Acquired resistance to IDH inhibition through trans or cis dimer–interface mutations

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Somatic mutations in the isocitrate dehydrogenase 2 gene (IDH2) contribute to the pathogenesis of acute myeloid leukaemia (AML). Enasidenib (AG-221) is an allosteric inhibitor that binds to the IDH2 dimer interface and blocks the production of 2HG by IDH2 mutants. In a phase I/II clinical trial, enasidenib inhibited the production of 2HG and induced clinical responses in relapsed or refractory IDH2-mutant AML. Here we describe two patients with IDH2-mutant AML who had a clinical response to enasidenib followed by clinical resistance, disease progression, and a recurrent increase in circulating levels of 2HG. We show that therapeutic resistance is associated with the emergence of second-site IDH2 mutations in trans, such that the resistance mutations occurred in the IDH2 allele without the neomorphic R140Q mutation. The in trans mutations occurred at glutamine 316 (Q316E) and isoleucine 319 (I319M), which are at the interface where enasidenib binds to the IDH2 dimer. The expression of either of these mutant disease alleles alone did not induce the production of 2HG; however, the expression of the Q316E or I319M mutation together with the R140Q mutation in trans allowed 2HG production that was resistant to inhibition by enasidenib. Biochemical studies predicted that resistance to allosteric IDH inhibitors could also occur via IDH dimer–interface mutations in cis, which was confirmed in a patient with acquired resistance to the IDH1 inhibitor ivosidenib (AG-120). Our observations uncover a mechanism of acquired resistance to a targeted therapy and underscore the importance of 2HG production in the pathogenesis of IDH1-mutant malignancies.

We investigated two patients with IDH2-mutant AML who developed acquired resistance to enasidenib, hypothesizing that we could identify molecular mechanisms of resistance to small-molecule IDH inhibition. The first patient with AML relapsed after induction with daunorubicin and cytarabine, and was refractory to subsequent decitabine treatment. The second patient had AML with t(11;19) and the R140Q IDH2 mutation. We treated the patient with ivosidenib, and the patient achieved a complete remission and maintained it for 17 months. At relapse, the patient had 100% blasts in the bone marrow and was refractory to a subsequent induction cycle with daunorubicin and cytarabine. We found the t(11;19) rearrangement and the R140Q IDH2 mutation together with the Q316E mutation in the other allele of the IDH2 gene. These findings suggest that the R140Q mutation in trans allows enasidenib resistance. We then treated the patient with enasidenib, and the patient achieved a complete remission and maintained it for 13 months. At relapse, the patient had 100% blasts in the bone marrow and was refractory to a subsequent induction cycle with daunorubicin and cytarabine. We found the t(11;19) rearrangement and the R140Q IDH2 mutation together with the Q316E mutation in the other allele of the IDH2 gene. These findings suggest that the R140Q mutation in trans allows enasidenib resistance. We then treated the patient with enasidenib, and the patient achieved a complete remission and maintained it for 13 months.

Fig. 1 | Acquired resistance to the mutant IDH2 inhibitor enasidenib (AG-221) associated with emergence of second-site mutations in IDH2. a–d, Clinical, laboratory and pathological features for patient A in relation to enasidenib (AG-221) treatment (blue box) and decitabine (DAC, grey box), including bone marrow blast percentage (a), blood absolute neutrophil count (ANC) (b), plasma 2HG concentration (c), and variant allele frequency (VAF) for mutations identified by targeted next-generation sequencing of bone marrow cells (d). e–h, Clinical, laboratory and pathological features for patient B in relation to enasidenib (AG-221) treatment (blue box) and other treatments (grey boxes), including blood absolute blast count (e), blood ANC (f), plasma 2HG concentration (g), and VAF for mutations identified by targeted next-generation sequencing of bone marrow cells (h). MLL, mixed lineage leukaemia; PTD, partial tandem duplication. Also see Extended Data Fig. 1.
(Fig. 1d). Treatment with enasidenib led to a decline in leukaemic blasts in the bone marrow (Fig. 1a and Extended Data Fig. 1a), a reduction in plasma 2HG concentration to less than 1 μM (Fig. 1c), and normalization of the absolute neutrophil count (Fig. 1b). The variant allele frequency (VAF) for IDH2R140Q remained in the 20–50% range (Fig. 1d), consistent with the observation that enasidenib promotes differentiation of IDH2-mutant blasts. After 9 months of therapy, the patient developed recurrent neutropenia and reappearance of leukaemic blasts consistent with disease progression (Fig. 1a, b and Extended Data Fig. 1a). Importantly, the plasma 2HG concentration had increased despite continuous treatment with enasidenib (Fig. 1c).

The second patient presented with AML who was refractory to cytarabine-based induction chemotherapy and subsequent therapy with an investigational DOT1L inhibitor (Fig. 1e). The patient had an increased blast count, neutropenia, and an increased plasma 2HG concentration of more than 5 μM (Fig. 1e–g and Extended Data Fig. 1b). Next-generation sequencing of bone marrow cells demonstrated the presence of an IDH2R140Q mutation (Fig. 1h). Treatment with enasidenib induced a considerable reduction in the peripheral blast count, an increase in the absolute neutrophil count, and a decrease in the plasma 2HG concentration to less than 1 μM (Fig. 1e–g and Extended Data Fig. 1b). The VAF for IDH2R140Q remained in the 15–40% range (Fig. 1h). After 6 months of continuous treatment with enasidenib, the patient developed progressive disease with a rise in leukaemic blasts (Fig. 1e and Extended Data Fig. 1b) and increased plasma 2HG levels (Fig. 1g).

