Towards personalised medicine for cancer

From initial therapy to follow-up

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Chapter 13: Radioprotection of IDH1-mutated cancer cells by the IDH1-mutant inhibitor AGI-5198


Abstract

Isocitrate dehydrogenase 1 (IDH1) is mutated in various types of human cancer to IDH1R132H, a structural alteration that leads to catalysis of α-ketoglutarate to the oncometabolite D-2-hydroxyglutarate (D2HG). In this chapter, we present evidence that small molecule inhibitors of IDH1R132H that are being developed for cancer therapy may pose risks with co-administration of radiotherapy. Cancer cells heterozygous for the IDH1R132H mutation exhibited less IDH-mediated production of NADPH, such that after exposure to ionizing radiation (IR) there were higher levels of reactive oxygen species, DNA double-strand breaks and cell death compared to IDH1 wild-type cells. These effects were reversed by the IDH1R132H inhibitor AGI-5198. Exposure of IDH1 wild-type cells to D2HG was sufficient to reduce IDH-mediated NADPH production and increase IR sensitivity. Mechanistic investigations revealed that the radiosensitivity of heterozygous cells was independent of the well-described DNA hypermethylation phenotype in IDH1-mutated cancers. Thus, our results argue that altered oxidative stress responses are a plausible mechanism to understand the radiosensitivity of IDH1-mutated cancer cells. Further, they offer an explanation for the relatively longer survival of patients with IDH1-mutated tumours, and they imply that administration of IDH1R132H inhibitors in these patients may limit irradiation efficacy in this setting.

Introduction

IDH1 and IDH2 are homodimeric enzymes that reversibly convert isocitrate to α-ketoglutarate (αKG) with concomitant reduction of NADP+ to NADPH in the cytoplasm and mitochondria, respectively.15 Somatic heterozygous hotspot mutations in IDH1/2 are observed in substantial percentages of various tumour types, such as glioma (80%), acute myeloid leukaemia (AML, 20%), cholangiocarcinoma (20%), chondrosarcoma (60%) and others.15

IDHMT cause metabolic changes in cancer.16 All IDH1/2 mutations, of which IDH1R132H is the most prevalent in glioma, cause loss of enzymatic wild-type IDH1/2 function.17,43,77 In addition, IDHMT has a neo-enzymatic (gain of function) activity: it converts αKG and NADPH to D2HG and NADP+. D2HG is an oncometabolite that is present in trace amounts in IDH1/2 wild-type cells but accumulates to levels up to 50 mM in IDH1/2-mutated cancers.13 Because of the chemical similarities between D2HG and αKG, D2HG competitively inhibits αKG-dependent dioxygenases, such as ten-eleven translocation factor 2 (TET2) and JmjC-domain containing histone lysine demethylases (JHKDMs).29,30 This results in a CpG island methylation phenotype (CIMP), which alters gene expression and induces malignant transformation.381

Patients with IDH1/2-mutated glioma and cholangiocarcinoma have up to 3-fold longer median overall survival times than IDH1/2 wild-type counterparts.12,17,20,43,289 Altered patient survival caused by differences in IDH1-mutated versus IDH1 wild-type tumour biology may be on the level of intrinsically-reduced malignancy, and/or altered responses to therapy. We previously showed in human glioblastoma samples that IDH1 mutations are associated with a 38% decrease in IDH-mediated NADPH production capacity.43 NADPH is the most important source of reducing power for cellular detoxification of oxidants, because it is an essential cofactor for the regeneration of reduced glutathione (GSH) from oxidised glutathione (GSSG) by glutathione reductase. Therefore, we and others proposed that altered redox responses result in increased responses to therapy in IDH1-
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123 This hypothesis is supported by in vitro studies, showing that overexpression of IDH1R132H sensitises glioma cells to IR or chemotherapy with carmustine (BCNU), cisplatin (CDDP) or temozolomide. 211,212,290 In addition, there is clinical evidence that IDH1MT sensitise glioma to a procarbazine, lomustine (CCNU) and vincristine (PCV) regimen 328 and sensitises glioblastoma to a combination of IR and temozolomide. 333

IDH1/2 mutations are early events in the formation of glioma, 54 chondrosarcoma, 71 and cholangiocarcinoma, 459 which makes IDH1/2 mutations attractive therapeutic targets. Inhibitors of mutant IDH1 and mutant IDH2 were recently developed. 130,158,281,382,383 Clinical trials of the IDH1R132H inhibitor AGI-5198 (ClinicalTrials.gov NCT02073994) are in progress in solid tumours. AGI-5198 inhibits IDH1R132H neo-enzymatic activity, which decreases D2HG production in IDH1-mutated cells, and thus inhibits carcinogenesis. 158 However, better prognosis of patients with established IDH1-mutated glioma or cholangiocarcinoma (which are past the carcinogenesis stage) may be related to increased anti-cancer therapy responses by virtue of increased oxidative stress in these tumours due to a lower NADPH production capacity. Thus, we argued that inhibition of mutant IDH1/2 decrease this stress and consequently increases cancer cell survival. 15,16

The aim of the present study is to provide in vitro evidence that the prolonged overall survival of glioma patients with IDH1-mutated tumours is caused by increases in oxidative stress, and in particular, reduced NADPH production capacity. We investigated whether this stress affects the response to IR and metformin. Moreover, we studied whether the IDH1R132H inhibitor AGI-5198 blocks this metabolic stress, thus interfering with the survival-prolonging properties of mutant IDH1.

