Towards personalised medicine for cancer

From initial therapy to follow-up

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Chapter 14: \(\text{IDH1/2} \) mutations sensitise acute myeloid leukaemia to PARP inhibition and this is reversed by \(\text{IDH1/2} \)-mutant inhibitors


Abstract
Somatic mutations in genes encoding for isocitrate dehydrogenase 1 and 2 (\(\text{IDH1/2} \)) occur in \(\sim 20\%\) of patients with myeloid neoplasms, including acute myeloid leukaemia (AML). \(\text{IDH1/2} \)-mutant enzymes produce D-2-hydroxyglutarate (D2HG), increase DNA damage levels and responses to chemo/radiotherapy and PARP inhibitors in solid tumour cells. Whether this also holds true for applicable to \(\text{IDH1/2} \)-mutated AML is yet unknown. Well-characterised primary human \(\text{IDH1} \)-mutated, \(\text{IDH2} \)-mutated and \(\text{IDH1/2} \) wild-type AML cells were analysed for DNA damage levels and therapy responses to daunorubicin, ionizing radiation and PARP inhibitors. \(\text{IDH1/2} \) mutations caused increased DNA damage and sensitization to daunorubicin, irradiation, and the PARP inhibitors olaparib and talazoparib in AML cells. \(\text{IDH1/2} \)-mutant inhibitors protected against these treatments. Combined treatment with a PARP inhibitor and daunorubicin had an additive effect on killing of \(\text{IDH1/2} \)-mutated AML cells. We provide evidence that the therapy sensitivity of \(\text{IDH1/2} \)-mutated cells was caused by D2HG-mediated downregulation of expression of the DNA damage response gene ATM and not by altered redox responses due to metabolic alterations in \(\text{IDH1/2} \)-mutated AML cells. In conclusion, \(\text{IDH1/2} \)-mutated AML cells are sensitive to PARP inhibitors as monotherapy but especially when combined with a DNA-damaging agent such as daunorubicin, whereas concomitant administration of \(\text{IDH1/2} \)-mutant inhibitors during cytotoxic therapy decrease the efficacy of both agents in \(\text{IDH1/2} \)-mutated AML cells. These results advocate in favor of clinical trials of PARP inhibitors either or not in combination with daunorubicin in \(\text{IDH1/2} \)-mutated AML and against these clinical trials in combination with \(\text{IDH1/2} \)-mutant inhibitors.

Introduction
Mutations in \(\text{IDH1/2} \) occur in various types of cancer, such as glioma, cholangiocarcinoma and certain myeloid neoplasms, including AML, myelodysplastic syndromes and myeloproliferative neoplasms. Wild-type \(\text{IDH1/2} \) converts isocitrate to \(\alpha\)-ketoglutarate (\(\alpha\KG\)) with concomitant reduction of NADP\(^+\) to NADPH. \(\text{IDH1/2} \) mutations result in a neomorphic function, where \(\text{IDH1/2} \)-mutant enzymes convert \(\alpha\KG\) and NADPH to D2HG and NADP\(^+\). D2HG accumulation is oncogenic because it inhibits various \(\alpha\KG\)-dependent dioxygenases involved in epigenetic regulation, thus inducing cellular dedifferentiation and leukaemogenesis. Appreciation of the causative role of \(\text{IDH1/2} \) mutantions in AML formation and maintenance led to the development of agents such as the \(\text{IDH1} \)-mutant inhibitor ivosidenib (AG-120) and the \(\text{IDH2} \)-mutant inhibitor enasidenib (AG-221), which was recently FDA approved for the treatment of relapsed/refractory \(\text{IDH2} \)-mutated AML. While ivosidenib or enasidenib monotherapy was effective in some patients with difficult-to-treat AML, the majority of treated patients either did not have deep responses, or did not have durable responses, indicating the need to combine these drugs with other anti-leukaemic agent(s).

Other effects of D2HG besides inhibition of \(\alpha\KG\)-dependent dioxygenases include the inhibition of the DNA repair enzyme alkB homolog (ALKBH) and the DNA damage response proteins lysine-specific demethylase 4A/B (KDM4A/B) and ataxia-telangiectasia mutated (ATM). Decreased ATM function, due to mutational inactivation, transcriptional repression, or posttranslational depletion, leads to increased DNA damage and sensitivity to DNA damage-inducing agents, including poly(ADP-ribose) polymerase (PARP) inhibitors, in various types of cancer such as prostate cancer, breast cancer, colorectal cancer, lung cancer, and lymphoma.
Accordingly, as compared to IDH1/2 wild-type cells, IDH1/2-mutated cells show increased levels of DNA damage and sensitization to the PARP inhibitors olaparib and talazoparib, either as monotherapy or in combination with DNA damage-inducing agents. These results have been described using genetically engineered cancer cells or murine haematopoietic stem cells (HSC), but not using models relevant for human AML. Therefore, we investigated the levels of DNA damage and sensitivity to PARP inhibitors and DNA damage-inducing chemotherapy in IDH1-mutated, IDH2-mutated and IDH1/2 wild-type primary AML cells.

