# Reducing D2HG by AGI-5198 does not affect the tumor cell population in short-term primary cultures of *IDH*-mutated gliomas

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Mutations in the *isocitrate dehydrogenase 1* (*IDH1*) gene have been frequently identified in various cancers including gliomas, acute myeloid leukemia (AML) and cholangiocarcinoma [1, 3, 9, 16]. In gliomas, over 90% of *IDH* mutations result in the R132H missense mutation within the catalytic domain of the enzyme. This mutation changes the substrate of the protein from isocitrate to α-ketoglutarate (αKG), which is then reduced to D2-hydroxygluratate (D2HG) [5]. Since mutations in *IDH* genes are amongst the most common identified in low grade gliomas (LGGs), and because of its clonality and mutation-specific enzymatic activity, it is considered a promising target for therapy. Indeed, inhibitors targeting the mutant-specific activity have been developed and these are currently being tested for clinical activity. Although some encouraging clinical responses have been reported in AML patients, preclinical evidence in gliomas shows limited growth inhibition [4, 11, 12, 14]. Further research into the oncogenic role of *IDH1* mutations in gliomas and efficacy of inhibitors is therefore required. However, there are only few IDH-mutant model systems for glioma and the cell cultures that have been established are predominantly derived from high-grade astrocytomas [2, 8, 10]. In this study, we report on a short-term *in vitro* assay of primary *IDH*-mutated LGG, including those with *1p19q* codeletion, and the effects of an IDH inhibitor on these cultures.

Glioma tissue samples were collected from the operating theatre and dissociated through mechanical and enzymatic digestion by incubating tumor fragments in DMEM-F12 medium (Gibco, Thermo Scientific, Rochester, USA) supplemented with DNAse (1%, Roche, Woerden, the Netherlands) and collagenase A (5%, Roche) for 1,5 hours in a 37°C shaking water-bath within 2 hours post resection. The dissociated cells were then re-suspended and seeded at a density of 10E4 cells/well in a 24-well plate in serum-free DMEM-F12 medium supplemented with 2% B27 (Invitrogen, Breda, the Netherlands), bFGF (5µg/ml, Tebu-Bio, Heerhugowaard, the Netherlands), EGF (5µg/ml, Tebu-Bio) and heparin (5 µg/mL, Sigma-Aldrich, Zwijndrecht, the Netherlands) in the presence of antibiotics (1% penicillin/streptomycin, Gibco). For all cultured glioma samples, written informed consent was obtained from each patient and approved by the institutional review board of the Erasmus Medical Center, Rotterdam.

Seventeen Grade II-III *IDH*-mutated astrocytomas or oligodendrogliomas were included in this study. A codeletion of *1p19q* was present in 7 of these tumors (*IDH* mutations and *1p19q* status of these tumors were determined as part of routine diagnosis in our institute using targeted next-generation sequencing (NGS) [6, 7] (Table 1).

**Table 1.** Overview of cultured *IDH*-mutated gliomas. ctr: DMSO control; VAF: variant allele frequency; BAF: B-allele frequency; F: faild analysis; nd: not done.

| | | | IDH status | | | | | | Other genetic changes | |
|---|---|---|---|---|---|---|---|---|---|---|
| Tumor | WHO 2016 | Grade | At diagnosis | 2 days | 10 days | VAF at diagnosis | VAF ctr | VAF inhibitor | At diagnosis | Ctr 10 days |
| 1 | astrocytoma | 3 | p.R132H | p.R132H | p.R132H | 41 | nd | nd | nd | nd |
| 2 | astrocytoma | 3 | p.R132H | p.R132H | WT | 36 | nd | nd | nd | nd |
| 3 | astrocytoma | 2 | p.R132C | p.R132C | p.R132C | 36 | nd | nd | nd | nd |
| 4 | astrocytoma | 3 | p.R132H | p.R132H | WT | 50 | nd | nd | nd | nd |
| 5 | astrocytoma | 2 | p.R132H | WT | WT | 42 | nd | nd | nd | nd |
| 6 | astrocytoma | 3 | p.R132H | WT | WT | 40 | nd | nd | nd | nd |
| 7 | astrocytoma | 2 | p.R132H | p.R132H | p.R132H | 42 | 21 | nd | TP53 p.R280G ATRX p.K1583fs | TP53 p.R280G ATRX p.K1583fs |
| 8 | astrocytoma | 2 | p.R132H | p.R132H | p.R132H | 43 | nd | nd | nd | nd |
| 9 | astrocytoma | 2 | p.R132H | p.R132H | p.R132H | 38 | 13 | 15 | TP53 p.R248W ATRX c.663-1G>A Imbalance Chr 7 | TP53 p.R248W ATRX c.663-1G>A |
| 10 | astrocytoma | 2/3 | p.R132H | p.R132H | p.R132H | 47 | 46 | 51 | TP53 p.R273H ATRX p.R1426* | TP53 p.R273H ATRX p.R1426* |
| 11 | oligodendroglioma | 2 | p.R132H | F | p.R132H | 35 | 9 | nd | none | none |
| 12 | oligodendroglioma | 2 | p.R132H | p.R132H | p.R132H | 40 | 12 | 17 | Partial imbalance Chr 7 | none |
| 13 | oligodendroglioma | 3 | p.R132H | WT | p.R132H | 45 | F | 3 | CIC p.R215Q Imbalance Chr 7 | F |
| 14 | oligodendroglioma | 3 | p.R132H | p.R132H | p.R132H | 39 | 10 | 9 | none | none |
| 15 | oligodendroglioma | 2 | p.R132H | p.R132H | p.R132H | 43 | 27 | 13 | none | none |
| 16 | oligodendroglioma | 2 | p.R132H | p.R132H | p.R132H | 28 | nd | nd | CIC p.R215W Imbalance Chr 9 | nd |
| 17 | oligodendroglioma | 2 | p.R132H | WT | WT | 40 | 4 | 5 | CIC p.R215Q CIC p.A253P | none |

