

Phosphorylation of ABL1 Tyrosine 204 is a Marker of ABL1 Activation

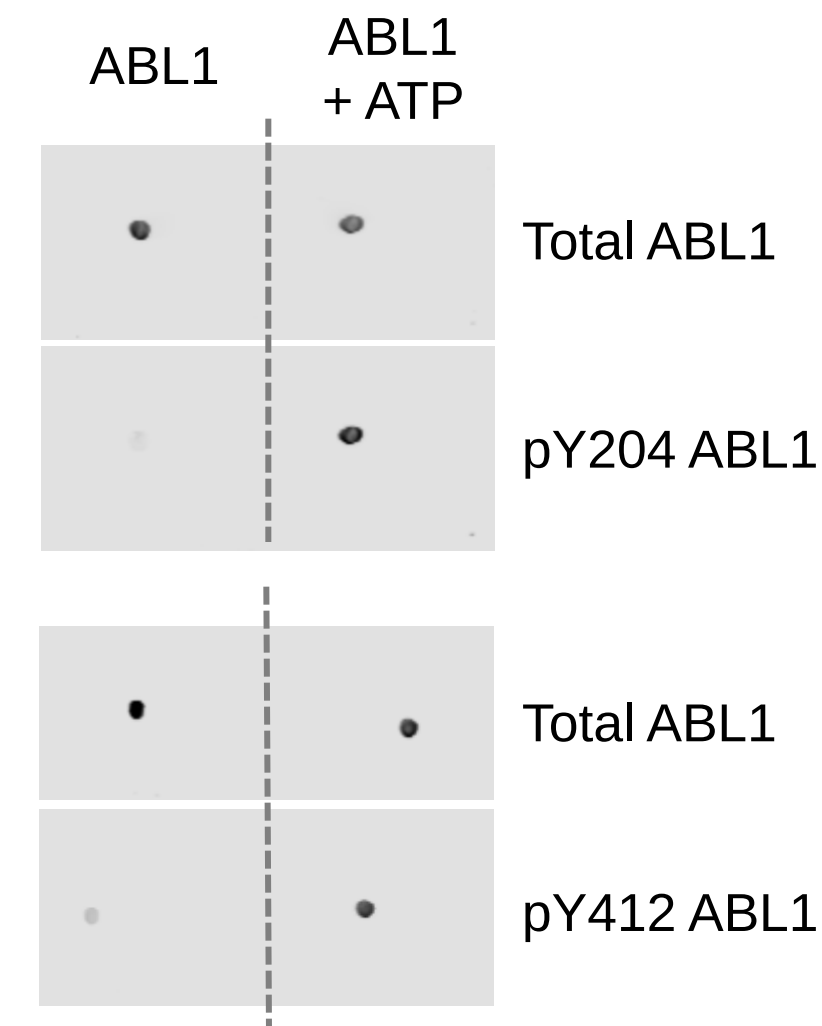
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- ABL1 is a tyrosine kinase activated by oxidative stress, which has been linked to Parkinson's disease [1]. ABL1 has been suggested to phosphorylate α -synuclein [2]. Multiple clinical trials for ABL1 inhibitors in neurodegenerative disease are ongoing.
- Activated ABL1 auto-phosphorylates at tyrosine 245 (Y245) and tyrosine 412 (Y412), and phosphorylation at these residues is used as a measure of ABL1 activation [3].
- ABL1 is expressed at low levels in common cellular models and mouse brain. Detection of total and auto-phosphorylated Y245 / Y412 ABL1 is difficult, limiting their use for both understanding kinase activation in conditions where the protein is not overexpressed, and determining *in vivo* target engagement of ABL1 inhibitors.
- We have identified phosphorylation at tyrosine 204 (Y204) of ABL1 as a biomarker of kinase activation and developed an ultra-sensitive assay for detection of endogenous phosphorylated Y204 ABL1 that offers increased sensitivity over Y245 and Y412 assays.