We hypothesized that clinical resistance to enasidenib in the setting of a progressive increase in 2HG levels might be due to acquisition of a new somatic mutation that mediates drug resistance. In both cases, we identified new mutations in the IDH2 gene at the time of acquired resistance, missense mutations that resulted in a substitution of glutamine 316 with glutamate (Q316E) in the first patient and substitution of isoleucine 319 with methionine (I319M) in the second patient (Fig. 1d, h). Droplet digital PCR (ddPCR) (Extended Data Table 1) demonstrated the secondary IDH2 mutations were not detectable before treatment with enasidenib. Thus, the secondary IDH2 mutations were either acquired during the course of treatment or originated in a rare subclone that was below the limit of detection of the ddPCR assay. The identification of second-site mutations in IDH2 in the setting of acquired resistance to enasidenib therapy raised the possibility of a resistance mechanism similar to those previously reported for tyrosine kinases such as BCR-ABL and EGFR. The Q316E and I319M mutations are encoded in exon 7 of the IDH2 gene, whereas the neo-morphic R140Q mutation is encoded upstream in exon 4 (Fig. 2a). To determine the allelic conformation of the different IDH2 mutations, we performed long-range PCR amplification of genomic DNA spanning exons 4–7 of IDH2 followed by subcloning and sequence analysis of individual clones (Fig. 2a–c). In the first patient, all clones with the IDH2R140Q mutation were wild type at position Q316 (Fig. 2b, d), whereas all clones with the IDH2Q316E mutation were wild type for R140 (Fig. 2b, d). We observed analogous results for the second patient, such that the IDH2I319M and IDH2I319Q mutations were observed exclusively in different clones (Fig. 2c, e). These data demonstrate that acquired resistance to enasidenib was associated with emergence of second-site mutations in trans on the IDH2 allele without the neomorphic R140Q mutation.

To investigate the potential significance of the Q316E and I319M mutations in IDH2, we mapped the mutations at Q316 and I319 to the recently published structure of an IDH2 dimer bound by enasidenib (Fig. 3a; RCSB Protein Data Bank (PDB) code 5I96). Q316 and I319 are located in the IDH2 dimer interface and are key residues that interact with enasidenib (Extended Data Fig. 2). Structural modelling predicted that the Q316E mutation disrupts hydrogen bonding with enasidenib (Fig. 3b), whereas the I319M mutation creates steric hindrance that would impede binding of enasidenib (Fig. 3c). Even though the dimer interface is symmetrical and enasidenib is not, identical residues on either side of the interface can make different, but important, interactions with the drug (Fig. 3a and Extended Data Fig. 2), allowing second-site mutations at the interface to function in trans (and potentially also in cis, see below).

We evaluated the effect of the Q316E and I319M mutations on IDH2 enzymatic function alone and in trans with the R140Q mutation. Expression of the Q316E or I319M mutations in Ba/F3 hematopoietic cells did not result in increased 2HG production, in contrast to the known effect of the R140Q mutation on neomorphic IDH2 function (Fig. 3d). Enasidenib dose-dependently reduced 2HG levels in Ba/F3 cells that co-expressed wild-type and R140Q-mutant IDH2 in trans, consistent with inhibition of mutant IDH2 enzymatic activity as previously described (Fig. 3e and Extended Data Fig. 3a). By contrast, Ba/F3 cells that expressed IDH2(R140Q) concurrently with either IDH2(Q316E) or IDH2(I319M) in trans continued to produce high levels of 2HG when exposed to enasidenib (Fig. 3e and Extended Data Fig. 3a), although this could be partially inhibited by higher doses of drug (Extended Data Fig. 3b).