**Methods**

**Patient population.** Peripheral blood and bone marrow samples were obtained from AML patients treated in the Cleveland Clinic. Diagnosis was confirmed according to the 2008 World Health Organisation (WHO) classification criteria. These samples were subjected to next-generation sequencing (NGS) and copy number variation (CNV) analysis targeting ~60 genes that are frequently mutated and/or lost in AML and genes involved in DNA damage response, including IDH1/2, ATM, BRC1, BRC2, XRCC2-5 and RAD50-52. Cancer and germline DNA was obtained from AML cells and paired CD3+ T cells or buccal swabs, respectively. Sequencing and bioinformatic analyses were conducted as previously described. Variant allelic frequencies (VAFs) were calculated as the fraction of mutated reads divided by the total number of reads for the gene. VAFs were adjusted to CNVs at the locus of each mutation. Informed consent was obtained from patients according to protocols approved by Cleveland Clinic Institutional Review Board and in accordance with the Declaration of Helsinki. Clinical details of the patients were obtained from medical records.

**Establishment of patient cohorts.** From the AML patients genotyped by NGS, we selected those with the following somatic mutation configurations: IDH1\textsuperscript{MUT}/IDH2\textsuperscript{WT}/TET2\textsuperscript{+/+}, IDH1\textsuperscript{WT}/IDH2\textsuperscript{MUT}/TET2\textsuperscript{+/+}, IDH1\textsuperscript{WT}/IDH2\textsuperscript{MUT}/TET2\textsuperscript{+/+}, or IDH1\textsuperscript{WT}/IDH2\textsuperscript{WT}/TET2\textsuperscript{+/+}, hereafter referred to as IDH1-mutated, IDH2-mutated, TET2\textsuperscript{+/+}, and IDH1/2 wild-type AML samples, respectively. Using copy number-adjusted VAFs, we reconstructed the clonal architecture of IDH1-mutated, IDH2-mutated, and TET2\textsuperscript{+/+} AML patients to select cases with the classifying mutations that were clonal/ancestral and the mutational load was >80%. Only IDH1/2 mutations that are known D\textsubscript{2}HG producers were included. TET2\textsuperscript{+/+} patients included those with hemi- or homozygous TET2 mutations (TET2\textsuperscript{MUT}/ or TET2\textsuperscript{MUT}/MUT).

**In vitro culture.** In all cell culture experiments, primary human AML cells from the aforementioned bone marrow samples were cultured in Iscove’s Modified Dulbecco’s Medium (Gibco, Life Technologies, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (HyClone, Thermo Fisher Scientific), 10 ng/ml interleukin-3 (IL-3), 50 ng/ml stem cell factor (SCF), 3 U/ml erythropoietin (EPO) and 10 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF), in 5% CO\textsubscript{2} at 37° C and were simultaneously used in various experiments (eFigure 1). For colony formation assays (CFAs), cells pretreated in the presence or absence of AGI-5198 (the preclinical version of the IDH1-mutant inhibitor ivosidenib\textsuperscript{158}), AGI-6780 (the preclinical version of the IDH2-mutant inhibitor enasidenib\textsuperscript{281}), 10 mM D\textsubscript{2}HG, or 5 μM N-acetyl cysteine (NAC) were seeded at a density of 1*10\textsuperscript{4}-1*10\textsuperscript{6} cells/ml in 3 ml Methocult methylcellulose medium (Stem Cell Technologies). The seeding density depended on the concentration of the cytotoxic agent. Cells were treated for 48 hours with 10-50 nM daunorubicin, 200-1000 nM cytarabine, 10-50 μM 5-azacytidine, 1-10 μM decitabine (all for 48 hours) or 2-6 Gy ionizing radiation (IR) using a \textsuperscript{137}Cs source. PARP inhibitors (0-25 μM olaparib or 0-25 nM talazoparib) were given during 48 hours before the start of the CFA and for 7 days during the CFA. Thus, treatment with all cytotoxic agents lasted for at least 48 hours, in which period >99% of investigated AML cells underwent at least one cell cycle (eFigure 2). Isogenic HCT116 IDH1\textsuperscript{WT}/WT and IDH1\textsuperscript{WT}/R132H knock-in cells, generated by AAV-targeting technology GENESIS,\textsuperscript{172} were kindly provided by Horizon Discovery and cell culture and CFAs were performed as described previously.\textsuperscript{163} Colonies (>50 cells) were counted at 7 days after treatment and results were analysed to determine the clonogenic fraction. This is the number of colonies counted, divided by the number of cells plated and corrected for the plating efficiency, as described previously.\textsuperscript{143} Cell
survival at 3 days after treatment was determined by MTT assays. AGI-5198 and AGI-6780 were purchased from MedChemExpress. D2HG, NAC, 5-azacytidine, cytarabine, daunorubicin, decitabine and MTT were purchased from Sigma-Aldrich. Olaparib (AZD-2281) and talazoparib (BMN-673) were purchased from SelleckChem.

**Enzyme activity measurements.** Quantitative enzyme cytochemistry (metabolic mapping) of AML cells was performed and analysed as described in Chapter 4 of this thesis. To detect the impact of cytoplasmic mutant IDH1 and mitochondrial mutant IDH2 on NADP+-dependent IDH1/2 activity, 1-methoxy-5-methylphenazinium methylsulfate (methoxy-PMS) and 5-methylphenazinium methylsulfate (PMS, both Sigma) were used, respectively, because the former does not pass mitochondrial membranes while the latter does.¹⁹⁹

**Cellular NADP⁺, NADPH, GSH, GSSG, ROS and D2HG measurements.** AML cells were analysed using a colourimetric NADP⁺:NADPH ratio assay (Abcam), a fluorometric GSH:GSSG ratio assay (Abcam) and a fluorometric CellROX Deep Red ROS assay (Life Technologies), in 96-well plates using a POLARStar Galaxy microplate reader (BMG Labtech). Enantiomer-specific mass spectrometry analysis of D2HG levels of AML cell lysates was performed as described before.⁴⁷⁸

**Quantitative real-time (qRT-PCR).** qRT-PCR was performed as previously described.⁴⁷⁹ Each sample was assayed in triplicate and normalised to ABL expression.⁴⁸⁰ Primers are listed in eTable 1.