| Other genetic changes | | | | 1p19q status | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **IDH inhibitor 10 days** | **VAF at diagnosis** | **VAF crt 10 days** | **VAF IDH inhibitor 10 days** | **At diagnosis** | **ctr 10 days** | **IDH inhibitor 10 days** | **tumor % based on BAF at diagnosis** | **tumor % based on BAF ctr 10 days** | **tumor % based on BAF IDH inhibitor 10 days** |
| nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| nd | 77 73 | 48 43 | nd | 19q LOH | 19q LOH | nd | 80 | 50 | nd |
| nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| TP53 p.R248W ATRX c.663-1G>A | 72 73 | 27 21 | 28 31 | wt | wt | wt | 0 | 0 | 0 |
| TP53 p.R273H ATRX p.R1426* | 45 59 | 46 58 | 49 67 | wt | wt | wt | 0 | 0 | 0 |
| none | none | none | none | LOH | LOH | nd | 80 | 20-30 | nd |
| Partial imbalance Chr 7 | none | none | none | LOH | LOH | LOH | 80-85 | 30-40 | 40 |
| CIC p.R215Q | 88 | F | 3 | LOH | F | wt | 90 | F | 0 |
| none | none | none | none | LOH | LOH | LOH | 75 | 20-30 | 10 |
| none | none | none | none | LOH | LOH | LOH | 85-90 | 50-60 | 30-40 |
| nd | 22 | nd | nd | LOH | nd | nd | 50 | nd | nd |
| none | 15 16 | none | none | LOH | wt | wt | 85 | 0 | 0 |

In 12 out of the 17 cultures, we were able to detect the *IDH1*-mutation after 2 days in our culture system using Sanger sequencing (Table 1 and Fig. 1a). We were unable to obtain Sanger sequencing data in one tumor at this time point. After 10 days in culture, we were able to detect the *IDH1* mutation in 12 cultures. In most cases (10/12) the mutant peak in the chromatogram was reduced indicating either loss of the mutation in tumor cells or a relative loss of the population of mutated cells (Fig. 1a). To discriminate between these options, we performed targeted-NGS on a subset of our cultures (n = 9 at 10 days in culture, analysis and scoring performed blinded to the original diagnosis data) using the identical panel as used for routine diagnostics. Using this assay, we were able to detect the *IDH1* mutation in all 9 tumors. Since *IDH1* mutations in gliomas are clonal and almost always heterozygous [15], the tumor cell percentage of each culture can be estimated based on the variant allele fraction (VAF) of the *IDH1* mutations (e.g. a VAF of 50 % $IDH1^{R132H}$ corresponds to 100 % tumor cell percentage). Similarly, the B-allele frequency (BAF) of single-nucleotide polymorphisms (SNP) located on 1p19q can also estimate the tumor cell percentage in tumors with 1p19q codeletion [7]. Since multiple SNPs are evaluated, a range is shown for the estimated tumor percentage based on BAF. We observed a lower VAF of the *IDH1* mutation in cultured gliomas compared to the original tumor at diagnosis in all but one tumor (tumor 10) (median = 12.5, range 4 - 46, suggesting 8 - 92 % tumor cells present in the culture). Lower VAF of other detectable mutations (*TP53*, *CIC,* Table 1) and a lower tumor cell percentage estimated based on BAF (Table 1 and Fig. S2-4) were also observed, which indicate that the fraction of tumor cells in our cultures was reduced rather than that the *IDH1*-mutation was lost from tumor cells. In tumor 9 for example, we detected mutations in *IDH1*, *TP53* and *ATRX*. For the homozygous mutations in *TP53* and *ATRX*, the VAF shows similar reduction as for *IDH* mutations. In tumor 17, we were unable to confirm the presence of additional mutations in *CIC*, but this was likely due to the very low tumor percentage in these cultures (VAF for *IDH1* was 4 %) combined with the fact that *CIC* mutations are subclonal. These data demonstrate that short-term cultures of glioma samples are feasible, including tumors with *1p19q* codeletion, but the fraction of tumor cells in these cultures is generally low.

We added an IDH1 inhibitor to 7 glioma cultures (continued presence from day 1 *in vitro*) to determine effects of the IDH inhibitor on these cultures. We first quantified the D2HG levels in culture medium using LC-MS/MS as previously described [13]. Indeed, in all 7 cultures tested, the D2HG levels were markedly elevated compared to the L2HG levels (with, at least in one sample, levels accumulating up to day 21, Fig. 1b and Fig. S1A,B). Addition of 10 µM AGI-5198 (Xcess Biosciences, Inc., San Diego, USA), a specific inhibitor of mutant IDH1 activity, resulted in reduced levels of D2HG in 6/7 cultures tested (Fig. 1c and Fig. S1C, D). Increasing levels of D2HG