1. Recombinant ABL1 auto-phosphorylates at pY204 *in vitro*

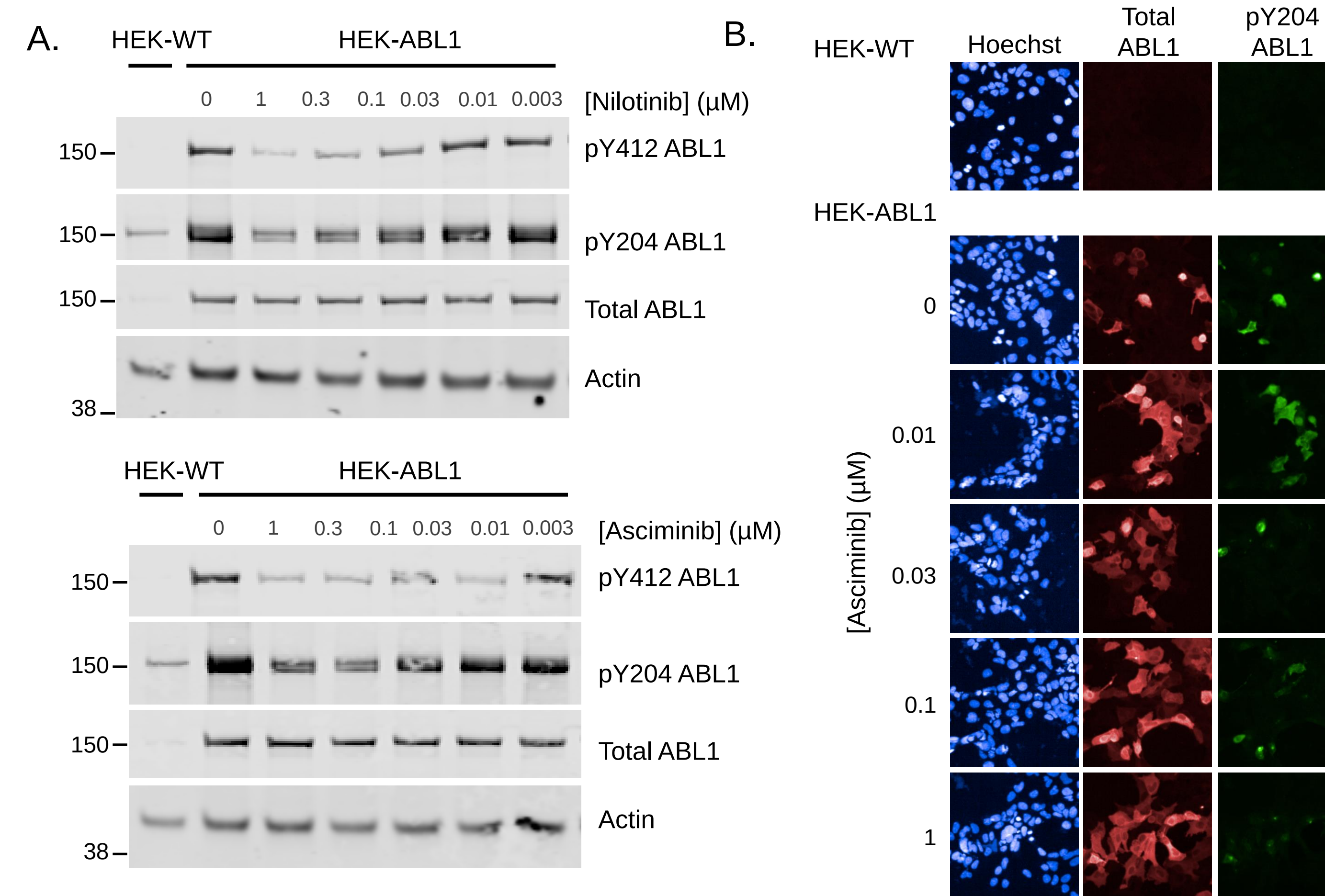
To determine whether active ABL1 auto-phosphorylates at Y204, recombinant ABL1 (amino acids 83-534) was incubated for 1 hr at room temperature in 50 mM HEPES, 10 mM MgCl₂, 0.01 % TX100, and with or without 2 mM ATP. 200 ng recombinant ABL1 was dotted onto nitrocellulose membrane, allowed to dry, and then probed with total and phosphorylated ABL1 antibodies.



pY204 ABL1 and pY412 ABL1 antibodies detected ABL1 incubated with but not without ATP, showing that *in vitro* ABL1 auto-phosphorylates both at Y204 and Y412.

2. Cellular ABL1 overexpression results in auto-phosphorylation, which is modulated by ABL1 orthosteric (nilotinib) and allosteric (asciminib) inhibitors

To investigate whether ABL1 can auto-phosphorylate at Y204 in cells, HEK293 cells were transiently transfected with ABL1 (ABL1 overexpression is sufficient to activate its auto-phosphorylation activity). Cells were treated with ABL1 inhibitors for either 1 hr (A. and C.) or 24 hrs (B.), and levels of phosphorylated ABL1 were determined by western blotting, immunofluorescence, ELISA and MSD assays.



Overexpressed ABL1 was phosphorylated at Y412 and Y204, which was decreased in a concentration-dependent manner by allosteric and orthosteric ABL1 inhibitors.