We next tested the effect of the Q316E and I319M mutations on the self-renewal of primary mouse haematopoietic stem/progenitor cells (HSPC) in which IDH2(R140Q) is expressed from the endogenous locus. Enasidenib inhibited the serial-replicating capacity of HSPCs that expressed both wild-type and R140Q mutant IDH2 in trans, consistent with inhibition of mutant IDH2-induced gain of self-renewal (Fig. 3f). However, primary mouse HSPCs that expressed IDH2(R140Q) in conjunction with Q316E or I319M in trans maintained serial replating in the presence of enasidenib (Fig. 3f). Similar findings were observed in a primary murine-derived leukaemia model, in which IDH2(R140Q) and the FLT3 internal tandem duplication (ITD) are expressed from...
Fig. 3 | Second-site mutations in IDH2 confer resistance to enasidenib in trans. a, Structure of the IDH2 dimer highlighting the binding pocket for enasidenib (AG-221; teal) at the dimer interface and the amino acids affected by second-site resistance mutations (Q316, I319; modelled from PDB code 5I96, see Methods). Second-site mutations are structurally distant from the catalytic active site containing the neomorphic R140Q mutation and NADP/H cofactor. b, Detailed view of the Q316E mutation (apostrophe denotes second dimer subunit) showing loss of a hydrogen bond that normally forms between the amino side chain of Q316 and a nitrogen in the diaminotriazine ring of AG-221. c, Detailed view of the I319M mutation, demonstrating steric effects from the bulky side chain of methionine predicted to hinder binding by AG-221. d, Intracellular 2HG levels in Ba/F3 cells that express IDH2 mutations R140Q (RQ), Q316E (QE) or I319M (IM) via retroviral transduction. Values are mean ± s.e.m. for triplicate cultures. e, Intracellular 2HG levels in Ba/F3 cells co-expressing IDH2 RQ plus wild-type (WT), QE or IM in trans and treated with vehicle (‘Veh’) or increasing doses of AG-221 (1, 10 or 100 nM). Data are mean ± s.e.m. for duplicate (CFU1) or triplicate (CFU2/3) cultures. f, In vivo 2HG rescue in IDH2 WT or IDH2 WT/Q316E colony-forming cells (CFU1/2/3) cultured in methylcellulose containing vehicle, AG-221 (50 nM) or AG-221 (50 nM) plus cell-permeable 2HG (octyl-2HG; 0.5 mM). Data are mean ± s.e.m. for duplicate (CFU1) or triplicate (CFU2/3) cultures. Asterisk indicates value of 0. Data are mean ± s.e.m. for triplicate cultures. h, Serial-replating of primary HSPCs from Idh2R140Q/Flt3ITD mice cultured in methylcellulose containing vehicle, AG-221 (50 nM) or AG-221 (50 nM) plus cell-permeable 2HG (octyl-2HG; 0.5 mM). Data are mean ± s.e.m. for duplicate (CFU1) or triplicate (CFU2/3) cultures. i, Intracellular 2HG (log2) levels in bone marrow mononuclear cells (see Fig. 1d). Similarly, the expression of the IDH2(Q316E) mutation in transduced cells increased despite enasidenib treatment (40 mg kg−1 twice daily) and assessed for WT or QE allele frequencies before and after treatment (i) or intracellular 2HG levels in bone marrow mononuclear cells (j). See Methods. Data are mean ± s.e.m. for n = 5 WT and n = 8 QE mice. P = 0.008 (i) or P = 4 × 10−7 (j) by two-tailed t-test. k–l, In vitro enzyme assays measuring absolute velocity (k) and relative activity (l) of NADPH-dependent reduction of α-ketoglutarate (α-KG) by haemagglutinin (HA)-precipitated IDH2 dimers purified from cells co-expressing IDH2 HA–RQ + Flag–WT, HA–RQ + Flag–QE, or HA–RQ + Flag–IM (see Methods). Reactions contained purified enzyme (10 μg ml−1), NADPH (0.3 mM), α-KG (5 mM) and AG-221 at 0.1, 0.3, 1, 3, 10 and 30 μM (k) or indicated concentrations (l). Data for k–l are mean ± 95% confidence intervals for replicate reactions. Results are representative of ≥3 (d, e, k–l, 2 (f–h), or 1 (i, j) independent experiments. See also Extended Data Figs. 2–4.

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their endogenous loci17 (Fig. 3g and Extended Data Fig. 3c, d). Cell-permeable 2HG exposure was sufficient to confer enasidenib-resistant serial-replating to Idh2R140Q/Flt3ITD-mutant HSPC (Fig. 3h), supporting the hypothesis that restored 2HG production contributes to the resistance mediated by the IDH2 Q316E and I319M mutations.

We determined the effect of expressing IDH2(Q316E) in trans on response to enasidenib in vivo (Fig. 3i, j). Bone marrow HSPCs expressing IDH2(R140Q) were transduced with either IDH2 WT or IDH2 Q316E and used to reconstitute the hematopoietic system of recipient mice. Recipient mice were treated for 2 weeks with enasidenib, and IDH2 WT and IDH2 Q316E mutant allele frequencies were assessed before and after treatment. Enasidenib treatment reduced the proportion of IDH2 WT-transduced cells, whereas the frequency of IDH2 Q316E-transduced cells increased despite enasidenib treatment (Fig. 3i and Extended Data Fig. 3e; P = 0.008), consistent with a fitness advantage of IDH2 Q316E-transduced mutant cells during enasidenib therapy (see Fig. 1d). Similarly, the expression of the IDH2(Q316E) mutation in trans conferred production of 2HG by IDH2 R140Q-mutant cells in vivo that was resistant to enasidenib treatment (Fig. 3j; P = 4 × 10−7), consistent with the in vitro studies (Fig. 3e–g).

To rule out indirect cellular effects by which the Q316E and I319M mutants might induce resistance to IDH2 inhibitors, we purified IDH2
enzymatic dimers constituted of R140Q–wild type, R140Q–Q316E, or R140Q–I319M and performed in vitro enzymatic reactions (Fig. 3k, l and Extended Data Fig. 5a) allowed for 2HG production that was not inhibited by enasidenib. In vitro enzymatic reactions with purified IDH2 dimers comprised of wild-type–R140Q/Q316E and wild-type–R140Q/I319M demonstrated that the enzymatic activity of these complexes was resistant to enasidenib (Fig. 4b and Extended Data Fig. 5b–g).

The experimental findings suggested that second-site mutations of IDH2 in cis also might mediate clinical resistance to enasidenib. Analysis of 14 patients with de novo resistance and 7 additional patients with acquired resistance to enasidenib did not identify any additional patients with second-site IDH2 mutations in either allele (Extended Data Table 2). Notably, we identified one patient with IDH1R132C, mutant AML who developed acquired resistance to the mutant IDH1 inhibitor ivosidenib (AG-120; blue box), including bone marrow blast percentage (c), plasma 2HG concentration (d), and VAF for mutations identified by targeted next-generation sequencing of bone marrow cells (e). f. Alignment of IDH1 and IDH2 protein sequences demonstrating that S280 of IDH1 corresponds to I319 of IDH2. g. Summary of Sanger sequencing results from patient X demonstrating that the IDH1R132C neomorphic mutation and the IDH1S280F mutation occur in cis on the same allele.

3, 10 or 30 μM). Data are mean ± 95% confidence intervals for triplicate reactions (duplicate reactions for WT + RQ/QE AG-221 3 μM and 30 μM). c–e, Clinical and laboratory features for patient X in relation to treatment with mutant IDH1 inhibitor ivosidenib (AG-120; blue box), including bone marrow blast percentage (c), plasma 2HG concentration (d), and VAF for mutations identified by targeted next-generation sequencing of bone marrow cells (e). f. Alignment of IDH1 and IDH2 protein sequences demonstrating that S280 of IDH1 corresponds to I319 of IDH2. g. Summary of Sanger sequencing results from patient X demonstrating that the IDH1R132C neomorphic mutation and the IDH1S280F mutation occur in cis on the same allele.