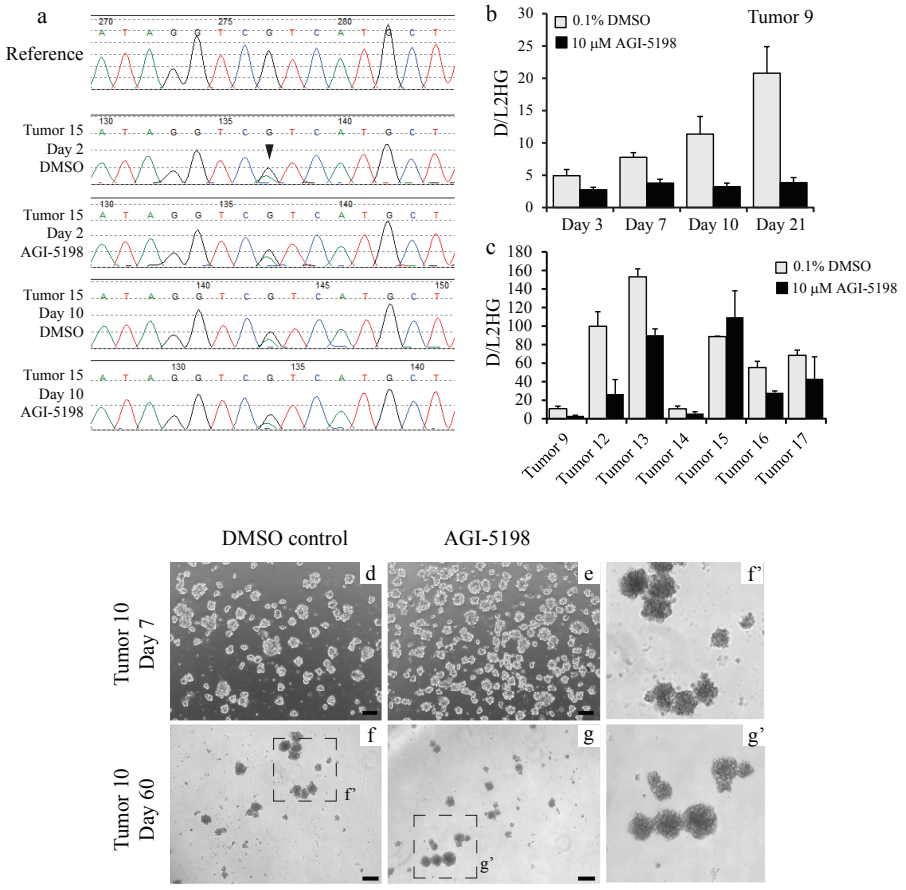



**Figure 1.** *IDH* mutation status is stable in our short-term primary glioma culture. a The *IDH* mutation is retained in our short-term culture. The figure shows sequencing data from the culture of tumor 15 after 2 and 10 days in culture. Top panel is the reference sequence and lower panels are the sequence of the culture in which a clear G>A mutation was observed (arrow head). Addition of AGI-5198, an IDH1 inhibitor did not affect the mutant peak in the chromatogram. b Temporal accumulation of D2HG levels in the culture of tumor 9. D2HG levels continued to accumulate up to day 21 in culture. Addition of an IDH1 inhibitor prevented the D2HG accumulation. c IDH1 mutant cultures have elevated levels of D2HG. In 6/7 cultures, these levels are reduced by the addition of an IDH1 inhibitor (inhibitor added at day 1 in culture). Morphology of tumor 10 culture after 7 days (d and e, scale bar 100μm) and 60 days(f. and g. scale bar 400μm) in culture did not show overt changes when treated with 10 μM AGI-5198.

in the absence of the inhibitor for 10 days indicate ongoing active metabolism of viable IDH1-mutant glioma cells in cultures. Production of D2HG was not completely inhibited in all cultured gliomas which could be due the fact that the inhibitor was not added instantly but after one day of culture; furthermore cell death (apoptosis/necrosis) could lead to release of cell content including D2HG into the medium where D2HG remains stably detectible.

Addition of the IDH1 inhibitor from day 1-10 *in vitro* did not lead to significant changes in the VAF of the *IDH1* mutation in any of the 6 cultures compared to control cultures at day 10 (Table 1) nor the cellular morphology (Fig. S6). In one culture, we added the inhibitor for up to day 60 and also did not identify significant changes in the cellular morphology and VAF of mutant *IDH1* (Fig. 1d and Table S1). These data demonstrate that inhibition of mutant IDH1 activity does not affect the relative number of tumor cells in our culture system.

In summary, we have established an assay for testing drugs in patient-derived primary *IDH1*-mutated LGG, including those with *1p19q* codeletion. We show that inhibiting mutant IDH activity in these cultures does not affect the tumor cell population, suggesting no lethal effect of the inhibitor in the short term.

