Evaluating the inhibition of ALK2 phosphorylation of SMAD1/5 by M4K lead compounds in DIPG patient-derived cells (SU-DIPG-IV, HSJD-DIPG-007, HSJD-DIPG-018 and SU-DIPG-XXI)

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Background:

The binding potency of M4K compounds to ALK2 has been assayed in biochemical assay and cellular assays in HEK293 or C2C12 myoblast cell lines. However, the potency of ALK2 inhibition by M4K compounds has not determined directly in DIPG patient-derived cell lines. While no major deviation from existing assay data is expected, direct experimental evidence is essential.

Experimental design:

DIPG cells will be trypsinized and resuspended in TSM-base medium without any growth factor for one-hour starvation. Subsequently, equal volume of TSM-base with 2X Activin A (200ng/mL) and M4K compounds or DMSO vehicle control will be added. After one-hour treatment, the cells will be pelleted and lysed in buffer with protease and phosphatase inhibitors for Western Blot analysis.

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)

Cell lysis buffer

Use whole cell lysis buffer without EDTA
50mM Tris-HCl pH7.4
150mM NaCl
1% Triton-X100
1 Protease inhibitor cocktail tablet per 10mL
25mM NaF
2mM Sodium Orthovannadate
1:1000 Benzonase (1ug/mL final concentration)

Cell lines to be used

DIPG line	ALK2 status	Histone H3 status
SU-DIPG-IV	G328V	K27M
HSJD-DIPG-007	R206H	K27M
HSJD-DIPG-018	R258G	K27M
SU-DIPG-XXI	G328W	K27M

Antibodies used

Antibody	Catalog no.	Dilution
Phospho-Smad1/5 (Ser463/465)	9516 (Cell Signalling)	1:1000
SMAD1	9743S (Cell Signalling)	1:1000
GAPDH	AM4300 (Life Technologies)	1:10000
Anti-rabbit-secondary-HRP	A6667-1ML (Sigma)	1:1000
Anti-mouse-secondary-HRP	GTX213111-01 (Insight Biotechnologies)	1:5000

Cell treatment

1. Cells were trypsinized, resuspended in TSM-base and 1mL cell suspension was seeded into each well of 96-well 2mL block.
2. After 1 hour incubation at 37 degree Celsius with 5% CO2, 1mL of TSM-base with 200ng/mL Activin A and double the desired M4K compound concentration or DMSO vehicle control was added to each well. Content of each well was mixed well by pipetting up and down a 3 times.
3. After 1 hour of treatment at 37 degree Celsius with 5% CO2, the 96-well block was centrifuged at 2000xG for 5 minutes at 4 degree Celsius.
4. 96-well block was placed on ice and most of supernatant was removed carefully without disturbing the cell pellets using a multichannel aspirator. Small amount of supernatant was left behind to not risk losing the cell pellets.

Sample processing

1. Cell pellets were resuspended in 200uL of PBS buffer and transferred to a 96-well PCR plate and kept on ice.
2. PCR plate was centrifuged at 2000xG for 5 minutes at 4 degree Celsius and supernatant was carefully removed using multichannel pipette.
3. Cell pellets were resuspended in 100uL of whole cell lysis buffer and kept on ice for 20 minutes.
4. Total protein concentration in each sample was quantified using Biorad DC protein kit.
5. Lysates were diluted with appropriate volume of whole cell lysis buffer to the same protein concentration.

Western blot

1. Cell lysates were boiled in sample buffer and separated in SDS-PAGE.
2. Proteins were transferred to PVDF membrane using Biorad Criterion wet transfer system for 1 hour.
3. After TBS-T rinse and 1 hour blocking in TBS-T 3% BSA, membrane was incubated with primary antibodies diluted in TBS-T 3% BSA overnight at 4 degree Celsius with agitation.
4. Next day, membrane was rinsed thrice for 15 minutes in TBS-T and incubated with secondary antibody diluted in TBT-T 3% milk.
5. After rinsing thrice for 15 minutes in TBS-T, membrane was imaged in ImageQuant LAS-4000 using SuperSignal West Pico PLUS Chemiluminescent Substrate (34580 Life Technologis).

Results:

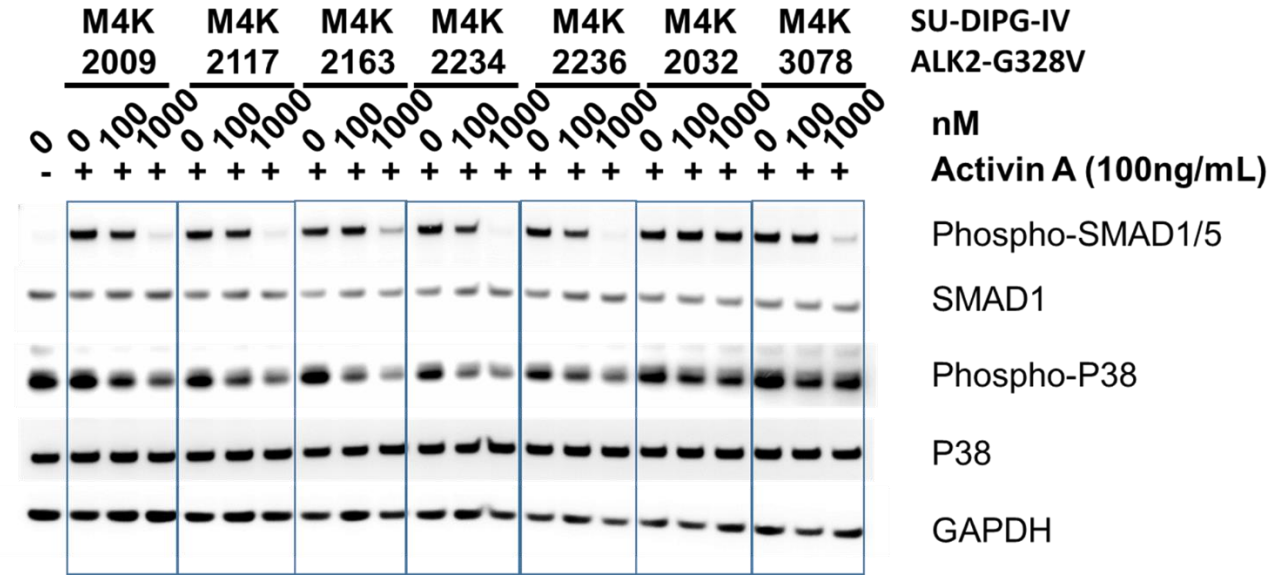

Figure 1. Inhibition of ALK2 phosphorylation of SMAD1/5 by M4K lead compounds in SU-DIPG-IV DIPG patient-derived cell line. Phosphorylated proteins were detected in Western Blot using specific antibodies.

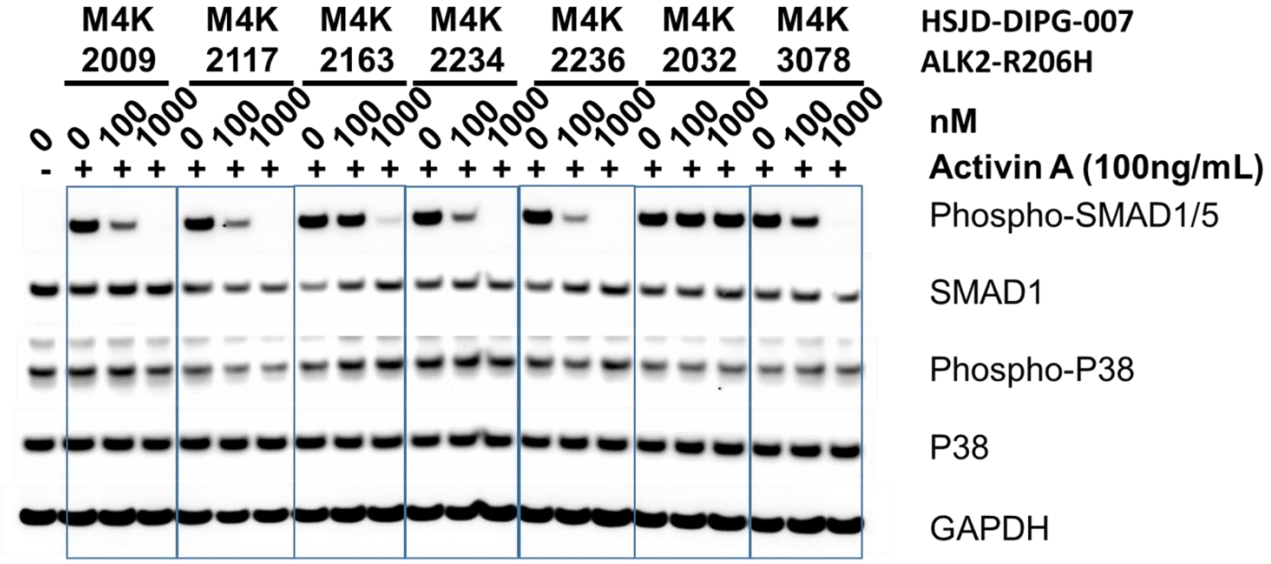

Figure 2. Inhibition of ALK2 phosphorylation of SMAD1/5 by M4K lead compounds in HSJD-DIPG-007 DIPG patient-derived cell line. Phosphorylated proteins were detected in Western Blot using specific antibodies.

