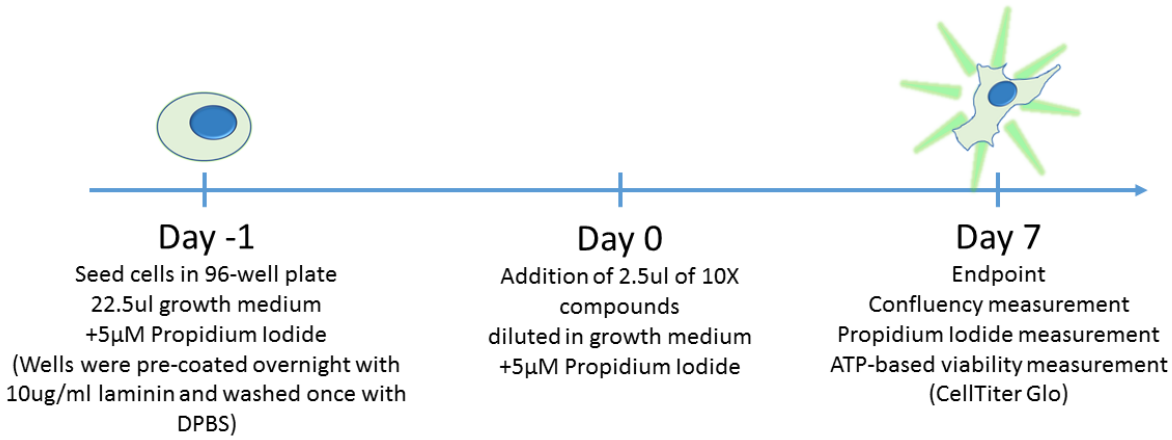


Figure 1. The timeline of this experiment.

Detailed protocol:

Medium composition

Tumour Stem Medium (TSM) Base

50% Neurobasal-A Medium (1X) (Thermofisher 10888022)

50% D-MEM/F-12 (1X) (Thermofisher 11320074)

10mM HEPES Buffer (Thermofisher 15630056)

1mM Sodium Pyruvate MEM (Thermofisher 11360039)

0.1mM MEM Non-Essential Amino Acids Solution (Thermofisher 11140035)

1X GlutaMAX-I Supplement (Thermofisher 35050038)

1X Antibiotic-Antimycotic (Thermofisher 15240062)

Additional components for TSM complete

1X B-27 Supplement Minus Vitamin A (Thermofisher 12587010)

20ng/ml Recombinant Human EGF (Peprotech AF-100-15)

20ng/ml Recombinant Human FGF-basic (Peprotech AF-100-18B)

10ng/ml H-PDGF-AA (Peprotech 100-13A)

10ng/ml H-PDGF-BB (Peprotech 100-14B)

2µg/ml Heparin (Sigma H3149-10KU)

Propidium iodide

5mM stock in water

5uM final concentration (1000X)

[P4864-10ML Sigma-Aldrich] store at 4 degree Celsius

Cell lines to be used

		Per well
SU-DIPG-IV	DIPG mutant ALK2	500
SU-DIPG-XXI	DIPG mutant ALK2	2500
HSJD-DIPG-07	DIPG mutant ALK2	500
SU-DIPG-VI	DIPG wild-type ALK2	1000