## REFERENCES

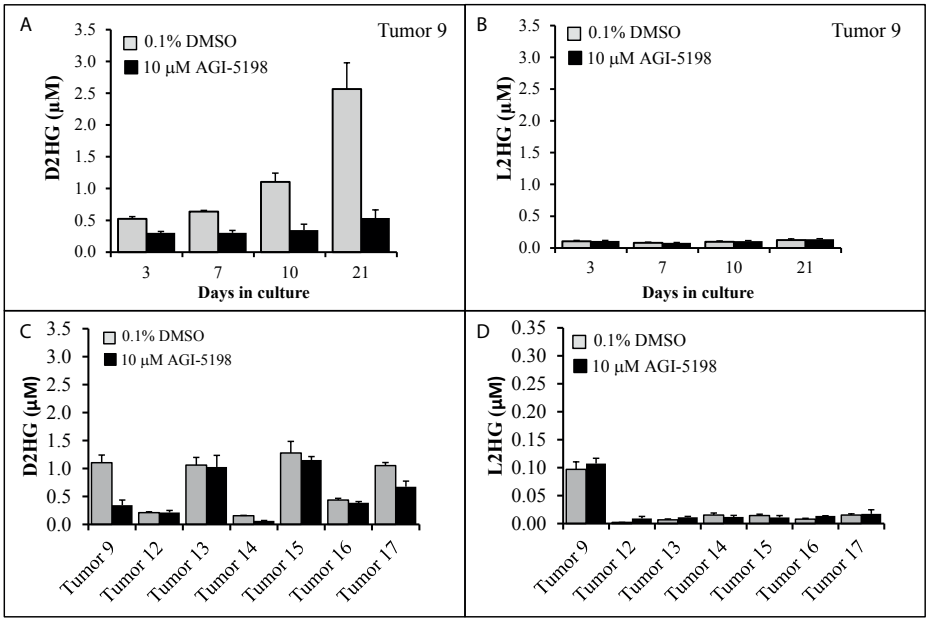

1. Amary MF, Bacsi K, Maggiani F, Damato S, Halai D, Berisha F, Pollock R, O'Donnell P, Grigoriadis A, Diss T, Eskandarpour M, Presneau N, Hogendoorn PC, Futreal A, Tirabosco R, Flanagan AM (2011) IDH1 and IDH2 mutations are frequent events in central chondrosarcoma and central and periosteal chondromas but not in other mesenchymal tumours. J Pathol 224:334-343
2. Balvers RK, Kleijn A, Kloezeman JJ, French PJ, Kremer A, van den Bent MJ, Dirven CM, Leenstra S, Lamfers ML (2013) Serum-free culture success of glial tumors is related to specific molecular profiles and expression of extracellular matrix-associated gene modules. Neuro Oncol 15:1684-1695
3. Boissel N, Nibourel O, Renneville A, Gardin C, Reman O, Contentin N, Bordessoule D, Pautas C, de Revel T, Quesnel B, Huchette P, Philippe N, Geffroy S, Terre C, Thomas X, Castaigne S, Dombret H, Preudhomme C (2010) Prognostic impact of isocitrate dehydrogenase enzyme isoforms 1 and 2 mutations in acute myeloid leukemia: a study by the Acute Leukemia French Association group. J Clin Oncol 28:3717-3723
4. Chaturvedi A, Cruz MMA, Jyotsana N, Sharma A, Yun HY, Gorlich K, Wichmann M, Schwarzer A, Preller M, Thol F, Meyer J, Haemmerle R, Struys EA, Jansen EE, Modlich U, Li ZX, Sly LM, Geffers R, Lindner R, Manstein DJ, Lehmann U, Krauter J, Ganser A, Heuser M (2013) Mutant IDH1 promotes leukemogenesis in vivo and can be specifically targeted in human AML. Blood 122:2877-2887
5. Dang L, White DW, Gross S, Bennett BD, Bittinger MA, Driggers EM, Fantin VR, Jang HG, Jin S, Keenan MC, Marks KM, Prins RM, Ward PS, Yen KE, Liau LM, Rabinowitz JD, Cantley LC, Thompson CB, Vander Heiden MG, Su SM (2009) Cancer-associated IDH1 mutations produce 2-hydroxyglutarate. Nature 462:739-744
6. Dubbink HJ, Atmodimedjo PN, Kros JM, French PJ, Sanson M, Idbaih A, Wesseling P, Enting R, Spliet W, Tijssen C, Dinjens WN, Gorlia T, van den Bent MJ (2016) Molecular classification of anaplastic oligodendroglioma using next-generation sequencing: a report of the prospective randomized EORTC Brain Tumor Group 26951 phase III trial. Neuro Oncol 18:388-400
7. Dubbink HJ, Atmodimedjo PN, van Marion R, Krol NMG, Riegman PHJ, Kros JM, van den Bent MJ, Dinjens WNM (2016) Diagnostic Detection of Allelic Losses and Imbalances by Next-Generation Sequencing: 1p/19q Co-Deletion Analysis of Gliomas. J Mol Diagn 18:775-786
8. Luchman HA, Stechishin OD, Dang NH, Blough MD, Chesnelong C, Kelly JJ, Nguyen SA, Chan JA, Weljie AM, Cairncross JG, Weiss S (2012) An in vivo patient-derived model of endogenous IDH1-mutant glioma. Neuro Oncol 14:184-191
9. Parsons DW, Jones S, Zhang X, Lin JC, Leary RJ, Angenendt P, Mankoo P, Carter H, Siu IM, Gallia GL, Olivi A, McLendon R, Rasheed BA, Keir S, Nikolskaya T, Nikolsky Y, Busam DA, Tekleab H, Diaz LA, Jr., Hartigan J, Smith DR, Strausberg RL, Marie SK, Shinjo SM, Yan H, Riggins GJ, Bigner DD, Karchin R, Papadopoulos N, Parmigiani G, Vogelstein B, Velculescu VE, Kinzler KW (2008) An integrated genomic analysis of human glioblastoma multiforme. Science 321:1807-1812
10. Piaskowski S, Bienkowski M, Stoczynska-Fidelus E, Stawski R, Sieruta M, Szybka M, Papierz W, Wolanczyk M, Jaskolski DJ, Liberski PP, Rieske P (2011) Glioma cells showing IDH1 mutation cannot be propagated in standard cell culture conditions. Br J Cancer 104:968-970
11. Pusch S, Krausert S, Fischer V, Balss J, Ott M, Schrimpf D, Capper D, Sahm F, Eisel J, Beck AC, Jugold M, Eichwald V, Kaulfuss S, Panknin O, Rehwinkel H, Zimmermann K, Hillig RC,

