ALK2 inhibitors display beneficial effects in preclinical models of ACVR1 mutant diffuse intrinsic pontine glioma

Diana Carvalho$^{1,2,11}$, Kathryn R. Taylor$^{1,2,3,11}$, Nagore Gene Olaciregui$^4$, Valeria Molinari$^{1,2}$, Matthew Clarke$^{1,2}$, Alan Mackay$^{1,2}$, Ruth Ruddle$^2$, Alan Henley$^2$, Melanie Valenti$^2$, Angela Hayes$^2$, Alexis De Haven Brandon$^2$, Suzanne A. Eccles$^2$, Florence Raynaud$^2$, Aicha Boudhar$^{5,6}$, Michelle Monje$^3$, Sergey Popov$^{1,2,7}$, Andrew S. Moore$^{8,9}$, Jaume Mora$^4$, Ofelia Cruz$^4$, Mara Vinci$^{1,2,10}$, Paul E. Brennan$^{5,6}$, Alex N. Bullock$^5$, Angel Montero Carcaboso$^4$ & Chris Jones$^{1,2}$

Diffuse intrinsic pontine glioma (DIPG) is a lethal childhood brainstem tumour, with a quarter of patients harbouring somatic mutations in ACVR1, encoding the serine/threonine kinase ALK2. Despite being an amenable drug target, little has been done to-date to systematically evaluate the role of ACVR1 in DIPG, nor to screen currently available inhibitors in patient-derived tumour models. Here we show the dependence of DIPG cells on the mutant receptor, and the preclinical efficacy of two distinct chemotypes of ALK2 inhibitor in vitro and in vivo. We demonstrate the pyrazolo[1,5-a]pyrimidine LDN-193189 and the pyridine LDN-214117 to be orally bioavailable and well-tolerated, with good brain penetration. Treatment of immunodeprived mice bearing orthotopic xenografts of H3.3K27M, ACVR1R206H mutant HSJD-DIPG-007 cells with 25 mg/kg LDN-193189 or LDN-214117 for 28 days extended survival compared with vehicle controls. Development of ALK2 inhibitors with improved potency, selectivity and advantageous pharmacokinetic properties may play an important role in therapy for DIPG patients.
Diffuse intrinsic pontine glioma (DIPG) is an incurable infiltrating glioma of the brainstem in children, with a median overall survival of 9–12 months\(^1\)-\(^3\). The mainstays of chemotherapy in histologically similar tumours of other anatomical brain regions are ineffective, in part due to the absence of factors which predict efficacy, such as MGMT promoter methylation and response to temozolomide\(^4\). The uniqueness of the underlying biology of DIPG is most readily demonstrated by the high prevalence (>80%) of lysine-to-methionine substitutions at position 27 (K27M) in genes encoding histone H3.1 (HIST1H3B, HIST1H3C) and H3.3 variants (H3F3A)\(^5\)-\(^9\). To date, radiotherapy provides the only therapeutic response, although >90% children suffer a relapse and die from their disease within 2 years\(^1\)-\(^3\).

Recent collaborative molecular sequencing initiatives have defined the genomic landscape of DIPG. We and others identified recurrent somatic activating mutations in the gene ACVR1 in ~25% of DIPG patients\(^6\)-\(^11\). Whilst H3.3 K27M mutations are also present in other midline regions such as the thalamus, spine and cerebellum (diffuse midline glioma with H3K27M mutation in the 2016 WHO classification scheme\(^12\)), ACVR1 mutations are associated with H3.1K27M substitutions, and appear restricted to DIPG\(^13\). In keeping with the clinicopathological differences between H3.3 and H3.1 K27M mutant subgroups\(^14\), ACVR1 mutations have been reported at a younger age of diagnosis and with longer overall survival in children with DIPG\(^11\)-\(^11\). Although apparently not found in any other human cancer, these variants are found in the germline of patients with the congenital malformation syndrome fibrodysplasia ossificans progressiva (FOP), in which soft tissue is remodeled to bone in response to (often trauma-related) inflammation\(^15\).

In the brain, activin A–ACVR1 signalling is involved in the process of myelination\(^16\)-\(^18\), an intriguing association given the putative oligodendroglial precursor origins of DIPG\(^18\)-\(^20\).