Wells coated with 1:100 (0.01mg/ml Laminin at 37 degree Celsius overnight)															
Plate 1		M4K2009 (214)			M4K3003 (135)			M4K3007 (139)			M4K2040 (65)					
		1	2	3	4	5	6	7	8	9	10	11	12		(1000X stock)	Dilution in DMSO
	A													10uM	10mM	24ulparent+36
	B													5uM	5mM	30ulA+30
	C													1uM	1mM	12ulB+48
	D													0.5uM	0.5mM	30ulC+30
	E													0.1uM	0.1mM	12ulD+48
	F													0.01uM	0.01mM	10ulE+90
	G													0.0001uM	0.0001mM	1ulF+99
	H													DMSO	DMSO	
Plate 2		M4K2117 (152)			M4K2118 (153)			M4K2143 (186)			M4K2149 (203)					
		1	2	3	4	5	6	7	8	9	10	11	12		(1000X)	
	A													10uM	10mM	24ulparent+36
	B													5uM	5mM	30ulA+30
	C													1uM	1mM	12ulB+48
	D													0.5uM	0.5mM	30ulC+30
	E													0.1uM	0.1mM	12ulD+48
	F													0.01uM	0.01mM	10ulE+90
	G													0.0001uM	0.0001mM	1ulF+99
	H													DMSO	DMSO	
Plate 3		M4K2163 (218)			M4K2032 (58)			M4K1055 (28)			M4K1196 (12)					
		1	2	3	4	5	6	7	8	9	10	11	12		(1000X)	
	A													10uM	10mM	24ulparent+36
	B													5uM	5mM	30ulA+30
	C													1uM	1mM	12ulB+48
	D													0.5uM	0.5mM	30ulC+30
	E													0.1uM	0.1mM	12ulD+48
	F													0.01uM	0.01mM	10ulE+90
	G													0.0001uM	0.0001mM	1ulF+99
	H													DMSO	DMSO	

Before starting (Day 1)

1) Prepare 100ml TSM complete and store at 4 degree Celsius.

2) Pre-coat wells of clear-bottom black 96-well plate (Greiner 655090) with 50µl of laminin diluted 1:100 in DPBS (final concentration of 0.01mg/ml) overnight at 37 degree Celsius.

3) Prepare 5mM (1000X) Propidium Iodide stock solution for dead cell staining by reconstituting lyophilised powder in appropriate volume of sterile water

Preparation of single cell suspension (Day 2)

For suspension DIPG cells (SU-DIPG-VI and HSJD-DIPG-007)

1) Collect all cells in a 50ml tube.

2) Pellet cells at 300xG for 5 minutes.

3) Save supernatant in a separate tube.

4) Resuspend cell pellet in 1ml of TrypLE express and incubate at 37 degree Celsius for 5 minutes.

5) Quench trypsin activity by adding supernatant to the cells resuspended in TrypLE.

6) Using a 5ml serological pipette triturate 5 times to break up cell clumps.

7) Pellet cells at 300xG for 5 minutes.

8) Discard supernatant and dislodge cell pellet by tapping.

9) Resuspend cells in 10ml TSM-complete.

10) Pass cell suspension through 50µm cell strainer to remove clumps.

11) Count cells and adjust to desired concentration.

For adherent DIPG cells (SU-DIPG-IV)

1) Transfer medium and dislodged cells to a 50ml tube.

2) Incubate flask with TrypLE express at 37 degree Celsius for 5 minutes (1ml for T25 flask and 3ml for T75 flask).

3) Add saved medium and dislodged cells back to the flask and collect everything in a 50ml tube.

4) Using a 5ml serological pipette triturate 5 times to break up cell clumps.

5) Pellet cells at 300xG for 5 minutes.

6) Discard supernatant and dislodge cell pellet by tapping.

7) Resuspend cells in TSM-complete.

8) Pass cell suspension through cell strainer to remove cell clumps.

9) Count cells and adjust to desired concentration.

Seeding cells into 96-well plate

1) Add 1µl of 5µM Propidium Iodide per ml of single cell suspension.

2) Wash wells once with 100µl DPBS right before adding cells.

3) Seed 90ul of cell suspension per well.

4) Incubate plates in a lunchbox with extra humidification to minimise evaporation in 37 degree Celsius incubator with 5% carbon dioxide.

Adding compounds to cells (Day 3)

1) Dilute compounds to 10X desired final concentration in TSM-complete supplemented with 5µM Propidium Iodide.

2) Add 10ul of 10X compounds per well.

Image-based quantification (Day 9 – after 7 days treatment)

1) Acquire images of all of the wells using Celigo imaging cytometer.

2) Imaging channels:

Brightfield: confluence

Red: Propidium Iodide (dead permeable cells)

3) Image all wells and perform the appropriate software analysis

End point CellTiter Glo 3D ATP measurement (Day 9 – after 7 days treatment)

1) Thaw CellTIter Glo reagent in 4 degree Celsius fridge overnight and aliquot into 10ml fractions. Extra tubes were frozen again at -20 degree Celsius.