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sequence analysis of individual clones. All clones with the IDH1R132C mutation also harboured the IDH1R132F mutation, demonstrating that the putative IDH1R132F resistance mutation was acquired in cis with the neomorphic IDH1R132C mutation (Fig. 4g).

In summary, we have identified a mechanism of acquired clinical resistance to a molecularly targeted therapy, in which a second-site mutation on the wild-type allele can cooperate with a heterozygous gain-of-function mutation on the other allele to induce therapeutic resistance. In theory, in trans resistance mutations may occur in other homomultimeric targets such as receptor tyrosine kinases. The possibility that in trans mutations of EGFR cysteine 797 (C797) could contribute to EGFR inhibitor resistance in cell lines has been reported, but it has not been observed to occur in vivo in the clinical context18. It remains unclear whether there may be a selective advantage to acquiring the IDH2R316F and IDH2R131M mutations in trans versus in cis; however, we would predict that in cis mutations may be identified as an alternative mechanism of acquired resistance to mutant IDH2 inhibitors. Consistent with this hypothesis, we identified a patient with acquired resistance to IDH1 inhibition who acquired an analogous IDH1 second-site mutation in cis. The identification of the IDH dimer-interface mutations credentials the importance of IDH as a therapeutic target and underscores the crucial role of 2HG in the pathogenesis of IDH-mutant malignancies.

Online content
Any Methods, including any statements of data availability and Nature Research reporting summaries, along with any additional references and Source Data files, are available in the online version of the paper at https://doi.org/10.1038/s41586-018-0251-7.

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Author contributions A.M.I., A.H.S., C.B.T., R.L.L. and E.M.S. conceived the project, designed the experiments, analysed the data, and wrote the manuscript. A.M.I., A.H.S., B.W. and A.N. performed the experiments with technical assistance from F.M.C., N.T., V.D., H.L. and J.R.C.; M.T. assisted with management of clinical data and specimens; M.E.A. and M.R. assisted with pathological assessment of biospecimens; A.S.R., S.K.A., G.A.P. and J.D.C. performed the structural modelling, N.F. and E.P. performed the mutational analysis; B.W., S.C., Z.D.K. and S.A.B. assisted with identification and analysis of the IDH1-mutant leukemia. All authors read and approved the manuscript.

Competing interests C.B.T. is a founder of Agios Pharmaceuticals and a member of its scientific advisory board. He also serves on the board of directors of Merck and Charles River Laboratories. R.L.L. is on the Supervisory Board of Qiagen. J.D.C. is a member of the scientific advisory board of Schrödinger. B.W., S.C., Z.D.K. and S.A.B. are employees of Agios Pharmaceuticals, Inc.

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METHODS

Clinical specimens. The patients described were enrolled on the phase I/Ii studies NCT02074839 or NCT0191549811. Enrolment was open to all patients with relapsed or refractory AML with a mutation in IDHI (NCT02074839) or IDH2 (NCT01915498) identified locally and confirmed centrally. Patients were required to be 18 years or older at the time of study entry. Both men and women were enrolled on the studies. Patients were required to have a performance status of 2 or better and adequate organ function as defined in the study protocols. Clinical data, blood and bone marrow samples from patients with AML were obtained after receiving written informed consent from patients. Approval was obtained from the Institutional Review Board at each institution participating in these clinical trials. Additional consent was obtained from participants at Memorial Sloan Kettering Cancer Center with analyses performed on the institutional biobanking protocol approved by the Institutional Review Board. Patient biospecimens were anonymized by creating unique identifiers with no associated protected health information (PHI) and keeping the key on a password-protected server. Data collection and research was performed in compliance with all relevant ethical regulations for human research participants. Absolute neutrophil counts and blasts percentages were determined by standard clinical assays. Next-generation sequencing and determination of VAF were performed as previously described12,13.

IDHI-mutant AML samples were assessed by the FoundationOne Heme panel as previously described12,20.

Allele-specific sequencing and PCR assays. Genomic DNA was isolated from bone marrow mononuclear cells using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer’s instructions. The following primers were used for IDH2 genomic region spanning exons 3–4: forward primer 5′-ATCTCCTGGCCCTCTTCTT-3′; reverse primer 5′-AGCCCGACATTTGCA-3′. The –2 kb PCR products were subcloned into a TA-cloning plasmid, followed by bacterial transformation, selection of individual bacterial colonies, isolation of plasmid DNA, and Sanger sequencing in the forward and reverse direction using the same primers. The IDH1 genomic region was cloned and sequenced in a similar manner with the following primers: forward 5′-ACCACAGCAAGACGTACCAA-3′; reverse 5′-CCTGGGAATGACCTGTCC-3′. IDH2 dPCR was performed with the following primers: R140F forward 5′-AGTCCAAATGGAATCTACG-3′, R140Q forward 5′-AGTCCCAATGGAATCTACG-3′, R140Q/R Reverse 5′-GGGGTTGAAGACCATTTGGA-3′, and with SYBR green reagent (Afyomatrix). cDNA was prepared using the Verso cDNA synthesis kit (ThermoFisher). For dPCR, an assay specific for each mutation was designed and ordered through Biorad. All reactions were performed on a QX200 ddPCR System (Biorad) and evaluated in technical duplicates. Reactions were partitioned into a median of ~16,000 droplets per well using the QX200 droplet generator and ordered through Biorad. All reactions were performed on a QX200 ddPCR System (Biorad) and evaluated in technical duplicates. Reactions were partitioned into a median of ~16,000 droplets per well using the QX200 droplet generator and run on a 96-well thermal cycler. Plates were then analysed with the QuantaSoft v1.7 System (Biorad) and evaluated in technical duplicates. Reactions were partitioned into a median of ~16,000 droplets per well using the QX200 droplet generator and evaluated in technical duplicates.

