

## Efficacy of inhibitor on wild-type ACVR1 and R206H mutant in C2C12 cells (by DLA)

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### Background:

There are concerns that compounds that are effective in inhibiting ALK2 by occupying its ATP-binding pocket might have reduced efficacy against mutant ALK2. That will be undesirable since the compounds should also target the mutant gain-of-function ALK2 in DIPG cells. The following experiment takes advantage of the fact that ALK2-R206H mutant confers a neofunction of being activated by Activin A (not activating towards wild-type ALK2). Therefore, using Activin A as the stimulation ligand, the effectiveness of inhibition on ALK2-R206H can be specifically determined. BMP6 which activates both wild-type ALK2 and ALK2-R206H is also included.

### Experimental details:

#### Day 1

5x10<sup>4</sup> C2C12 cells were seeded into each well of 2 6-well plates

6-well layout (for transfection)											
ACVR1 wild-type			ACVR1R206H			5x10 <sup>4</sup> cells per well					
						BRE=4000ngX12=48,000ng			ACVR1-C-FLAG=4000ngX5=20,000ng		
						RLTK=1000ngX12=12,000ng			ACVR1R206H-C-FLAG=4000ngX5=20,000ng		
						Lipofectamine=10X12=120ul			pCDNA=4000ngX2=8,000ng		
						OptiMEM=250ulX12X2=6,000ul					
96-well layout (for DLA)											
No LDN			LDN 0.01ug			LDN 0.1ug			LDN 1ug		
1	2	3	4	5	6	7	8	9	10	11	12
A											27x10 <sup>3</sup> cells needed for ACVR1 wild-type and R206H
B											ACVR1 wild-type Activin A 100ng/ml
C											ACVR1 R206H Activin A 100ng/ml
D											ACVR1 wild-type BMP6 250ng/ml
E											ACVR1 R206H BMP6 250ng/ml
F											ACVR1 wild-type BMP7 250ng/ml
G	pCDNA		ACVR1 wild-type		ACVR1R206H						ACVR1 R206H BMP7 250ng/ml
H											No stimulation/no LDN

#### Day 2

Cells in 6-well plates were transfected with RLTK (1000ng), BRE (4000ng) and either pCDNA control (4000ng), ACVR1-C-FLAG (4000ng) or ACVR1R206H-C-FLAG (4000ng)

#### Day 3

Cells were trypsinized and reseeded at 1x10<sup>3</sup> cells per well in 96-well plate  
Excess cells were lysed for WB to control for ACVR1 transfection/expression

#### Day 4

Cells were starved in DMEM 1%FBS for 6 hours

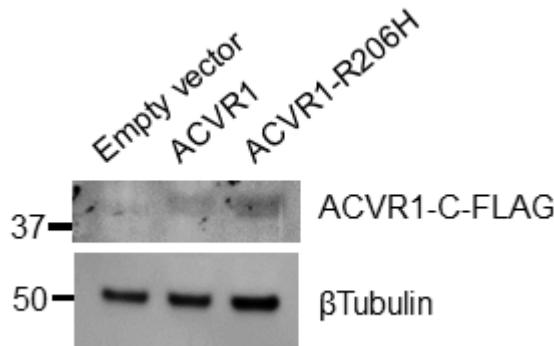
Cells were stimulated with Activin A (100ug/ml) or BMP7 (100ug/ml)