Materials and methods

Cell lines. HCT116 IDH1WT/R132H knock-in cells, generated by AAV targeting technology GENESIS, 172 were kindly provided by Horizon Discovery. HT1080 chondrosarcoma cells were a kind gift of Dr. Hamann (Department of Experimental Immunology, Academic Medical Centre). U251 and LN229 glioblastoma cells were stably transduced using lentiviral constructs encoding for IDH1 WT (wild-type) or IDH1R132H as described earlier. 460 Constructs contain a C-terminal biotin acceptor peptide and a HIS-tag, which allows distinction from endogenous wild-type IDH1 by molecular weight. IDH1WT and IDH1R132H expression was analysed by western blotting using a pan-IDH1 antibody (HPA0352428, Sigma-Aldrich) and a specific antibody recognizing IDH1R132H. 461 Western blots were analysed using a Odyssey system (Li-Cor Biotechnology). IDH1WT/R132H and IDH1WT/WT HCT116 cells were cultured in McCoy’s 5A medium ( Gibco, Life Technologies, Thermo Fisher Scientific) in 5% CO2 at 37°C. U251, LN229 and HT1080 cells were cultured in 5% CO2, 5% CO2 and 10% CO2, respectively, at 37°C in complete DMEM ( Gibco). All media were supplemented with 10% foetal bovine serum (HyClone, Thermo Fisher Scientific), 100 units/ml penicillin and 100 µg/ml streptomycin (both Gibco).

Reagents. AGI-5198 was purchased from MedChemExpress, L2HG, D2HG, αKG, coenzyme A, thiamine pyrophosphate and NAC were purchased from Sigma-Aldrich. Metformin was purchased from BioConnect.

Enzyme activity measurements. Metabolic mapping was performed as described in Chapter 4 of this thesis. We used supraphysiological substrate concentrations (up to 30 mM) because the viscous 18% polyvinyl alcohol-containing enzyme reaction incubation medium does not allow sufficient substrate diffusion at low concentrations. Thus, the determined enzyme activities do not reflect the in vivo situation at a given substrate concentration but are suitable for intra-experimental comparisons. 198 D2HG and L2HG inhibition experiments were performed in the presence of 1 mM isocitrate or 3 mM αKG and 30 mM D2HG or 30 mM L2HG or solvent only (double-distilled H2O, final concentration ≤3%) in the enzyme activity reaction medium. The 2HG:isocitrate and 2HG:αKG ratios used in these
experiments are in line with the pathophysiological conditions in human glioma where D2HG concentrations may be up to 100 to 1000-fold higher than isocitrate and αKG concentrations.  

**Colony-forming assays after IR.** Colony-forming assays after IR were performed and analysed as described previously. 462-500 cells/cm² were seeded; higher densities are needed at higher IR doses to obtain sufficient amounts of colonies. Cells were treated from 72 hours before to 4 hours after IR with D2HG, AGI-5198, the reactive oxygen species (ROS) scavenger N-acetyl cysteine (NAC) or solvent only (DMSO, ≤0.5%). Cells were irradiated with 1-6 Gy using a 137Cs source (Department of Experimental Oncology and Radiobiology, Academic Medical Centre) at 6 hours after plating in the presence or absence of 0-800 nM AGI-5198, 0-10 mM D2HG or 0-5 µM NAC. Cells were fixed and stained at 10 days after IR with a mixture of 0.05% crystal violet (Merck) and 6% glutaraldehyde (Merck) for ≥2 hours at room temperature. Clones consisting of ≥50 cells were manually counted using a stereoscope (Leica MZ6, Leica Microsystems). Data are expressed as clonogenic fraction, which is the number of colonies counted divided by the number of cells plated, corrected for the plating efficiency. D0 and n values were found by fitting a semilog line through the clonogenic fraction data points of the final slope. Dq values were found by solving the semilog line equation for 1.

**Epigenome-wide DNA methylation analysis.** Cells were lysed and genomic DNA was isolated as described previously. 463 Genomic DNA was bisulfite-converted using the EZ DNA Methylation Gold Kit (Zymo Research). Bisulfite-converted DNA was analysed for epigenome-wide DNA methylation analysis using an Infinium Human Methylation 450 BeadChip array (Illumina). This array includes over 450,000 CpG sites that cover approximately 99% of the RefSeq genes. Analysis was performed in the MinFI R-package (R statistical programming language) and samples were normalised using the SWAN method. 464 Normalised β values were evaluated. Values are between 0 and 1 which stand for a completely unmethylated and methylated probe, respectively.

**Cellular NADP+, NADPH, GSH, GSSG and ROS measurements.** 10⁶ cells were plated in a 6-well plate, incubated in the presence or absence of 800 nM AGI-5198 and treated with 0-2 Gy IR. After 60 minutes, cells were harvested, prepared and analysed for NADP+/NADPH ratios, GSH/GSSG ratios and ROS levels using a colourimetric NADP+/NADPH ratio detection assay kit (Abcam), a fluorometric GSH/GSSG ratio detection assay kit (Abcam) and a fluorometric CellROX Deep Red ROS detection assay kit (Life Technologies), respectively, in a 96-well plate using a POLARStar Galaxy microplate reader (BMG Labtech) according to manufacturers’ protocols. In addition, cells were analysed for ROS levels using a CellROX Deep Red ROS detection assay kit (Life Technologies) in an LSR Fortessa fluorescence flow cytometry analyser (BD Biosciences) according to the manufacturer’s protocol. Cells were counterstained using a SYTOX Blue Dead Cell stain (Life Technologies). Data was processed in FACSDiva (BD Biosciences) and analysed in FlowJo (FlowJo).