Cell culture and DNA constructs. Adherent 293T cells (purchased from ATCC) were maintained at low passage number in high glucose DMEM with 10% FBS, glucose 25 mM, glutamine 4 mM, penicillin 100 U ml −1, and streptomycin 100 μg ml −1 and split every 2–3 days before reaching confluence. Suspension Ba/F3 cells were harvested in M-PER buffer (ThermoFisher) without sonication, IDH2 tagged with HA or Flag antibodies were harvested using the internal standard (deuterated-2HG) peak area and cell number or protein quantified by BCA assay (ThermoFisher) and normalized for total protein concentration. Samples were separated by SDS-PAGE, then either stained directly with Coomassie Blue Reagent or transferred to nitrocellulose membranes (Life Technologies), blocked in 5% milk prepared in TBS with 0.1% Tween 20 (TBST), incubated with primary antibodies overnight at 4 °C then horseradish peroxidase (HRP)-conjugated secondary antibodies (GE Healthcare; anti-mouse, NA931V, sheep, 1:5,000; anti-rabbit, NA934V, donkey, 1:5,000) for 1 h the following day. After incubation with ECL (ThermoFisher or GE Healthcare), digital imaging analysis was performed using the SRX-101A (Konica Minolta). Primary antibodies used included: anti-Flag (Sigma, F1804; clone M2; mouse; 1:1,000), anti-GAPDH (Cell Signaling Technology, 5174; clone D16H11; rabbit; 1:1,000), anti-HA (Cell Signaling Technology, 2367S; clone E62; mouse; 1:1,000), anti-IDH2 (Abcam, ab55271; no clone name; mouse; 1:1,000), and anti-vinculin (Cell Signaling Technology, 4650; no clone name; rabbit; 1:1,000). For native gel electrophoresis, cells were harvested in M-PER buffer (ThermoFisher) without sonication, IDH2 enzyme complexes were purified with Pierce Anti-HA Agarose (ThermoFisher), separated by NativePAGE (ThermoFisher), and either stained directly with Coomassie reagent or transferred for western blotting as described above.

Enzyme assays. HA-tagged IDH2 enzymes were purified from transfected 293T cells using Pierce Anti-HA Agarose (ThermoFisher) according to the manufacturer’s instructions. HA-tagged enzymes were quantified by denaturable gel electrophoresis with Coomassie staining in reference to a defined quantity of recombinant human IDH2 (Abcam, ab198092). Purified enzymes were used at 7.5–10 μg ml −1 as indicated. NAPDH was used at 0.30 mM and α-ketoglutarate was used at 5 mM unless otherwise indicated. The enzyme reaction buffer consisted of HEPES 50 mM, pH 7.4, MgCl2 5 mM, and BSA 0.01%. Enzyme concentration was determined for each enzyme. Assays, reactions were conducted in UV-transparent 96-well plates (Corning) with reaction volumes of 200 μl. A SpectraMax Plus 384 Microplate Reader ( Molecular Devices) was used to monitor the absorbance at 340 nm every 30 seconds throughout the course of the reaction. For each condition, the mean rate of NDAPH consumption for triplicate control reactions without enzyme was subtracted from the rate of NDAPH consumption for triplicate experimental reactions with enzyme. Reaction velocities were calculated using an extinction coefficient for NDAPH at λmax of 340 and 6.220 M −1 cm −1 and pathlength of 0.56 cm for a 200 μl reaction volume in a standard 96-well plate.

Metabolite extraction and analysis. Metabolites were extracted with ice-cold 20:80 methanol:water containing 2 μM deuterated 2-hydroxylglutarate (n-2-hydroxylglutaric-2,3,3,4,4-d 5 acid; deuterated-2HG) as an internal standard. After overnight incubation at ~80 °C, cell extract was collected, sonicated and centrifuged at 21,000g for 20 min at 4 °C to precipitate protein. Extracts were then dried in an evaporator (Genevac EZ-2 Elite). For gas chromatography–mass spectrometry (GC–MS), metabolites were desalted by addition of 50 μl of methoxyamine hydrochloride (40 mg ml −1 in pyridine) and incubated at 30 °C for 90 min with agitation. Metabolites were further derivatized by addition of 80 μl of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) plus 1% 2,2,2-trifluoro-N-methyl-N-(trimethylsilyl)-acetamide, chlorotrimethylsilane (TCMS; Thermo Scientific) and 70 μl of ethyl acetate (Sigma) and incubated at 37 °C for 30 min. Samples were diluted 1:2 with 200 μl of ethyl acetate, then analysed using an Agilent 7890A GC coupled to Agilent 5975C mass selective detector. The GC was operated in splitless mode with constant helium carrier gas flow of 1 ml min −1 and with a HP-5MS column (Agilent Technologies). The injection volume was 1 μl and the GC oven temperature was ramped from 60 °C to 290 °C over 25 min with a hold time of 10 min. Peaks were integrated and identified by comparing mass to the extracted ion chromatograms and metabolite library. Data collection and processing was performed using MassHunter vB.08.00 (Agilent Technologies) and then normalized to both the internal standard (deuterated-2HG) peak area and cell number or protein content as applicable. Ions used for quantification of metabolite levels were
2HG m/z 247 (confirmatory ion m/z 349) and deuterated-2HG m/z 252 (confirmatory ion m/z 354). Peaks were manually inspected and verified relative to known spectra for each metabolite. Absolute metabolite quantitation was performed using an external calibration curve with deuterated-2HG internal standard and the resulting concentrations corrected for the total cell volume extracted. For IDH1-mutant AML patients, plasma 2HG concentrations were determined using a qualified liquid chromatography–tandem mass spectrometry (LC–MS/MS) method with a lower limit of quantitation of 30 ng ml⁻¹ as previously described.²²