#### Day 5

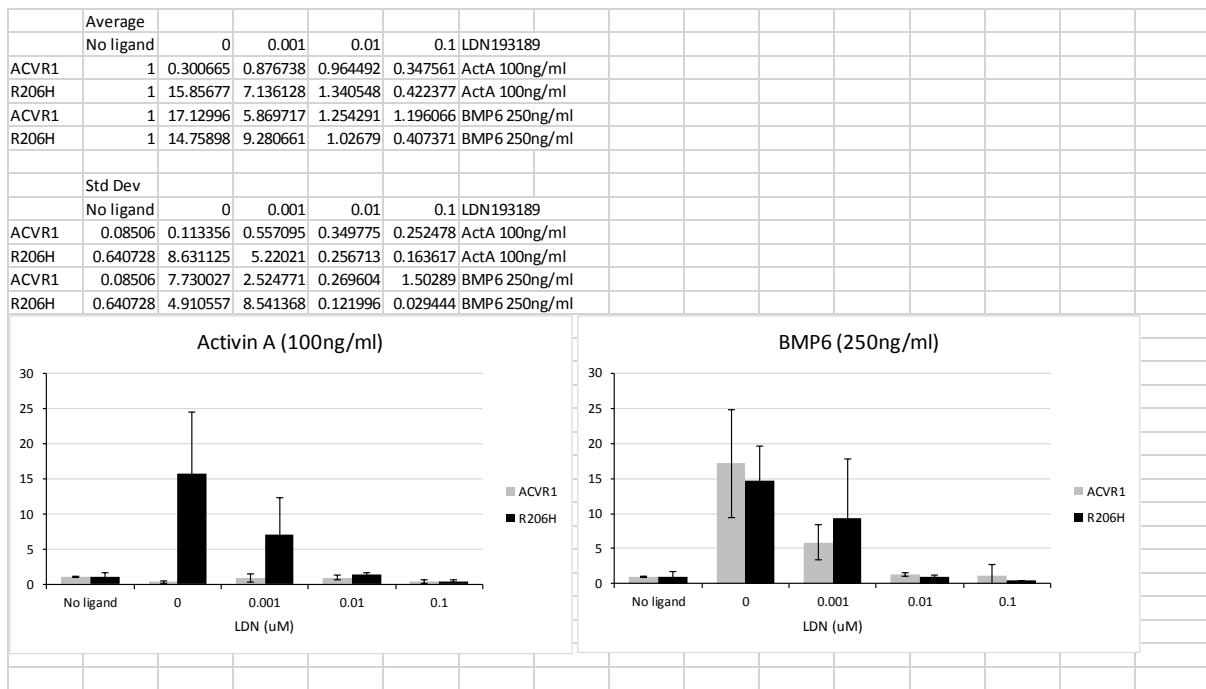
Cells were washed once in PBS, lysed in 1XPLB for 30 minutes and luciferase signals were measured in Pherastar FSX with two injectors as per kit instruction. (Promega Dual-Luciferase Reporter Assay System #E1060)

**Results:**

Wild-type ALK2 and ALK2-R206H expression control



Firefly luc	0	0	0	0.001	0.001	0.001	0.01	0.01	0.01	0.1	0.1	0.1	LDN 193189 (uM)
ACVR1	1415	1559	968	999	573	486	475	519	1661	3495	744	855	
R206H	99403	9461	91135	41182	15011	2553	1448	1626	3705	1001	671	1738	
ACVR1	89602	47458	94122	82041	26894	20627	3326	6344	2914	9832	716	644	
R206H	34676	38788	34053	78079	515	14257	429	478	2395	1398	1122	1368	
	424	435	412	474	458	427	461	520	837				
Renilla luc	0	0	0	0.001	0.001	0.001	0.01	0.01	0.01	0.1	0.1	0.1	LDN 193189 (uM)
ACVR1	7949	5111	6194	5841	631	619	544	670	4121	8079	9825	3706	
R206H	7475	2486	6696	5142	3303	2012	1864	2131	3506	5825	1754	6590	
ACVR1	5547	8045	6549	18267	4668	9256	3595	5988	4270	4741	4789	2013	
R206H	2827	3778	5624	6638	665	2621	744	654	3515	5236	4011	5645	
	603	594	646	614	652	655	522	587	4983				
Firefly/Re	0	0	0	0.001	0.001	0.001	0.01	0.01	0.01	0.1	0.1	0.1	LDN 193189 (uM)
ACVR1	0.17801	0.305028	0.15628	0.171032	0.908082	0.785137	0.873162	0.774627	0.403058	0.432603	0.075725	0.230707	
R206H	13.29806	3.805712	13.61036	8.008946	4.544656	1.268887	0.776824	0.763022	1.05676	0.171845	0.382554	0.263733	
ACVR1	16.15324	5.899068	14.37197	4.491214	5.761354	2.2285	0.925174	1.059452	0.682436	2.073824	0.149509	0.319921	
R206H	12.26601	10.26681	6.054943	11.76243	0.774436	5.439527	0.576613	0.730887	0.681366	0.266998	0.279731	0.242338	
	0.703151	0.732323	0.637771	0.771987	0.702454	0.651908	0.883142	0.88586	0.167971				
Relative t	0	0	0	0.001	0.001	0.001	0.01	0.01	0.01	0.1	0.1	0.1	LDN 193189 (uM)
ACVR1	0.251148	0.430355	0.220491	0.241304	1.281185	1.107726	1.231917	1.092897	0.568661	0.610346	0.106838	0.325497	
R206H	20.59615	5.894318	21.07984	12.40432	7.038801	1.965262	1.203151	1.181775	1.636718	0.266156	0.592503	0.408472	
ACVR1	22.7901	8.322811	20.27696	6.336513	8.128515	3.144122	1.305299	1.494748	0.962827	2.925894	0.210938	0.451366	
R206H	18.99769	15.90132	9.377946	18.21775	1.199453	8.424784	0.893063	1.132004	1.055305	0.413528	0.433249	0.375336	
	0.691082	0.708783	0.645658	1.089172	0.99107	0.919757	1.367817	1.372028	0.260155				



### Conclusion:

ACVR1-R206H mutant is not more resistant to inhibitor LDN193189 compare to wild-type ACVR1