**γ-H2AX immunofluorescence stainings and measurements.** DNA double-strand breaks (DSBs) kinetics were studied using γ-H2AX foci immunofluorescence staining. 465 Cells were plated on glass coverslips in a 6-well plate, incubated in the presence or absence of 800 nM AGI-5198 and treated with 0-2 Gy IR, washed with PBS and fixed after 30 minutes using 2% paraformaldehyde for 15 minutes. Cells were permeabilised with PTNBS (PBS containing 1% Triton X-100 and 1% FCS) for 1 hour and stained for γ-H2AX foci using a mouse monoclonal anti-γ-H2AX antibody (Millipore, diluted 1:100 in sTNBS (PBS containing 0.1% Triton X-100 and 1% FCS)) for 90 minutes at room temperature. Cover slips were washed with sTNBS and stained using secondary goat anti-mouse Cy3 antibody (Jackson ImmunoResearch, diluted 1:100 in sTNBS) for 30 minutes at room temperature in the dark. Lastly, cover slips were washed with sTNBS and nuclei were stained with DAPI (Millipore, 1:500) for 2 minutes at room temperature. Cover slips were mounted to microscopy slides using Vectashield (Vector Laboratories). Nail polish was used as sealant. The number of γ-H2AX foci per cell was determined using a Leica DM RA HC fluorescence microscope equipped with a CCD camera and a 100x objective Plan Apochromatic lens with 1.40 numerical aperture (Leica Microsystems). Cy3
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and DAPI signal were captured using excitation/emission wavelengths of 550/570 nm for 400 ms and 360/460 nm for 50 ms, respectively. Photomicrographs were obtained using custom-made software (Van Leeuwenhoek Centre for Advanced Microscopy, Academic Medical Centre). Stack images of at least 200 cells per sample were taken. One stack consisted of 40 slices with a 300 nm interval between the slices along the z-axis. The images were processed using deconvolution software and the number of γ-H2AX foci per cell was automatically scored using custom-made software.

**Statistical analysis.** Data were processed in Excel (Microsoft) and analysed using SPSS (IBM) and Prism 6 (GraphPad Software). Non-linear least squares were used to fit enzyme models. Data shown are representative of, or means ± SEM of, at least 3 independent experiments. P-values were calculated as described in the methods section and figure legends; (*) P value < 0.05; (**) P value < 0.01; (***) P value < 0.001; (****) P value < 0.0001.

**Results**

**IDH1 mutations reduce IDH-mediated NADPH production and IR tolerance in vitro.** NADP+-dependent IDH activity was significantly lower in IDH1WT/R132H HCT116 cells than in IDH1WT/WT HCT116 cells (Figure 1A and eFigure 1A). The reduced IDH-mediated NADPH production capacity of IDH1-mutated cells was confirmed in U251 and LN229 glioblastoma cell lines that stably overexpressed IDH1WT or IDH1R132H (Figure 1B-D). Cells overexpressing IDH1WT had a higher IDH-mediated NADPH production capacity than parental glioblastoma cells. The vulnerability of IDH1WT/R132H and IDH1WT/WT HCT116 cells to IR was investigated using colony-forming assays. Relative to IDH1WT/WT cells, we observed a significantly reduced surviving fraction of IDH1WT/R132H cells at all IR doses (Figure 1E), i.e. IDH1R132H radiosensitises HCT116 cells. We hypothesised that the increased radiosensitivity of IDH1WT/R132H HCT116 cells was caused by increased vulnerability to oxidative stress, which is a result of reduced IDH-mediated NADPH production capacity. To test this, we treated IDH1WT/R132H and IDH1WT/WT HCT116 cells with the NADPH surrogate and ROS scavenger NAC at 4 hours before IR. NAC equalised the surviving fractions of IDH1WT/R132H and IDH1WT/WT HCT116 cells (Figure 1F). This suggests that oxidative stress mediates the increased radiosensitivity of IDH1WT/R132H HCT116 cells. The culture medium McCoy 5A contains 0.5 mg/l reduced glutathione, which may affect redox potentials of HCT116 and therefore their IR response. We confirmed the increased radiosensitivity of IDH1WT/R132H HCT116 cells in DMEM medium, which contains no reduced glutathione (eFigure 1B). IDH1/2 mutations induce CIMP after long-term passaging of cells. CIMP has profound effects on gene expression and theoretically, this could alter IR sensitivity. We compared genome-wide methylation levels in early-passage (P3) and late-passage (P30) IDH1WT/R132H and IDH1WT/WT HCT116 cells and observed a relative CIMP in P30 compared to P3 IDH1WT/R132H HCT116 cells. Long-term culture did not induce CIMP in IDH1WT/WT HCT116 cells (Figure 1G). IR sensitivity of P30 IDH1WT/R132H HCT116 cells (Figure 1H) did not differ from IR sensitivity of P3 IDH1WT/R132H HCT116 cells (Figure 1E) and IR sensitivity is thus not related to CIMP. Statistics and Dφ, n and D0 values for all colony-forming assays are shown in eTables 1 and 2.