### Structural modelling

The structure of enasidenib (AG-221) bound to IDH2 was obtained from PDB code 5I96 retrieved from the RCSB.⁹ This structure has a R140Q mutation in both homodimer subunits. Residues I170, L298, S300, V315, Q316, S317, D318, I319, L320 and I170', V294', S300', V315', Q316', S317', D318', I319', L320', and AG-221 had two conformations resolved with different occupancies; here, residues with an apostrophe denote second dimer subunit. Two models were constructed: one for the high-occupancy conformations of all residues, and another for the low-occupancy conformations of all residues. Point mutants of interest (Q316E, I319M, Q316E' and I319M') were introduced in the structures of each of the two models using the 'mutate residues' functionality in Maestro 11.2 (Schrödinger)²³. and side-chain orientations were optimized using the 'predict side chains' functionality with default setting in Prime v4.8 (Schrödinger)²³–²⁵. This resulted in a total of eight single-mutant structures. Inspection of the mutated structures revealed potential differences in interactions between mutant and wild-type that could affect the binding of AG-221. These features were manually highlighted in figures produced using PyMOL v1.8.2.0 (Schrödinger; https://pymol.org/2/).

### Statistics and reproducibility

Significance was determined by two-tailed Student's t-test comparing the indicated condition to the corresponding wild-type or control. Open circles are individual data points. For quantitative measurements, n is provided in the figure legends. All results have been independently replicated at least twice, with the exception of the in vivo experiments shown in Fig. 3i, j. No statistical methods were used to predetermine sample size. The experiments were not randomized, and investigators were not blinded to allocation during experiments and outcome assessment.

### Reporting summary

Further information on experimental design is available in the Nature Research Reporting Summary linked to this paper.

### Data availability

Source Data for all figures are provided with the paper. Uncropped versions of blots are provided in Supplementary Figs. 1–3. Structural modelling studies referenced PDB accession code 5I96. Images, settings and scripts for the structural modelling are available at https://doi.org/10.6084/m9.figshare.5966878. Sequencing data are deposited in the European Nucleotide Archive (ENA) under accession number PRJEB26337.

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Extended Data Fig. 1 | Acquired clinical resistance to the mutant IDH2 inhibitor enasidenib (AG-221). a, b, Haematoxylin and eosin staining of bone marrow cells aspirated from patient A (a) and patient B (b) at indicated points in relation to treatment with AG-221. Remission images demonstrate decreased leukaemic blasts and increased myeloid differentiation that are reversed at the time of relapse. Images show 100× magnification. Images are representative fields of a single bone marrow aspiration performed at each time point.
Extended Data Fig. 2 | Structures illustrating potential interactions between IDH2 second-site mutations and enasidenib. a–h, Detailed view of the interactions between wild-type IDH2 Q316 (a, e) and Q316' (c, g) or mutant IDH2(Q316E) (b, f) and IDH2(Q316'E') (d, h) with AG-221 in the predicted dominant conformation (a–d) or a minor conformation (e–h). Hydrogen bonds are depicted in light green. Note the disrupted hydrogen bond (depicted as orange bar) in d resulting from the Q316E mutation in the IDH2' subunit.