ACVR1 encodes the receptor serine/threonine kinase ALK2, and in models of FOP, it has recently been reported that the characteristic mutations confer an aberrant sensitivity to the ligand activin A, produced as part of the inflammatory response, rather than the canonical BMPs\(^21\). This results in increased pathway activation and cell signalling via a canonical phosphorylated SMAD1/5/8-SMAD4 pathway to drive expression of target genes including ID1/2/3, SNAIL and HEY1\(^22\). Inhibition of this response provides the major impetus of preclinical development and clinical trials in FOP.

The first small molecule inhibitor of ALK2 was dorsomorphin, identified through a classical BMP centralization assay in zebrafish embryos\(^23\). The pyrazol[1,5-a]-pyrimidine scaffold of dorsomorphin provided the basis for further compounds demonstrated to target ALK2 and the related BMP receptors ALK3 and ALK6, without affecting the type I TGF-beta receptor ALK5\(^24\). Continued improvements in selectivity have been reported in a newer series based instead on a pyridine scaffold, the prototype of which is K22088\(^25\).

In the present study, we demonstrate ACVR1 mutations confer abnormal ligand responsiveness to activin A in DIPG cells, with spatiotemporal expression of activin A in neurodevelopment correlating with tumour origins. Screening mutant and wild-type DIPG cultures with a range of pyrazol[1,5-a]pyrimidine- and pyridine-based ALK2 inhibitors demonstrated differential effects on cell viability, recapitulating genetic knockdown with shRNA, and prolongation of survival in orthotopic patient-derived xenograft models of DIPG.

**Results**

**Clinical and molecular correlates of ACVR1 mutant DIPGs.** To assess the differences between distinct somatic variants, we re-examined data from a genomics meta-analysis comprising 212 DIPG cases for which ACVR1 status was available\(^14\). We identified 50/212 (23.6%) cases with ACVR1 mutation, and found no differences in age at diagnosis between R206H (n = 10, median = 5.35 years), R258G (n = 7, median = 5.2 years), G328E/V/W (n = 28, median = 5.5 years) and G356D (n = 5, 4.8 years) variants (p = 0.6957, ANOVA), though together, mutant cases were significantly younger than ACVR1 wild-type DIPG (5.25 vs 7.0 years, p < 0.0001, t-test) (Fig. 1a). The presence of any ACVR1 mutation conferred a longer overall survival (p = 0.00291, log-rank test), however this benefit was largely restricted to amino acid substitutions at the G328 residue (median survival = 16.0 months; p = 0.000646, log-rank test), with other mutations showing no significant differences in clinical outcome compared with ACVR1 wild-type patients (wild-type median survival = 10.0 months; R206H = 13.0 months; R258G = 13.1 months; G356D = 14.3 months) (Fig. 1b). Of eight long-term survivors (>24.0 months), five were ACVR1 mutant (62.5%), all of which were G328E/V/W; ranking DIPG patients by their overall survival, 10/21 (47.6%) of the top 10% survivors (>19.0 months) were ACVR1 mutant, all but one G328E/V/W.

Gene expression data were available across three independent platforms for 66 patients (Supplementary Fig. 1a), with an integrated analysis identifying 244 differentially expressed genes between ACVR1 mutant and wild-type DIPGs (adjusted p < 0.05, Mann–Whitney U test) (Supplementary Table 1). ACVR1 mutation was significantly associated with upregulation of known BMP/TGFβ target genes such as ID1 (p = 0.0261) and ID2 (p = 0.0101), as well as other regulators (EMILIN1, p = 0.0000218) and antagonists (GREM1, p = 0.00187), in addition to genes associated with neurogenesis (WIF1, p = 0.0000556), neuronal migration (RELN, p = 0.0292), differentiation (HOXD8, p = 0.000132) and pluripotency (LIN28B, p = 0.000714) (Fig. 1c). By gene set enrichment analysis, there was co-ordinated regulation of expression signatures associated with signalling through HIF1, TGFβ, WNT, STAT3 and TP53 pathways (Supplementary Fig. 1b).