**D2HG sensitises cells to IR and inhibits IDH-mediated NADPH production.** A hallmark of IDH1/2 mutations in cancer is D2HG accumulation13,15 and D2HG is known to induce oxidative stress in glia, neurons122-124 and Drosophila.466 Therefore, we considered the possibility that D2HG is responsible for the sensitisation of IDH1WT/R132H HCT116 cells to IR. IDH1WT/WT and IDH1WT/R132H HCT116 cells were exposed to D2HG from 4 hours before to 4 hours after IR treatment. D2HG significantly decreased the clonogenic fractions of IDH1WT/WT and IDH1WT/R132H HCT116 cells after IR (Figure 2A), i.e. D2HG radiosensitises HCT116 cells. Of note, the radiosensitising effect of D2HG was larger in IDH1WT/WT than in IDH1WT/R132H HCT116 cells, in line with pre-existing endogenous D2HG in IDH1WT/R132H HCT116 cells. D2HG and L2HG are competitive inhibitors of αKG-dependent enzymes.29-31 The structural similarity between isocitrate, αKG and D2HG led us to hypothesise that high levels of D2HG in IDH1/2-mutated cancers inhibit IDH-mediated NADPH production capacity. To study this, we performed enzyme activity experiments in the presence and absence of D2HG or L2HG. D2HG and
Figure 1. IDH1R132H mutations reduce IDH-mediated NADPH production and radioresistance.

(A) The NADP+-dependent IDH activity of early-passage (P3) IDH1WT/WT and IDH1WT/R132H HCT116 cells at various isocitrate concentrations was determined as absorbance of blue formazan produced from NBT per cell. (B) U251 and LN229 glioblastoma (parental) cell lines were stably transduced with lentiviral vectors harbouring IDH1WT and IDH1R132H genes. All open reading frames have a C-terminal biotin acceptor peptide and a HIS tag for detection and purification purposes (not used in this work), which is why two IDH1 bands appear on the blot. The lower band is endogenous IDH1, the upper band is the tagged IDH1. Anti-pan-IDH1 antibody is used to detect IDH1WT and IDH1R132H. A specific antibody against IDH1R132H is used for detection of the IDH1R132H mutant enzyme. GAPDH serves as a loading control. (C-D) As in (A), but with IDH1R132H- and IDH1WT-overexpressing glioblastoma cell lines U251 (C) and LN229 (D). To control for overexpression artefacts, enzyme activity of IDH1R132H- and IDH1WT-overexpressing cells was normalised relative to the enzyme activity of parental cells based on pan-IDH1 expression levels as determined by Western Blot. (E) Colony-forming assay after 0-6 Gy IR with IDH1WT/WT and IDH1WT/R132H HCT116 cells. The clonogenic fraction is the number of colonies counted divided by the number of cells plated, corrected for the plating efficiency. (F) As in (E), but in the presence or absence of 5 µM of ROS-scavenging NAC. (G) beta-values frequency plot of P3 and P30 IDH1WT/WT and IDH1WT/R132H HCT116 cells that were subjected to epigenome-wide DNA methylation analysis to determine whether IDH1R132H induced a DNA hypermethylator phenotype after long-term culture. β-values are between 0 and 1 and represent completely unmethylated and methylated probes, respectively. Inset: magnified plot of β-values between 0.8 and 1. (H) As in (A), but with P30 IDH1WT/WT and IDH1WT/R132H HCT116 cells. Y-axis in (E,F,H) is logarithmic. Abbreviations: v, versus; n.s., not significant; NAC, N-acetyl cysteine.
L2HG reduced the IDH-mediated NADPH production capacity in IDH1\textsuperscript{WT/WT} and IDH1\textsuperscript{WT/R132H} HCT116 cells, U251 glioblastoma cells and IDH1 wild-type and IDH1-mutated human glioblastoma tissue (n = 8; Figure 2B-E); this inhibition was dose-dependent (eFigure 2A). In addition, D2HG and L2HG inhibited the \(\alpha\)KGDH-mediated NADH production capacity in IDH1 wild-type and IDH1-mutated human glioblastoma tissue (n = 8; Figure 2E-F). For both IDH and \(\alpha\)KGDH, L2HG was a more efficient IDH inhibitor than D2HG. These results indicate that D2HG and L2HG are inhibitors of IDH-mediated NADPH production capacity. D2HG and L2HG did not affect G6PD-mediated NADPH production capacity or IDH-dependent NADH production capacity, indicating absence of off-target effects (eFigure 2B-C).