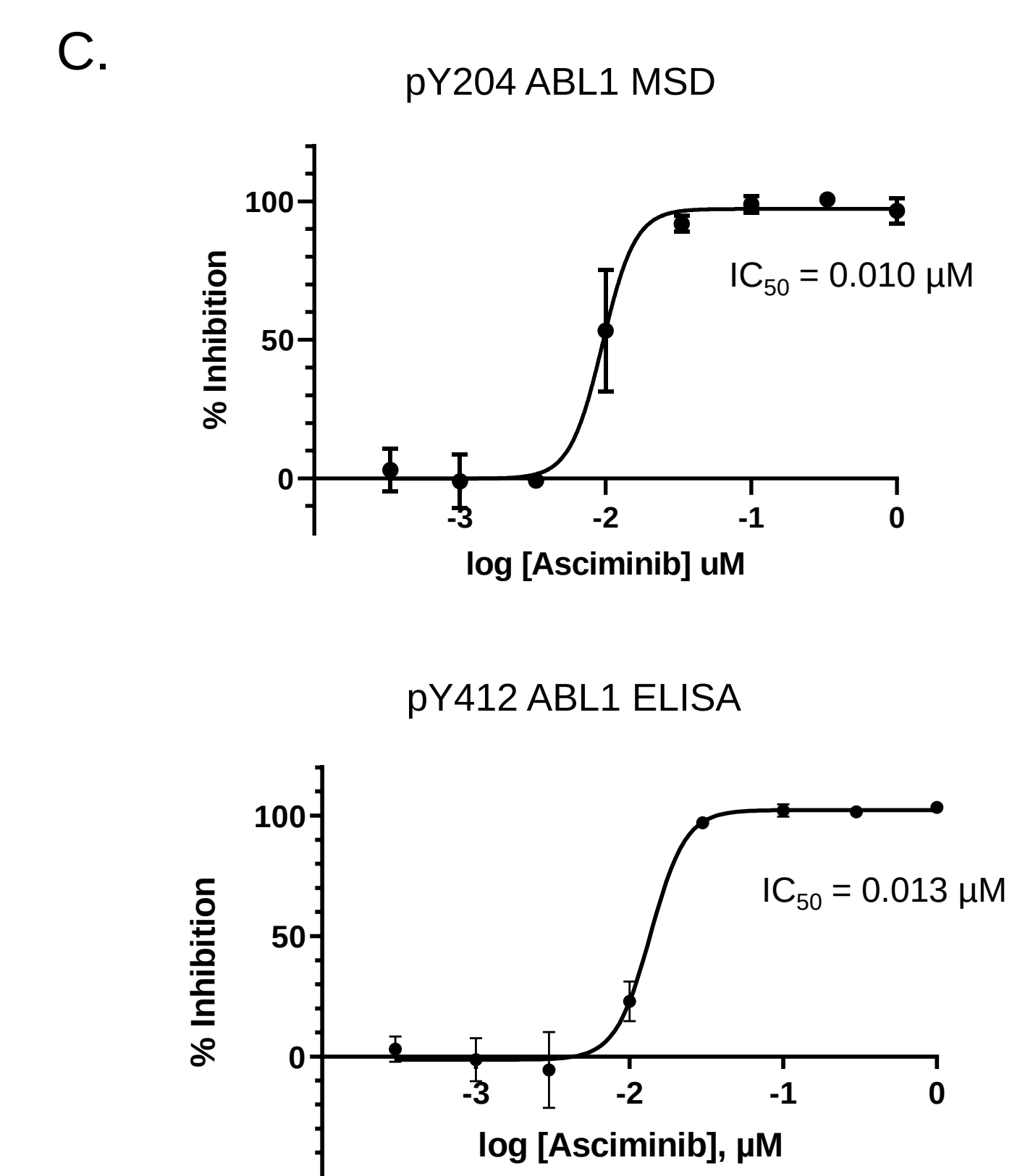
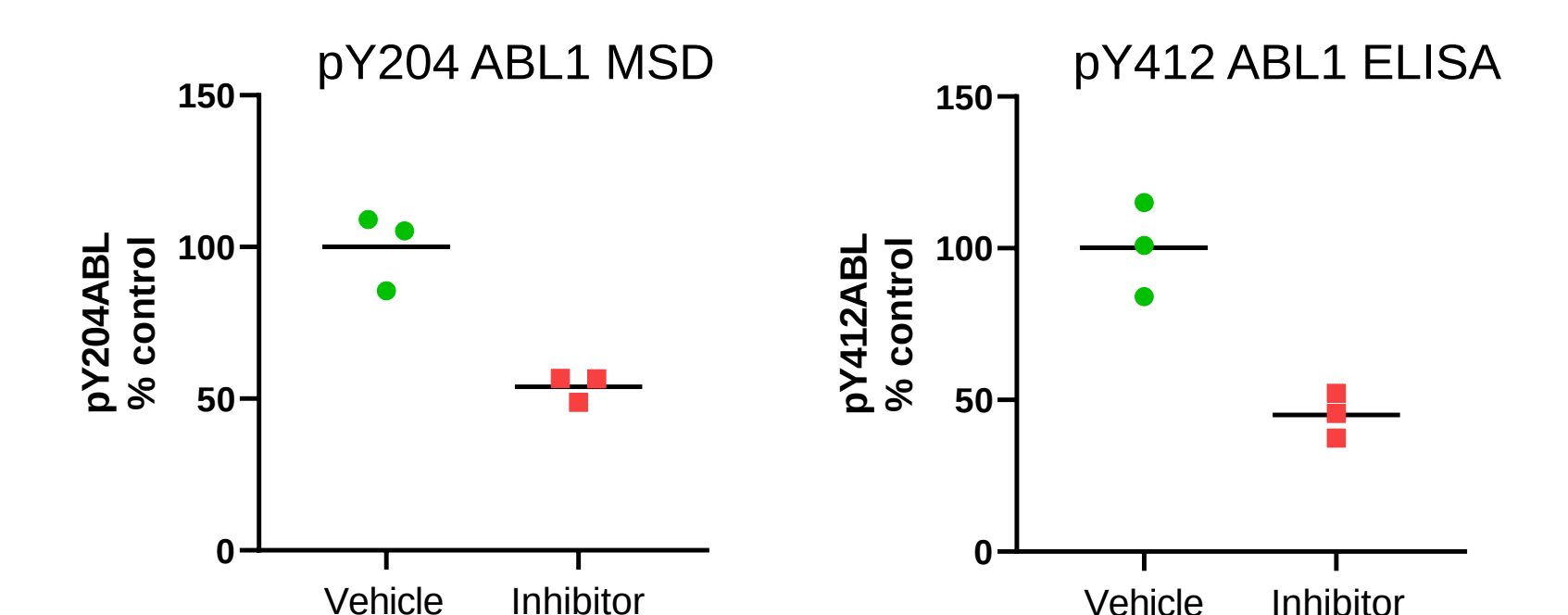