**Analysis of ATM protein levels and siRNA against ATM.** ATM protein expression was measured by immunoblotting using primary antibodies against ATM (Genetex) and β-Actin (Cell Signaling Technology). Immunoblots were analysed using a Li-Cor Odyssey system (Li-Cor Biotechnology). Two sets of “Silencer Select” siRNAs against ATM (s530444 and s5304445) and one negative control siRNA (catalog # 4390843) were obtained from Life Technologies and transfected into AML cells using standard protocols. siRNA efficacy was confirmed by immunoblotting against ATM. ATM-siRNA s530444 was selected to be used in CFAs.

**Analysis of TCGA data.** IDH1, IDH2 and TET2 mutational data and ATM, IDH1, IDH2 and G6PD mRNA expression data (RNASeq v2 RSEM or RPKM) for AML, lower-grade glioma, and glioblastoma cases were extracted from The Cancer Genome Atlas (TCGA) via cBioPortal¹⁷⁰,¹⁷¹ and correlated with each other as described previously.¹⁵⁹

**Generation of Tet1/Tet2 conditional knockout mice.** Tet1 and/or Tet2 were conditionally knocked out by generating Tet2floX/flox;MxCre and Tet1floX/flox-Tet2floX/flox;MxCre mice as described previously.⁴⁸¹ MxCre expression was induced by intraperitoneal injection of three doses of 300 mg polyinosine-polycytosine. Animal care was performed in accordance with institutional guidelines and approved by the local committees at the University of Miami and the Massachusetts Institute of Technology.

**RNA sequencing.** RNA-seq libraries were generated from duplicated samples using the Illumina TruSeq RNA Sample Preparation Kit v2. The RNA-seq libraries were sequenced as 50-cycle pair-end runs using Illumina HiSeq 2000 (Illumina). RNA-seq reads were aligned using TopHat v2.0.8 and differential RPKM expression values were extracted using Cuffdiff v2.2.1.²¹⁹

**γH2AX immunofluorescence staining and measurements.** DNA double-strand breaks (DSBs) were determined using immunofluorescent γH2AX staining (Millipore). γH2AX foci per cell were quantified from deconvoluted photomicrograph stacks with custom-made software, as described.²⁸⁰

**Statistical analysis.** Data were processed and analysed using R and visualised using Prism 6 (GraphPad Software). Two-sided tests were used with significance defined as α < 0.05.
**Results**

**Clinical characterization of primary AML cells.** The clinical and molecular characteristics of the selected IDH1-mutated, IDH2-mutated, TET2−, and IDH1/2 wild-type AML patient samples (n = 5 for each group) are shown in eTables 2 and 3. The clinical characteristics of the selected IDH1-mutated AML patients and IDH2-mutated AML patients were representative for those described in a previous cohort study of IDH1/2-mutated AML patients.280

**IDH1/2 mutations decrease ATM expression and increase DNA DSBs.** Motivated by earlier reports that genetically engineered and primary IDH1/2-mutated cancer cells have decreased levels of ATM expression338 and increased levels of DNA damage,143,338-340 we investigated these phenomena in primary human AML cells. We observed decreased ATM mRNA and protein expression in IDH1/2-mutated AML cells as compared to IDH1/2 wild-type AML cells. Administration of an IDH1/2-mutant inhibitor restored ATM expression in IDH1/2-mutated AML cells. Although ATM mRNA expression seemed decreased in TET2− AML cells D2HG concentrations in cell lysates of each IDH1/2-mutated

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**Figure 1. IDH1/2 mutations increase DNA DSBs and sensitize AML cells to PARP inhibitors.**

(A) IDH1-mutated, IDH2-mutated, IDH1/2 wild-type, and TET2− primary AML cells (n = 5 for each group) were incubated in the presence or absence of 1 µM AGI-5198 (an IDH1-mutant inhibitor) or AGI-6780 (an IDH2-mutant inhibitor) for 7 days, harvested, and analysed for ATM protein expression by immunoblotting. β-Actin served as loading control. Lanes were reordered horizontally for clarity. ATM mRNA expression by qRT-PCR was also measured in these cells. (B) D2HG levels as determined by enantiomer-specific mass spectrometry in cell lysates of 10^6 cells. Values were normalised to the D2HG concentration of untreated IDH1/2 wild-type cells and are shown as arbitrary units. (C) ATM mRNA expression data was taken from The Cancer Genome Atlas (TCGA) database and plotted on the basis of the IDH1, IDH2 and TET2 mutational status. Statistical comparisons were made using the one-way ANOVA test, comparing each group with the IDH1/2 wild-type group, with Dunnett correction for multiple comparisons. (D) IDH1/2-mutated and IDH1/2 wild-type primary human AML cells (n = 5 for each group) were incubated in the presence or absence of 1 µM IDH1/2-mutant inhibitor for 3 days or 7 days and pretreated with either 2 Gy IR or 25 nM daunorubicin. Cells were immunocytochemically stained for γH2AX/DSBs and DAPI/DNA content. Numbers of γH2AX+ foci per cell are shown (20 cells per patient sample). P values were obtained using one-way ANOVA on the difference between patient samples, using Tukey’s correction for multiple comparisons. Abbreviations: 3d, three days; 7d, seven days; IDH1MUT, IDH1-mutated; IDH2MUT, IDH2-mutated; IDH1/2WT, IDH1/2 wild-type; n.s., not significant; qPCR, quantitative polymerase chain reaction.
sample. D2HG concentrations were higher in IDH1-mutated AML cells than in IDH2-mutated AML cells, as has been described previously, and were potently suppressed by AGI-5198 and AGI-6780, respectively (Figure 1B). Using TCGA data, we confirmed that ATM mRNA expression is severely decreased in IDH1-mutated AML and not significantly decreased in IDH2-mutated and TET2-AML (Figure 1C). On the other hand, we observed significantly decreased Atm and Atm-Rad3-related (Atr) mRNA expression in Tet1+/Tet2- and Tet1+/Tet2- mice compared to control Tet1+/Tet2+ mice (Supplementary Figure S1). We observed more γH2AX+ foci (which recognise DNA DSBs) in IDH1-mutated AML cells than in IDH1 wild-type AML cells under steady-state conditions. Furthermore, the number of γH2AX+ foci was higher in IDH1/2-mutated AML cells than in IDH1/2 wild-type AML cells after IR or daunorubicin treatment. To confirm the causal relationship between IDH1/2 mutations and increased levels of DNA damage, we pretreated IDH1/2-mutated cells with an IDH1/2-mutant inhibitor prior to treatment with IR or daunorubicin, which reversed the number of γH2AX+ foci in IDH1/2-mutated AML cells to levels observed in IDH1/2 wild-type AML cells in a time-dependent fashion (Figure 1D).