There was an expected strong association of ACVR1 mutation with H3.1K27M (36/50 vs 8/50 H3.3 K27M and 6/50 H3 wild-type; p < 0.0001, Fisher’s exact test), however, the distribution of histone mutations differed with distinct ACVR1 variants. Notably, R206H mutant tumours contained equal proportions of H3.3 and H3.1K27M (Fig. 1d). DIPGs with more extensive genome or exome sequencing data (n = 154) allowed us to investigate additional co-segregating mutations, and identified a significant enrichment of PI3-kinase pathway alterations (PIK3CA and PTEN) in ACVR1 mutant compared with wild-type tumours (22/40, 55% vs 18/114, 15.8%; p < 0.0001), whilst conversely the TP53 pathway was targeted by mutations in TP53 and PPM1D significantly more commonly in ACVR1 wild-type cases (13/40, 32.5% vs 8/714, 76.3%; p < 0.0001, Fisher’s exact test) (Fig. 1e).

Taking the ACVR1 mutations separately, tumours with R258G trended towards fewer copy number aberrations (p = 0.0658, t-test), whilst R206H had significantly fewer somatic mutations (0.0257, t-test) (Supplementary Fig. 1c). There was enrichment for PIK3CA mutations in R258G tumours, and PIK3R1 in G356D (log2 odds ratios >2.5), however this failed to reach statistical significance. Screening all somatic mutations highlighted previously unreported co-segregation of ACVR1 G328E/V/W with variants in the cyclin-dependent kinase inhibitor CDKN2C (p = 0.0113, Fisher’s exact test) and the lysine demethylase KDM6B (p = 0.0238, Fisher’s exact test) (Supplementary Fig. 1d). Gene expression differences between the various ACVR1 mutations revealed an upregulation of genes involved in oligodendrocyte differentiation in G328E/V/W mutant tumours compared to R206H and G356D, including MOG (myelin oligodendrocyte...
DIPG cells were noted in the mouse pons/medulla compared to development (Fig. 2a) (Supplementary Fig. 2a). Notably, a similar pattern of differential expression for ACVR1 mutant (purple) vs wild-type cases (grey) in an integrated gene expression dataset (n = 66). A Kaplan–Meier plot of overall survival of cases separated by ACVR1 variant, p-value calculated by the log-rank test (n = 171). Boxplots representing gene expression differences between ACVR1 mutant (purple) vs wild-type cases (grey) in an integrated gene expression dataset (n = 66). Stacked barplot of distribution of H3 K27M mutations separated by WHO 2007 grade. H3 K27M, grey.
The most potent compound was LDN-193189, with GI50 values in the range 0.70–3.16 µM, although little selectivity was observed between ACVR1 mutant and wild-type cells. By contrast, the most selective compound was the pyridine LDN-214117, with GI50 values of 1.57 µM for R206H HSJD-DIPG-007 cells, 5.83–6.23 µM for G328V SU-DIPG-IV, and ACVR1 wild-type (but high phospho-SMAD1/5/8 expressing) SU-DIPG-VI cells, and 8.27 µM for ACVR1 mutant wild-type QCTB-R059 cells (p < 0.0001, t-test) (Table 1). Both compounds were seen to inhibit signalling through phospho-SMAD1/5/8 and the downstream effector ID1, with the first effects seen at 4 h with 0.1 µM, but more profound consequences seen at 1.0 µM (Fig. 4a, b) (Supplementary Fig. 3). Induction of apoptosis evidenced by an increase in cleaved PARP was seen in ACVR1 mutant, but not wild-type cells.

Such effects were not transient, with wash-out experiments for both compounds showing a persistent inhibition of downstream signalling 24 h post-treatment (Supplementary Fig. 6a, b). In the presence of ligands, there was no difference in GI50 values for LDN-193189 (Supplementary Fig. 6c), however, addition of Activin-A conferred enhanced sensitivity of R206H HSJD-DIPG-007 cells to LDN-214117 (Supplementary Fig. 6d). In order to assess whether treatment of DIPG with these compounds would afford a therapeutic window, we also assessed their effects on normal human astrocytes derived from the brainstem (NHA-BS). For both LDN-193189 (Supplementary Fig. 6f) and LDN-214177 (Supplementary Fig. 6i), there was a marked lack of sensitivity in the NHA-BS cells, with greater than 10-fold difference in effect on cell viability compared to the DIPG cultures.