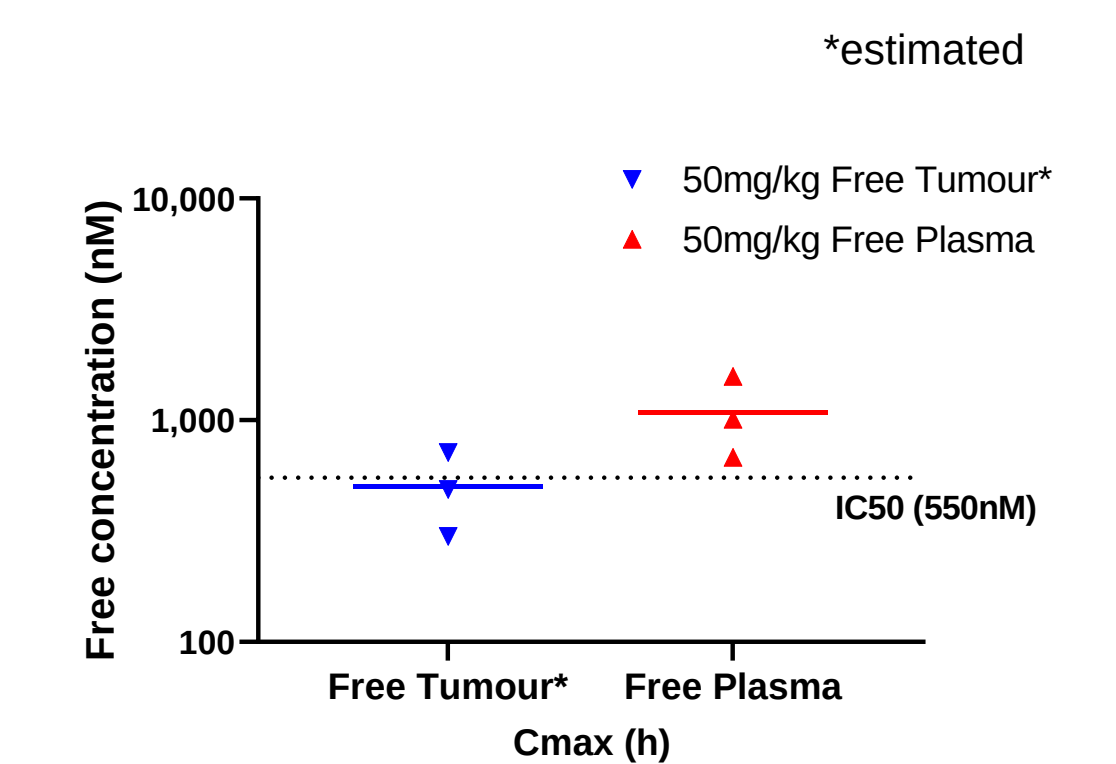