**Figure 2. IDH1/2 mutations sensitize AML cells to the PARP inhibitors olaparib and talazoparib.**

(A-B) Colony-forming assays with IDH1-mutated, IDH2-mutated, IDH1/2 wild-type, or TET2- primary AML cells (n = 5 for each group) after 48 hours pretreatment with, and during 7 days after plating with (A) 0-25 µM olaparib or (B) 0-25 nM talazoparib. (C) Pretreatment schedules for the IDH1-mutant inhibitor (AGI-5198) or the IDH2-mutant inhibitor (AGI-6780) for 3 or 7 days shown in panels D-I. Cells were exposed to daunorubicin or IR on day 7 and subsequently pretreated with daunorubicin for 48 hours or irradiated and plated for colony-forming assays. Throughout, squares are for control conditions, upward triangles for 3 days inhibition and downward triangles for 7 days inhibition. (C-H) Same as in (A-B), but after pretreatment or not with an IDH1/2-mutant inhibitor for the indicated period. Data are mean±SD from 3 independent experiments. The clonogenic fraction is the number of colonies counted, divided by the number of cells plated and corrected for the plating efficiency. Y-axes are on a logarithmic scale. Data obtained in control conditions are from the same experiments. Black significance indicators compare the indicated group with IDH1/2 WT AML cells. Coloured significance indicators compare the indicated group with its IDH1/2MUT inhibitor-untreated counterpart. P values are indicated as * < 0.05; ** < 0.01; ***< 0.005; ****< 0.001. Abbreviations: 3d, three days; 7d, seven days; c, control; IDH1MUT, IDH1-mutated; IDH2MUT, IDH2-mutated; IDH1/2WT, IDH1/2 wild-type.
**IDH1/2 mutations sensitise AML cells to PARP inhibitors.** The relationship between increased DNA damage, decreased ATM function and sensitivity to PARP inhibitors prompted us to compare the responses of IDH1/2-mutated and IDH1/2 wild-type AML cells to the PARP inhibitors olaparib and talazoparib. After treatment with olaparib or talazoparib, the surviving fraction of IDH1/2-mutated AML cells was lower than that of IDH1/2 wild-type AML cells in CFAs (Figure 2A-B). To investigate whether a causal relationship existed between IDH1/2 mutations and sensitization to PARP inhibitors, we pretreated IDH1-mutated AML cells with AGI-5198 and IDH2-mutated AML cells with AGI-6780 before cytotoxic treatment (Figure 2C). Pharmacological inhibition of mutant IDH1/2 for at least 7 days protected IDH1/2-mutated AML cells against PARP inhibitors (Figure 2D-G). In addition, IDH1/2-mutant inhibitors did not affect the sensitivity of IDH1/2 wild-type AML cells to PARP inhibitors (Figure 2H-I). We also observed reversible sensitivity to PARP inhibitors using another model of isogenic IDH1WT/R132H HCT116 cells, as compared to IDH1WT/WT HCT116 cells (Supplementary Figure S2).

**IDH1/2 mutations sensitise AML cells to irradiation and daunorubicin.** Given that IDH1/2 mutations decrease the DNA damage response and cause sensitivity to PARP inhibitors, we hypothesised that IDH1/2 mutations also sensitise AML cells to other DNA damage-inducing agents.

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**Figure 3. IDH1/2 mutations sensitise AML cells to irradiation and daunorubicin.**