Pharmacokinetics and pharmacodynamics of ALK2 inhibitors.
We next investigated which of the tested compounds possessed the desired biopharmaceutical profiles in terms of CNS penetration and in vivo effects on downstream signalling. Plasma drug exposure (area under the curve, AUC) was assessed for six compounds after oral and intravenous treatment, and bioavailability (F) was calculated. Those with the highest F were LDN-193189 (0.94), and to a lesser extent, LDN-214117 (0.75) (Supplementary Fig. 7). Both LDN-193189 and LDN-214117 were found to be well-tolerated in mice at 35 and 25 mg/kg, respectively, despite an initial weight loss with LDN-193189 (Fig. 5a, e). The concentrations of both compounds in the blood persisted over 8 h post oral administration (Fig. 5b, f), whilst importantly, brain levels were either even higher (LDN-193189 brain:plasma ratio = 1.34, 2 h post-dose, day 14) (Fig. 5c) or only slightly reduced compared with plasma (LDN-214117 brain: plasma ratio = 0.80, 2 h post-dose, day 14) (Fig. 5g). In both cases, the concentrations in mouse brain (3.37 and 10.94 µM) were considerably higher than the in vitro GI50 values observed (0.70 and 1.57 µM). Treatment at these concentrations also lead to a substantial reduction in pharmacodynamic biomarkers in HSJD-DIPG-007 xenograft tumours, showing a near-abolition of
phospho-SMAD1/5/8 and ID1 with LDN-193189, and to a lesser extent with LDN-214117 (Fig. 5d, h; Supplementary Fig. 3).

In vivo efficacy of targeting ALK2. Having identified two candidate compounds of distinct chemotypes with in vitro efficacy linked to effects on DIPG signalling, and brain penetration at doses sufficient to elicit a similar response in vivo, we next investigated their efficacy in orthotopic patient-derived xenografts. We treated established ACVR1 mutant HSJD-DIPG-007 and H3/ACVR1 wild-type (Supplementary Fig. 8a–c) brainstem xenografts at four and three weeks post-implantation, respectively, with either 25 mg/kg LDN-193189 or LDN-214117 for 28 days. No differences in survival were observed in wild-type control HSJD-GBM-001 tumours for either compound. By contrast, for both LDN-193189 and LDN-214177 we observed a significant prolongation of survival in ACVR1 R206H-mutant HSJD-DIPG-007-bearing animals, amounting to a median benefit of 15 days in both cases (82 vs 67 days, \( p = 0.0002 \), LDN-193189; and 75 vs 61 days, \( p = 0.003 \), LDN-214117, log-rank test) (Fig. 6a, b, e, f). After 28 days, there was a decrease in cellularity of the treated ACVR1 mutant tumours compared with controls, as assessed by counting of positive cells by immunohistochemical staining using anti-human nuclear antigen (Fig. 6c, d, g, h), though differences in proliferation (Ki67) (Supplementary Fig. 8d–g), and vascularisation (CD31) (Supplementary Fig. 8h–k) were not significant.

In summary, ACVR1 mutations elicit a dependency of downstream signalling in DIPG cells than can be inhibited by single agents of multiple chemotypes of inhibitor compounds.

**Table 1 Screening of ALK2 inhibitors**

|                  | HSJD-DIPG-007 | SU-DIPG-IV | HSJD-DIPG-018 | SU-DIPG-VI | QCTB-R059 |
|------------------|---------------|------------|---------------|------------|-----------|
| Histone H3       | H3F3A K27M    | HISTH38 K27M | HISTH38 K27M | H3F3A K27M | H3F3A K27M |
| ACVR1            | ACVR1 R206H   | ACVR1 G328V | ACVR1 R258G  | ACVR1 wild-type | ACVR1 wild-type |
| Dor somorphin    | >20 µM        | >20 µM     | >20 µM       | >20 µM     | >20 µM    |
| LDN-193189       | 0.70 ± 0.09 µM | 0.80 ± 0.02 µM | 3.16 ± 0.23 µM | 1.10 ± 0.20 µM | 0.89 ± 0.01 µM |
| LDN-212854       | 1.25 ± 0.05 µM | 1.30 ± 0.20 µM | 3.22 ± 0.22 µM | 1.70 ± 0.20 µM | 1.70 ± 0.00 µM |
| K02288           | 9.10 ± 0.26 µM | >20 µM     | 14.34 ± 113 µM | >20 µM     | >20 µM    |
| LDN-214117       | 1.57 ± 0.03 µM | 6.23 ± 0.30 µM | 16.38 ± 0.79 µM | 5.83 ± 0.18 µM | 8.27 ± 0.33 µM |
| LDN-213844       | 3.67 ± 0.66 µM | 12.79 ± 2.65 µM | 15.44 ± 0.94 µM | 12.70 ± 2.17 µM | >20 µM    |
| LDN-213819       | 3.27 ± 0.15 µM | 8.03 ± 0.31 µM | 11.18 ± 0.61 µM | 9.87 ± 0.94 µM | 10.99 ± 1.63 µM |
| Perhexiline      | 4.07 ± 1.31 µM | 4.70 ± 1.01 µM | 6.11 ± 0.12 µM | 4.90 ± 1.08 µM | 5.33 ± 1.07 µM |
| Saracatinib      | 17.76 ± 0.56 µM | >20 µM     | >20 µM       | >20 µM     | >20 µM    |
| Mometonitib      | 7.32 ± 0.99 µM | 14.23 ± 0.89 µM | 9.50 ± 0.38 µM | 10.42 ± 1.12 µM | 15.94 ± 1.44 µM |