Guenther J, Toschi L, Neuhaus R, Haegebart A, Hess-Stumpp H, Bauser M, Wick W, Unterberg A, Herold-Mende C, Platten M, von Deimling A (2017) Pan-mutant IDH1 inhibitor BAY 1436032 for effective treatment of IDH1 mutant astrocytoma in vivo. Acta Neuropathologica 133:629-644

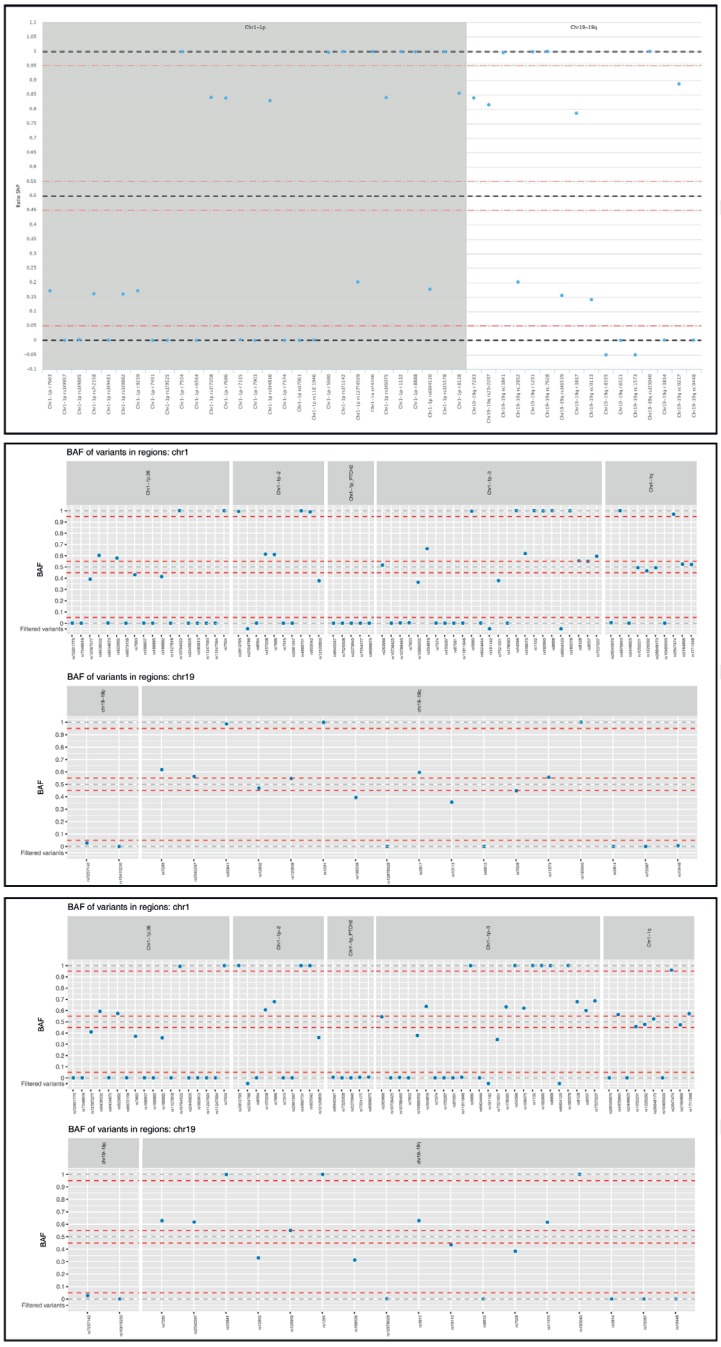
12. Rohle D, Popovici-Muller J, Palaskas N, Turcan S, Grommes C, Campos C, Tsoi J, Clark O, Oldrini B, Komisopoulou E, Kunii K, Pedraza A, Schalm S, Silverman L, Miller A, Wang F, Yang H, Chen Y, Kernytsky A, Rosenblum MK, Liu W, Biller SA, Su SM, Brennan CW, Chan TA, Graeber TG, Yen KE, Mellinghoff IK (2013) An inhibitor of mutant IDH1 delays growth and promotes differentiation of glioma cells. Science 340:626-630
13. Struys EA, Jansen EE, Verhoeven NM, Jakobs C (2004) Measurement of urinary D- and L-2-hydroxyglutarate enantiomers by stable-isotope-dilution liquid chromatography-tandem mass spectrometry after derivatization with diacetyl-L-tartaric anhydride. Clin Chem 50:1391-1395
14. Tateishi K, Wakimoto H, Iafrate AJ, Tanaka S, Loebel F, Lelic N, Wiederschain D, Bedel O, Deng G, Zhang B, He T, Shi X, Gerszten RE, Zhang Y, Yeh JJ, Curry WT, Zhao D, Sundaram S, Nigim F, Koerner MVA, Ho Q, Fisher DE, Roider EM, Kemeny LV, Samuels Y, Flaherty KT, Batchelor TT, Chi AS, Cahill DP (2015) Extreme Vulnerability of IDH1 Mutant Cancers to NAD + Depletion. Cancer Cell 28:773-784
15. Watanabe T, Nobusawa S, Kleihues P, Ohgaki H (2009) IDH1 mutations are early events in the development of astrocytomas and oligodendrogliomas. Am J Pathol 174:1149-1153
16. Yan H, Parsons DW, Jin G, McLendon R, Rasheed BA, Yuan W, Kos I, Batinic-Haberle I, Jones S, Riggins GJ, Friedman H, Friedman A, Reardon D, Herndon J, Kinzler KW, Velculescu VE, Vogelstein B, Bigner DD (2009) IDH1 and IDH2 mutations in gliomas. N Engl J Med 360:765-773