Colony-forming assays with IDH1-mutated, IDH2-mutated, IDH1/2 wild-type, or TET2/- primary AML cells (n = 5 for each group) after treatment with (A) 0-50 nM daunorubicin for 48 hours or (B) 0-6 Gy ionizing radiation. (C-H) Same as in (A-B), but after pretreatment or not with an IDH1/2-mutant inhibitor for the indicated period according to the pretreatment schedule shown in Figure 2C. Data are mean±SD from 3 independent experiments. The clonogenic fraction is the number of colonies counted, divided by the number of cells plated and corrected for the plating efficiency. Y-axes are on a logarithmic scale. Data obtained in control conditions are from the same experiments. Black significance indicators compare the indicated group with IDH1/2 wild-type AML cells. Coloured significance indicators compare the indicated group with its IDH1/2-mutant inhibitor-untreated counterpart. P values are indicated as * < 0.05; ** < 0.01. Abbreviations: 3d, three days; 7d, seven days; c, control; IDH1WT, IDH1-mutated; IDH2mut, IDH2-mutated; IDH1/2WT, IDH1/2 wild-type.
Relative to IDH1/2 wild-type AML cells and TET2⁻/⁻ AML cells, we observed a significantly reduced surviving fraction of IDH1/2-mutated AML cells after treatment with daunorubicin or IR in CFAs (Figure 3A-B). In addition, pharmacological inhibition of mutant IDH1/2 for at least 7 days protected IDH1/2-mutated AML cells, but not IDH1/2 wild-type AML cells, against subsequent treatment with daunorubicin or IR (Figure 3C-H). We confirmed these results in isogenic IDH1WT/WT and IDH1WT/R132H HCT116 colorectal cancer cells (Supplementary Figure S3). Pretreatment with the ROS scavenger NAC during 3 days did not affect the survival of IDH1/2-mutated AML cells after treatment with daunorubicin or IR (eFigure 3). We did not observe survival differences between IDH1/2-mutated AML cells and IDH1/2 wild-type AML cells after treatment with cytarabine, 5-azacytidine, or decitabine, which are antimetabolites and hypomethylating agents but do not induce DNA damage. We also did not observe survival differences between IDH1/2-mutated and IDH1/2 wild-type AML cells after treatment with daunorubicin or IR in short-term (3-day) cell viability assays (Supplementary Figure S4).

**PARP inhibitors further sensitise IDH1/2-mutated AML cells to cytotoxic therapy.** We hypothesised that combined treatment with a PARP inhibitor and a DNA-damaging agent had additive effects on IDH1/2-mutated AML cells. Combined treatment with olaparib or talazoparib and daunorubicin was more lethal to both IDH1/2 wild-type AML cells and IDH1/2-mutated AML cells than daunorubicin treatment alone, but the effect was significantly larger in IDH1/2-mutated AML cells (Figure 4).

**Increased sensitivity to DNA-damaging agents in IDH1/2-mutated AML cells is associated with decreased ATM expression.** To investigate causality between IDH1/2 mutations, ATM suppression and therapy sensitivity, we knocked down ATM in AML cells using siRNA (eFigure 4). ATM knockdown did not affect the sensitivity of IDH1/2-mutated AML cells to daunorubicin or IR (Figure 5A-D) but sensitised IDH1/2 wild-type AML cells to these treatments (Figure 5E-F). After 7 days of pretreatment with D2HG, untransfected IDH1/2 wild-type AML cells were sensitised to daunorubicin or IR, but IDH1/2 wild-type AML cells were not further sensitised when ATM was knocked down (Figure 5G-H). IDH1/2-mutant inhibitors protected untransfected IDH1/2-mutated AML cells against daunorubicin or IR (Figure 3C-F), but did not protect IDH1/2-mutated AML cells when ATM was knocked down (Figure 5I-J). Another siRNA with a lower knockdown efficiency of siRNA sensitised IDH1/2 wild-type AML cells less for daunorubicin or IR (eFigure 5).

**Figure 4. PARP inhibitors and daunorubicin have additive effects in IDH1/2-mutated AML cells.** Colony-forming assays with IDH1-mutated, IDH2-mutated, or IDH1/2 wild-type primary AML cells (n = 5 for each group) after 48 hours pretreatment before plating and during 7 days after plating with 0-25 µM olaparib or 0-25 nM talazoparib and with 0-15 µM daunorubicin. Data are mean±SD from 3 independent experiments. The clonogenic fraction is the number of colonies counted, divided by the number of cells plated and corrected for the plating efficiency. Y-axes are on a logarithmic scale. Black significance indicators compare the indicated group with IDH1/2 wild-type AML cells. Coloured significance indicators compare the indicated group with its IDH1/2-mutant inhibitor-treated counterpart. P values are indicated as * < 0.05; ** < 0.01; *** < 0.005; **** < 0.001. Abbreviations: IDH1MUT, IDH1-mutated; IDH2MUT, IDH2-mutated; IDH1/2WT, IDH1/2 wild-type.
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**IDH1/2 mutations decrease NADPH production but does not affect redox states in AML cells.** In glioma and colorectal cancer cells, mutant IDH1 inhibits wild-type IDH1/2 function, which perturbs redox states and sensitises these cells to irradiation. We interrogated whether or not IDH1/2 wild-type function and redox states played a role in the therapy sensitization of IDH1/2-mutated AML cells. NADP⁺-dependent IDH1/2 activity was significantly lower in IDH1/2-mutated AML cells than in IDH1/2 wild-type AML cells and TET2-/- AML cells. However, the impact of the decreased IDH1/2-mediated NADPH production capacity on the total cellular NADPH production capacity in AML cells was limited, because the NADPH production capacity by G6PD was ~4-fold larger than that of IDH1 and IDH2 combined (Figure 6A). IDH1/2 mutations were not associated with changes in NAD⁺-dependent IDH3 activity or NADP⁺-dependent G6PD activity in AML cells (Figure 6A-B). Pretreatment with an IDH1/2-mutant inhibitor for 3 days restored NADP⁺-dependent IDH1/2 activity in IDH1/2-mutated AML cells (Figure 6C). In addition, administration of 10 mM D2HG (which achieved D2HG levels in IDH1/2 wild-type AML cells similar to untreated IDH1/2-mutated AML cells [Figure 1B]) decreased NADP⁺-dependent IDH1/2 activity in IDH1/2 wild-type cells, which supports a causative role of D2HG accumulation in the suppression of IDH1/2-mediated NADPH production (Figure 6C). In agreement with the modest effects of IDH1/2 mutations on the total cellular NADPH production,}