Table of GI50 values for 11 compounds screened against four patient-derived DIPG cell cultures, with cell viability as the readout. Mean plus standard deviation are plotted from at least \( n = 3 \) independent experiments are provided.
which penetrate orthotopic tumours at concentrations that produce cell death and survival benefits in orthotopic mouse models as single agents at well-tolerated dosages.

**Discussion**

The discovery of somatic ACVR1 mutations in DIPG identical to those found in the germline of FOP patients has not only highlighted unexpected links between neurodevelopment and chondrogenesis, but also allowed for the fast-tracked evaluation of specific ALK2 inhibitors developed for the control of heterotopic ossification in the context of childhood brain tumours. Inhibition of ALK2, via pharmacologic or genetic means, led to inhibition of proliferation and induction of apoptosis, as well as a selective reduction in cell viability in...
patient-derived in vitro tumour models, and enhanced survival of mice bearing orthotopic ACVR1-mutant patient-derived DIPG xenografts in vivo.

A major challenge in the context of DIPG (unlike FOP) is the necessity for agents to penetrate the tumour behind what may be a particularly restrictive blood-brain barrier within the brainstem. Short-cutting drug development by using the reported off-target effects on ALK2 of drugs licensed for alternate targets and different indications holds promise given the urgency of the unmet clinical need, but appeared unwarranted for the compounds tested for different indications.

In our mouse models, derivatives of the earliest pyrazolo[1,5-a]pyrimidine- and pyridine-based compounds (LDN-193189 and LDN-214117, respectively) were found to be present within the tumours for LDN-214117, in both models, for anti-human nuclear antigen (HNA). Scale bar = 1000 µM. Magnified view of HNA staining (scale bar = 50 µM), and barplot quantifying cellularity by HNA-positive cells as a percentage of control. Mean and standard deviation plotted. **p < 0.01, t-test
at which DIPG arises. Notably, genes associated with oligoden- drocyte differentiation were elevated in G325E/V/W mutant tumors compared to R206H/G356D. Although Activin A con- fers enhanced signalling via the same phospho-SMAD1/5/8- SMAD4 axis as BMPs, it remains possible that various non- canonical pathways may also be activated within this specific context. Gene expression signatures point towards a delicate balance associated with hypoxia and stem cell niche maintenance that remain unexplored.

Notably, not all ACVR1 mutations appear to confer a better prognosis. The R206H mutation, which in the germline accounts for >95% of classical FOP patients, does not have the striking predilection for H3.1 K27M mutations seen with the other var- iants, and being found in roughly equivalent numbers of H3.3 K27M tumours likely accounts for the extremely poor survival of these patients. That LDN-193189 and LDN-214117 were incubated in complete media with vehicle or increasing concentrations of drug (0.1, 1, 10 µM) and protein was collected at 4 and 8 h post-treatment. For washout experiments, cells were incubated with 1 µM of LDN-193189 or LDN-21417 and either left in the culture media or washed out by media replacement for 2–24 h. Mouse brain samples were manually homogenised in protein cell lysis buffer. Samples were lysed by using lysis buffer (CST) containing phosphatase inhibitor cocktail (Sigma, Poole, UK) and protease inhibitor cocktail (Roche Diagnostics, Burgess Hill, UK). Following quantification using Pierce BCA Protein Assay Kit (Thermo Fisher), equal amounts of cell extracts were loaded for Western blot analysis. Membranes were incubated with primary antibody (1:10000) overnight at 4 °C, and horseradish peroxidase secondary antibody (Amersham Bioscience, Amersham, UK) for 1 h at room temperature. Signal was detected with ECL Prime western blotting detection agent (Amersham Biosciences), visualised using Hyperfilm ECL (Amersham Biosciences) and analysed using image J software.