i–p, Detailed view of the interactions between wild-type IDH2 I319 (i, m) and I319' (k, o) or mutant IDH2(I319M) (j, n) and IDH2(I319M') (l, p) with AG-221 in the predicted dominant conformation (i–l) or a predicted minor conformation (m–p). The solvent-excluded surface of AG-221 is shown transparently in grey. The van der Waals radius of the Cδ1 and Cγ2 atoms of I319/I319' or the Sδ and Cε atoms of I319M/I319M' are depicted as spheres. Unfavourable steric interactions between AG-221 and these atoms are depicted in red. Throughout the figure, the IDH2 subunit is depicted in blue-grey, the IDH2' subunit in purple, and AG-221 in teal. Non-polar hydrogen atoms are not shown. White, red, blue and yellow portions of stick structures indicate hydrogen, oxygen, nitrogen and sulfur atoms, respectively. All models were based on the AG-221–IDH2 structure (PDB code 5I96) (see Methods).
Extended Data Fig. 3 | Expression and activity of in trans IDH2 second-site mutations in haematopoietic cells. a, Allele-specific quantitative PCR (qPCR) showing similar expression of constructs in Ba/F3 cells co-transduced with IDH2<Sup>R140Q</Sup> (RQ) plus IDH2<Sup>WT</Sup> (WT), IDH2<Sup>Q316E</Sup> (QE), or IDH2<Sup>I319M</Sup> (IM) in trans. Control from IDH2<Sup>WT</Sup> human cell line (293T). Data are mean ± s.e.m. for triplicate reactions. b, Intracellular 2HG levels in Ba/F3 cells co-expressing RQ plus WT, QE or IM in trans and treated with vehicle or increasing doses of AG-221 (1 nM, 10 nM, 100 nM, 1 μM or 10 μM). Data are mean ± s.e.m. for triplicate cultures. c, Western blot showing IDH2 protein levels in primary HSPCs from Idh2<Sup>R140Q/Flt3<Sup>ITD</Sup></Sup> mice transduced with WT, QE or IM and untransduced control cells for comparison. GAPDH serves as a loading control. d, Intracellular 2HG levels in primary HSPCs from Idh2<Sup>R140Q/Flt3<Sup>ITD</Sup> mice transduced with WT, QE or IM and collected from the first passage of methylcellulose cultures containing AG-221 at 50 nM. Data are mean ± s.e.m. for triplicate cultures. e, Flow cytometry gating strategy for Fig. 3i. SSC-A, side scatter area; FSC-A, forward scatter area. DAPI is a viability dye. mCherry identifies retrovirally transduced cells. Results are representative of ≥2 (a–d) or 1 (e) independent experiments. For gel source data, see Supplementary Fig. 1.
Extended Data Fig. 4 | Purification and activity of IDH2(R140Q) dimers with wild-type IDH2 or mutants Q316E or I319M in trans. a, Schematic of experimental approach: 293T cells were co-transfected with HA-tagged IDH2(R140Q) plus Flag-tagged wild-type, Q316E or I319M. After 2 days, cells were lysed and enzyme complexes were purified by HA-immunoprecipitation. Reactions were performed with purified enzyme, NADPH, αKG and varying doses of AG-221 as detailed in Fig. 3. b–e, Purity and dimerization of HA-precipitated enzymes were assessed by denatured SDS–PAGE with Coomassie staining (b), denatured SDS–PAGE with western blotting (c), native PAGE with Coomassie staining (d), or native PAGE with western blotting for the indicated proteins (e). Separate membranes were used for western blots. f, In vitro enzyme assays measuring rate of NADPH consumption of IDH2 dimers purified as in b–e. Reactions contained purified enzyme (10 μg ml⁻¹), NADPH (0.3 mM), αKG (5 mM) and AG-221 at indicated concentrations. Data are mean ± 95% confidence intervals for triplicate reactions. Results are representative of ≥3 (b–d, f) or 2 (e) independent experiments. For gel source data, see Supplementary Fig. 2.
Extended Data Fig. 5 | Second-site IDH2 mutations in cis can confer resistance to enasidenib. a, Western blot showing IDH2 expression in Ba/F3 cells transduced with the indicated constructs. Vinculin serves as a loading control. The same membrane was probed for both IDH2 and vinculin. These are the same cells as in Fig. 4a. b–g, Purification and enzymatic activity of IDH2 WT–R140Q dimers with or without in cis second-site mutations. b, Schematic of experimental approach: 293T cells were co-transfected with HA-tagged wild-type IDH2 plus Flag-tagged IDH2 WT–R140Q, in cis double-mutant IDH2 R140Q/Q316E (RQ/QE) or in cis double-mutant IDH2 R140Q/I319M (RQ/IM). After 2 days, cells were lysed and IDH2 enzyme complexes were purified by HA-immunoprecipitation. c–e, Purity and dimerization of HA-precipitated enzymes were assessed by denatured SDS–PAGE with Coomassie staining (c), denatured SDS–PAGE with western blotting with the indicated antibodies (d), or native PAGE with Coomassie staining (e). Separate membranes were used for Western blots. f, g, In vitro enzyme assays measuring relative activity (f) and rate of NADPH consumption (g) by HA-precipitated IDH2 dimers. Reactions contained purified enzyme (7.5 μg ml⁻¹), NADPH (0.3 mM), αKG (5 mM), and vehicle or increasing doses of AG-221 (0.1, 0.3, 1, 3, 10 or 30 μM). Data are mean ± 95% confidence interval for triplicate reactions (duplicate reactions for WT–RQ/QE AG-221 3 μM and 30 μM). Results are representative of ≥3 independent experiments. For gel source data, see Supplementary Fig. 3.
## Extended Data Table 1 | ddPCR for IDH2 mutations in pre- and post-treatment samples

### Patient A

| Assay   | Sample | Day | Droplet Count Mutant | Droplet Count WT | Ratio (Mut/[WT]) |
|---------|--------|-----|----------------------|------------------|------------------|
| IDH2_Q316E | BM pre | -91 | 0                    | 5081             | 0.00             |
| IDH2_Q316E | BM pre | -14 | 0                    | 2666             | 0.00             |
| IDH2_Q316E | BM post| 364 | 1089                 | 1309             | 0.83             |
| IDH2_R140Q | BM pre | -91 | 2413                 | 3242             | 0.74             |
| IDH2_R140Q | BM pre | -14 | 1254                 | 1396             | 0.90             |
| IDH2_R140Q | BM post| 364 | 1232                 | 1365             | 0.90             |

### Patient B

| Assay   | Sample | Day | Droplet Count Mutant | Droplet Count WT | Ratio (Mut/[WT]) |
|---------|--------|-----|----------------------|------------------|------------------|
| IDH2_I319M | BM pre | -32 | 0                    | 8767             | 0.00             |
| IDH2_I319M | PB pre | -11 | 0                    | 11695            | 0.00             |
| IDH2_I319M | BM post| 465 | 800                  | 3684             | 0.22             |
| IDH2_R140Q | BM pre | -32 | 5086                 | 5718             | 0.89             |
| IDH2_R140Q | PB pre | -11 | 6270                 | 6389             | 0.98             |
| IDH2_R140Q | BM post| 465 | 2337                 | 2570             | 0.91             |
Extended Data Table 2 | Frequency of second-site IDH2 mutations in AML patients treated with AG-221

| Category                                                                 | N (%)   | Second-site IDH2 mutation (in subset) |
|-------------------------------------------------------------------------|---------|--------------------------------------|
| Total treated on AG-221 protocol at MSKCC and assessed by next-gen sequencing | 59 (100%) | 0/59 (0%)*                           |
| De novo drug resistance                                                 | 14/59 (24%) | 0/14 (0%)                            |
| Acquired clinical resistance                                            |         |                                      |
| • Normal blood 2HG                                                      | 5/59 (8.5%) | 0/5 (0%)                             |
| • Elevated blood 2HG                                                   | 4/59 (6.8%) | 2/4 (50%)                            |

*Pre-treatment/earliest sample.
Life Sciences Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form is intended for publication with all accepted life science papers and provides structure for consistency and transparency in reporting. Every life science submission will use this form; some list items might not apply to an individual manuscript, but all fields must be completed for clarity.