**Figure 2. D2HG radiosensitises cells and inhibits IDH-mediated NADPH production capacity.**

(A) Colony-forming assay after 0-4 Gy IR with IDH1\textsuperscript{WT/WT} and IDH1\textsuperscript{WT/R132H} HCT116 cells in the presence or absence of 10 mM D2HG. D2HG incubation was from 4 hours before to 4 hours after IR. The clonogenic fraction is the number of colonies counted divided by the number of cells plated, corrected for the plating efficiency. Y-axis is logarithmic. (B-D) The NADP\(^+\)-dependent IDH activity against 1 mM isocitrate of (B) IDH1\textsuperscript{WT/WT} and IDH1\textsuperscript{WT/R132H} HCT116 cells, (C) U251 glioblastoma cells and (D) IDH1 wild-type and IDH1-mutated human glioblastoma samples was determined in the presence or absence of 30 mM D2HG or 30 mM L2HG. Absorbance of blue formazan produced from NBT was measured per cell (HCT116 and U251 cell lines) or per 0.5 mm \(\times\) 0.5 mm tissue (human glioblastoma samples). In the latter case, serial sections were used. (E) Representative photomicrographs of serial sections of human glioblastoma samples after staining for NADP\(^+\)-dependent IDH activity against 1 mM isocitrate in the presence or absence of 30 mM D2HG or 30 mM L2HG. P values were obtained using one-way ANOVA using Tukey’s correction for multiple comparisons. Abbreviations: GBM, glioblastoma; IDH1\textsuperscript{WT}, IDH1 wild-type; IDH1\textsuperscript{MT}, IDH1-mutated; \(\alpha\)KGDH, \(\alpha\)-ketoglutarate dehydrogenase.
The IDH1-mutant inhibitor AGI-5198 restores IDH-mediated NADPH production in IDH1-mutated cells. Since the introduction of IDH1R132H increases metabolic stress via a reduced NADPH production capacity, we hypothesised that IDH1R132H inhibition reverses this effect. Indeed, after 72 hours of incubation in the presence of AGI-5198, IDH-mediated NADPH production capacity of IDH1WT/R132H HCT116 cells was restored to levels comparable with those of IDH1WT/WT HCT116 cells (Figure 3A). In contrast, no effect was detected after 4 hours of incubation with AGI-5198 (Figure 3B) or in IDH1WT/R132H HCT116 cells that were treated with AGI-5198 after adherence to microscopical glass slides (eFigure S3A). This suggests that AGI-5198 restores IDH-mediated NADPH production capacity after relieving IDH1WT/R132H cells of high D2HG concentrations. We validated these results in IDH1R132H U251 and LN229 cells (Figure 3C-D). IDH1R132H U251 cells needed the highest AGI-5198 doses for restoration of IDH-mediated NADPH production capacity, followed by IDH1R132H LN229 cells, followed by IDH1WT/R132H HCT116 cells. This may relate to higher IDH1R132H expression in U251 than in LN229 cells and higher IDH1R132H expression in IDH1R132H-overexpressing cells than in IDH1WT/R132H knock-in cells (Figure 1B and eFigure S1C). Larger amounts of IDH1R132H protein may require higher AGI-5198 doses for complete IDH1R132H inhibition. There was no effect after 72 hours incubation with AGI-5198 on NADP+-dependent IDH activity in IDH1WT/WT HCT116 cells (Figure 3E-F) or NAD+-dependent IDH3 activity in IDH1WT/R132H HCT116 cells (eFigure 3D), in agreement with AGI-5198 specificity for IDH1R132H.158 Because AGI-5198 inhibits IDH1R132C as well, although at higher concentrations than IDH1R132H,158 we confirmed our results in IDH1WT/R132C HT1080 cells (eFigure 3C-D).

Figure 3. The IDH1-mutant inhibitor AGI-5198 increases IDH-mediated NADPH production capacity in IDH1-mutated cells.

(A) The NADP+-dependent IDH activity of IDH1WT/WT and IDH1WT/R132H HCT116 cells at various isocitrate concentrations was determined as absorbance of blue formazan produced from NBT per cell after 72 hours cell culture in the presence or absence of AGI-5198. (B) As in (A), but after 4 hours cell culture in the presence or absence of AGI-5198 instead of 72 hours. (C) As in (A) but using parental, IDH1WT, overexpressing and IDH1R132H, overexpressing U251 cells. (D) As in (C) but using LN229 cells. (E-F) As in (A-B) but using IDH1WT/WT HCT116 cells only. All concentrations in the figure refer to AGI-5198.
Figure 4. *IDH1* mutations decrease NADPH levels and GSH levels, increase ROS levels and AGI-5198 attenuates this effect.

(A) Cells were incubated in the presence or absence of 800 nM AGI-5198 and treated with 0-2 Gy IR and were harvested, prepared and colourimetrically analysed for NADP+/NADPH ratios after 60 minutes. (B) As in (A), but with fluorometric analysis for GSH/GSSG ratios. (C) As in (A), but with fluorometric analysis for ROS levels. (D) as in (C), but with fluorescence-guided flow cytometry analysis for ROS levels (x-axis) and viable cells (y-axis). P values were obtained using one-way ANOVA on the difference between IR-treated and untreated cells using Tukey’s correction for multiple comparisons. All concentrations in the figure refer to AGI-5198.
**IDH1 mutations decrease NADPH levels, GSH levels and increase ROS levels and AGI-5198 attenuates this effect.** We investigated the effects of IDH1 mutations on cellular NADPH, GSH and ROS levels with and without pre-treatment with IR. Under steady-state conditions, IDH1<sup>WT/R132H</sup> HCT116 cells had similar NADP<sup>+</sup>/NADPH ratios, GSH/GSSG ratios and ROS levels as IDH1<sup>WT/WT</sup> HCT116 cells, as determined by colourimetric and fluorometric assays and fluorescence flow cytometry experiments (Figure 4A-D and eFigure S4). After treatment with 2 Gy IR, we observed an increase in the NADP<sup>+</sup>/NADPH ratio (owing to more NADP<sup>+</sup> and/or less NADPH), a decrease in the GSH/GSSG ratio (owing to less GSH and/or more GSSG) and an increase in ROS levels in all cell lines. Notably, the increase in the NADP<sup>+</sup>/NADPH ratio and ROS levels and the decrease in the GSH/GSSG ratio was larger in IDH1<sup>WT/R132H</sup> than in IDH1<sup>WT/WT</sup> HCT116 cells, and the IDH1-mutant inhibitor AGI-5198 attenuated this effect in IDH1<sup>WT/R132H</sup> HCT116 cells (Figure 4C-D). These findings suggest that compared with IDH1<sup>WT/WT</sup> HCT116 cells, the higher ROS levels in IDH1<sup>WT/R132H</sup> HCT116 cells after IR result in a higher GSH and NADPH consumption.