**Supplementary Table** 1. Addition of AGI-5198 for up to day 60 did not affect the VAF of *IDH1* mutations.

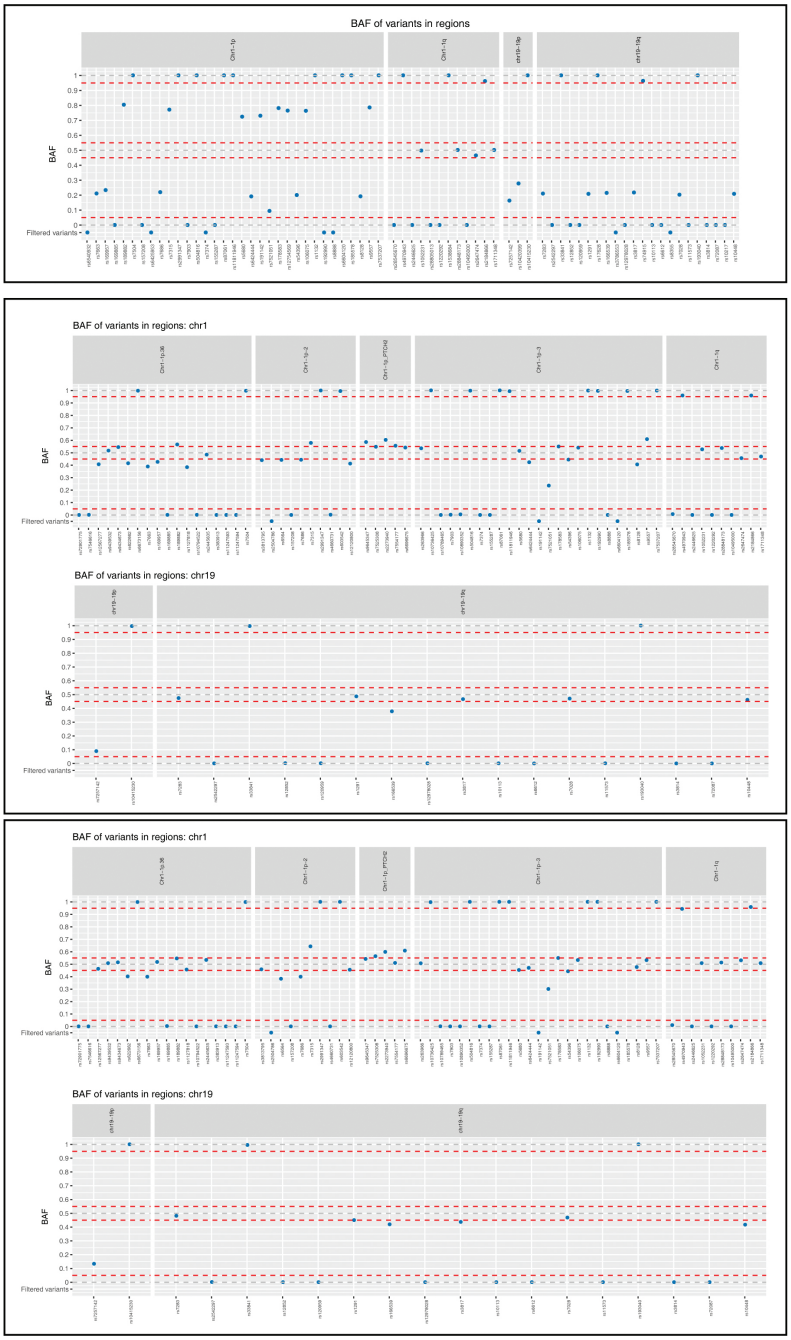
| Tumor | Days in culture | Condition | IDH status | VAF | TP53 status | VAF | ATRX status | VAF |
|---|---|---|---|---|---|---|---|---|
| 10 | 60 | DMSO control | IDH1 p.R132H | 46 | TP53 p.R273H | 36 | ATRX p.R1426X | 52 |
| 10 | 60 | 10μM AGI-5198 | IDH1 p.R132H | 46 | TP53 p.R273H | 33 | ATRX p.R1426X | 42 |



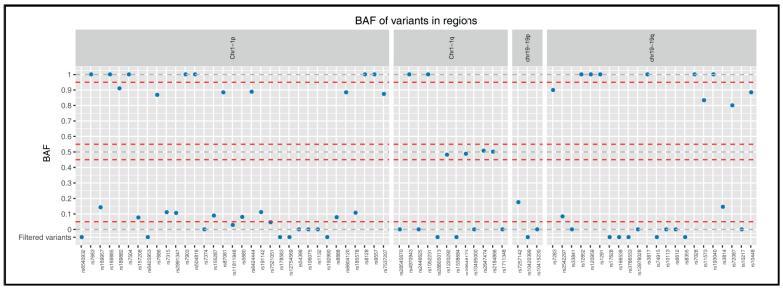

**Supplementary Figure 1** Addition of AGI-5198 decreases D2HG levels in the *IDH*-mutated cultures. A. Accumulated D2HG was detected in the culture medium of tumor 9 for up to 21 days, which was inhibited by the addition of an IDH1 inhibitor AGI-5198. B. No elevated L2HG was detected in the culture medium and addition of AGI-5198 did not affect the level of L2HG. C. Elevated levels of D2HG was detected in the medium of 7 *IDH*-mutated glioma cultures. Addition of AGI-5198 affected absolute concentration of D2HG in some cultures. D. Varied levels of L2HG were detected in the medium of the 7 glioma cultures, suggesting the absolute concentrations of D2HG should be corrected to the levels of L2HG in each culture.