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**Figure 5. ATM knockdown sensitises IDH1/2 wild-type AML cells to cytotoxic treatment, but not in the presence of D-2HG or an IDH1/2 mutation.**

(A-H) Colony-forming assays with IDH1-mutated, IDH2-mutated, or IDH1/2 wild-type AML cells (n = 5 for each group) after treatment with 0-50 nM daunorubicin for 48 hours or 0-6 Gy ionizing radiation in the presence or absence of siRNA against ATM and/or 10 mM D2HG and/or 1 µM AGI-5198 or 1 µM AGI-6780. ATM siRNA was controlled for using a negative control siRNA. Data are mean±SD from 3 independent experiments. The clonogenic fraction is the number of colonies counted divided by the number of cells plated and corrected for the plating efficiency. Y-axes are on a logarithmic scale. Significance indicators compare the adjacent group with untreated IDH1/2 wild-type AML cells. P values are indicated as "*" < 0.05; "**" < 0.01. (I) Model of IDH1 mutant-mediated therapy sensitization in AML cells and glioma cells, based on findings in this chapter and in Chapter 13, which studies IDH1 mutations in solid tumour cells (shaded part). Abbreviations: IDH1mut, IDH1-mutated; IDH2mut, IDH2-mutated; IDH1/2wt, IDH1/2 wild-type.
we observed similar NADP⁺:NADPH ratios, GSH:GSSG ratios and ROS levels between IDH1/2-mutated AML cells and IDH1/2 wild-type AML cells under steady-state conditions and after pretreatment with daunorubicin or IR (Figure 6D-F). In TCGA data, the mRNA expression of IDH1, IDH2 and G6PD enzymes was unchanged in IDH1/2-mutated AML versus IDH1/2 wild-type AML, whereas mRNA expression of these enzymes was lower in IDH1/2-mutated lower-grade glioma versus IDH1/2 wild-type lower-grade glioma (eFigure 6).

Discussion
We found that primary IDH1/2-mutated AML cells have reduced DNA damage responses and suppressed expression of ATM. As a consequence, they are sensitised to monotherapy with a PARP inhibitor, daunorubicin or IR and this is reversed by pretreatment with an IDH1/2-mutant inhibitor,

Figure 6. IDH1/2-mutated AML cells have decreased IDH1/2 activity but similar redox states as IDH1/2 wild-type AML cells.

(A) NADP⁺-dependent IDH1/2 activity, NAD⁺-dependent IDH3 activity and NADP⁺-dependent G6PD activity of IDH1-mutated, IDH2-mutated and IDH1/2 wild-type primary AML cells was determined using image analysis as absorbance of blue formazan produced from nitroBT per cell as a readout of NADPH production. Values were normalised to the NADP⁺-dependent IDH activity of IDH1/2 wild-type cells and are shown as arbitrary units. (B) Representative photomicrographs of NADP⁺-dependent IDH and G6PD activity in IDH1-mutated and IDH1 wild-type AML cells. Scale bars = 50 µm. (C) NADP⁺-dependent IDH1/2 activity after pretreatment in the presence or absence of 1 µM AGI-5198, 1 µM AGI-6780 or 10 mM exogenous D2HG; units are arbitrary and relative to IDH1/2WT rates under control conditions. (D) IDH1-mutated, IDH2-mutated and IDH1/2 wild-type AML cells (n = 5 for each group) were pretreated with 0-50 nM daunorubicin (DAU) for 48 hours or 0-2 Gy ionizing radiation and were harvested, prepared, and analysed, colourimetrically for NADP⁺:NADPH ratios and fluorometrically for GSH:GSSG ratios and for ROS levels. Data are means±SD from 3 independent experiments. P values are indicated as * < 0.05; ** < 0.01. Abbreviations: DAU, daunorubicin; IDH1MUT, IDH1-mutated; IDH2MUT, IDH2-mutated; IDH1/2WT, IDH1/2 wild-type; IR; ionizing radiation.
which also restores ATM expression and decreases DNA damage. Pharmacological inhibition of mutant IDH1/2 is unable to protect IDH1/2-mutated AML cells when ATM is knocked down, indicating a causal role of ATM suppression in increased IDH1/2-mutated AML therapy responses. D2HG mimics the effects of IDH1/2 mutations in IDH1/2 wild-type AML cells, suggesting that D2HG accumulation impedes ATM expression. Finally, combined treatment with a PARP inhibitor and daunorubicin has additive effects on the therapy response of IDH1/2-mutated AML. PARP inhibitor monotherapy is already an interesting perspective for personalised treatment of IDH1/2-mutated AML, because IDH1/2-mutant inhibitors restore the impaired DNA damage response in IDH1/2-mutated AML cells and thereby diminish the therapeutic index of PARP inhibitors. These findings in combination with earlier findings in IDH1-mutated glioma, are summarised in a model shown in Figure 4K.