**Methods**

**Bioinformatic analysis.** Curated gene-level copy number, expression and mutation data from DIPG patients were obtained from a recent meta-analysis of published and unpublished data and is available within the paediatric-specific implementation of the cbioPortal genomic data visualisation portal (pedcbioportal.org). Gene expression data from Agilent WG2.5, Affymetrix U133Plus2.0 or RNA sequencing was platform-centred, and log-transformed expression measures were combined and further normalised using pairwise loess normalisation. Gene Set Enrichment Analysis (software.broadinstitute.org/gsea) was performed using the GSEA java application based upon pairwise comparisons of the major subgroups in the merged dataset. Differential expression analysis was based on a Mann–Whitney U test for each chosen gene based on the values between the above-mentioned data. Spatiotemporal gene expression data from developing and adult brain samples were obtained from the Human Brain Transcriptome project (hbatlas.org).

**Cell culture.** All patient-derived material for cell culture was collected under IRB approval from Children’s Health Queensland Hospital. Patient-derived cultures HSJD-DIPG-007 (HSF3A K27M, ACVR1 R206H), HSJD-DIPG-018 (HIST1H3B K27M, ACVR1 R258G), SU-DIPG-IV (HIST1H3B K27M, ACVR1 G325V), SU- DIPG-VI, QCTB-B099 (both HSF3A K27M, ACVR1 wild-type), and HSJD-GBM-001 (HS/ACVR1 wild-type) were grown in stem cell media consisting of Dulbecco’s Modified Eagles Medium: Nutrient Mixture F12 (DMEM/F12), Neurobasal-A medium, HEPES Buffer Solution 1 M, sodium pyruvate solution 100 mM, non-essential amino acids solution 10 mM, Glutamax-I Supplement and Antibiotic- Antimycotic solution (all Thermo Fisher, Loughborough, UK). The media was supplemented with B-27 Supplement Minus Vitamin A (Thermo Fisher), 20 ng/ml Human-EGF, 20 ng/ml Human-FGF-basic-154, 20 ng/ml Human-PDGF-AA, 20 ng/ml Human-PDGF-BB (all Shennandoah Biotech, Warwick, PA, USA) and 2 µg/ml Heparin Solution (0.2%, Stem Cell Technologies, Cambridge, UK). Normal human astrocytes (NHA-BS) (Science Cell, #0413) coated flasks. Cell authenticity was verified using short tandem repeat (STR) DNA fingerprinting, and cells are available upon request.

**Western blot analysis.** For growth factor starvation, cells were washed twice with PBS and then incubated for 1 h in stem cell medium without growth factors. Stem cell medium supplemented with BMP4 (10 ng/ml, Peprotech, London UK) or Activin A (10 ng/ml, Thermo Fisher) was then added for 1 h and protein was collected. For treatment with LDN-193189 and LDN-214117, cells were incubated in complete media with vehicle or increasing concentrations of drug (0.1, 1, 10 µM) and protein was collected at 4 and 8 h post-treatment. For washout experiments, cells were incubated with 1 µM of LDN-193189 or LDN-21417 and either left in the culture media or washed out by media replacement for 2–24 h. Mouse brain samples were manually homogenised in protein cell lysis buffer. Samples were lysed by using lysis buffer (CST) containing phosphatase inhibitor cocktail (Sigma, Poole, UK) and protease inhibitor cocktail (Roche Diagnostics, Burgess Hill, UK). Following quantification using Pierce BCA Protein Assay Kit (Thermo Fisher), equal amounts of cell extracts were loaded for Western blot analysis. Membranes were incubated with primary antibody (1:10000) overnight at 4 °C, and horseradish peroxidase secondary antibody (Amersham Bioscience, Amersham, UK) for 1 h at room temperature. Signal was detected with ECL Prime western blotting detection agent (Amersham Biosciences), visualised using Hyperfilm ECL (Amersham Biosciences) and analysed using image J software.