Plate-based immunoassays showed a comparable dose-dependent reduction between pY204 and pY412 ABL1 following treatment with asciminib.

3. pY204 ABL1 is reduced by ABL1 inhibition *in vivo*

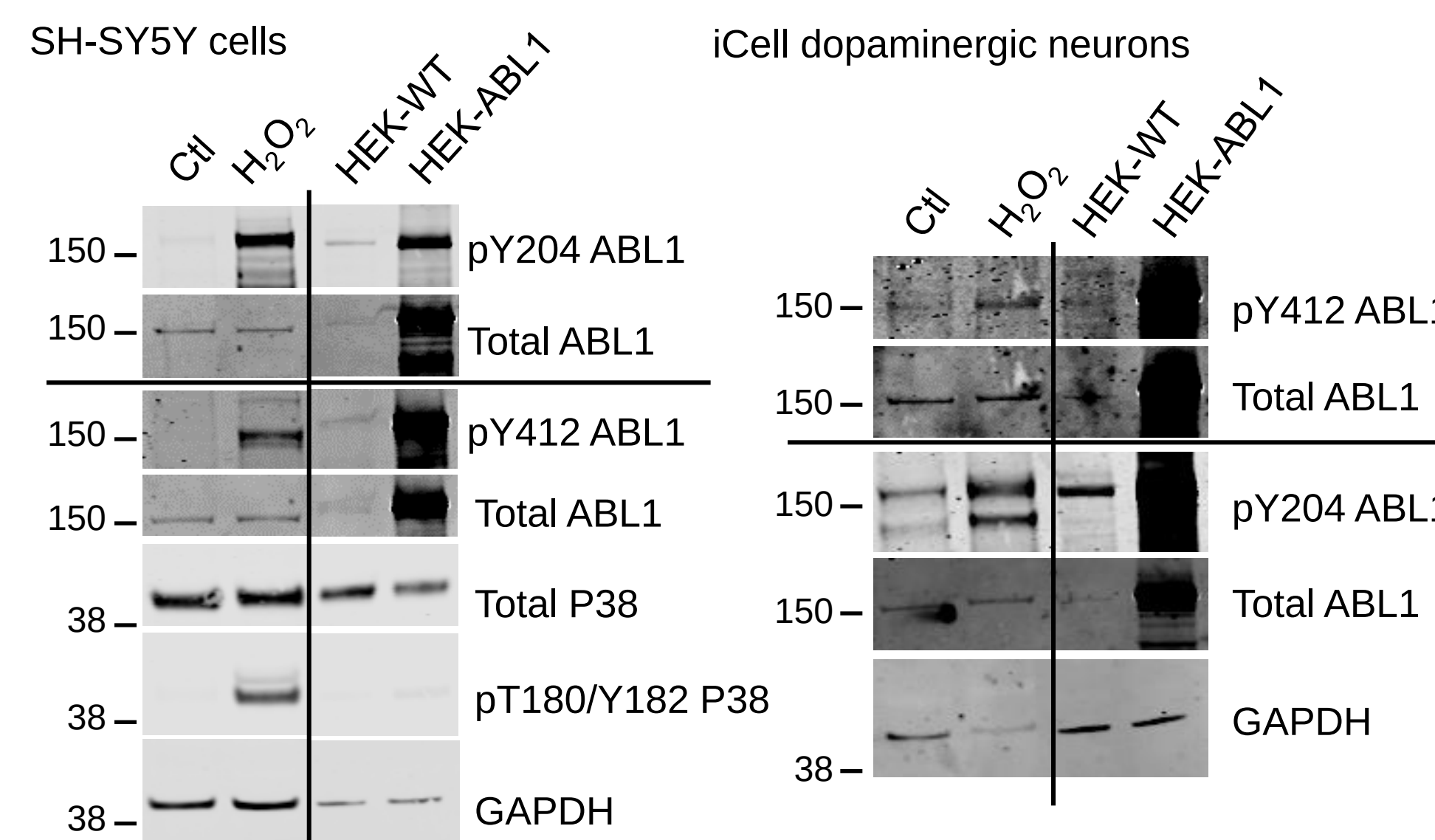
To determine whether pY204 ABL1 can be used as a marker of ABL1 activation *in vivo*, we used a BCR-ABL tumour xenograft mouse model. Male CB17 SCID mice bearing subcutaneous KCL-22 xenografts were given a single oral dose of an ABL1 inhibitor at 50 mg/kg. Animals were euthanised after 2 hrs and tumours immediately collected. Tumour lysates were analysed by ELISA and MSD assays.



pY204 ABL1 and pY412 ABL1 were detected in BCR-ABL tumours. pY204 ABL1 was reduced by ABL1 inhibitors to a similar extent as pY412 ABL1.