Our results corroborate other studies showing that compared to IDH1/2 wild-type counterparts, IDH1(2)-mutated human glioma, human colorectal cancer and murine HSCs are sensitised to treatment with daunorubicin, IR or PARP inhibitors,143,338-340 and in the case of glioma that are treated with chemotherapy this is supported by clinical evidence.284,328 Whereas IDH1/2-mutated AML cells are sensitised to cytotoxic therapy in CFAs, cell viability experiments failed to replicate this. CFAs better capture the long-term effects of treatment-induced DNA damage.462 The time scale of interest for DSBs is 24 hours, because the number of un-repaired DSBs remaining at 24 hours after irradiation is what correlates with cell survival.463 This is consonant with decreased ATM expression in IDH1/2-mutated AML cells versus IDH1/2 wild-type AML cells, as ATM is important in DNA damage signalling and its decreased expression is thus likely a contributor to the sensitivity of IDH1/2-mutated AML cells to PARP inhibitors, daunorubicin and IR. Pretreatment of IDH1/2-mutated AML cells with an IDH1/2-mutant inhibitor restored ATM expression, which suggests a link between mutant IDH1/2-induced D2HG accumulation and decreased ATM expression. Furthermore, a causal relation between mutant IDH1/2-mutated AML cells to PARP inhibitors does probably not function via D2HG-mediated TET2 inhibition, because the restoration of TET2 function sensitises rather than protects TET2 haploinsufficient AML cells to PARP inhibitors.362

What remains to be elucidated is how D2HG accumulation suppresses ATM mRNA expression. TET2 is a major downstream target of D2HG and our in vivo data suggests that ATM transcription may be mediated through TET1/2 inhibition, as Tet1/2−/− mice had decreased Atm mRNA levels, albeit not as extensive as in IDH1/2-mutated AML samples. However, knockdown of Tet2 in murine HSCs did not downregulate Atm in a previous study. Downregulation of ATM by D2HG may thus occur via more than just TET2 and another likely mediator is UTX/KDM6A.338,340 The sensitization of IDH1/2-mutated cancer cells to PARP inhibitors does probably not function via D2HG-mediated TET2 inhibition, because the restoration of TET2 function sensitises rather than protects TET2 haploinsufficient AML cells to PARP inhibitors.362

Several mechanistic results from IDH1/2-mutated AML cells in the present study contrast our earlier findings in IDH1-mutated glioma and colon carcinoma cells (Chapter 13), where pretreatment with an IDH1-mutant inhibitor for 3 days or NAC for 3 days achieved radioprotection due to restored production of NADPH and of the reduced antioxidant glutathione, which eliminates ROS. In IDH1/2-mutated AML cells, such protection against cytotoxic therapy required incubation with an IDH1/2-mutant inhibitor for 7 days and was not achieved by using the ROS scavenger NAC. IDH1/2 mutations have profound effects on cellular metabolism in glioma159,256 and this seems to be no different on the IDH1/2 enzymatic level in AML cells, where IDH1/2 mutations reduced IDH1/2-mediated NADPH production. However, it did not affect therapy responses in AML cells wherein IDH1/2 provides <20% of the cell's NADPH; in contrast, in glioma, IDH1/2 provides approximately two-thirds of the cell's capacity to produce NADPH.41 IDH1/2 mutations were associated with decreased mRNA expression of IDH1/2 and G6PD in glioma but not in AML, suggesting that IDH1/2
mutations may alter the metabolism of AML cells to a lesser extent than that of glioma. Relative to glioma and colon carcinoma cells, slower protection of IDH1/2-mutated AML cells by IDH1/2-mutant inhibitors is likely due to it being mediated by a different mechanism that involves slow epigenetic alterations needed to suppress ATM expression. Reversing redox states in glioma and colon carcinoma cells is likely to be much faster. Theoretically, the increased DNA damage in IDH1/2-mutated AML cells can be explained by cell cycle perturbations in IDH1/2-mutated AML cells versus IDH1/2 wild-type AML cells because differences in cell doubling times have been noted between IDH1/2-mutated cells and IDH1/2 wild-type cells and IDH1-mutant inhibitors are reported to affect cell cycle duration. However, we found no differences in doubling time between IDH1/2-mutated AML cells and IDH1/2 wild-type AML cells and increased γH2AX+ foci argue against cell cycle perturbations as being responsible for our results.

Patients with IDH1/2-mutated glioma and cholangiocarcinoma have longer survival times than IDH1 wild-type counterparts. This association is linked to improved responses to anti-cancer therapy. In contrast, such associations have not been shown for IDH1/2-mutated AML in retrospective clinical studies. As a possible explanation, low frequencies of daunorubicin treatment may not allow the putative predictive effects of IDH1/2 mutations to materialise into a significant prognostic association in retrospective studies. For example, only 30-40% of elderly AML patients (aged ≥65 years) are reported to receive any type of chemotherapy, of which not all receive intensive treatment regimens. We validated these data in the NCI program Surveillance, Epidemiology and End Results (SEER) and found that only ~60% of AML patients of all ages receive any type of chemotherapy (see Supplementary Methods and Results).

Impaired DNA repair was noted earlier in IDH1/2-mutated glioma, colorectal carcinoma and AML cells and this has been linked to improved responses to PARP inhibitors of the two former cell types, but not yet in AML. In summary, this study is the first to show that IDH1/2-mutated AML is vulnerable for PARP inhibition as monotherapy, but especially when combined with daunorubicin treatment. IDH1/2-mutant inhibitors protect IDH1/2-mutated AML cells against PARP inhibitors, daunorubicin or IR, which suggests that IDH1/2-mutant inhibitors should not be given concomitantly with DNA-damaging agents. Our data are crucial to the rational design and analysis of clinical trials with IDH1/2-mutant inhibitors, especially for clinical trials that investigate combinations of IDH1/2-mutant inhibitors with conventional chemotherapy for AML (ClinicalTrials.gov NCT02632708). Instead, our results show that exploiting impaired DNA repair in IDH1/2-mutated AML cells using a PARP inhibitor, ideally combined with a DNA-damaging agent, may be a better strategy for the treatment of IDH1/2-mutated AML.
Supplementary Figure S1. mRNA expression in Tet1+/+Tet2−/− or Tet1−/−/Tet2−/− mice compared to control Tet1+/+Tet2+/+ mice.