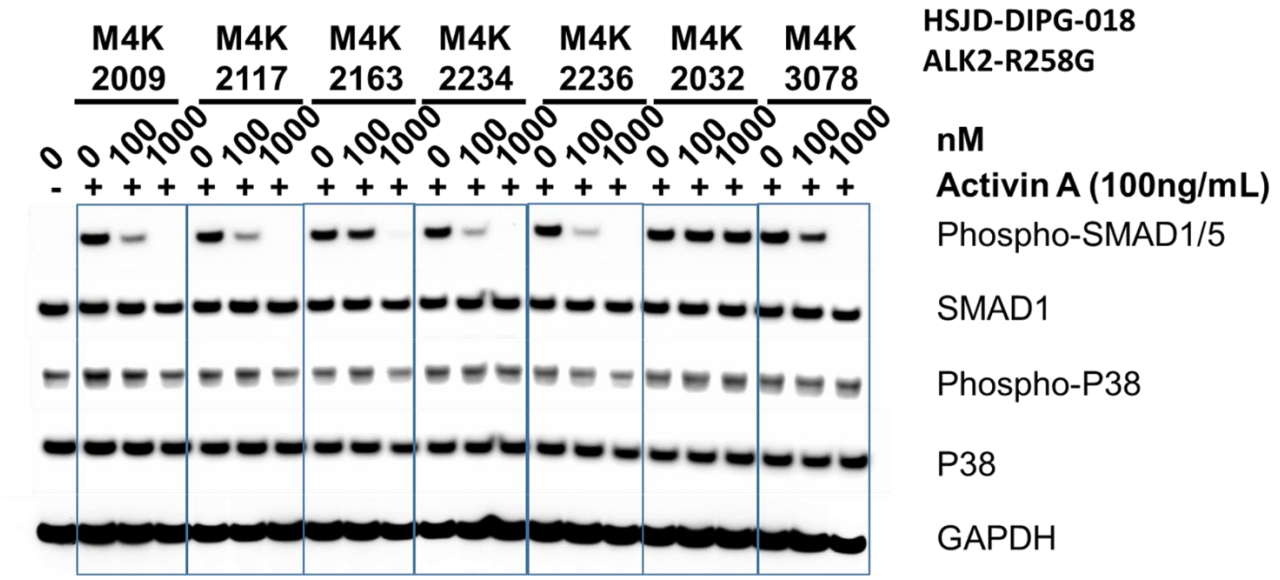

Figure 3. Inhibition of ALK2 phosphorylation of SMAD1/5 by M4K lead compounds in HSJD-DIPG-018 DIPG patient-derived cell line. Phosphorylated proteins were detected in Western Blot using specific antibodies.

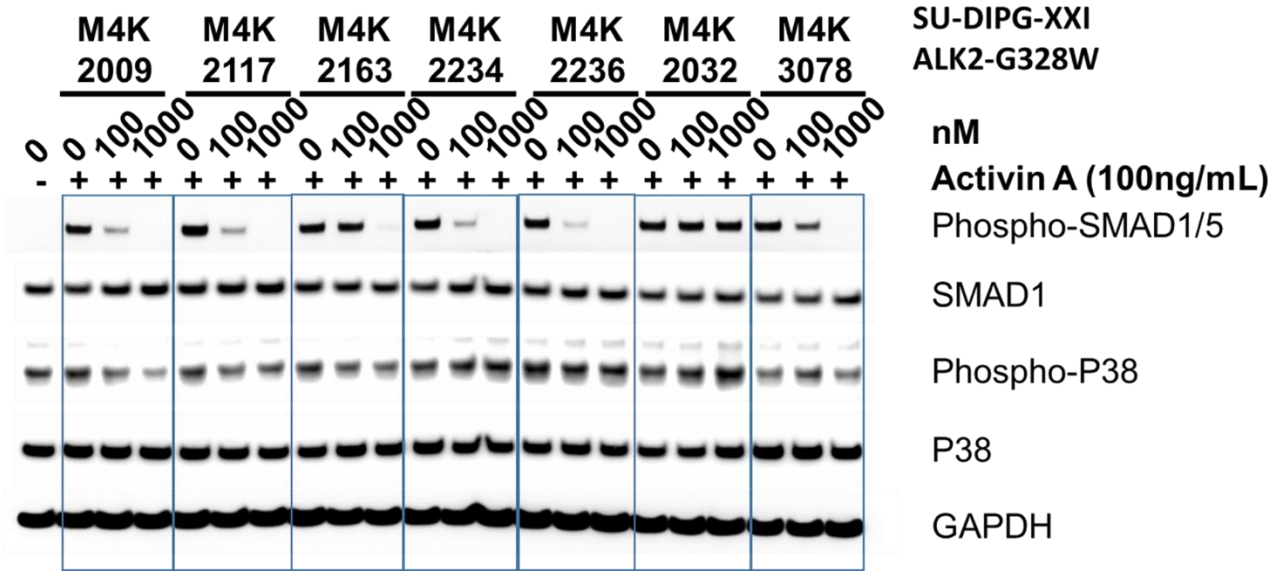

Figure 4. Inhibition of ALK2 phosphorylation of SMAD1/5 by M4K lead compounds in SU-DIPG-XXI DIPG patient-derived cell line. Phosphorylated proteins were detected in Western Blot using specific antibodies.

Findings:

1) All M4K lead compounds inhibited ALK2 kinase activity in DIPG cells.
2) M4K3078 appeared to be less potent even though its potency was highest when measured in biochemical assay and cellular target engagement assay in HEK293. Additional experiments are necessary to verify this result before hypothesis of the underlying reason can be developed.

In a nut shell:

On target ALK2 inhibition in DIPG patient-derived cell lines by M4K lead compounds had been verified using Western Blot.