**Compound efficacy assays.** Cells were plated at a density of 2000–4000 cells/well on laminin-coated 96-well plates in a minimum of triplicates. After three days of incubation, compound was added to each well in concentrations from 0.07–20 µM and incubated at 37 °C, in 5% CO2, 95% humidity for eight days (192 h). For Activin A / BMP4 studies, cells were supplemented with media containing BMP4 (10 ng/ml, Peprotech, London UK) or Activin A (10 ng/ml, Thermo Fisher) 24 h prior to adding the drug. Drug was added in media supplemented with Activin A (K03841b), LDN-212838 (K03449c) and K05907 were synthesised at the Structural Genomics Consortium, Oxford (see Supplementary Methods for chemical structures and synthesis). LDN-213819 was a kind gift from Paul Yu and Greg Cuny (Harvard University). Cell viability was assessed by the CellTiter-Glo luminescent cell viability assay (Promega, Madison, WI, USA) and Glc, values were calculated using GraphPad Prism version 6 as the concentration of compound required to reduce cell viability by 50%.
In vivo efficacy studies. A single cell suspension of each culture was made the day before implantation and cultured overnight. On the implantation day, small tumourospheres in exponential growth were harvested by mild centrifugation. 3-week-old female NOD.SCIID mice were anaesthetised with 100 mg/kg ketamine and 10 mg/kg xylazine and immobilized in a stereotaxic apparatus (Wood Dale, IL) at coordinates x=−0.5 and y=−5.4 from the bregma suture. 5×10^5 HSJD-DIPG-007 or HSJD-GRM-001 tumourospheres (5×10^5 cells), suspended in 5 µl matrigel (BD Biosciences) were injected at 3.1 mm depth (targeting the 4th ventricle) with a dull 22G needle attached to a 50 µl syringe (Hamilton, Bonaduz, Switzerland) using a stereotaxic arm. Mice were stratified (n=7–10 per group) and treated with 25 mg/kg of either either LDN-193189, LDN-214117 or vehicle for 28 days, starting 21–28 days post-inoculation. LDN-193189 was prepared each day in water whilst LDN-214117 was prepared in 10% DMSO diluted in saline. Mice were monitored by daily weighing and were sacrificed by cervical dislocation upon deterioration of condition or 20% weight loss from the maximum weight achieved, with tissue taken for further analysis. Mouse brains collected at the end of the efficacy study were processed for immunohistochemistry. Plasma and brain samples from treated and control mice were taken at 2 h post-dose at the end of the 28-day treatment for pharmacokinetic and pharmacodynamic analyses.

Immunohistochemistry. PFA-fixed mouse brains were paraffin embedded and sectioned (4 µm) for immunohistochemical analysis and stained with haematoxylin and eosin (H&E). For immunohistochemistry, sodium citrate (pH 6.0) heat-mediated antigen retrieval was performed and staining was carried out using an antibody directed against human nuclear antigen (HNA) (Millipore, #4383S, 1:100). Pressure antigen retrieval was performed and staining was carried out using antibodies directed against K67 (DAKO, #7240, 1:100), and CD31 (Abcam, #28364, 1:50). All primary antibodies were diluted into 1% Tris buffer solution with 0.05% Tween-20, except Ki67 which was diluted into Dako antibody diluent. #28364, 1:50). All primary antibodies were diluted into 1% Tris buffer solution with 0.05% Tween-20, except Ki67 which was diluted into Dako antibody diluent.

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Author contributions

D.C., K.R.T., A.M.C. and C.J. conceived the study; D.C., K.R.T., N.G.O., V.M., R.R., A.H., M.V., A.H., A.D.H.B., A.B. and M.V. carried out biological experiments; A.H., P.E.B. and A.N.B. generated novel chemical compounds; M.C. and S.P. carried out pathological assessment of samples; A.M. and C.J. carried out bioinformatic analysis; S.A.E., F.R., A.M.C. and C.J. supervised experiments; M.M., A.S.M., J.M., O.C. and A.M.C. generated patient-derived models; D.C. and C.J. wrote the manuscript; all authors approved the manuscript.

Additional information

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