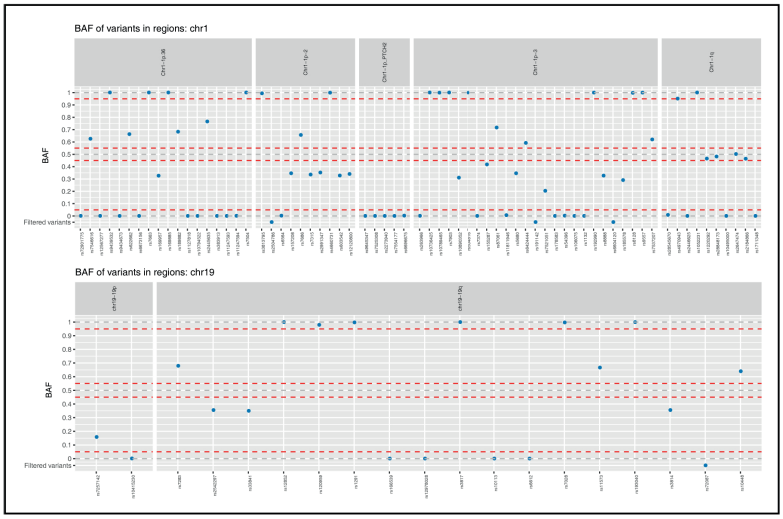

**Supplementary Figure 2.** SNP-based loss of heterozygosity (LOH) analysis for chromosomes 1 and 19 of tumor 12. Blue data points represent the BAF of individual SNP (a BAF of 0 equals absent of B-allele; 1 equals present of both B-alleles). A BAF of 15 and 85 corresponds to 80-85% tumor cells present at diagnosis. Only 30-40% tumor cells were present after 10 days in culture and the percentage of these cells was not affected by the addition of AGI-5198.



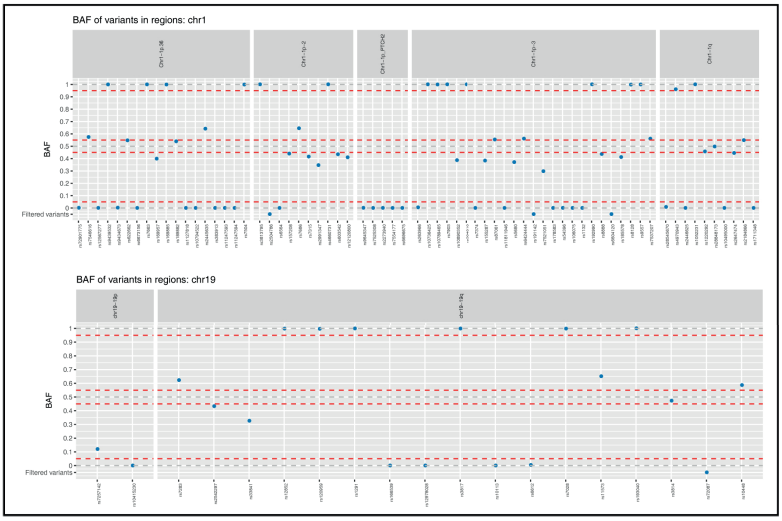

**Supplementary Figure 3.** SNP array data for chromosomes 1 and 19 of tumor 14 at diagnosis and 10 days in culture. Blue data points represent the BAF of individual SNP (a BAF of 0 equals absent of B-allele; 1 equals present a homozygous B-allele). A BAF of 25 and 75 corresponds to 75% tumor cells present at diagnosis. Only 20-30% tumor cells were present after 10 days in culture and the percentage of these cells was reduced to less than 10% by the addition of AGI-5198.