For further information on the points included in this form, see Reporting Life Sciences Research. For further information on Nature Research policies, including our data availability policy, see Authors & Referees and the Editorial Policy Checklist.

Experimental design

1. Sample size
   Describe how sample size was determined.
   No statistical methods were used to predetermine sample size. Sample size was based on experimental feasibility, sample availability, and N necessary to obtain definitive results.

2. Data exclusions
   Describe any data exclusions.
   None.

3. Replication
   Describe whether the experimental findings were reliably reproduced.
   All experimental findings were reproduced at least twice, with the exception of the in vivo data in Fig. 3i, j.

4. Randomization
   Describe how samples/organisms/participants were allocated into experimental groups.
   Randomization was not part of the design for the phase I/II trials NCT01915498 and NCT02074839. Randomization is not relevant to the current study as the object was to decipher the mechanisms of acquired clinical resistance to targeted therapies within individual patients.

5. Blinding
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   Blinding was not part of the design for the phase I/II trials NCT01915498 and NCT02074839. Blinding is not relevant to the current study as the object was to decipher the mechanisms of acquired clinical resistance to targeted therapies within individual patients.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters
   For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

   n/a Confirmed

   ☑ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
   ☑ A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
   ☑ A statement indicating how many times each experiment was replicated
   ☑ The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
   ☑ A description of any assumptions or corrections, such as an adjustment for multiple comparisons
   ☑ The test results (e.g. P values) given as exact values whenever possible and with confidence intervals noted
   ☑ A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
   ☑ Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.
7. Software

Describe the software used to analyze the data in this study.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

No restrictions.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

The antibodies described in the Methods section are widely used, commercially available antibodies validated by the companies and publications cited on the company websites.

Primary antibodies used included:
- anti-FLAG (Sigma, F1804; clone M2; mouse; 1:1000);
- anti-GAPDH (Cell Signaling Technology, 5174; clone D16H11; rabbit; 1:1000);
- anti-HA (Cell Signaling Technology, 2367S; clone 6E2; mouse; 1:1000);
- anti-IDH2 (Abcam, ab55271; no clone name; mouse; 1:1000);
- anti-vinculin (Cell Signaling Technology, 4650; no clone name; rabbit; 1:1000).

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

293T cells were purchased from ATCC. Ba/F3 cells were purchased from DSMZ.

b. Describe the method of cell line authentication used.

Short tandem repeat (STR) profiling.

c. Report whether the cell lines were tested for mycoplasma contamination.

Cells were routinely tested for mycoplasma.

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

No commonly misidentified cell lines were used in the study.

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

Mus musculus C57Bl/6 Mx-Cre(+) Idh2 (R140Q) and Mx-Cre(+) Idh2 (R140Q) Flt3 (ITD) mice were previously described (Shih et al, Cancer Discovery 2017). Hematopoietic stem/progenitor cells were harvested from female and male mice at age 8–10 months as previously described (Shih et al, Cancer Discovery 2017).
12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

The patients described were enrolled on the phase I/II studies NCT02074839 or NCT01915498 (Stein et al, Blood 2017). Enrollment was open to all patients with relapsed or refractory acute myeloid leukemia with a mutation in IDH1 (NCT02074839) or IDH2 (NCT01915498) identified locally and confirmed centrally. Patients were required to be 18 years or older at the time of study entry. Both men and women were enrolled on the studies. Patients were required to have a performance status of 2 or better, adequate organ function as defined in the study protocols. Clinical data, blood, and bone marrow samples from patients with acute myeloid leukemia were obtained after receiving written informed consent from patients. Approval was obtained from the Institutional Review Board at each institution participating in these clinical trials. Additional consent was obtained from participants at Memorial Sloan Kettering Cancer Center with analyses performed on the institutional biobanking protocol approved by the Institutional Review Board. Patient biospecimens were anonymized by creating unique identifiers with no associated PHI and keeping the key on a password-protected server. Data collection and research was performed in compliance with all relevant ethical regulations for human research participants.
Flow Cytometry Reporting Summary

Form fields will expand as needed. Please do not leave fields blank.

- **Data presentation**
  
  For all flow cytometry data, confirm that:

  1. The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
  2. The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
  3. All plots are contour plots with outliers or pseudocolor plots.
  4. A numerical value for number of cells or percentage (with statistics) is provided.

- **Methodological details**
  
  5. Describe the sample preparation. Blood was collected from mice and red blood cells were lysed. The remaining cells were pelleted and then analyzed for mCherry positivity.
  6. Identify the instrument used for data collection. BD LSRFortessa.
  7. Describe the software used to collect and analyze the flow cytometry data. Data was collected by BD FACS Diva software and analyzed using FCS Express 6 Flow.
  8. Describe the abundance of the relevant cell populations within post-sort fractions. No sorting was performed.
  9. Describe the gating strategy used. Gating was done on the DAPI– mCherry+ population.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.