**Figure 5.** IDH1-mutant inhibitor AGI-5198 dose-dependently radioprotects IDH1-mutated cells.

(A) Colony-forming assay after 0-4 Gy IR with IDH1<sup>WT/WT</sup> and IDH1<sup>WT/R132H</sup> HCT116 cells after long term (72 hours) incubation in the presence or absence of 0-400 nM AGI-5198. The normalised clonogenic fraction is the number of colonies counted divided by the number of cells plated, corrected for the plating efficiency and normalised to the clonogenic fraction of untreated, irradiated IDH1<sup>WT/WT</sup> HCT116 cells. (B) Colony-forming assay after 0-4 Gy IR with IDH1<sup>WT/WT</sup> HCT116 cells after long term (72 hours) incubation in the presence or absence of 0-400 nM AGI-5198. (C) Colony-forming assay after 0-4 Gy IR with IDH1<sup>R132H</sup>-overexpressing U251 cells after long-term (72 hours) incubation in the presence or absence of 800 nM AGI-5198. (D) Colony-forming assay after 0-4 Gy IR with IDH1<sup>WT/R132H</sup> HCT116 cells after short-term (4 hours) incubation in the presence or absence of 0-400 nM AGI-5198. (E) Colony-forming assay after 0-4 Gy IR with IDH1<sup>WT/WT</sup> and IDH1<sup>WT/R132H</sup> HCT116 cells after long-term (72 hours) incubation in the presence or absence of 0-400 nM AGI-5198 and/or 4 hours incubation in the presence or absence of 10 mM D2HG. Y-axis in (B-D) is logarithmic. All concentrations in the figure refer to AGI-5198. Abbreviations: v, versus; n.s., not significant.
AGI-5198 protects IDH1-mutated cells against IR. Because reduced NADPH production capacity in IDH1WT/R132H cells is associated with radiosensitisation (Figure 1), we hypothesised that by restoring NADPH production capacity in IDH1-mutated cells, the IDH1-mutant inhibitor AGI-5198 radioprotects IDH1-mutated cells. Therefore, we exposed IDH1WT/R132H and IDH1WT/WT HCT116 cells to AGI-5198 for 72 hours before IR. AGI-5198 did not affect radiosensitivity of IDH1WT/WT HCT116 cells, but reduced radiosensitivity of IDH1WT/R132H HCT116 cells, in a dose-dependent fashion, to radiosensitivity levels comparable with those of IDH1WT/WT HCT116 cells (Figure 5A-B). These data show that AGI-5198 radioprotects IDH1WT/R132H HCT116 cells and that high AGI-5198 doses completely block IDH1R132H-induced radiosensitivity. We confirmed these findings in IDH1R132H-overexpressing U251 glioblastoma cells (Figure 5C), where higher AGI-5198 doses were needed to reach a maximal effect. This is in agreement with our finding that higher AGI-5198 doses were needed to restore the IDH-mediated NADPH production capacity in U251 cells than in HCT116 cells (Figure 3A and 3C). No effect on radiosensitivity of IDH1WT/R132H HCT116 cells was observed after 4 hours of incubation with AGI-5198 (Figure 5D). AGI-5198 was unable to radioprotect IDH1WT/R132H or IDH1WT/WT HCT116 cells in the presence of D2HG (Figure 5E), so the radioprotective mechanism of AGI-5198 on IDH1WT/R132H HCT116 cells depends predominantly on the inhibition of IDH1R132H-mediated D2HG production.

IDH1 mutations increase numbers of DNA DSBs and AGI-5198 reverses this effect. DNA DSBs are important mediators of IR-induced cell death.465 We therefore hypothesised that IDH1-mutated cells are radiosensitised because they have increased numbers of DNA DSBs after IR. IDH1WT/R132H HCT116 cells had more γ-H2AX foci after treatment with 1 or 2 Gy of IR than IDH1WT/WT HCT116 cells, and AGI-5198 decreased the numbers of γ-H2AX foci after IR in IDH1WT/R132H, but not in IDH1WT/WT HCT116 cells (Figure 6A-B).

Figure 6. IDH1WT increase numbers of DNA DSBs and AGI-5198 reverses this effect. (A) Representative photomicrographs of cells that were plated on glass coverslips in the presence or absence of AGI-5198, treated with 0-2 Gy IR, and fixed after 30 minutes. Cells were stained immunocytochemically for γ-H2AX for DNA DSBs and with DAPI for DNA content. (B-C) Stack images (40 slices, 300 nm intervals) of at least 200 cells per sample were taken using a fluorescence microscope using custom-made software. Images were processed using deconvolution software and the number of γ-H2AX-positive foci per cell was automatically scored using custom-made software. 6th order smoothing (25 neighbours) was applied to the curves. (B) Frequency plot of γ-H2AX-positive foci per cell for cells treated with 1 Gy IR. (C) As in (B), but with 2 Gy IR. All concentrations in the figure refer to AGI-5198. P values were obtained using one-way ANOVA on the difference between treated and untreated cells using Tukey’s correction for multiple comparisons.
**IDH1 mutations increase sensitivity to metformin and AGI-5198 protects IDH1-mutated cells against metformin.** We wondered whether the increased sensitivity of IDH1WT/R132H HCT116 cells was specific to IR or also applicable to other treatment modalities that induce stress. IDH1-mutated cells are sensitised to antidiabetic biguanides such as metformin, which also depend on oxidative stress to induce cell death. We treated IDH1WT/R132H and IDH1WT/WT HCT116 cells with 0-6 mM metformin in a proliferation experiment. Relative to untreated cells, we observed less IDH1WT/R132H than IDH1WT/WT HCT116 cells after metformin treatment (Figure 7). Moreover, IDH1WT/R132H HCT116 cells that were treated with both AGI-5198 and metformin proliferated more than IDH1WT/R132H HCT116 cells treated with metformin alone. This indicates that IDH1WT/R132H HCT116 cells are sensitised to stress induced by IR or metformin and that AGI-5198 protects IDH1WT/R132H HCT116 cells against both treatment modalities.