4. Oxidative stress increases pY204 ABL1

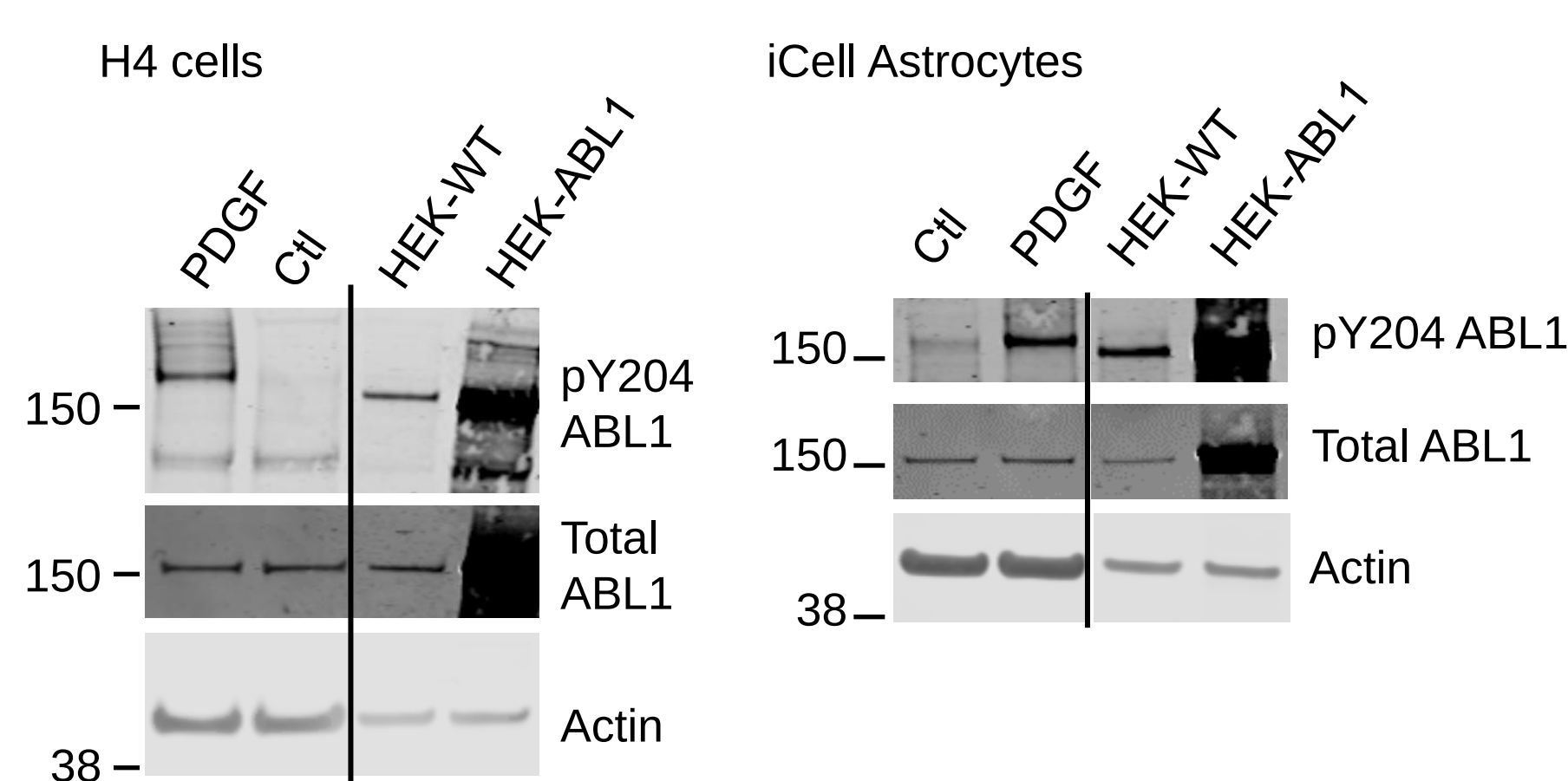
Oxidative stress activates ABL1 signalling pathway. To investigate the physiological relevance of ABL1 Y204 phosphorylation, 30 mM H₂O₂ treatment for 15 minutes was used to induce oxidative stress in SH-SY5Y and iCell dopaminergic neurons. Cells were harvested in 10% SDS lysis buffer and phosphorylation of ABL1 was determined by western blotting.



Phosphorylation of ABL1 at Y412 and Y204, and downstream p38 were increased in response to oxidative stress induced by H₂O₂.