Tet1 and/or Tet2 were knocked down or not in conditional Tet1+/+Tet2−/− and Tet1−/−/Tet2−/− knockout mice and bone marrow cells were subjected to RNA-seq. Shown are RPKM values normalised to values in Tet1+/+Tet2+/+ mice. Mre11a, Rad51 and Trp51 values are shown a separate Y-axis on the right.

Supplementary Figure S2. Isogenic IDH1WT/R132H HCT116 colorectal carcinoma cells are sensitised to treatment with PARP inhibitors and this is reversed by an IDH1MUT inhibitor.

Colony-forming assays with IDH1WT/WT and IDH1WT/R132H HCT116 cells after 48 hours pretreatment with 0-10 µM olaparib or talazoparib and during 7 days treatment with olaparib or talazoparib in the cell culture medium. "+ AGI-5198" indicates that cells were treated with daunorubicin or IR after pretreatment with an the IDH1MUT inhibitor AGI-5198 for 7 days. Data are mean±SD. The clonogenic fraction is the number of colonies counted, divided by the number of cells plated and corrected for the plating efficiency.

Supplementary Figure S3. Isogenic IDH1WT/R132H HCT116 colorectal carcinoma cells are sensitised to treatment with daunorubicin or irradiation and an IDH1MUT inhibitor protects IDH1WT/R132H HCT116 cells against these treatments.

(A-B) Colony-forming assays with IDH1WT/WT and IDH1WT/R132H HCT116 cells after treatment with (A) 0-50 nM daunorubicin for 48 hours or (B) 0-6 Gy ionizing radiation (IR). "+ AGI-5198" indicates that cells were treated with daunorubicin or IR after pretreatment with an the IDH1MUT inhibitor AGI-5198 for 7 days. Data are mean±SD. The clonogenic fraction is the number of colonies counted, divided by the number of cells plated and corrected for the plating efficiency.
Supplementary Figure S4. IDH1/2MUT AML cells are not sensitised to decitabine or 5-azacytidine in colony-formation assays or daunorubicin or irradiation in short-term cell proliferation experiments.

(A-B) Colony-forming assays with IDH1MUT, IDH2MUT, IDH1/2WT or TET2–/– primary AML cells (n = 5 for each group) after treatment with (A-B) 0-1000 nM cytarabine in the presence or absence of the IDH1/2MUT inhibitors AGI-5198 and AGI-6780, (C) 0-3 µM decitabine or (D) 0-50 µM 5-azacytidine. The clonogenic fraction is the number of colonies counted, divided by the number of cells plated and corrected for the plating efficiency. (E-F) Dose-response curves of cell survival of IDH1MUT, IDH2MUT, IDH1/2WT or TET2–/– AML cells at 3 days after treatment with (E) 10⁻⁸-10⁻³ M daunorubicin or (F) 0-6 Gy irradiation. Data are means±SD from ≥3 independent experiments. Note that IDH1/2MUT AML cells are more resistant to cytarabine than IDH1/2WT AML cells but that an IDH1/2MUT inhibitor only partly reverse this resistance.

Supplementary methods and results

Surveillance, Epidemiology and End Results registries. Given the low penetrance of daunorubicin treatment in elderly patients, we analysed records from all 18 registries of the Surveillance, Epidemiology and End Results (SEER) program of the National Cancer Institute. SEER provides incidence and survival data from population-based cancer registries, which cover ~28% of the United States population. SEER registry data are subjected to regular quality monitoring for completeness and data accuracy. Data from the November 2016 submission of all 18 SEER registries was queried using SEERaBomb, a previously validated package for the R statistical programming language, to identify a cohort of patients diagnosed with AML and treated with chemotherapy from 1973-2014. In addition, via an NCI-SEER custom data agreement, additional data on the use of chemotherapy was derived from the NCI desktop application SEER*Stat. In SEER, chemotherapy is recorded when a patient received chemotherapy in the first 3 months after diagnosis. Therefore, there is a high probability that chemotherapy use in the context of AML pertains to induction chemotherapy (likely/possibly including daunorubicin) and not second or third line therapy. R was used to merge the database acquired using SEERaBomb and SEER*Stat. For more info on SEER(aBomb), see chapter 17.

Selection of SEER patients. All patients ≥18 years or older with AML, defined as ICD-O-3 SEER codes (https://seer.cancer.gov/icd-o-3/) between 9840-9940 except 9866 (APL), were included.

Use of chemotherapy in AML patients in SEER. Among patients aged 18-65 years, 84% received chemotherapy in the first 3 months after diagnosis. On the other hand, only 48% of patients aged ≥65 years received chemotherapy. The latter outcome validates the numbers extracted by us from SEER because they compare with results that were reported elsewhere. Total use of chemotherapy in AML patients aged ≥18 years is given in the table below.

Supplementary Table S2. Receipt of chemotherapy in AML patients aged ≥18 years in the SEER database.

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<th>Age Group</th>
<th>Received chemotherapy</th>
<th>Did not receive chemotherapy</th>
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<td>4382 (16%)</td>
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<td>Ages ≥65</td>
<td>20046 (48%)</td>
<td>21873 (52%)</td>
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