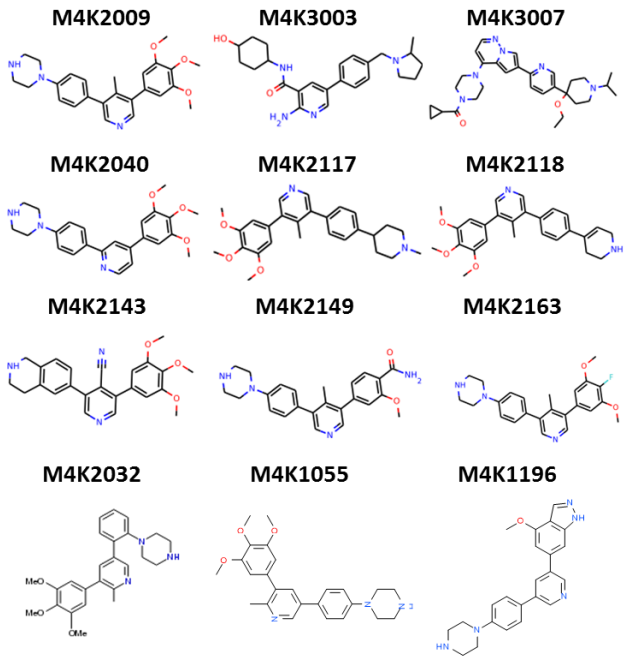
2) Add 100µl of room temperature CellTiter-Glo reagent to each well and shake at 200rpm for 5 minutes.

4) Incubate in the dark at room temperature for further 20 minutes without shaking.

5) Measure total luminescent signal using Clariostar plate reader.

6) EC50 values were determined by fitting the CellTiter Glo signal (RLU) against compound concentration in GraphPad Prism using [Iinhibitor] vs. response (three parameters) non-linear regression.

Results:



Compounds	Description
M4K2009	Best characterised
M4K3003	Patent literature
M4K3007	Patent literature
M4K2040	Negative control
M4K2117	More potent in ALK2 nanoBRET assay
M4K2118	More potent in ALK2 nanoBRET assay
M4K2143	More potent in ALK2 nanoBRET assay
M4K2149	Methoxyl to amide analog
M4K2163	Methoxyl to fluoro analog
M4K2032	Additional negative control
M4K1055	K03833/LDN-214117 – previously tested in literature
M4K1196	Less potent than M4K2009

DIPG patient cell line	Histone H3	ACVR1
SU-DIPG-VI	H3F3A-K27M	WT
SU-DIPG-IV	HIST1H3B-K27M	G328V
HSJD-DIPG-07	H3F3A-K27M	R206H

Figure 2. Chemical structures of the compounds tested in this experiment as well as the reasons they were selected (top table). The histone H3 and ACVR1/ALK2 mutation status of the different DIPG patient-derived cell lines is shown in bottom table.

SU-DIPG-VI (ALK2-WT)	ALK2 IC50 (nM)	ALK5 IC50 (nM)	Efficacy EC50 (nM)	95% CI (profile likelihood)	Reduction at 10000nM (% of DMSO)
M4K2009	48	3182	>10000		78
M4K3003	29	>10000	>10000		13
M4K3007	29	>10000	>10000		15
M4K2040	>5000	7970	>10000		100
M4K2117	31	3404	>10000		50
M4K2118	45	7532	>10000		85
M4K2143	33	2599	>10000		65
M4K2149	56	1365	>10000		5
M4K2163	98	7475	3356	2467 to 4718	100
M4K2032	>5000	>10000	>10000		41
M4K1055	251	>10000	>10000		87
M4K1196	244	1748	4467	2711 to 8107	100