**Discussion**

We showed that introduction of IDH1R132H results in D2HG accumulation, inhibits IDHWT function and sensitises cells to IR and metformin. The overall process can thus be described as a D2HG-NADPH-therapy sensitivity cascade for IDH1-mutated cancer cells (Figure 8). Inhibition of mutant IDH1 by AGI-5198 disrupts this cascade at the level of D2HG production, which enhances the capacity of IDH1-mutated cells to detoxify oxidative stress and protects them against IR and metformin.

Patients with glioma or cholangiocarcinoma tumours carrying IDH1/2 mutations have prolonged overall survival compared with IDH1/2 wild-type counterparts. This can be attributed to intrinsic (e.g. less aggressive tumours) and/or extrinsic (e.g. better response to therapy) differences in IDH1/2-mutated versus IDH1/2 wild-type cancers. Our data supports a correlation between the IDH1 mutation and response to therapy, which has been shown by others, both in vitro and retrospectively in the clinic. Our novel finding is that D2HG accumulation, as occurs in IDH1-mutated cancers, directly radiosensitises cancer cells via inhibition of IDH-mediated NADPH production capacity and that this is associated with increased numbers of DNA DSBs after IR. Thus, the prolonged survival effects of IDH1-mutated in glioma patients may, at least partly, be the result of a relative radiosensitivity of IDH1-mutated cancer cells.

**IDH1 mutations confer a worse prognosis in AML patients.** One difference between AML and glioma is that IR is typically not used to treat AML whereas it is routinely used as a treatment modality for glioma. Cytarabine and daunorubicin are used to treat AML and operate by DNA synthesis chain termination and topoisomerase activity, which cause cell death independent of ROS formation. Furthermore, D2HG accumulation in AML cells has likely less biological impact since NADPH production in leukocytes is largely attributable to activity of G6PD, not IDH1/2. In contrast, IDH1/2 is responsible for 65% of NADPH production in glioblastoma and IDH1 mutations decrease NADPH production capacity by 38%, making these tumours dependent on IDH1/2 for reducing power. In addition to glioblastoma and chondrosarcoma cell lines, we used HCT116 colorectal carcinoma cells as an in vitro model. Although IDH1 mutations are not as prevalent in colorectal carcinoma as in glioma, chondrosarcoma or cholangiocarcinoma, they do occur in 0.5% of patients. Thus, IDH1 mutations...
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may affect colorectal cancer cells similar to glioma, cholangiocarcinoma and chondrosarcoma cells. Because IDH1R132H functions as a heterodimer with wild-type IDH1, 1:1 IDH1R132H:IDH1WT expression in IDH1WT/R132H HCT116 cells is more true to nature than IDH1R132H overexpression. Previous reports have shown that the loss-of-function effects of IDH1 mutations are responsible for reduced IDH-mediated NADPH production capacity in IDH1-mutated tumours. In cancer cells, IDH1R132H mutations are heterozygous, i.e. cells lose one functional NADPH-producing allele. In addition, the net NADPH production in IDH1-mutated cells is further reduced via NADPH consumption by IDH1WT-IDH1R132H heterodimers. However, reductive hydroxylation of αKG by IDH1R132H occurs 100-1,000 times slower than the oxidative decarboxylation by wild-type IDH1/2, so such compounding is likely negligible. The present study shows that IDH1 mutations reduce NADPH production capacity through a third mechanism: IDH1R132H-produced D2HG inhibits wild-type IDH1/2. This supports earlier findings that IDH1 mutations inhibit catalytic wild-type IDH1 function in a dominant-negative fashion. We show that this dominant-negative inhibition is mediated by D2HG. Another, but not mutually exclusive, explanation is that this dominant-negative inhibition is mediated by dysfunctional IDH1WT-IDH1R132H heterodimers. In addition to wild-type IDH1/2, D2HG inhibits αKGDH and cytochrome C oxidase (complex IV of the electron transport chain), which further compromises tricarboxylic acid cycle metabolism in IDH1-mutated cells. AGI-5198 did not protect IDH1WT/R132H HCT116 cells against IR when exogenous D2HG was administered. This supports the notion that NADPH consumption by IDH1R132H does not radiosensitise IDH1WT/R132H HCT116 cells. Of note, L2HG was a more potent inhibitor than D2HG of IDH1/2 and αKGDH. This corroborates studies that showed that L2HG is a more potent inhibitor than D2HG of αKG-dependent enzymes, such as TET2, JHKDMs and EGLN.