SNPs in Tumor 15 at diagnosis

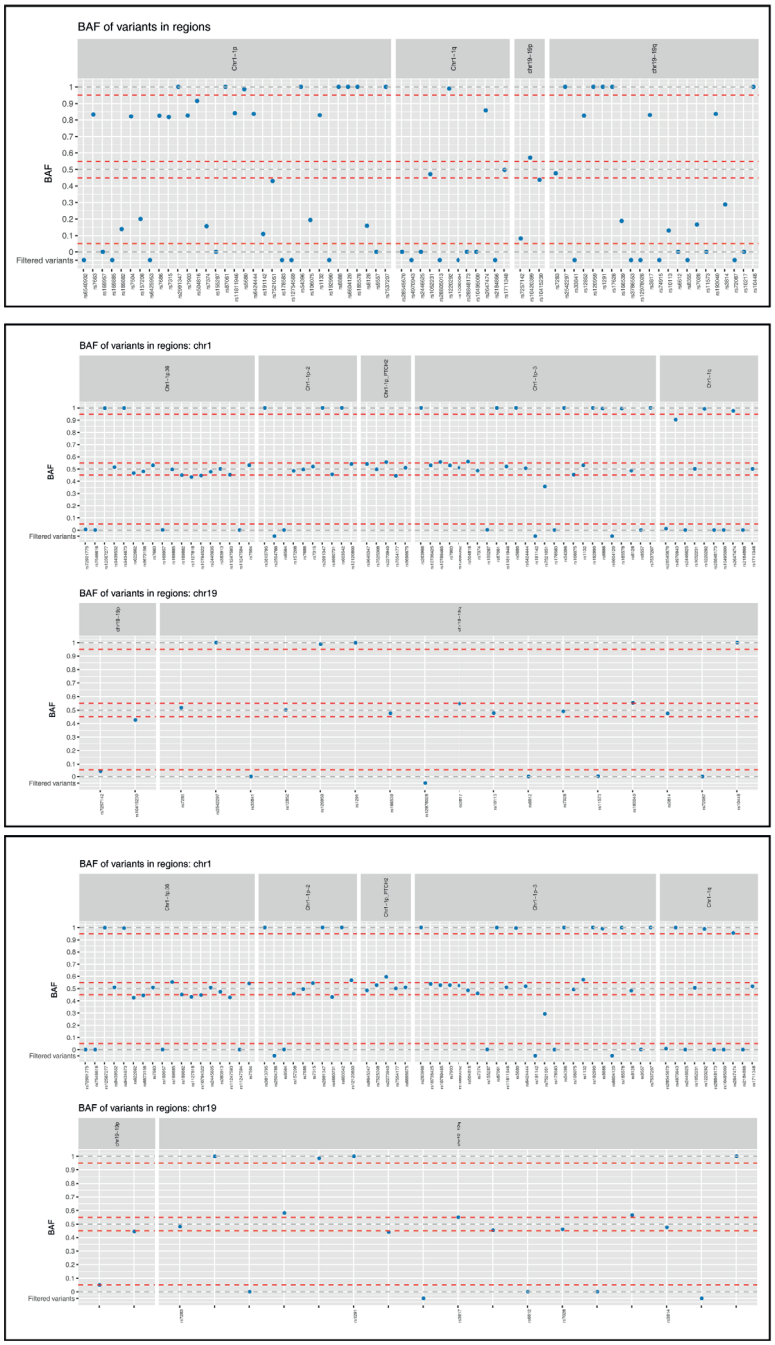



SNPs in Tumor 15 10 Days Control

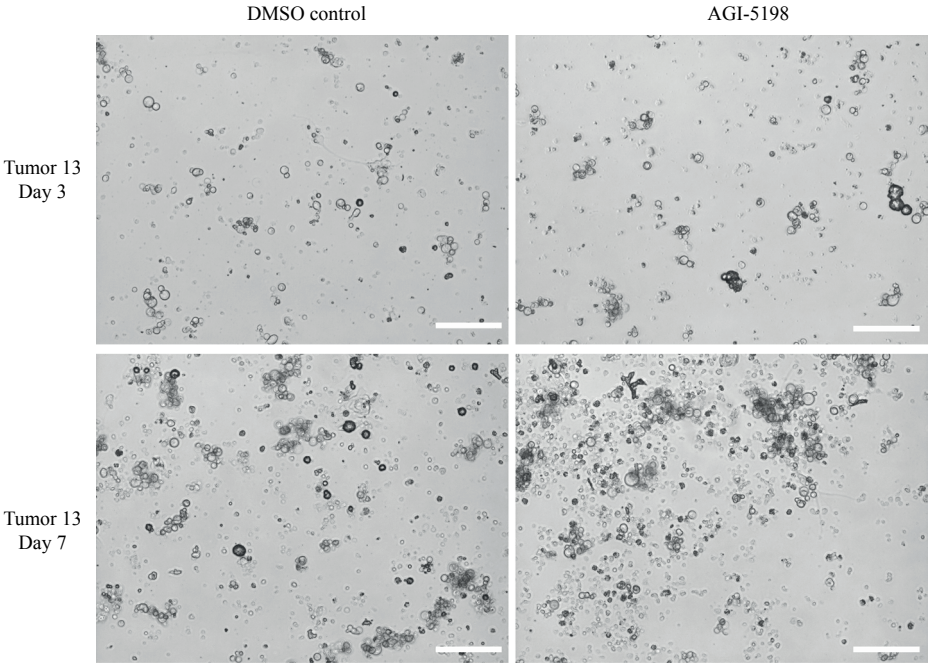



SNPs in Tumor 15 10 Days AGI-5198

**Supplementary Figure 4.** SNP array data for chromosomes 1 and 19 of tumor 15 at diagnosis and 10 days in culture. Blue data points represent the BAF of individual SNP (a BAF of 0 equals absent of B-allele; 1 equals present a homozygous B-allele). A BAF of 10 and 85 corresponds to 85-90% tumor cells present at diagnosis. About 60% tumor cells were present after 10 days in culture and the percentage of these cells was slightly reduced to about 40% by the addition of AGI-5198.




**Supplementary Figure 5.** SNP array data for chromosomes 1 and 19 of tumor 17 at diagnosis and 10 days in culture. Blue data points represent the BAF of individual SNP (a BAF of 0 equals absent of B-allele; 1 equals present a homozygous B-allele). A BAF of 15 and 85 corresponds to ~85% tumor cells present at diagnosis. No tumor cells were present in culture after 10 days and the percentage of these cells remained when treated with AGI-5198.




**Supplementary Figure 6.** Addition of AGI-5198 did not affect cellular morphology of cultured gliomas. Tumor 13 scale bar 50µm, tumor 12 scale bar 400 µm.