HSJD-DIPG-007 (ALK2-R206H)	ALK2 IC50 (nM)	ALK5 IC50 (nM)	Efficacy EC50 (nM)	95% CI (profile likelihood)	Reduction (% of DMSO)
M4K2009	48	3182	119	93.32 to 149.7	100
M4K3003	29	>10000	>10000		40
M4K3007	29	>10000	>10000		18
M4K2040	>5000	7970	432	339.3 to 546.4	100
M4K2117	31	3404	1107	965.3 to 1272	100
M4K2118	45	7532	502	427.4 to 587.6	100
M4K2143	33	2599	1660	1487 to 1858	99
M4K2149	56	1365	3045	2489 to 3781	99
M4K2163	98	7475	28	23.33 to 34.59	100
M4K2032	>5000	>10000	>10000		78
M4K1055	251	>10000	560	475.2 to 659.1	100
M4K1196	244	1748	188	141.2 to 247.9	100

SU-DIPG-IV (ALK2-G328V)	ALK2 IC50 (nM)	ALK5 IC50 (nM)	Efficacy EC50 (nM)	95% CI (profile likelihood)	Reduction (% of DMSO)
M4K2009	48	3182	4315	2360 to 9286	100
M4K3003	29	>10000	>10000		-6
M4K3007	29	>10000	>10000		-49
M4K2040	>5000	7970	>10000		100
M4K2117	31	3404	>10000		89
M4K2118	45	7532	>10000		90
M4K2143	33	2599	>10000		77
M4K2149	56	1365	>10000		10
M4K2163	98	7475	354	221.4 to 558.4	100
M4K2032	>5000	>10000	>10000		18
M4K1055	251	>10000	>10000		98
M4K1196	244	1748	6034	3065 to 15261	100

Figure 3. Efficacy of the compounds assayed compounds. EC50, 95% confidence interval of the EC50 values and maximal observed reduction in viability are shown for each compounds.

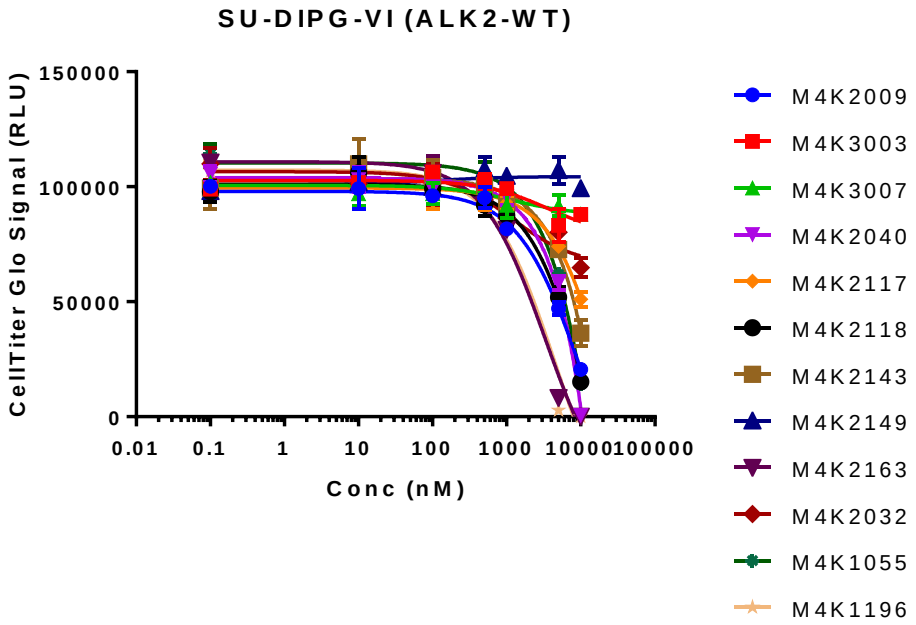


Figure 4. EC50 curves for SU-DIPG-VI cells treated with dilution series of each compound for 7 days.