AGI-5198 restored IDH-mediated NADPH production capacity and radioresistance of IDH1-mutated cells and both effects are likely the result of AGI-5198-inhibited D2HG synthesis. High-dose AGI-5198 completely abolishes D2HG accumulation and completely rescued the radiosensitivity of IDH1WT/R132H HCT116 cells. Oxidative stress is a likely mediator of IDH1WT/R132H radiosensitisation in HCT116 cells, since the ROS scavenger NAC normalised the radiosensitivity of IDH1WT/R132H HCT116 cells to levels of IDH1WTWT HCT116 cells. In addition, IDH1-mutated cells were also sensitised for treatment with metformin, whereas AGI-5198 protected IDH1-mutated cells against metformin. This is in agreement with the fact that mitochondrial inhibitors such as metformin depend on oxidative stress to induce cell death. Previous reports have shown that D2HG induces cellular oxidative stress, although the underlying mechanisms remained elusive thus far. Our data indicate that inhibition of IDH-mediated NADPH production capacity and αKGDH-mediated NADPH production capacity by D2HG and the resulting metabolic stress is, at least partly, responsible for this phenomenon. Our findings that D2HG and L2HG inhibit the activity of wild-type IDH1/2 and αKGDH suggest that this inhibition is due to the chemical similarities between D2HG, L2HG and αKG.

Long-lasting exposures of AGI-5198 (72 hours) radioprotected IDH1-mutated cells but short exposures (4 hours) did not. These data corroborate our quantitative enzyme histochemistry results where 72-hour exposure to high doses of AGI-5198 completely restored IDH-mediated NADPH production capacity.
production capacity of IDH1-mutated cells, but 4-hour exposures did not. This indicates that there is a delaying intermediate that causes lagged AGI-5198-induced restoration of IDH-mediated NADPH production capacity and radioresistance. As D2HG is metabolised slowly by D2HG dehydrogenase (D2HGDH), our findings suggest that D2HG is this intermediate. In addition, it is accordant with the finding that ML309, an IDH1-mutant inhibitor similar to AGI-5198, maximally suppresses D2HG concentrations when applied for at least 24 hours.

IDH1 mutations decreased NADPH and GSH levels and increased ROS levels, but only when cells were treated with IR. This corroborates earlier studies in which IDH1 mutations decreased NADPH and GSH levels and increased ROS in glioblastoma cells after treatment with temozolomide and CDDP, but not in a transgenic mouse model or glioblastoma cells under steady-state conditions. Taken together, our results suggest that in contexts of stress, such as after treatment with IR, the cellular demand for GSH increases. As a consequence, the demand for NADPH increases, but IDH1 mutations compromise the cellular NADPH production capacity and this restricts recycling of GSSG to GSH enough to cause higher ROS levels, i.e. it causes a scenario in which NADPH demand outweighs NADPH supply. In all, our results suggest that altered oxidative stress response is the most likely downstream element of D2HG that results in IDH1 mutant-mediated radioresensitivity. Oxidative stress can either directly induce cell death after treatment with IR, or indirectly via DNA DSBs. On average, IDH1WT/R132H HCT116 cells had twice the amount of DNA DSBs of IDH1WT/WT HCT116 cells after treatment with 2 Gy IR, but the amount of DNA DSBs was only slightly increased in IDH1WT/R132H compared with IDH1WT/WT HCT116 cells after treatment with 1 Gy IR. This resonates with a larger radiosensitivity of IDH1-mutated cells relative to IDH1 wild-type cells after treatment with ≥2 Gy IR than after treatment with 1 Gy IR. Further research is needed to assess whether this is due to increased generation of DSBs by oxidative stress, or decreased DNA DSB repair, and whether IDH1-mutated cancers are sensitised to anti-cancer therapy that targets DNA DSB repair, such as PARP inhibitors. CIMP did not affect the radiosensitivity of IDH1-mutated cells, which argues against a role for epigenetics in this phenomenon. Moreover, long-term AGI-5198 treatment does not reverse CIMP in IDH1-mutated glioma cells and this further precludes a link between CIMP and IDH1 mutant-mediated radiosensitivity, because AGI-5198 reverses IDH1 mutant-mediated radiosensitisation already after 72 hours.

Clinical trials with IDH1/2-mutant inhibitors have already started in patients with IDH1/2-mutated cancer. Our data show that AGI-5198 antagonises the possibly survival-prolonging radiosensitizing effects of IDH1 mutations in glioma. Our in vitro results suggest that concomitant administration of IDH1/2-mutant inhibitors and IR may result in an unfavourable clinical outcome. In vivo validation is urgently necessary as IDH1/2-mutant inhibitors are already in clinical trials. These limitations may also apply to other therapeutic strategies that include a combination of IDH1/2-mutant inhibition with anti-cancer agents whose activity are mediated by oxidative stress. More specifically, we warn against simultaneous treatment with IDH1/2-mutant inhibitors and IR. Instead, we envision treatments in which conventional treatment modalities are applied subsequently, but not concomitantly, with IDH1/2-mutant inhibitors. IDH1/2-mutant inhibitors may be of clinical use when patients are in periods of recovery from conventional anti-cancer drugs whose activity are mediated by oxidative stress. We propose that personalised medicine for IDH1-mutated solid tumours should aim to increase, not decrease, oxidative stress. This may be achieved by anti-tumour immune responses after immunisation of patients with an IDH1R132H peptide, causing inflammatory ROS, or physical exercise, causing mitochondrial ROS. In addition, other Achilles’ heels in IDH1-mutant metabolism could be exploited, such as mitochondrial dysfunction, increased dependence on glutaminolysis and oxidative phosphorylation. These vulnerabilities can be pharmacologically targeted via BCL-2 inhibitors, chloroquine and metformin, respectively. In summary, AGI-5198 radioprotecst IDH1-mutated cancer cells. Administration of an IDH1-mutant inhibitor during IR may thus decrease survival of IDH1-mutated glioma patients. We warn against multi-agent clinical trials with concomitant use of IDH1-mutant inhibitors and IR.