5. PDGF-mediated signalling increases pY204 ABL1

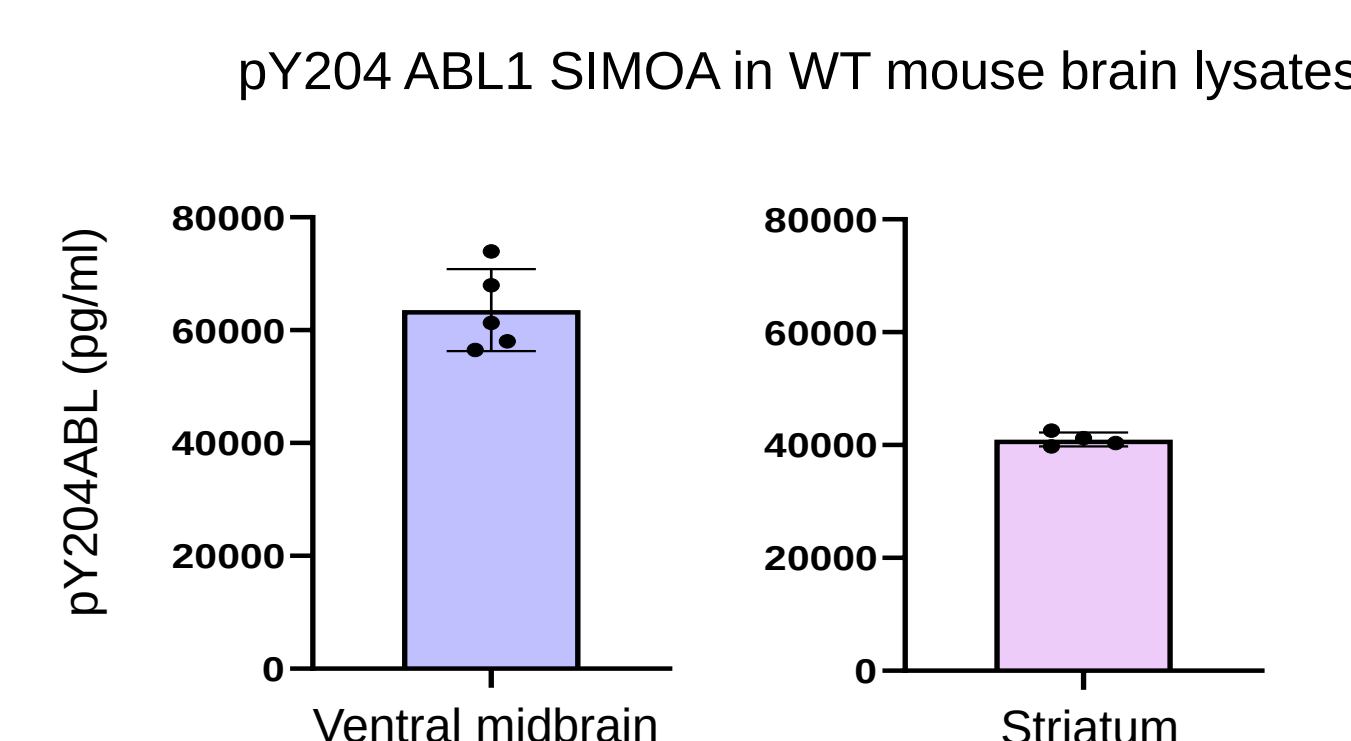
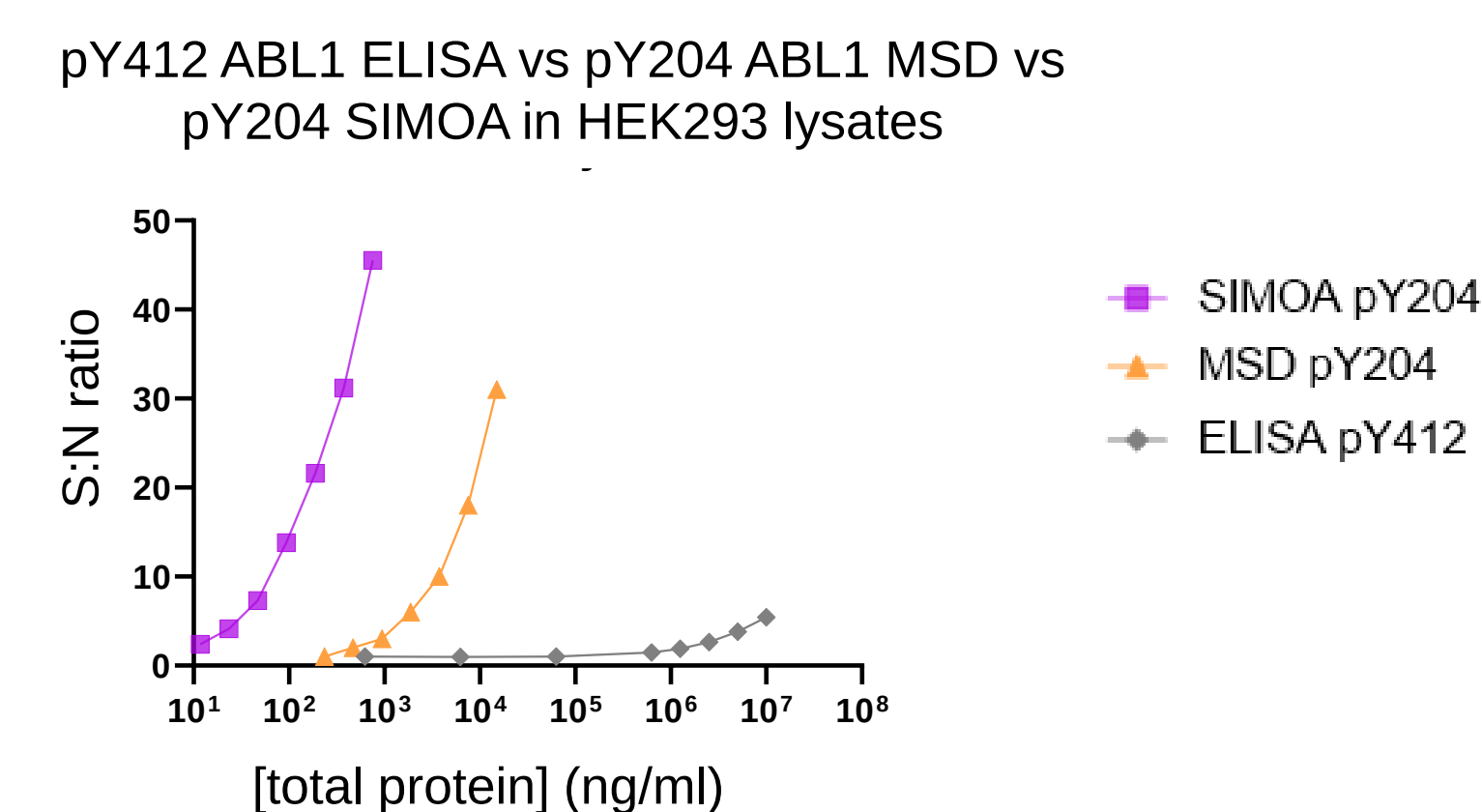
ABL1 signalling pathway is activated by Src family kinases. To investigate whether pY204 ABL1 phosphorylation is increased in response to PDGF-induced SRC kinase activation, H4 cells or iCell astrocytes were stimulated with 12.5 ng/ml recombinant PDGF for 10 minutes. Cells were harvested in 10% SDS lysis buffer and phosphorylation of ABL1 was determined by western blotting.