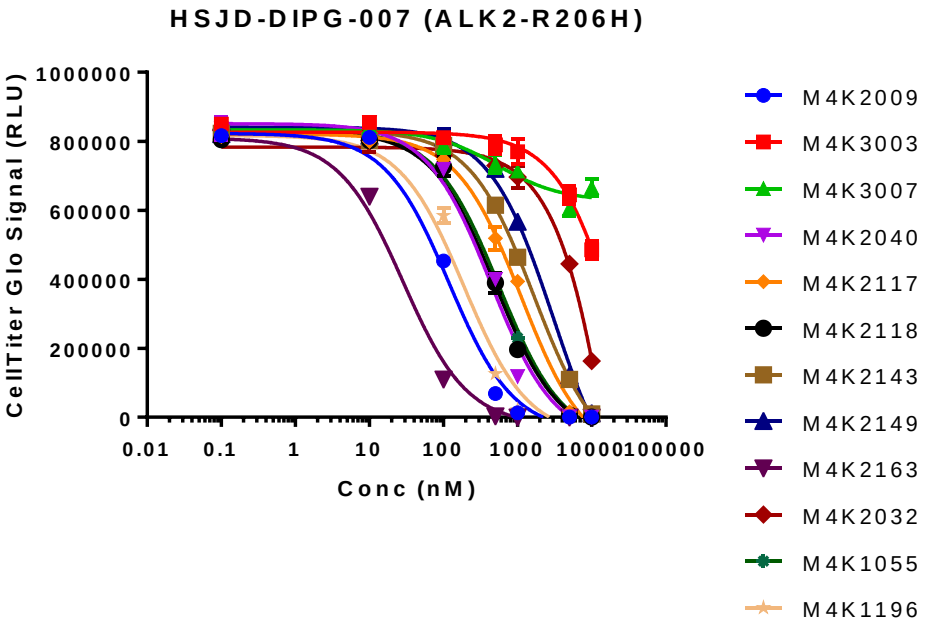


Figure 5. EC50 curves for HSJD-DIPG-007 cells treated with dilution series of each compound for 7 days.

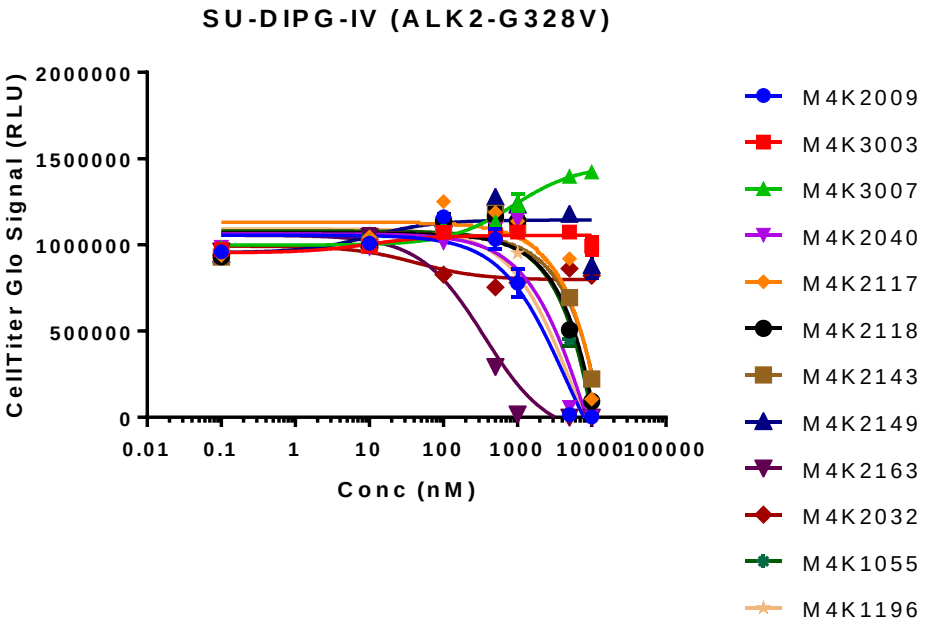


Figure 6. EC50 curves for SU-DIPG-IV cells treated with dilution series of each compound for 7 days.

Findings:

1) HSJD-DIPG-007 was sensitive toward most of the M4K compounds.

2) SU-DIPG-IV was not sensitive to most of the compounds.

3) M4K3003 and M4K3007 which were based on patent literature were not efficacious despite having decent potency towards ALK2.

4) M4K2009 was reduced the viability of DIPG lines with ALK2 mutation while M4K2163 showed efficacy in all of the DIPG cell lines.

In a nut shell:

More compounds still needs to be evaluated in more DIPG patient-derived cell lines in order to attempt to better establish relationship between ALK2 IC50, compound chemical structure and efficacy in reducing the viability of DIPG cells.