Phosphorylation of ABL1 at Y204 was increased in response to PDGF signalling.

6. Ultra-sensitive pY204 ABL1 assay

Detection of endogenous pY412 ABL1 in wild-type mouse brain was below the limit of detection using a range of different assays. To study ABL1 role in the brain, we developed an ultra-sensitive assay using the SIMOA platform to quantify the levels of endogenous pY204-ABL1. Assay validation using HEK293 cell lysates transfected with ABL1 showed increased sensitivity for pY204 ABL1 SIMOA compared to pY412 ABL1 ELISA.



pY204 ABL1 was detected and quantified in mouse brain using ultra-sensitive SIMOA assay.

Conclusions

- Auto-phosphorylation of ABL1 at Y204 mirrors the well-documented phosphorylation event at Y412 that occurs upon ABL1 activation.
- Phosphorylation at Y204 and Y412 were modulated by orthosteric and allosteric ABL1 inhibitors in a dose-dependent manner.
- Phosphorylation at Y204 was increased when the ABL1 pathway was activated via PDGF receptor activation or oxidative stress.
- pY204-ABL1 was detected in human BCR-ABL xenograft mouse model, and modulated by ABL inhibition to a similar degree as pY412-ABL1.
- Phosphorylation of ABL1 at Y204 can be detected in healthy mouse brain tissue with increased sensitivity using a SIMOA assay. The assay will have utility in the evaluation of Astex proprietary ABL1 inhibitors that have been designed using our fragment and structure-based drug discovery approaches.

References

- [1] T. Dawson & V. Dawson (2014) Neurodegener Dis 13:69-71
- [2] A. Mahul-Mellier (2014) Hum Mol Genet 23:2858-2879
- [3] B. Brasher & R. Etten (2000) J Biol Chem 275:35631-35637

